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Biophysical evaluation of the kinetics, thermodynamics, and structure-stability relationship of *Wuchereria bancrofti* glutathione transferase in comparison with human μ and π glutathione transferases

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Thesis

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Abstract

Lymphatic filariasis is an endemic disease caused mainly by the *Wuchereria bancrofti* parasite and has been classified as a major neglected tropical disease. The emergence of drug-resistant strains of *W. bancrofti* and the limited efficacy of the available drugs on adult worms threatens the eradication of the disease. *W. bancrofti* glutathione *S*-transferase (WbGST) is a homodimeric enzyme central to detoxifying electrophilic compounds in the parasite due to its lack of cytochrome P-450. Therefore, WbGST is a potential therapeutic target for lymphatic filariasis. Bromosulphophthalein (BSP) and epigallocatechin gallate (EGCG) were previously shown to inhibit glutathione *S*-transferase activity. In this study, the interaction of WbGST with BSP and EGCG in comparison with human glutathione *S*-transferase P1-1 (hGSTP1-1) and human glutathione *S*-transferase M1-1 (hGSTM1-1) isoforms was investigated. Soluble WbGST, hGSTP1-1 and hGSTM1-1 were recombinantly produced and purified successfully to homogeneity. Glutathione and 1-chloro-2,4-dinitrobenzene conjugation assay was employed to analyse the enzyme activity, kinetics and inhibitory potency of the compounds. Spectroscopic studies were employed to investigate the functional and structural impact of ligand binding to the enzymes. Both thermal and chemical stability studies were performed, and binding energetics were analysed using isothermal titration calorimetry. The activity of WbGST was predominantly inhibited, with IC_{50} values of 5 μ M for BSP and 12 μ M for EGCG. The EGCG displayed uncompetitive and mixed modes of inhibition towards WbGST with respect to glutathione and hydrophobic binding sites, respectively. Whereas BSP showed a mixed type of inhibition for both active sites of WbGST. Ligands reduced the turnover rates (k_{cat}) and the catalytic efficiencies (k_{cat}/K_M) of the enzymes. Upon ligand binding, 8-anilino-1-naphthalene sulphonate was displaced from WbGST and hGSTM1-1 by 67%_(BSP), 24%_(EGCG) and 72%_(BSP), 5%_(EGCG), respectively; suggesting that the ligands bind to the 8-anilino-1-naphthalene sulphonate binding site. Stability studies indicate that WbGST is the least stable of the three enzymes and that glutathione increases its stability. Isothermal titration calorimetry showed that BSP binds to multiple sites in WbGST with binding at site-1 (S_1) and site-2 (S_2), which are entropically and enthalpically driven, respectively. S_1 showed a higher affinity for BSP than S_2 . EGCG binding to WbGST was entropically driven. BSP had a higher affinity for the enzymes than EGCG. All the results indicated that the ligands significantly impact WbGST more than the human GSTs. Further investigations,

such as crystallography and molecular dynamics simulations, will shed more light on the ligand-protein interactions on a molecular level. Overall, this study suggests that BSP and EGCG are efficient inhibitors of WbGST that probably bind to both L and H-sites of WbGST, altering catalytic activity of the enzyme. The unique properties of the L-site are particularly suitable for rational drug design. Therefore, both ligands can be repurposed as new-generation therapeutics against filariasis.

Dedication

To יהוה (YHWH), I can never thank you enough.

"If I have seen further, it is by standing on the shoulders of Giants."

– Issac Newton

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List of abbreviations

3D	Three dimensional
ANS	8-anilino-1-naphthalene sulphonate
BSP	Bromosulphophthalein
CD	Circular dichroism
CDNB	1-chloro-2,4-dinitrochlorobenzene
C-terminal	Carboxylic acid terminal
ddH ₂ O	Deionised-distilled water
DDT	Dichloro-diphenyl-trichloroethane
dH ₂ O	Distilled water
ΔG	Change in Gibbs free energy
ΔH	Enthalpy change
ΔS	Entropy change
DSC	Differential scanning calorimetry
DTNB	5,5'-dithio-bis-(2-nitrobenzoic acid)
DTT	Dithiothreitol
EDTA	Ethylenediamine-tetra-acetic acid
EGCG	Epigallocatechin gallate
GSH	Glutathione (reduced form)
G-site	Glutathione binding site
GST	Glutathione S-transferase
hGSTM1-1	Human class Mu glutathione S-transferase with two type one subunits
hGSTP1-1	Human class Pi glutathione S-transferase with two type one subunits
H-site	Hydrophobic electrophilic substrate binding site
IC ₅₀	50% inhibitory concentration
IMAC	Immobilised metal affinity chromatography
IPTG	Isopropyl β -D-1-thiogalactopyranoside
ITC	Isothermal titration calorimetry
k_{cat}	Turnover number
$k_{\text{cat}}/K_{\text{M}}$	Catalytic efficiency

K_M	Michaelis-Menten constant
K_d	Dissociation constant
LB	Lysogeny broth
L-site	Non-substrate ligand binding site
MDA	Mass drug administration
ϵ	Molar absorption coefficient
M_r	Relative molecular mass
MW	Molecular weight
N-terminal	Amino-terminal
OD	Optical density
rpm	Revolution per minute
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SE-HPLC	Size exclusion-high performance liquid chromatography
TEMED	N, N, N', N'-tetramethylethylenediamine
Tris-HCL	Tris(hydroxymethyl)aminomethane-Hydrochloric acid
UV	Ultraviolet
V_{max}	Maximum velocity
v/v	Volume per volume
w/v	Weight per volume
WHO	World Health Organisation
WbGST	<i>Wuchereria bancrofti</i> glutathione S-transferase

Chapter 1

Introduction

1.1 Problem statement

Lymphatic filariasis is an endemic parasitic disease and has been classified as a major neglected tropical disease. The World Health Organisation (WHO) reported that in 2020, over 51 million people were infected, with more than 40 million people disfigured and disabled by the disease. However, it is concerning that more than 863 million people in 49 countries are at risk of infection (Cromwell *et al.*, 2020). Although there has been a reduction in the number of infected people in 17 countries, the disease is still endemic in most sub-Saharan African countries (except the Southern nations), Madagascar, Western Pacific Island nations, India, Southeast Asia and the South Americas (Lupenza *et al.*, 2022). Filariasis is a mosquito-borne disease caused by microscopic filarial nematodes, namely *Wuchereria bancrofti*, *Brugia malayi* and *Brugia timori*. Of these three filarioididea, *W.bancrofti* contributes 90% of global filaria infections. The *W. bancrofti* is transmitted by different mosquito species such as *Aedes spp.*, *Anopheles spp.*, *Culex spp.*, *Mansonia spp.*, and *Coquillettidia juxtamansonia* (Streit and Lafontant, 2008). Therefore, the geographical location highly influences the distribution of these mosquito species.

Most infections occur during childhood but do not manifest until eight to ten years. Many people who are infected remain asymptomatic and may never display any clinical symptoms (Routh and Bhowmik, 1993). However, the danger is that they will keep infecting other community members, who may show signs and develop severe lymphedema. Lymphedema occurs as a result of parasites damaging the lymphatic system. The damage results from the nesting and accumulation of adult worms in the inflammatory granuloma of the lymph, hence triggering lymphangitis and lymphadenitis with severe pain (Champion, 1979). Therefore, fluid accumulates in the lymph system resulting in swelling of the legs in most cases, but it can also occur in the arms and breasts. In addition, it may develop hydrocele or swelling of the scrotum in men (Edwards, 1979).

Consequently, the swelling and the diminished function of the lymph system also impacts negatively on the ability of the body to fight germs and infections. Thus, infected people often contract more bacterial infections on their skin and in their lymphatic system. Because of the severe infection, the physical appearance of the skin is tough and thick, resulting in what is known as elephantiasis (Ormsby and Montgomery, 1955). Furthermore, some infections in the Asian region can result in tropical pulmonary eosinophilia syndrome (Ottesen and Nutman, 1992), which occurs when white blood cells that help to fight diseases accumulate in abnormal concentrations. Symptoms of tropical pulmonary eosinophilia syndrome include coughing, shortness of breath, and wheezing (Vijayan, 2007). Besides the clinical disability from Lymphatic filariasis, the disease can also lead to financial disability. Hence it has been referred to as a disease of poverty, in addition to stigmatisation by members of society and, sadly, some health service providers (Person *et al.*, 2009).

At present, the first line of treatment is diethylcarbamazine. Unfortunately, it cannot be administered in countries with Onchocerciasis due to complications in Onchocerciasis patients (Lupenza *et al.*, 2022). However, the WHO recommended a combination therapy using Ivermectin, Albendazole and Diethylcarbamazine as a more effective treatment regimen. Unfortunately, drug resistance has developed in some countries, including resistance to Ivermectin in Ghana and Sudan (Ali *et al.*, 2002; Awadzi *et al.*, 2004a; Awadzi *et al.*, 2004b; Osei-Atweneboana *et al.*, 2007). This observation is reinforced by the elevated recurrence rate of genetic markers associated with resistance in other nematodes. It is particularly evident in populations with prolonged exposure to ivermectin compared to communities lacking previous exposure to ivermectin (Taylor *et al.*, 2010). Resistance to Albendazole, a derivative of Benzimidazole, is observed in nematode parasites resulting from single nucleotide polymorphism (Prichard, 2007). Studies among untreated populations in Burkina Faso and Ghana revealed that one of the single nucleotide polymorphisms is also present in *W. bancrofti* (Schwab *et al.*, 2005). Even after treating the population with a combination of Albendazole and Ivermectin, this mutation of a β -tubulin allele associated with benzimidazole resistance is still selected for higher frequencies in the two populations (Schwab *et al.*, 2007). Because the mechanism of action for diethylcarbamazine is not yet known, the existence of resistance cannot be adequately analysed.

Despite challenges to the elimination of filariasis, the WHO has made some progress through its mass drug administration program (MDA). However, this progress may be jeopardised if successfully treated populations are reinfected by drug-resistant *W.bancrofti* and if new effective drugs are not developed as soon as possible.

1.2 The rationale of study

There is no doubt that the emergence of drug-resistant strains of *W. bancrofti* is a considerable threat to the eradication of Lymphatic filariasis. Besides drug resistance challenges, the limited efficacy of available drugs on adult worms limits the efficacy of treating filariasis (Gyapong *et al.*, 2005; Norões *et al.*, 1997; Schwab *et al.*, 2005). Alongside adverse effects of diethylcarbamazine in Africa, side effects have also been observed for Albendazole and Ivermectin. These side effects include oedematous swelling of the limbs and face, urticaria, pruritus, myalgia, dermatitis, fever, and postural hypotension (Taylor *et al.*, 2010). Successful treatment requires an annual combination therapy over five to six years, while adult worm microfilariae are only reduced after 15 years of treatment. Thus, the treatment regimen is not cost-effective, and affected populations cannot afford it. Lapses in treatment, in turn, increase the probability of parasites developing drug resistance. These challenges underline the need to effectively develop alternative drugs to treat lymphatic filariasis.

The adult worms are the primary culprit in the life cycle of filaria infection. They nest within the lymphatic vessels, obstructing and damaging them. By producing many eggs, the adult worms maintain the microfilariae load. Microfilariae undergo several biochemical modifications, such as morphological and physiological changes, to survive within the host. This process triggers internal and external oxidative stresses arising from the physiological changes in the parasite and the host immune response, respectively. Therefore, to alleviate the impact of oxidative stress, the parasite depends on detoxification systems involving the reducing potentials of glutathione (GSH), thioredoxin, and disulphide oxidoreductases. Previous studies suggested that antioxidant enzymes are important for parasites to defend themselves against oxidative stress generated by the host (Brophy and Pritchard, 1992; Callahan *et al.*, 1988). A previous study implicated GST in protecting parasites against oxidative stress (Tracy *et al.*, 1983). The nematode detoxification system is limited to superoxide

dismutase, glutathione peroxidase, glutathione S-transferase (GST) and catalase (Ahmad and Srivastava, 2007; Brophy *et al.*, 1989). The GST is the central detoxification system in filarial parasites and replaces the absent cytochrome-P450 system (Brophy and Pritchard, 1994). GSTs support the conjugation of antibiotics, anthelmintics and other drugs, resulting in drug resistance (Ahmad and Srivastava, 2007; Brophy *et al.*, 1989). GST eliminates phase-II drugs in parasitic helminths such as *Setaria cervi* (Srivastava *et al.*, 1994), adult *Setaria digitata*, *W. bancrofti*, and *B. malayi*.

Interestingly, GST activity was substantially reduced when microfilaremia were treated with diethylcarbamazine (Bal and Das, 1996). In addition, *in vitro* studies indicate that diethylcarbamazine, butylated hydroxyanisole (BHA) and phenobarbitone induce the GST of adult *S. cervi* (Gupta *et al.*, 2005). Therefore, this suggests that GST could be vital for the survival of filarial parasites and is a potential drug target. This study thus focused on the inhibition of *W. bancrofti* GST (WbGST) as a preliminary step to identifying new drugs against lymphatic filariasis.

1.3 The novelty of study

While Bromosulphophthalein (BSP) is known to inhibit some GSTs, its effect on WbGST has not been tested, and the mode of inhibition remains unknown. The ability of epigallocatechin gallate (EGCG) to inhibit WbGST is also untested. The effect of BSP and EGCG on WbGST kinetics, energetics, and stability may help clarify its function and structure and evaluate their potential and suitability for drug repurposing. It is also important to study biophysical properties in the presence of the natural enzyme substrate (GSH) to support the successful design and optimisation of selective inhibitors of WbGST. The findings of this study may thus benefit the rational design of drugs against lymphatic filariasis.

1.4 Aims and objectives.

The functions and molecular properties emanating from protein-ligand interactions are critical in understanding complex biological regulations in living cells and systems. Hence, protein-ligand

interaction studies provide a theoretical basis for designing and discovering new drugs and druggable targets. This study aims to biophysically characterise *W. bancrofti* glutathione S-transferase (WbGST) inhibition by BSP and EGCG. This will aid alternative drug discovery for the treatment of lymphatic filariasis and knowledge base development on the structure-function relationship of apo WbGST and in complex with the ligands. Secondly, the impact of the ligands on two predominant human GST isozymes (hGSTP1-1 and hGSTM1-1) will be analysed and compared. The purpose of this research project will be achieved by accomplishing the following objectives using the stated methodologies:

- To heterologously produce recombinant WbGST and hGSTM1-1 using pET-11a expression vector and hGSTP1-1 in pET-15b vector in *E. coli* expression system and purify the recombinant proteins by immobilised metal affinity chromatography (IMAC).
- To determine the specific activity of the recombinant WbGST, hGSTM1-1 and hGSTP1-1 using GSH and 1-chloro-2, 4-dinitrobenzene (CDNB) conjugation assay.
- To structurally characterise the recombinant proteins using far-UV circular dichroism, intrinsic tryptophan fluorescence, and extrinsic 8-anilino-1-naphthalene sulphonate (ANS) fluorescence spectroscopy and size exclusion high-pressure liquid chromatography (SE-HPLC).
- To determine the IC_{50} of BSP and EGCG and obtain the kinetic parameters (K_M , k_{cat} , and k_{cat}/K_M) of WbGST, hGSTM1-1 and hGSTP1-1 in the presence and absence of BSP and EGCG using the GSH-CDNB assay.
- To thermally analyse the stability of WbGST, hGSTM1-1 and hGSTP1-1 and the impact of ligand binding on the protein secondary and oligomeric structure using far-UV CD, differential scanning fluorimetry (DSF) and differential scanning calorimetry (DSC).
- To chemically analyse the secondary and tertiary structure stability of WbGST using Urea unfolding studies.
- Thermodynamic parameters (ΔH , ΔS , ΔG , and K_d) will be explored using isothermal titration calorimetry (ITC) to determine the energetics of binding.

1.5 Structure of the thesis

The report commenced with a literature review in Chapter 2, which provided background information on Lymphatic filariasis, glutathione S-transferases, the ligands and an overview of some biophysical methods applied in this study. Chapter 3 covers the recombinant protein production, quantification and qualitative analysis for further downstream applications. This is followed by Chapter 4, reporting the recombinant proteins that have been functionally characterised by determining their enzyme activities and inhibitory capacities of the ligands. The kinetics of the enzymes were also determined. In Chapter 5, the structures of the proteins were characterised both in the presence and absence of the ligands. Chapter 6 captures the relationship between the recombinant protein structures and stability using thermal and chemical stability studies, while Chapter 7 accounts for protein energetics. Finally, Chapter 8 summarises the discussion and analysis of findings in this study, including the conclusion and suggested future work.

Chapter 2

Literature review

2.1 Filariasis

2.1.1 Background

Lymphatic filariasis, commonly known as elephantiasis, is a debilitating neglected tropical disease caused by *Wuchereria bancrofti*. The disease predates written history with various mythological accounts known from Egypt, India and equatorial Africa (Routh and Bhowmik, 1993). There is uncertainty and confusion surrounding the history and origin of the disease, including its etymology. Before the first diagnosis of the disease in the early 20th century, different authors described the clinical process in diverse ways. Benjamin Neisius from the University of Strassburg in Germany described it as the "Iliad of Diseases" resulting from numerous disease complications (Niesius *et al.*, 1927). Opinions diverge regarding the origin of the name elephantiasis, although etymologically, it derives from the Greek word for elephant. The name presumably derives from oversized limbs caused by the disease to resemble those of elephants (Skinner, 1961). Before the wide acceptance of the name elephantiasis, the disease was named differently by physicians and medical authors in various regions. Some names were based on places, underlying medical problems and resemblance to objects or affected body parts. Mason Good called it "bucnemia tropica" and classified it as a phlegmasia due to its inflammatory nature. Hillary, working on the West Indian island of Barbados, named it Barbados leg (Hillary, 1776), whereas Hendy called it a glandular disease of Barbados (Hendy, 1784). It was described as Yava leg by ancient Indian physicians (Dalton 1846), implying that it originates from a drink in the Polynesian Isles and as Galle leg in Sri Lanka. While practising in Cochin, Malabar district in India, William Twining and Clerk called it Malabar (Twining, 1832). In contrast, in Malabar's Southwest coast of India, it is called Kaal, meaning an elephant (Dalton, 1846).

However, the common aetiology of the disease condition remained mysterious until Jean Nicolas Demarquay discovered microfilariae in hydrocele fluids in 1863. Thereafter, in Brazil around 1868, Otto Wucherer also observed microfilariae in chyle urine. Timothy Lewis also found

microfilariae in peripheral blood in 1872 while working in Calcutta. In 1876, Joseph Bancrofti discovered an adult female parasite in Brisbane in a lymphatic abscess. The discovery by Otto Wucherer and Joseph Bancrofti led to the emergence of the name *Wuchereria bancrofti*. Later, it was observed that not all conditions of elephantiasis were caused by filaria transmission. Some were discovered to be hereditary, referred to as congenital elephantiasis. In contrast, others are non-filarial forms of elephantiasis, such as endemic big foot disease, which results from constant absorption of soil microparticles, elephantiasis of cayenne and elephantiasis chirurgica or surgical elephantiasis. The biggest contribution towards understanding filariasis was by Sir Patrick Manson between 1875 – 1879 while working in Amoy, China. First, he unravelled the connection between elephantiasis and endemic microfilaremia with other obstructive indications of the disease. Secondly, he discovered that the surge of microfilaria in the blood throughout the peak period of feeding promotes feasting on blood meals by culex mosquitoes. Lastly, he proposed that mosquitoes play a crucial role in sheltering the parasite during the development process to an infective stage (Strickland, 1991).

Lymphatic filariasis is among the primary causes of permanent disabilities in the tropics. Although the WHO launched a campaign in the year 2000 tagged Global Programme to Eliminate Lymphatic Filariasis (GPELF) with a target to eradicate filariasis by the year 2020. The campaign involves the annual mass drug administration (MDA) of anthelmintics in endemic populations. However, even in 2022, Lymphatic filariasis remains a burden for over 51 million people, with more than 40 million people disfigured and disabled by the disease. It is even more concerning that more than 86 million people in 49 countries are at risk of infection (Cromwell *et al.*, 2020). Although there has been a reduction in the number of infected people in 17 countries since the inception of the GPELF campaign, there are still many more grounds to cover, especially with the upsurge of drug-resistant strains. Furthermore, the disease is still endemic in most sub-Saharan African countries, Madagascar, Western Pacific Island nations, India, South-East Asia and South America (Lupenza *et al.*, 2022). According to global distribution studies (Figure 2.1), infection in sub-Saharan Africa is exceptionally heterogeneous, with more prevalent transmission across West Africa than affected Asian countries (Cano *et al.*, 2014).

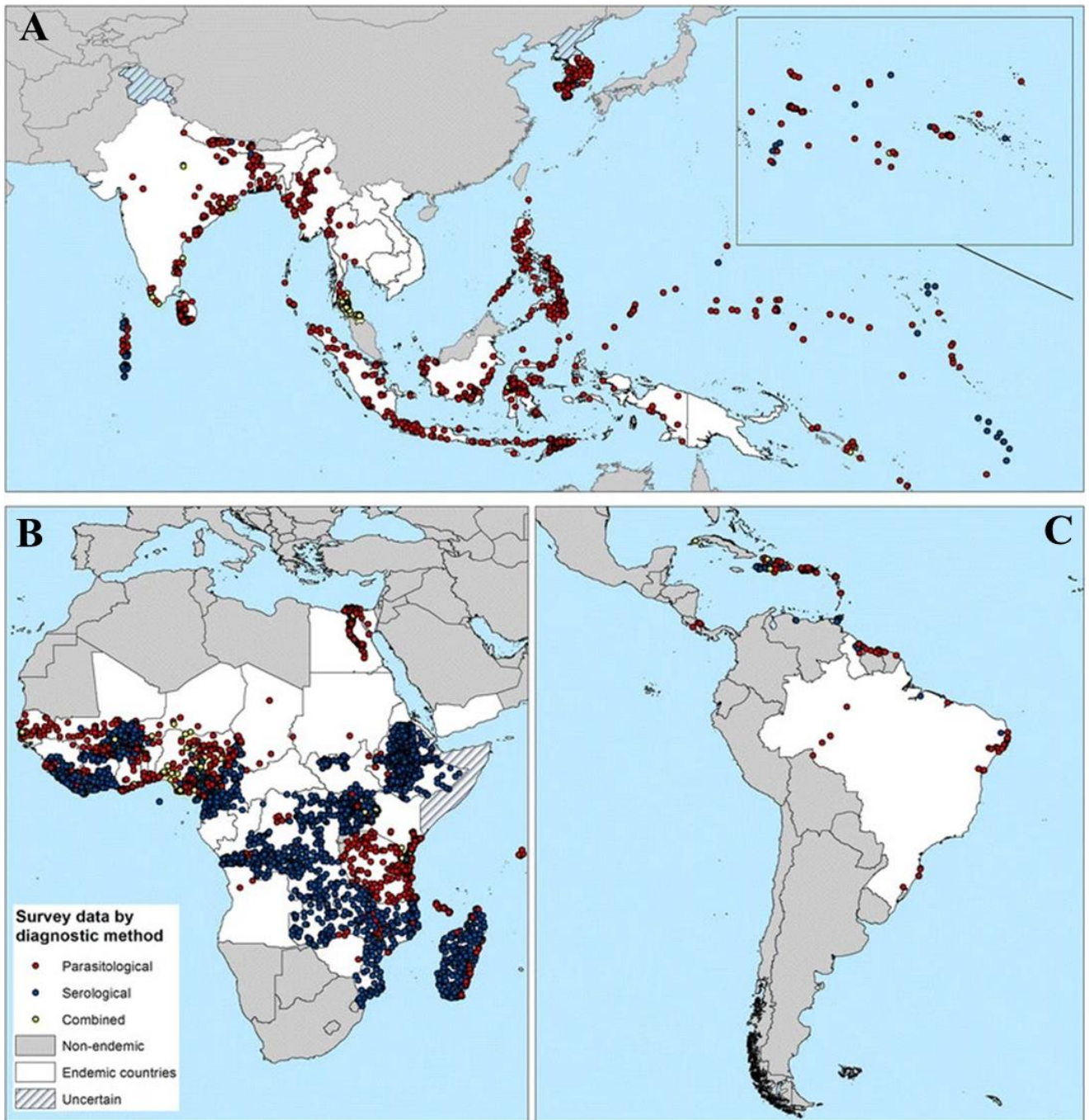


Figure 2.1 Global distribution of Lymphatic filariasis. Global map of Lymphatic filariasis based on the method of diagnosis. (A) South Asia and the Western Pacific are affected areas. (B) Some affected African countries, and (C) are regions in South America. Red, blue and yellow dots mark cases identified by parasitological methods, serological methods, and combined methods (Cano *et.al* 2014).

2.1.2 Life cycle

The life cycle of *W. bancrofti* is complex, comprising two different host systems. Humans are the definitive hosts, while the intermediate vector hosts are mosquitoes. Adult worms reside in lymphatic vessels where male and female worms mate and reproduce. The adult female worms are termed ovoviviparous because their eggs are laid and hatch inside the worm. Subsequently, the female worms release an embryonic brood known as microfilariae into the lymph from where they enter the bloodstream. These microfilariae mostly migrate to the thorax region. However, they are nocturnal, so they circulate the peripheral blood vessels at night, which is favourable for mosquitoes' feeding patterns. The microfilariae is the infective stage of the parasite. They usually survive for about 70 days in the human host and die if they are not transferred to the intermediate host. After a blood meal by mosquitoes from an infected human host, the microfilariae migrate to the midguts of the mosquitoes, where they remove their shielding sheath within 6 h (Chen and Shih, 1988). Afterwards, they penetrate the hemocoel and later move to the thoracic muscles within 24 h. Three stages of metamorphosis occur in the thoracic system; firstly, the shape of the microfilariae becomes short and thick, resulting in lethargic first-stage larvae (L1). Subsequently, within five to seven days, the short larvae moult into slender and stretched more active second-stage larvae. Finally, the stage-two larvae (L2) are exposed to another moulting and then transform to a more prolonged infective stage-three larvae (L3) between the 10th and 11th day. At this final stage, the larvae become highly active and oscillate between the mosquito head, thorax and abdomen. Subsequently, they migrate to the labium, also known as the proboscis, ready to be transmitted to a human host to complete the cycle (Paily *et al.*, 1995).

Transmission to the human host occurs when the mosquitoes bite and feed on human blood, thereby depositing the L3 on the skin. Afterwards, the larvae penetrate the skin through the bite wound and then move to the efferent lymphatic vessels. Upon entering the blood vessels, the fourth moulting stage occurs within nine to ten days. This is followed by the last developmental stage, where the stage-four larva moults and transforms into an adult worm. The lifespan of a female adult *W. bancrofti* is approximately ten years, with about five years of stable reproductivity (Vanamail *et al.*, 1990). Adult *W. bancrofti* vary in size, and male worms are about 23.8 - 30.6 mm in length and 90 - 120 µm in width. Female adult worms are more significant in size, ranging from 42.2 - 46.3 mm in length and 160 - 188 µm in width (Paily *et al.*, 1995)

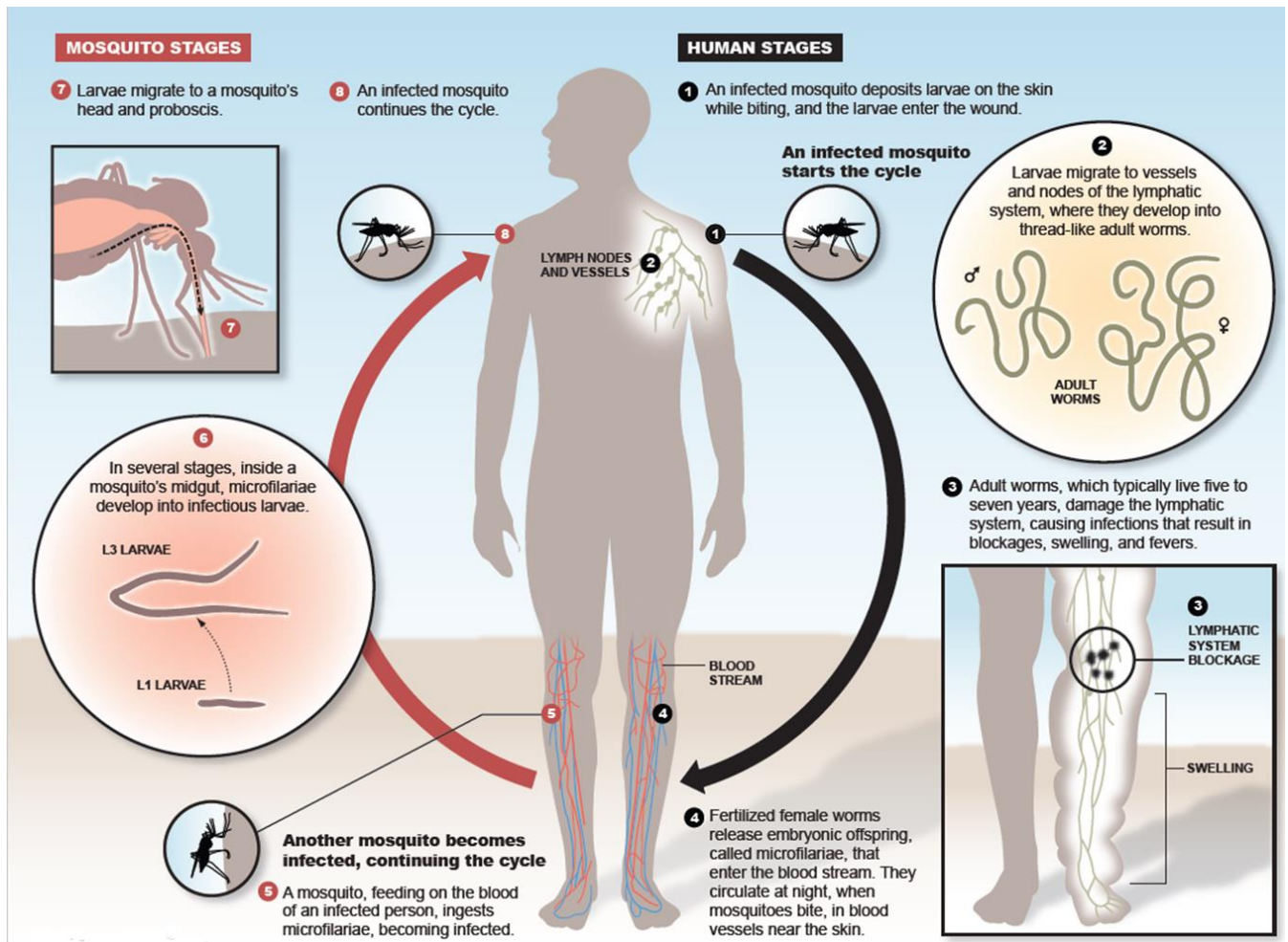


Figure 2.2 The life cycle of *W. bancrofti*. The two host stages are identified in red and black for mosquitoes and human hosts, respectively. The stages are numbered progressively, with (1) denoting the first stage, where infected mosquito feasts on humans and deposits the larvae on the skin, then the larvae enter the skin via the bite wound. (2) Larva relocates to the lymph nodes and vessels, developing into threadlike adult worms. (3) Adult worms damage the lymphatic system, resulting in infection and swelling. (4) Fertilised female worms release immature worms known as microfilariae, which migrate into the bloodstream where they circulate at night during the period of blood meal by the mosquitoes. (5) The cycle continues in the mosquitoes as they ingest the microfilariae after feeding on infected human blood (6) In the midgut of the mosquitoes, the larvae undergo three stages of transformation and develop into infective larvae. (7) The infective larva moves to the proboscis of the mosquito, ready to be transmitted, and finally, in (8), the cycle continues as an infected mosquito bites a human host. (Image from AI Granberg).

2.1.3 The pathogenesis of filarial disease

Despite the advanced studies on filariasis, there is still limited understanding regarding the pathogenesis of the disease. The contributing factors triggering the disease progression are still under speculation. The magnitude and frequency of exposure to an infective mosquito bite and the amount of antigens from adult worms accumulated in the lymphatic vessels appear to influence the pathogenesis of the disease (King, 2001). Another influencing factor is the response of the human host immunity followed by secondary bacterial and fungal infections (Freedman, 1998). In addition, the congenital traits of the human host influence filarial lymphedema susceptibility, besides the presence and concentration of microfilariae (Chesnais *et al.*, 2016; Choi *et al.*, 2003; Lammie *et al.*, 2002). Interestingly, clinical symptoms of lymphedema are usually more acute in infected people with undetectable microfilaremia, whereas in people with a visible circulation of microfilariae, no symptoms are often observed (Chesnais *et al.*, 2016; Dissanayake, 2001).

Filarial antigens trigger a Th2-type immune response at the onset of infection, resulting in the production of cytokines and interleukin 5, which elevate the level of immunoglobulin E (IgE) and immunoglobulin (IgG4). Subsequently, the upsurge of T lymphocytes is repressed, and the production of Th1 and Th2 cytokines are decreased, resulting in reduced modulation of host immunity to parasite antigens (Chesnais *et al.*, 2016). Perhaps this promotes the chronic manifestation of filarial infection. The suppression of immune response is typically observed in people with detectable microfilariae (Arndts *et al.*, 2012). The progression of chronic lymphatic pathology is linked to differential qualitative and quantitative parasite antigen IgG4 (Ottesen, 1992). Adult worms can initiate mechanical pathology by obstructing the lymph vessels. This does not necessarily cause inflammation but may be responsible for the observed subclinical lymphangiectasia and lymphatic dilatation in the initial stage of infection (Bennuru and Nutman, 2009).

Wolbachia is a symbiotic bacterium that lives within filarial nematodes in *W. bancrofti*, and they are primarily found in adult female worms. They have been linked to the pathogenesis of filariasis. Some studies proposed that the release of Wolbachia triggers an immune response leading to inflammation observed during the clinical progression of filariasis (Lammie *et al.*, 2002; Punkosdy *et al.*, 2003; Taylor and Hoerauf, 1999).

2.1.4 Clinical manifestations

Lymphatic filariasis is a major cause of acute and chronic morbidity, however, some individuals remain asymptomatic. Tropical pulmonary eosinophilia (TPE) is one of the acute clinical symptoms of filarial disease. It results from an abnormally high concentration of white blood cells in the body (Pani and Srividya, 1995). TPE usually leads to elevated levels of IgE and antifilarial antibodies. Acute adenolymphangitis (ADL) is generally observed at the onset of infection. It is characterised by abrupt fever and painful, abnormally enlarged lymph nodes (lymphadenopathy). ADL is often triggered by an immune response to dying, mature worms. In most cases, acute inflammation normally subsides within four to seven days and may reoccur one to four times per annum depending on the seriousness of lymphedema (Pani *et al.*, 1995). Another acute clinical manifestation is dermatolymphangioadenitis (DLA), which presents as oedema-like inflamed plaques. Typical symptoms include headache, fever, myalgia and chills. DLA arises from damaged skin infected by bacteria, which results in lesions mainly in the interdigital area (Dreyer *et al.*, 1999; McPherson *et al.*, 2006). Filarial fever occurs during early-stage infection and is sometimes mistaken for malaria symptoms in tropical regions.

One of the chronic clinical manifestations of lymphatic filariasis includes acute lymphedema of the limbs (Figure 2.3), referred to as elephantiasis and sometimes of the genitalia (hydrocele) resulting from a dysfunctional lymphatic vessel. Hydroceles are often not painful except when there is a complication due to bacterial infection. The accumulation of adult worms in the lymph of the spermatic cord can cause the cord to be thickened. Some studies have also reported the lymphatic filariasis of the ovary and mesosalpinx (Sethi *et al.*, 2001) and filariasis of the bilateral vulva (Chakraborty *et al.*, 2018). Lymphedema results when available lymph fluid is beyond the transport capacity of the lymphatic vessels. This leads to fluid accumulation in the interstitium (Witte and Bernas, 2020). The disparity in producing interstitial fluid and its transportation could result from a congenital malformation of the lymphatic system (the vessels or the lymph nodes), which can reduce the amount of lymph channels or block the accessible channels. Furthermore, a continuous buildup of lymphatic fluid increases the spread of adipocytes and the accumulation of collagen fibres in the extracellular matrix and all over the lymphatics.

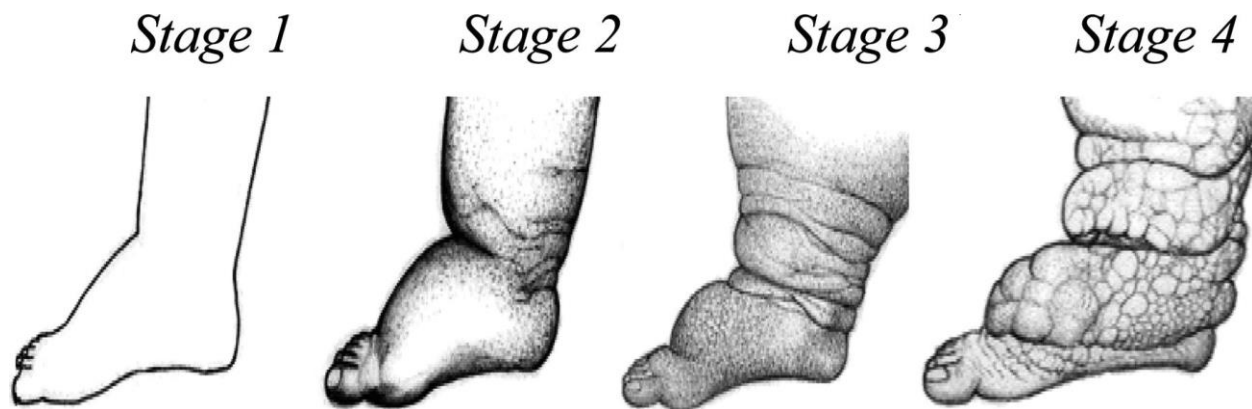


Figure 2.3 clinical manifestation of lymphatic filariasis. The first stage is characterised by a pitting oedema, often reversible. Stage two can be pitting or non-pitting, elevation of the affected limb is non-reversible with no skin change. Both stages three and four are non-reversible with skin thickening, with nodular or warty excrescences occurring in stage four (elephantiasis). (Mathieu *et al.* 2008).

Chyluria is one of the uncommon, chronic clinical manifestations of filarial disease. It is characterised by an intermittent leak of intestinal lymph into the renal pelvis leading to the passage of lymph fluid through urine, milky in colour (Franco-Paredes *et al.*, 2006). Individuals suffering from chyluria often lose substantial amounts of proteins and fat through urine, leading to anaemia and hypoproteinemia. In addition, in some individuals with detectable microfilariae, cases of hematuria and proteinuria have been reported (Dreyer *et al.*, 1992). Although the underlying cause is unclear, it is speculated to result from complex immune nephritis.

The ideal method for diagnosing filarial lymphatic infection includes detecting microfilariae in the blood, detecting filarial antigen or DNA in the blood and identification of adult worms in the lymphatics. The most basic diagnostic method is the microscopic examination of blood smears for the presence of microfilariae. Blood samples are usually collected at nighttime because the microfilariae circulation peak period is between 10:00 p.m. to 2:00 a.m. However, in South Pacific endemic regions, the microfilariae from *W. bancrofti* could be in circulation even during the daytime (Weller *et al.*, 1982). Blood samples are typically concentrated to increase microfilarial levels in the sample. Subsequently, the samples are stained and viewed under the microscope. The filarial species are then identified by their morphology. A second diagnostic approach is the

circulating filarial antigen (CFA) assay, which detects antigens released by mature worms. It is very effective even in individuals with a low circulation of adult worms (Dissanayake *et al.*, 1994).

Additionally, it is suitable because antigen levels do not fluctuate over time, so the test can be carried out at any time. However, it is unsuitable for progress evaluation because there are usually prolonged traces of the antigens circulating in the blood even after eradication of the worms (Weil *et al.*, 2011). The polymerase chain reaction technique has also been used to detect *W. bancrofti* in humans as well as assess the spread of mosquito microfilariae in likely endemic regions (Ramzy *et al.*, 1997). Another essential diagnostic tool is the antifilarial serologic test. This filarial antibody test detects high levels of IgG and IgG4, but it is unable to differentiate active infection from the previous infection. Therefore, it is primarily used in detecting filarial infections in individuals travelling from nonendemic regions.

Imaging techniques such as ultrasound and lymphoscintigraphy have also been employed in diagnosing lymphatic filariasis, specifically in detecting the existence of matured worms in the lymphatics. These imaging tools have successfully eliminated the diagnostic challenges posed by infected individuals with the absence of circulating microfilariae. Doppler ultrasounds can detect irregular motions of living worms in the lymphatic vessels because the worms move continuously, referred to as "filarial dance" (Mand *et al.*, 2003). Employing ultrasound to detect worm motions offers a valuable method for evaluating the progress and effectiveness of a treatment regime. Lymphoscintigraphy is a valuable means of assessing the magnitude of lymphatic damage (Freedman *et al.*, 1994). Lymphoscintigraphy is very efficient to such an extent that it can identify early lymphatic deformities even before the manifestation of clinical symptoms.

2.1.5 Treatment

More comprehensive knowledge of the disease and better diagnostic tools have improved treatment regimens. The aim is to both prevent the infection from spreading and to control morbidity (Ottesen *et al.*, 1997). Combination therapy of two different treatment regimens has proven to be effective in achieving and sustaining about 90 % success of the first strategy for at least one year (Gyapong *et al.*, 2005). The two-combination regime is (1) albendazole (400 mg)

with diethylcarbamazine (6 mg/kg) and (2) albendazole (400 mg) with ivermectin (200 mg/kg). The medications are to be administered for twelve days in the case of *W. bancrofti* (Ottesen, 2006). The second combination is used in populations co-endemic with onchocerciasis because diethylcarbamazine induces severe reactions in individuals suffering from onchocerciasis (Ottesen, 2000). However, before an interruption in transmission can be achieved in endemic areas, the microfilariae load in an endemic community must be reduced below the level required to sustain the spread for about four to six years. Therefore, either of the two drug combination regimes should be administered annually. Alternatively, it has been shown that substituting regular table salt for cooking with diethylcarbamazine-fortified salt effectively reduces microfilariae, thus interrupting transmission (Houston, 2000).

The second treatment strategy involves easing the sufferings triggered by the three main clinical manifestations of lymphatic filariasis, namely, lymphoedema (elephantiasis), hydrocele and acute dermatolymphangioadenitis (inflammatory episodes). In the case of elephantiasis, it is crucial to maintain good hygiene and proper skin care to prevent secondary bacterial infection, which exacerbates the disease (McPherson *et al.*, 2006). Other measures of caring for individuals suffering from elephantiasis include administering prophylactic antibiotics, elevation of the affected limb, and physical draining of the lymph vessels. Dermatolymphangioadenitis episodes often occur for four to eight days and could be managed by taking analgesics, antipyretics, systemic antibiotics, resting and elevating the affected limb (Dreyer *et al.*, 2002). Besides aspiration of the hydrocele fluid and injection of sclerosing substances, the most effective treatment method for lymphatic hydrocele is surgery. Unfortunately, the former is often ineffective with undesirable side effects.

Adult worms mainly cause lymphatic diseases. Unfortunately, none of the treatment regimens mentioned above can eliminate adult worms. For example, previous studies reported that Diethylcarbamazine could kill many but not all adult worms (Norões *et al.*, 1997; Ottesen, 1985). Similarly, a single dose of Albendazole was reported to affect the adult worms. However, the report did not state that the adult worms were killed or eliminated (Gyapong *et al.*, 2005). On the other hand, Ivermectin does not have an effect against adult filarial worms (Dreyer *et al.*, 1996).

Therefore, targeting and eliminating adult worms is a significant bottleneck towards treating and eradicating filarial diseases.

2.2 What is rational drug discovery, and why is it important?

Rational drug discovery refers to a multidisciplinary approach towards the synthesis of drug compounds that can bind to a target, such as proteins and nucleic acids (Stromgaard *et al.*, 2009). Drugs are developed based on prior knowledge of the structure and function of the target molecule. Therefore, the screening strategy of randomly testing hundreds of thousands of molecules is completely avoided. Hence, rational drug discovery applies a methodological process towards developing new drugs rather than blindly hoping that by some stroke of luck, one of the compounds could bind to a target and exert some therapeutic effect. This molecular-based drug design approach is obviously cost-effective and time-saving. Additionally, more success has been recorded in this century towards accurately targeting diseases with the lesser adverse effects caused by the drugs (Hongmao, 2015). Under this era of rational drug discovery, the following three main steps are often applied in developing new drugs:

- Before compound optimisation, researchers identify target receptors or enzymes associated with the disease.
- The structure of the target receptor or enzyme is resolved, and some insight towards the structure-function relationship is provided.
- Information obtained from number two is applied in designing and optimizing an efficient therapeutic molecule that can interact with the receptor or enzyme (Mavromoustakos *et al.*, 2011).

Other factors to be considered are the drug-compound affinity to the target molecule, and possibly its binding to other non-target molecules. In addition, the drug compounds' shape and charge must be analysed to ascertain how they influence binding to the target receptor and other properties such as absorption, distribution, metabolism, and elimination through the body (Stromgaard *et al.*, 2009). The detailed steps involved in rational drug discovery and the developmental process are summarised in Figure 2.4.

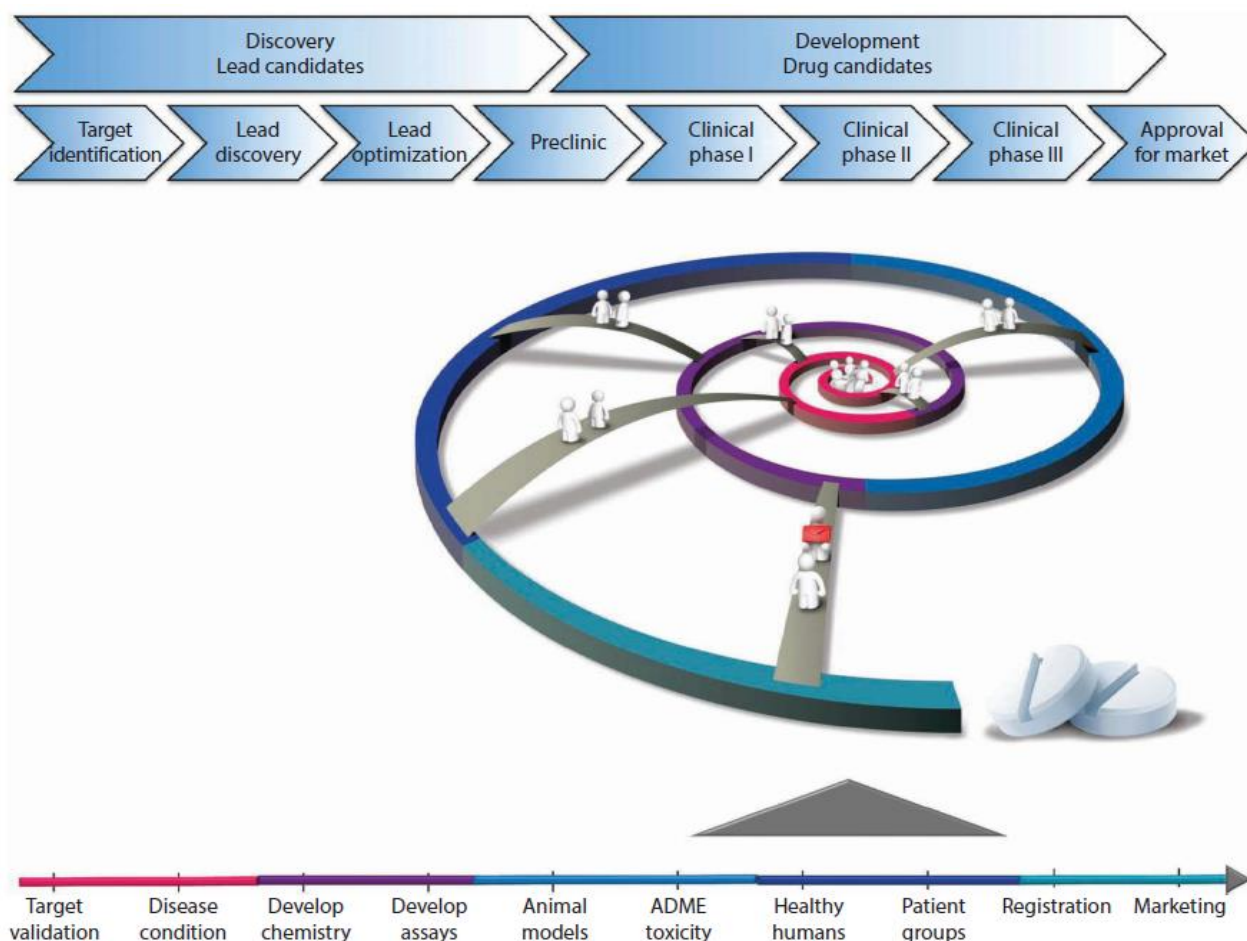


Figure 2.4 Steps in drug discovery. Outline of the drug discovery and development stages with an indication of individual steps (blue arrows). The different coloured arrow timeline below shows a continuous flow of the process which also comprises iterative processes at all stages. The spiral representation shows the involved forward and backward translation of knowledge by bridges between the different stages of development (Stromgaard *et al.* 2017).

In recent times, the concept of rational drug discovery has dramatically improved, facilitated by advances in biochemistry, molecular biology, protein crystallography and bioinformatics. Rational drug discovery integrates applied biological and chemical streams to simplify the drug discovery process, design, development, and optimisation. Therefore, it also addresses the limitations of the present structure-guided drug design (Mandal *et al.*, 2009). Two main approaches involved in rational drug discovery include (1) structure-based drug design and (2) ligand-based drug design. Structure-based drug design is an approach that involves the exploitation of structural knowledge and the active site information of a drug target, such as proteins, to develop a potential inhibitor. Hence, the first step is identifying the target receptor or protein structure (Anderson, 2003). This

is followed by identifying the active sites on the druggable targets, and the characterisation of these sites provides insight into the binding features. Knowledge of the amino acid residues in the active site and information regarding their functional group orientations are applied in designing ligands specific to that actual drug target (Gupta *et al.*, 2017). The ligand-based drug design is usually deployed in the absence of a structure of the drug target. This approach leverages the knowledge of the ligands or small molecules capable of binding to the druggable target. However, if the structural information of the biological target is available, the ligands can be optimised to obtain favourable drug properties. The ligands, often called lead compounds, can be identified computationally or experimentally. The computational method, also known as computer-aided drug discovery, involves three major categories: inspection, virtual screening, and de novo generation. The inspection method consists of the maximization of corresponding interactions of the identified small molecules on the binding sites of the target. This is in contrast to the virtual screening method that involves docking identified selected small molecules into a preferred binding region *in silico* with the predicted outcomes scored according to the level of interaction with the target site. In the de novo generation method, functional groups located within the binding site are scored and linked *in silico*. Subsequently, the resulting compound formed *in silico* from the attached fragments is then synthesized (Gupta *et al.*, 2017).

2.2.1 Rational discovery of druggable proteins in *Wuchereria bancrofti*

Before the 1960s, drug discovery programs were based solely on trial and error, which involved screening several thousand natural and synthetic compounds for drug development (Gershell and Atkins, 2003). Subsequently, selected potential compounds are optimised by the mere intuition of medicinal chemists by synthesising numerous related compounds. However, this drug discovery method is accompanied by a costly and risky process of developing a new drug. Various sources estimated the cost to be between \$161 million and \$1.8 billion annually (Morgan *et al.*, 2011). Despite vast invested funds and time spent annually in testing thousands of compounds, only a few new drugs (40 – 45 per year) are introduced to the market (Reddy and Parrill, 1999). Many of these new drugs are not unique because most pharmaceutical companies modify the molecular compounds of existing top-selling drugs. A limiting factor of a drug screening strategy is its inability to reveal the reasons behind the inactivity or activity of a compound and how it could be

optimised. Secondly, there is no guarantee that the selected active compound would specifically target a particular human protein (Mandal *et al.*, 2009). Most often than not, the lack of target specificity results in adverse side effects leading to a halt in drug development.

After successfully screening active target compounds, they are structurally optimised to enhance their potency and other valuable properties such as solubility, absorption, distribution, metabolism, excretion and toxicity. However, deciding when to progress from screening to synthesis is a challenge. Nonetheless, most previous existing drugs are products of the screening strategy. The screening strategy has many shortcomings, ranging from cost ineffectiveness to therapeutic inefficiencies. There is a drastic need for change in the approach towards compound optimisation, hence the advent of rational drug discovery.

2.2.2 Druggable protein in parasites

Interestingly, most therapeutic drugs available in the market are targeted against proteins (Whittle and Blundell, 1994). For example, nearly all target proteins for drug development are essential for the survival of infectious parasites or involved in a vital regulatory step in the human body. A critical requirement of rational drug discovery is that drug molecules must complement the shape, size, and electrostatic interactions at the active site of a target protein, thereby enhancing their binding affinity to the protein and obstructing the protein molecular function. Additionally, a progressive understanding of multi-protein complex dynamics predisposed to certain disease conditions is gaining momentum. Consequently, there has been an increased interest in targeting protein-protein interfaces in drug discovery.

Many human drugs target protein-protein interactions. For example, in preventing transplant rejection and treating autoimmune diseases, a drug called Ro26-4550 has been used successfully (Tilley *et al.*, 1997). This drug was targeted against cytokine receptor interactions by preventing the binding of interleukin-2 to its trimeric cytokine receptors. This interaction causes the proliferation and differentiation of cells and the mediation of immune disorders (Berard *et al.*, 1999; Nelson and Willerford, 1998). Similarly, T-cell activation can be controlled by preventing the cell surface of B7 proteins from binding to CD28 and cytotoxic T lymphocyte antigen-4 (Erbe

et al., 2002; Green *et al.*, 2003). In addition, some studies have reported the inhibition of anti-apoptotic proteins B-cell lymphoma-2 and B-cell lymphoma -X_L regulated by the binding of BAK, a pro-apoptotic factor (Kim *et al.*, 2001; Kutzki *et al.*, 2002; Tzung *et al.*, 2001). All the identified inhibiting small molecules bind at a hydrophobic groove formed by seven α -helices of the B-cell lymphoma proteins (Degterev *et al.*, 2001; Enyedy *et al.*, 2001; Jahnke *et al.*, 2003; Wang *et al.*, 2000). Similarly, the protein-protein interaction between the oncoproteins p53 and the murine double-minute-2 (MDM2) proteins in malignant cancer has also been targeted through ligand inhibition of MDM2 (Duncan *et al.*, 2001; Stoll *et al.*, 2001; Vassilev *et al.*, 2004; Zhao *et al.*, 2002). Furthermore, a small molecule called Ro 028-2750 has been identified to inhibit the binding of p75^{NTR}, a cell surface receptor, to the nerve growth factor (NGF) (Niederhauser *et al.*, 2000). The p75^{NTR} is pro-apoptotic, and its binding to the homodimeric NGF results in cell death as opposed to TRKA, a pro-growth cell receptor that also binds to NGF but on a different binding site.

In parasites, protein-protein interactions also constitute potential targets for rational drug discovery. For example, several protein-protein interactions (PPI) targets have been identified in *B. malayi* by Kumar *et al.* 2007. Some PPI targets include NOAH-1 and NOAH-2, bearing zona pellucida (ZP) domains, many plasminogen N-terminal (PAN) modules, serine-peptidase inhibitors comprising several Kunitz/Bovine trypsin inhibitor domains. It was speculated that the protease inhibitors may regulate the activity of peptidases like subtilisin, which process collagens of the cuticles and the activation of other forms of collagens responsible for either processing or atrophying enzymes. Another identified target is cysteine peptidase, specific to nematodes and encoded by conserved qua-1 genes. Its activity is autocatalysed by cleaving its own precursor (proenzyme or zymogen) to generate the active enzyme form, and it is vital for ecdysis and viability. In addition, two novel proteins encoded by *mlt-8* and *mlt-9* genes (MAM domain-containing proteins) were also identified to amplify endocrine signalling during new cuticle synthesis and associated with hypodermal signalling, respectively. Also, triacylglycerol lipase and adenosine deaminase were implicated in mediating moulting in nematodes. Similar to *B. malayi*, the Kunitz serine protease inhibitor targets are also found in *Onchocerca volvulus*, a related filarial parasite (Ford *et al.*, 2005).

Some targets were implicated in the function of the parasite structural components. A coiled-coil protein target named AJM-1 is required to elongate the embryo and preserve the epithelial integrity (Köppen *et al.*, 2001; McMahon *et al.*, 2001). Evolutionary conserved fibrous organelles encoded by *pat-12/gei-16* in filarial nematodes were also identified as potential targets. They help to attach muscles of the body wall and cuticle across the hypodermis to maintain a rigid body and enhance movement (Tsuboi *et al.*, 2002; Williams and Waterston, 1994). More structural PPI targets such as the chitin synthase, orthologs of UDP-galactopyranose mutase (UGM) and eight homologs of innexin were identified in filarial parasites (Foster *et al.*, 2005; Harris *et al.*, 2000; Kumar *et al.*, 2007). Chitin is a structural element consisting of polysaccharides critical for the growth and development of parasites. It is found in the pharynx and eggs of nematodes but is absent from the mammalian host (Zhang *et al.*, 2005). The UGM is an enzyme required for the biosynthesis of sugar galactofuranose, which is crucial for the viability and virulence of the parasites (Beverley *et al.*, 2005; Pan *et al.*, 2001). Both UGM and galactofuranose are lacking in mammals, making UGM a striking therapeutic target (Beverley *et al.*, 2005). Invertebrate innexins create intercellular channels connecting adjacent cells' cytoplasm, allowing electrical impulses to travel from cell to cell (Phelan, 2005). In addition, this is instrumental to movement, harmonised contraction of the pharyngeal muscular system, laying of eggs, and inhibition of oocyte development. Remarkably, the *unc-7* and *unc-9* innexin genes are essential for motion. They also moderate the response to ivermectin, an anthelmintic drug (Barnes and Hekimi, 1997; Dent *et al.*, 2000; Starich *et al.*, 1996). Several other nematode-specific PPI genes were also identified by Taylor *et al.* 2011, as potential drug targets.

On the other hand, individual parasite enzymes are attractive drug targets. Enzymes are often attractive mainly because of their substrate specificity, which has been leveraged in designing specific target inhibitors. Some studies have investigated the NADH-fumarate reductase system as a target against *Ascaris suum* (Ōmura *et al.*, 2001; Sakai *et al.*, 2011). It was discovered that Nafuredin inhibits NADH-fumarate and NADH-rhodoquinone oxidoreductase by binding to the quinone binding site (Ōmura *et al.*, 2001). These enzymes are essential for anaerobic electron transport in the mitochondria of helminths. The *Schistosoma mansoni* protein kinase “polo-like-kinases 1” (SmPlk1) regulates mitosis or meiosis in schistosome parasites (Long *et al.*, 2010). SmPlk1 plays a vital role in the parasite reproductive system, evident in the modifications observed

in the parasite gonad-altering oogenesis and spermatogenesis (Long *et al.*, 2010; Long *et al.*, 2012). Another prominent target is Topoisomerase II, which has been successfully inhibited by a methanol-like compound extracted from *Micrococcus luteus* BI252 (Kumar *et al.*, 2008; Misra-Bhattacharya *et al.*, 2004), slowing the growth of the parasite *Setaria cervi* (Sivasamy *et al.*, 2011). In *B. malayi* and *S. mansoni*, RNA helicase has been identified as a potential target that can interfere with the topological properties of the parasite nucleic acids (Singh *et al.*, 2012; Skinner *et al.*, 2012). Cathepsin B plays a vital role in the physiology of parasites and the interactions between the host and parasite by modulating diverse pathological events such as nutrient absorption, moulting, host immune evasion and tissue invasion. Cathepsin B is a cysteine protease and has been identified as a drug target in parasites like *S. mansoni*, *Fasciola hepatica*, and *Clonorchis sinensis* (Chen *et al.*, 2011; Jílková *et al.*, 2011). Then it was also discovered that vinyl sulphones K11017 and K11777 can inhibit the enzyme activity (Jílková *et al.*, 2011).

Another eminent antifilarial enzyme target in nematodes is the asparaginyl-tRNA synthetase (AsnRS) (Yu *et al.*, 2011). Five tirandamycins from *Streptomyces* sp. selectively inhibit AsnRS in *B. malayi*, effectively killing adult worms. Hence, it is a promising lead towards developing new antifilarial drugs. Lactate dehydrogenase is a potential drug target in liver fluke *C. sinensis* and *Taenia asiatica* with a phenolic yellow pigment known as gossypol as a possible lead small molecule (Huang *et al.*, 2009; Yang *et al.*, 2006). Trehalose phosphate phosphatase (TPP) helps synthesise trehalose, a nonreducing sugar used as a source of energy and structural protection in *C. elegans* and *B. malayi*. Inhibition of TPP resulted in the arrested development of the parasite larvae; hence, the enzyme is a potential drug target (Kushwaha *et al.*, 2012). The list of parasite enzyme targets cannot be complete without mentioning the glutathione S-transferase (GST). The filarial GST is an excellent drug target with numerous potential inhibitors (Awasthi *et al.*, 2009; Srinivasan *et al.*, 2009; Yadav *et al.*, 2010).

Aside from the enzyme targets, receptors and ion channel proteins are other sets of drug targets in parasites. Receptors are extremely ligand-specific and react according to their biological functions. One of the identified target receptors is the Nicotinic Acetylcholine Receptor (nAChR) of the nerves and muscles. The nAChR ion channels are triggered open by parasitic drugs like pyrantel and levamisole, resulting in the parasite protracted muscle spasm and spastic paralysis of the

parasite (Williamson *et al.*, 2009). Amino Acetonitrile Derivatives (AADs) are another class of newly synthesised anthelmintic drugs, which also take effect against nAChR (Kaminsky *et al.*, 2008). One of the classes of AADs, known as AAd-1566, has been used to target the nematode-specific DEG-3 gene, a subfamily of nAChR in *C. elegans* and *H. contortus* (Rufener *et al.*, 2009). Another group of targeted receptors that performs an important function in the signalling pathway are the G-protein coupled receptors (GPCRs). Many existing marketed drugs (more than 40%) target the GPCRs (Rana and Misra-Bhattacharya, 2013). Latrophilin is a neuronal "adhesion G-protein-coupled receptor that has been targeted in *H. contortus* by emodepside, a novel anthelmintics (Krüger *et al.*, 2009). In the same vein, latrophilin receptors are also targeted by a novel nematode anthelmintic ligand referred to as FMRFamide-like neuropeptides (Mühlfeld *et al.*, 2009). One more targeted receptor group is the nuclear receptor (NRs), which comprises exceptional transcription factors. They are involved in regulating some cellular processes like metabolism, differentiation, and growth in parasites because of their ability to detect metabolic and systemic hormonal signals. The activity of DAF-12, a conserved nuclear hormone receptor in parasitic nematodes, can be modulated by a ligand, dafachronic acid, in treating the spread of strongyloidiasis disease caused by *Strongyloides stercoralis*. DAF-12 regulates the signalling cascade in charge of nutrient metabolism and developmental stage of stage 3 infective larvae (iL3) in many nematodes such as *Ancylostoma* spp., *S. stercoralis*, and *Necator americanus* (Wang *et al.*, 2009). DAF-16 is another nuclear receptor found in *C. elegans* activated by a flavonoid compound, Apigenin. The activation of DAF-16 regulates larval development and longevity and decreases oxidative stress, and ultimately it signals DAF-2/insulin-like, thereby inhibiting the growth of the parasite larvae (Kawasaki *et al.*, 2010).

Ion channel receptors are multimeric proteins that transverse the cell membrane, regulating the passage of ions across the membrane. Ion channels are present in most neural and nerve cells controlling ion path and accumulating a voltage gradient for locomotion. Therefore, targeting the ion channels will paralyse the worms leading to their death. Most of the current drugs against helminth diseases target the ion channel protein receptors by inhibiting the movement of ions across the channel or continuously keeping the channel open, leading to hyperpolarization of the cell (Wolstenholme, 2011). Avermectinmilbemyacin and macrocyclic lactones are anthelmintics targeting the glutamate-gated chloride channels (McCavera *et al.*, 2009; Tandon *et al.*, 2006). In

contrast, the nicotinic agonists react against the nicotinic acetylcholine channel receptors. Intracellular calcium levels in schistosome parasites are regulated by voltage-dependent calcium channels (VDCCs), making them a striking prospective drug target (Salvador-Recatalà and Greenberg, 2010). Praziquantel impacts calcium homeostasis in *Schistosoma* parasites, and the drug is a prominent therapeutics in the treatment of schistosomiasis, although its mode of action is still unclear. The Cys-loop ligand-gated ion channels (CLGIC) are another significant group of essential targets in moderating neurotransmission (Williamson *et al.*, 2007). The CLGICs are gated by anthelmintic targeted receptors such as gamma-aminobutyric acid (GABA), 5-hydroxytryptamine (5-HT), acetylcholine receptor of cholinergic synapses, glutamate and glycine (Lees *et al.*, 2012). Changes in the receptor conformation resulting from binding neurotransmitters (inhibitors) usually open the integral chloride channel, thus reducing the excitation potential of the neurons (Hibbs and Gouaux, 2011).

Biochemical pathways are important therapeutic targets in parasites, and a better understanding of these pathways will aid in filling the existing knowledge gap regarding the mode of action of most anthelmintic drugs. Some anthelmintic drugs exert more impact when they affect certain cell physiological processes or are involved in the cell signalling pathway rather than a single molecular target. Therefore, medications like niclosamide used in tapeworm treatment target some vital cell signalling pathways. It has been reported that the drug inhibits Wnt3A-stimulated β -catenin stabilization and LEF/TCF reporter activity, stimulates Frizzled1 endocytosis and also deregulates the Dishevelled-2 protein (Chen *et al.*, 2009). In *S. japonicum*, a molecule referred to as SjIAP inhibits the apoptotic pathway and has been identified as a potential target on caspases to resist schistosomiasis (Peng *et al.*, 2010). In addition, other helminths signalling neuropeptide molecules such as neuropeptide FF (NPFF)-like, neuropeptide F (NPF)-like, FMRFamide-like (FLPs), myomodulin-like, and buccalin-like peptides have been proposed as novel drug targets in combating helminthic diseases (Marks and Maule, 2010; McVeigh *et al.*, 2009; Mousley *et al.*, 2010). These neuropeptides are widely spread within the nervous systems of helminths, making them very attractive for new therapeutic targets.

Furthermore, helminthic metabolic pathways could be an effective drug target. There are strategic differences between the helminths and host metabolism, thus, these differences could be harnessed

for novel drug discovery. For instance, the flatworm oxidation-reduction system is simply thiol-based and distinctive from humans (Otero *et al.*, 2010). The thioredoxin-glutathione reductase (TGR), a selenoprotein, is the only enzyme that supplies electrons to oxidized glutathione and thioredoxin in the parasites (Bonilla *et al.*, 2011). Hence TGR has lately been confirmed as a strategic drug target for Platyhelminthes diseases (Bonilla *et al.*, 2011; Boumis *et al.*, 2011; Martínez-González *et al.*, 2010). Additionally, a novel inhibitor derivative of 8-hydroxyquinoline-5-sulphonyl 1,4-diazepine was identified as a potential target against TGR enzyme in schistosomes (Eweas *et al.*, 2013).

The obligate endosymbiotic bacteria in the filarial parasite known as *Wolbachia* are crucial for the survival of adult female worms, including their fecundity and reproduction. Previous studies discovered that heme biosynthetic pathway enzymes are lacking in filarial parasites, but ferrochelatase is found in the final step of the pathway (Foster *et al.*, 2005; Wu *et al.*, 2009). Consequently, filarial parasites depend on the *Wolbachia* heme to activate their individual heme enzymes. This implies that the heme biosynthesis genes in *Wolbachia* are crucial for the survival of the host parasites. Globomycin is a pentapeptide, and it inhibits the signal peptidase II. Therefore, it acts against *Wolbachia* and *B. malayi*, a supposed *lspA* gene that was detected in the bacterial genome. Furthermore, the pentapeptide was also reported to be effective *in vitro* against the motility and survival of adult worms of *B. malayi* (Johnston *et al.*, 2010). Another prominent protein of *Wolbachia* is a GTPase referred to as the filamenting temperature-sensitive mutant Z (FtsZ), a prokaryotic homologue of the eukaryotic β -tubulin. This protein is present in the developmental stages of *B. malayi*, making it an attractive therapeutic target. Furthermore, a previous study showed that the GTPase activity of FtsZ was inhibited by berberine, a small bioactive molecule found in many plants (Li *et al.*, 2011). Therefore, FtsZ is a prospective drug target against filarial infection.

2.2.3 Rational discovery of druggable proteins in *Wuchereria bancrofti*.

The free β -tubulin in *Wuchereria bancrofti* is the primary target of albendazole, an anthelmintic drug. The binding of albendazole to the tubulin selectively inhibits the parasite microtubule polymerization and microtubule-dependent glucose absorption substantially (Shenoy *et al.*, 2000;

Simonsen *et al.*, 2010). Another potential druggable target identified in *W. bancrofti* is the deoxyribonucleic acid (DNA) topoisomerase II. The enzyme plays an important structural and functional role in the parasite DNA. Topoisomerase II enzyme is useful in the relaxation of DNA supercoils that accrue in the process of gene transcription on either side of the replication fork (Katiyar *et al.*, 2005). Besides, only topoisomerase II can decatenate replicated circular double-stranded DNA. The enzyme is also obligatory in modifying chromatin in mitosis (Nitiss, 1998). Topoisomerase II activity in *W. bancrofti* has been shown to be inhibited by a novel fatty acid methyl ester derivative, (Z) 15-tetracosenoic acid, present in *Micrococcus luteus* B1252 (Sivasamy *et al.*, 2011). Trehalose-6-phosphate phosphatase (TPP), a trehalose metabolic enzyme, has also been identified as a possible drug target. TPP removes phosphate molecules from trehalose-6-phosphate to yield free trehalose in the metabolic pathway (Matula *et al.*, 1971). Disaccharide trehalose is functional in the pleiotropy of nematodes, and its absence in mammals makes it an excellent target. Silencing the TPP gene in adult female worms negatively affected parasite viability, embryogenesis and larval infectivity (Kushwaha *et al.*, 2012). Asparaginyl-tRNA synthetase (AsnRS) is one of the aminoacyl tRNA synthetase (AARSs) enzymes, and it is crucial for parasite viability. The AsnRS is considered an attractive target in filarial parasites because it is expressed in both male and female worms, including larvae (Kron *et al.*, 1995; Nilsen *et al.*, 1988). The enzyme was selectively inhibited by tirandamycin B isolated from *Streptomyces* sp. 17944, indicating a new lead towards treating lymphatic filariasis (Yu *et al.*, 2011). Wolbachia are endosymbiotic bacteria that are vertically transmitted and also indispensable in filarial parasites. The difference in their biochemical properties with the human host makes them an attractive drug target. Although some studies have shown that antibiotics such as doxycycline, tetracycline, rifampicin, azithromycin and chloramphenicol are effective against the bacteria, large doses of these drugs are required for extended periods to achieve positive results (Hoerauf *et al.*, 2003; Hoerauf *et al.*, 1999; Hoerauf *et al.*, 2008; Rao and Weil, 2002; Supali *et al.*, 2008; Taylor, 2002; Taylor *et al.*, 2005). This has counter-indications on most individuals, hence the need for an alternative medicine against the bacteria (Rao and Weil, 2002).

Therefore, alternative drug targets like the enzymes participating in heme biosynthesis were indicated as viable options adding to the evolutionary divergence of the α -proteobacterial heme gene and their human homologues. N-methyl mesoporphyrin (NMMP) was reported to specifically

target and inhibit ferrochelatase activity, suggesting that the enzyme is a possible drug target (Wu *et al.*, 2009). The GSTs are among the recognised attractive drug targets in *W. bancrofti* owing to their crucial role in the parasite survival and the differences in the active site residues of the parasite and its human host. Some antifilarial compounds such as curcumin, capsaicin, piperine, geraniol ursolic acid and strychnine have been identified to be effective against the *W. bancrofti* GST (Nayak *et al.*, 2012; Saeed *et al.*, 2018). Similarly, the glutathione peroxidases are also part of the parasite defence system, hence they are regarded as potential drug targets.

2.3 Glutathione S-transferases

Glutathione transferases (GST, EC 2.5.1.18) comprise mainly cytosolic multi-gene isoenzymes known for their role in phase II detoxification (Mannervik *et al.*, 1992). The cytosolic GSTs are more ubiquitous than the microsomal, mitochondrial, and membrane-bound GSTs. They consist of two subunits with molecular weights ranging from 13 – 28.5 kDa each. GSTs help detoxify many exogenous and endogenous electrophilic compounds by glutathione (GSH) conjugation (Boydland and Chasseaud, 1967). They also support other activities such as the transport of molecules, isomerisation, prostaglandin and leukotriene biosynthesis, catabolism of aromatic amino acids, modulation or development of ion channels and control of signal transcription processes (Armstrong, 1991). In addition, GSTs are dimeric forming either homo or heterodimers. Moreover, protein stability is influenced by interactions at the dimer interface, which take place often via hydrogen bonds. Because GSTs are obligatorily dimeric, it follows that the interactions between the subunits are essential for appropriate folding and the retention of a stable quaternary structure (Rumfeldt *et al.*, 2008). Additionally, these interactions guarantee that heterodimers form spontaneously and that they only do so with members of the same class or the same type (Pettigrew and Colman, 2001). The transition from monomeric to dimeric form in GSTs is relevant to their optimal physiological functions (Fabrini *et al.*, 2009). Each dimer is made up of two domains (Figure 2.5) referred to as the thioredoxin-like domain, which houses the GSH-binding site (G-site) and a second domain consisting mainly of the α -helices and houses the hydrophobic electrophile binding site (H-site) (Mannervik *et al.*, 1988). The H-site has low specificity resulting in its ability to bind an array of xenobiotics, including steroids, quinones, halogenonitrobenzenes, $\alpha\beta$ -

unsaturated carbonyl compounds, organophosphorus compounds, aryl nitro compounds, aryl halides epoxides and isothiocyanates (Ketley *et al.*, 1975).

GSTs are categorised into three major groups based on their localisation: the mitochondrial GSTs, microsomal GSTs or MAPEG (membrane proteins associated with eicosanoid and glutathione metabolism) and cytosolic or canonical GSTs (Torres-Rivera and Landa, 2008). They can be classified into organism-specific and ubiquitous groups. The organism-specific class mostly exist in particular phyla, in plants as tau (τ), lambda (λ), phi (ϕ), and beta (β) in prokaryotes, and insect epsilon (ϵ), delta (δ) (Droog, 1997; Edwards and Dixon, 2005). The alpha (α), pi (π), sigma (σ), mu (μ), omega (ω), and zeta (ζ) classes are members of the ubiquitous cytosolic GSTs found in mammals (Mannervik *et al.*, 1992). The kappa (κ) is located in the mitochondrial GST group. Due to its localisation and high peroxidase activities, it contributes to the β -oxidation of fatty acids and lipid peroxidation. Four classes (I, II, III and V) of the microsomal GSTs have been identified by some studies, although they are not well defined or characterised like the other GSTs (Frova, 2006).

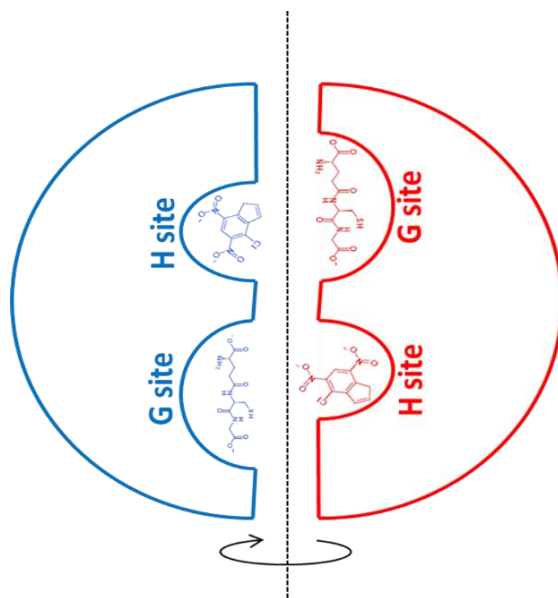


Figure 2.5 Schematic representation of GST. The illustration of a typical GST shows the two monomers in red and blue including the two active sites (the GSH binding site and the electrophile binding site).

2.3.1 Helminth glutathione S-transferases

The activity of glutathione S-transferases has been reported in adult worms and in the larvae of many helminths, namely, the cestodes (*Echinococcus multilocularis*, *Moniezia expansa*, *Hymenolepis diminuta*, *Echinococcus granulosus*, *Taenia solium*) (Brophy and Barrett, 1990b; Brophy *et al.*, 1990b; Brophy *et al.*, 1989; Fernández and Hormaeche, 1994; Liebau *et al.*, 1996a; Vibanco-Perez *et al.*, 1999); trematodes (*Fasciola hepatica*, *Schistosoma bovis*, *Schistosoma haematobium*, *Schistosoma japonicum*, *Schistosoma mansoni*) (Brophy and Barrett, 1990b; Brophy *et al.*, 1990b; Hughes, 1993; Salvatore *et al.*, 1995; Tiu *et al.*, 1988; Trottein *et al.*, 1992; Walker *et al.*, 1993; Wijffels *et al.*, 1992); and in nematodes (*Ascaris lumbricoides*, *Toxocara canis*, *Trichostrongylus colubriformis*, *Haemonchus contortus*, *Cooperia onchopora*, *Brugia spp*, *Wuchereria bancrofti*, *Onchocerca volvulus*) and many others (Brophy *et al.*, 1990a; Brophy *et al.*, 1994; Jaffe and Lambert, 1986; Liebau *et al.*, 1996a; Liebau *et al.*, 1994; Liebau *et al.*, 1996b; Rojas *et al.*, 1997; Salinas *et al.*, 1994; Sharp *et al.*, 1991). Interestingly, helminth GSTs exist in various forms and isoforms, suggesting heterogeneity in the function of the enzymes (Brophy and Barrett, 1990c; Howell *et al.*, 1988; Jaffe and Lambert, 1986; O'Leary and Tracy, 1988; Pemberton and Barrett, 1989).

GSTs are localised in some parts of the helminth parasites. For example, in *T. solium*, GSTs are found in the tegument of the parenchyma, in the cytoplasm of protonephridia, and cytons of the teguments (Vibanco-Perez *et al.*, 1999). The GST of *O. volvulus* is located in the hypodermis and spermatozoa (Salinas *et al.*, 1994) though a glycosylated GST was released by the worm *in vitro*. These two enzymes have different substrates, linked to their respective localisations (Liebau *et al.*, 1996b). One form of *A. summ* GST is localised in the intestine of the worm (Liebau *et al.*, 1997). In *F. hepatica*, some GST forms are localised in the lamellae of the epithelium of the intestine. At the same time, one was found both in the intestinal lamellae of adult worms and the parenchyma (Wijffels *et al.*, 1992). Some controversies exist regarding the localization of GST in *S. mansoni*. Two studies reportedly found GST in the tegument, protonephridia cells, cells of the subtegumental parenchyma, epithelium of the oesophagus, and the genitals of the parasite (Liu *et al.*, 1996; Taylor *et al.*, 1988). At the same time, another study reported that the enzyme was detected only in a subpopulation of parenchymal cells in all the stages of development (Porchet *et al.*, 1994). The GSTs in *Brugia spp* are primarily associated with the reproductive organs of adult

worms. In female worms, this is indicative of the susceptibility of embryos to oxidative stress (Rao *et al.*, 2000). The fact that the GSTs are widely distributed across different structures of the parasites and the likelihood of GST secretion indicates that GSTs play a vital role in the helminth metabolic process to detoxify both endogenous and exogenous toxic compounds.

Due to lack of studies, the effect of drugs on helminth GST production remains unclear. Drug compounds like phenobarbital and β -naphthoflavone elevate the activity of GST in the developmental stage of *Echinococcus granulosus* (Morello *et al.*, 1982), in adult *Hymenolepis diminuta* (Brophy and Barrett, 1990a), and *S. mansoni* (Vandewaa *et al.*, 1993). Additionally, 3-methylcholanthrene and butylated hydroxyanisole greatly induced GST activity in *S. mansoni*. The resistance against Cambendazole, an anthelmintic drug, appears to be linked to the activity of cytosolic GST in *Haemonchus contortus* (Kawalek *et al.*, 1984). The oxidative stress-sensitive Ov-GST_3 of *O. volvulus* is significantly induced by oxidative stress-related molecules (Liebau *et al.*, 2000; Perbandt *et al.*, 2005). In adult female *S. cervi*, the presence of diethylcarbamazine, butylated hydroxyanisole, and phenobarbital increased the GST activity. In the case of *S. cervi* microfilariae, exposure to butylated hydroxyanisole also increased the GST activity, whereas exposure to diethylcarbamazine resulted in a surprising decrease in activity (Gupta and Rathaur, 2005).

Furthermore, reports of helminth GST inhibition include, *A. suum* by heam byproducts, bile acids and inhibitors like cibacron blue, triphenyltin chloride and bromosulphophthalein (Liebau *et al.*, 1997). Some existing anthelmintics can inhibit GST activity in some parasites. A significant decrease in rAs-GST activity was observed in the presence of bithionol and hexachlorophene (Liebau *et al.*, 1997). Similarly, mebendazole was shown to inhibit *E. granulosus* GST activity in mice (Feng *et al.*, 1995) while the *P. westermani* GST was inhibited by albendazole (Hong *et al.*, 2000). The *F. hepatica* (Brophy *et al.*, 1990b) and *N. americanus* (Asojo *et al.*, 2007) GSTs were also found to be inhibited by anthelmintics. Compounds like bromosulphophthalein, cibacron blue and ellagic acid have been shown to inhibit GST activity in recombinant GSTs of *P. westermani*, *S. japonicum*, and *S. haematobium* (Akumadu *et al.*, 2020; Padi *et al.*, 2021; Platis *et al.*, 2021; Poee *et al.*, 2021). Besides, medicinal plant extracts were also reported to inhibit recombinant *A. suum* and *O. volvulus* GSTs (Fakae *et al.*, 2000).

Although helminth GSTs fall under the mammalian GST classes, most differ from the mammalian GST making a classification difficult. For instance, the GST isoforms in *M. expanza* differ biochemically from mammalian GST classes despite sharing N-terminal sequence identities with the α and μ classes (Brophy *et al.*, 1989). Even though the sequence of recombinant *E. multilocularis* is homologous to the mammalian μ GST class, however, there was no biochemical relationship observed between the parasite GST and the mammalian μ class (Liebau *et al.*, 1996a). On the other hand, in the genus *Schistosoma*, the recombinant 28 kDa GST from *S. mansoni* and the 26 kDa from *S. japonicum* shared some properties with the mammalian GST classes (α , μ and μ). Biochemical analysis of *S. mansoni* 28 kDa GST proposes that it is catalytically homologous to the α and μ class. The 26 kDa *S. japonicum* combines the characteristics of α and μ classes (Walker *et al.*, 1993). The *W. bancrofti* and *B. malayi* GSTs share 97 % sequence identity and are topologically related to the π -class GSTs. Ethacrynic acid, a known π -class GST ligand, is also bound preferentially to *W. bancrofti* GST (Rathaur *et al.*, 2003).

2.3.2 *Wuchereria bancrofti* glutathione S-transferase

Like other known GSTs, the *Wuchereria bancrofti* glutathione S-transferase (WbGST) is a homodimeric enzyme with 208 amino acid residues per subunit or ~25 kDa in size. The two subunits form a characteristic interface lined by 22 amino acid residues. Interdomain interactions linking chains A and B include, Phe47A - Lys127B, Arg68A - Asp88B, Glu81A - Arg72B, Thr85A - His69qB and Arg72A - Thr85B. The intersubunit interaction is critical in stabilising the tertiary structure. In some nematode GSTs, such as SjGST, a ligandin (L) site is located at the dimer interface (McTigue *et al.*, 1995). The dynamics of the L-site are not properly understood due to limited information about its structural and ligandin functions. Each subunit folds into distinct N and C-terminal domains. The N-terminal domain consists of amino acid residues 1 to 80 forming three α -helices that flank four β -strands. This N-domain retains the characteristic thioredoxin fold which includes the substrate-specific G-site (Figure 2.6), and this site is highly substrate-specific. The α -helices of the thioredoxin fold create a hydrophobic core that appears to facilitate the hydrophobic collapse of GSTs (Martin, 1995).

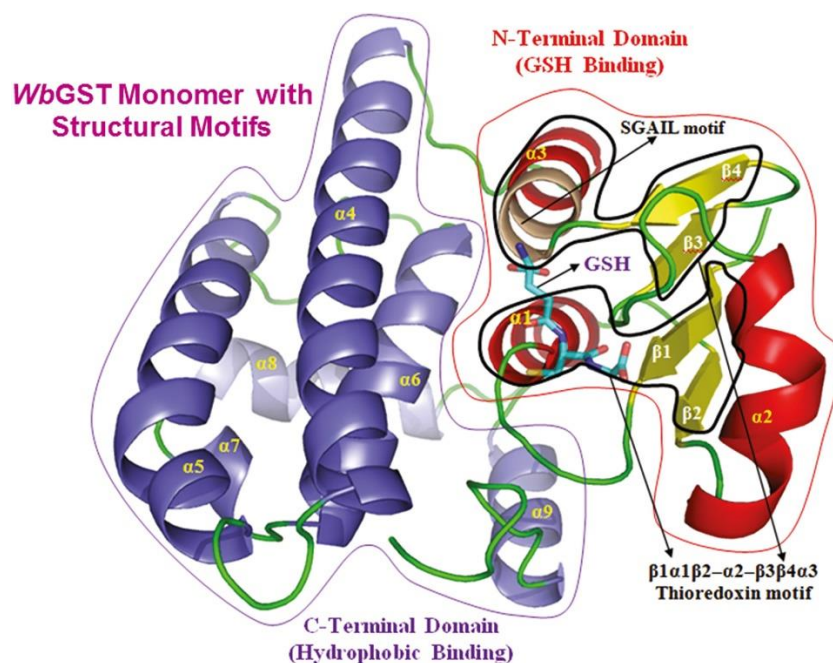


Figure 2.6 Monomeric ribbon representation of *W. bancrofti* GST. This structural motif shows the N-terminal domain and C-terminal domain presented in red and lilac, respectively. The TRX motif outlined in black shows two separate sub-motifs: the $\beta\alpha\beta$ and $\beta\beta\alpha$. The two sub-motifs are interconnected by the $\alpha 2$ -helix (Prabhu *et al.* 2018).

The N-domain of WbGST retains the canonical thioredoxin scaffold but lacks the reducing cysteine motif. The first β -strand is markedly twisted, compared to other thioredoxin proteins (Rajaiah Prabhu *et al.*, 2018). The catalytic Tyr7 that interacts with the thiol group of GSH is located towards the end of β -strand 1. In addition, the four β -strands of the N-terminus form parallel $\beta\alpha\beta$ and antiparallel $\beta\beta\alpha$ motifs connected by an α -helix. The loop connecting the two motifs sandwich the active site residues interacting with GSH, including Asp96 from α -helix 4 of the second subunit (Sheehan *et al.*, 2001). Other features in WbGST associated with GST proteins include the SNAIL/TRAIL motif (Koonin *et al.*, 1994) found in π -class GSTs with Gly64 replacing N/R (SGAIL/TGAIL) as the second residue. Secondly, WbGST has the conserved *cis*-Pro51 in the loop connecting $\alpha 2$ to $\beta 3$, stabilising the active site to increase enzyme activity. In addition, the notable hydrophobic lock and key of the π -class GSTs motif are also found in WbGST. The lock is formed by residues Gly93 in $\alpha 4$ as well as Lys127, Phe128, and Leu131 in $\alpha 5$ of the second subunit. At the same time, Phe47 forms the key, which descends from the loop of $\alpha 2$. Tyr49 replaces this Phe47 in human π -class GST counterpart (Rajaiah Prabhu *et al.*, 2018). Residues forming the G-site core include, Tyr7, Phe8, Leu13 (between loop of $\beta 1$ - $\alpha 1$), Gly48, Gln49,

Leu50, Pro51 (between loop $\alpha 2$ - $\beta 3$), Gln62, Ser63 (between loop $\beta 4$ - $\alpha 3$), Trp38 and Lys42 from $\alpha 2$.

The C-terminal domain is formed mainly by six alpha helices and comprises residues 81 to 208 (Figure 2.6). The H-site is located in the C-terminal domain, and the active site walls are surrounded by residues Ala14, Tyr07, Tyr106, Leu13, and Phe08. The internal core of the domain is lined by residues Ile10, Gly12, Ile33, Ala35, and Gly204 while Thr99, Thr102, His98, and Leu13 form the back. A closer look at the H-site of WbGST indicates that it is more accessible to electrophilic compounds than to the human enzyme (Rajaiah Prabhu et al., 2018). The accessibility of the H-site is increased due to Leu13 replacing arginine in human GST and providing an additional space of ~ 40 Å³. Similarly, Thr102 replaces Ile104 in humans further increasing the space. Compared to another filarial nematode GST like *O. volvulus*, the H-site of WbGST is wider and more accessible to electrophiles due to Thr99 and Ala35 in WbGST replacing Val99 and Lys35 in OvGST (Rajaiah Prabhu et al., 2018).

Structural differences between WbGST and human π -class GST include, $\beta 2$ in WbGST is a short strand formed by residues 26 – 32 with Arg32 as the C-terminus capping residue, which is exchanged with Val32 in human GSTs. Secondly, amino acid residues ranging from 37 – 42 formed a symmetrical α -helix in WbGST. In contrast, this region in the human π -class formed an irregularly shaped helix with the inclusion of Glu40 and Gly 41, which are absent in WbGST. It is interesting to note that these differences are found in the G-site region. Furthermore, residues 187 – 200 in human GSTs also formed an irregular $\alpha 9$ -helix which is regular in WbGST. Another significant difference is in residues 119 – 144 regions in WbGST, which creates a short-slanted α -helix in contrast to human GSTs (Figure 2.7). Overall, the human π -class GST has 42% sequence identity with WbGST (Figure 2.7A). The G-site and L-site structural differences that have been discovered imply that WbGST belongs to a novel GST class. Some scholars have also suggested that the nematode GSTs should be classified differently from humans (Vibanco-Perez *et al.*, 1999). In addition, the structural differences are advantageous in designing specific drugs targeted against the parasites.

On the other hand, other filarial GSTs from *B. malayi*, and *O. volvulus* display 98% and 79% sequence identity with WbGST, respectively. Although the G-sites in GST enzymes are usually conserved, these filarial GST residues have an 8% disparity from the human GSTs. Interestingly, the H-site of WbGST shows a 62% difference in sequence from that of humans, but the nematodes and human π -class GST H-site are layered with both hydrophobic and hydrophilic surfaces. Thereby increasing their capacity to recognise arrays of polar and non-polar xenobiotic compounds. In addition, residues Thr99 and Ala35 of WbGST are replaced by Val99 and Lys95 of OvGST, opening the WbGST H-site to become easily accessible to xenobiotic compounds compared to OvGST. Regarding the catalytic mechanism of WbGST, the binding and activation of GSH precede the conjugation of electrophilic compounds to GSH. Tyr initiates the activation of GSH via hydrogen bonding. Enzyme activity studies of GSTs have always been performed using the GSH-CDNB conjugation assays.

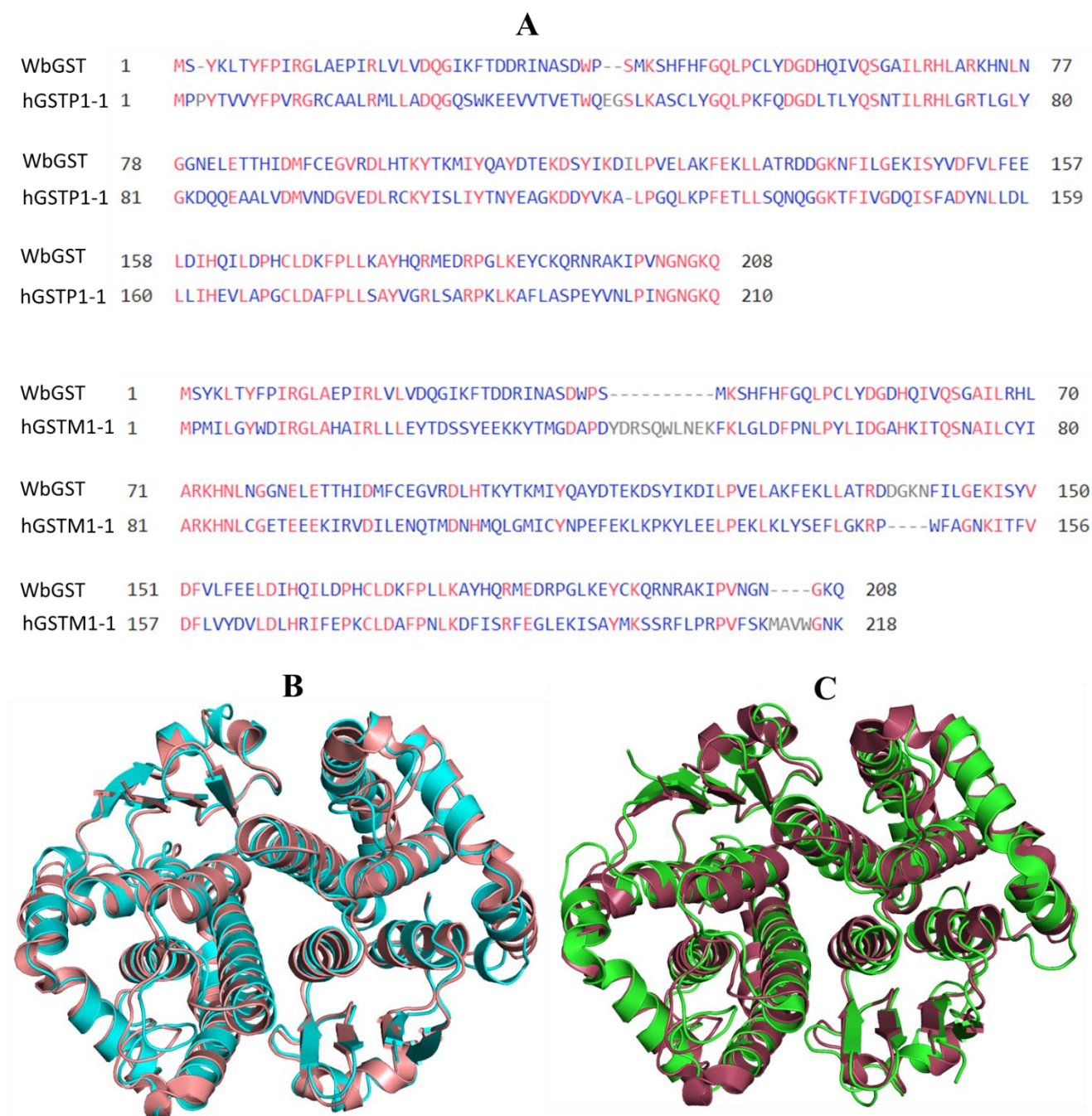


Figure 2.7 Structural differences between WbGST and the human GSTs. (A) Sequence alignment between WbGST and the human GSTs (hGSTP1-1 and hGSTM1-1). Similar residues are highlighted in red, hGSTP1-1 and hGSTM1-1 share 42 and 38% sequence similarity with WbGST. Ribbon representation of (B) WbGST (raspberry red) and hGSTP1-1 (cyan) with RMSD of 0.97, (C) WbGST and hGSTM1-1 (green) with RMSD of 1.55. The image was generated using PyMol.

2.4 Biophysical methods in early rational drug discovery

Biophysical methods are usually employed in the identification and characterisation of molecules and their targets in early drug discovery. Various techniques are applied to gain insight into the binding interactions of small molecules and target proteins. Secondly, physical parameters associated with binding interactions are quantitatively determined to aid high-throughput screening and lead progression. These biophysical methods complement other traditional biochemical approaches by providing the opportunity to obtain kinetics, thermodynamics and structural information using highly sensitive techniques. Some of these biophysical methods are highlighted below and are employed during the preliminary phase of rational drug discovery.

2.4.1 Enzyme inhibition

Enzymes play a crucial role in catalysing biological reactions and are highly regulated with remarkable specificity towards their substrates. They can be regulated through inhibition or induction, resulting in decreased or increased activity, respectively. The ability to modulate enzymes offers an opportunity to regulate cellular activities. Enzyme activators employ a more intricate approach to control catalysis. (Saboury, 2009). In contrast, enzyme inhibitors offer a simpler approach, directly targeting the enzyme as a catalytic moderator. This provides an opportunity to comprehend the enzyme's mode of action. Moreover, inhibitors have been instrumental in classifying certain GSTs (Mannervik *et al.*, 1988; Sheehan *et al.*, 2001), shedding light on enzyme catalysis, mechanisms and binding patterns. They also assist in understanding the contributions and substrate requirements for catalysis by WbGST. Consequently, the potential to inhibit enzyme activity makes them appealing as primary therapeutic targets. While many drugs work through enzyme inhibition, most are not substrates for their target enzyme. Enzyme assays, commonly used to study enzyme activity, involve determining reaction rates for enzyme-catalysed reactions, providing insights into enzyme kinetics and inhibition studies. The activity of GSTs is typically monitored through the GSH-CDNB (1-chloro-2,4-dinitrobenzene) conjugation reaction. Thus, in this study, WbGST activity is assessed using GSH-CDNB conjugation assays.

Enzyme inhibition kinetics

Enzyme kinetics investigates the reaction rate of enzyme-catalysed reactions to provide deeper insight into enzyme mechanisms. Kinetic parameters obtained from an enzyme assay are used to study the behaviour of the enzyme. Enzyme kinetics were first standardized by Leonor Michaelis and Maud Menten, resulting in so-called classical Michaelis-Menten kinetics (Michaelis *et al.* 1913). They proposed a comprehensive model that included an intermediate enzyme-substrate complex (ES) in equilibrium with the enzyme (E) and the substrate (S). The ES complex either continues towards a product (P) or dissociates to the free enzyme and substrate. The proposed model is:



The arrows indicate the reaction steps with associated rate constants indicated by the lower case k. The individual rate constants may be combined to yield the overall rate or define the Michaelis-Menten constant K_M (Equation 2.2). Rate constants with negative subscripts indicate reverse reactions. Following the formation of ES, the reaction may be reversed by the breakdown of the complex to E and S (k_{-1}) or it could progress to the formation of a product (k_2).

Because the reverse reaction will also be catalysed by the enzyme, E and P may return to the ES complex (k_{-2}). If we assume the reverse reaction (k_{-2}) to be negligible, then the process is simplified, especially if we only consider the initial reaction where the concentration of P is essentially non-existent. This model may be represented by:



The rate of ES complex formation is $k_1[E][S]$ while the rate of ES breakdown is $(k_{-1} + k_2)[ES]$. As long as sufficient substrate is available, the concentration of the ES intermediate will remain constant. Therefore, if the concentration of ES is assumed to remain constant, the rate of formation and breakdown of ES complex must balance such that:

$$k_1[E][S] = (k_{-1} + k_2)[ES] \quad (2.1)$$

To derive the Michaelis-Menten constant, K_M , equation 2.1 can be rearranged as follows:

$$\frac{[E][S]}{[ES]} = \frac{k_{-1} + k_2}{k_1} = K_M \quad (2.2)$$

Equation 2.2 indicates that K_M is not dependent on either the enzyme or substrate concentrations but instead, corresponds to the substrate concentration at which the catalytic activity is half its maximum rate ($V_{\max}$). K_M is unique for each enzyme/substrate combination due to the specificity of enzyme-substrate interactions, differences in active site properties, variations in catalytic efficiency, evolutionary adaptation, and the biological roles that enzymes play within cellular and biochemical processes. Therefore, K_M is a vital parameter in reactions involving enzymes and their substrates. The concentration of the ES complex can be derived by applying the Michaelis-Menten constant as follows:

$$[ES] = \frac{[E][S]}{K_M} \quad (2.3)$$

At low $[E]$, $[S]$ is initially not much affected by ES complex formation such that we can assume it to remain constant. The concentration of free enzyme or $[E]$ will correspond to the total enzyme concentration $[E]_T$ minus $[ES]$, such that equation 2.3 may be rewritten as:

$$[ES] = \frac{([E]_T - [ES])[S]}{K_M} \quad (2.4)$$

Rearranging equation 2.4 yields:

$$[ES] = \frac{[E]_T/K_M}{1 + [S]/K_M} = \frac{[E]_T[S]}{[S] + K_M} \quad (2.5)$$

This rearrangement describes the concentration of enzyme-substrate complex at steady state in terms of total enzyme concentration $[E]_T$, substrate concentration $[S]$, and Michaelis constant K_M . This implies that the rate of an enzyme-catalyzed reaction depends on substrate concentration and enzyme properties. Thus the rearranged equation shows the relationship between substrate concentration, Michaelis constant, total enzyme concentration, and the concentration of the enzyme-substrate complex.

Considering the relationship between the initial velocity of the reaction and the complex $[ES]$ ($v_0 = k_2[ES]$) and substituting it in equation 2.5 will give:

$$v_0 = k_2[E]_T \frac{[S]}{[S] + K_M} \quad (2.6)$$

When all enzyme active sites bind a substrate molecule, the maximum velocity (V_{max}) is attained. This leads to the simplification that $[ES] = [E]_T$ so that $v_0 = k_2[ES]$ changes to $V_{max} = k_2[E]_T$. Therefore, the resulting Michaelis-Menten equation is represented as:

$$v_0 = \frac{V_{max}[S]}{[S] + K_M} \quad (2.7)$$

where K_M is obtained at a very low enzyme concentration when $[E] \ll [S]$. For very low substrate concentrations, $[S] \ll K_M$, so that the initial velocity may be simplified to:

$$v_0 = \left(\frac{V_{max}}{K_M} \right) [S] \quad (2.8)$$

However, when the substrate concentration is higher than the K_M , the initial reaction rate becomes:

$$v_0 = V_{max} \quad (2.9)$$

The relationship changes when a substrate concentration equivalent to the K_M is used, we obtain the following:

$$v_0 = \frac{V_{max}}{2} \quad (2.10)$$

The above relationship (Equation 2.10) yields a hyperbolic plot (Figure 2.8A) of the Michaelis-Menten equation. It implies that K_M is the substrate concentration for which the velocity of the reaction is half V_{max} .

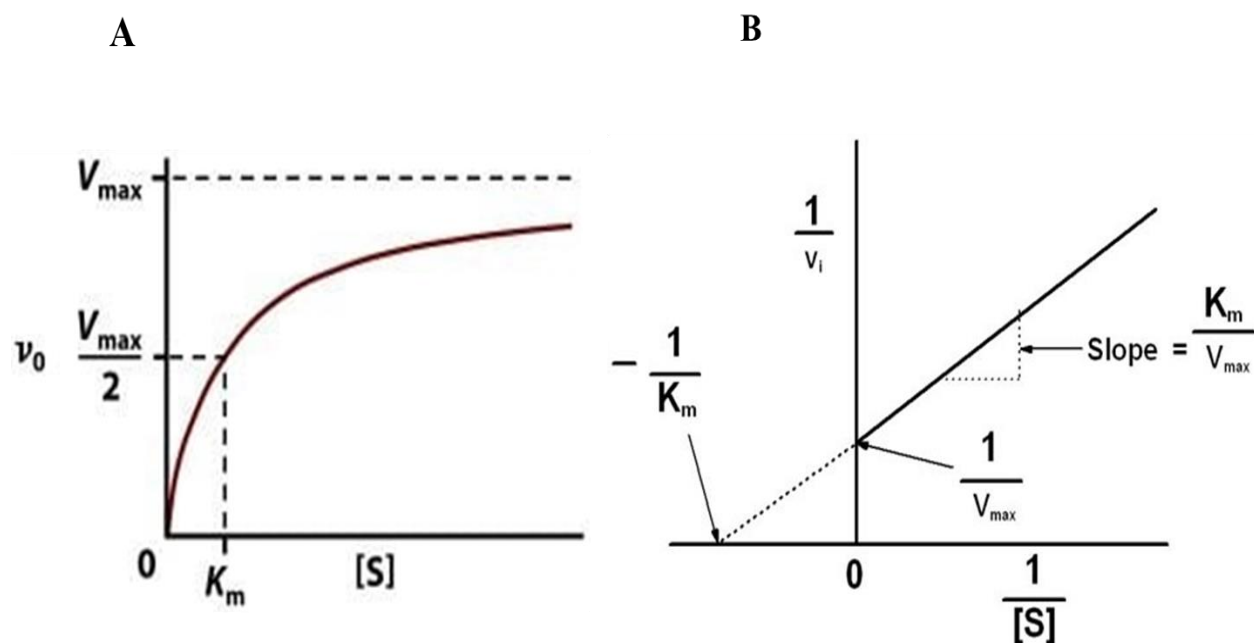


Figure 2.8 Enzyme kinetic plots. (A) The graphical illustration of the Michaelis-Menten equation shows the reaction velocity (v) plotted against substrate concentrations $[S]$. (B) Lineweaver-Burk double reciprocal plot of $1/v$ against $1/[S]$. Parameters like the K_M and V_{max} can be extrapolated from the x-axis and y-axis, respectively. During the reaction, enzyme concentrations are constant while substrate concentrations are varied (Timson 2015).

Before the emergence of computational software for curve fitting, deriving the K_M and V_{max} from Michaelis-Menten hyperbolic plots presented a considerable challenge. This challenge arose due to the incremental velocity that rises as substrate concentration increases while the reaction approaches its maximum velocity asymptotically. However, a strategy was developed to estimate these two constants through a plot known as the Lineweaver-Burk plot (Figure 2.9), or the double-reciprocal plot. This method involves rearranging the Michaelis-Menten equation into a linear form (Equation 2.11).

$$\frac{1}{v_0} = \left(\frac{K_M}{V_{max}} \right) \times \frac{1}{[S]} + \frac{1}{V_{max}} \quad (2.11)$$

The Lineweaver-Burk plot unfortunately tends to overestimate the velocity of the reactions at low substrate concentrations. Thereby dramatically inflating initial small errors in the measurement into huge errors on the linear plots. Nevertheless, the Lineweaver-Burk plot continues to be used in analysing the mode of enzyme inhibition.

Enzyme inhibition is a process where non-substrate molecules bind to the enzyme reducing its catalytic efficiency. These molecules or inhibitors may be classified as reversible or irreversible inhibitors (Saboury, 2009). Irreversible inhibitors bind covalently and hence irreversibly to the active site, blocking it and potentially altering the active site permanently. Often this sort of binding alters the conformation of the enzyme active site and sometimes the entire chemical structure. This mode of inhibition is utilised by certain therapeutics like chemotherapy drugs, antiepileptic drugs and as well as Viagra.

Reversible inhibitors by contrast bind to the enzymes but can also dissociate from the binding site. These reversible types of inhibitors interact with the enzymes noncovalently through hydrogen bonding, hydrophobic and electrostatic interactions. There are four modes of reversible inhibitors namely: competitive, noncompetitive, uncompetitive, and mixed mode of inhibition. Competitive inhibitors are often analogues of the enzyme substrates and bind at the enzyme active site. But in some cases, part of the functional groups on competitive molecules can mimick the substrate, allowing it to bind to the active site. Obstruction of the active site by the competitive inhibitor reduces the amount of ES complex formed thus decreasing the rate of enzyme catalysis. Competitive inhibitors bind variably to the target enzymes with different levels of affinity. The efficiency of competitive inhibitors is reduced by increasing substrate concentrations as being that the inhibitor needs to compete for the substrate binding site (Segel, 1975). Increasing the substrate concentration broadens the opportunity for the substrate to out-compete the inhibitor and bind to the enzyme. Therefore, achieving V_{max} in a competitive mode of inhibition often takes longer, and the K_M is increased as a result (Figure 2.9A).

In non-competitive inhibition, the inhibitors do not mimic the substrates but bind to sites different from the substrate binding site. Such inhibitors display an affinity for the free enzyme and the ES complex. In contrast to the competitive mode of inhibition, increasing the substrate concentration does not decrease inhibition (Figure 2.9B), resulting in a decrease in $V_{\max}$ while the apparent K_M remains unchanged. Secondly, the turnover number of the enzyme is decreased by noncompetitive inhibitors.

Concerning the uncompetitive type of inhibition, the inhibitors have an affinity towards the ES complex and not the free enzyme. Usually, binding of the substrate to the enzyme initiates conformational changes which enable inhibitor binding, and a formation of inactive ESI complex occurs (Segel, 1975). This type of inhibition decreases $V_{\max}$ but increases the substrate affinity towards the enzyme resulting in a decreased K_M (Figure 2.9C).

Mixed inhibition is a unique mode of inhibition where the inhibitor does not have an affinity for both the free enzyme and the ES complex. Rather the inhibitor binds at a distinct site other than the active sites (Saboury, 2009). The binding of the substrate influences the binding of the inhibitor and vice versa. Moreover, it is important to note that mixed inhibition does not imply a combination of types of inhibition. Instead, the K_M and $[S]$ remained the same, resulting from the conserved affinity of the enzyme to the substrate while $V_{\max}$ decreased (Figure 2.9D). Most parasite GSTs exhibit the mixed type of inhibition because they possess another binding site distinct from their active sites (van Bladeren and van Ommen, 1991). The double reciprocal plots of varied inhibitor concentrations indicate the mode of inhibition. Observing the changes in the kinetic graphs gives insight into the types of inhibition without numerical derivations. Qualitative analysis of enzyme inhibition is better analysed by thermodynamics and binding kinetic parameters.

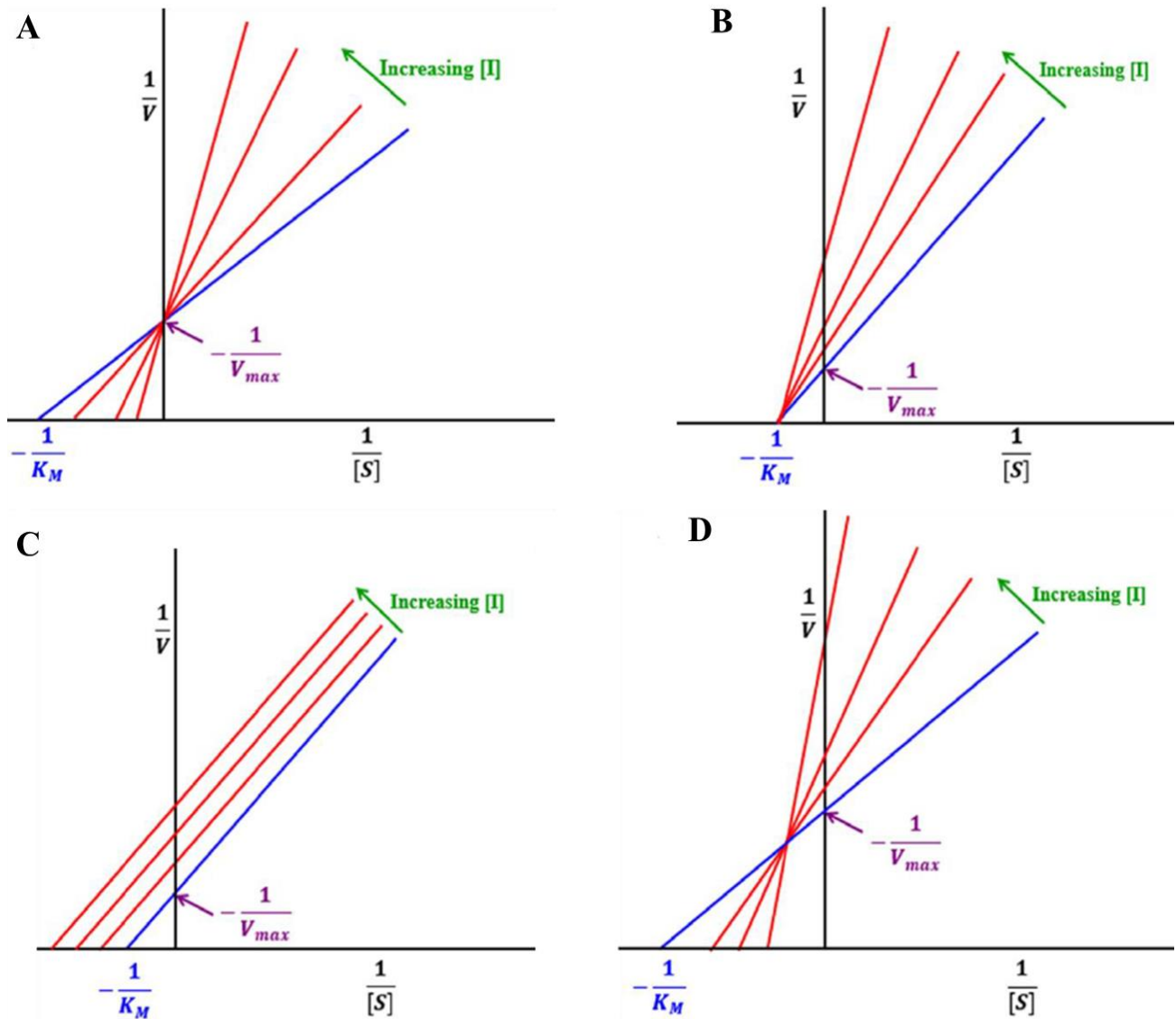


Figure 2.9 Illustration of enzyme modes of inhibition. Lineweaver-Burk plots representing different types of inhibition. Plots of $1/v$ against $1/[S]$ in the absence and the presence of different inhibitor concentrations: (A) Competitive inhibition, (B) noncompetitive (C) Non-Uncompetitive (D) Mixed inhibition.

2.4.2 . Thermodynamics of enzymatic interactions

The fundamental aspect of numerous biochemical interactions within living organisms revolves around 'energy'. Hence, it is imperative to understand the concept of 'energy' in comprehending the majority of biochemical interactions, including processes like bond formation, molecular conformations, and enzyme catalysis. Thermodynamics is a valuable tool that can be applied to gain insight into the energy involved in most biochemical reactions. Therefore, understanding the thermodynamic processes involved in enzyme catalysis and enzyme ligand interaction provides a better insight into binding kinetics and energetics. Despite the perceived chaos or disorderliness within cells or molecules, biological events appear organised and are subject to physical laws like the laws of thermodynamics. Thermodynamic processes best describe the change from one state to another of a catalytic system in a given surrounding. In this context, a 'system' refers to the amount of substance being analysed, and the properties of the substance both at the beginning and at the end of the analysis are described as 'state'. The environment where the 'system' undergoes a thermodynamic process is known as the 'surroundings'. It is important to note that the laws of thermodynamics differentiate between a 'system' and its 'surroundings'. The first law analyses energy flow, while the second law deals with the concept of entropy generation in a system. The first law states that "energy cannot be created or destroyed but can change into different forms", implying that the total energy of a system and its surroundings are constant. The second law states that in an isolated system, the entropy continuously increases over time, and energy is lost in the form of heat during conversions in a closed system. Even though living organisms seem to reduce entropy by assembling small molecules into larger structures, this effort produces heat, which in turn increases the entropy in the system.

Everything regarding the laws governing thermodynamics of chemical reactions appears to be correct, but not until Willard Gibbs published his article on the "Equilibrium of Heterogeneous Substances" in 1875-78. Gibbs argued that changes in the mole ratios of the reactants in a system should contribute to the change in internal energy (Gibbs, 1948). Hence, he introduced the concept of chemical potential, which is popularly known as Gibbs energy (G). This paved the way for better analysis of chemical reactions and provides the criteria for spontaneous change in equilibrium at constant temperature and pressure. In a chemical reaction, the Gibbs energy of the products must be lower than that of the reactants for a spontaneous reaction to occur (Figure

2.10A). Though a reaction may occur spontaneously, the transition from reactants to a product is constantly faced with a high free energy barrier (activation energy). Thus, enzymes break this high activation energy barrier by forming a transition state (Figure 2.10C). Enzymes, therefore, facilitate reactions without affecting the overall change in free energy change of the reaction. In other words, enzymes quicken the achievement of equilibrium but do not alter the point of equilibrium because the equilibrium is determined by the difference in free energy between the reactants and products. Conversely, inhibitors raise the activation energy barrier (Figure 2.10C), thereby slowing the reaction rate.

In a binding reaction between enzymes and substrates or ligands, the reaction could either release or absorb energy. This energy can manifest in different forms, but in this case, it is in the form of heat energy known as enthalpy (ΔH). When a change occurs in binding interactions, it is not usually detected solely as a change in affinity, owing to enthalpy-entropy compensation. This mechanism involves offsetting the released enthalpy resulting from improved bonding with an entropic penalty. Measuring the binding enthalpy often reveals the changes occurring during binding. During a reaction, if energy is released, the reaction is considered exothermic, characterized by a negative Gibbs free energy. This indicates a negative ΔH , suggesting that the enthalpy of the reactants is higher than that of the products (see Figure 2.10A). In contrast, the absorption of energy during a reaction is termed an endothermic process associated with a positive free energy. In an endothermic reaction, the enthalpy of the reactants is lower than that of the products. (Figure 2.10B).

The Gibbs free energy of binding is the most crucial parameter describing the binding thermodynamics of macromolecular complexes. It determines whether a particular biomolecular complex will form and how stable the complex will be. It is also a valuable tool for the qualitative description of structure-function relationships (Perozzo *et al.*, 2004). Several factors contribute to the binding free energy of a reaction. Enthalpic contributing factors such as the van der Waals interactions, hydrogen bonding, hydration, electrostatic and ionization effects, and contributions resulting from conformational changes, with entropic contributors such as conformational entropy, loss of translational and rotational degrees of freedom, must be accounted for individually (Åqvist *et al.*, 1994; Horton and Lewis, 1992). For instance, an observed Gibbs free energy change can

occur in binding interactions with both positive entropy and enthalpy changes, as well as in binding interactions with negative entropy and enthalpy changes. Secondly, binding reaction systems have the tendency to compensate entropic and enthalpic contributions to Gibbs free energy change. Hence, this makes free energy of binding less sensitive to certain detailed changes occurring during molecular interactions (Eftink *et al.*, 1983; Lumry and Rajender, 1970; Williams *et al.*, 1993). Therefore, to understand binding free energy, the change in enthalpy and entropy must be carefully considered and quantified.

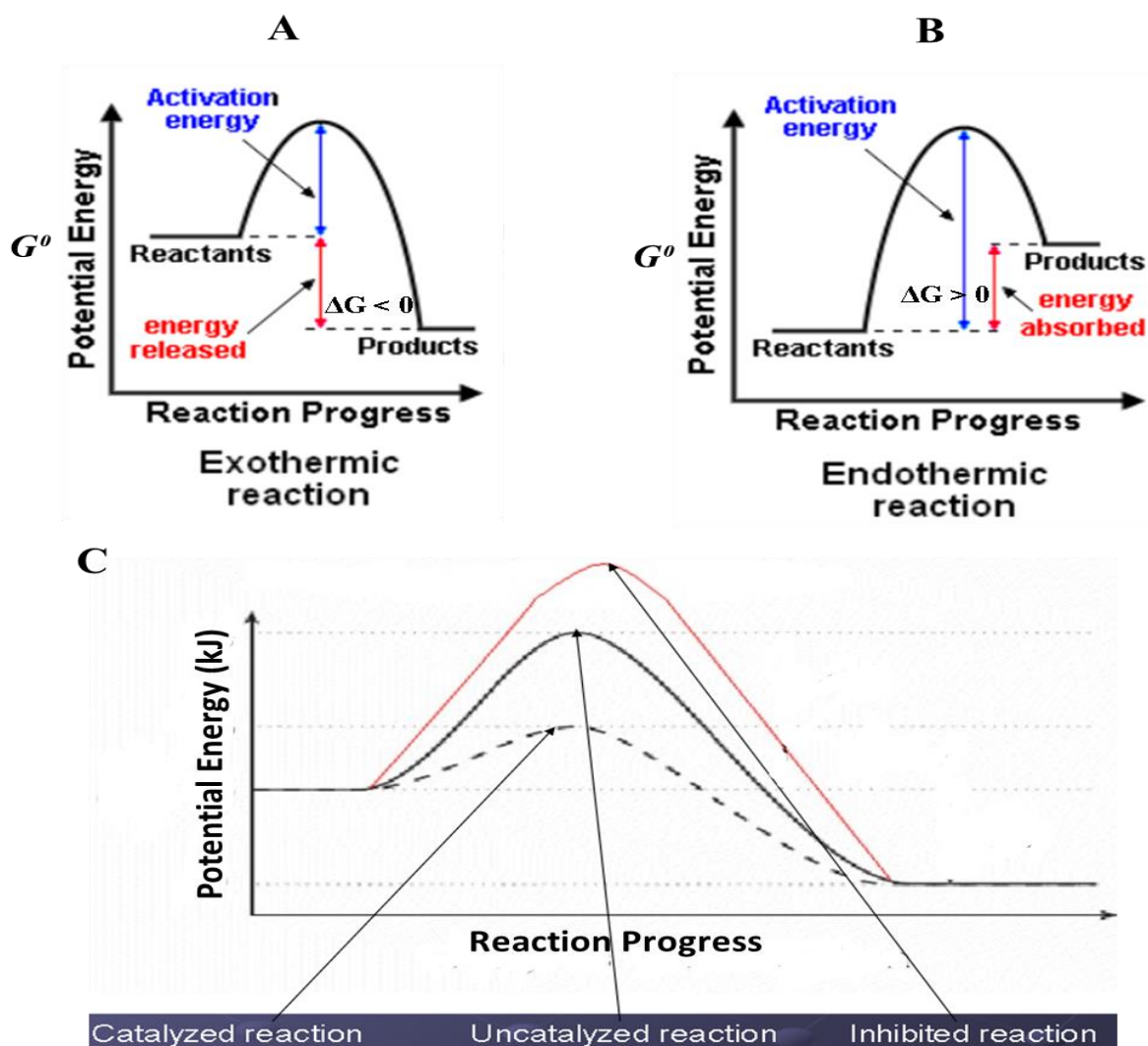


Figure 2.10 Potential binding energy profile of a binding reaction. The energy level of a chemical reaction shows the activation energy breaking the energy barrier for the reactants, the released products and the associated potential energy. Energy is released in exothermic reaction (A) and absorbed in endothermic reaction (B). When an enzyme reaction is inhibited, the energy barrier is raised higher by the inhibitor and activation energy is increased (C).

Considering the thermodynamics of a binding reaction at equilibrium where ligand (L) binds to a protein molecule (P) as described below:



If the protein has one binding site, the association constant, K_a , which is the reciprocal of the equilibrium dissociation constant (K_d), determines the ratio of bound and unbound species, which is represented as follows:

$$K_a = \frac{[PL]}{[P][L]} \quad (2.13)$$

The binding affinity is associated with the Gibbs free energy change as denoted in Equation 2.14:

$$\Delta G = -RT \ln K_a \quad (2.14)$$

where R represents the gas constant, and T is the absolute temperature. Moreover, the enthalpic (ΔH) and entropic (ΔS) contribution to the change in free energy of binding can be calculated using Equation 2.15 once ΔH has been determined experimentally.

$$\Delta G = \Delta H - T\Delta S \quad (2.15)$$

This is vital because two different binding interactions with the same affinities and structures can contribute different enthalpy and entropy compositions to their free energies. The enthalpy and entropy are the factors determining the binding affinity, thus combining equations 2.14 and 2.15 yields Equation 2.16:

$$\Delta G = -RT \ln K_a = \Delta H - T\Delta S \quad (2.16)$$

The enthalpy of a (binding) reaction, may be measured experimentally as a change in heat energy inside a calorimetric cell when a ligand is added to a solution of the protein. However, the measured heat may also be caused by side reactions such as protonation effects or heat contributions from non-specific binding events. Hence, all contributions to the observed enthalpy need to be identified and ideally eliminated by adjusting the experimental conditions. However, despite the corrections of the heat changes, they still comprise distinct heat contributions, resulting

in either apparent or observed enthalpy (Cooper and Johnson, 1994). Literally, change in enthalpy refers to the energy from non-covalent bonds (van der Waals, hydrogen bonds, electrostatic interaction) that occur during a binding reaction. However, this description does not capture all the observed heat energy values. Therefore, the observed enthalpy change of a system is a measure of individual breaking or formation of bonds because, in an aqueous medium bond, the formation does not occur without the breaking of bonds. Thus, the change in enthalpy of a binding reaction accounts for protein–solvent hydrogen bonds and van der Waals interactions, formation of protein–ligand bonds, salt bridges and van der Waals interactions, including solvent rearrangement near protein surfaces. These distinct interactions may result in favourable or unfavourable energy contributions to the system (Fisher and Singh, 1995).

A previous study performed using H₂O and D₂O reported that most of the observed changes in enthalpy resulted from the bulk hydration effect (Connelly *et al.*, 1993; Ladbury, 2001). The dimer interface of GST proteins and that of other protein complexes are largely lined by water molecules. These water molecules enhance the interaction between the protein subunits and also increase the hydrogen bond networks in the complex. Secondly, it increases the occurrence of favourable enthalpy; however, this is compensated by entropic penalties (Bhat *et al.*, 1994; Holdgate *et al.*, 1997; Ladbury, 1996). Apart from the resultant unspecified hydration effect during a binding interaction, other non-covalent bonds at the interacting interface contribute to the change in enthalpy. It is difficult to specify the individual contribution of the non-covalent interactions because the net enthalpy effect of a ligand binding results from an equilibrium between the binding enthalpy of the ligand to the protein and the solvent. Additionally, conformational changes at the binding site resulting from the binding incidents could also contribute to the net change in enthalpy. These contributions to enthalpy could either be favourable or unfavourable and combine their results to the net change in enthalpy (Liu *et al.*, 2012; Perozzo *et al.*, 2004).

The binding entropy measures the even distribution of heat energy throughout a thermodynamic system. In other words, entropy represents a combination of all positive and negative forces contributing to the free energy of a system. According to the second law of thermodynamics, heat energy often circulates from regions of higher temperature to lower temperature regions, hence decreasing the orderliness of the system. Therefore, entropy can be described as a measure of the

randomness and disorderliness of molecules in a thermodynamic system. A positive or negative sign in entropy change indicates the total increase or decrease in the degree of freedom in a system, respectively. The overall change in binding entropy can be expressed as the entirety of various contributing factors. The major factor emanates from a change in solvent entropy from hydration effects. Due to the large hydration entropy of polar and non-polar groups, and upon binding event, solvents are released from buried accessible surface areas, contributing large and positive entropy to the system. The second contributing factor results from the conformational freedom of the binding partners upon interaction, which could lead to a favourable or an unfavourable change in entropy since the degree of freedom of a protein-ligand complex can be lowered or increased when compared to the unbound components (Bronowska, 2011; MacRaild *et al.*, 2007).

Another factor originates from a reduction in the translational and rotational degree of freedom on the torsion angles of the interacting proteins and ligands side chains, thereby reducing the amount of particles in solution (Murphy *et al.*, 1995; Murphy *et al.*, 1994). However, this contributing factor is unfavourable towards the change in binding entropy. These contributions to entropy could be negative or positive. On the one hand, a negative entropy does not necessarily translate to an increased hydration interface or an unmodified interface. On the other hand, a positive change in entropy indicates that water molecules were released from the binding interface of the complex (Jelesarov and Bosshard, 1999). Essentially, the binding reaction must overcome the unavoidable entropic penalties, which are negative entropy changes resulting from a loss of translational and rotational degrees of freedom. Overcoming the entropic penalty could be achieved by a large positive solvent entropy gain or a favourable ligand binding to the protein, which results in a negative change in binding enthalpy (Liu *et al.*, 2012).

2.4.2.1 Isothermal titration calorimetry (ITC)

Thermodynamics governs the molecular recognition processes associated with intermolecular (ligand or substrate binding) and intramolecular (Protein-protein or protein-DNA interaction) formation of spatial arrangements of folded macromolecules. These types of molecular recognitions do not occur without being accompanied by some level of complexity depending on factors such as the size of the macromolecule, the specific folding pattern, and the environmental conditions. Thermodynamic principles govern the formation of spatial arrangements in folded

macromolecules. Achieving and maintaining the correct spatial arrangement often involves balancing competing energetic contributions, making the prediction of stable structures challenging. The thermodynamic profiles of protein molecules are associated with diverse, energetic interactions along their folding or unfolding pathway and even in complex formation with ligands. Therefore, ITC and DSC are designed with highly sensitive calorimeters and, as such, are the best methods for studying the thermodynamics associated with these diverse molecular recognition events. Understanding molecular interactions and the forces driving them is fundamental to every stage of the drug discovery process, from target identification to lead optimization and clinical development. This knowledge enables the rational design of safe and effective drugs with improved therapeutic outcomes.

The ITC is a gold standard biophysical technique for analysing intramolecular binding interactions, especially with the advent of new generation highly sensitive titration calorimeters. The technique is efficient in studying multiple binding interactions between macromolecules and ligands, thus conveniently analysing multiple stoichiometries and association constants simultaneously. The ITC measures the energetics of biomolecular interactions at a constant temperature and pressure. Despite the sophistication and advancement of computational molecular dynamics, it cannot provide detailed information regarding the full thermodynamic parameters and driving forces involved in complex formation (Chaires, 2008). Hence, an accurate binding affinity of a complex cannot be predicted except with an ITC technique.

Therefore, ITC is a vital and much-needed complementary technique to structural analysis in rational drug design (Judy *et al.*, 2016). The technique is advantageous because the energetics of free biomolecules can be measured in solution without being immobilised. Thus, this enables the assessment of macromolecular interactions in their native solution states and environments (Ghai *et al.*, 2012). A major factor that is always considered during ligand optimisation is the nature (entropic or enthalpic) and magnitude of the different forces propelling the ligand binding to the biomolecule. Moreover, the heat absorbed or released during a binding interaction, such as in ligand-protein, protein-protein, and protein-DNA interactions, is measured directly by the ITC technique.

Furthermore, the ITC technique measures the parameters that describe binding equilibrium namely: (i) the binding stoichiometry, n (ii) the binding affinity expressed as association constant, K_a (iii) in the presence of more than one binding site, the positive or negative binding cooperativity (iv) the description of entropic and enthalpic contributions to binding free energy and lastly (v) evaluating the binding equilibrium dependence on other environmental variables like the pH and ionic strength.

The ITC instrument contains two cells referred to as the sample cell and reference cell (Figure 2.11). Both cells are identically designed and are confined in an adiabatic jacket, thus reducing the contact of the cells with external environments. The instrument performs its analysis by comparing the temperature output of both the sample cell and the reference cell. The sample cell contains the measurement sample, and it is where the binding interaction occurs, while water or the analysis buffer is added to the reference cell. The temperature of the two cells must be the same throughout the experiment. The instrument is designed with a heat-sensing device which monitors and detects any temperature variation between the sample and reference cells. The heaters receive constant feedback from the heat sensing device once a binding interaction occurs and compensates for the difference in temperature between the two cells. Thereby returning the cells to a similar temperature. Adjacent to both cells are positioned thermopiles, which detect the thermal signals. Subsequently, electrical impulses are transmitted to a computing system, and then the deflected heat is displayed as an integrable peak for each injection. This results in a titration profile that will be fitted to a binding isotherm (Figure 2.11).

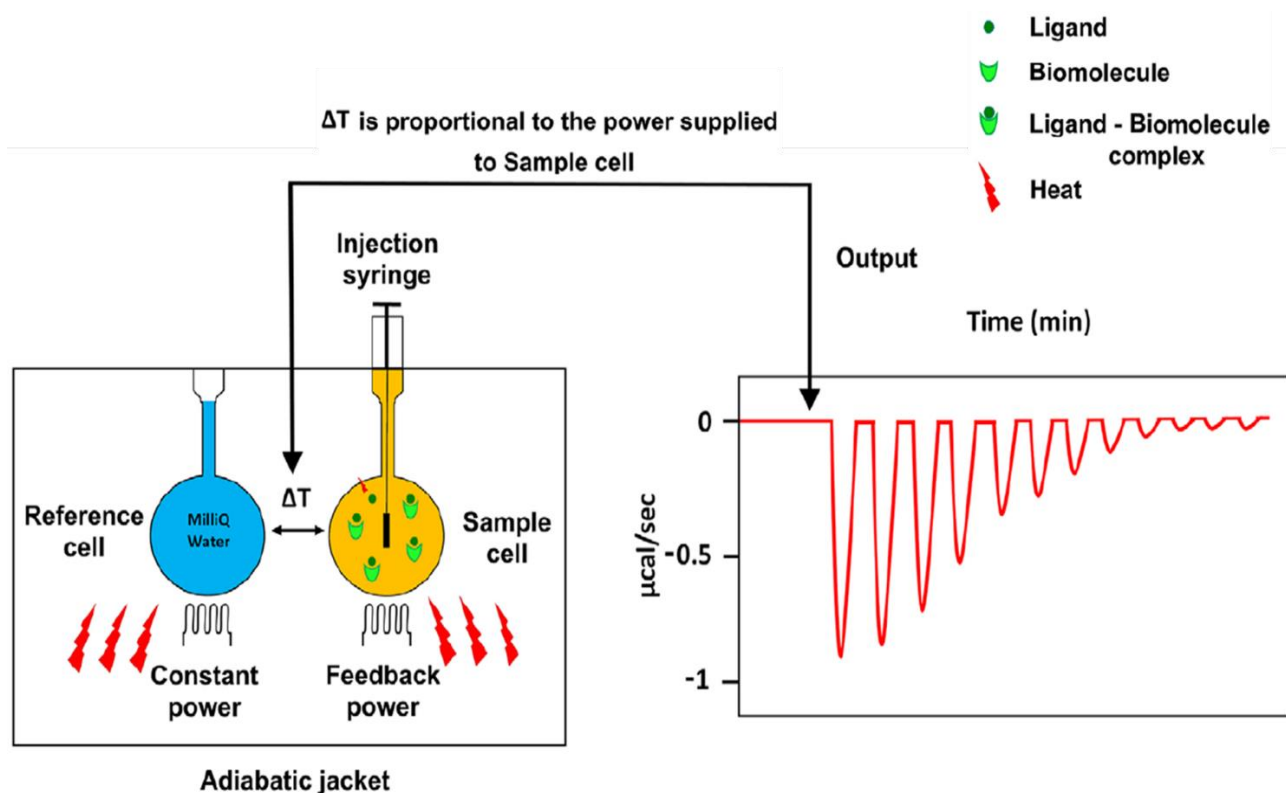


Figure 2.11 Principles of isothermal titration calorimetry. Principle of isothermal titration calorimetry. Illustration of an ITC instrumentation composed of a reference cell and a sample cell enclosed in an adiabatic jacket. The system can detect and balance differences in temperature between the cells ($\Delta T = 0$). A built-in heating system supplies power to both cells to maintain the temperature. The output from the instrument is the power ($\mu\text{cal}.\text{sec}^{-1}$) required to maintain $\Delta T = 0$ between the reference cell and the sample cell. Over time, ligand saturation of the biomolecules occurs, resulting in a reduced binding event and consequently, the change in heat decreases. When the protein becomes fully saturated with the ligand, the instrument detects the change in heat due to ligand dilution.

During an ITC experiment, a biochemical heat of reaction is triggered by the titration of the titrant (ligand) into the titrand solution. The associated heat results from the dilution of the ligand and its potential binding to the titrand, which changes the chemical equilibrium of the system. Following each injection of the titrant, an equilibrium of both bound and unbound titrant is achieved. Subsequently, a measurable heat of reaction is released (exothermic) or absorbed (endothermic), resulting in a temperature difference between the sample cell and reference cell (Figure 2.10). This difference in temperature signals the system to either increase or decrease the thermal power, as the case may be, to compensate for the temperature disparity. The experimental technique is

designed to give the system adequate time between injections to restore the balance in temperature, thus ensuring that the system equilibrium is accomplished. The associated reaction heat is monitored by the calorimeter, resulting in the generation of binding isotherms. The binding isotherms are characterised based on the heat of the reaction thereby allowing the estimation of enthalpic changes, including the equilibrium association constant. Towards the end of the injections, the binding sites of the macromolecule would have been saturated by the ligands (Figure 2.10), thereby enabling the determination of the observed association constant, K_a (Wiseman *et al.*, 1989). , the dissociation equilibrium constant, K_d , can be manually calculated by applying the following equation:

$$K_d = \frac{1}{K_a} \quad (2.17)$$

Similarly, the binding entropy resulting from desolvation and the conformational changes induced by complex formation can also be indirectly calculated as follows:

$$\Delta S = \frac{(\Delta H - \Delta G)}{T} \quad (2.18)$$

Furthermore, the heat associated with each injection is represented as:

$$q = V \times \Delta H \times \Delta[L_B] \quad (2.19)$$

where q is the heat released or absorbed as the concentration of bound ligand changes, $\Delta[L_B]$ is the change in concentration of bound ligands while V is the sample volume and ΔH is binding enthalpy per mole of ligand. Since q is proportional to the incremental amount of bound ligand, so as the titration progresses, the magnitude of q reduces due to ligand saturation of the macromolecule binding sites. The total cumulative heat associated with the binding interaction is directly proportional to the amount of bound ligand as represented in Equation 2.20:

$$Q = V\Delta H \sum \Delta[L_B] = V\Delta H[L_B] \quad (2.20)$$

where Q is the cumulative heat from each successive ligand injection and $[L_B]$ is the concentration of ligands bound to the macromolecule. When assessing the calorimetric data, the resulting heat can be analysed individually or cumulatively while considering the actual heat released or

absorbed as a function of the total ligand concentration. Thus, the binding equation for the experimental data analysis must account for the individual or cumulative associated heat for the sum of the ligand concentration since they are the experimentally derived variables.

2.4.2.2 Differential scanning calorimetry (DSC)

The DSC is one of the microcalorimetric techniques that is widely used in the biophysical characterisation of interactions amongst biomolecules. Besides, it is an important instrument that can be used during lead optimisation in drug discovery research and development (Chountoulesi *et al.*, 2019; Jelesarov and Bosshard, 1999; O'Brien *et al.*, 2001; Waterman and Adami, 2005). This is all thanks to its universal detection system, high sensitivity, rapid equilibration with stable baselines, and ability to apply the same instrumentation when studying diverse systems. When macromolecules are heated, the numerous interacting weak forces that stabilise them can be altered, leading to conformational changes or phase transitions, and the DSC can provide structural information about these changes (Chowdhry and Cole, 1989). The DSC technique directly determines the energetics governing the changes in structural conformations and transitional states of biomolecules (Plotnikov *et al.*, 1997). It measures the heat capacity as a function of temperature under a controlled incremental temperature range (Figure 2.12B). Furthermore, DSC enables the determination of thermodynamic mechanisms governing the equilibrium of conformation between the native form and unfolded form of proteins.

DSC offers various avenues for gaining structural insights into proteins by analyzing the thermal denaturation profiles, determining T_m (Melting Temperature) and observing the protein folding and unfolding transitions. Firstly, DSC measures the heat absorbed or released by a sample as a function of temperature. Proteins typically undergo thermal denaturation, where the native folded structure unfolds into a denatured state as temperature increases. The shape and characteristics of the DSC thermogram, such as the onset temperature, peak shape, and peak area, can provide information about the protein's stability and folding/unfolding transitions. Secondly, the temperature at which half of the protein molecules are unfolded, known as the melting temperature (T_m), is a key parameter obtained from DSC experiments. T_m is indicative of the protein's thermal stability. Higher T_m values suggest greater stability of the native structure, while lower T_m values indicate decreased stability or susceptibility to denaturation. DSC thermograms often exhibit

sigmoidal transitions corresponding to protein folding and unfolding events. Analysis of these transitions can provide insights into the energetics and kinetics of protein folding, including the presence of intermediate states and the cooperativity of the folding process. DSC can also be used to study the effects of ligand binding, such as small molecules or other proteins, on the thermal stability of proteins. Changes in T_m or thermogram shape upon ligand binding can provide information about the binding affinity, stoichiometry, and structural changes induced by binding. Additionally, DSC experiments conducted under different solution conditions (e.g., pH, salt concentration) can reveal the effects of these factors on protein stability and conformation. Lastly, the DSC can be used for comparative studies of protein mutants, variants, or different protein preparations (e.g., wild-type vs. engineered proteins). Differences in thermal stability and unfolding profiles between variants can highlight the impact of specific amino acid residues or modifications on protein structure and stability.

The DSC machine is designed with two identical cells (Sample and reference cells), and both cells function independently (Figure 2.12A). The volumes of the cells are in the range of 0.3 – 0.6 mL depending on the model and manufacturer. As in ITC, the cells are situated within an adiabatic chamber. Secondly, the DSC instrument works through a power compensation scheme comparable to the ITC operation principle. During an experiment, the temperature of the adiabatic chamber is increased at a constant rate. Molecular transitions in the sample cell result in heat being released or absorbed. Any disparity in temperature within the adiabatic shield is compensated for by additional or less heating of the sample cell. The difference in the power that is applied to the cell is associated with the change in heat capacity of both cells, which is recorded by the instrument as a function of temperature.

The DSC is often used to characterise protein thermal stability with regards to the melting temperature, T_m , the unfolding enthalpy, ΔH , and the change in heat capacity ΔC_p . These thermodynamic parameters can be measured directly from the DSC; consequently, the ΔG and ΔS can be derived. The van't Hoff enthalpy, ΔH_{VH} , can be obtained by fitting the DSC data to a model using software (NanoAnalyse), and the value obtained can differ from calorimetric enthalpy, ΔH_{cal} . The ΔH_{cal} refers to the entire enthalpic change of the system, which includes heat contribution from the entire unfolding processes. Secondly, the determination of ΔH_{cal} is not dependent on any

model, but the resultant ΔH_{vH} adopts a simple two-state transition model (Naghibi *et al.*, 1995). An adequately fitted DSC data can be informative regarding the cooperativity of an unfolding transition. This is made possible by comparing the change in the model-dependent, ΔH_{vH} with the observed ΔH_{cal} (Marky and Breslauer, 1987). A transition can be inferred as a two-state transition if the change in van't Hoff enthalpy is equal to the calorimetric change in enthalpy (Marky and Breslauer, 1987). Additionally, a quantitative insight towards the nature of the observed transition can be provided by the ratio of ΔH_{vH} and ΔH_{cal} . Hence, it measures the fraction of unfolded protein in a sole thermodynamic entity by describing the size of the cooperative portion.

Besides, the DSC can be used to study both the operational stability of a protein in relation to its shelf life and the stability in terms of the strength of the interactive forces maintaining the three-dimensional structure. The operational stability of protein molecules is controlled and quantified by equilibrium thermodynamics in addition to kinetic parameters. On the other hand, quantifying the energetics accountable for the maintenance of the folded structure solely requires the equilibrium thermodynamic evaluation. Also, a suitable mechanism to account for protein denaturation was proposed (Lumry and Eyring, 1954), and it is summed up as follows:



Where F is the folded native state, and U is the unfolded state of the protein, D is the denatured state of the protein that is irreversible. The transition between the folded and unfolded state is reversible and dictated by K_{eq} , an equilibrium constant while the rate of denaturation from the unfolded state is irreversible and is controlled by a rate constant k_2 . The shelf life of a protein relies on K_{eq} and k_2 whereas thermodynamic stability is solely determined by the K_{eq} . Regrettably, numerous proteins thermally unfold irreversibly, implying that their shelf-life is more limited. Thus, it is difficult and often unreliable to extract thermodynamic data from their thermal unfolding. Previous studies have been able to derive some thermodynamic treatments of irreversible unfolding from DSC data (Galisteo *et al.*, 1991; Sanchez-Ruiz, 1992; Sanchez-Ruiz *et al.*, 1988). Similar to the reversible thermodynamic unfolding systems the melting temperature, T_m , continues to be an exceptional excellent probe for comparing relative stabilities of protein

molecules (Remmele and Gombotz, 2000). An increase in the melting temperature of a transition depicts increased stability, while a decrease implies a destabilisation of the system.

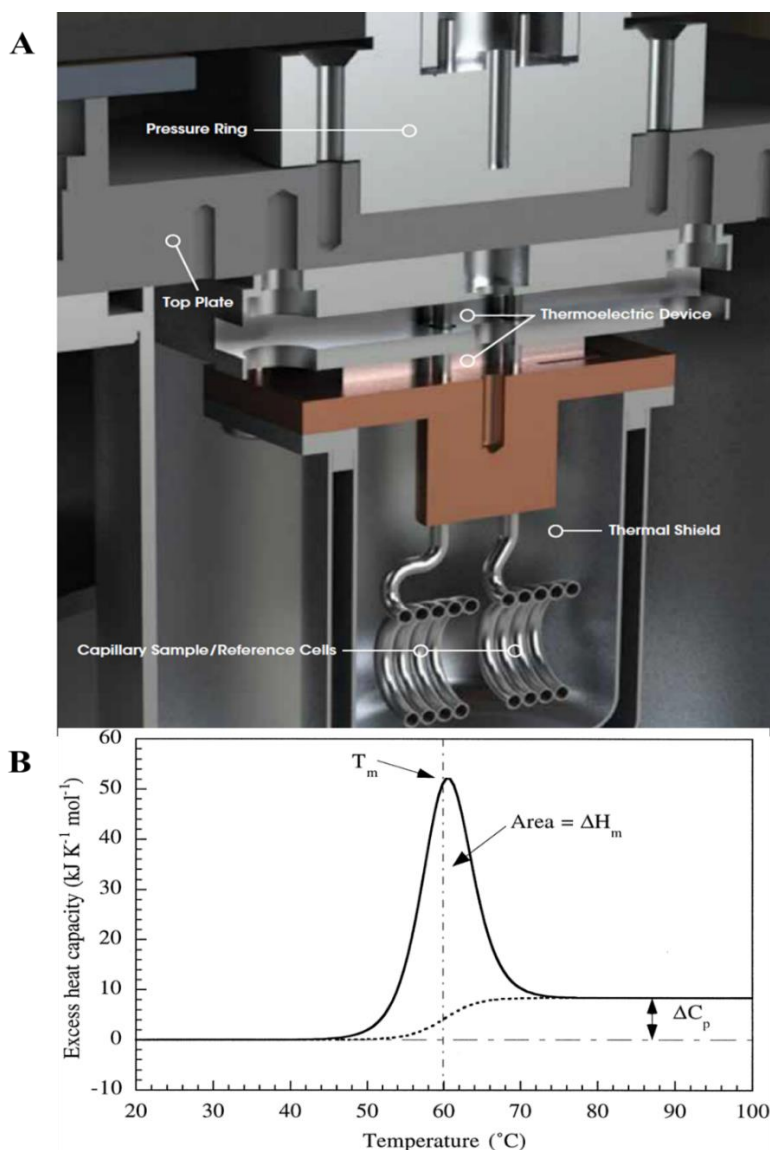


Figure 2.12 Schematic representation of differential scanning calorimetry. (A) The DSC instrument consists of a sample capillary and a reference capillary enclosed in a thermal shield. Both capillaries are maintained at almost the same temperature, and the heat flux is measured. (B). A simulated DSC experiment for globular protein two-state unfolding. The T_m is the apex of the heat capacity curve. The dashed lines represent linear extrapolations into the transition zone of the pre-and post-transition baselines. The ΔC_p is the difference between these values at T_m . The thick black line represents the theoretical 'progress' baseline generated by MiroCal software using the VP-DSC equipment. The calorimetric enthalpy, ΔH_{cal} , is the region between the DSC trace, and the progress baseline is the calorimetric enthalpy, ΔH_{cal} .

The T_m have been successfully utilised in some applications to analyse the specific and non-specific interactions, intrinsic and extrinsic stability of macromolecules, and oligomerization. Macromolecules can be stabilised by specific and non-specific binding, and the specific interactions can be probed using the T_m . Some of the examples include the binding of small molecules (ligands) to protein (Cooper *et al.*, 2001), binding of drugs to DNA (Hofr and Brabec, 2001), the binding and dissociation between Peptide Nucleic Acid (PNA) and their complementary single-strand DNA (Chakrabarti and Schwarz, 1999), peptide interactions with lipids (Liu *et al.*, 2002; Morein *et al.*, 2002) and protein-protein interdomain interactions (Brandts *et al.*, 1989). Furthermore, the T_m has been applied by researchers to optimise extrinsic factors like the pH, ionic strengths, and stabilising such as dithiothreitol to identify optimal protein conditions for downstream applications. Secondly, the measured T_m shifts are an excellent probe for monitoring the stability of protein molecules during the process of engineering the protein to further increase its intrinsic stability. Therefore, the T_m is very useful in predicting the stability of a protein and its susceptibility to aggregation over a long period of time, provided that the protein concentration is kept constant while measuring the T_m shifts.

Furthermore, the T_m of an unimolecular unfolding transition is supposed to be independent of the concentration of the measurement sample. A disparity in the T_m value is indicative of either a folded native multimer or an unfolded multimeric molecule. A two-state protein unfolding transition can be represented as follows:



Where $A(F)$ is the pre-transition state and $B(U)$ represents the post-transition state. Then, XO and YO denote the oligomeric states of the species. In a situation where the dissociation of a dimer to a monomer occurs, the XO will be larger than the YO, increasing T_m as the protein concentration increases. Whereas a decrease in T_m suggests that post-transition $B(U)$ is larger than the pre-transition $A(F)$. These observations are in accordance with Le Chatalier's principle and also provide insight to ascertain if a folded dimer is more stable than a monomer at elevated concentrations. Therefore, it can be deduced whether aggregation is a source of an irreversible protein unfolding, which will be indicated by a reduced T_m when the concentration is increased. However, in a situation whereby an apparent aggregation was observed while there was an increase

in T_m and concentration, dissociation must have taken place before aggregation (Ladbury and Doyle, 2004). Therefore, the protein can be termed a multimer, and thermodynamic principles may be applied despite the irreversibility of the unfolding process (Remmele *et al.*, 1998).

Assuming that a two-state unfolding process of a protein is reversible, it can be represented as follows:



Where F is the folded native state, and U is the unfolded state of the protein, K_{eq} is an equilibrium constant which can be expressed at any temperature. The equilibrium constant reveals the relative concentration of the native and unfolded protein in Equation 2.24:

$$K_{eq} = \frac{[U]}{[F]} \quad (2.24)$$

and the K_{eq} associated with the Gibbs free energy change, G , as shown in Equation 2.25:

$$\Delta G = -RT \ln K_{eq} = \Delta H - T\Delta S \quad (2.25)$$

The melting temperature, T_m , is the temperature at which the concentration of both the folded and unfolded are the same. This also describes the mid-point of the unfolding transition. The melting temperature is determined from the peak in the excess heat capacity of the macromolecule. Furthermore, the heat capacity, C_p , of the protein which is a measure of the amount of energy needed to heat the sample by one kelvin and it is associated with the enthalpic change as represented in Equation 2.26:

$$\Delta H = \int_{T_F}^{T_U} C_p dT \quad (2.26)$$

The change in enthalpy is derived from the integration of the extra heat capacity function, while the change in heat capacity is calculated as the pre-transition and post-transition baselines.

According to Le Chatelier's principle, ligand binding to the native state of a protein further stabilises the folded state; thus, the outcome of unfolding the protein will be unfavourable (Miller *et al.*, 2009). Therefore, the DSC can be applied in monitoring the impact of ligand binding by observing the effect on the T_m in the presence or absence of a ligand. Thus, in the presence of a ligand, the equilibrium constant of association (K_L) between the ligand (L) binding to the folded native state (F) of a protein is described as follows:

$$K_L = \frac{[FL]}{[F][L]} \quad (2.27)$$

Also, the equilibrium constant in the absence of the ligand, K_0 , in combination with K_L , can derive the value K_L at the T_m as represented in Equation 2.28:

$$K_{L(T_m)} = \frac{[K_{0(T_m)} - 1]}{[L]_{(T_m)}} \quad (2.28)$$

The K_0 can be transposed from the melting temperature in the absence of a ligand, T_0 , to the melting temperature upon ligand binding, T_m , to obtain:

$$K_{L(T_m)} = \frac{\left[\exp \left[\left(-\frac{\Delta H_{(T_0)}}{R} \right) \left(\frac{1}{T_m} - \frac{1}{T_0} \right) + \left(\frac{\Delta C_p}{R} \right) \left(\ln \left(\frac{T_m}{T_0} \right) + \left(\frac{T_0}{T_m} \right) - 1 \right) \right] - 1 \right]}{[L]_{(T_m)}} \quad (2.29)$$

Where $\Delta H_{(T_0)}$ is the change in unfolding enthalpy in the absence of a ligand, R is the gas constant, while ΔC_p is the change in heat capacity when the protein has unfolded. Moreover, assuming that the binding sites of the folded state of the protein are saturated, then the free ligand concentration, $[L]$, at the melting temperature can be derived from:

$$[L] = L_{tot} - \frac{nP_{tot}}{2} \quad (2.30)$$

Where $L_{tot} \geq nP_{tot}$, L_{tot} is the total ligand, n is the number of binding sites, and P_{tot} is the total protein concentration in the solution.

Therefore, the DSC technique is an excellent tool for studying macromolecular stabilities and protein unfolding transitions. Secondly, it can be applied towards obtaining more insight into the interactions between small molecules and macromolecules. Consequently, it is an interesting biophysical technique applied during lead optimisation in the process of drug design. Moreover, the introduction of hybrid DSC machines with autosamplers has made the technique even more efficient and attractive for the optimisation of formulations in liquid biopharmaceuticals.

2.4.2.3 Differential Scanning Fluorimetry

Differential scanning Fluorimetry (DSF), commonly known as thermofluor or thermal shift assay, is a spectroscopic method that evaluates the unfolding of protein molecules as a function of temperature. The DSF methodology is based on monitoring the fluorescence emitted by a dye, in this case, SYPRO Orange dye. The fluorescence of the dye is enhanced by its preferential binding to hydrophobic patches on the protein, which are further exposed upon the temperature-induced unfolding (Lo *et al.*, 2004; Pantoliano *et al.*, 2001; Poklar *et al.*, 1997). DSF is one of the favourite techniques used in pharmaceutical industries to assess the interaction of small molecules to target proteins. It is widely used for high-throughput screening of compound libraries to identify tight-binding small molecules that can be optimised into therapeutics (Lo *et al.*, 2004).

Several studies have implemented the DSF technique to discover ligands that can inhibit protein-protein interactions, like in the Hdm2-p53 interactions (Grasberger *et al.*, 2005; Koblish *et al.*, 2006; Parks *et al.*, 2005). It can also be applied in the assessment of ligand binding constants for enzymes like in the case of carbonic anhydrase (Klinger *et al.*, 2006; Matulis *et al.*, 2005). Furthermore, the DSF is a valuable technique for analysing the stability of recombinant proteins in various conditions such as pH, buffers, ionic strengths, and the propensity of the protein to aggregate under these conditions (Kervinen *et al.*, 2006; Mezzasalma *et al.*, 2007; Pantoliano *et al.*, 2001). Similarly, the impact of adding different pharmaceutical excipients during the formulation of therapeutics can also be monitored using DSF by observing the shift in T_m (Abad *et al.*, 2008). Secondly, it can be used to optimise various conditions suitable for protein crystallisation (Dupeux *et al.*, 2011; Ericsson *et al.*, 2006) and for the functional characterisation of new protein molecules (Carver *et al.*, 2005).

In DSF, fluorescent dyes are used as probes because they exhibit increased fluorescence intensity (Figure 2.13) in a non-polar environment like the hydrophobic patches found in unfolded protein molecules. Some examples of available probes include SYPRO Orange, dapoxyl sulphonic acid, Nile red, 8-anilidonaphthalene-1-sulphonic acid (1,8-ANS), and 4,4'-dianilino-1,1'-binaphthyl-5,5'-disulphonic acid (bis-ANS). However, SYPRO Orange has the most suitable properties for DSF experiments because it has a low signal-to-noise ratio. It also has a high excitation wavelength, which will reduce the likelihood of fluorescence quenching resulting from small molecules interfering with the optical properties of the dye (excitation at 465 nm and 590 nm, respectively). Leung *et al.* 1996, were the first to report on using environmentally sensitive dyes to observe thermal unfolding (Leung *et al.*, 1996). Subsequently, Pantoliano *et al.* 2001, modified the protocol by adopting a microplate set-up to facilitate high throughput assays (Pantoliano *et al.*, 2001). Unfortunately, the modified protocol was not readily available for use to the scientific community due to its proprietary. Later, a similar concept was tried using a thermocycler instrument designed for real-time polymerase chain reactions (Lo *et al.*, 2004) using the 96-well PCR plates (Niesen *et al.*, 2007).

Furthermore, the DSF possesses numerous biophysical attributes that meet the requirements of universal cross-target drug discovery assays. The resulting sigmoidal profile (Figure 2.13) from the analysis can be fitted to the Boltzmann equation (Niesen *et al.*, 2007). The equation can be used to determine the point of inflection of the transition known as the melting temperature, T_m , of the target protein.

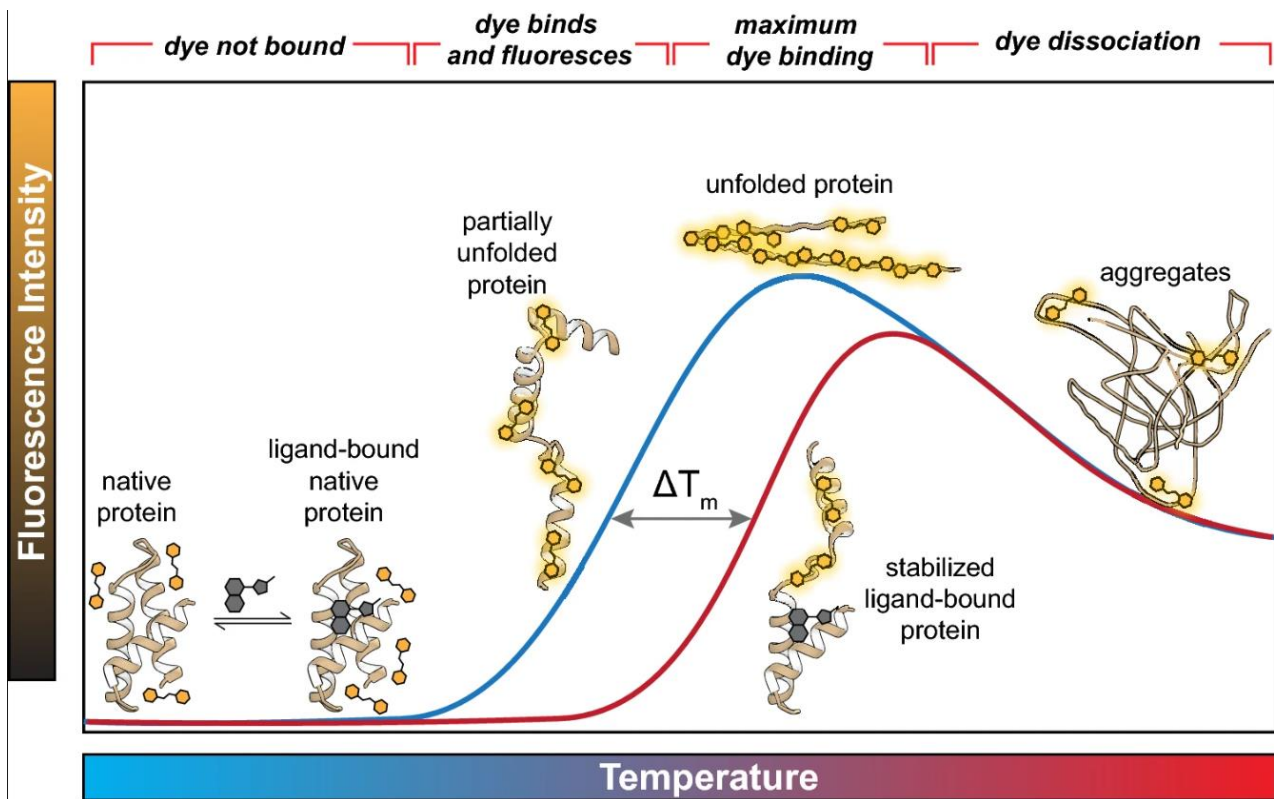


Figure 2.13 Principle of thermal shift assay. The thermal shift assay determines a protein melting temperature (T_m), which is the temperature at which 50% denaturation occurs. Protein denaturation is detected by a rise in the fluorescence of the SYPRO Orange dye, which binds to hydrophobic residues accessible when the target protein unfolds. Ligand attachment to the target protein frequently increases stability and raises the melting temperature (Eroll *et al.* 2021).

Moreover, the Gibbs free energy of the unfolding, ΔG_u , is associated with the melting temperature of the protein, in other words, the protein stability. In most cases, protein stability decreases alongside temperature, implying that an increase in temperature lowers the ΔG_u , which reduces to zero at equilibrium, where both folded and unfolded protein concentrations are equivalent. The temperature at this point of equilibrium is called the melting temperature, T_m . The binding of ligands to proteins induces conformational changes that further stabilise the protein molecules, and the ligand stabilising effects are proportionate to their concentration and affinity. This sort of interaction-induced stabilisation relies on the coupling of the energetics involved in ligand binding and the unfolding of the macromolecule. Thereby increasing the ΔG of the protein unfolding, resulting in an increased T_m . Consequently, this energetics causes the melting temperature, T_m , of the macromolecule to differ from the ligand complexed macromolecule. The resulting change in

T_m is referred to as a thermal shift, and it is proportional to the affinity (K_a) of the ligand towards the macromolecule (Pantoliano *et al.*, 2001). The T_m can be estimated by applying Equation 2.31:

$$F(T) = F_{post} + \frac{[F_{pre} - F_{post}]}{1 + \exp \left\{ \frac{-\Delta H_u}{R} \left(\frac{1}{T} - \frac{1}{T_m} \right) + \frac{\Delta C_{pu}}{R} \left[\ln \left(\frac{T}{T_m} \right) + \frac{T_m}{T} - 1 \right] \right\}} \quad (2.31)$$

The fluorescence intensity at temperature T is represented by $F(T)$, F_{pre} and F_{post} are the pre-transitional and post-transitional fluorescence intensities, respectively. The change in enthalpy of the unfolding is represented by ΔH_u R is the gas constant, and ΔC_{pu} is the change in the heat capacity of the unfolded protein. The ΔC_{pu} can be estimated from the molecular mass of the protein using the Robertson and Murphy method (Robertson and Murphy, 1997) or the simple sigmoid Boltzmann equation as follows:

$$y = F_{min} + \frac{(U_{max} - F_{min})}{1 + \exp \left(\frac{T_m - x}{a} \right)} \quad (2.32)$$

Where F_{min} and U_{max} are the minimum folded and maximum unfolded fluorescence intensity values, respectively. While a is the slope of the transition curve near T_m .

Ligand binding may occur to the folded or unfolded protein. In contrast to the stabilising effects of ligand binding discussed above, the binding of a ligand can also destabilise a protein. Unfortunately, ligand binding destabilisation of protein molecules is not well understood, as there is limited information regarding that. For example, the exact binding site of ANS to GSTs and Polo-box domain Plk1-PBD is unknown, and there are no crystal structures of ANS binding to these proteins. Similarly, the binding site of Zn^{2+} to porcine growth hormone (pGH) is also not known. It is important to note that some ligands may be destabilising to a particular protein and stabilising to others, this is vice versa for some proteins as well. Assuming that there are only two protein states at equilibrium, the linked ligand binding constant to the unfolded protein state is expressed as follows:

$$K_{bU} = \frac{[UL]}{[U][L]} \quad (2.33)$$

where K_{bU} is the ligand binding constant to unfolded protein $[UL]$ is the concentration of unfolded protein-ligand complex, $[U]$ is the concentration of free unfolded protein, and $[L]$ is the concentration of unbound ligand. The conservation of mass of the total protein may be expressed as:

$$P_t = [F] + [U] + [FL] + [UL] \quad (2.34)$$

$$L_t = [L] + [FL] + [UL] \quad (2.35)$$

Where P_t is the total protein and L_t is the total ligand concentration. $[F]$ is the concentration of folded free protein while $[FL]$ is the concentration of folded protein-ligand complex. The fraction unfolded, f_U can be represented as:

$$f_U = \frac{[U] + [UL]}{P_t} \quad (2.36)$$

Therefore, the total added ligand concentration can be expressed as follows:

$$L_t = (f_U + K_U(f_U - 1)) \times \left(\frac{P_t(K_{bF} + K_{bU}K_U)}{K_U(K_{bU} - K_{bF})} + \frac{1}{K_UK_{bU} - f_U(K_{bF} + K_{bU}K_U)} \right) \quad (2.37)$$

Where K_U is the equilibrium constant of protein unfolding in the absence of a ligand, and K_{bF} is ligand binding constant to the folded native protein. But considering that at T_m , half of the protein is folded and the other half is unfolded ($f_U = 0.5$), equation 2.37 can be simplified, and the relationship between the equilibrium constant of ligand binding to the folded protein and binding to the unfolded protein can be represented as:

$$L_t = (1 - K_{U_{T_m}}) \times \left(\frac{P_t (K_{bF_{T_m}} + K_{bU_{T_m}} K_{U_{T_m}})}{2K_{U_{T_m}} (K_{bU_{T_m}} - K_{bF_{T_m}})} + \frac{1}{K_{U_{T_m}} K_{bU_{T_m}} - K_{bF_{T_m}}} \right) \quad (2.38)$$

The subscript, T_m , of the equilibrium constants, represents the value of each constant at the temperature, T_m . But in a situation where the ligand binds more to the unfolded protein and binds weakly to the folded native state, satisfying the following conditions $K_{bU} \gg K_{bF}$ and $K_U K_{bU} \gg K_{bF}$ then equation 2.38 can be simplified as:

$$L_t = (1 - K_{U_{T_m}}) \times \left(\frac{P_t}{2} + \frac{2}{K_{U_{T_m}} K_{bU_{T_m}}} \right) \quad (2.39)$$

Furthermore, ligand binding to unfolded or partially unfolded proteins also results in a saturation effect. The T_m increases linearly with increasing concentration of the ligand. However, there are instances where the T_m stops increasing (Cimpmperman *et al.*, 2008; Cooper *et al.*, 2001), and this is referred to as the saturation effect.

Chapter 3

Protein production, purification, and analysis

3.1 Introduction

Technologies for the large-scale production of recombinant proteins have dramatically improved the biophysical and biochemical characterisation of protein samples. Furthermore, genes can be sequenced and cloned into a vector, enabling the overexpression and study of the encoded genome. The use of *Escherichia coli* as an important host for cloning and production of recombinant proteins has been well-established and widely used as a protein expression tool. This is because the cells grow very fast and double the rate of growth in about 20 min using low-cost components. Secondly, it is easy to produce high-cell-density cultures and transform them with external DNA (Makrides, 1996). Many expression vectors are available, and the choice of an appropriate one depends on the specific properties of the vector. Attributes such as the type of replicon, promoter, selection marker, affinity tags, and tag removal should be considered. The pET vector, which uses the T7 expression system, was used in this research because of its ability to express more of the target protein (Graumann and Premstaller, 2006).

The next step following a successful protein expression is the purification step. This is crucial for successfully performing other downstream applications such as crystallisation and functional characterisations. Chromatography techniques such as affinity, ion exchange, hydrophobic interaction, and size exclusion chromatography are mainly applied during protein purification. These purification steps can be either automated or manually performed using a high-performance liquid chromatography machine or gravity method, respectively. Most recombinantly expressed proteins are not usually purified to homogeneity by the first purification step, so a second or even a third step may be required using different methods. It is essential to determine the quality and quantify the purified protein. Qualitative techniques like sodium dodecyl sulphate polyacrylamide gel electrophoresis can be used to resolve purified protein samples. The result obtained can show if there are contaminants in the sample, and it will also indicate whether the size is as expected. UV-absorbance spectroscopy is one of the easiest ways to estimate the amount of protein in the

sample. It can also be used to check for DNA contamination and the presence of cofactors (FAD, NAD, etc.), which may have been purified along with the protein of interest.

3.2 Materials

The table below shows the non-standard chemicals used in this chapter. All the other reagents and buffers used were of analytical grade.

Table 3.1 Non-standard materials and suppliers.

Materials	Source	Location
pET-11a expression vector	Novagen	Darmstadt, Germany
T7 Express Competent Cells	New England Biolabs	Ipswich, MA, USA
Yeast extract, Tryptone, and Agar	Sigma-Aldrich	St. Louis, MO, USA
Ampicillin sodium salt	Sigma-Aldrich	St. Louis, MO, USA
Chloramphenicol	Sigma-Aldrich	St. Louis, MO, USA
Lysozyme (chicken egg white)	Sigma-Aldrich	St. Louis, MO, USA
Molecular weight markers	Sigma-Aldrich	St. Louis, MO, USA
Isopropyl β -D-1-thiogalactopyranoside	Sigma-Aldrich	St. Louis, MO, USA
Coomassie Brilliant Blue G250	Sigma-Aldrich	St. Louis, MO, USA
Dithiothreitol (DTT)	New England Biolabs	Massachusetts USA

The glycerol stock of *Escherichia coli* T7 cells was transformed with the pET-15b vector (Figure 3. 1) encoding hGSTP1-1, which was received from Prof H. W.Dirr.

3.3 Methods

3.3.1 Design of expression vectors

The WbGST and hGSTM1-1 genes were inserted into a pET-11a vector, while pET-15b was used for hGSTP1-1. Professor Ikechukwu Achilonu prepared the vectors. The gene fragments containing the WbGST and hGSTM1-1 were cloned into the pET-11a vector (Figure 3.1) between

the *Nde* I and *Bam*H I restriction sites. The multiple cloning sites are controlled by an inducible T7 promoter, which is repressed because of the *lacI* gene, which encodes for a repressor protein. A hexahistidine tag was incorporated at the N-terminus of the sequence to facilitate IMAC purification, and a gene that confers ampicillin antibiotic resistance to assist target gene selectivity. The pET-11a also contains various cloning sites enabling restriction enzymes to insert target gene fragments, therefore facilitating the expression of the target proteins.

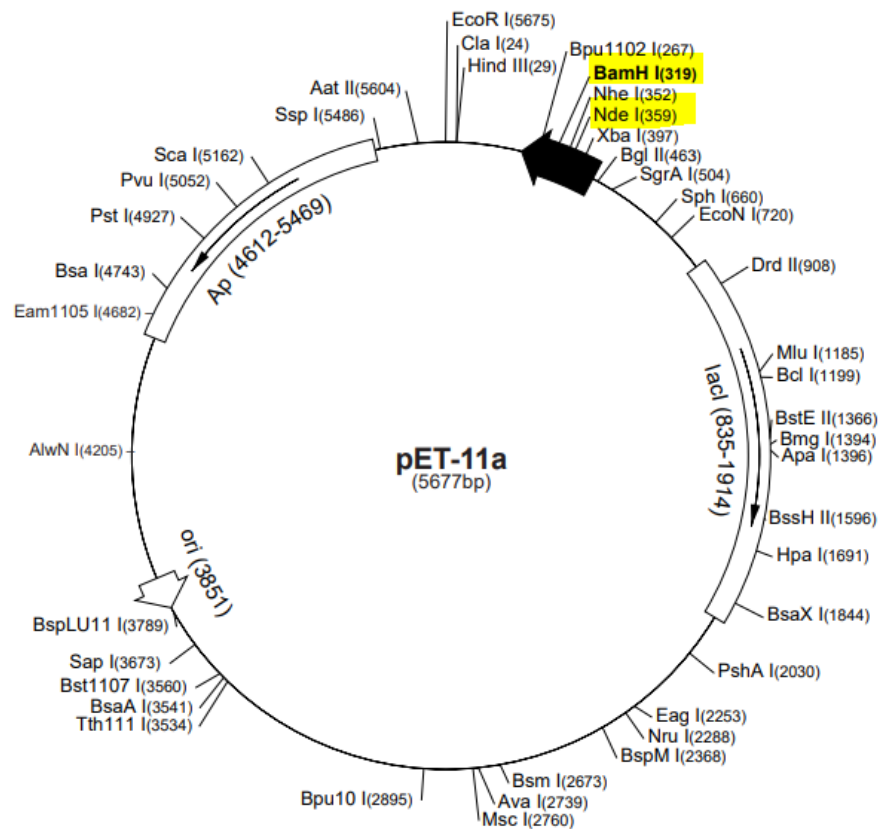


Figure 3.1 Map of a pET-11a expression vector. The illustration indicated the essential sites on the vector, and highlighted in yellow are the insertion sites of the target sequences. Also displayed are the ampicillin-resistant gene and multiple cloning sites (pEt-11a DNA Novagen).

3.3.2 Transformation of competent *Escherichia coli* T7 express

Preceding the actual transformation step, vials containing 4 µg of lyophilised pET-11a vectors with inserts of the complementary deoxyribonucleic acid (cDNA) sequences for WbGST and

hGSTM1-1 were centrifuged at $6000 \times g$ for one minute at 4 °C. Using cDNA for protein expression studies offers greater efficiency, precision, and controlled expression compared to using gDNA. It ensures that only the biologically relevant coding sequence of the gene is transferred to the expression vector, leading to more reliable and reproducible experimental results. Centrifugation step was performed to avoid the loss of some of the lyophilisates at the initial opening of the vials after shipment. Then, the lyophilised DNA was reconstituted in 100 µL sterilised and filtered Milli-Q water and vortexed for 1 min. After that, 100 ng.µL⁻¹ of the vector WbGST or hGSTM1-1 was added to 100 µL T7 Express *E. coli* competent cells. The mixture was kept on ice for 30 min, and afterwards, it was heat shocked for 45 s to increase the porosity of the cell membranes for uptake of the vector DNA. After that, the cells were placed on ice for 2 min, and 900 µL super optimal broth with catabolite repression (SOC) media (Appendix A) was added to the cells and incubated for 1 h at 37 °C at 250 rpm at a New Brunswick Excella E24/E24R incubator (Eppendorf, Hamburg, Germany). The cells were plated on lysogeny broth agar plates with 34 µg.mL⁻¹ chloramphenicol and 100 ug.mL⁻¹ ampicillin. The number of cells in the plates was diluted to allow for a more accessible selection of individual colonies by spreading two additional plates without flaming the spreader. The plates, including a negative control, were incubated at 37 °C for 16 h. Following the growth of the cells, a single colony was selected from each plate and was grown in a 2× YT media (Appendix A) for 16 h at 37 °C while shaking at 250 rpm in a New Brunswick Excella E24/E24R incubator. Glycerol stocks of the cells were prepared by concentrating 1 mL of cell sample down to 200 uL, adding 800 uL of 80% v/v sterilised glycerol, and storing at -80 (C).

3.3.3 Heterologous over-expression of the recombinant proteins

The *E. coli* expression systems can be engineered to produce a high protein expression yield sufficient for downstream applications. In this study, overexpression of recombinant pET-11a-WbGST, pET-11a-hGSTM1-1, and pET-15b-hGSTP1-1 was controlled by the T7 promoter and *lac* promoter, which is needed for the expression of T7 RNA polymerase. The promoter can be activated by adding a lactose analogue to the cell culture, in this case, isopropyl β-D-1-thiogalactopyranosides (IPTG). Adding IPTG induces overexpression of the gene of interest by

suppressing the action of the repressor protein, which allows *E. coli* cells to express T7 RNA polymerase that binds to the T7 promoter (Smith and Johnson, 1988).

Single colonies of T7-pET-11a-WbGST, T7-pET-11a-hGSTM1-1, and a 1 mL glycerol stock of T7- pET-15b-hGSTP1-1 were grown in 50 mL 2× YT media supplemented with 100 µg.mL⁻¹ ampicillin and 34 µg.mL⁻¹ chloramphenicol at 37 °C for 16 h while shaking at 250 rpm. After that, the overnight cell culture was diluted in 1:50 100 mL 2× YT with 100 µg.mL⁻¹ ampicillin and 34 µg.mL⁻¹ chloramphenicol in baffled shake flasks (Duran Erlenmeyer flasks (Sigma Aldrich, Missouri, USA). The cells were grown at 37 °C, 250 rpm until the mid-log growth phase of ~0.6 OD₆₀₀ was attained. Cold induction was performed by cooling the cell culture on ice before adding 0.25 mM IPTG to T7-pET-11a-hGSTM1-1 and T7- pET-15b-hGSTP1-1 to induce overexpression. An induction trial was conducted for T7-pET-11a-WbGST because the protein had not been overexpressed in our Lab previously. Varying concentrations of IPTG (0, 0.25, 0.5, and 1 mM) were used to determine the optimal IPTG concentration required to induce overexpression of WbGST, and the optimal concentration (0.25 mM) was the same as that of the other two human GST proteins. Overexpression of the target proteins was performed by incubating the induced cell culture at 30 °C for 6 h while shaking at 200 rpm. However, overexpression of WbGST was repeated at 20 °C for 18 h. Subsequently, the cells were harvested by centrifugation (4 °C, 7500 × g, 10 min) using the Thermo Fischer Scientific Sorvall LYNX 4000 Superspeed Centrifuge. The obtained pellet was resuspended in 35 mL resuspension buffer (Appendix A) per 1 L culture and frozen at -20 °C.

3.3.4 Protein purification

Affinity chromatography is a separation technique in which the target biomolecules bind to an immobilised ligand while the other unwanted molecules flow through the system. The IMAC technique, which immobilises metal ions to a solid phase by chelation using nitrilotriacetic acid or iminodiacetic acid, binds and coordinates the metal ions while still making room for the protein tags to bind (Porath *et al.*, 1975). The target proteins contain a hexahistidine tag at the N-terminus, which allowed the use of the IMAC purification method. First, the cells were thawed in water at 20 °C, and then lysozyme and deoxyribonuclease I (DNase I) were added to a final concentration

of 0.1 mg.mL^{-1} and 0.01 mg.mL^{-1} respectively. Next, the cells were incubated on ice for 30 min to optimise cell lysis, followed by sonication at 50% amplitude for 30 s and resting on ice for 60 s to prevent heating of the cells. Finally, the resulting lysate was centrifuged at $23\,000 \times g$, $4\text{ }^{\circ}\text{C}$ for 30 min to separate the soluble fraction from the pellets.

Subsequently, the obtained soluble protein fractions were loaded using a flow rate of 4 mL.min^{-1} , 0.3 kPa onto a 5 mL HiTrap IMAC columns attached to an ÄKTAprime plus liquid chromatography purification system (Cytiva Life Sciences, Chicago, USA). Before loading the soluble protein fractions into the columns, the columns were equilibrated with ten-column volumes of equilibration buffer (Appendix A). After loading the column with the soluble fraction, unbound and contaminant proteins were washed off by passing ten-column volumes of equilibration buffer. This was followed by a second wash step using the wash buffer (Appendix A), and it was rinsed with ten-column volumes of the equilibration buffer. Afterwards, the bound target protein was eluted in fractions of 2 mL using the elution buffer. Both WbGST and hGSTM1-1 were purified using Ni^{2+} charged columns, while the Co^{2+} charged column was used for hGSTP1-1 purification. A different column was used for hGSTP1-1 because it was observed in our laboratory that it binds better on the Co^{2+} charged column.

3.3.5 Qualitative and quantitative analysis of the recombinant proteins

Following the purification of the recombinant proteins, it is essential to assess the quality of the purified fractions and determine the actual amount of the target protein in the solution for downstream applications.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was utilised to ascertain the overexpression of recombinant WbGST, hGSTM1-1, and hGSTP1-1 and their purity, size, and homogeneity. SDS-PAGE is an analytical procedure that allows the separation of proteins or other molecules based on electrophoretic mobility. The migration of protein molecules depends on the size of the protein when exposed to an electric field on polyacrylamide gel (Laemmli, 1970). SDS is an anionic detergent. It unfolds the protein and changes its native conformation by breaking the non-covalent bonds. Hence, the electrophoretic

mobility of the protein molecules becomes a function of the size. Shorter polypeptide chains will migrate further through the gel than the longer ones (Maurer, 2011). Furthermore, adding β -mercaptoethanol to the protein samples enhances denaturation by breaking down disulphide bonds within the protein, thus achieving a further elongated structure. This technique was used to determine the homogeneity, identity, and purity of the protein, which is inferred by the appearance of a single band on the gel lane and the size calculated based on the distance migrated alongside a lane of protein standards of known size.

Reducing SDS-PAGE was performed as described by Laemmli (1970) using a PowerPac Basic Bio-Rad electrophoresis system and accessory unit. The gel casting unit was assembled, and the running gel solution (prepared according to Table 3.2) was applied between the glass plates. It was overlaid with water to prevent inhibition of acrylamide polymerisation. Redox agents, such as dithiothreitol (DTT) or β -mercaptoethanol, can inhibit acrylamide polymerization by reducing any oxygen present in the reaction mixture. Oxygen can act as a radical initiator for polymerization reactions, and removing or reducing its concentration inhibits polymerization. When the mixture polymerised, stacking gel solution (Table 3.2) was applied to the upper region of the glass plates. The gel comb was inserted, and the mixture was allowed to polymerise. Protein samples (10 μ L) were prepared in two-fold dilution by mixing with the reducing sample buffer (Appendix A). They were warmed in a hot water bath for 10 minutes to ensure the protein was adequately denatured. 10 μ L of the samples were loaded on the gel wells, and the first well was loaded with 10 μ L of prestained Precision Plus Protein standards from Bio-Rad (California, USA). The unit was connected to the power pack, and electrophoresis was performed at 120 V till the gel front was about 0.5 cm to the bottom. The gel was stained with the staining solution (Appendix A) and was destained in a destaining solution (Appendix A). The electrophoretogram was analysed using the Bio-Rad Gel Doc XR+ system. The molecular mass was obtained by plotting a graph of the log molecular weight of the standards against the relative distance migrated by the protein bands on the gel.

Table 3.2 Recipe for preparing gel solutions.

Reagents	Separating gel 12.5% (w/v)	Stacking gel 4% (w/v)
Monomer solution ^a	6.52 mL	940 µL
Separating gel buffer ^a	3.75 mL	
Stacking gel buffer ^a		1.75 mL
10% SDS	150 µL	70 µL
Distilled water	4.75 mL	4.3 mL
Initiator reagent ^a	75 µL	35 µL
TEMED ^a	15 µL	15 µL

^aProtocols for preparing gel buffers are detailed in Appendix A.

Ultraviolet-visible (UV-vis) spectroscopy

After confirming that the purified recombinant proteins are pure and of the expected size, additional qualitative and quantitative analysis was performed by (UV-vis) spectroscopy. This analytical procedure is based on the amount of light absorbed or transmitted when light is passed through a sample. It was used to determine the concentration of the protein sample by applying the extinction coefficient of the chromophores (Perkins, 1986). Tryptophan and tyrosine exhibit more absorbance at 280 nm, while cysteine and phenylalanine contribute much less. The extinction coefficient at 280 nm of WbGST, hGSTM1-1, and hGSTP1-1 was calculated as described by Perkins (1986):

$$\epsilon_{280} (\text{WbGST}) = 5550 \sum \text{Trp} + 1340 \sum \text{Tyr} + 150 \sum \text{Cys} \quad (3.1)$$

$$= 5550 (1) + 1340 (10) + 150 (4)$$

$$= 19\,550 \text{ M}^{-1} \text{ cm}^{-1} \text{ (per monomer)}$$

$$\epsilon_{280} (\text{hGSTM1-1}) = 5550 \sum \text{Trp} + 1340 \sum \text{Tyr} + 150 \sum \text{Cys}$$

$$= 5550 (4) + 1340 (12) + 150 (4)$$

$$= 17\,020 \text{ M}^{-1} \text{ cm}^{-1} \text{ (per a monomer)}$$

$$\epsilon_{280} (\text{hGSTP1-1}) = 5550 \sum \text{Trp} + 1340 \sum \text{Tyr} + 150 \sum \text{Cys}$$

$$= 5550 (2) + 1340 (12) + 150 (4)$$

$$= 27\,780 \text{ M}^{-1} \text{ cm}^{-1} \text{ (per a monomer)}$$

Absorbance increases as path length increases. The Beer-Lambert law describes the relationship between absorbance and concentration and absorbance and path length as follows:

$$A = \epsilon \ell C \quad (3.2)$$

$$C = \frac{A}{\epsilon \ell} \quad (3.3)$$

where: A is absorbance (a dimensionless number)

ϵ is the proportionality constant called the molar extinction coefficient or molar absorptivity ($M^{-1}cm^{-1}$) at 280 nm measured in water. A value of 20 400 (per-monomer) was obtained for WbGST and was determined using the Expasy ProtoParam algorithm (Gasteiger *et al.*, 2005).

ℓ is the path length (1 cm)

C is concentration (M)

The purified recombinant WbGST, hGSTMu1-1, and hGSTP1-1 were quantified by dilution series and analysed using Jasco V-630 spectrophotometers (Jasco Inc., Tokyo, Japan), monitoring absorbance at 280 and 340 nm. To prepare protein sample for UV-vis spectroscopy analysis, fractions of the purified proteins were dialysed against the storage buffer (Appendix A) for 16 h using a SnakeSkin dialysis tubing (MWCO of 10 kDa) from Thermo Fisher Scientific (Massachusetts, USA). The first dilution sample was prepared by adding 50 μ L protein to 950 μ L dialysis buffer (1:20 dilution), and subsequent samples were prepared by halving the concentration. Absorbance measurements were recorded between 240 and 400 nm wavelengths at 20 °C. A calibration plot of corrected absorbance versus dilution factor was used to obtain a gradient, which was applied in the equation below (Eqn. 3.4) to calculate the protein concentration.

$$conc\ mg/ml = \frac{slope \times Molecular\ mass}{\epsilon \times path\ length\ (cm)} \quad (3.4)$$

The absorbance ratio at 280 nm to 260 nm was determined to analyze the proportion of protein relative to DNA. Additionally, absorbance at 340 nm was measured for each dilution to assess protein aggregation.

3.4 Results

Overexpression of recombinant proteins

E. coli T7 Express cells were transformed with pET-11a and pET-15b vectors containing genes that encode WbGST, hGSTM1-1 and hGSTP1-1. When the cell culture had an OD600 of ~0.6, protein production was induced with 0.25 mM IPTG for hGSTM1-1 and hGSTP1-1 and continued for 6 h at 30 °C, varying concentrations of IPTG were used for WbGST. However, the overexpression of WbGST using the above condition mainly produced insoluble proteins (Figure 3.2 A). Reducing the overexpression temperature to 20 °C allowed almost half of the protein to be overexpressed solubly (Figure 3.2 B). The amount of protein produced was sufficient to proceed with downstream applications. Figures 3.2 and 3.3 indicate that 0.25 mM IPTG induced sufficient protein expression resulting in thick bands at the expected molecular sizes. As for the induction trial performed on WbGST, IPTG concentrations of 0.25, 0.5, and 1 mM had no direct effect on the total amount of protein produced (Figure 3.2 A). No background expression was observed in uninduced cells, indicating that the promoter is tightly controlled. The amount of wet cells obtained from one litre of WbGST culture was ~8.9 g.L⁻¹.

Recombinant protein purification

Soluble recombinant cell lysates were purified using Ni²⁺ or Co²⁺ charged IMAC-FPLC, as described in section 3.3.4. The supernatant was loaded onto the column, and non-specific proteins and other contaminants, which do not have an affinity for the metal ions in the column, resulted in an increase in absorbance at 280 nm, indicated as peak-1 on the elution profile (Figure 3.3). Applying the equilibration buffer decreased the peak to the initial baseline. But a 1% tween-20 wash also triggered the slight increase in absorbance labelled as 2 (Figure 3.3) due to the flow-through of remaining non-specifically bound contaminants. A single-step elution showing a symmetrical peak (peak 3) was observed as each recombinant fusion protein was eluted from the columns and collected in fractions.

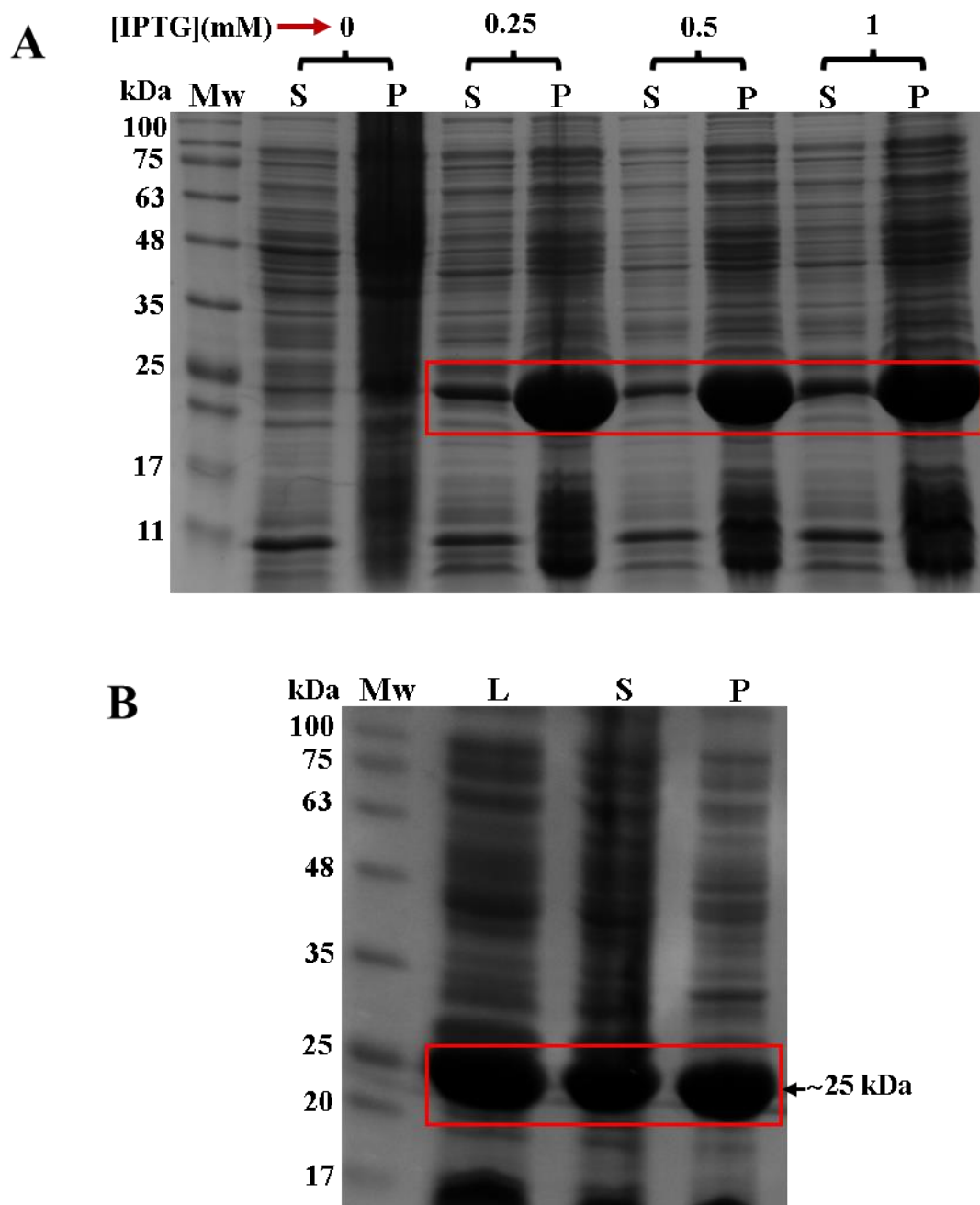


Figure 3.2 The SDS-PAGE of recombinant WbGST overexpression trial. (A) T7-pET-11b-WbGST cells were induced (lanes 4 to 9) and un-induced (lanes 3 and 4) to express WbGST at 30 °C for 6 h at varying concentrations of IPTG (0.25, 0.5, and 1 mM). Cells were harvested and fractionated into soluble (Supernatant) and insoluble cell fractions (Pellets). (B) Expression trial at 20 °C, 18 h with 0.25 mM IPTG. Lane 1 (Mw) is the molecular weight marker, while lanes 2-4 represent the cell lysate, soluble cell fractions of cells, and insoluble cell fractions, respectively. All lanes contain an approximately equal number of cells. The overexpressed protein band resolved on the (12% acrylamide) gel at about 25 kDa. The letters S, P, and L represent supernatant, pellet, and lysate, respectively.

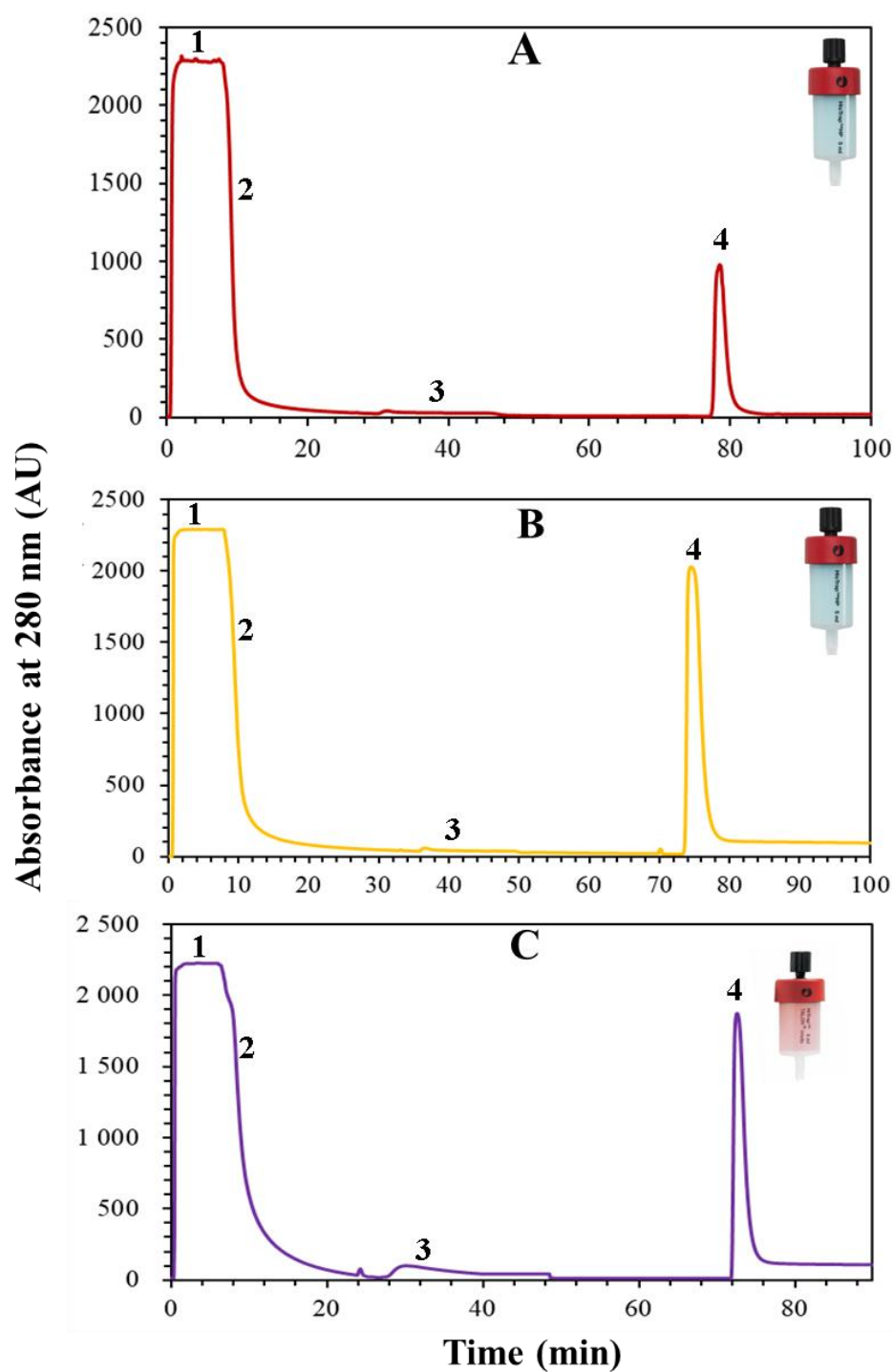


Figure 3.3 Protein purification profile. The profile was obtained by purifying the recombinant WbGST (A), hGSTM1-1 (B) and hGSTP1-1 (C). Loading supernatant of the homogenised T7 Express *E. coli* cells to the IMAC column (1) and the contaminant proteins flow through resulted in an increased absorbance at 280 nm (2). Following column equilibration is a detergent wash step (3), using 1% tween-20, and then a 10-column volume equilibration to rinse the detergent. Subsequently, the tagged proteins were eluted through a single-step elution (4).

Qualitative and quantitative evaluation

Collected fractions were analysed for purity and molecular weight using 12% acrylamide SDS-PAGE. The following samples were resolved on the gel: the homogenised cell lysate, supernatant, resuspended and washed pellets, and flow through and eluted fractions (Figure 3.4 A, B, C). The absence of a target protein in the flow-through indicates that the tagged fusion proteins are quantitatively bound to the Ni^{2+} or Co^{2+} charged IMAC columns (Figure 3.4). The obtained electrophoretogram suggests that the protein is pure, as observed on lanes f1-f5 (Figure 3.4). Densitometry analysis of the purified sample revealed the purity of WbGST and hGSTMu1-1 to be 98.9% and 98.7%, respectively, while hGSTP1-1 eluted with 96% purity. The molecular size of the purified proteins was calculated by extrapolating the distance migrated by the protein to that of the known protein standards under similar conditions (Figure 3.4 B, D, F). The observed monomeric mass of WbGST, hGSTM1-1, and hGSTP1-1 was calculated to be 24, 23, and 26 kDa, respectively (Figure 3.4), which are roughly in line with the theoretical monomeric masses of 25.3, 26.9 and 23.4 kDa for WbGST, hGSTM1-1 and hGSTP1-1, respectively.

The amount of purified target protein obtained was assessed by UV-Vis absorbance spectroscopy. This technique can also serve to detect nucleic acid contamination and assess protein sample aggregation (Sheehan, 2013). UV-Vis absorbance ranging from 240 nm to 350 nm was monitored. Each target protein exhibited a characteristic spectrum featuring a trough at 260 nm, a peak at 280 nm, and a stable flat line at 340 nm (Figure 3.5). The average yield per 1 L of cell culture was 6, 11, and 12 mg.mL^{-1} for WbGST, hGSTM1-1 and hGSTP1-1, respectively. The average obtained volume ranges between 5-10 mL.

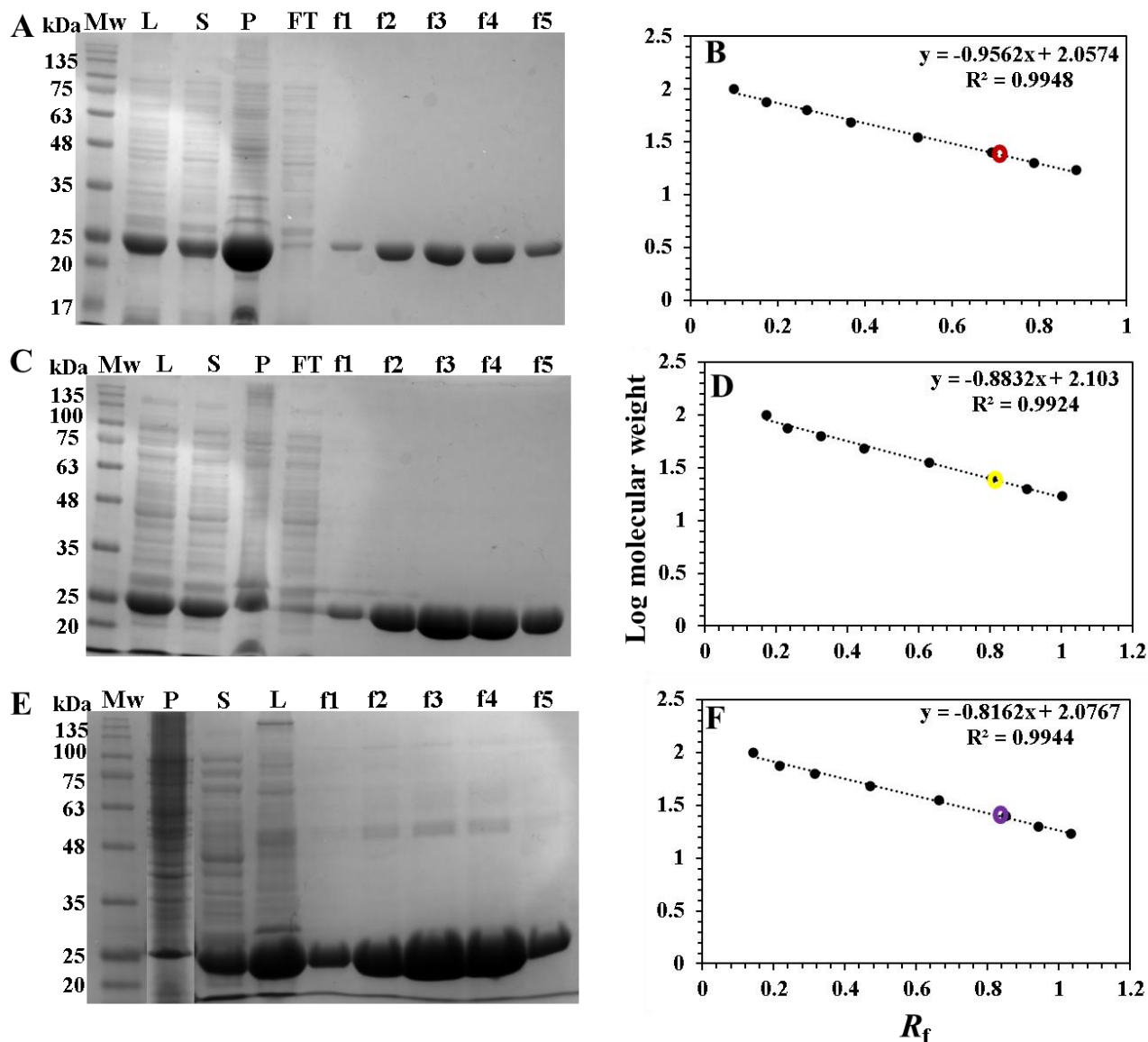


Figure 3.4 Protein purity and size verification. The purified proteins were analysed using SDS-PAGE. Gels of the target proteins, WbGST (A), hGSTM1-1 (C), and hGSTP1-1 (E), were analysed on Image Lab™ software, and densitometry analysis reveals that the target proteins were $\geq 96\%$ pure. The molecular weight maker (Mw) was loaded on the first lane, followed by the lysates (L), supernatants (S), pellets (P), and flow-through (FT) except for hGSTP1-1 which has no FT and with the lanes for L and P interchanged. Lane labelled f1 -f5 represents the purified eluted fractions of the target proteins. Calibration curves for WbGST (B), hGSTM1-1 (D), and hGSTP1-1 (F) were obtained from plotting the logarithm of protein standards molecular weights against the relative fronts (R_f). The molecular weight of the target proteins, WbGST, hGSTM1-1, and hGSTP1-1, were extrapolated to be 24 kDa, 23 kDa, and 26 kDa, respectively.

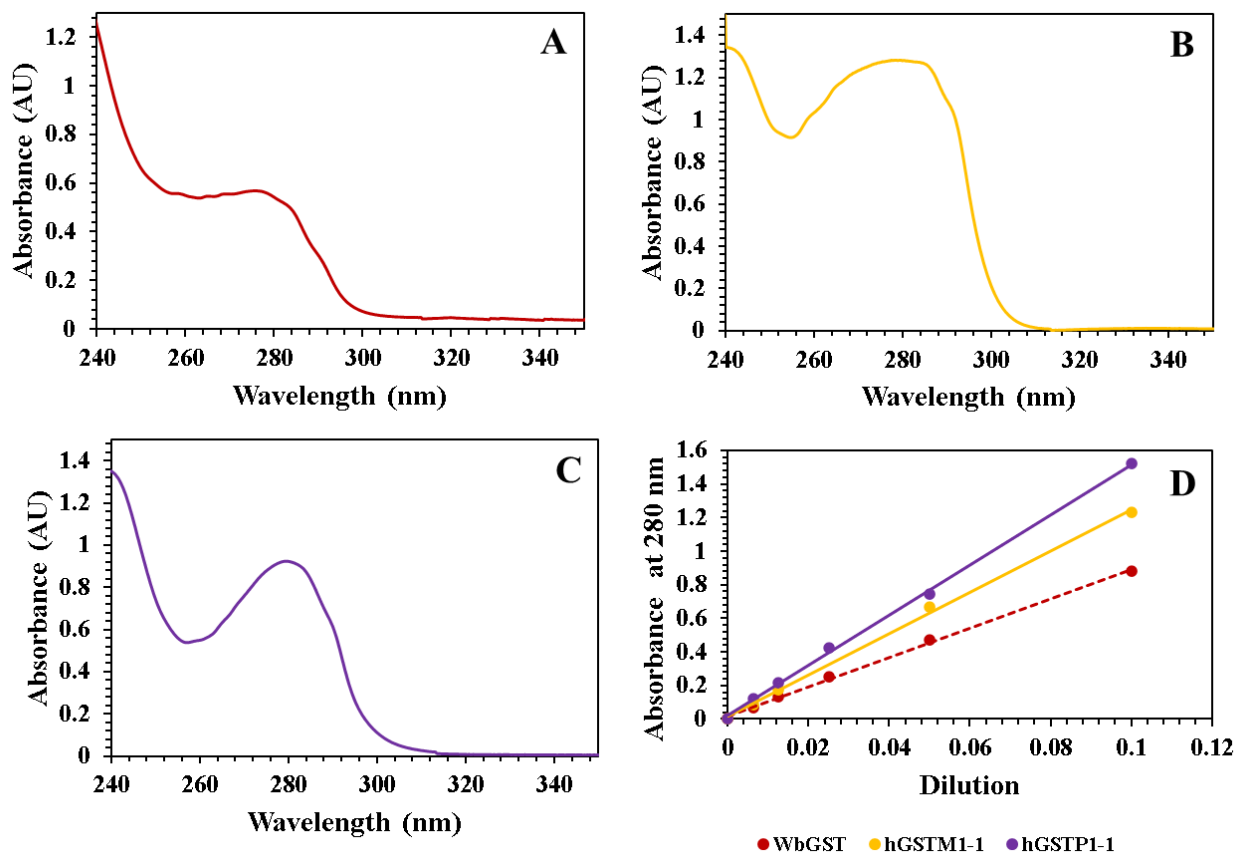


Figure 3.5 Protein quantification and quality assessment. UV-vis absorbance of WbGST (A), hGSTM1-1 (B), and hGSTP1-1 (C) were measured from 240 – 350 nm wavelength. A flat baseline at 340 nm indicates the absence of protein aggregation, and a trough at 260 shows that the proteins are free of nucleic acid contamination. Although phenylalanine residues present in the target proteins and disulphide bonds formed by cysteine residues can contribute to the increased absorbance at 260 nm, the proteins were dialysed into a buffer containing a reducing agent. Therefore, the contribution of absorbance by disulphide bonds can be ruled out and attributed to phenylalanine residues. (B) The concentration of the target proteins was calculated by serial dilution, the absorbance was monitored at 280 nm and 340 nm, and a linear regression graph was plotted using corrected absorbance at 280 nm. The resulting slopes ($y = 8.7419x + 0.0188$ for WbGST, $12.313x + 0.0177$ for hGSTM1-1, and $y = 14.946x + 0.0218$ for hGSTP1-1) were implemented in the Beer-Lambert equation to calculate the concentration of the target proteins. The concentration for WbGST, hGSTM1-1, and hGSTP1-1 was determined to be 10 mg.mL^{-1} , 11 mg.mL^{-1} , and 12.1 mg.mL^{-1} , respectively with $R^2 = 0.99$.

3.5 Discussion

Encoding the cDNA of WbGST, hGSTM1-1, and hGSTP1-1 sequences into the pET-11a and pET-15b vectors, respectively, was successful, as well as the transformation of the T7 *E. coli* express system. Results obtained from the overexpression of recombinant T7-pET-11a-WbGST, T7-pET-11a-hGSTM1-1, and T7-pET-15b-hGSTP1-1 using the following conditions: 20 °C, 18 h for WbGST and 30 °C, 6 h for hGSTM1-1 or hGSTP1-1 and shaking at 200 rpm indicates a successful overexpression (Figures 3.2). Although the initial overexpression of WbGST was unsuccessful at 30 °C despite applying the cold induction method, at a reduced temperature of 20 °C, half the protein was produced solubly. It is common for most recombinant proteins expressed using the T7 expression system to form aggregates and inclusion bodies when induced and expressed at higher temperatures (30-37 °C) but become more soluble when induced at a lower temperature (15-25 °C). This is one of the characteristics of the T7 system. Overexpression of recombinant proteins at low temperatures reduces the protein production rate and consequently allows sufficient time for proper folding of the protein as the transcription process progresses (Vera *et al.*, 2007; Weickert *et al.*, 1997).

Purification of the hexahistidine-tagged target protein was successfully achieved with a high purity level (by one-step purification using a Ni²⁺ or Co²⁺ IMAC (section 3.3.4). This is evident in the gels (Figure 3.4) because single bands closely corresponding to the expected monomeric molecular mass were observed on eluted fraction lanes (f1-f5). Moreover, the target proteins were not seen in the flowthrough samples, indicating a successful cross-linking between the available binding sites on Ni²⁺ or Co²⁺ and the imidazole rings of the hexahistidine tag. Eluting WbGST required a higher concentration of imidazole and 10 – 20 min of incubating the column in an elution buffer, enabling a better detachment of the target protein from the resin. The increased affinity of the target protein to the column could be because WbGST has many surface histidine residues.

The SDS-PAGE densitometry analysis indicates that a purity level above 95 % was achieved following the purification of the target proteins. However, the resulting molecular weight deviated slightly from the theoretical weight obtained from the ExPASy ProtParam tool (<https://web.expasy.org/cgi-bin/protparam/protparam>). The predicted molecular weight for

WbGST and hGSTM1-1 was 1.4 and 3.9 kDa higher than the theoretical mass, respectively, while hGSTP1-1 was 2.6 kDa lower. Nonetheless, the quaternary size obtained from the HPLC analysis corresponds with the predicted size of the enzyme protein. Thus confirming the identity of the proteins. The behavioural deviation in the electrophoretic mobility of the proteins was probably impacted by the compositions of amino acids of the different proteins and the degree to which they bind SDS (Rath *et al.*, 2009). Furthermore, it was initially estimated that the maximum protein-SDS complex was 1.4 g SDS per gram of protein, but this has been refined to 0.7 – 2 g SDS per gram of protein, depending on the amino acid composition. Therefore, this contributes to deviations observed between the predicted and expected molecular protein weights (Reynolds and Tanford, 1970).

Absorbance spectroscopy measures the interaction of light and molecules in a solution. It is a better tool to accurately determine the concentration of protein in a solution and the purity from nucleic acid contamination and aggregation of protein in the sample. The ratio of absorbance at 260 and 280 nm of a protein-free from nucleic acid contamination is expected to be below 1.7. Therefore, the three protein samples obtained in this study are not contaminated by nucleic acid. Although a higher absorbance at 260 nm was observed for WbGST and hGSTM1-1 when compared to hGSTP1-1 (Figure 3.5), it can be attributed to the contribution in absorbance by the number and position of phenylalanine residues (WbGST: F = 22, hGSTMu1-1: F = 26, hGSTP1-1: F = 14), which absorbs light maximally at 257 nm. Disulphide bonds can also contribute to the absorbance at 260 nm wavelength. Still, because the protein samples were dialysed into a buffer containing a reducing agent (DTT), the increased absorbance observed cannot be attributed to disulphide bond formation (Schmid, 2001). Furthermore, the absence of Rayleigh scatter between 320 – 350 nm indicates no aggregation in the protein samples, enabling the calculation of protein concentration with higher accuracy. Ultimately, this chapter captures the successful production of pure homogenous samples of the recombinant WbGST, hGSTM1-1, and hGSTP1-1, which will be used for downstream applications.

Chapter 4

Protein functional characterisation

4.1 Introduction

Following successful production and purification of the target proteins, they were dialysed into dialysis buffer 1 (Appendix A). Subsequently, the activity of the target enzymes was assessed using a GSH-CDNB conjugation assay. The colourimetric assay involves monitoring the change in the substrate or product concentration in an enzyme-catalysed reaction in a specified period. This change in concentration is observed spectrophotometrically because the substrate and product possess unique spectroscopical characteristics. Therefore, the enzyme activity and other underlying enzyme kinetics reactions resulting in changes in the substrate structure which leads to the formation of products having chromophores can be monitored by UV-vis absorbance spectroscopy.

A variety of xenobiotic substances has been utilised in monitoring GST enzyme activity. However, 1-chloro-2,4-dinitrobenzene (CDNB) has been proven to be efficient and most uncomplicated in observing the progress of the enzymatic reactions. On the other hand, GSH, a natural substrate for GSTs, can conjugate CDNB through the replacement of chlorine by the group of GSH, leading to the formation of the product 1-(S-glutathionyl)-2,4-dinitrobenzene (GS-DNB) (Habig *et al.*, 1974). However, in the absence of GST, this reaction proceeds slowly, but adding GST catalyses the reaction (Figure 4.1). First, apparent substrate affinity (K_M) to the enzymes and maximum velocity (V_{max}) were accessed using the Michaelis Menten model. Next, the potential inhibition of the reaction was monitored in the presence of the inhibitors (BSP and EGCG). Following inhibition confirmation, the half-maximal inhibitory concentration of the ligands (IC_{50}) and the inhibition constant (K_i) were determined by varying ligand concentrations following the same assay principle. Subsequently, the mode of their inhibition was determined by employing the double reciprocal of Lineweaver-Burk plots derived from the Michaelis Menten curves.

4.2 Materials

The list of nonstandard materials used in this chapter is provided in Table 4.1 below. All the other reagents and buffers used were of analytical grade.

Table 4.1 Non-standard materials and suppliers

Materials	Source	Location
GSH	Sigma-Aldrich	St. Louis, MO, USA
CDNB	Sigma-Aldrich	St. Louis, MO, USA
BSP	Sigma-Aldrich	St. Louis, MO, USA
EGCG	Angene	Hyderabad, India
Snakeskin dialysis tubing	ThermoFisher Scientific	Waltham, MA, USA
Dithiothreitol (DTT)	New England Biolabs	Massachusetts USA

4.3 Methods

4.3.1 Dialysis or buffer exchange

Dialysis is a separation technique based on the principle of diffusion. Usually, small molecules in a solution diffuse out of a semi-permeable membrane until equilibrium is achieved in the system, leaving the larger molecules in the volume enclosed by the membrane. After successfully purifying the target proteins, the next step involved performing buffer exchange to remove imidazole from the elution buffer and potentially replace it with a buffer suitable for individual downstream applications. Therefore, eluted fractions containing the target proteins were dialysed against 2 L dialysis buffer-2 (Appendix A) at 4 °C for 4 h, followed by 1 L of dialysis buffer-2 for 16 h, using a 10 kDa molecular weight cut-off dialysis tubing (ThermoFisher Scientific, Massachusetts, USA) to reduce the amount of imidazole in the protein sample.

4.3.2 Enzyme-specific activity determination

1-chloro-2, 4-dinitrobenzene (CDNB) is a substrate commonly used for detecting GST activity, which can be indicative of specific GST isozymes. Different isozymes of GSTs may exhibit

varying affinities and catalytic efficiencies toward CDNB and other substrates. Therefore, by measuring the activity of GSTs using CDNB as a substrate, the presence and activity levels of specific GST isozymes can be assessed. Monitoring the reaction progress is also very easy (Habig et al., 1974). CDNB undergoes conjugation with GSH through an aromatic nucleophile substitution reaction, where the thiol group of GSH replaces the chlorine atom in CDNB. This reaction produces a dinitrophenyl thioether and chloride ion (Figure 4.1). Formation of the product (1-(S- glutathionyl)-2,4-dinitrobenzene) is followed by the appearance of an absorption band at 340 nm, enabling a spectrophotometric assay for GST activity. The absorption of light at 340 nm by 1-(S-glutathionyl)-2,4-dinitrobenzene is likely due to the presence of conjugated systems, functional groups like nitro groups, and chromophores within its molecular structure. These features allow the molecule to absorb photons with energies corresponding to that wavelength, leading to the observed absorption band. Thus, the presence of GST leads to a gradual increase of dinitrophenyl thioether chromophores. The reaction of CDNB and GSH is slow relative to the enzyme-catalysed reaction. The activity of WbGST, hGSTM1-1, and hGSTP1-1 was assayed in the presence and absence of BSP and EGCG using the GSH-CDNB conjugation assay.

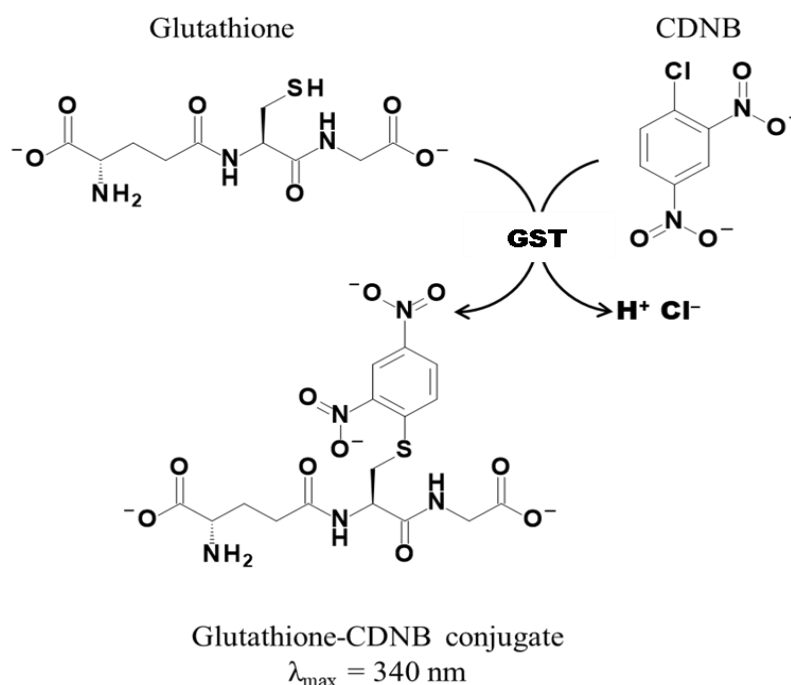


Figure 4.1 GSH-CDNB conjugation reaction. The reaction is catalysed by GST, and Glutathione-CDNB is conjugated, resulting in the release of products (1-(S-glutathionyl)-2,4-dinitrobenzene (GS-DNB), which is monitored at 340 nm wavelength.

The assayed samples were prepared by varying the concentration of the target proteins (0 – 500 nM for WbGST, 0 – 50 nM for both hGSTM1-1 and hGSTP1-1) and incubated in 1 mM GSH using the enzyme activity assay buffer (Appendix A). The reaction was initiated by adding 1 mM CDNB in a Herasil quartz cuvette with a path length of 1 cm (Hellma, Müllheim, Germany) while ensuring a final assay volume of 3 mL using the assay buffer (Appendix A). The formation of GS-DNB ($\epsilon = 9600 \text{ M}^{-1}.\text{cm}^{-1}$) was monitored at 20 °C, 340 nm for 60 s by measuring changes in absorbance using UV-visible light spectrophotometer (Jasco V-630, Jasco Inc., Tokyo Japan). The assay was also performed in the presence of 50 μM BSP and EGCG to assess their potential inhibitory capacity. Each sample was examined in triplicate, and the non-enzymatic response was adjusted using the blank samples. A linear progress curve was plotted as a change in absorbance at 340 nm for 60 s, and the slope represents the reaction rate. After that, the specific activity ($\mu\text{mol}.\text{min}^{-1}.\text{mg}^{-1}$) was obtained by plotting the slope of the initial velocity of complex formation against the mass of the enzyme (mg).

4.3.3 IC₅₀ and K_i determination

In studying enzyme inhibition, both inhibition constant (K_i) and half-maximal inhibitory concentration (IC_{50}) are important parameters used to describe the interaction between inhibitors and enzymes or other biological targets. These values provide valuable insights into the potency and effectiveness of inhibitors but are used in slightly different contexts. The IC_{50} evaluates or determines the potency of a substance in inhibiting a specific biochemical activity. It estimates the concentration of an inhibitor at which the response or its capacity to bind is reduced by 50% of maximal binding. In terms of enzyme activity, IC_{50} is the inhibitor concentration that reduces the enzyme activity to half-maximal activity. On the other hand, the K_i quantifies the binding affinity between enzymes and their inhibitors, providing insights into enzyme kinetics, mechanisms of inhibition, and applications in drug development and biochemical research. It quantifies how effectively the inhibitor binds to the target and reduces its activity. Understanding K_i is fundamental for unravelling the intricacies of enzymatic reactions and their regulation in biological systems

The IC₅₀ of BSP and EGCG on the enzymes was performed by monitoring the enzyme activity in the presence of varying concentrations of the ligands using the GSH-CDNB conjugation assay described in section 4.3.1. All samples were analysed in triplicate using a final protein concentration of 500 nM WbGST, 50 nM for both hGSTM1-1 and hGSTP1-1 incubated with varying concentrations of the ligands for 1 h, and the progress curves were monitored spectrophotometrically at 340 nm for 60 s. All progress curves were blankly corrected, and standard deviations were also calculated for each data point. The reaction rate for each sample was converted to a percentage of enzyme activity as follows:

$$\text{Enzyme activity (\%)} = \frac{(\text{Reaction rate: inhibitor})}{(\text{Reaction rate: no inhibitor})} \times 100\% \quad (4.1)$$

The IC₅₀ was obtained by plotting percentage enzyme activity values against the log inhibition concentration, and the data were fitted using the dose-response curves inhibition equation (Equation 4.2) on GraphPad Prism.

$$Y = \frac{100}{(1 + 10^{((\text{LogIC}_{50} - X) * \text{HillSlope}))}} \quad (4.2)$$

where:

IC₅₀ is the concentration of agonist that gives a response halfway between the Bottom and Top parameters. The Top and Bottom parameters describe the values at which the curve reaches a plateau, coming infinitely close, but never quite reaching these values. HillSlope describes the steepness of the curves, as a result, a higher HillSlope value would result in a steeper curve and *vice versa*.

To obtain the apparent K_i value, dose-response curves obtained from the IC₅₀ were analysed by selecting the appropriate inhibition model for each mode of inhibition observed. Subsequently, the apparent K_i obtained from the initial fit was adjusted to the true K_i . This adjustment is necessary because the apparent K_i can be affected by the concentration of the substrate used in the assay.

$$\text{True } K_i = \frac{\text{Apparent } K_i}{(1 + \frac{[S]}{K_M})} \quad (4.3)$$

where:

Apparent K_i is the K_i obtained from the initial fit.

[S] is the concentration of the substrate used in the assay.

K_M is the Michaelis-Menten constant, which represents the affinity of the enzyme for the substrate.

4.3.4 Enzyme kinetic analysis

The mechanism of reactions catalysed by enzymes is usually calculated through kinetic measurements on enzyme-substrate reaction systems. This includes evaluating the enzyme-catalysed reaction rates at different enzyme and substrate concentrations (Bowden, 1995). Although enzymatic reactions are usually observed using a spectrophotometer to monitor the colour development over time, a spectrofluorimeter could be used by applying a fluorescence probe. The maximum velocity (V_{max}) is a kinetic parameter that indicates the maximum rate of reaction progress with an increasing concentration of a substrate using a constant concentration of enzyme. The K_M is the concentration of substrate needed to produce a velocity of one-half V_{max} . In a situation where an enzyme K_M value is small, the enzyme will achieve maximum catalytic efficiency at a low substrate concentration. Therefore, the smaller the K_M value, the more efficient the enzyme is.

Steady-state kinetic measurements for the WbGST, hGSTM1-1, and hGSTP1-1 were analysed using the GSH-CDNB conjugation assay described previously in section 4.3.2. To obtain the apparent V_{max} and K_M of GSH the enzymes were assayed with CDBN fixed at a non-saturating concentration while varying concentrations of GSH (0-10 mM). For that of the CDBN, the enzymes were assayed with GSH at a non-saturating concentration and varying concentrations of CDBN (0-5 mM for WbGST, 0-10 mM for hGSTM1-1 and hGSTP1-1). Samples were analysed both in the absence and presence of different concentrations of BSP ([2.5, 5 and 7.5 μ M] for WbGST, [4, 12 and 25 μ M] for hGSTM1-1, and [40, 110 and 250 μ M] for hGSTP1-1). Concentrations of the ligand were chosen based on their IC_{50} for the different proteins. The assays were performed in triplicate, and the data points were fitted to Michaelis Menten single substrate model using GraphPad Prism. The mode of inhibition was obtained using the Lineweaver-Burk

plot. A double reciprocal plot of the initial inverse velocity as a function of inverse substrate concentration for both GSH and CDNB yielded a linear plot.

$$V = \frac{V_{max}[S]}{K_M + [S]} \quad (4.4)$$

Taking the reciprocal of the Michaelis Menten equation above (Eqn. 4.4) gives equation 4.5:

$$\frac{1}{V} = \frac{1}{V_{max}} + \frac{K_M}{V_{max}} \cdot \frac{1}{[S]} \quad (4.5)$$

where:

V = reaction velocity

K_M = Michaelis Menten constant

V_{max} = Maximum velocity

$[S]$ = Substrate concentration

The k_{cat} is the turnover number of the enzyme, which is the micromole of products made per minute per micromole of an enzyme. It is the 1st order reaction rate constant for converting the enzyme-substrate complex to a product. The k_{cat}/K_M is the kinetic efficiency of the enzyme (specificity constant). It is the 2nd-order rate constant for the reaction of an enzyme with a substrate to produce a product measured in $M^{-1}min^{-1}$. It enables the ranking of enzymes according to how efficient they are with different substrates.

The k_{cat} was obtained by varying the concentration of the enzymes from 0 - 0.5 μM for WbGST and 0 - 0.05 μM for the human GSTs, while keeping substrate concentrations constant at 1 mM in the absence or presence of 20 μM BSP and EGCG. The linear plot of velocity versus the enzyme concentration yielded the k_{cat} (sec^{-1}). The k_{cat}/K_M was obtained experimentally by varying substrate concentrations, using concentrations far below the K_M values in the absence and presence of different BSP and EGCG concentrations. A linear plot of the reaction velocity versus the substrate concentration was obtained. All data points are average of triplicate experiments and were blank corrected.

4.4 Results

Enzyme activity assay

The specific activities of WbGST, hGSTM1-1, and hGSTP1-1 were assessed using the GSH-CDNB conjugation assay with or without 50 mM BSP and EGCG. The progress curve (Figure A1) for each triplicate data was monitored at A_{340} for 60 s, and the slope for each reaction curve provides the initial velocity (v_0), which was plotted against each enzyme concentration. Subsequently, the calculated activity for each enzyme concentration while implementing the extinction coefficient ($\epsilon_{340\text{nm}} = 9600 \text{ M}^{-1}\text{cm}^{-1}$) of the product (GS-DNB) was plotted against each mass of enzyme (mg) to obtain the specific activity (Figure 4.2). The specific activity of WbGST, hGSTM1-1, and hGSTP1-1 is 1.2, 35, and 59 $\mu\text{mol min}^{-1}.\text{mg}^{-1}$ respectively. The activity of the enzymes was reduced in the Presence of BSP and EGCG (Table 4.2). More details about how the specific activity was calculated are described in the Appendix section.

Table 4.2 Enzyme specific activity

	Specific activity ($\mu\text{mol min}^{-1}.\text{mg}^{-1}$)		
	No Inhibitor	BSP	EGCG
WbGST	1.2 ± 0.016	0.03 ± 0.002	0.77 ± 0.003
hGSTM1-1	35 ± 0.62	10 ± 0.32	28 ± 0.31
hGSTP1-1	59 ± 0.74	28 ± 0.42	58.6 ± 0.81

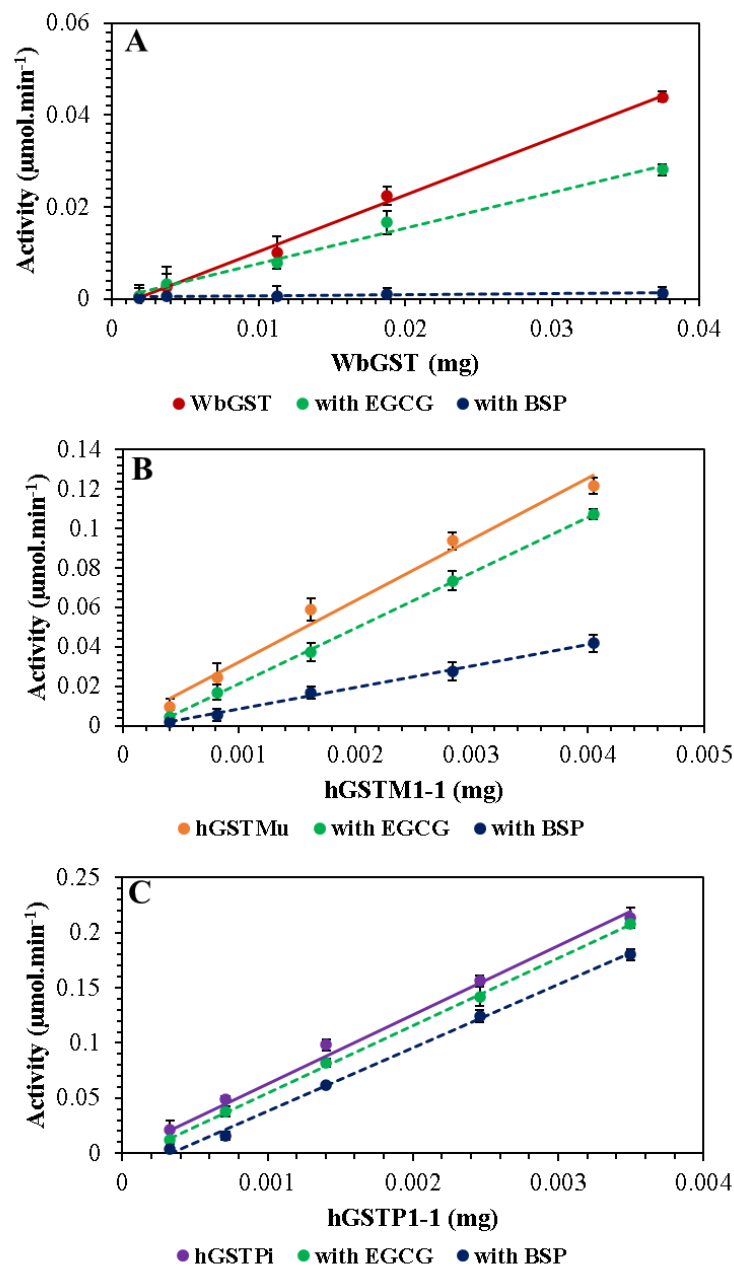


Figure 4.2 Enzyme activity and inhibition. These enzymes catalyse the reaction between reduced GSH and CDNB, resulting in a coloured GS-DNB product with an absorbance at 340 nm. Therefore, the increase in absorbance will be directly proportional to the specific activity of the enzymes. **(A)** Specific enzyme activity obtained for WbGST 1.2 $\mu\text{mol.min}^{-1}.\text{mg}^{-1}$ (red line), in the presence of BSP and EGCG: 0.03 $\mu\text{mol.min}^{-1}.\text{mg}^{-1}$ (Green line) and 0.77 $\mu\text{mol.min}^{-1}.\text{mg}^{-1}$ (blue line), respectively. **(B)** Specific activity obtained for hGSTM1-1 35 $\mu\text{mol.min}^{-1}.\text{mg}^{-1}$ (Orange line), in the presence of BSP and EGCG, 10 $\mu\text{mol.min}^{-1}.\text{mg}^{-1}$ (Green line) and 28 $\mu\text{mol.min}^{-1}.\text{mg}^{-1}$ (blue line), respectively. **(C)** Specific enzyme activity obtained for hGSTP1-1 is 59 $\mu\text{mol.min}^{-1}.\text{mg}^{-1}$ (Purple line), in the presence of BSP and EGCG: 28 $\mu\text{mol.min}^{-1}.\text{mg}^{-1}$ (Green line) and 58.6 $\mu\text{mol.min}^{-1}.\text{mg}^{-1}$ (blue line), respectively. Each data point was obtained in triplicate.

IC₅₀ and K_i determination

Following the confirmation that BSP and EGCG inhibit the activity of the target enzymes, the IC₅₀ and K_i of the enzymes were determined. GSH-CDNB conjugation assay was employed to determine the amount of BSP, and EGCG required to inhibit the activity of WbGST, hGSTM1-1, and hGSTP1-1 by 50% (Figure 4.3). An IC₅₀ curve was constructed by varying ligand concentrations. The IC₅₀ value of BSP towards WbGST, hGSTM1-1, and hGSTP1-1 was determined from the curve to be 5 µM, 13.5 µM, and 110.1 µM, respectively. Similarly, for EGCG, the IC₅₀ towards the enzymes was determined to be 13 µM, 138.4 µM, and 361.2 µM for WbGST, hGSTM1-1, and hGSTP1-1, respectively (Table 4.3). The apparent K_i obtained from the initial fit of IC₅₀ curves while applying the model for each inhibition mode was adjusted to the true K_i (Table 4.3). This adjustment is necessary because the apparent K_i can be affected by the concentration of the substrate used in the assay.

Table 4.3 IC₅₀ and K_i results of BSP and EGCG on the recombinant enzymes.

	IC ₅₀		K _i	
	BSP (µM)	EGCG (µM)	BSP (µM)	EGCG (µM)
WbGST	5 ± 0.51	13 ± 0.4	1.21 ± 0.4	9.4 ± 0.8
hGSTM1-1	13.5 ± 0.3	138 ± 1.2	11.5 ± 0.12	131 ± 1.4
hGSTP1-1	110 ± 1.5	361.2 ± 1.02	107.4 ± 1.4	359.4 ± 0.6

Enzyme kinetic analysis

The enzyme reaction rate and impact of BSP and EGCG on the mechanism of WbGST, hGSTM1-1, and hGSTP1-1 catalysed reaction was further analysed using kinetic evaluation of the enzyme-substrate reaction systems (using GSH-CDNB conjugation assay). The progress curves of the enzyme activity were monitored spectrophotometrically at A₃₄₀ for 60 s each. Enzyme activity was monitored using different substrate concentrations, in this case, GSH or CDNB, in the presence of varying concentrations of BSP and EGCG. The reactions for GSH and CDNB followed the Michaelis Menten kinetics. A plot of the reaction velocity as a function of the substrate concentration (GSH and CDNB) was fitted using a hyperbolic function implemented in GraphPad Prism. The calculated V_{max} and K_M obtained are shown in Table 4.4.

The mode of inhibition of both ligands for the two substrates was determined using the Lineweaver Burk plots. Analysis of WbGST-catalysed GSH-CDNB (varying [GSH]) conjugation reaction in the presence of BSP shows that BSP does not compete with GSH. Rather a mixed type of inhibition was observed (Figure 4.4D). The plots did not intersect on either the x -axis or y -axis, which is typical of a mixed inhibition. Furthermore, BSP inhibition of the hGSTP1-1 and hGSTM1-1 with regards to the G-site was noncompetitive (Figure 4.4E and F). Analysing the mode of EGCG inhibition of WbGST towards the G-site indicates an uncompetitive mode of inhibition, while that of the hGSTP1-1 and hGSTM1-1, is indicative of a noncompetitive mode of inhibition and non-competitive (Figure 4.5). On the other hand, varying the concentration of CDNB as a substrate indicated that BSP and EGCG inhibited the H-site of WbGST in a mixed form of inhibition, with the lines intersecting below the negative side of the x -axis. In contrast, BSP inhibition of the hGSTP1-1 and hGSTM1-1 at the H-site was both non-competitive, with the lines intercepting on the x -axis. Similarly, the inhibition of hGSTP1-1 by EGCG at the G-site and H-site was observed to be also non-competitive, while that of hGSTM1-1 indicated a mixed type of inhibition.

To probe further the impact of BSP and EGCG on WbGST and the two human GST variants, catalytic efficiency, and the rate of product formation, the k_{cat} and k_{cat}/K_M were determined. GSH-CDNB conjugation assay was also used to perform the reaction using GSH as the substrate in the presence and absence of inhibitors. To determine the k_{cat}/K_M , a GSH concentration below the K_M was varied, and the progress curve of the velocity was plotted against the various substrate concentrations (Figure 4.9). For k_{cat} , GSH concentration was kept constant, while protein concentration was varied in the presence or absence of BSP and EGCG (Figure 4.8). The obtained results (Table 4.4) indicate that the inhibitors reduced both the turnover and kinetic efficiency of WbGST.

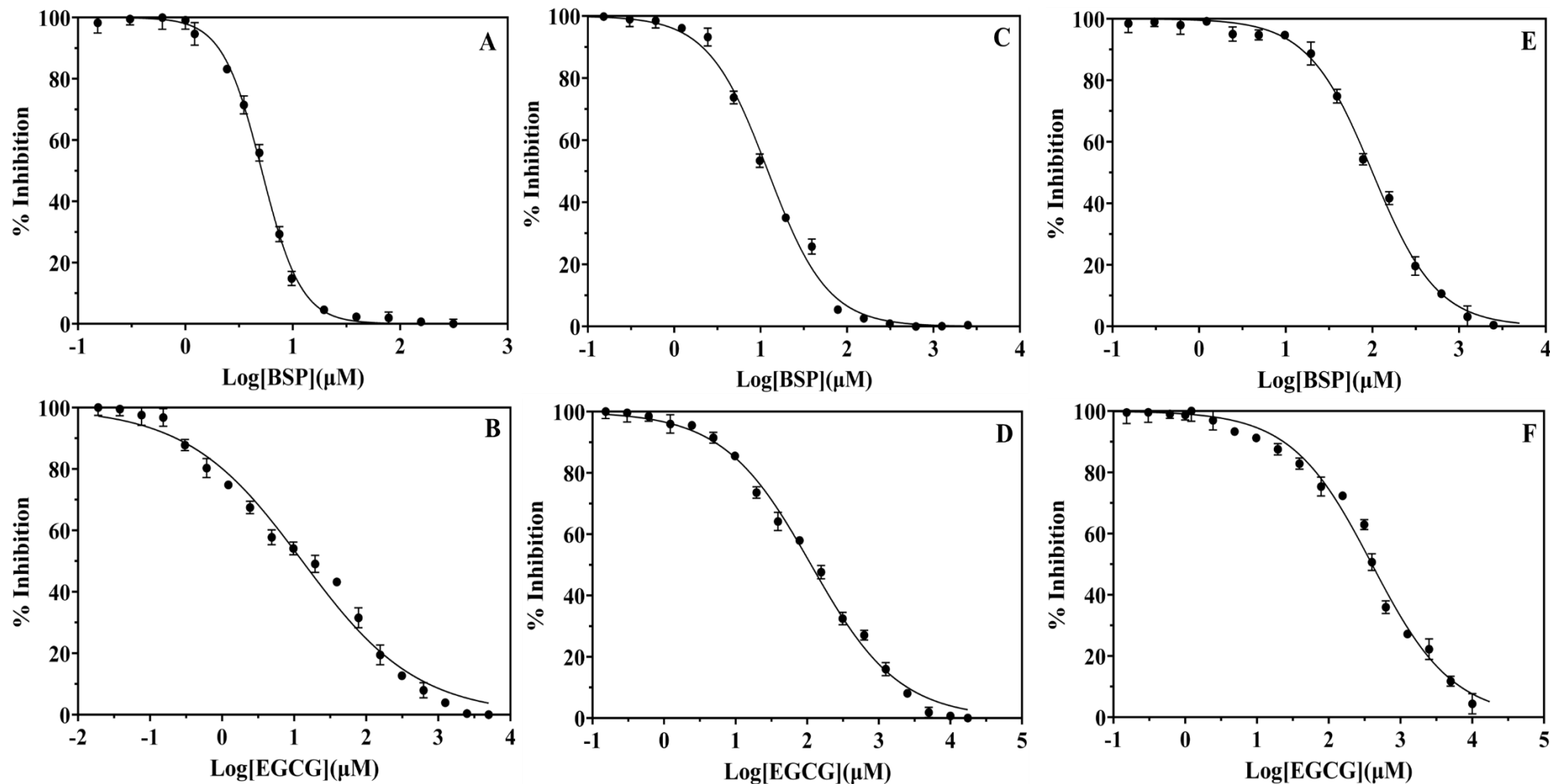


Figure 4.3 The IC_{50} of BSP and EGCG. GSH-CDNB assay was performed in varying concentrations of BSP and EGCG. The reaction rate for each data point was converted to percentage enzyme activity, plotted against the logarithm of inhibitor concentration. The IC_{50} was obtained by fitting the data to the sigmoidal dose-response curves - inhibition parameter on GraphPad Prism. It was observed that BSP inhibited WbGST with an IC_{50} of 5 μM (A), hGSTM1-1 13.5 μM (B), and hGSTP1-1 110 μM (C). The IC_{50} of EGCG for WbGST is 13 μM (D), hGSTM1-1 is 138 μM (E) and hGSTP1-1 is 361.2 μM (F). Each data point is representative of three separate experiments. The error bars reflect the standard deviation of each replication experiment.

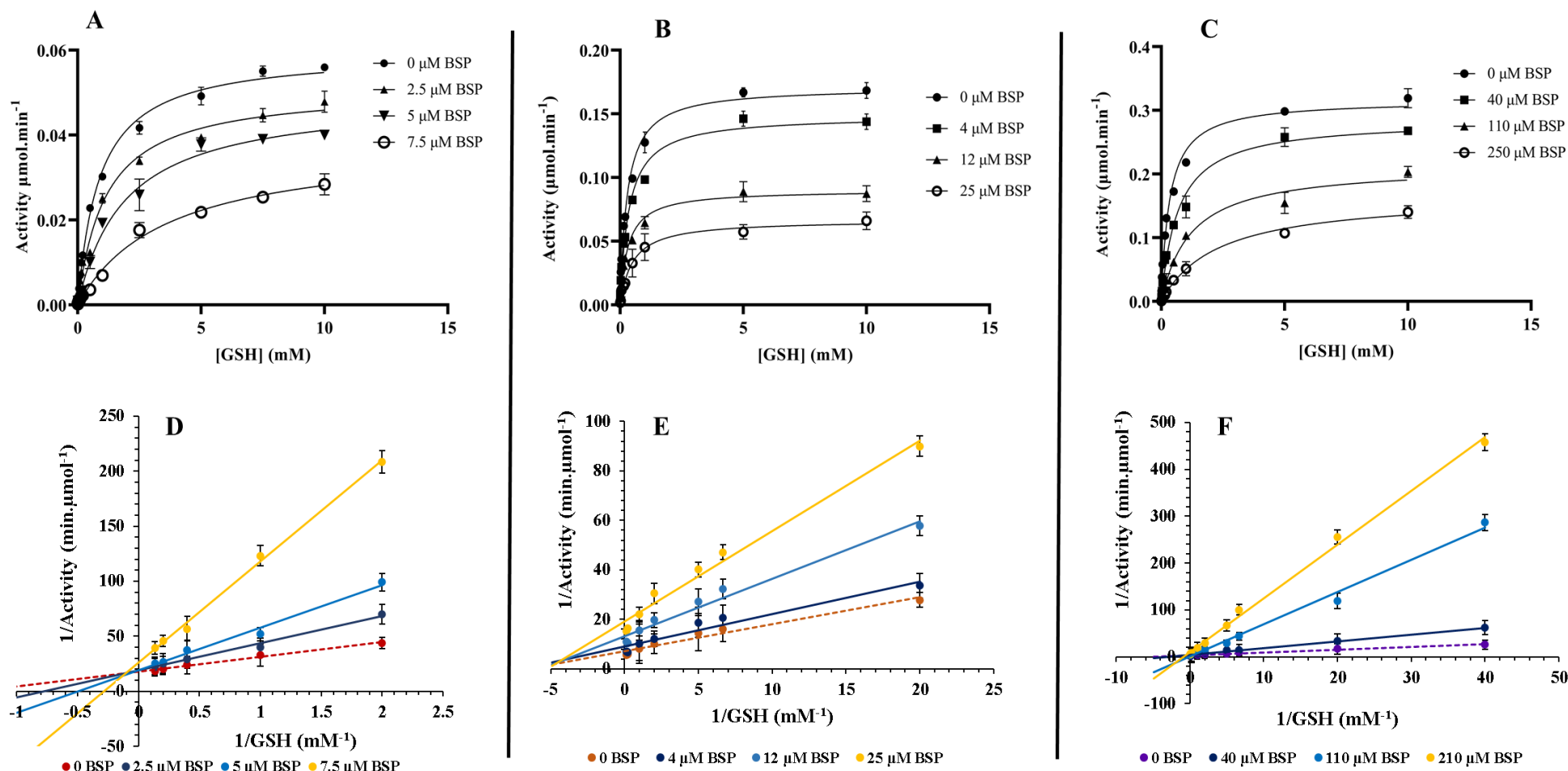


Figure 4.4 Michaelis Menten curves of GSH-BSP and accompanying transformations. Michaelis Menten curves of WbGST (A), hGSTM1-1 (B), and hGSTP1-1 (C), with varying concentrations of GSH in the presence of varied BSP concentrations. While keeping the CDNB concentration constant at 1 mM, the reaction was monitored at 340 nm for 60 s. Hyperbolic curves (●) represent enzyme reactions in the absence of BSP, (■) represents 2.5, 4, and 40 μM BSP incubated in WbGST (500 nM), hGSTM1-1 and hGSTP1-1 (50 nM) respectively. (▲) represents incubation of the enzymes (WbGST, hGSTM1-1, and hGSTP1-1) with 5 μM, 12 μM, and 110 μM BSP, respectively. (○) is the curve obtained when 7.5 μM, 25 μM, and 250 μM of BSP were added. The reaction velocity was plotted against a varying concentration of GSH. Lineweaver Burke double reciprocal plot of the rate of inactivation of WbGST (D), hGSTM1-1 (E), and hGSTP1-1 (F) activity as a function of the concentration of BSP using GSH as the substrate was obtained by transforming the Michaelis Menten curves. Mixed inhibition of WbGST was observed (D) while noncompetitive inhibition was observed for the hGSTM1-1 (E) and hGSTP1-1 (F) towards GSH. The error bars reflect the standard deviation of each replication experiment

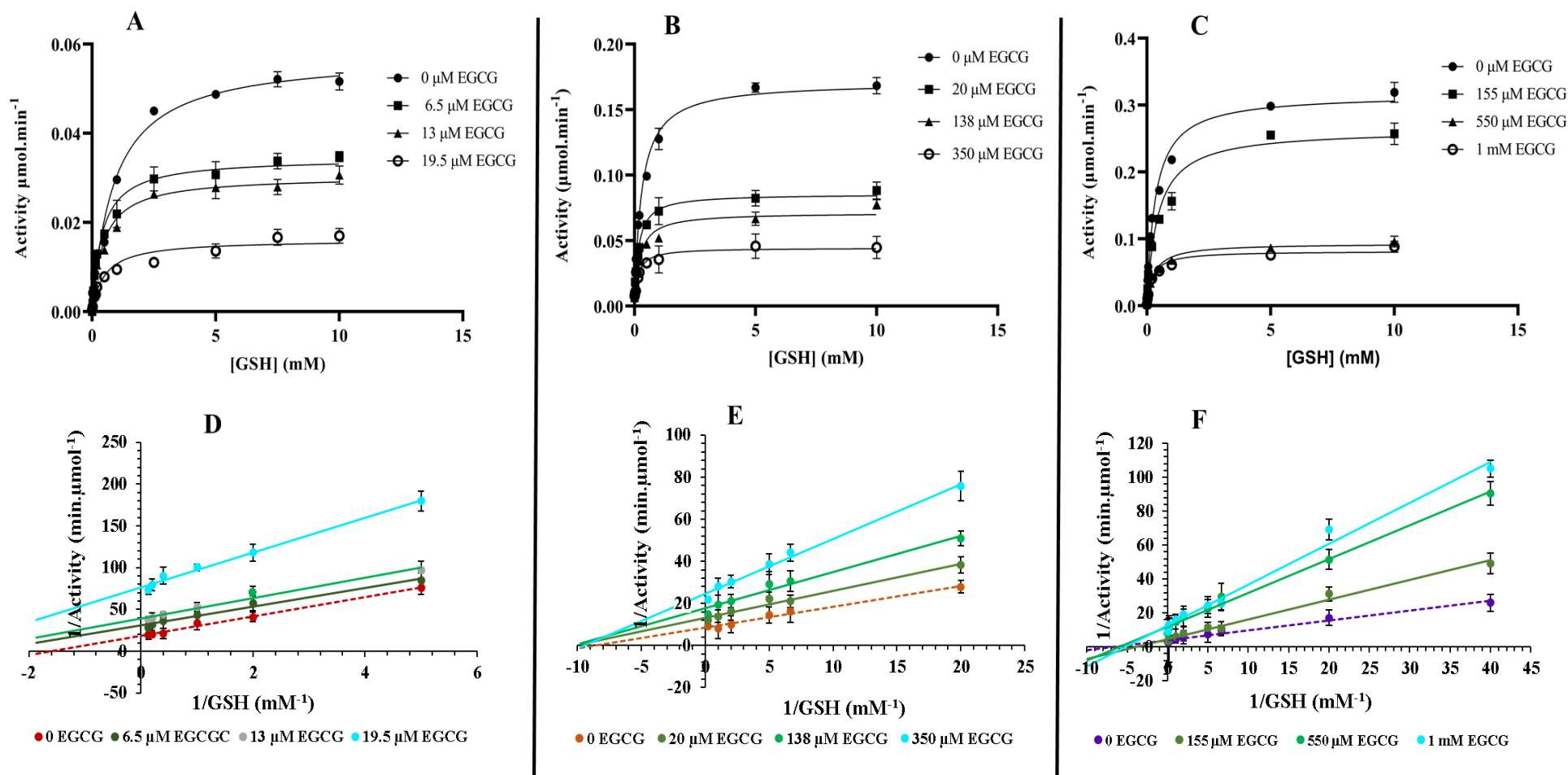


Figure 4.5 Michaelis Menten curves of GSH-EGCG and accompanying transformations. Michaelis Menten curves of WbGST (A), hGSTM1-1 (B), and hGSTP1-1 (C), with varying concentrations of GSH in the presence of varied EGCG concentrations. While keeping the CDNB concentration constant at 1 mM, the reaction was monitored at 340 nm for 60 s. Hyperbolic curves (●) represent enzyme reactions in the absence of EGCG, (■) represents 6.5, 20, and 155 μM EGCG incubated in WbGST (500 nM), hGSTM1-1 and hGSTP1-1 (50 nM) respectively. (▲) represents incubation of the enzymes (WbGST, hGSTM1-1, and hGSTP1-1) with 13 μM , 138 μM , and 550 μM EGCG respectively. (○) is the curve obtained when 19.5 mM, 350 mM, and 1 mM of EGCG were added. The reaction velocity was plotted against a varying concentration of GSH. Lineweaver Burke double reciprocal plot of the rate of inactivation of WbGST (D), hGSTM1-1 (E), and hGSTP1-1 (F) activity as a function of the concentration of EGCG using GSH as the substrate was obtained by transforming the Michaelis Menten curves. An uncompetitive mode of inhibition of WbGST (D) by BSP was observed, whereas, for the hGSTM1-1 (E), and the hGSTP1-1 (F) the noncompetitive inhibition was observed for both enzymes. The error bars reflect the standard deviation of each replication experiment.

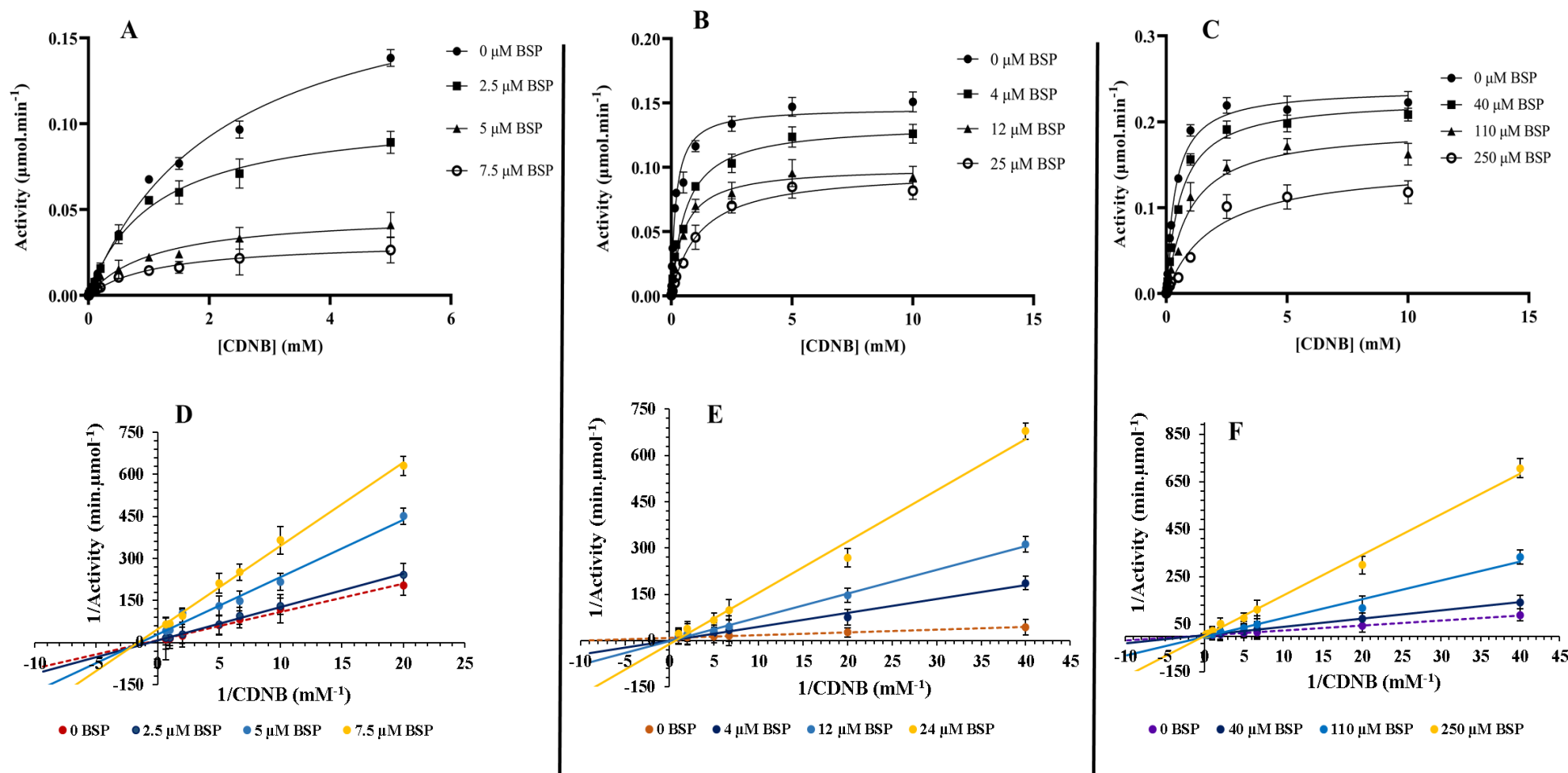


Figure 4.6 Michaelis Menten curves of CDNB-BSP and accompanying transformations. Michaelis Menten curves of WbGST (A), hGSTM1-1 (B), and hGSTP1-1 (C), with varying concentrations of CDNB in the presence of varied BSP concentrations. While keeping the GSH concentration constant at 1 mM, the reaction was monitored at 340 nm for 60 s. Hyperbolic curves (●) represent enzyme reactions in the absence of BSP, (■) represents 2.5, 4, and 40 μM BSP incubated in WbGST (500 nM), hGSTM1-1 and hGSTP1-1 (50 nM) respectively. (▲) represents incubation of the enzymes (WbGST, hGSTM1-1, and hGSTP1-1) with 5 μM , 12 μM , and 110 μM BSP, respectively. (○) is the curve obtained when 7.5 μM , 25 μM , and 250 μM of BSP were added. The reaction velocity was plotted against a varying concentration of CDNB. Lineweaver Burke double reciprocal plot of the rate of inactivation of WbGST (D), hGSTM1-1 (E), and hGSTP1-1 (F) activity as a function of the concentration of BSP using CDNB as the target substrate was obtained by transforming the Michaelis Menten curves. Mixed inhibition of WbGST (D) by BSP was observed, while the BSP exhibited a noncompetitive mode of inhibition towards both hGSTM1-1 (E) and hGSTP1-1 (F). The error bars reflect the standard deviation of each replication experiment.

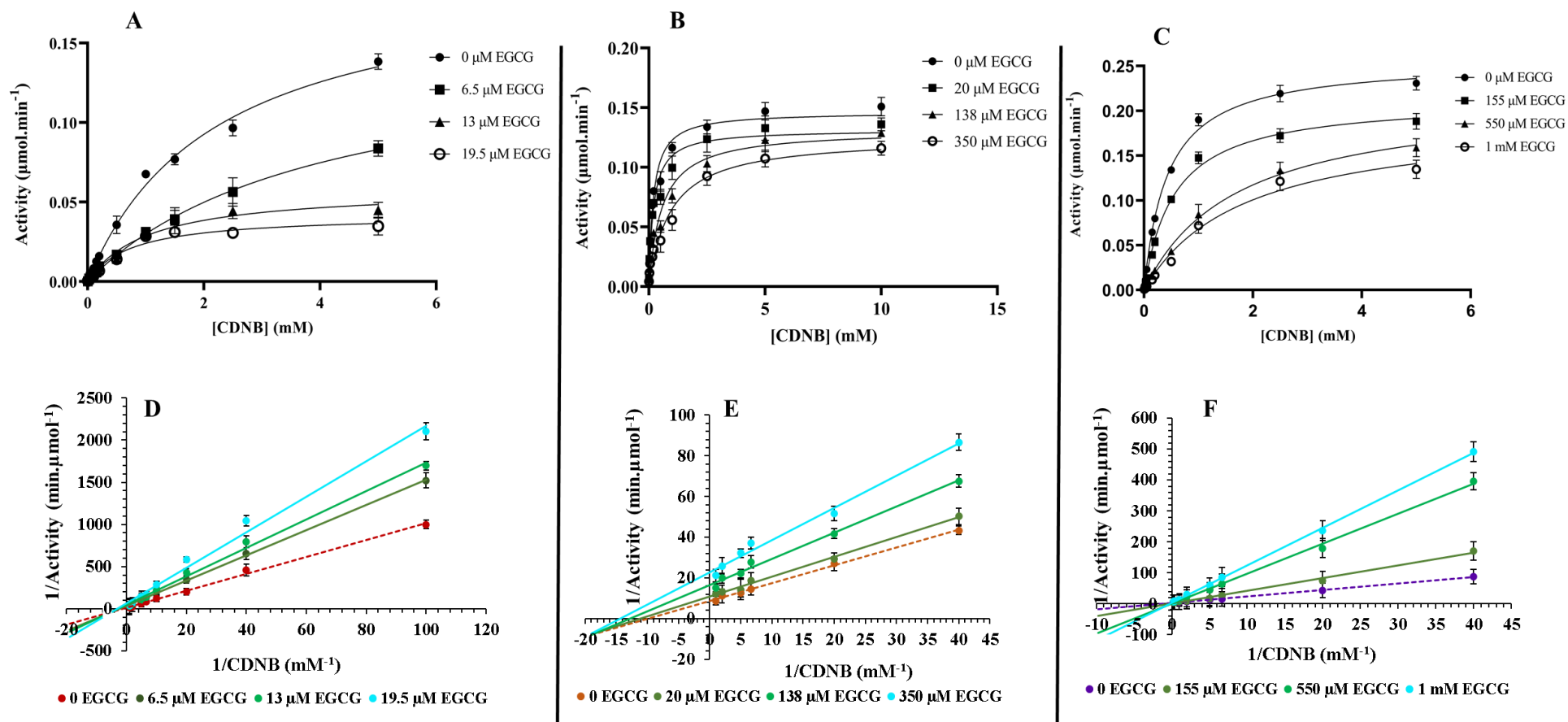


Figure 4.7 Michaelis Menten curves of CDNB-EGCG and accompanying transformations. Michaelis Menten curves of WbGST (A) hGSTM1-1(B) and hGSTP1-1 (C) with varying concentrations of CDNB in the presence of varied EGCG concentrations. While keeping the GSH concentration constant at 1 mM, the reaction was monitored at 340 nm for 60 s. Hyperbolic curves (●) represent enzyme reactions in the absence of EGCG, (■) represents 6.5, 20, and 155 μM EGCG incubated in WbGST (500 nM), hGSTM1-1 and hGSTP1-1 (50 nM) respectively. (▲) represents incubation of the enzymes (WbGST, hGSTM1-1, and hGSTP1-1) with 13 μM , 138 μM , and 550 μM EGCG respectively. (○) is the curve obtained when 19.5 mM, 350 mM, and 1 mM of EGCG were added. The reaction velocity was plotted against a varying concentration of CDNB. Lineweaver Burke double reciprocal plot of the rate of inactivation of WbGST (D), hGSTM1-1 (E), and hGSTP1-1 (F) activity as a function of the concentration of EGCG targeting CDNB as the substrate was obtained by transforming the Michaelis Menten curves. A mixed mode of inhibition of both WbGST (D) and hGSTM1-1 (E) by EGCG was observed, while noncompetitive inhibition was observed in hGSTP1-1 (F). The error bars reflect the standard deviation of each replication experiment.

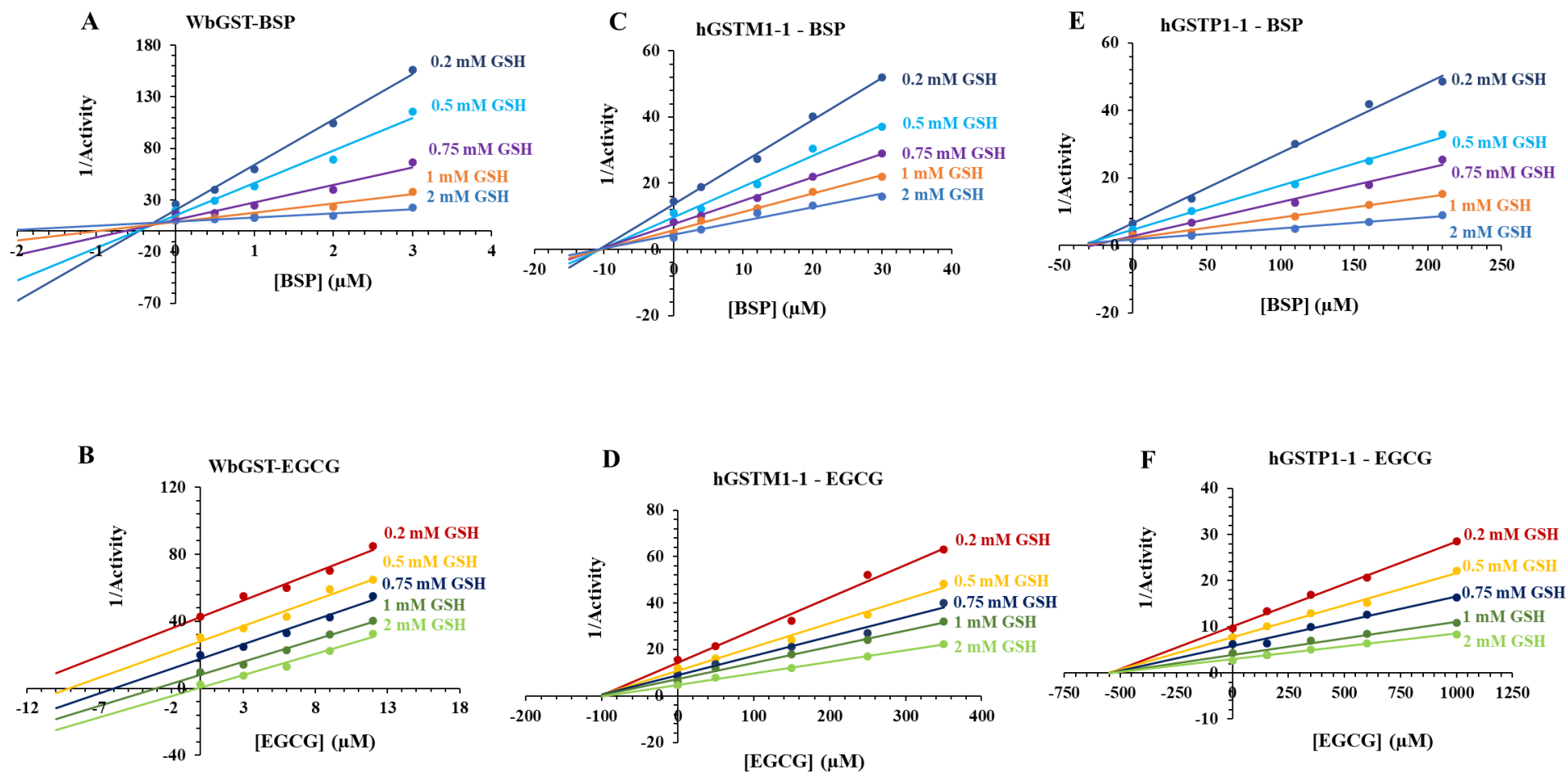


Figure 4.8 Dixon Plot. Mixed inhibition of WbGST (D) by BSP was observed, while in the case of hGSTM1-1 (C), and the hGSTP1-1 (E) noncompetitive inhibition was observed for both enzymes using the Dixon plot. An uncompetitive mode of inhibition of WbGST (B) by EGCG was observed, whereas, for hGSTM1-1 (D), and hGSTP1-1 (F) noncompetitive inhibition was observed.

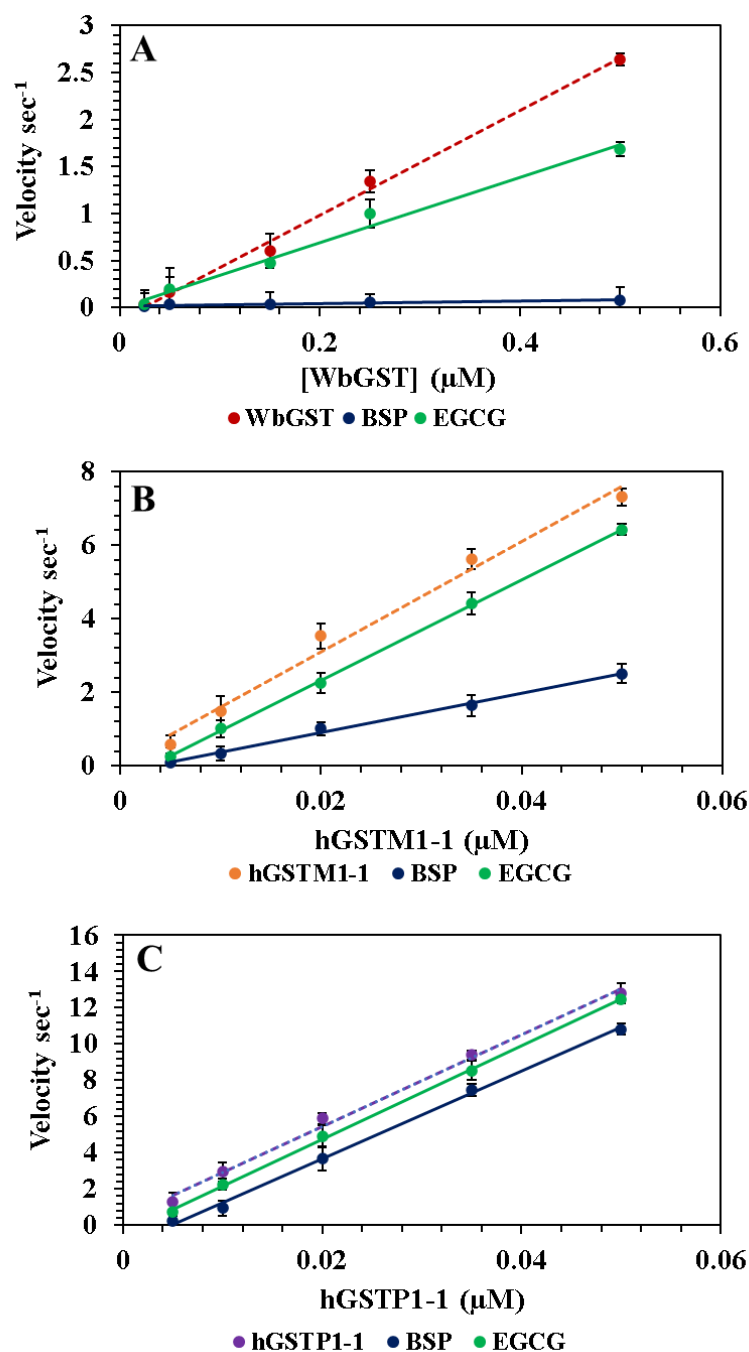


Figure 4.9 k_{cat} analysis of the enzymes. The k_{cat} was derived from the linear plots of varied enzyme concentrations against their velocities. The k_{cat} (0.002 sec^{-1}) of WbGST (**A**) in the absence of the ligand is represented in the red dotted line. The green and blue lines represent the k_{cat} in the presence of EGCG (0.001 sec^{-1}) and BSP (0.0004 sec^{-1}), respectively. (**B**) A k_{cat} of 0.0418 sec^{-1} was observed for the hGSTM1-1 (orange line), 0.038 sec^{-1} , and 0.015 sec^{-1} , the presence of EGCG (green line), and BSP (blue line), respectively. (**C**) The k_{cat} of hGSTP1-1 is 0.071 sec^{-1} (purple dotted line), 0.072 sec^{-1} , and 0.067 sec^{-1} for EGCG (green line) and BSP (blue line), respectively. The error bars reflect the standard deviation of each replication experiment.

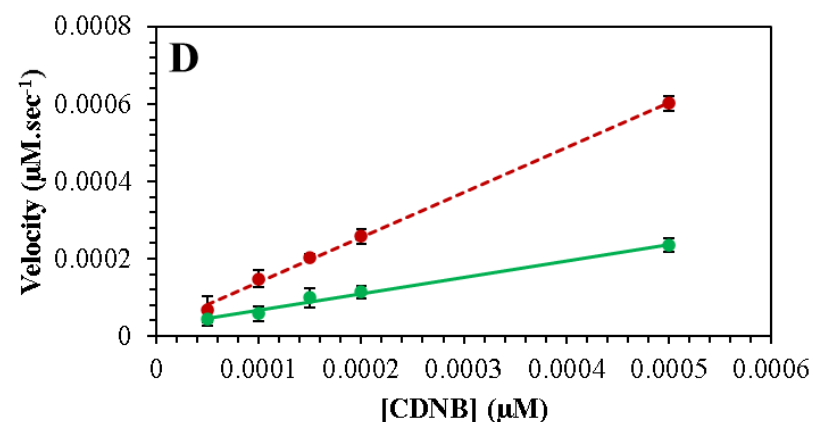
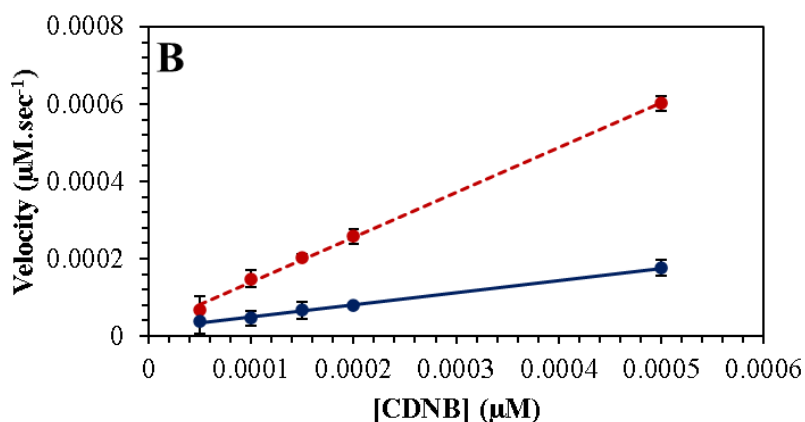
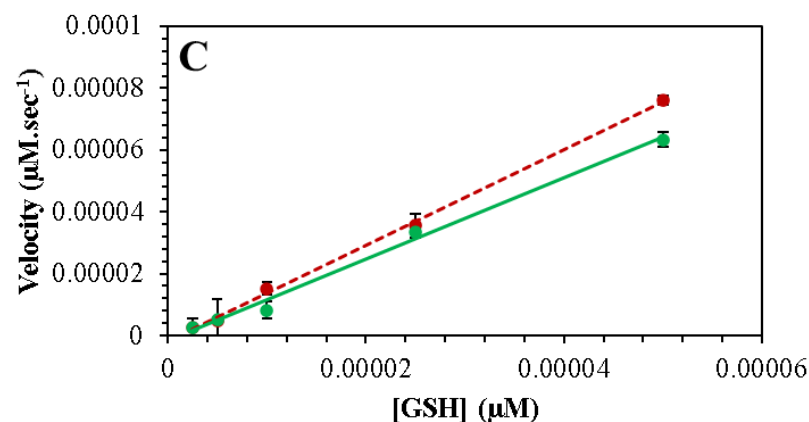
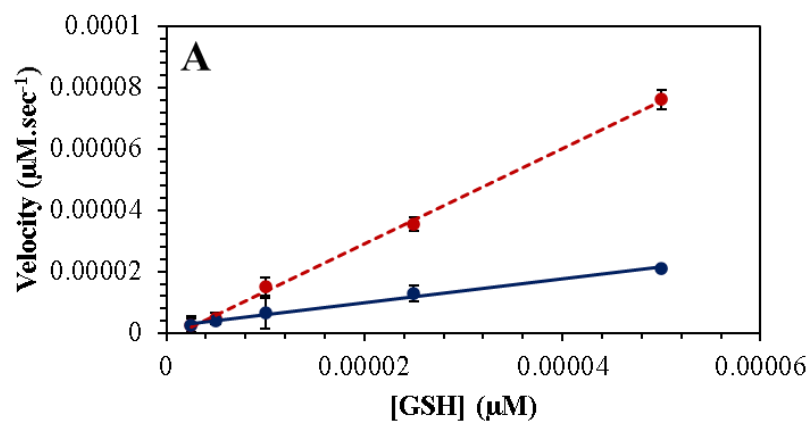


Figure 4.10 Experimental analysis of k_{cat}/K_M for WbGST. The k_{cat}/K_M was obtained experimentally in a condition where $[S] \ll K_M$ by varying substrate concentrations in the presence or absence of BSP and EGCG. The velocity was plotted as a function of substrate concentrations, and the resulting slope was used to calculate the k_{cat}/K_M . Varying concentrations of GSH (A) and CDNB (B) in the absence and absence of BSP (blue) and EGCG (green) in (C and D). The k_{cat}/K_M of WbGST concerning varied GSH and CDNB concentration in the absence of ligand is 3.1 and $2.32 \text{ M}^{-1}.\text{sec}^{-1}$, respectively. These values were observed to be reduced in the presence of BSP and EGCG. Results for hGSTM1-1 and hGSTP1-1 are in Appendix B (Figure S2)

Table 4.4 Enzyme kinetics parameters of GSH and CDNB towards the WbGST, hGSTM1-1 and hGSTP1-1

		GSH			CDNB			k_{cat} (sec ⁻¹)
		V_{max} ($\mu\text{mol.min}^{-1}$)	K_M (mM)	k_{cat}/K_M (M ⁻¹ .sec ⁻¹)	V_{max} ($\mu\text{mol.min}^{-1}$)	K_M (mM)	k_{cat}/K_M (M ⁻¹ .sec ⁻¹)	
WbGST	[BSP] (μM)							
	0	0.062	0.61	0.011	0.195	2.21	0.008	5.6
	2.5	0.051	1.14		0.107	1.09		
	5	0.049	1.91		0.048	1.09		
	7.5	0.038	3.59	0.003	0.032	1.16	0.002	0.13
	[EGCG] (μM)							
	0	0.062	0.61	0.011	0.195	2.21	0.008	5.6
	3.5	0.034	0.45		0.149	3.99		
	13	0.031	0.45		0.059	1.04		
	19.5	0.016	0.4	0.009	0.042	0.75	0.003	0.98
hGSTM1-1	[BSP] (μM)							
	0	0.171	0.29	0.4	0.146	0.19	0.77	150.4
	4	0.485	0.36		0.136	0.6		
	12	0.091	0.32		0.1	0.51		
	25	0.067	0.5	0.11	0.098	1.14	0.12	53.2
	[EGCG] (μM)							
	0	0.171	0.29	0.4	0.146	0.19	0.77	150.4
	20	0.086	0.16		0.131	0.21		
	138	0.071	0.21		0.131	0.56		
	350	0.044	0.15	0.12	0.125	0.93	0.4	137
hGSTP1-1	[BSP] (μM)							
	0	0.316	0.33	0.71	0.239	0.37	0.47	253
	40	0.285	0.68		0.227	0.59		
	110	0.213	1.19		0.194	0.97		
	250	0.167	2.29	0.08	0.152	2.01	0.076	241.8
	[EGCG] (μM)							
	0	0.316	0.33	0.71	0.256	0.43	0.47	253
	155	0.263	0.43		0.213	0.56		
	550	0.093	0.28		0.217	1.7		
	1000	0.082	0.21	0.3	0.193	1.83	0.09	257.7

4.5 Discussion

The GSH-CDNB assay is widely used to access the functionality of most GST proteins *in vitro*. The assay involves enzyme catalysation of the reaction between the GST natural substrate (GSH) and a xenobiotic compound (CDNB). The resulting coloured product (GS-DNB) absorbs light at 340 nm wavelength (Habig *et al.*, 1974). In this study, the specific activity, IC_{50} , mode of inhibition, and enzyme kinetics of WbGST, hGSTM1-1, and hGSTP1-1 were analysed using this assay method. The enzymes exhibited activity towards CDNB, which was evident by the observed increase in absorbance at 340 nm wavelength. The specific activity of WbGST is $1.2 \mu\text{mol min}^{-1}.\text{mg}^{-1}$ and it is comparable to the previously obtained value (Rathaur *et al.*, 2003). The comparable specific activity also indicates proper folding of the purified recombinant WbGST. Although WbGST exhibits lower activity when compared with hGSTM1-1 ($35 \mu\text{mol min}^{-1}.\text{mg}^{-1}$), hGSTP1-1 ($59 \mu\text{mol min}^{-1}.\text{mg}^{-1}$), this may be influenced by the differences in the H-site amino acid residue composition and the 'in' and 'out' possible conformational changes of Try7. It was also observed that the specific activity values obtained for the human GSTs (hGSTP1-1 and hGSTM1-1) were comparable to previous reports (Goodrich and Basu, 2012; Habig and Jakoby, 1981; Huang *et al.*, 2008). Subsequently, incubating the enzymes in the presence of BSP and EGCG shows enzyme activity inhibition (Figure 4.2).

Following the confirmation of the inhibition potential of the ligands, their IC_{50} was assessed by varying ligand concentrations while monitoring the GSH-CDNB conjugation catalysis of the enzyme. The IC_{50} value indicates the ligand 50% inhibition capacity towards the enzyme. An IC_{50} value of 5 μM and 13 μM were obtained for BSP and EGCG, respectively, towards WbGST (Figure 4.3 A and D). The values obtained are in the lower micromolar range of inhibition, indicating that BSP and EGCG are suitable candidates for effective inhibition of WbGST and could be modified for better efficiency. The IC_{50} of BSP and EGCG towards WbGST has not been previously determined, but the value obtained for BSP is comparable with the value obtained for a helminth GST, *Schistosoma Japonicum* (Padi *et al.*, 2021; Pooe *et al.*, 2021). On the other hand, the human GSTs were less inhibited by the ligands (Figure 4.3) with BSP showing more inhibitory capacity when compared to EGCG. Furthermore, the hGSTM1-1 was more susceptible to inhibition by the ligands than the hGSTP1-1. The IC_{50} values of BSP obtained for the human GSTs

in this study are consistent with those obtained in previous reports (Hamed *et al.*, 2011; Hirrell *et al.*, 1987; Mannervik *et al.*, 1988; Prapanthadara *et al.*, 2000). Although a study by Awasthi *et al.* reported that inhibition of human erythrocyte GST by BSP was not observed in their studies, instead, BSP appeared to be a substrate to the enzyme (Awasthi and Singh, 1984). On the other hand, there is limited information regarding the impact of EGCG on the activity of GSTs. However, the IC_{50} of EGCG towards equine liver GST was in the lower micromolar value of 1.57 μ M (Boušová *et al.*, 2012).

It is well-known that GST reactions adhere to the Michaelis-Menten equation concerning one substrate at a constant concentration of the other substrate. Due to time constraints caused by the COVID-19 pandemic and lockdown, other enzyme-catalysed reaction rate equations for two-substrate reactions were not considered in this study. The Albery general rate equation and Dalziel equation were also derived to study two-substrate enzyme reactions. The steady-state kinetics of the enzyme inhibition were analysed using GSH as a probe toward the G-site and CDNB as a probe for the H-site. The BSP and EGCG concentrations were varied based on their various IC_{50} values. Hyperbolic Michaelis-Menten kinetic plots (Figures 4.6 and 4.7) were obtained, indicating that there was no cooperativity of binding between the G-site and H-site of the enzymes (Berg *et al.*, 2015). The Michaelis constant (K_M) is a crucial aspect of the interaction between enzymes and their substrates, and it does not depend on the concentration of either the enzymes or the substrates. Therefore, K_M is the concentration value of the substrate equivalent to half of the maximum rate of reaction. This implies that half of the active sites are occupied. Secondly, the binding affinity of the Enzyme and substrates can be ascertained by the K_M values, where a high value indicates weak interaction and a low value signifies strong binding. The K_M values obtained for WbGST show that GSH (0.89 mM) has a higher affinity for WbGST than CDNB (2.21 mM). This attribute is consistent with what was observed in the hGSTM1-1 and hGSTP1-1. Likewise, most GSTs have been previously reported to display a similar pattern of affinity towards the two substrates (Armstrong, 1991). This phenomenon could be attributed to GSH being a natural enzyme substrate. However, hGSTM1-1 and hGSTP1-1 exhibit more affinity towards GSH than WbGST. This may result from the mutations in the active site of WbGST, Tyr49, and Cys47 in hGSTP1-1 being replaced by Phe49 and Phe47 in WbGST. On the other hand, the maximum rate of reaction, V_{max} , is the amount of substrate converted to product by a molecule of an enzyme per time

following enzyme saturation by the substrate. The $V_{\max}^{\text{GSH}}$ for WbGST ($0.06 \mu\text{mol. min}^{-1}$) is consistent with what has been observed in most parasite GSTs (Akumadu *et al.*, 2020; Padi *et al.*, 2021; Poee *et al.*, 2021). In contrast, the two human GSTs analysed in this study have a higher rate of converting substrate to products ($V_{\max}$) than WbGST (Table 4.4). This may explain why the specific activity observed for the human GSTs is much higher than that of WbGST (Figure 4.2).

Furthermore, the amount of substrate molecules converted by an enzyme into a product per time before the enzyme reaches maximum saturation with a substrate was also determined. This is also known as the turnover number of an enzyme k_{cat} attributed to $V_{\max}$. The human GSTs have the capacity to convert more substrate to the product compared to WbGST, as reflected in the higher value of their k_{cat} (Table 4.4 and Figure 4.9). Similarly, the kinetic efficiency, $k_{\text{cat}}/K_{\text{M}}$ (Figure 4.10), of WbGST towards CDNB and GSH was lower than what was observed for the human GSTs. This is in line with what was observed in the K_{M} values of the enzymes because the level of affinity of the substrates towards the enzyme influences their kinetic efficiency. Thus, the higher the substrate affinity, the higher the enzyme efficiency. However, the kinetic efficiency of the enzymes was reduced in the presence of the inhibitors, with BSP having the most effect.

The BSP and EGCG mode of inhibition towards the enzymes was determined by transforming the Michaelis Menten data into Lineweaver-Burke plots. Lineweaver-Burke plots are prone to errors due to the inflation of the reciprocals of $[S]$ derivations at low substrate concentrations. Thus, the overall shape of the graph and the mathematical derivatives are prone to flaws (S Ochs, 2010). Therefore, Dixon plots were obtained for comparison (Figure 4.8). The BSP showed a mixed form of inhibition towards the G-site as well as the H-site of WbGST. A mixed type of inhibition does not necessarily mean that the inhibitor is bound on two different active sites. Rather it is an indication that the binding of the inhibitor hampers substrate binding and decreases the k_{cat} of the enzyme (Berg *et al.*, 2015). In other words, BSP does not compete with either GSH or CDNB binding. However, BSP binding induces conformational changes that can affect substrate binding to the active sites. On the other hand, EGCG inhibited WbGST in an uncompetitive mode with respect to the G-site. This indicates that EGCG binds to the enzyme-substrate (ES) complex and has no affinity towards the free enzyme. Furthermore, the presence of EGCG tends to pull the system away from free WbGST towards the ES complex, consequently reducing the K_{M} (Lorsch,

2014). Hence, the binding of BSP and EGCG will not prevent GSH or CDNB from binding to their respective active sites on WbGST. Rather the inhibitors could bind at the H-site at the same time together with CDNB or at an alternative site often referred to as the L-site.

Naturally occurring plant phenolic compounds have been reported to inhibit GSTs primarily by mixed and uncompetitive modes of inhibition. For example, ellagic acid and curcumin inhibited GST isoforms in a mixed and uncompetitive manner for both G-site and H-site probably because they also bind at the dimer interface (Akumadu *et al.*, 2020; Burg and Mulder, 2002; Hayeshi *et al.*, 2007). Secondly, previous studies have reported alternative ligand binding sites on GSTs, especially at the dimer interface of parasite GSTs (Akumadu *et al.*, 2020; McTigue *et al.*, 1995). Thus, both BSP and EGCG can bind at the electrophilic active site and also at the dimer interface of WbGST, resulting in the observed modes of inhibition.

Regarding the human GSTs, both BSP and EGCG showed non-competitive inhibition towards the hGSTM1-1 and hGSTP1-1 active sites, except for EGCG which displayed the mixed type of inhibition towards the H-site of the hGSTM1-1. This implies that the ligands may not bind to the active sites in both human isozymes. However, owing to the promiscuous properties of the H-site and the small size of CDNB, it is possible that the site can accommodate both the inhibitors and CDNB concurrently. Similarly, a previous study suggested that BSP binds to the hGSTP1-1 on two sites located within the H-site, this second site is different from the buffer binding site previously identified (Oakley *et al.*, 1999). The observed mixed inhibition of hGSTM1-1 by EGCG suggests that the binding of EGCG to the H-site could result in some conformational changes, making the dimer interface accessible and allowing the binding of EGCG. Studies have shown that the GSTM1-1 isoenzyme possesses a ligandin binding site located at the dimer interface similar to what has been observed in some parasite GSTs (Hornby *et al.*, 2002).

Both Lineweaver-Burke and Dixon plots showed similar modes of inhibition. But Dixon plots displayed clearer intersection points on the plots.

Chapter 5

Protein structural characterisation

5.1 Introduction

This chapter describes the effect of BSP and EGCG on the structure of WbGST, hGSTM1-1, and hGSTP1-1. The ability of an enzyme to catalyze a chemical reaction is intricately linked to the sequence of its amino acids, the resulting tertiary structure and how individual amino acid residues interact with substrates or non-substrate molecules (Segel, 1975). The inherent features and exact order of these molecular interactions also help to define their catalytic mechanism. However, all these cannot be elucidated without the structural knowledge of the enzymes and at large proteins. This chapter analyses the major structural levels observable in proteins, namely: secondary, tertiary, and quaternary structures. Various spectroscopic methods applied in this study provided a wealth of structural knowledge about the interactions of BSP and EGCG with the enzymes.

Secondary structural characterisations of WbGST, hGSTM1-1, and hGSTP1-1 in the presence or absence of the inhibitors were performed by using Far-UV circular dichroic (CD) spectropolarimetry. This technique measures the difference between the absorption of right and left circularly polarised light of the polypeptide backbone chiral chromophores (Campbell and Dwek, 1984). Different secondary structural elements (β -strands, α -helices, and loops) produce distinct spectra resulting from the interaction of circularly polarized light. The tertiary structure was characterised by intrinsic and extrinsic fluorescence spectroscopy. Tryptophan residues were used as a probe for the intrinsic fluorescence, while 8-anilino-1-naphthalenesulphonic acid (ANS) was used as an extrinsic probe. Fluorophores such as tryptophan and ANS absorb radiation energy at a particular wavelength and, in turn, emit the energy at a longer wavelength. Concerning intrinsic protein fluorescence, the indole ring of tryptophan is sensitive to the polarity of its surrounding environment. Thus, its location can give insight into structural changes observed in proteins. ANS is a fluorescent dye that can provide further insight into the structural attributes of a protein. Like tryptophan, ANS is also susceptible to changes in the surrounding environment which is a valuable characteristic of fluorophores. Therefore, ANS is a suitable extrinsic probe for hydrophobic patches on proteins and a

probe of the active site of enzymes. Change in fluorescence properties of the dye is usually observed consequent to the interaction of the dye with a hydrophobic pocket on the protein. The resulting information obtained from the observed fluorescence changes in the presence or absence of the inhibitors provided an understanding of possible structural conformation and the effect of ligand binding to the proteins. Estimation of the quaternary structure was achieved by using high-pressure liquid chromatography (SE-HPLC) and dynamic light scattering (DLS) techniques. The SE-HPLC separates molecules according to their molecular sizes (Porath and Flodin, 1959). Thus, the technique is dependent on the molecular separating properties of utilised permeable materials. Considering that the size accuracy of protein separation using SE-HPLC may be affected by the overall compactness (shape) of the protein and hydrophobic column interactions, the DLS technique was also utilised. DLS detects fluctuations of scattered light intensity resulting from the Brownian motion of molecules in solution. The DLS experiment provides insight into the diffusion coefficient of scattering particles, resulting in the determination of the hydrodynamic radius of molecules (Schmitz and Phillies, 1991).

5.2 Materials

Table 5.1 is a list of some chemical reagents that were used in this chapter, and all other reagents are of analytical grade.

Table 5.1 List of materials and their respective suppliers.

Materials	Source	Location
ANS	Sigma-Aldrich	St. Louis, MO, USA
GSH	Sigma-Aldrich	St. Louis, MO, USA
BSP	Sigma-Aldrich	St. Louis, MO, USA
EGCG	Angene	Hyderabad, India
Snakeskin™ dialysis tubing	ThermoFisher Scientific	Waltham, MA, USA
Dithiothreitol (DTT)	New England Biolabs	Massachusetts USA
Gel filtration standards	Bio-Rad	Hercules, CA, USA

5.3 Methods

5.3.1 Secondary structure content characterisation

Circular dichroism (CD) between 180 and 240 nm was used to analyse the secondary structure content of WbGST, hGSTM1-1, and hGSTP1-1 in the presence or absence of GSH, BSP, and EGCG. The technique provides conformational studies of biomolecules and biopolymers. The backbone conformation of a protein is chiral such that proteins absorb left and right circularly polarised light to different degrees depending on the wavelength (Greenfield, 2006). Information about secondary structural components of the protein, namely the α -helices, and β -sheets, was deduced from the data obtained. Circular dichroism analysis can be conducted in either the far-UV CD region or the near-UV CD region. For this experiment, far-UV-CD analysis was employed, which covers wavelengths from 180 to 240 nm. This range is particularly useful for analyzing the secondary structure of proteins because the protein-peptide backbones, specifically amino acids like tryptophan and tyrosine, absorb circularly polarized light in this region. In proteins predominantly composed of α -helices, absorption occurs at 208 and 222 nm with negative ellipticity, and at 190 nm with positive ellipticity (Figure 5.1). Conversely, proteins with β -sheet conformations absorb right polarized light at 218 nm with negative ellipticity and at 195 nm with positive ellipticity.

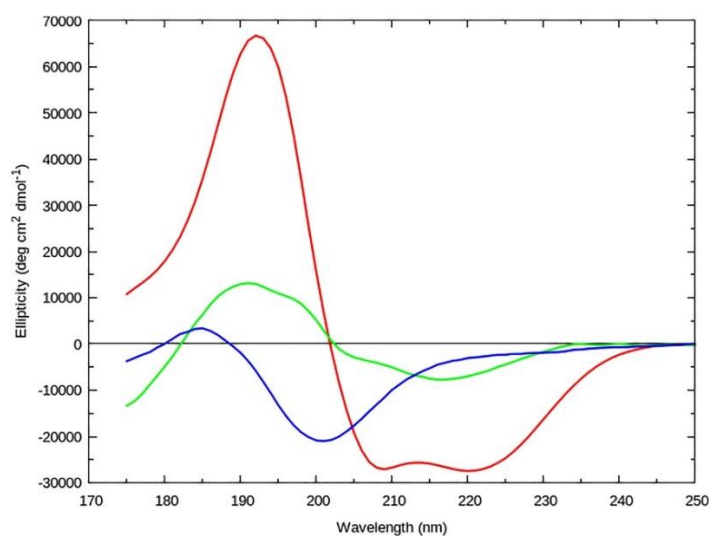


Figure 5.1 Illustration of CD Spectra depicting three secondary structures. CD spectrum for a predominantly α -helical protein (red line), β -sheet protein (green line), and random coil protein (blue line).

In this study, far-UV CD spectra of the three proteins of this study were measured at 20°C on a Jasco J-810 spectropolarimeter between 180 and 250 nm at 20°C. The accompanying software Spectra Manager version 1.5.00 was used to monitor the progress of the experiment. Protein samples were dialysed against 5 mM sodium phosphate pH 7.4, 0.01% (w/v) NaN₃, 10% (v/v) glycerol, and 1 mM TCEP to reduce the signal-to-noise ratio. Subsequently, 2 µM of each protein sample were analysed in the absence and presence of 1 mM of GSH, 30 µM BSP, and 30 µM EGCG. All spectra were recorded at a scan rate of 200 mm.min⁻¹, data pitch of 0.2 nm, a bandwidth of 0.5 nm, and 1 s response time, using a 2 mm pathlength quartz cuvette. The spectra were captured as an average of 5 accumulations and were all buffer-corrected. Mean residue ellipticities $[\theta]$ (θ.cm².dmol⁻¹) were normalised using the formula below:

$$[\theta] = \frac{100 \times \theta}{cnl} \quad (5.1)$$

where:

θ is the measured ellipticity signal in mdeg.

c is protein concentration in mM.

n is the total number of residues in the protein chain.

l is the path length in cm.

Percentages of secondary structural elements for each protein were estimated using the Dichroweb algorithm server (Whitmore and Wallace, 2008).

5.3.2 Tertiary structure characterisation

Intrinsic tryptophan fluorescence spectroscopy

Certain molecules can absorb electromagnetic radiation of particular energies inducing an energetically higher excitation state. Some of the absorbed energy may under specific conditions be re-emitted as electromagnetic radiation, and occasionally as visible light. The emitted light always has a longer wavelength (lower energy) than that of the absorbed photons. Fluorescence spectroscopy allows the energy of the emitted radiation to be measured. The difference in energy between excitation and emission peaks is called Stoke's shift. It results from the loss of energy due to vibrational relaxation within the excited molecule. This technique is usually accurate at a lower

concentration of protein sample because fluorescence intensity increases as sample concentration increases. The excitation wavelength of the tryptophan indole group, which is highly sensitive to the polar environment, is usually 295 nm, and the emission is between 300 nm and 350 nm. In tryptophan, stoke shifts usually occur, which implies that the emission wavelength is dependent on the tryptophan environment to a large extent and it can be affected by exposure to a hydrophilic environment. When the tryptophan residues are buried (hydrophobic environment) they exhibit high quantum yield, hence high fluorescence intensity occurs. The three-dimensional structure of WbGST was characterised in comparison to those of the hGSTM1-1 and hGSTP1-1, using this technique. The profile of the spectrum depends on the polarity of tryptophan residues. WbGST contains one tryptophan residue on each subunit on the N-terminus domain as shown in (Figure 5.2). Whereas the hGSTM1-1 and hGSTP1-1 have three and two tryptophan residues per monomer, respectively.

The fluorescence spectra of the protein samples were monitored using Jasco FP-6300 with Spectra Manager software v1.5.00 (Jasco Inc., Tokyo, Japan). Samples were analysed in the presence or absence of GSH, BSP, and EGCG at 20°C, using a 10 mm path length quartz cuvette. Fluorescence emission spectra of 3 µM protein in PBS storage buffer (Appendix A), were recorded in triplicate and an average of three accumulations. To probe the effect of BSP and EGCG on WbGST, hGSTPM1-1, and hGSTP1-1, 30 µM of the inhibitors were incubated with 3 µM of protein in the presence or absence of GSH for 30 min and the fluorescence emission spectra recorded afterwards. Emission spectra were obtained using excitation wavelength at 295 nm, 200 nm.min⁻¹ scan speeds, slit widths of 5 nm (excitation), and 2.5 nm (emission). Fluorescence emission was collected between 280 nm and 500 nm (to show Rayleigh scatter) and all spectra were also buffered. By using equation 5.2, the fluorescence intensities in the presence of BSP and EGCG were also normalized to account for the inner filter effect for any absorption of excitation (primary) and emission (secondary) of light:

$$F_{corr} = F_{obs} \times 10^{\left(\frac{OD_{ex} + OD_{em}}{2}\right)} \quad (5.2)$$

where F_{corr} and F_{obs} are the corrected and observed fluorescence intensities, respectively. While OD_{ex} and OD_{em} are the optical density measurements at excitation and emission wavelengths, respectively.

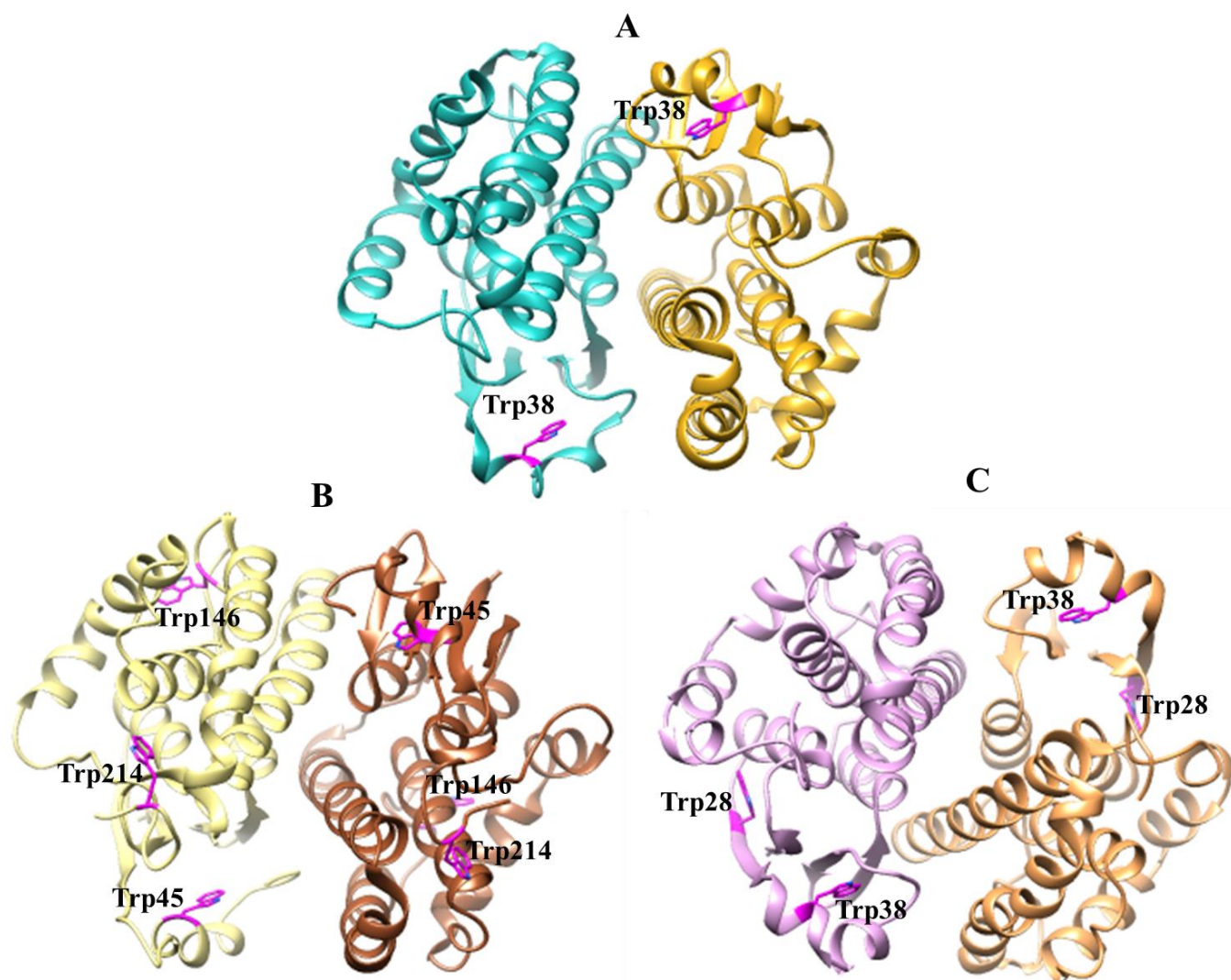


Figure 5.2 Positions of tryptophan residues on the proteins. A ribbon representation of WbGST (A), hGSTM1-1 (B), and hGSTP1-1 (C) showing the positions of the tryptophan residues indicated in pink rings (PDB ID: 5D73, 1XW6, and 2GSS for WbGST, hGSTM1-1, and hGSTP1-1, respectively).

Extrinsic ANS-fluorescence spectroscopy

Extrinsic ANS-fluorescence spectroscopy was used to probe ANS-accessible hydrophobic binding cavities in WbGST, hGSTM1-1, and hGSTP1-1. The fluorescence dye 8-anilino-1-naphthalene sulphonic acid (ANS) is an extrinsic fluorescent dye applied in various aspects of protein analysis, such as the characterisation of protein folding intermediates, measuring surface hydrophobicity and detecting aggregation or fibrillation (Hawe *et al.*, 2008). ANS has low fluorescence intensity in a polar environment, but upon interacting with proteins the intensity is increased. Electrons of the dye molecules are excited by absorbing light and they move from the ground state to the higher state (excited state). Processes such as vibrational relaxation and/or internal conversion, solvent relaxation, and twisted intra-molecular charge transfer usually compete with fluorescence. This may lead to loss of energy and consequently a red shift of fluorescence emission due to absorption (Stokes shift). The change in polarity will increase the intensity and quantum yield ($\Delta\Phi$), resulting in a blue shift between 500 nm and 480 nm wavelengths.

Initially, ANS (10 mM) stock solutions were prepared (Appendix A), and the concentration was measured spectroscopically by monitoring its absorbance at 340 nm. An extinction coefficient (ϵ) of 5000 M⁻¹cm⁻¹ (Weber and Young, 1964) at 350 nm, was used to calculate the concentration. The solution was wrapped in foil paper to prevent photodecomposition, and it was stored at 4°C. Subsequently, 3 µM of protein samples were incubated with 100 µM ANS in the PBS storage buffer (Appendix A), for 1 h in the dark to enhance the proper binding of ANS to the proteins. Blank samples containing only ANS were also prepared. All the samples were analysed using Jasco FP-6300 fluorimeter with excitation at 390 nm, 20 °C, with 5 nm slit widths, and emission was monitored between 400-600 nm wavelengths at a scan speed of 200 nm.min⁻¹. The spectra of free ANS were subtracted from ANS-bound protein spectra in the presence and absence of BSP and EGCG.

5.3.3 Quaternary structure characterisation

The hydrodynamic volume of the proteins was deduced by using size exclusion-high pressure liquid chromatography (SE-HPLC) and dynamic light scattering techniques (DLS). Analytical SE-HPLC is a technique that separates proteins based on hydrodynamic size, surface properties, and

diffusion coefficient. This technique monitors the retention of the protein in comparison with a set of protein standards. It is often preferred to size exclusion chromatography (SEC) due to its higher resolution and rapid molecule separation (Tayyab *et al.*, 1991). However, tiny-sized protein molecules permeate through the pores in the beads, and their movements become retarded. In contrast, the larger proteins migrate freely around the beads and elute before the smaller molecules. The diameter of the column (TOSOH Bioscience HPLC column) is 4.6 mm, and a length of 30 cm filled with permeable silica beads arranged 3-dimensionally to retard the migration of the molecules through the stationary phase of the column.

Initially, the column was coupled to a TSKgel UP-SW3000 DC Guard Column connected to a Shimadzu Prominence HPLC system (SPD20A). Subsequently, the column was equilibrated with the equilibration buffer (Appendix A) using a flow rate of 0.25 mL.min⁻¹ maintained for all sample analyses at 20 °C. Following column equilibration, 20 µL of 50 µM protein sample was centrifuged and loaded into the column. Afterwards, column calibration was performed using Bio-Rad Gel Filtration standards containing Bovine thyroglobulin (670 kDa), γ-globulin (150 kDa), Ovalbumin (44.3 kDa), Ribonuclease A type I-A (13.7 kDa) and P-aminobenzoic acid (pABA). To ascertain the effect of BSP and EGCG on the native size of the proteins, 50 µM of protein samples were incubated with 30 µM of each inhibitor for 1 h. Then the mixture was loaded into the column and equilibrated with the equilibration buffer containing 30 µM BSP or EGCG. The quaternary structure of WbGST, hGSTM1-1, and hGSTP1-1 analysed both in the presence and absence of the inhibitors were calculated using a calibration curve of the gel filtration standards used to calibrate the column. All samples were analysed in triplicate.

On the other hand, dynamic light scattering (DLS) is a non-invasive technique for measuring the translational diffusion coefficient (DT) of a macromolecule undergoing Brownian motion in solution (Ferré-D'Amaré and Burley, 1994; Schmitz and Phillies, 1991). Usually, samples at equilibrium are illuminated with monochromatic light, resulting in light scattering based on concentration and density fluctuations. The scattered light acquires a power spectrum mirroring the temporal evolution of the fluctuations (Schmitz and Phillies, 1991). Therefore, by scattering light from small particles, their geometrical structure and state of motion can be measured

(Goldburg, 1999). Hydrodynamic methods such as DLS can be applied to characterise the dynamic properties and particle size of proteins in solution.

5.4 Results

Maintaining structural integrity is crucial for proteins to function optimally. Hence, it's equally important to explore how the interaction between BSP and EGCG influences the various structural levels of the proteins examined in this study.

Evaluating the Secondary structure of the target proteins

The far-UV circular dichroism spectroscopy was used to assay the secondary structure content of the target proteins. CD spectra (Figure 5.3) for WbGST, hGSTM1-1 and hGSTP1-1 show prominent troughs respectively at 209 and 223 nm, 208 and 220 nm, 209 and 219 nm. The CD spectra most closely resemble those of predominantly α -helical proteins. Data deconvolution on DichroWeb using the CONTIN method of analysis suggests that the α -helical and β -sheet content of around 53.4 and 8.3%, 52.6 and 8.8%, 51.8 and 14.6% for WbGST, hGSTM1-1 and hGSTP1-1, respectively. This is in correlation with their crystal structures: (i) PDB: 5D73 (WbGST, 55 and 8.7%), (ii) PDB: 1XW6 (hGSTM1-1, 51 and 9.2%), and (iii) PDB: 2GSS (hGSTP1-1, 55 and 8.7%). The hGSTP1-1 showed the least ellipticity at the two prominent troughs, followed by WbGST, and hGSTM1-1 had the most ellipticity. The addition of BSP to WbGST causes a marginal increase in ellipticity at 209 nm and a decrease at 223 nm, both in the presence and absence of GSH. In contrast, EGCG induces an increase in ellipticity at both wavelengths and also in the presence or absence of GSH. In hGSTM1-1, BSP reduces the ellipticity in the absence and presence of GSH, and the reduction in ellipticity occurred only at 220 nm. On the other hand, an increase in ellipticity was observed in hGSTP1-1 bound to BSP and EGCG in the presence or absence of GSH. The results are tabulated in Table 5.2.

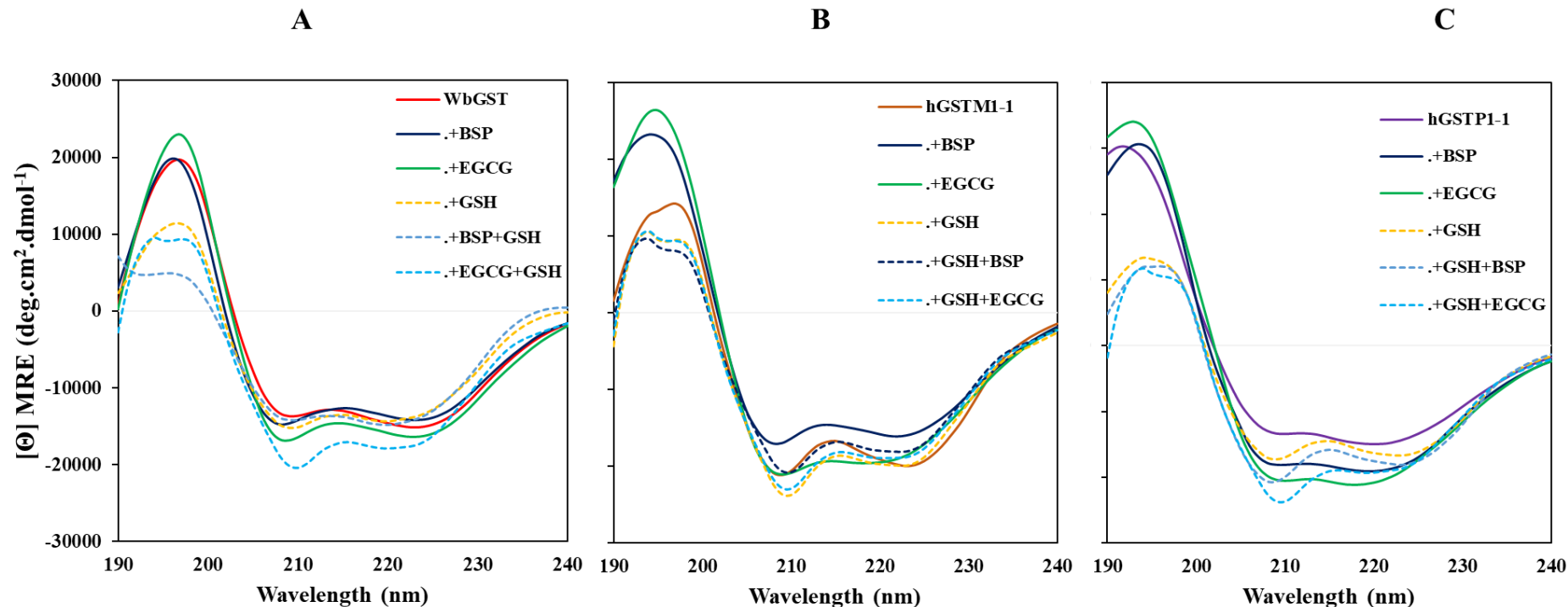


Figure 5.3 Assessing the secondary structure of the target proteins upon ligand binding. The secondary structure of the target proteins (2 μ M) was obtained in the presence or absence of the ligands and GSH at 20°C by far-UV CD in 5 mM Na_2HPO_4 pH 7.4, 1 mM DTT, and 0.01% NaN_3 . All spectra were measured in triplicate and, in the absence of the ligands, showed two prominent troughs at 208 nm and 222 nm, indicative of α -helix predominance. The presence of BSP did not change the secondary structure content of WbGST (A), but the α -helical content of hGSTP1-1 (C) was increased, and a reduction was observed for hGSTM1-1 (B). On the other hand, EGCG boosts the α -helical content of the target proteins

The secondary structural components of WbGST, hGSTM1-1, and hGSTP1-1 were derived from the DichroWeb program using the CONTIN algorithm and are tabulated in Table 5. 2.

Table 5.2 Secondary structural contents of the recombinant proteins.

		Structural motifs / %contents			
		Helix (%)	Strands (%)	Turns (%)	Disordered (%)
WbGST	Native	53.4	8.3	18.1	27.8
	BSP	54	7.3	18.6	27.6
	EGCG	62.9	9.5	15.5	25.5
	GSH	44.7	15	17.4	30.3
	GSH+BSP	51.9	13.1	19.1	30.7
	GSH+EGCG	49.9	4.8	18.5	34.2
hGSTP1-1	Native	51.8	14.6	18.1	25.3
	BSP	62.4	6.4	19.7	22.4
	EGCG	67.2	5.5	16.9	21.2
	GSH	53.2	9.2	49.3	28.2
	GSH+BSP	55.9	5.2	18.8	30.1
	GSH+EGCG	60.1	2.3	16	31.6
hGSTM1-1	Native	52.6	8.8	17.2	26.4
	BSP	47.4	8.6	16.4	27.5
	EGCG	54.6	5.4	16.3	23.8
	GSH	54	3.1	17.4	25.4
	GSH+BSP	48.4	3.5	16.2	31.8
	GSH+EGCG	51.3	3.4	16.8	28.5

Intrinsic tryptophan fluorescence as a probe of tertiary structure

Fluorescence spectroscopy was used to probe the microenvironment of tryptophan residues in the target proteins. WbGST has one tryptophan residue per subunit and has a fluorescence emission maximum at 344 nm. Corresponding maxima for hGSTM1-1 and hGSTP1-1 are observed at 341 and 342 nm, respectively (Figure 5.4). The emission maxima indicate a partial exposure of the tryptophan residues to a polar environment. Tryptophan residues are highly fluorescent, and the surrounding environment can influence their fluorescence properties. In polar environments, the fluorescence emission of tryptophan may be altered due to changes in the polarity and dielectric constant of the solvent. For example, the fluorescence intensity may increase or decrease, and the emission wavelength may shift depending on the specific properties of the polar environment. Trp38 in chain A has an accessible surface area (ASA) of 37.49 Å², whereas chain B has an ASA of 42.74 Å². As a result, the tryptophan residue in chain A may contribute more to the fluorescence emission peak at 344 nm. BSP binding to WbGST in the presence or absence of GSH induced a redshift in the emission maximum and decreased the quantum yield. EGCG similarly decreased the quantum yield but did not affect the peak wavelength.

Similarly, binding of the ligands decreased the fluorescence intensity of hGSTM1-1. However, a blueshift and redshift were observed in the presence of BSP and EGCG, respectively. There was no shift in the presence of GSH. There are four tryptophan residues (Trp7, Trp45, Trp146, and Trp214) per monomer of hGSTM1-1, and they are noted to have ASA of 29.55, 7.59, 24.34, and 51.66 Å² respectively. On the other hand, no shift was observed by the binding of the ligands to hGSTP1-1, but a decrease in quantum yield was observed. Furthermore, in the presence of GSH, a blueshift was observed in the binding of BSP, and no shift occurred concerning the binding of EGCG. There are two tryptophan residues (Trp28 and Trp38) per monomer in hGSTP1-1, and they have ASA of 52.67 and 26.87 Å², respectively.

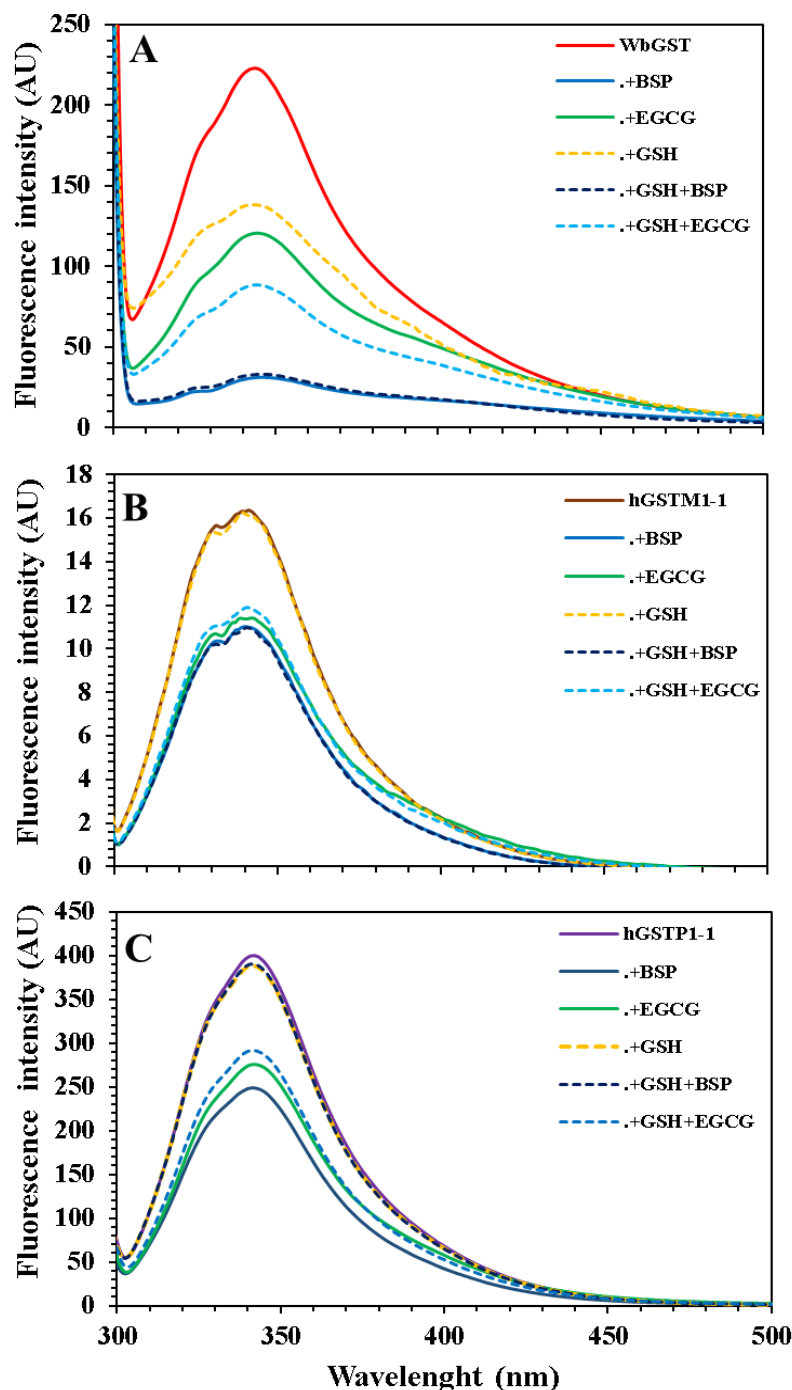


Figure 5.4 Intrinsic fluorescence of the target proteins. The tertiary structure of WbGST (A), hGSTM1-1 (B), and hGSTP1-1 (C) were analysed in the presence or absence of BSP, EGCG, and GSH. The analysis was performed using 5 μ M WbGST, 2 μ M of hGSTM1-1 and hGSTP1-1 in 20 mM Na_2HPO_4 pH 7.4, 2 mM DTT, 1 mM EDTA and 0.01% (w/v) NaN_3 at 20°C. The samples were excited at 295 nm, and ligand binding inner filter effects were corrected. Fluorescence spectra show that the binding of the ligands changes the polarity of the local environment of the tryptophan residues. The spectra represent the average of triplicate buffer-corrected experiments.

Table 5.3 Protein intrinsic fluorescence properties. This table summarises the fluorescence properties of WbGST, hGSTM1-1, and hGSTP1-1 and the effect of GSH, BSP, and EGCG binding on their fluorescence properties.

		Maximum emission wavelength (λ) nm	Shift relative to Apo (nm)	Type of shift relative to Apo	Quantum yield ($\Delta\Phi$) relative to Apo
WbGST	Apo	344	Baseline	Baseline	Baseline
	BSP	346	2	Redshift	Decrease
	EGCG	344	0	None	Decrease
	GSH	342	2	Blueshift	Decrease
	GSH+BSP	346	2	Redshift	Decrease
	GSH+EGCG	344	0	None	Decrease
hGSTM1-1	Apo	341	Baseline	Baseline	Baseline
	BSP	340	1	Blueshift	Decrease
	EGCG	342	1	Redshift	Decrease
	GSH	339	2	Blueshift	Baseline
	GSH+BSP	341	0	None	Decrease
	GSH+EGCG	341	0	None	Decrease
hGSTP1-1	Apo	342	Baseline	Baseline	Baseline
	BSP	342	0	None	Decrease
	EGCG	342	0	None	Decrease
	GSH	341	1	Blueshift	Baseline
	GSH+BSP	341	1	Blueshift	Baseline
	GSH+EGCG	342	0	None	Decrease

Extrinsic ANS fluorescence spectroscopy

Hydrophobic pockets of the recombinant target proteins were probed using ANS. It is an anionic dye that exhibits a lower quantum yield in an aqueous environment. However, when ANS binds to a hydrophobic pocket, the quantum yield increases in addition to a blueshift of the maximum wavelength. These changes offer an understanding of polarity and changes in the binding pockets of the target protein. The three target proteins (WbGST, hGSTM1-1, hGSTP1-1) in this study were

established to bind ANS, hence validating the presence of accessible hydrophobic pockets on the native conformation of the proteins. Exciting ANS bound to the target proteins at 390 nm increased the quantum yield with a blueshift from 513 nm to 478 nm, 496 nm, and 489 nm for WbGST, hGSTM1-1, and hGSTP1-1, respectively (Figure 5.5). This shift towards a shorter wavelength and increased quantum yield are typical of ANS binding to a hydrophobic patch on a protein (Gasymov and Glasgow, 2007; Stryer, 1965).

In the presence of both BSP and EGCG, a decrease in the quantum yield of ANS bound to WbGST was observed (Figure 5.5A). Upon binding of EGCG, a redshift relative to ANS-WbGST wavelength occurred, while no shift was observed for BSP. However, in the presence of GSH, the binding of BSP resulted in a blueshift and a redshift for EGCG. In contrast, the binding of the ligands to hGSTM1-1 resulted in a further blueshift from the wavelength of the ANS-hGSTM1-1 complex (Figure 5.5B). The quantum yield decreased upon binding of BSP and EGCG, but in the presence of GSH, the binding of BSP reduced the quantum yield while EGCG triggered an increase. As for the hGSTP1-1, the binding of both ligands to the protein resulted in an increased quantum yield with a blueshift occurring in the presence of BSP and none for EGCG (Figure 5.5C). However, in the presence of GSH, ligand binding decreased quantum yield with a redshift of the maximum emission wavelength. Table 5.4 shows a tabulated result of the ANS analysis.

Furthermore, the binding of the ligands resulted in the displacement of ANS. It was noted that BSP and EGCG displaced ANS from WbGST with an efficiency of 67 and 24%, respectively (Figure 5.6A). However, in the presence of GSH, the efficiency of ANS displacement by EGCG reduced to 14%, while BSP increased to 69%. Similarly, BSP and EGCG displaced ANS from hGSTM1-1 by 72% and 5%, respectively. Also, in the presence of GSH, 73% ANS displacement efficiency by BSP occurred, while no displacement by EGCG was observed. Interestingly, ANS was not displaced in hGSTP1-1 by the ligands, but a slight displacement was observed in the presence of GSH.

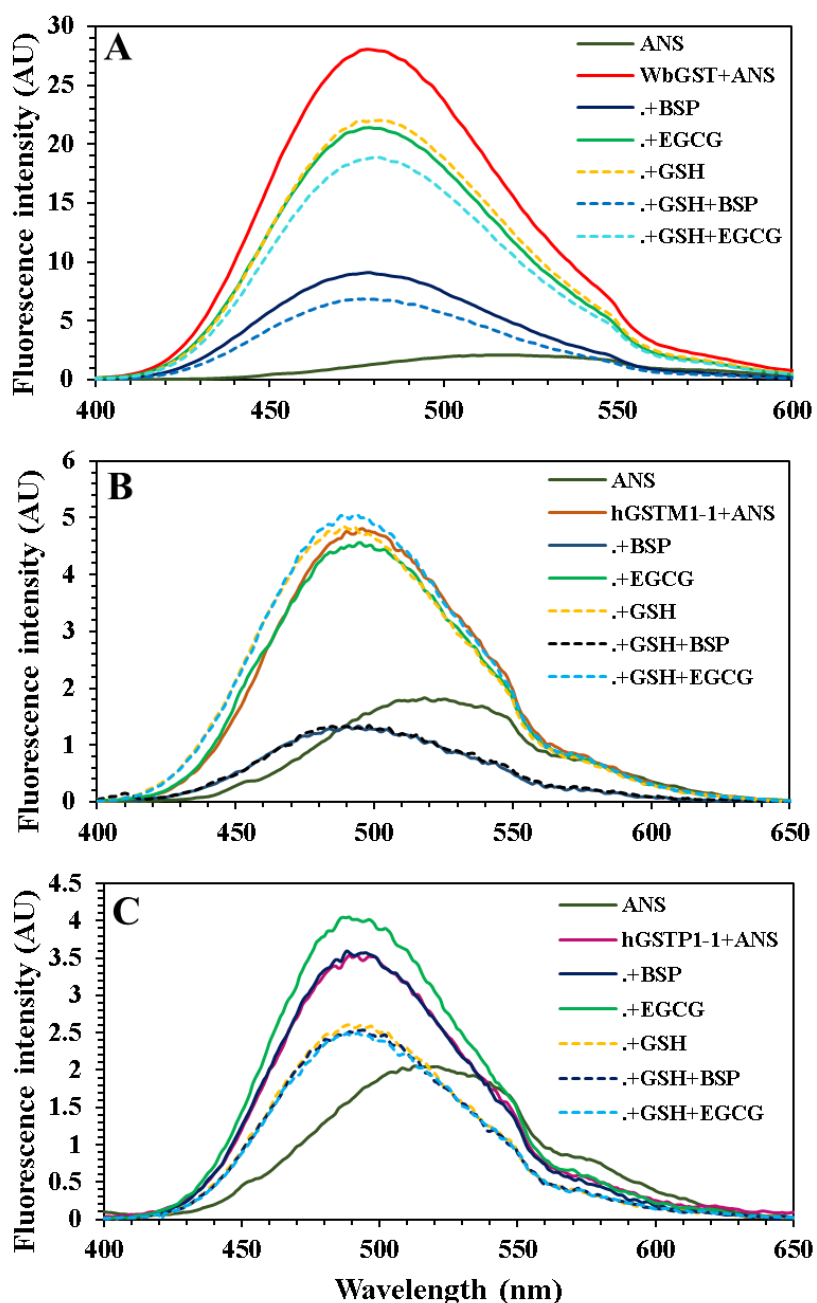


Figure 5.5 Extrinsic ANS fluorescence of the target proteins. Binding of ANS to WbGST (A), hGSTM1-1 (B), and hGSTP1-1 (C) in the presence or absence of BSP, EGCG, and GSH. The analysis was monitored using 2 μ M of protein incubated with 100 μ M ANS in 20 mM Na_2HPO_4 pH 7.4, 2 mM DTT, 1 mM EDTA and 0.01% (w/v) NaN_3 at 20°C. ANS was excited at 390 nm, and the binding of ANS to the proteins resulted in an increased quantum yield and blue shift. The binding of ligands to WbGST interfered with ANS binding, resulting in a decreased quantum yield, while the impact on hGSTM1-1 and hGSTP1-1 varies. The fluorescence intensity of WbGST bound to ANS was more than 80% higher than that of hGSTM1-1 and hGSTP1-1, indicating that WbGST has more accessible hydrophobic pockets. The spectra represent the average of triplicate buffer and inner filter effect corrected experiments.

Table 5.4 Extrinsic ANS fluorescence properties upon binding to target proteins. The table presents the impact of ANS binding to the target recombinant proteins in the presence or absence of GSH, BSP, and EGCG.

		^a Maximum λ (nm)	^b Quantum yield (Φ)	^c F/F ₀	$\Delta\Phi$ relative to Apo (nm)	Type of shift relative to Apo
WbGST	Free ANS	513	2.06	1	Baseline	Baseline
	No ligand	478	28.03	13.61	Baseline	Blueshift*
	BSP	478	9.11	4.43	Decreased	None
	EGCG	479	21.38	10.38	Decreased	Redshift
	GSH	483	22.02	10.69	Decreased	Redshift
	GSH+BSP	475	6.89	3.35	Decreased	Blueshift
	GSH+EGCG	481	18.88	9.17	Decreased	Redshift
hGSTM1-1	No ligand	496	4.81	2.34	Baseline	Blueshift*
	BSP	490	1.34	0.65	Decreased	Blueshift
	EGCG	495	4.56	2.22	Decreased	Blueshift
	GSH	489	4.85	2.36	Increased	Blueshift
	GSH+BSP	485	1.34	0.65	Decreased	Blueshift
	GSH+EGCG	488	5.04	2.45	Increased	Blueshift
hGSTP1-1	No ligand	489	3.53	1.72	Baseline	Blueshift*
	BSP	489	3.58	1.74	Increased	None
	EGCG	489	4.05	1.96	Increased	None
	GSH	489	2.6	1.26	Decreased	None
	GSH+BSP	493	2.53	1.23	Decreased	Redshift
	GSH+EGCG	492	2.48	1.21	Decreased	Redshift

^aWavelength (λ) at maximum fluorescence intensity

^bMagnitude at the maximum fluorescence intensity

^cF/F₀ ratio at maximum fluorescence intensity

*Blueshift of ANS bound to apo protein relative to free ANS

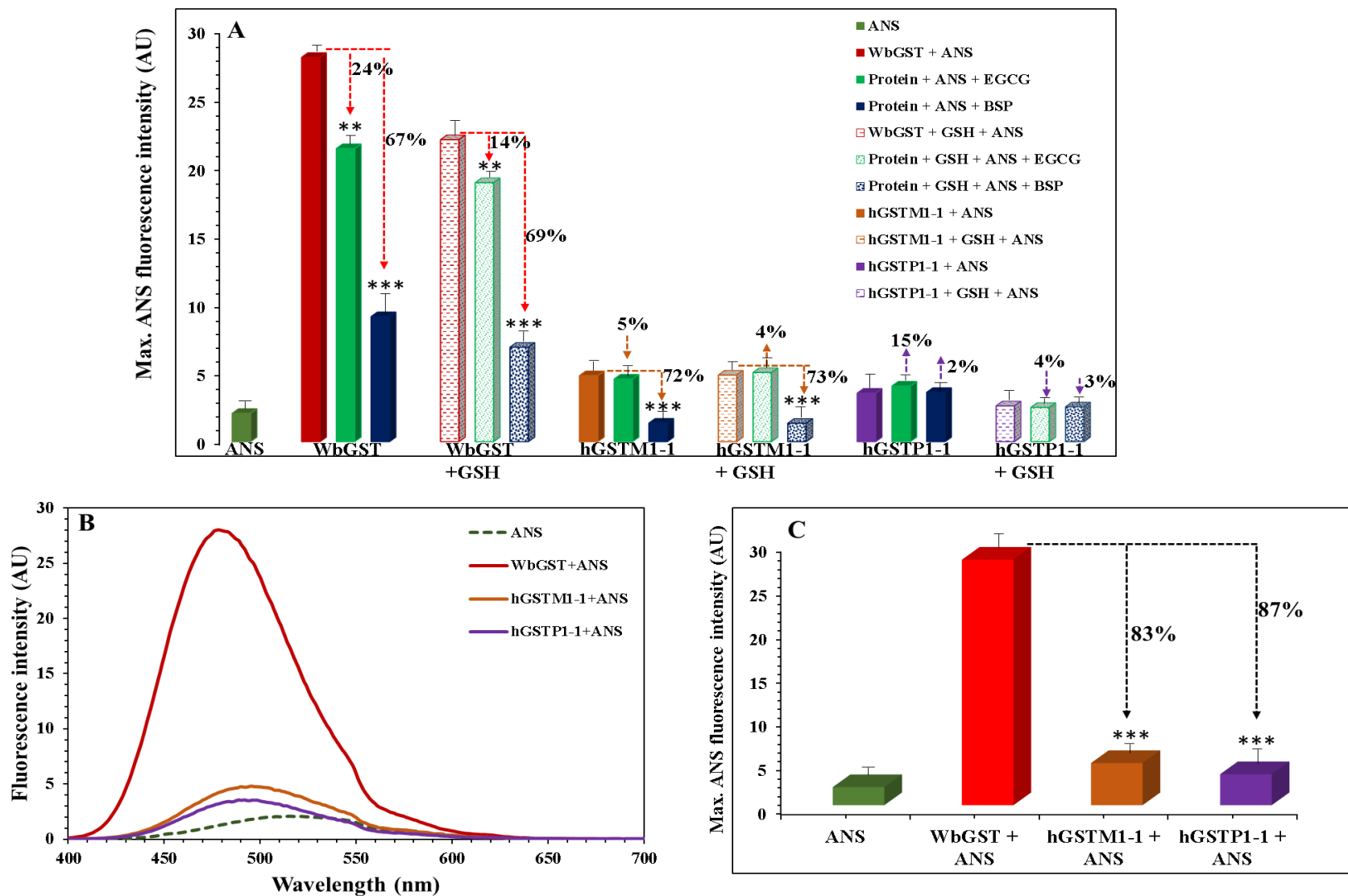


Figure 5.6 Ligand ANS displacement and comparison of ANS-protein quantum yield. Extrinsic ANS analysis was monitored using 2 μ M of protein incubated with 100 μ M ANS in 20 mM Na_2HPO_4 pH 7.4, 2 mM DTT, 1 mM EDTA and 0.01% (w/v) NaN_3 at 20°C. (A) Binding of EGCG and BSP to WbGST displaces ANS with an efficiency of 24% and 67%, respectively, while in the presence of GSH, 14% (EGCG) and 69% (BSP) efficiency was observed. Similarly, 72% displacement of ANS by BSP was observed while 5% was for EGCG binding, but in the presence of GSH, 72% was observed for BSP with non-by EGCG. In contrast, ligand binding did not displace ANS from hGSTP1-1. However, in the presence of GSH, a 4% (EGCG) and 3% (BSP) ANS displacement was observed. (B) WbGST has more accessible hydrophobic pockets than hGSTM1-1 and hGSTP1-1, as indicated by the high fluorescence intensity. Therefore, WbGST binds ANS more efficiently than hGSTM1-1 and hGSTP1-1 by 83% and 87%, respectively (C).

Evaluating the quaternary structures of the recombinant target proteins.

The oligomeric state of WbGST, hGSTM1-1, and hGSTP1-1 was assessed using SE-HPLC. Column calibration was performed by analysing gel filtration standards, and the resulting chromatogram was used to plot a standard curve of log molecular mass against retention time (Inset of Figure 5.7D). The resulting chromatogram for WbGST (Figure 5.7A) shows a single peak eluting at 11.5 ± 0.03 min in the presence of GSH and 12.1 ± 0.04 min in the absence of GSH. These retention times were estimated to be equivalent to ~ 48 kDa for the WbGST-GSH complex and ~ 26 kDa in the absence of GSH using the standard curve. Without DTT, WbGST undergoes oxidative aggregation, shown on the chromatogram with several peaks (Figure 5.7B). The first peak depicts a higher-ordered oligomer (soluble aggregate), while the second is eluted at 10.6 min. The binding of BSP to WbGST induces the formation of soluble aggregates eluting at 7.7 ± 0.02 min and a second peak at 12.4 ± 0.003 min. Consequently, the molecular weight of the peaks was estimated to be ~ 897 kDa for the soluble aggregates and ~ 18 kDa for the second peak. In contrast, the binding of EGCG did not initiate the formation of a higher-ordered oligomer. However, most of the protein eluted at a retention time (14.2 min) lower than the apo WbGST. Therefore, the estimated size of the first and second peaks on the calibration curve is ~ 26 kDa and ~ 2 kDa, respectively.

The SE-HPLC chromatogram for hGSTM1-1 (Figure 5.8A) shows a single peak both in the presence and absence of GSH, which eluted at 11.4 and 12 min, respectively. Consequently, the calculated molecular weight in the presence and absence of GSH was ~ 52 and ~ 36 kDa, respectively. Upon binding to BSP, the retention time was extended to 13 min, resulting in a lower molecular weight of ~ 17 kDa (Figure 5.8B). The chromatogram for the binding of EGCG to hGSTM1-1 indicates the presence of two peaks eluted at 13.6 and 15 min. Therefore, the calculated molecular size for both peaks is ~ 12 and ~ 2 kDa. In the case of hGSTP1-1 (Figure 5.8C), retention time in the absence of GSH was 11.8 and 11.4 min in the presence of GSH, with an estimated molecular weight of ~ 40 and ~ 47 kDa, respectively. Ligand binding resulted in the formation of two peaks (Figure 5.8D); the first and second peaks upon BSP binding eluted at 13.5 and 15.6 min, respectively. Thus, the estimated size was ~ 13 and ~ 2 kDa. As for EGCG, peaks one and two retention times are 13.7 and 15.4 min, with an estimated molecular weight of ~ 14 and ~ 5 kDa, respectively.

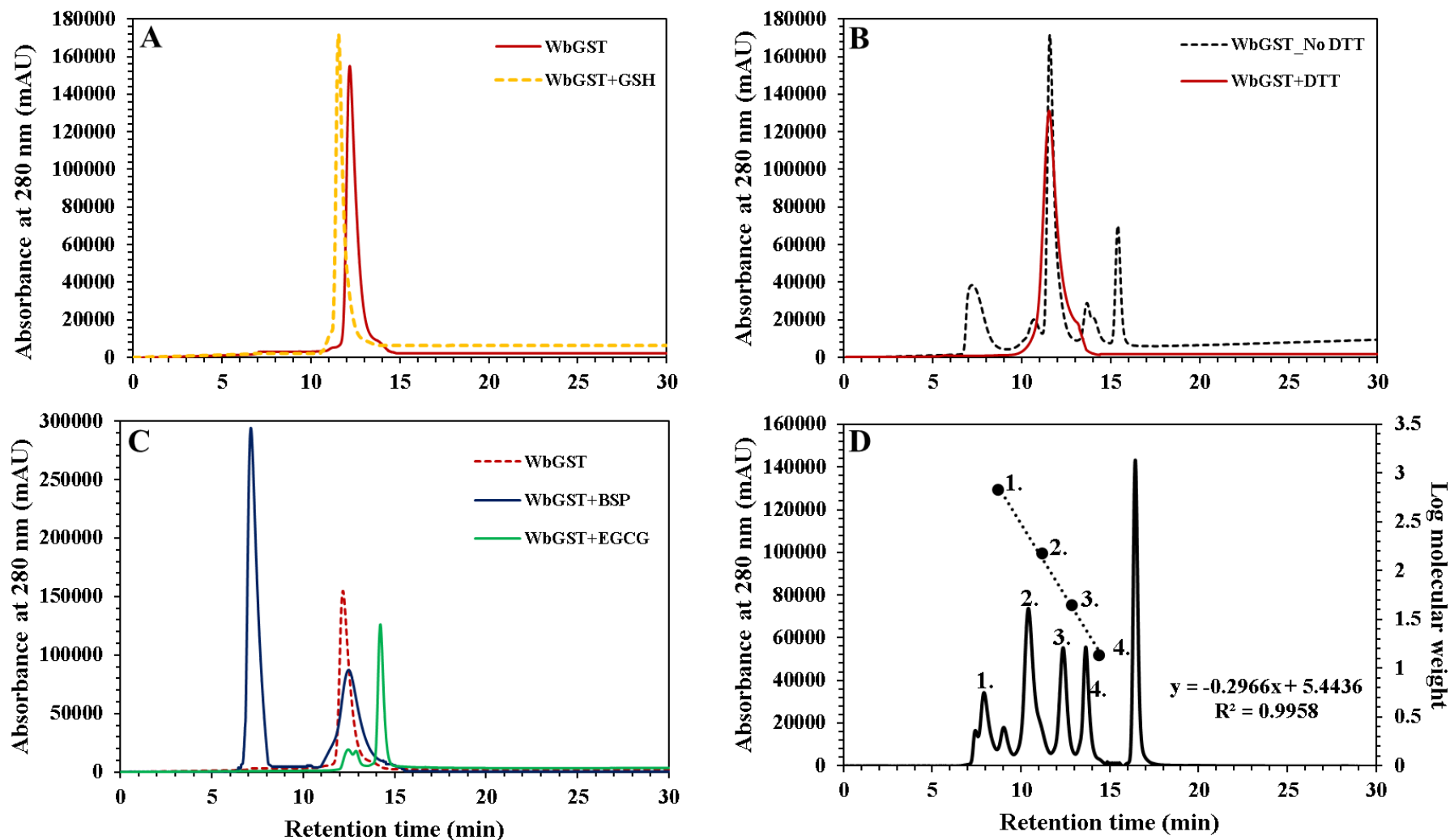


Figure 5.7 Verifying the tertiary structure of WbGST. SE-HPLC was used to verify the oligomeric state of WbGST in the presence or absence of GSH (A), DTT (B), ligands (C), and the gel filtration standards with an inset calibration curve (D). 50 μ M of WbGST was analysed in 20 mM Na₂HPO₄ pH 7.4, 500 mM NaCl, 5 mM DTT, 1 mM EDTA and 0.01% (w/v) NaN₃ at 20°C. 20 μ L of protein sample was loaded onto an SEC column at 0.3 mL.min⁻¹ flow rates. In the presence of GSH, the molecular mass of WbGST was ~48 kDa, while the size in the absence of GSH was ~26 kDa (A). Without GSH, WbGST exhibited anomalous behaviour in the SEC because of the variance observed between the expected and predicted molecular weight. There are four Cysteine residues in WbGST, hence the formation of higher-order oligomeric soluble proteins and monomeric peaks in the absence of DTT (B). Ligand binding changes the oligomeric state of WbGST (C). The molecular mass was calculated from a calibration (inset D) using (1) thyroglobulin (670 kDa), (2) γ -globulin (150 kDa), (3) Albumin chicken egg (44.3 kDa), (4) Ribonuclease A type (13.7 kDa) and (5) P-aminobenzoic acid (0.137 kDa).

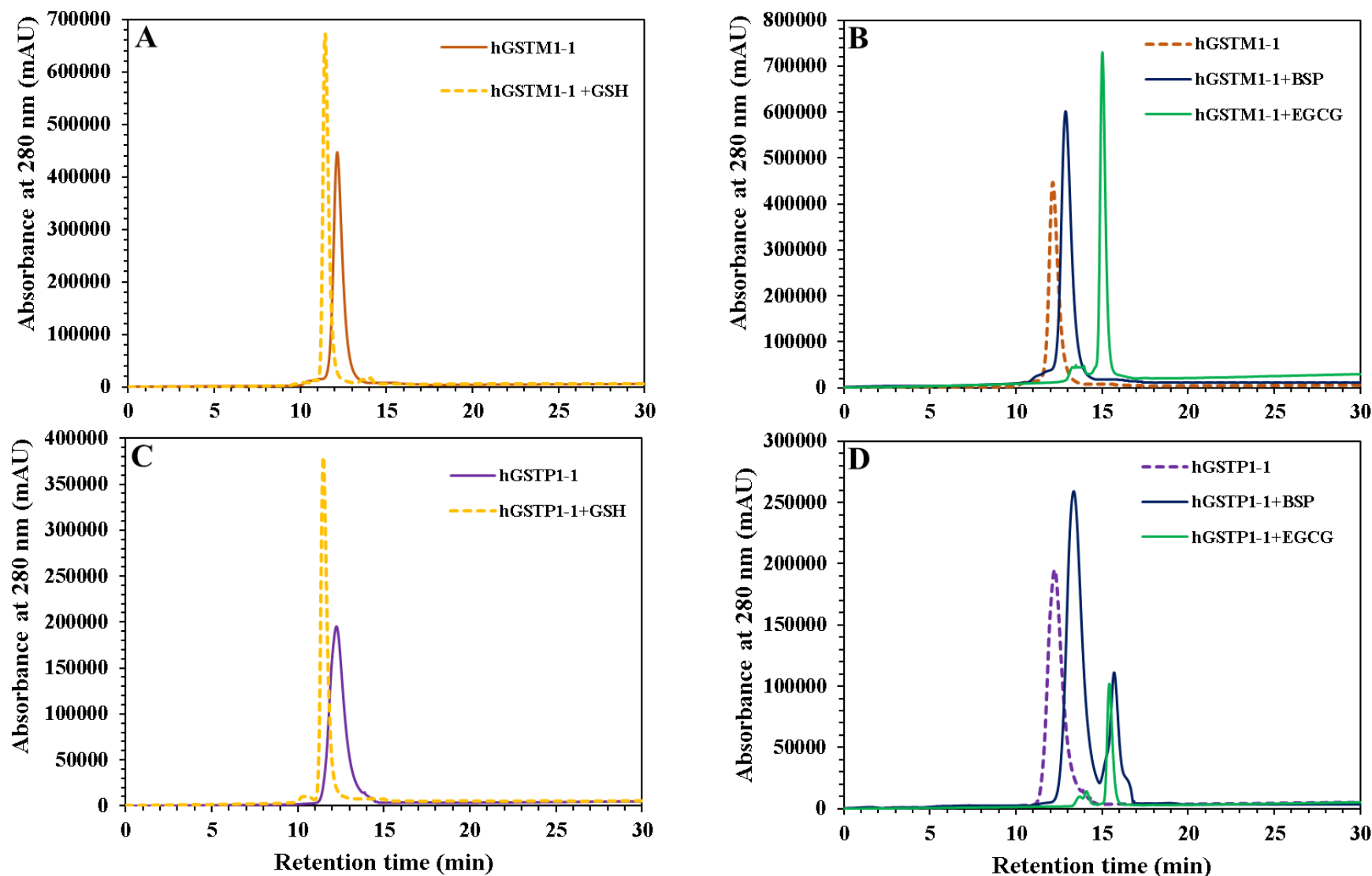


Figure 5.8 Verifying the tertiary structure of hGSTM1-1 and hGSTP1-1. SE-HPLC was used to verify the oligomeric state of hGSTM1-1. (A and B) and hGSTP1-1 (C and D) in the presence or absence of GSH and ligands, respectively. 50 μ M of the human GSTs were each analysed in 20 mM Na₂HPO₄ pH 7.4, 500 mM NaCl, 5 mM DTT, 1 mM EDTA and 0.01% (w/v) NaN₃ at 20°C. 20 μ L of protein sample was loaded onto an SEC column at 0.3 mL.min⁻¹ flow rates. The molecular mass of ~36 and ~40 kDa was estimated for hGSTM1-1 and hGSTP1-1, respectively. In the presence of GSH, the molecular mass of ~52 and ~48 kDa was obtained for hGSTM1-1 and hGSTP1-1, respectively. However, monomeric mass was obtained in the presence of the ligands. The target proteins exhibited anomalous behaviour in the SEC without the GSH.

5.5 Discussion

GSTs are globular proteins consisting of the N-terminal and C-terminal domains. The N-terminus of GST is like the thioredoxin domain with a combination of helices and β -strands (β 1- α 1- β 2- α 2- β 3- β 4- α 3), while the C-terminal domain is mainly α -helical. The predominantly α -helical profile (Figure 5.2) obtained for the recombinant target proteins in this study correlates to their obtained crystal structures. Further information regarding the nature of helices in α -helical proteins can be ascertained by analysing the difference in the ellipticity of the two prominent troughs. Therefore, when the ratio 222/208 nm is either ≤ 0.86 or ≥ 1 , the helices are said to be primarily isolated or coiled-coil α -helices, respectively (Lau *et al.*, 1984). The ratio obtained for WbGST, hGSTM1-1, and hGSTP1-1 indicates that their secondary structure comprises more of the coiled-coil α -helices. However, the binding of BSP and EGCG to WbGST changed the nature of the helices, making them mainly isolated. EGCG triggered more than a 10% increase in helical contents of the target proteins, while a substantial increase caused by BSP was observed only in hGSTM1-1. In contrast, EGCG caused more than a 50% decrease of β -strands content in the presence of GSH. Perhaps the interaction of the ligands with amide groups of amino acid backbone residues could result in some conformational changes, causing these observed fluctuations in the secondary structure content of the target proteins. Nevertheless, the binding of the ligands did not substantially affect the overall secondary structure of the target recombinant proteins.

The ability of protein molecules to absorb ultraviolet light and their intrinsic fluorescence properties are ascribed to aromatic residues such as phenylalanine, tryptophan, and tyrosine. However, protein fluorescence emission is mainly contributed by tryptophan residues because they possess the highest molar extinction coefficient and considering the FRET phenomena whereby excitation (Ex.) energy can be transferred from phenylalanine to tyrosine and lastly to tryptophan (Wu and Brand, 1994). Then again, the fluorescence of the indole ring of tryptophan is very sensitive to the polarity of its local environment. Therefore, tryptophan is an ideal probe to monitor changes in tertiary structures of the recombinant target proteins and their interaction with ligands. The maximum fluorescence emission of WbGST, hGSTM1-1, and hGSTP1-1 was observed at 344 nm, 341 nm, and 342 nm wavelengths, respectively (Figure 5.4). These observed emission maxima corroborate the phenomena that when found in a polar environment, tryptophan residues exhibit

maximum fluorescence around 350 nm wavelength (Lakowicz, 2006). Hence, the tryptophan residues present in the three target proteins in this study exist in a more solvent-exposed environment.

Furthermore, tryptophan fluorescence has been proven to be a valuable tool in studying ligand binding to protein molecules and protein conformational changes (Akbar *et al.*, 2016; Dahmane *et al.*, 2018; Yammine *et al.*, 2019). Changes in fluorescence intensity and shifts either to higher wavelength (decreased hydrophobicity) or lower wavelength (increased hydrophobicity) provide insight into the form of conformational changes occurring around the local tryptophan environment (Chen *et al.*, 2014). First, the binding of the ligands to WbGST resulted in a decrease in fluorescence intensity (Figure 5.4A). This is consistent with previously observed results in other parasite GSTs (Akumadu *et al.*, 2020; Padi *et al.*, 2021). Interestingly, GSH binding to apo WbGST also leads to quenching of fluorescence intensity with a blue shift. This is because the tryptophan residue in WbGST is located at the G-site, but it is also in close proximity to the H-site. Therefore, the binding of GSH possibly increased the hydrophobicity of the local tryptophan environment hence the observed blueshift and quenched fluorescence intensity. Secondly, the interaction between GSH and Asp96 can change the orientation of the residue, which may contribute to the quench in fluorescence because arginine and asparagine are typically responsible for the quenching of tryptophan fluorescence.

Structural conformation of protein molecules influences variations observed in tryptophan fluorescence emissions. Thus, the amino acid residues near the tryptophan residues may induce the quenching of indole and not just the local environment of the solvent. For example, charged amino groups and electron acceptors like the protonated carboxyl groups can impact the quenching of tryptophan fluorescence. However, the location of the interacting groups or atoms determines the stability and level of occurring electron transfer quenching. Consequently, tryptophan quenching by BSP and EGCG (Figure 5.4A) could be caused by the interaction between the functional groups of the ligands and active site residues.

In the case of hGSTM1-1 and hGSTP1-1, the binding of GSH did not induce a change in the fluorescence intensity (Figures 5.4 B & C). However, slight blueshifts occurred, indicating an

increase in the hydrophobicity of the surrounding tryptophan residues. Upon binding of BSP and EGCG, a quench in the fluorescence intensity was observed. It appears that the tryptophan residue contributing the most to the fluorescence intensity observed in hGSTP1-1 (Trp28, with ASA of 52.67 Å²) became more buried upon ligand binding, which resulted in the decreased fluorescence intensity (Figure 5.4C). Therefore, no shift in the baseline was observed, and the hydrophobicity of the indole environment was not impacted. The flexible Mu-loop of hGSTM1-1 connects β2 and α2 where Trp 7 and Trp 45 are located, respectively, at the G-site, and was reported to promote substrate affinity (Ji *et al.*, 1992). The binding of GSH can possibly initiate a movement of the loop resulting in a change in conformation, which increases the hydrophobicity of the tryptophan residues hence the blueshift. The redshift observed upon binding EGCG resulted in a conformational change of the neighbouring residues covering the tryptophan residues. This implies that the tryptophan residues became partially exposed to the surrounding solvents.

Complementary to the intrinsic fluorescence probe, extrinsic fluorophores such as ANS can be used to characterise hydrophobicity in native protein structures. Like tryptophan, fluorescence quantum yield is usually quenched in a solvent-exposed environment but enhanced when ANS binds to accessible hydrophobic patches. This increased quantum yield typically accompanies a blueshift in maximum fluorescence emission wavelength (Stryer, 1965; Uversky *et al.*, 1998). The three target proteins in this study could bind ANS, thus indicating the presence of accessible hydrophobic pockets on the surfaces of the proteins in their native conformations (Figure 5.6B). Previous studies on GSTs have also reported the ability of ANS to bind GST proteins without a significant impact on their structure and conformational stability (Erhardt and Dirr, 1995; Sluis-Cremer *et al.*, 1996; Stevens *et al.*, 1998). However, the binding of ANS to WbGST yielded more than an 80% increase in quantum yield accompanied by a larger blueshift than the hGSTM1-1 and hGSTP1-1 (Figure 5.6B & C). Hence, suggesting that WbGST has more accessible hydrophobic pockets burying a more significant portion of the naphthalene moiety.

Considering that both BSP and EGCG are hydrophobic molecules, it is probable that they could bind at the same site as ANS. So, ANS displacement is possible if the ligands have a higher affinity towards the ANS binding site. Moreover, previous studies proposed that ANS binds to the H-site and possibly at a putative L-site, which could be located at the dimer interface (Nishihira *et al.*,

1992; Oakley *et al.*, 1999; Prade *et al.*, 1997). However, Oakley *et al.* (1999) argued that the location of the L-site at the dimer interface of hGSTP1-1 is unlikely because of the polar environment of the region. The dimer interface of parasite GST is comparable to hGSTP1-1 because they are made up of a mixture of polar and non-polar amino acid residues and have been confirmed to bind ligands. Although parasite GSTs contain more non-polar amino acid residues than human GSTs. For instance, Praziquantel and ellagic acid have been shown to bind at the dimer interface of *Schistosoma japonicum* GST (Akumadu *et al.*, 2020; McTigue *et al.*, 1995). This study observed that both BSP and EGCG could displace ANS from WbGST by 3-fold and 1-fold, respectively (Figure 5.6A). Similarly, previous studies have reported the displacement of ANS from parasite GSTs (Akumadu *et al.*, 2020; Padi *et al.*, 2021; Poole *et al.*, 2021). BSP demonstrated greater affinity than EGCG, as seen in the higher degree of ANS displacement by BSP. Perhaps protonation of the BSP sulphate group may have excluded water molecules from the site, thereby increasing the hydrophobicity of the site and resulting in increased affinity. The ligands may bind at both the H-site and L-site (Rajaiah Prabhu *et al.*, 2018). However, considering the promiscuous nature of the H-site towards electrophilic molecules, weak and transitory interactions may occur. ANS binding in the presence of GSH resulted in a redshift relative to the maximum fluorescence intensity of the WbGST-ANS complex, thus changing the hydrophobicity of the active site. The changes may have influenced the shift in wavelength in the network of water molecules and protonation of the sulphur atom of glutathione (Prade *et al.*, 1997). Alternatively, being that the G-site is adjacent to the H-site and L-site, it is possible that the binding orientation of GSH could be blocking some part of the ANS binding site. Therefore, resulting in reduced accessibility of ANS to its binding site.

On the one hand, a blueshift was observed in WbGST:GSH:ANS:BSP complex, suggestive of an increased hydrophobic binding site induced by BSP. On the other hand, a redshift occurred in the WbGST:GSH:ANS:EGCG complex implying that the hydrophobicity of the binding environment became reduced. Since WbGST has substantial hydrophobic patches (Figure S6), ANS could bind at alternative pockets. At the same time, BSP and EGCG can also bind at the putative non-catalytic site described by Prabhu *et al.* (2018). Secondly, the observed enormous difference in ANS fluorescence between WbGST and the human GSTs (Figure 5.6B and C) can be attributed to the substitution of Arg13 in humans with Leu13 in WbGST. This substitution developed an extended

space of $\sim 40 \text{ \AA}^3$ making the H-site more open for electrophiles, whereas, in humans, Arg13 interacts with Glu97 to form two hydrogen bridges.

In the case of hGSTM1-1, the F/F_0 ratio (Table 5.4) was less than 1, indicating that ANS was utterly displaced by BSP both in the presence or absence of GSH. However, a slight increase of ANS fluorescence occurred when GSH was bound to hGSTM1-1 (Figure 5.5B) with a larger blueshift, suggesting that GSH induced conformational changes of the active site residues, which resulted in the exclusion of some water molecules. Furthermore, it seems that this phenomenon resulted in the formation of transient sub-pockets in hGSTM1-1:GSH:ANS:EGCG complex, therefore increasing accessible binding pockets for ANS and hence the increase in ANS fluorescence with a larger blueshift. However, in the absence of GSH, EGCG could displace ANS slightly and not entirely, as in the case of BSP.

The hGSTP1-1 had the least accessible hydrophobic pockets (Figure 5.6B). There was an increase in ANS fluorescence intensity upon the binding of BSP and EGCG in the absence of GSH. It appears that ANS binds at a site different from that of the ligands, and ligand binding induces orientational changes in nearby residue functional groups. Thus, enabling the binding of more ANS molecules hence the increased fluorescence. The possibility of hGSTP1-1 possessing high and lower affinity ANS binding pockets throughout the enzyme surface has been previously reported (Ralat and Colman, 2004).

Furthermore, it was reported that the binding of MES on the putative L-site influences ANS binding to hGSTP1-1 (Prade *et al.*, 1997). Therefore, the non-displacement of ANS by BSP and EGCG in the absence of GSH indicates more than one ANS binding site on hGSTP1-1. On the contrary, binding of the ligands in the presence of GSH resulted in a slight displacement of ANS with a reduction in the hydrophobicity of the site (redshift). This means that GSH interaction with the active site residues induced a conformational change, which increased solvent accessibility of ANS binding sites. Consequently, reducing ANS affinity towards the binding sites. In short, besides the H-site, the existence of alternative binding sites for these hydrophobic molecules (ANS, BSP, and EGCG) on the three target proteins in this study cannot be ruled out.

Evaluating the hydrodynamic volume of the target proteins using the SE-HPLC provided more information about the protein oligomeric states and the ligand impact. Using SE-HPLC, the molecular weight of WbGST in the absence of GSH was predicted to be ~26 kDa (Figure 5.7A), similar to a monomeric size. Nevertheless, the monomeric size obtained from reduced SDS-PAGE (24 kDa) agrees with the expected dimeric size obtained in the WbGST-GSH complex (48 kDa). It is possible that the binding of GSH changes the global conformation of the protein to become more compact and globular. Consequently, it enables the protein to elute at a lower retention time in its dimeric size. In contrast, the absence of GSH results in a less compact conformation of WbGST, which could lead to increased secondary interactions between the exposed polar functional groups of the analyte and the diol groups on the stationary phase (Harris, 2010; Podzimek, 2011). Under non-reducing conditions, more than one peak was observed, with the first peak eluting at a void volume (Figure 5.7B). WbGST consists of four cysteine residues per monomer, which can quickly form cystines under non-reducing conditions through the formation of disulphide bridges by the sulphhydryl groups. Thereby forming higher-order oligomers (soluble aggregates) that elute at void volume. The binding of BSP to WbGST induced the formation of soluble aggregates, which eluted at the void volume while the second peak eluted at a monomeric size. While there was no formation of aggregates in the presence of EGCG, it is evident that the global conformation of WbGST was impacted. This is because most of the protein eluted in the second peak at ~2 kDa.

Regarding the human GSTs, hGSTM1-1 eluted in its dimeric form both in the presence and absence of GSH. However, in the absence of GSH, the dimeric size (36 kDa) was lower than the expected theoretical size. This was similar to the size previously observed in our Lab by Wiid *et al.* (2018). But then, when bound to GSH, the hGSTM1-1 elutes at a size (52 kDa) almost that of the theoretical size. Similarly, hGSTP1-1 eluted at its theoretical size (47 kDa) when bound to GSH. However, it also eluted in a dimeric size in the absence of GSH but at a size (~ 40 kDa) lower than the estimated theoretical size. Moreover, in the presence of both BSP and EGCG, the dimeric size of both human GSTs was not retained as they eluted in monomeric sizes. Concerning EGCG, besides the conformational changes resulting from ligand binding, the moieties of EGCG could increase the interaction with the stationary phase. Hence the observed higher retention time within the column.

Thus, with reference to the obtained SE-HPLC data, it has been shown that WbGST is dimeric. Furthermore, it can be categorically stated that the presence of GSH increases the compactness of the globular structure of the target proteins, thus reducing the retention time in the column. Secondly, the binding of BSP and EGCG changes the native oligomeric conformation of the three target proteins. Nevertheless, other aspects, such as protein concentration, buffering system, ionic strength, and pH, may also influence the outcome of SE-HPLC protein size evaluation.

Chapter 6

Structure-stability relationship

6.1 Introduction

One of the significant challenges that protein scientists have been trying to unravel for decades is the subject of protein folding. Understanding the complexities surrounding the rearrangement of amino acid sequences resulting in secondary, tertiary, and quaternary structures leading to the formation of multidomain functional structures is very crucial. This will explicate the extensiveness of biological processes and give insights towards a better knowledge of numerous disease states (Ferguson and Fersht, 2003) because unsuccessful protein folding may eventually lead to cellular dysfunction resulting in disease. Hsien Wu (1931) was the first scientist to understand the fundamental relationships between the folded (native) and unfolded (denatured) states of proteins. He proposed that protein structures are well-ordered and very compact and that the structures are principally linked together by interactions among the polar groups of the primary chain and the polar side functional moieties (Edsall, 1995). Therefore, these forces holding the folded protein molecules together in a defined configuration are similar to those inducing macroscopic crystal packing of the protein molecules.

On the other hand, denaturing the protein molecules induces the unfolding of the packed molecules into a more open chain, leading to the assumptions of numerous possible conformations (Rose, 2002). This results in a loss of function and specificity, though the structure and function of the protein molecule can still be restored by refolding. This chapter describes the folding mechanisms of WbGST and its stability. This was evaluated using urea equilibrium unfolding studies and was monitored spectroscopically by far-UV CD, intrinsic tryptophan fluorescence, and ANS fluorescence emissions. The loss or gain of isolated helical secondary structural content at increasing urea concentrations was observed using far-UV CD spectropolarimetry. Changes in the local tertiary structure were accessed by fluorescence emission using tryptophan and ANS as probes.

In the same vein, the stability of a protein molecule can be evaluated by chemical or thermal denaturation. The stability of protein native conformation can be monitored by applying the equilibrium process of the two states (native and denatured states). However, this does not often apply to thermal denaturation because most thermal denaturation is usually irreversible. In this study, the stability of WbGST was analysed chemically by following Urea induced unfolding and thermally by differential scanning calorimetry (DSC), differential scanning fluorometry (DSF), also called the thermal shift assay (TSA) and spectroscopically using far-UV CD spectropolarimetry.

6.2 Materials

The following analytical grade materials listed in Table 6.1 were utilized in this chapter

Table 6.1 List of reagents and suppliers

Materials	Source	Location
Urea	Sigma-Aldrich	St. Louis, MO, USA
SYPRO orange dye	Sigma-Aldrich	St. Louis, MO, USA
ANS	Sigma-Aldrich	St. Louis, MO, USA
GSH	Sigma-Aldrich	St. Louis, MO, USA
BSP	Sigma-Aldrich	St. Louis, MO, USA
EGCG	Angene	Hyderabad, India
Dithiothreitol (DTT)	New England Biolabs	Massachusetts USA

6.3 Method

6.3.1 Differential scanning fluorometry (DSF)

Fluorogenic dyes have been used in previous studies to monitor protein unfolding and folding. Frequently used dyes such as ANS and TNS (naphthalene-6-sulphonic acid) experience a substantial increase in quantum when they bind to hydrophobic regions (Hawe *et al.*, 2008). As a result, the process of protein denaturation exposes further buried hydrophobic pockets, allowing

more dye binding, followed by a measurable fluorescence. Unfortunately, ANS and TNS do not have fluorescence properties (wavelengths) within the range detectable by PCR instruments. Therefore, SYPRO orange dye was used to probe the melting temperature (T_m) of the target recombinant proteins (WbGST, hGSTM1-1, and hGSTP1-1) because its excitation/emission wavelength (λ_{ex} 470 nm / λ_{em} 570 nm) are compatible with the available filters on the real-time PCR machines (Niesen *et al.*, 2007). The T_m was assessed both in the presence or absence of GSH, BSP, and EGCG to evaluate how these ligands impact the protein stability and inhibition mode.

Before the commencement of the assay, a 50× stock solution of Sypro Orange dye was prepared by diluting 10 μ L of 5 000× concentrate into 1 mL Milli-Q water. Then, 10 μ L protein which amounts to a final concentration of 1.5 mg/mL, was aliquoted into a 96-well PCR microplate (Bio-Rad). Subsequently, 5 μ L (10× final concentration) of 50× SYPRO Orange stock solution was added to each well. PBS buffers were prepared in the presence or absence of GSH and adding 10 μ L buffer gave a 5 mM final concentration of GSH. For assays performed in the presence of BSP or EGCG, 5 μ L ligand and 5 μ L buffer were added. The ligands were transferred from a mother plate to the PCR plate with final concentrations ranging from 0.2 to 200 μ M. Columns G and H on the plates were used for blank samples, and all samples were analysed in triplicate. A total assay volume of 25 μ L was centrifuged at 1000× g for 30 s while sealing the plates with a Microseal 'B' PCR Plate Sealing Film (Bio-Rad). The reaction was monitored using a Real-Time PCR Detection System (Bio-Rad CFX96 Touch) by heating the thermal cycler from 10 °C to 90 °C at increments of 0.5 °C.

6.3.2 Differential scanning calorimetry

Differential scanning calorimetry (DSC) is a unique, label-free method used to study the stability of proteins and other macromolecules in solution or complex with ligands, proteins, or even nucleic acids. Proteins usually unfold when heated, and the induced thermal transitions, such as energy changes in the solution, can determine absolute thermodynamic parameters. Parameters such as changes in enthalpy (ΔH°) and entropy (ΔS°) which sums up as the change in free energy (ΔG°) used to analyse the stability of folded or unfolded state of protein molecules (Bruylants *et al.*, 2005). The occurring change in energy, as well as the protein melting temperatures, are

measured using DSC. The machine comprises a sample cell and a reference cell that does not undergo phase transition near the desired temperature range, unlike the sample cell. Throughout the experiment, the control system tries to maintain the temperature of both cells at zero. When the sample undergoes any form of transition resulting in a temperature change, the system quickly compensates (by increasing or decreasing supplied heat) to ensure that constant temperature is maintained between the sample and reference cells (McElhaney, 1982). So, the DSC records the surplus-specific heat or heat disparity as a temperature function.

Therefore, the heat capacities of WbGST, hGSTM1-1, and hGSTP1-1 were evaluated in the presence or absence of the ligands as a function of temperature using a high-precision Nano-DSC microcalorimeter (TA Instruments, Delaware, USA). Before loading samples, they were degassed to prevent the formation of bubbles within the cell capillaries. Samples containing 1.5 mg.mL^{-1} of protein were prepared in the presence or absence of 5 mM GSH, 50 μM of BSP or EGCG were prepared in PBS buffer, and 500 μL was loaded into the sample cell. The thermal unfolding profiles were analysed by linearly heating the cells from 20 °C to 90 °C at the rate of 1°C.min^{-1} . NanoAnalyze software was used to fit the obtained data using the non-linear least squares model described by (Marky and Breslauer, 1987).

6.3.3 Thermal stability of target proteins monitored by far-UV CD spectropolarimetry.

The ability of protein molecules to perform their physiological functions is highly dependent on the accurate folding of emerging amino acid chains into the biological native conformation of the protein. The folded and unfolded states of protein molecules typically exist in equilibrium. The equilibrium can be leaned towards the unfolded portion by specific environmental changes such as increased temperature, high concentrations of denaturants, and changes in the ionic strength and pH (Murphy, 2001). Unlike the native structural conformation of proteins, it is assumed that unfolding or denaturing a protein molecule induces the loss of secondary, tertiary, and quaternary structures. Hence the speculation that denatured proteins consist of random coil polypeptide chains.

On the contrary, experimental reports indicate that it is rare to denature protein because residual structures are frequently observed totally. Thus, a protein molecule can be considered unfolded rather than denatured (Shirley, 1995). Nevertheless, the ability of a protein molecule to maintain a state of equilibrium between unfolded and folded native states can be described as its conformational stability. The difference between the two states is considered Gibbs's free energy change in thermodynamics.

Since the stability of protein molecules are often dependent on molecular interactions such as hydrogen bonding, van der Waals force, disulphide bonds, and salt bridges. Consequently, the binding of ligands to protein molecules can induce changes in the composition of these interacting forces. Therefore, comparing the unfolding profiles obtained in the presence and absence of the ligands can provide insights into the impact of the ligand on protein stability (England and Haran, 2011). In contrast to the reversible nature of chemical unfolding by chaotropic agents, most thermally unfolded proteins do not often refold. This is because most proteins aggregate at high temperatures due to increased hydrophobic interactions of close adjacent molecules (Rocco *et al.*, 2008). Therefore, far-UV CD thermal unfolding often provides qualitative insight into protein conformational stability by monitoring unfolding transition temperatures (T_m).

To monitor the thermal unfolding of WbGST, hGSTM1-1, and hGSTP1-1, 5 μ M protein in PBS buffer was analysed in the presence or absence of 5 mM GSH, 50 μ M BSP, and 50 μ M EGCG. The unfolding was evaluated using far-UV CD spectropolarimetry by measuring the change in the circular dichroism ellipticity at 222 nm while increasing the temperature from 20 to 80 degrees Celsius at 1 degree Celsius per minute. All measurements were performed in a Suprasil quartz cuvette with a path length of 0.2 cm (Hellma, Müllheim, Germany) and monitored using the Jasco J-1500 spectropolarimeter that was coupled to a Peltier temperature controller (Jasco, Pfungstadt, Germany). The unfolded data were normalized by estimating the fraction of unfolded protein using equation 6.1 below:

$$F_u = \frac{(\theta - \theta_{unfold})}{(\theta_{fold} - \theta_{unfold})} \quad (6.1)$$

where:

- F_u = fraction of unfolded protein
- θ = the ellipticity at a particular temperature
- θ_{fold} = ellipticity of folded protein and
- θ_{unfold} = ellipticity for unfolded protein

Afterwards, the data were fitted to the sigmoidal three-parameter model in GraphPad Prism.

6.3.4 Urea-induced equilibrium unfolding of WbGST

Protein molecules undertake different pathways during the folding process from their unfolded polypeptide chains to achieve a functional native state. This pathway comprises various discrete numbers of folded proteins existing in a state of equilibrium (Walters *et al.*, 2009). Therefore, equilibrium unfolding studies aim to analyse these discrete points along the folding pathway by denaturing the protein chemically or physically (Pace, 1986). In this study, the fraction unfolded was determined spectroscopically using far-UV CD spectropolarimetry, intrinsic tryptophan fluorescence, and extrinsic ANS fluorescence emission. The folding mechanism was ascertained by plotting the fraction unfolded against a range of urea concentrations. Various parameters such as the folding mechanism, cooperativity (m), conformational stability (ΔG_{H20}), and denaturation midpoint ($[Urea]_{1/2}$) of WbGST were characterised. Our lab has extensively studied the equilibrium unfolding mechanism of hGSTM1-1 and hGSTP1-1.

The unfolding studies were performed using urea as the chemical denaturant. Prior to each experiment, a stock solution of 10 M urea was freshly prepared, and the final concentration was obtained using a refractometer as described by Pace (1986). The urea samples were prepared in PBS and 20 mM sodium phosphate buffer pH 7.4 for unfolding assessed by fluorescence emission and far-UV CD spectropolarimetry, respectively. All samples were prepared with proteins denatured in urea with concentrations ranging from 0 to 8 M varied at 0.25 M intervals. Samples containing final concentrations of 3 μ M and 5 μ M of WbGST for fluorescence spectroscopy and far-UV CD, respectively, were prepared in triplicate and incubated at room temperature for 1 h before analyses.

Urea equilibrium refolding

A stock solution of 10 M urea was prepared as described above (section 6.3.4). Subsequently, samples were prepared by diluting a concentrated WbGST-Urea solution as opposed to a WbGST-buffer solution during unfolding. The protein sample was concentrated and then denatured in 10 M urea with pH adjusted to pH 7.4, making up a stock solution. This solution was incubated at room temperature for 1 h to allow the proteins to unfold. Following the unfolding of the stock sample, it was diluted to calculated volumes of urea and buffer (PBS or sodium phosphate) to arrive at the required concentrations of WbGST and urea, as mentioned in section 6.3.4. After that, the samples were incubated for 1 h to enable the protein to refold to equilibrium. The recovery of the secondary and tertiary structure of WbGST was probed by far-UV CD and fluorescence spectroscopy.

Spectroscopic probes for urea-induced equilibrium folding studies.

Possible secondary structural alterations were assessed using a Jasco J-1500 CD spectropolarimeter (Jasco, Pfungstadt, Germany) while helical contents were monitored at 222 nm. Tryptophan fluorescence was used to monitor changes in the tertiary structure using a Jasco FP-6300 fluorescence spectrophotometer (Jasco Inc., Tokyo, Japan), as reported before (see section 5.3.2). Samples were excited at 295 nm, and emission was captured at wavelengths ranging from 280 nm to 500 nm.

ANS was also utilized as an extrinsic probe to track changes in the protein tertiary structure. The ANS stock concentration was calculated using the Beer-Lambert equation ($\epsilon_{350} = 4950 \text{ M}^{-1} \cdot \text{cm}^{-1}$) (Weber and Young, 1964). With a Jasco FP-6300 fluorescence spectrophotometer linked to a Peltier temperature controller set at 20 °C, ANS binding was monitored using an excitation wavelength of 390 nm and emission from 400 nm to 700 nm. The acquired spectra are an average of three accumulations per triplicate sample, and all spectra have been adjusted for unbound ANS in the buffer. The relative ANS binding was computed using the methods described by (Chaudhuri *et al.*, 1993).

6.4 Data fitting

The unfolding data obtained from monitoring tryptophan fluorescence and far-UV CD was fitted by regression modelling. This was performed using an iterative process of nonlinear curve fitting estimations while constraining the provided user-defined parameters. The iterative process was

performed until a good fit convergence was achieved using GraphPad Prism 9 and the fit computed thermodynamic parameter of folding. In this experiment, two states ($N_2 \leftrightarrow 2U$) and three states ($N_2 \leftrightarrow I_2 \leftrightarrow 2U$) unfolding model were applied in the curve fitting.

For ease of data comparison and better visualisation, all data were normalised before performing curve fitting using the following equation:

$$Y_{normalised} = \frac{Y_{obv} - Y_U}{Y_f - Y_U} \quad (6.2)$$

where Y_{obv} represents the readings obtained from either far-UV CD or intrinsic tryptophan fluorescence emission at a specific concentration of urea, Y_U is the signal of the unfolded protein obtained by a linear fit of data from the pre-transition region and extrapolated to zero denaturants, while Y_N is the signal of the native protein obtained from the linear fit of data in the post-transition region and extrapolated to zero denaturants.

Two-state dimeric model ($N_2 \leftrightarrow 2U$)

The unfolding reaction in a two-state dimeric model starts with a native dimer and ends with two unfolded monomers. It is assumed that proteins in native or unfolded states majorly populate the two-state folding pathway. Hence, the two-state mechanism of unfolding can be summed up to unity and is represented as follows:

$$F_{U^2} + F_{f_2} = 1 \quad (6.3)$$

where F_{U^2} is the fraction of unfolded monomers, and F_{f_2} is the fraction of folded dimer or the native dimer fraction of the protein. The two predominant species contribute to each signal obtained at any point during the experiment:

$$Y_{obv} = Y_f F_{f_2} + Y_U F_{U^2} \quad (6.4)$$

where Y_{obv} is the signal for each spectroscopic probe, Y_f is the signal (y-value) for the folded state and can be extrapolated from the pre-transition region of the obtained data F_{f_2} is the fraction of folded proteins, Y_U represents the signal (y-value) from the unfolded state, which can be extrapolated from the post-transition region of the unfolding data and F_{U^2} is the fraction of

unfolded monomers So, combining equations (6.3) and (6.4) fraction of unfolded protein can be obtained as:

$$F_{f_2} = \frac{Y_f - Y}{Y_f - Y_U} \quad (6.5)$$

and similarly, the native protein or the fraction of folded protein can be expressed as:

$$F_{f_2} = \frac{Y_U - Y}{Y_U - Y_f} \quad (6.6)$$

then, the equilibrium constant for the unfolding reaction can be described as:

$$K = \frac{F_{U^2}}{F_{f_2}} \quad (6.7)$$

and consequently, by substitution of equations (6.5) and (6.6) into (6.7):

$$K = \frac{Y - Y_{f_2}}{Y_{U^2} - Y} \quad (6.8)$$

and Gibbs-Helmholtz free energy change (ΔG) can be expressed as:

$$\Delta G = -RT(K) \quad (6.9)$$

where ΔG represents the free energy of unfolding, R is the gas constant ($1.987 \text{ cal.mol}^{-1}.\text{K}^{-1}$), T is the absolute temperature measured in Kelvin, and K is the equilibrium constant for the reaction. Assuming that ΔG linearly depends on the denaturant concentration $[D]$, then for each varying concentration of urea (Pace, 1986), the free energy change in the absence of denaturant can be determined as:

$$\Delta G = \Delta G_{H_2O} - m[D] \quad (6.10)$$

where ΔG_{H_2O} represents the absence of a denaturant to destabilise the protein, m is the cooperativity of the reaction, which correlates to the solvent-accessible surface area of the protein during unfolding, and $[D]$ is denaturant concentration. Thus, substituting equation (6.9) into equation (6.10) gives:

$$F_U = \frac{2[Y_{f_2}] + Y_U \cdot e^{-(\Delta G_{H_2O} - m[D])/RT}}{1 + e^{-(\Delta G_{H_2O} - m[D])/RT}} \quad (6.11)$$

The data obtained (F_U) was plotted against [urea]. The fitted data was used to obtain the ΔG_{H_2O} and m values of unfolding, including the $[\text{urea}]_{1/2}$.

Three-state dimeric model ($N_2 \leftrightarrow I_2 \leftrightarrow 2U$)

The three-state dimeric model assumes that the folding pathway is populated by protein in a native (N_2), or folded state (f_2), unfolded ($2U$) form, and stable intermediate (I_2) conformation. Consequently, the native of the folded state accrued intermediate and unfolded fractions of protein can be summed up to unity as:

$$F_{U^2} + F_{I_2} + F_{f_2} = 1 \quad (6.12)$$

where F_{U^2} , F_{I_2} and F_{f_2} are the fractions of the unfolded intermediate and the native protein, respectively. Both the unfolded, intermediate, and folded states contribute to the observed signals as:

$$Y_{obv} = Y_f F_{f_2} + Y_I F_{I_2} + Y_U F_{U^2} \quad (6.13)$$

where Y_{obv} is the value of the signal obtained for each spectroscopic probe, Y_f is the signal (y-value) for the folded state and can be extrapolated from the pre-transition region of the obtained data Y_I is the signal of the intermediate obtained from the linear fit of data from stable intermediate extrapolated to zero denaturants, Y_U represents the signal (y-value) from the unfolded state, which can be extrapolated from the post-transition region of the unfolding data. The first of the two equilibrium constants can be described as the $N_2 \leftrightarrow I_2$ transition:

$$K_{N_2 \leftrightarrow I_2} = \frac{[I_2]}{[f_2]} = \frac{F_{I_2}}{F_{f_2}} \quad (6.14)$$

and the second intermediate $I_2 \leftrightarrow 2U$ transition:

$$K_{I_2 \leftrightarrow 2U} = \frac{[U^2]}{[I_2]} = \frac{F_{U^2}}{F_{I_2}} \quad (6.15)$$

where $K_{N_2 \leftrightarrow I_2}$ and $K_{I_2 \leftrightarrow 2U}$ are the equilibrium rate constants for the native to intermediate transition and intermediate to two unfolded transitions, respectively.

The Gibbs-Helmholtz free energy change (ΔG) for each equilibrium constant, corresponding to equation (6.9), may be expressed as:

$$\Delta G_{N_2 \leftrightarrow I_2} = -RT \ln(K_{N_2 \leftrightarrow I_2}) \quad (6.16)$$

and:

$$\Delta G_{I_2 \leftrightarrow 2U} = -RT \ln(K_{I_2 \leftrightarrow 2U}) \quad (6.17)$$

Assuming that ΔG for each step of the reaction is linearly dependent on the denaturant concentration (Pace, 1986), then the free energy change in the absence of denaturant for each transition can be calculated as:

$$\Delta G_{N_2 \leftrightarrow I_2} = \Delta G_{H_2O N_2 \leftrightarrow I_2} - m_{N_2 \leftrightarrow I_2} [D] \quad (6.18)$$

and:

$$\Delta G_{I_2 \leftrightarrow 2U} = \Delta G_{H_2O I_2 \leftrightarrow 2U} - m_{I_2 \leftrightarrow 2U} [D] \quad (6.19)$$

where $\Delta G_{N_2 \leftrightarrow I_2}$ and $\Delta G_{I_2 \leftrightarrow 2U}$ are the free energy changes in the absence of denaturant for $N_2 \leftrightarrow I_2$ and $I_2 \leftrightarrow 2U$ transitions, respectively. While $m_{N_2 \leftrightarrow I_2}$ and $m_{I_2 \leftrightarrow 2U}$ represents the cooperativity of the reaction for the $N_2 \leftrightarrow I_2$ and $I_2 \leftrightarrow 2U$ transitions, respectively. This m -value correlates with the solvent-accessible surface area of the protein during unfolding. And $[D]$ refers to the concentration of denaturant. Furthermore, rearranging equations (6.12) through (6.19) represents F_U and it is expressed as:

$$F_U = \frac{Y_{N_2} + Y_{I_2} \cdot e^{\frac{-(\Delta G_{H_2O N_2 \leftrightarrow I_2} - m_{N_2 \leftrightarrow I_2} [D])}{RT}} + Y_{U^2} \cdot e^{\frac{-(\Delta G_{H_2O N_2 \leftrightarrow I_2} - m_{N_2 \leftrightarrow I_2} [D])}{RT}} \cdot e^{\frac{-(\Delta G_{H_2O I_2 \leftrightarrow 2U} - m_{I_2 \leftrightarrow 2U} [D])}{RT}}}{1 + e^{\frac{-(\Delta G_{H_2O N_2 \leftrightarrow I_2} - m_{N_2 \leftrightarrow I_2} [D])}{RT}} + e^{\frac{-(\Delta G_{H_2O N_2 \leftrightarrow I_2} - m_{N_2 \leftrightarrow I_2} [D])}{RT}} \cdot e^{\frac{-(\Delta G_{H_2O I_2 \leftrightarrow 2U} - m_{I_2 \leftrightarrow 2U} [D])}{RT}}} \quad (6.19)$$

The obtained F_U was plotted against the denaturant concentration (urea). After that, the resulting fitted data was used to calculate the $\Delta G_{H_2O N_2 \leftrightarrow I_2}$, $\Delta G_{H_2O I_2 \leftrightarrow 2U}$, $m_{N_2 \leftrightarrow I_2}$, and $m_{I_2 \leftrightarrow 2U}$ of refolding. The $[\text{urea}]_{1/2}$ for $N_2 \leftrightarrow I_2$ and $I_2 \leftrightarrow 2U$ transitions.

Individual data set curve fitting and global curve fitting

The data sets obtained from equilibrium unfolding studies monitored by far-UV CD and intrinsic tryptophan fluorescence were curve fitted individually, and global curve fitting was also performed. Parameters for a two-state ($N_2 \leftrightarrow 2U$) and three-state ($N_2 \leftrightarrow I_2 \leftrightarrow 2U$). The dimeric folding model was applied to each data set to obtain the best fit. Parameters for the two-state model include the Y_U and Y_N . Each value represented the unfolded protein signal determined from the linear fit of data in the pre-and post-transition region and extrapolated to zero, respectively. The three-state model had an additional parameter, I_2 , which referred to the linear fit of the data in the region of the stable intermediate, extrapolated to zero. The model fit was accepted if the R^2 -value for the fit was >0.95 . This indicates that the variance of the regression model accounts for at least 95 % of the variance of the observed values, meaning that the fitted curve significantly modelled a two- or three-state folding mechanism.

Global curve fitting was performed to calculate the thermodynamic parameters for each equilibrium unfolding study (incorporating both unfolding and refolding data for fitting) monitored by either far-UV CD or intrinsic tryptophan fluorescence. Global curve fitting allows for the simultaneous analysis of multiple data sets. For instance, data for all unfolding and refolding studies obtained from both spectroscopic techniques were analysed in one fitting to derive global parameters that are consistent with all data sets under the same experimental conditions, such as ΔG_{H_2O} , m and $[\text{urea}]_{1/2}$ over the same range of $[\text{urea}]$ (Herman and Lee, 2012; Koshland Jr *et al.*, 1966).

6.5 Results

Differential scanning fluorimetry (DSF) or Fluorescence thermal shift assay

Fluorescence thermal shift assay is one of the thermal denaturing techniques that can be applied in monitoring how ligands modulate the stability of recombinant protein molecules. This assay was performed using SYPRO Orange dye, which binds to exposed hydrophobic patches as the protein unfolds due to increasing temperature, consequently increasing the fluorescence quantum yield. It was observed that at 50 μM ligand concentration, the proteins were destabilised in the absence of GSH except EGCG in complex with WbGST, which was further stabilised (Figure 6.1). Melting temperature (T_m) observed for the recombinant proteins were 37.5 $^{\circ}\text{C}$, 49 $^{\circ}\text{C}$, and 50 $^{\circ}\text{C}$ for WbGST, hGSTP1-1, and hGSTM1-1, respectively (Figure 6.1 and Table 6.2). However, in the presence of GSH, the proteins became more stabilised, as confirmed by a thermal shift of 6 $^{\circ}\text{C}$, 2 $^{\circ}\text{C}$, and 4 $^{\circ}\text{C}$ for WbGST, hGSTM1-1, and hGSTP1-1, respectively. In the presence of 50 μM BSP, the T_m decreased to 35 $^{\circ}\text{C}$, 50.5 $^{\circ}\text{C}$, and 49.5 $^{\circ}\text{C}$ for WbGST hGSTM1-1 and hGSTP1-1, respectively. Similarly, upon binding EGCG to hGSTM1-1 and hGSTP1-1, a decrease in the T_m was observed with lesser destabilising effects than BSP. A T_m of 37 $^{\circ}\text{C}$, 51 $^{\circ}\text{C}$, and 50 $^{\circ}\text{C}$ were obtained in the presence of EGCG for WbGST, hGSTM1-1, and hGSTP1-1, respectively. The observed melting temperatures are tabulated in Table 6.2.

Ligand binding affinity can be estimated by measuring the effect of the inhibitor on protein stability by monitoring the thermal shifts. This is usually successful when the ligand involved is a stabilising ligand. However, in the case of BSP and EGCG, both ligands seem to stabilise the protein at lower concentrations and destabilisation was observed slightly from 25 μM – 200 μM (Inset Figure 6.2A). Hence, it was impossible to calculate the inhibitor binding affinity towards the protein using this technique.

Furthermore, information obtained from the DSF T_m while varying inhibitor concentration was utilised to determine the mechanism of inhibition with respect to GSH complexed protein. The thermal shifts between the control and inhibitor-containing samples in the presence and absence of GSH were plotted against the log concentration of inhibitors. In this instance, concentration values within the range of observable stabilization were chosen. Both BSP and EGCG increased the thermal shift at a concentration below 25 μM . The presence of GSH induces an enhancement to the baseline due to its stabilising effects (Figure 6.2). In the presence of GSH, EGCG, upon binding to hGSTM1-1 and hGSTP1-1, produced similar profiles to those obtained in its absence.

A similar profile was also observed for BSP bound to hGSTP1-1. Therefore, the stabilising effect of EGCG on the human GSTs and BSP on hGSTP1-1 appeared to be independent of the corresponding effect observed in the presence of GSH. A parallel trend was observed in WbGST-BSP as the enhanced thermal shift produced in the presence of GSH became compressed to a difference of 0.6 °C at higher BSP concentrations. BSP produced a diverging profile with hGSTM1-1, there was no significant increase (<0.1 °C) in stabilisation in the absence of GSH. However, when GSH was added, the thermal stabilization effect was enhanced to 1.6 °C. Hence it seems that the thermal stabilisation, in this case, depends on the presence of GSH as a co-substrate. An interesting profile was observed WbGST- EGCG (Figure 6.2B) with a convergence of the GSH-present and absent samples occurring at lower concentrations of EGCG. Then upon enhanced stabilisation of the protein at increased EGCG concentration GSH-WbGST curve became parallel with the GSH-absent curve. Surprisingly, the WbGST-EGCG curve in the absence of GSH shows higher stabilisation, unlike the trend observed with the other profiles.

Table 6.2 Thermal shift melting temperatures of proteins complexed with EGCG or BSP in the presence or absence of GSH.

		Melting temperature (T_m) (°C)	
		No GSH	GSH
WbGST	No ligand	35.5	42.5
	BSP	34.5	40.5
	EGCG	37.5	41.5
hGSTM1-1	No ligands	51	53
	BSP	49	51
	EGCG	50	51
hGSTP1-1	No ligands	49.5	53
	BSP	46.5	49.5
	EGCG	50	53.5

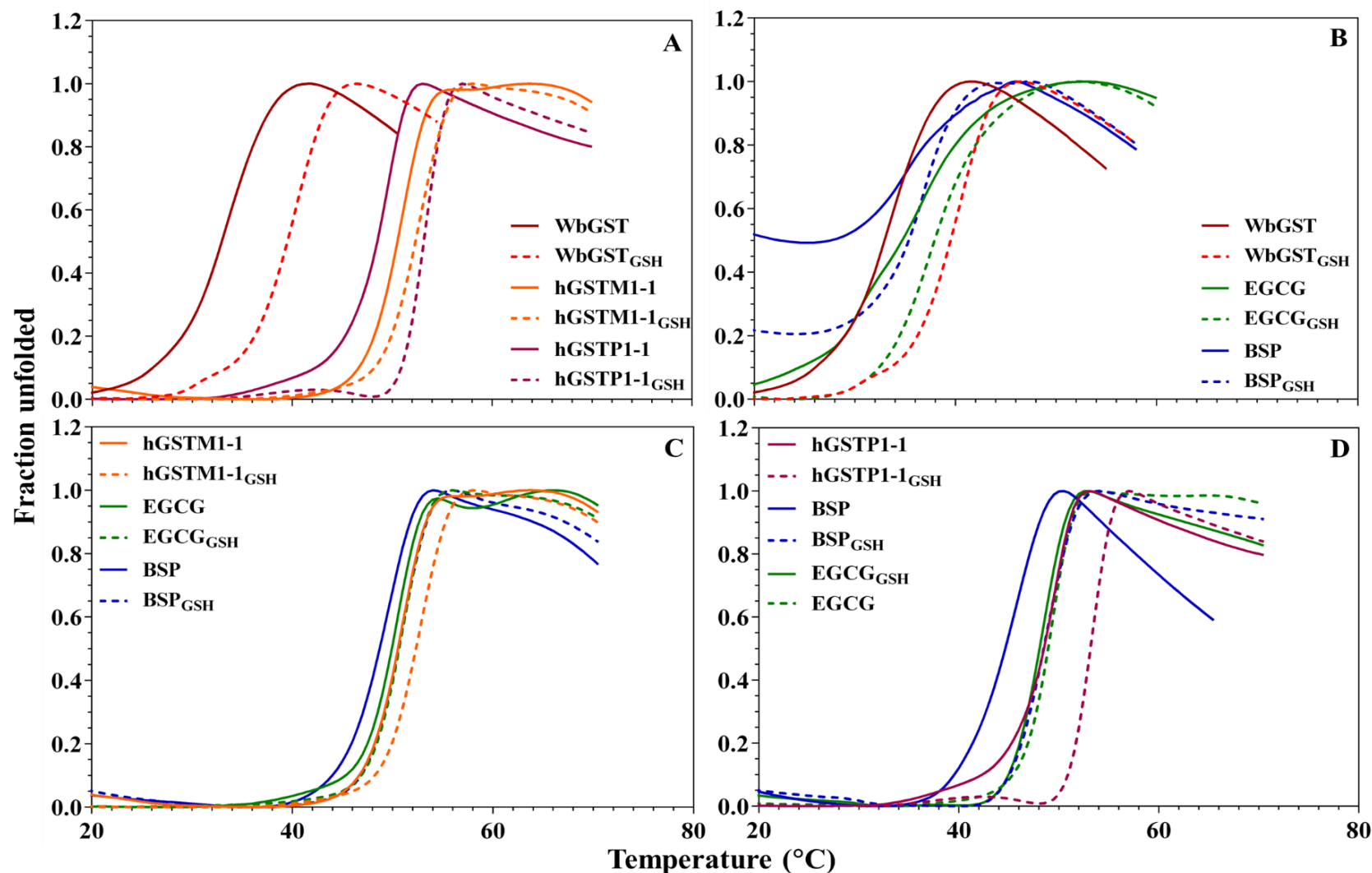


Figure 6.1 Thermal denaturing curves of the recombinant protein. The thermal fluorescence shift analysis of the recombinant protein stability and ligand interactions was monitored using SYPRO Orange dye in PBS with 50 μ M BSP and EGCG. (A) is the melting curves of the proteins in the absence of the ligands. The melting curves are represented in red, orange, purple, blue, and green for WbGST (B), hGSTM1-1 (C), hGSTP1-1 (D), BSP, and EGCG, respectively. The thermal unfolding was analysed in the absence (solid lines) and presence (dotted lines) of 5 mM GSH. As the temperature increases, fluorescence emission also increases, producing a sigmoidal curve showing the protein unfolding transition. The short initial unfolding transition observed for WbGST indicates that the dye already started binding the protein-exposed hydrophobic patches prior to heating. As observed in the unfolding transition in the presence of GSH, a rightward shift indicates increasing stability. The concentration of ligands used in GSH-absence destabilized the proteins except in the case of WbGST-EGCG.

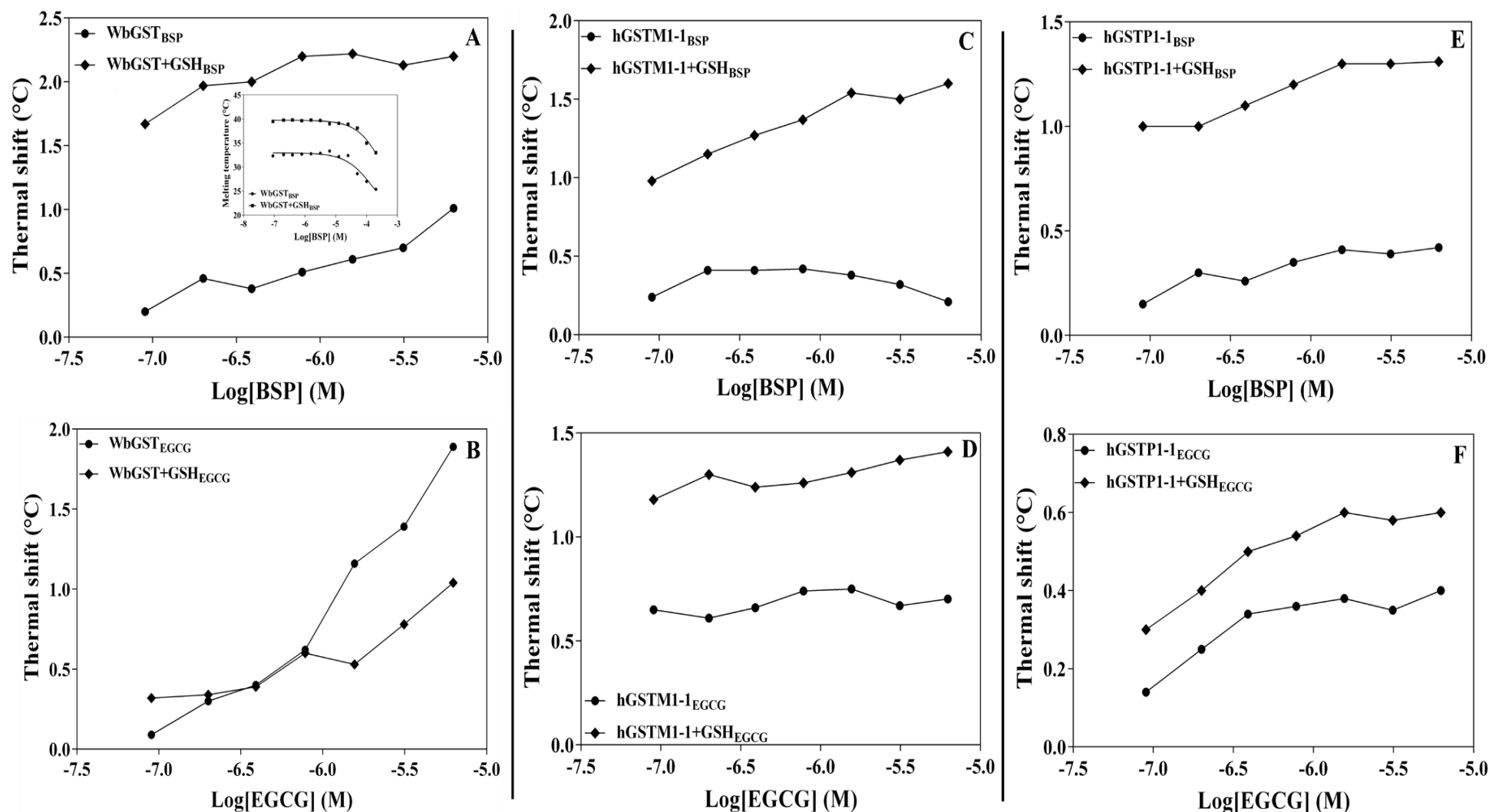


Figure 6.2 Thermal shift concentration-response curves. The recombinant WbGST, hGSTM1-1, and hGSTP1-1 were analysed with lower ligand concentrations ranging from 0.2 μ M – 20 μ M in the presence and absence of GSH. Thermal shifts represent the differences in T_m between the control (Protein without ligand) and ligand-containing samples. The obtained profiles can provide insight into the cooperativity of GSH towards stabilising the protein by the ligands. (A) The profile for WbGST-BSP followed a parallel trend. (B) EGCG showed higher affinity to WbGST in the absence of GSH. Parallel curves obtained in (A, D, E, F) indicate that ligand affinity to the protein is independent of GSH stabilising effects. The divergent trend observed in (C) suggests conjugate formation, which reduces product formation upon BSP binding to the hGSTM1-1 isozyme.

Differential scanning calorimetry

The DSC was also employed to analyse the stability of the proteins by measuring the heat capacity as a function of temperature. The structural stability of the recombinant proteins in this study was compared in the presence or absence of GSH, BSP, and EGCG. The stabilities obtained with the DSC are comparable to those of DSF with T_m values of 37.8 °C for WbGST and 51.8 °C for both hGSTM1-1 and hGSTP1-1 (Figure 6.3). The T_m increased to 44.5 °C, 53.9 °C, and 55.4 °C for WbGST, hGSTM1-1 and hGSTP1-1, respectively. Similar to the observation in DSF, GSH increased the stability of the proteins both in the presence or absence of the ligands. All the melting curves obtained exhibited a two-state transition with single transition temperatures, and the unfolding process was also cooperative, as indicated by the symmetric narrow picks. After that, TA Instruments validated the supposed two-state behaviour by fitting the curves to the van't Hoff equation implemented on the NanoAnalyze™ software. The resulting van't Hoff enthalpies were more prominent than the calorimetric enthalpies indicative of multimeric protein molecules (Marky and Breslauer, 1987). However, the complete thermodynamic characterisation could not be reliably obtained because the unfolding of the recombinant proteins was irreversible. Hence only the transition midpoint, T_m , is considered. The obtained data are tabulated in Table 6.3.

Table 6.3 DSC fitted thermodynamic parameters. The parameters were obtained by fitting the melting curves to the van't Hoff equation.

		T_m (°C)		ΔH (kJ.mol ⁻¹)		ΔS (kJ.K ⁻¹ .mol ⁻¹)	
		No GSH	GSH	No GSH	GSH	No GSH	GSH
WbGST	No ligand	37.8	44.5	435.4	897	1.55	2.2
	BSP	36.5	42.5	430.4	744.3	2.19	1.52
	EGCG	38.5	44.4	474	777.1	1.52	2.89
hGSTM1-1	No ligands	51.8	54.4	738.9	831	2.71	2.94
	BSP	51.3	53.9	632	783.1	2.19	2.31
	EGCG	52.3	53.9	729.4	730.5	2.53	2.61
hGSTP1-1	No ligands	51.8	55.9	880.9	969	2	2.56
	BSP	50.9	54	788.3	809	2.19	2.31
	EGCG	51.7	55.4	822.2	943	2.53	2.61

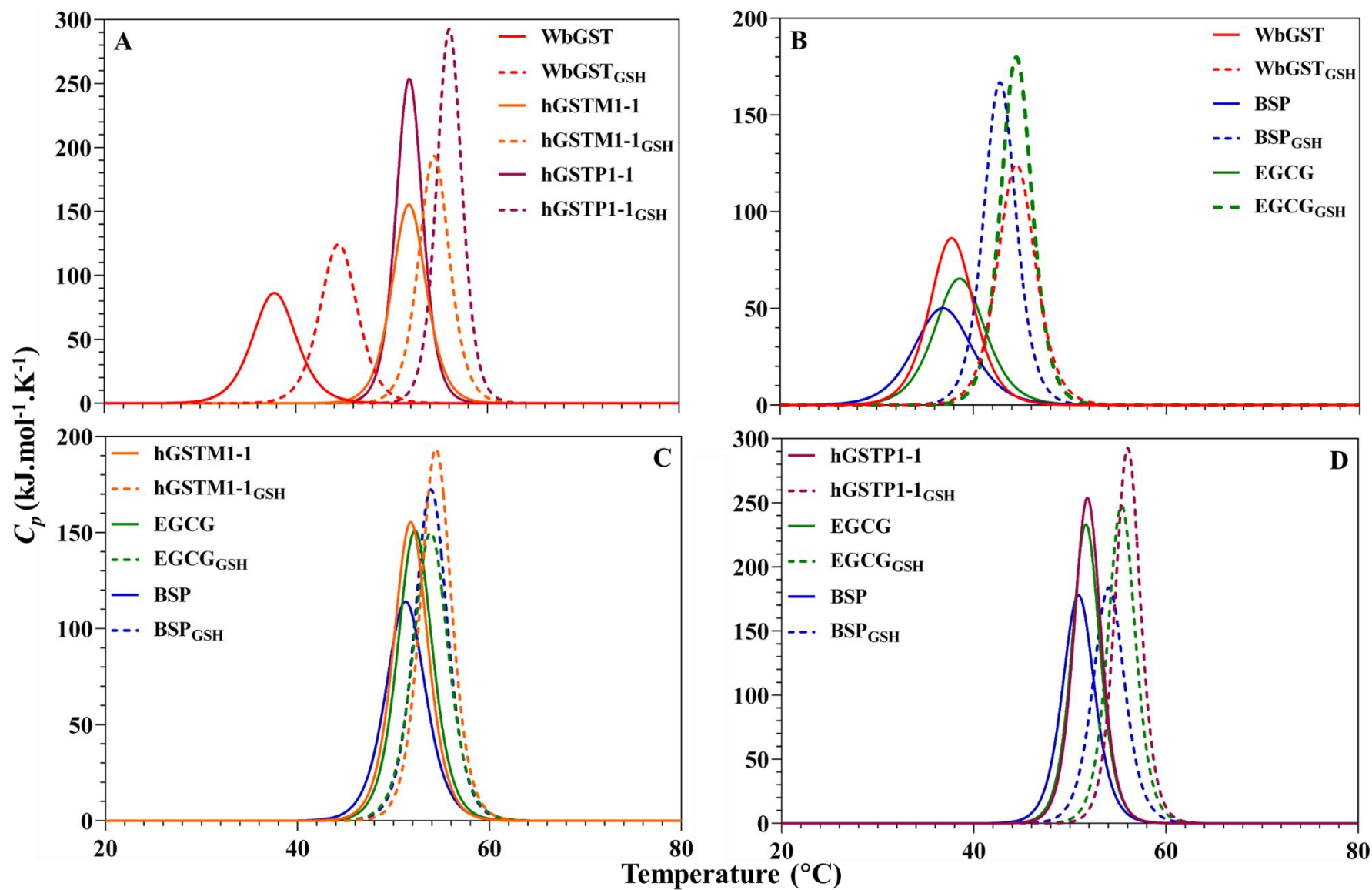


Figure 6.3 Deconvoluted DSC thermograms. The plots of WbGST (Red), hGSTM1-1 (Orange) and hGSTP1-1 (Purple), curves in dotted lines represent samples analysed in the presence of GSH. BSP (Blue) and EGCG (Green). Prior to fitting and plotting the data, they were baseline corrected and converted to molar heat capacity by applying the dimer concentrations of the proteins. The presence of GSH increased the stability of the proteins (A), as indicated by a higher ΔT_m . EGCG increased the stability of WbGST (B), while BSP produced a destabilising effect in the absence of GSH. BSP decreases the stability of hGSTM1-1 (C) and hGSTP1-1 (D).

Evaluating thermal stability of target proteins by far-UV CD spectropolarimetry

To gain insight towards the stability contributions of the secondary structural contents of WbGST, hGSTM1-1 and hGSTP1-1, thermal denaturation was performed and monitored using far-UV CD spectropolarimetry. The protein samples were unfolded over a temperature range of 20 °C – 80 °C and data were recorded at 1 °C increments. A single unfolding transition ($N \rightarrow U$) was observed for the three recombinant proteins both in the presence and absence of ligands. The observed T_m in the absence of ligands was 43 °C, 52.9 °C and 52.8 °C for WbGST, hGSTM1-1, and hGSTP1-1, respectively (Figure 6.4). Similar to other previous unfolding observations, GSH also increased the stability of the secondary structural content of the proteins. The various T_m values are tabulated in Table 6.4. Unfortunately, other thermodynamic parameters, such as enthalpy of unfolding (ΔH_m), melting temperature (T_m), and entropy of unfolding (ΔS_m), could not be correctly established because the unfolding process was irreversible.

Table 6.4 Melting temperatures obtained from far-UV CD spectropolarimetry of WbGST, hGSTM1-1, and hGSTP1-1 in the presence and absence of GSH, BSP, and EGCG

		Melting temperature (T_m) (°C)	
		No GSH	GSH
WbGST	No ligand	38	44
	BSP	42	48
	EGCG	38.6	48.2
hGSTM1-1	No ligands	52.9	53.8
	BSP	49	52.5
	EGCG	48	53.5
hGSTP1-1	No ligands	52.6	55.7
	BSP	52.7	52.4
	EGCG	52.6	57

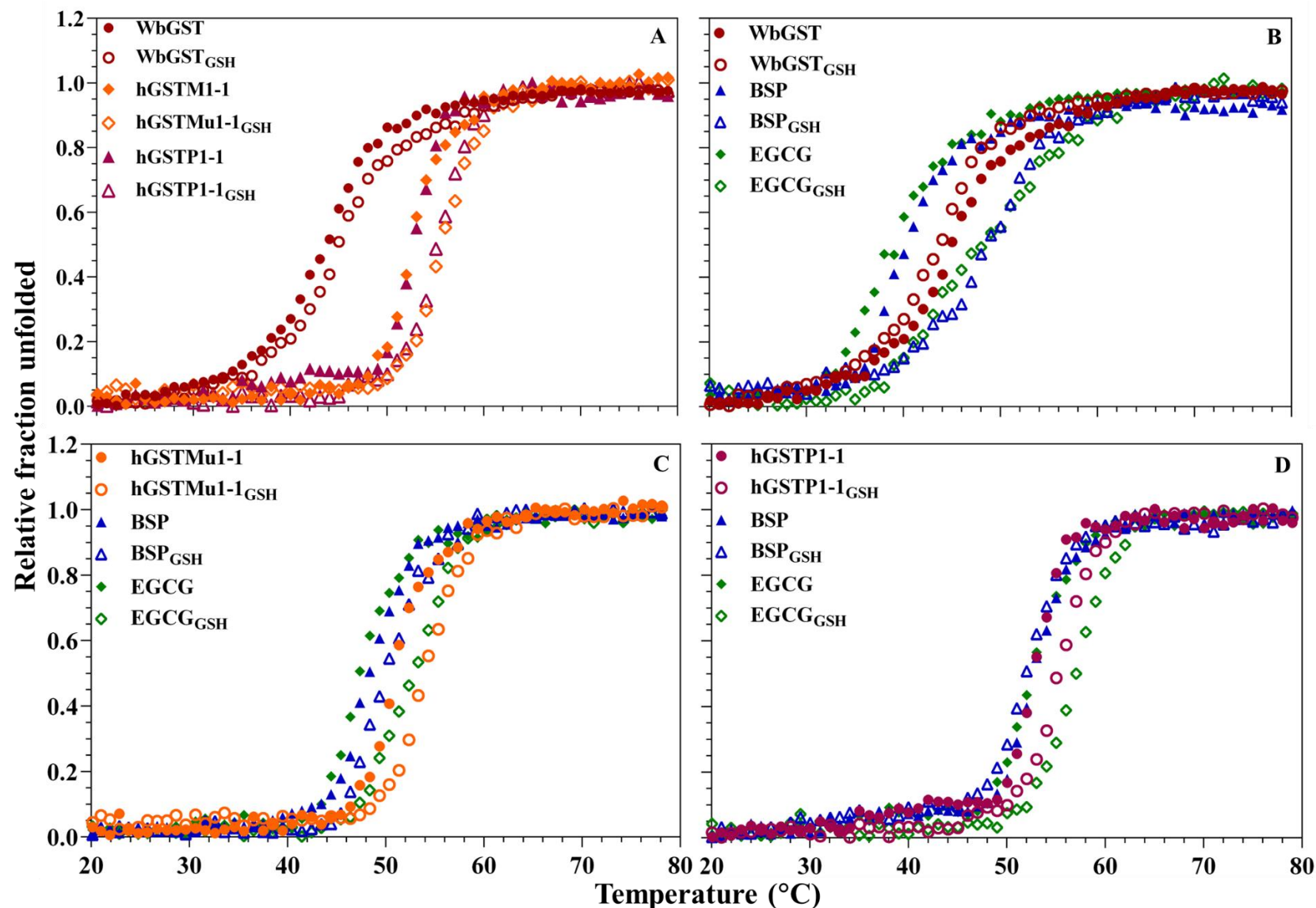


Figure 6.4 Recombinant protein far-UV CD thermal unfolding. (A) The unfolding of WbGST (Red), hGSTM1-1 (Orange) and hGSTP1-1 (Purple) in the presence and absence of GSH was observed to follow a single-step transition. The obtained melting temperatures are as follows: 43 °C (WbGST), 44 °C (WbGST_{GSH}), 52.9 °C (hGSTM1-1), 53.8 °C (hGSTM1-1_{GSH}), 52.8 °C (hGSTP1-1), and 55.2 °C (hGSTP1-1_{GSH}). (B) WbGST curves have the shortest pre-transition region as well as the lower T_m . (C) hGSTM1-1 and (D) hGSTP1-1 appear to be the most thermally stable. The three proteins were analysed both in the presence and absence of BSP (Blue) and EGCG (Green).

Urea-induced equilibrium unfolding

Protein polypeptide chains adopt various folding pathways to attain a native folded conformation. Protein folding pathways can be populated with a distinct number of folded intermediates at equilibrium. During the unfolding process, proteins are unfolded using discrete concentration values of denaturants along the protein folding pathway. The equilibrium unfolding and refolding pathways can be monitored using a variety of probes. Spectroscopic probes such as far-UV CD can observe changes in secondary structure content. At the same time, tryptophan fluorescence can monitor global and localised tertiary structural changes and ANS fluorescence to observe the changes in the formation of hydrophobic patches and intermediates. Equilibrium unfolding studies of WbGST were performed to characterise its folding cooperativity (m), conformational stability (ΔG_{H_2O}) and the denaturant midpoint ($[Urea]_{1/2}$). Trp38 was excited at 295 nm and emissions at 342 nm and 351 nm wavelengths were employed to monitor the tertiary structural changes. The two emission wavelengths represent the maximum folded and unfolded wavelengths, respectively. Secondary structural changes were analysed by scanning ellipticity values at 222 nm wavelength.

Reversibility and Recoverability of WbGST unfolding.

Thermodynamic parameters cannot be reliably determined when the unfolding transition is irreversible (Pace, 1986). Therefore, it is important to establish the reversibility and the recoverability of the protein to its native state after denaturation. The ability of WbGST to refold and recover its native secondary and tertiary conformation was evaluated by far-UV CD spectropolarimetry and intrinsic tryptophan fluorescence, as previously described in section 6.3.4. Spectroscopic data obtained for the refolded WbGST was comparable to that of the native protein (Figure 6.5). Percentage native signal recovery of ~98 % and ~89 % was obtained for the intrinsic tryptophan fluorescence and far-UV CD, respectively. Furthermore, the overlapping of both unfolding and refolding curves is an indication of a successful unfolding-refolding process (Figure 6.6). Therefore, confirming the reversibility of denatured WbGST to its native state.

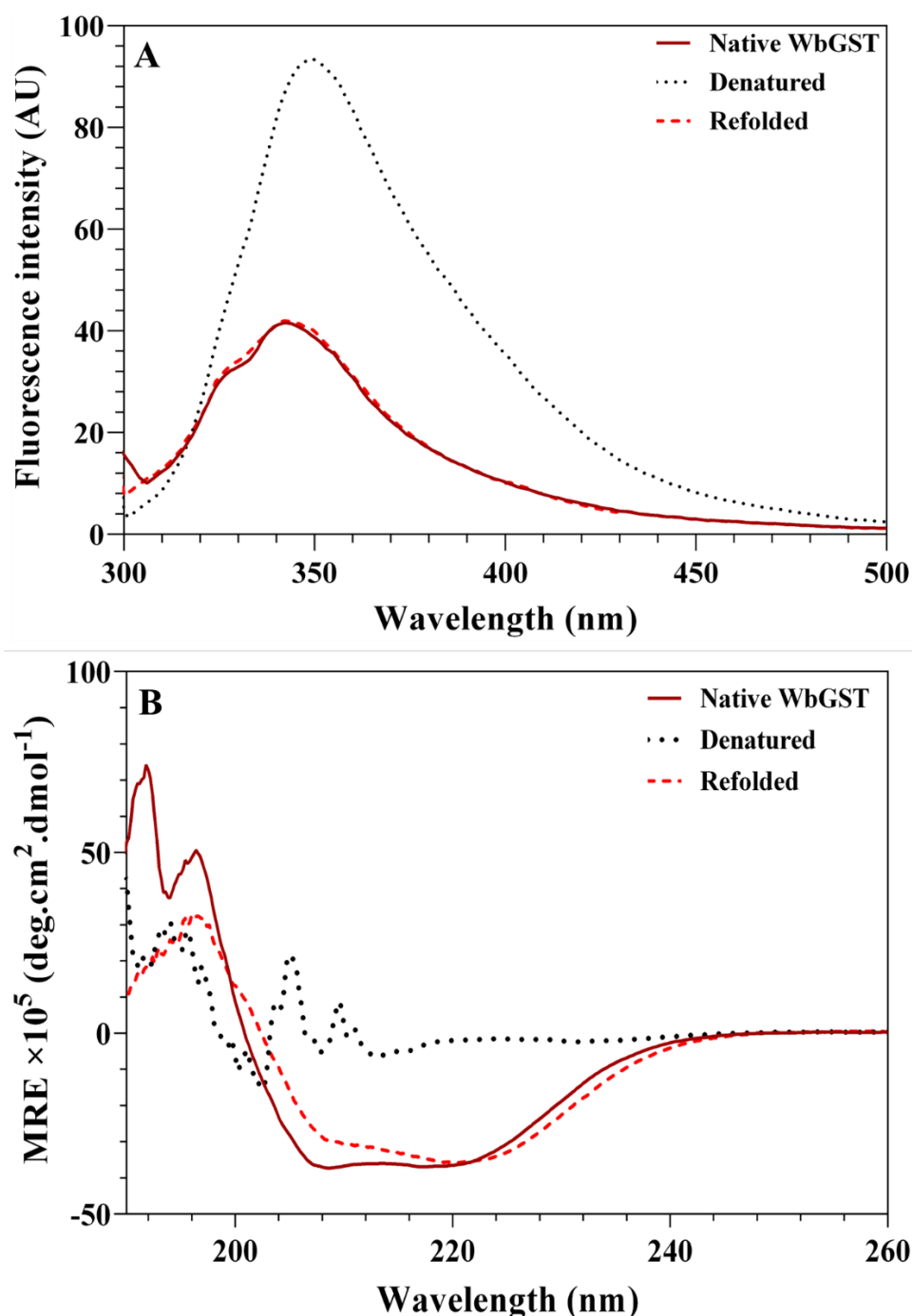


Figure 6.5 Urea-induced unfolding recovery of WbGST tertiary and secondary structures. (A) Unfolding and refolding spectra of the intrinsic tryptophan fluorescence are overlaid, indicating the recovery of the tertiary structure of WbGST. Tryptophan was excited at 295 maximum emissions at 342 nm and 351 nm wavelengths for the folded and unfolded proteins, respectively. The percentage recovery of the tertiary structure was ~98 %. (B) Secondary structure recovery was monitored by far-UV CD using ellipticity values at 222 nm wavelength. The ellipticity at 222 nm for both native and refolded proteins are identical. The percentage recovery of the native signal obtained was 89 %. Native spectra are represented in solid red lines, denatured in black dotted lines, while recovered spectra are in red dotted lines.

The unfolding of WbGST monitored by tryptophan fluorescence and far-UV CD

Changes occurring at the local environment of Trp38, located at the active site of WbGST, is a reliable probe to assess the changes in the tertiary structure of the protein as the unfolding process progresses. Protein samples (3 μ M) were unfolded in urea ranging from 0 – 8 M for 1 h at room temperature. Tertiary structural changes of WbGST were monitored by intrinsic tryptophan fluorescence excited at 295 nm and emission signals at 340 nm and 351 nm wavelengths. The resulting profile revealed that unfolding and refolding occurred over a single transition both in the presence and absence of GSH (Figure 6.6A and C). However, in the lack of GSH, changes in the tryptophan environment started at the first urea concentration (0.25 M), whereas in the presence of GSH, it began after 1 M urea. Data for unfolding and refolding are superimposable and satisfy a two-state curve fitting model with $R^2 = 0.99$ for both unfolding and refolding curves. Similarly, a global fit of the curves using the two-state curve fit model was successful (Figure 6.6B and D). The following thermodynamic parameters resulted from the fit: $\Delta G_{H_2O} = 11.7 \pm 1.6 \text{ kcal.mol}^{-1}$, $\Delta G_{H_2O(GSH)} = 15.8 \pm 1.4 \text{ kcal.mol}^{-1}$, $m = 4.3 \pm 0.2 \text{ kcal.mol}^{-1}.\text{M}^{-1}$, $m\text{-GSH} = 4.5 \pm 0.4 \text{ kcal.mol}^{-1}.\text{M}^{-1}$, $[\text{Urea}]_{1/2} = 2.7 \text{ M}$ and $[\text{Urea}]_{1/2\text{GSH}} = 3.5 \text{ M}$.

Secondary structural unfolding monitored using far-UV CD followed a single transition model from native to unfolded state (Figure 6.7A). In contrast, the refolding process in the absence of GSH exhibited two transitions, observed between 3.5 M and 5.25 M urea and the second between 1.25 M and 2.5 M urea (Figure 6.7A). A stable secondary structure intermediate was observed between 2.5 M and 3.25 M. Data obtained for unfolding was fitted to a two-state model curve ($R^2 = 0.99$) using regression modelling and a three-state model curve ($R^2 = 0.99$). The folding mechanism of the protein in the presence of GSH satisfied a two-mechanism (Figure 6.7B). The following thermodynamic unfolding parameters were obtained when globally fitted: $\Delta G_{H_2O(GSH)} = 16.2 \pm 0.7 \text{ kcal.mol}^{-1}$, $m\text{-GSH} = 4.5 \pm 0.32 \text{ kcal.mol}^{-1}.\text{M}^{-1}$, and $[\text{Urea}]_{1/2\text{GSH}} = 3.6 \text{ M}$. Conversely, the folding mechanism in the absence of GSH was disparate. Secondary structure GSH absent unfolded through a two-state mechanism $N_2 \rightarrow 2U$ and then refolded according to a three-state mechanism $2U \rightarrow I_2 \rightarrow N_2$. Therefore, it is difficult to reliably determine the thermodynamic parameters of the secondary structure unfolding in the absence of GSH, as the unfolding and refolding curves are not superimposable.

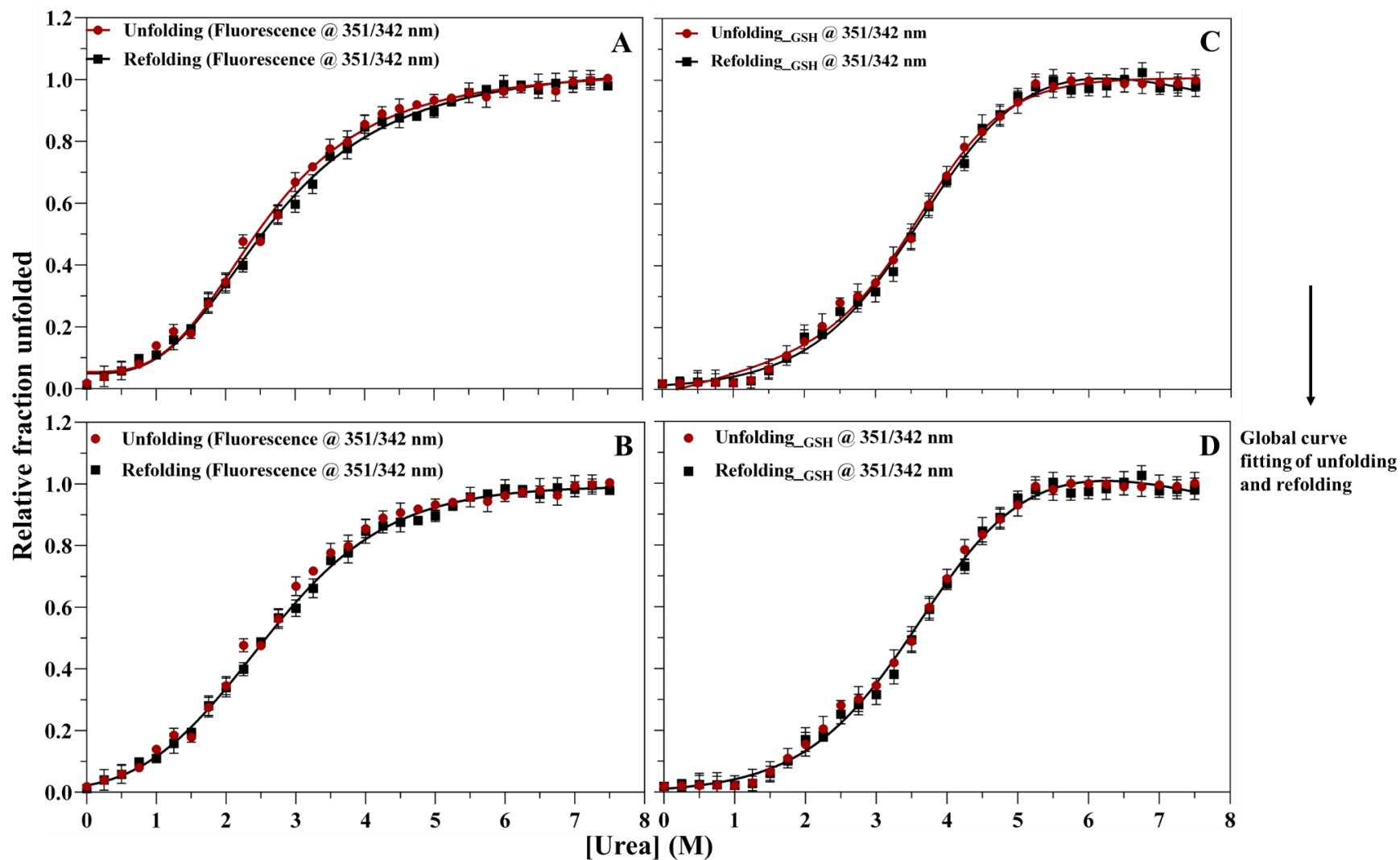


Figure 6.6 Unfolding mechanisms monitored by intrinsic tryptophan fluorescence. The obtained intrinsic tryptophan fluorescence (F_{350/342}) was excited at 295 nm wavelength. The fraction of unfolded WbGST (3 μM) was plotted against the urea concentration with error bars indicating standard errors of the mean. (A) Unfolding (red circles) and refolding (black square) curves of WbGST in the absence of GSH data were fitted to $N_2 \leftrightarrow 2U$ two-state unfolding curves ($R^2 = 0.99$). (B) Global fitting of the unfolding and refolding data of 'A' to a two-state folding mechanism with the following thermodynamic parameters: $\Delta G_{H_2O} = 11.7 \pm 1.6 \text{ kcal.mol}^{-1}$, $m = 4.3 \pm 0.2 \text{ kcal.mol}^{-1}.\text{M}^{-1}$, $[\text{Urea}]^{1/2} = 2.7 \text{ M}$. (C) Overlay of unfolding (red circles) and refolding (black square) of WbGST in the presence of 5 mM GSH. Data for both unfolding and refolding was fitted to $N_2 \leftrightarrow 2U$ two-state unfolding curves ($R^2 = 0.99$), and both have similar folding pathways. (D) Global fitting of unfolding and refolding data of 'C' to a two-state folding mechanism. The following thermodynamic parameters were obtained $\Delta G_{H_2O}(\text{GSH}) = 15.8 \pm 1.4 \text{ kcal.mol}^{-1}$, $m_{\text{GSH}} = 4.5 \pm 0.4 \text{ kcal.mol}^{-1}.\text{M}^{-1}$, and $[\text{Urea}]^{1/2}_{\text{GSH}} = 3.5 \text{ M}$.

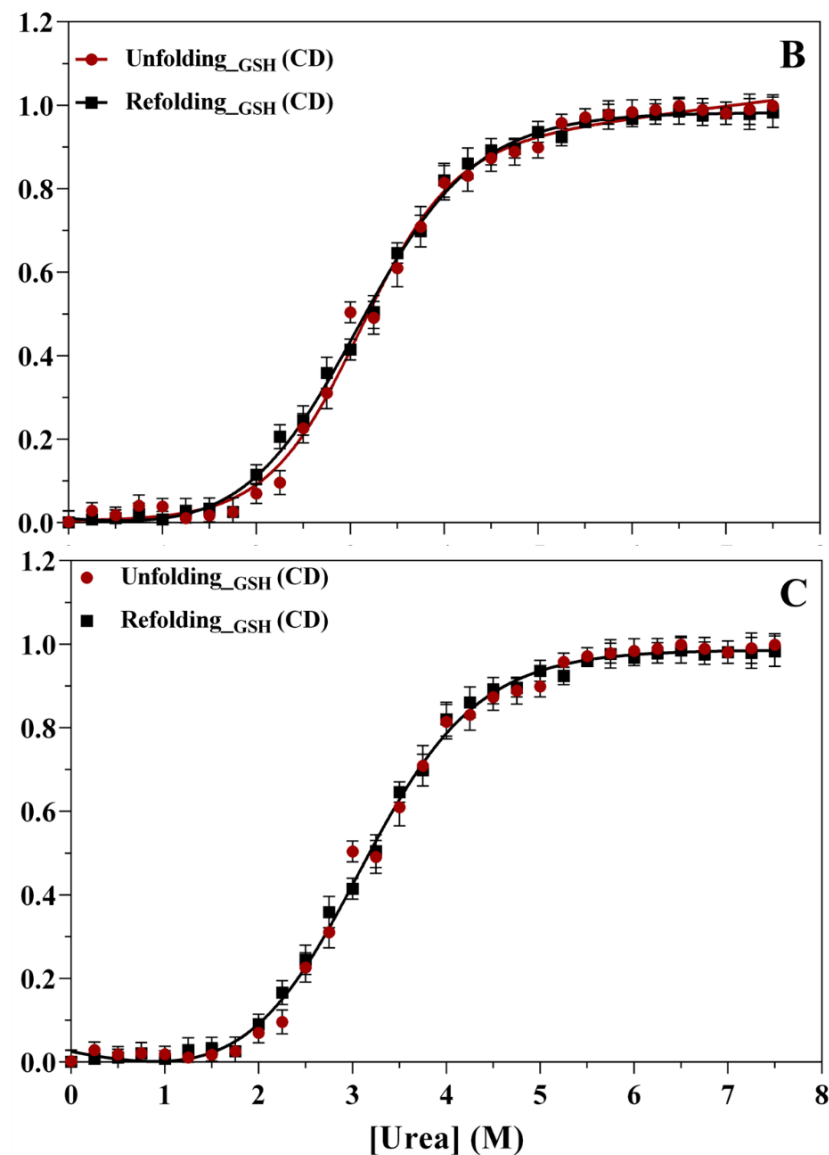
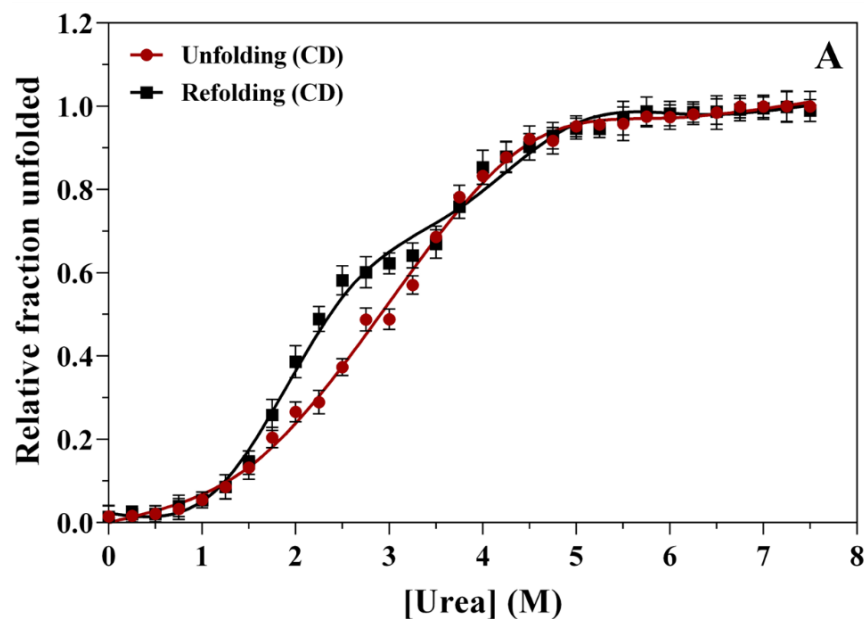


Figure 6.7 Unfolding of WbGST secondary structure. The data was obtained by monitoring ellipticity at 222 nm. The fraction of unfolded WbGST (5 μ M) was plotted against the urea concentration with error bars indicating standard errors of the mean. (A) Unfolding (red circles) and refolding (black squares) of WbGST in the absence of GSH. The unfolding data was fitted to a two-state $N_2 \rightarrow 2U$ folding mechanism ($R^2 = 0.99$), while the refolding was fitted to a three-state $2U \rightarrow I_2 \rightarrow N_2$ folding mechanism ($R^2 = 0.99$). (B) In the presence of GSH, both the unfolding and refolding profiles overlaid and data points were fitted to a two-state mechanism $N_2 \leftrightarrow 2U$ with $R^2 = 0.99$. (C) Global fitting of the unfolding-refolding ($_{GSH}$) data to a two-state regression model. With the following thermodynamic parameters: $\Delta G_{H_2O(GSH)} = 16.2 \pm 0.7 \text{ kcal.mol}^{-1}$, $m_{-GSH} = 4.5 \pm 0.32 \text{ kcal.mol}^{-1}.\text{M}^{-1}$, and $[\text{Urea}]^{1/2}_{GSH} = 3.6 \text{ M}$.

Table 6.5 Folding thermodynamic parameters of the global fitting.

		ΔG_{H2O} (kcal.mol ⁻¹)	<i>m</i> -value (kcal.mol ⁻¹ .M ⁻¹)	[Urea] _{1/2} (M)
Intrinsic tryptophan fluorescence	WbGST	11.7 ± 1.6	4.3 ± 0.2	2.7
	WbGST _{GSH}	15.8 ± 0.9	4.5 ± 0.4	3.5
Circular Dichroism	WbGST Unfolding	11.4 ± 2.1	3.8 ± 0.3	3
	WbGST Refolding _(2U→I2)	13.4 ± 1.9	3.2 ± 0.12	4.2
	WbGST Refolding _(I2→N2)	10.2 ± 1.1	6 ± 1.1	1.7
	WbGST _{GSH}	16.2 ± 0.7	4.5 ± 0.32	3.6

Unfolding monitored by ANS binding.

ANS binding was also used to monitor unfolding and to detect changes at the active site and its surroundings. The anionic dye has been previously reported to bind at the H-site and adjacent to the dimer interface of native GSTs. In addition, ANS can be used to probe hydrophobic patches emerging as the unfolding process proceeds. The emission wavelength of ANS in water (521 nm) shifts to 487 nm when ANS is bound to WbGST with a considerably high quantum yield. ANS does not bind unfolded protein. There was no increase in intensity as the protein unfolded, meaning that the level of ANS binding did not increase (Figure 6.8). This is contrary to what is typically observed with most GSTs and multimeric proteins that unfold into stable monomers first, thus exposing more hydrophobic patches before unfolding the monomers entirely (Abdalla and Hamed, 2006; He *et al.*, 2009; Hornby *et al.*, 2000). The level of ANS binding in the absence of GSH was higher at lower urea concentrations but started decreasing gradually from 0.5 M urea concentration, but in the presence of GSH, stability was observed from 0 M - 1.5 M urea. An overlay of the two curves was observed at 3.25 M urea, and no ANS binding was observed from 6 M urea concentration.

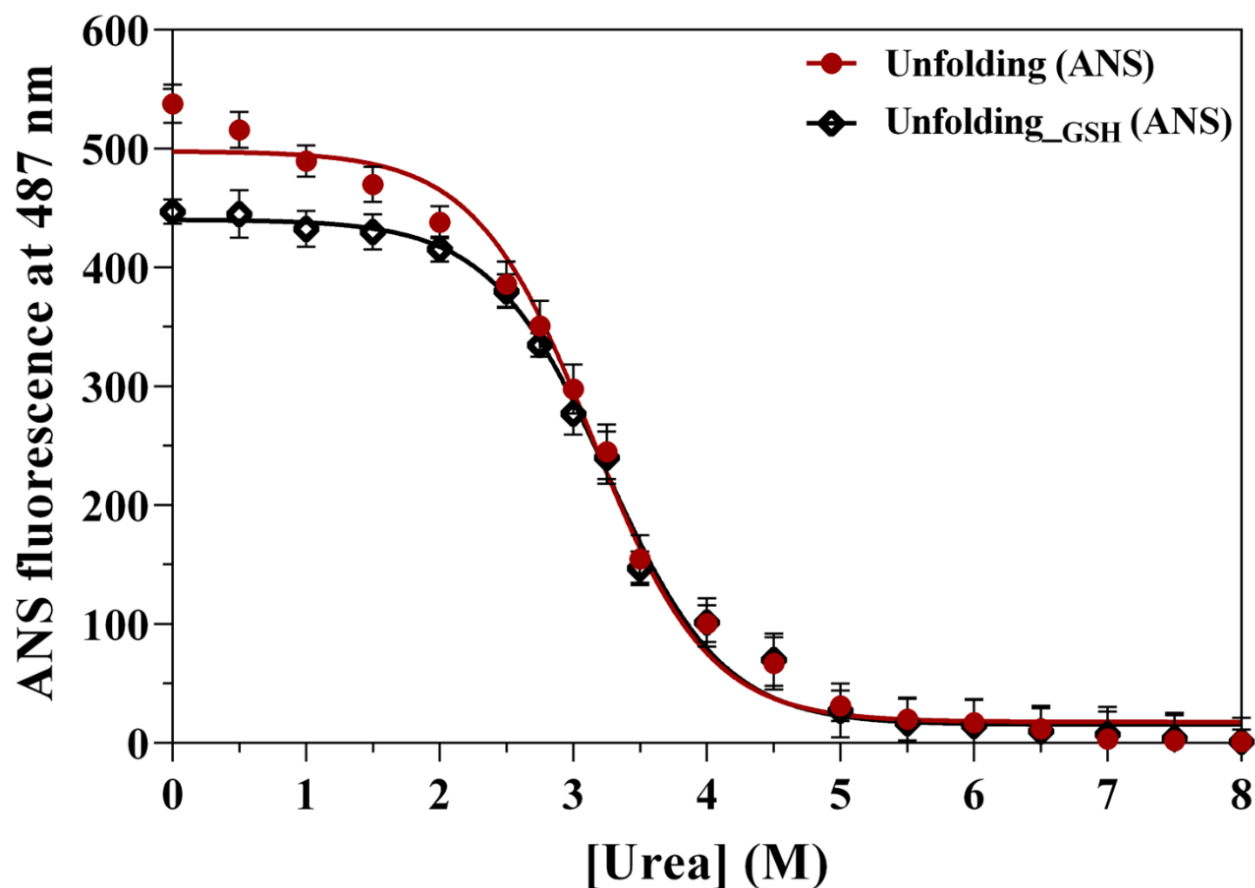


Figure 6.8 Urea unfolding of WbGST monitored by ANS binding. ANS fluorescence was selectively excited at 390 nm, and emission at 487 nm for WbGST in the absence of GSH (red circles) and presence of GSH (black diamonds) was plotted against a range of urea concentration with error bars indicating standard errors of the mean. The concentration of ANS used was 100 μ M with 3 μ M of WbGST and 5 mM GSH. The unfolding data of WbGST in complex with GSH and the absence of GSH did not overlay at lower urea concentrations. However, at 3.25 M urea, the data sets overlay until the protein ultimately unfolds at about 5 M urea concentration. There was no binding of ANS from 6 M urea, indicating that the protein has been completely unfolded. The unfolding transition is typical of a two-state unfolding mechanism with no observable stable intermediate.

6.6 Discussions

The stability of protein molecules is crucial for functional optimisation and effective downstream applications, especially in protein-drug applications. Therefore, protein conformational stability may be evaluated by altering the existing equilibrium between the native and unfolded states using denaturants such as temperature, chemicals, pH, and pressure. The use of temperature in combination with SYPRO Orange was employed to monitor the stability of WbGST in the presence or absence of BSP and EGCG in differential scanning fluorimetry (DSF). SYPRO Orange dye is an environmentally sensitive dye which experiences a substantial increase in fluorescence intensity upon binding to hydrophobic patches in proteins (Hawe *et al.*, 2008). This increase in fluorescence correlates with increasing temperature as the unfolding proceeds with the formation of a sigmoidal curve. The T_m of the protein can be extrapolated from the curve inflection point, and an increase in T_m values corresponds to increased stability. The hGSTM1-1 exhibited a higher T_m value and, thus, higher stability when compared to hGSTP1-1 and WbGST. However, upon binding of GSH to the recombinant proteins, hGSTP1-1 displayed the highest T_m value. GSH binding initiated a more stabilising effect on WbGST, with a 19.7 % increase in T_m followed by hGSTP1-1 (7 %), and the hGSTM1-1 (3.8 %) had the least noticeable impact (Figure 6.1A). This trend is similar to what was observed in ANS spectroscopy, where GSH binding reduced ANS fluorescence the most in WbGST and the least in hGSTM1-1. In like manner, analogues of GSH have been noted to exert stabilising effects on proteins (Lea and Simeonov, 2012).

The binding of ligands to proteins usually increases protein stability. When ligand binding is coupled to protein unfolding, the Gibbs free energy of the protein unfolding is also increased. This is characterised by increased T_m and can also indicate the ligand ability to bind the protein. However, some ligands are destabilising ligands and bind mainly to the unfolded state of the protein molecules (Cimpmperman *et al.*, 2008). BSP binding had the most destabilising effect on the recombinant proteins. Even at room temperature, the destabilising effect of BSP on WbGST is pronounced (Figure 6.1B) and evident in the increased pre-transition region fluorescence (high baseline). Similarly, the SYPRO Orange could access certain hydrophobic regions on apo WbGST prior to heating. The binding of ligands to unfolded protein states is not adequately understood. Destabilising ligands can bind to sites other than the active site. They may bind to intermediate

species occurring as the protein unfolds. To my knowledge, there are no crystal structures of unfolded proteins. ANS is an example of a ligand that binds to the unfolded state of proteins (Cimpmperman *et al.*, 2008). Hence the exact binding site of ANS is still under speculation because there is no crystal structure of ANS bound to protein. The destabilising effect of ANS increases as the concentration increases, and a similar trend was observed with BSP and EGCG.

Furthermore, information obtained from DSF can provide insight towards the categorization of inhibitors and their disparate mechanisms. This was achieved by measuring protein thermal shifts in the presence of varying concentrations of the ligands. Evaluation of the profiles obtained in the presence and absence of GSH depicted each inhibitor mechanism of action (Figure 6.2). Parallel trend lines were observed in the binding of BSP and EGCG to the recombinant proteins in this study, except for the binding of BSP to the hGSTM1-1, which is divergent. The parallel trend indicates non-competitive inhibition towards the G-site, which corresponds to the observation made during the GSH-CDNB inhibition assay earlier in this study. Secondly, the parallel trend depicts that both inhibitor stabilising effects can occur equally without GSH. A similar trend was observed in the binding of particular ligandin types of inhibitors having planar aromatic compounds with anionic functional groups (Lea and Simeonov, 2012). The diverging trend observed in BSP-hGSTM1-1 shows that the stabilising effect of BSP on hGSTM1-1 was greatly enhanced by GSH binding (Figure 6.2C). This suggests that the BSP is enhanced in the presence of GSH and that BSP may have some sort of interaction with GSH. EGCG binding to WbGST displayed an interesting trend in the absence of GSH, with the thermal shift converging and exceeding that of WbGST-GSH (Figure 6.2B). Therefore, the EGCG stabilising effect is independent of GSH, but the presence of GSH tends to reduce its stabilising effect. This implies that GSH may be reducing the occupancy of EGCG since both active sites are adjacent.

In addition, the DSF dilution series of the inhibitors also revealed that their stabilising effect occurs at a lower concentration range below 25 μ M. Therefore, increasing the ligand concentration destabilised the proteins, as evident in the observed decreasing T_m values (Figure S3). This observation may be helpful when deciding the concentration of inhibitor to use during protein crystallisation because it is difficult to crystallise a partially folded protein.

Further stability studies as a function of heat capacity were performed on the recombinant proteins in the presence and absence of the ligands using DSC. This technique assesses the proportion of the interacting forces stabilising the native structure of protein molecules. These forces result from the interaction within the various atoms of the amino acid sequence and also between the protein and its surrounding solvent molecules, thus controlling protein stability (Connelly, 1994). Perturbation of native proteins monitored in an under-regulated range of temperatures can be used to understand the impact of heat on these intra-molecular and inter-molecular interactions. The binding of ligands to proteins changes the protein heat capacity, resulting in a change in the profile. The binding of ligands preferentially to the native state of protein results in a T_m higher than that of the apoprotein. The reverse is the case when a ligand binds more to an unfolded protein. Therefore, DSC can directly measure if the binding of a ligand to a protein molecule is stabilising or destabilising in nature. Besides changes in T_m , the profiles of ligand complexed proteins can differ in shape compared to that of apoproteins. This depends majorly on the formed complex heat capacity and the ligand impact on the stability of the protein. Indeed, striking distortions of ligand complexed protein thermograms have been previously observed (Lin *et al.*, 1994; Shrake and Ross, 1990). In light of the above, the DSC of apo WbGST, hGSTM1-1 and hGSTP1-1 and in complex with the ligands were analysed. The resulting profiles depict cooperative unfolding transitions. In the Apo state, WbGST had the lowest T_m value, and in complex with GSH, it exhibited the most stabilising effect with a ΔT_m value of 6.9 °C followed by hGSTP1-1 with a ΔT_m of 4 °C (Figure 6.3A). Therefore, the binding of GSH to these recombinant proteins propels them into a more energetically stable state, and these trends are in agreement with DSF results.

The binding of BSP reduced the T_m values of the three proteins more than EGCG. In fact, EGCG stabilised WbGST as an increase in T_m value was observed (Figure 6.3B). It appears that protein-stabilising enthalpic interactions, such as hydrophobic interactions and hydrogen bonds, were easily disrupted by the binding of BSP. Protein structural conformational changes induced by the binding of BSP and EGCG enhance the exposure of non-polar side chains and displacement of water molecules during heating and, in turn, impact the heat absorption of the protein. The lower T_m values (Table 6.3) induced by ligand binding as opposed to increased T_m values are indicative of destabilisation similar to what was observed in DSF. Other thermodynamic parameters were also obtained (Table 6.3), but due to the inability of the protein samples to refold, the values are

not sufficient to obtain accurate calculations. Notwithstanding, the substantial difference between the unfolding enthalpy of WbGST and the human GSTs gives an insight into the compactness of folding and the accessibility of hydrophobic side chains. The higher unfolding enthalpies of the human GSTs imply that the non-polar moieties of the protein were more tightly packed. Thus, more bonds were broken during the unfolding process, unlike WbGST, which is more flexible with larger accessible hydrophobic pockets, as confirmed by the ANS binding studies. Secondly, the higher unfolding enthalpies observed in GSH complexed proteins correspond with the observed increased T_m values, inferring increased stability.

Subsequently, thermal monitoring of the secondary structure unfolding using far-UV CD yielded gradients indicative of unfolding cooperativity. The unfolding process was irreversible, and consequently, the thermodynamic parameters of unfolding could not be calculated. Nevertheless, the obtained T_m values (Table 6.4) gave insight into the stability of the secondary structure contents of the recombinant proteins. The secondary structure contents of WbGST were the least stable (Figure 6.4). Ligand complexed proteins were characterised by decreased stability except hGSTP1-1 (Figure 6.4D). Interestingly, protein-EGCG complexes induced the most flexibility on the protein structures, contrary to their impact on their tertiary and quaternary structures. Increased T_m values were observed in protein-GSH complexes (Figure 6.4A), comparable to the increase in stability of tertiary and quaternary structures observed in DSF and DSC.

In addition to the thermal processes of evaluating protein unfolding, chemical denaturation of proteins can give insight into the folding pathways, with a better chance of reversibility than thermally denatured proteins. The folding pathways of WbGST were monitored by fluorescence spectroscopy and far-UV CD spectropolarimetry. The chemical unfolding of human GSTs has been studied extensively, so only WbGST was analysed. Denaturation of WbGST was induced over a gradient of increasing urea concentration. The urea unfolding of WbGST was utterly reversible and ~98 % recoverable when analysed by fluorescence spectroscopy (Figure 6.5A). While ~89 % of the secondary structural elements were recovered when monitored by far-UV CD (Figure 6.5B). The discrepancy observed in the percentage of recoverability may have resulted from technical errors from the imprecision of protein concentration of the folded and refolded protein samples. This is because the protocol applied for analysing the recoverability of WbGST

involves two dilution steps. The first dilution was with urea followed by the buffer, so it is very likely to accumulate some pipetting errors during sample preparations. While this may be the case, it is also possible that the difference in recoverability implies that not all secondary structure components could refold to their native conformations in the presence of urea. Besides, the refolding of the secondary structure in the absence of GSH followed a pathway different from the WbGST-GSH complexed sample (Figure 6.7A). This may also indicate that the stabilising effect of GSH contributes to proper refolding and reassumption of the native secondary structure of WbGST.

(Un)folding studies of WbGST monitored by intrinsic tryptophan fluorescence both in the presence and absence of GSH was fitted to a two-state mechanism ($R^2 = 0.99$) (Figure 6.6). Conversely, secondary structure refolding in the absence of GSH did not assume the two-state mechanism. However, in the presence of GSH, both unfolding and refolding assumed the two-state mechanism (Figure 6.7). It is known that the majority of globular monomers, small dimeric proteins, GSTs and even proteins similar to GSTs fold in a two-state mechanism (Erhardt and Dirr, 1995; Gildenhuis *et al.*, 2010; Gloss and Matthews, 1998; Kaplan *et al.*, 1997; Wallace *et al.*, 1998). Secondly, it is not uncommon to observe stable monomeric intermediates populating the unfolding pathways of GSTs. For instance, the rGSTM1-1, bbGSTP1-1 and *Proteus mirabilis* GSTB1-1 unfold via a stable monomeric intermediate (Hornby *et al.*, 2000; Sacchetta *et al.*, 1993; Sacchetta *et al.*, 1999). In addition, the unfolding of GST proteins via more than a single stable intermediate has equally been reported (Gildenhuis *et al.*, 2010; Stevens *et al.*, 1998). Unfolding monitored by intrinsic fluorescence in the absence of GSH started from the lowest urea concentration at 0.25 M (Figure 6.6A), unlike in the presence of GSH, which started at 1.25 M (Figure 6.6C). This indicates that the binding of GSH increased the stability of the intrinsic interactions surrounding the only tryptophan residue (Trp38) in WbGST. Moreover, the tryptophan residue is located at the highly dynamic helix $\alpha 2$ walling off the G-site, which is situated at the N-terminal domain of the protein (Rajaiah Prabhu *et al.*, 2018). In addition, the tertiary structure unfolding was more sensitive to urea during the equilibrium unfolding process, possibly because of the tryptophan location. WbGST is a pi-class GST, and the hGSTP1-1 also possesses the flexible helix $\alpha 2$, which is very flexible in the absence of GSH or analogues of GSH. In fact, it was almost impossible to obtain a density of helix $\alpha 2$ in apo crystal structures of hGSTP1-1 due to its

promiscuousness (Oakley *et al.*, 1998). However, binding of a G-site substrate or inhibitor stabilises helix $\alpha 2$ by making it immobile. A similar phenomenon has been reported in hGSTP1-1 and the sigma class GSTS1-1 (Gildenhuis *et al.*, 2010; Stevens *et al.*, 1998). Therefore, the observed difference in unfolding stability of WbGST and WbGST-GSH complexed samples could result from the helix $\alpha 2$ characteristics.

On the other hand, the three-state mechanism ($N_2 \rightarrow I_2 \rightarrow 2U$) of secondary structure refolding observed in the absence of GSH (Figure 6.7A) may have occurred because of the high instability of the helix $\alpha 2$. Hence, resulting in an increased population of soluble aggregates at lower urea concentrations. Secondly, it is possible that the dynamic helix $\alpha 2$ may have assumed a different conformation from the native structure, resulting in a different refolding pathway. This is a possibility because a two-state mechanism was observed in all the other equilibrium unfolding pathways analysed in this study. DSC has been used extensively as a technique to establish the presence or absence of equilibrium unfolding intermediates along the unfolding pathways (Freire, 1994; Kaplan *et al.*, 1997; Privalov and Potekhin, 1986; Sturtevant, 1987). The DSC thermogram shows that WbGST unfolded via a single transition, and the calculated van't Hoff ΔH ($\Delta H_{vH} = 435.4 \text{ kJ.mol}^{-1}$) is in agreement with the calorimetric ΔH ($\Delta H_{cal} = 446.5 \text{ kJ.mol}^{-1}$) supporting the proposition of a two-state mechanism. The ratio $\Delta H_{vH} / \Delta H_{cal}$ in the absence of GSH is 0.98, which indicates approximately a two-state behaviour (Sturtevant, 1987). Additionally, ANS, which has been predicted to bind at the active site or the ligandin site adjacent to the active site (Dirr *et al.*, 2005; Kinsley *et al.*, 2008) was also used to probe hydrophobic surfaces that emerge as the protein unfolds (Abdalla and Hamed, 2006; He *et al.*, 2009; Hornby *et al.*, 2000; Stevens *et al.*, 1998). ANS binding gradually decreased from 0.5 M urea concentration in the absence of GSH (Figure 6.8). In contrast, the binding of ANS to the WbGST-GSH complex displayed more steady fluorescence at lower urea concentrations prior to decreasing at 2.5 M urea. Remarkably, the level of ANS binding did not increase at lower urea concentrations, as observed for some GST proteins and some dimeric proteins. These proteins associated with an initial increase in ANS binding usually separate into structured intermediates (monomers), exposing more hydrophobic surfaces before unfolding entirely. Conversely, the ANS mode of binding to WbGST reflects that of GST proteins, which have been reported to unfold from their native dimer to denatured monomers (Erhardt and Dirr, 1995; Wallace *et al.*, 1998).

The conformational stability of protein molecules associated with the urea-induced unfolding in the presence of water molecules is indicated by the value of ΔG_{H_2O} (Pace, 1986). The value of ΔG_{H_2O} (11.6 kcal.mol⁻¹) is quite lower than the range (19.8 – 26.8 kcal.mol⁻¹) previously reported for some dimeric GST proteins (Erhardt and Dirr, 1995; Gildenhuis *et al.*, 2010; Kaplan *et al.*, 1997; Wallace *et al.*, 1998). However, these dimeric GST proteins were unfolded at a lower pH (6.5), whereas WbGST unfolding was performed at a physiological pH (7.4). The difference in pH values during unfolding can influence the ΔG_{H_2O} by about 5 kcal.mol⁻¹ (Timm *et al.*, 1994). Previous studies of ΔG_{H_2O} unfolding as a function of pH in both monomeric RNase A and a dimeric growth factor were found to be pH-dependent. This pH dependency was primarily attributed to the impact of protein folding on the pK of a small number of ionizable groups (Pace, 1990; Timm *et al.*, 1994). Furthermore, concerns have been raised regarding the exact nature of protein unfolded states, which can contribute to underestimating ΔG_{H_2O} . Unfortunately, this issue has not been adequately tackled, although (Peng *et al.*, 1993) were able to estimate residual structure in the solvent using NMR. Therefore, the discrepancy in the obtained ΔG_{H_2O} value could result from traces of residual protein structure remaining at a high urea concentration, which may result in low artificial values of ΔG_{H_2O} . This is a possibility considering that WbGST has eight cysteine residues which could form disulphide bonds and perhaps lead to the existence of more residual structure in the denatured state. Notwithstanding the seeming underestimation of ΔG_{H_2O} , the ΔG_{H_2O} of the WbGST-GSH complex is higher than that of WbGST in the absence of GSH. This indicates that the WbGST-GSH complex is more stable, and the higher C_m value further confirms this assertion ([urea]_{1/2}) of WbGST-GSH samples.

As the protein native structure unfolds, the exposed surface area of the protein increases. The m -value obtained from the fitted curves measures the amount of exposed accessible surface area (ASA) (Alonso and Dill, 1991; Myers *et al.*, 1995). The ΔASA of WbGST was calculated to be 39269 by using the following equation from Myers *et al.* (1995).

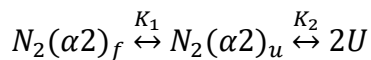
$$(n \times 93) - 907 = \Delta ASA \quad (6.20)$$

where n is the number of amino acid residues of the protein. The theoretical m -value of denaturation using urea can be estimated based on the calculated ΔASA using the following equation:

$$(\Delta ASA \times 0.11) + 374 = m \quad (6.21)$$

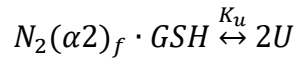
Applying the above equation, the predicted m -value for WbGST is 4.7 kcal.mol⁻¹.M⁻¹ of urea (Myers *et al.*, 1995). This predicted m -value of WbGST is comparable to the experimental value (4.5_{GSH} and 4.3 kcal.mol⁻¹.M⁻¹) (Table 6.5). Therefore, indicating that the equilibrium unfolding of WbGST is in accordance with a two-stage mechanism. Furthermore, the obtained m -value is similar to those obtained for other GST isoforms of pi and alpha classes (Erhardt and Dirr, 1995; Kaplan *et al.*, 1997; Wallace and Dirr, 1999; Wallace *et al.*, 1998). However, in the absence of GSH, the far-UV CD m -value for unfolding was lower (3.8 kcal.mol⁻¹.M⁻¹) than that of the tertiary structure unfolding. This could be due to the early unfolding of helix $\alpha 2$ forming a partially dimeric protein at the beginning of the unfolding transition, which precedes the dissociation of the two monomers. Moreover, considering that a wholly folded helix $\alpha 2$ contributes ~0.5 kcal.mol⁻¹.M⁻¹ to the m -value of the native protein.

Following the information gathered from the unfolding data of WbGST, two basic models for the equilibrium (un)folding pathway can be assumed. Considering the unfolding data and profiles (Figures 6.6A, 6.7A and 6.8) obtained in the absence of GSH, which shows the unfolding of helix $\alpha 2$ at lower urea concentrations, the first model can be represented as a three-state mechanism of unfolding:



where N_2 represents the native dimeric WbGST with folded helix $\alpha 2$, while f and u represent the folded but mobile and unfolded conformations of helix $\alpha 2$, respectively. $K_1 = [N_2(\alpha 2)_u]/[N_2(\alpha 2)_f]$ is the equilibrium constant for the unfolding of mobile helix $\alpha 2$ and $K_2 = [U]^2/[N_2(\alpha 2)_u]$ represents the equilibrium constant for the second unfolding reaction. Although helix $\alpha 2$ is highly dynamic without a G-site substrate, it is primarily folded (Hitchens *et al.*, 2001) prior to adding the first urea concentration. A similar unfolding mechanism was also observed in

hGSTP1-1, an isozyme of WbGST (Gildenhuis *et al.*, 2010). The second proposed model is based on the observed less dynamic conformation of helix $\alpha 2$ induced by ligand binding to the G-site. This induced stability of helix $\alpha 2$ has been reported in other pi-class GST proteins (Caccuri *et al.*, 1998; Erhardt and Dirr, 1996; Oakley *et al.*, 1998). According to the data obtained for the WbGST-GSH complex (Figure 6.6C, 6.7B and 6.8), unfolding followed a highly cooperative mechanism with the immobilization of helix $\alpha 2$ resulting in a substantial reduction in the population of $N_2(\alpha 2)_u$ along the unfolding pathway. Therefore, model two is represented as follows:



where $N_2(\alpha 2)_f$ represents the native dimer of WbGST with folded helix $\alpha 2$ (subscript f), while $K_u = [U]^2/[N_2(\alpha 2)_f \cdot GSH]$ is the equilibrium constant for the biomolecular reaction. It is also noteworthy that the stabilisation of helix $\alpha 2$ by G-site substrates does not influence the stability of the global dimeric structure (Erhardt and Dirr, 1996; Gildenhuis *et al.*, 2010).

Chapter 7

Energetics of BSP and EGCG binding

7.1 Introduction

Isothermal titration calorimetry (ITC) is a technique for measuring the energetics of biochemical reactions occurring at a constant temperature. These reactions result from molecular interactions such as the binding of ligands to proteins, interactions between enzymes and substrates, protein-protein interactions, or other interacting macromolecular complexes. Reactions monitored by ITC are usually initiated by a change in the chemical compositions of the molecule of interest by titrating the desired reactant. One of the distinctive attributes of the ITC technique is that it can determine all binding parameters simultaneously in a single experiment. Binding parameters such as the binding association constant (K_a), binding enthalpy (ΔH) and stoichiometry of binding (n) can be directly obtained from a global fitting of the data. Other thermodynamic parameters which can be derived from the data include the entropy (ΔS), Gibbs free energy (ΔG) and the equilibrium dissociation constant (K_D). In addition to confirming direct interaction between the reacting titrants and the analyte, analysed thermodynamic parameters can also provide an understanding of the contributing noncovalent interaction forces. Typically, enthalpic favourable reactions are exerted by polar interactions, but entropically driven interactions are more hydrophobic in nature.

Previously obtained results from enzyme activity and kinetic studies confirmed that BSP and EGCG bind to WbGST, hGSTM1-1 and hGSTP1-1. However, it is crucial to quantitatively describe the nature of the forces governing the ligand-protein interactions. Interactions between hydrogen donors and acceptors, electrostatic interactions, dipole interactions and van der Waals interactions provide binding specificity. This information contributes to the knowledge-based optimisation of small molecules to repurpose them as new medicinal compounds. Thus, this chapter captures the binding interaction between BSP, EGCG and the three recombinant proteins of this study. The materials and method employed will be elucidated, followed by the results and the discussion of the obtained data.

7.2 Materials

The table below shows the non-standard chemicals used in this chapter. All the other reagents and buffers used were of analytical grade.

Table 7.1 List of materials and suppliers

Materials	Source	Location
GSH	Sigma-Aldrich	St. Louis, MO, USA
BSP	Sigma-Aldrich	St. Louis, MO, USA
EGCG	Angene	Hyderabad, India
TCEP	New England Biolabs	Massachusetts USA

7.3 Methods

Biochemical reactions are usually accompanied by the release or absorption of heat, which is best quantified using the ITC technique. All the thermodynamic parameters associated with the interaction of biomolecules can also be conveniently quantified (Sheehan, 2013). One of the advantages of using ITC is its ability to characterise biomolecular interactions with high-affinity binding constants ranging from $10^8 - 10^9 \text{ M}^{-1}$ without bias from reporter labels. By employing the ITC technique, the energetics of molecular reactions and the resulting thermodynamic parameters are quantified at a constant temperature. Therefore, the binding equilibrium can be described by ascertaining the number of binding sites, also known as stoichiometry (n), followed by quantifying the binding affinity of the often referred to as the association constant (K_a) and the change in Gibbs free energy ΔG . Lastly is the characterisation of the enthalpic and entropic contributions to Gibbs free energy of binding (Freire *et al.*, 1990). ITC analyses how forces of interaction, namely, hydrophobic and electrostatic interactions, contribute to the thermodynamics of protein and ligand binding. Thus, ITC provides information regarding the thermodynamics and structure-function relationship of protein and ligand interactions which cannot be gathered merely by analysing protein structures (Ladbury, 2001).

Instrumentation of ITC offers the technique its unique attributes. ITC machines are designed with two cells surrounded by an adiabatic jacket. The first is referred to as the sample cell where the binding reaction occurs, and the second is referred to as the reference cell typically filled with buffer. The two cells must be maintained at the same temperature throughout the experiment. This is because heat changes occur during binding and the heat sensing device relays the information to the heaters, reimburses for the temperature difference and then returns both cells to a similar temperature. Ligands are often loaded into a syringe fitted to an injecting device, and the sample cell is loaded with protein. Subsequently, the injecting device is inserted into the sample cell, and aliquots of the titrant are titrated into the protein in a stepwise manner. Upon a binding event, heat is generated or absorbed in the system depending on the nature of the heat change, and the heat-sensing device compensates accordingly. Consequently, the observed heat flow for each injection reflects the sum of power utilized to regulate the constant temperature in the system. This is also proportional to the enthalpy of binding, and the stoichiometry for the interaction can also be derived. After that, the peak per injection is integrated and analysed against the molar ratio of the titrant to protein. Then the resulting isotherm is fitted to a suitable binding model to obtain the binding thermodynamics (Blandamer, 1998).

The ITC experiment was performed by titrating 1 mM BSP or EGCG into 60 μ M WbGST and 100 μ M hGSTM1-1 or hGSTP1-1 in the presence or absence of 2 mM GSH using 20 mM sodium phosphate pH 7.4, 137 mM NaCl, 2 mM EDTA, 2 mM TCEP and 10% (v/v) glycerol. WbGST aggregated in the titration of 1 mM EGCG concentration and was later reduced to 600 μ M. The analysis was monitored by the Affinity ITC, 1 mL standard volume (TA Instruments, New Castle, USA) at 20 °C. The experiment was initiated by first injecting 2 μ L of the ligand into the sample with 300 s injection intervals while stirring at 250 rpm with subsequent injections set at 5 μ L. All protein and ligand samples were degassed before analysis to prevent bubbles from forming during the experiment. Afterwards, the resulting isotherm obtained from the data was fitted and analysed by the NanoAnalyze software. But prior to the data fitting, integrated heat for each injection was corrected for heat of dilution.

Considering a binding reaction at equilibrium between a biomolecule and a ligand:



where P is the protein and L is the ligand. Assuming a single binding site is present, the binding affinity or association constant determines the partition of the reacting species into unbound and bound species, which is given by:

$$K_a = ([PL])/([P][L]) \quad (7.2)$$

where K_a is the association constant. Then the binding affinity is associated with the free energy of binding by:

$$\Delta G = -RT \ln K_a \quad (7.3)$$

where ΔG is the free energy change, R is the gas constant, and T is the absolute temperature. However, the free energy of binding can also be expressed as:

$$\Delta G = \Delta H - T\Delta S \quad (7.4)$$

where ΔH represents the enthalpy and ΔS is the entropy of the system. Therefore, the free energy of binding can be integrated as:

$$\Delta G = -RT \ln K_a = \Delta H - T\Delta S \quad (7.5)$$

7.4 Results

Using spectroscopy and fluorescence studies, it has been established that BSP and EGCG bind to WbGST, hGSTM1-1 and hGSTP1-1. Therefore, biocalorimetry was performed to validate the observed binding interaction and obtain the BSP and EGCG binding thermodynamics to the recombinant proteins. In addition, it is paramount to understand the nature of the forces driving this binding interaction because this will contribute towards achieving successful ligand optimisation. The obtained ITC profile for both BSP and EGCG binding to the target proteins are indicative of exothermic reactions ($0 > \Delta H$). All the binding events between the recombinant proteins and the ligands were spontaneous ($0 > \Delta G$) at pH 7.4 and 20 °C with reference to the negative free energies observed (Table 7.2). The data obtained for BSP binding to WbGST was

fitted to a multiple-site model using NanoAnalyze (Figure 7.1). The resulting fit indicated that approximately one molecule of BSP binds at the first binding site ($n_1 = 1.1$). In contrast, approximately six molecules of BSP are bound at the second binding site ($n_2 = 5.75$). However, in the presence of GSH (WbGST-GSH-BSP), a different stoichiometry was observed for the first binding site ($n_1 = 2.1$, $n_2 = 6.01$). In addition, binding site-1 displayed more affinity towards BSP than binding site-2, as shown by the K_d values ($K_{d1} = 18.8 \mu\text{M}$, $K_{d2} = 112 \mu\text{M}$). Moreover, BSP binding to the first binding site (S_1) in the absence of GSH was not enthalpically favourable but entropically favourable, and the overall reaction was entropically driven. In contrast, the second site (S_2) in the absence of GSH was enthalpically driven with an unfavourable entropy ($T\Delta S = -7.8 \text{ kJ.mol}^{-1}$). Similarly, in the presence of GSH, BSP binding displayed more affinity towards WbGST in S_1 ($K_{d1} = 7.2 \mu\text{M}$, $K_{d2} = 68.2 \mu\text{M}$) and with a much lower K_d value for S_2 , compared to binding in the absence of GSH. Unlike BSP binding in the absence of GSH the entropy and enthalpy of binding for S_1 and S_2 were both favourable (Table 7.2). However, S_1 was entropically driven while S_2 was enthalpically driven. Details of the obtained results are tabulated in Table 7.2.

On the other hand, the binding of EGCG to WbGST was fitted to an independent binding model (Figure 7.2) and EGCG bound to WbGST with a stoichiometry of $n = 1.86$ in the absence of GSH and $n = 1.89$ in the presence of GSH. The binding interactions were enthalpically driven with slightly favourable entropy. In the presence of GSH, EGCG displayed more affinity towards WbGST ($K_d = 32.9 \mu\text{M}$, $K_{d\text{GSH}} = 20.3 \mu\text{M}$). Regarding the human isoforms, both BSP and EGCG bound to the hGSTM1-1 with stoichiometry approximately to one (Figures 7.3 and 7.4). Furthermore, the binding of both BSP and EGCG to the hGSTM1-1 were all enthalpically driven with unfavourable and favourable entropy (Table 7.2) in the absence and presence of GSH, respectively. In the case of the hGSTP1-1, BSP bound to the protein with a stoichiometry of $n = 1.88$ and $n_{\text{GSH}} = 2.13$, while EGCG bound with a stoichiometry of $n = 1.79$ and $n_{\text{GSH}} = 1.87$. The affinity of $K_d = 26.7 \mu\text{M}$, $K_{d\text{GSH}} = 22.8$ for BSP binding and $K_d = 87.9 \mu\text{M}$, $K_{d\text{GSH}} = 64.2 \mu\text{M}$ for EGCG binding to hGSTP1-1 was observed. The binding of BSP was shown to have both favourable enthalpy and entropy. However, the overall BSP binding event both in the presence and absence of GSH was entropically driven (Figure 7.5). EGCG bindings were enthalpically driven with a favourable entropy in the absence of GSH, and entropically unfavourable in the presence of GSH (Figure 7.6). All the binding isotherms with their corresponding fitted data

displayed profiles typical of an exothermic reaction ($-\Delta H$). Furthermore, all the ligand binding events to the recombinant proteins occurred spontaneously ($-\Delta G$).

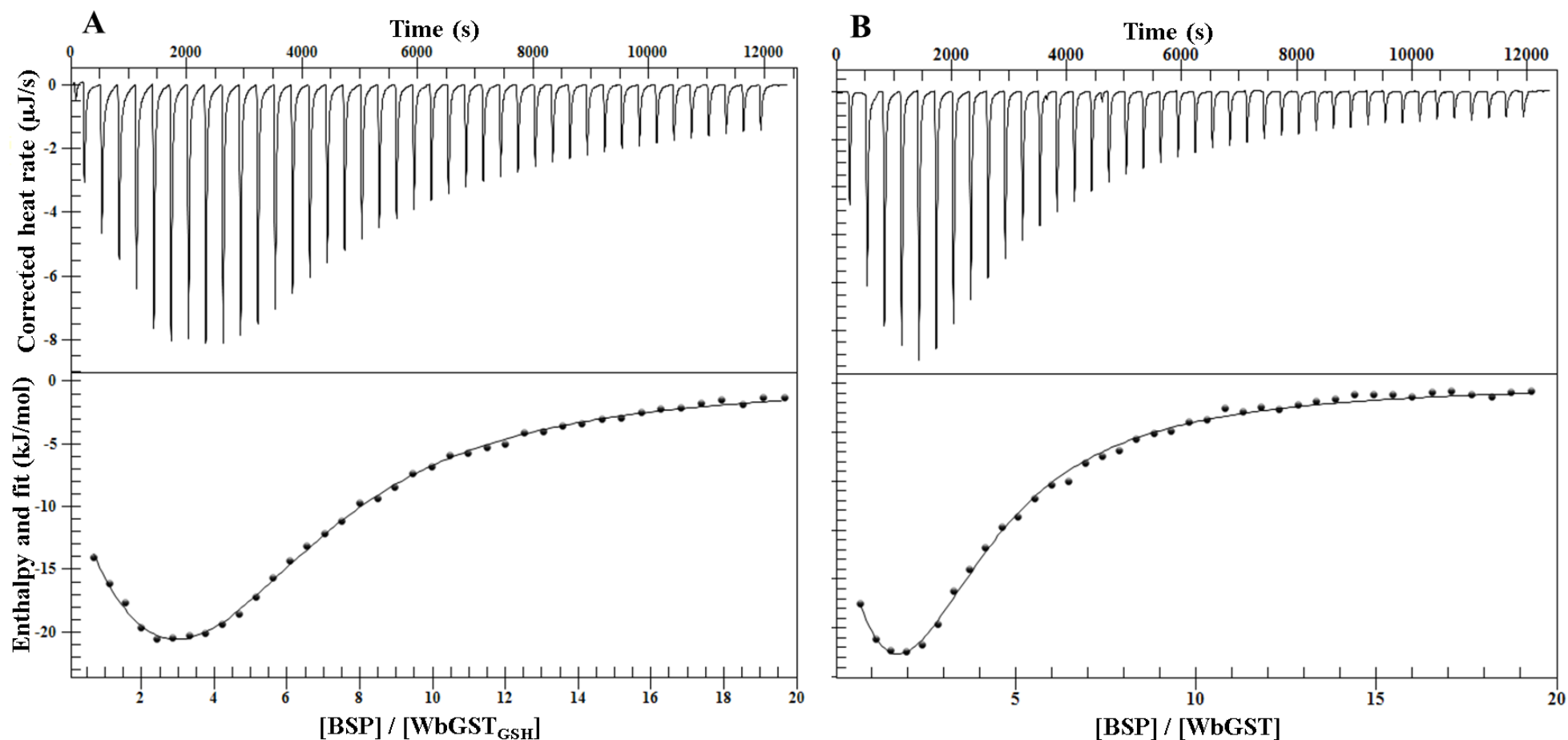


Figure 7.1 Isothermal titration calorimetry of BSP and WbGST. The thermodynamic parameters were obtained in the presence (A) and absence (B) of GSH (2 mM) by titrating 4.7 mM BSP to 50 μ M WbGST. Each titration was performed at 20 °C with a stirring rate of 250 rpm buffered in 20 mM sodium phosphate pH 7.4, 137 mM NaCl₂, 0.02% (w/v) NaN₃, 2 mM EDTA and 2 mM TCEP. The data were fitted using the multiple-site binding model algorithm implemented in the NanoAnalyze software. The solid lines represent the non-linear best fit of the experimental data resulting in the following thermodynamic parameters for (A): $n_1 = 2.1$, $n_2 = 6.01$, $K_{d1} = 7.2 \mu\text{M}$, $K_{d2} = 68.2 \mu\text{M}$, $\Delta H_1 = -2.1 \text{ kJ.mol}^{-1}$, $\Delta H_2 = -15.3 \text{ kJ.mol}^{-1}$, $T\Delta S_1 = 26.73 \text{ kJ.mol}^{-1}$, $T\Delta S_2 = 8.06 \text{ kJ.mol}^{-1}$ and for (B): $n_1 = 1.1$, $n_2 = 5.75$, $K_{d1} = 18.8 \mu\text{M}$, $K_{d2} = 112 \mu\text{M}$, $\Delta H_1 = -104.6 \text{ kJ.mol}^{-1}$, $\Delta H_2 = -29.5 \text{ kJ.mol}^{-1}$, $T\Delta S_1 = 131.15 \text{ kJ.mol}^{-1}$, $T\Delta S_2 = -7.8 \text{ kJ.mol}^{-1}$.

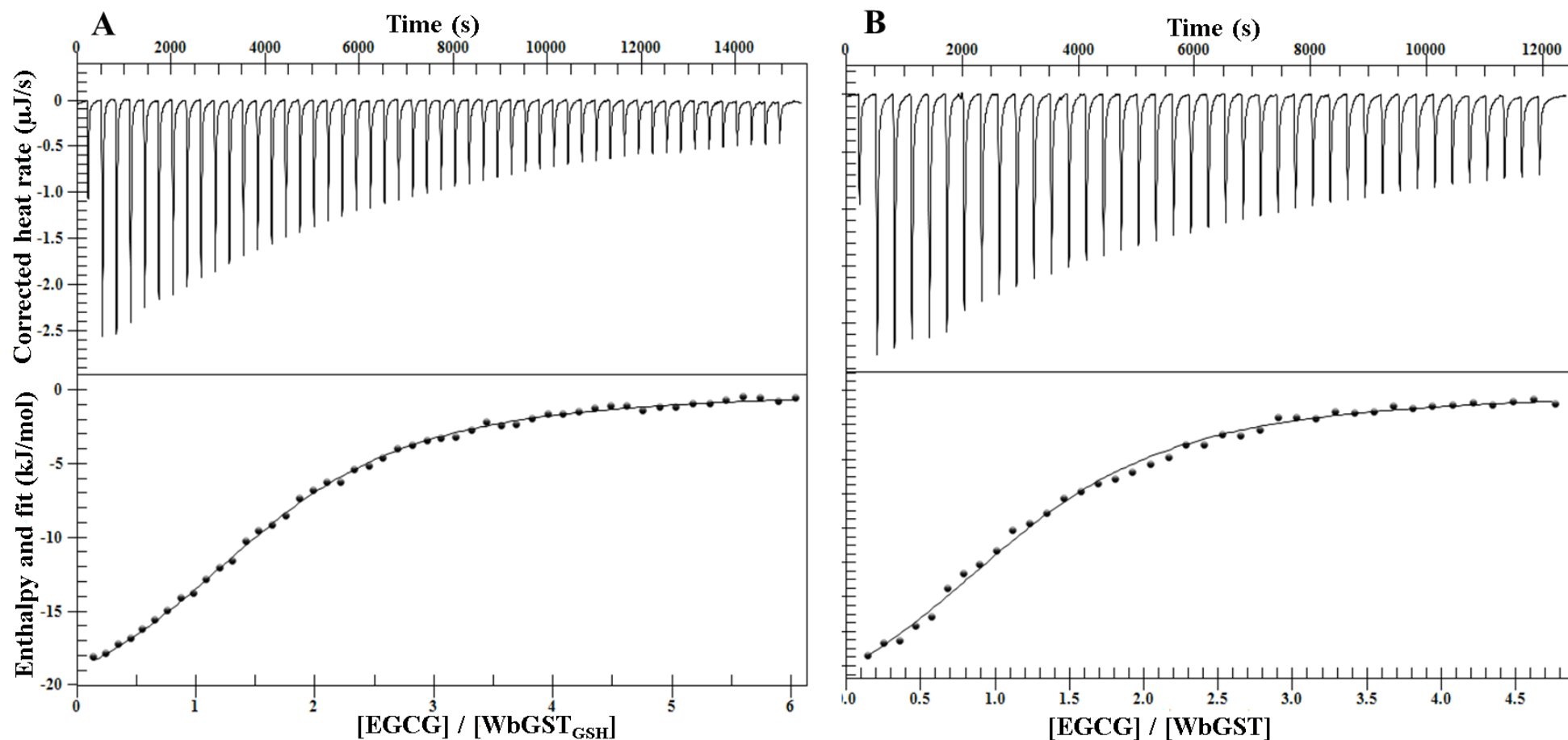


Figure 7.2 Isothermal titration calorimetry of EGCG and WbGST. Using ITC, the thermodynamic parameters were obtained in the presence (A) and absence (B) of GSH (2 mM) by titrating 0.68 mM EGCG to 50 μ M WbGST. Each titration was performed at 20 $^{\circ}$ C with a stirring rate of 250 rpm buffered in 20 mM sodium phosphate pH 7.4, 137 mM NaCl₂, 0.02% (w/v) NaN₃, 2 mM EDTA and 2 mM TCEP. The data were fitted using the independent binding model algorithm implemented in the NanoAnalyze software. The solid lines represent the non-linear best fit of the experimental data resulting in the following thermodynamic parameters for (A): $n = 1.89$, $K_d = 20.3 \mu$ M, $\Delta H = -23.14 \text{ kJ.mol}^{-1}$, $T\Delta S = 3.2 \text{ kJ.mol}^{-1}$ and for (B): $n_1 = 1.86$, $K_d = 32.9 \mu$ M, $\Delta H = -24.8 \text{ kJ.mol}^{-1}$, $T\Delta S = 0.35 \text{ kJ.mol}^{-1}$.

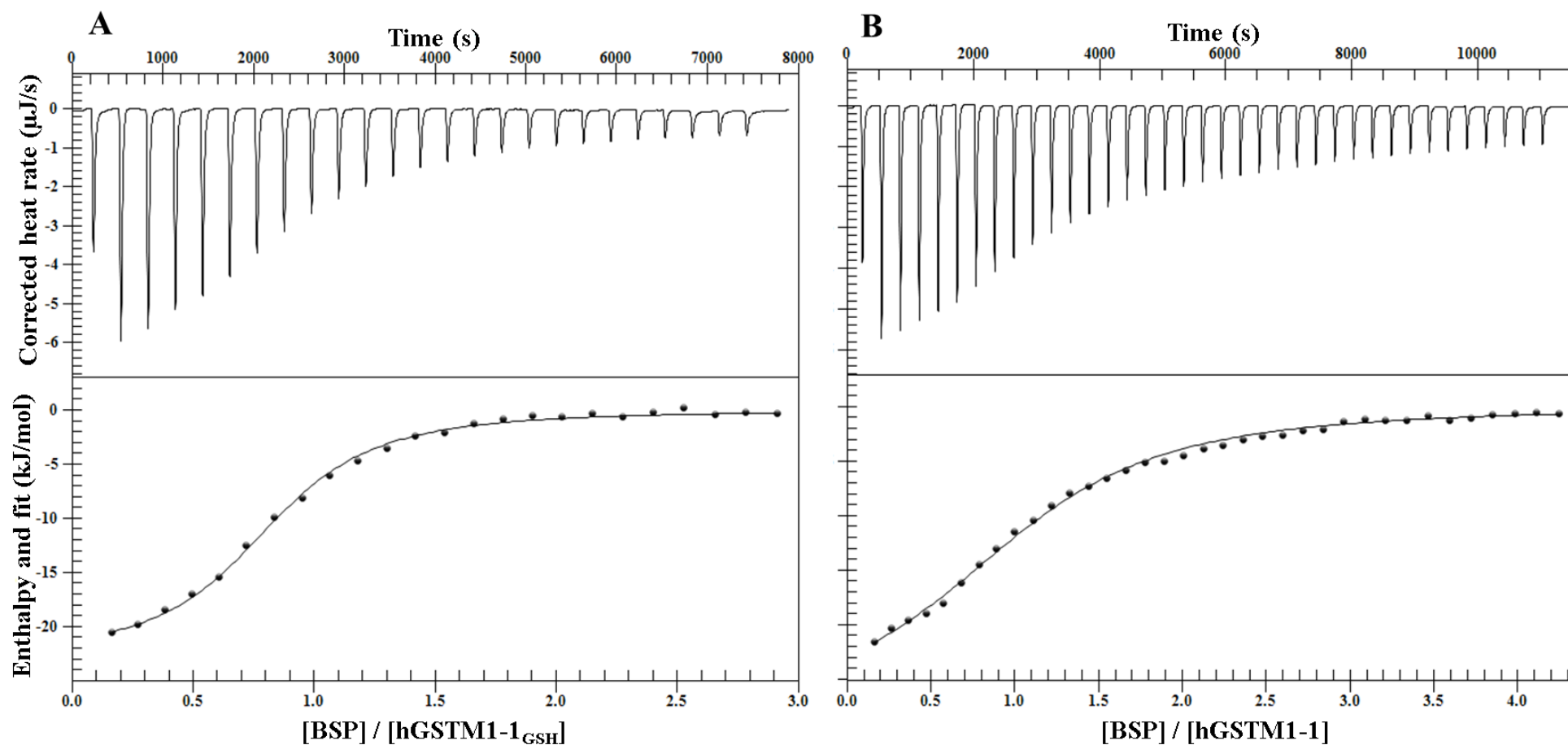


Figure 7.3 Isothermal titration calorimetry of BSP and hGSTM1-1. Using ITC, the thermodynamic parameters were obtained in the presence (A) and absence (B) of GSH (2 mM) by titrating 1.7 mM BSP to 100 μ M hGSTM1-1. Each titration was performed at 20 $^{\circ}$ C with a stirring rate of 250 rpm buffered in 20 mM sodium phosphate pH 7.4, 137 mM NaCl₂, 0.02% (w/v) NaN₃, 2 mM EDTA and 2 mM TCEP. The data were fitted using the independent binding model algorithm implemented in the NanoAnalyze software. The solid lines represent the non-linear best fit of the experimental data resulting in the following thermodynamic parameters for (A): $n = 1.01$, $K_d = 17.5 \mu\text{M}$, $\Delta H = -23.51 \text{ kJ.mol}^{-1}$, $T\Delta S = 3.18 \text{ kJ.mol}^{-1}$ and for (B): $n_1 = 1.11$, $K_d = 38.5 \mu\text{M}$, $\Delta H = -35.65 \text{ kJ.mol}^{-1}$, $T\Delta S = -10.85 \text{ kJ.mol}^{-1}$.

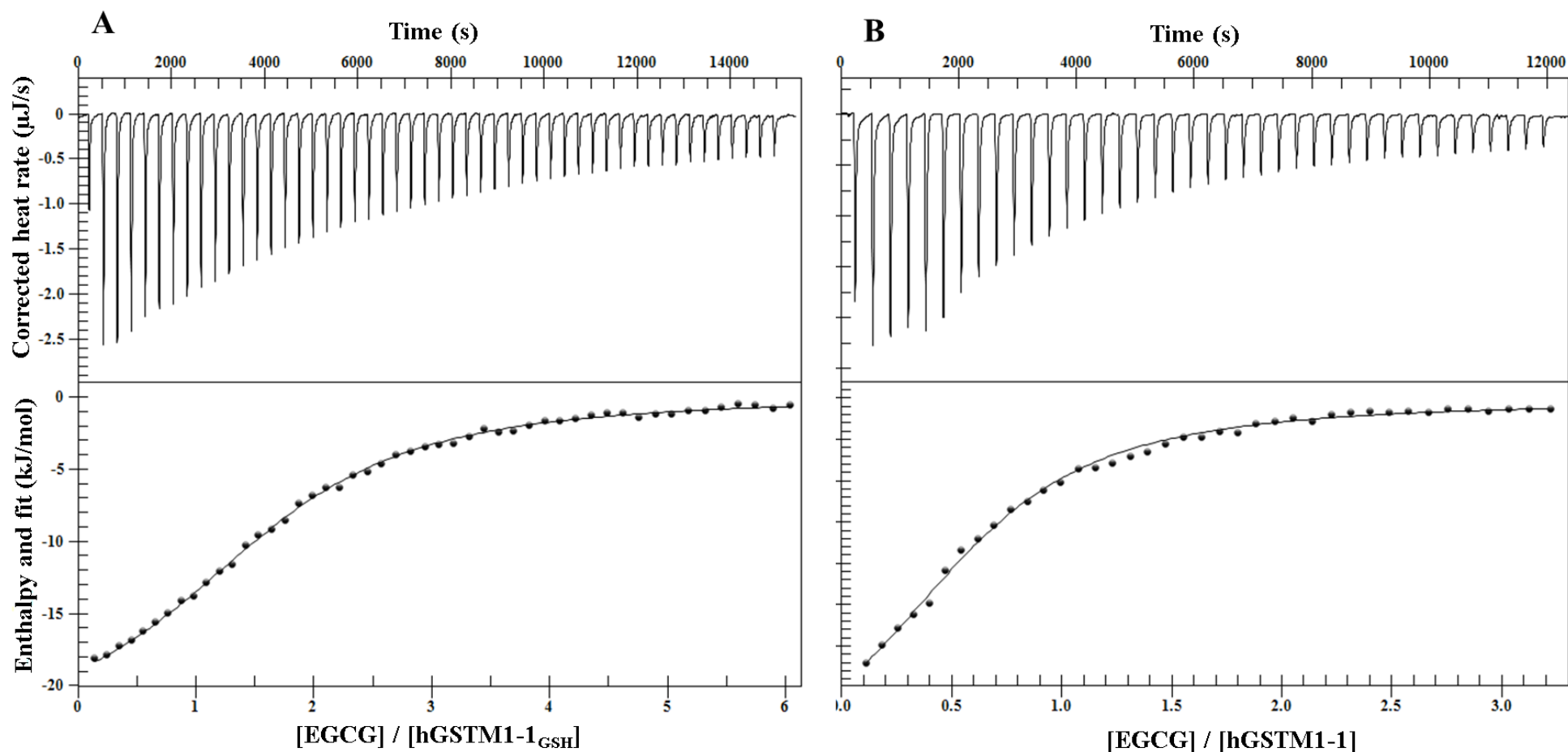


Figure 7.4 Isothermal titration calorimetry of EGCG and hGSTM1-1. Monitoring the Isothermal titration calorimetry of the interaction between EGCG and hGSTM1-1. Using ITC, the thermodynamic parameters were obtained in the presence (A) and absence (B) of GSH (2 mM) by titrating 1.75 mM EGCG to 100 μ M hGSTM1-1. Each titration was performed at 20 $^{\circ}$ C with a stirring rate of 250 rpm buffered in 20 mM sodium phosphate pH 7.4, 137 mM NaCl_2 , 0.02% (w/v) NaN_3 , 2 mM EDTA and 2 mM TCEP. The data were fitted using the independent binding model algorithm implemented in the NanoAnalyze software. The solid lines represent the non-linear best fit of the experimental data resulting in the following thermodynamic parameters for (A): $n = 1.1$, $K_d = 35.8 \mu\text{M}$, $\Delta H = -21.12 \text{ kJ.mol}^{-1}$, $T\Delta S = 3.82 \text{ kJ.mol}^{-1}$ and for (B): $n_1 = 1.02$, $K_d = 42.3 \mu\text{M}$, $\Delta H = -30.22 \text{ kJ.mol}^{-1}$, $T\Delta S = -5.68 \text{ kJ.mol}^{-1}$.

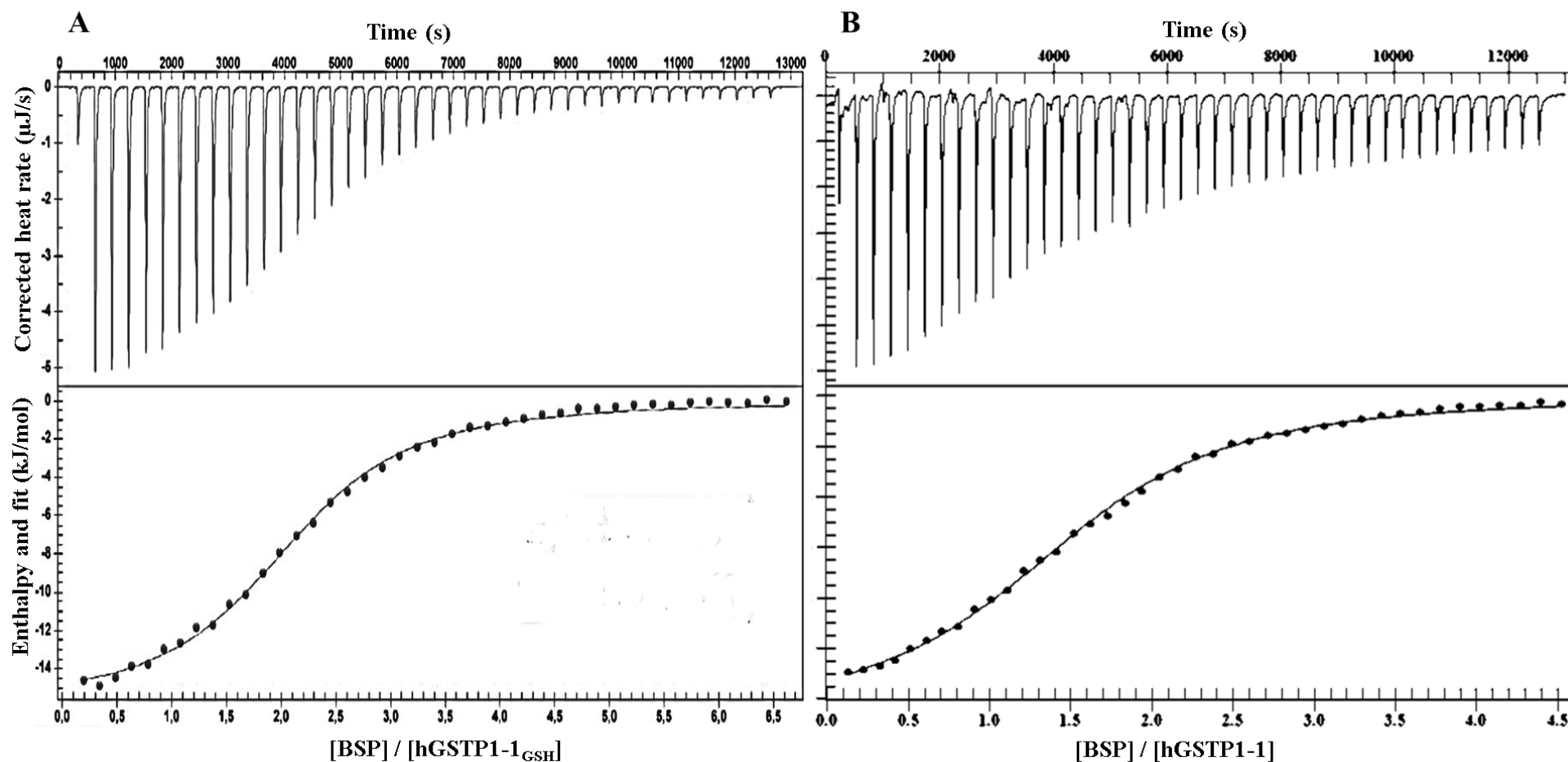


Figure 7.5 Isothermal titration calorimetry of EGCG and hGSTM1-1. Monitoring the Isothermal titration calorimetry of the interaction between EGCG and hGSTM1-1 Using ITC, the thermodynamic parameters were obtained in the presence (A) and absence (B) of GSH (2 mM) by titrating 1.7 mM BSP to 100 μM hGSTM1-1. Each titration was performed at 20 °C with a stirring rate of 250 rpm buffered in 20 mM sodium phosphate pH 7.4, 137 mM NaCl₂, 0.02% (w/v) NaN₃, 2 mM EDTA and 2 mM TCEP. The data were fitted using the independent binding model algorithm implemented in the NanoAnalyze software. The solid lines represent the non-linear best fit of the experimental data resulting in the following thermodynamic parameters for (A): $n = 2.13$, $K_d = 22.8 \mu\text{M}$, $\Delta H = -6.03 \text{ kJ.mol}^{-1}$, $T\Delta S = 20.02 \text{ kJ.mol}^{-1}$ and for (B): $n_1 = 1.88$, $K_d = 26.7 \mu\text{M}$, $\Delta H = -11.43 \text{ kJ.mol}^{-1}$, $T\Delta S = -14.24 \text{ kJ.mol}^{-1}$

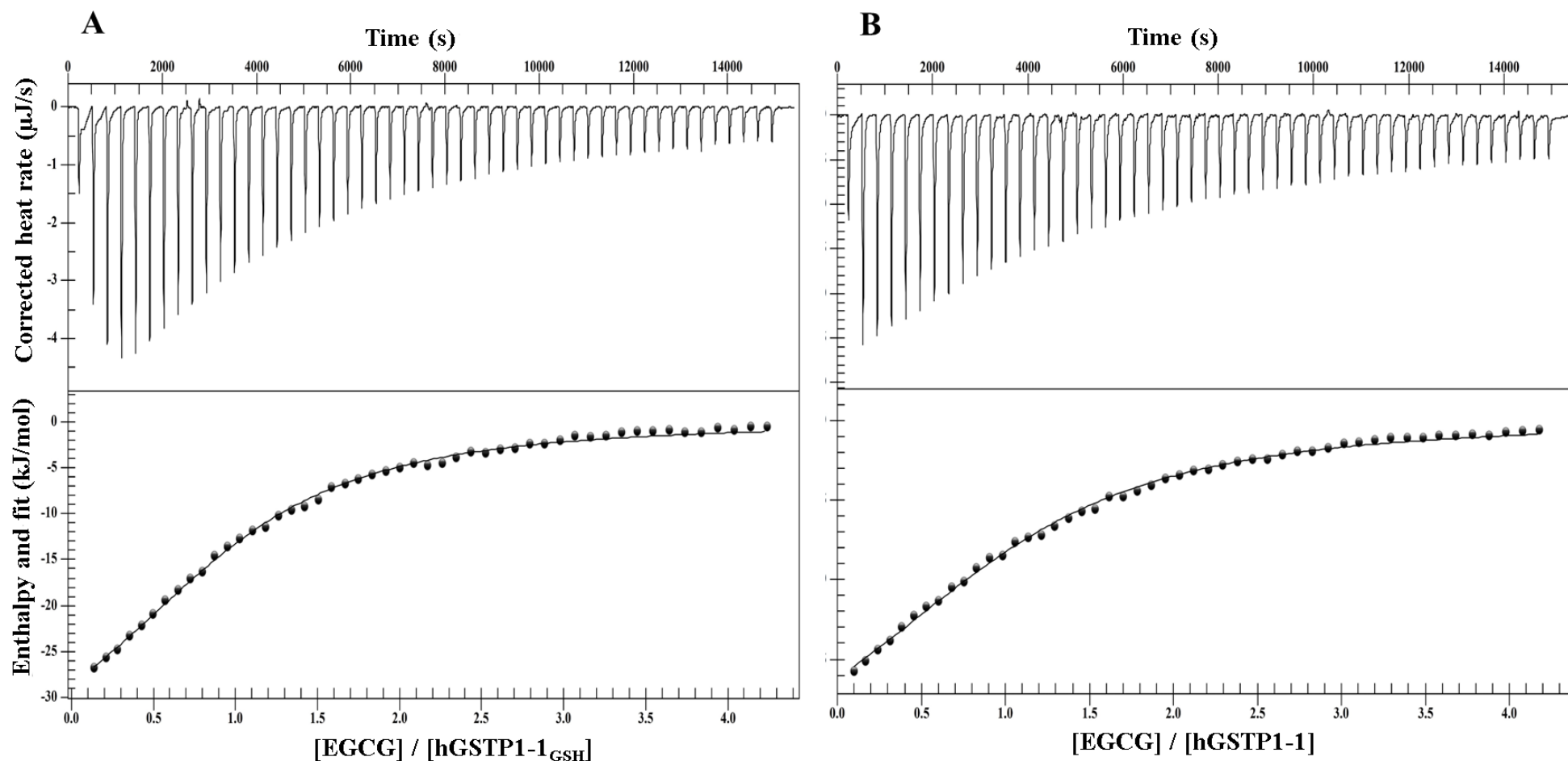


Figure 7.6 Isothermal titration calorimetry of EGCG and hGSTP1-1. Using ITC, the thermodynamic parameters were obtained in the presence (A) and absence (B) of GSH (2 mM) by titrating 1.75 mM EGCG to 100 μ M hGSTP1-1. Each titration was performed at 20 $^{\circ}$ C with a stirring rate of 250 rpm buffered in 20 mM sodium phosphate pH 7.4, 137 mM NaCl₂, 0.02% (w/v) NaN₃, 2 mM EDTA and 2 mM TCEP. The data were fitted using the independent binding model algorithm implemented in the NanoAnalyze software. The solid lines represent the non-linear best fit of the experimental data resulting in the following thermodynamic parameters for (A): $n = 1.87$, $K_d = 64.2 \mu\text{M}$, $\Delta H = -33.07 \text{ kJ}\cdot\text{mol}^{-1}$, $T\Delta S = -9.54 \text{ kJ}\cdot\text{mol}^{-1}$ and for (B): $n_1 = 1.79$, $K_d = 87.9 \mu\text{M}$, $\Delta H = -15.7 \text{ kJ}\cdot\text{mol}^{-1}$, $T\Delta S = 7.06 \text{ kJ}\cdot\text{mol}^{-1}$.

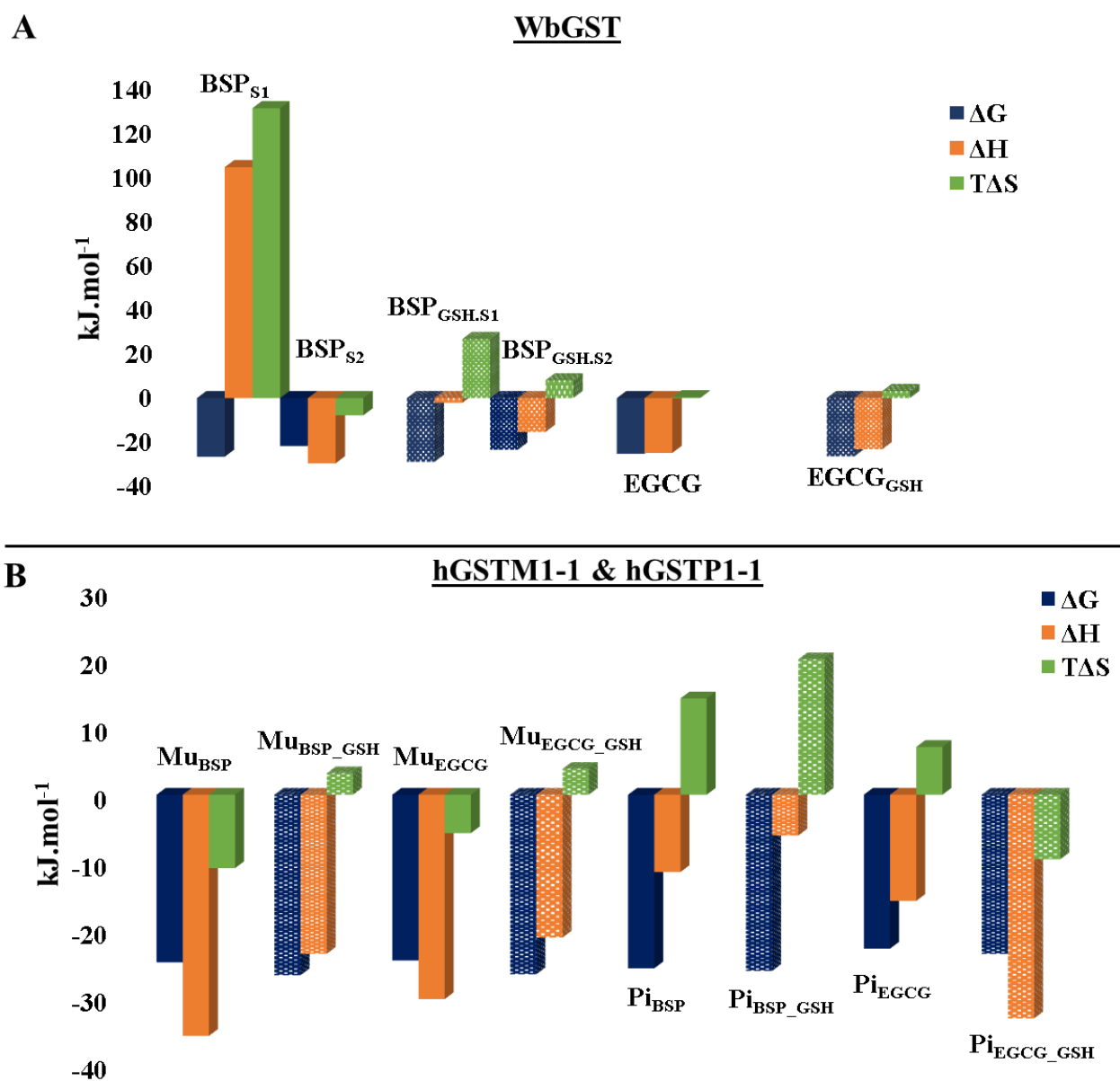


Figure 7.7 Summary of the obtained thermodynamic parameters. The parameters were obtained from WbGST (A), the human GST mu and Pi isoforms (B) binding interaction with BSP and EGCG.

Table 7.2 Thermodynamic parameters obtained from the ITC. Thermodynamic parameters obtained from ITC analysis of the interaction between BSP, EGCG and WbGST, hGSTM1-1 and hGSTP1-1, all in the presence or absence of GSH. The binding isotherm for BSP interaction with WbGST was fitted using the multiple-site binding model.

			<i>n</i>	<i>K_d</i> (μM)	ΔG	ΔH	$T\Delta S$
					kJ.mol ⁻¹		
WbGST	BSP	S ₁	1.1 ± 0.2	18.8 ± 0.2	-26.55 ± 0.2	104.6 ± 0.5	131.15 ± 1.8
		S ₂	5.75 ± 0.1	112 ± 0.3	-21.69 ± 0.8	-29.5 ± 1.5	-7.8 ± 1.5
	BSP _{GSH}	S ₁	2.1 ± 0.3	7.2 ± 0.2	-28.83 ± 0.2	-2.1 ± 0.7	26.73 ± 1.7
		S ₂	6.01 ± 0.2	68.2 ± 0.5	-23.36 ± 0.3	-15.3 ± 2.1	8.06 ± 1.9
	EGCG		1.86 ± 0.2	32.9 ± 0.2	-25.16 ± 0.9	-24.8 ± 0.3	0.35 ± 0.2
	EGCG _{GSH}		1.89 ± 0.1	20.3 ± 0.3	-26.34 ± 0.8	-23.14 ± 0.2	3.2 ± 0.1
hGSTM1-1	BSP		1.11 ± 0.1	38.5 ± 0.1	-24.78 ± 0.4	-35.65 ± 0.3	-10.85 ± 0.2
	BSP _{GSH}		1.01 ± 0.1	17.5 ± 0.6	-26.69 ± 0.3	-23.51 ± 0.6	3.18 ± 0.1
	EGCG		1.02 ± 0.3	42.3 ± 0.1	-24.51 ± 0.5	-30.22 ± 1.1	-5.68 ± 0.3
	EGCG _{GSH}		1.1 ± 0.1	35.8 ± 0.2	-24.94 ± 0.4	-21.12 ± 1.5	3.82 ± 0.2
hGSTP1-1	BSP		1.88 ± 0.2	26.7 ± 0.3	-25.67 ± 0.8	-11.43 ± 0.1	14.24 ± 0.2
	BSP _{GSH}		2.13 ± 0.1	22.8 ± 0.2	-26.05 ± 0.4	-6.025 ± 0.5	20.02 ± 0.3
	EGCG		1.79 ± 0.1	87.9 ± 0.5	-22.76 ± 0.2	-15.7 ± 0.3	7.06 ± 0.1
	EGCG _{GSH}		1.87 ± 0.1	64.2 ± 0.2	-23.53 ± 0.3	-33.07 ± 0.4	-9.54 ± 0.2

7.5 Discussion

The ITC analysis provides a quantitative understanding of the thermodynamics driving ligand binding to proteins and the vital role it plays in ligand optimisation cannot be overemphasised. WbGST interacts with BSP at multiple sites. The obtained stoichiometry for WbGST-BSP indicates that one molecule of BSP binds to one molecule of WbGST at the first binding site while approximately six molecules of BSP bind at the second site. It appears that the first binding site is the primary binding site seeing that it exhibits more affinity for BSP than the second site. This primary binding site is likely the L-site often located at the dimer interface of

parasite GSTs. However, multiple ($n_2 = 5.75$ and $n_{2_GSH} = 6.01$) numbers of BSP were shown to bind at the second binding site, therefore indicating that two molecules of BSP could be bound to each H-site per monomer and to other alternative L-sites. However, BSP is a hydrophobic molecule and could also possibly bind on the multiple hydrophobic pockets found on WbGST (Supplementary Figure S6 A) as predicted using the DoGSiteScorer tool (Volkamer *et al.*, 2010; Volkamer *et al.*, 2012) on ProteinsPlus server and also highlighted in ANS displacement by BSP. Conversely, six molecules of BSP bound to the second site in the presence of GSH. Moreover, some of the BSP molecules could be bound at a prominent pocket located in-between the tail end of the C-terminus domain monomer-1 and N-terminus domain of monomer-2, identified using the DoGSiteScorer tool. Seeing that the presence of GSH stabilises WbGST as observed in the stability studies, therefore, GSH binding enhances the interaction of the two monomers resulting in the formation of this tail-end pocket (Figure S2). In contrast to BSP, EGCG was shown to interact with WbGST with a stoichiometry of $n = 1.89$ and $n = 1.86$ in the presence and absence of GSH respectively. This implies that two molecules of EGCG bind per dimer of WbGST.

The binding of BSP molecules to the first binding site of WbGST was enthalpically unfavourable and entropically favourable, but the overall binding interaction was entropically driven (Figure 7.1). This binding entropy is a sum of contributions arising from other physical attributes resulting in a change in the dynamics of the system (Ladbury and Doyle, 2004; Perozzo *et al.*, 2004):

$$\Delta S_{bind} = \Delta S_{solv} + \Delta S_{conf} + \Delta S_{comp} \quad (7.6)$$

where ΔS_{bind} represents the total change in the binding entropy of the system, ΔS_{solv} is the entropy change resulting from localised water molecules on the active site and ligand surfaces, ΔS_{conf} is the change in conformational degrees of freedom of the protein and ligand and ΔS_{comp} represents a loss of rotational, translational and vibrational degrees of freedom of molecules in the protein-ligand complex. This loss in ΔS_{conf} and ΔS_{comp} results from the inability of the side chains to assume different rotameric states and often leads to unfavourable entropy in most cases. Moreover, the ΔS_{solv} is the main force propelling a favourable change in the entropy of the system (Ladbury, 1996).

Considering that BSP is a hydrophobic molecule, the largely favourable entropy arising from BSP binding to WbGST-S₁ could be attributed to the hydrophobic effect. The desolvation of both BSP and WbGST upon binding will contribute remarkably to the disorder of the system, thereby compensating for the enthalpic penalty of ligand binding to WbGST. This resulted in an overall entropically driven binding of BSP at the S₁ of WbGST (Figure 7.1). Although the binding event one, S₁, rendered an unfavourable enthalpy in the absence of GSH ($\Delta H = 104.6 \text{ kJ mol}^{-1}$), however, a favourable enthalpy was observed in the presence of GSH ($\Delta H = -29.5 \text{ kJ mol}^{-1}$). Interestingly, BSP shows a higher affinity at S₁ despite the larger unfavourable ΔH compared to S₂. This implies that enthalpy-entropy compensation mitigates enthalpic penalties such that the ligand affinity is unaffected despite the decreased binding interaction (large positive ΔH). Secondly, the release of entropically unfavourable waters was further enhanced by BSP binding at S₁, hence the larger positive enthalpy change.

The binding enthalpy is associated with the breaking and/or formation of bonds. The bond formation is indicative of a favourable ΔH , often related to non-covalent interactions such as hydrogen bond formation, van der Waals interactions and a variety of electrostatic interactions. Also, the ΔH can proffer insight towards ligand specificity seeing that a favourable ΔH is indicative of specific interactions between the ligand and the protein. Even though the observed ΔH represents the heat change within a system, it is important to note that a binding event can involve either the uptake or release of a proton. Moreover, the ionisation for some buffer systems can also involve the release of a proton into the solvent, so in reference to Le Chatelier's principle the change in the protonation state resulting from an interaction can shift the equilibrium and hence contribute to the heat of ionisation (ΔH_{ion}) for the buffer system (Perozzo *et al.*, 2004). Therefore, the ITC experiment was performed with phosphate buffer because it is known to have only a negligible enthalpy of ionization ($\Delta H_{\text{ion}} \approx 1 \text{ kcal} \cdot \text{mol}^{-1}$) in combination with only a minor pKa change with increasing temperature ($\Delta \text{pKa}/dT = -0.0028 \text{ K}^{-1}$). This is to ensure that the binding of the ligands to the target proteins in this study was not coupled to protonation events and heat of ionisation (ΔH_{ion}) of the buffer system, but that the observed ΔH was due to the protein-ligand interaction. Thus, the observed ΔH will be essentially the binding enthalpy.

Conversely, the binding of BSP at the S₂ was enthalpically driven ($\Delta H = -29.5 \text{ kJ mol}^{-1}$) with an unfavourable entropy ($T\Delta S = -7.8 \text{ kJ mol}^{-1}$). This favourable enthalpy suggests that BSP forms a more binding interaction with WbGST at S₂ when compared with the interaction at S₁.

Moreover, the unfavourable entropy is consistent with the binding enthalpy because the binding is expected to restrict the degrees of freedom of the flexible groups of both the ligand and the protein at the binding site. It is becoming more and more apparent that ligand binding to protein molecules is not solely driven by the release of entropically unfavourable waters, but also by a dominant favourable change in enthalpy. A typical example was demonstrated using the major urinary protein (MUP) with a minimal desolvation penalty upon ligand binding to its rich hydrophobic pocket (Portman *et al.*, 2014). Consequently, there is less water molecule to be displaced upon ligand binding hence less entropic contribution, resulting in a predominant binding enthalpy. Thus, Portman *et al.* 2014, demonstrated that the binding energetics are controlled by two desolvation processes with relatively different thermodynamic signatures. The first is ligand desolvation, which follows the classical hydrophobic effect phenomenon (Tanford, 1978) involving the release of water to bulk solvent of highly ordered water molecules from the nonpolar ligand surface as observed in S_1 (positive ΔS and positive ΔH). The second is the protein binding site desolvation associated with the liberation of “high energy” surface water molecule networks back into solution as observed at the BSP second binding event, S_2 (negative ΔS and negative ΔH). These two desolvation processes collectively underpin the foundations of broad ligand binding selectivity. Additionally, a dipole moment of a ligand can impact the arrangement of the water network at a binding site (Jelesarov and Bosshard, 1999). So, if a strong hydrogen-bond network is formed with the first hydration shell of the protein-ligand complex, then this would contribute significantly to a favourable change in the binding enthalpy (Duff and Howell, 2015). Therefore, the formation of multiple hydrogen bonds between BSP and several groups at the WbGST binding site may explain the enthalpically driven binding at S_2 , often accompanied by an entropic penalty.

The observed negative enthalpy change associated with negative entropy change is typical of an enthalpy-entropy compensation (Bronowska, 2011; Chodera and Mobley, 2013; Du *et al.*, 2016). Because increased bonding results in negative ΔH at the cost of increased orderliness leading to a more negative ΔS . Moreover, the limited measurable range of ΔG (-15 to -50 kJmol⁻¹) by the ITC may help to elucidate the observation of enthalpy-entropy compensation in many biomolecular interactions (Cooper *et al.*, 2001). Nonetheless, the entropic penalty for the second binding event (S_2) between BSP and WbGST exhibited a weaker binding affinity at S_2 ($K_{d2} = 112 \mu\text{M}$) compared to the K_d of S_1 ($K_{d1} = 18.8 \mu\text{M}$) despite the more favourable ΔH . Furthermore, the relatively large ΔH has also been observed for inhibitors binding at different subsites in stromelysin (Sarver *et al.*, 1999) similar to the multiple binding of BSP at S_2 of

WbGST ($n_2 = 5.75$). Ultimately, because the binding energetics for the BSP molecules bound at the low-affinity binding site, S_2 , could not be individually resolved from the calorimetric trace, therefore, the presence of six separate low-affinity sites cannot be ruled out if a stoichiometry of one BSP molecule per site is assumed.

In contrast to BSP, EGCG was shown to interact with WbGST with a stoichiometry of $n = 1.86$ and $n_{\text{GSH}} = 1.89$, implying that two molecules of EGCG bind per dimer of WbGST. The binding sites are probably located at the H-site of each monomer or even at the dimer interface considering the uncompetitive and mixed mode of inhibition towards the G-site (Figure 4.5 D) and H-site (Figure 4.7D) substrates, respectively. The binding interactions were observed to be enthalpically favourable (Figure 7.2) both in the presence and absence of GSH with lower favourable entropy both in the absence and presence of GSH, respectively. The low favourable entropic contribution possibly resulted from the desolvation of a few water molecules at the binding site. On the other hand, the ΔH can provide context towards ligand specificity considering that a more favourable ΔH is an indication of specific interactions between the binding partners (Bronowska, 2011; Chen *et al.*, 2016; Du *et al.*, 2016; Ross and Subramanian, 1981). The interaction between the ligands and the hGSTP1-1 revealed that EGCG made more binding contacts with WbGST compared to BSP, considering the observed values of ΔH (Table 7.2). Therefore, this indicates that although the interaction between the WbGST and BSP was sufficient to overcome the interactions that the individual molecules had made with the solvent in order to facilitate binding, the WbGST showed a greater degree of specificity for EGCG. This specificity can be attributed to a greater number and/or strength of non-covalent interactions between WbGST and the functional groups of EGCG. Despite this, EGCG displayed a lower affinity towards WbGST when compared to BSP binding at S_1 . Ultimately, the overall ligand binding event in WbGST proceeded spontaneously ($\Delta G < 0$) and it must be highlighted that ΔG depends mostly on the subtle balance between ΔH and ΔS .

The binding interaction of the ligands with the hGSTP1-1 and hGSTM1-1 was also investigated to understand these interactions from a thermodynamic perspective. Especially with the consideration that WbGST is more topologically related to the Pi-class GST (42% sequence similarity), but its biochemical attributes are more similar to the Mu-class as observed in both functional and structural analysis in this study. It was shown that one molecule of BSP binds one molecule of hGSTM1-1 ($n = 1$) whereas approximately two molecules of BSP bound per dimer of the hGSTP1-1 ($n = 2$). A similar, trend was observed in the stoichiometry of EGCG

binding to both human proteins. The stoichiometry observed in BSP binding to hGSTP1-1 is concurrent with what was observed in the crystal structure of BSP complexed hGSTP1-1, which shows binding of a molecule of BSP per monomer in the H-site (Oakley *et al.*, 1999). Regarding the hGSTM1-1, the location of the ligand binding site is unclear, because it was presumed that BSP and EGCG bind at the electrophilic bin site (H-site) of each monomer, which should be $n = 2$. However, it is possible that the ligands bind at the dimer interface of the enzyme. Moreover, a previous theoretical analysis of BSP complexed hGSTM1-1 showed that the best docking pose was observed at the dimer interface of the protein (Valli and Achilonu, 2022). Secondly, studies have shown that the GSTM1-1 isoenzyme possesses a ligandin binding site located at the dimer interface similar to what has been observed in some parasite GSTs (Hornby *et al.*, 2002). Unfortunately, the ligandin functions of class Mu GSTs have not been well studied, hence very little is known about them.

On the other hand, previous studies have suggested that most GST proteins bind one molecule of ligand per dimer (CACCURI *et al.*, 1990; Ketley *et al.*, 1975), and this corresponds to ITC stoichiometry ($n = 1$) observed for the hGSTM1-1 in this study. The crystal lattice might be unable to differentiate between the bound and unbound monomers of a dimer bound to one ligand therefore, the electron density for the ligand is averaged out in both binding sites. Consequently, it follows that the ligand occupancy of each site in the dimer will be equivalent to or less than half which fits in with the estimated occupancy (Oakley *et al.*, 1999). This probably could explain the apparent paradox surrounding the actual number of bound ligands in crystallised dimeric GSTs. One explanation behind the possibility that the ligand could bind at only one H-site per dimer is due to conformational changes induced at the second binding site following ligand binding to the first H-site. Previous studies have reported such conformational changes observed in the G-site of the hGSTP1-1 and bearing in mind that the H-site is closely situated to the G-site (Bello *et al.*, 1998; Oakley *et al.*, 1998; Ricci *et al.*, 1995).

Both the BSP and EGCG binding to the hGSTM1-1 were enthalpically favourable with an unfavourable and favourable entropy in the absence and presence of GSH, respectively. Although the magnitude of the favourable entropies was quite low ($T\Delta S_{\text{BSP-GSH}} = 3.2 \text{ kJ.mol}^{-1}$ and $T\Delta S_{\text{EGCG-GSH}} = 3.8 \text{ kJ.mol}^{-1}$) and probably contributed mostly by GSH-induced conformational changes. However, the overall binding of both BSP and EGCG was enthalpically driven. The observed ΔH pattern (Table 7.2) implies that the most contribution

to the binding interactions can be ascribed to non-covalent protein-ligand interactions and probably ligand solvent interactions. The desolvation of the BSP and EGCG binding site on the hGSTM1-1 would have contributed to the observed unfavourable entropy change. Therefore, indicating the occurrence of reduced conformational degrees of freedom of the hGSTM1-1 and the ligands upon binding leading to $\Delta S_{\text{conf}} + \Delta S_{\text{comp}} > \Delta S_{\text{solv}}$. This marginal desolvation event could also impact the ligand by decreasing the proportion of buried hydrophobic surface area of BSP and EGCG. Furthermore, in the absence of structural data, it cannot be ruled out that the interaction between both BSP and EGCG with the hGSTM1-1 was mediated by water molecules and in the event of an orderly arrangement of these water entropic penalties will be inevitable. However, there was a less entropic penalty in the presence of GSH for both BSP and EGCG binding, resulting in higher affinity. This could be a result of induced conformational changes on the active sites after the release of water molecules upon GSH binding (Patskovsky *et al.*, 2006).

In the case of the hGSTP1-1, BSP and EGCG binding to the protein were both enthalpically and entropically favourable except for EGCG binding in the presence of GSH which had an unfavourable entropy. Overall, the binding of both BSP and EGCG to hGSTP1-1 was entropically driven. The physiochemical properties of BSP indicated that the compound is non-polar with an AlogP value of 5.10 (Table S1, Appendix B). Therefore, the desolvation of BSP and probably the hGSTP1-1 binding site resulting from the hydrophobic effect would have contributed to an increased state of disorder at the binding site. Thus, compensating for the enthalpic penalty of BSP binding results in an overall entropically driven interaction. Besides, BSP binding had lower values of ΔH compared to EGCG, probably because BSP binding resulted largely from van der Waals interactions. In fact, no hydrogen bond interaction was involved in the binding of BSP to hGSTP1-1 (Oakley *et al.*, 1999). Secondly, the crystal structure of BSP complexed hGSTP1-1 indicated that no density was observed for the two benzyl sulphonic acid moieties implying a reduced binding contact between the ligand and the protein (Oakley *et al.*, 1999). In contrast, EGCG binding showed more binding interaction than BSP as indicated by its more negative ΔH but with lower binding affinity indicating that binding affinity is independent of binding enthalpy. Overall, BSP displayed more affinity towards the recombinant proteins compared to EGCG both in the presence or absence of GSH. Furthermore, the binding free energy and consequently the binding affinity of both BSP and EGCG binding to hGSTP1-1 showed higher affinity in the presence of GSH (Table 7.). This is concurrent with the observed increased stability of the studied proteins in the presence of GSH.

The release of water molecules upon GSH binding may have increased polar interactions between the protein atoms and solvent molecules thereby increasing stability as well as ligand affinity.

Chapter 8

General discussion and conclusion

Comparable to most other cytosolic GSTs, WbGST performs detoxification reactions in filarial parasites, compensating for the absence of the cytochrome-P450 system in adult worms (Brophy and Pritchard, 1994; Precious and Barrett, 1989). The lack of an effective alternative xenobiotic metabolism system in parasitic helminths identifies GSTs as their sole identifiable detoxification enzyme (Perally *et al.*, 2008). Consequently, GST becomes an attractive therapeutic target, particularly for the rational design of anthelmintics (Arora *et al.*, 2004; Brophy and Pritchard, 1994; Gupta and Srivastava, 2006; Rajaiah Prabhu *et al.*, 2018; Rao *et al.*, 2000). Additionally, the ligandin function of GSTs enables these enzymes to bind various electrophilic compounds. One of such compound analysed for its inhibitory potency on WbGST is BSP, an amphipathic hydrophobic anion dye primarily used in liver function tests (Schwenk *et al.*, 1976). Moreover, BSP's capacity to modulate the intracellular concentration of GSH led to its use in probing the catalytic properties of GSTs (Cui *et al.*, 2020). Furthermore, BSP presents a potential avenue for rational drug discovery of next-generation anthelmintics against filariasis, as previous studies have shown its inhibition of enzyme activity in (i) *Schistosoma* parasite GSTs (Padi *et al.*, 2021; Poee *et al.*, 2021; Yassin *et al.*, 2004), (ii) Anopheles GST Isozymes (Prapanthadara *et al.*, 2000) (iii) porcine class pi GST (Bico *et al.*, 1995) (iv) the human class pi and alpha GSTs (Kolobe *et al.*, 2004; Oakley *et al.*, 1999). On the other hand, EGCG, a naturally occurring plant polyphenol primarily found in green tea, induces apoptosis and demonstrates anti-proliferative and antioxidant activities in certain cancer cell lines (Sharma *et al.*, 2012; Steele *et al.*, 2000; Yang and Wang, 2011, 2016; Yang *et al.*, 2009). Previous studies have also reported EGCG antiplasmodial activity (Peter *et al.*, 2017; Sannella *et al.*, 2007; Zininga *et al.*, 2017) and its ability to inhibit GST activity (Boušová *et al.*, 2012). These prior investigations into BSP and EGCG form the foundation of this current research, particularly in an era where repurposing drugs to target neglected tropical diseases emerges as a significant research focus.

Thus, this study demonstrates that both BSP and EGCG inhibit the WbGST-mediated GSH-CDNB conjugation reaction, with BSP exhibiting greater potency than EGCG. Similarly, the inhibitory effects of these compounds were evaluated on the human pi and mu class GSTs, with both human proteins showing lesser inhibition compared to the parasite GST, as indicated by the obtained IC50 values (Table 4.3). Additionally, the kinetics data reveal that BSP and EGCG inhibit the activity of

WbGST and the two human GST isoforms in a dose-dependent manner. BSP exhibits a mixed-type inhibition mode for both GSH and CDNB with WbGST, suggesting additional binding affinity at sites other than the active sites (Saboury, 2009). EGCG displayed an uncompetitive mode of inhibition for GSH and a mixed mode for CDNB. These observed inhibition modes are typical of parasite GSTs. Regarding the studied human GSTs, the mode of inhibition of hGSTP1-1 by BSP and EGCG appears to be non-competitive for GSH and CDNB. This similarity between BSP and EGCG mode of inhibition of hGSTP1-1 could provide insight towards identifying EGCG actual binding site and possible binding contacts because the crystal structure of BSP-hGSTP1-1 is already available. The mode of inhibition observed for hGSTM1-1 is similar to that of the hGSTP1-1 except for CDNB inhibition by EGCG which displayed the mixed type of inhibition.

Based on empirical deductions in this study, it can be hypothesized that the observed inhibition results from the interaction of BSP and EGCG at both the H-site and the L-site of WbGST. Previous research suggests that the L-site in parasite GST is situated at the dimer interface of the homodimeric enzyme, a characteristic not commonly found in human GSTs. The binding behaviour of the two human GST isoforms investigated in this study implies that both BSP and EGCG bind at a different site than the speculated H-site, considering that other binding sites have been previously identified on hGSTP1-1. However, the crystal structure of hGSTP1-1:BSP and hGSTP1-1:CB3GA has shown that the H-site can accommodate both the ligands and CDNB simultaneously (Oakley *et al.*, 1999), despite the bulkiness of BSP. Unfortunately, there is limited information available regarding the ligandin site of hGSTM1-1, necessitating further studies to fully comprehend the ligandin function of this GST isoform and potentially identify the location of the L-site.

Furthermore, ANS was systematically employed to investigate the potential binding sites of both BSP and EGCG on WbGST and the human GSTs. It was observed that both BSP and EGCG displaced ANS, indicating competition between these compounds and ANS for the same binding site, which could be located at both the H-site and the L-site. BSP binding to WbGST in the presence of ANS led to an increase in the hydrophobicity of the binding site, while EGCG binding had the opposite effect. Additionally, ANS binding studies revealed that WbGST has a greater number of accessible hydrophobic pockets compared to the human isoforms, as evidenced by the substantial differences in maximum fluorescence intensity upon ANS binding. Specifically, the WbGST-ANS fluorescence intensity was 83% and 87% higher than that of hGSTM1-1 and hGSTP1-1, respectively.

Concerning the structural integrity of WbGST and the human GSTs, ligand binding did not exert a significant influence on the secondary structural contents of the enzymes in this study. However, size-exclusion high-performance liquid chromatography (SE-HPLC) data suggested that the oligomeric state of WbGST was affected by both BSP and EGCG binding in the absence of GSH. Moreover, the data obtained for GSH-complexed WbGST, as well as those of the human isoforms in the absence of ligands, revealed that the enzymes assumed more compacted oligomeric conformations in the presence of GSH. Changes in protein stability possibly correlate with alterations in the compactness of the protein induced by ligand binding.

GST stability is influenced by contacts at the dimer interface, and since GSTs function as dimers, interactions between subunits are crucial for correct folding and stability of quaternary structures. Stability investigations using differential scanning fluorimetry (DSF), differential scanning calorimetry (DSC), and far-ultraviolet circular dichroism (far-UV CD) revealed that WbGST is the least stable among the isoforms, while hGSTP1-1 is the most stable. Binding of GSH enhances the stability of the enzymes, with WbGST-GSH showing the greatest degree of stabilisation. Typically, ligand binding increases protein stability, but DSF results suggest that increasing concentrations of both BSP and EGCG had a destabilizing effect on WbGST, particularly in the absence of GSH. Thus, GSH binding propels these recombinant proteins into a more energetically stable state. The observed destabilization at high ligand concentrations likely contributed to the unsuccessful crystallisation attempts of ligand-complexed WbGST in this study. Thermal unfolding studies using DSC revealed two-state cooperative unfolding transitions, consistent with observations from chemical unfolding studies of WbGST using urea, except for the refolding process in the absence of GSH monitored by far-UV CD.

In addition, Isothermal Titration Calorimetry (ITC) provides insights into changes in interacting groups by monitoring the enthalpy of binding at the protein-ligand interface, which may be challenging to detect using other functional assays without three-dimensional structural data. With only the three-dimensional structure of BSP complexed hGSTP1-1 available, ITC was conducted to understand the interacting forces and binding energetics in the absence of the enzymes' three-dimensional structures in complex with BSP and EGCG.

ITC results revealed that BSP binds to multiple sites on WbGST, including high and low-affinity sites, suggesting binding at both the dimer interface and the H-site. The low-affinity site showed binding of multiple BSP molecules ($n = 6$), indicating the presence of many accessible hydrophobic pockets observed in ANS binding studies and by the DoGSiteScorer application, capable of binding

BSP molecules. Both BSP and EGCG bound moderately to the enzymes in the micromolar range, suggesting reversible interactions without covalent bonding or tight binding.

While BSP binding at the higher affinity site of WbGST was driven by entropy, it exhibited the most affinity compared to binding to human GSTs and EGCG. Ultimately, both ligands showed greater affinity towards WbGST compared to human GSTs, consistent with IC₅₀ results. Moreover, in line with the impact of GSH observed in spectroscopic studies, ITC results demonstrated that the ligands displayed higher affinity for the enzymes in the presence of GSH.

In conclusion, the findings and insights from this study represent an initial step towards addressing the design of new-generation anti-filarial therapeutics through rational drug design. By identifying WbGST as a potential target in the *Wuchereria bancrofti* parasite with BSP and EGCG as effective inhibitors, the groundwork for rational drug discovery has been laid. Both BSP and EGCG show promise as starting points for systematically exploring drug candidates against filariasis. Furthermore, the ligandin function of WbGST can be leveraged in rational drug design, as ligands like BSP and EGCG can influence the activity of catalytic active sites. This suggests potential targeting of the L-site, which likely overlaps with the H-site, offering a broad binding surface for ligands and enabling highly specific drug design. Despite WbGST being classified under the pi class GSTs, biochemical analyses indicate differences from hGSTP1-1. This underscores the dynamic nature of GSTs, with physiological, biochemical, and functional characteristics often deviating from the textbook narrative.

Future investigations will involve crystallising WbGST in complex with BSP and EGCG to elucidate the exact binding sites and contacts of the ligands with the enzyme. Alternatively, if obtaining diffractable crystals proves challenging, NMR spectroscopy can provide information on binding site residues involved in the interaction. Computational studies such as induced fit docking and molecular dynamics simulations will also aid in rational drug discovery and design in the absence of 3D structures. These approaches collectively contribute to the ongoing efforts to develop effective treatments against filariasis.

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Appendices

Appendix A

Reagents and Buffer Preparations

LB-agar [1.5% (w/v) Agar, 1% (w/v) Tryptone, 0.5% (w/v) NaCl, and 0.5% (w/v) Yeast Extracts].

3.0 g agar, 2.0 g tryptone, 1.0 g NaCl and 1.0 g yeast extract were dissolved in 200 mL of dH₂O with continuous stirring. The media was autoclaved, and appropriate antibiotics were added once the agar started cooling.

2xYeast-Tryptone (2xYT) [1.6% (w/v) Tryptone, 1% (w/v) Yeast Extract and 0.5% (w/v) NaCl].

1.6 g tryptone, 1.0 g yeast extract, and 0.5 g NaCl were completely dissolved in 100 mL dH₂O with continuous stirring. The media was autoclaved and allowed to cool at room temperature.

SOC Medium [2% (w/v) Tryptone, 0.5% (w/v) Yeast Extract, 10 mM NaCl, 2.5 mM MgCl₂, 10 mM MgSO₄ and 20 mM Glucose].

0.5 g tryptone, 0.25 g yeast extract, and 0.03 g NaCl were dissolved in 25 mL of dH₂O and autoclaved. The media was cooled to approximately 55 °C, after which 125 µL MgCl₂·6H₂O, 500 µL MgSO₄, and 1 mL glucose were added. The volume was made up to 50 mL with autoclaved dH₂O, aliquoted into sterile Eppendorf tubes and stored at −20 °C.

Antibiotics

A stock solution of ampicillin (100 mg/mL^{−1}) was prepared in ddH₂O, while chloramphenicol (35 mg/mL^{−1}) was dissolved in 100% (v/v) methanol. Solutions were filtered using a 0.22 µm syringe filter into sterile Eppendorf tubes and stored at −20 °C.

Phosphate Buffered Saline (PBS) [137 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄, and 2 mM KH₂PO₄, pH 7.4]

8.0 g NaCl, 0.2 g KCl, 1.15 g Na₂HPO₄, and 0.2 g KH₂PO₄ were dissolved in 800 mL of dH₂O. pH was adjusted to 7.4 and volume was made up to 1 L with dH₂O. Buffer was stored at room temperature.

Resuspension Buffer [PBS, 25 mM Imidazole, 0.01 M PMSF, 10% (v/v) glycerol, 0.01% (w/v) NaN₃, pH 7.4].

1.7 g imidazole and 0.2 g NaN₃ were dissolved in 800 mL of PBS buffer, 100 mL of glycerol was added, and pH was adjusted to 7.4 while topping up the volume to 1 L with PBS solution. Buffer was stored at 4 °C temperature.

Equilibration Buffer [PBS, 25 mM Imidazole, 10% (v/v) glycerol, 0.01% (w/v) NaN₃, pH 7.4].

2.55 g imidazole and 0.15 g NaN₃ were dissolved in 1.3 L PBS buffer, and 100 mL of glycerol was added pH was adjusted to 7.4 while topping up the volume to 1.5 L with PBS solution. Buffer was stored at room temperature.

Wash Buffer [0.1% (v/v) Tween-20 and Equilibration Buffer, pH 7.4].

0.5 mL tween-20 was made up to 500 mL with equilibration buffer and stored at room temperature.

Elution Buffer [PBS, 350 mM Imidazole, 2 mM DTT, 10% (v/v) glycerol, 0.01% (w/v) NaN₃, pH 7.4].

23.85 g imidazole, 0.31 g DTT, and 0.1 g NaN₃ were dissolved in 800 mL PBS buffer, 100 mL of glycerol was added, and pH was adjusted to 7.4 while topping up the volume to 1 L with PBS solution. Buffer was stored at room temperature.

Dialysis Buffer 1 [PBS, 5 mM EDTA, 2 mM DTT, 10% (v/v) glycerol, 0.01% NaN₃, pH 7.4].

1.20 g NaH₂PO₄, 0.74 g EDTA, 0.31 g DTT, and 0.10 g NaN₃ were dissolved in 800 mL of ddH₂O, 100 mL of glycerol was added, and the pH was adjusted to 7.4, and volume made up to 1 L with ddH₂O. Buffer was stored at room temperature.

Dialysis Buffer 2 [20 mM NaH₂PO₄, 2 mM EDTA, 2 mM DTT, 10% (v/v) glycerol, 0.01% NaN₃, pH 7.4].

1.20 g NaH₂PO₄, 0.74 g EDTA, 0.31 g DTT, and 0.10 g NaN₃ were dissolved in 800 mL of ddH₂O, 100 mL of glycerol was added, and the pH was adjusted to 7.4, and volume made up to 1 L with ddH₂O. Buffer was stored at room temperature.

Activity assay Buffer [100 mM sodium phosphate, pH 6.5, 2 mM EDTA, 2 mM DTT, 0.01% NaN₃].

8.22 g NaH₂PO₄, 4.47 g Na₂HPO₄, 0.74 g EDTA, and 0.10 g NaN₃ were dissolved in 900 mL of ddH₂O, pH was adjusted to 6.5, and volume made up to 1 L with ddH₂O. Buffer was stored at room temperature.

Monomer solution [30% (w/v) acrylamide, and 2.7% (w/v) bis-acrylamide].

73.0 g acrylamide and 2.0 g bis-acrylamide were dissolved in dH₂O, and the volume was made up to 250 mL. The solution was filtered using Whatman No. 1 filter paper and stored in an amber bottle at 4 °C.

Reducing Sample Buffer [25.0% (v/v) Tris-HCl, 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) β -mercaptoethanol, pH 6.8].

2.5 mL Tris-HCl (0.5 M), 4 mL 10% SDS, 2 mL glycerol, and 1 mL β-mercaptoethanol were mixed thoroughly, and volume was made up to 10 mL with dH₂O. 35 µg of bromophenol blue was added as a tracking dye and stored at room temperature.

Tank/ Electrophoretic buffer [250 mM Tris-HCl, 1.92 M glycine, 0.1% (w/v) SDS, pH 8.3].

15.0 g Tris-HCl and 72.0 g glycine were resuspended in 5 L of dH₂O and stirred. Before use, 1.0 g SDS was added to 900 mL of the buffer. pH adjusted to 8.3 and volume made up to 1 L.

Coomassie Stain Solution [0.1% (w/v) Coomassie dye in 1:5:4 (v/v/v) acetic acid-methanol-water solution].

0.1 g Coomassie dye was dissolved in 1:5:4 (v/v/v) acetic acid-methanol-water solution and stored at room temperature.

Destain Solution [10% (v/v) Glacial Acetic Acid, 40% (v/v) Methanol, 50% (v/v) distilled water].

50 mL of glacial acetic acid, 200 mL methanol, and 250 mL dH₂O were mixed and stored at room temperature.

Preparation of competent *E. coli* T7 cells

In 200 mL of autoclaved LB-agar, 35 µg/mL⁻¹ of chloramphenicol was added, and agar was poured into plates and allowed to solidify. An *E. coli* T7 glycerol stock solution was then spread

across plates and incubated for 16-24 h at 37 °C. A single colony was selected afterwards and inoculated into 50 mL of 2xYT media overnight at 37 °C in a rotating incubator at 250 × g. The overnight culture was diluted with freshly prepared media in the ratio of 1/20 and grown to an optical density of about 0.6 ($OD_{600\text{ nm}} \approx 0.6$) at 37 °C with shaking at 250 rpm. The culture was centrifuged at 5 000 rpm for 10 min at 4 °C. Pelleted cells were resuspended in 10 mL of $MgCl_2$ (100 mM) and incubated on ice for 30 min, followed by centrifugation at 5 000 rpm for 10 min at 4 °C. Afterward, cells were resuspended in 10 mL of $CaCl_2$ (100 mM), incubate on ice for 4 h, and centrifuged at 3 000 × g for 10 min at 4 °C. Cells were finally resuspended in 1 mL $CaCl_2$ and 1 mL glycerol (80% (v/v)). 100 µL of the suspension was aliquoted into cold sterile Eppendorf tubes using cold sterile pipette tips. The cells were quickly frozen in liquid nitrogen and stored at −80 °C.

Gel Preparation and set-up

SDS gel was set up using 12.5% (w/v) separating gel and 4% (w/v) stacking gel prepared according to the protocol in Table 3.2. TEMED and ammonium persulphate were only added just before pouring the solution into the gel casting unit. The gel-casting unit (Bio-Rad mini-PROTEAN® vertical electrophoresis apparatus) and the glass plates (81.5 mm × 101.5 mm and 72.5 mm × 101.5 mm) were assembled, and the gel was set up by pouring separating gel solution first into the glass plate casting unit to a height of about 6cm from the base and covered with a little water, to prevent oxidative inhibition of polymerisation of the acrylamide. Upon polymerisation, the water cover was removed, and the glass plate was topped up to the brim with the stacking gel, followed by the immediate insertion of a 1.5 mm gel comb into the stacking gel and allowed to polymerise. Afterwards, the glass plate was removed from the casting unit and then transferred into the electrophoresis tank. The gel comb was removed, and samples were loaded (5 µL of standard Molecular weight marker and 10 µL protein sample) into the gel wells.

ANS (8-anilino-1-naphthalenesulphonic acid)

50 mg of ANS was dissolved in 1 mL ddH₂O and vortexed for ~1 h in the dark. This was followed by centrifugation at 13,000 × g for 5 min. The supernatant was collected, and the concentration was determined at 350 nm via a UV-Vis Spectrophotometer (Jasco V-630) using molar extinction coefficients 5000 M^{−1} cm^{−1}. All ANS preparation procedures were carried out in foil to limit light absorption.

SYPRO Orange Dye.

50x SYPRO Orange stock concentration was prepared from 5000x concentration by diluting 10 μ L of 5000x concentration into 1 mL of ddH₂O.

SE-HPLC Buffer [750 mM NaCl, 10 mM NaH₂PO₄, pH 7.4, 0.01% (w/v) NaN₃, 2 mM DTT].

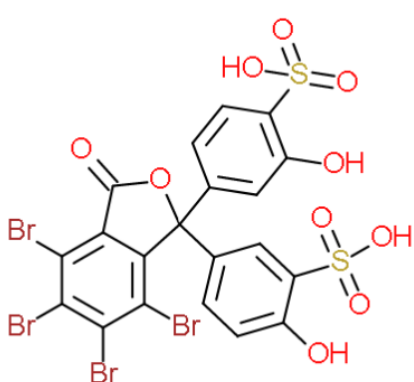
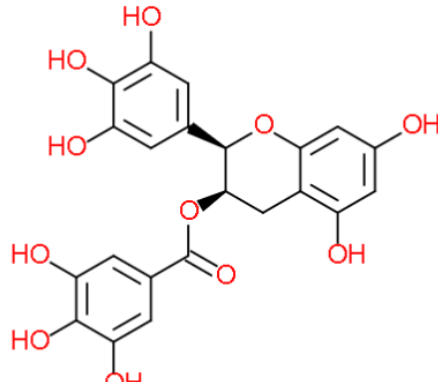
21.92 g NaCl, 0.59 g NaH₂PO₄, 0.31 g DTT, and 0.05 g (NaN₃) were dissolved in 450 mL of ddH₂O. pH was adjusted to 7.4 and volume was made up to 500 mL with ddH₂O. The prepared buffer was subjected to degassing to prevent the introduction of air bubbles and subsequent interference with normal column functioning.

Gel Filtration Standards

The lyophilised mixture containing approximately 18 mg of protein (5.0 mg thyroglobulin, 5.0 mg g-globulin, 5.0 mg ovalbumin, 2.5 mg myoglobin, and 0.5 mg Vitamin B12) per vial of molecular weight markers, was reconstituted with 0.5 mL of deionised water. The vial was swirled gently to mix thoroughly and thereafter it was left on ice for about 2–3 min, followed by another round of mixing. The hydrated mixture was aliquoted and stored at –20 °C. Before usage, the standards are always centrifuged to remove any larger particles such as aggregated protein.

Appendix B
Supplementary data

Table S1. Physio-chemical properties of BSP and EGCG

Chemical properties		BSP	EGCG
Chemical structure			
Chemical formular		$C_{20}H_{10}Br_4O_{10}S_2$	$C_{22}H_{18}O_{11}$
Molecular weight (g.mol ⁻¹)		838	458.4
Molecular shape volume (Å ³)		431.4	347.3
Polar surface area (Å ²)		175.5	197.37
AlogP		5.10	2.23
No. hydrogen bond acceptors		8	11
No. hydrogen bond donors		4	8
No. rotatable bonds		4	4
Stereo centre count		0	2

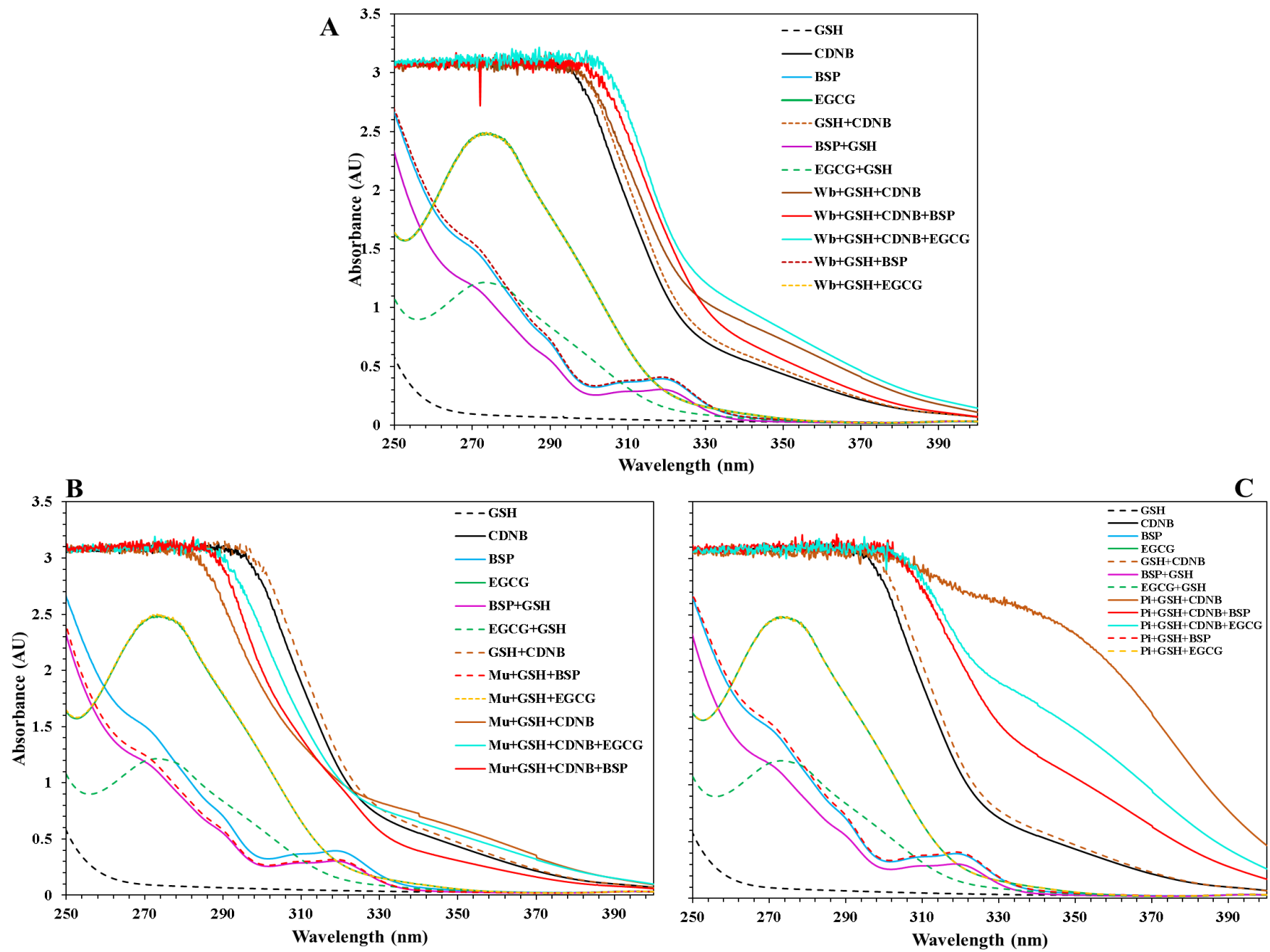


Figure S1. Spectroscopic and physio-chemical properties of BSP and EGCG. The UV-Vis absorbance spectroscopic properties of BSP and EGCG in the presence of WbGST (A), hGSTM1-1 (B), and hGSTP1-1 (C) were monitored by using 100 μ M of either compound in activity assay buffer (Appendix A). All data were corrected by subtracting the contributions caused by the buffer. The BSP and EGCG had an absorbance maximum at 319 nm and 272 nm, however, BSP displayed an additional characteristic slopy absorbance between 266-299 nm. BSP and EGCG share a similar number of rotatable bonds (4). However, the AlogP value, where P is the partition coefficient that defines the ratio of the concentration of a solute between two solvents with different polarities, is different between BSP and EGCG. The AlogP value for BSP is larger than that of EGCG and therefore indicates that BSP is more hydrophobic. Furthermore, as opposed to BSP, EGCG has a stereo centre which may account for the different UV-Vis absorbance spectroscopic properties.

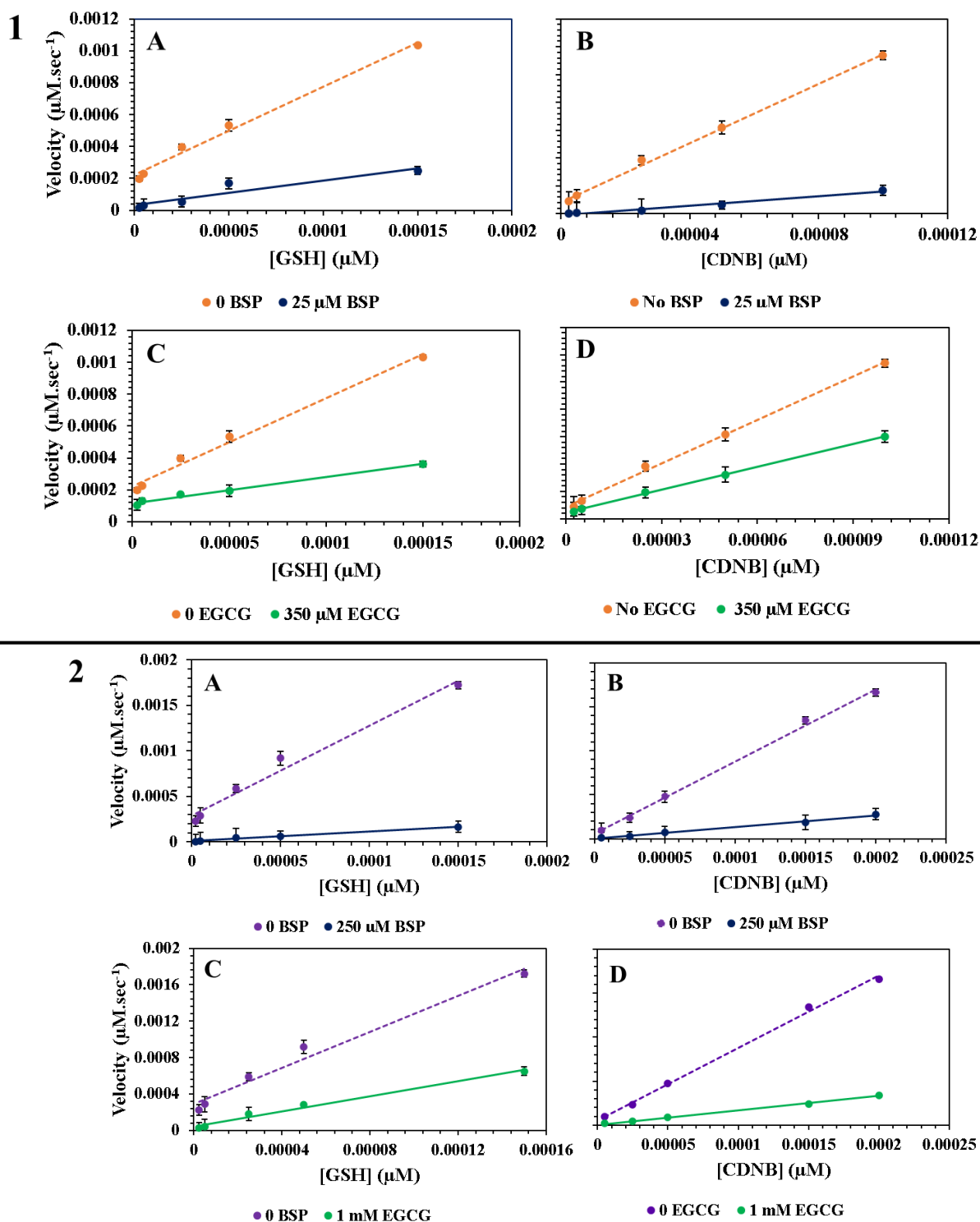


Figure S2 Experimental analysis of k_{cat}/K_M . The k_{cat}/K_M of hGSTM1-1 (**panel 1**) and the hGSTP1-1 (**panel 2**) were obtained experimentally in a condition where $[S] \ll K_M$ by varying substrate concentrations in the presence or absence of BSP and EGCG. The velocity was plotted as a function of substrate concentrations, and the resulting slope was used to calculate the k_{cat}/K_M . Varying concentrations of GSH (A) and CDNB (B) in the absence and absence of BSP (blue) and EGCG (green).

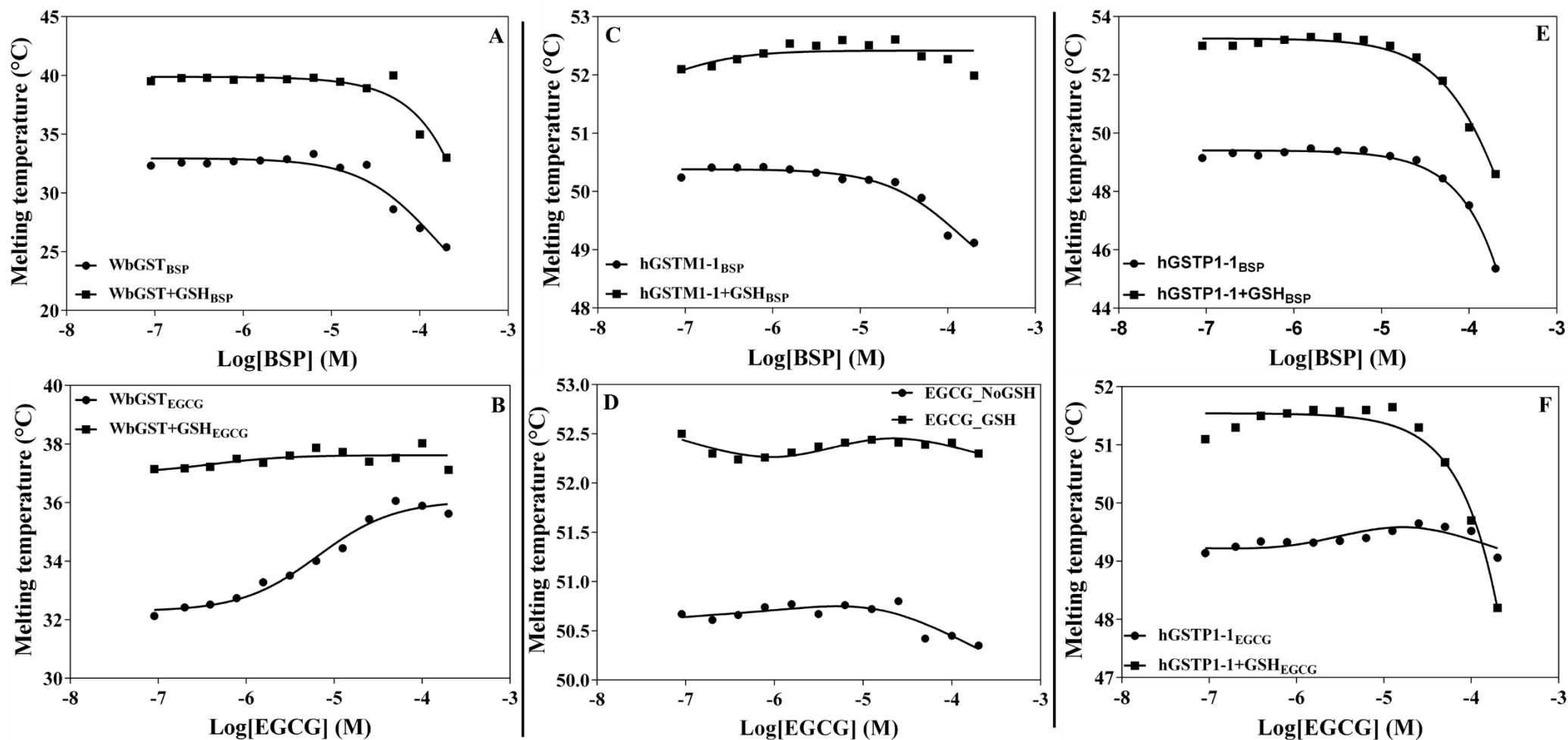


Figure S3. Monitoring ligand stabilisation effect using the DSF. The stabilising effect of BSP on WbGST (A), hGSTM1-1 (C), and hGSTP1-1 (E) was accessed in the presence and absence of GSH by varying BSP concentrations from 0-200 μ M. A similar analysis was performed with EGCG binding to WbGST (B), hGSTM1-1 (D), and hGSTP1-1 (F). The profiles are mostly hyperbolic, showing that initially at lower concentrations the melting temperatures were slightly increased. The most increase was observed for WbGST-EGCG in the presence of GSH. (B). At higher ligand concentrations, a decrease in the melting temperatures started progressively.

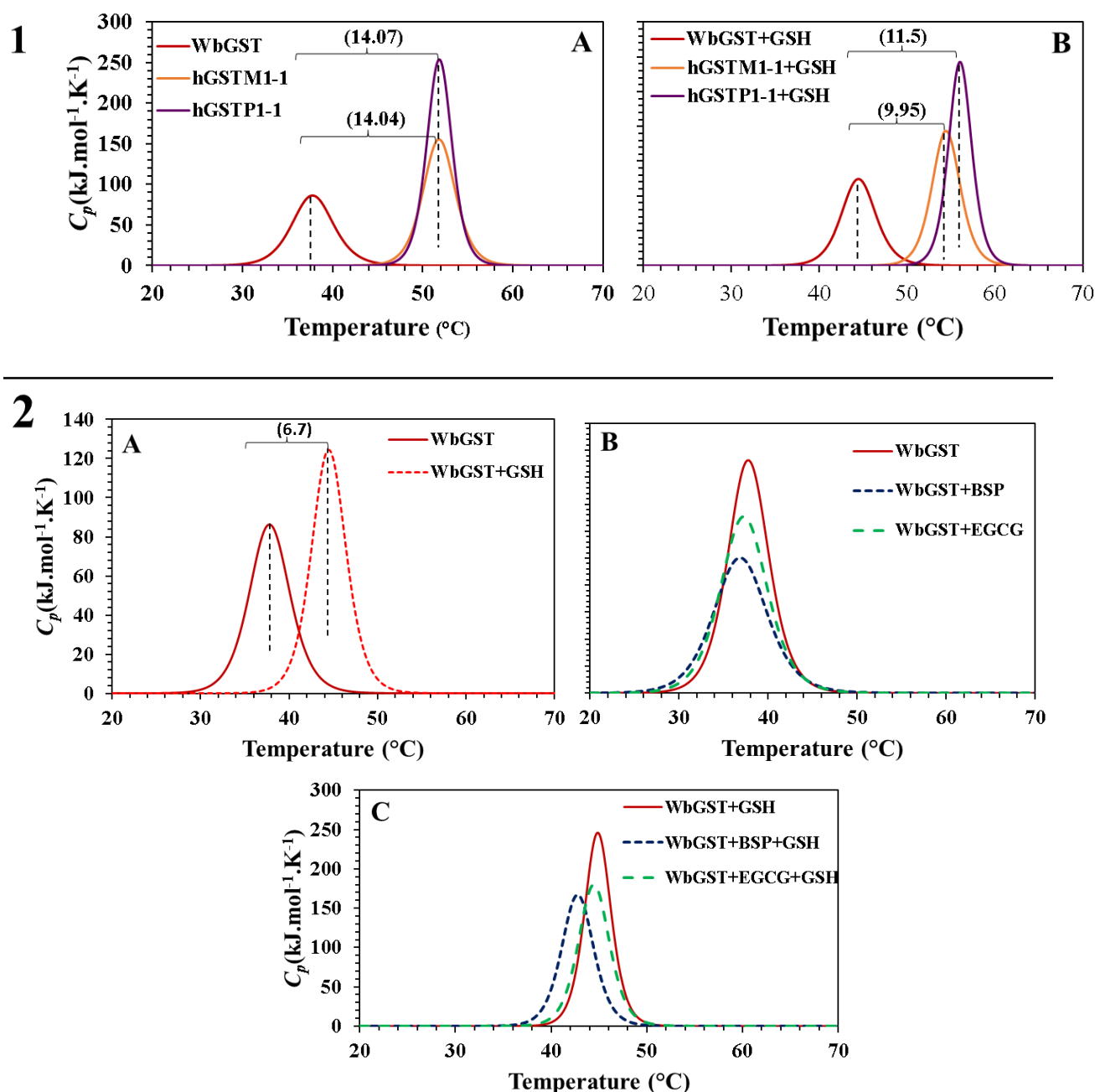
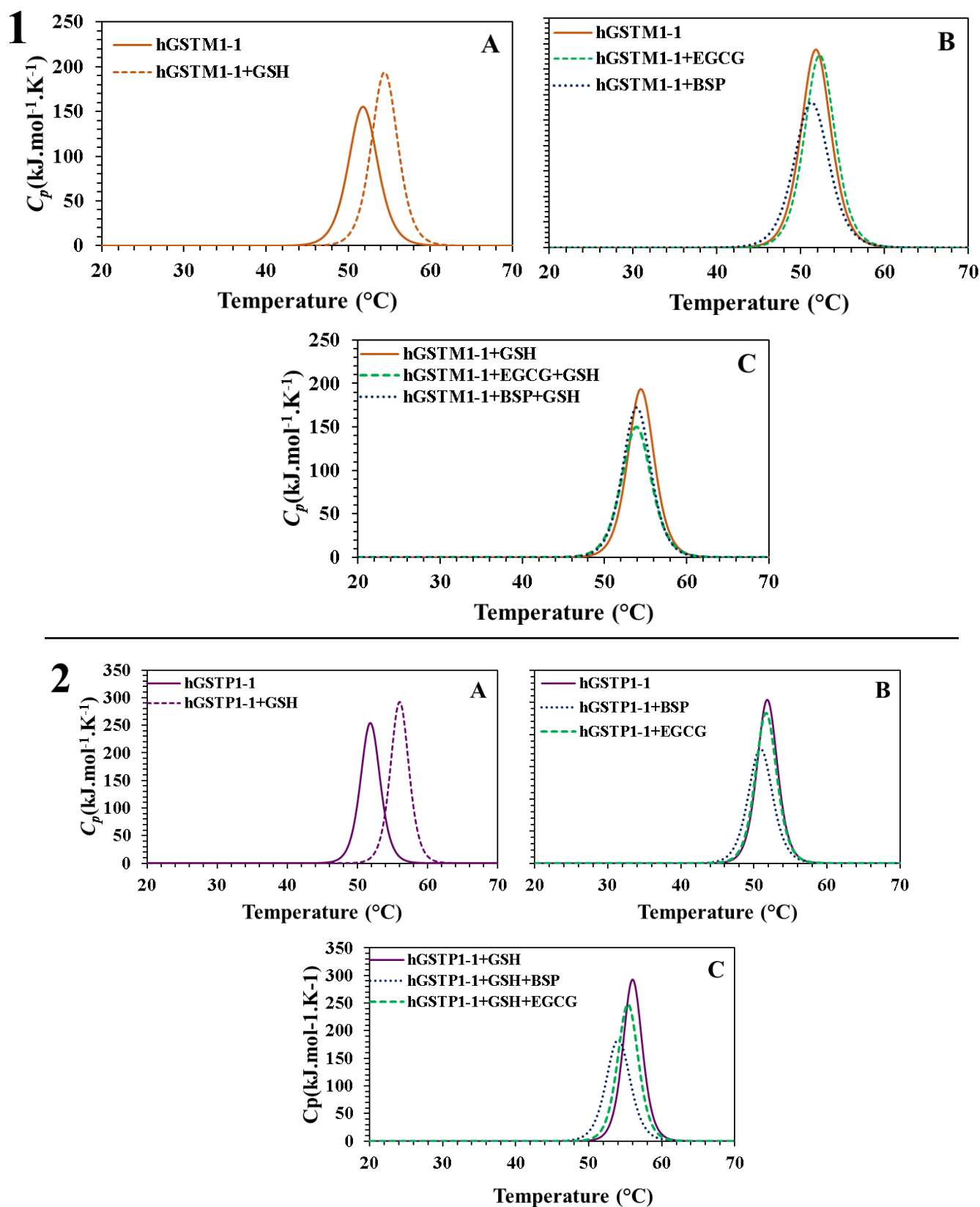


Figure S4 Deconvoluted DSC thermograms. Panel one compares ΔT_m (in brackets) of WbGST (Red), with the hGSTM1-1 (Orange) and hGSTP1-1 (Purple). WbGST shows a lower stability (14 °C less) compared to the human isozymes in the absence of GSH and 11.5 °C and 9.95 °C lower than the hGSTP1-1 and hGSTM1-1, respectively. The curves in dotted lines represent samples analysed in the presence of GSH while BSP (Blue) and EGCG (Green). Prior to fitting and plotting the data, they were baseline corrected and converted to molar heat capacity by applying the dimer concentrations of the proteins. The presence of GSH increased the stability of the proteins (A), as indicated by a higher ΔT_m . EGCG increased the stability of WbGST (2B), while BSP produced a destabilising effect in the absence of GSH. BSP decreases the stability of hGSTM1-1 (C) and hGSTP1-1 (D).



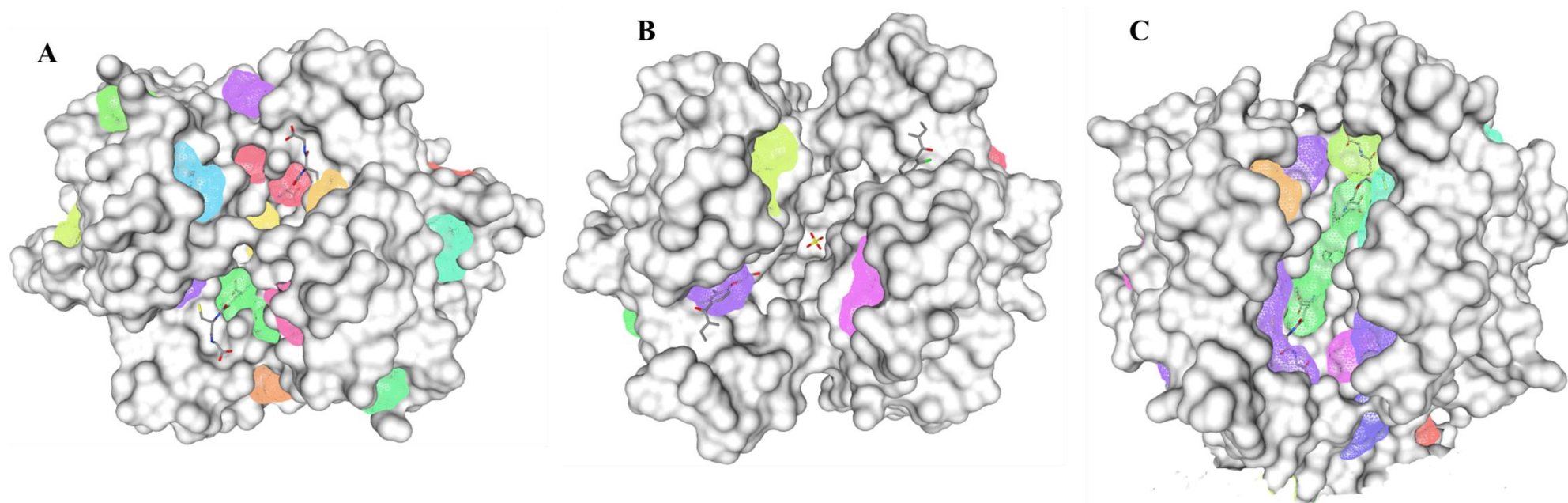


Figure S6. Surface representations of predicted possible binding sites. (A) shows 25 predicted possible binding pockets on WbGST. While (B) is the hGSTP1-1 with 8 binding pockets and (C) is the hGSTM1-1 showing 12 binding pockets. The G-site per monomer is shown with bound GSH molecules shown in a stick representation. The images were generated using DoGSiteScorer on Proteins Plus