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UNIVERSITY OF THE WITWATERSRAND

PURIFICATION AND CHARACTERISATION OF STARCH METABOLIZING ENZYMES FROM *STREPTOCOCCUS SANGUIS 1MC 204*

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A dissertation submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg, in fulfilment of the requirement for the degree of Master of Science

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ABSTRACT

An attempt has been made to purify and isolate a starch endo-hydrolase enzyme produced by *Streptococcus sanguis*, an organism that may be implicated in dental caries.

In order to isolate the enzyme by affinity binding, a chemically modified amylopectin was prepared, similar to the preparation of chromogenic substrate for the assay of α-amylase, but without dye. The amylopectin was treated with 10 percent ammonium sulphate at 55°C for 45 minutes.

A crude enzyme extract was prepared from concentrated culture medium and by precipitation with 60 percent ammonium sulphate. The concentrated culture medium was added to the modified amylopectin substrate and the mixture was incubate for 30 minutes at 37°C and centrifuged. The precipitate was resuspended in 20 mM hepes buffer pH 6.5 which contained 0.02 M KCl to release the enzyme from the enzyme-substrate complex. The suspension was tested for enzyme activity and the presence of proteins.

More than 50 percent of the yield of the enzyme was achieved by this process, after three assays, from both crude enzyme extracts and enzyme serum samples. A 5.2 fold purification was obtained from the extraction process of the crude enzyme extract and a protective enzyme activity effect was noticed in the presence of ammonium sulphate.

The analytical methods selected for the activity assay were mainly used for the activity evaluation of α-amylases and carbohydrate hydrolysing enzymes. The result showed carbohydrate interference.

The isolation method proved sensitive and highly specific for the isolation of a starch metabolizing enzyme produced from *Streptococcus sanguis 1 MC 204.*

The purification of the enzyme by gel filtration and its characterization by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) showed that this enzyme was a glycoprotein. SDS-PAGE was performed with mucin and heparin in the presence of other proteins as markers, and stained with the periodic acid- alcian prestained silver method. This gave one transparent band around the phosphorylase b marker and four other more slowly running clear bands.

Further, the comparison of scans of several proteins and glycoproteins with the scan of the eluted sample of the amylopectin extracted enzyme showed a similarity with the UV scan of mucin and confirmed that the enzyme was a glycoprotein.

It may be further characterized by selecting methods that take into account the carbohydrate content of the protein and by eliminating the carbohydrate interference in the assays.

DECLARATION

I declare that this dissertation is my own, unaided work. It is being submitted for the degree of master of Science in the University of the Witwatersrand, Johannesburg. It has not been prepared under the aegis or with the assistance of any other body or organisation or person outside the university of the Witwatersrand, Johannesburg.

BENJAMIN BOGUO I.
(Name of candidate)

29 day of MARCH 1996.

LIST OF PUBLICATIONS

The following publications have arisen from material included in this thesis.

Boguo L.B., Coogan M.M. and Manchester K.L.(1994), Isolation of a Starch Hydrolysing Enzyme from *Streptococcus sanguis 1 MC 204*, Proceedings of the International Association for Dental research, XXVIII Scientific Congress.

Boguo L.B., Coogan M.M. and Manchester K.L.(1995), Isolation of a Starch Hydrolysing Enzyme from *Streptococcus sanguis 1 MC 204*, Proceedings of the South African Biochemical Society, THIRTEENTH Congress.

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CHAPTER I.

INTRODUCTION

I. DENTAL CARIES

Dental caries in the simplest terms is the slow destruction of teeth resulting from the loss of the hydroxyapatite crystals which results in the reduced structural integrity of the teeth. It is one of the oldest diseases known to man and is still without a remedy. It affects an important part of the world population irrespective of social class. Current understanding supports the theory that dental caries has a bacteria etiology which depends upon interaction between host defense systems, dietary factors and time. Dental caries, which is selective and relatively slow, requires time for induction and progression through the enamel. The host defense system inhibits the development of caries through the removal of bacteria. This has been supported by observing the effect of hyposalivation on the induction of dental caries (Wolonsky, 1988; Hardie, 1992; Ooshima et al.,1992). In addition the dental anatomical contours and arch form may favour the development of dental caries in man.

Although interest in the diagnosis, aetiology, prevention, and treatment of the dental caries has increased dramatically over the past two decades, the problems underlying the disease process are not clearly understood. For example, the relationship between root caries and coronal caries needs to be studied (Billings et al., 1993).

Numerous studies undertaken into the different factors show clearly the high complexity of the problem of dental caries. The diversity of species of the oral microflora combined with

the multifactorial aspects of dental diseases has led to problems understanding of the complex processes causing this disease. For this reason the control of dental caries has not been successful.

II. MAIN FACTORS IMPLICATED IN DENTAL DISEASES

II. A) DIETARY FACTORS

Research work in this area was focused mainly on the role of carbohydrates as a substrate. Initially researchers investigated cariogenic and acidogenic sugars which may be used as a substrate by the oral flora. Most studies indicate that sucrose, hydrolysable starch and pure mono and polysaccharides are cariogenic and acidogenic (Edgar, 1993; Imfeld, 1993; Marthaler, 1990).

A.1 SUGARS IN FOOD AND DENTAL CARIES

The accumulated evidence which shows a relationship between dietary sugar consumption and caries in animals seems unclear in human studies. In spite of the effects of fluorides on caries reduction, there is a poor correlation between sugar intake and dental caries in humans (Burt, 1993). Nevertheless, sucrose has been shown to play a more dominant role in the development of smooth surface caries than in fissures caries (Burt,1993; Marthaler, 1990). Several studies suggest that the metabolism of sucrose by the *Streptococci* leads to the synthesis of polysaccharides. These carbohydrates increase the adherence of these bacteria to the smooth enamel surface of the tooth and thereby implicates them in dental caries (Burt, 1993; Newbrun, 1992b; Rolla et al., 1985).

A.2 SUGARS SUBSTITUTES

Most studies on the cariogenic potential of pure mono- and disaccharides indicate that the major food sugars, glucose, fructose, and maltose are almost similar to sucrose (Edgar, 1993).

Lactose however less cariogenic. A study on milk shows that it reduces the adsorption of glucosyltransferase onto the tooth surface as well the expression of the adsorbed enzyme (Vacca-Smith and Bowen, 1995).

Studies on sugar substitutes were undertaken to study their bacteriostatic aspects. The stimulation of saliva flow in the absence of significant acid production can lead to the remineralization of enamel (Marsh, 1993) on one hand where as the selective effects can influence the composition of the oral microflora on the other hand (Grenby and Saldanha, 1986; Best and Brown, 1987; Scheie, 1989; Rogers et al., 1991; Newbrun, 1992). Dietary factors, which play an important role in the development of the microbial population of the oral cavity have been considered the second most important agent in the induction of human dental caries.

II. B) MICROBIAL FACTORS

B.1 DENTAL PLAQUE

Since dental caries as well as dental diseases have been shown to be a multifactorial etiology, several clinical longitudinal studies have shown that dental plaque has a clear relationship with dental caries and periodontal disease (Sanz, 1988).

a) COMPOSITION

Dental plaque consists predominantly of bacteria rather than food remnants (Sanz, 1988; Socransky et al., 1963). Bacteria make up approximately 70 to 80% of the plaque material in an extremely complex arrangement and proliferating microorganisms along with a scattering of epithelial cells, leucocytes, and macrophages in adherent intercellular matrix form the rest of dental plaque. Microorganisms like mycoplasma, fungi, protozoa and viruses may be found in dental plaque.

b) FORMATION

On basis of the gingival margin, dental plaque is differentiated into supragingival (plaque forming above gingival) and subgingival plaques. In both types, the pellicle constitutes the first layer of organic materials that adsorb on the tooth surface.

The important part of these materials are provided by:

- Saliva mucin which participates in the formation of the protective pellicle covers tooth enamel and consists mainly of large glucoproteins (Slomiany et al., 1993, 1986 and 1987b; Sanz, 1988).
- Polymers secreted by bacteria of the *Mutans streptococci* group which are

enzymatically active in the extracellular medium even when aggregated by the saliva glucoproteins (Vacca-Smith and Bowen, 1995; Schilling and Bowen, 1988).

- The synthesis of polysaccharides from the activity of this enzyme on sucrose. The polysaccharides have sticky properties and are thought to partake in the cell adherence on the tooth surface (Coogan, 1990; Koga et al., 1986; Morisaki et al., 1986; Shockman et al., 1986 and Wolinsky, 1988).

The further development of dental plaque depends on different factors such as bacteria, bacterial products, interbacterial matrix, host and diet in which electrostatic forces and hydrophobic interactions are associated.

B.2 'THE CARIOGENICITY OF A COMMUNITY'

Streptococcus mutans and other *Streptococcal* strains have been strongly implicated as the main agents in the initiation and induction of human and animal dental caries.

The ability of microorganisms to grow and survive in an acidic environment, to ferment dietary carbohydrates and to lower the pH, suggests they may be considered cariogenic microorganisms (Hamada and Slade, 1980; Hamada, 1986). However, this assumption is no longer upheld by many researchers today. An idea which has developed recently is the concept of **'The cariogenicity of a community'**.

This theory suggests that the interaction of the oral microflora may play an important role in dental caries rather than the direct effect of any single species or type (Hardie, 1992; Wolinsky, 1988). The presence of *Lactobacilli* and *Mutans streptococci*, specifically

Streptococcus sobrinus which is strongly aciduric, together with *Streptococcus mutans* in dental plaque and on the teeth of caries prone subjects strongly supports this assumption (Hallgren et al., 1992; Van Houte, 1980; Almedy et al., 1993; Loeche, 1986; Bratthall, 1991; Lindquist et al., 1991a). Nevertheless, *Streptococcus mutans* and other *Streptococci* are considered the most cariogenic bacteria.

B.3 UNDERSTANDING THE ROLE OF *MUTANS STREPTOCOCCI*

a) CLASSIFICATION

Studies on the *Mutans streptococci* showed they were an heterogenous group of microorganisms. In order to classify these organisms, serological specificity of the cell wall carbohydrate antigens and DNA homology methods were used and revealed eight serotype strains and five groups in the *Mutans streptococci*. Serotype c,e and f form *Streptococcus mutans* strain and serotype d and g belong to *Streptococcus sobrinus* strain (Saarela et al, 1993). Serotype c contains the most common strains found in dental plaque of man and *Streptococcus mutans* and *Streptococcus sobrinus* are regularly isolated from humans (Hardie, 1992).

Restriction endonuclease digestion and agarose gel electrophoresis methods have shown that genetic diversity exists between serotype as well as among isolated strains of the same serotype (Saarela et al., 1993). The *Mutans streptococci* can be divided in two subgroups based on extracelluar polysaccharides synthesis. However the ability to produce a particular polysaccharide is not associated with a specific serotype.

Extracelluar glucosyltransferase-s is associated with the production of water soluble glucans and glucosyltransferase-I with insoluble glucans synthesis. Furthermore, an insoluble glucan,

designated amyloglucan, which may participate in dental plaque formation and the adherence of streptococcal cells (Coogan, 1990) has also been observed.

b) THE ROLE OF THE ENZYME ON CARIES INCIDENCE

1) SALIVA ENZYMES

Saliva is the main host factor that affect caries incidence. It play an active and passive role in the host defence system. The active role is fulfilled by the enzymes like lysozyme, lactoperoxidase and lactoferrin which are involved in the cell wall lysis of types of gram positive bacteria, and the inhibition of *Streptococcal* glycolytic enzymes and bacterial growth by Fe removal respectively (Slomiany, 1993; Wolinsky, 1988; Sanz, 1988).

The passive role is fulfilled as buffer in:

- neutralization of lactic and acetic acids produced by bacteria and reduction of enamel demineralization (buffer system bicarbonate - carbonate);
- the formation of the pellicle protecting tooth enamel and soft mucosa;
- and in the promotion of bacterial aggregation and clearance from the oral cavity through mucus glucoproteins and host protection through salivary immunoglobulin (IgA) which acts as a specific agglutinin (Wolinsky, 1988; Sanz, 1988; Slomiany, 1993).

2) STREPTOCOCCAL ENZYMES

A) CELL ASSOCIATED AND EXTRACELLUAR ENZYMES PRODUCTION

Studies on the cell walls of the *Streptococci* have revealed that, in addition to mechanical, shape and protective functions, they also have nutritive and transport functions (Chockman et al., 1986). Some of the polymers that are secreted become part of the extracelluar media whereas others may remain loosely or tightly associated with the cell wall itself. These polymers, which are composed mainly of carbohy- es, proteins, glycosylproteoglucan, lipoteichoic acids and some glycopeptides, have enzyme activity and are thought to be involved in the cell adherence processes and leads to the hypothesis of dental plaque formation (Coogan, 1990; Wolinsky, 1988; Koga et al., 1986; Morisaki et al., 1986 and Chockman et al., 1986).

B.4 ENZYME ACTIVITIES

A) INTRODUCTION

Several studies on the role of enzymatic activity on initiating as well as inducing dental caries show that glycolytic activity, enzymatic polysaccharide hydrolysis and glucan synthesis are the most implicated in this process (Walker, 1966; Coogan, 1990; Boyer et al., 1991; Tanaka et al., 1993).

Research into sugar and starch hydrolysis by *Mutans streptococci* has shown that the activity of external cell associated enzymes and extracelluar enzymes on these carbohydrates leads to the synthesis of polysaccharides (Ciardi, 1976; Russell, 1979; Boyer, 1971; Coogan, 1992 and Mukassa, 1989).

B) SUCROSE and STARCH METABOLISM

It is well established that the action of glucosyltransferase (GTF, EC 2.4.1.5) on sucrose leads to the formation of insoluble glucan (Chassy, 1983) and the endo-hydrolase activity of α-amylase on the starch leads to glycolytic hydrolysing activities (Coogan and Russell, 1992; Mizokami, 1988; De Cordts, 1993). Therefore the expression of glucosyltransferase, transglucosylase and glucosyltransferase in different studies on identification and purification of these enzyme(s) show that they were responsible for the production of glucans. For sake of clarity the word glucosyltransferase will be used for the enzymes which synthesize polysaccharides from sucrose whereas α-amylase will be used for the enzymes that hydrolyse carbohydrates.

1) Sucrose Metabolism

Indeed, work by Ciardi et al (1976) on *Streptococcus mutans* G 715 has shown that the enzymes that produce water soluble and insoluble glucans can be stained for protein and glucoprotein respectively. The enzyme extract of the water insoluble glucan gives a large number of protein bands on 6% SDS-PAGE. The results of this study suggest that the glucosyltransferases are glycoproteins and can occur in multimolecular forms (Ciardi et al., 1976).

Russell (1979) in his work on identifying and purifying the enzyme responsible for the production of mutans from *Streptococcus mutans* strain Ingbritt, found that glycosyltransferase enzymes which synthesize polymers from sucrose may:

- exist in multiform according to their difference in charge, molecular size or antigenic structure.

- have a marked tendency to form high molecular weight aggregates with the result that most of the activity fails to enter even low concentration gels.

Although the SDS-PAGE separation gave four proteins bands, only the 200 kDa enzyme associated band was separated by gel filtration and assayed for enzyme activity. The study of α-amylase activity was not undertaken in this paper (Russell, 1979).

Mukassa in 1989 has shown, with the same strain of *Streptococcus mutans*, that most glucosyltransferase (GTF) synthesizing insoluble glucan was cell-associated and probably may contribute to their colonization of the human tooth surface. The enzyme was extracted with SDS and purified by using a (1-3)α-D-glucan gel column. Although the amount of GTF-I activity in cell associated and supernatant fractions varied greatly, the total activity however remained constant and the molecular weight of both surface and extracelluar GTF-I were similar, suggesting that the enzymes are identical (Mukassa et al., 1989).

2) Starch Metabolism

Boyer (1971), in his work with *Streptococcus equinus 1091* noticed that the extracelluar α-amylase (EC 3.2.1.1.) and transglycosylase had an hydrolysing activity on amylose. The transglycosylase may synthesize large maltodextrins in the culture medium in the presence of maltose whereas α-amylase produces maltose, maltotriose maltotetraose and glucose at the early stage of amylose hydrolysis. The activity assay was undertaken by measuring the colour reduction of both starch blue value in 10 min at pH 7 and 37°C for transglycosylase and amylose blue value in the presence of glucose (Boyer and Hartman, 1971).

The extracelluar proteins from *Streptococcus sanguis 1 MC 204* and *Streptococcus mitis MC 101* have high glycosidic hydrolysing activity when incubated with amylopectin; high synthetic activity resulting in amyloglican production when amylopectin was present (Coogan, 1990 and Coogan et al., 1992). The endo-hydrolase from *Streptococcus sanguis* culture filtrate was separated on SDS-PAGE containing amylopectin and shown to have a clearance activity at the region of 120 KDa. Meanwhile the glycosyltransferase activities appeared at the region of 150 KDa (Coogan, 1990). Further work by Coogan (1991) on a starch degrading enzyme and structural studies on the polymer produced from starch and its derivatives showed that the amyloglucan as well as the soluble glucan originated from enzyme activity (Coogan, 1991 and Wolinsky, 1988). Later studies suggested that the large polysaccharide can be formed from the smaller oligosaccharides, provided by the first endo-hydrolase reaction of α-amylase (Coogan et al., 1994).

Many workers were not successful in characterizing these enzymes and elucidating pathways because they found they had difficulty in obtaining a pure enzyme extract which was highly active. The finding that a polysaccharide amyloglucan which is synthesized by *Mutans streptococci* sticks to glass surfaces, was seen as a means to elucidate metabolic pathways and to gain an understanding of enzymatic activity. This led to structural studies on amyloglucan and soluble glucan as well as research into the degradation of starch and its derivatives. This information was considered valuable in understanding caries initiation and the adherence of bacterial cells to the tooth surface (Manners, 1985; Coogan, 1990; Coogan et al., 1991; 1992 and 1994).

Although some specificity has been detected in enzyme activity, the mechanism of carbohydrate hydrolysis on the one hand and the pathway of amyloglucan synthesis by *Mutans*

streptococci on the other remains to be elucidated. The isolation and the characterisation of each enzyme taking part in these enzymatic activities may lead to the understanding of the metabolic pathways, of cell adherence to the tooth surface as well as the cariogenicity of the *Mutans streptococci*.

III. THE PRESENT STUDY

A- SEPARATION AND PURIFICATION OF EXTRACELLUAR ENZYMES

Studies undertaken on the purification of α-amylases from other *Streptococcal* strains or on enzyme activity indicates that the enzyme has a great affinity for its substrate (Huaru Li, 1992; Barry, 1980; El Hassan, 1992). Mizokami (1988) purified in one step α-amylase from an extracelluar crude protein extract of *Streptococcus bovis* by using its affinity for soluble and raw starch (Mizokami, 1988). Most of the chromogenic substrate, used for carbohydrate metabolizing enzyme purification methods or for quantitative activity assay of endo-hydrolase, exploit the adsorption principle of the enzyme to the substrate (De Cordts, 1993; McCleary, 1980; Mizokami, 1988; Rinderknecht, 1967; Barry, 1980).

Many substrate that were developed were prepared by chemically modifying the carbohydrate structure before treatment with a dye. This modification opens the macrostructure and increases the solubility of the polysaccharide.

A chemically modified amylopectin without dye can be prepared similar to the chromogenic substrate for the assay of α-amylase, by treating amylopectin with ammonium sulphate. This can be used for the isolation of a starch metabolizing enzyme produced from *Streptococcus sanguis 1 MC 204* on a chemical defined media (Janda and Kuramitsu, 1976).

B- CHARACTERIZATION OF THE ENZYME

The purified enzyme extracts were tested for the presence of protein and enzyme activity. In addition the molecular weight was determinate by gel filtration chromatography and SDS-Polyacrylamide Gel Electrophoresis.

Research on bacterial α-amylases and on glycoproteins leads to anomalous behaviour of these

biomolecules on SDS-PAGE methods and that were encountered during the protein assay, SDS-PAGE was extended further by using methods for glycoprotein detection.

For example, *Bacillus subtilis* α-amylase, which had a molecular weight of 48 kDa by ultracentrifugation or gel electrophoresis yielded an apparent molecular weight over 150 kDa with SDS-PAGE. This behaviour was associated with the lesser capacity of the enzyme to bind Dodecyl Sulphate (Mitchell et al., 1973). Furthermore the enzyme, produced by *Bacillus subtilis NA-64*, has shown to contain about 7% glucosamine and about 2% neutral carbohydrate (Yamane et al., 1973).

Because of its behaviour on polyacrylamide gel electrophoresis in the presence of dodecyl sulphate, *Aspergillus orysae* α-amylase was chosen to study the ability of dodecyl sulphate to denature proteins (Kubo and Takagi , 1995).

A positive exploitation of this behaviour was undertaken by Pszkeiwics (1995) when he used sodium dodecyl sulphate-polyacrylamide gel electrophoresis as preparative method to improve the purity of porcine gastric mucin.

Much research shows anomalous behaviour of bacterial α-amylases and glycoproteins when purification was undertaken or when molecular weight determination were performed using Sodium Dodecyl Sulphate PolyAcrylamide Gel Electrophoresis. This indicated that the behaviour was partly associated with the reduced capacity of the enzyme to bind or to resist dodecyl sulphate attack (Mitchell et al., 1973; Kubo et al., 1995). Others factors include the large size of the molecule, the proportion of sialic acid residues and the hydrodynamic behaviour of the glycoprotein-SDS complex (Paszkiewicz- Gadek et al., 1995; Møller et al.,

1993; Strayer et al., 1980; Segrest et al., 1971). The glycosylated proteins charges and shape may also interfere with the formation of the dye complex and yield the possible explanation of poor binding of Coomassie blue or others dye used in classic protein staining methods (Moller, 1993 and 1995).

In addition, work on glucosyltransferase from *Streptococcus mutans* has shown that the enzyme is a glycoprotein. The molecular weight of major protein bands decreased with an increase in the gel concentration of acrylamide because carbohydrates in the enzyme preparation are not tightly associated or covalently linked to the protein (Ciardi at al., 1976; Pazur, 1972; Segrest et al., 1971). Although this hypothesis cannot explain fully all the anomalous behaviour encountered in molecular weight determination by SDS-PAGE, it gives some explanation of the multiple protein bands seen on SDS-PAGE. However, the hydrodynamic behaviour of the glycopeptide-SDS complex could not be correlated with the amount of carbohydrate in the glycoprotein (Strayer et al., 1980).

C- THE AIM

The aim of this study was:

1- to isolate an amylopectin hydrolysing enzyme, from a crude extract of extracellular proteins, by complexing the enzyme with a chemically modified amylopectin substrate.

2- to purify and to characterise this enzyme by gel filtration chromatography and sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

CHAPTER II

MATERIALS AND METHODS

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CHAPTER II

MATERIALS AND METHODS

A) MATERIALS

- A crude extracelluar protein extract from culture media of *Streptococcus sanguis* Type 1 strain MC 204 was used as source of the unknown protein.
- A chemically modified medium (Janda, 1976) was used as culture medium with a change in casein content and vitamin composition. (Casein hydrolysate content was reduced to 3% and vitamins were replaced by 0.1% yeast extract).
- A pure grade corn amylopectin was used for a chemically modified amylopectin preparation (Sigma).
- Dye labelled amylopectin (Sigma) was used as chromogenic substrate for the assay of α-amylase activity. α-Amylase from *Bacillus subtilis* (Boehringer)
- Pure bovine serum albumin fraction V and phosphorylase b from rabbit muscle and blue dextran were used as protein markers for SDS-PAGE and gel permeation chromatography.
- Purified gastric Mucin from porcine stomach (BDH) and heparin solution 5000 Unit/ml (Flow Laboratories) were used as glycoproteins standards.
- UV-spectrophotometer Shimadzu 160 was used for protein evaluation at 280 nm and protein scanning between 200 and 320 nm.

- Sephadex G-25, Sephadex G-75 and Ultrogel AcA 34 were used as separating materials for gel filtration chromatography.

B) METHODS

1-CHEMICALLY DEFINED MEDIUM

Salts and bases: 3 g ammonium sulphate; 0,15 g adenine; 0,05 g ferrous sulphate; 0,1 g guanine; 0,05 g manganous sulphate; 1,125g trisodium citrate; 30,0 g sodium acetate; 0,05 g sodium chloride; 15,75 g disodium hydrogen phosphate; 10,25 g sodium hydrogen phosphate; 0,15 g uracil; 2,2 g potassium dihydrogen phosphate; 1,5 g dipotassium hydrogen phosphate; 15,0 g casein hydrolysate; 5 g yeast extract were dissolved in 5000 ml distilled water and autoclaved at 115°C for 20 min.

Final medium: In 1000 ml of salts and bases solution add 10 ml 50% glucose; 1 ml 20% magnesium sulphate; 0,25 g of cysteine HCl and 2,2 g of sodium carbonate.

The glucose and the magnesium sulphate were prepared separately and autoclaved at 115°C for 20 min.

The cysteine HCl and sodium carbonate were fresh prepared respectively before use and mixed under sterile conditions. They were dissolved in small quantity of distilled water and sterilized by passing through a filter membrane with a pore size of 0,2 micron.

Note: The modified chemical defined medium is similar to the chemical defined medium of

Janda and Karamitsu (1976) however, the vitamin solution is replaced by yeast extract (0.1%) and the casein content is reduced to 3 g/l.

2-PREPARATION OF MODIFIED AMYLOPECTIN

Amylopectin is one of the main components of starch. Amylose and amylopectin coexist in starch granules. Research undertaken into amylose and amylopectin about their structures remain complex and has made the study the properties of the starch difficult. In spite of this, amylose has been shown to be a long unbranched chain of (1-4)α-d-glucopyranose unit which is rapidly and extensively degraded by both α- and ß- amylase.

The fine structure of amylose has been shown to be helical structure with each turn containing six glucose units. The helical structure are characterised by a hydrogen bond between the C_2 hydroxyl groups of one α-D-glucopyranosyl unit and the hydroxyl at C_3 of the following sugar unit into a linear amylose structure (Hanes, 1937; Sarko and Marchessault, 1967 and Coogan, 1990).

On the other hand amylopectin has been shown to be a highly branched carbohydrate molecule which contains chains of about 27 glucose units each terminated by a non-reducing glucose unit. The investigation of overall molecular structure of amylopectin proposes several models. The results of partial debranching of amylopectin with pullulanase suggest that a Cluster model was a more accurate representation of the macromolecule (French, 1973 and 1984; Maners, 1985a and Coogan, 1990).

Referring to the Cluster model of the amylopectin macromolecule (Manners, 1985), a chemically modified amylopectin could have a slightly opened branch structure. The opened

structure may provide both the binding sites of the enzyme on the substrate and allow a number of enzymes to act at the same time.

A proposed structure may be as below (Fig 1)

The preparation of chemically modified amylopectin was obtained by treating the crude amylopectin with 10% ammonium sulphate, 5% Tri- base at 55°C and washing several times with distilled water and alcohol before drying.

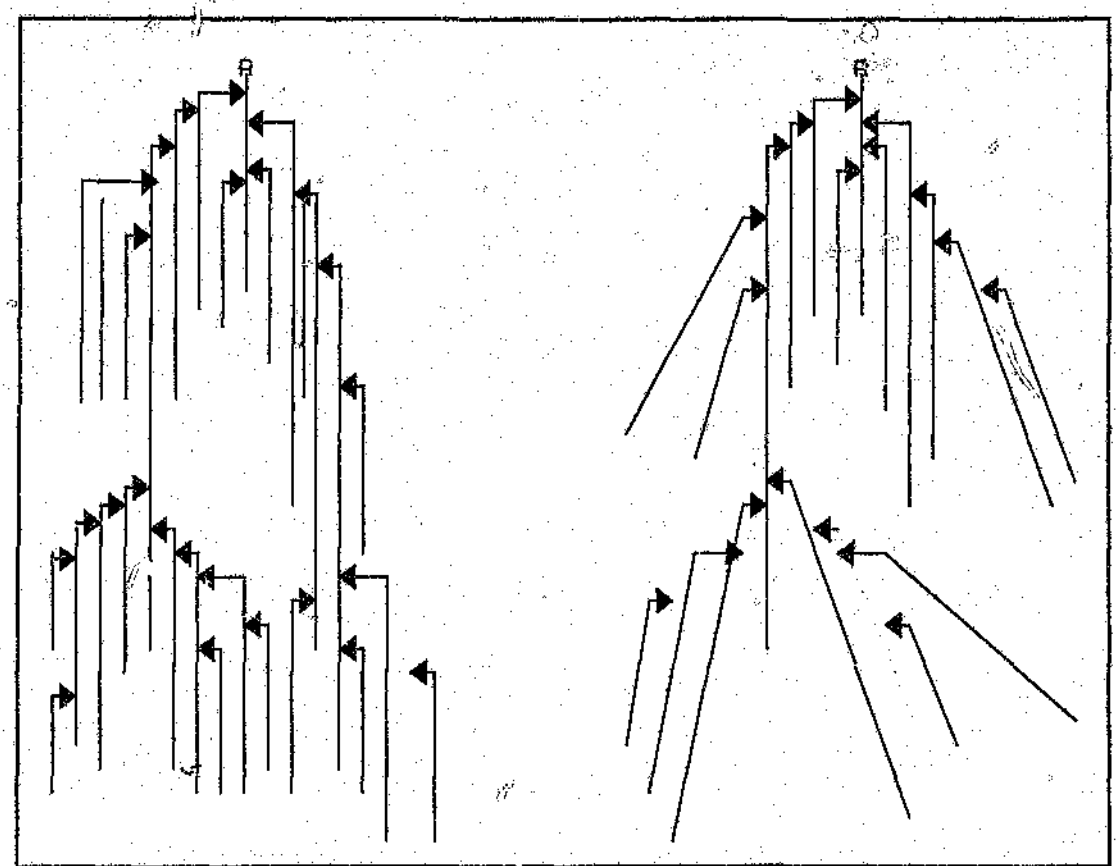


Figure 1

Figure 1: Structures of normal (Cluster model) and modified amylopectin. The modified amylopectin shows a structure opening between the chains after a chemical treatment of the normal amylopectin

Protocol:

1-Weigh 15 g of amylopectin and suspend it in 100 ml of distilled water.

2-Stir at 50°C for 45 minutes

3-Cool and slowly add 10% ammonium sulphate and stir to complete dissolution.

4-Add 5% of solid Tris to bring pH to 9 and stir for 30 more minutes.

5-Centrifuge at 3000 g for 10 minutes and discard the supernatant.

6-Wash the pellet twice with 100 ml of a mixture of water-ethanol(1:1) to precipitate partially gelatinized amylopectin.

7-Wash it again with 96% ethanol and dry it in vacuum desiccator.

Note:
- A small portion of alcohol in the amylopectin suspension leads to the gel at 55°C even if ammonium sulphate is omitted.
- If the temperature is not maintained at 50-55°C, the gelatinization process of amylopectin starts immediately at the temperature over 55°C, with ammonium sulphate.
- The drying temperature can gelatinize the modified amylopectin if over 30°C.

3-INOCULATION AND INCUBATION

a) Inoculum preparation

A blood agar plate was inoculated with a pure culture and incubated for 24 hours at 37°C under CO_2. The colonies were harvested from the plate and placed in 2 ml sterile PBS (pH 7,2). One percent of this suspension was used to inoculate the culture broths.

b) Culturing

Two hundred ml of final medium was poured into a 500 ml bottle, inoculated with *Streptococci* and placed into candle jar for incubation under CO_2. The culture was incubated under CO_2 for 24 hours at 37°C and aerobically for further 48 hours. After the incubation cultures were shaken vigorously, poured into 250 ml centrifuge tube and centrifuged at 12000 g for 30 minutes. Supernatants were collected, placed in a dialysis tube and concentrated against polyethylene-glycol (60% volume concentration). A small aliquot of this concentrated cultured medium was retained for analysis. Half of the concentrated medium was stored at 4°C for enzyme isolation. The remainder was precipitated with 60% ammonium sulphate and centrifuged at 14000 g for 30 minutes. The pellet was resuspended in Tris-HCl buffer and stored at 4°C for enzyme isolation.

4) ENZYME ISOLATION

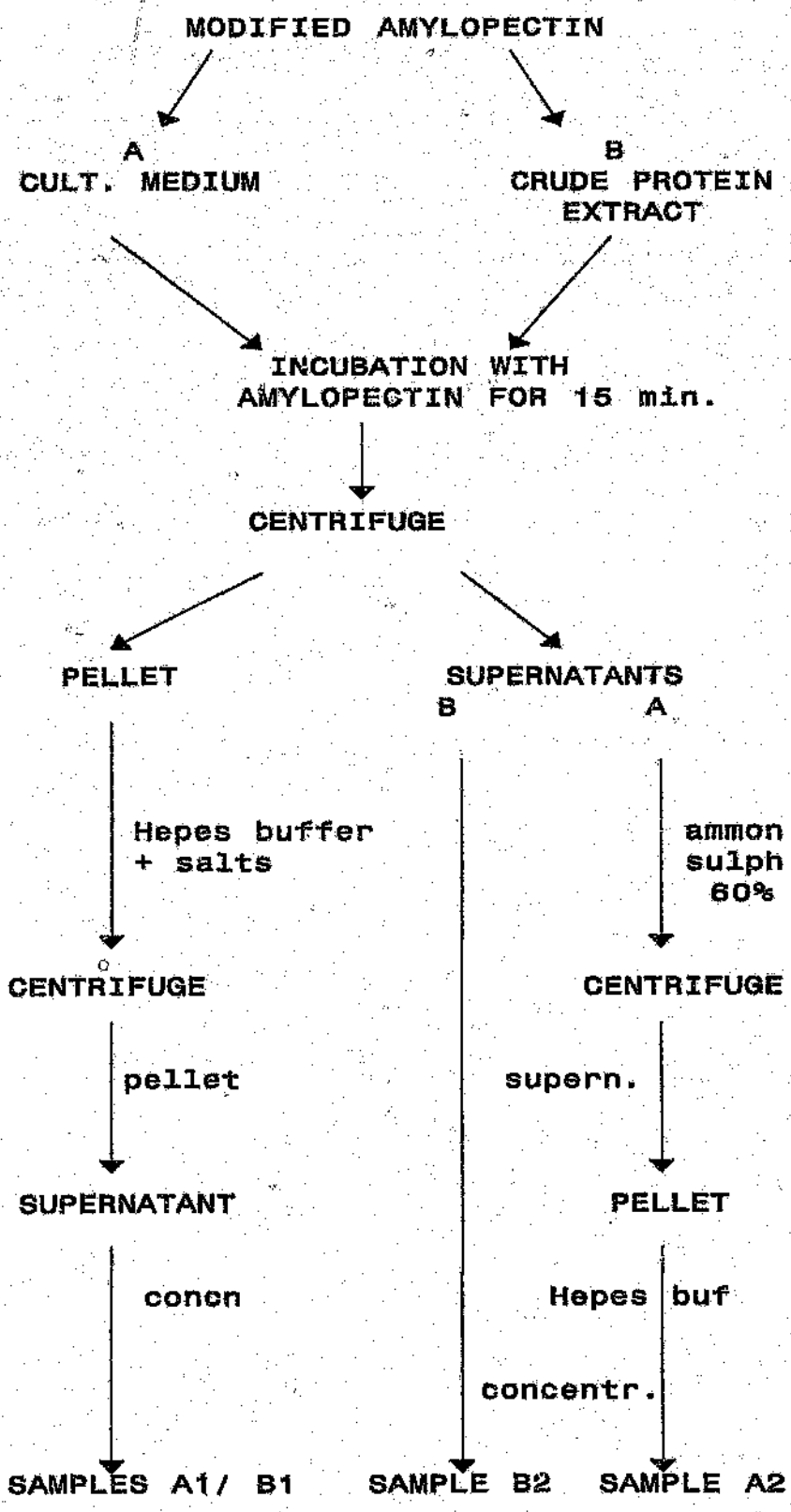
a) ISOLATION

1- A crude enzyme extract was added to 1% modified amylopectin; the mixture was incubated for 30 minutes at 37°C and centrifuged at 3000 g for 10 minutes.

2- The supernatant was removed and used for the next extraction step. The pellet was resuspended in hepes buffer pH 6 containing 0,02 M KCl. A 0.2% SDS Tris-HCl buffer to release the enzyme by denaturing.

3- The amylopectin was removed by centrifugation and the supernatant was concentrated against polyethylene-glycol, precipitated with 60% ammonium sulphate, desalted and tested for enzyme activity and the presence of protein.

There may be an interaction between SDS and modified amylopectin because the enzyme-substrate complex remained in suspension and reduced the possibility of separation by centrifugation. The enzyme extraction continued as below (see page 35).


MODIFIED AMYLOPECTIN
A
CULT. MEDIUM
B
CRUDE PROTEIN EXTRACT
INCUBATION WITH AMYLOPECTIN FOR 15 min.
CENTRIFUGE
PELLET
SUPERNATANTS
B
A
Hepes buffer + salts
ammon sulph 60%
CENTRIFUGE
CENTRIFUGE
pellet
supern.
SUPERNATANT
PELLET
concn
Hepes buf
concentr.
SAMPLES A1/ B1
SAMPLE B2
SAMPLE A2

b) PROTEIN PRESENCE AND ENZYME ASSAY

- Protein was determining by Lowry and UV-ultraviolet methods.
- Three analytical methods were selected for the evaluation of the enzyme activity of the samples. Iodine staining substrate complex and methods measuring the reducing end sugar of Somogoyi and Bernfeld (Street, 1989).

1- Measurement of amylase activity by starch-Iodine complex method.

A) Substrate solution (1%)

Weight 2.5 g amylopectin

Add 5 ml of ethanol and stir

Add 8 ml of 10% NaOH and stand for 30 minutes

Add 20 ml of distilled water

Add 6.76 g of $CaCl_2$ and stir for 1 hour

Stand for 10 minutes and stir and add buffer gradually until a complete dissolution of the amylopectin

Adjust the pH with HCl and bring the volume to 250 ml.

Autoclave and keep it at 37°C.

B) Iodine solution stock

Weight 2 g KI and dissolve in 100 ml of distilled water;

add 0.9 g of Iodine

Stir at the room temperature till complete dissolution and add H_2SO_4 5 mM to make up to 1 litre. Keep the solution in a dark bottle.

C) Hydrolysing enzyme assay

Prepare numbered test tubes

Put 1 ml of amylopectin substrate into each tube;

add 50 microlitre enzyme solution to each tube sequentially and vortex.

Incubate these tubes at 37°C overnight.

Thereafter prepare new numbered test tubes;

Dil Iodine solution stock (20 fold).

Pour 5 ml of this diluted Iodine solution in each tube and add 0.1 ml of the digest sample to each tubes and vortex.

Wait for 15 minutes and read the absorbency at 650 nm.

The activity of the enzyme was calculated from the formula:

Units of amylase= n (Do-Ds)/Do

Where Do: Absorbency of the substrate-Iodine complex in the absence of the enzyme.

Ds: Absorbency of the digest sample

n: dilution factor of the enzyme.

Note: For a rapid enzyme assay the incubation time was reduced to 15 minutes and the assay was carried as below:

After 15 minutes incubation of the digest sample, remove 100 µl of the digested mixture and add it to the Iodine solution and vortex. Wait for 15 minutes and read the absorbency at 534 nm.

If the enzyme concentration was low, 250 µl of enzyme solution was used for the digestion.

Volume activity = $[(A_o - A)/A_o] * K * 1/t * v * n$

Where A_o Absorbency of the substrate-Iodine complex in the absence of the enzyme.

A Absorbency of the digest sample

t incubation time of the digest mixture

v total digest volume

n dilution factor of the enzyme.

K = m substrate/MW glucose * 0.1 ml digest used/v

= 6.16 µmole

2- NELSON SOMOGOYI METHOD (Street, 1989)

Copper reagents:

reagent A: 5 g anhydrous sodium carbonate, 5 g sodium potassium tartrate, 4 g sodium bicarbonate and 40 g anhydrous sodium sulphate dissolved in 200 ml distilled water.

reagent B: dissolve 6 g cupric sulphate pentahydrate in 40 ml of acidulated water (one drop H_2SO_4).

reagent C: dissolve 5 g ammonium molybdate in 90 ml distilled water to which 4 ml of sulphuric acid was added; dissolve 0.6 g of sodium arsenate heptahydrate separately in 5 ml of water. This was added slowly to the above solution with constant stirring. The whole was made up to 100 ml and warmed carefully for 30 minutes in a water bath at 55°C.

Reagent D: mix 1 ml reagent B with 25 ml of reagent A.

Assay procedure

To 0.25 ml of the substrate add 0.25 ml of the enzyme solution and incubate for 10 minutes at 37°C. Add 0.5 ml of a fresh reagent D, boil it for 10 minutes and cool in cold running water. Add 1 ml of reagent C and shake until CO_2 is no longer evolved. Wait 10 minutes and add 3 ml of water. Read the absorbency at 520 nm.

A blank was made up by using 0.25 ml of the substrate and 0.25 ml of water. Read against a standard curve for calibration.

Units of amylase (U) = micromole of maltose/10 min.

3- BERNFELD METHOD (Street, 1989)

Measure of sugar liberated during the amylopectin hydrolysis

amylopectin + n H2O → (n + 1) - X) fragment + X maltose

1) Buffer substrate solution (1% amylopectin solution)

To 1 g amylopectin add 2 ml ethanol; 4 ml 10% NaOH and stand for 30 min. Add 20 ml of buffer and stir to complete dissolution, adjust the pH at 7.2 with HCl 10% and bring the volume to 100 ml. This solution must be prepared fresh or prepared and autoclaved and kept at 37°C.

2) Standard solution (7 µmole maltose/ ml)

Weight 63,06 mg of maltose and dissolve in 25 ml distilled water and filter it with 0.2 µm filter.

3) Colour reagent

Weight 1 g 3,5-dinitrosalicylic acid, 30 g sodium potassium tartrate and dissolve in 100 ml 1 M NaOH solution

Enzyme assay

Put 0,25 ml substrate in each tube and incubated at 37°C for 3 minutes.

Add 0.25 ml of enzyme (serially) and incubate for 15 minutes.

Add 0,5 ml of colour reagent to all test tubes and mix.

Place it in the boiling water bath for 5 min more, cool and add 5 ml distilled water. Read the absorbency at 540 nm against blank.

Unit of enzyme activity = 1 µmole maltose/min at 25°C

4- ENZYME ASSAY WITH CHROMOGENIC SUBSTRATE (Wahlefeld, 1989).

Assay procedure:

A- Make up 1 litre of phosphate buffer 0.02 M pH 7.0

solution A: 3.58 g Na_2HPO_4. 12 H_2O in 500 ml of water

solution B: 1.38 g NaH_2PO_4. H_2O in 500 ml of water

mix 39 ml of A with 61 ml of B and add 20 mg of NaN_3 and 0.292 g of NaCl;

B- Prepare fresh 1% of amylopectin dye labelled with buffer solution;

C- Prepare 1.2 M sodium hydroxide;

D- Dissolve 1 mg of pure α- amylase in 10 ml of buffer (solution A); dilute 1: 100 with the same buffer before use.

Act= 1 µg/ml = 18 U/ml

Activity assay

Pipette 2 ml of buffered substrate (in triplicate) into test tube; add 0.1 ml of sample or standard enzyme into two tubes and 0.1 ml of distilled water to the last tube.

Mix and incubate for 10 minutes at 37°C and add 0.5 ml of NaOH.

Scan between 620 - 570 nm and take the maximum reading (ABS at 595 nm).

Act = (ABS sample/ABS standard)* 18 U/ml

-Total carbohydrate determination (Dubois, 1956).

-Qualitative determination of RNA (orcinol method) and DNA (Diphenylalamine method) (Robyt, 1984).

5) CHROMATOGRAPHY

Because of their complementarity Gel filtration and Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis were selected in this study.

a) <u>GEL FILTRATION</u>:

Gel filtration is placed at the top of the techniques used in the purification of biomolecules because it protects biological and enzymatic activities of proteins.

The discovery of new gel beads makes the method acceptable for protein separation and for molecular weight determination of a large range of molecular sizes (Scopes, 1988; Wolf, 1969).

-Gel filtration chromatography columns were performed with Sephadex G-25 (1.1 x 30 ml) and Sephadex G-75 (1.1 x 70 ml) according to the method described by Stenesh (Stenesh, 1984), and Ultrogel AcA 34 (1.1 x 30 ml). Samples was eluted with 0.05 M Tris-HCl buffer

pH 7,5 containing 0,15 M KCl.

Before column packing, the Sephadex G-75 (G-25) was suspended in 0.05 M Tris-HCl buffer pH 7.5 was degassed using water aspirator for one hour. An other column was packed with Sephadex G-75 which was washed with repel silane first. The repel silane was used under a hood, the gel was slurred with 0.05 M Tris-HCl buffer and degassed at the room temperature for two hours. The filling of the column was undertaken through plastic tubing. A third of the column was filled with buffer containing 0.15 M KCl and the gel was delivered continuously into the column and the excess was eliminated by overflow. The delivery head of the tube was under the buffer surface during the process to avoid trapping air bubbles. The packed column was equilibrated for 24 hours by eluting with Tris-HCl buffer pH 7.5 containing 0.15 M KCl in the cold room.

Proteins markers and blue dextran solution were mixed with equal volume buffer. The crude enzyme extract was not mixed with the buffer because of there being a high concentration of salts in the concentrated culture medium and the ammonium sulphate precipitated samples. A feeding tank was used for the addition of buffer. The position of this tank was not modified because a change in the hydrostatic pressure may affect the column.

Samples of 1.5 ml of the elute were collected automatically using a fraction collector at 4°C. The absorbency of fraction was read at 280 nm in UV-Spectrophotometer.

b) SODIUM DODECYL SULPHATE-POLYACRYLAMIDE GEL ELECTROPHORESIS(SDS-PAGE):

Many researchers consider SDS-PAGE as an important and powerful tool for high-resolution protein separation method. It facilitates the study of complex mixtures of proteins as well as inter- and intra- specific proteins homologies, physiological roles and post-translational modifications of polypeptides and glycosylated proteins (Prussak et al., 1989; Allore et al., 1984; Sandri et al., 1993 and Paszkeiwics et al., 1995). Recent improvement in the gel staining method also optimizes the detection of proteins as well as proteoglucans and glycoaminoglucan using Coomassie blue (Møller et al., 1993 and 1995; Fernandez-Patron et al., 1995). This last improvement makes SDS-PAGE highly sensitive and selective.

- SDS-Polyacrylamide Gel Electrophoresis was performed according to Laemmli one dimensional SDS-PAGE method (Laemmli, 1970);
- The modified Russell method (Russell, 1976 and 1979) and gradient gel of 5-25% method (Garfin, 1990) for proteins, enzyme hydrolysing activity and protein molecular weight determination was also used.

The modified Gel electrophoresis method to detect enzymes that modify amylopectin (Russell, 1976 and 1979) used the method of Laemmli with the incorporation of 0.5% amylopectin in the gels. Gradient gel of 5 to 25% was performed in a plate gel apparatus and the separating gel has a total volume of 25 ml.

Experimental information

Solution stock of:

- Acrylamide and Bisacrylamide were prepared by dissolving 60 g Acrylamide and 1.6 g bisacrylamide in 100 ml distilled water.
- 1.5 M Tris-HCl buffer was prepared by dissolving 45 g Tris in 200 ml and pH 8.8 was adjusted with 2 M HCl solution.
- 10% Sodium Dodecyl Sulphate (10 g in 100 ml distilled water).
- 10% ammonium persulphate was prepared freshly before the gel preparation.

A- Preparation of the gels:

1) SDS-PAGE containing amylopectin

- Weight 125 mg amylopectin in a Buckner flask, add 10 ml distilled water and boil it while stirring with a magnetic stirrer until complete dissolution.
- After cooling, add 3 ml of Acrylamide and Bisacrylamide solution, 9 ml Tris-HCl pH 8.8 and 2.5 ml distilled water.
- Degas and then add 250 µl 10% SDS; 10 µl Temed and 100 µl 10% ammonium persulphate.
- Pour the mixture between the glass plates prepared according to Laemmli (1970) and overlay it with water (0.5 cm).
- Allow the gel to polymerise and drain off the water.
- Pour 4 ml Stacking gel solution prepared by mixing 4 ml SDS stacking solution (Laemmli, 1970) with 20 µl 10% ammonium persulphate.
- Insert the sample comb, overlay with water and allow the gel to polymerise.

The gel was prepared one day before use and kept in the cold room.

2) SDS-PAGE gradient gel

Two mixtures were made up for separating gel preparation:

"Light" mixture(5% T): mix 0.97 ml Acrylamide-Bisacrylamide solution; 4.5 ml Tris-HCl buffer; 5 ml 0.25% amylopectin solution and 3 ml distilled water.

"Heavy" mixture(25% T): mix 4.87 ml Acrylamide-Bisacrylamide solution; 4.5 ml Tris-HCl buffer; 3 ml 0.25% amylopectin solution.

Degas these mixtures, pipette the "heavy" mixture into the rear chamber and the "light" into the front chamber of the gradient mixing vessel. Under continuous stirring, add 120 µl 10% Sodium Dodecyl Sulphate, 10 µl Temed in both chambers and 100 µl 10% ammonium persulphate in the front chamber and 50 µl 10% ammonium persulphate into the rear chamber. Open the link between the two chambers and let the mixture flows gravitationally between the gel plates. After 2 minutes overlay the gel with 0,5 ml water and allow it to polymerise. If the gel was made up without amylopectin, the proportion of amylopectin was replaced by water in the two mixtures. The stacking gel is made up according to Laemmli (1970).

B- Preparation of the samples:

In an effort to improve the analysis with SDS-PAGE normal samples were prepared using generally accepted methods as well as concentrated samples using TriChloroacetic, phenol-ether and polyethylene-glycol.

Normal samples

Proteins markers were prepared by mixing 5 µl of each of the protein solution with 30 µl of the sample buffer. The remaining proteins samples of 25 and 30 µl were mixed with 25 and 20 µl of the sample buffer respectively.

Sample for detecting GTF activity were incubated at 45°C water bath for 2 hours before the loading of the gel. Other protein samples were heated to 95 - 100°C for 5 minutes and cooled to room temperature before loading.

Concentrated samples

1- The TriChloroacetic concentrate samples were prepared as below:

1 ml sample are precipitated with 10% TCA in a 1.5 ml microfuge tube, centrifuged at 5000 g and the supernatant was discarded. The pellet was dissolved into 25 µl sample buffer, neutralised 0.1 M NaOH, heated to 95 - 100°C for 5 minutes and cooled to room temperature before loading.

2- The phenol ether concentration was performed by following the procedure below:

- mix 0.5 ml water-saturate phenol with 1 ml protein sample, vortex for 20 second at maximum speed, centrifuge at 12000 g for 5 minutes and discard the upper phase;
- add 1 ml ether to the phenol phase, vortex and centrifuge as above;
- discard the upper phase and repeat this step;
- dry the lower aqueous phase by centrifugation under reduced pressure.

The dried samples were dissolved in 30 µl sample buffer, heated at 95 - 100°C for 5 minutes and cooled to room temperature before loading (Sauvé, 1995).

3- The polyethylene-glycol concentrated sample were placed in a dialysis tube and concentrated against polyethylene-glycol.

C- Electrophoresis

Electrophoresis was carried out at room temperature at 150 volt (+/- 20 mA) for 4 hours. To detect enzyme modifying amylopectin, the gels were washed extensively by shaking in 0.05 M Tris-HCl pH 7.5 and then incubated for 1 day at 37°C in 0.05 M phosphate buffer pH 6,5 containing 0.5% amylopectin, 2.5% sucrose, 1% Triton X-100, 0.01% Dextran T10 and 1% Nystatin to prevent the growth of microorganisms.

D- Staining procedures

Immediately after electrophoresis, the gels were fixed in 75% ethanol (v/v) for 30 minutes or immerse for 2 hour in Coomassie brilliant blue G-250 solution, and transferred to destaining solution.

I) The fixed gel was stained with Periodic acid-Schiff stain as described below:

- immerse the gel for 1 hour in 0.7% periodic acid, 5% acetic acid solution;
- replace this solution by 0.2% sodium metabisulfite, 5% acetic acid solution and shake for 1 hour (several changes).
- place the gel in 5% dilute solution of Schiff reagent for 30 seconde and stop the reaction with 0.5% sodium metabisulfite solution consisting of a mixture of acetic acid: ethanol: water (10:45:45).

The gel stained pink and clear zones indicated enzyme hydrolysing activity.

2) For silver staining, the fixed gel was oxidized with periodic acid and sodium metabisulphite as above and

* wash twice in distilled water
* transfer to $AgNO_3$ (1 g/l), 1,5 ml 37% HCOH/litre solution for 30 minutes
* rinse with distilled water
* develop with Na_2CO_3 (30 g/l), 1,5 ml 37% HCOH/litre, $Na_2S_2O_3.5\ H_2O$ (2 mg/l) solution for 2 - 5 minutes
* stop the reaction with 10% acetic acid in water.

If glycoproteins are present, the intense protein bands appear in 2 minutes. When alcian blue is used, the gel was prestained with 0.125% alcian blue, 10% ethanol, 5% acetic acid in water for 15 minutes and washed three times with water before silver staining.

c) AGAROSE GEL

-Agarose gel was used for determination of RNA contamination.

The agarose gel was prepared as follows:

* Dissolve 0.3 g agarose in electrophoresis buffer to a total volume of 30 ml. Melt it in the microwave for about 3 minutes and ensure that it is completely melted. Check that the volume is still 30 ml and add hot water if necessary. Add ethidium bromide to a final concentration of 0.5 µg/ml.
* Allow the agarose to reach 55°C before pouring it onto the gel platform.
* Seal the gel platform at both ends, pour in the agarose and insert the comb ensuring that there are no air bubbles.
* After the gel has set remove the tape and withdraw the comb, taking care not

to tear the sample wells.

* Place the gel casting platform into the electrophoresis tank and add electrophoresis buffer to a depth of about 1 mm. Release any air that may become trapped in the wells.
* Load the RNA into the wells using a pipet. Do not overload the wells and avoid mixing between wells.
* Load molecular weight marker.

After running the electrophoresis the agarose gel was placed under UV light. The fluorescence of ethidium bromide indicated proteins or nucleic acids bands which can be photographed.

CHAPITER III

RESULTS

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CHAPITER III

RESULTS

I. SAMPLES DEFINITION

Cultures were grown and the following preparations studied:

So- Concentrated cultured medium

A- Cultured medium concentrated with polyethylene glycol after removal of cells.

B- Crude protein extract precipitated with 60 percent ammonium sulphate.

A1- Protein extracted with amylopectin from culture medium

A2- Protein remaining in culture medium after amylopectin extraction, precipitated with ammonium sulphate.

B1- Protein from crude protein extract, extracted with amylopectin.

B2- Protein remaining in crude protein extract after amylopectin extraction.

These extracts were added to modified amylopectin for enzyme extraction as shown in the diagram (see page 35).

II.RESULTS

1) ENZYME EXTRACTION

The evaluation of the presence of protein by Lowry method showed that 29% of enzyme were extracted from concentrated cultured medium (A) and 18% from crude protein extract precipitated with 60% ammonium sulphate (B) (front range Fig. 2). The test for enzyme activity of the above samples showed otherwise that 88.5% enzyme hydrolysing activity were recorded with A1 and 67% from B1 respectively (rear range Fig. 2).

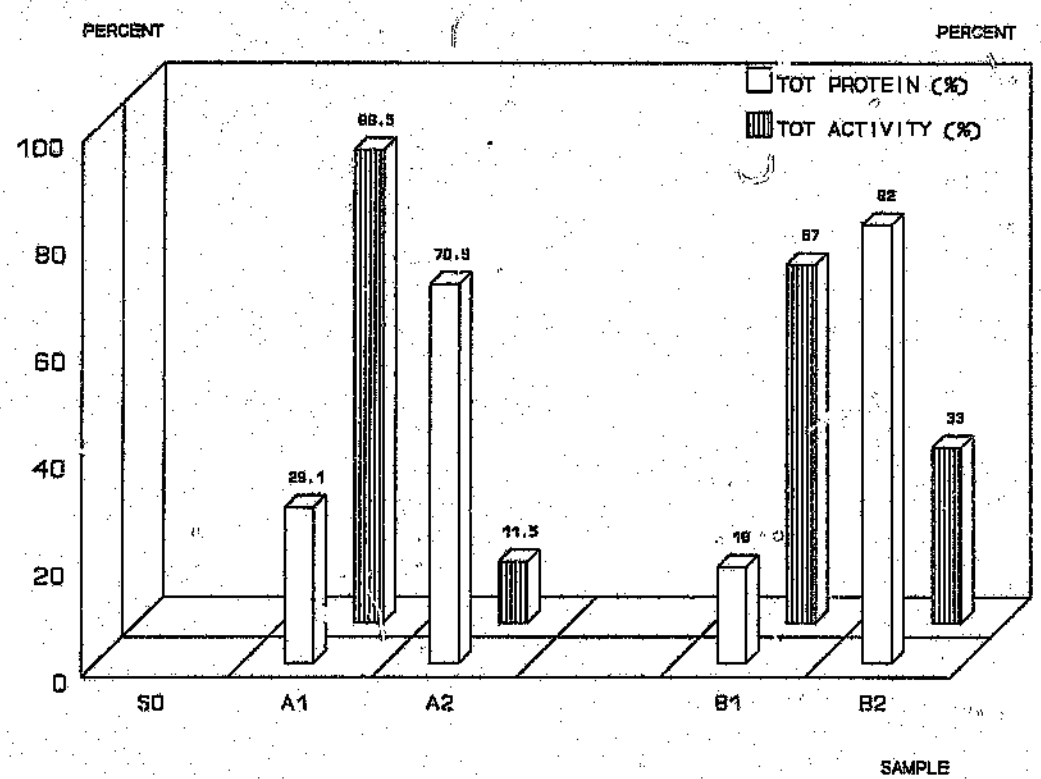

Figure 2: Protein determination (Lowry method) and activity assay (Somogoyi method). By considering that total protein of extract A and B correspond to the total amount of protein from A1 and A2, and from B1 and B2 respectively, the percentage of each fraction can be calculate by A1%= A1/(A1+A2). Ditto for the activity assay.

2- ACTIVITY ASSAY

Three analytical methods, widely used in α-amylase activity assay, were selected for the evaluation of the enzyme activity of the samples.

a) Iodine substrate complex staining method (Table 1)

143% and 76% of activity were recorded for samples A1 and B1 after overnight incubation at 37°C. A purification of 2.6 fold was recorded for A1 whereas B1 extract gave 1.2 fold.

Sample	Volume (ml)	Total protein mg	Volume activity (U/ml)	Total activity (U)	Specific activity (U/mg)	Yield	Fold purification
S0	20	54.20	12.02	240.45	4.44		
A1	70	30.16	4.90	343.06	11.4	1.43	2.57
A2	50	75.36	12.87	720.2	9.60	2.99	2.21
B1	25	35.09	7.28	181.87	5.20	0.76	1.17
B2	75	169.75	8.11	603.25	4.28	2.84	0.96

Table 1: All values are average of three experimental results. Volume activity= n(Do - Ds)/Do; Total activity= U/ml* volume; specific activity= tot. act/tot. protein; Yield= total activity fraction/total activity So; Fold purification= Spec. activity fraction/Spec. activity So.

b) Bremfeld method (Table 2)

The Bremfeld method gave a recovery of activity of 282% for A1 and 178% for B1 with purification of 5.2 fold for A1 and 1.9 for B1.

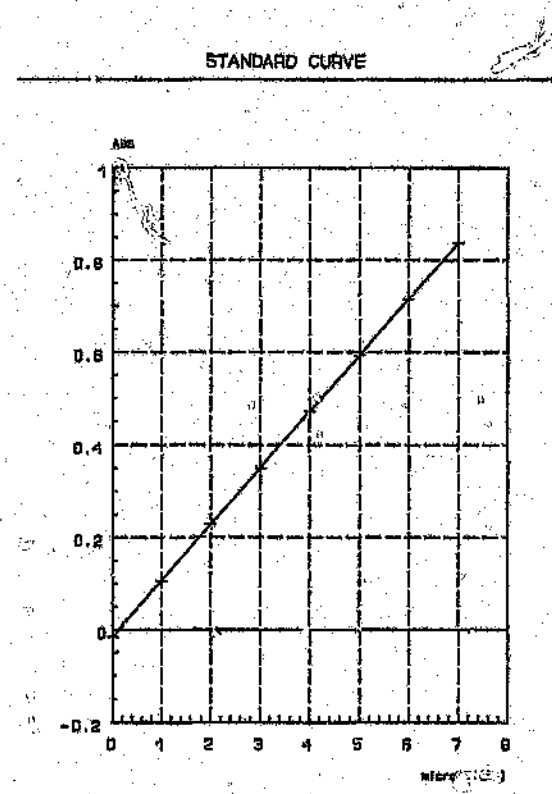

Figure 3: The standard curve was made by using 7 µmole/ml maltose. Average of three experimental values.

Sample	Volume (ml)	Total protein (mg)	Volume activity (U/ml)	Total activity (U)	Specific activity (U/mg)	Yield	Fold purification
So	20	54.2	0.036	0.720	0.013		
A1	70	30.1	0.029	2.03	0.067	2.82	5.15
A2	56	73.38	0.029	1.45	0.020	2.03	1.54
B1	25	35.0	0.051	1.28	0.037	1.78	2.85
B2	57	159.75	0.027	2.03	0.013	2.82	1.0

Table 2: All values are the average of three experimental results. Volume activity= standard curve reading/15; Total activity= U/ml* volume; specific activity= tot. act/tot. protein; Yield= total activity fraction/total activity So; Fold purification= Spec. activity fraction/Spec. activity So.

c) Nelson Somogoyi method (table 3)

The Somogoyi method gave a recovery of activity of 466% for A1 and 85% for B1 with 8.4 fold and 2.9 fold respectively (see table 3).

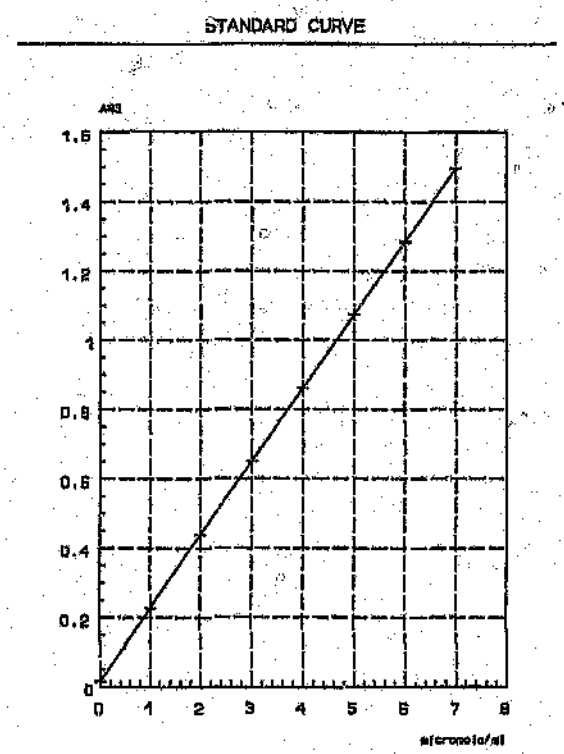

Figure 4: The standard curve was made by using 7 µmole/ml maltose. All values are the average of three experimental values.

Sample	volume (ml)	Tot prot. mg	Volume activity (U/ml)	Total activity (U)	Specific activity (U/mg)	Yield	Fold purification
So	20	54.2	0.13	20.60	0.048		
A1	70	30.1	0.173	12.11	0.402	4.68	8.38
A2	56	73.36	0.028	1.57	0.021	0.60	0.34
B1	25	35.0	0.088	2.20	0.063	0.85	1.31
B2	75	159.75	0.050	3.75	0.023	1.44	0.48

Table 3: All values are the average of three experimental results. Volume activity= standard curve reading/10; Total activity= U/ml* volume; specific activity= tot. act/tot. protein; Yield= total activity fraction/total activity So; Fold purification= Spec. activity fraction/Spec. activity So.

3) CHROMATOGRAPHY

A) ENZYME AMYLOPECTIN EXTRACTED

1) GEL FILTRATION

The ammonium sulphate concentrated protein (60% ammonium sulphate) gave two protein peaks when desalted on Sephadex G-25 column. The UV scan of these eluted fractions between 200 and 300 nm gave no specific protein absorption peak at 280 nm for the first eluted protein and a maximum absorption peak at 260 nm for the second eluted protein. Both peaks were confirmed by the Lowry method (result not shown).

To improve the sample purification, a column with Sephadex G-75 was set up and loaded with 2 ml of amylopectin enzyme extract (sample A1).

A sample of 1,5 ml was collected (0,2 ml/min) and the elution profile gave two peaks (see Fig. 5).

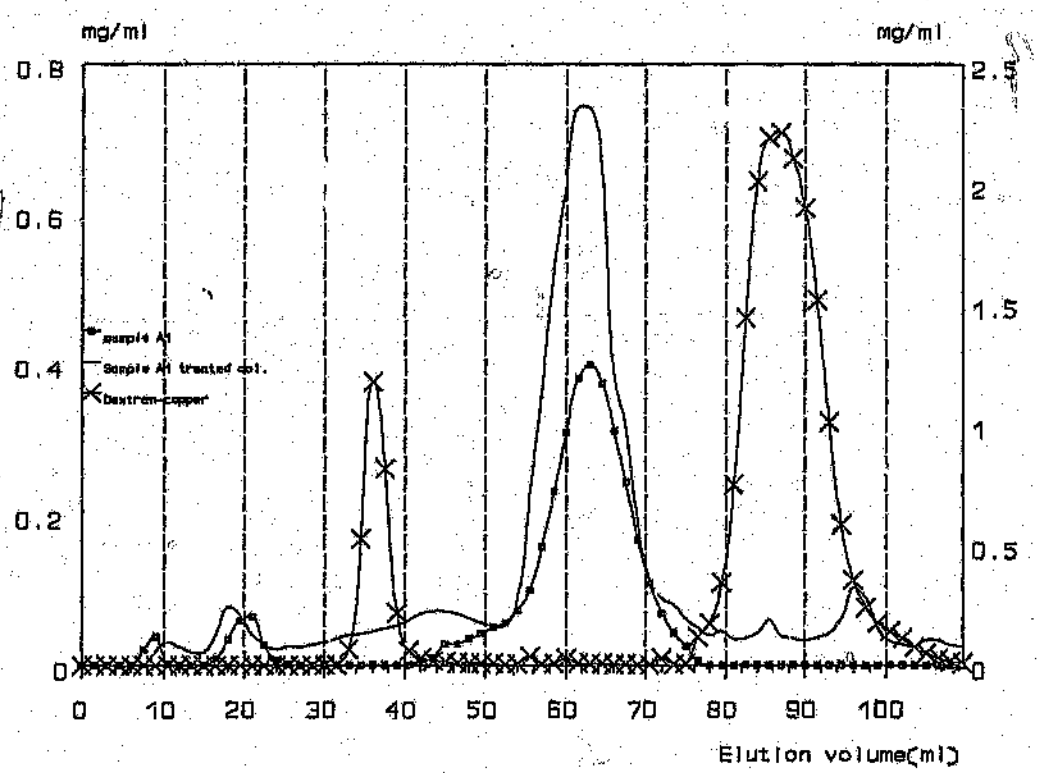

Figure 5: Elution profile of enzyme sample extracted with amylopectin through Sephadex G-75 column and Sephadex G-75 repel silane treated column respectively.

The eluted protein was evaluated by UV spectrophotometry at 280 nm and scanned between 200 and 300 nm for protein characterization. The other scanned proteins were bovine serum albumin (BSA), β-phosphorylase, alcohol dehydrogenase, α-amylase from *Bacillus subtilis*, and Blue dextran, heparin and mucin from bovine gastric.

The result showed that pure protein absorb at 280 nm Blue dextran and mucin absorb at 263 and 260 nm respectively, and heparin had a hyperbolic curve. The sample presenting high absorbency at 280 nm gave a scanning graph with a maximum at 260 nm besides a huge peak at 220 nm. There was a similarity between the UV scan of mucin and amylopectin extracted enzyme (see Fig. 6).

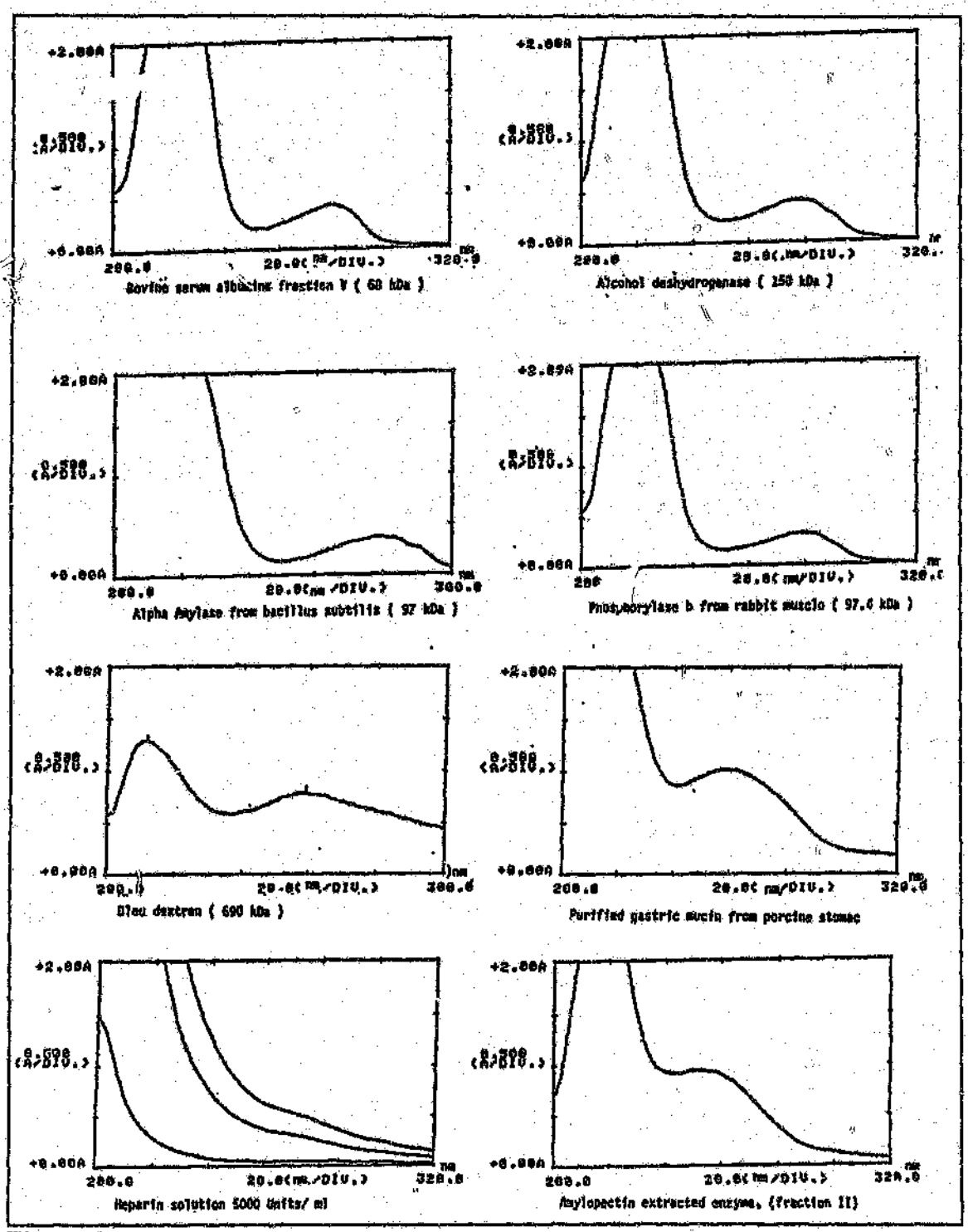

Figure 6

Figure 6: Scanning graphs of protein, enzymes and glycoproteins compared with the eluted amylopectin extracted sample (fraction 2).

2) AGAROSE GEL

An agarose gel, which is very sensitive for nucleic acids, showed only protein bands (see Fig. 7). This result suggested the presence of glycoproteins.

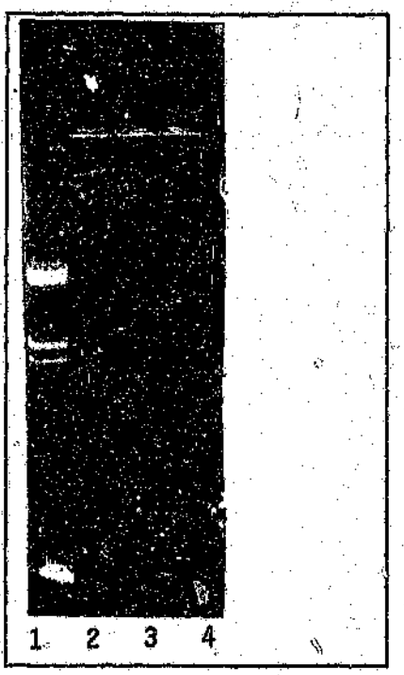

Figure 7: Agarose gel performed with eluted sample A1 (fraction II). Lane 1: DNA Molecular weight markers number III range from 21,226 to 125 (Boehringer Mannheim); Lanes 2, 3, 4: Protein bands of enzyme amylopectin extracted after elution chromatography.

B) CRUDE PROTEIN EXTRACT

The elution profile of either the crude protein extract, precipitated with 60% ammonium sulphate, the amylopectin extracted enzyme and the supernatant that remained (sample A2) as seen below showed that the protein eluted in the amylopectin extracted sample was the main ammonium sulphate extracted protein from the concentrated culture medium (see Fig. 8).

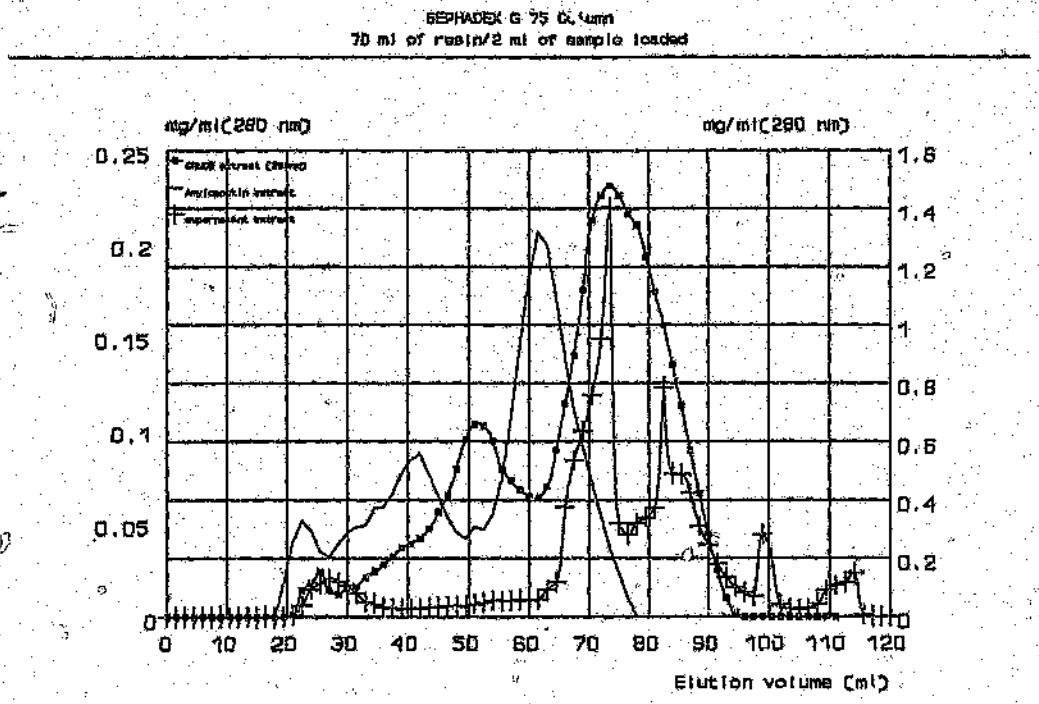

Figure 8: Elution profile of crude protein extract, amylopectin enzyme extract and protein remaining in crude protein extract after amylopectin extraction.

C) CHARACTERISATION OF THE ENZYME

1)Protein estimation

The comparison of protein estimation by absorbency at 280 nm and using the correction formulae of protein concentration (Layne, 1957) showed that (Fig. 9) the estimated absorbency was ten times higher than the result obtained with the corrected formula.

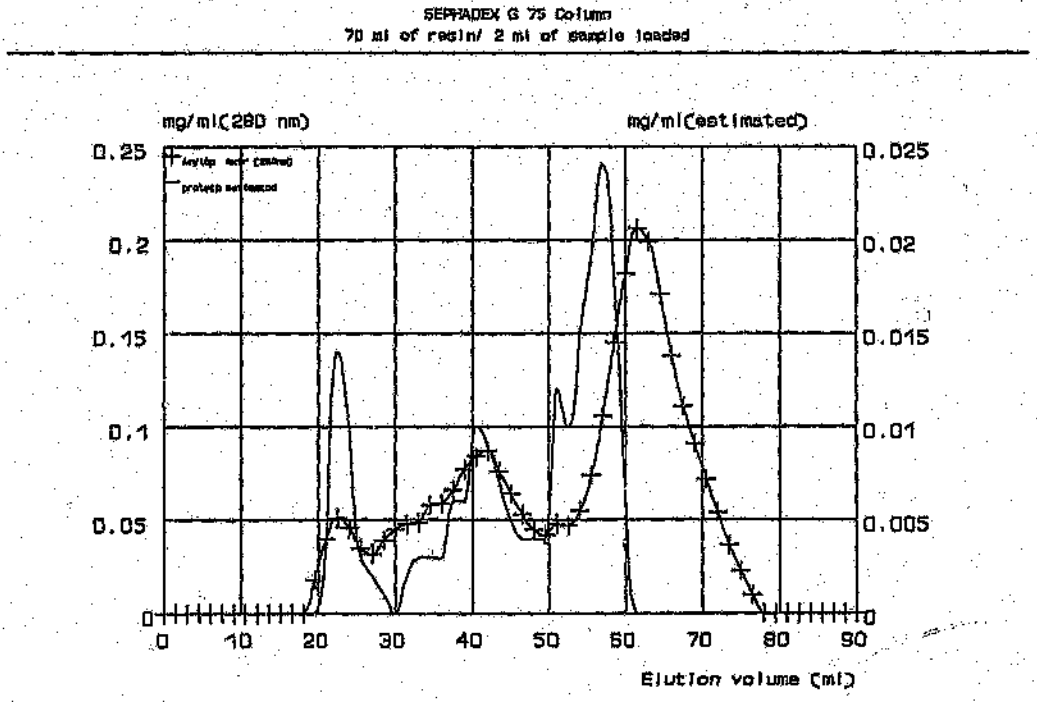

Figure 9: Graphs comparing protein estimated by absorbency at 280 nm (+++) and by protein concentration formulae ($1.45*A_{280} - 0.74*A_{260}$) (---).

2) Enzyme assay with chromogenic substrate (Table 4) (Wahlefeld, 1989)

A comparison of the iodine complex staining method and the chromogenic substrate method for the assay of α-amylase activity on the eluted samples showed there was synthesizing

than the blank.

The absorbency of the fraction S1, S2 and S3 were higher at 490 nm than with the iodine staining method whereas hydrolysing activity was displayed when the chromogenic substrate was used.

SAMPLE	IODINE STAINING METHOD <ABS>	<ACT>U/ml	CHROMOGENIC METHOD <ABS>	<ACT>U/m
Blank	0.361	-	-	-
Standard	-	-	0.217	18
So	0.204	0.971	0.288	23.89
A1	0.423	0	0.047	8.91
A2	0.458	0	0.050	9.47
S1	0.540	0	0.079	6.58
S2	0.469	0	0.035	2.93
S3	0.430	0	0.018	1.46
A1a	0.368	0	0.153	12.69
A1b	0.271	0.561	0.238	26.54

Table 4: Activity evaluation with iodine substrate complexing and chromogenic substrate methods respectively. Samples S1, S2 and S3 are from So after elution on a G-75 column and concentration. Samples A1a and A1b are from A1 after elution and concentration. Standard used 0.01 mg/ml of a pure solution of α-amylase from *Bacillus subtilis*.

3) Molecular weight determination

The molecular weight determination of this enzyme was also measured by comparison with the profile of molecular weight markers which were eluted first (Fig. 10). These results suggest that the sample had a small molecular weight. A comparison of the separation of the enzyme on Sephadex G-75 and Ultrogel AcA 34 showed that although the amount of the protein collected from the treated G-75 column was considerably higher than with normal

Sephadex G-75 column, the profile remained the same.

The UV scan of the samples collected after the elution of the crude protein extract through the Ultrogel column did not give a maximum protein absorbency at 280 nm. The elution profile however gave the same result with the crude protein extract (see Figs. 11 and 12).

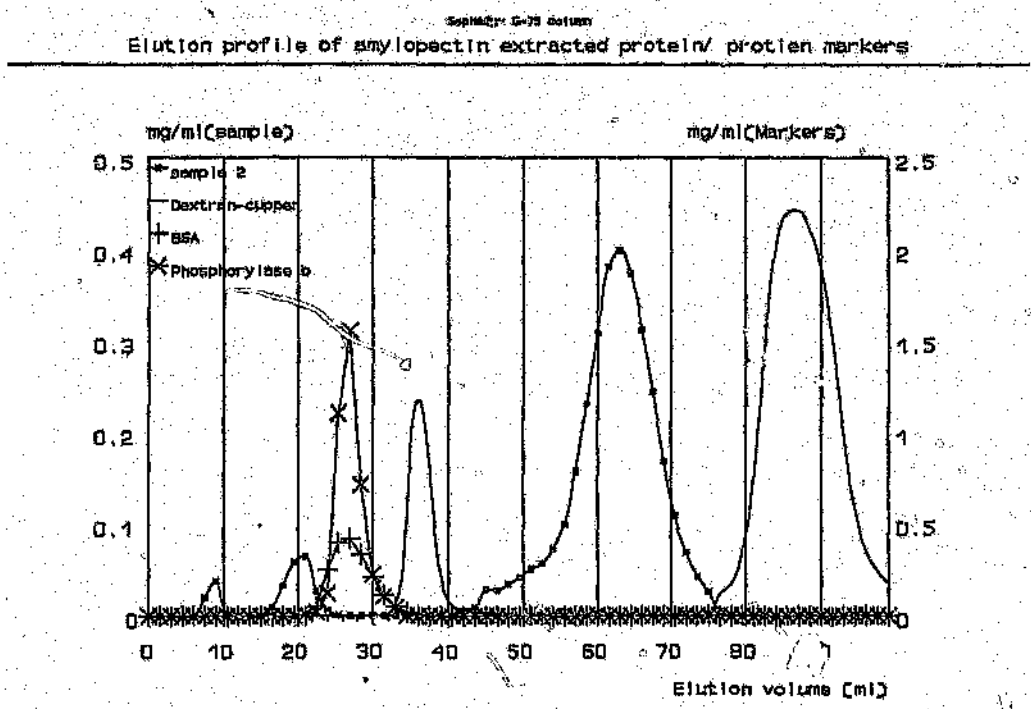

Figure 10: Elution profile of amylopectin extracted sample, BSA, phosphorylase b and blue dextran and copper sulphate as calibration scale.

A second column was prepared with Ultrogel AcA 34. The amylopectin extracted sample was applied to these column, eluted and the fractions were collected (see Fig. 11).

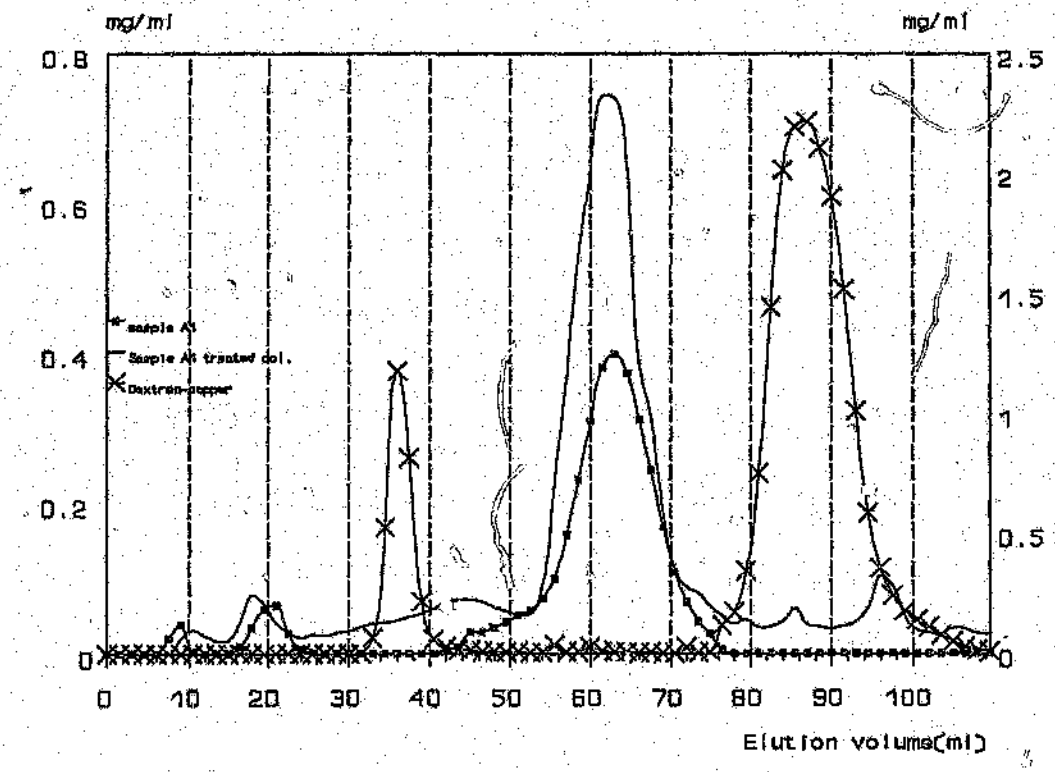

Figure 11: Elution profile comparison of amylopectin extracted sample on a normal and a repel silane treated G-75 column.

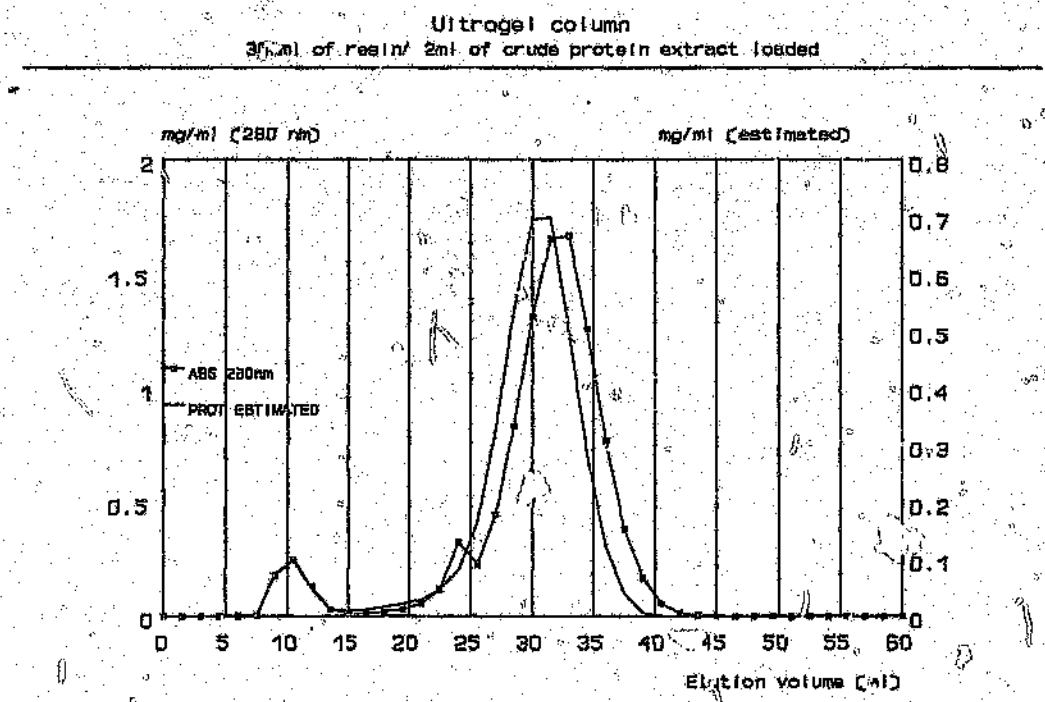

Figure 12: Elution profile of crude protein extract through Ultrogel column (30 ml).

The investigation with SDS was performed on Sephadex G-75 column. The normal amylopectin extracted sample and sample treated with 1% solution SDS was loaded. There was no difference between the elution profile of the normal sample and the sample treated with SDS (see Fig. 13).

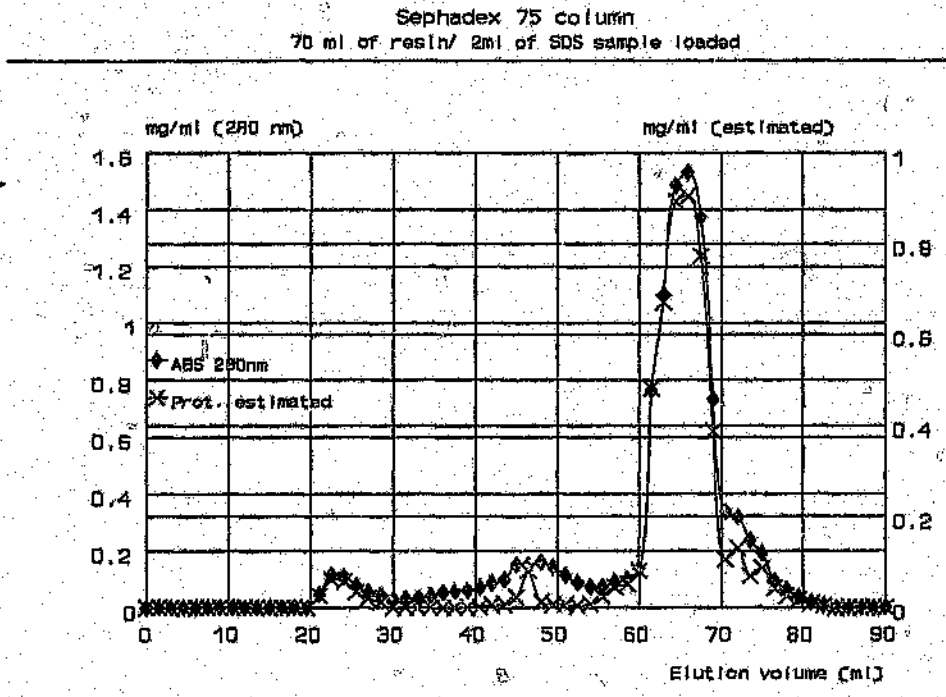

Figure 13: Elution profile of 2 ml amylopectin extracted sample mixed with 1% sodium dodecyl sulphate before loading.

4) SODIUM DODECYL SULPHATE-POLYACRYLAMIDE GEL ELECTROPHORESIS

a) Normal Samples

A preliminary study of the enzyme from a crude protein extract incubated with different carbohydrate substrate gave similar bands on Laemmli SDS-PAGE and clearance at the top of the lanes on a Russell gel (Fig. 14).

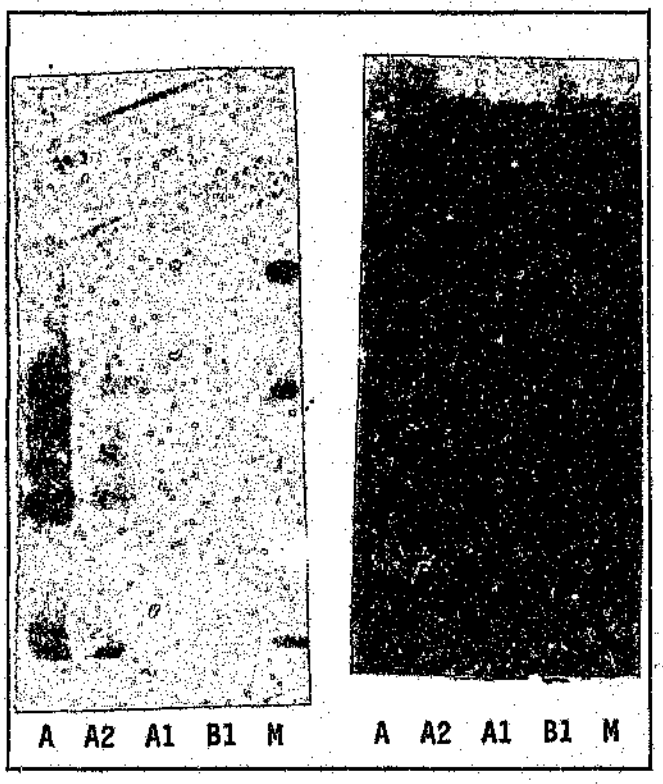

Figure 14: Gel showing protein bands of crude enzyme extract (lane 1), remaining in crude extract after amylopectin extraction (lane 2), protein extracted with amylopectin (lane 3, 4) and protein markers bands (phosphorylase b and BSA) and hydrolysing clearance activity of amylopectin hydrolysing enzyme (1 and 4).

Experiment with the enzyme extracted with amylopectin also showed hydrolysing activity at the top of the lanes (Fig. 15). The same gel stained with Coomassie blue gave protein band around BSA band for the crude enzyme extract and protein remained. Any Coomassie blue stain band was seen with amylopectin extracted enzyme at this zone (lane 6). The protein remained left in the supernatant as well as the extracted protein gave similar clear bands with SDS-PAGE.

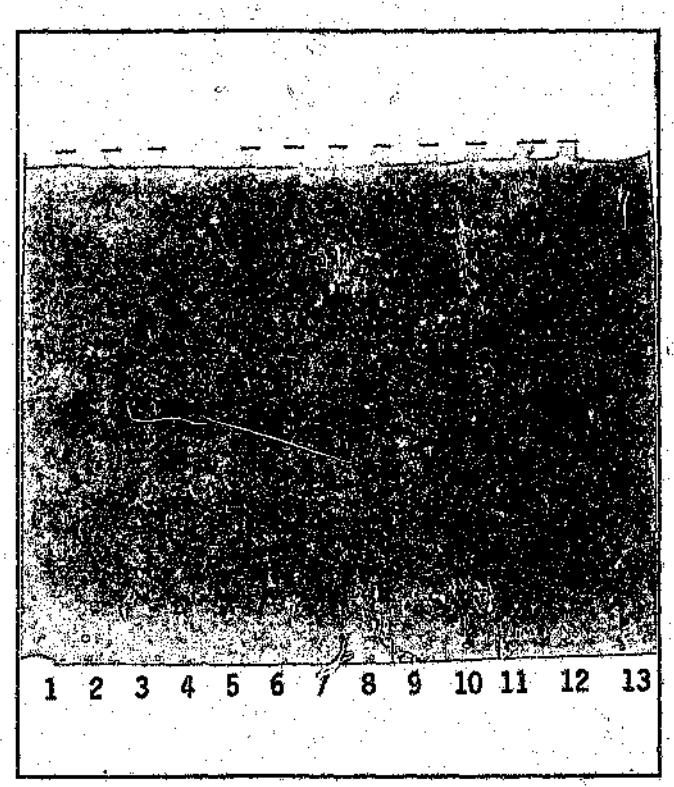

Figure 15: Gel showing enzyme clearance activity on the top of the gel. Coomassie blue staining gave protein bands around BSA band for crude enzyme extract and protein remaining after the extraction, but amylopectin extracted enzyme did not give protein band (Lane 6).

Similar gels stained with the modified silver staining method gave the result below (Fig. 16). The gel was a 7.5% acrylamide gel containing 0,25% amylopectin and pretreated with sodium metabisulfite before silver staining. The protein markers α-macroglobulin (lanes 1, 7, 13); supernatant (2, 3, 4 and 10,11,12); crude proteins extract (lanes 6 and 8) and lanes 5 and 9 showed multi protein bands (samples amylopectin extracted).

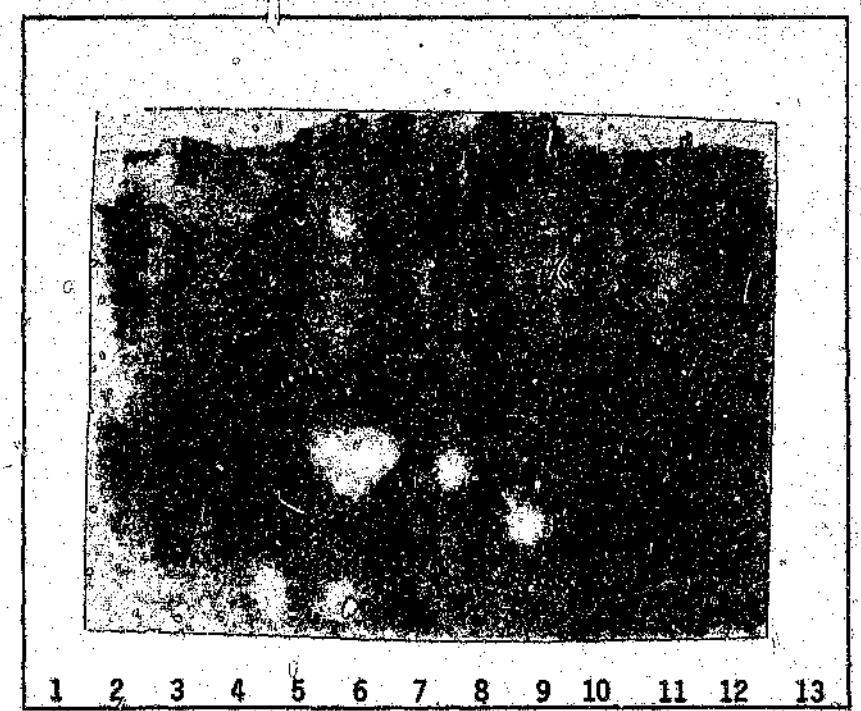

Figure 16: Gel showing proteins bands stained silver staining method. The gel was a 7.5% acrylamide gel containing 0.25% amylopectin and pretreated with sodium metabisulfite before silver staining. The protein markers α-macroglobulin (lanes 1, 7, 13); supernatant (2, 3, 4 and 10,11,12); crude proteins extract (lanes 6 and 8) and lanes 5 and 9 shows multi protein bands (samples amylopectin extracted).

b) Concentrated Samples

A 7,5% gel performed with these eluted samples (S1, S2 and S3) showed no protein bands

with Coomassie blue staining. The trichloroacetic (TCA) concentrated sample on the gel gave a straight blue line without specific band. Phenol-ether concentrated samples gave a band at 68 kDa and a heavily stained band at the front of the gel with the periodic acid-silver staining method (Sauvé et al., 1995). A slightly band was seen with Coomassie blue staining if carbohydrate was incorporated in the gel.

Samples concentrated against polyethylene-glycol and dialysed against 5 mM Tris-HCl buffer gave sharpe protein bands above 78 kDa and another at 68 kDa with periodic acid- silver stain (Fig. 17).

Samples that were incubated for 2 hours at 45°C before being loaded onto the gel, gave bands above 97 kDa and at 75 kDa respectively using the periodic acid alcian blue prestaining silver method (Fig. 18).

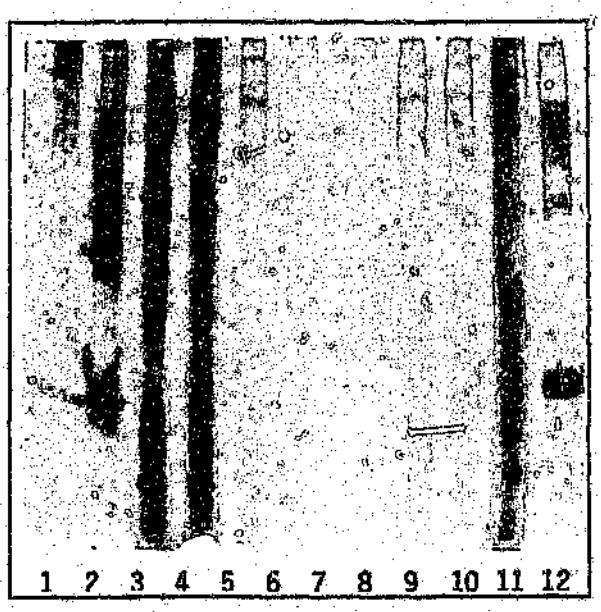

Figure 17: Gel was 7.5% acrylamide gel stained as above and loaded with markers (1 and 2), sample phenol-ether concentrated (3 and 4), normal samples and polyethylene-glycol concentrated (5, 6, 7, 8, 9 and 10).

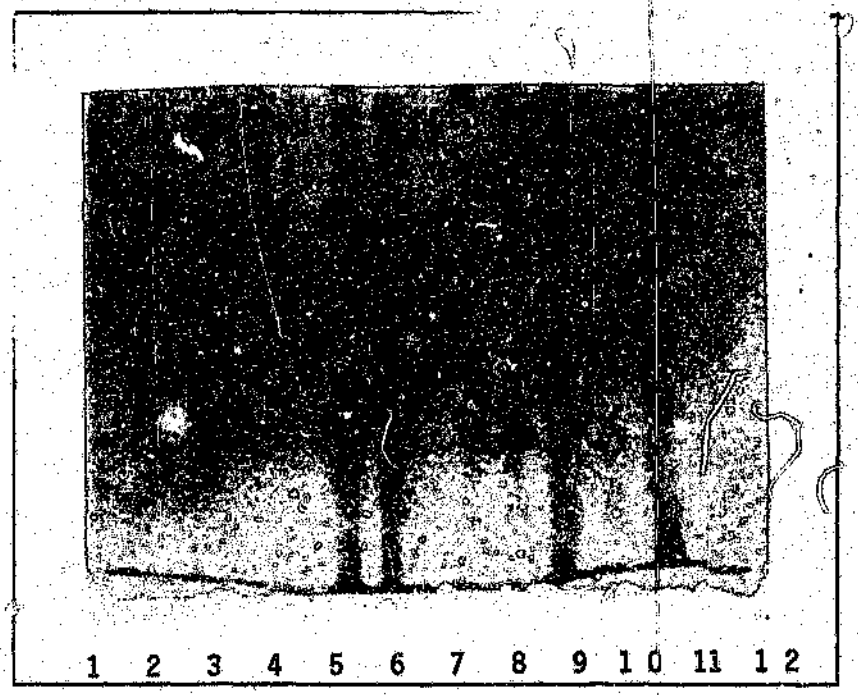

Figure 18: Gel was 7.5 % acrylamide gel containing 0.25% amylopectin, stained as above and loaded with markers (7 and 8),normal samples beside sample phenol-ether concentrated A1 and B1 (3,4 and 5, 6 and 9,10,11 and 12).

The gel performed with same sample in the presence of α-amylase from *Bacillus subtilis* (2 μg) and stained with Coomassie blue stain did not show any bands for the sample but bands were seen for α-amylase. Staining was only at the front of the gel (see Fig. 19). The gel was a 7.5 % acrylamide gel stained with Coomassie blue stain and loaded with BSA as markers (4 and 8), 20 μl of 0.1 mg/ml α-amylase from *Bacillus subtilis* (2 μg) (1,2, and 3) and amylopectin extracted enzyme (5,6 and 7).

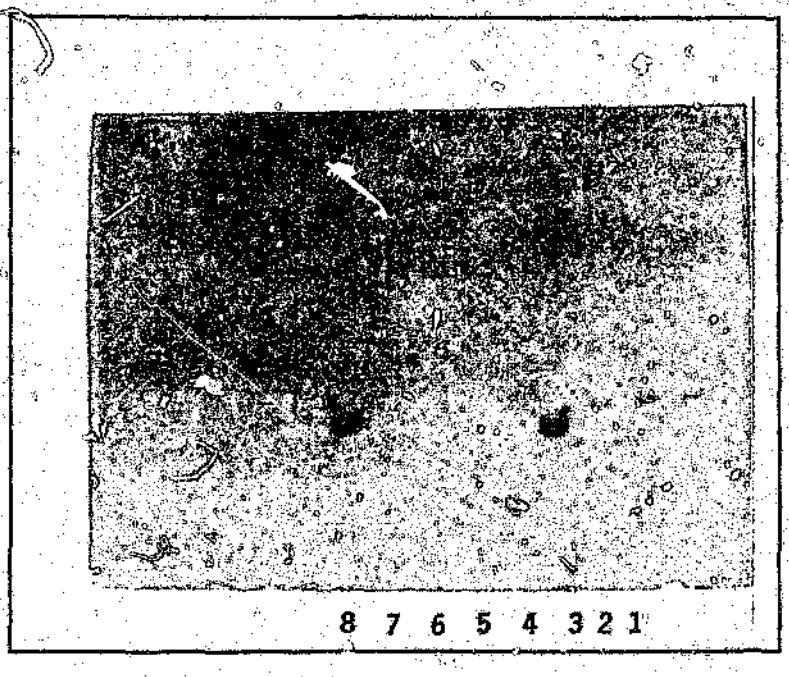

Figure 19: Gel was a 7.5 % acrylamide gel stained with Coomassie blue stain and loaded with BSA as markers (4 and 8), 20µl of 0.1 mg/ml α-amylase from *Bacillus subtilis* (2 µg) (1,2, and 3) and amylopectin extracted enzyme (5,6 and 7).

CHAPTER IV

DISCUSSION

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CHAPTER VI

DISCUSSION

1) ENZYME EXTRACTION

These results show that protein extracted from culture medium and from crude protein extract were specifically the amylopectin hydrolysing enzyme. The residual enzyme activity in the supernatant showed that the extraction process must be repeated at least three times to extract 80% of the enzyme in the presence of ammonium sulphate.

2) ACTIVITY ASSAY

Together, these tables showed a recovery of enzyme activity from the protein samples extracted with amylopectin. No correlation was found between the three analytical methods. Even if the iodine substrate complex method did not correlate with the reducing end sugar methods, because of the difference of the process, the reducing end sugar methods should present correlation. Some explanation may be found, however, by considering that the reagents of Somogoyi method can react with neutral carbohydrate which is present in enzyme aliquot used.

Although this problem requires a particular attention for determining the limit of each method, the collected results contain a lot of information that is worthy of note:

- Both methods showed that the amylopectin hydrolysing enzyme from *Streptococcus sanguis* can be isolated from concentrated culture medium or crude protein extract by using a chemically modified amylopectin. With the three analytical methods,

purification of extract A1 was several times higher than B1 extract. This may be understood by considering that the presence of some salt in the culture medium enhance the activity of the enzyme while ammonium sulphate used in crude enzyme extract reduce its activity.

- However the extraction from crude enzyme extract might be the most suitable method for the enzyme recovery. This may be due to the presence of salt in the amylopectin extracted enzyme because the enzyme in this sample may remain active for a long period if it is not desalted after the extraction process.
- The poor extraction result of crude enzyme extract may be explained by the tendency of α-amylase or glycoprotein to aggregate in the concentrate solution.

The enzyme isolation method proved sensitive and highly specific for the endo-hydrolase and could facilitate the high degree of purification if combined with a chromatography methods.

Otherwise, the iodine complex staining method, which is widely used for the α-amylase activity assay, could not be selected for this enzyme activity assay because of its overnight period of incubation.

A comparative study with this method when the incubation time was reduced to 15 minutes showed that:

- 50 µl of 0,01 mg/ml pure α-amylase hydrolysed 0,5 ml of 1% amylopectin in 15 minutes (no iodine staining was observed). While with the same amount of amylopectin extracted enzyme high iodine blue staining absorbency are observed (see table 4). This might come from a synthesis enzyme activity as well as from the carbohydrate part of the enzyme (Yamane et al., 1973). But as the enzyme amount

used is 100 times small than the substrate this interference could be insignificant.

3) CHROMATOGRAPHY

A) ENZYME AMYLOPECTIN EXTRACTED

1) GEL FILTRATION

The comparison of the scans of proteins, glycoproteins with the amylopectin enzyme extract suggests that this enzyme is not a pure protein (Fig. 6).

The scan of amylopectin extracted enzyme was seen with nucleic acid contaminated samples.

Tests for nucleic acid undertaken on these eluted samples gave a negative answer for orcinol, which is specific for RNA and a positive answer with diphenylalamine, which is used to detect the presence of DNA or reducing sugar as deoxypentose. An agarose gel, which is very sensitive for nucleic acids, showed protein bands only (see Fig. 7). This result led one to suspect the presence of glycoproteins.

B) CRUDE PROTEIN EXTRACT

The scans of the sample from the main peaks of the crude protein extract and the protein that remained in the supernatant were similar to those obtained with the amylopectin enzyme extract, shown on fig. 6 above.

This result was possibly explained by comparing the scans of several proteins. The result showed firstly that the carbohydrate part of the glycoprotein interfered with protein absorption

and shifted the maximum absorption to 260 nm; secondly, the scanning curve of the eluted sample of the amylopectin extracted enzyme was similar to mucin and this suggests that it may be a glycoprotein (see Fig. 6).

C) CHARACTERISATION OF THE ENZYME

1)Protein estimation

Protein estimation by formula using absorbency at 280 nm and 260 nm had to be modified for glycoproteins and glycoaminoglucans. The comparison of protein estimation by absorbency at 280 nm and using the correction formula of protein concentration (Layne, 1957) showed that (Fig. 9) the estimated absorbency was ten times higher than the result obtained with the corrected formula. For that, coefficients for the above formula could be recalculate by selecting a pure glycoprotein sample which can provide the specific absorption coefficients at the wavelengths specified (Manchester, 1996). The absorbency reading also seemed high when these results were compared to those that were obtained when the Lowry method for protein determination was used.

2) Enzyme assay with chromogenic substrate (Table 4)

A comparison of the iodine complex staining method and the chromogenic substrate method for the assay of α-amylase activity on the eluted samples showed there was synthesizing activity when the iodine staining method was used because the sample absorbency was higher than the blank. Furthermore, hydrolysing activity was also present when the chromogenic substrate was incubated with the same enzyme sample.

This may be explained by considering the interference of the carbohydrate moiety of the glycoprotein with iodine complex formation.

The method using chromogenic substrate may be more suitable for measuring reducing sugars than iodine solution because carbohydrates interferences is eliminated.

The presence of carbohydrate in fraction S1, S2 and S3 was confirmed by determining the total carbohydrate present in these fractions using the phenol-sulphuric acid assay (Dubois et al., 1956; Spiro, 1966 and Chaplin, 1986). The result was higher absorbency at 490 nm compared to the standard.

3) Molecular weight determination

The molecular weight determination of this enzyme was also measured by comparison with the profile of molecular weight markers which were eluted first (Fig. 10). These results suggest that the sample has a small molecular weight. However, the enzyme was precipitated with 60% ammonium sulphate and precipitation only occurred when a relatively high molecular weight protein or an aggregate of proteins was present (Scopes, 1988). The next step in this study was to compare the separation of the enzyme on Sephadex G-75 and Ultrogel. Sephadex G-75 was treated with repel silane and packed into the column. Although the amount of the protein collected from the treated G-75 column was considerably higher than with normal Sephadex G-75 column, the profile remained the same.

The UV scan of the samples collected after the elution of the crude protein extract through the Ultrogel column gave the same result with crude protein extract (see Fig. 12).

From this result it can be conclude that:

- Although the enzyme affinity was detectible when Sephadex G-75 was used, the position of the peaks did not change which suggests that there were no other enzyme present which adsorbed to the column.

- This suggests that the study should be expanded in order to characterize the eluted proteins.

Some workers suggest that SDS may be used to solve the problem of protein carbohydrate aggregation on gel filtration columns (Ciardi et al., 1976; Pazur, 1972 and Segrest et al., 1971). However, in the present investigation with SDS there was no difference between the elution profile of the normal sample and the sample treated with SDS (see Figs. 11 and 13).

The presence of detergent up to a concentration of 1% during the extraction process was tolerated by the enzyme. Any concentration above this level destroyed the activity of the enzyme. The protein amylopectin extract gave intense bands similar to those of the protein crude extract obtained from the supernatant. This may be the effect of SDS-complex formation on proteins (Sandri et al., 1993) because at low concentration and pH around 7 SDS can bind partially to proteins. SDS can be removed by preparing a mixture of SDS and sample, loading the mixture onto a gel filtration column and eluting it with Tris-HCl elution buffer containing 0.15 M KCl. This enables KDS to precipitate and release the denatured protein (Sandri et al., 1993).

In a further study a sample of amylopectin extracted enzyme was eluted on a Sephadex G-75 column, after it was concentrated against polyethylene-glycol and loaded into SDS-PAGE. The bands that were obtained did not differ from the original sample (Fig. 13).

4) Sodium Dodecyl Sulphate-PolyAcrylamide Gel Electrophoresis

a) Normal Samples

The above suggested SDS-PAGE can be used for determining the molecular weight and studying hydrolysing activity which was associated with the enzyme.

Experiment with the enzyme extracted with amylopectin also showed hydrolysing activity at the top of the lanes (Fig. 15). The same gel stained with Coomassie blue gave protein bands around BSA band for crude enzyme extract and the remaining protein. Any Coomassie blue stain band was seen with amylopectin extracted enzyme at this zone (lane 6). The protein that was left in the supernatant as well as the extracted protein gave similar SDS-PAGE clearance bands which suggests that the enzyme was large.

For this reason an experiment with gradient gel was undertaken.

The bands showed that the extracted enzyme may have a higher molecular weight than bovine serum albumin (Fig. 15). This result did not agree with the data obtained from gel filtration study which gave results that suggest a small molecular weight.

The same gels stained with the modified silver staining method gave the result seen on Figure. 16.

b) Concentrated samples

A 7,5% gel performed with these eluted samples (S1, S2 and S3) showed no protein bands with Coomassie blue staining. The trichloroacetic (TCA) concentrated sample on the gel gave a straight blue line without specific band. Phenol-ether concentrated samples gave a band at 68 kDa and a heavily stained band at the front of the gel with the periodic acid-silver staining method (Sauvé et al., 1995). A slight band was seen with Coomassie blue staining if

carbohydrate was incorporated in the gel.

Samples concentrated against polyethylene-glycol and dialysed against 5 mM Tris-HCl buffer gave sharper protein bands above 78 kDa and another at 68 kDa with periodic acid- silver stain (Fig. 17).

Samples that were incubated for 2 hours at 45°C before being loaded onto the gel also gave bands (Fig. 18).

Although the protein contents of these samples were evaluated by UV and confirmed by the Lowry method, the presence of heavy proteins bands in these concentrated samples suggested protein aggregates or glycoproteins. However the result from protein scanning above (Fig. 6) suggested that the enzyme was a glycoprotein (Ciardi et al., 1976).

Furthermore, SDS-PAGE was performed with mucin and heparin in the presence of others proteins and stained with the periodic acid- alcian prestained silver method. This gave a high molecular weight band for mucin and heavily stained band at the elution front for heparin while clear bands indicated the position of the pure proteins.

This shows that the method is more sensitive to the presence of glycoproteins than to pure proteins and can be used for a rapid evaluation of the nature of the proteins (Møller et al., 1995). The purified sample obtained from the amylopectin extracted enzyme yielded one silver stained band around phosphorylase b clear band and 4 clear bands. Two were between phosphorylase b and BSA clear bands, one at 68 kDa and the last at 35 kDa. The gel performed with same sample in the presence of α-amylase from *Bacillus subtilis* (2 μg) and stained with Coomassie blue stain did not show any bands for the sample but bands were seen for α-amylase. Staining was only at the front of the gel (see Fig. 19).

5) ENZYME PROPERTY

At this stage of purification, analysis for determining the optimum pH and the action of some salts such as KCl, NaCl, $CaCl_2$ and ammonium sulphate was studied. These salts were used in the incubation buffer in Russell method (Russell, 1976). Ammonium sulphate has shown on this paper to play a role in enzyme activity. The optimum pH of the amylopectin hydrolysing enzyme could not be defined at this stage of purification because of interference by the carbohydrate moiety of the glycoprotein. Salts tested had a inhibiting effect on the enzyme. The enzyme was kept in the buffer and its activity may be reduced by the lack of activating cations.

The conditions of the activity protection of the extracted enzyme should therefore be established and the enzyme characterisation should continue.

6) CONCLUSION

This study showed that a modified amylopectin can be used for the isolation of a starch metabolizing enzyme from a crude extract of extracelluar protein of *Streptococcus sanguis I MC 204, a strain of oral Streptococci.*

This method appears to work for the isolation of a starch metabolizing enzyme produced from *Streptococcus sanguis.* More than 50 percent of the yield of enzyme was achieved by this process, after three assays, from both crude enzyme extracts and enzyme serum samples. Many-fold purification was obtained from the extraction process of the crude enzyme extract and a protective enzyme activity effect was noticed in the presence of the ammonium sulphate. The extraction from crude enzyme extract prepared by 60 percent ammonium precipitation from cultured medium was the most suitable method for the enzyme recovery.

The result of a combination of amylopectin extraction method with gel filtration chromatography and SDS-PAGE suggests that a starch metabolizing enzyme isolated from *Streptococcus sanguis* is a glycoprotein.

By comparing the scans of several proteins, glycoproteins with the scan of the eluted sample of the amylopectin extracted enzyme, the result suggests that it may be a glycoprotein.

The recognition that the starch metabolizing enzyme isolated from *Streptococcus sanguis* is a glycoprotein may open up new avenues and lead to an understanding of the role of enzymes in dental caries and solve problems caused by this disease.

Consequently, protein estimation using absorbency at 280 nm and 260 nm may have to be adapted for glycoproteins and activity assay could be estimated by a method which would not interfere with the carbohydrate moiety of the glycoprotein. In the present study the method using chromogenic substrate was more suitable for measuring reducing sugars than iodine solution because carbohydrates interference is eliminated.

The similarity found between the UV scan of mucin and amylopectin extracted enzyme (See Fig. 6), may answer some of the question about caries initiation as well as adhesion of microorganism to the tooth surface because:

Firstly, salivary mucin may occur in two forms. As a large and highly glycosylated glycoprotein it may participate in the formation of a protective pellicle covering tooth enamel. As a low molecular weight glycoprotein it may aggregate bacteria thus assisting with their clearance from the mouth (Slomiany et al., 1993; 1986; 1987b).

Secondly, the knowledge that *Mutans streptococci* cell walls and its components, for example, peptidoglucan exhibit a variety of immunological activities, the starch metabolising enzyme which is a glycoprotein could aggregate bacteria and attach to the mucin protective pellicle where it would be enzymatically active (Vacca-Smith and Bowen, 1995; Schilling and Bowen, 1988; Hamada et al., 1986, Coogan et al., 1992).

This aggregated form of the enzyme was observed as a clear zone at the top of the amylopectin containing gel (Fig. 14). In this respect it acts like a glycoprotein because most of the enzyme remains at the top of lane whereas a few dissociated enzymes may have reached the separating gel and hydrolysed the amylopectin that was present in the gel.

The knowledge that bacterial α-amylases may have glucosamine and neutral carbohydrate in their structure (Yamane et al., 1973) and that the same carbohydrates are tightly and covalently link to polypeptide, may explain the presence of multi protein bands seen on SDS-polyacrylamide gel.

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