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PURIFICATION OF N-ACETYL-β-D-GLUCOSAMINIDASE ISOENZYMES FROM BABOON KIDNEY

By

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A dissertation submitted to the Faculty of Science,
University of the Witwatersrand, Johannesburg
for the degree of
MAGISTER SCIENTIAE

November 1988

Supervisor : Dr. M.M.J. Oosthuizen

I declare that this dissertation is my own, unaided work.
It is being submitted for the degree of Master of Science at
the University of the Witwatersrand, Johannesburg.
It has not been submitted for any degree or examination at
any other University

H.A.G. Beukes

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17 th day of November 1988

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ABSTRACT

1. The N-Acetyl-β-D-glucosaminidase isoenzyme B was partially purified, while the isoenzyme A was isolated to apparent homogeneity which was verified by one band on SDS-PAGE and anionic PAGE, as well as PAG-IEF after treatment with Endoglycosidase D or Glycopeptidase F.

2. NAG A was separated into 2 distinct isoenzymes containing 30% and 17% total carbohydrate and 6% and 1% sialic acid respectively.

3. Amino acid analyses revealed 320 amino acids resulting in a peptide molecular weight of 36.4 kD. When corrected for the carbohydrate content the MW was calculated to be 52.0 kD. NR-SDS-PAGE gave a molecular weight of 52.1 kD. Determination of molecular weight using sedimentation equilibrium centrifugation revealed an aggregation phenomenon of the molecule whereby it was not possible to acquire the MW.

4. Isoelectric focusing revealed a pI of 4.97 for the NAG A isoenzyme.

5. A pH optimum of 4.55 was found for both NAG A and B.

Optimum temperature for NAG A and B was 50°C and 40°C respectively. NAG B was more high temperature resistant than NAG A

6. The K_m value for NAG A was 0.497 mmol/L for the substrate 4-Nitrophenyl-N-acetyl-β-D-Glucosaminide. Reaction specificity resided in the β-D-glycosidic bond. Substrate specificity was mainly for the N-Acetyl-Glucosamine residue but the N-Acetyl-Galactosamine residue showed an 80% equal substrate specificity in terms of the PNP-NAG substrate. The ions Pb, Ag and acetate had the highest inhibitory effect towards NAG with Ki-values of 3.6, 8.5, and 23-31 mmol/L respectively.

7. Subcellular distribution of the major NAG isoenzymes A and B was very similar with NAG A more localized in the lysosomes, while NAG B was found in both lysosomes and mitochondria in equal quantities.

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SYMBOLS AND ABBREVIATIONS

A

A	Absorbance
A	*Ampére*
AA	Amino Acid
Ac	Acetate
Ala	Alanine
Arg	Arginine
Asn	Asparagine

B

BA	Bicinchoninic acid
Bisacrylamide	N,N'-methylene-bis acrylamide

C

C	Degrees Celsius
Cd	Cadmium
Cl	Chlorine
cm	Centimeter
Con	Concanavalin
Cr	Chromium
Cys	Cysteine

D

D	Dalton
DEAE	Diethylamino-ethyl
Dol	Dolichol

E

E	Enzyme concentration
e.g.	For example
eq.	Equation
ELISA	Enzyme linked immuno-sorbant assay
ER	Endoplasmic Reticulum

F

Fe	*Iron*
Fuc	Fucose

G

g	Gram
G	Gravitational acceleration (9.8 m/s^2)
Gal	Galactose
GDP	Guanosine di-phosphate
Glc	Glucose
Glu	*Glutamine*
Gly	Glycine

H

h	Hour
HCl	Hydrochloric acid
HTP	Hydroxylapatite
H_2SO_4	Sulphuric acid
H_2O	Water

I

i.e.	that is
IEF	Isoelectric focusing

K

k	Kilo
kD	Kilo Daltons (Molecular weight)
KCl	Potassium chloride
Ki	Dissociation constant of the inhibitor-enzyme complex
Km	Michaelis constant
KOH	Potassium hydroxide
KNO_3	Potassium Nitrite

L

L	Litre
Leu	Leucine
Li	Lithium
Log	Logarithm

L

Lys	Lysine

M

M	Molarity
m	Metre
m	Milli
Man	Mannose
Met	Methionine
Mg	Magnesium
min	Minute
Mw	Molecular weight
Mn	Manganese

N

NAG	N-Acetyl-β-D-glucosaminidase
N	Normality
NaCl	Sodium chloride
NaOH	Sodium hydroxide
Neu	Neuraminic acid
No	Number

O

O.D.	Optical Density

P

PAGE	Polyacrylamide gel electrophoresis
PAG	Polyacrylamide gel
PB	Polybuffer
Pb	Lead
pI	*Isoelectric point*
pH	Negative log of hydrogen ion concentration
Phe	Phenylalanine
PNP-NAG	4-Nitrophenyl-N-acetyl-B-D-glucosaminide
PNP	Para-nitrophenyl
Pro	Proline
Pt	Platinum

R

r	Radius
R	Universal Gas Constant
RER	*Rough endoplasmic reticulum*
Rf	Relative movement of item
rpm	Revolutions per minute

S

s	Second
S.A.	Sialic acid
SDS	Sodium dodecyl sulphate
Ser	Serine
Std.	Standard

T

TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	1,2,3 tri-hydroxymethyl-2-*aminomethane*
Trp	Tryptophan
Thr	*Threonine*
Tyr	Tyrosine

U

µ	Micro
UHQ	Ultra high quality
U.V.	Ultra Violet

V

V	Volt
v/v	Volume per volume
Val	Valine
Vis	Visual
vs	Versus

W-Z

w/v	Weight per volume
Xyl	Xylose
Zn	Zinc

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CHAPTER 1 GENERAL INTRODUCTION

1.1. Motivation for this Study on the Enzyme N-Acetyl-β-D-Glucosaminidase (NAG)

Urinary components such as albumins, globulins, creatinine, and glycoproteins such as NAG have been used as indicators of metabolic abnormalaties[47-64]. NAG was compared to creatinine, globulins, neopterin and glucosidases[340-344] and found to be an early indicator of kidney damage. NAG is a kidney enzyme, and since baboon kidneys were freely available, this animal source was exploited for the isolation of NAG. In order to determine the chemical and physical characteristics of this enzyme, it was necessary to isolate the protein to homogeneity. Previous attempts have been made by various authors to purify this glycoprotein to homogeneity[270-284], but without confirmation.

Only NAG A is found in low levels in normal urine. With necrosis and kidney disease NAG B appears in urine, along with an increase in urinary NAG A levels. The appearance of NAG B in urine with kidney disease is thought to be brought about by damage to the lysosomes.

Therefore, it was important to assess the subcellular localization of this enzyme in the kidney and to understand the mechanism(s) involved in the release of the isoenzymes during necrosis or other damage brought about in the kidney. By elucidating the mechanism(s) involved in releasing NAG into urine, it would be possible to adapt

medication towards the specific locus in the kidney where the dysfunction occurs.

CHAPTER 2 LITERATURE SURVEY

2.1. Overview

Knowledge of glycoproteins was limited, until it was discovered that glycoconjugates of the cell membranes are profoundly modified in cancer cells[1]. The observation that the activities of hormones, enzymes, and transport substances that are glycoproteins, are diminished or inhibited by chemical modification of the glycan moieties, gave birth to the view that glycans are not by coincidence the products of different glycosyltransferases, but represent signals for cell to cell recognition, or of proteins and cells interactions. More than 20 years ago it was found that the serological specificity of blood groups resided in the glycan structure of the cell. In 1955, Gottschalk[2,14] demonstrated that the elimination of sialic acid from red blood-cell membranes prevented the fixation of the influenza virus on them[7]. In 1963, Aub and co-workers[3] observed that the ability of a cancer cell to agglutinate is profoundly modified by lectins or phytoagglutinins. Gesner and Ginsberg[4] showed that rat lymphocytes treated with fucosidase migrate to the liver instead of the spleen. The fucose present at the lymphocyte surface thus constitutes a recognition signal of these cells by a receptor present on the spleen cell-membranes.

At the beginning of research on the biological role of glycoproteins, some authors considered glycan association with proteins to be irrelevant. This hypothesis (upheld by Gottschalk[2]), stated that the

biosynthesis of glycans took place coincidentally when the following conditions were fulfilled:

(a) The presence in the peptide chains of a 'coded sequence' of amino acids, for example the tripeptide Asn-X-Ser (or Tyr), which codes for the conjugation of the first 2-acetamido-2-deoxy-D-glucose residue on L-Asn in the case of N-Glycosylproteins, or the sequence Gly-X-Hyl-Gly-Y-Arg, which directs the linkage of a residue of D-Gal with hydroxyl-L-Lysine (Hyl), X and Y being diverse amino acids.

(b) The presence in the cells of specific glycosyltransferases and glycosylnucleotide precursors. Under these conditions, the composition and structure of the glycan would depend on the relative concentration of the 'sugar nucleotides'. If this hypothesis was correct, the structure of the glycans would depend on coincidence and chance, and would never be definite. Follow-up experiments suggested that the glycans play an important biological role[3-7].

The following hypotheses are currently under investigation:

(1) The induction of protein conformation based on glycan-glycan, and glycan-protein interactions via the interplay of ionic forces, i.e. repulsive and attractive.

(2) Protection of proteins against proteolytic attack based on the observation that numerous glycoproteins lose their resistance to proteases on treatment with neuraminidases.

(3) Control of membrane permeability based on the observation that chemical or enzymic modification of the glycan β-Gal-(1-3)-α-GalNAc-(1-)-Tyr of the 'antifreeze glycoprotein' of an antarctic

fish abolishes the function of this protein, which lowers the freezing point of blood by 2°C'. It was hypothesized that at the level of cell membranes, the orientation and concentration of water, and in consequence, the movement of mineral ions and organic substances of low molecular weight is linked to the glycans of membrane glycoconjugates. More particularly, it is linked to the relative number of hydrophilic sialic acid residues, and hydrophobic fucose residues[8]. Any modification of the composition and distribution of the cell surface glycoconjugates could lead to the disturbances observed in transformed cells and cancer cells[8].

(4) The 'Exit passport' hypothesis: In 1966 Eylar[8] observed that most extracellular proteins are glycosylated and intracellular proteins rarely so. According to this author, the carbohydrate unit in a biological active glycopeptide has a general role and he proposed that the carbohydrate acts as a chemical label which, upon interaction with a membrane receptor or carrier, promotes the exocytosis of a newly synthesized glycoprotein into the extracellular environment. He also believed that the carbohydrate unit plays no further functional role in biologically active proteins.

(5) The Recognition-signal concept proclaims that intercellular recognition and association by proteins are because of specific carbohydrate groups that the cells carry, which play the role of 'antennae' towards membrane receptors[9].

Findings which support this theory are:

(a) Erythropoietin, which stimulates the formation of red-blood

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Findings which support this theory are:

(a) Erythropoietin, which stimulates the formation of red-blood

corpuscles in the bone marrow, and certain hypophyseal hormones become 'blind' after treatment with neuraminidase, and are then incapable of recognizing their target cells, or of acting on the regulatory system of adenyl cyclase[10].

(b) The elimination of the terminal sialic acid residues from numerous glycoproteins, in particular of serum glycoproteins diminished their circulating life time[10]. For example, when al-acid-glycoprotein, which possesses terminal galactosyl groups, was exposed to β-D-Galactosidase, and then injected into animals, it prolonged the circulating time. The elimination of the terminal galactosyl groups with β-D-Galactosidase, which exposes N-Acetylglucosyl groups in the terminal positions, maintains the asialo-agalacto-al-acid glycoprotein in the plasma. The terminal galactosyl groups are thus the recognition signals of these sialoglycoproteins for the hepatocytes and for binding the asialo-al-acid glycoprotein onto the hepatocyte-membrane proteins. A highly specific hepatocyte-membrane receptor for galactoproteins, a sialylglycoprotein has been isolated, that loses its property of recognition of Gal if it is desialylated by neuraminidase[11-12]. Therefore a 'galactoglycan' carries the recognition signal of a sialoglycan glycoprotein embedded in the hepatocyte plasma membrane. Any modification to the glycan makes both types of glycoproteins lose their acceptor and receptor sense.

(c) Treatment of erythrocytes with neuraminidase reduces their lifetime from 25 to 2 days in the dog, and is followed by an uptake of the 'asialo-erythrocytes' in the liver and spleen[9].

(d) Some types of virus, such as influenza virus and myxovirus, can

attach to cells with the aid of sialic acid residues bound to cell-membrane glycoconjugates, with the latter playing a part in the infection of cells with viruses.

Glycoproteins play two important roles:

The first is of a physico-chemical nature. It concerns:

(1) The conformation of a peptide chain of glycoproteins and its protection against proteolytic attack, and

(2) The orientation and concentration of water molecules, and the movement of mineral ions and organic compounds at the cell-surface membranes.

The second is of a biochemical nature based on the concept that glycans carry a recognition signal. The biological roles of glycans and glycoconjugates are:

(1) Glycoconjugates are cell-surface antigens, and their structure and function are modified in transformed cells and in cancerous cells.

(2) They play an important role in intercellular adhesion and recognition, and in cell-contact inhibition.

(3) They are part of receptor sites for enzymes, hormones, protein, and viruses.

(4) Glycan groups permit the export of proteins from the cell.

(5) The sugar component regulates the catabolism of circulating proteins by different tissues, and the lifetimes of proteins and even cells.

2.2. Glycoproteins

Glycoproteins are widespread, and they can be classified in various ways (see table 1 page 9). The diversity in the nomenclature of these compounds is, in many instances, confusing. In addition to the terms glycoprotein and glycopeptide, such terms as sialoglycoprotein, acid glycoprotein, mucin, mucoprotein, and fucopeptide are used, often without adequate restriction in their meaning. The term glycoprotein describes a relatively low molecular weight compound that is predominantly peptide-like in its properties and composition, with sugar compounds covalently bonded at specific amino acid residues.

Glycoproteins serve a vast number of functions. There are glycoprotein enzymes, hormones, and immunoglobulins. Glycoproteins are found in blood and secretions, in cell membranes, and in connective tissue. They are components of the structure of blood vessels and skin, and are commonly found in epithelial secretions. Most of the serum proteins contain carbohydrates, as do many of the proteins present in milk and egg white. The gelatinous fluids of certain tumors are rich in glycoproteins[14].

A given glycoprotein may be homogeneous with regard to its amino acid sequence, but heterogeneous with respect to its carbohydrate components[15-19]. The sugars that commonly occur in glycoproteins include Gal, Man, Glc, N-acetylglucosamine, N-acetylgalactosamine, sialic acid, Fuc, and Xyl. The carbohydrate content of a glycoprotein may vary from quite low values, 0.8% for collagen, to ± 50% in the

TABLE 1

Classification of Carbohydrate Containing Proteins

Group of compounds	Definition	Alternative nomenclature	Examples
Glycoproteins	Substances of high molecular weight having many of the physical properties of a protein, but containing covalently bonded carbohydrate *component(s)*	Muco*proteins* Mucosubstances Mucins	*Ovalbumin* Thyroglobulin Ceruloplasmin
Acid glycoprotein	Glycoproteins containing a high concentration of sialic acid and having a low isoelectric point	Sialoglycoproteins	*Orosomucoid*
Protein polysaccharides	Substances of high molecular weight having many of the physical properties of a polysaccharide, but containing covalently bonded protein component(s)	Mucosubstances *Mucopolysaccharides* Mucins Mucoproteins	Blood group-substances Intrinsic substances
Glycopeptides	Substances of low molecular weight having *many of* physical properties of a peptide, but containing *covalently* bonded carbohydrate component(s)	Mucopeptides Mucins	Muramyl peptides Products of enzymic degradation of *glycoproteins.*
Peptide-*oligosaccharides*	Substances of low molecular weight having many of the physical properties of an oligosaccharide, but containing covalently bonded peptide component(s)	Mucopeptides Fucopeptides Glycopeptides	Products of enzymic degradation of *glycoproteins*

blood group substances[14]. The amino sugars are almost invariably N-acetylated, the hexosamines with acetyl groups, and sialic acid with acetyl or glycosyl groups[12-13]. Brain tissue yields a large number of glycoproteins, many of which are associated with synaptosomes, microsomes, and axons[20]. Urine contains a number of glycoproteins that are identical with, or closely related to, serum glycoproteins[19], and others that are compositions similar to those of mammalian glycoproteins in general. Uronic acid may sometimes be a component of such glycoproteins[22]. The Tamm-Horsfall protein found in urine, which has a relatively large proportion of carbohydrate as part of its structure, was originally part of the renal-tubule cells[21].

2.3. Heterogeneity in Glycoproteins

Many macro-homogeneous glycoproteins are micro-heterogeneous due to their carbohydrate moieties. Micro-heterogeneity may arise due to genetic variations as well as in the mechanism of sequence biosynthesis of the sugar chain. Catabolism of glycoproteins may also contribute to micro-heterogeneity as well as deamidation. The separation of glycoproteins containing carbohydrate moieties that are structurally very closely related requires highly refined techniques.

Differences in the structure of glycoproteins manifest themselves in a number of ways: The polypeptide chain to which the oligosaccharide moieties become attached may differ[23-25], for example, a given amino acid may be present either unsubstituted or glycosylated. Ribonuclease A and B appear to differ solely by virtue of the latter

having an oligosaccharide moiety attached through L-Asn-34 from the N-terminal end[24]. Only about 35% of the heavy chains of rabbit gamma-G immunoglobulin in a form where a glycosyl group[25] is bound to L-threonine. In collagen[26], the carbohydrate moiety occurs in part as 2-O-alfa-D-glucopyranosyl-O-β-D-Galactopyranosyl and in part as O-β-D-galactopyranosyl groups, that is, both with and without addition of *the terminal D-glucopyranosyl groups*. Variations of this type in the structure of glycoproteins are sometimes referred to as peripheral heterogeneity[29]. Analogous variations in the structure of various oligosaccharides in other glycoproteins occur; these include fetuin[30], transferrin[31], β_{2A}-glycoprotein[32], and human-serum alkaline phosphatase[33]. The extents of their heterogeneity, as revealed by electrophoresis in PAGE gels, were in all instances markedly decreased after removal of their sialic acid residues by treatment with neuraminidase.

In addition to heterogeneity of this type, which is primarily concerned with the nonreducing, terminal sugar residue(s), there is a more complicated variation in the structure of the carbohydrate moieties of glycoproteins. For example, the single oligosaccharide that occurs in hen's-egg albumin can contain differing or varied concentrations of D-Man and GlcNAc[34-35].

In summary, a polypeptide chain may have one or more oligosaccharide units attached to it. The size of the carbohydrate chain attached at different points along the polypeptide may differ radically, as in calf thyroglobulin[36], which is reported to contain nine carbohydrate chains consisting of five residues, and 14 larger chains, per

molecule of protein. The same characteristic is encountered in ox-aorta glycoprotein[37], and ovomucoid[38]. Structural variations may also occur within a carbohydrate chain attached at a specific position to the polypeptide as found in egg albumin[39]. Other variations in structure may also occur in that sialic acid or other types of sugars may be present or absent[40]. The main contributor to micro-heterogeneity on PAGE and PAG-IEF is due to the charged carboxylic group of sialic acid.

2.4. Chemico-Physico Properties of Glycoproteins

2.4.1. Carbohydrate Component

The carbohydrate composition and nature of the monosaccharide residues in typical glycoenzymes can be seen in table 2 page 14. The content of carbohydrate in most glycoproteins ranges between 2 and 50%, with the commonest residues being D-Man and 2-acetamido-2-deoxy-D-glucose. The D-Glu and D-Gal residues are next in abundance, and L-Fuc, D-Xyl, L-Ara, and sialic acid also occur in a number of glycoproteins. There does not seem to be a direct correlation between the content of carbohydrate and the types of carbohydrate moieties present in glycoenzymes, nor does there appear to be any correlation between the types of carbohydrate present and the biological origin of the glycoprotein[22].

There is a wide variation in the number of sugar residues present in glycoproteins, varying from 8 in ribonuclease B to 800 in invertase. Typical molecular structures of the carbohydrate moieties of

glycoproteins are represented in figure 1 page 15. N-Acetyl-glucosamine or D-Man residues occur at the reducing end of the carbohydrate chain as well as D-Xyl. The latter is also called a bridge-carbohydrate residue. Formulas (a) and (b) in figure 1 page 15 depict highly branched structures and are commonly found as components in mammalian glycoproteins, fungal amylase and pineapple bromelain[9]. The terminal D-mannose residues in these glycoproteins can be removed by an α-D-mannosidase, indicating that these terminal D-mannosyl residues are α-D-linked.

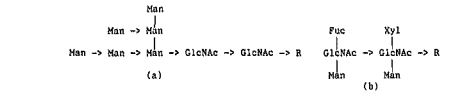
TABLE 2

Carbohydrate content of some Glycoproteins.

Enzyme	Biological source	Carbohydrate %	Monosaccharides	Ref.
alpha-Amylase	Aspergillus oryzae	3	Man, GlcNAc, Gal, Xyl, Ara	67
Glucoamylase I	Aspergillus niger	14	Man, Glc, Gal	68
Glucoamylase II	Aspergillus niger	23	Man, Glc, Gal	68
Invertase	Yeast	50	Man, GlcNAc	69
beta-D-Glucosiduronase	Bovine liver	6	?	70
alpha-D-Galactosidase I	Broad bean	25	?	71
alpha-D-Galactosidase I	Broad bean	3	?	71
Ribonuclease B	Bovine pancreas	9	Man, GlcNAc	72
	Porcine pancreas	35	Man, GlcNAc, Gal, Fuc, Sialic acid	73
Deoxyribonuclease A	Bovine pancreas	3	Man, GlcNAc	74
Deoxyribonuclease B	Bovine pancreas	4	Man, GlcNAc, Gal, Sialic acid	74
Deoxyribonuclease C	Bovine pancreas	3	Man, GlcNAc	74
Bromelain II	Pineapple	3	Man, GlcNac, Fuc, Xyl	75
Bromelain III	Pineapple	2	Man, GlcNAc, Fuc, Xyl	75
Protease b	Snake venom	16	Man, GlcNAc, Gal, Sialic acid	76
Protease A	Saccharomyces carlbergensis	7	Man, Glc	76
Protease B	Saccharomyces carlbergensis	4	Man, Glc	76
Protease C	Saccharomyces carlbergensis	20	Man, Glc	76
Protease D	Saccharomyces carlbergensis	13	Man, Glc	76
Glucose oxidase	Aspergillus niger	16	Man, GlcNAc, Gal	77
Chloroperoxidase	Caldariomyces fumago	25	GlcNac, Ara	78
Monoamine oxidase	Bovine plasma	5	?	79

FIGURE 1

Diagrammatic representation of the structure of some carbohydrate moieties of glycoproteins



Man -> Man -> Man -> Man -> Man -> Man -> GlcNAc -> GlcNAc -> R
(c)

$(Man)_{8-9}$ ->GlcNAc -> R
(d)

Man -> Man -> R
(e)

Gal -> Man -> Man -> R
(f)

R = Protein

Many glycoproteins have more than one kind of oligosaccharide chain per molecule. This is substantiated by the isolation of different types of glycopeptides from hydrolyzates of glycoproteins[73-74,97]. Variations also exist in the sequence and residues of the carbohydrate portion of the molecules[74]. The oligosaccharides are often branched since each hexose has four hydroxyl groups and each hexosamine has three hydroxyl groups available for substitution.

In some glycoproteins, such as bovine RNase B, the carbohydrate residues occur as a single chain attached to a particular amino acid namely asparagine-34. The carbohydrate chain of ribonuclease B is an octasaccharide (formula (c) in figure 1 page 15) composed of six residues of D-mannose and two of N-Acetyl-glucosamine[98]. Five of the D-mannose residues can be removed from the octasaccharide by α-D-mannosidase, indicating that the D-Man residues are α-D-linked where as the linkage between the N-Acetylglucosamine residues is β-D[90-98].

RNase from mammalian sources have several carbohydrate chains in their structures. RNase from porcine pancreas contains at least three carbohydrate chains which are attached at Asn residues 21, 34, and 76. The carbohydrate chains present at residues 21 and 76 are considerably more complex than at residue 34[87-89]. The carbohydrate chains of the porcine pancreas isoenzymes of DNase and RNase are similar[97]. Since both are from the same organ, it may be possible that the same pathways and enzymes are utilized for the biosynthesis of their carbohydrate chains.

Glycoenzymes such as invertase, D-Glucose-oxidase, and chloroperoxidase contain many carbohydrate chains. Invertase contains carbohydrate chains of high molecular weight, whose core moiety is depicted diagrammatically by formula (d) in figure 1 page 15. Two other carbohydrate core moieties of glycoproteins are illustrated by formula (e) and (f) in figure 1 page 15; these types are di- and tri-saccharide fragments of neutral monosaccharides. The carbohydrate component of glucoamylase contains oligosaccharide fragments of this type[80-84]. Many such carbohydrate fragments must be present in glucoamylase, see tables 2 and 3 pages 14 and 18 respectively. Terminal D-Gal residues have been detected in glucoamylase mainly by use of the D-Gal oxidase reaction[78].

TABLE 3

Number and Types of Carbohydrate residues for some Glycoenzymes

Enzymes	GlcNAc	Man	Glc	Gal	S.A.*	Ara	Ref.
Glucoamylase I	-	69	16	2	-	-	68
Glucoamylase II	-	128	20	3	-	-	68
Glucoamylase R	20	67	-	-	-	-	68
Invertase	40	760	-	-	-	-	94
Ribonuclease B (bovine)	2	6	-	-	-	-	95
Deoxyribonuclease A	2	6	-	-	-	-	74
Deoxyribonuclease B	3	5	-	1	1	-	74
Deoxyribonuclease C	2	5	-	-	-	-	74
Protease b (Snake venom)	14	10	-	30	9	-	79
Glucose oxidase	19	128	10	3	-	-	96
Chloroperoxidase	5	-	-	-	-	68	78

S.A.* Sialic acid (NeuAc)

TABLE 3

Number and Types of Carbohydrate residues for some Glycoenzymes

Enzymes	GlcNAc	Man	Glc	Gal	S.A.*	Ara	Ref.
Glucoamylase I	-	69	16	2	-	-	68
Glucoamylase II	-	128	20	3	-	-	68
Glucoamylase R	20	67	-	-	-	-	68
Invertase	40	760	-	-	-	-	94
Ribonuclease B (bovine)	2	6	-	-	-	-	95
Deoxyribonuclease A	2	6	-	-	-	-	74
Deoxyribonuclease B	3	5	-	1	1	-	74
Deoxyribonuclease C	2	5	-	-	-	-	74
Protease b (Snake venom)	34	10	-	30	9	-	79
Glucose oxidase	19	128	10	3	-	-	96
Chloroperoxidase	5	-	-	-	-	68	78

S.A.* = Sialic acid (NeuAc)

2.4.1.1.The Size and Number of Carbohydrate Chains

The carbohydrate chain may be as simple as a single sugar residue, or a disaccharide, or as complex as 15 sugar residues[42-45]. The carbohydrate unit in a glycoprotein is generally less than 30.0 kD, consisting of repeating units. There are exceptions, such as soybean hemagglutinin with a value of 45.0 kD[46]. The factors responsible for limiting the length of the carbohydrate chains are as yet unknown. Certain structural features such as terminal fucosyl, sialyl, α-D-galactosyl or N-Acetyl galactosyl residues, may play a role, also kinetic factors, and the distribution of the various activated sugar transferases during the passage of the nascent protein through the cisternae of the endoplasmic reticulum.

2.4.1.2.The Concept of a 'Core'

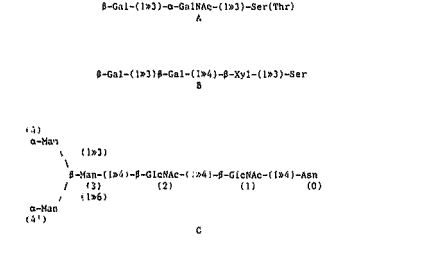
Certain sugar residues immediately adjacent to the protein carbohydrate linkage are referred to as the linkage region of the core fragment. These sequences appear to be exclusively associated with a particular type of protein-carbohydrate linkage see figure 2 page 20, i.e. Xyl-Ser, GlcNAc-Asn or GalNAc-Ser(Thr).

2.4.1.3.Significance of the Carbohydrate Moieties

The question of function for the carbohydrate chains in glycoproteins falls into two categories; biological and structural.

FIGURE 2

Oligosaccharide `Inner' Cores of Glycoproteins



(0) = Peptide Attachment
(1) = The 'Bridge' -Carbohydrate Peptide Linkage Region
(2)-(3) = Core Region
(4) = Antennae Region

2.4.1.4. Biological Function

(i) Transport

Carbohydrate residues play a role in membrane transport of glycoproteins. Many proteins destined to be secreted are glycoproteins, whereas cellular proteins are not.

The carbohydrate chain signals that the glycoprotein is destined for secretion and also signals for molecules to enter a cell. In the latter process, neuraminic acid and D-Gal residues are important as discussed before.

(ii) Immunological

Glycans of glycoproteins present on the surface of cell membranes contribute to the antigenicity of the proteins[125-128].

(iii) Inter Cell Interactions

Glycans of glycoproteins are important in the interadhesion of cells[129-131].

2.4.1.5. Structural Function

Carbohydrate residues affect intrinsic viscosity, frictional ratio, diffusion coefficient, and solubility[72] of the glycoproteins. It has been suggested that the carbohydrate residues function as protective agents for the protein moiety of glycoproteins, rendering them less susceptible to proteolysis and might also help in maintaining the tridimensional structure required for activity in glycoenzymes. A

peptide fold in the chain may be held in position through the strategic location of carbohydrate chain along the peptide, and the molecular transformation occurring with denaturation might be hindered[102-103]. In the case of susceptibility to proteolysis, the carbohydrate residues interfere with the formation of the enzyme-substrate complex, and hydrolysis of the molecule cannot occur. Removal of some of the carbohydrate residues from the glycoprotein often makes the molecule much more susceptible to enzymic hydrolysis. However, the glycoproteins that are degraded in the lysosomes appear to be an exception; the peptide components of certain glycoproteins are degraded at the same rate by lysosomal hydrolases, regardless of whether or not the oligosaccharide chains from the molecule are removed[103].

1.4.1.6. Sialic Acids

The name Sialic acid (NeuAc) was created in 1957 by Blix, Gottschalk, and Klenk. Sialic acids are also called acylneuraminic acids[141]. Sialic acids are absent from most bacteria, and are rare in viruses. Most viral sialic acids seem to be synthesized by the enzymes of the host cell.

Sialic acid mainly occur as a component of oligosaccharides, polysaccharides, and glycoconjugates. Neu Ac forms an α-glycosidic bond with galactose, N-Acetyl galactose or N-Acetyl glucose. Sialic acids are most frequently linked to Gal by α-(2->3) or α-(2->6) linkages. Sialic acids are usually the terminal residues of oligosaccharide chains of glycoproteins and other glycoconjugates of

the cell. Secreted glycoproteins occurring in serum, urine, and especially products from the mucous glands contain a considerable proportion of sialic acid[142].

Sialyltransferases are widely distributed in animals. In the transfer reactions, different α-glycosidic linkages are formed, and various sugars are known to be binding partners of the sialic acid residues.

(a) Sialidases

Sialidases, or neuraminidases, initiate the breakdown of sialoglycoconjugates and sialo-oligosaccharides. The primary product of the hydrolytic reaction is the α-anomeric form of free sialic acid, which in the case of Neu5Ac, mutarotates in aqueous solution, yielding mainly the β anomer[143]. Because sialoglycoconjugates are essential components of cells and body fluids, and are involved in specific, biological functions, sialidases may become 'toxic' enzymes when present in non-physiological amounts. Low levels of this leads to diseases, such as mucolipidosis and sialidosis. Sialidases have a wider distribution in nature than have the sialic acids. They are found in the ortho- and para-myxo viruses[144], which usually do not contain sialic acids.

(b) Function due to the Negative Charge of Sialic acids

On the basis of the accumulation of the negatively charged sialic acid residues on cell membranes, it may be expected that

these compounds strongly influence the behaviour of cells and glycoproteins. It is believed that more than 10^7 Neu residues are bound to the surface of a single human-erythrocyte[148]. This estimate agrees with the 1.8×10^7 negative charges on the surface of one human erythrocyte. Membrane sialic acid prevents aggregation due to electrostatic repulsion in blood platelets and erythrocytes. Sialic acids also contribute to the rigidity of the cell surface, as shown by the increase in the deformability of sarcoma cells after enzymic removal of sialic acid residues.

Sialic acids may initiate the binding of cationic compounds to macromolecules and cells; Sialic acids on the surface of L1210 mouse-leukemia cells have been found to influence transport of potassium ions through the cell membrane[147]; the uptake of 2-amino-2-methylpropanoic acid by HeLa cells is decreased after treatment with sialidases. Sialic acid residues are also important Ca^{2+}-binding sites in muscle cells[147].

(c) Influence of Sialic Acids on Macromolecular Structure

Removal of sialic acids from submandibular-gland glycoproteins drastically lowers their viscosity. Sialic acid residues cause repulsion of the oligosaccharide chains from the glycoprotein core, giving some glycoproteins a rod-like structure[147], and an increase in intrinsic viscosity[148].

The influence of sialic acids on the macromolecular conformation

seems to be the reason for the proteolytic resistance of several glycoproteins. An example is the loss of proteolytic resistance of the intrinsic-factor and its binding capacity for vitamin B_{12} after removal of sialic acid. Similarly the protection of dopamine β-hydroxylase by sialyl residues against proteases has been demonstrated[149].

(d) Anti-Recognition Effect of Sialic Acids

The anti-recognition effect of NeuAc has been well established on a molecular level since the discovery that it masks the D-Gal residues of serum-glycoproteins[150].

Another site, except the liver, for the recognition of desialylated glycoproteins is the bone marrow[151]. Sialic acid is involved not only in the regulation of the life-time of soluble serum glycoproteins but also of mammalian blood cells. Desialylated T-lymphocytes and thrombocytes are reversibly trapped in the liver and reappear in circulation after resialylation of their membrane glycoconjugates[152-153].

The chain length of the sialo-glycan seems not to be involved in this function, because shortening of the side-chains does not affect the viability of erythrocytes. It is considered that the charged carboxyl group plays the main protective role on sialic acid residues found in erythrocytes. The sialic acid and membrane-carbohydrate content decreases in the course of the erythrocyte life-time[154] making it more susceptible for

degradation.

There is evidence for a masking-effect of the glycoprotein sialic acids in kidney glomerular-membranes. A decrease in the sialic acid of the glomerular membranes is observed in some renal diseases, and is presumed to be related to immunological injuries to the glomeruli[153-154].

2.4.2. Protein Component

The amino acids of glycoproteins are those found in typical proteins. Most of the isoglycoenzymes have identical amino acid compositions and differ only in their carbohydrate components[83].

2.4.2.1. Conformational Structure

RNase A and B possess the same amino acid composition and sequence, and the same catalytic activity[100,117-118], but the two isoenzymes differ in that RNase B contains a carbohydrate chain at Asn-34.

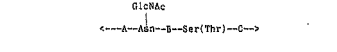
The enzyme gluco-amylase have many short oligosaccharides chains along its polypeptide. It is thought that the spacing of the chains along the polypeptide folds it into a rigid position[119]. Glycoproteins having this type of structure are extremely stable[119]. Isoenzymes of invertase denature at slower rates when the carbohydrate contents are increased[103]. Therefore, isoglycoenzymes having a high content of carbohydrate are quite resistant to denaturation.

2.4.3. Protein-Carbohydrate Linkages

The principle linkages found between the carbohydrate and the protein component of glycoproteins are of the N-glycosyl and the O-glycosyl types. The N-glycosyl bond is formed between the side-chain amino group of Asn and the hemiacetalic hydroxyl group of a N-Acetyl glucosamine at the reducing end of the carbohydrate chain. Glycopeptides having an N-glycosyl linkage between the carbohydrate and an amino acid have been isolated from many glycoenzymes, such as RNase B[27]. The O-glycosyl linkage in glycoproteins is formed between the hydroxyl group of L-Ser or L-Thr residues, and the hemiacetal hydroxyl group of the carbohydrate residue at the reducing end of the carbohydrate chain. The simplest 'glycopeptide' component of a glycoprotein would thus be an O-glycoserine or O-glycothreonine residue. The AA sequences of several glycoenzymes around the point of attachment of the carbohydrate have been worked out. (see figure 3 page 27). Several different types of glycopeptides can be isolated from the same glycoenzyme, indicating a heterogeneity for the protein-carbohydrate linkages in a single glycoprotein.

FIGURE 3

Bridge Residues for for Asn



The AA sequence around the N-glycosyl linkages is generally of the type seen in figure 3 page 27. In this depiction, A and B are unspecified amino acids. L-Ser and L-Thr residues specify the transferases responsible for the attachment of a carbohydrate chain to the polypeptide. The hydroxyl groups of L-Ser and L-Thr, together with the amide group of L-Asn, may be the acceptor substrate group in the formation of the enzyme-substrate complex. The nature of residue B in the peptide chain also affects the type of carbohydrate that becomes attached to the L-asparagine residue. If the B residue is polar, a carbohydrate moiety of greater complexity is found attached to Asn^{72}. In glycoenzymes containing O-glycosyl bonds, the carbohydrate chain e.g. in α-amylase is linked to L-Ser residues of the protein[27]. Some glycoproteins are known to contain both the N-glycosyl and the O-glycosyl bonds[116].

2.5. Biosynthesis of Glycoproteins

2.5.1. Cellular Locale and Reactions

In mammalian systems, studies with the perfused liver[104] and with isotopically labeled carbohydrates have shown that the liver is the major site of glycoprotein synthesis. Other mammalian organs and tissues, e.g. the pancreas[105], submaxillary gland[106], thyroid, retina, kidney, and mammary gland[107], are also involved in glycoprotein synthesis.

The biosynthetic pathway of glycoproteins can be divided into three distinct phases:

(1) Assembly of the Polypeptide

This takes place on the ribosomes of the rough endoplasmic reticulum (RER) via the normal routes and reactions of protein synthesis[108].

(2) The 'Bridge'-Carbohydrate Peptide Linkage

The linkage sugars of a $Glc_3Man_9GlcNAc_2$-PP-Dol unit are coupled to specific amino acid residues of the polypeptide chains by transferases using the appropriate nucleoside 5'-(glycosyl pyrophosphates). This is done while the polypeptide chain is still attached to the ribosomes[109] and is sometimes still being synthesized on the ribosomes[110]. Nucleotidyl transferases are responsible for the activation of the hexosamine by way of uridine 5'-(N-Acetyl-glucopyranosyl)[111], and glycosyl transferases[112] for the attachment of the residues to the polypeptide. The glycosyl transferases are membrane bound enzymes[113] found in the ER and Golgi.

(3) Completion of the Glycoprotein

The glycosylation of the glycoprotein is completed by the stepwise addition of carbohydrate residues from nucleoside 5'-(glycosyl pyrophosphates) by appropriate transferases[112] to the dolichol mediated partially glycosylated polypeptide. Some of the addition occurs in the region of the RER, but takes place mainly in the Golgi[114-118]. The completed glycoprotein accumulates in the Golgi prior to secretion into the circulatory system[120-121].

2.5.2. Glycosylation

2.5.2.1. Fundamental Requirements for Glycosylation

(a) Peptide-Chain Conformation

A specific amino acid sequence for linkage is necessary, but is not sufficient for carbohydrate attachment. A second requirement is that certain regions of the peptide chains must have a specific secondary structure, since, glycans are located in the loops of the peptide[132]. Loops are generally located at the surface of globular proteins, making Asn accessible to glycosyltransferases. Additional proof indicates that the carbohydrate moieties are positioned on the outside of the glycoprotein, and is in agreement with the role of recognition attributed to some glycans[132]. It is also possible that the protection against proteolysis is due to the masking of loops by the carbohydrate[133-135].

(b) Glycan Primary Structure

Glycan structures revealed certain features and for N-acetyl-lactosamine glycans the following would apply:

(1) NeuAc is bound to the C-3 and/or C-6 of the terminal Gal.

(2) Fuc is coupled to C-3 of GlcNAc when the latter is part of the N-acetyl-lactosamine chain but to C-6 of GlcNAc when the last-named is linked to Asn.

(3) The C-4 of the terminal β-Man is coupled to a GlcNAc.

(4) Substitution in the Man-4 by supplementary sugars are on C-4 for Man-4 and on C-6 for Man-4 (see Fig 2c page 20).

(c) Termination of Glycosylation

In most glycoprotein structures it is observed that the conjugation of a residue with sialic acid prevents further substitution of the glycan moiety. The same can be said for fucosyl additions, except that the fucosyl residue can itself be fucosylated. The act of sulphation of sugar residues is also alleged to be a final act of modification.

The locus operandi of glycosylation is difficult to determine. As soon as the protein is in the ER, the signal peptide (if present) is cleaved, and the first of the series of glycosylation can commence.

2.5.2.2. The Dolichol Oligosaccharide Transfer Mechanism

Dolichols are long-chain poly-isoprenoids (13-22 units) found in eukariots[157]. The dolichol kinases needed for the activation of dolichol is found in several mammalian-cell types[158-159]. The phosphate donor is CTP. The newly synthesized dolichol phosphate is then glycosylated by either GDP-Man or UDP-GlcNAc, to form the monosaccharide lipid derivatives[160]. Dolichols differ in chain lengths, and therefore dolichol glycosyltransferases differ[161]. The α-isoprene unit of polyprenol phosphate acts as the acceptor of glycosyl moieties[162].

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GDP-Man + Dol-P --> Man-P-Dol + GDP

UDP-Glc + Dol-P --> Glc-P-Dol + UDP

UDP-GlcNAc + Dol-P --> GlcNAc-PP-Dol + UMP

UDP-Glc + Dol-P --> Glc-PP-Dol + UMP

The transfer of the lipid-linked fragment oligosaccharide from Dol-PP to the appropriate Asn on the polypeptide is catalyzed by oligosaccharyltransferase[170-171].

(a) Synthesis of the Donor Oligosaccharide Fragment

The dolichol linked D-mannosyl, D-glucosyl, and GlcNAc residues are used for the synthesis of the fragment. Fourteen sugars are transferred stepwise from nucleotide or dolichol-phosphate carriers to the poly-isoprenoid, Dol-P, to generate $Glc_3Man_9GlcNAc_2$-PP-Dol. The synthesis of the oligosaccharide-lipid donor proceeds in an ordered fashion[163-166]. The lipid-linked oligosaccharide biosynthesis occurs in the ER. $Man_5GlcNAc_2$-PP-Dol is synthesized on the cytoplasmic side of the ER, and then translocated to the lumenal side. The mature precursor, $Glc_3Man_9GlcNAc_2$-lipid, is then completed on the lumenal side where it serves as the donor in polypeptide glycosylation.

(b) Transfer of Donor Oligosaccharide Fragment to the Peptide

The donor oligosaccharide fragment from $Glc_3Man_9GlcNAc_2$-PP-Dol is transferred to the polypeptide. Other fragments as small as $GlcNAc_2$-PP-Dol and $Man_1GlcNAc_2$-PP-Dol can also serve as donors[171-173].

Transfer of the dolichol donor to the acceptor protein takes place in the lumen of the RER. For this reaction to occur the polypeptide must be translocated from the cytoplasm into the lumen[100].

(c) Trimming of the Transferred Dol-Donor Glycan

Transfer of the donor fragment $Glc_3Man_9GlcNAc_2$ to the protein is followed by trimming to a glycan containing only three mannose residues. To the trimmed donor fragment sialic acid, Gal, and GlcNAc is stepwise added directly by transferases. The subcellular localization of these enzymes responsible for glycan trimming is the ER[177-183].

(d) Branching

The final steps in the synthesis of lipid-linked oligosaccharide are the coupling of two Glc residues via α 1,3 and α 1,2 to terminal Man using Dol-P-$Glc^{1\alpha 3-1\alpha 2}$-Glc[184].

(e) Addition of Rare Sugar Moieties

The further elongation of the carbohydrate chain after the attachment of the trimmed Dolichol fragment occurred via enzymic transfer of single sugar residues from glycosyl esters or nucleotides to the non-reducing, terminal positions of the growing chain[45-49]. Transferases acting upon uridine 5'-(D-galactopyranosyl pyrophosphate)[50], and 5'-(D-glucopyranoside pyrophosphate)[51] result in the formation of β-D-galactosyl and β-D-glycosyl groups being respectively added to the carbohydrate chain.

The incorporation of sulphate into the carbohydrate chain of glycoproteins occurs enzymically, after the sugar chain was completed, and is a post-ribosomal event[52-54].

(f) Maturation of Glycans

Finally, the chain-branches NeuAc-Gal-GlcNAc and Fuc are added prior to the appearance of the mature glycoprotein at the plasma membrane[139-140].

2.5.2.3. Topography of Glycosylation

Oligosaccharide transferases and the $Glc_3Man_9GlcNAc_2$-PP-Dol are located on the lumen of the RER[184-192]. Peptides are only glycosylated if they are inserted into microsomal vesicles and secreted into the lumen[185-190].

2.5.3. Carbohydrate-Peptide Linkages Specificity

A fairly high degree of specificity is involved in the coupling of the carbohydrate moiety. An L-Asn moiety in a polypeptide is an acceptor of only GlcNAc[47]

2.5.3.1. Synthesis of N-linked Glycans

The oligosaccharide is transferred from the Dol-carrier as a fragment to an Asn residue of protein in the lumen.

2.5.3.2.Synthesis of O-Linked Glycans

The total number of Man residues found in O-linked oligosaccharides approximates that found in the N-linked glycans. The first Man is transferred with inversion of configuration, from Dol-P-Man to Ser or Thr in the ER. Up to four Man can then be transferred from GDP-Man in the Golgi[174]. Further elongation and modification of the glycan seems to equivalent these processes found for N-linked glycans.

2.5.4.Glycoprotein Sorting and Secretion

An important aspect of the metabolism and synthesis of glycoproteins is the secretion of these molecules through the cellular membrane. Some of the terminal sugar residues become attached to the glycoprotein as the protein passes through the plasma membrane. For example, in the synthesis of gamma-globulin by lymph-node cells, the terminal N-acetylneuraminic acid residue is added to the molecule, both inside and outside the cell, at essentially the same rate[107]. The sialic acid is thus added to the glycoprotein at the plasma membrane during passage of the macromolecule through the membrane[122]. Similar, the D-glycosyl transferase involved in attaching terminal D-glycosyl residues in the biosynthesis of collagen, is found exclusively on the plasma membrane[123].

The carbohydrate portion of a glycoprotein is thus a marker for the transport and acts as a director of these molecules to their final destination.

There are two different mechanisms whereby complete glycoprotein molecules are secreted from cells. (i) Molecules accumulate in the Golgi membranes and are exocytosed as molecule packets by way of Golgi vesicles[108]. (ii) Glycoproteins are transported through channels in a continuum of membranes, passing sequentially from RER to SER, to Golgi bodies, and to the plasma membrane. There occurs a successive addition of terminal carbohydrate residues to the molecule with terminal sialic acid being added at the last membrane before the glycoprotein is released into the circulatory system[124].

With the exception of the mitochondrial and some nuclear proteins, synthesis of all proteins destined for noncytoplasmic locations, including those retained in the endoplasmic reticulum and the Golgi, begins in the ER.

ER ---> Golgi body ---> Vesicles ---> Cell surface.

2.6.Glycoenzymes

2.6.1.Introduction

Glycoenzymes are glycoproteins, most being isoglycoenzymes, which differ only in the carbohydrate portion of the molecules. The hydrolase group of enzymes contains the largest number of glycoenzymes, with examples such as yeast invertase, fungal amylase, glucoamylase, various ribonuclease, and gluco- and galacto-sidases[63-65].

The interglycosidic bonds of the carbohydrate chains in most glycoenzymes have the α-D (L) conformation. Structures that are highly branched are often encountered in the carbohydrate chains of glycoenzymes.

2.6.2.N-Acetyl-β-D-Glucosaminidase; NAG

2.6.2.1.Introduction

The enzyme N-Acetyl-β-D-glucosaminidase (E.C.3.2.1.30, NAG) is a sialylglycoprotein that is present in abundance in the epithelium of the proximal renal tubule. NAG is involved in the catabolism of glycolipids, glycoproteins, and glycosaminoglycans[217].

N-Acetyl-β-D-glucosaminidases are known to exist in multiple forms. Various isoenzyme forms have been isolated in urine and tissue[201]. The biochemical function of the different forms of NAG remain unknown, and there is no clear relationship between the different forms. Interest in the isoenzymes were greatly stimulated by the demonstration of a deficiency of one or both of the A and B isoenzymes in certain diseases, and also by the appearance of NAG in the urine as a kidney pathological indicator[201].

2.6.2.2.Physical Properties and Occurrence

NAG is widely distributed in many tissues, abundant in organs where high rates of mucoid turnover might be expected, particularly rich in the kidney[222]. Within the nephron, NAG activity is very high mainly

in the epithelium of the proximal convoluted tubule[223]. NAG is a hydrolase[213] with molecular weight of 112.0 kD[216] to 140.0 kD[223], determined by exclusion chromatography, and 27.2 kD for Streptomyces griseus[335] determined by sedimentation equilibrium analysis.

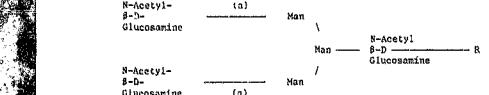
At least eight isoenzymes have been identified in tissues and body fluids on the basis of molecular charge, thermal stability, and pH optimum[201]. The two major isoenzymes, called isoenzyme A and B respectively, have been isolated by electrophoresis[201,222,214-218]. The sialic acid contents and stability are different from each other. By ion-exchange chromatography at least up to six different fractions having NAG activity has been isolated from human urine[214-219,223]. Up to date and to my knowledge no NAG isoenzyme has been purified to homogeneity. In Tay-Sachs disease NAG A is missing but the activity of NAG B is increased[202-204,234]. In Sandhoff's disease, both the A and B isoenzymes are lacking[203-204,234].

2.6.2.3. Biochemical Functions

NAG is important in the breakdown of mucopolysaccharides and of glycoproteins. NAG catalyses the hydrolysis of the terminal N-Acetylglucosaminyl moieties from glycopeptides and polysaccharides, or from synthetic substrates in which the amino sugar is linked to a chromophoric group by a β-glycosidic linkage[199-200]. NAG is believed to specifically catalyze the hydrolysis of a terminal N-acetyl-β-D-glucosamine residue from a β-D-glycosidic linked Man[251] (refer to figure 4).

FIGURE 4

The substrate site of Glucosamine susceptible to NAG catalytic attack



R = Rest of Carbohydrate Chain
(a) = Bond cleaved by the hydrolytic action of NAG

It has been suggested that terminal N-Acetylglucosamine of glycoproteins serve as a lysosomal recognition marker of lysosomal hydrolases[205].

Structural studies of oligosaccharides released in the urine of patients with oxoglycosidase deficiencies have disclosed that all these compounds possess in common the structural feature of Man(β1-4)GlcNAc at their reducing end[255]. On this basis it is postulated that the cellular degradation of N-glycans is initiated by the splitting of a glycan moiety by N-acetyl-β-D-glucosaminidase[256-259].

2.6.2.4. NAG as a Urinary Indicator Enzyme

With the description of increased activities in the urine of patients with kidney diseases the use of urinary enzymes for diagnostic purposes was introduced[236,244]. It was realized that various kidney diseases - tumors excluded - may cause increased urinary enzyme

levels. At least 45 enzymes for the diagnosis of urorenal diseases have been identified so far[217].

Urinary NAG, known to increase in patients with renal disease, was investigated as an indicator in diagnosis of various types of renal diseases[210,224-226], as an indicator of rejection after kidney transplantation[227-228], and also in the assessment of nephrotoxicity of drugs in man[229-233,253-254]. The urinary excretion of NAG has been considered to have diagnostic usefulness in renal disturbances in association with various other diseases, i.e. diabetes mellitus[262] and hypertension[233]. Urinary NAG thus gained importance, in addition to other urine parameters, such as α_1- and β_2-microglobulins[245], retinol binding protein, albumin, transferrin, immunoglobulins, and the classical creatinine[245,246].

Investigations of Prince[242] in 200 patients with renal transplants and 1500 determinations of NAG in urine revealed that in 70% of the cases with an acute rejection of the transplant[240,241] an increase of NAG was evident 1 to 3 days before any other test parameter turned positive. Neither immunosuppresives, diuretics, antibiotics, nor hypotensive substances - except Gentamycin[251] - increased urinary NAG activity[247]. There is still controversy over the question if *Cyclosporin elevates urinary NAG levels*[260].

Definitive diagnosis of acute renal transplant rejection is often difficult, and may pose impossible if oliguria follows transplantation. The renal tubular enzyme NAG is released into the urine following renal injury, and may be seen as an index to tubular

damage. Daily measurement of urinary NAG has been proposed as an aid in the diagnosis of transplant rejection[209]. Urinary NAG reflects not only renal injury, but also blood sugar levels or blood sugar control, and even indicates development of microangiopathic changes[211-213].

Drugs such as Cyclosporin, aminoglycoside antibiotics, and other drugs which are known to cause renal tubular damage[220-221], has been studied. No specific tendency is observed. NAG increases after administration but returns to the normal levels[222,220-241].

The importance of NAG measurement in the urine is:

(a) Early detection of tubular kidney damages, e.g. diabetes, hypertension, ect.

(b) Monitoring of acute and chronic renal diseases.

(c) Monitoring of renal function during therapy with nephrotoxic drugs.

(d) Testing for rejection reactions after renal transplants.

2.7. Nephrotoxicity

A combination of biochemical events contribute to the susceptibility of the kidney to nephrotoxins[262]. Investigations have revealed that frequently the initiating chemical moiety is a metabolite of the ingested toxin. This metabolite may be produced in the kidney as a result of renal metabolism of the ingested toxin[264]. The key event in nephrotoxicity of certain compounds appears to be due to the ability of the kidney to accumulate compounds to concentrations sufficient to

induce organ-specific damage[206]. The biochemical function of the kidney renders it susceptible to a variety of nephrotoxins. Nephrons are present in abundance and the human can function well with only one kidney. This remarkable reserve capacity poses great difficulties in the experimental detection of tubular stresses at low doses, and at the present there is no satisfactory method to evaluate subtle tubular toxicity in man.

The kidney is uniquely susceptible to chemical toxicity, partially because of its disproportional high blood flow - 25% of cardiac output[207]. The proximal tubule is the predominant site of action of nephrotoxins[262]. The mechanisms by which chemicals produce renal damage are numerous and complex. Various biochemical events contribute to the susceptibility of the kidney to several classes of nephrotoxicity. With damage only low molecular weight proteins derived from serum, such as β_2 micro globulin, lysozyme, retinol binding protein, and α-microglobulin, that can pass through the glomerulus are released into urine. Because urinary NAG with such a large molecular weight is too large to pass through the renal glomeruli[118], it is assumed to be derived from the parenchyma of the tubuli[206,214].

In cases with nephrotoxic syndrome urinary NAG is significantly higher. A possible explanation is that the degeneration and breakdown process of renal tubular epithelial cells is inversely related to the process of reabsorption through renal tubuli of small proteins filtered through the glomeruli[214]. If the elevated urinary NAG levels are really due to the breakdown process, then urinary β_2 micro-

globulin, (an index of tubular damage), would also be elevated because no reabsorption can occur[215]. In practice elevated urinary NAG levels were accompanied with normal to slightly elevated urinary β_2 micro-globulin levels. Thus, urinary NAG in nephrotoxic syndromes could not be due to tubular damage alone. Nephrotoxic syndrome is also caused by damage of the glomerular basal membrane and urinary NAG most likely originates from the glomeruli[216-217].

The importance of measurement of NAG in tissue and in urine is to be of interest in the early detection of tubular kidney damages, for the monitoring of renal function during therapy with nephrotoxic drugs, and for the testing of rejection reactions after renal transplants[220]. In pathological conditions in the tubular interstitial region of the kidney, an elevation of the NAG concentration can be seen in the urine. The rise in excretion of NAG is accompanied by the appearance of the B isoenzyme of NAG, which is normally absent from urine and serum, although present in kidney tissue[210].

A method to detect subpathogenic stress would also permit the investigation of nephrotoxin activity modification in humans. The use of a urinary enzyme in renal toxicology is an old idea that has never found acceptance due to the failure of various advocates to deal adequately with a number of difficulties inherent in this application[206].

CHAPTER 3 EXPERIMENTAL RESULTS

3.1. Experimental Approach to the Isolation and Purification of Baboon Kidney NAG

One of the problems continually facing biochemists is the separation and purification of biological compounds from a mixture of compounds. Although the biochemist may be primarily interested in studying processes at the molecular level, his findings must be related to and interpreted at the subcellular and cellular level, the level of the organ, tissue, and whole organism. In order to determine the composition, mode of action and role of these compounds, sophisticated qualitative and quantitative analytical techniques have to be employed.

Important in the isolation and purification of any compound, is to yield a product that is biologically inviolated, and thus as close as possible to the natural occurring compeer. One of the most convenient methods for achieving such separations is the use of chromatographic techniques. Several chromatographic methods may be used sequentially to achieve purification of a compound, such as:

(a) Adsorption chromatography
(b) Countercurrent distribution and partition chromatography
(c) Ion-exchange chromatography
(d) Exclusion chromatography and
(e) Affinity chromatography.

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Glycoproteins tend to have a charisma for some techniques, and an

animosity for others. Depending on various features of glycoproteins, one being carbohydrate content and composition, one has to select from all the techniques available the most applicable ones, in order to ensure that certain criteria are met for the isolation and purification procedures. Homogeneity can be determined using various techniques, but once again, these techniques all have limitations, with deviations occurring when certain requirements are overhauled. It is thus of the uttermost importance to evaluate results in this respect, and also to try and consolidate results so as to gain the most information out of it.

3.2.Subcellular Distribution of NAG Isoenzymes

3.2.1.Collection and Storage of Baboon Kidneys

Kidneys were obtained from Baboons (Papio ursinus) used in transplant experiments. The mean wet weight of one baboon kidney was 25 g. Intact kidneys were snap-frozen in liquid nitrogen, and stored at -20°C until sufficient material was obtained. The use of frozen kidneys was preferred since this added to the process of disrupting cell membranes.

3.2.1.1.NAG Enzyme Assay

NAG activity was determined using the Para-Nitrophenol (PNP) method where the PNP released from the PNP-NAG Substrate, (4-Nitrophenyl-N-acetyl-β-D-glucosaminide, Boehringer Mannheim GmbH), was measured by photometry in alkaline solution at 405 nm. The procedure is as

follows: The substrate solution contained 1 mg PNP-NAG substrate in 1 ml citrate buffer, 100 mmol/l, pH 4.5. The reaction was carried out at 37°C as follows: Preincubate ½ ml. of Citrate buffer, 100 mmol/l pH 4.5, and 100 µl of the sample to be determined, at 37°C for 5 minutes. Add ½ ml. of the substrate solution, and allow the reaction to continue for a fixed time between 5 to 30 minutes. Add 2. ml of Boric acid buffer, 200 mmol/l pH 9.8 and measure the absorbance at 405 nm, using a Pye-Unicam SP8-400 UV/VIS Spectrophotometer. Distilled water was used in the place of the sample and substrate solution, in order to obtain sample and substrate blanks respectively. Activity was calculated using the following equation:

eq. 1

$$\text{NAG activity (U/L)} = \frac{\text{Abs.}_{405\ nm} \times \text{Total vol. (ml)} \times 1000}{\varepsilon \times \text{Time (min)} \times \text{Sample vol.(ml)} \times \text{Lpl(cm)}}$$

$\text{Abs.}_{405\ nm} = \text{Abs.}_{\text{Sample}} - \text{Abs.}_{\text{blank}}$
Total volume = 3.1 ml.
$\varepsilon_{405} = 18.5\ \text{mmol}^{-1}\ \text{cm}^{-1}$ for PNP
Time = Reaction time in minutes.
Sample volume = 100 µl.
Light path length (lpl) = 1 cm.
1000 : A coefficient for conversion from U/ml to U/l.

3.2.1.2. Modification of NAG Enzyme assay for Microtiter Plates

To perform the above NAG enzyme assay in a microtiter plate well, the enzyme assay was scaled down so that the total reaction volume did not exceed 0.16 ml, the well volume. A simple way was to divide all of the volumes of the standard assay by twenty, yielding a total volume of 0.155 ml. In one microtiter plate 96 reactions could be

performed concurrently. Plates were incubated between two Shandon water cooling/heating plates, connected to a constant temperature water bath. A four channel microplate pipettor (NICHIRYO Model 8400) was used to make transfer times between wells almost negligible. The plates were then read using a EASY ELIZA Reader EAR 400 (SLT Laboratory Instruments Austria), fitted with a 405 nm light filter.

In order to ensure that values obtained from the Microtiter plate method, using the EASY reader, correlate with the standard NAG enzyme method, 25 NAG enzyme assays were concurrently determined in duplicate. Using Pearsons correlations coefficient, the data had a r value of 0.9972 for N=25. Because the correlation between the two methods was so good the microplate enzyme determination method was accepted and used for determination of enzyme activity of column effluents, and also for kinetic studies.

3.2.1.3. Protein Concentration Determination by Bicinchoninic Acid

Protein concentration was determined using the Bicinchoninic acid (BCA) method of Smith et al[248]. The method is more specific, stabile, and sensitive than the Lowry method for protein determination[248].

3.2.1.4. Materials and Methods

Reagent A consisted of an aqueous solution of 1% BCA, 2% Na_2CO_3, 0.16% Na-tartrate, 0.4% NaOH and 0.95% $NaHCO_3$. The pH was adjusted with 0.1 M NaOH to pH 11.25. Reagent B consisted of 4% $CuSO_4$ in distilled water. Albumin, (Albumin Bovine, Sigma A-7030, MW=68.0 kD)

A standard working reagent (SWR) was prepared by mixing 100 volumes of Reagent A with 2 volumes of Reagent B.

The standard assay procedure consisted of mixing 1 volume sample (standard or unknown) with 20 volumes of SWR. The solution was incubated at 30°C for 30 min. Samples were cooled to room temperature and their absorbances were measured at 562 nm versus a reagent blank. Concentration of unknowns were determined from a plot of concentration (20 to 200 µg protein) against absorbances obtained for the standard protein solution.

3.2.2. *Preparation of the Subcellular Fractions*

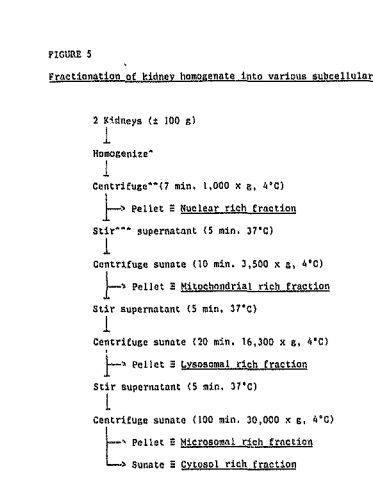
Separation of the kidney into subcellular fractions was done by modification of the methods of Shibko & Tappel[267], and Williams and Wilson[269].

Two frozen kidneys, approximately 100 g, were homogenize for 3 min. at high speed in 10% w/v 10 mM Tris-HCl buffer, pH 7.4, containing 0.25 M sucrose, 3 mM $CaCl_2$, and 1 mM PMSF, in a Waring blender at 10°C. Differential centrifugation was carried out according to figure 5 page 49.

Each of the obtained subcellular rich fractions were then suspended in 10 mM Tris-HCl pH 7.4 buffer containing 0.4 M NaCl, and stirred for 2 hours at 37°C.

FIGURE 5

Fractionation of kidney homogenate into various subcellular fractions



^ 10% w/v 10 mM Tris-HCl buffer pH 7.4, 0.25 M sucrose, 1 mM PMSF, 3 mM $CaCl_2$
^^ MSE-50 Superspeed, angle rotor.
^^^ IKA Stirrer (Janke & Kunkel)

Fractions were subjected to centrifugation for 10 min. at 1000 x g, and the supernatants retained. Each pellet was then washed twice with aliquots of 10 mM Tris-HCl pH 7.4 buffer without NaCl, and centrifuged as before. Centrifugatic, was done for 10 min. Sunates

were combined and dialyzed overnight against the Tris-HCl buffer without NaCl and centrifuged as before.

Protein concentration was determined using the BCA method[248], and total NAG enzyme activity by the standard NAG enzyme assay.

The two major NAG isoenzymes, A and B, were separated by using a Whatman ion-exchange DE 81 disk fitted in a Swinnex-Millipore filter holder. The sunate of each fraction was washed through the DE 81 filter three times. This caused NAG A to adhere to the filter paper. The NAG B rich rinses were accumulated. NAG A was released from the filter paper by washing it twice with aliquots of 10 mM Tris-HCl buffer, pH 7.4, containing 0.8 M NaCl. Total protein concentration and NAG enzyme activity was then determined for all the fractions.

3.2.2.1. Results and Discussion

Protease activity was minimized by the addition of 1 mM PMSF in the homogenization buffer. Isotonic sucrose and $CaCl_2$ was used to prevent the swelling and bursting of subcellular particles.

The release of membrane associated NAG from the subcellular particles was obtained by stirring it in the said buffer containing 0.4 M NaCl as a chaotrophic agent. Dialysis was performed against the same NaCl free buffer since NaCl prevents the binding of NAG-A to DE-81 as well as to precipitate high ionic soluble proteins. Results expressed as specific activity is presented in table 4 page 51.

TABLE 4

Percentage Specific Activity Distribution of NAG

Subcellular rich fraction	NAG A	NAG B	Total NAG	Ratio NAG A:B
Nucleus	3	3	6	1 : 1
Mitochondria	10	15	25	1 : 1.5
Lysosomes	22	18	40	1.2 : 1
Microsomes	11	5	16	2 : 1
Cytosol	4	9	13	1 : 2

From figure 6 page 52 it can be seen that the highest level of NAG was found in the lysosomes (40%). It appears from the ratio of NAG A:B that the mitochondria contains more of B and the lysosomes or microsomes more of A. The idea that A comes exclusively from lysosomes and B from the cytosol was not supported by the results and therefore the hypothesis that A is a secretory product and B an necrosis product was not substantiated.

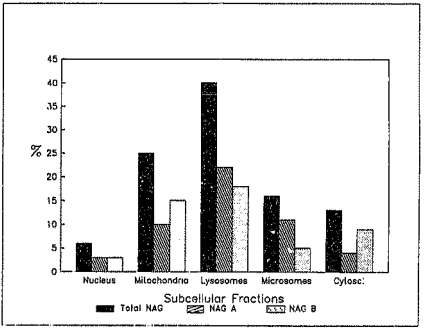


FIGURE 6

A histogram depicting the subcellular distribution of total NAG, NAG A isoenzyme species, and NAG B isoenzyme species. Note that the total NAG enzyme activity was found predominantly in the lysosomal fraction.

CHAPTER 4

PURIFICATION OF THE NAG ISOENZYMES

NAG was isolated from the kidney since it has been found in abundance in the epithelium of the proximal renal tubule[270-272]. Purification of the NAG isoenzymes was applied in such a way as to make use of the differences in molecular charge of the isoenzymes[273-276]. This was best achieved by using ion exchange chromatography[277-281].

Step 1:Homogenization and Extraction procedures

Twelve frozen kidneys, with a total average mass of 330 g, were allowed to thaw to room temperature. 25% w/v ELGASTAT UHQ (Ultra high quality) distilled H_2O containing 1 mM PMSF was added, and the mixture was homogenized at 10°C for 5 min at maximum speed, with a Waring blender. The solution was then stirred at 37°C at 300 rpm, with an IKA stirrer. After 2 hours dry NaCl was added to the solution to reach a final concentration of 0.4 M. The solution was once again stirred for 2 hours at 37°C[269].

Centrifugation was done at speeds corresponding to 30,000 x g for 40 min. in either a MSE Superspeed 50 centrifuge (25,000 rpm for 50 min.) or an MSE Hi-Spin 21 centrifuge (20,000 rpm for 1 h), both fitted with an 8 x 50 ml. angle rotor. Supernatants were accumulated, transferred to dialysis tubes (MW. cut-off between 12.0 - 14.0 kD) and concentrated with polyethylene glycol (PEG 10.0 kD; Merck) to a volume of 200 ml.

4.1:1.Results and Discussion

Inactivation of serine type proteases was achieved with the addition of PMSF. Water as hypotonic agent was used to burst the cells.

Dehydration of the supernatant against PEG was used as a milder concentration technique since freeze drying was found to inactivate NAG.

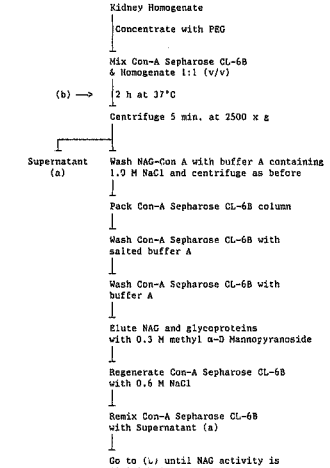
Step 2:Con-A Sepharose Affinity Chromatography.

Concanavalin-A Sepharose (15g Con-A Pharmacia) was preparatively washed with a 20 mM acetate buffer pH 5.8, containing 2 mM $MnCl_2$ and 2 mM $MgCl_2$ (buffer A). Concentrated supernatant from Step 1 was mixed in a 1:1 ratio with the Con-A Sepharose. This solution was carefully stirred for 2 hours at 37°C, and then centrifuged on a Beckman desktop refrigerated TJ-6 centrifuge for 5 min at 2500 x g. The supernatant were kept and repeatedly subjected to affinity adsorption by Con-A Sepharose. The Con-A gel pellet was washed by mixing it with buffer A containing 1 M NaCl (Salted buffer) to remove unabsorbed proteins. The Con-A was then packed in a 2 x 20 cm column. Two column volumes of salted buffer and 2 column volumes of buffer A were passed consequently through the column at a flow rate of 30 ml/h.

Adsorbed NAG and glycoproteins were eluted from the Con-A Sepharose with buffer A containing 0.3 M methyl α-D Mannopyranoside (Merck M 3752). Fractions of 3.0 ml were collected and monitored at 280 nm. NAG enzyme activity was determined using the microplate method.

FIGURE 7

Flowchart for Con-A Sepharose affinity extraction of NAG from the homogenate



Buffer A:
20 mM Acetate buffer pH 5.8, containing 2 mM $MnCl_2$ and 2 mM $MgCl_2$

Salted Buffer A:
Buffer A containing 0.4 M NaCl

Tubes containing NAG activity were combined and concentrated to a final volume of 5 ml using a Millipore CX-30 ultra concentrator.

The Con-A was regenerated by washing it with 0.6 M NaCl and equilibrated with buffer A. The supernatant subjected previously to Con-A Sepharose adsorption was once again mixed with the regenerated adsorbant. This process was repeated 2-3 times, designated as C_1 to C_3, until no NAG enzyme activity was detected in the remaining supernatant - see figure 7 page 55 for detail.

4.2:1.Results and Discussion

Con-A specifically binds glycoproteins that contain the trimannose N-linked structure Man α1->6 (Man α1->3) Man[207], and thus particularly binds α-D-mannosidyl, α-D-glucosidyl, and other sterically similar residues[291]. Lloyd has shown that bi-antennary complex structures, and not tri- or tetra-antennary complexes or linear carbohydrate chains, bind to Concanavalin-A[292].

Con-A Sepharose was used to extract glycoproteins from the crude kidney mixture. $MnCl_2$ and $MgCl_2$ were added to the buffer since Con-A Sepharose CL-6B contains a binding site for Mn^{2+} and Mg^{2+} ions. Removal of these ions inactivates the lectin.

The Con-A Sepharose CL-6B was washed with the NaCl rich buffer before Man rich glycoproteins were released since NaCl in the buffer (0.1M to 1.0M NaCl) sets glycoproteins with low affinity for Con-A Sepharose CL-6B free[293].

Adhered glycoproteins were eluted from the column with methyl α-D Mannopyranoside. Eluted profiles C_1, C_2 and C_3 differed in peak height, and profile symmetry, with the C_1 peak being more distinct and less tailing than C_3, while the C_2 profile was just the opposite in having a flat peak and long tail (See figures 8 and 8(b) pages 58 and 58(b)). Attempts were made to selectively elute glycoproteins from the column with linear methyl α-D mannopyranoside gradients (0 -> 0.6 M) but this resulted in broad, tailed peaks. Chromatography of the second wash of the kidney homogenate on Con-A, designated C_4, can be seen in figure 8 page 58.

Poorer separation and elution of the glycoproteins from Con-A Sepharose C_L-6B were obtained when a 50 mM Tris HCl pH 7 buffer substitute buffer A.

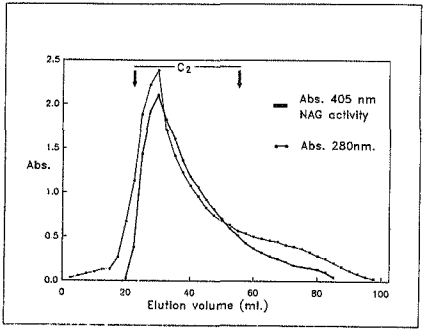


FIGURE F

Con-A Sepharose affinity chromatography of the kidney extractions resulted in the release of mannose rich glycoproteins (including NAG) upon column elution with methyl α-D-mannopyranoside.

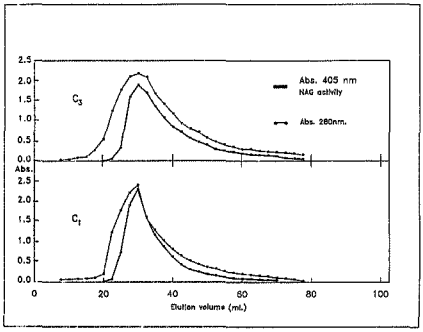


Figure 8(b)

Con-A Sepharose affinity chromatography of the kidney extractions C_1 and C_3

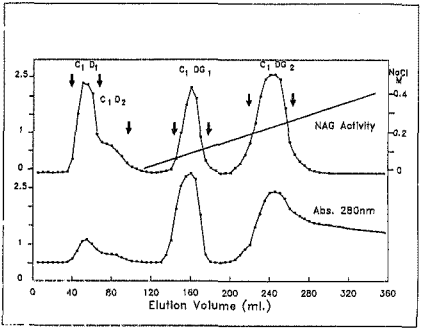


FIGURE 9

Chromatography on DEAE-Trisacryl M of the Con-A Sepharose NAG active peak resulted in the separation of the two isoenzymes. NAG B eluted as one major peak (C_1D_1) having a shoulder peak (C_1D_2) as a minor contaminant. NAG A eluted from the column with a linear NaCl gradient, resulting in two NAG A fractions designated as C_1DG_1 and C_1DG_2.

Step 3:Separation of NAG Isoenzymes on DEAE-Trisacryl M

DEAE-Trisacryl M (LKB) was washed with 20 mM potassium phosphate pH 6.0 and packed in a 2 x 40 cm column, regenerated with the same buffer containing 0.5 N NaCl and afterwards equilibrated with the same buffer without the NaCl.

Each of the C_1 to C_3 concentrates was individually submitted to DEAE-Trisacryl M anion exchange chromatography. Three millilitre fractions were eluted with 20 mM potassium phosphate pH 6.0 buffer (buffer B). The flowrate was 30 ml/hour. After 120 ml was collected, a linear NaCl gradient (0 -> 0.4 M) was applied (100 ml buffer B + 100 ml buffer B containing 0.4 M NaCl). The fractions were monitored at 280 nm, and for enzyme activity. Collected peaks were concentrated to a maximum volume of 5 ml with a Millipore CX-10 ultra concentrator. Protein concentration, using the Bicinchoninic acid method[26a], and total NAG activity, using the standard NAG enzyme assay, were determined for all the peaks.

4.3:1.Results and Discussion

Glycoproteins carrying a negative charge at neutral pH will bind to DEAE-trisackryl M. Glycoproteins with high Sialic acid content are usually strongly bound and elute with high salt concentrations. Charge heterogeneity due to variation in Sialic acid content can give rise to broadening of the peaks[30a].

Separation of the isoenzymes on the anion exchanger DEAE-Trisacryl M is due to the difference in molecular nett charge, resulted in the NAG B isoenzyme not binding to the exchanger at all. NAG B resulted in a large symmetical peak C_1D_1 and a small shoulder peak C_1D_2. Selective desorption of the retained NAG A isoenzyme was done with the application of a NaCl gradient. Two clear-cut symmetrical NAG A peaks, C_1DG_1 and C_1DG_2 were eluted - see figure 9 page 59. The respective C_2 and C_3 fractions differed slightly in symmetry and area of the peaks.

DEAE Sepharose CL-6B was used in previous isolations, but was found to be less effective in isoenzyme separation.

4.3:2.Pooling of Isoenzymes

Matching fractions were combined, eg. $C_1D_2 + C_2D_2 + C_3D_2$ were combined to form $C\text{-}D_2$, and were then concentrated to a maximum volume of 5 ml using a Millipore CX-10 ultra concentrator. Total NAG activity and protein concentration were determined as before.

Step 4:Purification procedure for NAG A

4.4:1.Rechromatography of the NAG A Isoenzymes on DEAE-Trisacryl M

The NAG A isoenzyme fractions, i.e. $C\text{-}DG_1$, and $C\text{-}DG_2$, were subjected to DEAE-Trisacryl M rechromatography with a shallow NaCl gradient (150 ml 20 mM buffer B + 150 ml buffer B containing 400 mM NaCl). A flowrate of 30 ml/h was applied. The NAG active peaks were pooled,

concentrated, and activity and protein content was determined as before.

4.4:2. Results and Discussion

Rechromatography on DEAE-Trisacryl M of the A isoenzyme fractions, C-DG_1 and C-DG_2, with a shallow NaCl gradient eliminated some inactive protein peaks. Active peaks were designated with an additional (R) to indicate rechromatography on DEAE. See figure 10 page 63 for DEAE-Trisacryl M rechromatography of C-DG_1, and figure 11 page 64 for DEAE-Trisacryl M rechromatography of C-DG_2, yielding C-DG_1(R) and C-DG_2(R) respectively.

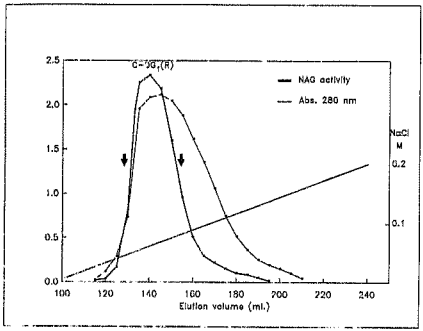


FIGURE 10

Rechromatography of the NAG A isoenzyme fraction C-DG$_1$ on DEAE - Trisacryl M. A more shallow NaCl gradient of 20 mM (150 ml) to 200 mM (150 ml) was applied to the column resulting in one single NAG enzyme active peak C-DG$_1$(R) to be eluted.

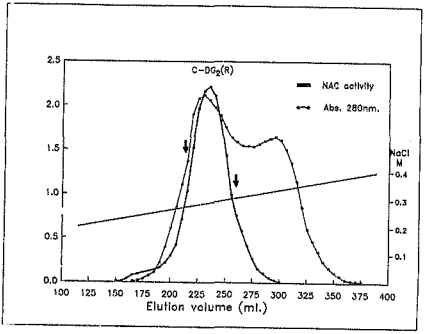


FIGURE 11

Rechromatography of the NAG A isoenzyme specie C-DG_2 on DEAE-Trisacryl M. Enrichment of the NAG A isoenzyme fraction C-DG_2 was achieved by rechromatography with a shallow NaCl gradient of 200 mM (150 ml) to 400 mM (150 ml). One NAG enzyme active peak C-DG_2(R) was eluted from the column.

Step 5:Hydroxylapatite Chromatography

Bio-Rad Hydroxylapatite (HTP) was suspended in 20 mM buffer B and allowed to settle for 30 min. A cloudy upper colloid was decanted, and a 2 x 60 cm column was packed. Two column volumes of buffer, at a flowrate of 20 ml/h were pumped through the column. The NAG A isoenzyme fraction C-DG_1(R) was applied to the column and 3.0 ml fractions were collected. After one column volume of buffer B had passed through the column (± 210 ml), a linear gradient of 20 mM to 1.0 M potassium phosphate pH 6.0 buffer (200 ml of each) was applied to the column. The collected fractions were monitored at 220 nm, and NAG enzyme activity was analyzed using the microplate method. When all material had been displaced from the column, the HTP column was washed with one column volume of buffer B containing 0.4 M NaCl, and followed by two column volumes of buffer B.

Fraction C-DG_2(R) was subsequently applied to the HTP column as was done for C-DG_1(R). Active peaks were pooled and concentrated as before. Protein content and NAG activity were determined as before.

4.5:1.Results and Discussion

HTP proved to be able to purify complex proteins[200]. According to Bernardi and Kawasaki neutral and acidic glycoproteins compete for cationic groups on the adsorbent with the phosphate ions of the buffer[200].

HTP chromatography of each of C-DG_1(R) and C-DG_2(R) resulted in a

large NAG active peak (H_1) with a smaller adjacent NAG active peak (H_2). Prior to these peaks, 2 to 3 unknown inactive protein peaks appeared. For C-DG_1(R) all protein eluted from the column between 150 mM and 300 mM of the applied 20 mM to 1.0 M potassium phosphate pH 6.0 gradient. For C-DG_2 however, protein eluted from the column between 400 mM and 800 mM of the applied 20 mM to 1.0 M potassium phosphate pH 6.0 gradient. The gradient was always prevailed to 1.0 M phosphate buffer in order to ensure that all protein was eluted from the HTP. The applicable peaks were named accordingly, e.g. C-DG_2(R)-H_1 and C-DG_2(R)-H_2 in the case of C-DG_2(R). See figures 12 and 13 pages 68 and 69 respectively.

4.5:2. Rechromatography of NAG A Isoenzymes on HTP

The 2 x 60 cm column was reconditioned by washing with one column volume of 1.2 M potassium phosphate buffer, and then two column volumes of buffer B.

The four A isoenzymes obtained so far, being C-DG_1(R)-H_1 (figure 12 page 68), C-DG_1(R)-H_2 (figure 12 page 68), C-DG_2(R)-H_1 (figure 13 page 69), and C-DG_2(R)-H_2 (figure 13 page 69), were subjected to HTP rechromatography. The same flowrate, fraction volumes, and buffers as for the original HTP chromatography was used. More shallow phosphate gradients were used as indicated on the profiles. Active peaks were concentrated. NAG activity and protein content was determined as before.

4.5.3. Results and Discussion

By varying phosphate gradient levels the four A isoenzymes were each eluated in such a manner as to allow clear-cutting of the profiles, eliminating contaminants. The obtained fractions (see figures 14 to 17, pages 70 to 73), designated with and additional (R), were subjected to anionic PAGE (see figures 20.1 and 20.2 pages 82 to 83).

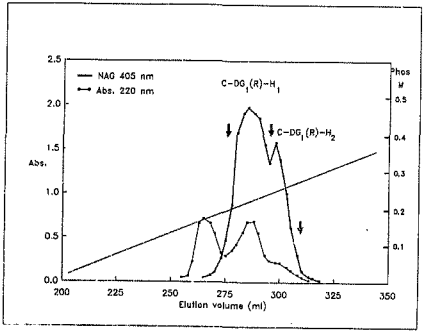


FIGURE 12

HTP chromatography of NAG A isoenzyme specie C-DG_1(R). After application of the sample and passage of one column volume (± 210 ml), a 20 mM (200 ml) to 1.0 M (200 ml) phosphate pH 6.0 gradient applied to the column resulted in all protein to be released between 150 mM and 300 mM of the gradient. NAG enzyme activity appeared in one major peak C-DG_1(R)-H_1, and in a smaller adjoining peak C-DG_1(R)-H_2.

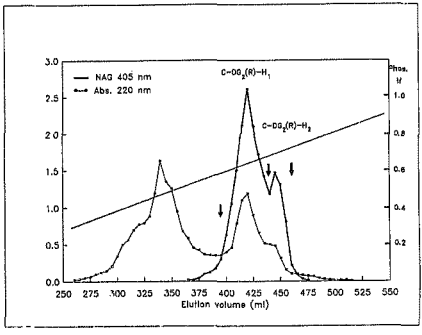


FIGURE 13

HTP chromatography of NAG A isoenzyme specie C-DG$_2$(P'). One column volume (± 210 ml) of buffer B (20 mM potassium phosph te pH 6.0) was passed from the column after sample application. The applied gradient (20 mM (200 ml) to 1.0 M (200 ml) potassium phosphate pH 6.0) resulted in all protein to be released from HTP between 400 mM and 500 mM of the gradient. NAG enzyme activity appeared in one major peak C-DG$_2$(R)-H$_1$, and in a adjoining peak C-DG$_2$(R)-H$_2$.

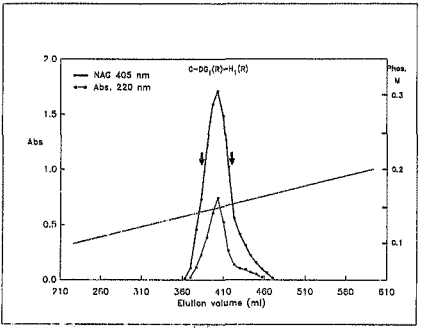


FIGURE 14

Rechromatography of NAG A isoenzyme specie C-DG$_1$(R)-H$_1$ on HTP. After sample application and the passing of one column volume (± 210 ml) of buffer B (20 mM potassium phosphate pH 6.0), a 100 mM (200 ml) to 200 mM (200 ml) potassium phosphate pH 6.0 gradient applied to the column resulted in a single NAG enzyme active peak C-DG$_1$(R)-H$_1$(R).

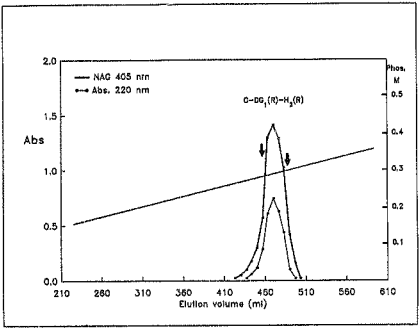


FIGURE 15

Rechromatography of NAG A isoenzyme specie C-DG$_1$(R)-H$_2$ on HTP. One column volume (± 210 ml) of buffer B was passed through the column after application of the sample. A linear gradient of 150 mM (200 ml) to 350 mM (200 ml) potassium phosphate pH 6.0 subsequently applied to the HTP column resulted in the retrieval of NAG enzyme activity in one peak designated as C-DG$_1$(R)-H$_2$(R).

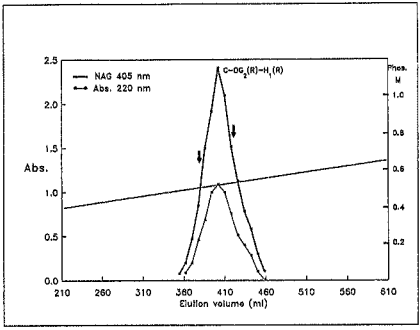


FIGURE 16

Rechromatography of NAG A isoenzyme specie C-DG$_2$(R)-H$_1$ on HTP. After sample application, one column volume (± 210 ml) of buffer A was passed through the column before a 400 mM (200 ml) to 650 mM (200 ml) potassium phosphate pH 6.0 gradient was applied to the column. This resulted in one NAG enzyme active peak designated as C-DG$_2$(R)-H$_1$(R).

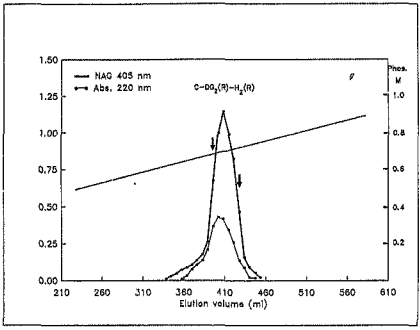


FIGURE 17

Rechromatography of NAG A isoenzyme specie C-DG$_4$(R)-H$_2$ on HTP. One column volume (± 210 ml) of buffer B was passed through the column after the sample was applied to the column. The application of a 500 mM (200 ml) to 850 mM (200 ml) potassium phosphate pH 6.0 gradient applied to the column resulted in one single peak C-DG$_3$(R)-H$_2$(R).

Step 6:Purification Procedure for NAG B

4.6:1.Rechromatography of the B Isoenzyme on DEAE - Trisacryl M

Rechromatography of the fractions C-D_1 and C-D_2 were performed on the regenerated DEAE - Trisacryl M column. Each fraction was loaded onto the column and the B isoenzyme was eluded with a 20 mM potassium phosphate pH 6.0 buffer. A flowrate of 30 ml per hour was maintained, and 3.0 ml fractions were collected. Active fractions were pooled, concentrated with a Millipore CX-10 ultra concentrator. Protein concentration and NAG activity were assessed in the same manner as before.

4.6:2.Results and Discussion

Rechromatography of the NAG B isoenzymes on DEAE - Trisacryl M indicated more isoenzymes. Absorbance 220 nm profiles coincide on the NAG B isoenzyme activity profiles (405 nm). The resulting peaks were given an extra (R) designation to indicate rechromatography, with a subscript 1 or 2 indicating the peak origin. The fractions were labeled C-$D_1(R)_1$, (figure 18 page 75) and C-$D_2(R)_1$ and C-$D_2(R)_2$ (figure 19 page 76).

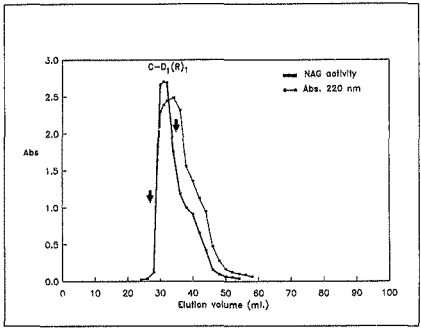


FIGURE 18

Rechromatography of the NAG B specie C-D_1 on DEAE-Trisacryl M. The NAG B isoenzyme was eluted directly from the column with a 20 mM potassium phosphate pH 6.0 buffer. All the NAG enzyme activity (Abs.405 nm) appeared in one peak. Absorbance 220 nm profiles coincide on the NAG B isoenzyme activity profile (405 nm), except to the right where there is a continuation in the absorbance at 220 nm even after a decrease in the NAG B isoenzyme C-D_1 enzyme activity.

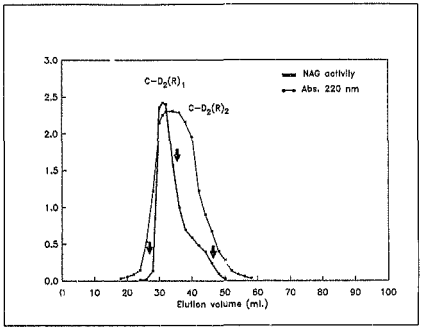


FIGURE 19

Rechromatography of the NAG B specie C-D_2 on DEAE-Trisacryl M. A 20 mM potassium phosphate pH 6.0 buffer was used to elute the NAG B isoenzyme specie C-D_2. NAG enzyme activity appeared in one major peak C-$D_2(R)_1$, and a smaller adjoining peak C-$D_2(R)_2$.

Step 7:Attempts to purify the B isoenzyme on CM - Sepharose CL-6B

Isoenzyme B fractions were subjected to cation exchange chromatography. CM - Sepharose CL-6B (Pharmacia) was packed into a 2 x 40 cm column, and subsequently respectively loaded with the four NAG B isoenzyme fractions. Buffer B was used to collect 3.0 ml fractions. After the eluant of one column volume, the column was subjected to a 0 -> 0.8 M NaCl gradient prepared in buffer B. NAG active peaks were pooled and concentrated. Protein content and NAG enzyme activity were determined as before.

4.7:1.Results and Discussion

The subjection of the NAG B isoenzyme fractions to CM Sepharose chromatography resulted in no significant separation or elimination of any inactive peaks. Protein peaks (220 nm) overlapped completely with NAG active peaks. Variation of the NaCl gradient slope, column size, different buffers with different pH values, and also with different eluant speeds made no difference in profiles. This purification step was omitted.

Step 8:Exclusion Chromatography

Both NAG A and B fractions obtained after rechromatography on DEAE, were submitted to exclusion chromatography on Sephacryl S-200 (2 x 40cm.). Fractions of 3.0 ml were collected at a flowrate of 25 ml/h. Absorbance at 220 nm was read for all tubes collected, and the microplate method was used for enzyme activity determination.

4.8:1. Results and Discussion

The isoenzymes lost between 80% to 95% activity with the submission of NAG to this kind of exclusion chromatography. Even with the use of different buffers with various pH values, or with Sephadex G200 as an alternative gel, enzyme activity was lost. It is thus understandable why this purification method and molecular weight determination method was declined. Similar separation results were obtained by Gibbons[29].

4.8:2. Polyacrylamide Gel Electrophoresis of Isoenzyme fractions

All the obtained isoenzyme fractions separated and collected up to this stage were subjected to a modified anionic Laemmli continuous PAGE method[296,297].

4.8:3. Preparation of the Electrophoretic Gel Chamber

Using a Hoofer Model SE 600 vertical gel unit, two 150 mm spacers were assembled between two 18 x 16 x 0.3 cm glass plates in a sandwich like manner in a casting stand. Having been sealed at the bottom with Parafilm, the two moulds were rinsed with a 0.2% v/v Patterson anti-static wetting agent. The moulds were turned upside down and allowed to dry.

4.8:4. Preparation of the Separation Gel (10% T, 2,7 % C)

TABLE 5

Composition of the 10% T, 2.7% C Separating Gel

Stock Solutions	Volume
Acrylamide / Bisacrylamide (30% T, 2,6% C)	30 mL
1.5 M Tris-HCl pH 8.8	22.5 mL
H_2O (ELGA)	16 mL
Ammonium Persulphate (10% w/v)	600 µL
TEMED	20 µL

The separating gel was prepared according to table 5 page 79, except for the addition of ammonium persulphate. Prepared in a 125 mL vacuum flask, with a magnetic stir bar placed inside, the solution was deaerated, and stirred for 10 min. at room temperature.

While swirling the flask, the required volume of ammonium persulphate was added. The gel solution was carefully poured inside the sandwich mould, and a comb was immediately inserted into the top of the mould. A small water layer was placed on top of the gel, and it was left to polymerize with the aid of a fluorescent lamp.

The gel was allowed to polymerize and after 4 hours the comb was removed. Each well was then rinsed with ELGA distilled water, and then filled with the tank buffer.

4.8:5.Pre-Electrophoresis

Gels were mounted in the 10°C precooled Hoofer electrophoresis tank containing 4 liters of 25 mM Tris / 192 mM Glycine pH 8.3 tank buffer. With a Hamilton syringe, 10 µl 10% bromophenol blue solution was injected onto one of the wells and used as a tracking dye.

A constant current of 15 mA was applied per gel, with the voltage and wattage settings at maximum output. This process was terminated when the dye was a ½ cm from the bottom of the gel.

4.8:6.Electrophoresis

Samples, dissolved in the mentioned tank buffer, containing 45 µg protein, were applied with a 50 µL Hamilton syringe. Since two gels were run simultaneously, a total current of 60 mA was applied to the gels. Electrophoresis was stopped when the tracking due was within ½ cm from the bottom of the gel. Another 10 µL dye was placed onto the same gel channel, and electrophoresis was resumed. The gels were finally released from electrophoresis when the dye was once again within 0.5 cm from the bottom of the gel.

4.8:7.Gel Staining and Destaining

Gels were left overnight in a 17.5% TCA solution. The gels were then once rinsed in distilled water, and stained using the Picrate & Coomassie blue method of Stephano, Gould et al[208]. For 1 to 2½ hours the gel was soaked in a 250 mL. 0.1 N picric acid solution (adjusted

to pH 7.0 with NaOH) containing 50 ml of 2% Coomassie Brilliant Blue R250, dissolved in 45% methanol, and 10% acetic acid. Destaining was achieved overnight by rinsing gels frequently in warm tap water to a transparent background with blue bands.

4.8:8 Results and Discussion

Results can be seen in figure 20 pages 82 to 83. All the B isoenzyme fractions showed a high degree of heterogeneity, and it is for this reason that purification was directed to the A isoenzyme.

The four NAG A isoenzyme fractions show some similarity on PAGE, especially between C-$DG_1(R)$-$H_1(R)$ and C-$DG_1(R)$-$H_2(R)$ (see figure 20 page 82 to 83). The C-$DG_2(R)$-$H_1(R)$ fraction formed two discrete bands which was the reason why it was onwards used for purification.

Step 9:Purification of C-$DG_2(R)$-$H_1(R)$ by PAGE and Gel Slicing

As can be recalled from PAGE (figure 20.1 page 82) the fraction C-$DG_2(R)$-$H_1(R)$ contained 2 bands and it was decided to use semi-preparative PAGE and gel slicing to separate them.

4.9:1.Procedure for Gel Slicing

A PAGE gel was prepared as described in table 5 page 79. Deviation to the method occurred when a toothless comb was used to create a trough rather than a multi well slot. The C-$DG_2(R)$-$H_1(R)$ fraction (500 µg)

FIGURE 20

Anionic PAGE of the for NAG A and four NAG B species.

FIGURE 20.1

A 10% T, 2.7% C anionic PAGE of the 4 NAG A species and the 4 NAG B species. Note that the NAG A isoenzyme specie C-$DG_2(R)$-$H_1(R)$ appears as only two distinct protein bands (Lane 7).

NAG B :

1 C-$D_1(R)_1$
2 C-$D_1(R)_2$
3 C-$D_2(R)_1$
4 C-$D_2(R)_2$

NAG A :

5 C-$DG_1(R)$-$H_1(R)$
6 C-$DG_1(R)$-$H_2(R)$
7 C-$DG_2(R)$-$H_1(R)$
8 C-$DG_2(R)$-$H_2(R)$

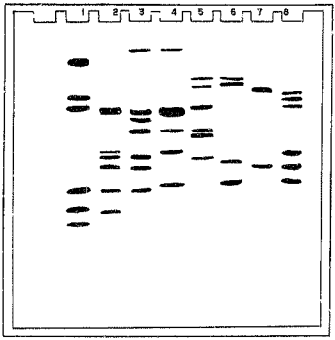
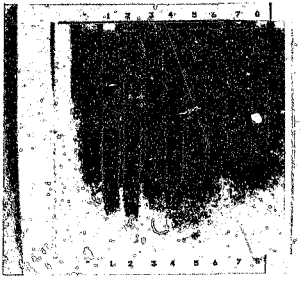


FIGURE 20.2

A photographic reproduction of the 10% T, 2.7% C anionic PAGE gel of the 4 NAG A species and the 4 NAG B species as depicted in figure 20.1 on page 82. The index is the same as for figure 20.1.



was loaded into this well, making sure that the sample was evenly spread. Electrophoresis was carried out as before.

One of the glass plates were removed, a 1 cm wide vertical strip was cut off on both sides and kept for colour development. The rest of the gel was sliced with a surgical blade into 2 mm wide horizontal slices, working from the top to the bottom. Each gel slice was then carefully removed and placed in a separate glass tube containing 1 ml of a 100 mM citric buffer, pH 4.5. With a glass rod each gel slice was pushed underneath the buffer. The tubes were shaken on a orbital shaker overnight at 4°C in order to remove as much enzyme from the gel as possible.

The border vertical strips were subjected to fixation, staining and destaining as described before. The gel strip was then scanned at 540 nm on a Gelscanner accessory of the Pye-Unicam spectrophotometer. NAG enzyme activity was determined with the Microtiter plate method for each of the slices. NAG activity together with the gel scanning profile was plotted against tube number - see figure 21 page 85.

Two peaks revealed NAG activity and were pooled seperately. Each fraction was filtered through Whatman Nr 1, to remove gel particles, and then concentrated as before only after an aliquot for re-PAGE was taken.

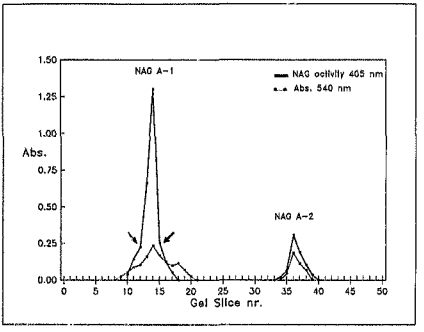


FIGURE 21

A plot of gel-sliced numbers vs the Absorbance at both 540 nm (scan of stained protein bands) and 405 nm (NAG enzyme activity) of the NAG A specie $C\text{-}R_{0.4}(R)\text{-}H_1(R)$

4.9:2.Results and Discussion

Scanning of the stained border vertical gel strips resulted in 2 prominent peaks, which coincided with the NAG activity profiles. The resulting two NAG A fractions on PAGE from C-DG_2(R)-H_1(R), were then designated as follows: The major peak NAG A-1, and the minor one as NAG A-2. (see figure 21 page 85). Re-PAGE of each of these fractions resulted in a single band, corresponding to their original Rf values. NAG A-2 had only 21% enzyme activity in comparison to NAG A-1. NAG A-1 was then assessed for homogeneity and chemical - physical characteristics.

CHAPTER 5

PHYSICO-CHEMICAL CHARACTERISTICS AND HOMOGENEITY ASSESSMENT OF NAG A-1

Glycoproteins with high carbohydrate content tend to be asymmetric molecules with high frictional ratios, high intrinsic viscosities, and large virial coefficients[295]. Since micro-heterogeneity is found in a high degree in glycoproteins[295], this phenomenon causes paucidispersity of these molecules due to a variation in carbohydrate composition, mostly in sialic acid and mannose content[295]. Paucidispersity is the appearance of the same macromolecule in different ionic species in an analytical procedure due to deamidation of Asn and Gln, as well as a variation in the carbohydrate - sialic acid content[295]. Polydispersity may also occur and is revealed with molecular weight determinations. Polydispersity arises from a slight difference in molecular shape, charge, and molecular interactions which contribute to heterogeneity.

The NAG A-1 isolated isoenzyme was subjected to PAGE in step 9 (page 81) and one single band was obtained on anionic PAGE. Using IEF (Isoelectric focusing), NR-SDS-PAGE (Non Reducing SDS-PAGE) and R-SDS-PAGE (Reducing SDS-PAGE), it was possible to assess the homogeneity of the isoenzyme, and also to derive chemical and physical characteristics regarding this molecule.

5.1.Physical Characteristics

5.1.1.PAG-Isoelectric Focusing of NAG A-1

In IEF amphoteric substances like proteins, are separated due to the properties of the ampholites which creates a pH gradient in an electrical field. Proteins migrate to the point at which they possess no nett charge, which is their isoelectric point (pI).

5.1.1.1.Preparation of a 5% T, 3% C Polyacrylamide Gel

Assembly of the gel casting mould was done according to the procedure described in the LKB application note (LKB 2117). Preparation of the gel was done according to table 6 page 90. After deaeration of the solution, 1.5 ml ammonium persulphate solution (1% w/v) and 30 µl of TEMED were added. The solution was swirled in a 50 ml flask, and then poured into the mould (115 x 230 x 1 mm), and left to polymerize for one hour in front of a fluorescent light source.

5.1.1.2.Mounting and Profocusing of the Gel on the Apparatus

A Specht Scientific cooling bath, set at 10°C, was connected to a LKB Flat Bed 2117 Multiphor 2 Electrophoresis Unit. A LKB 2297 Macrodrive 5 Power unit was used as the power source. The prepared gel was placed onto the cooling plate after 2 ml of liquid paraffin was spread across the cooling plate. An anode electrode paper strip soaked in 1 M phosphoric acid, and cathode electrode paper soaked in 1 M sodium hydroxide, were applied to the gel to accommodate the

platinum electrodes across the length of the gel.

Prefocusing of the gel was performed for at least 1½ hours until the pH gradient was established. This was observed when the current applied settled at constant value. The power supply settings to the gel were as follows: 25 Watts, with current and voltage settings at maximum.

5.1.1.3. Sample Application and Gel Running

Six IEF markers from a Sigma IEF marker kit (IEF MI, pH range 3.55 - 9.3) and Methyl red as a tracking dye (pI = 3.75), see table 7 page 90, were applied as standards in a 50 µg/15µl concentration, with a Hamilton microsyringe onto LKB sample applicator paper strips. Samples were applied in 200µg/20µl, 100µg/20µl, and 50µg/20µl concentration. Electrophoresis was carried out in the same manner as for prefocusing. After 45 min. of electrophoresis, the sample applicator paper strips were carefully removed, and electrophoresis was resumed for another hour.

5.1.1.4. Fixing, Staining, and Destaining of the Gel

The gel, left on a glass plate, was fixed for one hour in a 3.5% sulphosalicylic acid and 11.5% trichloroacetic acid (w/v) fixation solution. Gels were then destained in a destaining solution consisting of 25% ethanol and 8% acetic acid (v/v). The gels were stained for one hour in a staining solution consisting of 460 mg Coomassie Brilliant Blue R250 per 400 ml destaining solution.

TABLE 6

Preparation of a 5% T, 3% C IEF-Polyacrylamide Gel

Stock Solution	Volume (ml)
29.1% Acrylamide stock	10.0
0.9% Bis stock	10.0
87% (v/v) Glycerol	7.0
Pharmalyte pH 2.5 - 5	1.4
Pharmalyte pH 5 - 8	1.4
Water (Elgastat)	30.2

TABLE 7

pI Calibration Standards for IEF

Nr	Sample		pI	Migration Distances (mm)
1	Amyloglucosidase	Std.	3.55	18
2	Trypsin Inhibitor	Std.	4.55	40
3	β-Lactoglobulin A	Std.	5.13	60
4	Carbonic Anhydrase B (Bovine)	Std.	5.85	82
5	Carbonic Anhydrase B (Human)	Std. *	6.57	108
6"	Myoglobin	Std.	6.76	111
6"	Myoglobin	Std.	7.16	116
7	NAG A-1		4.97	57

Std. = IEF Standards from Sigma
* Standards run on the same tract

Destaining was achieved by leaving the gel overnight in the destaining solution.

5.1.1.5. Determination of the pI for the NAG A-1 isoenzyme

The different distances migrated for the different pI standards and samples are listed in table 7 on page 90. Migration distances against pI values were plotted in order to determine the pI for the NAG A-1 isoenzyme (see figure 28 page 110).

5.1.1.6. Results and Discussion

The NAG A-1 isoenzyme did not appear as one single band on PAG-IEF, but showed one major band at pI 4.97 and 18 bands having pI ranging from 4.55 to 5.85.

5.1.2. Homogeneity Assessment

5.1.2.1. Homogeneity Assessed by PAG-IEF

Due to the high degree of micro-heterogeneity found on PAG-IEF for NAG A-1, and since NAG A-1 appeared as only one band on anionic PAGE, three commercial enzymes were used to remove carbohydrate moieties from the NAG A-1 isoenzyme to eliminate charge micro-heterogeneity.

5 ment of NAG A-1 with Glycosidases

Glycosidases hydrolyses internal glycosidic bonds, releasing in part, or all of the carbohydrate moiety. The ultimate aim was thus to selectively remove some or all of the carbohydrate moieties from the NAG A-1 glycoprotein that are believed to be responsible for the appearance of the 19 bands between nearly one pH unit on IEF, and then to resubmit it to PAG-IEF.

5.1.2.3.Choice of Glycosidases

Endoglycosidase D, (Boehringer 752991, 0.1U, optimum pH 6.5 and MW is 280.0 kD) hydrolyses $(Man)_3(GlcNac)_2$ from glycoproteins containing the trisaccharide (Man) α1->3 (Man) β1-4 (GlcNAc), as part of the carbohydrate residue. The non-reducing terminal α-mannosyl residue not substituted by any other sugar is essential for specificity. It hydrolyses sugar chains that are linked to N-acetylglucosamine, or (Fuc) α1->6 (GlcNAc), or (GlcNAc) -> Asn, or to (Fuc) α1->6 (GlcNac) -> Asn.

Endoglycosidase H, pH optimum of 5.5, (Boehringer 886424, 0.1U) hydrolyses $(Man)_3(GlcNAc)_2$ from glycoproteins containing the tetrasaccharide (Man) α->3 (Man) α1->6 (Man) β1-4 (GlcNAc) as part of the carbohydrate chain. It also hydrolyses sugar chains linked to N-acetylglucosamine, to N-acetylglucosaminitol, and to GlcNAc->Asn.

Glycopeptidase F, (Boehringer 903337, 20U, pH optimum 7-8, MW of 35.5 kD) cleaves high Mannose glycans from glycoproteins between Asn

and GlcNAc.

5.1.2.4. Incubation of NAG A-1 with Glycosidases

The procedure of Kobata[212] was used. Since each of the glycosidases had a different optimal pH for activity, a 50 mM Citric buffer pH 5.5 was used for Endoglycosidase H, a 20 mM potassium phosphate buffer pH 6.5 was used for Endoglycosidase D, and a 20 mM Tris-HCL buffer pH 7.4 was used for the Glycopeptidase F.

In an Eppendorf 1 mg of NAG A-1, and 0.1 U of the glycosidase was made up to 0.5 ml buffer. This was done for each of the three glycosidases. The mixtures were carefully shaken. Out of each Eppendorf 125 µl was removed, and this was added together in another Eppendorf. The four eppendorf vials were sealed, and then placed in a Labotech incubator at 37°C for 60 h.

5.1.2.5. IEF of NAG A-1 treated with Glycosidases

Since each of the Glucosidases were present in such a low concentration, it was reasoned that they would not be visible on PAG-IEF. An IEF gel was prepared as described in section 5.1.1 page 88. Out of each eppendorf vial 40µg/20µl was applied to the IEF gel. The gel was run, removed, and stained in the usual manner.

5.1.2.6. Results and Discussion

The glycosidases had the following effect on NAG A-1 as seen on PAG-

IEF:

Endoglycosidase D eliminated micro-heterogeneity since one single band appeared on IEF (see (b) on figure 22 page 96). This means that NAG A-1 had α-Man residues occurring as unsubstituted sugars on the non-reducing terminal of a carbohydrate fragment having a trisaccharide (Man) α1->3 (Man) β1-4 (GlcNAc) in its moiety.

Endoglycosidase H had a selective effect on NAG A-1, since 12 of the original 19 bands remained on PAG-IEF (see (c) on figure 22 page 96). This means that the remaining 12 bands had carbohydrate fragments that were not susceptible to Endoglycosidase H, indicating that the carbohydrate moiety was not a tetrasaccharide.

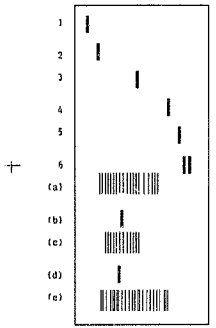
Glycopeptidase F, like Endoglucosidase D, eliminated all occurrences of heterogeneity, since only one peak appeared on IEF (see (d) on figure 22 on page 96). This indicates through the specificity of Glycopeptidase F, that the carbohydrate moiety on NAG A-1 is linked to the peptide via GlcNAc unit Asn. It can be accepted that carbohydrate moieties on NAG A-1 are high in Man, and complex in structure.

The action of the combination of the three glycosidases on NAG A-1 on IEF was not successful in elimination of heterogeneity (see (e) on figure 22 page 96) since more bands appeared under these conditions than for NAG A-1 subjected to Endoglycosidase H. All of these glycosidases are glycoproteins, and it is likely that they have exerted some complicated catalytic action upon each other, or the substrate enzyme.

NAG enzyme activity was determined after the subjection of NAG A-1 to the different glycosidases. No enzyme activity for NAG A-1 could be detected after subjection of NAG A-1 to Glycopeptidase F, however, after subjection of NAG A-1 to Endoglycosidase H or Endoglycosidase D no loss of enzyme activity was found. The combination incubation showed a 17% loss in activity. Loss in activity was believed to be due to the complete removal of the carbohydrate portion(s) of the glycoprotein, which may have resulted in a conformational change or denaturation of the isoenzyme.

FIGURE 22

PAG-IEF pattern of NAG A-1 untreated, and treated with Endoglycosidase D, Endoglycosidase H, and Glycopeptidase F individually and together. Standards used for the determination of the pI of the different NAG A-1 species are also displayed.



Index

1 - 6 : pI standards (See table 7 page 90)

(a) : Untreated NAG A-1

(b) : NAG A-1 treated with Endoglycosidase D

(c) : NAG A-1 treated with Endoglycosidase H

(d) : NAG A-1 treated with Glycopeptidase F

(e) : NAG A-1 treated with Endoglycosidase D and H, and Glycopeptidase F

5.1.3.Molecular Weight Determination of NAG A-1

The molecular weight of the NAG A-1 isoenzyme was determined using both sodium dodecyl sulphate -PAGE (SDS-PAGE) and sedimentation equilibrium analytical centrifugation.

5.1.3.1.Molecular Weight Determination by SDS-PAGE

Both reducing SDS-PAGE (R-SDS-PAGE) and non-reducing SDS-PAGE (NR-SDS-PAGE) were used in determining the molecular weight of the NAG A-1 isoenzyme. Shapiro[320], and Weber and Osborn[321] reported that proteins dissolved in SDS exhibit electrophoretic mobilities in polyacrylamide gels which are a direct function of their molecular weight *(NR-SDS-PAGE) or of their sub-unit(s) molecular weight (R-SDS-PAGE)*[320-326].

5.1.3.2.Procedure for MW Determination using SDS-PAGE

A modified Laemmli[296-297] continuous buffer system was employed for SDS-PAGE. Apparatus and assembly of the glass plates were done according to the method previously described for PAGE as on page 78.

5.1.3.3.Preparation of the 10% T, 2.7% C Gel

The separating gel solution was prepared in a 250 ml flask according to table 8 page 100, except for the addition of ammonium persulphate. The solution was deaerated under vacuum for 10 min, whereafter the

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ammonium persulphate was added. The flask was gently swirled, and the solution was poured into the mould. The gel was left to polymerize.

5.1.3.4. Preparation of Molecular Weight Standards and NAG A-1

Electran molecular weight markers for SDS-PAGE (BDH 44264) with molecular weight range from 12.3 kD to 78.0 kD (see tables 9 and 10 pages 100 and 101 respectively), were used as molecular weight standards. For R-SDS-PAGE one milligram of the mixed marker solution or NAG A-1 was dissolved in 1 ml of treatment buffer (0.125 M Tris-HCL pH 6.8, 4% SDS, 20% glycerol, 10% mercaptoethanol). For NR-SDS-PAGE the samples and standards were dissolved in a treatment buffer not containing the mercaptoethanol. Samples were incubated for one hour at 60°C in a water bath, where after the standards were cooled and kept on ice until needed.

5.1.3.5. Electrophoresis Conditions

With a Hamilton syringe, 25 µg/25µl standard and NAG A-1 were loaded in a glycerol dense layer under the buffer onto the gel. Phenol red (0.1%, 15 µL) was used as a tracking dye. Gels were subjected to electrophoresis as described under 'Electrophoresis' on page 80.

5.1.3.6. Results and Discussion

The migration distances of the BDH MW standards obtained from the SDS-PAGE gel (see figures 23 and 24 pages 102 to 104) were plotted against their $\log_{10}$ MW's as stated in tables 9 and 10 pages 100 and

ammonium persulphate was added. The flask was gently swirled, and the solution was poured into the mould. The gel was left to polymerize.

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5.1.3.6. Results and Discussion

The migration distances of the BDH MW standards obtained from the SDS-PAGE gel (see figures 23 and 24 pages 102 to 104) were plotted against their $\log_{10}$ MW's as stated in tables 9 and 10 pages 100 and

101 respectively, to give the linear graphs as found in figures 29 and 30 pages 111 and 112 respectively.

Subjection of NAG A-1 to NR-SDS-PAGE resulted in a single band appearing at a migration distance of 33 mm from the sample well which gave a calculated MW for NAG A-1 of 52.1 kD. Since only one band appeared on NR-SDS-PAGE this was taken to be another confirmation of homogeneity of NAG A-1. This MW of 52.1 kD obtained correlates with the molecular weight derived from the amino acid composition, which was 36.5 kD and multiplied by 1.43 to account for the 30% contribution of the carbohydrate moiety. Various authors[330-333] have shown that SDS-PAGE is subject to unpredictable errors in molecular weight determination with glycoproteins having more than 5% carbohydrates. They show that glycoproteins migrate more slowly on SDS-PAGE than standard polypeptides of the same mass do. This more slow behaviour of glycoproteins on SDS-PAGE results from both aberrant hydrodynamic behaviour and from decreased binding of SDS compared to polypeptides of equal mass[332].

An unusual and unexpected phenomenon occurred when NAG A-1 was subjected to R-SDS-PAGE. Two rather high MW bands appeared at a migration distance of 6 mm and 9 mm from the sample wells. From the standard curve molecular weights of 88.8 kD and 83.5 kD was calculated (see table 10 page 101, figure 24 pages 103 and 104, and figure 30 page 112). These high molecular weight species appearing under reducing conditions (10% 2-mercaptoethanol), may have resulted from aggregation due to distinct combinations of the glycoprotein. This phenomenon could be explained from the documented observations

that under reducing conditions glycoproteins modify their rigidity and flexibility, and in general their molecular shape which may result in aggregation[324-329].

TABLE 8

Composition of the 10% T, 2.7% C Separating Gel

Stock Solutions	Vol.
Acrylamide / Bisacrylamide (30% T, 2.5% C)	30 mL
1.5 M Tris-HCl pH 8.8	22.5 mL
H_2O	36 mL
Ammonium Persulphate	600 µL
TEMED (10% v/v)	30 µL
SDS (10%)	900 µL

TABLE 9

Migration Distances for NR-SDS-PAGE Standards and NAG A-1

	Protein	Migration Distance from well (mm)	MW (D)	Log MW
1	Myoglobin	79	17,200	4.22
2	Carbonic Anhydrase	54	30,000	4.46
3	Ovalbumin	38	45,000	4.68
4	Albumin	24	66,250	4.82
5	Ovotransferrin	21	76,000	4.88
	NAG A-1	33	52,100	4.71

TABLE 10

Migration Distances for R-SDS-PAGE Standards and NAG A-1

	Protein	Migration Distance from well (mm)	MW (D)	Log MW
1	Myoglobin	62	17,200	4.22
2	Carbonic Anhydrase	38	30,000	4.46
3	Ovalbumin	26	45,000	4.68
4	Albumin	15	66,250	4.82
5	Ovotransferrin	12	76,000	4.88
	NAG A-1	6	88,800	4.95
	NAG A-1	8	83,500	4.92

FIGURE 23

Non-Reducing SDS-PAGE of NAG A-1

FIGURE 23.1

Photographic reproduction of the NR-SDS-PAGE gel of NAG A-1



Index of lanes to both figures 23.1 and 23.2

(a) NAG A-1

(b) Standards - refer to table 9 page 100

FIGURE 23.2

Graphic reproduction of the NR-SDS-PAGE gel of NAG A-1

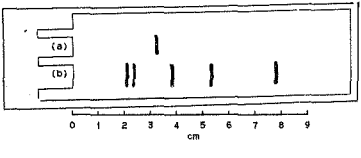
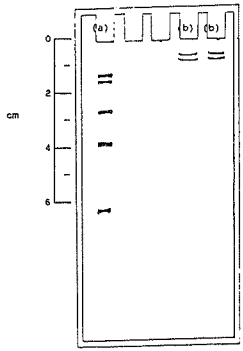


FIGURE 24

Reducing SDS-PAGE of NAG A-1

FIGURE 24.1

Graphics reproduction of the R-SDS-PAGE gel of NAG A-1



Index to both figures 24.1 and 24.2

(a) Standards - refer to table 10 page 101

(b) NAG A-1

FIGURE 24

Reducing SDS-PAGE of NAG A-1

FIGURE 24.2

Photographic reproduction of the R-SDS-PAGE gel of NAG A-1



Index

(a) Standards - refer to table 10 page 101

(b) NAG A-1

5.1.4.Analytical Ultracentrifugation

Another method employed for NAG A-1 molecular weight determination was sedimentation equilibrium ultracentrifugation. This was kindly done at the Department of Biochemistry of the University of Port Elizabeth by Dr. D. Litthauer on a Beckman L2-75 B preparative ultracentrifuge equipped with a scanning attachment (280 nm filter)

A double sector cell (12 mm) with quartz windows was used in a Beckman An-D rotor at 20°C.

5.1.4.1.Determination of the Sedimentation Coefficient for NAG A-1

A total of 5.4 mg of freeze dried NAG A-1 protein was dissolved in 1 ml of a 50 mM potassium phosphate buffer pH 6.8 containing 0.1 M KCl. To the reference cell 400 µl of buffer was added, while the sample cell contained 50 µL of FC-43 oil and 350 µL of the 5.4 mg/ml NAG A-1 solution at 20°C. The ultracentrifuge was run at 45,000 rpm for two hours to achieve high speed velocity. The first scan was taken at 13.5 min., and then again almost every 15 minutes. A total of 7 scans were used to plot time against displacement ($\log_{10}$ X) of the specific scan (see figure 25 page 107), resulting in the determination of the sedimentation coefficient (s).

5.1.4.2.Calculation of the Sedimentation Coefficient (s) for NAG A-1

The value of s was calculated using least squares linear regression, resulting in S=3.37729.

$$s = 1/W^2 \qquad \text{eq.2}$$

$$1 S \sim 10^{-13} \text{ Sec.} \qquad \text{eq.3}$$

5.1.4.3. Sedimentation Equilibrium Ultracentrifugation

Meniscus depletion was achieved by overspeeding for 2 hours at 45,000 rpm, followed by the equilibrium speed 10,000 rpm for 68 h. Calculation of the MW was derived from the plot of $\log_{10} A_{280nm}$ against r^2, using the gradient (d log A/d r^2) applied to the equation

$$MW = 2 \times \frac{2,303\ RT}{(1-vp)} \times \frac{1}{w^2} \times \frac{d \log_{10} A}{dr^2} \qquad \text{eq.4}$$

$$MW = \frac{4.606 \times RT}{(1-vp)w^2} \times gr \qquad \text{eq.5}$$

v = Partial specific volume (cm^3/g)
p = Solution density (g/cm^3)
R = Universal gas constant, 8.314158×10^7 ergs/degree mole
T = Temperature (293°K)
w = Rotor angular velocity in radials per second ($2\pi r.p.m/60$)
A = Absorption at 280 nm.
r = Distance of radius (cm) from rotor centre.
gr = gradient = (d log A/d r^2)
(1-vp) = 0.680 (assumed for NAG A)

The sedimentation equilibrium plot resulted in a non-linear relationship between r^2 and $\text{Log}_{10} A_{280}$, indicating that NAG A-1 underwent self-association to form aggregates of different molecular weights. This observation, as described by Stone and Reynolds[337], occurs as a result of interactions of peptide and carbohydrate moieties with the solvent[338]. The resulting average molecular weight of 37.6 kD calculated from experimental data was subsequently disregarded - refer to figures 26 and 27 on pages 108 and 109.

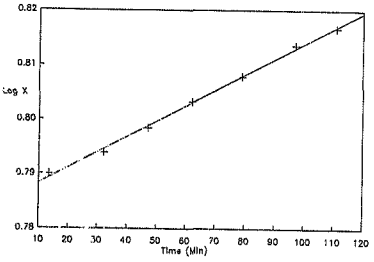


FIGURE 25

Plot of time (min) against Log X for the determination of the sedimentation coefficient (s) for NAG A-1

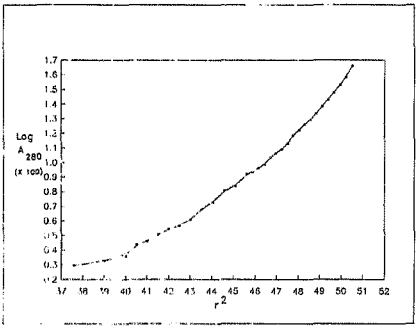


FIGURE 26

Plot of r^2 (r=radial distance between the particle and the centre of rotation in cm) against log A280 nm x 100. Non-linearity of the sedimentation equilibrium plot can be indicative of heterogeneity, polydispersity or of molecular interactions (ref 295). For NAG A-1 the molecular weight average for the original undisturbed sample was obtained through integration over the whole cell.

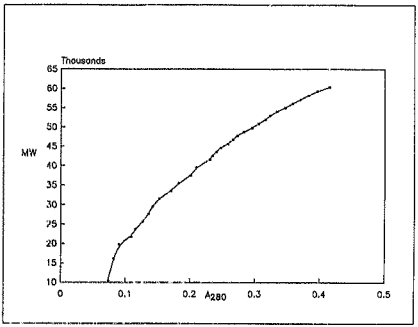


FIGURE 27

Plot of A280 nm against MW (kD). A less parabolic appearance of the plot is once again indicative of heterogeneity, polydispersity or of molecular interactions.

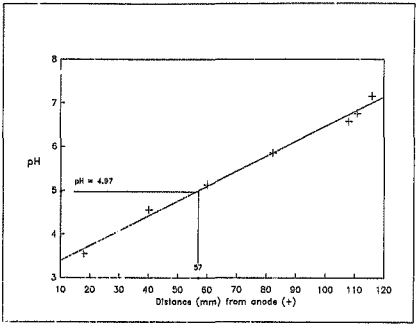


FIGURE 28

Calibration curve to determine pI of NAG A-1 by PAG-IEF. Migration distances in mm from the anode against the known pI values for certain proteins were used to derive a pI value of 4.97 for NAG A-1.

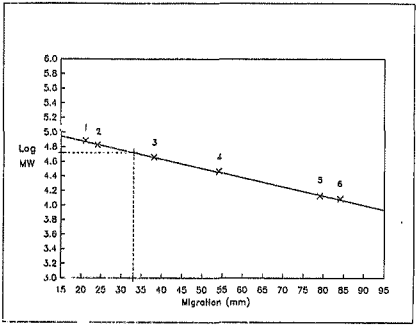


FIGURE 25

Non-Reducing SDS-PAGE for NAG A-1 molecular weight determination. Plot of the molecular weight of the proteins standards mentioned in table 9 page 100 against their migration distance in mm. This resulted in NAG A-1 having a molecular weight of 52.1 kD.

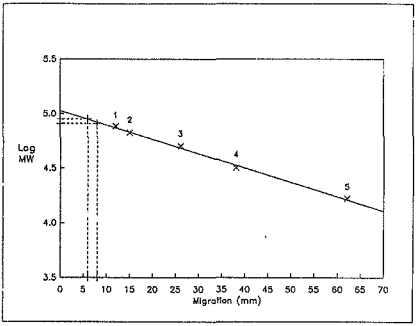


FIGURE 30

Reducing SDS-PAGE for NAG A-1 molecular weight determination. Plot of the molecular weight of the proteins standards mentioned in table 10 page 101 against their migration distance in mm. The appearance of two high MW bands (88.8 kD at 6 mm and 83.5 kD at 8 mm), is most likely due to the aggregation of NAG A-1 under the reducing conditions.

5.2.Chemical Characteristics

5.2.1.Amino Acid Composition of NAG A-1

5.2.1.1.Preparation and Detection of Amino Acids

The NAG A-1 isoenzyme fraction (±500 µg) was dissolved in 200 µl of 4 N methane sulphonic acid (MSA), and then hydrolyzed for 20 hours at 110°C in an evacuated hydrolysis tube[214]. Thereafter 80 µl of 10 N KOH was added to stop the hydrolytic action of the MSA. A pH adjustment of the NAG A-1 hydrolysate was made to pH 2.2, resulting in a final protein concentration of ±500 µg / 330 µl. The amino acid composition of 250 µl of the hydrolysate was then determined on a Beckman 118BL amino acid analyzer by the method of Spackman[215] et al at the Department of Biochemistry of the University of Port Elizabeth under the supervision of Dr. R. Naude.

5.2.1.2.Results and Discussion

The amino acid composition of the NAG A-1 isoenzyme is presented in table 11 on page 115. A total of 320 amino acids yields a minimum molecular weight of 36.4 kD for the peptide. There is a high Asp (30), Ser(18), Thr(16), and Glu(54) content. These amino acids are vital for glycan attachment to the peptide. The number of Pro(15) residues is average, since high Pro numbers in the area of glycosylation jeopardizes the attachment of a carbohydrate moiety to the peptide[216].

There is a total of 27 basic amino acids (Lys=15, Arg=12), and 84 acidic amino acids (Gln=54, Asn=30) proving the peptide to be acidic. Micro-heterogeneity is enhanced if any deamidation of the Asn (30) and Gln (54) residues occurred. The hydrophobic (nonpolar) amino acids total to 102 (Ala=20, Val=24, Leu=25, Ileu=14, Met=6, Tyr=13), while the polar amino acids total 118 (Ser=18, Thr=16, Asn=30, Gln=54), explaining some of the hydrophilic character of the glycoprotein. This is also affected and influenced by the carbohydrate content, especially by the sialic acid content of the carbohydrate moiety[217].

TABLE 11

Amino Acid Composition of the NAG A-1 isoenzyme

Amino Acid	Molar Ratio		MW
TRP	6	(5.6)	1053
HIS	7	(7.1)	981
LYS	15	(15.2)	1956
ARG	12	(11.6)	1820
ASN	30	(29.6)	3417
THR	16	(15.7)	1593
SER	18	(17.7)	1548
GLU	54	(54.4)	7027
PRO	15	(15.1)	1468
GLY	22	(21.7)	1239
ALA	20	(20.2)	1441
½ CYS	2	(1.5)	157
VAL	24	(24.1)	2388
MET	6	(5.9)	776
ILEU	14	(13.7)	1553
LEU	25	(24.6)	2788
TYR	13	(13.4)	2195
PHE	21	(20.9)	3083
NH_2	152	-	-
Total	320		36483

5.2.2.Determination of the Carbohydrate Content of NAG A-1 and A-2

5.2.2.1.Procedure for Total Carbohydrate Content Determination

The carbohydrate content of the NAG A-1 and A-2 isoenzymes were determined using the method of Graff[218] for unhydrolyzed glycoproteins. Standards of 50 to 200 µg D(+) glucose versus A620 nm were used to compile a standard graph which was used to determine the total carbohydrate content of NAG A-1, and also of the NAG A-2 fraction obtained from PAGE. Standards and samples were determined in triplicates. The protein content was determined by the BCA method of Smith[248].

Of each glucose standard 4 ml was carefully mixed with 8 ml of the anthrone reagent (0.4 g anthrone / 200 ml 96% sulphuric acid) and left to stand for 20 min. to cool at room temperature. After an internal zero calibration at 820nm was made to compensate for the viscosity of the sulphuric acid for each sample, the A620 was read and a standard curve was compiled (see figure 31 page 117).

Horse radish peroxidase, with a total carbohydrate content of 18%, was used as a test of accuracy for the method[219]. With the protein concentration determined with the A220nm - A235nm method, and carbohydrate content determined with the anthrone method, Horse radish peroxidase was found to contain 16.3% carbohydrate.

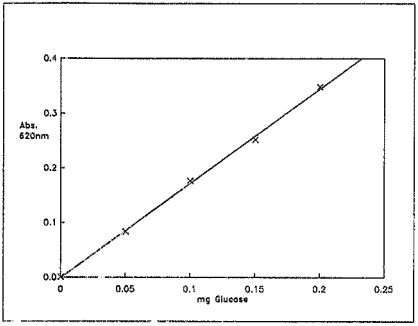


FIGURE 11

Standard curve for total carbohydrate content determination of NAG A-1 and A-2 isoenzymes. Five glucose standards were used to compile the graph following the method of Graff[218]

The carbohydrate content of the isoenzyme NAG A-1 and NAG A-2 were determined in the following way: 1 mg of the NAG isoenzyme was dissolved in 1 ml of 10 mM sodium bicarbonate solution pH 8.5. Dilution of 1, 10, and 25 µg/4ml were used in the determination.

5.2.2.2. Results and Discussion

NAG A-1 contained 30,5% total carbohydrate and NAG A-2 had a content of 17,4%. This high carbohydrate content for the NAG A-1 glycoprotein may explain some of the awkward phenomena experienced during isolation and purification of NAG. Glycoproteins with more than a 5% carbohydrate content exert strange and unusual behavior on various analytical and preparative techniques[330-333], for example, ion exchange and exclusion chromatography of these glycoproteins deviate with regard to their eluant volumes, retention times and peak profiles[330].

5.2.3. Determination of Sialic Acid Content of NAG A-1 and A-2

The total sialic acid content for NAG A-1 and NAG A-2 were determined since neuraminic acid residues influence the nett charge of glycoproteins.

5.2.3.1. Method for Sialic Acid content Determination

Only selective hydrolysis of the glycoprotein is necessary for the release of sialic acids. Samples (1 mg/ml) were hydrolyzed in 0.1 M NaOH at 25°C for 30 min.

The method of Aminoff[324-328] was used for total sialic acid determination[324-328]. The samples (1, 10, and 25 µg/ml) and a blank were treated with 0.25 ml periodate reagent (0.025 M periodic acid in 62.56 mM H_2SO_4, pH 1.1) for 30 min. in a 37°C water bath. Sodium arsenite, 1 ml of a 2% w/v sodium arsenite solution in 0.5 N HCl, was added to reduce the excess periodate. After 2 min. 2 ml of the thiobarbituric acid reagent (0.1 M 2-thiobarbituric acid in water, pH adjusted to pH 9.0 with NaOH) was added, covered and heated in a boiling water bath for 10 min. The colored solutions were cooled on ice and then shaken with 5 ml of acid butanol. Centrifugation was applied to separate the two phases. The butanol phase was read at 549 nm.

A relationship of 10 mM of N-acetylneuraminic acid giving an absorbance of 0.35 at 549 nm was used to calculate the sialic acid content[324-328].

5.2.3.2. Results and Discussion

The NAG A-1 isoenzyme had a total of 6.1% sialic acid content, while the NAG A-2 fraction had a 0.8% sialic acid content. Sialic acids in mammalians occur always as terminal non-reducing residues[326]. Since the sialic acid content influences the nett charge of glycoproteins, micro-heterogeneity of a molecule may arise due to the inconsistent removal of sialic acid residues during the life span of the molecule[327].

CHAPTER 6

ENZYMIC AND KINETIC PROPERTIES OF NAG A AND B ISOENZYMES

Enzymic activity is affected by factors such as pH, temperature, and chemical components such as anions, cations, metals, co-factors and substrate analogues. For NAG A the $C\text{-}DG_a(R)\text{-}H_a(R)$ fraction, and for NAG B the $C\text{-}D_a\text{-}(R)_a$ fraction was used right through the enzymic and kinetic determinations, in order to correlate results.

6.1. Enzymic Properties

6.1.1. Effect of pH on NAG A and B Isoenzyme Activity

Glycoproteins are rich in Glu, Asn, Lys, Arg, and sialic acids. These molecules are all greatly affected by pH. This could lead to inactivation and denaturation of the enzyme[299-300].

6.1.1.1. Methods and Procedure for pH optimum determination

The standard NAG activity procedure was modified in the following way. A 22 mM Citric acid and a 20 mM Tri-Sodium Citrate stock solutions were individually prepared. The citric acid solution was titrated against the tri-sodium citrate. The wide range started from pH 2.5 to pH 8, having 0.5 pH increments. The process of titration was controlled with the aid of a Phillips 2 digit pH meter. A narrow pH range was also prepared with the starting and ending pH values of

pH 4.0 and 5.0 respectively, having increments of 0.05 pH units.

The PNP-NAG substrate (0.29 mol/L) and 0.2 mol/L borate buffer (NaOH titrated) was prepared as before.

Reaction were carried out in triplicates in microtiter plates as described on page 46. Double concentration citric acid buffers was used to compensate for the dilution caused by substrates made up in water.

The enzyme fractions were diluted to have moderate reaction times to assess pH optimum. Reaction were performed at different time periods (1, 5, 15, 30, 45 min.)

6.1.1.2. Results and Discussion

A typical bell like plot was obtained for NAG A and B. Different pH spectrums of activity for the two isoenzymes were obtained. The wide pH spectrum was used to indicate the pH spectrum for pH optimum. The narrow pH range was used to demonstrate the optimal pH point specifically.

Both the isoenzymes had an optimum pH at 4.55 as derived from the narrow pH range, see figure 33 page 126. Differences in the wide pH range (see figure 32 page 125) were as follows: The A isoenzyme showed a sharp decline in activity between pH 3.5 and pH 4.0, while activity was still found at pH 8.0. The B isoenzyme showed the same declination but between pH 3.0 and pH 2.5. Activity for B was also

more drastically diminished at the higher pH values than for the A isoenzyme.

6.1.2.Effect of Temperature on NAG A and B Isoenzymes

Temperature has a twosome effect on any enzyme: First on the catalytic reaction itself, and secondly thermal inactivation, and denaturation. Inactivation by temperature becomes important only at high temperatures, and is negligible at low temperatures[203-204].

6.1.2.1.Procedure for Temperature Optimum Determination for NAG Isoenzymes

The standard assay for NAG was used for the optimum temperature determination. A constant temperature waterbath, accurate to within two degrees Celsius, and calibrated with two ChemLab 0-100°C thermometers, was used. Triplicate reactions were performed at 5°C intervals starting at 15°C and ending at 70°C. The reaction mixture was incubated separate from the enzyme, at the specific temperature for 5 min. prior to the addition of the enzyme. Termination was achieved after 15 min. or 30 min. with the addition of the boric acid buffer.

6.1.2.2.Results and Discussion

The optimal temperature for enzyme activity for the two isoenzymes differed by 10°C (see figure 34 page 127). The optimal temperature was 50°C for isoenzyme A, and 40°C for isoenzyme B. The B isoenzyme

was more high temperature resistant, while the A isoenzyme was more low temperature resistant. Differences in optimal temperature spectra may be attributed to differences in total carbohydrate content for the isoenzymes.

6.1.3.Heat Stability of NAG A and B Isoenzymes

Heat stability of the NAG A and B isoenzymes were assessed by determining at which temperatures the isoenzymes can tolerate heat for a fixed time duration without losing enzyme activity.

6.1.3.1.Method for Determination of Heat Stability

Isoenzyme activity was determined as before but after storage at a certain temperature conditions. Isoenzymes were frozen at -12°C, or were freeze dried, or snap frozen with liquid nitrogen, and then afterwards exposed to room temperature (19°C) for certain time intervals. NAG was also incubated at temperatures 30 to 70°C, in 5°C intervals, for one hour before redetermination of the enzymic activity.

6.1.3.2.Results and Discussion

NAG isoenzymes purified up to this level appear to be well protected against temperatures above freezing point and below 40°C. Temperature below freezing point caused inactivation. Temperatures above 50°C and 40°C for NAG A and B respectively had identical results as for the optimum temperature determination.

6.2. Kinetic Properties

The rate of the enzyme-substate formation is dependent on affinity and specificity of the enzyme for a given substrate[305]. It is important to determine K_m as a characteristic of the enzyme and to determine the substrate specificity.

6.2.1. Determination of K_m for the NAG Isoenzymes

6.2.1.1. Method used for K_m Determination

K_m was determined from the Lineweaver-Burke plot. The standard NAG assay was modified for this purpose. Isoenzyme preparations were incubated with the substrate concentrations varying from 0.1 mM to 3.0 mM at 37°C for 30 min. Determinations were done in triplicate. Different isoenzyme concentrations were used to determine the intercept for K_m determination.

6.2.1.2. Results and Discussion

NAG A and B followed typical Michaelis-Menten kinetics. The reciprocal values of absorbance at 405 nm. taken as NAG activity, and the reciprocal of substrate concentrations in mmol/L, were plotted. K_m values were confirmed using the Hanes plot. Identical K_m values of 0.497 mmol/L with PNP-NAG as substrate was found for both isoenzymes. (see figure 35 page 128).

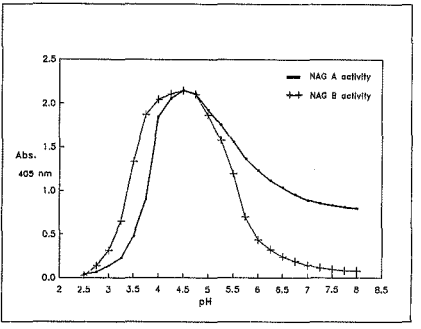


FIGURE 32

Wide pH range for the determination of the pH optimum for NAG A and B isoenzymes.

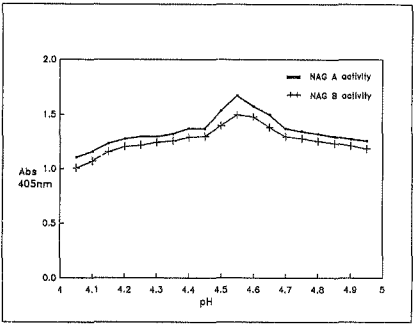


FIGURE 33

Narrow pH range for the determination of the narrow pH optimum for NAG A and B isoenzymes.

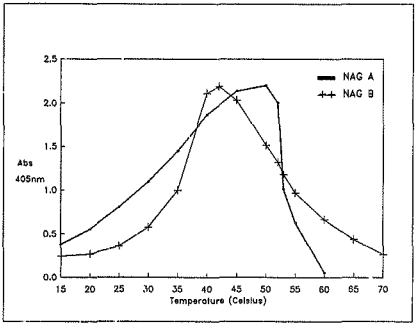


FIGURE 14

Optimum temperature plot of NAG isoenzyme activity for the determination of the optimum temperature for NAG A and B isoenzymes.

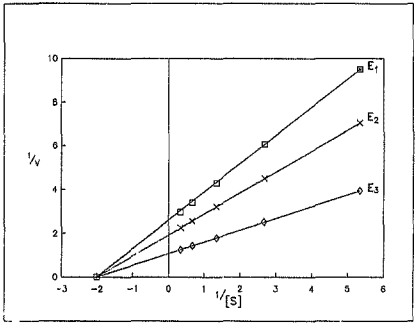


FIGURE 35

Lineweaver - Burk plot for the Km determination of NAG isoenzymes. Three different NAG isoenzyme concentrations (E1, E2, and E3), and five different 4-Nitrophenyl-N-acetyl-β-D-glucosaminide substrate concentrations (0.187, 0.375, 0.75, 1.5, 3.0 mM) were used for the Km determination.

6.2.2.Determination of Substrate Specificity for NAG Isoenzymes

Different substrates were systematically varied from the NAG-PNP substrate and tested for NAG activity. The ten chosen substrates were tested on both isoenzymes. Enzymes may have wide or narrow specificities in terms of reaction as well as substrate structure. Trypsin, for example, has a wide reaction specificality (i.e. it hydrolysis either peptide -, ester - or amide bonds) but a narrow structural specificity (i.e. it hydrolysis only bonds formed by Lys and Arg)[207-212].

6.2.2.1.Determination of Substrate and Reaction Specificity

The microplate method for NAG activity was adapted. The PNP-NAG substrate was substituted each time with one of the listed systematically related substrates. Reaction times were varied from 5 min. up to 45 min. in 10 minute intervals. Substrate concentrations were varied from 0.32 to 0.0752 M. Incubation of the plates were made for 5 min. at 37°C prior to enzyme addition. Lineweaver-Burk plots were used to obtain K_m values.

6.2.2.2.Results and discussion

No difference in substrate specificity was found between the two isoenzymes. The K_m values for a given substrate was identical for both isoenzymes (see table 12 page 131). In table 12 the relative rates of hydrolysis are compared with PNP-NAG taken as 100%.

As seen from table 12 on page 131 NAG shows a very narrow reaction specificity, i.e. the hydrolysis of β-D-glycosidic bonds with no affinity for α-D-glycosidic bonds. The structural specificity was narrow in terms of N-Acetyl substitution but less specific in terms of the hexose moiety for instance the substitution of Glc by Gal reduced the relative activity only by 20%.

TABLE 12

Percentage relative rate and K_m values for various substrates for isoenzymes A and B

Substrate	% Relative Rate	K_m*
P-Nitrophenyl-N-Acetyl-β-D-Glucosaminide (PNP-NAG)	100	0.497
P-Nitrophenyl-N-Acetyl-α-D-Glucosaminide	5	9.938
P-Nitrophenyl-β-D-glucopyranoside	0	∞
P-Nitrophenyl-β-D-Galactopyranoside	0	∞
P-Nitrophenyl-β-D-Fucopyranoside	0	∞
P-Nitrophenyl-β-D-Mannopyranoside	0	∞
P-Nitrophenyl-N-Acetyl-β-D-Galactosaminide	79	0.627
P-Nitrophenyl-N-Acetyl-α-D-Galactosaminide	0	∞
P-Nitrophenyl-2-Acetamido-2-deoxy-3-O-β-D-Galacto-pyranosyl-β-D-Glucopyranoside	8	6.213
P-Nitrophenyl-N-Acetyl-1-Thio-β-D-Glucosaminide	36	1.381

* Km in mmol/L compared to the PNP-NAG substrate.
∞ Infinite.

6.3.Effect of Inorganic and Organic ions on the Activity of NAG A and B isoenzymes

Metallic ions may undergo valence changes in accepting or donating electrons to or from substrates or enzymes in order to allow a certain enzymic reaction to occur[207]. In some peptide splitting systems, metallic ions joins with groups on the substrate molecule in a chelating manner to destabilize the bond that is to be split[207]. Another example is the requirement for Mg^{2+} in the formation of the N-Glycosidic bond[207] between adenine and ribose-5-phosphate.

6.3.1.Method for Testing the Influence of Compounds

By adapting the NAG microplate method, the effect of 26 different compounds, each at 5 different concentrations, and each of those at 4 different substrate concentrations for both the A and B isoenzymes was determined. The effect of compounds was tested at 100 mM, 50 mM, 25 mM, 10 mM, and 5 mM. Compounds were each first prepared as stock solutions, and then dissolved in 0.1 M citric buffer pH 4.5. Some of the citrate buffer and compounds to be tested formed precipitates due to the chelating effect of the citrate buffer. These compounds were tested either at low concentrations, or were omitted from the assays.

Substrate concentrations used were varied between 0.1875 mM to 3.0 mM 4-Nitrophenyl-N-acetyl-β-D-glucosaminide. The complete reaction mixture was prepared, except for the addition of the isoenzyme, and incubated at 37°C for 5 min. prior to the addition of the enzyme. After reactions termination absorbance was read at 405 nm. as before.

6.3.1.1.Results and Discussion

K_a values, expressed in mmol/l, were calculated from Lineweaver-Burk and Dixon plots. See figures 36(a) and 36(b) pages 136 and 136(b). A computer program EZ-FIT (Version 1.1., Medical Products Dept., E.I. Du Pont de Nemours & Co) was used for the analysis of all the enzyme data in this dissertation.

All inhibition encountered was uncompetitive (refer to figures 36(a) and 36(b) pages 136 and 136(b) indicating that the metal ions do not compete with the substrate for the active centre. Uncompetitive inhibition occurs when an inhibitor combines reversibly only with the enzyme-substrate complex to form a enzyme-substrate-inhibitor complex, which cannot yield product.

There was a great similarity of inhibition between NAG A and B when the activities were compared (see tables 13 and 14 pages 134 and 135 respectively). This could be due to identical catalytic centre or amino acid sequences.

From tables 13 to 15 (pages 134 and 135) it can be seen that it is possible to derive the following:

(1) Neither Na, K, Al, Li, Cl, NO_3, or SO_4 are inhibitors of NAG.
(2) Ag followed by Pb were relatively strong inhibitors.
(3) Acetate, F and Cr were moderate inhibitors.
(4) The divalent ions Mn, Mg, Cu, Sn, Fe and Ca were poor inhibitors but it could be due to the chelating effect of the citrate buffer.

TABLE 13

K_a values and percentage inhibition of uncompetitive inhibitors of NAG A isoenzymes

Inhibitor Compound	K_a (mmol/L)	Inhibitor concentration 5	10	25	50	100 (mM)
		Percentage Inhibition				
$AgNO_3$	3.6	5	9	24	47	94
Pb-Ac	8.5	4	7	18	35	71
Cd-Ac	23.3	3	5	13	26	51
Co-Ac	23.6	3	5	13	26	51
Zn-Ac	24.4	3	5	13	25	50
NH_4-Ac	27.4	2	5	12	24	48
K-Ac	27.9	2	5	12	24	47
Na-Ac	28.1	2	5	12	24	47
NaF	31.3	2	4	11	22	44
$CrCl_3$	33.8	2	4	11	21	42
$MnCl_2$	56.7	1	2	6	12	25
$MgCl_2$	61.2	1	2	5	10	20
$CuSO_4$	64.9	1	2	5	9	19
$SnCl_2$	81.3	0	1	2	4	7
$FeCl_2$	86.5	0	0	1	2	4
$CaCl_2$	88.4	0	0	1	1	2

Ac = Acetate

TABLE 14

K_a Values and percentage inhibition of uncompetitive inhibitors of NAG B isoenzymes

Inhibitor Compound	K_a (mmol/l)	Inhibitor concentration 5	10	25	50	100 (mM)
		Percentage Inhibition				
$AgNO_3$	3.4	5	10	24	48	97
Pb-Ac	7.1	3	7	17	35	70
Cd-Ac	29.5	2	5	11	23	46
Co-Ac	23.0	3	5	13	26	52
Zn-Ac	21.9	3	5	13	26	53
NH_4-Ac	28.8	2	5	12	23	46
K-Ac	23.2	3	5	13	26	51
Na-Ac	20.6	3	5	13	27	54
NaF	27.7	2	5	12	24	47
$CrCl_3$	17.5	2	4	10	20	39
$MnCl_2$	61.4	1	2	5	11	22
$MgCl_2$	62.1	1	2	5	11	21
$CuSO_4$	60.2	1	2	6	11	22
$SnCl_2$	88.4	0	0	1	1	2
$FeCl_2$	89.2	0	0	0	1	2
$CaCl_2$	78.3	0	1	2	5	9

Ac = Acetate

TABLE 15

Compounds found to have no inhibition on NAG activity

$AlCl_3$	CHAPS	HEPES	KCl	KNO_3	LiCl
$LiSO_4$	Na_2SO_4	NaCl	$NaNO_3$	Triton X-100	

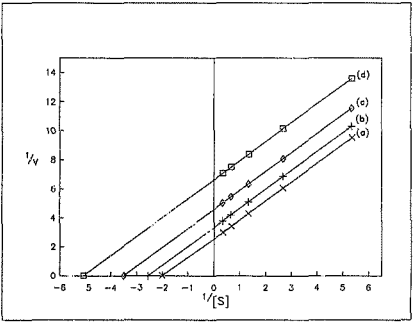


FIGURE 36(a)

Reciprocal plots of reaction velocity (v) and substrate concentration (s) of the NAG A specie without (a) and with the uncompetitive inhibitor NaF at (b) 10 mM, (c) 25 mM, and (d) 50 mM NaF.

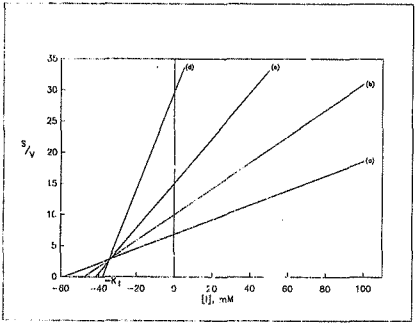


FIGURE 36(b)

Dixon plot of the NAG A specie at concentrations of 0, 5, 10, 25, 50, and 100 mM $CrCl_3$ at four different substrate concentrations (0.375(a), 0.75(b), 1.5(c), 3(d) mM PNP-NAG). All inhibition encountered was uncompetitive.

CHAPTER 7 CONCLUSION

Baboon kidney NAG isoenzymes were isolated by adapting and modifying previous attempts of other workers[270-281]. To my knowledge purification of the isoenzyme was not achieved nor homogeneity confirmed. The purification of NAG A is summarized in figure 37 on page 140, and the enrichment of purification is presented in table 16 page 141. Con A-Sepharose affinity- DEAE- and HTP- ion-exchange chromatography and selective rechromatography resulted in two distinct bands on 10% T, 2.7% C anionic PAGE with a 109 fold purification. By performing semi-preparative anionic PAGE followed by gel slicing, the two bands were individually extracted from the gel. The major band proved to be homogeneous on NR-SDS-PAGE with a molecular weight of 52.1 kD and a purification of 316 fold. On R-SDS-PAGE two high molecular weight aggregates of NAG A were obtained with molecular weights of 88.8 kD and 83.5 kD respectively. Using sedimentation equilibrium ultracentrifugation NAG A also revealed an aggregational phenomenon.

On PAG-IEF the NAG A-1 fraction displayed micro-heterogeneity between pH 4.55 and pH 5.85 where 19 distinct bands could be observed. Selective removal of certain carbohydrate moieties with the use of glycosidases resulted in a selective reduction of 7 bands by Endoglycosidase H (12 bands vs 19 bands) while Endoglycosidase D and Glycopeptidase F consolidated all bands into one (pI 4.97) to confirm homogeneity and can micro-heterogeneity be assigned to variation of the carbohydrate moieties.

The amino acid composition revealed a total of 329 amino acids

yielding a peptide molecular weight of 36,483 kD. Amino acids involved in carbohydrate attachment, like Asn, Ser, Thr, and Glu, were prominent in numbers. The peptide was classified as hydrophilic with an acidic character.

NAG A-1 revealed a 30% carbohydrate content including 6% sialic acids. The high sialic acid content is most likely responsible for the heterogeneity on PAG-IEF. The high carbohydrate content may also explain the unconventional behaviour of the enzyme on ion-exchange chromatography, PAG-IEF, and sedimentation equilibrium centrifugation.

For the calculation of kinetic parameters, a V^{-1} against S^{-1} plot resulted in a K_m value of 0.497 mmol/L. Substrate and reaction specificity was assessed by subjecting 9 structural related substrates to PNP-NAG isoenzymes A and B. The p-Nitrophenyl-N-Acetyl-β-D-Galactosaminide substrate had a 80% relative activity in comparison to the PNP-NAG substrate revealing a specific requirement for N-Acetyl amino hexoses but not so specific for the kind of hexo-pyranose moiety (Glucose vs Galactose) The reaction specificity was very specific being only for β-D glycosidic bond and not for α-D bonds.

The effect of inorganic ions on NAG A and B activity was investigated. It was found that silver, lead and acetate were the most potent uncompetitive inhibitors to NAG.

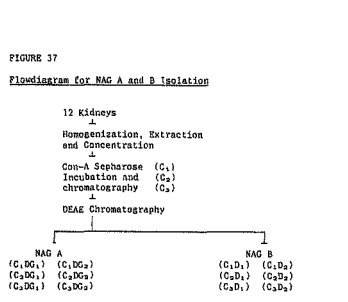
A pH optimum of 4.55 was found for both the NAG A and B isoenzymes.

The optimum temperature for the NAG isoenzymes was 50°C for A and 40°C for B. The difference in heat stability for the A and B isoenzymes indicated that NAG B was stable at high temperature.

Assessment of the subcellular distribution of the two major NAG isoenzymes in the kidney revealed that the A isoenzyme was found profoundly in the lysosomal fraction, while the B isoenzyme was distributed equally amongst the lysosomal and mitochondrial fraction.

FIGURE 37

Flowdiagram for NAG A and B Isolation



* No purification ** Inactivation of enzyme

TABLE 16

Purification of N-Acetyl-β-D-Glucosaminidase A and B

Purification Step	Total Units (x 1000)	Total Proteins (mg)	Specific Activity (U/mg protein)	Yield (%)	Enrich-ment
Extraction	8965	43000	208	100	1
Con-A Chr.	5895	894	6593	66	32
NAG A					
DEAE Chr.	2302	221	10416	26	49
DEAE Rechr.	1956	156	12[illegible]	22	60
HTP Chr.	1504	67	22447	17	107
HTP Rechr.	1370	57	24035	15	115
Gel Slicing:					
Iso A-2	274	12	22833	3	109
Iso A-1	165	2.5	66000	2	316
NAG B					
DEAE Chr.	1160	267	4344	15	20
DEAE Rechr.	986	226	4362	11	21
CM Chr.	986	226	4362	11	21

A Unit (U) of enzyme activity was defined as nmol of product formed per hour determined by the standard assay.

Chr. Chromatography
Iso. Isoenzyme

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