GLYCOSYLATION OF BOVINE α-LACTALBUMIN

A thesis presented in partial fulfilment of the requirements
for the degree of
Master of Philosophy in Biochemistry,
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1999

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ABSTRACT

Bovine α-lactalbumin exists in four different forms. These are the F, M, S_1 and S_2 forms named after their positions on native gels. F, S_1 and S_2 are minor components representing 15% of the total α-lactalbumin fraction whereas M is the major component. S_1 and S_2 have been shown to be glycoforms of α-lactalbumin and although there are potentially three glycosylation sites in the protein, only asparagine 45 appears to be glycosylated. It has been suggested that F differs from M by the replacement of an amide group.

The glycoforms of α-lactalbumin (S_1 and S_2), and the non-glycosylated proteins (M and F) were isolated and purified using selective precipitation, affinity chromatography, size exclusion chromatography and preparative electrophoresis.

The potential glycosylation sites were investigated using selective proteolysis in conjunction with Edman sequencing and electrospray mass spectrometry (ES/MS).

It was found that although the main fraction (M) or non-glycosylated protein contained no covalently bound carbohydrate, selected ion monitoring experiments showed that there appeared to be a lactosamine sugar associated with the protein.

A number of methods were investigated for analysing and separating the glycoforms of α-lactalbumin. These included high pH anion exchange chromatography with pulsed amperometric detection (HPAEC/PAD), fluorophore assisted gel electrophoresis (FACE) and derivatisation of the oligosaccharides with 1-(p-methoxy)phenyl-3-methyl-5-pyralozone (PMPMP) and subsequent separation by RP-HPLC. Electrospray mass spectrometry was used to confirm the results of these various techniques.

Although it was not firmly established which of the three possible sites were glycosylated, refinement of the purification protocol resulted in several different glycans being identified on the basis of the ES/MS and FACE results. It would appear that there are up to 15 different glycoforms of α-lactalbumin, some of which are highly sialated. It is difficult to determine whether the simpler structures represent breakdown products of the more complex structures, or whether they are present naturally.

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ABBREVIATIONS

API	Atmospheric pressure ionisation
ANTS	8-amino-napthalene-1,3,6 trisulphonic acid
AUFS	Absorbance units at full scale
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
cAMP	Cyclic 3', 5'-adenosine monophosphate
CAP	3-(Cyclohexylamino)-propanesulphonic acid
CBR-250	Coomassie Brilliant Blue R-250
CID	Collision induced decomposition
Con A	Concanvalin A
3-D	Three dimensional
Da	Dalton
DDT	Dithiothretitol
DEAE	Diethylaminoethyl
Dp	Degree of poymerization
EDTA	Ethylenediamine tetra-acetic acid (di-sodium salt)
ENGase	Endo-N-acetyl-β-D-glucosaminidase or endoglycosidase
ER	Endoplasmic reticulum
ES/MS	Electrospray mass spectrometry
FACE	Fluorophore assisted carbohydrate electrophoresis
FPLC	Fast protein liquid chromatography
GPI	Glycosylphosphatidylinositol
HEWL	Hen egg-white lysozyme
H^1 NMR	Hydrogen nuclear magnetic resonance
HPAEC/PAD	High performance anion exchange chromatography with pulsed amperometric detection
IgGs	Immunoglobulins

IS	Ion spray voltage
IQ	Q exit lens
α-LA	α-lactalbumin
β-LG	β-lactoglobulin
M+H	Molecular ion (protonated)
PAGE	Polyacrylamide gel electrophoresis
PNGase	Peptide-N4-(N-acetyl-β-D-glucosaminyl) asparagine amidase
PMPMP	1-(p-methoxy)phenyl-3-methyl-5-pyrazolone
PVDF	Polyvinylidene difluoride
OR	Orifice
RER	Rough endoplasmic reticulum
RP-HPLC	Reverse phase-high performance liquid chromatography
RNG	Ring voltage
SDS	Sodium dodecyl sulphate
TFA	Trifluoroacetic acid
TLC	Thin layer chromatography
Tris	Tris(hydroxymethyl) aminomethane
UDP	Uridine diphosphate

Sugar abbreviations

Gal	Galactose
Man	Mannose
Fuc	Fucose
GlcNAc	N-acetylglucosamine
GalNAc	N-acetylgalactosamine
NeuNAc	N-acetylneuraminic acid (sialic acid)

The 20 common amino acids

A	Ala	alanine
C	Cys	cysteine
D	Asp	aspartic acid
E	Glu	glutamic acid
F	Phe	phenylalanine
G	Gly	glycine
H	His	histidine
I	Ile	isoleucine
K	Lys	lysine
L	Leu	leucine
M	Met	methionine
N	Asn	asparagine
P	Pro	proline
Q	Gln	glutamine
R	Arg	arginine
S	Ser	serine
T	Thr	threonine
V	Val	valine
W	Trp	tryptophan
Y	Tyr	tyrosine

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CHAPTER I

INTRODUCTION

1.1 Outline

This section will provide a brief overview of the relationship between glycosylation and bovine α-lactalbumin. Firstly, protein glycosylation will be reviewed looking at the different glycans that are found covalently linked to proteins, the manner in which they are attached to the protein, and how effectively certain recognition sites are glycosylated. Secondly, the functional roles of these oligosaccharides will be discussed. Finally, there will be a detailed discussion about α-lactalbumin and glycosylated α-lactalbumin.

1.2 Glycoproteins

Glycoproteins are proteins which possess an oligosaccharide moiety that is covalently attached to either the amide side chain of asparagine via an N-glycosidic bond or the hydroxyl side chain of serine, threonine, hydroxy proline or hydroxy lysine via an O-glycosidic bond. The plethora of glycosyltransferases present in the endoplasmic reticulum (ER) and the Golgi apparatus results in the biosynthesis of a large heterogeneous population of structurally related oligosaccharides that can be attached to one or more sites on any one protein. These oligosaccharides can be different in their primary structure as well as their anomeric (α or β) and isomeric (links through the 2, 3, 4, or 6 position) configurations. Each sugar has an absolute configuration (D or L) and can have a different ring size (furanose or pyranose). Additional groups (usually phosphate or sulphate) can also be attached at various positions around the ring (Figure 1.1).

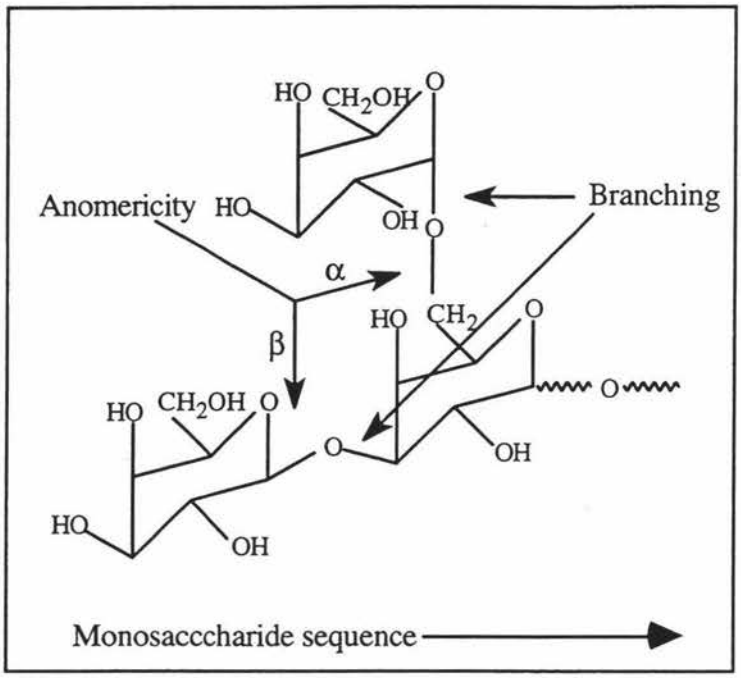
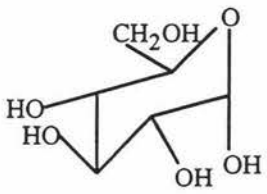


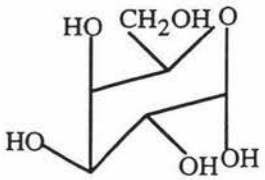
Figure 1.1 *Origins of structural diversity.*

Oligosaccharides are produced by the sequential addition of monosaccharide units to a core structure. Figure 1.2 illustrates the different types of monosaccharide units commonly found in glycoproteins.

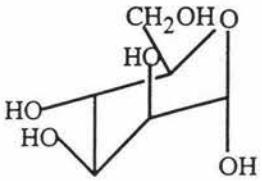
In general the oligosaccharides of glycoproteins can be classed into two groups by the way in which they are bound to the protein. N-linked oligosaccharides are linked via a chitobiose unit to the amide nitrogen of an asparagine residue. O-linked oligosaccharides are linked to a hydroxyl oxygen in the side chain of an amino acid, most frequently a serine or threonine residue. A third type of sugar chain is a component of the glycophosphatidylinositol (GPI) anchor, which links some cell surface proteins to the lipid bilayer.



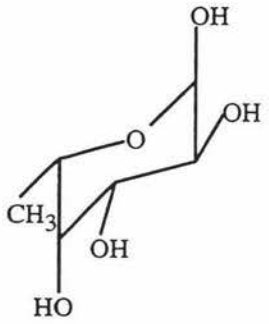
α-D-Glucose



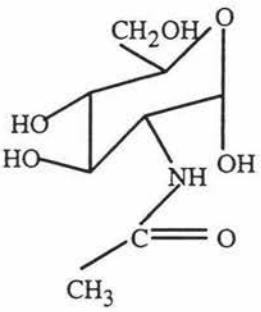
α-D-Galactose



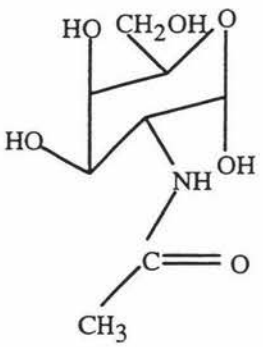
α-D-Mannose



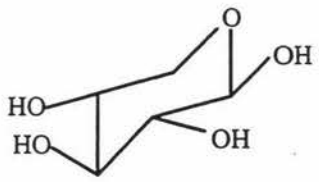
α-L-Fucose



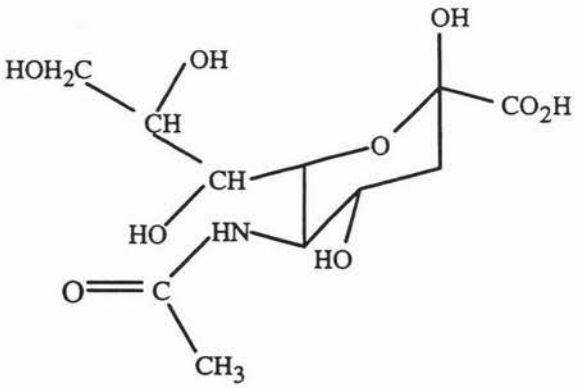
N-Acetyl-α-D-glucosamine



N-acetyl-α-D-galactosamine



β-D-Xylose



α-N-Acetylneuraminic acid

(Sialic acid)

Figure 1.2 *Structures of monosaccharides commonly found in eukaryotic glycoproteins.*

1.3 N-linked glycosylation

One of the most common types of eukaryotic protein modification is N-linked glycosylation. The biosynthesis of N-linked oligosaccharides begins with the synthesis of a lipid linked oligosaccharide moiety ($Glc_3Man_9GlcNAc_2PPDol$) that is transferred onto the nascent polypeptide chain in the rough endoplasmic reticulum (RER). The acceptor is the amino group of an asparagine side chain that is part of a tripeptide recognition sequence Asn-X-Ser/Thr. A series of trimming and elongation reactions is then catalysed by glucosidases, mannosidases and an array of glycosyltransferases (N-acetylglucosaminyl-, fucosyl-, galactosyl-, sialyltransferases) in the RER and the Golgi apparatus to produce a wide range of glycans that are specific to each cell type, tissue type and species. Glycoproteins which contain more than one glycan site can carry different N-glycan structures at each recognition sequence (Rademacher *et al.*, 1988).

1.3.1 Structure of the N-linked oligosaccharides

Structurally, the N-linked oligosaccharides can be classified into three sub groups:

(1) High mannose

(2) Complex

(3) Hybrid

All three classes contain an identical core pentasaccharide as shown below:

(1) High mannose type

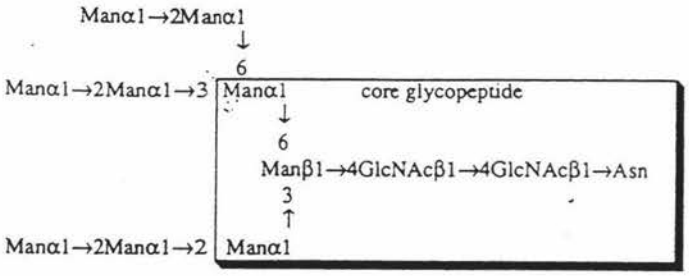


Figure 1.3 *High mannose type N-linked glycan (Mort and Pierce, 1995).*

Oligosaccharides that contain only mannose and N-acetylglucosamine residues are known as high mannose type sugar chains (Figure 1.3).

(2) Complex type

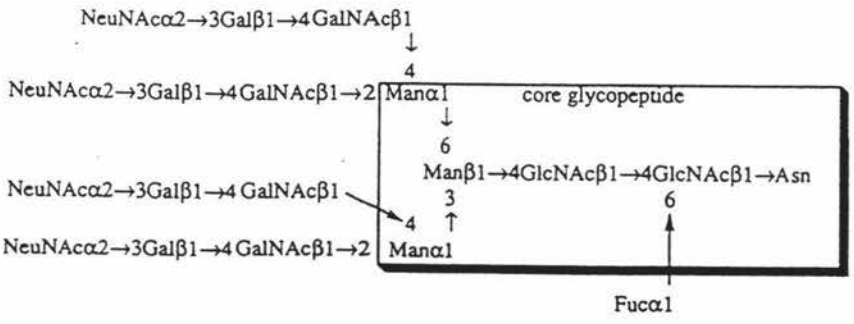


Figure 1.4 *Complex type N-linked glycan (Mort and Pierce, 1995).*

Complex-type sugar chains are formed when the oligosaccharide contains monosaccharides other than mannose and N-acetylglucosamine. Further structural variation is afforded by the presence or absence of fucose residues linked at the C-6 or C-3 positions of the proximal N-acetylglucosamine residue. Complex type oligosaccharides are known to be present in α-lactalbumin (Tilley *et al.*, 1991).

(3) Hybrid type

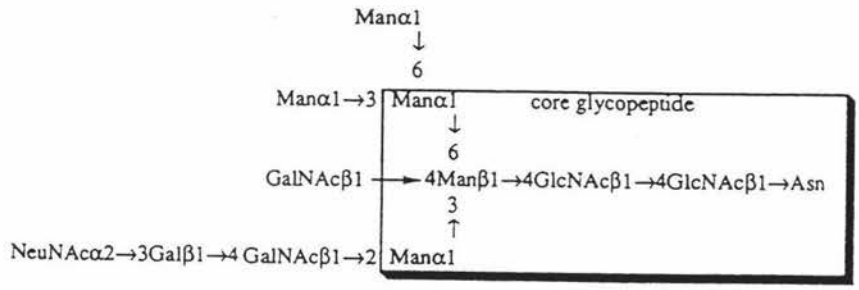


Figure 1.5 *Hybrid type N-linked glycan (Mort and Pierce, 1995).*

The hybrid type was found during a comprehensive study of the large sugar chains isolated from hen egg albumin (Kobata and Endo, 1993). As shown above, these sugar chains are classified as the hybrid type because they have structural features that are characteristic of both the high mannose type and complex type sugar chains.

1.3.2 Influence of local amino acids on core-glycosylation

N-linked glycosylation occurs only when the asparagine residue is part of the sequon Asn-X-Ser/Thr. There are some reports that amino acids near an asparagine residue are important determinants of core glycosylation efficiency. These include amino acids within the sequon itself (i.e: serine, threonine and X) as well as amino acids which flank the sequon.

(a) The influence of the hydroxy amino acids

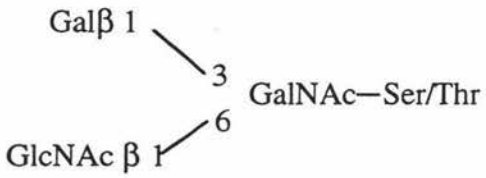
Studies using a variety of experimental approaches have shown that the hydroxy amino acid within the sequon can influence its potential glycosylation. Surveys of N-linked glycans have revealed that Asn-X-Thr sequons are 2-3 times more likely to be glycosylated than Asn-X-Ser sequons. These results were confirmed by experiments with a series of peptides that showed Asn-X-Thr sequons are more efficient oligosaccharide acceptors compared to Asn-X-Ser sequons (Kaplan *et al.*, 1987). A mechanism was proposed in which the hydroxy amino acid plays a direct, catalytic role in the glycosylation reaction through a series of hydrogen bond transfer reactions (Imperialli *et al.*, 1997). This involves an oligosaccharyltransferase, the hydroxy amino acid side chain, and the β amino group of asparagine. Interestingly it was also shown that the sulfhydril group of cysteine can support glycosylation at Asn-X-Cys sequons in peptides, although the oligosaccharide acceptor activity of such peptides is low (Shakin-Eshleman, 1996). Glycosylated Asn-X-Cys sequons have been observed in bovine and human protein C (Miletich and Broze, 1990), human von Willebrand factor (Titani *et al.*, 1986) and human α-lactalbumin (Giuffrida *et al.*, 1997). Only serine, glutamine, alanine and isoleucine have been observed at the X position in this sequon. The presence of proline at the X position seems to prevent glycosylation (Bause, 1983).

(b) The influence of the X amino acid

While there are instances of all the common amino acids apart from proline in position X of glycosylated sequons, some appear to be less favourable than others for glycosylation to occur. These amino acids include cysteine, tryptophan, aspartic acid and glutamic acid. Large hydrophobic amino acids at position X (e.g: tryptophan) also impair glycosylation, as do negatively charged amino acids (e.g: glutamic acid and aspartic acid). In contrast, small amino acids (e.g: glycine) or positively charged amino acids (e.g: lysine, arginine, histidine) in the X position seem to favour efficient glycosylation (Shakin-Eshleman, 1996).

1.4 O-linked glycosylation

Sugars may also be linked to proteins through a serine or threonine residue. In mammals, this link is commonly to an α-N-acetylgalactosamine (αGalNAc-Ser/Thr) residue and is known as a mucin type linkage. Mammalian O-linked glycoproteins have either linear chains formed by an extension of the oligosaccharide chain through a β1,3-linked galactose (Gal β1-3 GalNAcα-Ser/Thr) or branched structures by additional extension through β1,6-N-acetylglucosamine linked to the N-acetylgalactosamine as follows:



1.5 Functions of the oligosaccharide moiety

Many different functions have been attributed to the carbohydrate side chains that are covalently attached to the majority of eukaryotic membrane and secreted proteins (Varki, 1993, Lis and Sharon, 1993), all of which are not yet fully understood. The effect of carbohydrates on the physiochemical properties of proteins such as viscosity, isoelectric pH, the degree of hydration, solubility, thermal stability, and resistance to proteolysis, have been known for some time. Similarly, it has also been known that the oligosaccharide structures of glycoproteins are frequently different in various pathological states, including malignancy. Recently it has been shown that glycosylation has an important effect on protein folding and is involved in targeting within the cell. The first clear-cut demonstration of the functional significance of the carbohydrate moiety of a glycoprotein was in the blood group substances, where immunological specificity was found to be dependent on the structure of the oligosaccharide chains linked to the glycoproteins of the erythrocyte cell membrane. A major breakthrough in the search for functional significance of carbohydrates linked to proteins occurred when it was fortuitously discovered that the removal of sialic acid from the glycan

of proteins resulted in the rapid clearance of that glycoprotein from circulation. It was found that removal of sialic acid exposes the penultimate galactose residues at the non-reducing termini of the carbohydrate chains. These specifically bind to a Gal/GalNAc-binding receptor on hepatocytes and the glycoprotein becomes internalised by receptor-mediated endocytosis. Other specific carbohydrate-binding receptors or lectins for GlcNAc/Man and L-fucose were subsequently found to be located in the reticuloendothelial system. Carbohydrate-lectin interactions are also involved in lymphocyte migration, since treatment of lymphocytes with exoglysosidases or inhibitors of oligosaccharide-processing enzymes has been shown to alter their migratory or so called homing properties. Mammalian sperm and egg interaction has been shown to involve the binding of a sperm protein with a zona pellucida protein, ZP-3. In this case O-linked and not N-linked oligosaccharides on ZP-3 have been shown to be involved in binding (Paulson and Colley, 1989).

The variety of biological phenomena, such as the alteration of the carbohydrate structure of glycoproteins in cellular growth, development, and differentiation, and in various disease states, allude to the importance of the role that glycans play in biology. For some proteins these roles have been elucidated by studying the function of the protein in its glycosylated and deglycosylated states. For example when the glycan of the hormones human chorionic gonadotropin (hCG), lutropin (LH), and thyrotropin (TSH), is removed enzymatically, the receptor binding activity of the hormones is not affected but the ability of the deglycosylated hormones to stimulate cAMP and steroidogenesis is drastically reduced.

1. 6 Deglycosylation

1.6.1 PNGases

A number of endoglycosidases have been isolated that recognise and hydrolyse the N-linked glycans found on glycoproteins. Peptide-N-(N-acetyl-β-glucosaminyl)-asparagine amidase, or PNGase, selectively hydrolyses the β-asparatylglucosylamine bond of most known types of N-linked oligosaccharides as shown in Figure 1.6 (Tarentino *et al.*, 1985). The hydrolysis of this bond occurs in two steps, as proven by H^1 NMR and kinetic studies (Risley and Van Etten, 1985). In the first step, the carbohydrate is cleaved from the protein/peptide and the asparagine residue is converted to aspartic acid. The intermediate oligosaccharide retains the amino group. This intermediate then slowly, non-enzymatically degrades to an intact oligosaccharide and free ammonia (Risley and Van Etten, 1985, Tarentino *et al.*, 1982). Substrates for these enzymes include N-linked, high mannose, hybrid, and bi-, tri- and tetraantennary complex glycans (Tarentino *et al.*, 1982). It has been shown however, that PNGase F will not act on an oligosaccharide made up of less than two GlcNAc residues, or one that has a fucose linked 1-3 to the proximal GlcNAc (Chu, 1986). The asparagine has to be part of a peptide of at least five amino acids. Optimum deglycosylation occurs when the asparagine is near the middle of this peptide. Table 1.1 shows PNGases that have been isolated to date.

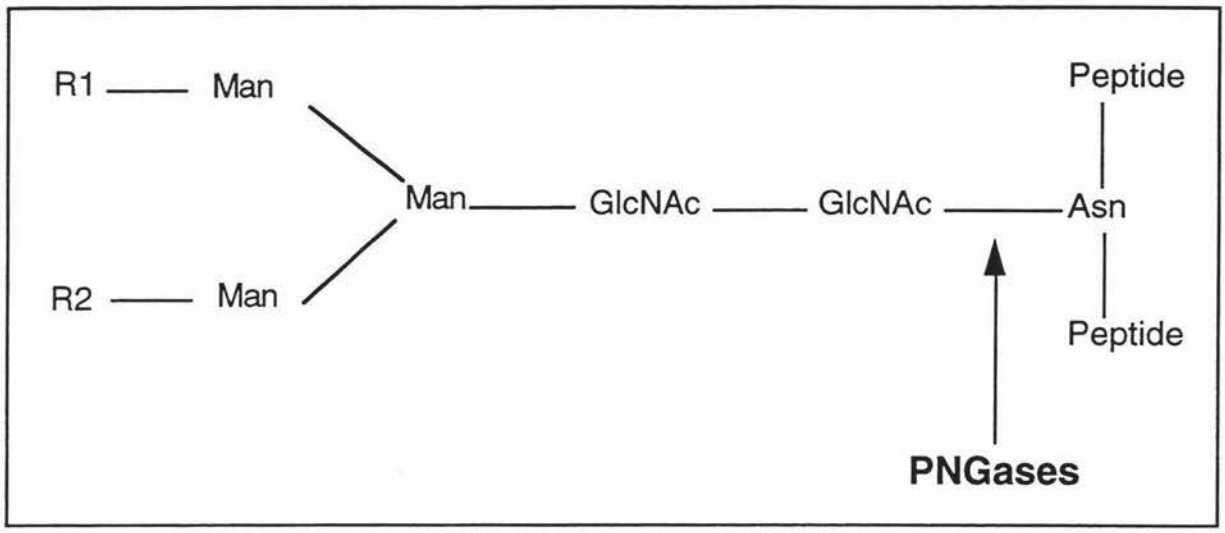


Figure 1.6 *The β-asparartylglucosylamine bond cleaved by PNGases.*

Table 1.1 *PNGases and their source.*

Enzyme	Source	Reference
PNGase A	Almond	Takahashi, 1977
PNGase F	Bacterium	Plummer *et al.*, 1984
PNGase L-929	Mouse fibroblast	Suzuki *et al.*, 1994
PNGase Se	White campion	Lhernould *et al.*, 1992
PNGase	Oryzias latipes	Seko *et al.*, 1991
PNGase J	Jack beans	Sugiyama *et al.*, 1983
PNGase P	Pea	Plummer *et al.*, 1987
PNGase R	Radish	Beger *et al.*, 1995
PNGase	Various mouse organs	Kitajima *et al.*, 1995
PNGase	Humans, chickens	Suzuki *et al.*, 1995
PNGase HO	Hen oviduct	Suzuki *et al.*, 1997
PNGase GM	Soybean	Kimura and Ohno, 1998

1.6.2 ENGases

A more specific enzyme that recognises only high mannose structures is endo-β-N-acetylglucosaminidase H (Endo H) which hydrolyses the linkage between the two N-acetylglucosamine (GlcNAc) residues in the chitobiose core (Tai *et al.*, 1977). This reaction cleaves the glucosidic bond in the chitobiose core, leaving a GlcNAc residue on the glycoprotein and liberating the oligosaccharide with one less reducing end GlcNAc. The site of cleavage catalysed by this enzyme is shown in Figure 1.7. A number of ENGases have been isolated from various sources as shown in Table 1.2.

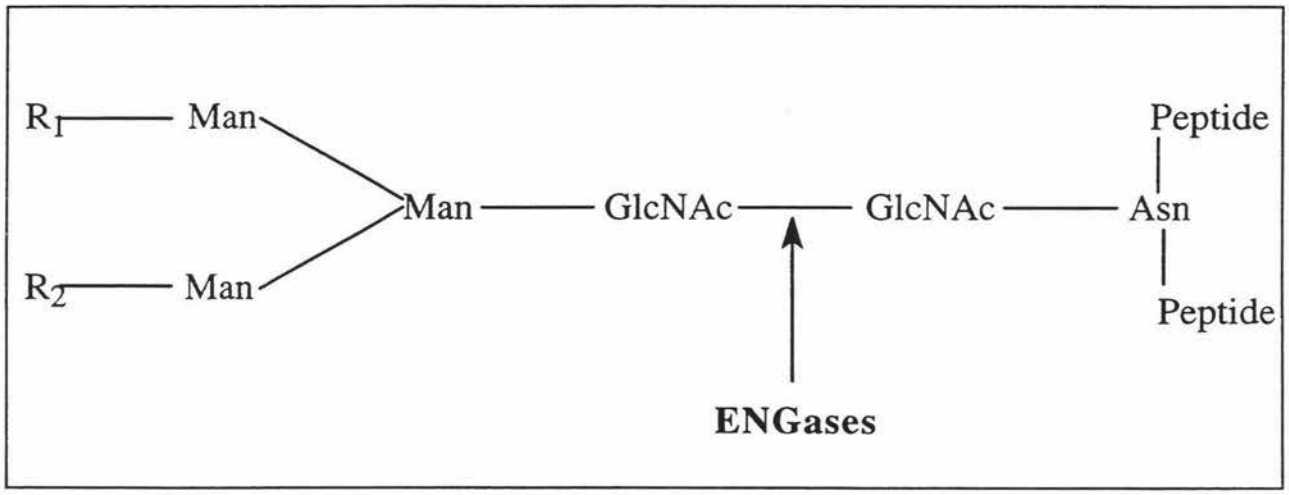


Figure 1.7 *Site cleaved by ENGases.*

Table 1.2 *Some ENGases and their source.*

Enzyme	Source	Reference
Endo B	Fungi	Bouquelet *et al.*, 1980
Endo C	Bacterium	Ito *et al.*, 1975
Endo D	Bacterium	Muramastu, 1971
Endo F1, F2, F3	Bacterium	Plummer *et al.*, 1984
Endo H	Bacterium	Tarentino and Maley, 1974
Endo L	Bacterium	Tarentino and Maley, 1974
Endo S	Fungi	Freeze and Etchinson, 1984

1.7 α-Lactalbumin

α-Lactalbumin, a globular whey protein consisting of 123 amino acid residues with a molecular weight of around 14 kDa, is produced in the lactating mammary gland. Three genetic variants (A, B, and C) are known to exist, although B is the predominant variant in bovine milk. The B variant differs from the A variant by a substitution of arginine for glutamic acid at position 10 (Gordon *et al.*, 1968). The results of investigations into α-lactalbumin have shown that it has an interesting role as a specificity regulator of lactose synthase (Brodbeck *et al.*, 1967). It has also been shown to have unexpected homology to the c-type lysozymes (Brew *et al.*, 1967). Although their functions are different both contain 51 homologous residues (including four disulphide bonds), and 24 additional residues with similar properties. It has been suggested that α-lactalbumin and lysozyme may have evolved from the same ancestral genes by a process of gene duplication and divergence. More recently, α-lactalbumin was shown to bind Ca^{2+} (Hiroaka *et al.*, 1980) and Zn^{2+} (Musci and Berliner, 1985), and is denatured at pH 3.5 due to the dissociation of these calcium ions from the protein (Desmet *et al.*, 1987). Later, it was shown that the α-lactalbumin molecule contained one tight binding site for Ca^{2+} and one weaker binding site for Zn^{2+} (Fitzgerald and Swaisgood, 1989).

1.7.1 Biological role of α-lactalbumin

The elucidation of the role played by α-lactalbumin in the synthesis of lactose represents a significant biochemical discovery. While studying the enzyme lactose synthase, Brodbeck and Ebner (1966) determined that it consisted of two components that were designated as "A-protein" and "B-protein". One year later, it was determined that α-lactalbumin was indeed the B protein and that it functioned in concert with the A protein as a "specifier" protein in the catalytic function of lactose synthase, possessing no catalytic function itself (Brodbeck and Ebner, 1966). Brew *et al.*, (1968) identified the A-protein as a galactosyltransferase.

In the absence of α-lactalbumin, galactosyltransferase transfers galactose from uridine diphosphate-galactose (UDP-galactose) to N-acetylglucosamine. In lactating mammary tissue, in the presence α-lactalbumin, the transfer of UDP-galactose is to glucose rather than to N-acetylglucosamine forming the milk sugar, lactose as shown in Figure 1.8. α-Lactalbumin has been found in milk of all species in which lactose is the principal sugar, and occurs in glycosylated and non-glycosylated forms.

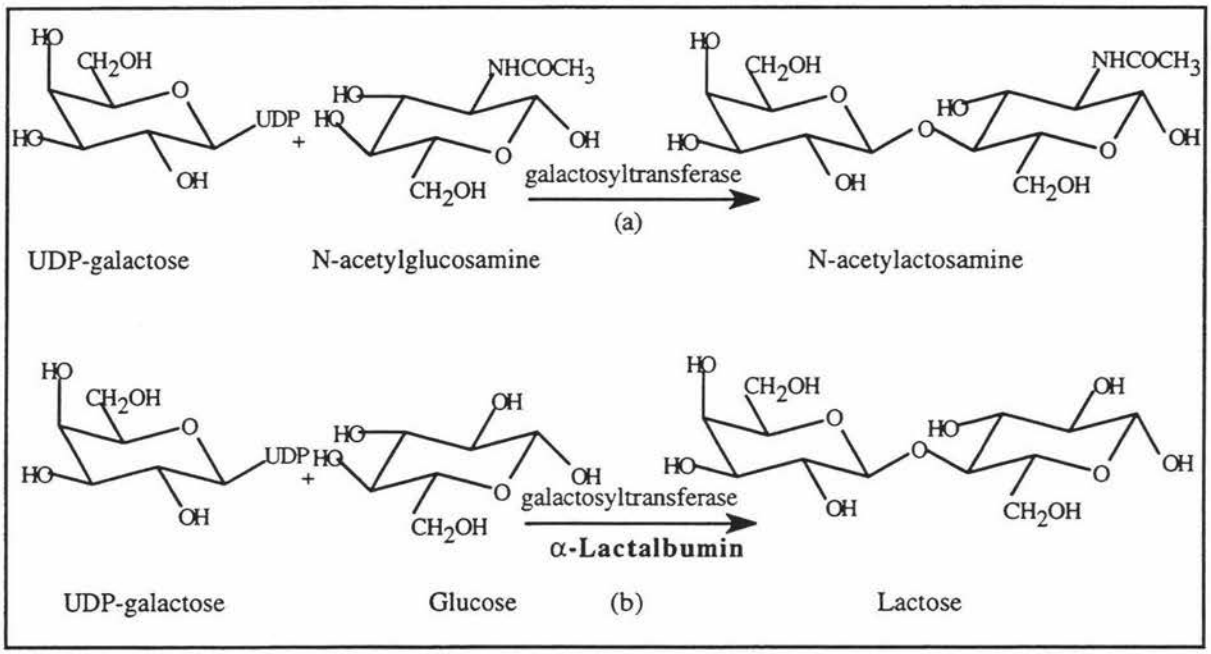


Figure 1.8 *Reactions catalyzed by galactosyltransferase. (a) In the absence of α-lactalbumin. (b) In the presence of α-lactalbumin.*

1.7.2 The importance of α-lactalbumin in infant nutrition

The complete primary structures of both the bovine and human forms of α-lactalbumin have been reported (Brew *et al.*, 1970, Findlay and Brew, 1972). Both consist of 123 amino acids that show 72% sequence homology. An additional 6% of the amino acid sequence are similar. Because of its amino acid composition α-lactalbumin is a remarkable protein. It has a very high chemical score and is rich in essential amino acids. The high concentration of α-

lactalbumin in human milk is largely responsible for its distinctive amino acid composition.

In the food industry the "humanising" of bovine milk for infant formulae has become an important process. Bovine milk has a different composition from human milk in that it has a lower content of α-lactalbumin, lactoferrin, lysozyme, and immunoglobulins (IgGs) (Hambraeus, 1977). In humans, α-lactalbumin comprises 28% of the total protein, whereas in

bovine milk α-lactalbumin represents only 4% of the total protein (Heine *et al.*, 1991). β-lactoglobulin is the major whey protein in bovine milk but it is present at only low levels in human milk. It has been claimed that β-lactoglobulin is one of the main causes of the allergy to bovine milk exhibited by some infants (Kuwata *et al.*, 1985). The aim of "humanising" milk is to achieve an adequate supply of essential and semi-essential amino acids but to limit the oversupply of non-essential amino acids. In order to produce infant formulae with a more similar composition to that of human milk, a proportion of β-lactoglobulin needs to be removed and α-lactalbumin needs to enriched.

1.7.3 The structure of α-lactalbumin

The primary structure of α-lactalbumin was reported by Brew *et al.*, (1970) (Figure 1.9), who drew scientists' attention to aspects of its structure that were similar to hen's egg-white lysozyme (HEWL). Direct X-ray crystallographic studies of the three dimensional structure of α-lactalbumin have been difficult for two reasons. First, it was difficult to find suitable crystals for data collection (Aschaffenburg *et al.*, 1972a,b, Fenna, 1982) and secondly, it was difficult to prepare heavy-atom derivatives necessary to solve the structure. Baboon (*Papio cynocephalus*) α-lactalbumin was found to give diffraction quality crystals and low resolution structures (6.0 Å and 4.5 Å) were reported (Smith *et al.*, 1987). However to obtain a useful isomorphous derivative, it proved necessary to chemically modify the protein by breaking the disulphide bond between cysteines 6 and 120 to provide a specific binding site for a mercury ion. X-ray crystallographic studies of baboon α-lactalbumin at high resolution revealed a

novel calcium binding loop and confirmed that in the native state, α-lactalbumin and c-type lysozyme have highly similar three dimensional structures (Stuart *et al.*, 1986., Acharya *et al.*, 1989). The Ca^{2+} seems to be important, as its removal results in conformational changes in the tertiary structure and may therefore affect the catalytic properties of the lactose synthase complex (Acharya *et al.*, 1991). It is proposed that Ca^{2+} binding may be important in controlling the release of α-lactalbumin from the Golgi membrane, a process necessary for the induction of lactation (Stuart *et al.*, 1986).

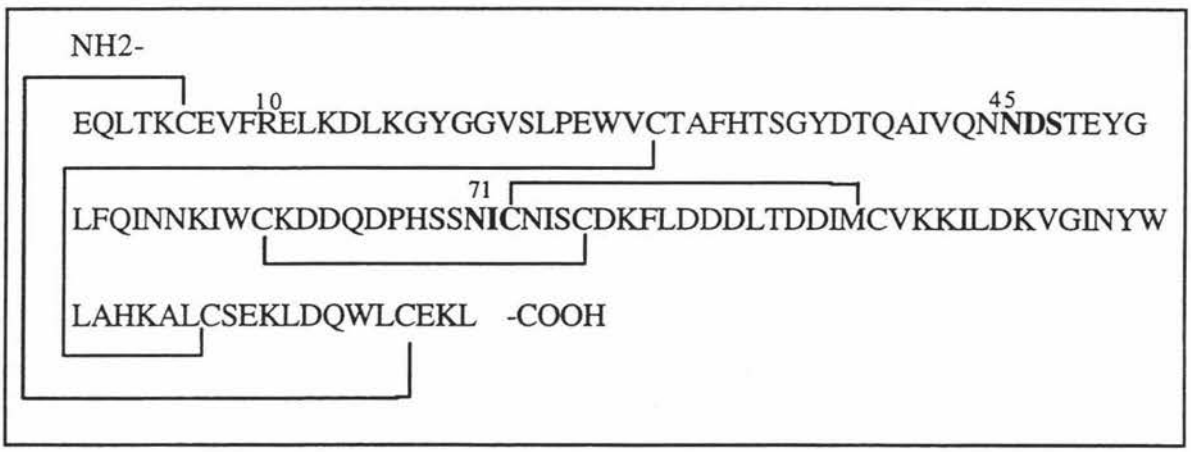


Figure 1.9 *Primary sequence of bovine α-lactalbmin. Intramolecular disulphide bridges are indicated between residues 6 - 120, 28 - 111, 61 - 77 and 73 - 91. Amino acids in bold represent the possible glycosylation sites.*

A comparison of the three dimensional crystal structures of human α-lactalbumin refined at 1.7 Å resolution with the baboon α-lactalbumin also refined at 1.7 Å (Acharya *et al.*, 1991, Acharya *et al.*, 1989) showed them to be very similar. The two proteins are known to have more than 90% amino acid sequence identity and crystallise in the same orthorhombic space group, $P2_12_12$.

In order to investigate the molecular basis of α-lactalbumin's function in lactose synthase, the three dimensional structures of guinea-pig, goat and a recombinant bovine α-lactalbumin were determined (Pike *et al.*, 1996). These structures were solved by molecular replacement techniques. Overall, the structures are very similar reflecting their high degree of amino acid sequence identity (66-94%). There is however, a part of the molecule, (residues 105-110) that does show a number of distinctly different conformations in the various structures. This region has been shown to be important in the function of the molecule and lies adjacent to two residues (Phe 31 and His 32) that are thought to be involved in stabilising the binding of the monosaccharide glucose to lactose synthase.

A comparative analysis of the structures showed that these different conformations significantly affected the enviroment of Phe 31 and His 32. It has been hypothesised that changes in the enviroment of the functional groups of these residues could affect the way in which they interact with the monosaccharide-lactose synthase complex. Interestingly, both residues are located in a region of the molecule that has relatively high thermal parameters. While this is indicative of disorder, it is also indicative of flexibility. So that each different conformation observed in each structure may represent a "snapshot" of one of many conformations adopted by the α-lactalbumin at any one time. Such flexibility may be important for the regulation of lactose synthase activity, an idea that is supported by the observation that metal ions have an enormous effect on the conformation of α-lactalbumin in solution (Pike *et al.*, 1996).

1.7.4 Glycosylation of α-lactalbumin

Minor amounts of bovine α-lactalbumin in milk have been isolated which are electrophoretically distinct from each other and have different carbohydrate contents. Some investigators obtained fractions that contained only hexosamine (Gordon *et al.*, 1968) whereas Barman, (1970) isolated a so-called glyco-α-lactalbumin from bovine milk using ion-exchange chromatography that contained mannose, fucose, galactose, galactosamine, and

sialic acid. Proctor and Wheelock, (1974) obtained at least two different carbohydrate containing fractions with varying amounts of the aforementioned monosaccharides. These species appear to have both the same specifier protein activity and the same amino acid sequence as the non-glycosylated form. However, the primary structures of the carbohydrate moieties have not yet been established. It has been suggested that all α-lactalbumins are glycosylated on secretion but are deglycosylated after leaving the secretary cells (Barman, 1970), but this suggestion has not been confirmed. The point of attachment of the carbohydrate group is probably at asparagine 45 (Tilly *et al.*, 1991) but this also remains to be confirmed. The sequence in this region of bovine α-lactalbumin is Asn-Gln-Ser, a glycosylation sequon (Marshall, 1972).

Studies on the glycosylation of the protein using *in vitro* systems are under active investigation. Although bovine α-lactalbumin contains two tripeptide sequences of -Asn_{45}-Asp_{46}-Ser_{47}- and Asn_{74}-Ile_{75}-Ser_{76}- glycosylation seems to only occur at aspargine 45 under *in vitro* conditions (Struck *et al.*, 1978). There is also a Asn_{71}-Ile_{72}-Cys_{73} sequence, although there have been no reports confirming the presence of glycans at this site.

Bovine, sheep, goat, rabbit, human, rat and wallaby α-lactalbumin all have a consensus N-glycosylation sequence (Asn-X-Ser) at residues 45-47. In bovine (and probably in other ruminants) a small proportion (around 15%) of molecules are glycosylated, whereas rabbit and rat α-lactalbumins appear to be uniformly glycosylated at this site (Prasad *et al.*, 1980). This difference has been attributed to the Asn-Gly-Ser sequence at this site in the rabbit and rat being a more effective substrate for N-glycosylation than the corresponding ruminant sequence Asn-Asp-Ser (Prasad *et al.*, 1982). Recently it was reported that asparagine 71 was glycosylated in human α-lactalbumin. This is an Asn-Ile-Cys sequon and only a relatively small proportion (about 1%) of the protein is glycosylated at this site (Guiffrida *et al.*, 1997).

Bovine α-lactalbumin possesses two main genetic variants, A and B, differentiated by one amino acid. The variant normally present in European dairy herds is variant B (Lyster, 1972). When these variants were studied using ion-exchange chromatography both variants behaved in a similar way except that the α-lactalbumin from variant A eluted at a higher salt concentration than variant B (Hopper and McKenzie, 1973). Homozygous genetic variants of bovine α-lactalbumin (i.e: AA or BB) were shown to contain a major component (component M) and three minor components (components F, S_1 and S_2) when analysed by gel electrophoresis. AB heterozygotes consist of two major and six minor components. F, M, S_1 and S_2 are named because of their position on starch gels after electrophoresis at pH 7.7 as shown in Figure 1.10 and Table 1.3. Only the S_1 and S_2 components have been shown to be glycosylated (Hopper and McKenzie, 1973). Analysis showed that S_1 contained 6 to 7 hexosamines, 6 hexoses, 1 deoxyhexose, and sialic acid. The S_2 protein had an identical carbohydrate composition except that it contained no sialic acids.

Hopper and McKenzie (1973), concluded that component F does not contain any carbohydrate residues, but suggested that it differs from component M because of an additional amide group, which accounts for its greater mobility on PAGE gels. Component F makes up approximately 5% of the total α-lactalbumin obtained from whole milk while S_1 and S_2 make up 3 to 5% of the total α-lactalbumin isolated from whey with component M making up the rest.

Starch gel electrophoresis

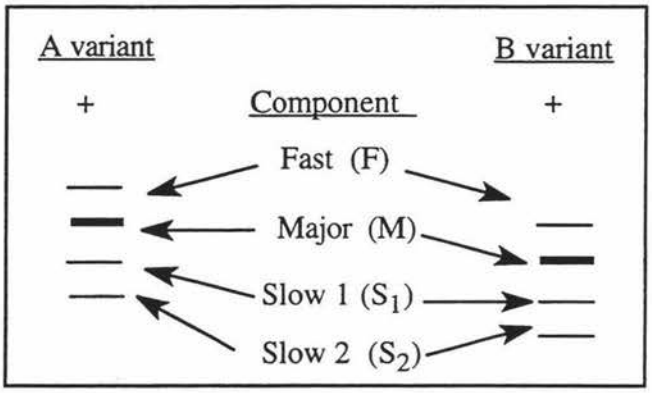


Figure 1.10 *Summary of the electrophoretic behaviour and designation of the components of bovine α-lactalbumins from A and B variants (adapted from Hopper and McKenzie, 1973).*

A recent abstract gave the structure of one of these glycoforms but did not detail the conditions used or indicate which particular glycoform (S_1 or S_2) was sequenced, it was shown to have the following structure (Tilly *et al.*, 1991) (Figure 1.11).

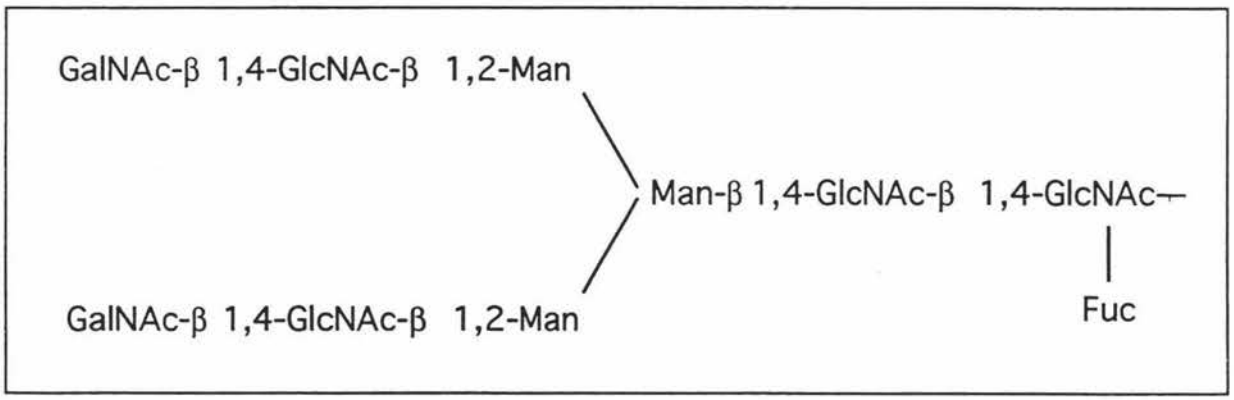


Figure 1.11 *Structure of one of the bovine α-lactalbumin glycans.*

There is no report as yet of the structure of a second glycoform that was found. Because it runs faster on native polyacrylamide gels, it is believed to contain a sialic acid residue. Glycoforms containing sialic acid should run faste, as sialic acid contains a negative charge.

Recent developments in the field of glycobiology suggest that the glycan of α-lactalbumin could have a variety of functions. Because of the current concern in improving human milk substitutes, the determination of the functional role of the glycan on α-lactalbumin is of interest. However the elucidation of the oligosaccharide structures must first be more thoroughly investigated to see whether or not the different glycan structures have different effects on protein function.

1.7.5 Incomplete glycosylation of bovine α-lactalbumin

It is unclear why all of the bovine α-lactalbumin in milk is not glycosylated at any of these possible glycosylation sequons since it has been proposed (Marshall, 1972) that glycosylation is a requirement for secretion of glycoproteins. Several possible reasons have been proposed:

1. All α-lactalbumins are glycosylated on secretion but are deglycosylated by endogenous ENGases or PNGases. Deglycosylation by ENGases would leave one GlcNAc residue attached to the glycosylated Asn.

2. The rate of synthesis, folding and secretion of α-lactalbumin could be sufficiently rapid to prevent glycosylation. Indeed, some other secreted proteins are also found in both glycosylated and non-glycosylated forms, for example, ribonuclease (Catley, 1973). Interestingly glycosylation has been shown to regulate the activity of ribonuclease.

3. The sequence is poorly glycosylated because of physical reasons due to the sequence near the vicinity of the sequon, such as:
   (a) The presence of aspartic acid at the X position.
   (b) The proximity of disulphide bonds.

1.8 Aims of current research

1.8.1 Objective 1

Deglycosylation of S_1 and S_2 using crude PNGase F (prepared in house) produced two bands in native gels. The major fraction ran to the same position as the F component of α-lactalbumin, while a faint band ran the same as the M component. This suggested that in the F protein, asparagine was replaced by aspartic acid as a consequence of the action of the deglycosylating enzyme PNGase. An ENGase, on the other hand, would leave a single N-acetylglucosamine on the protein. The presence of this single sugar inhibits further PNGase F digestion which requires a minimum of two monosaccharide units attached to a peptide for cleavage to occur. There is therefore the interesting possibility that the M component of α-lactalbumin may contain a single hexosamine attached to asparagine 45. Asparagine 71 and 74 sites are not known to be glycosylated under *in vitro* conditions (Struck *et al.*, 1978). Therefore the first objective of this current research was to analyse the M component of α-lactalbumin to investigate whether the main fraction contains polypeptide chains with one hexosamine per protein molecule attached to asparagine 45 or not.

1.8.2 Objective 2

Recent work carried out at Massey University, indicated that the S_1 and S_2 fractions actually represented families of glycoforms, not just two different glycoforms. Mass spectrometry and gel electrophoresis showed that these may be families of glycoforms with and without sialic acid (Chilcott, 1996). The second objective was designed to take the work one step further and analyse the glycosylated α-lactalbumin:

1. To confirm the number of glycans linked to glycosylated α-lactalbumin.

2. To separate and analyse the different oligosaccharide chains bound to α-lactalbumin.

1.8.3 Objective 3

The F fraction is to be sequenced to see whether it contains an aspartic acid residue at the asparagine 45 glycosylation site. This is to investigate whether it has been deglycosylated by endogenous PNGases. This requires a modified methodology due to the low concentration of the F fraction present in bovine milk.

CHAPTER II

MATERIALS AND METHODS

2.1 Materials

(a) Raw Milk

Raw milk was obtained fresh from the Massey University No 1 Dairy Farm.

(b) $FeCl_3$ precipitation

$FeCl_3$ (Ajax Chemicals, Sydney, Australia)
NaOH (BDH Laboratory Supplies, Poole, England)

(c) Ammonium sulphate precipitation

Ammonium sulphate (Riedel-deHaën, Germany)

(d) (Native and SDS)-PAGE

Glycine (BDH Chemical Ltd, Poole, England)
Dithiothreitol (DTT) (Sigma Chemicals Co, St Louis, U.S.A)
Tris (hydroxymethyl)-aminomethane (Serva Feinbiochemica, Heidelberg, New York)
Acrylamide (BIO-RAD Laboratories)
Coomassie Brilliant Blue R-250 (Fluka Chemie AG CH-9470 Buchs, Switzerland)
Methanol and acetic acid (BDH Laboratory Supplies, Poole, England)
Sodium dodecyl sulphate (SDS) (BIO-RAD Laboratories)

(e) Anion exchange chromatography

DEAE-Sephadex A-50 (Phamacia Fine Chemicals, AB, Upsala, Sweden)
Dialysis tubing (10,000 MW cut-off, The Scientific Instrument Centre Ltd, Number one, Leeke Street, London)
Tris HCl (Sigma Chemicals Co, St Louis, U.S.A)
NaCl (AR) (BDH Laboratory Supplies, Poole, England)

(f) Lectin affinity column chromatography

Concanvalin A-Sepharose CL-4B (Pharmacia)
Methyl α-D mannopyranoside (Sigma Chemicals Co, St Louis, U.S.A)
Glucose (Ajax Chemicals, Sydney, Australia)
$MnCl_2$ (M & B Laboratory Chemicals, May & Baker Ltd, Dagenham, England)
$CaCl_2$ (J. T. Baker Chemical Co, Phillipsburg)
$MgCl_2$ (Prolabo, France)
Ammonium acetate (Ajax Chemicals, Sydney, Australia)

Loading buffer: 20mM NH_4Ac, 1mM $CaCl_2$, 1mM $MnCl_2$, 1mM $MgCl_2$ (pH 6.5)
Elution buffer: 0.5M Methyl α-D mannopyranoside in loading buffer.
Cleaning buffer A: 0.1M Tris, 0.5M NaCl (pH 8.5)
Cleaning buffer B: 0.1M NaAc, 0.5M NaCl (pH 4.5)

(g) Gel filtration chromatography

Superdex 75 HR (10/30) (Pharmacia)
$(NH_4)_2HPO_4$ (Ajax Chemicals, Sydney, Australia)
NaCl (AR) (BDH Laboratory Supplies, Poole, England)

(h) Reduction and alkylation

Guanidine HCl, N-ethylmorpholine and acetic acid (AR) (BDH Laboratory Supplies, Poole, England)
β-mercaptoethanol (J. T. Baker Chemical Co, Phillipsburg)
4-Vinylpyridine (Aldrich-Chem, Steinheim, West Germany)
Dialysis tubing (3,500 MW cut-off) (Spectrummedical Industries, Inc., Los Angeles)

(i) Enzymatic cleavage

α-Chymotrypsin, TLCK treated (Sigma Chemicals Co, St Louis, U.S.A)
NH_4HCO_3 (Ajax Chemicals, Sydney, Australia)
$CaCl_2$ (J. T. Baker Chemical Co, Phillipsburg)

(j) RP-HPLC

Vydac, C_{18} column (6.4 x 250mm) (Alltech, Deerifield, IL)
TFA (Perkin Elmer) (Applied Biosystem division, Great Britain)
Acetonitrile (Mallinckrodt)

(k) ES/MS

Acetonitrile (Mallinckrodt)
Formic acid (BDH Chemical Ltd, Poole, England)

(l) HPAEC/PAD

50% NaOH solution (AR) (BDH Laboratory Supplies, Poole, England)
Anhydrous sodium acetate (BDH Laboratory Supplies, Poole, England)
Acetic acid (AR) (BDH Laboratory Supplies, Poole, England)
Ribonuclease B (Sigma Chemicals Co, St Louis, U.S.A)
Eluent A = 200mM NaOH (made up from 50% NaOH)
Eluent B = 1M sodium acetate in 100mM NaOH
Eluent C = 20mM NaOH
Eluent D = 100mM NaOH
Columns: Carbopac PA-100 Analytical (4 x 250mm) (Dionex)
Carbopac PA-100 Guard (4 x 50mm) (Dionex)

(m) PNGase F treatment

PNGase F (recombinant) (kind gift from Dr. G. E. Norris, Massey University)
Toluene (BDH Chemical Ltd, Poole, England)

(n) FACE

Cation exchange cartridge (Alltech, Deerifield, IL) (kind gift from Paul McJarrow, New Zealand Dairy Research Institute)
100% ethanol, acetic acid, dimethyl sulphoxide (AR) (BDH Laboratory Supplies, Poole, England)
8-amino-napthalene-1,3,6 trisulphonic acid (ANTS) (Molecular Probes, Inc.) (kindly donated by Prof. P. Sullivan, Massey University)

Cyanoborohydride (Fluka Chemie AG CH-9470 Buchs, Switzerland) (kindly donated by Prof. P. Sullivan, Massey University)
Standard glucose ladder (Seikagaku Corporation, Japan) (kindly donated by Prof. P. Sullivan)
Glycine (BDH Laboratory Supplies, Poole, England)
Tris (hydroxymethyl)-aminomethane (Serva Feinbiochemica, Heidelberg, New York)
Glycerol (Sciencetific Supplies Ltd, Auckland)
Ribonuclease B (Sigma Chemicals Co, St Louis, U.S.A)

(o) Derivatisation of oligosaccharides with PMPMP

p-methoxy-phenylhydrazine hydrochloride (Aldrich Chemical Co, Milwauke, U.S.A)
Sodium acetate trihydrate (AR) (Sigma Chemicals Co, St Louis, U.S.A)
Toluene (AR) (Ajax Chemicals, Sydney, Australia)
Column matrix (Silica gel 60, Merck) (Sigma Chemicals Co, St Louis, U.S.A)
TLC plates (Silica gel 60 F254, Merck) (Sigma Chemicals Co, St Louis, U.S.A)
Methanol, ethanol, ethylacetate (AR) (BDH Laboratory Supplies, Poole, England)
Filter papers (Whatman chromatgraphy paper, 3mm, Whatman Paper Ltd, England)
NaOH, HCl (AR) (BDH Laboratory Supplies, Poole, England)
Na_3PO_4 (Ajax Chemicals, Sydney, Australia)
Acetonitrile (Mallinckrodt)
Ribonuclease B (Sigma Chemicals Co, St Louis, U.S.A)

(p) PVDF membrane blotting for N-terminal sequencing

CAPS (Sigma Chemicals Co, St Louis, U.S.A)
PVDF membrane (Applied Biosystem division, Great Britain)
Methanol (AR) (BDH Laboratory Supplies, Poole, England)

(q) Purification of derivatised oligosaccharides

Nylon syringe filter (0.45µm, 25mm dia., Nylon Acrodisc, Gelman Science)
Acetonirile (Mallinckrodt)

(r) Protein determination assay

Bicinchoninic acid (Sigma Chemicals Co, St Louis, U.S.A)
Bovine serum albumin (Sigma Chemicals Co, St Louis, U.S.A)

(s) Buffers and reagents

Solutions for RP-HPLC, FPLC work were filtered through a 0.22μm filter before use. The pH was measured using an Orion digital pH meter, model 410A.

(t) Instrumentation

FPLC (Pharmacia)
RP-HPLC (Philips PU4100 HPLC equipped with a Philips PU4110UV/VIS detector and Spectra Physics SP 8800 HPLC equipped with a SP8490 UV/VIS detector)
Triple quadrupole electrospray mass spectrometer (Perkin-Elmer-Sciex, API-300 Benchtop LC/MS/MS)
HPAEC/PAD (DX500) (Dionex)
Protein sequencer (Applied Biosystems automatic sequencer, model 476A)
BIO-RAD "Mini-PROTEAN" electrophoresis gel apparatus

2.2 Methods

2.2.1 Methods for objective 1

The following experimental steps were used:

1. Purification of M component (non-glycosylated) of α-lactalbumin and analysis by ES/MS.

2. Reduction and alkylation of the purified M component of α-lactalbumin.

3. Digestion of non-glycosylated α-lactalbumin (M component) using chymotrypsin.

4. Separation of the peptides generated in step 3 using reverse phase high performance liquid chromatography (RP-HPLC).

5. Identification of the peptides using Electrospray Mass Spectrometry (ES/MS).

6. Confirmation of the presence of the correct peptide by using Edman microsequencing techniques.

(1) The purification of non-glycosylated α-lactalbumin

(a) Separation of whey proteins

Acid whey was prepared from 4 litres of raw milk obtained fresh from Massey No 1 Dairy Farm. The milk was centrifuged for 40 minutes at 17,000 x g at 4°C and the fat which formed a plug at the top was removed manually with a spatula. The remaining whey was passed through a cheesecloth to remove any remaining fat particles. Casein was precipitated by slowly lowering the pH to 4.6 at 30°C using 5M HCl and constant stirring. The casein was removed by first filtration through cheesecloth and then centrifugation at 17,000 x g for a further 40 minutes to remove any residual casein. The yellow whey was gently poured off the casein pellet.

(b) $FeCl_3$ precipitation

The pH of the acid whey was lowered to 4.2 and $FeCl_3$ was added slowly at room temperature to give a final concentration of 7-7.5 mM. The pH was maintained at 4.2 during $FeCl_3$ addition, by the dropwise addition of 3M NaOH. The temperature of the solution was lowered by stirring on ice for 15 minutes before being left overnight to settle at 4°C. The precipitate was removed by centrifugation at 17,000 x g for 20 minutes. The yellow supernatant was poured off and the pH adjusted to 8.5.

(c) Ammonium sulphate precipitation

Finely powdered ammonium sulphate (264g per litre of solution) was slowly added to the supernatant from the previous step with stirring at 0°C, over a period of 35-45 minutes. The solution was let to equilibrate for 2 hours at 4°C. After this time, the white precipitate that had formed was removed by centrifugation, leaving an orange coloured supernatant, due to excess $Fe(OH)_3$. Examination of the supernatant and the precipitated proteins by PAGE revealed hardly any non-glycosylated α-lactalbumin remained in the supernatant.

(d) Anion exchange chromatography

The ammonium sulphate precipitate was dissolved in Milli Q water and dialysed for 3-7 days against deionised water, followed by 10mM Tris HCl pH 8. Dialysed whey protein (100mL of 0.75mg/mL protein) was applied to a pre-equilibrated DEAE-Sephadex A-50 column (1.5cm x 8cm) in the 10mM Tris HCl, pH 8 buffer and eluted with a 0-1M NaCl gradient in the same buffer. The elution was monitored by measuring the absorbance of each fraction (5mL) at 280nm and plotting the absorbance verses the fraction number. Peaks were analysed by native-PAGE.

(e) Lectin affinity column chromatography

Concanvalin A (Con A) is a plant lectin which binds strongly to mannose residues. It is a metalloprotein and requires several divalent metal ions such as Mn^{2+}, Mg^{2+}, and Ca^{2+} to form the lectin-sugar complex. Glycoproteins containing several mannose residues are therefore expected to bind. The more mannose groups present, generally, the tighter the binding to the affinity column. Con A was used to separate the glycosylated protein from non-glycosylated proteins, as all N-linked glycans contain at least 3 mannose residues that are part of the core pentasaccharide (Figures 1.3-1.5).

α-Lactalbumin containing fractions were pooled, dialysed against loading buffer (20mM ammonium acetate, 1mM Ca^{2+}, 1mM Mn^{2+}, 1mM Mg^{2+}, pH 6.5), concentrated by ultra filtration and applied to a column (12x3 cm). Non-glycosylated proteins were eluted with loading buffer and the elution monitered at 280nm. Peak fractions were analysed by native-PAGE, and those containing α-lactalbumin were lyophilised.

(f) ***Size-exclusion chromatography (Gel filtration chromatography)***

The lyophilised fractions were further purified using size-exclusion chromatography and FPLC according to the following procedure:

Portions of the lyophilised protein (25mg) were dissolved in a minimum of distilled deionised water and filtered through a 0.22μm filter before being applied to a Superdex 75 column (HR10/30) using a Pharmacia FPLC. A 500μL sample of protein solution (10mg/mL) was injected onto the column which had been equilibrated in phosphate buffer (20mM sodium phosphate, 150mM NaCl, pH 7.0) at room temperature and eluted using a flow rate of 0.4mL/min. Fractions of 1.2mL were collected and the elution monitored by measuring the absorbance of the eluent at 280nm. Multiple injections of 500μL were then carried out, using the same conditions as above.

(2) Reduction and alkylation

1mg of protein was dissolved in 1mL of 0.5M N-ethylmorpholine/acetate buffer (pH 8.3). 1g of guanidine HCl and 4mg of dithiothreitol (DTT) was added to the dissolved protein. The mixture was stirred under nitrogen at room temperature for 4 hours to ensure complete reduction had occured. 8μL of 4-vinylpyridine was then added and alkylation was allowed to proceed overnight under nitrogen at room temperature. The reaction mixture was diluted by the addition of 3 volumes of deionised distilled water and dialysed against 0.2M ammonium bicarbonate using 3,500 cut-off dialysis tubing to remove all unreacted reagents (Shively, 1986). During this time the protein precipitated. The dialysis tube was then equilibrated in 0.2M ammonium bicarbonate.

(3) Enzymatic Cleavage

The reduced and alkylated insoluble protein was digested with chymotrypsin at an enzyme/substrate ratio of 1:50 (w/w) in 0.2M ammonium bicarbonate at 37°C overnight. Theresulting solution was then subjected to RP-HPLC.

(4) Reverse phase high performance liquid chromatography (RP-HPLC)

RP-HPLC was performed using a Vydac, C_{18} column (6.4mm x 250mm). A Philips PU4100M liquid chromatograph was used to effect the separation, and a Philips PU4110UV/VIS detector used to monitor the eluent at 214nm. For RP-HPLC analysis, samples were dissolved in 0.1% trifluoroacetic acid in deionised distilled water. The digest was filtered through a 0.22μm filter, and 500μL samples were applied to the column.

A linear gradient, 0-60% buffer B over 60 minutes, was used at a flow rate of 1mL/minute (Buffer A: 0.1% (v/v) trifluoroacetic acid in deionised distilled water, Buffer B: 0.08% (v/v) trifluoroacetic acid in acetonitrile). Peptide peaks were collected manually and dried under vacuum for further analysis.

(5) Electrospray ionisation mass spectrometry (ES/MS)

ES/MS has proven to be extremely useful in the analysis of large, labile biomolecules up to 200,000 Da with a precision of better than 0.01% (Fenn *et al.*, 1989). For protein and peptides within the range of 100 to 100,000 Da, it is possible to identify post-translational modifications such as phosphorylation, glycosylation, acetylation, methylation and hydroxylation solely by the increase in mass. Subunits or prosthetic groups that are not covalently bound will be dissociated and can be measured as separate species (SIM-Separate Ion Monitoring). Conversely, covalently bound groups will result in a characteristic increase in mass. Also, the mass change produced by a single amino acid substitution or deletion may often be used to establish or confirm the presence of variants.

The electrospray ionisation process produces intact protein molecules in an ionised form from a dilute protein solution by nebulisation in the presence of a strong electric field. As a consequence of the strong electric field between the end of the capillary and a counter electrode, the sample solution emerging from the capillary is dispersed into an aerosol of highly charged droplets. The fine droplets formed carry excess positive or negative charge (depending on the polarity of the electric field). The droplets diminish in size by evaporation, assisted by a dry gas and/or heat. A point is reached where multiply charged ions are released free of solvent resulting in multiply ionised proteins in the gas phase. These are directed into the mass analyser (usually a quadrupole) held under high vacuum for mass to charge ratio (m/z) analysis (Figure 2.1).

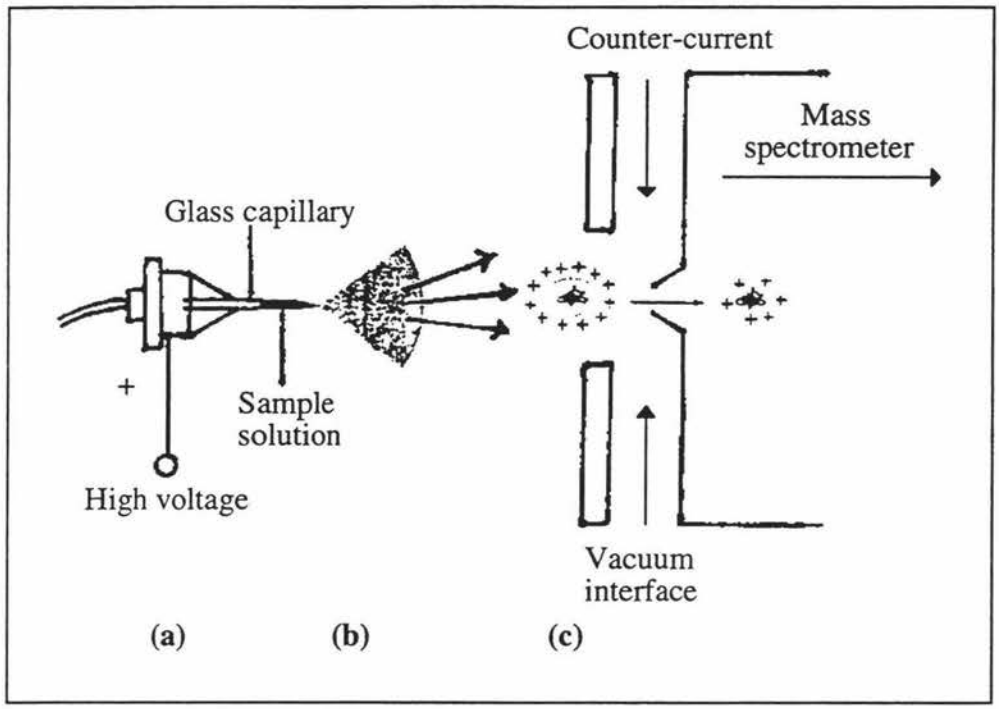


Figure 2.1 *The three principal steps of electrospray mass spectrometry (ES/MS) (Fenn et al., 1989). (a) Formation of small highly charged droplets by electrostatic dispersion of a solution under the influence of a high electric field. (b) Desorption of protein ions from the droplets into the gas phase. (c) Mass analysis of the ions in a mass spectrometer.*

A characteristic of an ES mass spectrum is the production of intact ions with extensive multiple charging, resulting in a mass spectra containing a family of charge states arising from a single protein.

Proteins are normally analysed as positive ions where the charges are produced by added protons. The ions have the general form:

$$(M+nH)^{n+}$$

Where M is the molecular mass of the protein
n is an integer number of protons (charges)
and H is the mass of a proton (1.00794)

The mass to charge ratio (m/z) of each peak is given by:

$$m/z = \frac{M+nH}{n}$$

Electrospray mass spectra were acquired on a triple quadrupole electrospray mass spectrometer operating with an API ion source in the positive ion electrospray mode. Peptide samples were diluted into acetonitrile/0.2% formic acid (1:1, by volume), to a concentration of 20pM/μL and infused at 5μL/min, using a syringe pump (Harvard Apparatus 55-1111 (Canada))

The monosaccharides in the glycoproteins were detected selectively by SIM based on diagnostic oxonium ions $HexNAc^+$ produced by GalNAc or GlcNAc, m/z 204), $NeuAc^+$ (N-acetylneuraminic acid, m/z 292), and $Hex\text{-}HexNAc^+$ (produced by Gal-GlcNAc, Man-GlcNAc or Gal-GalNAc, m/z 366). The abundance of these low-mass marker ions in electrospray mass spectra was enhanced by collision-induced decomposition of the parent ions. This was accomplished by stepping the potential which controls the extent of collision-induced decomposition of source-produced ions from a high voltage, to maximise fragment-ion production during acquisition of low m/z ions and at a lower voltage to yield intact ionised molecules during the remainder of scan (Huddleston *et al.*, 1993). The experimental information is shown in appendix A.

(6) Peptide Sequencing

An Applied Biosystems model 476A protein sequencer with an in-built PTH (phenylthiohydantoin) analyser was used to obtain sequence information from the automated Edman degradation.

2.2.2 Methods for objective 2

The summary of experimental procedures used for objective 2 are as follows:

1. Purification of glycosylated α-lactalbumin.

2. Digestion of purified protein with PNGase F.

3. Separation and identification of PNGase F released oligosaccharides using the following techniques:

(a). High pH anion-exchange chromatography with detection by pulsed amperometry (HPAEC/PAD).

(b). Fluorophore-assisted carbohydrate electrophoresis (FACE).

(c). Derivatisation of oligosaccharides using 1-(p-methoxy)phenyl-3-methyl-5-pyrazolone (PMPMP) and separation using RP-HPLC..

These methods were developed using the glycosylated protein ribonuclease B before being used to analyse bovine α-lactalbumin.

4. An electrospray mass spectroscopic (ES/MS) study of purified glycosylated α-lactalbumin.

(1) Purification of glycosylated α-lactalbumin

(a) Lectin affinity column chromatography

In order to purify glycosylated α-lactalbumin the following procedure was carried out. Two litres of fresh bovine milk was used to make one half litre of acid whey. This was then dialysed against loading buffer overnight and directly loaded onto a column (12x3cm) that had been equilibrated in loading buffer. All the unbound protein was removed by washing with loading buffer until the absorbance at 280nm was less than 0.1. The same procedure was carried out on three separate occasions with milk taken at different times of the milking season. A significant difference between these three preparations was the use of aseptic conditions in preparation 3 (Prep 3). This action was taken to prevent the possible degradation of glycans by any microbial action that may have occured in Prep 1 and Prep 2. Bound glycosylated proteins were eluted from the Con A column by an isocratic elution using either 0.5M methyl α-D mannopyranoside in loading buffer or 2.0M glucose in loading buffer. The protein content of the fractions from the Con A affinity chromatography were estimated using the absorbance at 280nm, and a plot of fraction number verses absorbance at 280nm was drawn. Peaks were analysed by native-PAGE. Those containing α-lactalbumin were pooled and lyophilised.

(b) Size-exclusion chromatography

The samples recovered from the Con A chromatography purification were further purified using size-exclusion chromatography and FPLC as described in objective 1.

(2) PNGase F Treatment

Figure 2.2 shows the mode of action of PNGase F. This enzyme releases the oligosaccharide with the amino group of the asparagine residue still attached to the oligosaccharide. The glycosyl amine (Figure 2.2, structure II) is unstable, particularly under acidic conditions, and usually only the reducing oligosaccharides are observed (Figure 2.2, structure III). PNGase F releases most types of oligosaccharide chains, thus its discovery and characterisation represents a major analytical tool for the study of glycoprotein glycans.

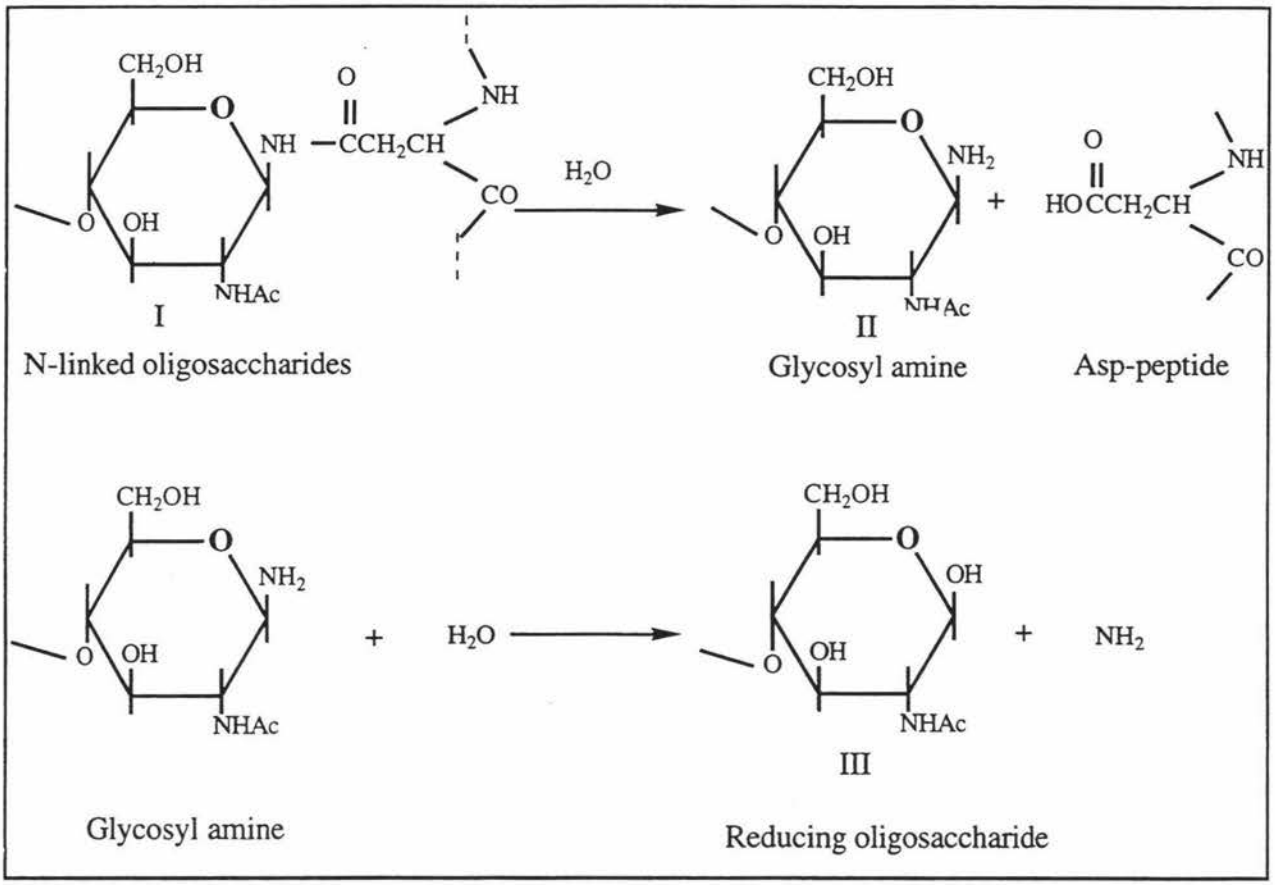


Figure 2.2 *Action of PNGase F*

(a) Preparing the PNGase F digest

Table 2.1 was used as a guide to set up the digest in sterile eppendorf tubes. All reagents were filtered through 0.22μm filters before use to minimise the likelihood of microbial contamination. Reagents were added using sterile tips and dispensed into sterile containers using aseptic procedures where possible. Samples were incubated at 37°C for 18 hours at near neutral pH. 5μL of toluene was added to each tube to prevent bacterial growth. The digestion was monitored by PAGE.

Table 2.1 *PNGase F digestion of bovine α-lactalbumin*

Tube	α-lactalbumin (5mg/mL) (μL)	Water (μL)	Enzyme (1mg/mL) (μL)	Comments
1	0	100	0	Water blank
2	100	0	0	Substrate only
3	0	100	100	Enzyme only
4	100	0	100	Digest

(b) Conversion of the glycosyl amine in PNGase F digestion.

After overnight incubation, 6μL of 174mM acetic acid were added to each sample vial and incubated for 2 hours at room temperature. All tubes were vacuum concentrated to dryness at ambient temperature to remove excess acetic acid. Each sample was then dissolved in 200μL of water and analysed using FACE, HPAEC/PAD and RP-HPLC.

(3) Separation of oligosaccharide mixtures

(a) High-pH anion-exchange chromatography with detection by pulsed amperometry (HPAEC/PAD)

The separation and detection of complex carbohydrate mixtures by high-performance liquid chromatography (HPLC) is difficult owing to the polar, hydrophobic nature of this class of analytes and the fact that they do not possess an ultra violet (UV) chromophore. The problem is further complicated by the need to separate closely related structural isomers that can differ only by a single linkage position or their geometry. Carbohydrates may be separated from a mixture as oxanions at high pH (>12) by high performance anion exchange chromatography (HPAEC) and detected by pulsed amperometry. The elution order of the oligosaccharide is dependent on their molecular size, sugar composition, linkages of the monosaccharides and the relative acidity of the substituted hydroxyl groups (hemiacetal > 2-OH > 6-OH > 3-OH > 4-OH). The highly basic conditions necessary for the separation of the carbohydrates is also amenable to pulsed amperometric detection (PAD) using a gold electrode. The PAD utilises a repeating sequence of three applied potentials that are applied for specific durations.
The resulting total current is the sum of:

1. The carbohydrate oxidation current.

2. The current due to the charging of the electrode surface, and

3. The current caused by the oxidation of the gold electrode.

HPAEC/PAD was used to separate oligosaccharides liberated from ribonuclease B and α-lactalbumin after PNGase F treatment. This technique has been found to be particularly useful for analysing sialated lactosamine-type oligosaccharides (Townsend *et al.*, 1989). These separations are marked by large differences (~15min) in retention times between the mono-, di-, tri-, and tetrasialated species. Within each iso-charged complex, resolution has been achieved on the basis of sialic acid linkage α(2-3) versus α(2-6) and/or the branch position of these residues (Townsend *et al.*, 1989). Importantly, HPAEC with PAD obviates the requirement for derivatisation under acidic conditions that can hydrolyse sialic acid residues.

(i) Instrument configuration

The chromatograph system (Dionex) used consists of a gradient pump, a detector (PAD-II), and an eluent degas module (EDM). The EDM is used to sparge and pressurise the eluents. The system is controlled and data is collected using Dionex AI450 software. Sample injection is via a Rheodyne microinjection valve equipped with a 25µL sample loop and a Tefzel rotor seal to withstand the alkalinity of the eluents. The pulsed potentials for the PAD-II were E_1=0.05V, t_1=480 msec, E_2=0.75V, t_2=120 msec, and E_3=-0.15V, t_3=60msec. The time constant was set to 3 seconds. Oligosaccharides were separated using a Carbopac PA100 column (4 x 250mm) (Dionex) equipped with a Carbopac guard column (4 x 50mm).

(ii) Preparation of eluents

The eluents used were as follows:

Eluent A = 200mM NaOH
Eluent B = 1.0M sodium acetate in 100mM NaOH
Eluent C = 20mM NaOH
Eluent D = 100mM NaOH

In HPAEC chromatography, it is extremely important to minimise contamination with carbonate, a divalent anion at pH >12, because it binds strongly to the column and interferes with carbohydrate chromatography, causing a loss of resolution and efficiency. Commercially available NaOH pellets are covered with a thin layer of sodium carbonate and should not be used. Rather, a 50% (w/w) NaOH solution is the preferred source for NaOH as it is much lower in carbonate. It is essential to use high quality water. Water should be collected and stored in a glass reservoir without a plastic tubing extension, which can be a site of microbial contamination.

Eluent A was prepared by diluting 10.7 mL of a 50% NaOH into 1 litre of filtered water. Water was sparged for 15 minutes before adding NaOH. Eluents C and D were also prepared using the 50% NaOH, as described above for eluent A. Eluent B, 1M sodium acetate in 100mM NaOH, was prepared as follows. It is important to filter eluent B before installation, so that the valves of the high performance liquid chromatography (HPLC) pump are protected from particulate matter that may be present in commercial preparations of salts. Therefore 500mL of 2M sodium acetate was prepared and vacuum filtered through a 0.45µm pore size nylon filter. After filtration of the solution, water was added to a final level of 994.65mL and sparged for 15 minutes. 5.35mL of 50% NaOH was added to give a final concentration of 100mM NaOH. All eluents were kept blanketed under helium (5 to 7 psi).

(iii) Preparation of the column

The column was washed in 100% eluent D at a flow rate of 1mL/min for 30 minutes. The longer time was necessary to achieve good separation of the various oligosaccharides. The column was equilibrated in 98% eluent D, 2% eluent B.

(iv) Method of separation

The samples were eluted using the gradient shown in Table 2.2.

Table 2.2 *Timed eluent and set up for separation of oligosaccharides on Carbopac PA 100 column.*

Time (min)	Flow (mL/min)	%A	%B	%C	%D
Wash	1.00	100.0	0.0	0.0	0.0
0.00	1.00	100.0	0.0	0.0	0.0
10.00	1.00	100.0	0.0	0.0	0.0
10.10	1.00	0.0	0.0	100.0	0.0
20.10	1.00	0.0	0.0	100.0	0.0
21.10	1.00	0.0	0.0	100.0	0.0
35.00	1.00	0.0	2.0	0.0	98.0
85.00	1.00	0.0	20.0	0.0	80.0

(b) Fluorophore Assisted Carbohydrate Electrophoresis (FACE)

Polyacrylamide gel electrophoresis of fluorophore labelled carbohydrates is a method by which complex carbohydrates can be profiled in a similar way to proteins (Jackson, 1994). The carbohydrate on the protein is first labelled with the fluorophore 8-amino-napthalene-1,3,6, trisulphonic acid (ANTS) (Figure 2.3).

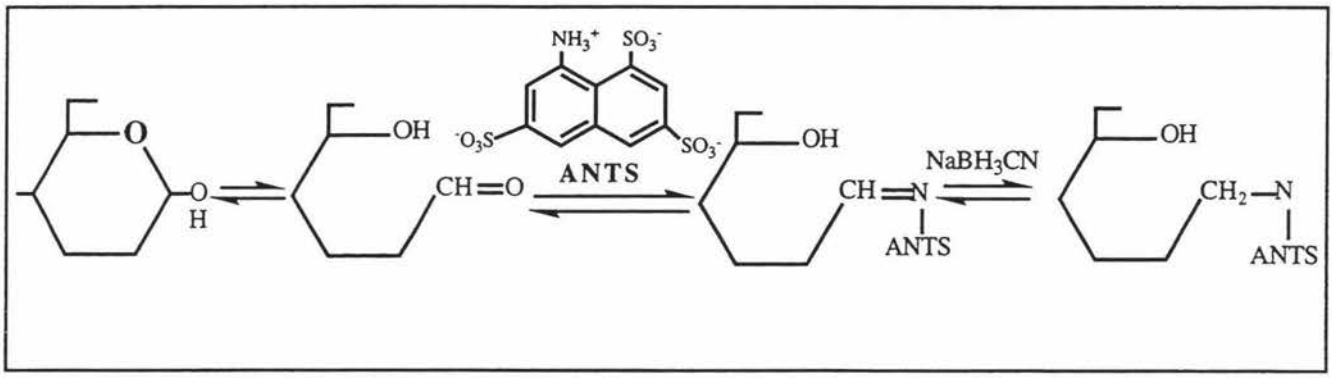


Figure 2.3 *Schematic diagram of fluorophore labelling of oligosaccharides by reductive amination.*

ANTS reacts with the aldehydic reducing carbon to form a Schiff base. The Schiff base is reduced by addition of cyanoborohydride to form a stable secondary amino derivative. The labelled samples are then separated on highly concentrated polyacrylamide gels, typically 32-35% acrylamide at 5-8°C. The charge associated with the ANTS groups allows electrophoresis of neutral oligosaccharide derivatives.

After electrophoresis the gels are visualised under UV light which excites the ANTS fluorophore resulting in a fluorescent band. The mobility of the ANTS derivatised polysaccharide is dependent mainly on the oligosacchride size, but is also affected by the anomericity of the links between monosaccharides.

(i) Sample preparation

The high ionic strength associated with 10-100mM NaOH and 0-1M sodium acetate gradients used with HPAEC are incompatible with FACE techniques. Therefore cation exchange cartridges were used for get rid of the high concentration of sodium ions in the samples.

50μL (1mg/mL) of recombinant PNGase F was added to 1mL (1mg/mL) of glycosylated α-lactalbumin and left overnight to digest at 37°C. The digest was monitored by PAGE, and when complete, the reaction was stopped and the protein precipitated by adding 3 volumes of cold 100% ethanol and left to stand for 10 minutes at 0°C. The precipitated protein was removed by centrifugation and the supernatant containing the released glycans retained. The supernatants were then dried by evaporation under vacuum (Speed Vac, Savant, SVC 100H).

(ii) Labelling and PAGE running conditions

The protocols used in this study were based on the procedures described previously (Jackson, 1994). Typically 4μL of ANTS solution (0.15M in 15% acetic acid) was added to 10-20μg of oligosaccharide followed by 4μL of cyanoborohydride solution (74mg/mL in dimethyl sulphoxide) and the reaction incubated at 37°C overnight. After incubation the reaction mixture was dried in a speed-vac then resuspended in an appropriate volume of loading buffer (80% glycerol, 20% water). Typically 1-2μg of oligosacchride were loaded per lane. 4μL of a standard glucose ladder was used as a marker. Electrophoresis was performed at 5-8°C for about 1.5 hours with running buffer (192mM Glycine, 25mM Tris, pH 8.5). This was achieved by immersing the electrophoresis chambers in ice. After running the gel, the bands were visualised under UV light, and the image recorded using an Alpha Innotech gel documentation system.

(c) Separation of 1-(p-methoxy)phenyl-3-methyl-5-pyrazolone (PMPMP) labelled oligosacchrides using RP-HPLC.

This method can be used to label sialic acid-containing oligosaccharides without causing any desialylation (Kakehi *et al.*, 1991). The active hydrogen of this reagent reacts with the carbonyl group of the reducing carbohydrates under slightly basic conditions (pH 8.2) to form a bis-PMPMP derivative (Figure 2.4). The PMPMP derivative can be detected at 249nm with a sensitive UV detector.

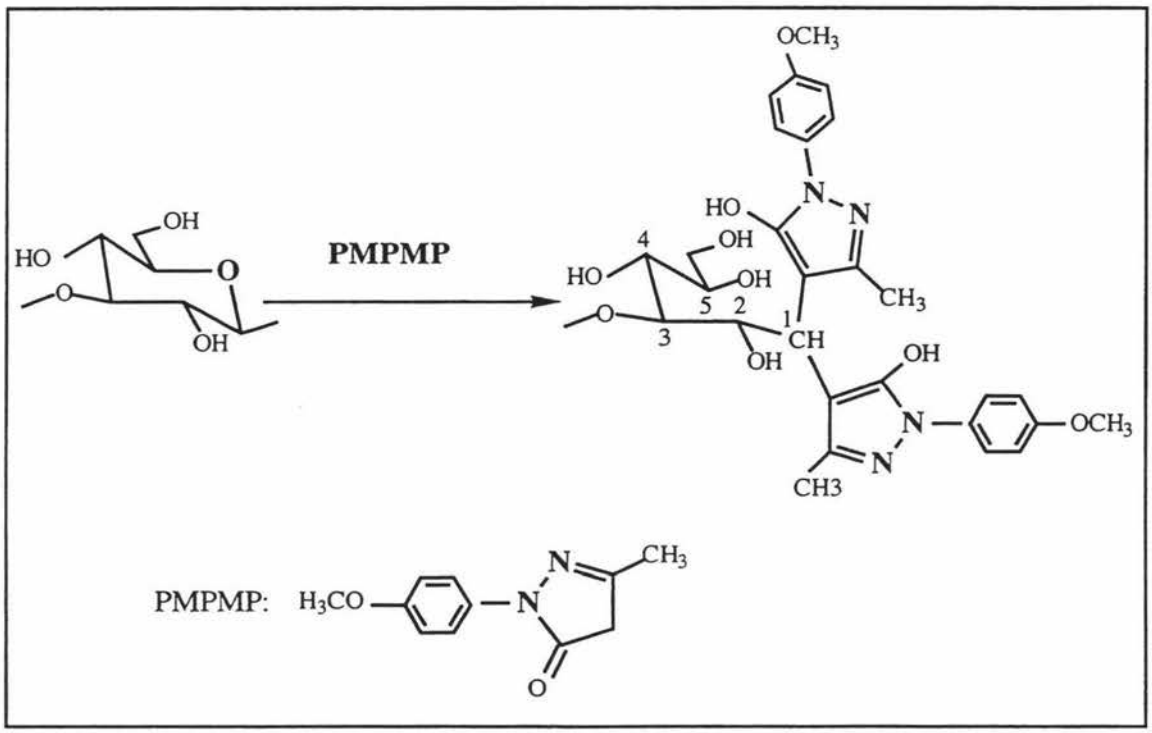


Figure 2.4 *Derivatisation with PMPMP*

(i) Preparation of 1-(p-methoxy)phenyl-3-methyl-5-pyrazolone

Preparation of 1-(p-methoxy)phenyl-3-methyl-5-pyrazolone was performed by modification of the method of Kakehi *et al.*, (1991). An ethanolic solution (40mL) of p-methoxy-phenylhydrazine hydrochloride (5.6g, 32mM), sodium acetate trihydrate (5.45g, 40mM), and ethyl acetoacetate (4.16g, 32mM) was heated under reflux for 2 hours. After cooling, the mixture was evaporated to dryness, and the residue dissolved in 10mL of ethanol. The insoluble material was removed by filtration, and the filtrate was evaporated to dryness. The residue left was dissolved in a small volume of 4:1(v/v) toluene/ethyl acetate and fractionated on a column of silical gel (150g, silica gel 60, Merck) pre-equilibrated with the same solvent.

In total 100 fractions of 10mL were collected and analysed by TLC (silica gel 60 F254, Merck) using the same solvent. Fractions 40-70, which showed a single component on TLC (Rf 0.41, detected by UV absorbance), were collected and evaporated to dryness. Crystallisation from methanol yielded 24.5% (w/w) of the compound with a UV λ_{max} at 249nm. PMPMP was analysed by mass spectrometry and nuclear magnetic resonance (NMR).

(ii) Derivatisation and RP-HPLC of oligosaccharides with PMPMP

Oligosaccharide samples obtained by PNGase F digestion of glycosylated proteins were derivatised as follows:

To a sample of oligosaccharide (cleaved from 1mg of protein) in a screw capped polypropylene tube were added 0.3M NaOH (20µL) and 0.5M PMPMP in methanol (20µL). The pH of the mixture was about 8.3 (as measured on universal indicator paper). The mixture was incubated for 20 minutes at 70°C to allow derivatisation to occur, and was then cooled and neutralised with 0.3M HCL (20µL). To the mixture were added 200µL of water-saturated ethyl acetate and 200µL of water, and the mixture was shaken vigorously. The organic layer was carefully removed using a syringe, and the extraction was repeated four more times. The aqueous layer was evaporated to dryness, and the residue dissolved in 200µL of a mixture of water and acetonitrile (85:15, v/v). 100µL of this solution was used for HPLC analysis. A column (250 x 4.6mm) (Vydac, C_{18}) was run with a mixture of 100mM phosphate buffer (pH 7.0) and acetonitrile (85:15, v/v) at a flow rate of 0.6mL/min. The eluent was monitored at 249nm.

(iii) Purification of derivatised oligosaccharides

To get accurate ES/MS data the separated derivatised oligosaccharides had to be desalted. This was accomplished using a nylon syringe filter disk (0.45µm, 25mm dia., Nylon Acrodisc, Gelman Science). A 5mL disposable plastic syringe was cut at the 2mL mark to use it as a funnel. The nozzle was plugged lightly with glass wool in order to prevent air locks during filtration The filter was primed with acetonitrile:water (95:5) using a syringe and then the pre-cut syringe was attached to the filter. The filter was rinsed with 2 x 1mL of acetonitrile:water (95:5) using the gravity flow method. Derivatised samples were diluted with 1mL of acetonitrile:water (95:5) and mixed. The diluted reaction mixture was transferred as such without any centrifugation onto the filter and allowed to flow through. The filter was washed with 2 x 1mL of acetonitrile:water (95:5) or 1-2mL in sample aliquots. Both flow through and washes were discarded, and the bound oligosaccharides were eluted with 2 x 0.5 mL of acetonitrile:water (20:80) into a clean test tube and mixed.

2.2.3 Methods for objective 3

The following experimental steps were used in order to identify peptides containing asparagine 45:

1. In-gel proteolytic digest of non-glycosylated α-lactalbumin using chymotrypsin and extraction of the peptides.

2. Separation of the peptides by RP-HPLC.

3. PVDF membrane blotting of the peptides for N-terminal sequencing.

(1) Identification of Asn_{45} containing peptides from the F band of α-lactalbumin

In-gel proteolytic digestion of acrylamide gel separated protein was the method used for generating peptide fragments for the purpose of identifying Asn_{45} containing peptides of the F fraction by Edman degradation and electrospray mass spectrometry (Moritz *et al.*,1996).

(a) In-gel S-Pyridylethylation of elecrophoretically separated proteins

Native-PAGE gel electrophoresis was performed stained with Coomassie Brilliant Blue R-250 (CBR-250) in acid solution to fix the protein. The total gel slab containing CBR-250 stained proteins was washed extensively with water (x 3 changes) for approximately 1.5 hours in total and then immersed in 50mL of reaction buffer (10mM DDT, 0.2M Tris-HCl, 6.3M guanidine hydrochloride, pH 8.4, containing 2mM EDTA) at 40°C for 2 hours. After reduction, 4-vinylpyridine (2% v/v) was added to the reduction buffer and the incubation was continued, in the dark, at 25°C for 1 hour. Alkylation was halted by the addition of excess β-mercaptoethanol (2%) and the gel slab was washed extensively with water for 1 hour. Gels were restained with CBR-250 following alkylation. For in-gel digestion, the CBR-250 stained protein bands of interest were excised with a scalpel and placed in eppendorf tubes. The CBR-250 was removed by two washings with 0.2M NH_4HCO_3/50% acetonitrile, for 30 mins at 30°C. The gel bands were then dried completely by centrifugal lyophilisation.

(b) In-gel protein digestion

For *in situ* chymotrypsin cleavage, the gel bands were rehydrated with a small aliquot of 0.2M NH_4HCO_3/0.5mM $CaCl_2$, containing 0.5µg chymotrypsin. A further 200µL of 0.2M NH_4HCO_3/0.5mM $CaCl_2$, was added and the mixture incubated at 37°C for 16 hours.

(c) Peptide extraction

The incubation buffer was collected and the following protocol was used to remove the peptides from the gel:

(1) 200µL of 1% TFA was added to the eppendorf tubes containing the gel bands. This was then sonicated at 40°C for 30 minutes then the was extract removed.

(2) A further 200µL of 0.1% TFA, 60% acetonitrile were added to the tubes, which were again sonicated for 30 minutes at 40°C before the extract was removed.

(3) The extracts were pooled and concentrated by centrifugal lyophilisation (care was taken not to dry the samples to completion).

(2) RP-HPLC

The peptide mixture was fractionated by RP-HPLC using a Phenomenex s/no 219475, C_{18} column (4.6mm x 250mm). A Spectra Physics SP 8800 HPLC was used and the eluent monitored at 214nm using a SP8490 UV/VIS detector. 200µL samples were injected manually using a Rheodyne model 7125 injector equipped with a 1mL injection loop. A linear gradient was used to differentially elute the peptides (0-100% buffer B in 60 minutes at a flow rate of 1mL/min). Buffer A was aqueous 0.1% v/v TFA and Buffer B was 60% acetonitrile containing aqueous 0.09% v/v TFA. Fractions were collected manually in eppendorf tubes and stored at 0°C.

(3) PVDF membrane blotting for N-terminal sequencing

A sample of whey protein containing the fast band (F) was subjected to electrophoresis on a 12% native gel but was not stained with Coomassie Blue R 250. The blotting apparatus was prepared according to the manufacturer's instructions using 10mM CAPS/10% methanol, pH 11 as the transfer buffer. The PVDF membrane was equilibrated in methanol for a few seconds followed by transfer buffer, then placed with the gel in a sandwich made of filter paper (Whatman no. 3).

The sandwich was placed in the apparatus and electrophoresed for one and half hours at 200mA. Afterwards the membrane was rinsed in water then stained with 0.1% Coomassie Blue/50% methanol/10% acetic acid and finally rinsed in water before air drying. Individual bands were cut out for N-terminal sequencing on the automated protein sequencer (Applied Biosystem).

2.2.4 General Methods

*(a) **Protein determination assay***

(i) The Bicinchoninic acid method

The bicinchoninic acid (BCA) method used was as described by Smith *et al.*, (1985). The bicinchoninic acid was obtained from Sigma. Bovine serum albumin was used (1mg/mL) to construct a standard curve from 0-100μg. All solutions were made up to 100μL and then 2mL of protein determination reagent was added and mixed. The mixtures were left for 30 minutes at 37°C. After cooling to room temperature the absorbances were measured at 562nm.

*(b) **Polyacrylamide gel electrophoresis (PAGE)***

(i) Native-PAGE

The purification procedures were monitored by running samples on native-PAGE. The major whey components were identified by comparison with literature results (Hopper and McKenzie, 1973).

The resolving gel contained 15% (w/v) acrylamide in 0.375M Tris HCl buffer, pH 8.8. The stacking gel contained 4% (w/w) acrylamide in 0.125M Tris HCl, pH 6.8. The reservoir buffer contained 0.025M Tris and 0.192M glycine, pH 8.3. Samples taken for native gel electrophoresis were added to an equal volume of sample buffer (0.125M Tris HCl, pH 6.8, 10% glycerol (v/v) and bromophenol dye). All gels were run at a constant voltage of 200V. Once the bromophenol blue dye had reached the bottom of the plate, the gels were run for a further 15 minutes to achieve optimum separation of the α-lactalbumin. Gels were stained in 0.125% Coomassie R-250 in methanol:acetic acid:water (5:1:4) and destained in methanol: acetic acid:water (3:1:10). Glycoproteins on the native gels were visualised by pre-soaking the gels in 0.05% SDS for 30 minutes before staining with Coomassie Blue.

(ii) SDS-PAGE

For SDS-PAGE of protein samples, the resolving gel contained 15% (w/v) acrylamide in 0.375M Tris HCl, pH 6.8, and 0.1% (w/v) SDS. The reservoir buffer contained 0.025M Tris, 0.192 M glycine and 0.1% (w/v) SDS, pH 8.3. SDS-PAGE molecular weight standards, low range, (catalogue number 161-0304, BIO-RAD) were used. All gels were run at a constant voltage of 200V. Gels were stained in 0.125% Coomassie R-250 in methanol:acetic acid:water (5:1:4) and destained in methanol:acetic acid:water (3:1:10).

CHAPTER III

RESULTS AND DISCUSSION

3.1 Results of Objective 1

3.1.1 Purification of non-glycosylated α-lactalbumin

(a) $FeCl_3$ precipitation for non-glycosylated α-lactalbumin

β-lactoglobulin is the predominant whey protein in bovine milk and hence a major contaminant in the α-lactalbumin purification. When the pH of whey was adjusted to 4.2 (the isoelectric point of α-lactalbumin) in the presence of 7-7.5 mM $FeCl_3$, a significant proportion of β-lactoglobulin, and bovine serum albumin (BSA) as well as some of the immunoglobulins (IgGs) were eliminated from the whey as a precipitate as seen from PAGE gels. There were however, significant amounts of β-lactoglobulin and Immunoglobulin remaining.

(b) Ammonium sulphate precipitation

The ammonium sulphate precipitation of the protein removes the excess ferric ions. The white precipitated proteins were removed by centrifugation leaving an orange supernatant, indicating a separation of the excess $Fe(OH)_3$. Comparison of the supernatant and the precipitated fractions by native-PAGE revealed that hardly any non-glycosylated α-lactalbumin remained in the supernatant.

(c) Anion exchange chromatography

Figure 3.1 shows a typical elution profile of ammonium sulphate precipitated proteins on a DEAE-Sephadex A-50 column as described in methods section 2.2.1 (1d). The elution was monitored by measuring the absorbance at 280nm and collected fractions were examined by native-PAGE (Figure 3.2). Peak fractions 18-21(lanes 2-5) showed that α-lactalbumin was the major protein component while fractions 22-24 (lanes 6-8) mainly contain β-lactoglubulins.

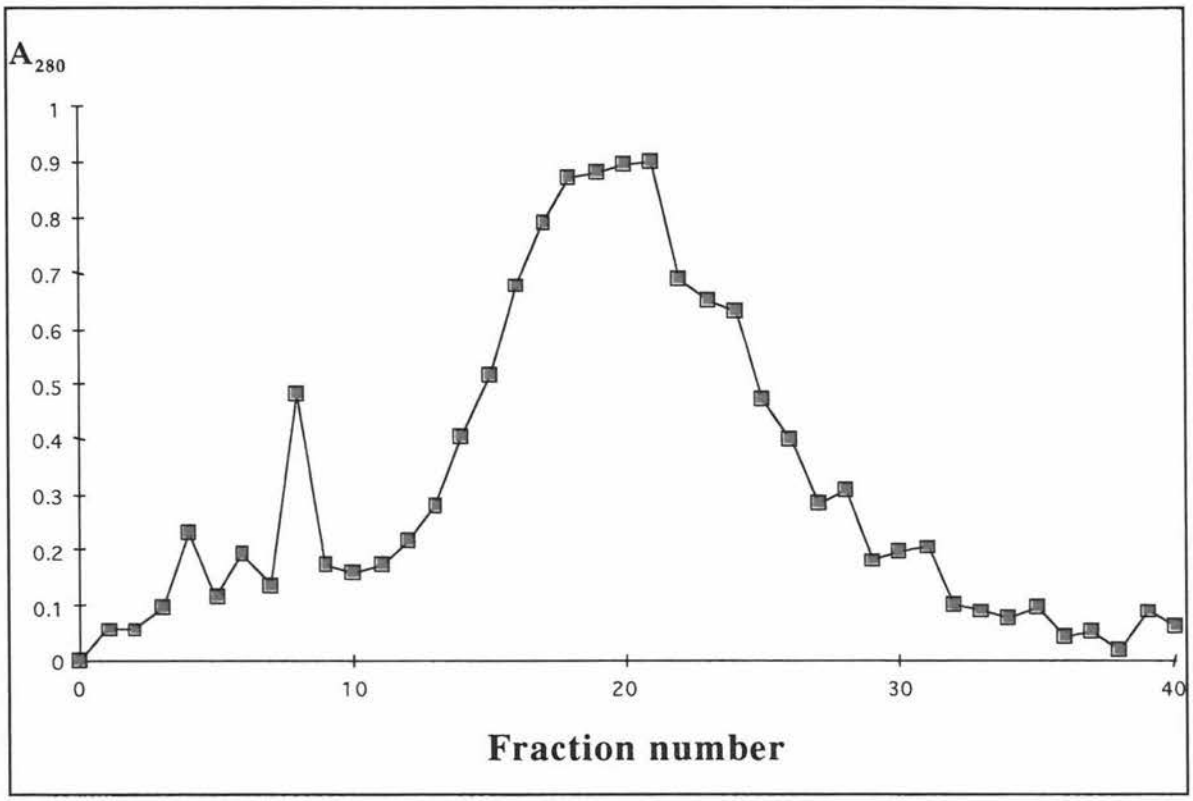


Figure 3.1 *Elution profile of ammonium sulphate precipitated proteins on the DEAE anion exchange column.*

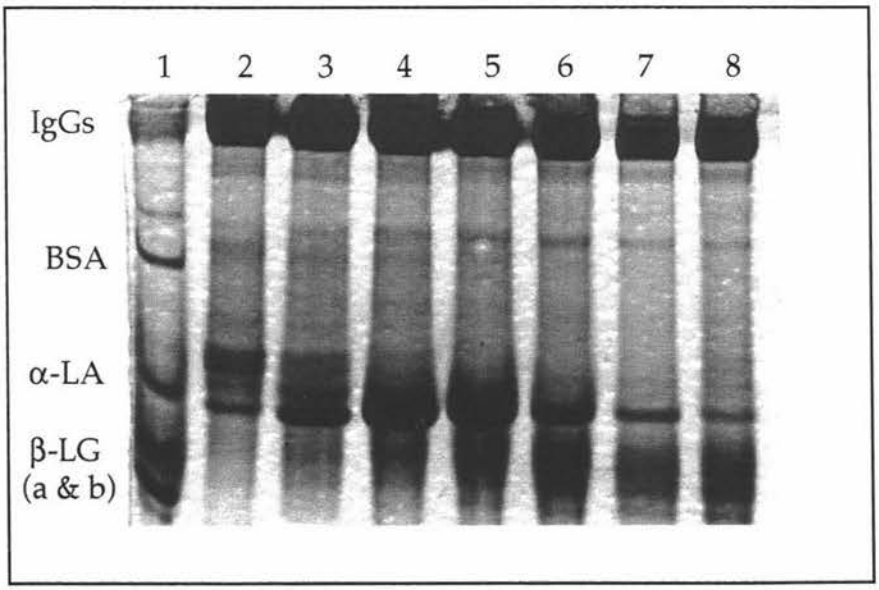


Figure 3.2 *Native-PAGE of peak fractions collected from anion exchange chromatography of ammonium sulphate precipitated proteins. Lane 1 represents the marker, Lanes 2-8 correspond to fractions 18-24 collected from the chromatogram shown in Figure 3.1.*

However, these fractions were still contaminated with glycosylated α-lactalbumin, IgGs and BSA.

(d) Lectin affinity column chromatography for non-glycosylated α-lactalbumin

A Con A column was used to separate mannose containing glycosylated proteins from the non-glycosylated proteins. Fractions 18-21 from the anion exchange chromatography (section (c)) were combined, concentrated by lyophilisation and applied to a Con A column. Non-glycosylated α-lactalbumin (M fraction) failed to bind to the column and was collected during the wash. Native-PAGE analysis showed the presence of other contaminants such as β-lactoglobulins and immunoglobulins, that also did not bind to the column.

(e) *Size exclusion chromatography*

The sample collected from the Con A column was applied to a gel filtration column as described in methods section 2.2.1 (1f) to get rid of other contaminants from the non-glycosylated α-lactalbumin. The elution was monitored by measuring the absorbance at 280nm. Two large peaks were observed in fraction numbers 73 and 77 respectively (Figure 3.3). From native-PAGE analysis it became apparent that peak 1 mainly contained IgGs while peak 2 contained non-glycosylated α-lactalbumin (Figure 3.4).

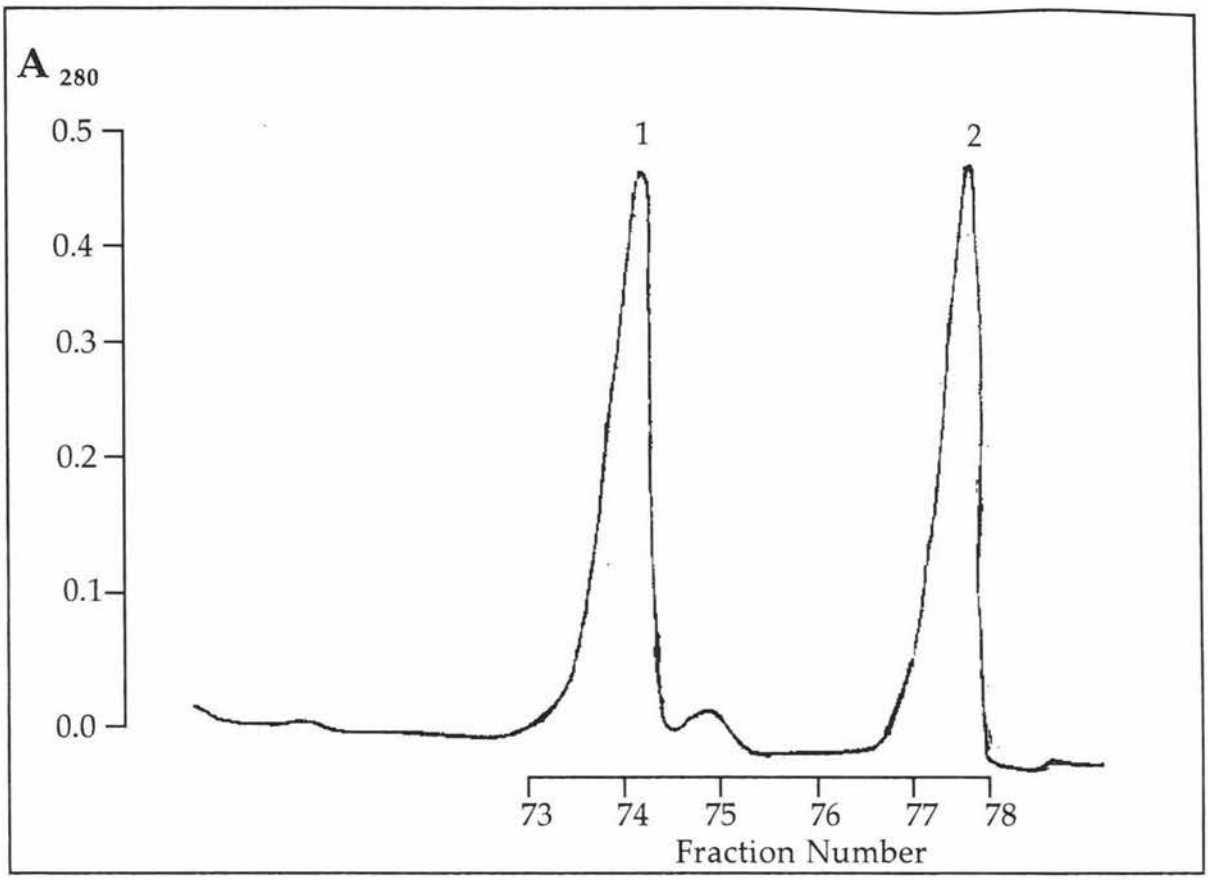


Figure 3.3 *Chromatogram of the elution from gel filtration of the M fraction of α-lactalbumin.*

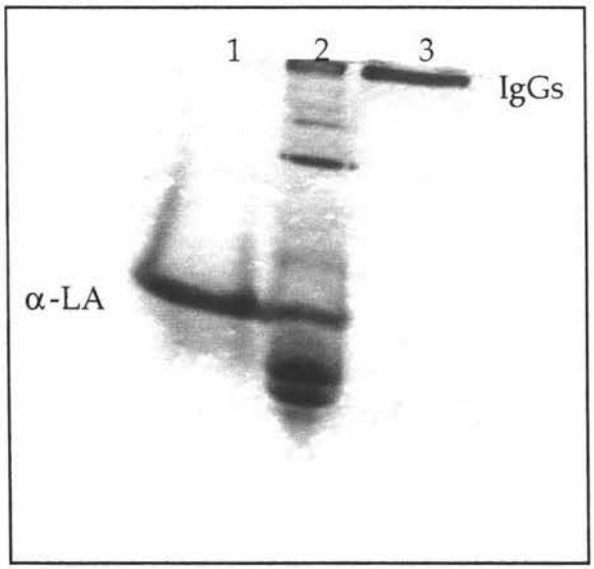


Figure 3.4 *Native gel of gel filtration fractions. Lane 1: Fraction 77 (peak 2), Lane 2: Marker, Lane 3: Fraction 73 (peak 1).*

The α-lactalbumin purified by the method above indicated a purity of greater than 95% when analysed by SDS-PAGE (Figure 3.5) and was deemed to be sufficiently pure for use in further experiments.

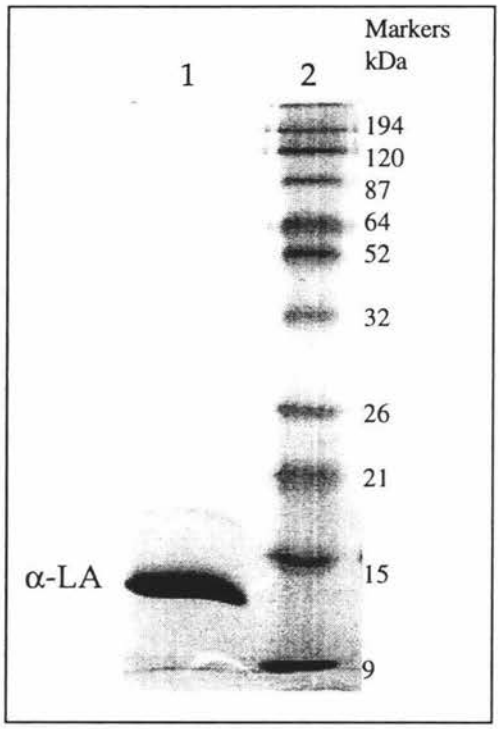


Figure 3.5 *SDS gel of purified non-glycosylated α-lactalbumin.*

3.1.2 Reduction and alkylation

Purified non-glycosylated α-lactalbumin (M fraction) was reduced and alkylated as described in methods section 2.2.1 (2). When the reduced and alkylated protein was desalted using low molecular weight cut-off dialysis tubes, the protein precipitated.

3.1.3 Enzymatic cleavage and RP-HPLC

The precipitated protein was recovered and resuspended in 0.2M ammonium bicarbonate solution as the protein did not fully dissolve in the solution, a more prolonged enzyme digest time was required for complete digestion. Chymotrypsin cleaves at the carboxyl-terminal peptide bonds of aromatic and bulky hydrophobic residues (tryptophan, tyrosine and phenylalanine). Peptide bonds preceded by other hydrophobic residues may also be cleaved, but generally at a slower rate.

Chymotrypsin hydrolysis of α-lactalbumin should theoretically result in the production of 25 peptides as shown in Table 3.1. When the digest of α-lactalbumin was applied to a C_{18} column, and eluted using the conditions described in method section 2.2.1 (4), about 30 peaks were resolved in a typical peptide map (Figure 3.6). The additional peaks observed could have been be due to incomplete hydrolysis of the protein, resulting in additional intermediate peptides containing chymotrypsin susceptible bonds which were not cleaved.

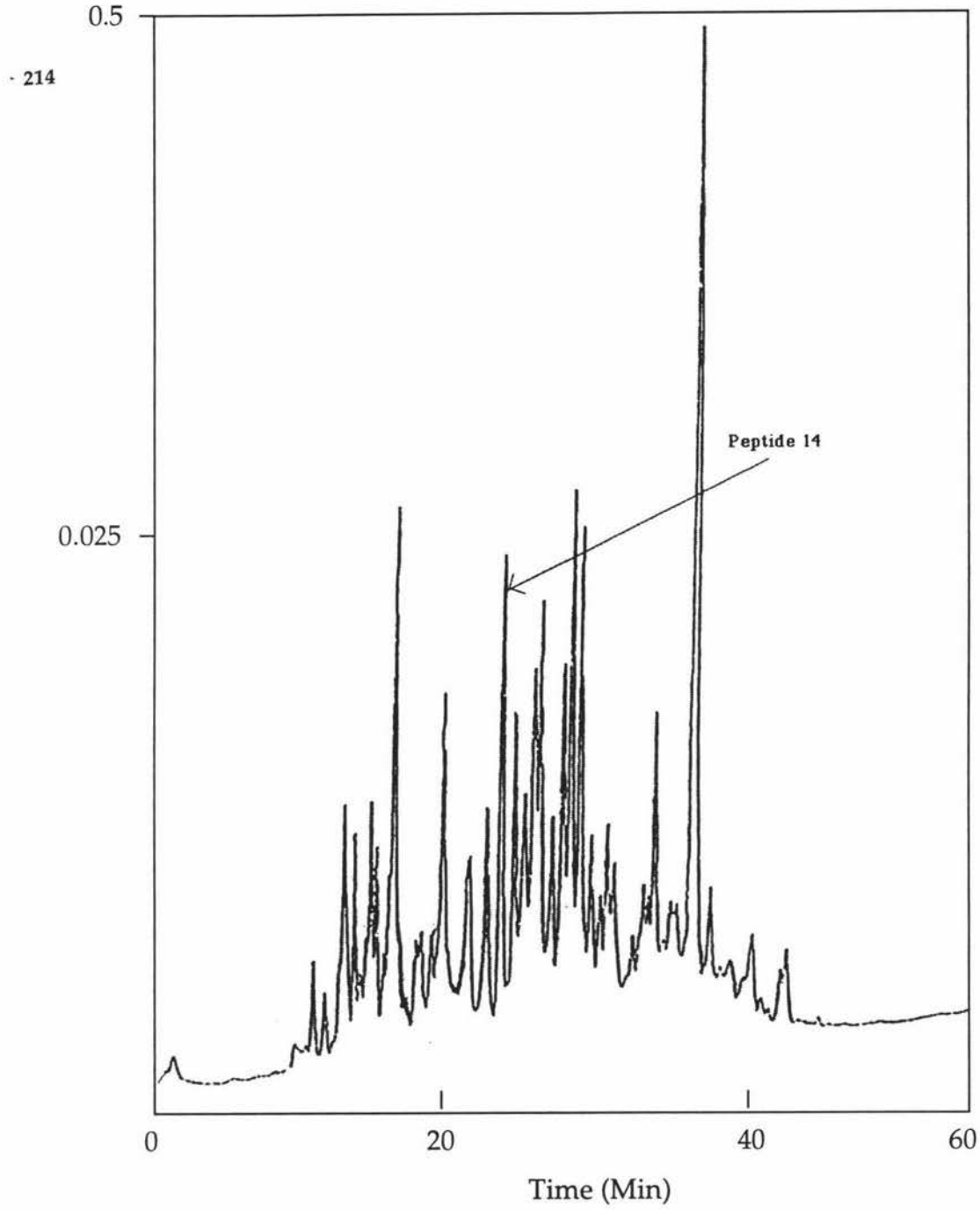


Figure 3.6 *A typical peptide map of the M fraction of bovine α-lactalbumin. Samples of Chymotryptic digest (500μL) were applied to a Vydac, C_{18} column and eluted with a linear gradient of 0-60% acetonitrile in water/0.08% TFA.*

Table 3.1 *Expected chymotryptic peptides from bovine α-lactalbumin and their calculated average masses.*

Fragment	Mass(MH^+)	Sequence
104-104	205.24	W
51-53	336.41	GLF
27-31	540.66	VCTAF
32-36	564.58	HTSGY
119-123	605.78	LCEKL
19-26	844.94	GGVSLPEW
54-60	916.07	QINNKIW
27-36	1086.22	VCTAFHTSGY
1-9	1097.28	EQLTKCEVF
10-18	1122.31	RELKDLKGY
51-60	1233.46	GLFQINNKIW
19-31	1366.58	GGVSLPEWVCTAF
37-50	**1598.62**	**DTQAIVQNNDSTEY**
105-118	1642.92	LAHKALCSEKLDQW
104-118	1829.14	WLAHKALCSEKLDQW
37-53	1916.01	DTQAIVQNNDSTEYGLF
10-26	1948.23	RELKDLKGYGGVSLPEW
32-50	2144.17	HTSGYDTQAIVQNNDSTEY
1-18	2200.57	EQLTKCEVFRELKDLKGY
105-123	2229.68	LAHKALCSEKLDQWLCEKL
61-80	2270.49	CKDDQDPHSSNICNISCDKF
81-103	2641.08	LDDDLTDDIMCVKKILDKVGINY
81-104	2827.30	LDDDLTDDIMCVKKILDKVGINYW
54-80	3167.54	QINNKIWCKDDQDPHSSNICNISCDKF
61-103	4892.55	CKDDQDPHSSNICNISCDKFLDDLTDDIMCVKKILDKVGINY

The 37-50 peptide highlighted contains asparagine 45.

3.1.4 *ES/MS and peptide sequencing*

The peptides separated by RP-HPLC were collected manually and identified using ES/MS and a peptide mass library for bovine α-lactalbumin. Peptide peak 14 (Figure 3.6) showed a M+2H ion at m/z 799.7 and M+H ion at 1598.9 (Figure 3.7) which corresponds to the calculated mass of 1598.62 for peptide 37-50 (Table 3.1). Edman sequencing of this peptide confirmed that it had the sequence DTQAIVQNNDSTEY. These results show there is no GlcNAc residue attached to asparagine 45 of the bovine α-lactalbumin.

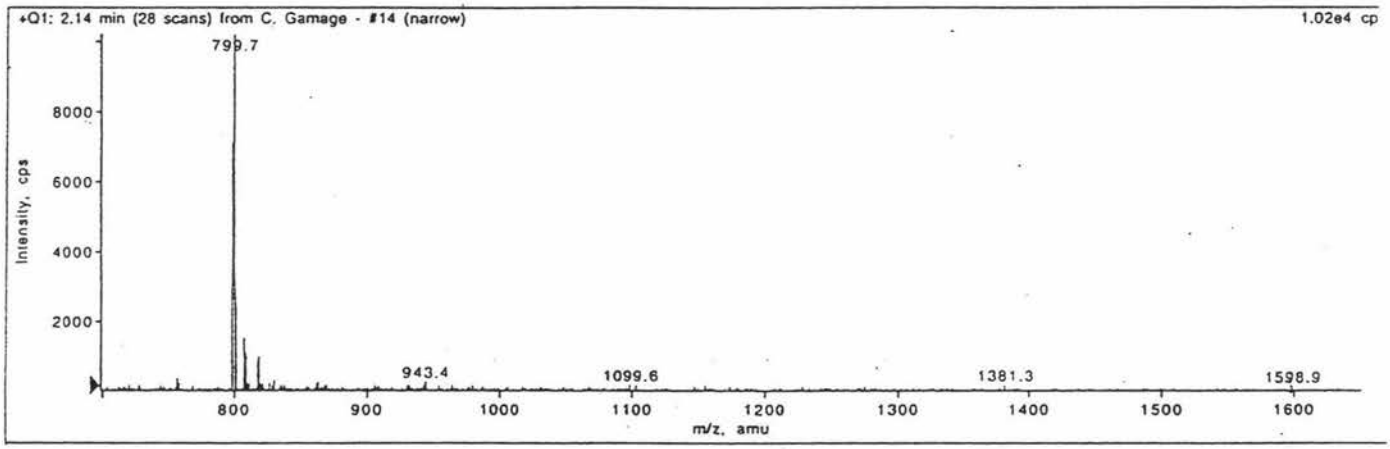


Figure 3.7 *The ES/MS spectrum of isolated peptide peak 14 (37-50 peptide). (at OR=30V, IS=5000V, RNG=140V and IQ1=-11V).*

Mass spectroscopic analysis of the purified M component of α-lactalbumin showed a single species with an M+H ion at m/z 14179.0 (Figure 3.8 (a)), which corresponds to the mass calculated from the published sequence. While this result indicates that these are no covalently linked sugar residues i.e: none of the possible glycan sites has a sugar, SIM analysis shows that there is a carbohydrate non-covalently associated with the protein. A strong peak was observed at 366 amu (Figure 3.8(b)) in the positive ion mode. The instrument was set to successively monitor specific m/z values after subjecting the protein solution to alternatively high and low energy potentials at the orifice. This peak corresponds to the molecular weight of a hexosamine such as Gal-GlcNAc, Man-GalNAc or Gal-GalNAc. As these sugars are known to be present in milk, it is not unlikely that they may bind to the protein. A longer monitoring time devoted to these values means allowed these to be detected with much higher sensitivity than in the usual scanning mode.

3.1.5 Discussion of Objective 1 Results

There was no evidence of any glycans covalently attached to the M fraction of α-lactalbumin. However from the ES/MS data there appeared to be mass that corresponded to the disaccharide Hex-HexNAc (Gal-GalNAc, Gal-GlcNAc or Man-GlcNAc) associated with the protein. Due to the "soft ionisation" techniques involved in electrospray, sugars may stay associated with the protein by non-covalent interactions or be found as a separate species. If this is the case, one would expect to see a mass corresponding to that of α-lactalbumin +366 or if the attachment was very weak a species of mass 366 should be seen in the raw mass spectrum, a small peak at 381 was observed in the original spectrum (Figure 3.8 (a)). This requires further investigation as it is unlikely that the lactosamine found was an external contaminant derived from any buffer or an artefact of the purification scheme. The binding site for sugars is usually in a cleft between two domains. In proteins, this binding is typically mediated by hydrogen bonds between the sugar hydroxyl groups and the polar groups of the protein. There are also non-polar interaction between aromatic side chains and the sugar rings (Quiocho, 1986).

Given its close structural relationship with hen egg white lysozyme (HEWL), it is not unreasonable that α-lactalbumin may also bind sugars, especially as it is involved in the biosynthesis of lactose. It is believed that binding of these sugars in the cleft region may stabilise the sugar substrate in the lactose synthase complex. While galactosyltransferase is itself unable to catalyse the biosynthesis of lactose under physiological conditions because of its poor affinity for glucose, α-lactalbumin interacts with galactosyltransferase to promote glucose

binding by 1000-fold. The exact nature of this interaction is not known. However it has not been shown that α-lactalbumin is able to bind sugars in isolation.

(a) Comparison between α-lactalbumin and lysozyme

The similarities and differences between α-lactalbumin and lysozyme are striking. Lysozyme has been extensively characterised in both its structure and enzyme activity for many years. A few of the features of α-lactalbumin and lysozyme are shown in Table 3.2.

Table 3.2 *Comparison of α-lactalbumin and hen egg white lysozyme (HEWL)*

	α-lactalbumin	**HEWL**
Molecular weight	14.2kDa	14.6kDa
Amino acids	123	129
Disulphide bridges	4	4
3-D structure	2 domains with binding cleft	2 domains with binding cleft
Catalytic activity	Unknown, acts as "specifier" protein in lactosye synthase complex.	Hydrolyses specific β1-4 glycosidic bonds.
Localisation	Mammalian milk and colostrum	Ubiquitous in various tissues and secretions

(b) The binding sites of lysozyme and α-lactalbumin

Lysozyme hydrolyses the β1-4 linkages between N-acetylmuramic acid and N-acetylglucosamine. The optimum size of the oligosaccharide substrate is six residues in length. Oligosaccharides of less than 5 residues are only slowly hydrolysed and can act as competitive inhibitors of the enzyme. A study using an oligosaccharide consisting of 3 GlcNAc residues found that this inhibitor associated with lysozyme through strong hydrogen bond interactions via the acetamido groups of the end GlcNAc residues with both side chains and main chain atoms of the protein as well as by making close-fitting hydrophobic contacts.

The cleft region of α-lactalbumin is very similar to that of lysozyme. The residues in α-lactalbumin (Phe 31, His 32 and Leu 110) are structural equivalents to those found in lysozyme that are involved in saccharide binding. Direct evidence to support this has been provided by site-directed mutagenesis on recombinant bovine α-lactalbumin. Amino acid substitutions in this region reduced α-lactalbumin's ability to promote lactose synthesis by the lactose synthase complex (Grobler *et al.*, 1994).

(c) Possible mode of action of α-lactalbumin in the lactose synthase complex

Although α-lactalbumin is not known to have any enzymatic activity in itself the ability to bind small oligosaccharides may directly assist the activity of galactosyltransferase in the lactose synthase complex. It may be possible that α-lactalbumin acts as a "sugar carrier" protein. The binding site could perhaps carry small sugars (1-2 residues) and then present these in a suitable conformation to galactosyltransferase. The association of α-lactalbumin with the enzyme determines the specificity (possibly by altering the conformation of the donor sugar in a situation reminiscent of lysozyme). The proximity of α-lactalbumin (with a non-covalently bound sugar) to galactosyltransferase may not only alter the enzymes affinity for glucose but α-lactalbumin could enhance the delivery of the appropriate sugars to the enzyme.

Lactosamine was found associated with α-lactalbumin, this is produced when the galactosyltransferase is not associated with α-lactalbumin. The sugars that may commonly be found in association with α-lactalbumin are likely to be substrates and/or products of the galactosyltransferase.

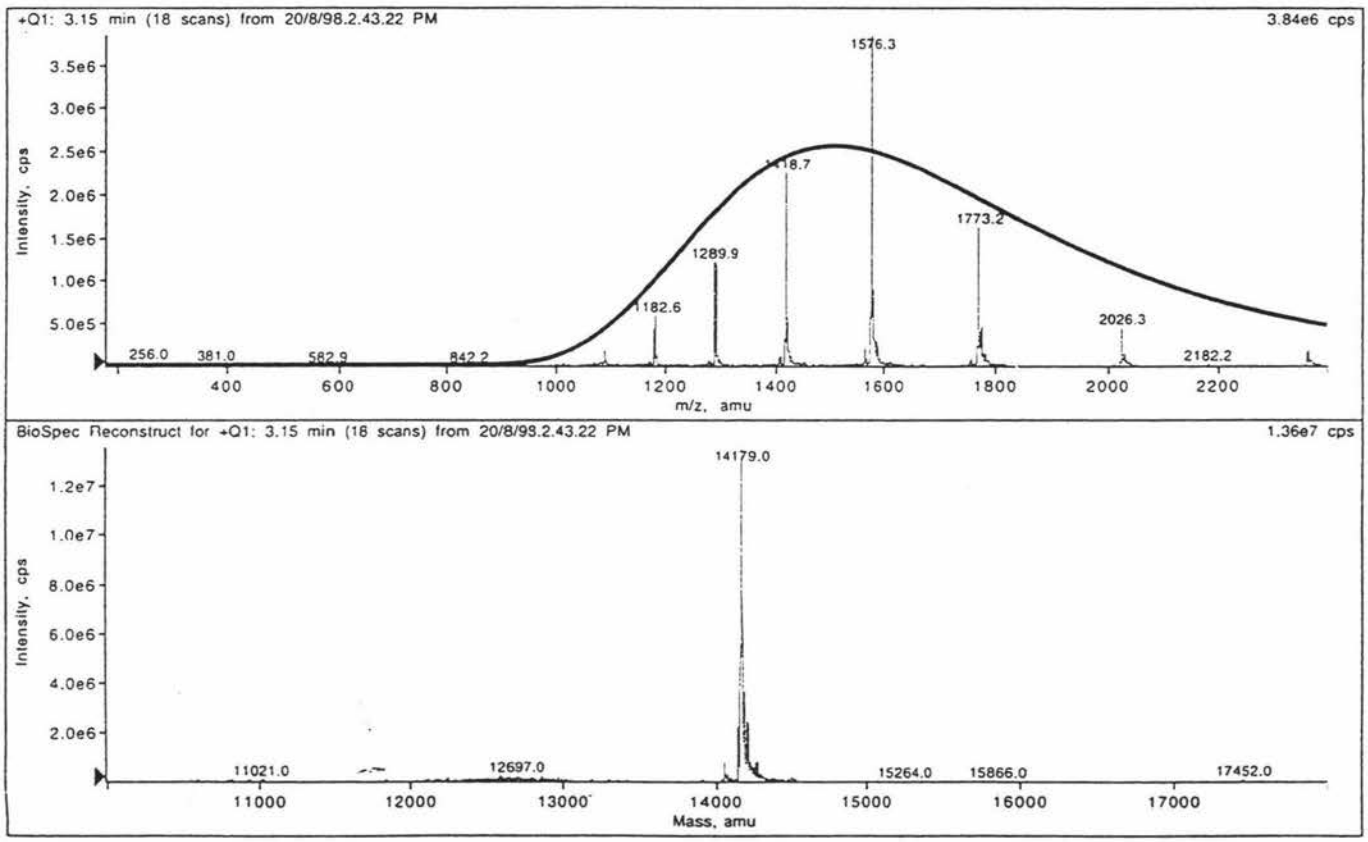


Figure 3.8 (a) *ES/MS spectrum of a sample of purified M fraction of bovine α-lactalbumin (at OR=30V, IS=5000V, RNG=140V and IQ1=-11V).*

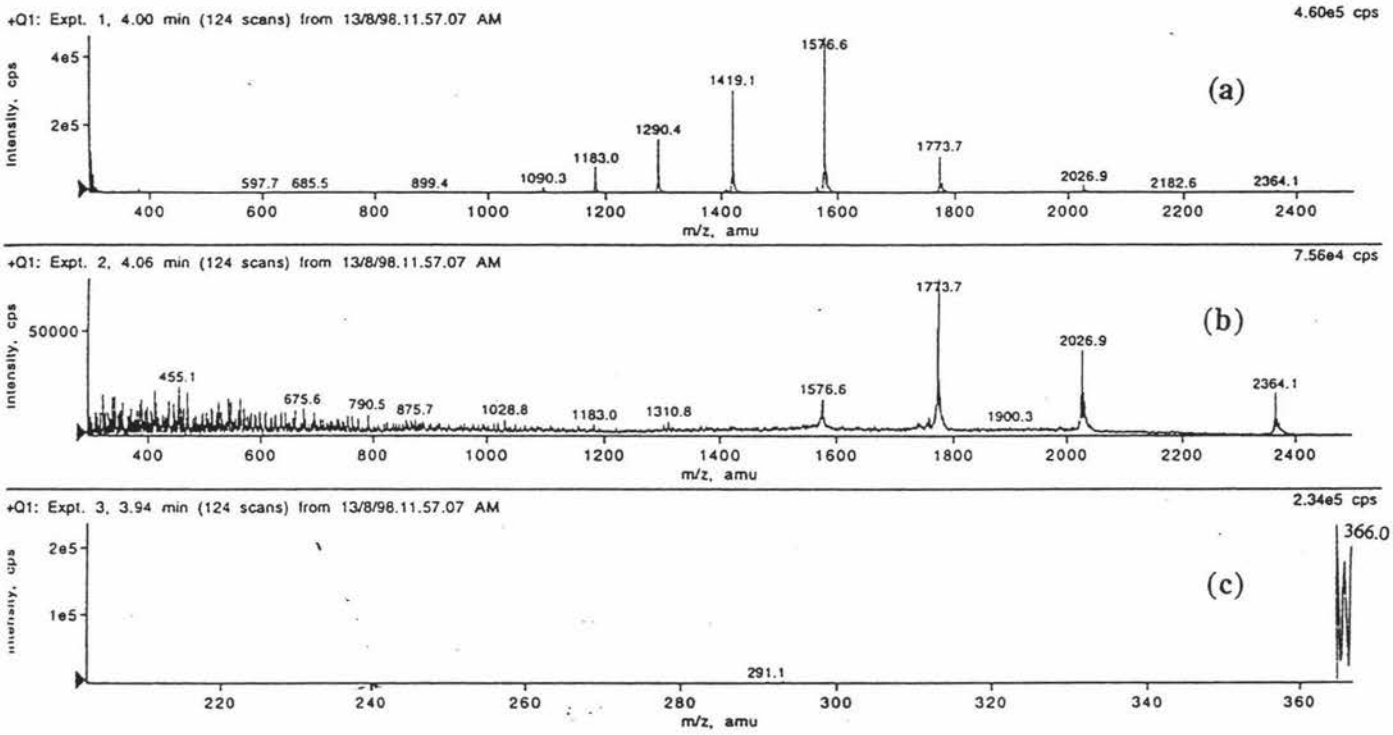


Figure 3.8 (b) *The ES/MS spectrum of the carbohydrate marker ion at m/z 366 for the sample of non-glycosylated α-lactalbumin. This data was observed in the positive ion mode, at high orifice potential. (a) low energy (at OR=30V, IS=5000V, RNG=140V and IQ1=-11V) (b) high energy (at OR=90V, RNG=400V, and IQ2=-20V). (c) monitoring at 162, 203, 366 and 291 amu (at OR=200V, RNG=400V, IQ1 and IQ2=-15V).*

3.2 *Results of Objective 2*

3.2.1 *Purification of glycosylated α-lactalbumin.*

(a) Lectin affinity column chromatography for glycosylated α-lactalbumin

Three glycosylated α-lactalbumin samples were prepared from three separate samples of milk collected at different times of the milking season, these samples are referred to throughout as Prep 1, 2 and 3. Dialysed acid whey was directly applied to a Con A column without $FeCl_3$ precipitation. Bound glycosylated proteins were eluted from the Con A column using 0.5M methyl α-D mannopyranoside or 2M glucose in the same buffer. Using this method the glycosylated proteins were separated from the bulk of the proteins in the acid whey. The elution was monitored by measuring the absorbance at 280nm and collected fractions were analysed by native-PAGE. The fractions containing α-lactalbumin were pooled, dialysed against Milli Q water and concentrated by lyophilisation. Native-PAGE analysis showed that there was bovine serum albumin (BSA) and immunoglobulin (IgGs) contamination with the glycosylated α-lactalbumin (Figure 3.10, lane 1). IgGs are glycoproteins and may be expected to bind, but BSA is not glycosylated and is most likely non-covalently associated with the glycosylated proteins.

(b) Size exclusion chromatography

The glycosylated α-lactalbumin was further purified by size exclusion chromatography as described in methods section 2.2.1 (1f). The elution was monitored by measuring the absorbance at 280nm (Figure 3.9) and fractions were analysed by native-PAGE (Figure 3.10). Lane 1 shows the sample from Prep 1 that was loaded onto the column. This contained a high proportion of the S_1 glycoform and very little S_2. Lanes 3-5 were enriched with the S_1 glycoform and there was some indication of another possible glycoform in lane 4. This ran at a position close to that expected for S_2, but appears to be a different species when compared to the marker. Identical peaks from a number of runs were pooled together, concentrated and rechromatographed on Superdex-75 to remove any faint traces of immunoglobulin and other contaminants. The samples were then lyophilised for later digestion by PNGase F.

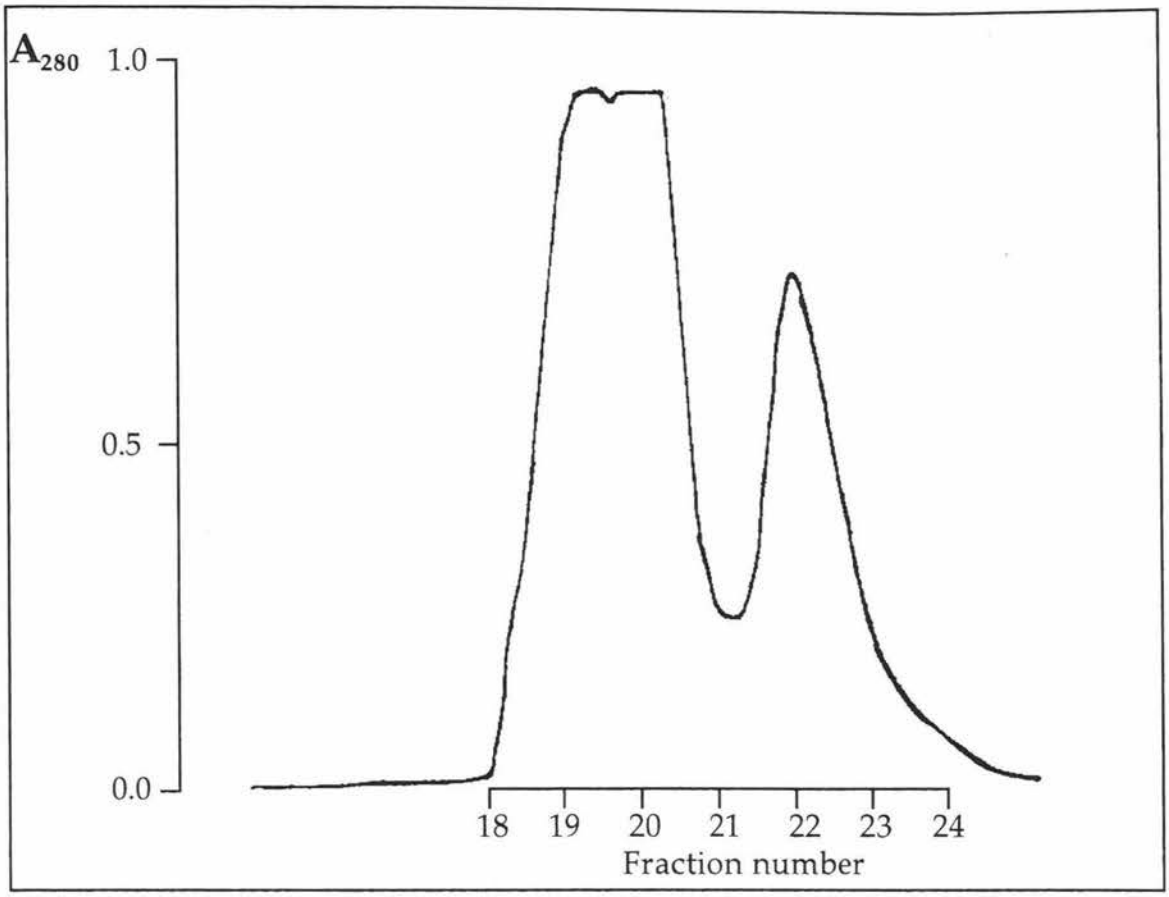


Figure 3.9 *Chromatogram of the elution from Superdex-75 of glycosylated α-lactalbumin from Prep 1.*

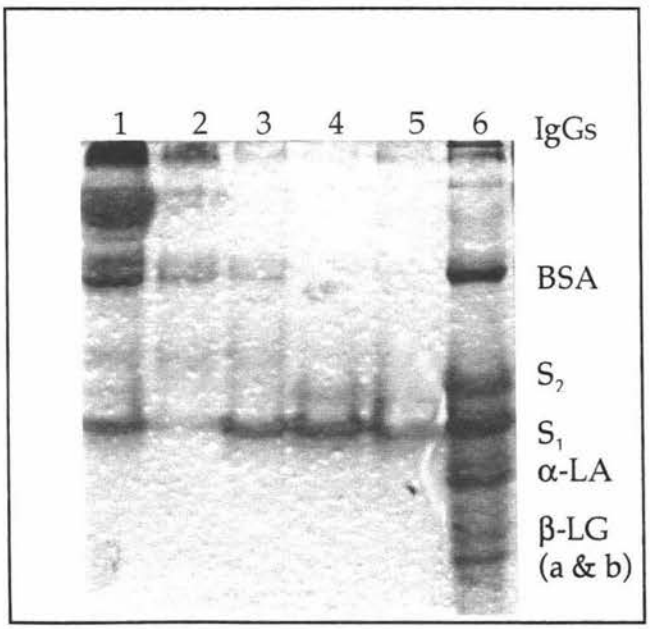


Figure 3.10 *Fractions from size exclusion chromatography. Lane 1: sample after Con A chromatography (load). Lane 2: Fraction 21. Lane 3: Fraction 22. Lane 4: Fraction 23 Lane 5: Fraction 24. Lane 6: Marker.*

3.2.2 PNGase digestion of glycoproteins

Ribonuclease B was used as a control to test all methods prior to using them to analyse the oligosaccharides of α-lactalbumin. There are five known glycoforms of ribonuclease B ($Man_5GlcNAc_2$-$Man_9GlcNac_2$). Each is different by one extra mannose residue. These have already been extensively studied and characterised making the protein an ideal model.

(a) PNGase F digest of ribonuclease B

SDS and native PAGE gels were used to check that the PNGase F digestion was complete. The digest condtions were according to methods section 2.2.2 (2). Figure 3.11 shows the PNGase F digestion results for ribonuclease B (SDS gel).

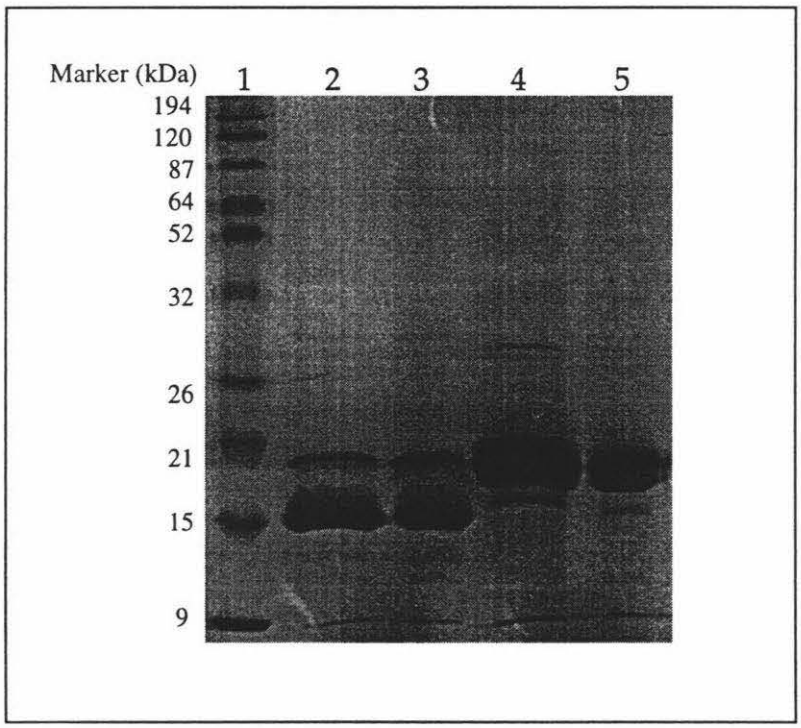


Figure 3.11 *SDS gel of PNGase F digestion of ribonuclease B. Lane 1: molecular weight marker. Lane 2: Ribonuclease B after PNGase F digestion (10μl loading). Lane 3: same as lane 2 (5μl loading). Lane 4 : Ribonuclease B (10μl loading). Lane 5 : same as lane 4 (5μl loading).*

Lanes 4 and 5 contain glycosylated ribonuclease B. There appears to be a double band which may reflect a number of different glycoforms. A faint band below these could possibly be some naturally occuring non-glycosylated ribonuclease B. After the addition of PNGase F in lanes 2 and 3 there is a strong band that represents deglycosylated ribonuclease B at about 15 kDa. A faint band at around 17kDa remained indicating that deglycosylation was not fully complete.

(b) PNGase F digest of glycosylated bovine α-lactalbumin

The concentrated and freeze dried samples of glycosylated α-lactalbumin from the size exclusion chromatography were digested with PNGase F. Figure 3.12 shows the PNGase F digestion results of bovine α-lactalbumin (native gel).

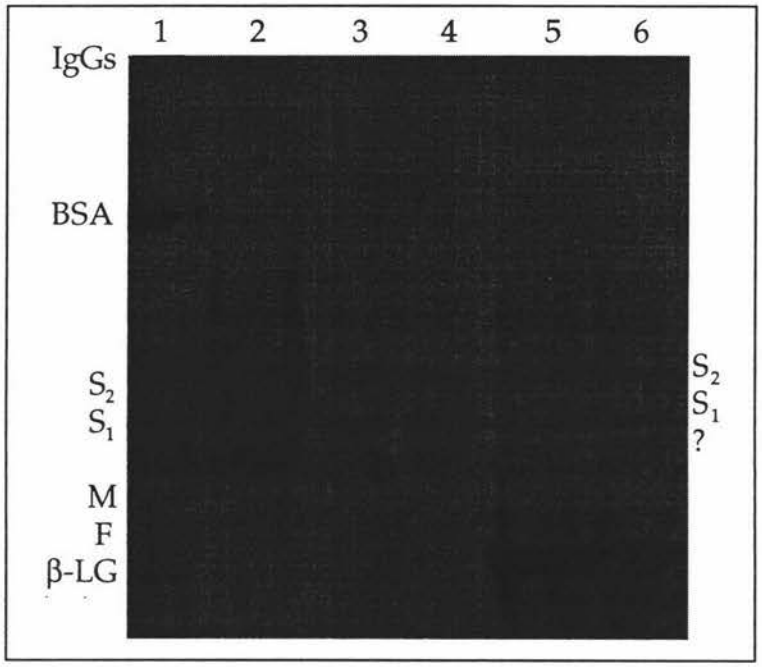


Figure 3.12 *Native gel of PNGase F digestion of glycosylated α-lactalbumin from Prep 1. Lane 1 : Marker, Lanes 2, 3 and 4: Glycosylated bovine α-lactalbumin at different loadings (20μl, 5μl, 10μl), Lanes 5 and 6: After PNGase F digestion of glycosylated bovine α-lactalbumin.*

In lanes 2-4 there are different loadings that clearly show the two glycoforms S_1 and S_2 are both present in Prep 1. After deglycosylation with PNGase F a strong single band was evident with a similar mobility to the F band in lane 1. However the bands are difficult to compare directly because of the heavy loading of the sample in lanes 5 and 6. Measuring from the top of the bands is the optimum way of comparing these. The gel shows deglycosylation was not complete with some S_1 and S_2 remaining as well as a band just below the S_1. The identity of this band is unknown.

3.2.3 Separation of Oligosaccharide mixtures

(a) High-pH anion-exchange chromatography with detection by pulsed amperometry (HPAEC/PAD)

(i) Analysis of high mannose-type oligosaccharides obtained from ribonuclease B

Figure 3.13 shows the oligosaccharide map obtained for the oligosaccharides of ribonuclease B. The PNGase digest mixture contained ribonuclease B, enzyme and a mixture of oligosaccharides. When this mixture is loaded (dissolved in water) onto the Carbopac PA-100 column it allows clean separation of these diverse molecules. The first to elute are the proteins (ribonuclease B and PNGase F) within the first 5 minutes. The oligosaccharides elute later according to their size and conformation. Identification of these was done by a comparison of the elution times with a previous study (Kakehi *et al.*, 1991). There are 5 main peaks. The first reasonably significant peak, which eluted at approximately 18 minutes, is $Man_5GlcNAc_2$ (peak 1) the most retained peak is $Man_9GlcNAc_2$ (peak 5). Between these peaks are $Man_6GlcNAc_2$, $Man_7GlcNAc_2$, and $Man_8GlcNAc_2$ respectively. $Man_7GlcNAc_2$ eluted as two peaks, because of the different branch positions of the terminal mannose residues (Kakehi *et al.*, 1991). Other small peaks that eluted before these oligosaccharides may be due to degradation products.

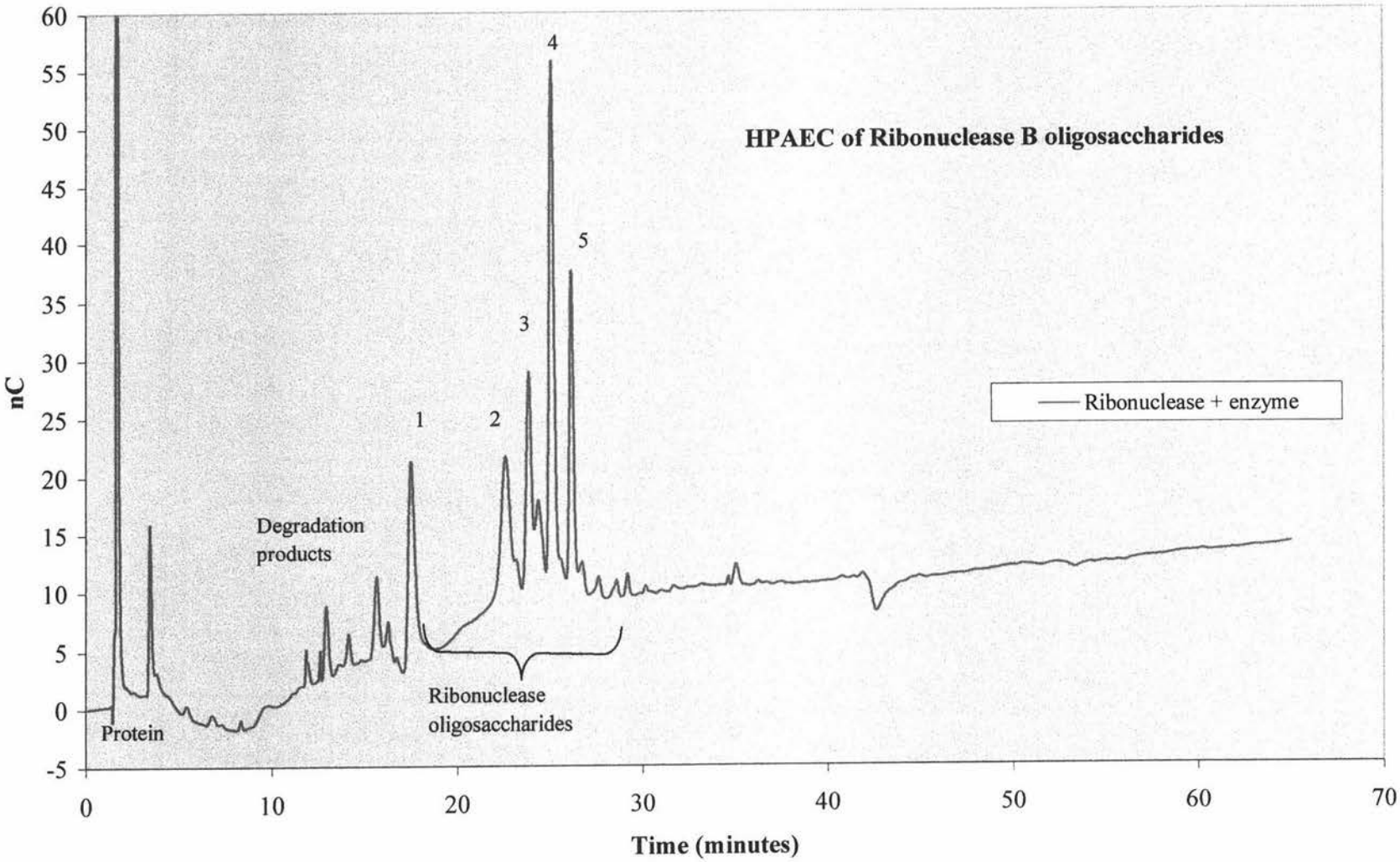


Figure 3.13: *Oligosaccharide map obtained from the PNGase-released oligosaccharides of Ribonuclease B*

(ii) Analysis of oligosaccharides obtained from bovine α-lactalbumin

Figures 3.14 (a) and (b) show the HPAEC/PAD profiles of α-lactalbumin oligosaccharides, from Prep 1 and 3 respectively that were enzymatically released from bovine glycosylated α-lactalbumin using PNGase F. From their elution positions it was apparent that the oligosaccharides of α-lactalbumin can be separated into two groups: neutral and sialic acid containing species. The sialic acid containing oligosaccharides are probably represented by the S_1 band of α-lactalbumin due to their expected higher mobility on native PAGE. This is further evidence to support the previous work done on the glycoforms of α-lactalbumin suggesting that there are two families (Chilcott, 1996). Seven peaks (1-7) were collected from the Prep 1 profile (Figure 3.14 (a)). In addition there were at least seven other minor peaks that were not collected due to their low concentration. The FACE technique was used to analyse the collected peaks. An oligosaccharide profile of Prep 3 (Figure 3.14 (b)) showed over twenty peaks could be separated. This was significantly more than the number seen in Prep 1 due to the different methods of preparation although both had two distinct groups based on their retention times. Appropriate controls showed that these peaks were not a results of contaminating substances in the solutions (Figure 3.14 (b)).

Although HPAEC analysis of acidic and neural oligosaccharides is possible in a single run, it is better to conduct separate analyses in order to avoid long retention times that could favour the alkaline degradation of oligosaccharides.

Because HPAEC gives sufficient chromatographic resolution for all classes of oligosaccharides associated with glycoproteins there is a high probability that each peak represents a single oligosaccharide structure; thus, HPAEC is a useful "oligosaccharide mapping" method.

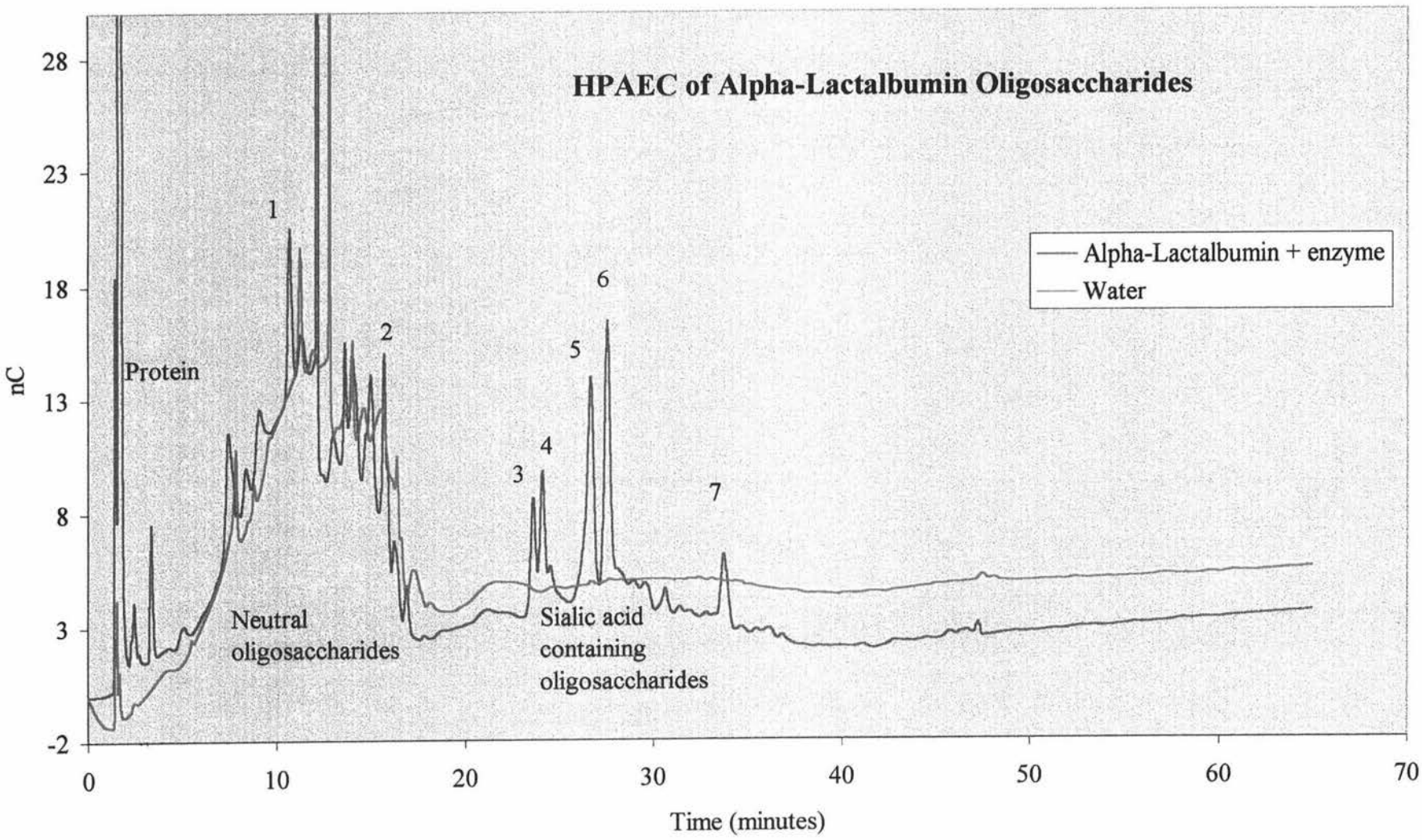


Figure 3.14 (a): *Finger printing of bovine α-lactalbumin oligosaccharides from preparation 1 .*

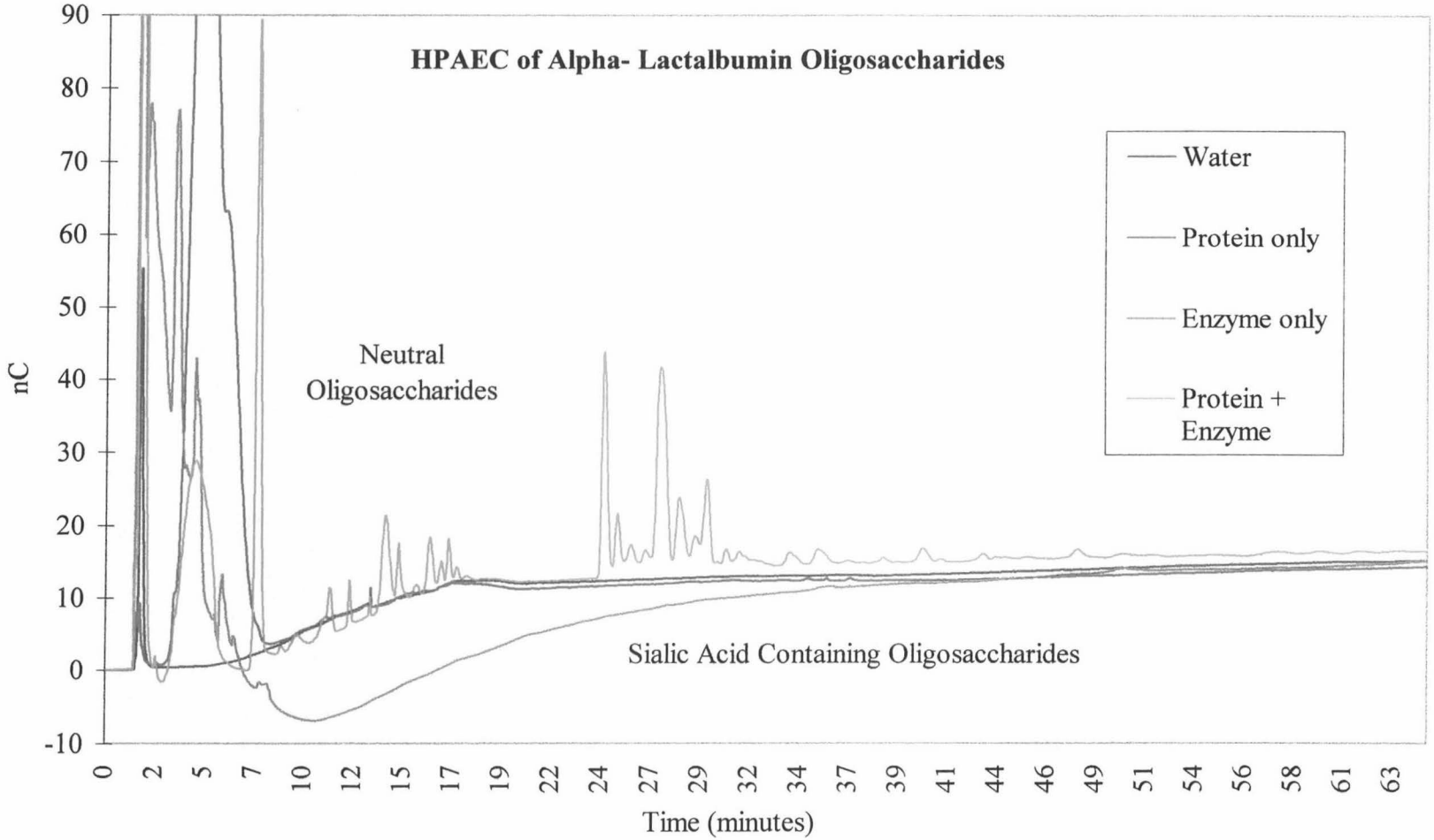


Figure 3.14 (b): *Finger printing of bovine a-lactalbumin oligosaccharides uing HPAEC with PAD detection (Preparation 3)*

(b) Fluorophore assisted carbohydrate electrophoresis (FACE)

The principle of FACE separation of carbohydrates is based on both size and charge. 8-amino-napthalene-1,3,6, trisulphonic acid (ANTS) labelling of an oligosaccharide not only converts the molecules into a fluorescent product but also adds three negative charges which allows a previously neutral sugar to migrate in an electric field. Because of the very high percentage of acrylamide used in the FACE system (30-35%), only carbohydrates are able to move into the gel, whereas high molecular-weight molecules such as proteins are not. Consequently, if a carbohydrate binds to a protein its electrophoretic mobility should be retarded.

(i) Analysis of high mannose-type oligosaccharides obtained from ribonuclease B

The FACE pattern of oligosaccharide released from ribonuclease B is shown in Figure 3.15. Lane 2 shows the size-based separation of neutral oligomannose oligosaccharides released from ribonuclease B, $Man_5GlcNAc_2$ is the lowest major band, with $Man_5GlcNAc_2$-$Man_9GlcNAc_2$ forming the ladder. The band seen at the bottom of the lane is belived to be a degradation product of the ribonuclease oligosaccharides. A standard ladder containing linear glucose polymers from 2-8 glucose units was run as a marker in the other lane (lane 1).

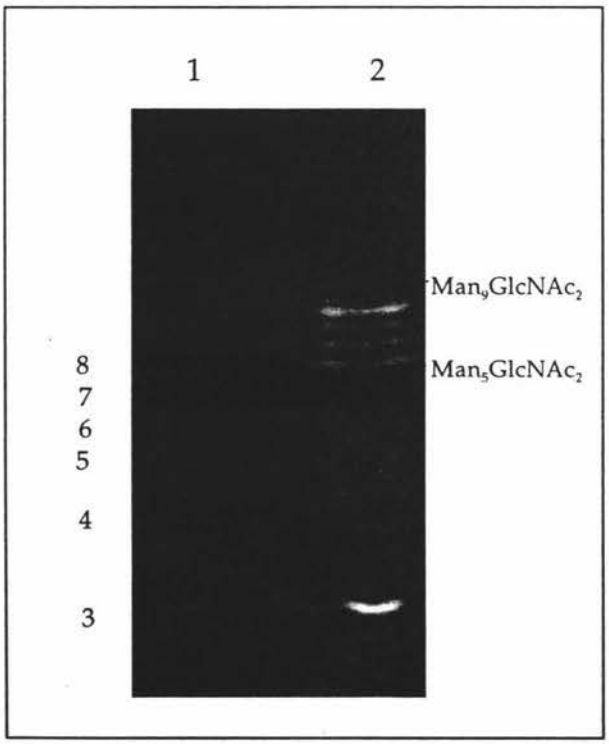
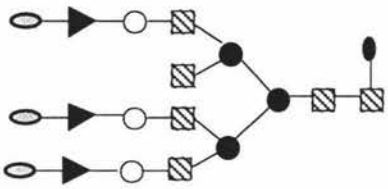
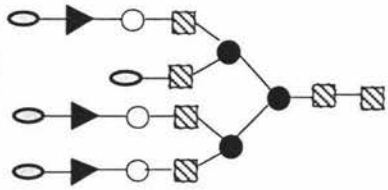
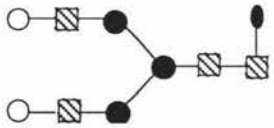
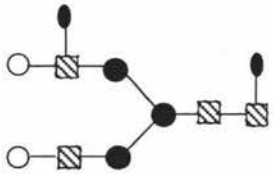
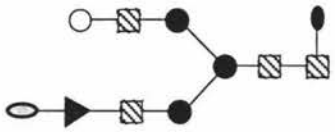


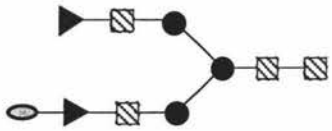
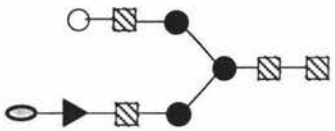
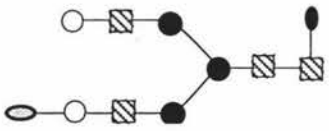
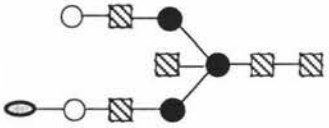
Figure 3.15 *FACE pattern of oligosaccharides released from ribonuclease B Lane 1: Standard glucose ladder. Lane 2 : Oligosaccharides released from ribonuclease B.*

(ii) Analysis of oligosaccharides obtained from bovine α-lactalbumin

Figure 3.16 (a) and (b) shows the FACE separation of oligosaccharides released from bovine α-lactalbumin from Prep 1 and Prep 3 respectively. Actually eight glycoforms could be observed as well as two other faint bands which may represent another two glycoforms in small quantities between the two FACE gels shown. These results were from the combined results of the three different preparations of glycosylated α-lactalbumin. The bands toward the bottom of each lane are probably formed by degradation products. A standard ladder containing linear glucose polymers from 2-8 glucose units was run as a marker in the other lane. The sample bands that ran between the marker bands indicate that monosaccharides other than glucose made up the oligosaccharides. The relative position of the band to a glucose ladder is used to compare different FACE gels. The position of the band can also provide some tentative predictions of the oligosaccharide composition and structure. This is based on their Dp (degree of polymerisation) values relative to the glucose ladder (Hu, 1995 and Starr *et al.*, 1996) (Table 3.3).

Table 3.3 *Correlation between the FACE gel values and the proposed basic glycan structure (Hu, 1995 and Starr et al., 1996).*

Bands observed in FACE gels Figures 3.16 (a) and (b)	Approximate observed Dp values			Proposed glycan structures	Calculated Dp values
	Prep 1	Prep 2	Prep 3		
a	>8	>8			15.33 ± 2
b	>8				14.35 ± 2
c	7.9-8.0		7.9-8.2		7.9 ± 2
					8.3 ± 2
					8.25 ± 2

Bands observed in FACE gels Figure 3.16 (a) and (b)	Approximate observed Dp values			Proposed glycan structures	Calculated Dp values
	Prep 1	Prep 2	Prep 3		
d	6.7	6.9-7.8	6.9-7.8		7.8 ± 2
					7.55 ± 2
					6.7 ± 2
					6.9 ± 2
e			6.0		
f			5.1		
g			4.8	Conserved trimannosyl core fuc	4.8 ± 2
h			3.3	Manα1-6Manβ1-4GlcNAcβ1-4GlcNAc	3.3 ± 2
i	2.5		2.5	Manβ1-4GlcNAcβ1-4GlcNAc	2.5 ± 2

▨ GlcNAc ● Mannose ▶ Galactose ○ GlcNAc ● Fucose ⬭ Sialic acid

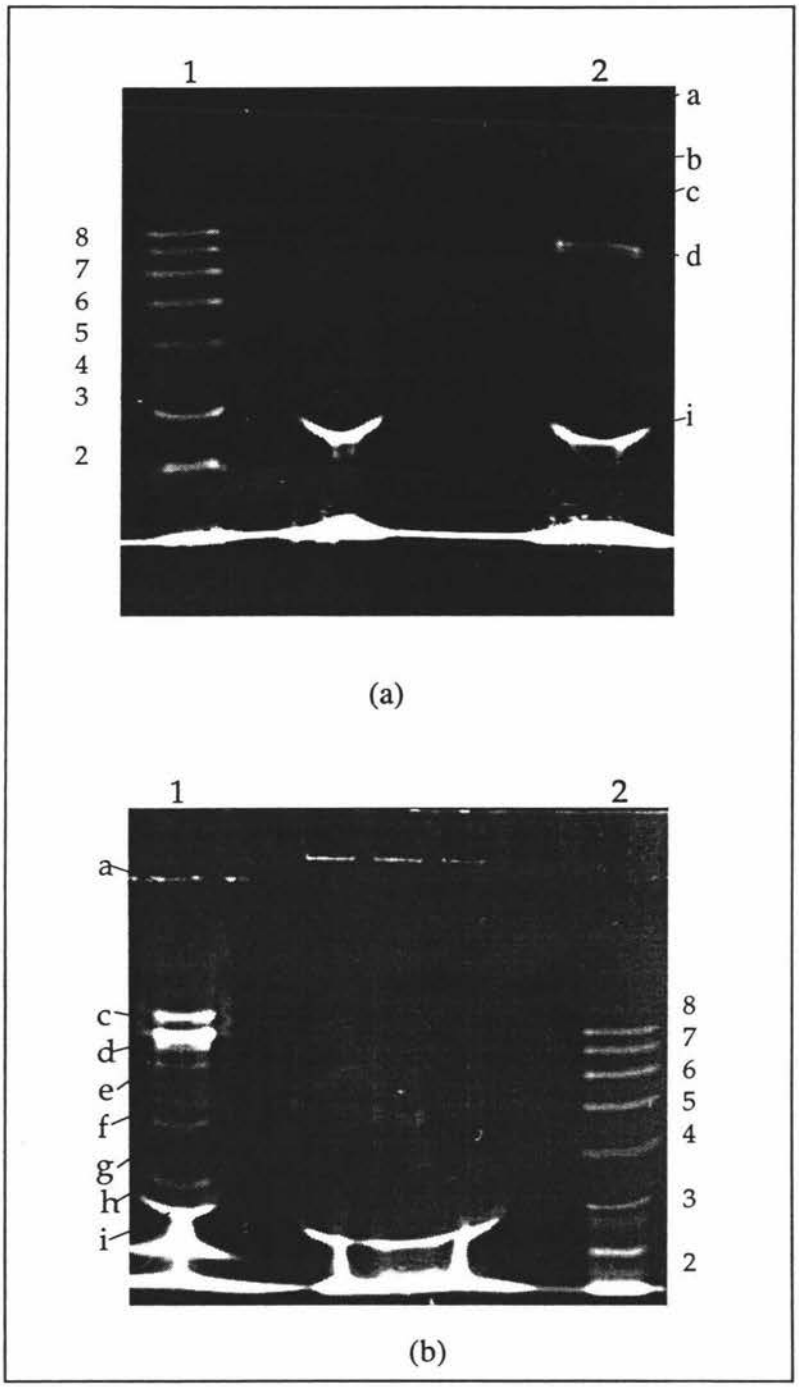


Figure 3.16 *FACE pattern of oligosaccharides released from bovine α-lactalbumin (a) Prep 1: Lane 1: Standard glucose ladder. Lane 2: Oligosaccharides released from bovine α-lactalbumin. (b) Prep 3: Lane 1: Oligosaccharides released from bovine α-lactalbumin. Lane 2: Standard glucose ladder.*

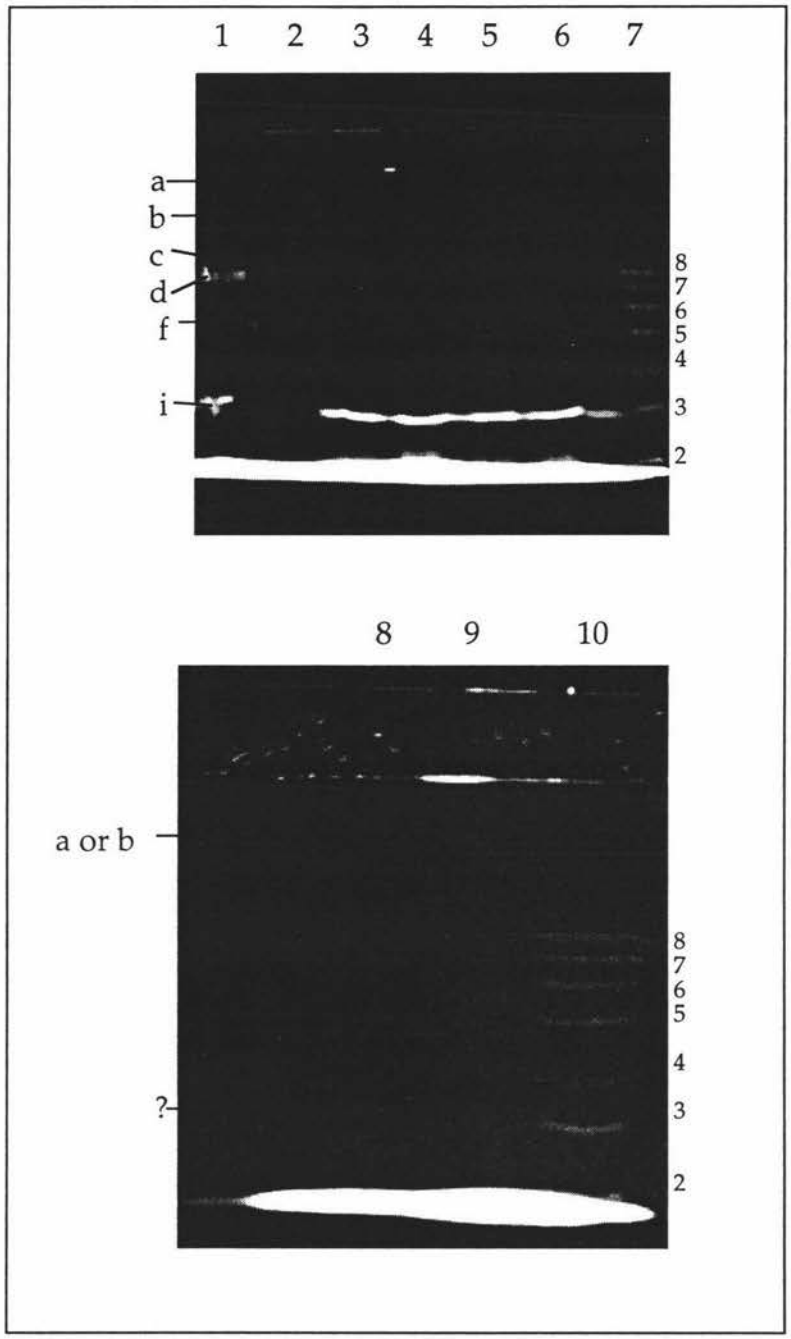


Figure 3.17 *FACE gel of HPAEC/PAD separation of bovine α-lactalbumin (Prep 1, Figure 3.15 (a)). Lane 1: Oligosaccharides released from bovine α-lactalbumin. Lanes 2-9 represent peaks 1-7. Lanes 7 and 10: Standard glucose ladder.*

The FACE technique was used to analyse the peaks observed from bovine α-lactalbumin oligosaccharides (Prep 1) using the Dionex HPAEC/PAD system (Figure 3.14(a)). Figure 3.17 shows the results obtained from the FACE method. Although some separation was observed

for several of the bands the concentration was insufficient to yield clear results.

Two faint bands around Dp=5.6 and 7.8 in lane 2 can be seen. Lanes 3-6 have intense lower bands that are located at approximately Dp=3.0, which could contain $Man_2GlcNAc_2$ species. The band seen in lane 8 around Dp=3.2 is unknown. It chould be a low molecular weight tri- or tetrasialated species according to the Dionex elution time. The band in lane 9 shows a high molecular weight species that has been observed in Figure 3.16 (a). According to ES/MS results there is evidence for such large molecules (Table 3.4). The elution position of this molecule corresponds to trisialated or tetrasialated species according to the HPAEC/PAD results. Therefore this gives strong evidence that this species is tri- or tetrasialated and the structures are postulated in Table 3.4 (Structures 13 or 14)

(c) Derivatisation of Oligosaccharides with PMPMP and Separation by RP-HPLC

(i) Preparation of 1-(p-methoxy)phenyl-3-methyl-5-pyrazolone (PMPMP)

PMPMP was prepared according to the method described in methods section 2.2.2 (3c),.PMPMP was purified and recrystallised from methanol. The yield was 24.5% (w/w) of the compound and gave a UV λ_{max} at 249nm as shown in Figure 3.18. The shape of the peak indicates that the sample may not be pure.

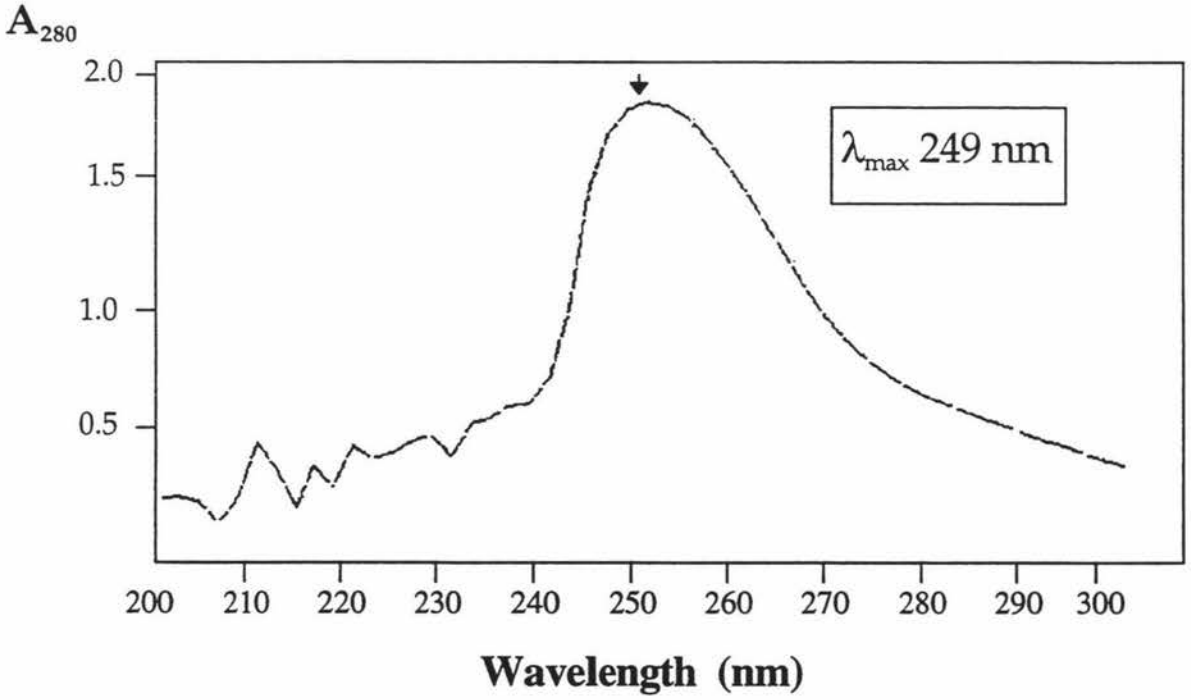


Figure 3.18 *UV spectrum of PMPMP.*

(ii) Derivatisation of oligosaccharides with PMPMP and RP-HPLC

The oligosaccharide mixtures from ribonuclease B and bovine α-lactalbumin were derivatised separately with PMPMP as described in methods section 2.2.2 (3c). Samples were loaded onto a C_{18} column to produce the chromatograms shown in Figure 3.19 and Figure 3.20 respectively. Analytical conditions are described in methods section 2.2.2 (3c). The peaks between 3-17 mins are due to the reagent blank and artefacts as these were also present in a control sample.

(iii) ES/MS results of PMPMP derivatives of separated ribonuclease B oligosaccharides

Five distinct peaks were collected from the ribonuclease B profile of the PMPMP derivatives. These were identified by comparison with a previous study (Kakehi *et al.*, 1991). Confirmation of this was achieved by the analysis of peak 4 by ES/MS. From the ES/MS data, peak 4 isolated from ribonuclease B showed an intense signal of M+H ion at m/z 1887.8 (Fig 3.21) which gives evidence of the octasaccharide structure ($2PMPMP + Man_6GlcNAc_2 + 2Na^+$) as two PMPMP molecules react with one molecule of oligosaccharide as shown previously (Figure 2.4). The molecular weight of PMPMP is 204 but the ES/MS data for purified PMPMP gave an M+H ion at m/z 233 (Figure 3.22). Therefore the molecular weight of PMPMP was considered as 232Da. This is probably due to an additional ethyl group as suggested by NMR and ES/MS data (Dr David Officer - personal comunication).

(iv) ES/MS results of PMPMP derivatives of separated bovine α-lactalbumin oligosaccharides

Unfortunately the analysis of the oligosaccharides derived from bovine α-lactalbumin (Prep 1) did not yield any useful results. All peaks 1-8 that were collected, gave the same ES/MS data of an M+H ion at m/z 1217 (Figure 3.23) which offers evidence of the tetrasaccharide structure ($2PMPMP+Man_2GlcNAc_2+Na^+$). This structure is common to all complex type

oligosaccharides, and could be produced by the fragmentation of the sample ions in the mass spectrometer. This species was also seen in most of the peaks separated by HPAEC/PAD that when analysed by a FACE gel and had a Dp value of around 3 (band i).

Overall the derivatisation of oligosaccharides with PMPMP did not adequately provide a superior method of separating and detecting the α-lactalbumin glycans, although it was successfully used with ribonuclease. Further investigation into the procedure with α-lactalbumin needed to be carried out, but this was not possible within the time frame of this study.

(v) Advantages of Derivatisation

- The derivatisation itself can be performed in a single step and can be used to label sugars with free reducing ends even in complex mixtures of sugars and proteins.
- The presence of sialic acids is not altered by the derivatisation treatment.
- The extra mass of 2PMPMP units can allow better detection of small sugars by ES/MS.
- Standard RP-HPLC procedures can be used to separate and detect a range of oligosaccharides by UV monitoring.

(vi) Disadvantages of Derivatisation

The modification of sugars by derivatisation is not desirable if further analysis of the primary oligosaccharide structure is to be done. Enzymatic or chemical digestions of the derivatised sugar will not be as effective due to the size and hydrophobic nature of the labelling groups attached. Attempts to underivatise can also modify and destroy structures that are labile.

While the best methods are those that do not alter the structure, detection of these unmodified sugars requires sensitive and expensive detector arrays. Ideally a mass spectrometer that is "on-line" to the separating system would allow quicker analysis and avoid losses that can occur in sample handling procedures.

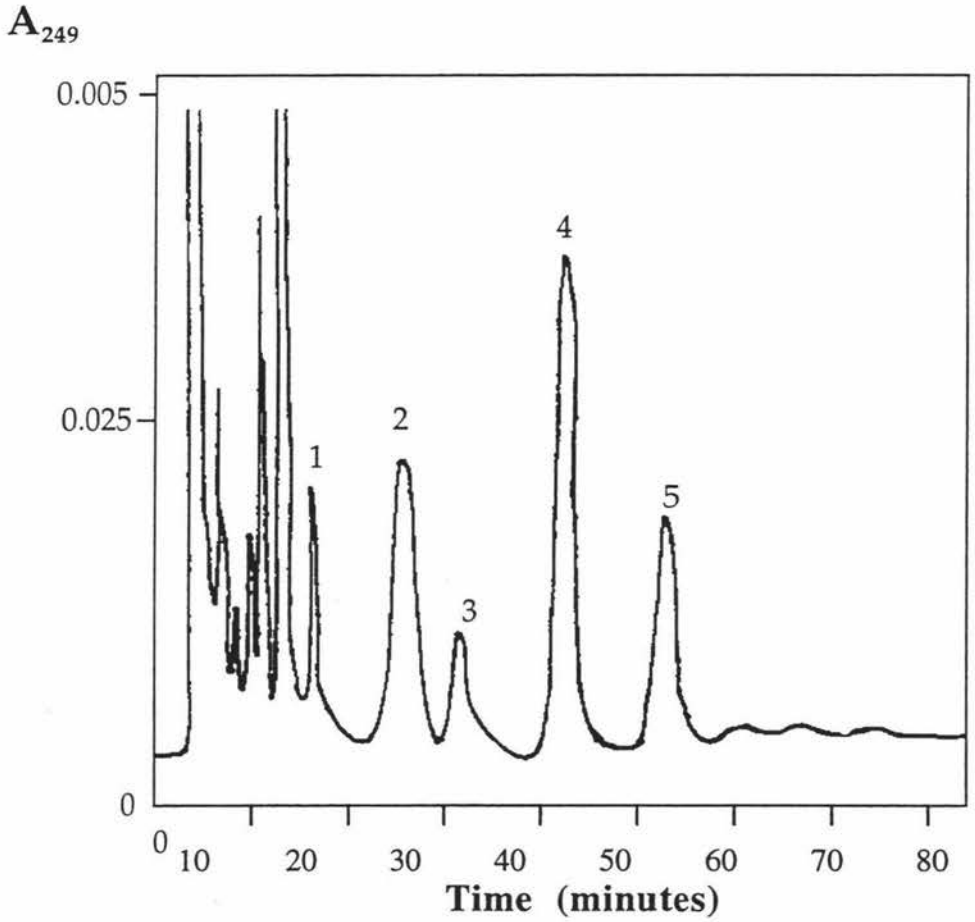


Figure 3.19 *Analysis of high mannose type oligosaccharides obtained from ribonuclease B as their PMPMP derivatives. The amount of sample injected corresponded to 1mg of protein. Column: Vydac, C_{18} (4.6 x 250mm), eluent: 85 : 15 (v/v) mixture of 0.1M phosphate buffer (pH 7.0)/acetonitrile, flow rate of 0.60ml/min.*

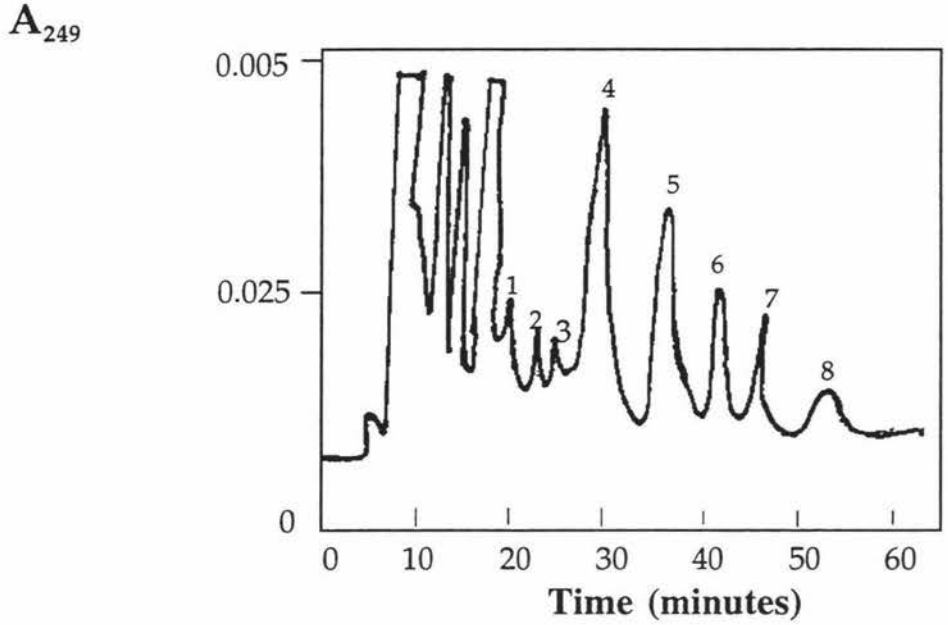


Figure 3.20 *Analysis of complex type oligosaccharides obtained from bovine α-lactalbumin as their PMPMP derivatives. Analytical conditions were the same as those described in the legend to Figure 3.19.*

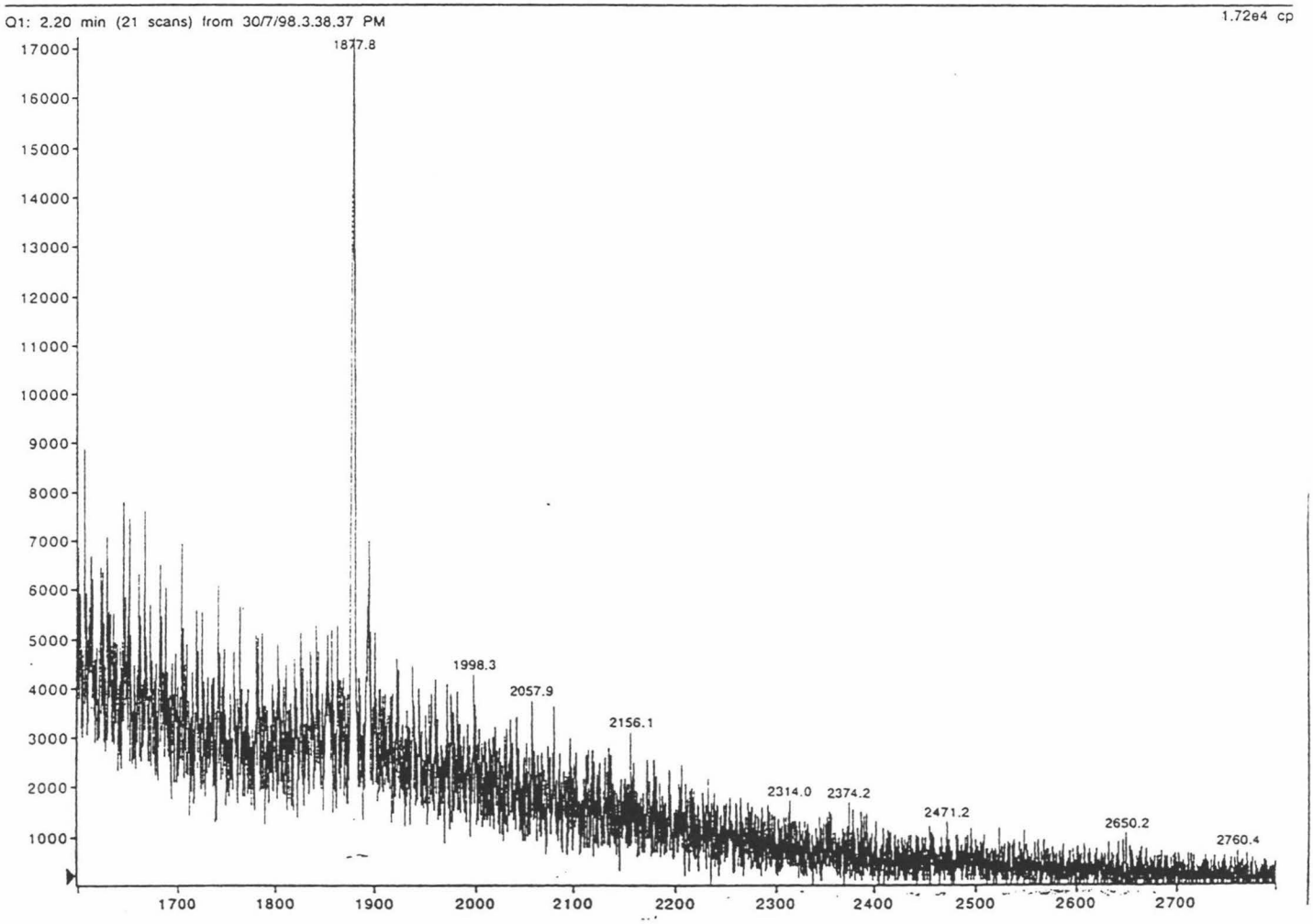


Figure 3.21 *ES/MS spectrum of peak 4 isolated from PMPMP derivatives of ribonuclease B oligosaccharides (at OR=30V, IS=5000V, RNG=140V and IQ1=-11V).*

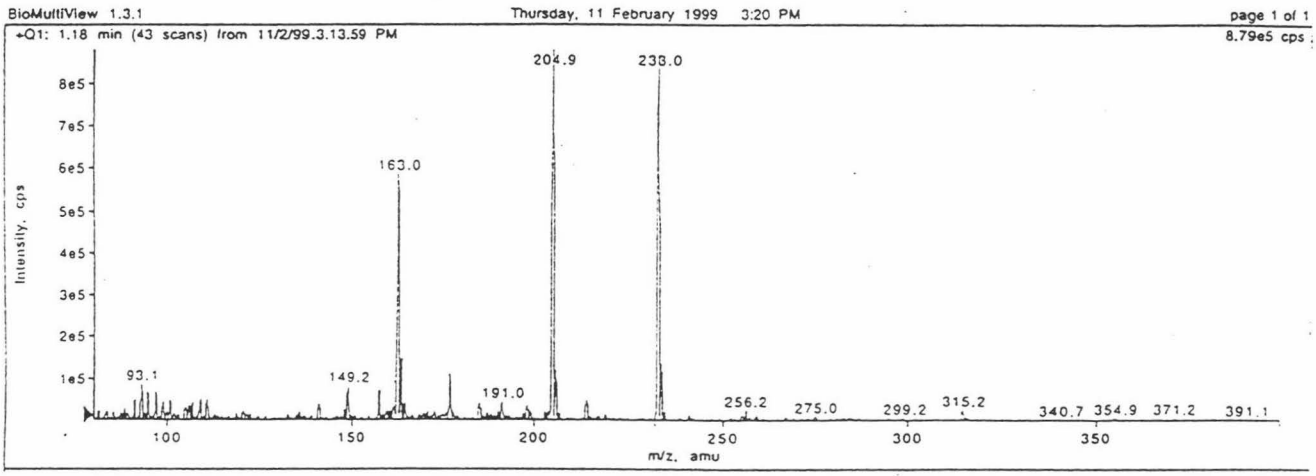


Figure 3.22 *ES/MS spectrum of purified PMPMP (at OR=30V, IS=5000V, RNG=140V and IQ1=-11V).*

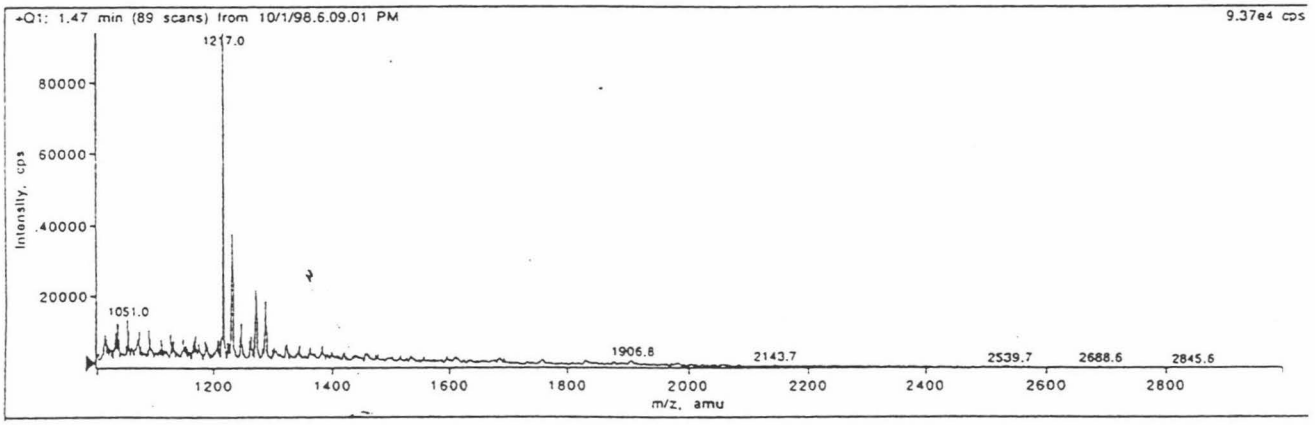


Figure 3.23 *ES/MS spectrum of peaks 1-8 isolated from PMPMP derivatives of bovine α-lactalbumin oligosaccharides (at OR=30V, IS=5000V, RNG=140V and IQ1=-11V).*

3.2.4 An electrospray mass spectroscopic (ES/MS) study of glycosylated α-lactalbumin.

From the literature it was known that the glycans attached to bovine α-lactalbumin are N-linked and to consist of mannose, galactose, N-acetylglucosamine, N-acetylgalactosamine, fucose and sialic acid residues. The purified glycosylated α-lactalbumin showed microheterogeneity with mass components ranging from 15843 to 18137 Da in three different preparations as shown in Figures 3.24 (a), (b), (c) and Table 3.4. Carbohydrate marker ions at m/z 163 (Gal or Man), 204 (GalNAc or GlcNAc), 292 (N-acetylnuraminic acids) and 366 (produced by Gal-GlcNAc, Man-GlcNAc, Gal-GalNAc) were observed in the positive ion mode, using separate ion monitoring (SIM) techniques (Figure 3.25).

Given the above monosaccharide composition and the known core structure for N-linked oligosaccharides, the structures of 15 different α-lactalabumin glycoforms could be postulated. There is no way to determine the actual composition or the linkages between monosaccharide units from these results. Further work needs to be done using specific exoglycosidases in conjunction with analysis by high pH anion exchange chromatography with pulsed amperometric detection and mass spectrometry of analysed PNGase F released oligosaccharides to determine the sequence. However, using these masses, and the results from the FACE gels and HPAEC, a set of structures that may represent the bovine α-lactalbumin glycans have been postulated. Table 3.4 shows the summary of the results from the mass spectroscopy data in three different preparations of α-lactalbumin and the correlation between the mass spectrometry results and the FACE results.

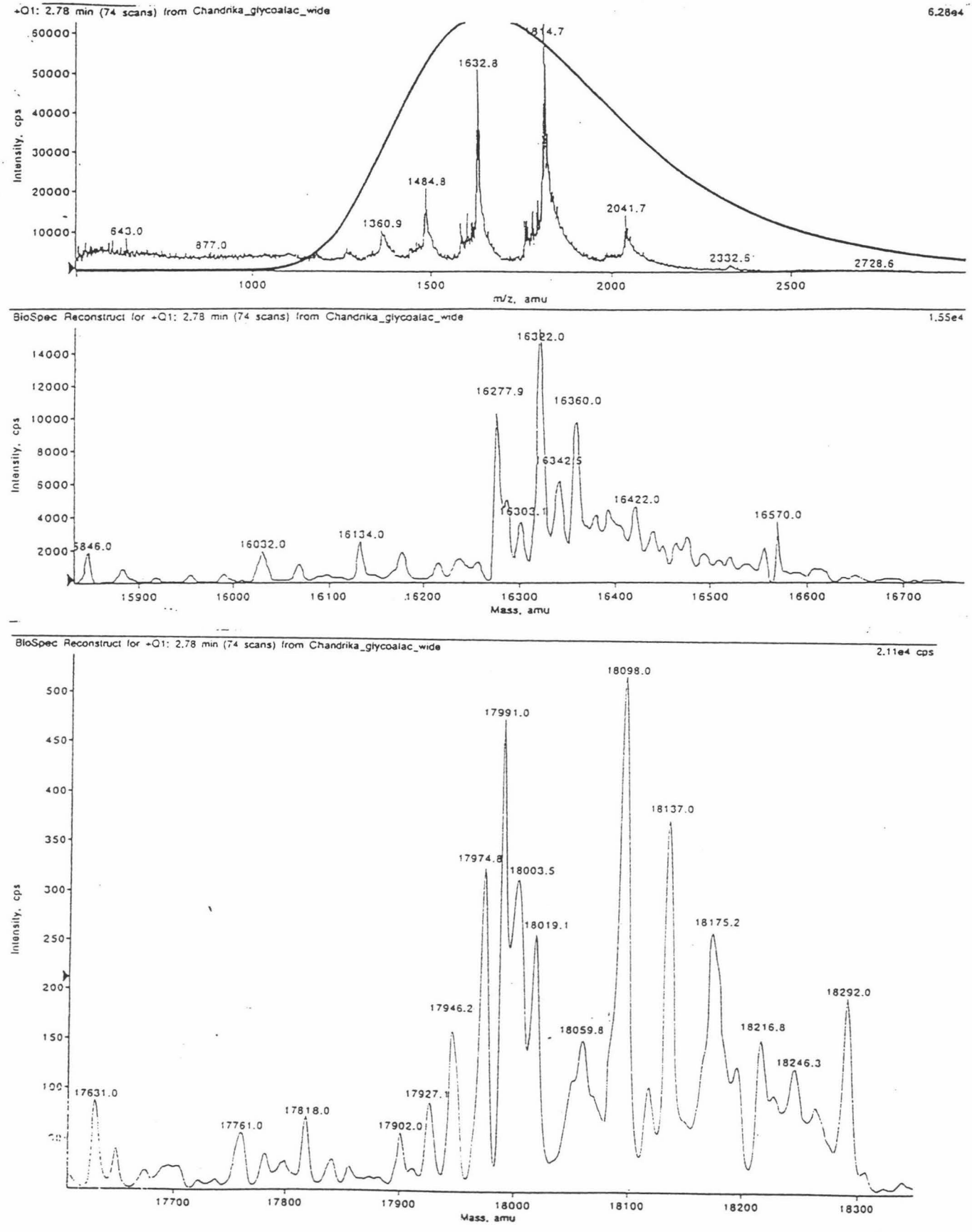


Figure 3.24 (a) *ES/MS spectrum of a sample of purified glycosylated α-lactalbumin (Prep 1) (at OR=30V, IS=5000V, RNG=140V and IQ1=-11V).*

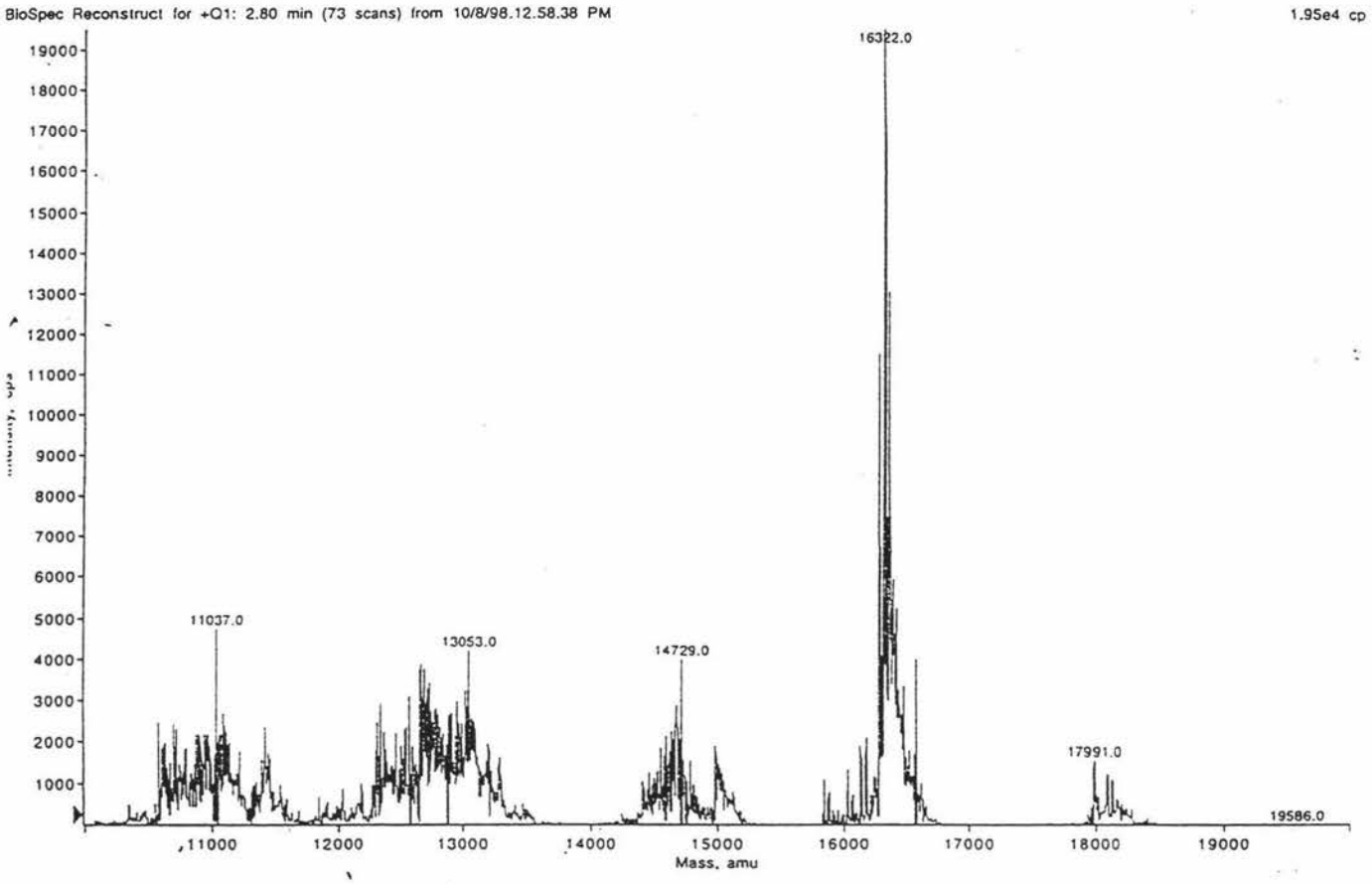


Figure 3.24 (b) *ES/MS spectrum of a sample of purified glycosylated α-lactalbumin (Prep 2) (at OR=30V, IS=5000V, RNG=140V and IQ1=-11V).*

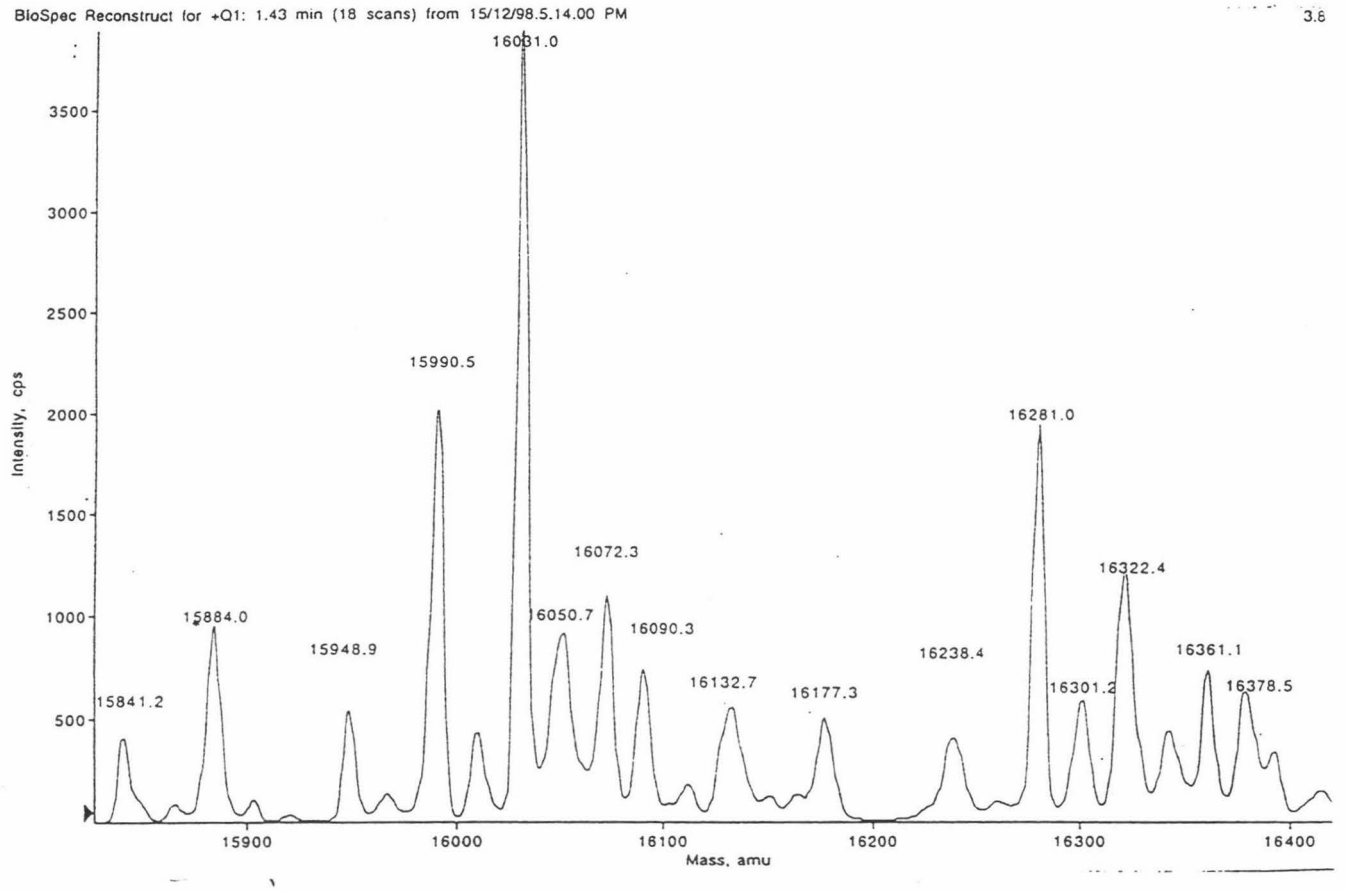


Figure 3.24 (c) *ES/MS spectrum of a sample of purified glycosylated α-lactalbumin (Prep 3) (at OR=30V, IS=5000V, RNG=140V and IQ1=-11V).*

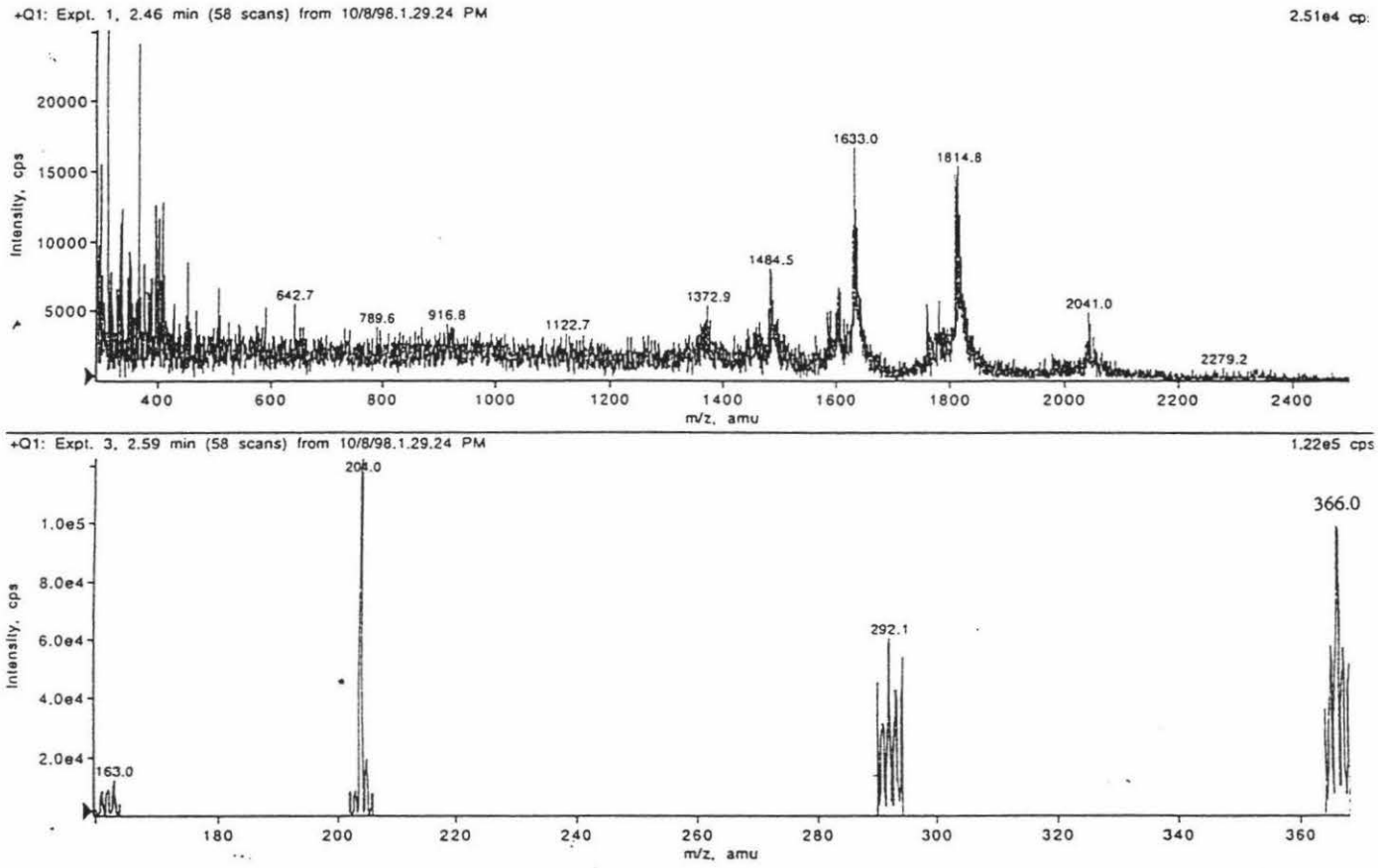
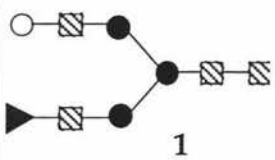
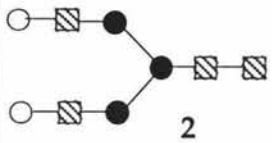
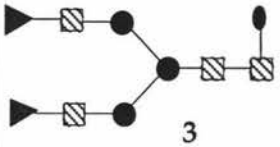
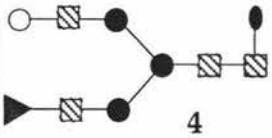
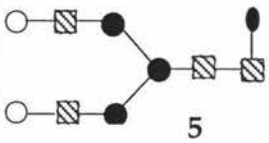
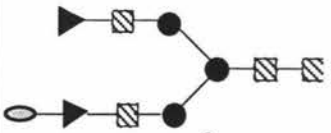
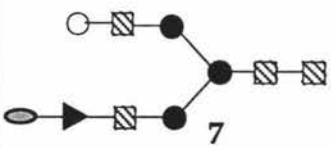
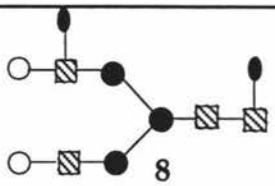
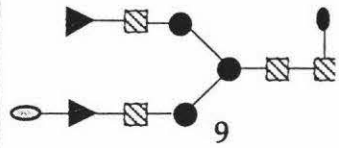
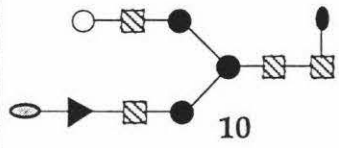
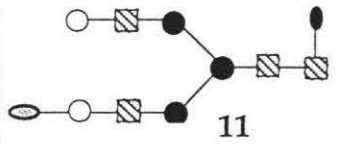
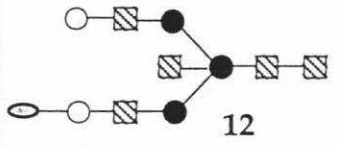
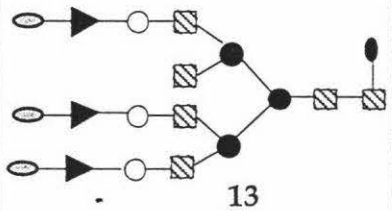
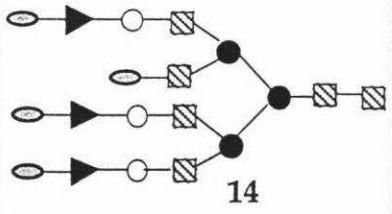


Figure 3.25 *ES/MS spectrum of monosaccharides found in glycosylated α-lactalbumin. (at OR=200V, RNG=400V and IQ1 and IQ2=-15V).*

Table 3.4 *The correlation between the mass spectrometry results and FACE gel results.*

Molecular weight predicted by ES/MS			Glycan mass			Proposed glycan structures (1-15)	Calculated FACE gel values and observed bands[a]
Prep 1	Prep 2	Prep 3	Prep 1	Prep 2	Prep 3		
15846.0		15841.2	1670.0		1667.1	 1	8.55 ± 2
		15884.0			1708.0	 2	7.1 ± 2
		15948.9			1772.9	 3	9.5 ± 2
		15990.5			1814.5	 4	9.25 ± 2
16032.0		16031.0	1856.0		1854.0	 5	7.7 ± 2 (band c)
		16090.3			1914.3	 6	7.8 ± 2
16134.0		16132.7			1956.7	 7	7.55 ± 2
	16177.3				2001.3	 8	8.3 ± 2

Molecular weight predicted by ES/MS			Glycan Mass			Proposed glycan structure	Calculated FACE gel values and observed bands[a]
Prep 1	Prep 2	Prep 3	Prep 1	Prep 2	Prep 3		
		16238.4			2062.4	 9	8.5 ± 2
16277.9		16281.0	2101.9		2105.0	 10	8.25 ± 2
16322.0	16322.0	16322.4	2146.0	2146.0	2146.4	 11	6.7 ± 2 (band d)
		16378.5			2202.5	 12	6.9 ± 2
17990.0	17991.0	17992.0			3816.0	 13	15.35 ± 2 (band a)
18137.0					3961.0	 14	14.35 ± 2 (band b)
		14526.7			350.7	 15	

Glycan mass = Observed glycoforms mass- observed non-glycosylated α-lactalbumin mass (14176.0).

▨ GlcNAc ● Mannose ▶ Galactose ○ GlcNAc ● Fucose ◉ Sialic acid

[a] bands derived from FACE gels shown in Figures 3.16 (a-h)

In all three preparations there was a very intense signal with a glycan mass of 2146.0 Da and a very intense band in the FACE gels of around Dp=7 (Figure 3.16, band d). ES/MS data from Prep 2 showed only this species and a high molecular weight glycan (3816.0 Da). In the FACE gel of Prep 2, there were only two bands that correspond to these two species. From the ES/MS data and the calculated FACE gel values (Hu, 1995 and Starr *et al.*, 1996), it can be concluded that the main bovine α-lactalbumin glycan containing sialic acid (representing S_1) is the structure shown in Figure 3.26 (a).

There is another significant signal which corresponds to the molecular weight of the bovine α-lactalbumin glycan (1854.0 Da) reported by Tilley *et al.*, (1991). This was observed in both Prep 1 and Prep 3 (Figure 3.24, Figure 3.26 and Table 3.4) and as an intense band in FACE gels with a Dp value of approximately 8 (Figure 3.16 (a) and (b)). From the ES/MS data and the calculated FACE gel values (Hu, 1995 and Starr *et al.*, 1996), this species should be the main neutral glycoform represented by S_2 (Figure 3.26 (b)).

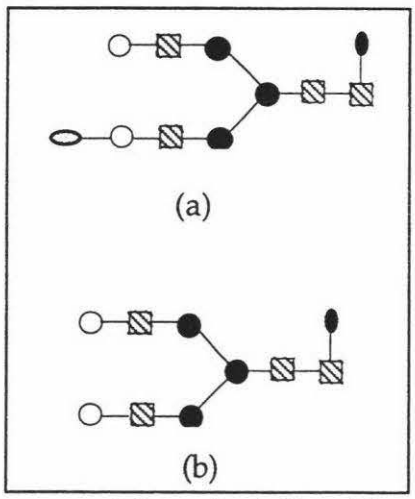


Figure 3.26 *(a) Proposed structure of the main sialic acid containing bovine α-lactalbumin glycan. (b) Proposed structure of the neutral bovine α-lactalbumin glycan.*

▨ GlcNAc ● Mannose ▶ Galactose ○ GlcNAc ● Fucose

⬭ Sialic acid

It is interesting to note that the two main glycans differ by only one sialic acid (NeuAc) residue (Figure 3.26).

The smallest glycan with an ES/MS signal at 14526.7Da represents the α-lactalbumin protein with a GlcNAc-Fuc residue attached. This glycan would not have been seen in a FACE gel as the PNGase F is unable to cleave less than two GlcNAc residus from a protein. The detection of this Asn-GlcNAc-Fuc species by ES/MS may be the result of an endogenous exoglycosidase found in the mammary gland.

3.2.5 Discussion of Objective 2 Results

The purification scheme for obtaining glycosylated α-lactalbumin from milk involved fewer initial steps than those used for the non-glycosylated form. The lectin affinity step could be used with a crude acid whey fraction to selectively separate glycosylated protein from non-glycosylated contaminants. As expected, immunoglobulins were the primary glycoprotein contaminant although other proteins such as BSA seemed to co-elute. It can only be assumed that there is some non-covalent attachment of BSA with these glycosylated proteins under the conditions used for the separation. The size exclusion chromatography gave a clean separation between α-lactalbumin and the immunoglobulins as well as the other contaminants after several repeat runs.

In total three different glycosylated α-lactalbumin preparations were examined. The combined results of these indicated a complex mix of glycans, that could be separated by HPAEC/PAD which proved to be an excellent analytical tool. The running conditions used meant that no separation of protein from the glycan components was necessary, eliminating a step that may have resulted in oligosaccharide losses. If suitable oligosaccharide standards were available, it would be possible to quickly identify the different species separated. The main advantage of this method was the detection of underivatised sugars. Unfortunately, the high ionic strength associated with 10-100mM NaOH and 0-1M sodium acetate gradients used with HPAEC/PAD are not well suited for electrospray mass spectrometry, which not only is the method of choice for identifying small quantities of oligosaccharides but can also be used to look at the composition of the oligosaccharides using collision induced decomposition (CID) fragmentation. Anion micromembrane suppression (AMMS) can be used in conjuction with HPAEC to remove Na^+ for the determination of oligosaccharides before ES/MS. However, this facility was not available at the time.

The FACE system was useful for the analysis of the sugars. The results would have been more meaningful if a suitable ladder of 2-16 glucose units had been available rather than only 2-8 units. Nevertheless the results did show larger oligosaccharides were present. A tentative assignment of the glycan composition could be made by comparison to the glucose ladder standards. The samples for the FACE gels were prepared according to methods section 2.2.2 (3b). Loss of samples may have been incurred during the use of the cation exchange cartridges to remove salts from the peaks collected from HPAEC/PAD as well as in the 100% ice cold ethanol preciptation step (i.e: oligosaccharides can also be precipitated with protein). The FACE gel information was more useful when compared with other data, especially HPAEC/PAD and ES/MS.

Methods for separating oligosaccharides have always been limited by the detection system utilised. Carbohydrates do not naturally contain any chromophores or fluorophores and therefore underivatised glycans cannot be sensitively detected by the usual methods of UV absorbance unless ultra-pure solvents can be obtained.

Derivatisation has become a standard method for detection of carbohydrates and a variety of labels are available from UV chromophores, fluorophores, and even radioactive isotopes. The derivatives used in this experiment was PMPMP because it offers the convenience of standard UV detection coupled with RP-HPLC. The ablity of 2PMPMP molecules to bind to a single oligosaccharides also enhances detection. The method appeared to work effectively enough with regards to ribonuclease B and this was confirmed by ES/MS. However a very unusual result occured with the α-lactalbumin oligosaccharides. It seems all of the oligosaccharides separated by RP-HPLC had "decomposed" when tested by ES/MS to a common tetrasaccharide structure ($Man_2GlcNAc_2$). This species was ubiquitously found in all experiments and appears to be a possible degradation product.

3.3 Results of Objective 3

Only one whey sample (pre-prepared) was found with the F band. No F band was found in any of the three preparations that were investigated. *In-situ* gel digestion was carried out on the whey sample which contained the F band. PVDF membrane blotting was also carried out so that N-terminal sequencing of the F band could be done, but the concentration of the protein was insufficient for sequencing data to be obtained. Unfortunately the concentration of peptide peaks were insufficient for further analysis to be conducted and so this experiment was not continued.

3.4 Overall Conclusions

3.4.1 Objective 1

The mechanism by which α-lactalbumin modifies the galactosyltransferase activity in the lactose syntase complex has not yet been established. When purified α-lactalbumin was examined by ES/MS a signal representing a dissacharide was also discovered that could have been non-covalently attached to the protein. α-lactalbumin has close structural homology to the sugar binding sites of the enzyme lysozyme, but α-lactalbumin has no known catalytic activity. It may be possible that α-lactalbumin utilises its sugar binding capacity to directly assist the galactosyltransferase when α-lactalbumin binds to form the lactose synthase complex.

3.4.2 Objective 2

The most sensitive method for detecting glycoprotein was the use of ES/MS. This method could confirm the results of the HPAEC/PAD and FACE methods as well as provide some unexpected findings:

(i) The structures of the main sialic acid containing glycan and neutral glycan were postulated, which represent S_1 and S_2 respectively (Figure 3.26 (a) and (b)). It is interesting to note that these molecules differ by only one sialic acid residue.

(ii) The detection of the Asn-GlcNAc-Fuc species by ES/MS could be the result of an endogenous exoglycosidase (ENGase)that is found in the mammary gland.

(iii) Two large glycans that have not been reported before with molecular weights of 3816.0 and 3961.0 are proposed, which represent trisaialated and tetrasialated species respectively. These species also only differ by one sialic acid residue.

(iv) The structure of 15 different bovine α-lactalbumin glycans were postulated.

A comparison of the three different preparations of α-lactalbumin showed a variety of glycoforms apparant in each preparation that may have indicated that losses had occured due to experimental procedures or they could genuinely be due to changes in glycosylation patterns. This could be due to changes in the distribution of certain glycoforms throughout the stages of lactation as glycans are known to serve as biological markers. The physiological roles of the glycans of α-lactalbumin has yet to be investigated.

CHAPTER IV

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APPENDIX A

Experiment Information
Experiment Name: EX_LOWOR
Last Modified: Fri, 7 Aug 1998, 8:06:06 AM
Scan Type: Q1
Scan Time: 00:03.672 secs
Peak Hopping : Disabled
Comment: LOW ORIFICE SCAN
Mass Defect: 0 mmu/100amu
Pause Time: 5.000 msec

Mass Range Information
Mass Range 1

Start(amu)	Stop(amu)	Step(amu)	Dwell(msec)	Param	Start	Stop
300.000	2500.000	0.300	0.500	CR	20.000	40.000
				RNG	200.000	300.000
				IQ2	-20.000	-60.000

Experiment Information
Experiment Name: EX_HIGH ORIF
Last Modified: Fri, 7 Aug 1998, 8:06:09 AM
Scan Type: Q1
Scan Time: 00:03.672 secs
Peak Hopping : Disabled
Comment: HIGH ORIFICE SCAN
Mass Defect: 0 mmu/100amu
Pause Time: 5.000 msec

Mass Range Information
Mass Range 1

Start(amu)	Stop(amu)	Step(amu)	Dwell(msec)	Param	Start	Stop
300.000	2500.000	0.300	0.500	CR	90.000	90.000
				RNG	400.000	400.000
				IQ2	-20.000	-60.000

Experiment Information
Experiment Name: sim cho#2
Last Modified: Thu, 1 Oct 1998, 5:26:09 PM
Scan Type: Q1
Scan Time: 00:08.220 secs
Peak Hopping : Disabled
Comment: SIM forglycopeptide fragment ions
Mass Defect. 0 mmu/100amu
Pause Time: 5.000 msec

Mass Range Information
Mass Range 1

Mass(amu)	Width(amu)	Step(amu)	Dwell(msec)	Param	Start	Stop
204.000	4.000	0.100	50.000	CR	200.000	200.000
				RNG	400.000	400.000
				IQ1	-15.000	-15.000

Mass Range 2

Mass(amu)	Width(amu)	Step(amu)	Dwell(msec)	Param	Start	Stop
292.100	4.000	0.100	50.000	CR	200.000	200.000
				RNG	400.000	400.000
				IQ2	-15.000	-15.000

Mass Range 3

Mass(amu)	Width(amu)	Step(amu)	Dwell(msec)	Param	Start	Stop
366.100	4.000	0.100	50.000	CR	200.000	200.000
				RNG	400.000	400.000
				IQ2	-15.000	-15.000

Mass Range 4

Mass(amu)	Width(amu)	Step(amu)	Dwell(msec)
162.000	4.000	0.100	50.000

Method Information
Method Name: LCMS LO_HI_CHO#2
Last Modified: Wed, 5 Aug 1998, 11:18:16 AM
Comment: Method for detecting Glycopeptides by LC/MS

Command	Description	Time (sec)	Reps	Duration (min)	Total Time (hh:mm:ss)
Scan	Mode: Profile Threshold: 1.0 x 10 E1 cps Pause: 0.0 sec Expt: State: Q1 Cal: Q3 Cal:	??	1	0.053	-
Scan	Mode: Profile Threshold: 1.0 x 10 E1 cps Pause: 0.0 sec Expt: State: Q1 Cal: Q3 Cal:	??	1	0.053	-
Scan	Mode: Profile Threshold: 1.0 x 10 E1 cps Pause: 0.0 sec Expt: State: Q1 Cal: Q3 Cal:	??	1	0.052	-
Loop	Loop Low OR/HighOR/SIM CHO	9.577	595	95.000	01:35:00