STUDIES ON LYSOSOMAL ALPHA-D-MANNOSIDASE OF HUMAN PLACENTA

A thesis presented

by

JYOTI. V. NAIR

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A C K N O W L E D G E M E N T S

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Jyoti. V. Nair

JYOTI. V. NAIR

C E R T I F I C A T E

I Jyoti. V. Nair hereby certify that I had personally carried out the work depicted in the thesis entitled "Studies on lysosomal alpha-D-mannosidase of human placenta" except where external help sought and acknowledged.

Signature: Jyoti. V. Nair

JYOTI. V. NAIR

Date: 29-6-1988

D E C L A R A T I O N

This is to certify that Smt. Jyoti. V. Nair in the division of Neurochemistry this Institute, has fulfilled the requirements of the regulations relating to the nature and prescribed period of research for the Ph.D degree of the Sree Chitra Tirunal Institute for Medical Sciences and Technology, Trivandrum. The work relating to her thesis entitled "STUDIES ON LYSOSOMAL ALPHA-D-MANNOSIDASE OF HUMAN PLACENTA" was carried out under my direct supervision.

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(Guide)

The thesis

entitled

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(Name of thesis examiners)

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ABBREVIATIONS

Gal	Galactose
Glc	Glucose
Man	Mannose
Fuc	Fucose
GalNAc	N-acetyl-D-galactosamine
GlcNAc	N-acetyl-D-glucosamine
NeuAc	N-acetyl neuraminic acid
Man-6-P	Mannose-6-phosphate
Dol-P	Dolichol phosphate
RER	Rough endoplasmic reticulum
ConA	Concanavalin A
WGA	Wheat germ agglutinin
JSA	Jack fruit seed agglutinin
RCA	Ricinus communis agglutinin
Dansyl chloride/ Dns-Cl	Diaminonaphthosulfonyl chloride

CHAPTER I

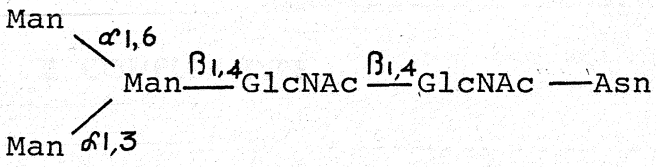
INTRODUCTION

Glycoproteins are a diverse group of biopolymers which contain oligosaccharide chains covalently linked to the polypeptide backbone. They are ubiquitous constituents of living organisms. Glycoproteins are found in soluble form in the extracellular fluids, and in insoluble form in membrane and intercellular matrix components. This class of compounds include enzymes, hormones, immunoglobulins, lectins, toxins, carriers and structural proteins. The monosaccharides commonly occurring in glycoproteins include D-Gal, D-Glc, D-Man, L-Fuc, GalNAc, GlcNAc, NeuAc and L-Ara. The number of oligosaccharide chains attached to the glycoprotein are varied. Glycoproteins such as ovalbumin and bovine ribonuclease B contain a single oligosaccharide chain per molecule, whereas ovine submaxillary mucin contains over 100 such chains per molecule.

Proteoglycans are classified as a different group of macromolecules. They differ from glycoproteins in several structural characteristics: a) most proteoglycans have very long polysaccharide chains b) carbohydrate moieties contain uronic acid c) presence of the characteristic repeating disaccharide units d) the saccharide - peptide linkage region of proteoglycans is characterized by Xyl - Ser.

The oligosaccharide chains of most glycoproteins are linked to the polypeptide backbone by two types of covalent linkages: the N-glycosidic type and the O-glycosidic type (1,2).

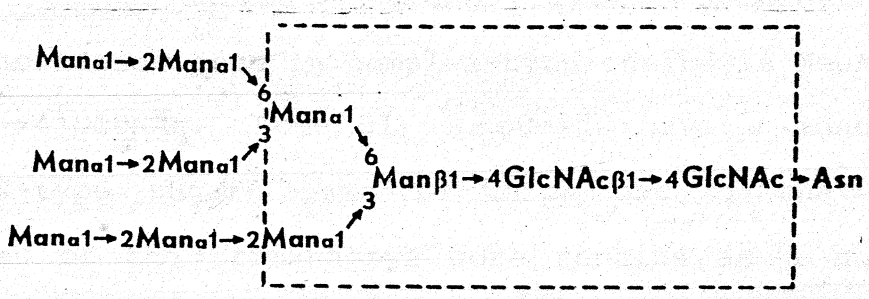
The first linkage to be recognised was the N-glycosidic type between the anomeric carbon atom of N-acetyl-D-glucosamine and the amide nitrogen of asparagine in the polypeptide chain (3). The Asn - GlcNAc linkage is of wide distribution. The carbohydrate units linked to asparagine contain a common pentasaccharide core as suggested by Montreuil (1).



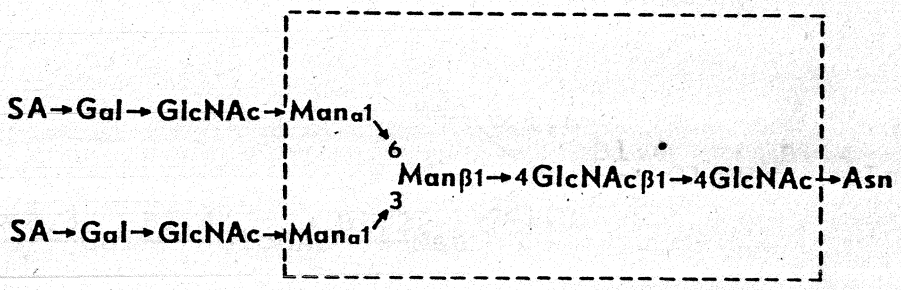
Depending on the type of additional saccharide residues attached to the pentasaccharide core, the N-glycoproteins are of three types (Fig.1).

The first group of glycoproteins which contain only mannose residues attached to the core are known as oligomannosidic or high mannose-type. The total number of mannose residues in this type ranges from 6 to 12 and the chains are often branched.

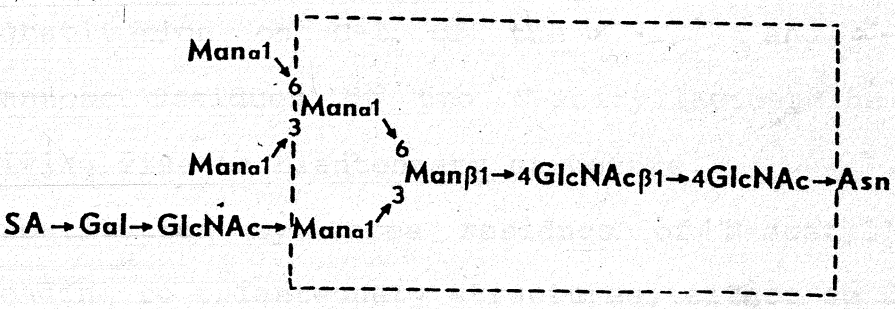
The second group contains the disaccharide N-acetyl-lactosamine (Galβ1→4GlcNAc) attached to the core. Sialic acid residues may or may not be linked to Gal. Such chains are called N-acetyllactosamine or complex-type. The most



I. HIGH-MANNOSE TYPE



II. COMPLEX TYPE

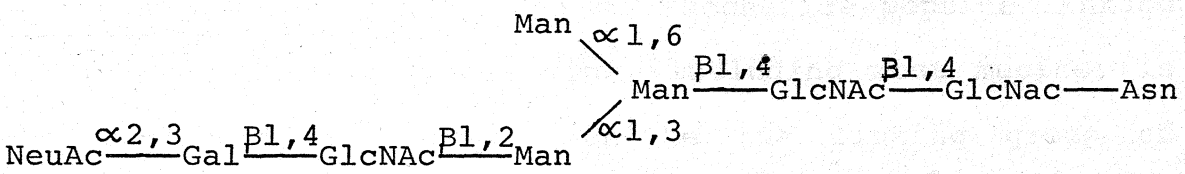


III. HYBRID TYPE

Figure 1. Structures of the oligosaccharide chains of the asparagine-linked glycoproteins.

Adapted from Elbein, A.D. (1984) CRC. Crit. Rev. Biochem., 16, p.22.

common trisaccharide sequence found attached to the pentasaccharide core in complex-type chains is NeuAcα(2→3)Galβ(1→4)GlcNAc. Some glycoproteins have a monoantennary complex-type sugar chain in which one of the α-mannosyl residues of the trimannosyl core remains as a nonreducing terminal. In such cases, the outer chains are located on Manα-1, 3-branch of the core.



Various other carbohydrate structures that have been found in complex-type chains are the following:

a) substitution on C-2 of the α-1,3- and α-1,6-linked mannose residues by two N-acetyllactosamine residues, giving rise to biantennary structure.

b) substitution by three residues of N-acetyllactosamine leading to triantennary structures, either on C-2 and C-4 of α-1,3- linked mannose and on C-2 of α-1,6- linked mannose or on C-2 of α-1,3- linked mannose and on C-2 and C-6 of α-1,6- linked mannose.

c) substitution by four residues of N-acetyllactosamine on C-2 and C-4 of α-1,3- linked mannose residue and on C-2 and C-6 of α-1,6 - linked mannose residue leading to the tetraantennary structure.

d) substitution by GlcNAc residue on C-4 of the β-linked mannose residue resulting in 'bisected' form.

e) substituion on C-6 of the innermost GlcNAc residue in the core by a fucose residue. However, variations on these basic structures are also known (1).

Finally, a third group of glycoproteins was described by Kobata and coworkers. This type which contains features of both high mannose and complex-type structures was called hybrid - type or mixed oligomannosidic-N-acetyllactosamine type (4,5).

The second major type of saccharide-peptide linkage occurring in many glycoproteins, including most mucins, is the O-glycosidic linkage. Unlike the linking group of N-glycosidic units which is always GlcNAc — Asn, the linking groups of O-glycosidic units are of several types.

i) the GalNAc - Ser/Thr linkage is found mainly in mucins, blood group active glycoproteins, in certain plasma glycoproteins and membrane glycoproteins.

Oligosaccharide units linked O-glycosidically via GalNAc - Ser/Thr vary in size and structure. The simplest ones are the disaccharides such as NeuAcα2→6 GalNAc found in most submaxillary mucins and Galβ1→3 GalNAc found in the antifreeze glycoprotein of antarctic fish (6). More complex structures based on the same core disaccharide are present as in case of porcine submaxillary mucins.

ii) the O-glycosidic linkage between Gal and Hyl is unique to collagens.

iii) O-glycosidic linkage between L-Ara and Hyp has been

found only in plant and algal glycoproteins.

iv) the linkage between Gal and Ser has been recognized in earthworm cuticle collagen and plant cell walls.

Glycoproteins perform many vital roles in living systems. The contribution of the carbohydrate moieties to their functions is still a subject of speculation. However, the wide distribution and conserved structure of the carbohydrate moieties of glycoproteins in animals suggest their importance in an undefined, but universal physiological process.

The carbohydrate part is found to stabilize the three dimensional structure of glycoproteins and also effect the properties of the molecule. The physicochemical effects of the carbohydrate moieties of glycoproteins are evident in various cases. Studies with salivary glycoproteins that are rich in sialic acids have led to the conclusion that this sugar is responsible for the high viscosity of the mucin solution. Removal of sialic acid residues result in deterioration of the viscosity property (7). The antifreeze glycoproteins of antarctic fish depend on the integrity of the disaccharide units for their activity. The ability of these glycoproteins to depress the freezing point of water is lost on removal or modification of the saccharide side chains (8). The carbohydrate plays a vital role in the physical property of the glycoprotein.

Glycoproteins rich in sugar are relatively resistant to proteolysis (9). This was supported by the finding that

the enzymatic removal of terminal NeuAc from ovine submaxillary mucin enhanced its susceptibility to trypsin digestion. Analysis of the effect of tunicamycin on glycoprotein synthesis led to the conclusion that carbohydrate protects protein against proteolysis in vivo, in the course of their biosynthesis. Nonglycosylated fibronectin synthesized in chick embryo fibroblasts in presence of tunicamycin was degraded intracellularly at a much faster rate than the glycosylated protein (10).

Ashwell and Morell demonstrated that removal of sialic acid from circulating glycoproteins by sialidase led to an enhancement in the rate of their clearing from the system (11). The asialoglycoproteins are rapidly taken up and catabolized by the liver. Uptake depends on the recognition by the liver cells of the exposed Gal residues on glycoproteins. Removal of Gal or replacement of the missing sialic acid from the desialylated glycoproteins extends the time they remain in circulation (12,13). A glycoprotein that binds asialoglycoproteins has been isolated from rabbit liver membranes and has been termed hepatic binding protein (14). A GlcNAc —— specific recognition system has been found in experiments on the clearance of infused rat lysosomal enzymes from the circulatory system (15). Another clearance system based on recognition of terminal nonreducing L-Fuc has been described in mice (16).

A different system in which carbohydrates on soluble

glycoproteins serve as determinants of recognition is that responsible for the intracellular segregation and selective pinocytosis of certain lysosomal glycosidases by fibroblasts. The Man-6-P recognition marker on the lysosomal enzymes function as an intracellular traffic signal to direct acid hydrolases to lysosomes (17).

The blood group substances A,B,H and Lewis (Le^a and Le^b) are glycoproteins (18). The carbohydrate moieties are the immunodeterminants of these blood type specific antigen on human erythrocytes. The inhibition of agglutination of human erythrocytes by influenza virus was abolished by treatment of the cells with neuraminidase (19). These facts demonstrated the role of cell surface saccharides in biological function.

Changes in the sugar moieties on cell surface occur when normal cells are transformed into malignant ones. The most widely recognized phenomenon correlated with the malignant transformation of cells is the appearance of Fuc-containing glycopeptides (20,21).

Many interesting facts concerning the developmental changes of the surface glycoproteins have also been reported. Sharon found that the number of glycoproteins that bind to peanut agglutinin changes during lymphocyte maturation in mouse and man (22). Normal development of embryonic cells is dependent on growth, response to signal molecules, adhesiveness, migration and recognition. It is assumed that glycoproteins play an important role in early embryogenesis.

Work of a number of laboratories has provided an outline of the elaborate pathways that led to the synthesis of N-glycosidically and O-glycosidically linked oligosaccharide chains of glycoproteins (23-27).

First observation indicating the involvement of Golgi apparatus in a process related to the synthesis of a 'carbohydrate-protein complex' was made by Hersh, which was confirmed by Leblond (28,29). Conclusive evidence for a role of the Golgi apparatus in the synthesis of glycoproteins was provided by autoradiographic visualization of the incorporation of various ^{3}H - labeled monosaccharides into proteins. Application of more refined techniques regarding the subcellular sites and organization of glycosylation steps has been reviewed by Roth (30).

Great progress has been made in clarifying the cellular and enzymatic pathways of glycoprotein biosynthesis. This started with the discovery by Leloir of sugar nucleotides and their role as donors in the formation of oligosaccharides (31). The nucleotide sugars play a major role in the interconversion of monosaccharides and in the biosynthesis of glycosidic linkage. The nucleotide-linked sugars can undergo several types of modification reactions, or transformations including epimerization, oxidation, decarboxylation, reduction and rearrangement.

The enzymatic pathways of glycoprotein biosynthesis was elucidated by the study of various glycosyltransferases that catalyze the transfer of sugars from nucleotides to the

saccharide chains of glycoproteins (32).

Another major development was the finding that monosaccharides and oligosaccharides linked to a lipid carrier (Dolichol - P) serve as intermediates in the assembly of N-glycosidically linked carbohydrate units of glycoproteins and for the monosaccharide - lipids, Dol-P-Man and Dol-P-Glc (33). Dolichol-P is a phosphorylated polyprenol containing 14-21 isoprene units in which the α-isoprene unit is saturated. They are synthesized from mevalonic acid. This has been established using the drug compactin, that blocks the synthesis of mevalonic acid. The lipid - linked intermediates which are hydrophobic, serve for the transport of activated sugars from the cytoplasm through the endoplasmic reticulum membrane during the assembly of the precursor oligosaccharide - lipid into the lumen of the endoplasmic reticulum.

Glycoprotein biosynthesis pattern varies according to the linkage type. The synthesis of Asn-linked oligosaccharides proceeds in distinct steps as shown in Fig.2. Initiation of the synthesis of Asn-GlcNAc type oligosaccharide occur by preassembly of the core oligosaccharide as a lipid intermediate with subsequent transfer of the oligosaccharide to the nascent polypeptide (34,35). It has been reported that both high mannose and complex-type chains have a common biosynthetic origin, $Glc_3Man_9GlcNAc_2$-P-P-Dol (36).

Oligosaccharide - lipid synthesis begins with the transfer of a GlcNAc-P moiety from UDP-GlcNAc to Dol-P, to

yield GlcNAc-P-P-Dol. This reaction results in the formation of a pyrophosphate linkage between the lipid and sugar. Tunicamycin inhibits the synthesis of GlcNAc-P-P-Dol (37). The discovery of specific inhibitors of protein glycosylation, especially the antibiotic tunicamycin, has facilitated the study of the role of Asn-linked oligosaccharides in the synthesis, intracellular transport and secretion of glycoproteins.

The addition of the second GlcNAc residue to yield $GlcNAc_2$-P-P-Dol is the next step in the oligosaccharide - lipid synthesis. A trisaccharide lipid is then synthesized by the addition of a β-linked mannosyl residue from GDP-Man. The trisaccharide - lipid is elongated by the addition of eight α-linked mannosyl residues that form the high mannose structure. Two different mannose donors are involved in this process. The first four Man residues are added directly from GDP-Man to yield $Man_5GlcNAc_2$-P-P-Dol. The remaining four mannose residues are added from the monosaccharide-lipid, Dol-P-Man. The discovery that GDP-Man and Dol-P-Man act as donors for different mannosyl residues in the oligosaccharide - lipid is based on studies with mutant animal cell lines that are unable to synthesize Dol-P-Man. In these cells, $Man_5GlcNAc_2$ accumulates as the major lipid - linked oligosaccharide (38). When membranes from these cells are supplemented with Dol-P-Man in vitro, $Man_5GlcNAc_2$ is elongated to $Man_9GlcNAc_2$ suggesting that the last four mannosyl residues are added from the monosaccharide - lipid. Inhibition of Dol-P-Man synthesis

is observed with the antibiotic amphomycin, fluorodeoxyglucose and EDTA (39). Treatment with these compounds results in accumulation of $Man_5GlcNAc_2$-P-P-Dol.

The precursor oligosaccharide - lipid is completed with the addition of three glucosyl residues to yield $Glc_3Man_9GlcNAc_2$-P-P-Dol. The donor for these transfers is Dol-P-Glc (40). Addition of glucosyl residues to lipid - linked $Man_5GlcNAc_2$ and $Man_8GlcNAc_2$ has been observed in mutant cell lines that accumulate these species and in normal cells under conditions where $Man_5GlcNAc_2$ accumulates.

Assembly of lipid-linked oligosaccharides occur on both the cytoplasmic and luminal surface of the rough endoplasmic reticulum (41). The final product of the assembly line, $Glc_3Man_9GlcNAc_2$-P-P-Dol is present on the luminal side of the RER. This is the site where it becomes transferred en bloc to the nascent, ribosome-bound polypeptide. This transfer of the oligosaccharide is mediated by a membrane-associated oligosaccharyltransferase which is located in the RER (42). In most cases, the oligosaccharide transfer is a co-translational event (25).

Removal of glucose from the lipid-linked oligosaccharide completely abolishes the donor activity. This result indicated that the presence of Glc in the lipid-linked oligosaccharide serves as a signal for the transfer of oligosaccharide chain to a protein.

Analysis of glycoprotein sequences has established that glycosylated Asn residues always occur in the sequence Asn-X-Ser/Thr, where X can be any amino acid except probably

Pro and Asp (43). This is an important, but not the sole factor necessary for glycosylation since a survey of secretory protein sequences has shown that only one-third of all potential sites are glycosylated (44).

Mature Asn-linked oligosaccharides are synthesized by extensive modification of the precursor oligosaccharide after it is transferred to protein. This processing is accomplished by the stepwise removal and additon of saccharide residues. Membrane-bound glycosidases and glycosyltransferases in both ER and Golgi apparatus carry out the modifications on glycoproteins (Fig.3).

Processing reaction in ER entail removal of all three Glc residues which occur in a stepwise fashion from new glycoproteins soon after the transfer of the precursor oligosaccharide (45). The first reaction involves removal of the terminal α-1,2-linked Glc by the enzyme glucosidase I (46). Glucosidase II acts after glucosidase I and removes the two inner α-1,3-linked Glc residues.

Several lines of evidence suggest that further processing of the oligosaccharide occurs when the glycoprotein is still present in the ER. Thyroglobulin, contained in the ER fraction prepared from bovine thyroid slices was found to contain $Man_8GlcNAc_2$ oligosaccharide (47). The ER α—mannosidase, which cleaves Man from $Man_9GlcNAc_2$ species has been characterized by Bischoff and Kornfeld (48,49). Another mannosidase from calf-liver was described which acts immediately after the removal of all three Glc residues and therefore, seems to be present in the

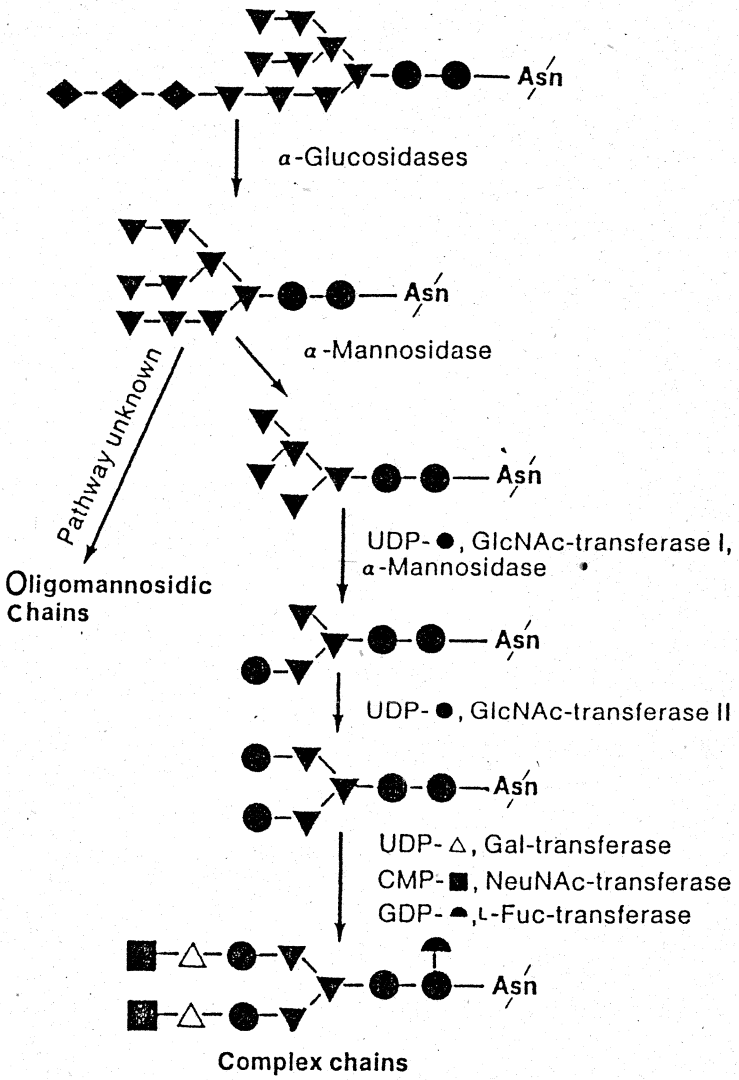


Figure 3. Enzymes involved in the trimming and elongation of N-linked glycoproteins.
◆ = Glc ; △ = Gal ; ▼ = Man; ◓ = L-Fuc;
● = GlcNAc ; ■ = NeuNAc.

Adapted from Sharon, N. and Lis, H. (1981) Chem. Engg. News, 59, p.36.

ER (50). Calf liver α- mannosidase cleaves three of the four α - 1,2 linked mannoses from $Man_9GlcNAc_2$ oligosaccharide.

The partially processed glycoproteins from the ER next enter the Golgi apparatus. In case of glycoproteins, which are destined to become lysosomal enzymes, a highly specific phosphorylation reaction on mannose residues occur. This pathway is unique to acid hydrolases (Fig.4). Two enzymes are involved in the processing. First a GlcNAc-1-P residue from UDP-GlcNAc is transferred to C-6 position of selected mannose residues of high mannose-type oligosaccharide. This reaction is brought about by N-acetylglucosaminylphosphotransferase (51-53). In the next step, an uncovering enzyme, N-acetylglucosamine-1-phosphodiester-α-N-acetylglucosaminidase, removes GlcNAc residue from the phosphodiester to yield mannosyl phosphomonoester (54,55). The Man-6-P residues present on lysosomal enzymes act as recognition marker. Uptake of both newly made and extracellular enzymes into lysosomes is accomplished by binding of the recognition marker to a specific Man-6-P receptor. Phosphorylation of high mannose oligosaccharides blocks further processing since α-mannosidase cannot act on these species.

Processing reaction on high mannose-type oligosaccharides from secretory and membrane glycoproteins take place by trimming of α-1,2-linked mannose residues to yield $Man_5GlcNAc_2$ structure. This is accomplished by the action of Golgi α-1,2-mannosidases IA and IB (56-58).

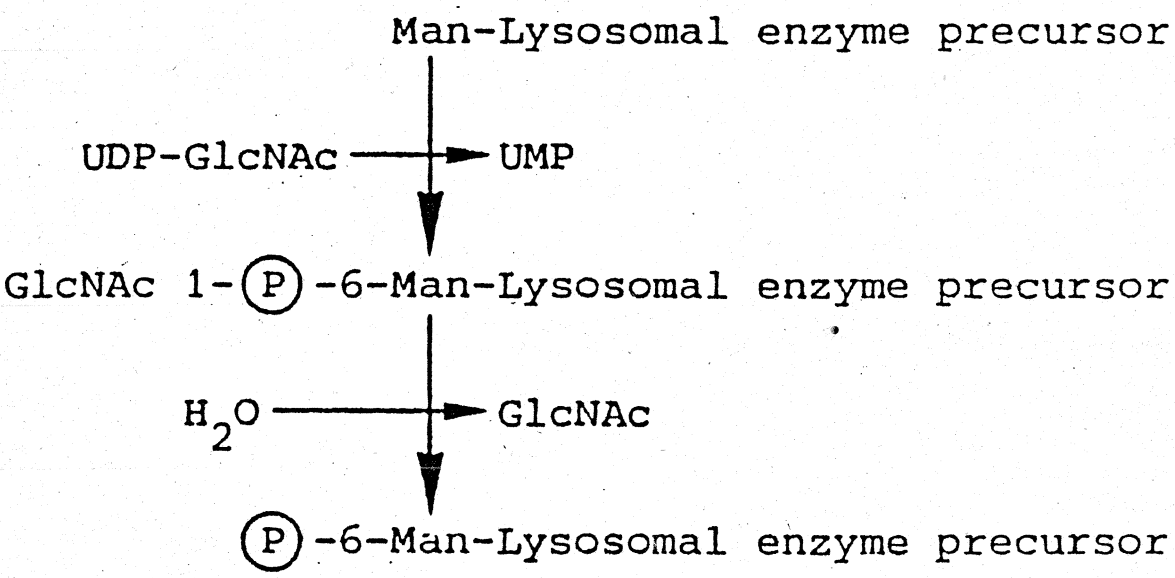


Figure 4. Two step biosynthesis of Man-6-P residues in lysosomal enzymes.

Adapted from von Figura, K. and Hasilik, A. (1986) Ann. Rev. Biochem., 55, p.174.

The direct precursor in the synthesis of complex and hybrid structures is served by $Man_5GlcNAc_2$. The initial step in the conversion process is the addition of a GlcNAc residue to $Man_5GlcNAc_2$ by GlcNActransferase I (59,60). Evidence for the importance of this step comes from a mutant cell line that lacks this enzyme. This line does not synthesize complex oligosaccharides and accumulates large amounts of $Man_5GlcNAc_2$. The next enzyme to act in the synthesis of complex-type chains is Golgi α-mannosidase II which cleaves the $\alpha(1\rightarrow3)$-and $\alpha(1\rightarrow6)$-linked mannose residues to yield GlcNAc $Man_3GlcNAc_2$ structure (56,59). It is highly specific for $GlcNAcMan_5GlcNAc_2$ and is found to have no activity against $Man_5GlcNAc_2$. The role of this enzyme in vivo has been confirmed using the alkaloid swainsonine. Golgi α-mannosidase II activity is inhibited by swainsonine and therefore, it blocks the synthesis of complex oligosaccharides in vivo, leading to the accumulation of $GlcNAcMan_5GlcNAc_2$ (61).

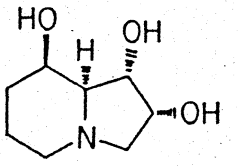
Further glycosylation reaction takes place resulting in the addition of more GlcNAc residues to mannose residues. The assembly of biantennary complex type oligosaccharide is intiated by GlcNAc transferase II which transfers a GlcNAc residue to the α-1,6- linked mannose residue. Addition of a second GlcNAc residue to the α-1,3- linked mannosyl residue by GlcNAc transferase IV initiates pathway for triantennary complex-type oligosaccharides. An additional modification that occurs at this stage of processing is the transfer of an α-Fuc residue to the innermost GlcNAc residue of the core

by fucosyltransferase enzyme. GlcNAc transferase III adds a β(1→4)- linked GlcNAc residue to the β-linked mannose residue of the inner core. This GlcNAc residue is linked between the two α-linked mannosyl branches, and therefore it is termed "bisecting". GlcNAc transferase III has been shown to act on $GlcNAcMan_5GlcNAc_2$ yielding a hybrid type structure and on $GlcNAc_2Man_3GlcNAc_2$ yielding a bisected complex structure. Addition of the "bisecting" GlcNAc residue alters the conformation of the inner core and prevents Golgi α- mannosidase II, GlcNAc transferase II and fucosyltransferase from acting and thus ensures that the hybrid-type structures are not processed further.

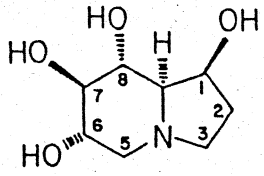
Complex and hybrid chains are completed with the addition of Gal, Fuc and NeuAc residues to the nonreducing ends of the oligosaccharide branches. The glycosyltransferase enzymes involved in these addition reaction exhibit high degree of substrate specificity. Extensive studies by Hill and co-workers with the use of highly purified glycosyltransferases and defined substrates have shown a definite order for the terminal glycosylation (32). The action of a number of these enzymes are mutually exclusive, for example, addition of α-2,6 - linked NeuAc to Galβ(1→4)GlcNAc of transferrin oligosaccharide blocks fucosylation of GlcNAc residue. The glycosyltransferases involved in oligosaccharide completion, such as galactosyl, fucosyl and sialyltransferase have been found to be highly enriched in Golgi fraction.

Biosynthesis of N-linked oligosaccharides have been examined by using inhibitors that block these pathways at specific steps (Fig.5). Tunicamycin, which is a nucleoside antibiotic, inhibit the first reaction in the lipid-linked saccharide pathway, that is transfer of GlcNAc-1-P from UDP-GlcNAc to Dol-P to yield GlcNAc-P-P-Dol. Tunicamycin bears a close structural resemblance to UDP-GlcNAc, hence it was suggested to be a competitive inhibitor of GlcNAc-1-P transferase (62). Amphomycin, a lipopeptide antibiotic blocks the transfer of Man from GDP-Man, Glc from UDP-Glc and GlcNAc-1-P from UDP-GlcNAc to Dol-P. Amphomycin has been utilized as an inhibitor of lipid-linked saccharides in membrane preparations from brain tissue (63). It may form a complex with Dol-P and thus obstruct glycosylation reactions. The inhibitory action of bacitracin in eukaryotic systems is due to the formation of a complex with the lipid carrier, Dol-P. Thus bacitracin blocked incorporation of both Man and GlcNAc from their sugar nucleotide derivatives into lipid-linked monosaccharides. Showdomycin and diumycin completely suppressed the formation of chitobiosyl- and mannose-containing trisaccharide lipids but not that of GlcNAc-P-P-Dol.

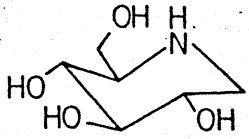
Analogues of glucose and mannose, such as 2-deoxyglucose and 2-fluoro-2-deoxy-D-glucose have been shown to interfere with protein glycosylation. When 2-deoxyglucose is given to cells in culture, it is converted to UDP-dGlc and GDP-dGlc, as well as Dol-P-dGlc. GDP-dGlc



Swainsonine



Castanospermine



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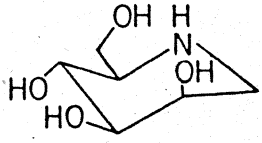
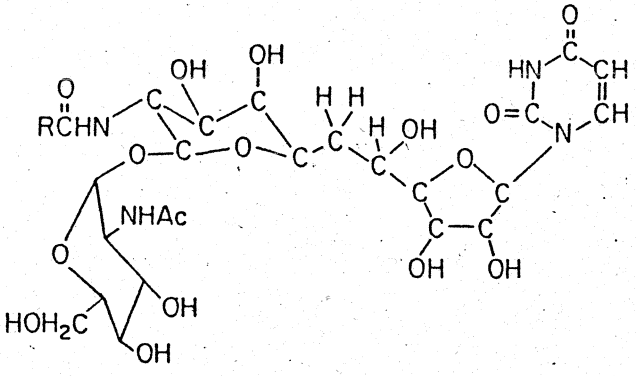


Deoxymannojirimycin



I : R =	$(CH_3)_2CH(CH_2)_7CH=CH-$	VI : R =	$(CH_3)_2CH(CH_2)_{11}-$
II :	$(CH_3)_2CH(CH_2)_8CH=CH-$	VII :	$(CH_3)_2CH(CH_2)_{10}CH=CH-$
III :	$CH_3(CH_2)_{10}CH=CH-$	VIII :	$CH_3(CH_2)_{12}CH=CH-$
IV :	$CH_3(CH_2)_{11}CH=CH-$	IX :	$CH_3(CH_2)_{13}CH=CH-$
V :	$(CH_3)_2CH(CH_2)_9CH=CH-$	X :	$(CH_3)_2CH(CH_2)_{11}CH=CH-$

Tunicamycin and its analogues

Figure 5. Structures of inhibitors of N-linked glycoprotein biosynthesis.

Adapted from Elbein, A.D. (1987) Ann. Rev. Biochem., 56, p.502 and p.516.

was found to inhibit the formation of lipid-linked oligosaccharides (64). Under these conditions, dGlc-$GlcNAc_2$-P-P-Dol is accumulated. Addition of Man residues to this trisaccharide - lipid was not possible. Fluoroglucose and fluoromannose inhibit the in vivo formation of Dol-P-Glc and Dol-P-Man. In chick embryo cells treated with fluoroglucose, formation of lipid-linked oligosaccharides was not completed and chains with decreased amounts of Glc and Man were formed (65).

Another group of inhibitors have been reported that inhibits the trimming of $Glc_3Man_9GlcNAc_2$ oligosaccharide after its transfer from lipid donor to protein. Nojirimycin (5-amino-5-deoxy-D-glucose) and deoxynojirimycin (1,5-dideoxy - 1,5-imino-D-glucitol) are inhibitors of glucosidase I and II that trim Glc from $Glc_3Man_9GlcNAc_2$(66). 1-Deoxymannojirimycin (1,5-dideoxy-1,5-imino-D-mannitol) is an inhibitor of Golgiα-mannosidase IA and IB activities and blocks conversion of high mannose to complex oligosaccharides (67). Swainsonine (8aβ-indolizidine-1α,2α, 8β-triol) a toxic plant alkaloid is a potent inhibitor of Golgi α-mannosidase II and lysosomal α-mannosidase (61,68). It prevents the formation of complex glycoproteins by the inhibition of Golgi mannosidase II.

Castanospermine (1,6,7,8-tetrahydroxyoctahydroindolizine) is a plant alkaloid that was found to be an inhibitor of α- glucosidase I, thereby preventing glycoprotein processing. Bromoconduritol (6-bromo-3,4,5-

tri- hydroxycyclohex-1-ene) is an active-site directed covalent inhibitor of glucosidase II. This inhibitor prevented the trimming of the innermost Glc residue from $Glc_3Man_9GlcNAc_2$ thereby preventing the formation of complex oligosaccharides.

The biosynthesis of O-glycosidically linked oligosaccharide chains occur by a sequence of classical glycosyltransferase catalysed reactions. The carbohydrate - peptide linkage, GalNAcα-1-O-Ser/Thr is formed by the transfer of GalNAc residue from UDP-GalNAc to Ser or Thr residues on the polypeptide. This is in contrast to the synthesis of N-linked oligosaccharides where a lipid-linked oligosaccharide precursor is assembled, followed by the enbloc transfer of the oligosaccharide to the polypeptide. There is no specific polypeptide sequence around the Ser or Thr residues which serve as a recognition sequence for the initiation of O-glycosylation. The UDP-GalNAc:polypeptideα-1,3-GalNAc transferase which initiates O-glycosylation has been purified and characterized (69,70). Evidences have been provided to indicate that the core O-glycosylation starts in the cis side of the Golgi apparatus of intestinal goblet cells and not in the ER. Therefore O-glycosylation is a post-translational event.

The initial O-glycosylation step is followed by the sequential addition of single sugar residue from nucleotide sugar donor substrates. Detailed studies have characterized the specificity of the glyocosyltransferases involved in

chain elongation and the preferred order of their action. There is an important branch point immediately after incorporation of GalNAc residue on Ser or Thr residue of the protein. If incorporation of NeuAc occurs to form NeuAcα(2→6)GalNAc disaccharide, further attachment of carbohydrate cannot take place, as found in ovine submaxillary mucin. Incorporation of Gal to form Galβ(1→4)GalNAc disaccharide proceeds towards synthesis of the various oligosaccharide chains present in bovine submaxillary mucin. The relative proportion of the sialyl- and galactosyltransferases may control this branch point.

Genes control the synthesis of oligosaccharides by being structural genes for a large variety of glycosyltransferases. Each enzyme is specific for one sugar nucleotide donor. The types of acceptor specificity observed with glycosyltransferases fall into two categories. All these enzymes have preference for a specific acceptor, either an amino acid or another sugar which serves as the attachment of the glycosyl residue. In addition, many of these enzymes will not function with low molecular weight acceptors having appropriate structure and are thus limited to macromolecular acceptors, that is glycoproteins. The most important feature of this system of glycosyltransferases is that the product of one transfer reaction is the substrate for the next transferase in the series. This process is a nontemplate mechanism of information transfer and is subject to a higher incidence of

errors than a template mechanism. This fact is one of the factors responsible for microheterogeneity widely found in the sugar chains of glycoproteins (71).

Post-translational factors that may affect glycosylation include a) the rate of synthesis of nuecleotide sugar donors, b) the intracellular transport of donor substrates, c) the accessibility and structure of acceptor substrates, d) the concentration of inhibitors such as nucleotide products of glycosyltransferase reaction, e) competition with other glycosyltransferases of overlapping specificity and f) depletion of substrates by degradative enzymes such as pyrophosphorylases or glycosidases.

Enzymes capable of splitting various linkages found in glycoproteins are widely distributed in nature. They include a variety of glycosidases and proteases. In animals, many of these enzymes have been shown to be constituents of lysosomes, organelles with a full complement of hydrolases that is necessary for the complete degradation of glycoproteins.

LYSOSOME

The lysosome concept was initiated by de Duve et al (72). Biochemically defined, lysosomes are cytoplasmic organelles containing a variety of hydrolases, most of which have maximal activities at acid pH. The lysosomal enzymes display latency when the organelles are properly isolated.

Lysosomes appear to be an universal constituent of animal cells. They are membrane-bound organelles of variable size, shape and structure. The membrane is usually tripartite. There are two types of lysosomes: primary lysosomes are packages of acid hydrolases that are newly synthesized by the cell, whereas secondary lysosomes are sites of digestive processes in which the hydrolases are active. When secondary lysosomes have accumulated undigested or indigestible molecules, they are referred to as residual bodies.

Digestion of biosynthetic material appears to be the main function of lysosomes. The numerous hydrolytic enzymes present in these organelles usually exert their action inside the cell, either on exogenous material engulfed by endocytosis or on endogenous cell constituent segregated by autophagy. However, lysosomal enzymes may also be excreted by exocytosis and be active outside the cell in the degradation of some extracellular components.

Endocytosis is the general term used for the internalization of extracellular fluid or particles by invagination or pinching off of the plasma membrane. A distinction is made between engulfment of large particles (phagocytosis) and small particles, solutes and fluids (pinocytosis). When introduced into the cytoplasm, the foreign material is completely surrounded by a membrane that originates from the cell membrane. This newly formed vacuole is called a heterophagosome. Fusion of hetero

phagosomes with a lysosome initiates digestion.

In the autophagic function of lysosomes, a piece of cell's own cytoplasm become segregated by a membrane and separates it from the rest of the cell. These vacuoles contain mitochondria, ER and other cytoplasmic entities. The resulting autophagosome is converted to an autolysosome by fusion with a lysosome. Autophagy occurs continuously in normal cells. Therefore, it plays an important role in the turnover of cell constituents.

Farquhar and coworkers observed another type of autophagy in secretory cells (73). It involves the direct fusion of a lysosome with a secretory granule without preliminary segregation. In this mechanism, lysosomal digestion takes care of the secretory products formed in excess over the needs, and thus accomplishes a regulatory function. The term "crinophagy" has been suggested by de Duve for this process (74).

The lysosomal membrane contains a characteristic set of proteins that reflects its unusual properties (75). In addition to sequestering a high concentration of hydrolytic enzymes, the lysosomal membrane is responsible for the acidification of the lumen, the selective transport of degradation products and metabolites from the lumen to the cytoplasm and the regulation of fusion events between lysosomes and other organelles (76).

BIOGENESIS OF LYSOSOMES

Lysosomal enzymes carry a special recognition marker that enables them to be selectively bound by a receptor. Studies by Kaplan et al confirmed the presence of Man-6-P as the recognition marker (77). Evidences indicate that the Man-6-P recognition marker is acquired in two steps. In the first step GlcNAc-1-P is transferred from UDP-GlcNAc to the C-6 position of mannose residues on high mannose-type chains of newly synthesized acid hydrolases (51). The second step involves the enzymatic release of GlcNAc, which exposes the 6-phosphomonoester (54).

The findings in I-cell disease fibroblasts led to the suggestion that all enzymes are normally "secreted" into the extracellular medium and delivered to lysosomes by receptor mediated recapture. This proposal came to be known as "secretion-recapture hypothesis". However, when experiments were conducted, presence of Man-6-P, a competitive inhibitor in the growth medium, did not decrease the intracellular levels of lysosomal enzymes (78). These results suggested that secretion-recapture pathway is not the major pathway by which acid hydrolases reach the lysosomes. Sly and Stahl proposed an intracellular pathway for the phosphomannosyl receptor mediated segregation of lysosomal enzymes (17). The biosynthesis, sorting and transport of lysosomal enzymes has been summarized recently by Kornfeld (79). Lysosomal enzymes are synthesized in the RER (Fig.6). The proteins then move, by vesicular transport, to the Golgi stack where

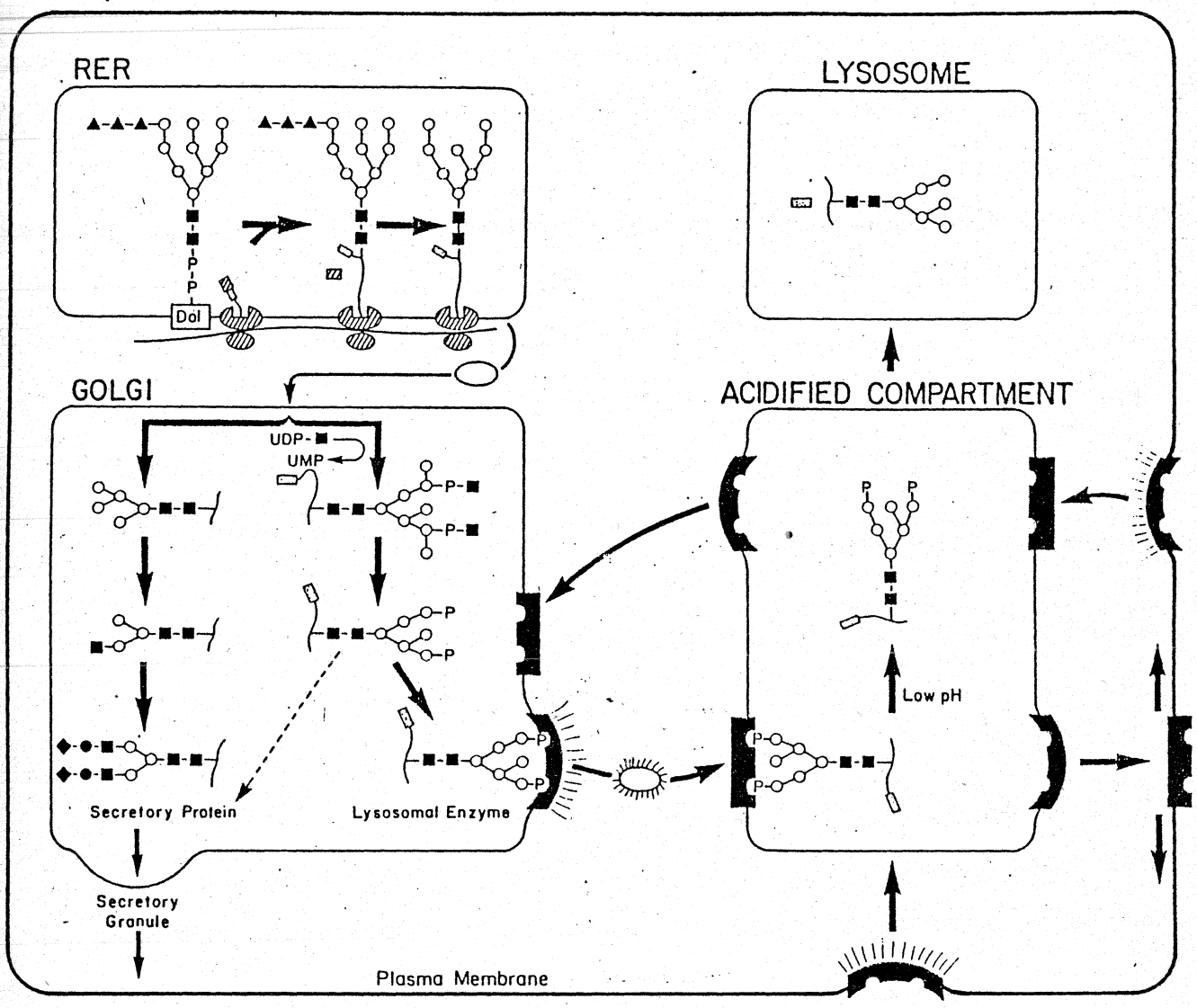


Figure 6. Schematic pathway of lysosomal enzyme targeting to lysosomes.
= mannose-6-phosphate receptors; = coated pits ; ■ = GlcNAc; ○ = Man; ▲ = Glc ; ● = Gal ; ◆ = NeuAc.

Adapted from Kornfeld, S. (1987) FASEB J., 1, p.463.

they undergo a variety of post-translational modifications. The lysosomal enzymes acquire phosphomannosyl residues. The resultant phosphomonoesters serve as a recognition marker that permits high-affinity binding to Man-6-P receptors in the Golgi. The ligand-receptor complex then exits the Golgi via a coated vesicle and is delivered to an acidified prelysosomal compartment. The low pH here facilitates the dissociation of the enzyme from the receptor. The receptor then recycles back to the Golgi for further rounds of enzyme transport while the lysosomal enzymes are packaged into lysosomes. A small amount of lysosomal enzymes, about 5-20% is secreted before its delivery to lysosomes. So the extracellular lysosomal enzymes are not obligatory intermediates in the pathway to lysosomes, rather they reflect the escape of some enzymes from segregation. Two Man-6-P receptors are known. These receptors can be differentiated by dependence on divalent cations. The cation-independent receptor has been extensively characterized (80). Existence of a second cation-dependent receptor is reported by Hoflack and Kornfeld (81).

As soon as lysosomes were recognized as bearers of acid hydrolases, and their digestive function suspected, the inference was made that the interior of these organelles must be acid. Many attempts were made to determine the intralysosomal pH. The best method was developed by Ohkuma and Poole (82). Their method was based on the pH dependence of the fluorescence excitation spectrum of fluorescein. A

pH of the order of 4.7 was observed in the lysosomes of mouse peritoneal macrophages. An ATP-driven proton pump has been identified in lysosomes (83). The acidity of the lysosome is maintained by this proton pump. The support for this proton pump was provided by the ability of lysosomes to concentrate chloroquine, neutral red and other weakly basic components. This accumulation was attributed to proton trapping. Lysosomotropic substances like ammonium chloride and methylamine have been found to raise the intralysosomal pH. The rise in endolysosomal pH is caused by the inhibitory effects that they exert on lysosomal digestion, and on endosomal sorting and translocation process.

The battery of enzymes enclosed in the lysosomes allows complete digestion of most biological polymers which include proteins, nucleic acids, lipids and glycoconjugates. Lysosomes are capable of digesting proteins through the combined action of a series of endo- and exopeptidases. Hydrolases acting on bonds between amino acid residues are collectively called cathepsins. Cathepsin A is a carboxypeptidase which attacks polypeptide chains at the carboxyl-terminal and sequentially releases one amino acid after the other. Cathepsin C removes sequentially one terminal dipeptide after the other from the NH_2-terminal of the polypeptide chains. Cathepsins B and D are endopeptidases. Other proteinases include acid carboxypeptidase which removes carboxy-terminal amino acid from various tripeptides, a dipeptidylaminopeptidase II

which catalyzes cleavage of NH_2-terminal dipeptides from tripeptides and a dipeptidase that splits various dipeptides containing Gly at the carboxy-terminal position. Barret has reviewed the properties of these enzymes in detail (84).

The hydrolysis of nucleic acids and various lipids are confined to lysosomes as the enzymes involved in the degradation of these molecules appear to be restricted to these organelles alone (85,86).

Majority of the enzymes capable of hydrolysing glycosyl bonds in mammalian tissues are localized in lysosomes. These acid glycosidases form the single largest group of enzymes in these organelles. Glycohydrolases are implicated in the digestion of polysaccharides and of the carbohydrate chains of glycoproteins, glycolipids and proteoglycans.

The digestive activity of highly purified liver lysosomes at acid pH on several glycoproteins has been studied in vitro by Aronson and de Duve (87). The role of lysosomal neuraminidase appear to be the removal of terminal NeuAc residues from the nonreducing end of the carbohydrate chains of glycoproteins. This reaction paved way for the action of other glycosidases involved in the complete degradation of these macromolecules.

Lysosomal β-galactosidase with pH optimum 4.0 — 4.5 is widely distributed in mammalian tissues. The acid β-galactosidase is capable of cleaving the terminal β-D-galactoside residue from the GM_1-ganglioside (88). This

hydrolysis is stimulated by an activator protein. β-Galactosidase deficiency leads to GM_1-gangliosidosis which results in the storage of GM_1 and GA_1-gangliosides within the lysosomes. N-acetyl-β-Hexosaminidases of lysosomes catalyze the hydrolysis of terminal β-linked GlcNAc and GalNAc residues of glycoconjugates (89). Deficiency of hexosaminidases leads to various types of GM_2-gangliosidoses (90,91).

α-L-Fucosidase located in lysosomes is capable of cleaving Fuc residues at the nonreducing terminus of the oligosaccharide chains of glycoproteins. Fucosidosis is a consequence of the functional deficiency of lysosomal α-L-Fucosidase resulting in the accumulation of Fuc-containing molecules. The role of lysosomal α-glucosidase is well understood by the fact that congenital absence or decreased activity of the enzyme leads to a serious disorder of glycogen metabolism resulting in the accumulation of excessive glycogen within lysosomes of many tissues.

Multiple forms of α-D-mannosidase having acid, neutral and intermediate activities have been described. The acid α-mannosidase with pH optimum 4.0 — 4.5, a typical lysosomal glycohydrolase, is widely distributed in mammalian and plant tissues (92). The lysosomal α-mansosidase has been purified and characterized from various mammalian organs (93-98). The enzyme causes removal of terminal nonreducing α-D-mannopyranosyl residues from glycopeptides

(96,99). Lysosomal α-mannosidase hydrolysed α-(1→2), α-(1→3) and α-(1→6)-linked mannose units (99,100). This broad specificity of the enzyme is expected since it is located in the lysosome which functions in the breakdown of macromolecules into their monomeric components. Man-containing oligosaccharides have been observed to accumulate in tissues of patients with a storage disease termed mannosidosis (101). Patients with mannosidosis are characterized by the deficiency of acid α-mannosidase. Oligosaccharides containing single GlcNAc residue with varying number of Man molecules have been isolated from urine of patients suffering from this storage disease. Acid α-Mannosidase completely hydrolyses the terminal α-linked mannose from the storage product, Manα(1→3)Manβ(1→4)-GlcNAc. This is a strong evidence that mannosidosis is a lysosomal storage disease resulting from the deficiency of acidic α-mannosidase (102).

Swainsonine, a plant toxin has been isolated from leguminous plants of the genus Swainsona (103). This alkaloid strongly inhibits lysosomal α-mannosidase (67). Swainsonine is considered to be the agent responsible for the neurological disease seen in livestock after prolonged ingestion of the plant. Swainsonine toxicosis is a phenocopy of mannosidosis of human and Angus cattle (104). It was suggested that swainsonine owes its inhibitory activity to its conformational similarity to the mannosyl cation intermediate of mannoside hydrolysis (67).

Swainsonine offers the possibility of inducing and terminating a lysosomal storage disease and so it is an useful tool that can be used in the study of pathogenesis and reversibility of storage - mediated tissue injury.

AIMS AND OBJECTIVES

Lysosomal α-D-mannosidase (EC 3.2.1.24) is an important enzyme involved in the catabolism of macromolecules containing α-D-mannopyranosyl residues. The physiological importance of lysosomal acid α-mannosidase is indicated by the fact that its deficiency is the cause of the lysosomal storage disease Mannosidosis, which occurs in both human and bovine species. Individuals with this genetic disease Mannosidosis, accumulate mannose - rich oligosaccharides in the tissues and body fluids.

The purification and characterization of lysosomal α-mannosidase from several mammalian sources have been described. The review of the literatures revealed a considerable gap in the informations on the structure - function relationship in the enzyme. The present study included investigations on the structure and function of lysosomal α-mannosidase purified from an useful hospital waste, human placenta. The role of zinc ion in the structure and biological activity of the enzyme. Inhibition studies with several substrate analogues were investigated to obtain informations about the active site of the enzyme. Chemical modification of amino acid residues as a probe was used to investigate the involvement of the various amino

acid residues at the active site. N-terminal sequencing of the enzyme was carried out. Composition, structure and contribution by the oligosaccharide chains to the biological function of the enzyme was studied. The immunological properties of the enzyme from developing and adult human tissues have been discussed.

CHAPTER II

MATERIALS AND METHODS

p-nitrophenyl derivatives of α-D-man, β-D-Gal, β-D-Glc, α-L-Fuc, β-D-GlcNAc, crystalline bovine serum albumin, ovalbumin, mannosamine hydrochloride, mannonic acid -γ-lactone, mannose-6-phosphate, methyl-α-D-mannopyranoside, galactose, GlcNAc, wheatgerm, chitin, trinitrobenzene sulfonic acid, maleic anhydride, dithiobis-(2-nitrobenzoic acid), p-hydroxymercuribenzoate, N-acetylimidazole, trifluoro acetic acid, acrylamide, N,N'-methylene-bis-acrylamide, N,N,N',N'-tetramethylenediamine, Coomassie brilliant blue R 250 and G 250, sodium dodecyl sulfate, dansyl chloride and standard dansyl amino acid kit were purchased from Sigma Chemical Co., St.Louis, USA. Iodoacetic acid, N-ethylmaleimide and reduced glutathione were obtained from Serva Feinbiochemica, Heidelberg, West Germany. The molecular weight marker proteins were purchased from Pierce Chemical Co., Rockford, U.S.A. Phenylglyoxal hydrate, 1,2-cyclohexanedione, 2-methoxy-5-nitrobenzyl bromide, 2-hydroxy-5-nitrobenzyl bromide and phenylisothiocyanate were the products of Fluka, Buchs, Switzerland. Sepharose-4B, CM-Sephadex C-50 Sephadex G-200 and Blue

dextran 2000 were purchased from Pharmacia Fine Chemicals, Sweden. Swainsonine (8a β -indolizidine-1α-,2α-,8β- triol) was a gift from Prof.P.Dorling, Murdoch University, Australia. Synthetic Man analogues 5-amino-5-deoxy-D-mannopyranose hydrogen sulphite and 1-deoxymannojirimycin (1,5-dideoxy-1,5-imino-D-mannitol) were gifts from Prof.G.Legler, Institute for Biochemistry, University of Cologne, Federal Republic of Germany. 5-amino-5-deoxy-D-mannopyranose was prepared from 5-amino-5-deoxy-D-mannopyranose sulfite adduct according to the method of Legler and Julich (105). Other chemicals used were of analytical grade and obtained from local sources. The seeds of Canavalia gladiata or Canavalia ensiformis, Artocarpus integrifolia, Ricinus communis and Psophocarpus tetragonolobus were obtained locally.

ENZYME ASSAY

The standard assay system for α-mannosidase contained 200 μmoles of citrate-phosphate buffer pH 4.2, 0.5μmole of p-nitrophenyl α-D-mannopyranoside, 100 μgm of bovine serum albumin, 1μmole of $ZnSO_4$ and suitable amount of enzyme in a total volume of 0.5 ml. The mixture was incubated at 37°C for 30 min. The reaction was terminated by heating at 100°C for 30 sec. The contents, after cooling were mixed with 2.5ml of 0.4 M Glycine-NaOH buffer pH 10.5 and centrifuged at 2000xg for 5 min. The yellow colour formed due to the liberation of p-nitrophenol was measured at 405 nm.

One unit of enzyme is defined as the amount of enzyme required to liberate one nmole of p-nitrophenol per minute. Other glycosidases were assayed according to the method of Bossmann (106).

POLYACRYLAMIDE GEL ELECTROPHORESIS

The purity of the enyzme protein was evaluated by polyacrylamide gel electrophoresis. Electrophoresis was carried out on 7.5% (w/v) acrylamide gel at pH 4.3 in β-alanine-acetic acid buffer according to the method of Davis (107). Each tube contained 30-50 μgm of protein. The gels were run at 3mA per tube at room temperature. The gels were stained with Coomassie brilliant blue R and destained with methanol: acetic acid: water (1:1.5:7.5, v/v)(108).

PROTEIN ESTIMATION

Protein was estimated by the method of Lowry et al with crystalline bovine serum albumin as standard (109) or by the method of Bradford with ovalbumin as standard (110).

CARBOHYDRATE ESTIMATION

The total neutral sugar was estimated by phenol-sulfuric acid method of Dubois et al with galactose as standard (111).

MOLECULAR WEIGHT DETERMINATION

Molecular weight of the enzyme was determined by gel

filtration as described by Andrews on a Sephadex G-200 column (2x65cm) (112). The void volume of the column was determined with blue dextran 2000 and the column was calibrated with the following standard proteins - ovalbumin (45,000), bovine serum albumin (67,000), aldolase (158,000), catalase (240,000) and ferritin (540,000).

The subunit molecular weight of the enzyme was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis on a 10% slab gel according to the method of Laemmli (113). Each well contained 5-10 μgm protein. Bovine serum albumin (67,000), ovalbumin (45,000), aldolase (39,500) and winged bean agglutinin (35,500) were used as standard proteins. The slab gel was run at 10mA at room temperature. The gel was stained with Coomassie brilliant blue R (108) or with silver nitrate according to the method of Wray et al (114).

AFFINITY COLUMNS

Concanavalin A was purified from Canavalia gladiata or Canavalia ensiformis according to the method of Surolia et al (115). Sepharose-4B was activated by 30mg of cyanogen bromide per ml of packed gel according to the method of Axen et al (116). Con A (10mg/ml gel) in 0.1M $NaHCO_3$, pH 8.5 was reacted with activated Sepharose-4B at 4°C for 18 h. The unreacted active groups were blocked by treatment with ethanolamine (1ml/100ml gel). The coupled gel was washed successively with a) 0.1 M $NaHCO_3$ b) 1M NaCl c) distilled water d) 0.02 M Tris-HCl buffer, pH 7.4 containing 1mM $CaCl_2$,

1 mM $MgCl_2$ and 1mM $MnCl_2$ and finally with e) 0.02 M Tris-HCl, pH 7.4, to remove excess metals. The amount of ConA bound to the gel was calculated from the amount of unbound protein in the washing. 7-9mg of ConA was bound per ml gel.

Wheat germ agglutinin was purified on a chitin column according to Bloch and Burger (117). Jack fruit seed agglutinin was purified from Artocarpus integrifolia according to Sureshkumar et al (118). The coupling of WGA and JSA to activated Sepharose-4B was carried out as described earlier for ConA. The coupled gels were washed successively with 0.1M $NaHCO_3$, distilled water and the respective equilibration buffer. Protein A was isolated from Staphylococcus aureus (Cowan I strain) and coupled to Sepharose-4B according to the method of Hjelm et al (119). Ricinus communis agglutinin was purified and immobilised according to the method of Appukuttan et al (120). Winged bean agglutinin was isolated from Psophocarpus tetragonolobus according to the method of Appukuttan and Basu (121).

CHEMICAL MODIFICATION OF AMINO ACID RESIDUES AND OLIGOSACCHARIDES

Amino groups were modified with trinitrobenzene sulfonic acid (122). Eight milligram of enzyme protein in 1 ml of 0.2 M borate buffer, pH 9.5 was treated with 0.02 ml of 1.1 M trinitrobenzene sulfonic acid. The reaction was stopped after 5 min. by the addition of 2 ml of 0.1M NaH_2PO_4. The protein was dialysed against 100 volumes of 0.02 M phosphate

buffer, pH 7.0 for 6 h with three changes.

For maleylation, 80 µg protein in 1 ml of 0.2 M borate buffer, pH 9.0 was treated with 1 mg maleic anhydride overnight at 23°C (123). The modified protein was dialysed against phosphate buffer, pH 7.0 for 6h with three changes to remove excess maleic anhydride. Total number of free amino groups in the enzyme sample was estimated by the method of Habeeb (124).

Arginine residues of the enzyme were modified by treatment with phenylglyoxal hydrate according to the method of Takahashi (125). Eight microgram of enzyme in 0.5 ml of 0.2 M N-ethylmorpholine acetate buffer, pH 8.0 was treated with 1% phenylglyoxal hydrate, and incubated overnight at 23°C. The modified protein was dialysed against 100 volumes of 0.02 M phosphate buffer pH 7.0 for 6h with three changes.

Arginine residues were also modified with 1,2-cyclohexanedione (126). Eighty microgram of protein in 1 ml of borate buffer, pH 8.5 was treated with 15-fold molar excess of 1,2-cyclohexanedione for 4h at 37°C. Modified enzyme was dialysed against 100 volumes of borate buffer, pH 8.5 for 6h with three changes.

Modification of histidine residues was carried out with diethylpyrocarbonate according to the method of Miles (127). Eight microgram of enzyme protein in 1 ml of 0.1M phosphate buffer, pH 6.5 was treated with 0.25mM diethylpyrocarbonate for 1h at 23°C. The protein was freed from excess reagent by dialysis against PBS, pH 7.4 for 6h with three changes.

Tryptophan residues were modified with active benzyl halides according to the method of Horton and Koshland (128). Eighty microgram of enzyme protein in 0.02 M phosphate buffer, pH 6.0 was treated with 7mM 2-hydroxy-5-nitrobenzylbromide for 5 min at 23°C. In another experiment, similar amount of protein was incubated with 2-methoxy-5-nitrobenzyl bromide overnight at 23°C. Excess reagent was removed by dialysis against 100 volumes of PBS, pH 7.4 for 6h with three changes. Tryptophan residues were determined by measuring the absorbance at 250nm and 280 nm in PMQIII spectrophotometer before and after oxidation with a mixture of dimethylsulfoxide, concentrated HCl and glacial acetic acid as described by Savige and Fontanna (129).

Tyrosine modification with N-acetylimidazole was carried out according to the method of Riordan and Vallee (130). Eight microgram of protein in 0.5 ml of O.1M phosphate buffer, pH 7.5 was acetylated by treating with 400-fold molar excess of N-acetylimidazole for 1h at 23°C. The sample was dialysed against 100 volumes of PBS pH 7.4 for 6h with three changes. Eight hundred microgram per ml solution of enzyme in 0.1 M phosphate buffer, pH 7.5 was used for tyrosine estimation. The number of O-acetylated tyrosine residues was calculated from the absorbance change at 278 nm on treatment with 1M hydroxylamine.

Total SH groups were estimated according to the modified method of Habeeb (131). The number of disulfide

bonds in the protein was assayed with disodium 2-nitro-5-thiosulfobenzoate (NTSB) using oxidised glutathione as standard (132). Chemical modification of SH groups were carried out with a variety of reagents (133,134). 8 µgm of enzyme protein in 0.02 M phosphate buffer, pH 6.5 was incubated separately with 10^{-2}—10^{-3}M of p-hydroxymercuribenzoate, N-ethylmaleimide and iodoacetic acid for 1h at 23°C. Modified proteins were dialysed against PBS pH 7.4 for 6h with three changes.

Periodate oxidation of 80 µg protein with 10mM and 100mM periodate was done in citrate-phosphate buffer, pH 4.0 for 1h at 4°C in the dark (135). Reaction was quenched with 0.05ml of 1M ethylene glycol and dialysed against phosphate buffer pH 7.0 for 6h with three changes.

A suitable protein control was treated under similar conditions without the modifier. The modified and control samples were assayed under standard conditions.

DETERMINATION OF N-TERMINAL SEQUENCE

The N-terminal sequence of the protein was determined by the Dansyl-Edman method (136). Twelve nmoles of the protein was taken in 100 µl of water. Five microlitre was set aside for dansylation. To the remainder protein, added 100 µl of 1% (v/v) phenylisothiocyanate in pyridine. The tube was flushed with N_2, covered with parafilm, stirred thoroughly and left to react for 1h at 45°C. After the reaction period, the sample was dried in vacuo. Two hundred µl of anhydrous

trifluoroacetic acid was added to the dried residue, and the tube flushed with N_2 and sealed with parafilm. Reaction was allowed to proceed for 30 min. at 45°C, and the trifluoroacetic acid removed in vacuo. The nonvolatile by-products were removed by extraction with n-butyl-acetate. The dried residue was suspended in 500 µl of the organic phase (top) of the n-butylacetate/water mixture and then 250 µl of the aqueous phase (bottom) was added. After thorough mixing, the two phases were separated by centrifugation. The organic phase was removed and discarded. The aqueous phase was reextracted twice with 500 µl aliquots of the organic phase. The aqueous phase was dried in vacuo and the protein suspended in 100 µl of water. A 20 µl aliquot was removed for dansylation. The remainder was put through the cycle of Edman degradation.

A 100 µl of 4 mM lithium carbonate buffer, pH 9.5 was added to the aliquots removed at each cycle. Five microlitre of Dansyl chloride (2.5 mg/0.1 ml acetonitrile) was added to give a final concentration of 5 mM Dns-Cl. The contents were mixed well, and the reaction allowed to proceed for 1h at 37°C. The contents were dried and 200 µl of 6 N HCl was added. The ampule was flushed with N_2, sealed and hydrolysed at 105-108°C for 18h. After the acid hydrolysis, tubes were opened and the contents dried.

The dansyl amino acids were separated by HPLC using gradient elution on a 5 µm Lichrosorb RP-18 column (4x250mm), according to the method of Marquez et al (137).

Dansyl amino acids were monitored by their fluorescence (Ex max 333 nm, EM max 510 nm) in a Shimadzu Spectrofluorophotometer. Retention time for standard dansyl amino acids were determined.

SDS-PAGE of the native and swainsonine treated enzyme (50 μgm) was carried out on 10% tube gels according to the method of Laemmli (113). One tube was stained with Coomassie brilliant blue R. Corresponding protein subunits were cut out from other tube gels and extracted with Laemmli chamber buffer. The subunits were dansylated and the fluorescent dansyl amino acids detected after acid hydrolysis.

PREPARATION OF IMMUNOGLOBULINS

Control serum from rabbit was collected before immunization. Five hundred microgram protein was mixed with 1ml of Freund's complete adjuvant and injected intramuscularly to the rabbit. Two booster doses of the enzyme with Freund's incomplete adjuvant were given at ten days interval. The rabbit was bled ten days after the last injection. Immunoglobulins were purified from the resulting serum by ammonium sulfate precipitation and dialysed against PBS, pH 7.4.

SEPARATION OF CLASS OF IMMUNOGLOBULIN

Three milligram of purified α-mannosidase immunoglobulin was applied on Protein A-Sepharose column (1x7cms) equilibrated with 0.1M phosphate buffer, pH 7.0. The column was washed

with 10 ml of the same buffer. Unbound immunoglobulins from Protein A-Sepharose column was collected and passed through JSA-Sepharose column (1x6cms). Immunoglobulin bound to Protein A-Sepharose column was eluted with 0.3 M Glycine-HCl, buffer pH 3.0. The pH of the eluted protein was restored to 7.0 with 2 M Tris, dialysed against PBS, 7.4 and concentrated by ultrafiltration. JSA-Sepharose column was washed with 10 ml of PBS, pH 6.5 and the unbound immunoglobulin collected. The bound protein from JSA-Sepharose column was eluted with 100 mM Gal in PBS, 6.5.

IMMUNODIFFUSION

Immuonodiffusion was carried out by the Ouchterlony double diffusion technique. One percent agarose in PBS, 7.4 and 0.1% sodium azide was used to prepare immunodiffusion plates. The enzyme protein, (25 μg in 10 μl) was loaded in the central well. Ten microlitre each of Protein A-Sepharose eluate, JSA-Sepharose eluate and the unbound from JSA-Sepharose column were loaded in the peripheral wells. The immunodiffusion plate was kept covered in a petri dish over humid atmosphere at 23°C for 24 h.

EXTRACTION OF α-MANNOSIDASE FROM HUMAN FETAL ORGANS

Human fetus was collected in ice from SAT hospital, Trivandrum Medical College within 10-12 h after medical termination of pregnancy. Various fetal organs were dissected out, cleaned and stored at -20°C until use. The fetal organs were homogenised with 0.02 M phosphate buffer

pH 7.0 containing 0.1M NaCl, stirred for 30 min. at 4°C and centrifuged. Supernatents containing α-mannosidase activity were used as antigens in immunological cross-reactivity.

CHAPTER III

RESULTS AND DISCUSSIONS

α-D-Mannosidase (α-D-mannoside mannohydrolase, EC 3.2.1.24) is one of the glycosidases involved in the hydrolysis of α-mannosidic bonds in glycoconjugates. Multiple forms of α-D-mannosidase having acid, neutral and intermediate activities have been described. Acid α-mannosidase with pH optimum 4.0-4.5, a typical lysosomal glycohydrolase is widely distributed in animal and plant tissues (92). Another enzyme with pH optimum 6.0-6.5, the neutral form localized in the cytosol has also been isolated (138). α-Mannosidases purified from rat liver Golgi membrane has an intermediate pH optimum. Three Golgi α-mannosidases, IA, IB and II, have been reported. They account for the trimming of six mannosyl residues from the biosynthetic intermediate, $Man_9GlcNAc_2$. The cleavage of α-1,2-linked mannosyl residues was accounted for by Golgi membrane α-mannosidases, IA and IB (57,58). α-Mannosidase II cleaves α-1,3- and α-1,6-linked mannosyl residues from $GlcNAcMan_5GlcNAc_2$ to produce $GlcNAcMan_3GlcNAc_2$ (58). A calcium-activated α-mannosidase that specifically hydrolyses α-1,2-linked mannose residues has been partially purified

from rabbit liver microsomes (139). The purified enzyme has requirement of nonionic detergents or of specific phospholipids for its activity. An α-mannosidase has been identified in rat liver endoplasmic reticulum which was shown to be distinct from previously described Golgi α-mannosidases IA, IB, II and lysosomal α-mannosidase (140). The enzyme was solubilized with deoxycholate and separated from other α-mannosidases by passage over ConA-Sepharose to which it does not bind. The ER α-mannosidase cleaves α-1,2-linked mannoses from high mannose oligosaccharides prior to its entry into the Golgi complex. The presence of an unique α-mannosidase in the ER can account for the earlier observation that partially processed $Man_8GlcNAc_2$ oligosaccharides occur on glycoproteins which have not reached the Golgi apparatus (47). Different mannose residues are removed from $Man_9GlcNAc_2$ oligosaccharide at Asn 402 and Asn 563 in the processing of human IgM (141). The soluble α-mannosidase of rat liver, described as the cytoplasmic α-mannosidase has the same enzymatic properties as the ER membrane α-mannosidase. The soluble and membrane bound ER α-mannosidases are the two forms of the same enzyme in their immunological cross-reactivity (48). This phenomenon, identical activities present in the soluble and membrane fractions has been observed with yeast α-mannosidase activity (142). A processing mannosidase, Man_9-mannosidase, distinct from other processing mannosidases has been purified from calf liver microsomes

(50). Man_9-mannosidase cleaves three of the four α-1,2-linked mannose residues from $Man_9GlcNAc_2$ oligosaccharide and is not active against aryl α-mannosides, indicating a possibily specific functional role in the N-glycoprotein processing pathway.

Lysosomal α-D-mannosidase is an important enzyme involved in the metabolic breakdown of macromolecules containing α-D-mannopyranosyl residues. The dependence of α-mannosidase activity or stability on the presence of Zn^{2+} has been reported for the rat epididymal enzyme and other mammalian acid α-mannosidases (99,143 -145). Snaith has characterized jack-bean α-mannosidase as a metalloenzyme (93). Mannosidosis, a lysosomal storage disease, resulted from a genetic deficiency of lysosomal α-D-mannosidase. It was characterized by excretion in the urine and accumulation in the tissues of mannose-rich oligosaccharides. Mannosidosis has been described in humans, bovine and feline species (101,146,147). The patterns of neutral urinary oligosaccharides in bovine, feline and human mannosidosis are different suggesting differences in the catabolic pathways of glycoproteins among these species (148). Swainsonine, an indolizidine alkaloid occurs in Swainsona canescens, spotted locoweed, Astragalus lentiginosus and the fungus, Rhizoctonia leguminicola (103,149,150). It has been considered to be the agent in plants responsible for the neurological disease in livestock resembling the hereditary lysosomal α-mannosidosis (24). This conclusion

has been confirmed by a direct comparison of the effects of swainsonine and locoweed in the pig (151). Swainsonine blocks the synthesis of complex N-linked glycoproteins by inhibiting Golgi mannosidase II, which acts on glycoproteins containing $GlcNAcMan_5GlcNAc_2$ species (61).

The different types of α-mannosidase from mammalian sources differ in their physico-chemical and immunological properties. Immunological studies on the acid, neutral and intermediate forms of the enzyme support the view that they are three distinct proteins of different genetic origin (96). The immunological cross-reactivity between neutral and membrane ER α-mannosidase suggested that they may be two forms of the same enzyme (48). The bovine and human acidic α-mannosidases have very similar properties, however, the antiserum raised against the human enzyme did not cross-react with the bovine enzyme and vice versa (152).

Lysosomal α-D-mannosidase has a broad substrate specificity. The enzyme from most sources is capable of hydrolysing α-(1→2), α-(1→3), α-(1→4) and α-(1→6) linkages of oligomannosides (96,100). This observation is in accordance with the fact that the enzyme is located in a cellular organelle which functions in the breakdown of macromolecules into their component monomers.

Specific chemical modification of amino acid residues and its correlation with the enzymatic activity is used as a probe to investigate the involvement of these residues at the active site. Chemical modification of lysine and

cysteine residues in proteins is based upon the ability of the ϵ-NH_2 groups and sulfhydryl groups to react as nucleophiles (122, 123, 133). Reaction of diethylpyrocarbonate with histidine residues results in substitution at one of the nitrogen atoms of the imidazole ring (127). Phenylglyoxal and 1,2 - cyclohexanedione primarily modify guanidino groups of arginine residues (125, 126). The tyrosine residues may be selectively acetylated by reaction with N-acetylimidazole (130). Active benzyl halides are the specific modifiers of tryptophan residues. This reaction has been used to introduce environmentally sensitive chromophores, absorbing in the visible region, into proteins (128). Periodate oxidation is generally used in the structural investigation of oligosaccharide moiety in glycoproteins (135).

Reports on the investigation of amino acid residues and groups associated with α-mannosidase activity are very few in the literature. Human kidney acid α-mannosidase was inhibited by thiol group modifying agents suggesting a possible role of thiol group in the enzymatic function (95). Evidences for essential carboxyl groups and tryptophan residues was obtained for α-mannosidase from Phaseolus vulgaris (153, 154). The plant enzyme was shown to be a glycoprotein and had an optimum pH of 4.5. There was no indication that tyrosine, histidine and cysteine residues were involved in the enzymic activity. In all the above respects, the plant enzyme was comparable to that of placental α-mannosidase.

Most of the lysosomal enzymes are glycoproteins. The carbohydrate moieties of lysosomal enzymes consist of Asn-linked complex and high mannose-type of oligosaccharide chains. This is indicated by sugar composition, binding to certain lectins, partial sensitivity to endo-β-N-acetyl-glucosaminidase H and inhibition of glycosylation by tunicamycin (155-158). The high mannose oligosaccharides of lysosomal enzymes carry one or more phosphate groups (51,159). In a few studies, evidence for the presence of both high mannose and complex oligosaccharides in lysosomal enzymes have been shown (160-164).

Investigations on the structure of the oligosaccharide sequences of glycoproteins is facilitated by the use of lectins as a screening system. Lectins are divalent or multivalent, carbohydrate - binding proteins or glycoproteins of non immune origin. They agglutinate cells and precipitates glycoconjugates. The lectins can also distinguish complex structural features of glycoconjugates than single sugar residues. This property of lectins provide the basis for the technique of lectin affinity chromatography.

It has been well established by several workers that ConA - Sepharose column fractionate Asn-linked oligosaccharides into three classes. The unbound fractions consists of tri- and tetraantennary complex N-linked oligosaccharides as well as O-linked oligosaccharide chains. The weakly bound fraction which could be displaced by 10mM α-methyl

glucoside consist of biantennary complex types. The tightly bound fraction eluted with 500mM α-methyl mannoside or with 100mM α-methyl mannoside at 60°C contains high-mannose and hybrid type of chains (165-170). ConA presents high affinity for the trimannosidic core, Manα1→6(Manα1→3)Man, substituted by two GlcNAc residues (171). Wheat germ agglutinin is a GlcNAc-specific lectin. An intact N,N'-diacetylchitobiose moiety is required for strong binding to immobilized WGA (172). Hybrid-type glycopeptides were retarded on WGA-Sepharose column. GlcNAc β1→4Man-β1→4GlcNAcβ 1→4GlcNAc—Asn structure is essential. The lectin does not present any affinity for oligomannosidic-type glycopeptides. WGA was found to interact with NeuAc of glycoconjugates (173). Monsigny et al termed this interaction as "charge effect" (174). Ricinus communis agglutinin interacts with highest affinity with bi- and triantennary Asn-linked oligosaccharides that contain terminal galactose residues (169). Bisected biantennary oligosaccharides with two terminal gal have also been reported to bind to or retarded by immobilized RCAI (175,176).

Inferences on the structure based on chromatographic behaviour of glycoconjugates on lectin affinity columns can expedite subsequent detailed structural analysis.

PURIFICATION OF LYSOSOMAL α-MANNOSIDASE

Lysosomal α-mannosidase was purified from human placenta by a modification of the method of Khan and Basu (98). Human placental tissue was collected in ice. It was cleaned free of membrane and other connective tissue, washed thoroughly in distilled water and kept frozen at -20°C until use. All operations were carried out at 0-4°C unless otherwise stated.

Frozen tissue (500 gms) was cut into small pieces and homogenised with 2.5 l of 0.02 M phosphate buffer pH 7.0 containing 0.1 M NaCl in a Sorvall Omnimixer for 3 min at full speed. The homogenate was stirred for 30 min and centrifuged at 20,000 x g for 20 min in Sorvall RC-5B centrifuge. The supernatent was collected.

Solid ammonium sulphate (490 gms/litre) was added to the supernatent with constant stirring. The pH of the solution was adjusted to 7.0 with ammonia solution and stirred for 30 min. The suspension was centrifuged at 20,000 x g for 20 min. The precipitated enzyme was dissolved in minimum volume of 0.05 M phosphate buffer pH 7.0 containing 0.1M NaCl and dialysed against the same buffer for 18 h with two changes. The precipitate formed during dialysis was discarded by centrifugation. The NaCl concentration was raised to 0.5 M by the addition of solid NaCl.

The enzyme sample was loaded on a ConA-Sepharose column (3x14 cms) at a flow rate of 40 ml/h. The column was washed with 0.05M phosphate buffer pH 7.0 containing 0.5M NaCl at 25°C until the washings had O.D. less than 0.05 at 280nm. The bound enzyme was eluted at 25°C with the above buffer containing 0.5M methyl α-D-glucopyranoside. 10 ml fractions were collected. Active fractions (7-22) were pooled and was precipitated by the addition of solid ammonium sulphate (560 gm/l). The pH of the solution was maintained at 7.0 as before. The precipitate collected by centrifugation was dissolved in minimum volume of 0.02 M phosphate buffer pH 7.0 containing 0.1M NaCl and dialysed against the same buffer with three changes for 20 h.

The enzyme sample was made 1mM Zn^{2+} and then heated at 65°C for 1 h. The protein solution after cooling was centrifuged at 100,000 x g for 40 min in Beckman ultra-centrifuge. Supernatent was collected and dialysed against 0.02M phosphate buffer pH 7.0 containing 0.1M NaCl with three changes for 20 h.

The enzyme was mixed with anhydrous pyridine (10:1, v/v) at 23°C for 1h, and then dialysed against 0.02M phosphate buffer pH 7.0 containing 0.1M NaCl to remove pyridine. The dialysate was centrifuged at 20,000 x g for 20 min to remove any precipitate.

The clear supernatent (6ml containing 15-18 mg protein) was applied on a CM-Sephadex C-50 column (1.8 x 10 cms) equilibrated with 0.05M citrate-phosphate buffer pH 4.5 at a flow rate of 10 ml/h. The column was washed with 50 ml of the equilibrating buffer, followed by 50 ml of the same buffer containing 0.1 M NaCl. The enzyme was eluted with same buffer containing 0.3M NaCl. Four-ml fractions were collected and the active fractions (3-12) pooled. The pH was adjusted to 6.5 with 2M Tris and the enzyme sample was concentrated by ultrafiltration through Amicon YM-10 membrane.

RESULTS

The "materials and methods" not described here have been discussed earlier in chapter II. The lysosomal α-mannosidase was purified from the homogenate obtained from 500 gm of human placenta with an overall recovery of 23%. The experimental procedure is described above. Table 1. summarizes the purification steps involved. The yield of acid α-mannosidase was low when isolated according to the method of Khan and Basu (98). Pyridine treatment step, followed by an ion-exchange chromatography on CM-Sephadex C-50 at pH 4.5 introduced after the heat treatment step increased the yield considerably.

The neutral α-mannosidase was separated from the acidic activity at an early stage in the purification by chromatography on ConA - Sepharose. The neutral enzyme was not adsorbed on the affinity column. Selective heat treatment at 65°C for one hour in presence of 1mM Zn^{2+} caused complete inactivation and precipitation of β-galactosidase, α-L-fucosidase and β-D-glucosidase, with only about 10-12% loss in α-mannosidase activity. Pyridine treatment at 25°C for one hour selectively precipitated the hexosaminidase activity. Ion-exchange chromatography on CM-Sephadex C-50 at pH 4.5 and elution with 0.3M NaCl gave a single peak of enzyme activity. The purified placental α-mannosidase was free from all the other glycosidase activities.

TABLE-I

PURIFICATION OF α-MANNOSIDASE FROM HUMAN PLACENTA

Enzyme fraction	Total activity (units)	Total protein (mg)	Specific activity
Homogenate	26,500	31,000	0.85
Supernatent	23,400	21,800	1.1
Ammonium sulfate	20,000	15,500	1.3
Con A-Sepharose eluate	16,500	450	37
Heat treatment at 65°C	14,000	190	74
Pyridine treatment	11,000	120	92
CM-Sephadex C-50 chromatography	6,000	5	1200

Homogeneity of the final preparation was established by electrophoresis on 7.5% polyacrylamide gel at pH 4.3. A single band of protein was detected when stained with Coomassie brilliant blue, (Fig.7).

Molecular weight of the placental α-mannosidase was determined by gel filtration on Sephadex G-200 column. A molecular weight of 300 kDa was observed for placental acidic α-mannosidase.

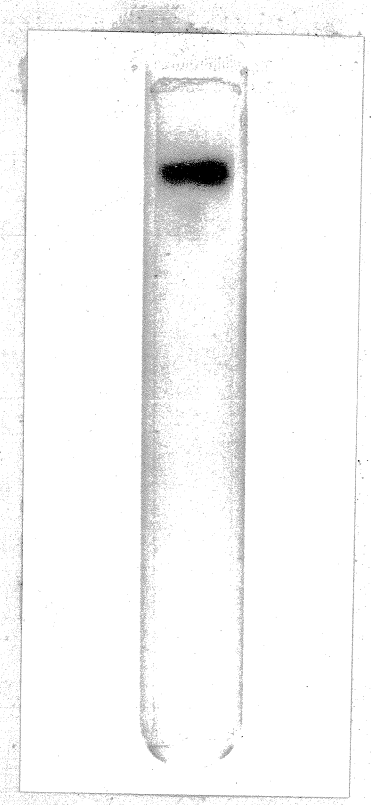


Figure 7. Polyacrylamide gel electrophoresis of purified α-mannosidase at pH 4.3.

Sodium dodecyl sulphate/polyacrylamide gel electrophoresis of proteins was carried out on 10% slab gel as described earlier. ∝-D-Mannosidase migrated as a single band corresponding to a molecular weight of 78 kDa indicating that the native enzyme is a tetramer of identical subunits (Fig.8). Dissociation of the enzyme into subunits is observed in presence and absence of β-mercaptoethanol.

The purified enzyme had a pH optimum of 4.2 with p-nitrophenyl ∝-D-mannoside as substrate, Km value obtained was 2mM. Zinc had no effect on pH optimum of the enzyme. Presence of 2mM Zn^{2+} in the assay medium caused upto 20-25% activation at all stages of purification.

Figure 9 illustrates the effect of pH and Zn^{2+} on the stability of the enzyme. Purified ∝-mannosidase was incubated at 37°C in phosphate buffer pH 6.5 and citrate-phosphate buffer pH 4.2, respectively. An aliquot was taken out at one hour interval and assayed under conditions in which Zn^{2+} was not included in the standard assay system. The enzyme retained its original activity even after 24 h of incubation at pH 6.5. However, there was gradual loss of enzyme activity when enzyme alone was incubated at pH 4.2 and 37°C unless Zn^{2+} was added to the incubation sample. Addition of Zn^{2+} to an inactivated enzyme did not restore the original activity. The externally added Zn^{2+} protected the enzyme from further inactivation as illustrated in Fig.9.

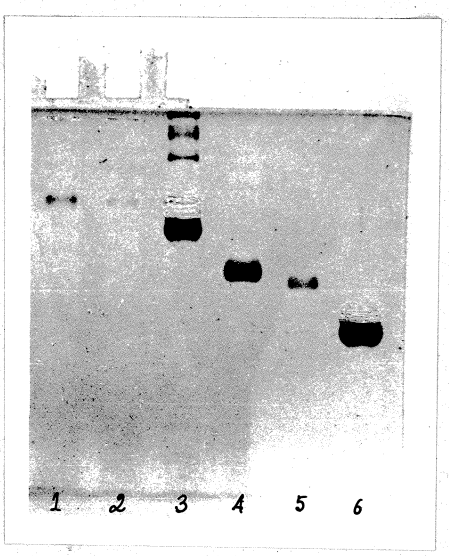


Figure 8. SDC-PAGE of purified placental -mannosidase on 10% acrylamide gel.
1. and 2. α-mannosidase; 3. bovine serum albumin; 4. ovalbumin; 5. aldolase; 6. winged bean agglutinin.

Details of the experiment are described in Chapter II.

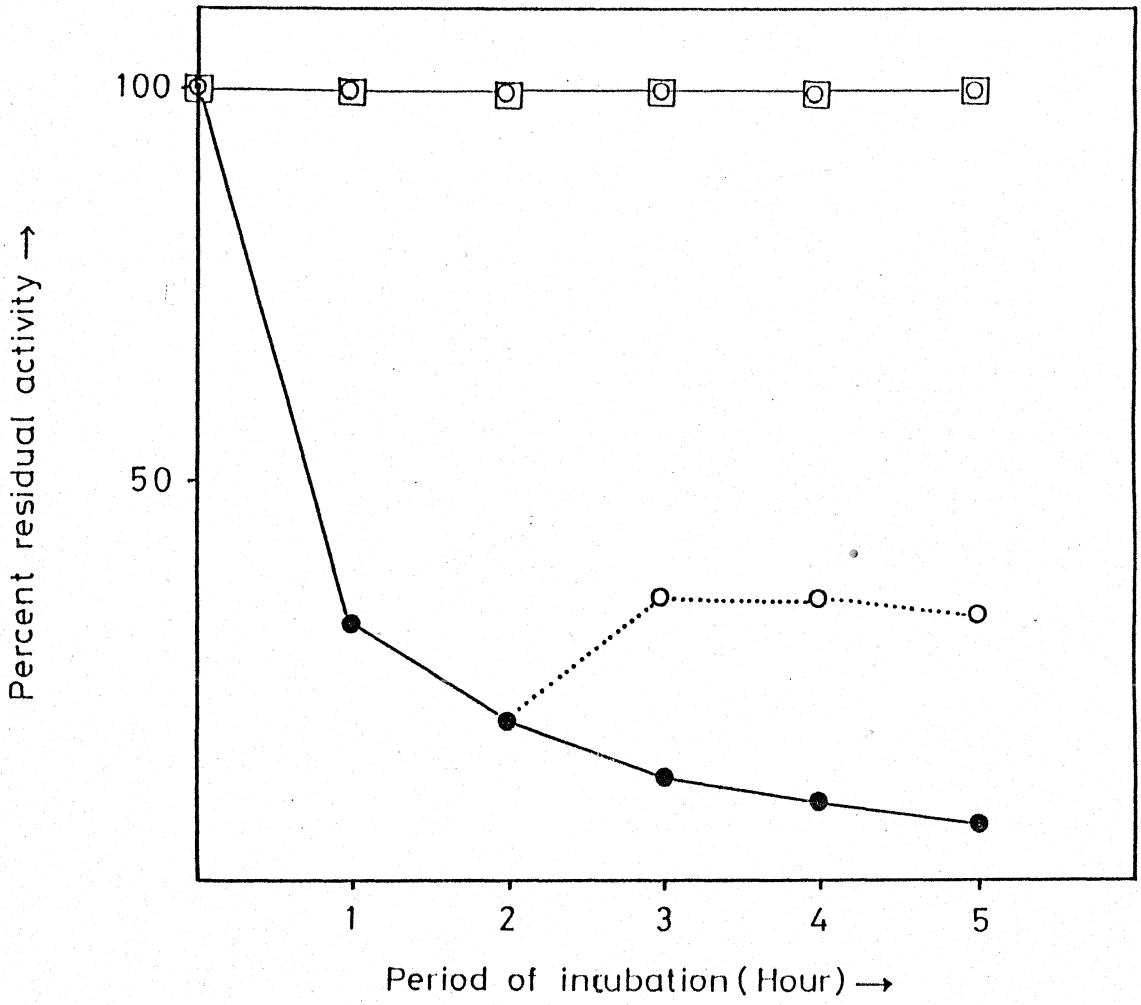


Figure 9. Effect of pH and Zn^{2+} on placental ∝-mannosidase activity

□—□ = enzyme alone at pH 6.5

○—○ = enzyme + lmM $ZnSO_4$ at pH 4.2

●—● = enzyme alone at pH 4.2

○···○ = enzyme + lmM $ZnSO_4$ (added after 2 hours of incubation) at pH 4.2

Results are expressed as a percentage of the activity of the enzyme preparation at 0 hour.

In another experiment, the purified placental enzyme was preincubated with EDTA (4mM) at 25°C in citrate-phosphate buffer pH 4.2 for 20 min. The EDTA - treated enzyme when assayed was found to have 47% of the activity of the control. Addition of excess of Zn^{2+} (8-20mM) to an EDTA-inactivated enzyme caused complete restoration of the original activity instantaneously. The reactivation of an EDTA-treated enzyme was observed even in presence of the substrate, suggesting that the substrate cannot combine with the metal - free enzyme protein.

Table II shows the effect of various substrate analogues on α-mannosidase activity. The enzyme was preincubated with the substrate analogues for 20 min at 25°C and then assayed under standard assay conditions. Mannonic acid - γ-lactone (1mM) and mannose-6-phosphate (10mM) did not inhibit enzyme activity. 100mM methyl α-D-manno-pyranoside caused a decrease of 33% of the placental enzyme. Mannosamine hydrochloride (20mM), before and after adjustment of its pH to 7.0, produced 50 and 74% inhibition of the enzyme activity, respectively.

Legler and Julich studied the inhibitory properties of 5-amino-5-deoxymannopyranose and 1-deoxymannojirimycin with α- and β-mannosidases from several sources (105). 0.6mM 1-deoxymannojirimycin and 0.25mM of 5-amino-5-deoxy-mannopyranose inhibited 50% and 100% of the placental α-mannosidase activity, Table II. 1-Deoxymannojirimycin is a relatively weak inhibitor.

TABLE II

EFFECT OF SUBSTRATE ANALOGUES ON ∝-MANNOSIDASE ACTIVITY

Substrate analogue	Final concentration (M)	% inhibition
Mannonic acid- ϒ -lactone	10^{-3}	0
Mannose-6-phosphate	10^{-2}	0
Methyl- ∝-D-mannopyranoside	10^{-1}	33
Mannosamine hydrochloride	2×10^{-2}	50
1 -Deoxymannojirimycin	6.0×10^{-4}	50
5 -amino-5-deoxy-mannopyranose	2.5×10^{-4}	100

The pioneering work of Dorling and associates led to the identification of swainsonine as a potent inhibitor of lysosomal ∝-D-mannosidase (67). Complete inhibition of placental enzyme was observed with 10^{-7} M concentration of Swainsonine. Ki for swainsonine from the Dixon plot was found to be 2×10^{-8}M, Fig.10 (177). Kinetics of inhibition show that it is a competitive inhibitor.

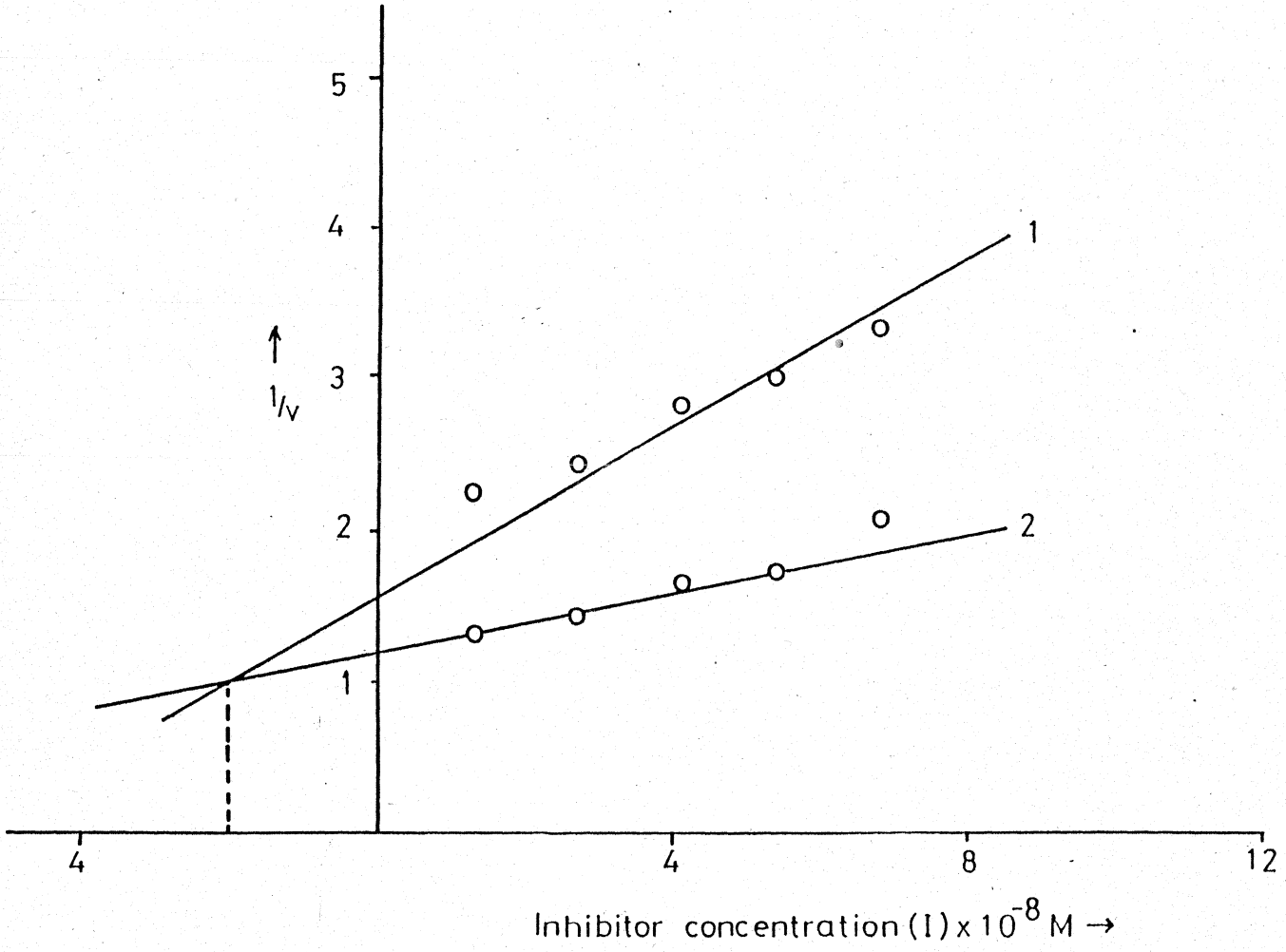


Figure 10. Dixon plot for the determination of Ki for swainsonine with p-nitrophenyl -mannoside as substrate (1). 1mM p-nitrophenyl -mannoside (2) 2mM p-nitrophenyl -mannoside. Details of the experiment are in the text.

The effect of chemical modifications of amino acid residues of α-mannosidase of human placenta are listed in Table III. One mole of the enzyme contained 152 moles of amino groups. The enzyme lost 60% of its activity when amino groups were reacted with trinitrobenzene sulfonic acid. Maleylation completely inactivated the enzyme, suggesting the essentiality of the amino groups for the biological activity of the enzyme. Eight moles of tryptophan residues were estimated per mole of the enzyme. Chemical modification of these residues with active benzyl halides completely abolished enzymatic activity suggesting a possible role of tryptophan residues in the active site of the enzyme. Phenylglyoxal hydrate and 1,2-cyclohexanedione, which primarily modify guanidino groups of arginine caused 90% and 40% inhibition of enzyme activity respectively.

One mole of the enzyme contained 24 moles of tyrosine residues, however O-acetylation of the tyrosine residues had no effect on enzymic activity. Specific modification of histidine residues with diethylpyrocarbonate did not result in any loss of enzymatic activity. The enzyme contained 16 sulfhydryl groups and 6 disulfide bonds per mole of the enzyme. However, sulfhydryl group modifiers like p-hydroxymercuribenzoate, N-ethylmaleimide and iodoacetic acid had no effect on the enzyme activity. These results suggested the non-involvement of tyrosine, histidine and SH groups in the catalytic activity of the enzyme.

TABLE III

CHEMICAL MODIFICATION OF AMINO ACID GROUPS AND OLIGOSACCHARIDE CHAINS

Modifying Agent	Amino acid group or oligosaccharide modified	% Residual Activity
Trinitrobenzene sulfonate	$-NH_2$	40
Maleic Anhydride	$-NH_2$	0
2-Hydroxynitrobenzyl bromide	Trp	0
2-Methoxynitrobenzyl bromide	Trp	0
Phenylglyoxal hydrate	Arg	10
1,2-Cyclohexanedione	Arg	60
Diethylpyrocarbonate	His	100
N-Acetylimidazole	Tyr	100
p-hydroxymercuribenzoate	-SH	100
Iodoacetic acid	-SH	100
N-Ethylmaleimide	-SH	100
Periodic acid 10^{-1}M	Oligosaccharides	0
10^{-2}M		90

Periodate oxidation of oligosaccharide chains at 10mm and 100mM concentration of the reagent inhibited 10 and 100% of the control activity, respectively, Table III. These results suggest a possible role of the oligosaccharide chains in the biological activity of the enzyme.

The N-terminal amino acid sequence of the enzyme was carried out by the dansyl-Edman procedure. The N-terminal sequence was found to be 'Serine-Lysine-Methionine-'. Dansylation of the protein subunits resulted in the modification of the α-NH_2 group of the N-terminal amino acid, serine, as well as all the ϵ-NH_2 group of the lysine residues. The Dns-ϵ-Lys peak was negligible in case of swainsonine treated enzyme when compared to the native enzyme subunit (Fig. 11)

LECTIN AFFINITY CHROMATOGRAPHY

The structure of the oligosaccharide sequences of the enzyme was investigated by affinity chromatography on various immobilised Lectins. Placental α-mannosidase (600 units) was subjected to affinity chromatography on a ConA-Sepharose column (1x8cms) equilibrated with 0.02M phospate buffer pH 7.0 containing 0.5 NaCl at $4^{o}C$. The column was washed with 50 ml of the same buffer at $25^{o}C$ and eluted with the above buffer containing 10mM and 500mM methyl α-D-mannopyranoside respectively at $25^{o}C$. One-ml fractions were collected. The elution pattern from ConA-Sepharose is shown in Fig.12. 10mM and 500mM methyl α-D-mannopyranoside eluate contained 20% and 54% of the applied activity.

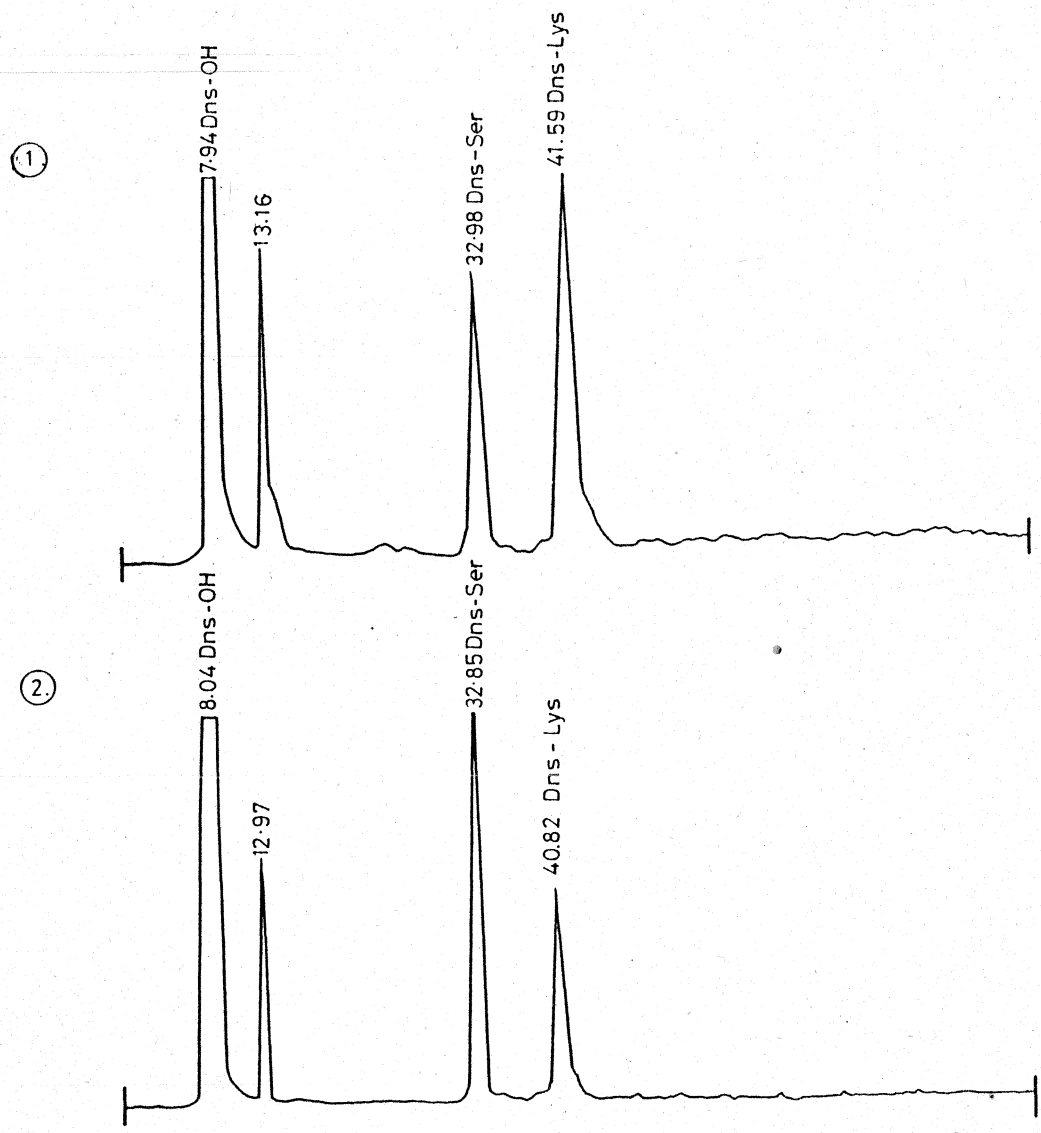


Figure 11. HPLC of dansylated subunits of (1) native and (2) swainsonine bound ∝-mannosidase. Volume applied to the HPLC column were 10 µl and 20 µl respectively.

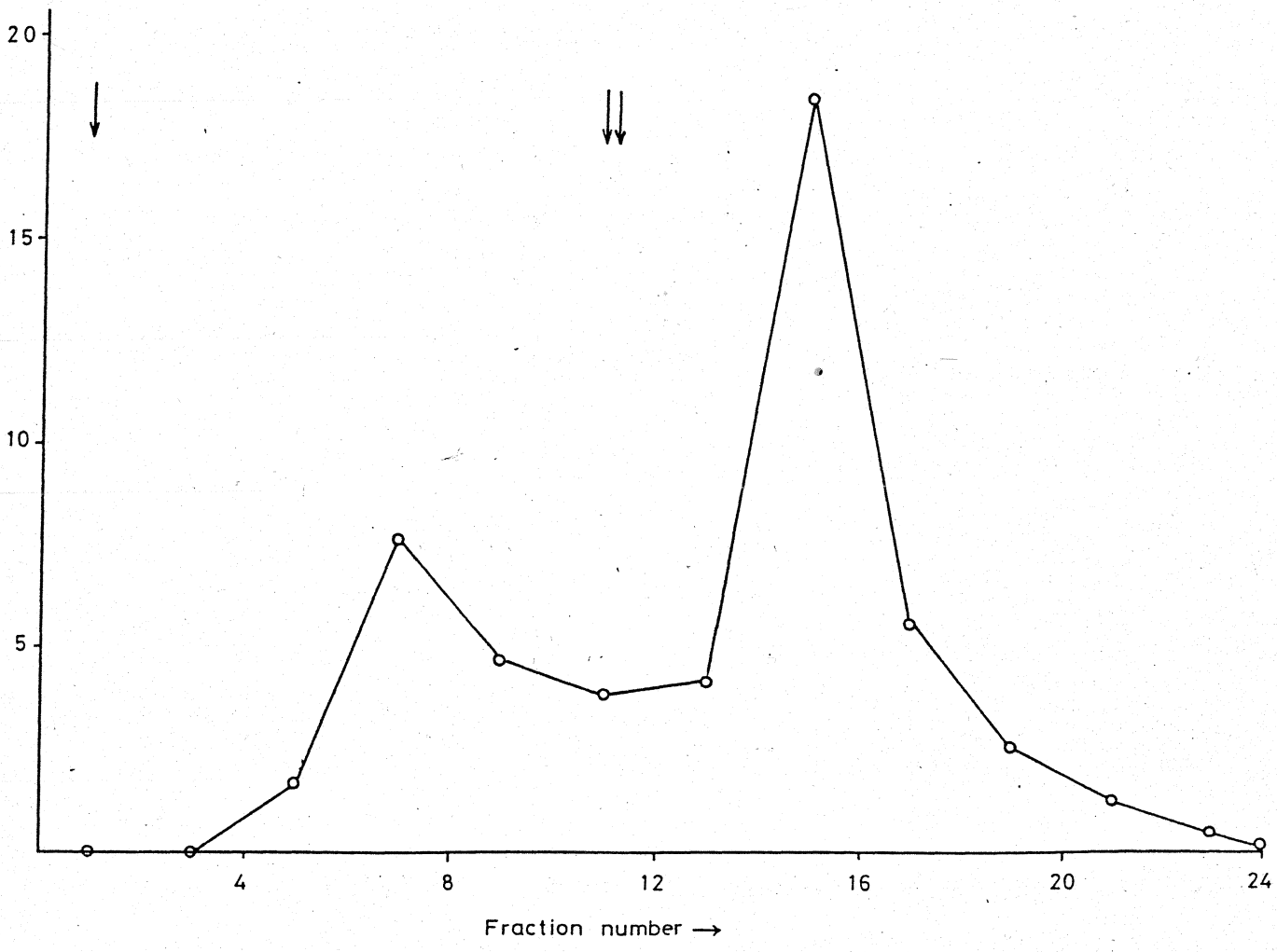


Figure 12. Affinity chromatography of placental α-mannosidase on Con A-Sepharose column. Elution of the bound enzyme was carried out with 10mM (↓) and 500mM (⇊) methyl α-D-mannoside. o = enzyme activity (units)

Details of the experiment are in the text.

The enzyme containing 250 units of activity was applied to a WGA-Sepharose column (1x8cms) equilibrated with 0.02M phosphate buffer pH 7.0 containing 0.1M NaCl. The column was washed with 15ml of the same buffer followed by elution with 0.05M GlcNAc in the same buffer. One-ml fractions were collected. The elution profile from WGA-Sephorose is as shown in Fig.13. The unbound fraction contained 15% of the applied activity while 55% of the activity was recovered in the bound fraction.

Placental enzyme containing 250 units of activity was loaded on RCA-Sepharose column (1x6cm) equilibrated with 0.02M phosphate buffer pH 7.0 containing 0.1M NaCl. The column was washed with 20ml of the same buffer. One-ml fractions were collected. No enzyme activity could be detected in the unbound fractions. Elution of the bound enzyme was tried with the above buffer containing 1) 100mM galactose 2) 0.2M lactose + 1M NaCl 3) glycopeptides prepared from placental ConA-Sepharose eluate. The enzyme could not be eluted from the RCA-Sepharose column. The RCA-Sepharose bound α-mannosidase retained enzymic activity for eighteen months when stored at $4^{o}C$.

IMMUNOLOGICAL STUDIES

Antibody was raised in rabbit against placental α-mannosidase. Immunoglobulins purified from the resulting serum was passed successively through Protein A-Sephorose and JSA-Sepharose columns which specifically bind IgG and

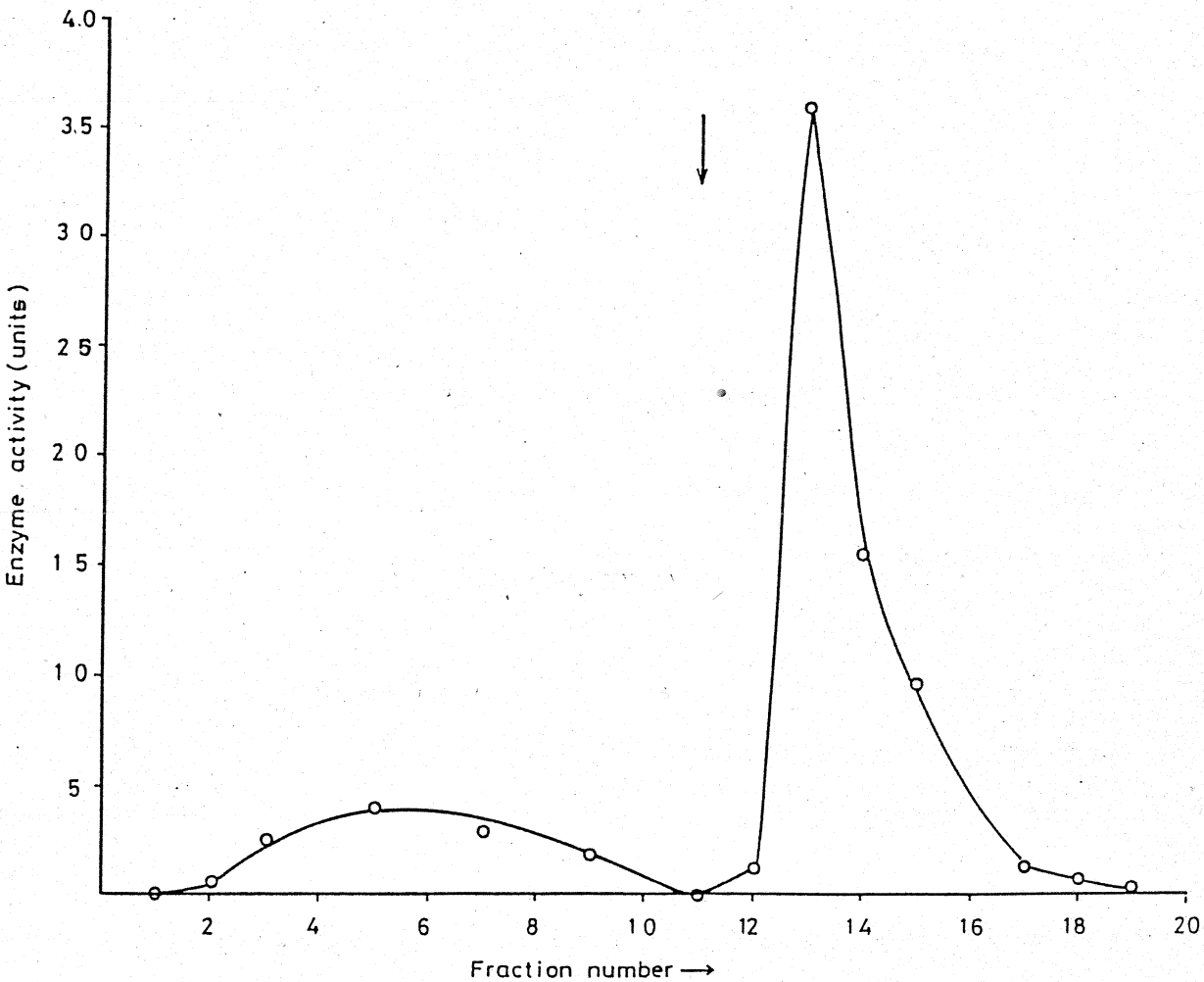


Figure 13. Affinity chromatography of placental α-mannosidase on WGA-Sepharose column. The bound enzyme was eluted with 0.05M GlcNAc (arrow). o = enzyme activity (units).

Details of the experiment are described in the text.

IgA class of immunoglobulins respectively (119, 178). Immunodiffusion of the immunoglobulins eluted from the two columns was carried out against the placental enzyme by Ouchterlony double diffusion technique, Fig.14. IgG eluted from the Protein A-Sepharose column precipitated the enzyme protein. No such immunoprecipitation was observed with the unbound and bound immunoglobulins (IgM and IgA respectively) from JSA-Sepharose column.

In another experiment, the enzyme was incubated with its antibody at 25°C for 1h. The enzyme-antibody complex isolated and washed was found to retain complete enzymic activity. Incubation of the active enzyme-antibody complex with swainsonine (10^{-7}M) resulted in complete loss of enzymic activity.

The soluble extracts containing α-mannosidase activity was prepared from human fetal organs. The antiserum raised against purified human placental α-mannosidase was tested against the α-mannosidase activity in human fetal tissues by the Ouchterlony double diffusion technique. Immunological cross reativity of the placental enzyme antibody was observed with α-mannosidase activity from human fetal heart, liver, brain, kidney, placenta and skeletal muscle, Fig.15.

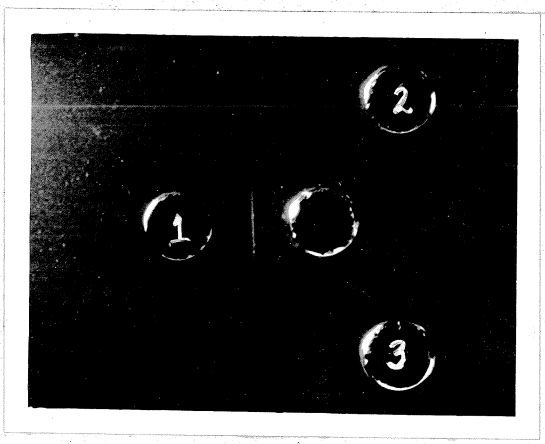


Figure 14. Immunodiffusion of placental ∝-mannosidase against immunoglobulins of the enzyme.

Central well - placental ∝-mannosidase.

1. IgG (eluted from Protein A-Sepharose column),
2. IgA (eluted from JSA-Sepharose column),
3. IgM (unbound from JSA-Sepharose column).

Details of the experiment are described in the text.

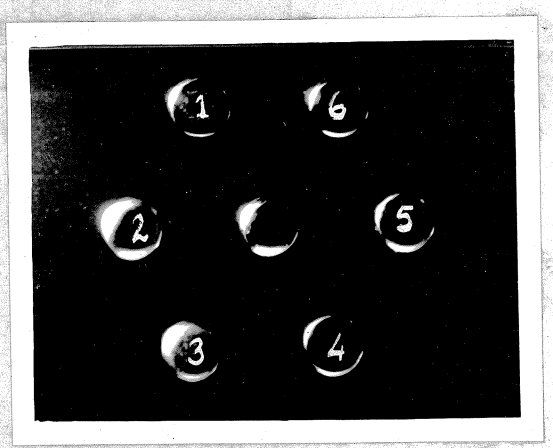


Figure 15. Immunological cross-reactivity of placental α-mannosidase antibody with α-mannosidase activity in human fetal organs.

Central well - placental α-mannosidase antibody,

1. Fetal placenta;
2. Fetal brain;
3. Fetal liver;
4. Fetal heart;
5. Fetal kidney;
6. Fetal skeletal muscle.

Details of the experiment are described in the text.

DISCUSSIONS

The lysosomal acid α-mannosidase was purified from human placenta by a modification of the method of Khan and Basu (98). The yield of the enzyme was considerably increased by the modified method of purification. Ion-exchange chromatography on CM-Sephadex C-50 at pH 4.5 and elution with 0.3M NaCl suggested strong basicity of the enzyme. This is in agreement with its lack of mobility in alkaline pH electrophoresis and high mobility in acid pH electrophoresis (98). The purified enzyme on polyacrylamide gel electrophoresis at pH 4.5 revealed a single band when stained with Coomassie blue.

Molecular weight of the native placental α-mannosidase was found to be 300 kDa by gel filtration method. A molecular weight of 180 kDa was obtained for human kidney α-mannosidase while that of bovine kidney was between 275 and 300 kDa (144, 95). Human liver acid α-mannosidase A was found to have a molecular weight of 220 kDa and that of B form 300 kDa (94). Determination of subunit molecular weight by SDS-PAGE with and without 2-mercaptoethanol revealed a single band of protein corresponding to a molecular weight of 78kDa. These results suggest that the enzyme is a homotetramer of noncovalently associated subunits. Human kidney α-mannosidase dissociated

into two subunits with molecular weight of about 58 kDa and 30 kDa respectively. The enzyme is assumed to have two heavy and two light chains (95).

pH optimum of the placental α-mannosidase was found to be 4.2 and was unaffected in presence of Zn^{2+}. In case of human liver and rat epididymal enzymes, Zn^{2+} lowered the pH optimum (94,143). The dependence of α-mannosidase enzymic activity for stability in the presence of Zn^{2+} has been reported for several mammalian acid α-mannosidases (99,143-145). The effect of varying the pH on the stability of placental α-mannosidase is markedly influenced by the addition of Zn^{2+}. At pH 6.5 the enzyme was quite stable, but at pH 4.2, there was gradual loss of enzyme activity in the absence of zinc ion. If the instability of α-mannosidase below neutral pH was only due to a simple dissociation of the protein-zinc complex, it would be expected that the addition of Zn^{2+} to an inactivated sample would cause restoration of the original activity as observed in case of jack-bean α-mannosidase and rat epididymal enzyme (93,143). In the present study, however, the externally added Zn^{2+} could not restore the original activity.

One of the most striking indications of the importance of Zn^{2+} for the enzyme activity was obtained with EDTA-treated enzyme. Treatment of the purified enzyme with EDTA retained only 47% of the original activity. Addition of excess of Zn^{2+} to an EDTA-treated enzyme restored the original activity. Reactivation of an EDTA-treated enzyme

by Zn^{2+} in presence of the substrate suggested that the substrate cannot combine with the metal-free enzyme protein. This pattern of behaviour with EDTA and Zn^{2+} has been found in rat liver lysosomal and rat epididymal α-mannosidases (96, 143). Smith has reported that jack-bean α-mannosidase is a true zinc metalloprotein (93).

There was no inhibition of enzymic activity by several substrate analogues. Swainsonine completely inhibited placental enzyme activity at 10^{-7}M concentration. The reversible inhibition of jack bean and lysosomal α-D-mannosidases has previously been suggested to be similar in nature but "quite complex" (67). However, specific differences in the action of swainsonine on these two enzymes and on Golgi mannosidase II have been reported by Tulsiani et al (179). The stearic similarities between swainsonine and mannosyl ion may account for the specificity of swainsonine for α-mannosidase. 5-amino-5-deoxy-mannopyranose and 1-deoxymannojirimycin which are mannose analogues inhibited the placental enzyme activity. 5-amino-5-deoxymannopyranose completely inhibited the enzyme at 2.5×10^{-4}M concentration. Its Ki was found to be 14mM (180). 1-Deoxymannojirimycin with dehydroxylation at C-1 position, was a weaker inhibitor. Substitution of the hydroxyl group at position 5 in case of 5-amino-5-deoxymannopyranose, did not impair its specificity for the enzyme. 1-Deoxymannojirimycin inhibited Golgi α-mannosidase IA and IB in vivo and thus blocked the conversion of high

mannose to complex oligosaccharides (68). Man_9 mannosidase from calf liver microsomes was inhibited by 1-deoxy-mannojirimycin with a Ki of 7 μM(48).

The role of amino acid residues and groups involved in the active centre of an enzyme can be studied by the application of specific modifying agents. The loss of enzymic activity by amino group modifications by trinitrobenzene sulfonic acid treatment and maleylation suggested a role of this group at the catalytic site. Reaction of α-mannosidase with tryptophan modifying reagents, 2-hydroxy - and 2-methoxy-5-nitrobenzyl bromide resulted in loss of enzyme activity. Phenylglyoxal hydrate and 1,2-cyclohexanedione which modify arginine residues inhibited α-mannosidase activity. These results suggest the involvement of lysine, tryptophan and arginine residues at the active site of the enzyme. There was no indication of the role of cysteine, tyrosine and histidine residues in the enzymatic activity.

The N-terminal amino acid sequence of α-mannosidase was found to be 'Serine-Lysine-Methionine-' by the sequential degradation method of Edman using phenylisothiocyanate and identification of the newly released amino-terminal residues as dansyl derivatives at each cycle by reaction with dansyl chloride (136). The N-terminal amino acid was reported to be blocked in case of α-mannosidase purified from Phaseolus vulgaris (181). Dansylation of the enzyme subunits resulted in the modification of all the ϵ-NH_2 groups

of the lysine residues as well as the α-NH_2 group of the N-terminal amino acid, serine. The Dns-ϵ-lysine peak was negligible in case of swainsonine bound enzyme than in the native enzyme subunit. In presence of swainsonine, the lysine groups are not free for dansylation. This observation is in accordance with the results of chemical modification studies, which suggest that lysine residues are involved at the active site of the enzyme.

The enzyme contained 8% sugar as estimated with galactose as standard. NeuAc,Glc,Gal,Man,GlcNac and GalNAc have been reported from this enzyme (180). Periodate oxidation at 10 and 100mM concentration inhibited the enzyme activity by 10 and 100% respectively, of the control. On this basis, it may be suggested that oligosaccharides may have some role to play in placental α-mannosidase activity. Affinity chromatography of the enzyme on immobilised ConA, WGA and RCA indicated the presence of high-mannose, hybrid and complex-type of oligosaccharide chains. The 10mM α-methyl mannoside eluate from ConA - Sepharose column predominantly contained biantennary complex type of chains, whereas 500mM α-methyl mannoside eluate contained high-mannose and hybrid-types of chains. The unbound and bound fractions of the enzyme from WGA-Sepharose column contained high mannose and hybrid type of chains, respectively. There was irreversible binding of the enzyme on immobilised RCA, and this enzymic activity was retained for eighteen months when stored at $4^{o}C$. The unusual

behaviour of the enzyme on RCA-Sepharose cannot be explained at present. Acid α-mannosidase from monkey brain, however, has been separated on RCA-Sepharose into bound and unbound forms (160). Lysosomal acid hydrolases are glycoproteins containing high mannose oligosaccharides capable of binding to the lectin ConA (182,183). Instances of lysosomal glycosidases containing both high mannose and complex oligosaccharides have been indicated (160-164).

The antibody raised to plecental α-mannosidase was passed in tandem through Protein A - Sepharose and JSA - Sepharose columns (119,178). The IgG-specific protein A - Sepharose eluate precipitated the enzyme protien. The IgA - specific JSA — Sepharose eluate and the unbound immunolgobulin (IgM) from JSA-Sepharose did not show any reactivity with the enzyme, suggesting that the placental α-mannosidase antibody belonged to the class IgG.

The enzyme-antibody complex retained full enzymic activity, and incubation of the active enzyme-antibody complex with swainsonine completely inactivated the enzyme. It may be interpreted that the antigenic determinant site and the catalytic site of the enzyme are located at different positions of the polypeptide chain.

Immunological cross reactivity of the placental α-mannosidase antibody was observed with acid enzyme activities from human fetal heart, liver, brain, kidney, placenta and skeletal muscle. This suggests the presence of antigenic determinants for the specific antibody in the

early stages of development. The antiserum raised against human liver acid α-mannosidase precipitated acidic α-mannosidase activity from brain, kidney, leucocytes as well as liver (152). Considering these results it is unlikely that the enzyme exists in organ-specific forms that are immunologically distinct.

SUMMARY

The widespread occurrence of α- linked D - mannose residues in mammalian glycoproteins and the recognition of the deficiency of lysosomal acid α-mannosidase in human and bovine mannosidoses created an interest in the mammalian enzyme. The present study included investigations on the structure and function of lysosomal α-mannosidase purified from an useful hospital waste, human placenta.

Lysosomal α-D-mannosidase was purified from frozen human placenta by modifying the earlier procedure developed in this laboratory. The enzyme was homogeneous on polyacrylamide gel electrophoresis at pH 4.3 and it did not move at pH between 7.0 to 8.3. The native enzyme had a molecular weight of 300 kDa by gel filtration on Sephadex G-200. SDS - polyacrylamide gel electrophoresis of the enzyme, in presence and absence of 2 -mercaptoethanol, revealed a single band corresponding to a molecular weight of 78 kDA. It may be tentatively concluded that the placental α-mannosidase is a homotetramer of non covalently associated subunits. The enzyme was optimally active at pH 4.2. The biological activity of the placental

α-mannosidase gradually decreases at this pH range, unless Zn^{2+} was added. The catalytic activity of the enzyme also decreased to half on treatment with the chelating agent, EDTA. Addition of excess of Zn^{2+} to an EDTA-treated enzyme restored the original activity.

Substrate analogues like mannonic acid-γ-lactone and mannose - 6 - phosphate had no effect on enzyme activity. Methyl α-D-mannopyranoside and mannosamine inhibited 33% and 50% of the enzyme activity at 0.1 M and 0.02 M concentration respectively. 5 -amino-5-deoxymannopyranose completely inhibited the enzyme activity at 2.5 x 10^{-4}M concentration, whereas 1-deoxymannojirimycin inhibited only 50% of the enzyme activity at 6 x 10^{-4}M. Swainsonine was found to be the most potent inhibitor of the enzyme with a Ki of 2 x 10^{-8}M. It was observed to be a competitive inhibitor.

Modification of amino groups with maleic anhydride and trinitrobenzene sulfonic acid inhibited the enzyme activity. Tryptophan modifying agents like 2-hydroxy and 2-methoxy-5-nitrobenzyl bromide completely abolished the enzyme activity. Reaction of α-mannosidase with arginine modifiers, phenylglyoxal hydrate and 1,2-cyclohexanedione resulted in loss of biological activity. These results suggested the involvement of lysine, tryptophan and arginine residues at the catalytic site of the enzyme. Modification of cysteine, tyrosine and histidine had no effect on the enzymic activity. The N-terminal amino acid sequence of the enzyme was found to be 'Serine-Lysine-Methionine-'.

Placental α-mannosidase is a glycoprotein. The neutral sugar content was 8%. Periodate oxidation of the oligosaccharides at 10mM and 100mM concentration inhibited 10% and 100% of the enzyme activity respectively. This suggested a possible role of the oligosaccharide chains in the biological activity of the enzyme. Affinity chromatography of the placental enzyme on immobilised ConA, WGA and RCA indicated the presence of high mannose, complex and hybrid-type of Asn-linked oligosaccharide chains.

The placental enzyme antibody raised in rabbit belonged to IgG class. Retention of catalytic activity by the enzyme-antibody complex and abolishment of the same on incubation with swainsonine suggested that the antigenic determinant site and catalytic site are topologically distinct. Human placental α-mannosidase antibody cross-reacted with the same enzyme from human fetal organs.

BIBLIOGRAPHY

1. Montreuil, J. (1980). Adv. Carbohydr. Chem. Biochem., 37, 158.

2. Zinn, A.B., Planter, J.J., and Carlson, D.M. (1977) in "The Glycoconjugates" (Horowitz, M.I. and Pigman, W., eds.), Vol. I, p.69, Academic Press, New York.

3. Kornfeld, R. and Kornfeld, S. (1976) Ann. Rev. Biochem., 45, 217.

4. Tai, T., Yamashita, K., Setsuko, I. and Kobata, A. (1977) J. Biol. Chem., 252, 6687.

5. Yamashita, K., Tachiubana, Y. and Kobata, A. (1978) J. Biol. Chem., 253, 3862.

6. Shier,W.T., Lim, Y., and DeVries, A.L. (1972) Biochim. Biophys. Acta., 263, 406.

7. Gottschalk, A.(1960) Nature, 186, 949.

8. Vandenheede, J.R., Ahmed, A.I. and Fecney, R.E. (1972) J.Biol. Chem., 247, 7885.

9. Gottschalk, A . and Fazekas de St. Groth, S. (1960) Biochim. Biophys. Acta., 43, 513.

10. Olden, K., Pratt, R.M. and Yamada, K.M. (1978) Cell, 13, 461.

11. Ashwell, G. and Morell, A.G. (1977) Trends Biochem. Sci., 2, 76.

12. Hickman, J., Ashwell, G., Morell, A.G., Vanden Hamer, C.J.A. and Scheinberg, I.H. (1970) J. Biol. Chem., 245,759.

13. Morell, A.G., Irvine, R.A., Sternlieb, I., Scheinberg, I.H. and Ashwell, G. (1968) J. Biol. Chem., 243, 155.

14. Kawasaki, T. and Ashwell, G. (1976) J. Biol. Chem., 251, 1296.

15. Stahl, P., Six, H., Rodman, J.S., Schlesinger, P., Tulsiani, D.R.P. and Touster, O. (1976) Proc. Natl. Acad. Sci., 73, 4045.

16. Prieels, J.P., Pizzo, S.V., Glasgow, L.R., Paulson, J.C., and Hill, R.L. (1978) Proc. Natl. Acad. Sci., 75, 2215.

1. Montreuil, J. (1980). Adv. Carbohydr. Chem. Biochem., 37, 158.

2. Zinn, A.B., Planter, J.J., and Carlson, D.M. (1977) in "The Glycoconjugates" (Horowitz, M.I. and Pigman, W., eds.), Vol. I, p.69, Academic Press, New York.

3. Kornfeld, R. and Kornfeld, S. (1976) Ann. Rev. Biochem., 45, 217.

4. Tai, T., Yamashita, K., Setsuko, I. and Kobata, A. (1977) J. Biol. Chem., 252, 6687.

5. Yamashita, K., Tachiubana, Y. and Kobata, A. (1978) J. Biol. Chem., 253, 3862.

6. Shier,W.T., Lim, Y., and DeVries, A.L. (1972) Biochim. Biophys. Acta., 263, 406.

7. Gottschalk, A.(1960) Nature, 186, 949.

8. Vandenheede, J.R., Ahmed, A.I. and Fecney, R.E. (1972) J.Biol. Chem., 247, 7885.

9. Gottschalk, A . and Fazekas de St. Groth, S. (1960) Biochim. Biophys. Acta., 43, 513.

10. Olden, K., Pratt, R.M. and Yamada, K.M. (1978) Cell, 13, 461.

11. Ashwell, G. and Morell, A.G. (1977) Trends Biochem. Sci., 2, 76.

12. Hickman, J., Ashwell, G., Morell, A.G., Vanden Hamer, C.J.A. and Scheinberg, I.H. (1970) J. Biol. Chem., 245,759.

13. Morell, A.G. , Irvine, R.A., Sternlieb, I., Scheinberg, I.H. and Ashwell, G. (1968) J. Biol. Chem., 243, 155.

14. Kawasaki, T. and Ashwell, G. (1976) J. Biol. Chem., 251, 1296.

15. Stahl, P., Six, H., Rodman, J.S., Schlesinger, P., Tulsiani, D.R.P. and Touster, O. (1976) Proc. Natl. Acad. Sci., 73, 4045.

16. Prieels, J.P., Pizzo, S.V., Glasgow, L.R., Paulson, J.C., and Hill, R.L. (1978) Proc. Natl. Acad. Sci., 75, 2215.

17. Sly, W.S. and Stahl, P. (1978) Life Sci. Res. Rep., 11, 229.

18. Watkins, W.M. and Morgan, W.T.J. (1952) Nature, 169, 825

19. Paulson, J.C., Sadler, J.E. and Hills, R.L. (1979) J. Biol. Chem., 254, 2120.

20. Meezan, E., Wu,H., Black, P. and Robbins, P.W. (1969) Biochemist , 8, 2518.

21. Glick, M.C. and Warren, L. (1974) Meth. Memb. Biol., 2, 157.

22. Sharon, N. (1979) in "Structure and Function of Biomembranes" (K. Yagi., ed.), p.63, Japan Scientific Society Press, Tokyo.

23. Schachter, H. and Roseman, S. (1980) in "Biochemistry of Glycoproteins and Proteoglycans" (Lennarz, W.J., ed.), p. 85, Plenum Press, New York.

24. Hubbard, S.C. and Ivatt, R.J. (1981) Ann. Rev. Biochem., 50, 555.

25. Kornfeld, R. and Kornfeld, S. (1985) Ann. Rev. Biochem., 54, 631.

26. Beyer, T.A., and Hills, R.L. (1982) in "The Glycoconjugates" (Horowitz, M.I., ed.), Vol.3, p.25, Academic Press, New York.

27. Hanover, J.A. and Lennarz, W.J. (1981) Arch. Biochem. Biophys., 211, 1.

28. Hersh, I. (1949) Arch. Pathol., 47, 99.

29. Leblond, C.P. (1950) Am. J. Anat., 86, 1.

30. Roth, J. (1987) Biochim. Biophys. Acta., 906, 405.

31. Behrens, N.H., and Leloir, L.F. (1970) Proc. Natl. Acad. Sci., 66, 153.

32. Beyer, T.A., Sadler, J.E., Rearick, J.I., Paulson, J.C. and Hill, R.L. (1981) Adv. Enzymol., 52, 23.

33. Behrens, N.H., Parodi, A.J. and Leloir, L.F. (1971) Proc. Natl. Acad. Sci., 68, 2857.

34. Schachter,H. (1978) in "The Glycoconjugates" (Horowitz, M.I. and Pigman, W., eds.), vol. 2, p.87, Academic Press, New York.

35. Beyer, T.A. and Hill, R.L. (1982) in "The Glycoconjugates" (Horowitz, M.I., ed.). Vol.3. p.25, Academic press, New York.

36. Li, E., Tabas, I. and Kornfeld, S. (1976) J. Biol. Chem., 253, 7762.

37. Struck, D.K. and Lennarz, W.J. (1977) J. Biol. Chem., 252, 1007.

38. Kornfeld, S., Gregory, W. and Chapman, A. (1979) J. Biol. Chem., 254, 11649.

39. Elbein, A.D. (1984) CRC Crit. Rev. Biochem., 16, 21.

40. Staneloni, R.J., Ugalde, R.A. and Leloir, L.F. (1980) Eur. J. Biochem., 105, 275.

41. Perez, M., and Hirschberg, C.B. (1986) J. Biol. Chem., 261, 6822.

42. Welply, J.K., Kaplan, A., Shenbagamurthy, P., Naider, F. and Lennarz , W.J. (1986) Arch. Biochem. Biophys., 246, 808.

43. Marshall, R.D. (1972) Ann. Rev. Biochem., 41, 673.

44. Kroquist, K.E., and Lennarz, W.J. (1978) J. Supramol. Struct., 8, 51.

45. Tabas, I., Schleisinger, S. and Kornfeld, S. (1978) J. Biol. Chem., 253, 716.

46. Elting, J.J., Chen, W.W. and Lennarz, W.J. (1980) J. Biol. Chem., 255, 2325.

47. Godelaine, D., Spiro, M.J. and Spiro, R.G. (1981) J.Biol. Chem., 256, 10161.

48. Bischoff, J. and Kornfeld, S. (1986) J. Biol. Chem., 261, 4758.

49. Bischoff, J. and Kornfeld, S. (1986) J. Biol. Chem., 261, 4766.

50. Schweden, J., Legler, G. and Bause, E. (1986) Eur. J. Biochem., 157, 563.

51. Tabas, I. and Kornfeld, S. (1980) J. Biol. Chem., 255,6633.

52. Hasilik, A., Waheed, A., and von Figura, K. (1981) Biochem. Biophys. Res. Commun., 98, 761.

53. Reitman, S.L. and Kornfeld, S. (1981) J. Biol. Chem., 256, 4275.

54. Varki, A. and Kornfeld, S. (1980) J. Biol. Chem., 255, 8398.

55. Waheed, A., Hasilik, A., and von Figura,K. (1981) J. Biol. Chem., 256, 5715.

56. Tulsiani, D.R.P., Opheim, D.J. and Touster, O. (1977) J. Biol. Chem., 252, 3227.

57. Tabas, I. and Kornfeld, S. (1979) J. Biol. Chem., 254, 11655.

58. Tulsiani, D.R.P., Hubbard, C., Robbins, P.W. and Touster, O. (1982) J. Biol. Chem., 257, 3660.

59. Tabas, I. and Kornfeld, S. (1978) J. Biol. Chem., 253, 7779.

60. Harpaz,N. and Schachter, H. (1980) J. Biol. Chem.,255, 4885.

61. Tulsiani,D.R.P., Harris, T.M. and Touster, O. (1982) J.Biol.Chem., 257, 7936.

62. Lehle, L. and Tanner, W. (1976) FEBS Lett., 71, 167.

63. Banerjee, D.K., Scher, M.G. and Waechter, C.J. (1981) Biochemistry, 20, 1561.

64. Datema, R. and Schwarz, R. (1978) Eur. J. Biochem., 90, 505.

65. Datema, R., Schwarz, R. and Jankowski, A.W. (1980) Eur. J. Biochem., 109, 331.

66. Saunier, B., Kilker, R.D., Tkacz,J.S., Quaroni,A. and Herscovics, A. (1982) J.Biol. Chem., 257, 14155.

67. Fuhrman, U., Bause, E., Legler, G. and Ploegh, H. (1984) Nature, 307, 755.

68. Dorling, P.R., Huxtable, C.R. and Colegate, S.M. (1980) Biochem. J., 191, 649.

69. Saquira, M., Kawasaki, T. and Yamashina, I. (1982) J.Biol. Chem., 257, 9501.

70. Elhammer, A. and Kornfeld, S. (1986) J.Biol. Chem., 261, 5249.

71. Terentino, A., Plummer, T.H. and Maley, F. (1970) J. Biol. Chem., 245, 4150.

72. de Duve, C., Pressmann, B.C., Gianetto, R., Wattiaux, R. and Applemans, F. (1955) Biochem. J., 60, 604.

73. Farquhar, M.G. (1969) in "Lysosomes in Biology and Pathology" (Dingle, J.T. and Fell, H.B., eds.), Vol.2, p.462, North-Holland Publ., Amsterdam.

74. de Duve, C. (1969) Ann. N.Y. Acad. Sci., 166, 602.

75. Lewis,V., Green, S.A., Marsh, M., Vihko, P., Helemins, A. and Mellman, I. (1985) J. Cell Biol., 100, 1839.

76. Mellman, I., Fuchs, R. and Helemins, A. (1986) Ann. Rev. Biochem., 55, 663.

77. Kaplan, A., Achord, D.T. and Sly, W.S. (1977) Proc. Natl. Acad. Sci., 74, 2026.

78. Neufeld, E.F., Sando, G.N., Garvin, A.J. and Rome, L.H. (1977) J. Supramol. Struct., 6, 95.

79. Kornfeld,S (1987) FASEB J., 1, 462.

80. Sahagian, G.G., Distler, J., and Jourdian, G.W. (1981) Proc. Natl. Acad. Sci., 78, 4289.

81. Hoflack, B. and Kornfeld, S. (1985) Proc. Natl. Acad. Sci., 82, 4428.

82. Ohkuma, S. and Poole, B. (1976) Proc. Natl. Acad. Sci., 75, 3327.

83. Schneider, D.L. (1981) J. Biol. Chem., 258, 3858.

84. Barrett, A.J. (1968) in "Lysomes in Biology and Pathology" (Dingle, J.T. and Fells, H.B., eds.), Vol.2, p.245, North-Holland Publ., Amsterdam.

85. Arsenis, C., Gordon, J.S. and Touster, O. (1970) J. Biol. Chem., 245, 205.

86. Vaes, G. (1973) in "Lysosomes and storage diseases" (Hers, H.G. and Hoof, V.F., eds.), p.62, Academic Press, New York.

87. Aronson, N.N. Jr., and de Duve, C. (1968) J. Biol. Chem., 243, 4564.

88. Ho, M.W., Cheetham, P.S.J. and Robinson, D. (1973) Biochem, J., 136 351.

89. Nisisawa, K. and Aashimoto, Y. (1970) in "The Carbohydrates" (Pigman, W. and Horton, N., eds.), Vol.2, Academic press, New York.

90. Okada, S. and O' Brien, J.S. (1969) Science, 165, 698.

91. Sandhoff, K. (1969) FEBS Lett., 4,351.

92. Snaith, S.M. and Levvy, G.A. (1973) Adv. Carbohydr. Chem. Biochem., 28, 401.

93. Snaith, S.M. (1975) Biochem. J., 147, 83.

94. Phillips, N.C., Robinson, D. and Winchester, B.G. (1976) Biochem. J., 153, 579.

95. Marinkovic, D.V. and Marinkovic, J.N. (1976) Biochem.J., 155, 217.

96. Opheim, D.J., and Touster, O. (1978) J. Biol. Chem., 253, 1017.

97. Mathur, R. and Balasubramanian, A.S. (1981) Indian J., Biochem. Biophys., 18, 334.

98. Khan, F.A. and Basu, D. (1982) J. Biosci., 4, 133.

99. Okumura, T. and Yamashina, I. (1970) J. Biochem. (Tokyo), 68, 561.

100. Okumura, T. and Yamashina, I. (1973) J. Biochem. (Tokyo), 73, 131.

101. Ockerman, P. (1967) The Lancet, 2, 239.

102. Norden, N., Lundblad, A. and Jolly, R. (1973) FEBS Lett., 35, 209.

103. Colegate, S.M., Dorling, P.R. and Huxtable C.R. (1979) Aust. J. Chem., 32, 2257.

104. Dorling, P.R., Huxtable, C.R. and Vogel, P. (1978) Neuropathol. Appl. Neurobiol., 4, 285.

105. Legler, G. and Julich, E. (1984) Carbohydr. Res., 128, 61.

106. Bossmann, H.B. (1972) Biochim. Biophys. Acta., 258, 265.

107. Davis, B.J. (1964) Am. N.Y. Acad. Sci., 121, 404.

108. Weber, K. and Osborn, M. (1969) J. Biol. chem., 244, 4406.

109. Lowry. O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem., 193, 265.

110. Bradford, M.M. (1976) Anal. Biochem., 72, 248.

111. Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A. and Smith, F. (1965) Anal. Chem., 28, 350.

112. Andrews, P. (1964) Biochem. J., 91, 222.

113. Laemmli, U.K. (1970) Nature, 227, 680.

114. Wray, W., Boulikas, T., Wray, V.P., and Hancock, R. (1981) Anal. Biochem., 118, 197.

115. Surolia, A., Prakash, N., Bishayee, S. and Bachhawat, B.K. (1973) Indian J. Biochem. Biophys., 10, 145.

116. Axen,R., Porath, J. and Ernback, S. (1967) Nature, 214, 1302.

117. Bloch, R. and Burger, M.M. (1974) Biochem. Biophys. Res. Commun., 58, 13.

118. Suresh kumar, G., Appukuttan, P.S. and Basu, D. (1982) J. Biosci., 4, 257.

119. Hjelm,H., Hjelm,K. and Sjoquist, J. (1972) FEBS Lett., 28, 72.

120. Appukuttan, P.S., Surolia, A. and Bachhawat, B.K. (1977) Indian J. Biochem. Biophys., 14, 382.

121. Appukuttan, P.S. and Basu, D. (1981) Anal. Biochem., 113, 253.

122. Fields, R. (1972) Meth. Enzymol., 25, 464.

123. Butler, P.J.G., Harris, J.I., Hartley, B.S. and Liberman, R. (1969) Biochem. J., 112, 679.

124. Habeeb, A.F.S.A. (1967) Arch. Biochem. Biophys., 119, 264.

125. Takahashi, K. (1968) J. Biol. Chem., 243, 6171.

126. Smith, E.L. (1977) Meth. Enzymol., 47, 156.

127. Miles, E.W. (1977) Meth. Enzymol., 47, 431.

128. Horton, H.R. and Koshland, D.E. (1972) Meth. Enzymol., 25, 468.

129. Savige, W.E. and Fontanna, A. (1977) Meth. Enzymol., 47, 442.

130. Riordan, J.F. and Vallee, B.L. (1972) Meth. Enzymol., 25. 500.

131. Habeeb, A.F.S.A. (1972) Meth. Emzymol., 25, 457.

132. Thannhauser, T.W., Konishi, Y., and Scherage, H.A. (1984) Anal. Biochem., 138, 181.

133. Riordan, J.F. and Vallee, B.L. (1972) Meth. Enzymol., 25, 449.

134. Gurd, F.R.N. (1967) Meth. Enzymol., 11, 532.

135. Marshall, R.D. and Neuberger, A. (1972) in "Glycoproteins" (Gottschalk, A., ed.), Vol.5, Part A, p.331, Elvesier Publ. Co., Amsterdam.

136. Gray, W.R. and Hartley B.S. (1963) Biochem. J., 89. 379.

137. Marquez, F.J., Quesada, A.R., Sanchez-J.F., and Nunez de Castro, I. (1986) J. Chromatogr., 380, 275.

138. Shoup, V.A. and Touster, O. (1976) J. Biol. Chem., 251, 3845.

139. Forsee, W.T. and Schutzback, J.S. (1981) J. Biol. Chem., 256, 6577.

140. Bischoff, J. and Kornfeld, R. (1983) J. Biol. Chem., 258, 7907.

141. Chapman, A. and Kornfeld, R. (1979) J. Biol. Chem., 254, 816.

142. Jelenik-K.S., Akiyama, T., Saunier, B., Tkacz, J.S. and Herscovics, A. (1985) J. Biol. Chem., 260, 2253.

143. Snaith, S.M. and Levvy, G.A. (1969) Biochem. J., 114, 25.

144. Philips, N.C., Robinson, D., Winchester, B.G., and Jolly, R.D. (1974) Biochem. J., 137, 363.

145. Philips, N.C., Robinson, D. and Winchester, B.G. (1974) Clin. Chim, Acta. 55, 11.

146. Hocking, J.D., Jolly R.D. and Batt, R.D. (1972) Biochem. J., 128, 69.

147. Vandevelde, M., Faukhauser, R., Bichsel, P., Weismann, U. and Herschkowitz, N. (1982) Acta Neuropathol. (Berlin), 58, 64.

148. Abraham, D., Blakemore, W.F., Jolly, R.D., Sidebotham, R. and Winchester, B. (1983) Biochem. J., 215, 573.

149. Molyneux, R.J. and James, L.F. (1982) Science, 216, 190.

150. Schneider, M.J., Ungemach, F.S., Broquist, H.P. and Harris, T.M. (1983) Tetrahedron, 39, 29.

151. Tulsiani, D.R.P., Broquist, H.P., James, L.F. and Touster, O. (1984) Arch. Biochem. Biophys., 232, 76.

152. Phillips, N., Robinson, D, and Winchester, B. (1975) Biochem. J., 151, 469.

153. Paus,E. (1978) Biochim. Biophys. Acta., 526, 507.

154. Paus, E. (1978) Biochim. Biophys. Acta., 533, 446.

155. Strawser, L.D. and Touster, O. (1980) Rev. Physiol. Biochem. Pharmacol., 87, 169.

156. Fiddler, M.B., Ben-Y, Y. and Nadler, H.L. (1979) Biochem. J., 177, 175.

157. von Figura, K. and Klein, U. (1979) Eur. J. Biochem., 94, 347.

158. von Figura, K., Rey, M., Prinz, R., Voss, B. and Ullrich, K. (1979) Eur. J. Biochem., 101, 103.

159. Varki, A. and Kornfeld, S. (1980) J. Biol. Chem., 255, 10847.

160. Mathur, R., Alvares, K. and Balasubramanian, A.S. (1984) Biochem. Biophys. Res. Commun., 123, 1185.

161. Hasilik, A. and von Figura, K. (1981) Eur. J. Biochem., 121, 125.

162. Howard, D.R., Natowicz, M. and Baenziger, J.U. (1982) J. Biol. Chem., 257, 10861.

163. Vladutiu, G.D. (1983) Biochim. Biophys. Acta., 760, 363.

164. Miller, A.L., Kress, B.C., Stein, R., Kinnon, C., Kern, H., Schneider, J.A. and Harms, E. (1981) J. Biol. Chem., 256, 9352.

165. Cummings, R.D., and Kornfeld, S. (1982) J. Biol. Chem., 257, 11235.

166. Krusius, T., Finne, J. amd Rauvala, H. (1976) FEBS Lett., 71, 117.

167. Ogata, S., Muramatsu, T. and Kobata, A. (1975) J. Biochem. (Tokyo), 78, 687.

168. Narasimhan, S., Freed, J.C. and Schachter, H. (1986) Carbohydr. Res., 149, 65.

169. Baenziger, J.U. and Fiete, D. (1979) J. Biol. Chem., 254, 2400.

170. Goldberg, D.E. and Kornfeld, S. (1981) J. Biol. Chem., 256, 13060.

171. Debray, H., Decont, D., Strecker, G., Spik, G. and Montreuil, J. (1981) Eur. J. Biochem., 117, 41.

172. Yamamoto, K., Tsuji, T., Matsumoto, I. and Osawa, T. (1981) Biochemistry, 20, 5894.

173. Bhavanandan, V.P. and Katlic, A.W. (1979) J. Biol. Chem., 254, 4000.

174. Monsigny, M., Roche, A.C., Sene, C., Maget-D, R. and Delmotte, M. (1980) Eur. J. Biochem., 104, 147.

175. Yoshima, H., Tsuji, T., Tagami, S., Yamamoto, K. and Osawa, T. (1981) Biochemistry, 20, 560.

176. Narasimhan, S., Freed, J.C. and Schachter, H. (1985) Biochemistry, 24, 1694.

177. Dixon,M. and Webb, E.C. (1979) Enzymes 3rd ed., p.332, Academic Press, London.

178. Roque-B, M.C. and Compos-N, A. (1985) J.Immunol., 134, 1740.

179. Tulsiani, D.R.P., Broquist, H.P. and Touster, O. (1985) Arch. Biochem. Biophys., 236, 427.

180. Farhat, A.K. (1984) Ph.D. Thesis, Aligarh University.

181. Paus, E. (1977) Eur. J. Biochem., 73, 155.

182. Sly, W.S. and Fischer, H.D. (1982) J. Cell Biochem., 18, 67.

183. Dulaney, J.T. (1979) Mol. Cell Biochem., 21, 43.

SYNOPSIS

Glycoproteins are a group of polymers which contain oligosaccharide chains covalently linked to the polypeptide backbone. Many soluble and membrane-bound glycoproteins contain N-glycosidic type of saccharide-peptide linkage. These are of three types according to the saccharide composition, (a) high mannose (b) complex and (c) hybrid. Another type of saccharide-peptide linkage is the O-glycosidic type.

In most cases, nucleotide sugars are the donor of the protein bound saccharide units. Leloir et al had shown the role of polyprenol phosphates in mammalian glycoprotein synthesis (1). N-glycosidic units are synthesized by the en bloc transfer of the oligosaccharide, $Glc_3Man_9GlcNAc_2$ from the lipid carrier, dolichol phosphate, to an asparagine residue of the nascent polypeptide chain, followed by a series of modifications. The synthesis of O-linked oligosaccharide proceeds by stepwise addition of nucleotide-linked monosaccharides directly to the polypeptide.

Enzymes capable of cleaving various linkages in glycoproteins are mainly localized in lysosomes. Most of the lysosomal enzymes are glycoproteins in nature and display optimum biological activity at acid pH. Lysosomal α-D-mannosidase (EC 3.2.1.24) is one of the acid

glycosidases involved in the hydrolysis of α-mannosidic bonds in glycoconjugates. Multiple forms of α-mannosidase having acid, neutral and intermediate activities with different localisations have been reported in the literature (2-6).

AIMS AND OBJECTIVES

The widespread occurrence of α-linked D-Man residues in mammalian glycoproteins and recognition of the deficiency of lysosomal acidic α-mannosidase in human and bovine Mannosidosis (7,8) created interest in the mammalian enzyme.The isolation and characterization of lysosomal α-mannosidase from various mammalian tissues have been reported (9-12). However, there is scanty reports on the structural analysis of the enzyme. The present study included the investigations on the structure and function of lysosomal α-mannosidase purified from an useful hospital waste, human placenta. Amino acid residues involved at the active site of the enzyme were examined. Composition, structure and contribution by the oligosaccharide chains to the biological function of the enzyme were studied. Immunological properties of the enzyme has also been discussed.

RESULTS AND DISCUSSION

Lysosomal α-mannosidase was purified from frozen human placenta by a modification of the method of Khan and

Basu (13). The enzyme was prepared upto the heat treatment step as in the previous method. The enzyme was then treated with anhydrous pyridine at 25°C and dialysed against phosphate buffer pH 7.0 with 100mM NaCl to remove pyridine. The enzyme was subjected to ion-exchange chromatography on a CM-Sephadex C-50 column equilibrated with 50mM citrate-phosphate buffer, pH 4.5. The enzyme was eluted with 300mM NaCl in the same buffer. Active fractions were pooled, pH restored to 6.5 and finally concentrated by ultrafiltration.

The homogeneity of the purified enzyme was established by polyacrylamide gel electrophoresis at pH 4.3. The native enzyme had a molecular weight of 300 kDa by gel filtration. Subunit molecular weight determination by SDS-polyacrylamide gel electrophoresis yielded a single protein band corresponding to a molecular weight of 78kDa.

The effect of pH on the stability of the enzyme was markedly influenced by the presence of zinc. At pH 6.5 and 37°C, the enzyme retained its original activity. There was gradual loss of activity at pH 4.2. However, presence of Zn^{2+} in the incubation mixture helped to retain the enzymic activity. The enzyme, incubated with EDTA (4mM) was found to have 47% of its original activity, but addition of 8-20mM Zn^{2+} to the assay mixture, restored the initial activity.

Enzyme activity was not significantly inhibited by its substrate analogues, such as mannonic acid-γ-lactone, mannose-6-phosphate, methyl α-D-mannopyranoside and mannosamine. 1-Deoxymannojirimycin and 5-amino-5-deoxy

mannopyranose inhibited 50% and 100% of the enzyme activity at 6 x 10^{-4}M and 2.5 x 10^{-4}M concentration, respectively. Swainsonine was shown to be a potent inhibitor of lysosomal ∝-mannosidase by Dorling et al (14). It was found to be a competitive inhibitor in the present study with a Ki of 2 x 10^{-8}M.

There are no information in the literature about the amino acid residues involved in the active site of the enzyme. In the present study, chemical modification of the amino acid residues were carried out to obtain information on the amino acids at the active site (15-18). Amino group modifying agents like trinitrobenzene sulfonic acid and maleic anhydride inhibited the enzymic activity. Modification of tryptophan residues with active benzyl halides resulted in complete loss of enzyme activity. Phenylglyoxal hydrate and 1,2 - cyclohexanedione which specifically modifies arginine residues also inhibited the enzymic activity. These results suggested the involvement of lysine, tryptophan and arginine residues at the catalytic site of the enzyme.

The N-terminal amino acid sequence was determined by dansyl-Edman method (19). The dansyl derivative of the amino acids were separated by HPLC on a 5μm Lichrosorb RP-18 column by the method of Marquez (20). The detection of the dansyl amino acids were carried out by measuring the fluorescence using a Spectrofluorophotometer. The N-terminal sequence of the enzyme was found to be 'Serine-Lysine-Methionine-'.

The subunit of the native and swainsonine treated enzyme was prepared by SDS-PAGE. The protein bands after extraction were dansylated and the amino acids were detected as mentioned above. Two peaks corresponding to serine and lysine were observed. Lysine peak on swainsonine treatment was found to be negligible. Lysine residues were involved at the catalytic site as mentioned earlier.

The total carbohydrate content estimated by phenol-sulphuric acid method with galactose as standard was found to be 8%. Periodate oxidation inhibited the enzyme activity. The oligosaccharide chains may have some role in the biological activity of the enzyme.

The structure of the oligosaccharide sequences of the enzyme was investigated by affinity chromatography on various immobilised lectins. ConA-Sepharose chromatography and elution with 10mM and 500mM methyl-α-D-mannopyranoside showed that the protein contained biantennary, high mannose and hybrid types of oligosaccharides. The enzyme was separated on WGA-Sepharose as bound and unbound forms. This confirmed the presence of hybrid-type of oligosaccharides in the bound form and high mannose-type in the unbound form. There was irreversible binding of the enzyme on immobilised RCA and the enzymic activity was maintained for eighteen months at 4°C.

Polyclonal antibody against the enzyme was raised in rabbits. IgG class of immunoglobulins were found to cross-react with the enzyme protein. Retention of α-mannosidase

activity by the enzyme-antibody complex suggested that the antigenic determinant site and catalytic site were topologically distinct on the enzyme molecule. The placental α-mannosidase antibody cross-reacted with the soluble extracts containing same enzymic activity from various other foetal human organs.

REFERENCES

1. Behrens, N.H., Parodi, A.J. and Leloir, L.F. (1971) Proc. Natl.Acad. Sci., 68, 2857.
2. Snaith, S.M. and Levvy, G.A. (1973) Adv. Carb. Chem. Biochem., 28, 401.
3. Shoup, V.A. and Touster, O.(1976) J.Biol, Chem., 251, 845.
4. Dewald, B. and Touster, O. (1973) J. Biol. Chem., 248, 223.
5. Forsee, W.T. and Schutzback, J.S. (1981) J. Biol. Chem., 256, 6577.
6. Bischoff, J. and Kornfeld, R. (1983) J.Biol. Chem., 258, 7907.
7. Ockerman, P. (1967) Lancet, 2, 239.
8. Hocking, J.D., Jolly, R.D. and Batt, R.D. (1972) Biochem. J., 128, 69.
9. Phillips, N.C., Robinson, D. and Winchester, B.G. (1976) Biochem. J., 153, 579.
10. Marinkovic, D.V. and Marinkovic, J.N. (1976) Biochem. J., 155,217.

11. Mathur, R. and Balasubramanian, A.S. (1981) Indian J. Biochem. Biophys., 18, 334.

12. Opheim, D.J. and Touster, O. (1978) J. Biol. Chem., 253, 1017.

13. Khan, F.A. and Basu, D. (1982) J. Biosci., 4, 133.

14. Dorling, P.R., Huxtable, C.R. and Colegate, S.M. (1980) Bicchem.J., 191, 649.

15. Fields, R. (1972) Meth. Enzymol., 25, 464.

16. Horton, H.R. and Koshland, D.E., Jr. (1972) Meth. Enzymol., 25, 468.

17. Takahashi, K. (1968) J. Biol. Chem., 243, 6171.

18. Smith, E.L. (1977) Meth. Enzymol., 47, 156.

19. Gray, W.R. (1972) Meth. Enzymol., 25, 338.

20. Marquez, F.J., Quesada, A.R., Sanchez-Jimenez, F. and Decastro, I.N. (1986) J. Chromatogr., 380, 275.