STRUCTURE AND PROPERTIES OF β-GALACTOSIDASE AND β-GALACTOSIDE-BINDING PROTEIN OF HUMAN PLACENTA

A THESIS PRESENTED

by

P. MADHUSOODANA NAMBIAR,

to

THE DIVISION OF NEUROCHEMISTRY IN
PARTIAL FULFILMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN THE SUBJECT OF

BIOCHEMISTRY

SREE CHITRA TIRUNAL INSTITUTE
FOR
MEDICAL SCIENCES AND TECHNOLOGY
TRIVANDRUM

JUNE—1988

CERTIFICATE

I, MADHUSOODANA NAMBIAR. P hereby certify that I had personally carried out the work depicted in the thesis entitled "STRUCTURE AND PROPERTIES OF β-GALACTOSIDASE AND β-GALACTOSIDE-BINDING PROTEIN OF HUMAN PLACENTA", except where external help sought and acknowledged.

Signature

P. MADHUSOODANA NAMBIAR

Date: 27 JUN 1988

Prof. Debkumar Basu Neurochemistry division

DECLARATION

This is to certify that Sri. Madhusoodana Nambiar. P in the division of Neurochemistry this Institute, has fulfiled 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 his thesis entitled "STRUCTURE AND PROPERTIES OF β-GALACTOSIDASE AND β-GALACTOSIDE-BINDING PROTEIN OF HUMAN PLACENTA", was carried out under my direct supervision.

Signature

Debkumar Basu

Prof. Debkumar Basu
(Guide)

The thesis
entitled

STRUCTURE AND PROPERTIES OF β-GALACTOSIDASE AND β-GALACTOSIDE-BINDING PROTEIN OF HUMAN PLACENTA

Submitted
by

MADHUSOODANA NAMBIAR. P

for

Doctor of Philosophy

in

BIOCHEMISTRY

of

SREE CHITRA TIRUNAL INSTITUTE
FOR
MEDICAL SCIENCES AND TECHNOLOGY
TRIVANDRUM

Evaluated and approved
by

__________ __________ __________ __________

Name of the
(Guide)

(Names of thesis examiners)

CONTENTS

			PAGE
Acknowledgements			v-vi
CHAPTER - I		GENERAL INTRODUCTION	1-32
		Lysosomal acid hydrolases	1-28
		Animal lectins	28-32
AIMS AND OBJECTIVS			33-34
CHAPTER - II		MATERIALS AND GENERAL METHODS	35-49
		Materials	35-36
		General Methods	36-49
CHAPTER - III		RESULTS AND DISCUSSION	50-89
		Acid β-galactosidase of human placenta	50-68
		Introduction	50-57
		Methods	57-59
		Results	59-65
		Discussion	65-68
		Beta-galactoside-binding protein of human placenta	69-89
		Introduction	69-73
		Methods	74-78
		Results	78-82
		Discussion	82-89
CHAPTER - IV		GENERAL DISCUSSION AND SUMMARY	90-104
		General Discussion	90-100
		Summary	101-104
BIBLIOGRAPHY			105-121
SYNOPSIS			122-129

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

It has been my privilege to carry out this work under the guidance of Prof. Debkumar Basu. I take this opportunity to acknowledge my indebtedness to him for his sustained interest, sound counsel and constant encouragement as Guide and Chairman of my advisory committee for successful completion of the present work.

I wish to express my sincere thanks to Dr. P.S. Appukuttan for the timely suggestions and constructive criticisms, which have been invaluable. The excellent assistances of Mrs. K.I. Annamma and Mrs. Lizamma Antony are thankfully acknowledged. I would like to thank all my colleagues of Neurochemistry Division for their co-operation during the course of this study. I thank Mr. J. Abraham for drawing the figures and Mr. P.J. George for photography. Excellent typing of the thesis by Miss. Usha. B is highly appreciated.

I express my gratitude to Prof. M.S. Valiathan, Director of the Institution for providing me opportunity to carry out the work. The author acknowledges his appreciation to the members of Doctoral Advisory Committee for their valuable advise and encouragement. I wish to convey my thanks to Dr. M.R. Das, Centre for Cellular and Molecular Biology, Hyderabad, for providing the facilities

Prof. G. Legler, University of Cologne, W. Germany, for the generous gift of galacto-1-deoxynojirimycin. I am indebted to the Superintendent, S.A.T. Hospital, Medical College, Trivandrum for allowing me to collect the tissue materials for the study.

The financial support from Department of Science and Technology, New Delhi, is gratefully acknowledged.

MADHUSOODANA NAMBIAR. P.

ABBREVIATIONS

Man	Mannose
Glc	Glucose
Gal	Galactose
Fuc	Fucose
GalNAc	N-Acetylgalactosamine
GlcNAc	N-Acetylglucosamine
NeuNAc	N-Acetylneuraminic acid
UDP	Uridine diphosphate
GDP	Guanosine diphosphate
CMP	Cytidine monophosphate
Galacto-1-deoxynojirimycin	1,5-dideoxy-1,5-imino-D-galactitol
PNP-ß-D-Gal	p-Nitrophenyl-ß-D-galactopyranoside
MeUmb-ß-D-Gal	4-Methylumbelliferyl-ß-D-galactopyranoside
Con A	Concanavalin A
WGA	Wheat germ agglutination
RCA	Ricinus communis agglutinin
WBA	Winged bean agglutinin
JSA	Jack seed agglutinin
PHA	Phaseolus vulgaris hemagglutinin
TNBS	Trinitrobenzene suphonic acid
DTNB	5,5'-Dithiobis(2-nitrobenzoic acid)
pHMB	para-Hydroxymercuribenzoate
NEM	N-ethylmaleimide
NBS	N-bromosuccinimide
DMSO	Dimethylsulphoxide
PNP	para-Nitrophenol
SDS	Sodium dodecyl sulphate
PAGE	Polyacrylamide gel electrophoresis
DNS-Cl	Dansyl chloride (1-dimethylamino-naphthalene-5-sulfonyl chloride)
PBS	20mM Sodium phosphate buffer containing 150mM NaCl.

CHAPTER - I

GENERAL INTRODUCTION

INTRODUCTION

LYSOSOMAL ACID HYDROLASES

The process of degradation of cellular constituents is one of the main features in maintenance of the 'steady state'. Enzymes capable of cleaving various linkages found in glycoproteins are primarily localized in lysosomes, organells with a complete complement of hydrolases to degrade almost all cellul constituents (1). They exhibit optimum activity in acidic medium. It has been observed that all the lysosomal hydrolases including those acting on glycoprotein substrates are themselves glycoproteins (2). This might explain the unusual ability of these enzymes to remain stable at an acid pH and in the presence of lysosomal proteases.

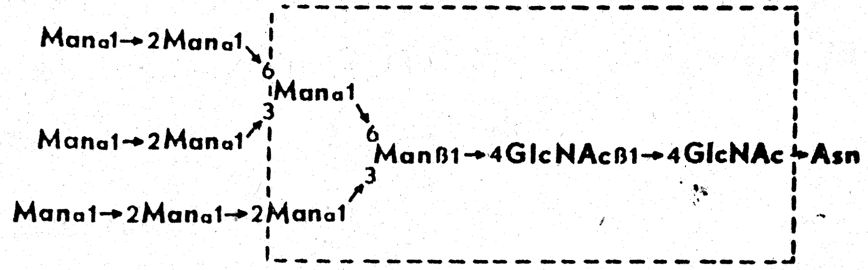
Glycoproteins are most diverse group of biological polymers that are constituents of almost all living organisms with the possible exception of bacteria (3). Included in this class of compounds are enzymes, immunoglobulins, hormones, toxin lectins and structural proteins. The carbohydrate units vary in size and structure from that of a mono-or disaccharide to branched oligosaccharides consisting of about 20 or more monosaccharide residues. The number of such chains in different glycoproteins ranges between 1 and over 100 and they are distributed unevenly along the polypeptide backbones.

The carbohydrate units are classified according to the nature of their carbohydrate peptide linkages. Oligosaccharide chains may be linked N-glycosidically from GlcNAc to Asn or

Asn-linked oligosaccharides contain a common pentasaccharide core, Manα1→3(Manα1→6)Manβ1→4 GlcNAcβ1→4GlcNAc. To the pentasaccharide core are attached additional residues resulting in the formation of three classes of N-glycosidic chains (Figure -1). (i) Oligosaccharide chains which contain only 6-12 Man residues are attached to the core. These chains are often branched and are known as 'high mannose-type'. (ii) Those which contain the disaccharide Galβ1→4GlcNAc with or without NeuNac linked to Gal are known as 'complex-type'. According to the number of Galβ1→4GalNAc units they have been designated as bi-, tri- and tetraantennary complex oligosaccharides. (iii) Oli saccharide unit in which both Man residues and Galβ1→4GlcNAc are attached to the core is classified as 'hybrid-type'.

Variations on these basic structures are known. Thus Fuc may be attached in an α1→6 linkage to the Asn-linked GlcNAc or in an α1→3 linkage to a GlcNAc of one of the Galβ1→4 GlcNAc units. Sometimes, the core Man is substituted by a GlcNAc residue. Non-carbohydrate substituents also occur in N-glycosidic units. Phosphate residues are present on C-6 of Man. Sulfated glycoproteins have been isolated from various animal sources (4).

Oligosaccharide chains attached to the peptide by O-glycosidic linkage from GalNAc/Gal to the hydroxyl group of Ser or Thr. The GalNAc-Ser/Thr linkage is found mainly in mucins, blood group active glycoproteins and certain membrane glycoproteins. The simplest O-glycosidically linked oligo



I. HIGH-MANNOSE TYPE

SA→Gal→GlcNAc→Manα1

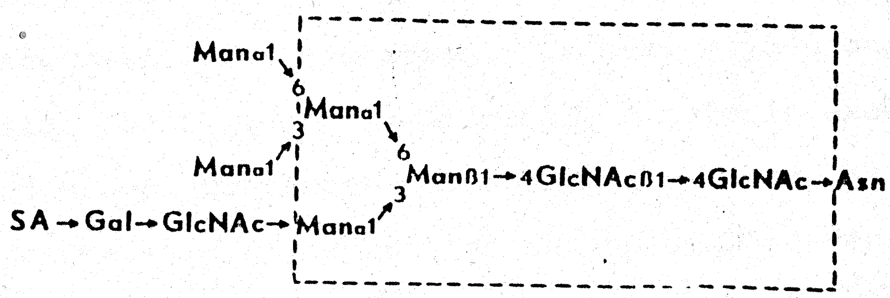
6

Manβ1→4GlcNAcβ1→4GlcNAc→Asn

3

SA→Gal→GlcNAc→Manα1

II. COMPLEX TYPE



III. HYBRID TYPE

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

Fig.1. Structures of the major classes of asparagine-linked oligosaccharides. The box delineated by the broken line indicates the common pentasaccharide core structure.

Adopted from Elbein, A.D. (1984) CRC. Critical. Rev. Biochem. 16, 22.

mucins and Galβ1$\rightarrow$4GalNAc found in antifreeze glycoprotein of Antarctic fish (5). More complex O-glycosidic oligosaccharides are present in many glycoproteins. O-Glycosidic linkage through Hyl is confined exclusively to collagens (6). Xyl-Ser is typical of glycosaminoglycan linkage to peptides.

Functions of the oligosaccharides

Glycoproteins perform many vital roles in living systems. In spite of the remarkable advances in our knowledge of glycoprotein structure and metabolism, little is known about the contribution of the carbohydrate moieties for their functions. It is apparent that carbohydrates in glycoproteins do not perform a single function but act in a variety of ways. In particular, they modify the physicochemical properties of proteins by changin hydrophobicity, electrical charge, mass and size. Glycosylation is not a prerequisite for secretion. In most glycoproteins oligosaccharide chain is not immunogenic; a notable exception are the blood group substances. Carbohydrates serve as important recognition markers on glycoproteins and on cell surfaces.

Studies with salivary glycoprotein that are rich in sialic acid have led to the conclusion that this saccharide is responsible for the high viscosity of mucous solutions (7). The antifreeze glycoproteins of Antarctic fish depend for their activity on the integrity of their disaccharide units (8). It has been known for a long time that, glycoproteins especially if they are sugar rich, are relatively resistant to proteolysis. The resistance is probably due to the fact that, the oligosacchar

a local shield that hinders the approach of proteolytic enzymes to the peptide chain. It has also been suggested that saccharide units may function in directing the protein to its most stable conformation (9).

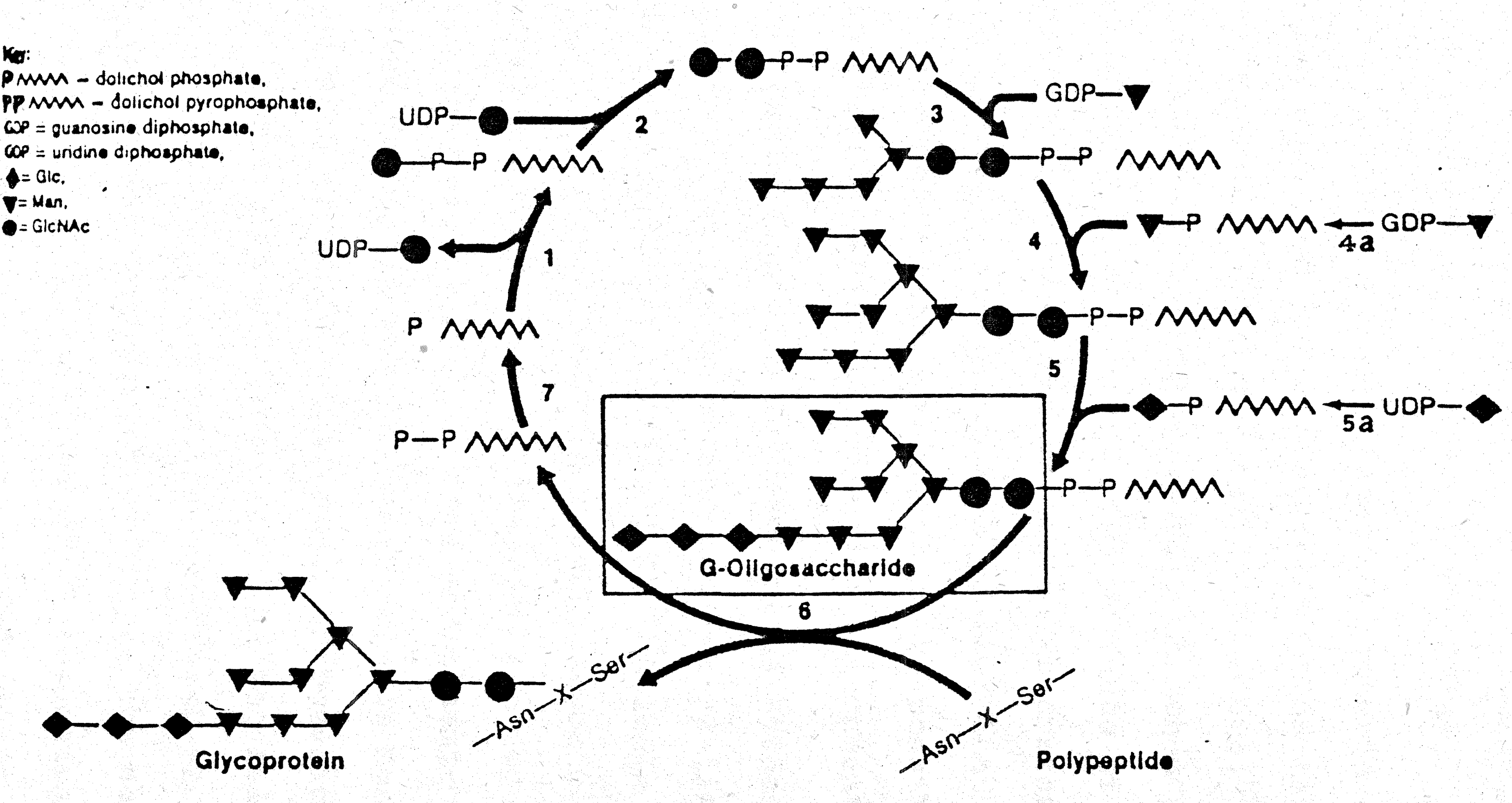
Asymmetrically distributed saccharides of cell membrane is in the form of glycolipids and glycoproteins. The membrane glycoproteins play a part in what has often bee referred to as the social behaviour of cells. Saccharides a important in determining the life span of circulating glyco-proteins and cells. The classic work of Ashwell and Morell has demonstrated that removal of sialic acid from circulatin glycoproteins leads to a dramatic enhancement in the rate of their clearance from the circulatory system (10). Uptake depends on the recognition by the liver cells of the exposed Gal residues on the glycoprotein. A different system in which saccharides serve as a recognition marker for the intracellular seggregation and pinocytosis of certain glyco-sidases will be discussed later.

Biosynthesis

The polypeptide chains of glycoproteins are synthe-sized by the same machinery that produces non-glycosylated proteins. Attachment of the carbohydrate chain starts during translation, although some residues are incorporated post-translationally. The biosynthesis of N-glycosidic units proceeds by a mechanism different from that of O-glycosidic carbohydrate chains, although the primary sugar

are sugar nucleotides and the enzymes involved are glycosyltransferases (11). Glycosyltransferases add with great precision, single sugar residues or oligosaccharides from their activated donor substrates to suitable acceptor molecules. These enzymes are most conveniently classified according to the sugar transferred. Elongation of the oligosaccharide chain proceeds by sequential action of the transferases, the product of one glycosyltransferase serving as the substrate for the next one.

The biosynthesis of Asn-linked oligosaccharide is a multistep process which involves, (i) assembly of a lipid-linked oligosaccharide intermediate, (ii) transfer of the oligosaccharide from the lipid carrier to the growing peptide chain, and (iii) trimming of the carbohydrate unit and addition of peripheral saccharides. Through the pioneering work of Leloir and his co-workers, strong evidence was provided for the transfer of Glc, Man and GlcNAc from their respective nucleotides to Dol-P, a phosphorylated polyprenol containing 14-21 isoprenol units, with α-isoprenol unit saturated (12). The lipid-linked oligosaccharide synthesized in the endoplasmic reticulum consists of $Glc_3Man_9GlcNAc_2$ linked to Dol via a pyrophosphate. The biosynthetic pathway of the so called dolichol phosphate cycle is schematically illustrated in Figure 2. Synthesis begins with the reversible transfer of a GlcNAc moiety from UDP-GlcNAc to Dol-P . (Reaction.1), followed by the irreversible transfer of a



= dolichol phosphate,
= dolichol pyrophosphate,
= guanosine diphosphate,
= uridine diphosphate,
= Glc,
= Man,
= GlcNAc
UDP
P–P
2
3
GDP
UDP
P–P
UDP
1
P
4
P
GDP
4a
P–P
7
5
P
UDP
5a
P–P
P–P
G-Oligosaccharide
6
—Asn—X—Ser—
—Asn—X—Ser—
Glycoprotein
Polypeptide

directly from GDP-Man (Reaction.3). Addition of the last four Man and all three Glc proceed via the corresponding Dol-p derivatives (Reaction.4 and 5), formed from UDP-Man and UDP-Glc respectively (Reaction.4a and 5a).

The presence of the tripeptide sequence Asn-X-Ser/T (where X can be any amino acid except Pro and Asp) is a necessary condition for glycosylation to occur. The saccha ride chains seems to be preferentially attached to peptide sequences forming ß-turns (13). The presence of Glc in the lipid linked oligosaccharide serves as a signal for its transfer to protein. It has been suggested that rapid folding of the polypeptide chain may be responsible for the lack of saccharide chains at potential attachment sites (14

The processing of oligosaccharides attached to the protein begins with removal of terminal α1→2 linked Glc residue by the enzyme glucosidase I in the lumen of rough endoplasmic reticulum (15). The enzyme glucosidase II acts after glucosidase I and removes stepwise the two inner α1→ linked Glc residues (Figure 3). The endoplasmic reticulum α-mannosidase catalyzed the removal of single Man residue from $Man_9GlcNAc_2$ to yield $Man_8GlcNAc_2$. Processing continue by further trimming of α1→2 Man residues to yield Man_5GlcN This is accomplished by the action of Golgi α1→2 mannosidase IA and IB (16). The transfer of GlcNAc to the Man residue linked α1→3 to the ß-linked core Man is essential before α-mannosidase removes the two Man residue linked to Man,

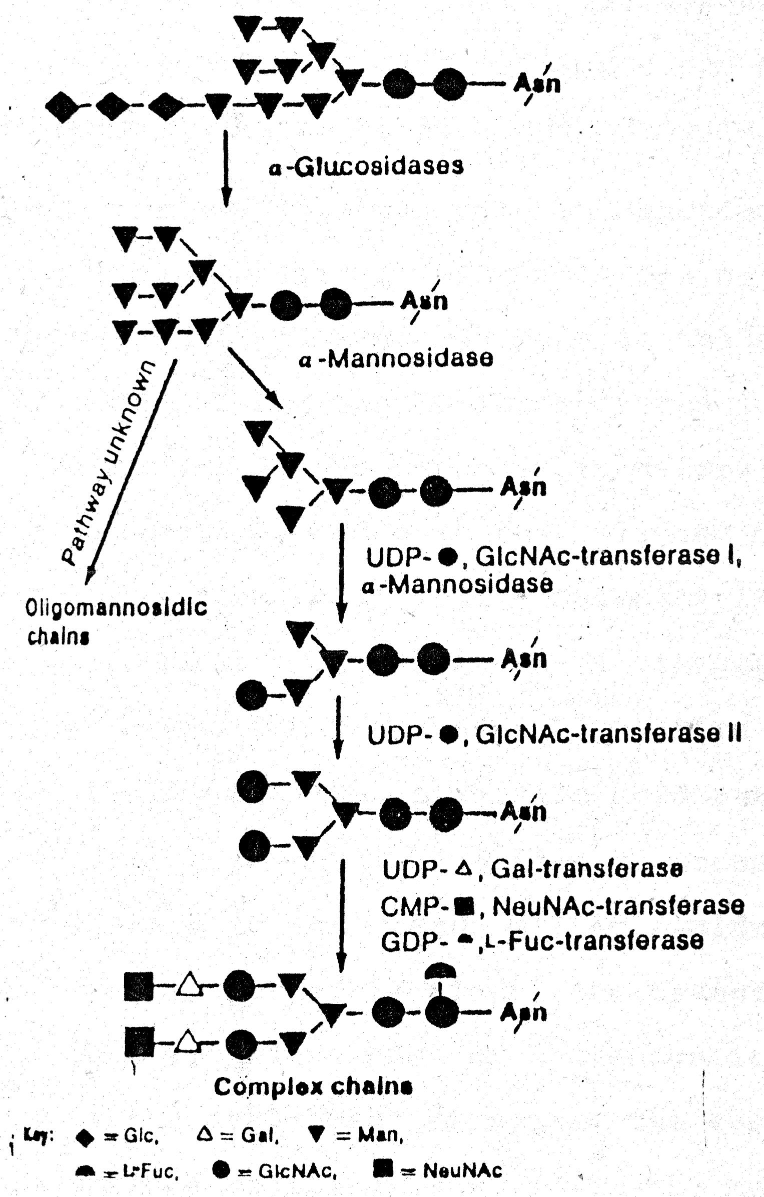


Fig.3. Schematic representation of the processing reactions in the synthesis of complex-type asparagine-linked oligosaccharides.

Adopted from Sharon, N. and Lis, H. (1982) Mol. Cell. Biochem. 42, 177.

added by GlcNAc-transferase I in $\beta 1\rightarrow 2$ linkage to the $\alpha 1\rightarrow 3$ linked Man, and this reaction is essential for normal processing (17). Golgi α-mannosidase II removes the non core $\alpha 1\rightarrow 3$ and $\alpha 1\rightarrow 6$ Man residues to yield GlcNAc Man_3 $(GlcNAc)_2$.

The assembly of complex-type oligosaccharides is initiated by GlcNAc transferase II which adds the next GlcNAc residue to $\alpha 1\rightarrow 6$ linked Man. As soon as the second GlcNAc residue is added, L-Fuc can be incorporated into the GlcNAc nearest the Asn residue by L-fucosyltransferase. Another GlcNAc residue may be added to the β-linked Man of the core by GlcNAc transferase III (18). Galactosyltransferase catalyzes the addition of Gal in $\beta 1\rightarrow 4$ linkage to the terminal GlcNAc residue of the branches. Elongation of the chain is completed by the addition of sialic acid residues in $\alpha 2\rightarrow 3$ or $\alpha 2\rightarrow 6$ linkage to the Gal residue or occassionally of L-Fuc in $\alpha 1\rightarrow 3$ linkage to the branch GlcNAc residues. Unlike the other common sugar nucleotides, the transfer of CMP-NeuNAc takes place with inversion of the anomeric carbon atom. Subsequent to the attachment of sugars the saccharide chain may undergo various modifications (19). The Man-6-P recognition marker is synthesized by the transfer of GlcNAc-1-P from UDP-GlcNAc to the C-6 hydroxyl group of a Man residue and hydrolysis of the covering GlcNAc residue.

The biosynthesis of oligosaccharides linked through GalNAc-Ser/Thr occurs by sequential addition of one sugar at a time to the growing glycoproteins (19). The first step

aminoacid in the peptide back bone, catalyzed by GalNAc transferase. There is an important branch point immediately after incorporation of GalNAc. If incorporation of sialic acid occurs, to form NeuNAcα2$\rightarrow$6GalNAc, further attachment of carbohydrate cannot take place. If Gal is incorporated to form Galβ1$\rightarrow$3GalNAc the pathway proceeds towards the synthesis of various saccharides.

Some of the Hyl residues of collagen are glycosylated by the action of galactosyltransferase to form Gal-β-Hyl. The Gal residue may serve as an acceptor for a specific glucosyltransferase forming Glcα1$\rightarrow$2Gal (20). The glycosylation of collagen Hyl apparently occurs in concert with the hydroxylation of Lys in the endoplasmic reticulum and ceases when the collagen chain folds in to triplex.

Regulation of glycoprotein biosynthesis

There are a number of potential sites of regulation of the N-glycosidically linked glycoprotein biosynthesis. These include regulation of the level of (1) initial glycosyl donors, sugar nucleotides; (2) glycosyl acceptor, Dol-P; (3) Glycosyltransferases and glycosidases that catalyse these reactions and (4) acceptor protein. There are at least two regulatory mechanisms known to affect the synthesis of nucleotide sugars essential for glycoprotein synthesis. The first of these involves inhibition of D-fructose-6-phosphate:L-glutamine amidotransferase by

the synthesis of hexosamine from hexose and therefore controls the availability of UDP-GlcNAc, UDP-GalNAc and CMP-sialic acids. The second control point involves inhibition by CMP-sialic acid of UDP-GlcNAc-2-epimerase, an important enzyme in sialic acid biosynthesis (22). Enzymes which degrade nucleotide sugars are present in mammalian tissues and may play a role in controlling glycoprotein synthesis. Thus liver plasma membrane carries on its outer surface an enzyme which acts as a nonspecific pyrophosphatase capable of cleaving various nucleotide sugars (23).

The donor nucleotide sugars for all glycosyltransferase reactions involved in glycoprotein synthesis are formed in cytosol a site distinct from that of glycosyltransferase action. Once formed the nucleotide sugars are transported to Golgi and are utilized for glycoprotein synthesis. The products of glycosyltransferase action, 5'-nucleotides, are potent inhibitors of nucleotide sugar transport, which potentially modulate glycoprotein synthesis. A rather bewildering array of inhibitory and stimulatory effects have been described in studies on the actions of nucleotides on glycosyltransferases. Some of these effects are probably the result of allosteric interaction between nucleotides and glycosyltransferase, while the others are caused by the protection of nucleotide sugar from hydrolysis by pyrophosphatases (24). These studies suggest that intracellular nucleotide sugar concentration may play an important role

level of GDP-Man may affect the level of glycoprotein synthesis, since Man is uniquely found in glycoproteins.

Two specific sites of regulation of protein glycosylation would be those involving Dol-P synthesis and regeneration of Dol-P from Dol-PP. Possible sites for specific regulation of synthesis of Dol-P are either those involving condensation of isopentenyl units with farnesyl pyrophosphate or the reactions involved in the final structura alterations to the complete chain (25). One of the end product of the reaction between oligosaccharide-P-P-Dol and acceptor protein is Dol-PP. Presumably for Dol-PP to 'recycle' and serve as an acceptor, it must be enzymatically converted to Dol-P. An enzyme that acts on Dol-PP has not been discovered in eukaryotes (79). Another possible site of regulation is the interconvertion of Dol and Dol-P. Although a phosphatase that acts on Dol-P has not been reported, a kinase that converts Dol to Dol-P has been detected (26).

Quantitative or qualitative modification of glycosyltransferases also controls glycoprotein biosynthesis. They can be broadly classified as (1) factors which affect the availability of substrates, cofactors and cations, (2) factors which activate, inhibit or modify enzyme activity, and (3) factors which affect the synthesis and catabolism of enzyme protein. Genes control the synthesis of oligosaccharides primarily by being structural genes for

about the mechanism that regulate the expression of glycosyltransferases.

Williams and Lennarz have obtained evidence that indicates that the substrate specificities of the processing enzyme can differ between tissues and or species (27). These findings indicate the control of processing involves more sophisticated mechanisms than simple exposure of the oligosaccharide chains. It may be that interaction of protein signals with certain signal glycosyltransferases, which mediate the terminal steps in oligosaccharide assembly, determines which of the many possible structures the mature oligosaccharide will have. Alternatively, differences in the interaction of the oligosaccharide with the underlying peptide may determine the nature of the final structure (28).

One determinant of oligosaccharide processing is the location of the protein in the cell. The glycoproteins resident in endoplasmic reticulum, not having been exposed to Golgi processing enzymes, would be expected to have only high mannose units; HMG-CoA reductase has $Man_6(GlcNAc)_2$ and $Man_8(GlcNAc)_2$ chains (29). These findings indicate that the physical accessibility of the oligosaccharide to processin enzymes can control processing. The interaction of protein subunits may also influence oligosaccharide processing. Pollack and Atkinson noted that the location of glycosylation sites in the polypeptide chain correlates with processing (30)

Inhibitors of Glycosylation

Great interest has been focussed on compounds that can prevent glycosylation of Asn-linked glycoprotein or cause alterations in the structure of the oligosaccharide chains. Inhibition of the transfer of GlcNAc-1-P to the lipid carrier can prevent glycosylation of the protein, where as inhibition of other reactions in the formation of lipid-linked saccharide can modify the oligosaccharide structure. Alternatively, inhibition of the synthesis of the carrier, Dol-P will also prevent glycosylation of the protein. Another way to prevent glycosylation is to modify the amino acids in Asn-X-Ser/Thr, the tripeptide that carries the oligosaccharide chain. Several plant alkaloid and sugar analogues, with nitrogen in the ring instead of oxygen block the processing pathway at various stages, giving rise to modified oligosaccharide structure. Finally, the transport of glycoproteins from the endoplasmic reticulum through the various Golgi stacks is inhibited by certain ionophores; this effectively stops terminal glycosylation.

Tunicamycin, an inhibitor of lipid-linked oligosaccharide formation is a nucleoside antibiotic composed of uracil, a fatty acid and two glycosidically linked sugars. This antibiotic inhibited the first step in the lipid-linked saccharide pathway, i.e, the transfer of GlcNAc-1-P from UDP-GlcNAc to Dol-P, to form Dol-PP-GlcNAc (31). Tunicamycin acts as a tight binding, reversible inhibitor, and might be

may inhibit protein synthesis, and any observed effects on glycoprotein function or cellular physiology must be considered in that light (32). Tunicamycin has been used in great number of studies to examine the role of oligosaccharides in the properties and function of various glycoproteins.

Several other antibiotics have been shown to inhibit the formation of lipid-linked saccharides in cell free extracts of various tissues. Amphomycin inhibited the formation of Dol-P-Man. In extracts of brain, amphomycin inhibited the formation of Dol-P-Glc, Dol-P-Man and Dol-PP-GlcNAc, showing that the inhibitor might form a complex with Dol-P, thereby inhibiting these reactions (33). Tsushimycin is a lipopeptide antibiotic that appears to have the same site of action as amphomycin. Bacitracin, a peptide antibiotic, also inhibits lipid-linked saccharide formation. The action of bacitracin is due to its ability to form a complex with polyisoprenyl phosphates. Thus bacitracin appears to effect all of the reactions in which polyisoprenyl phosphate participate (34). Diumycin was found to inhibit Dol-P-Man synthesis. The antibiotic also blocked the transfer of the second GlcNAc (35). The antibiotic flavomycin also interferes with the formation of lipid-linked monosaccharides. Showdomycin probably inhibits the lipid-linked saccharide pathway because of the reactivity of of its maleimide groups towards -SH groups on the sensitive enzymes (36).

2-deoxyglucose, the sugar is converted to both UDP-2-deoxyglucose as well as to Dol-P-2-deoxyglucose. The major compound involved in the inhibition of protein glycosylation is GDP-2-deoxyglucose that serves as a sugar donor for the formation of Dol-PP$(GlcNAc)_2$-2-deoxyglucose, which cannot be further elongated (37). The accumulation of such abnormal lipid-linked saccharides may prevent glycosylation and may also tie up all the available Dol-P. The UDP and GDP derivatives of 2-deoxy-2-fluoromannose were also found to inhibit lipid-linked saccharide synthesis. A number of sugar analogues including 4-deoxy-4-fluoro-D-Glc, 4-deoxy-4-fluoro-D-Man, 2-deoxy-2-fluoro-D-Man and 6-Deoxy-6-fluoro-D-Man appeared to inhibit the synthesis of lipid-linked saccharide (38).

Glucosamine inhibits an early stage in the assembly of the lipid-linked oligosaccharides, but the inhibition requires intact cells (39). Mannosamine was also an inhibitor, and appears to inhibit at a different step in the biosynthetic pathway than glucosamine (40). One problem with the use of sugar analogues is that they may be metabolized in the cell, leading to alterations that indirectly affect protein glycosylation. Some uncouplers of oxidative phosphorylation and Glc starvation also effects glycosylation (41).

Oligosaccharide processing inhibitors have specific sites of action on the glycosidases, leading to altered oligosaccharide structure. Swainsonine was shown to be a potent inhibitor of lysosomal α-mannosidase (42). It was

from the structural similarity of its protonated from to the mannosyl cation. The liver Golgi α-mannosidase II was decreased Castanospermine, a plant alkaloid, was found to be a strong competitive inhibitor of α-glucosidases (43). It also inhibited β-glucosidase although less effectively in a competitive manner.

Deoxynojirimycin as well as nojirimycin is a Glu analogue with an amino group substituted for the oxygen atom in the pyranose ring, were found to be potent inhibitors of α-glucosidases (44). Based on the inhibition of glucosidase by deoxynojirimycin, the Man analogue of this compound was synthesized chemically and shown to be an inhibitor of α-mannosidase IA and IB (45). Glycosylmethyl-p-nitrophenyltriazenes irreversibly inactivate lysosomal β-galactosidase and β-glucosidase <u>in vitro</u> (46).

Threonine analogue, β-hydroxynorvaline inhibited the glycosylation by modification of the amino acid sequence at the Asp residue that becomes glycosylated (47). Threo-β-fluoroasparagine, markedly inhibited glycoslylation by its incorporation in to protein (48). Several inhibitors of protein synthesis also have been found to effect the synthesis of lipid-linked oligosaccharide. Inhibitors of Dol-P formation, like compactin and 25-hydroxycholesterol inhibit the synthesis of lipid-linked saccharides and in turn protein glycosylation (49,50).

differ in the size and composition of their carbohydrate units, a phenomenon known as microheterogeneity (51). Individual glycosylation sites tend to have characteristic oligosaccharides, and when microheterogeneity is encountered it usually consists of a family of closely related oligosaccharides which may differ from oligosaccharides at another site on the same protein. The single carbohydrate unit of ovalbumin is not identical in all molecules of a single preparation (52). Although microheterogeneity has apparently not been demonstrated on a single preparation of ribonuclease B, the glycoprotein isolated in different laboratories by different procedures has been reported to have variable ratio of Man to GlcNAc (53).

Microheterogeneity is believed to be an outcome of the mechanism of biosynthesis of the carbohydrate units (3), which are secondary gene products and are attached to the nascent polypeptide chain by a co-post translational modification. It is evident from the preceeding discussion that a key factor in determining the synthesis of a particular N-linked oligosaccharide is the level of expression of the various glycosyltransferases. Difference in the relative activity of these enzymes among species and tissues can account for many of the variations in oligosaccharide structure that occur. Further, the substrate specificities of the transferases determine the structural identity of the oligosaccharide sequence. This process is a non-template

fact is believed to be one of the factors responsible for the microheterogeneity of glycoproteins. Thus the microheterogeneities found in many glycoproteins represent oligosaccharides in varying stages of completion, or resulting from transglucosylations that do not have absolute specificity (54). The chain terminating mechanism may also play a role in the phenomenon of heterogeneity; however, we have no knowledge of this mechanism. As a result of microheterogeneity it is often very difficult to establish the structure of carbohydrate moieties of glycoproteins. Immobilized lectins have facilitated the isolation and purification of such glycoproteins and helped in coping with the problem of microheterogeneity.

The degradation of glycoproteins may be initiated by the action of either proteolytic enzymes or glycosidases in the lysosomes. The lysosomal concept was initiated when de Duve et al noted that several acid hydrolases were concentrated in a particulate fraction from rat liver, sedimenting between the mitochondria and microsomes (55). These hydrolases display latency if the particles are properly isolated. Integrity of the membrane shield surrounding the particle is essential for latency of the enzyme. An injury to the membrane by known abnormal situations could liberate the contained enzymes and lead to the digestion of the cell itself (56).

Lysosomes, unlike the other intracellular organells

size, shape and internal structure, because of the great diversity of these properties. Primary lysosomes are granules of acid hydrolases, newly synthesized by the cell and they have not yet been active in molecular degradation. Their proteins are synthesized by ribosomes attached to rough endoplasmic reticulum and channelled to the smooth surfaced particles of this system, and then to the Golgi apparatus appearing finally within small Golgi vesicle (57). Several types of lysosomes arose from a specialized region of smooth endoplasmic reticulum at the inner surface of Golgi stack named (GERL) (58). GERL appeared to form coated vesicles which may act as primary lysosomes. The subsequent fate of a primary lysosome is to fuse with a phagosome containing the material to be digested, thus forming a secondary lysosome, or with a secondary lysosome providing it with additional enzyme molecule, or exceptionally, with the cell membrane, discharging its enzyme in to the extracellular environment.

Lysosomes can have heterophagic or autophagic function depending on whether the substrate for intralysosomal digestion originates from outside or inside the cell respectively (59). In the heterophagic function exogeneous materials are engulfed by phagocytosis or pinocytosis, ending up with in a cytoplasmic vesicle surrounded by a membrane. Endocytotic vacuoles becomes digestive vacuoles when they receive lysosomal hydrolases. This involves,

In the autophagic function of lysosomes, a piece of cell's own cytoplasm becomes segregated by a membrane, which eventually surrounds it completely, and separates it from the rest of the cell. These vacuoles contain mitochondria, endoplasmic reticulum, microbodies, particulate glycogen and other cytoplasmic entities that show various degrees of disorganization. Having obtained its battery of lysosomal hydrolases an autophagic vacuole becomes, a secondary lysosome.

A special kind of autophagy occurs in secretory cells (60). It involves direct fusion of a lysosome with a secretory granule without preliminary seggregation and leads to the intracellular breakdown of the secretion products. The term 'crinophagy' has been suggested by de Duve for this process to distinguish it from autophagy.

Lysosomal enzymes may also be secreted by exocytosis, and be active outside the cell in the degradation of some extracellular components (61). It is thought that this is an essential condition for the efficiency of the hydrolytic process, because it allows the cell to create a microenvironment suitable for digestion. Lysosomes are equipped with ATP-driven proton pump which maintains the lysosomal acidity. Some progress has been made in the characterization of this system (62). The lysosomotropic agents, better named acidotropic agents have found to raise intralysosomal pH and impairs the delivery of enzymes

The final products of intralysosomal digestion, if sufficiently small diffuse or transported across the lysosomal membrane to enter cellular metabolism. The non-digestable products that are unable to pass through the lysosomal membrane will accumulate in lysosomes causing pathological overloading of these organells. Most of the cells, appear unable to discharge the residue of their lysosomal digestion. Their lysosomes are recycled through numerous consecutive digestive events, and in the process become laiden with increasing amount of residues. This phenomenon is accelerated in various storage diseases characterized by the congenital lack of one or more of the lysosomal hydrolases.

Man-6-P was discovered as the recognition marker in the uptake of lysosomal enzymes (64). The functions of phosphorylated recognition marker depends on their interaction with Man-6-P receptors. Two types of Man-6-P receptors are known, (a) cation independent receptor (MPR) and (b) cation dependent receptor (65). The binding to receptor followed by selective packaging into vesicles, separates the newly synthesized enzymes from other products. Receptor-ligand complex dissociates below pH 5.7. In vivo the same mechanism appears to be utilized for dissociation of receptor-ligand complexes (66). CURL (compartment for uncoupling receptor and ligand) is a likely candidate as the site where receptors are physically separated from

into specific vesicles in the CURL, which fuse with existing lysosomes. A more likely possibility is gradual transition of CURL elements to lysosomes (67). Man-6-P independent mechanism also contributes to targetting lysosomal enzymes in normal tissues (68).

Lysosomes effect the digestion of biological material through the large collection of hydrolytic enzymes acting on most of the bonds found in proteins, nucleic acids, lipids, oligosaccharides and polysaccharides. Lysosomes have been shown to be capable of digesting proteins extensively both in vivo and in vitro through the synergistic action of a series of endo- and exopeptidases that release peptides and free amino acids. The cathepsins are important among the peptidases of lysosomes. Cathepsin A is a carboxypeptidase, and its action is slowed down by the presence of carboxy-terminal basic amino acids (69). Cathepsin B is found to be an endopeptidase of broad substrate specificity (70). Cathepsin C or dipeptidylaminopeptidase I attacks polypeptide chains at the amino terminal to sequentially remove one terminal dipeptide after the other (71). Cathepsin D is the major acid protease of animal tissues with a specificity similar to that of pepsin (72). The cathepsins are involved in the extensive hydrolysis of proteins at an acid pH including the peptide moiety of glycoproteins (73). A complete hydrolysis of nucleic acids to free pyrimidine and purine nucleosides and inorganic

A lipase acting on fatty esters of glycerol is present in the lysosomes (74). Lysosomes contain phospholipase A1, which splits the acyl ester bond at C-1 of the phosphoglyceride, and phospholipase A2, active on the ester bond that involve C-2 (75).

An acid sphingomyelinase was found in brain and it hydrolyses sphingomyelin into ceramide and phosphoryl choline was appeared to be restricted to lysosomes (76,77). Lysosomes from brain was also shown to contain ceramidase which hydrolyze the acyl bond in ceramide (78). Enzymes capable of releasing free hexose from galactocerebroside or from glucocerebroside have been located in the lysosomes (79) Lysosomal arylsulfatases are involved in the desulfation of sulfatids.

Gangliosides contain more complex oligosaccharides. The digestion of gangliosides requires the sequential action of neuraminidase, β-galactosidase, N-acetyl-β-hexosaminidase β-cerebroside-β-glucosidase and ceramidase. All these enzymes have optimal activity at acid pH. Neuraminidase releases sialic acid from di-and trisialogangliosides but not from monosialoganglioside (GM_1) and from Tay-Sachs ganglioside (GM_2) (80). Four distrinct β-galactosidases are active in the degradation of glycolipids: Galactocerebroside -β-galactosidase, monosialoganglioside-β-galactosidase, lactosyl ceramide-β-galactosidase and digalactosyl glucosyl ceramide-α-galactosidase. A crude preparation of β-galacto-

TABLE - 1

ACID HYDROLASE DEFICIENCY IN SOME OF THE LYSOSOMAL STORAGE DISEASES

DISEASE	SIGNS AND SYMPTOMS	MAJOR LIPID ACCUMULATION	ENZYME DEFEC
FARBER'S DISEASE	HOARSENESS DERMATITIS SKELETAL DEFORMATION MENTAL RETARDATION	CERAMIDE OH SPHINGOSINE O C N-H FATTY ACID	CERAMIDASE
GAUCHER'S DISEASE	SPLEEN AND LIVER ENLARGEMENT EROSION OF LONG BONES AND PELVIS MENTAL RETARDATION ONLY IN INFANTILE FORM	GLUCOCEREBROSIDE CERAMIDE N GLUCOSE	GLUCOCEREBROSIDE β-GLUCOSIDASE
KRABBE'S DISEASE (GLOBOID LEUKODYSTROPHY)	MENTAL RETARDATION ALMOST TOTAL ABSENCE OF MYELIN GLOBOID BODIES IN WHITE MATTER OF BRAIN	GALACTOCEREBROSIDE N GALACTOSE	GALACTOCEREBROSID β-GALACTOSIDASE
METACHROMATIC LEUKODYSTROPHY	MENTAL RETARDATION PSYCHOLOGICAL DISTURBANCES IN ADULT FORM NERVES STAIN YELLOW BROWN WITH CRESYL VIOLET DYE	SULFATIDE N OSO_3 GALACTOSE 3-SULFATE	SULFATIDASE
CERAMIDE LACTOSIDE LIPIDOSIS	SLOWLY PROGRESSING BRAIN DAMAGE, LIVER AND SPLEEN ENLARGEMENT	CERAMIDE LACTOSIDE N GLUCOSE GALACTOSE	NEUTRAL β-GALACTOSIDASE
FABRY'S DISEASE	REDDISH PURPLE SKIN RASH, KIDNEY FAILURE PAIN IN LOWER EXTREMITIES	CERAMIDE TRIHEXOSIDE N GLUCOSE GALACTOSE GALACTOSE	CERAMIDETRIHEXOSID α-GALACTOSIDASE
TAY SACHS DISEASE	MENTAL RETARDATION, RED SPOT IN RETINA, BLINDNESS MUSCULAR WEAKNESS	GANGLIOSIDE GM_2 N GALACTOSE GalNAc GLUCOSE NeuAc	HEXOSAMINIDASE A
TAY SACHS VARIANT	SAME AS TAY SACHS DISEASE BUT PROGRESSING MORE RAPIDLY	GLOBOSIDE (AND GANGLIOSIDE GM_2) N GLUCOSE GALACTOSE GalNAc GALACTOSE	HEXOSAMINIDASE A AN
GENERALIZED GANGLIOSIDOSIS	MENTAL RETARDATION LIVER ENLARGEMENT SKELETAL DEFORMITIES ABOUT 50 PERCENT WITH RED SPOT IN RETINA	GANGLIOSIDE GM_1 N GALACTOSE GalNAc GALACTOSE GLUCOSE NeuAc	β-GALACTOSIDASE
FUCOSIDOSIS	CEREBRAL DEGENERATION MUSCLE SPASTICITY THICK SKIN	H ISOANTIGEN N GALACTOSE GLUCOSE GALACTOSE N ACETYL GLUCOSAMINE FUCOSE	α-FUCOSIDASE

Adapted from, Brady R.O. (1979) in "Glycoconjugate Research". (Gregory, J.D. and Jeanlog, R.W. eds) Vol.II, pp-856, Academic Press, New York.

position of GM_1 ganglioside and GA_1 ganglioside, the two glycolipids that accumulate in GM_1 gangliosidosis (81). Dawson and Stein (82) have shown the deficiency of lactosyl ceramide-β-galactosidase, the enzyme that releases the terminal Gal from lactosyl ceramide in a congenital disease with the accumulation of lactosyl ceramide. The enzyme that is deficient in Fabry's disease was identified as α-galactosidase. Table.1 gives a collection of various inborn lysosomal disorders. Two different form of N-acetyl-β-hexosaminidases are involved in the hydrolytic degradation of gangliosides and that of globoside.

Majority of the enzymes that hydrolyze glycosyl bonds in mammalian tissues are located in lysosomes and they form the single largest group of enzymes in these organells. Glycosidases are implicated in the digestion of oligosaccharides and carbohydrate chains of mucopolysaccharides, glycolipids and glycoproteins. Glycosidases are of two types (1) endoglycosidases which act within the main chain and (2) exoglycosidases which mediates stepwise attack at the non-reducing end groups of the polymorphic carbohydrate structures. Many of these hydrolases have been assayed on synthetic substrates and that their action on natural substrates are often less well known.

Several disaccharides, cellobiose, gentibiose, turanose and trehalose, which all resisted digestion by macrophage lysates caused the vacuolization of the lysosomal system

could be hydrolyzed by the macrophage α-glucosidase or β-galactosidase did not. Thus, it appears that some disaccharides are digested in vivo in lysosomes forming monosaccharide products that can diffuse freely out of these organells. Liver lysosomes also contain an α-glucosidase that is able to hydrolyze the α1→4 glycosidic linkages of maltose and glycogen (83). This enzyme is deficient in type II glycogenosis (Pomp's disease) an inborn error of metabolism characterized by the accumulation of glycogen in lysosomal vacuoles (84).

In the digestion of proteoglycans hydrolysis of protein core may be as important as that of carbohydrate chains. An acid hyaluronidase with optimal activity around pH 6.0 has been demonstrated in various tissues (85) and found to be associated with lysosomes (86). In vitro purified lysosomal hyaluronidase acts on both chondroitin 4-and 6-sulfate although less rapidly than on hyaluronic acid (87). It was shown that hyaluronate was first degraded by the endohexosaminidase action of hyaluronidase to oligosaccharides which are then attacked by two exoglycosidases, β-glucuronidase and N-acetyl-β-glucosaminidase, both of lysosomal origin (88). The hydrolysis of keratan sulfate, proceeds through a pathway similar to that of hyaluronate degradation but involving the action of specific sulfatase found in lysosomes (89), as well as that of β-galactosidase, a lysosomal enzyme which is deficient in

Complete degradation of glycoprotein with in cells requires as many glycosidases as there are distinct glycosidic bonds in the molecule, an unknown number of proteolytic enzymes and a specific enzyme cleaving the carbohydrate to protein linkage. Lysosomes contain enzymes acting on practically every glycosidic linkage found in glycoproteins. Carbohydrate portion of glycoproteins split off sequentially from the non-reducing end by exoglycosidases present in lysosomes, and the absence of a particular exoglycosdase prevents the action of enzyme next in line. Schemes for the catabolism of complex N-glycosidic chains are given in Figure.4. The digestive activity of highly purified lysosomes at an acid pH on several glycoproteins has been studied in vitro by Aronson and de Duve (90). With orosomucoid or fetuin as substrate, both containing oligosaccharide chains with the sequence NeuNAcα2$\rightarrow$3Galβ1$\rightarrow$4GlcNAc linked to the core, peptide cleavage started rapidly up to the splitting of about 50% peptide bonds, before the attack on the oligosaccharide chains.

The first step in the degradation of the carbohydrate chains of glycoproteins involved the removal of terminal sialic acid by a lysosomal neuraminidase. Gangliosides were also hydrolyzed by the lysosomal neuraminidase. Apart from general catabolism of glycoproteins, tissue neuraminidases might conceivably function in the control of the biological activity and life of glycoproteins (91,92).

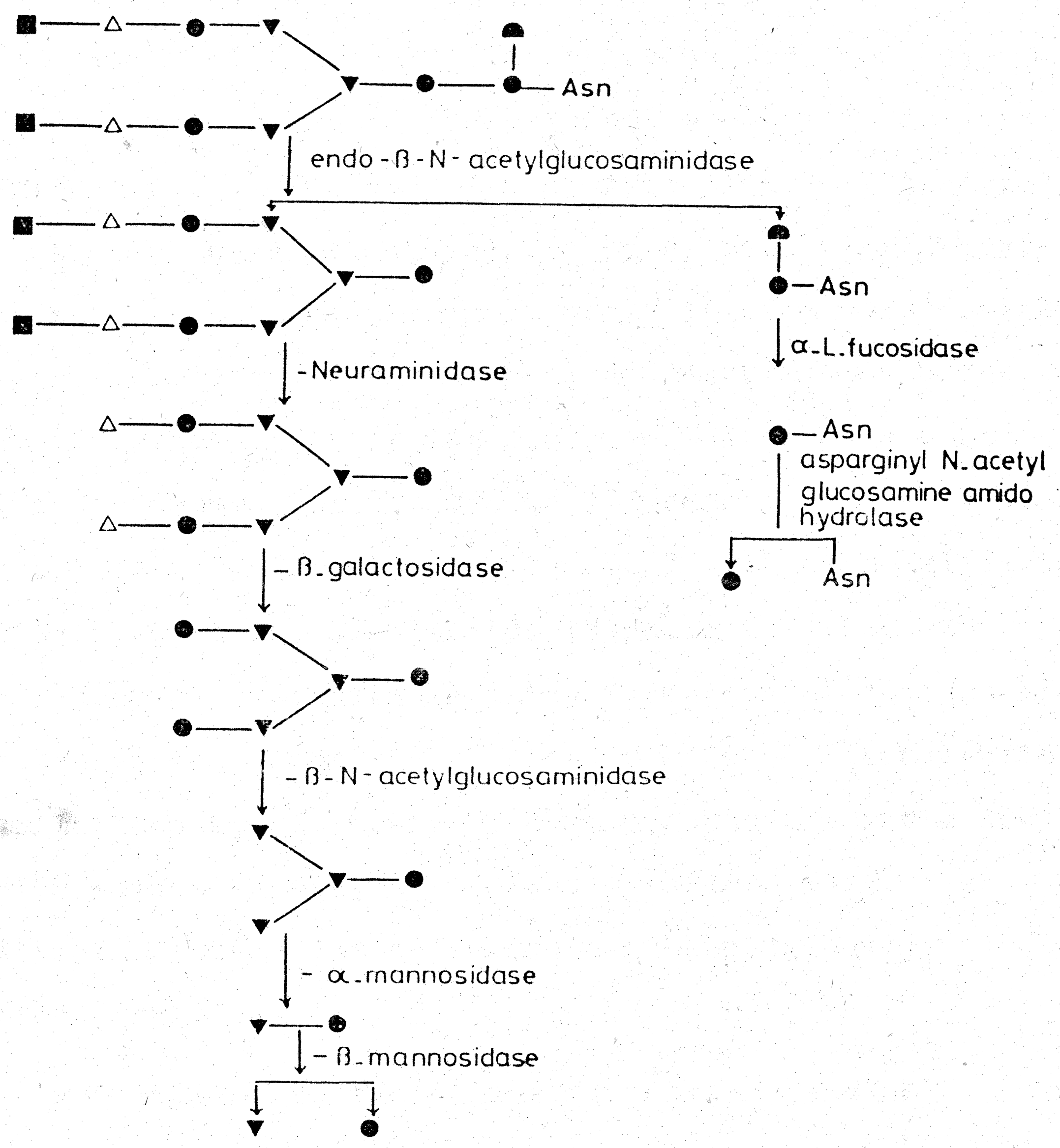


Fig.4. Proposed pathway for the catabolism of asparagine-linked oligosaccharide chains.

Modified from Sharon, N. and Lis, H. (1982)

The proteins (Neurath, H. and Hill, R.L. ed.) Vol.V, p-98
Academic Press, New York.

After the complete removal sialic acid, β-galactosidase acts to release Gal from the oligosaccharides of glycoproteins. The acid β-galactosidases are optimally active in the pH range 4.0-4.5, is believed to be of lysosomal origin. The hydrolysis of terminal Gal residues from GM_1 ganglioside is stimulated by an activator protein (93 The absence or reduced catalytic activity of β-galactosidase has been associated with an inherited metabolic disorder GM_1 gangliosidosis (94). The acid β-galactosidase appear to be under common genetic control, distinct from that of neutral β-galactosidase, which is found with a normal range of activities in GM_1 gangliosidosis patients (95). In humans, acid β-galactosidase activity exists as low and high molecular weight forms (96). Both forms were thermolabile and stimulated by Cl^- ions. A common subunit for the low and high molecular weight form was described (97). Two forms of the enzyme was shown to be immunologically identical (98).

N-Acetyl-β-hexosaminidase hydrolyzes terminal GlcNAc from the oligosaccharide chains of glycoproteins. Lysosomal N-acetyl-β-hexosaminidase hydrolyzed the β-glycosidic linkage of both N-acetylglucosaminides and N-acetylgalactosaminides (99). Two isoenzymes of N-acetyl-β-hexosaminidase has been demonstrated in human tissues (100). The two forms of the enzyme shows similar catalytic activities. Form B is not inactivated at 50°C for 4h,

inactivated under these conditions. Both forms of the enzyme share a common subunit. Deficiency of N-acetyl-β-hexosaminidase A was found in Tay-Sachs disease. Mammalian N-acetyl-β-hexosaminidase has been shown to release terminal GlcNAc from hyaluronic acid or chondroitin-4-sulfate.

Alpha-linked Man residues in the oligosaccharide chains were hydrolyzed by a lysosomal α-mannosidase and the enzyme was found to be deficient in α-mannosidosis, an inborn error of metabolism where Man rich glycoproteins are found to accumulate in tissues. Zn^{2+} was found to be essential for the enzyme activity (101). The β-linked Man residue present in the core region of Asn-linked oligosaccharides is hydrolyzed by a lysosomal β-mannosidase (102).

L-Fucose a frequent constituent of glycoproteins is released by the action of a lysosomal α-L-fucosidase. It was found to be deficient in an inborn error of metabolism, fucosidosis, in which Fuc containing glycoproteins and glycolipids accumulate in tissues.

Carbohydrate-peptide linkages found in glycoproteins were hydrolyzed through the action of lysosomal enzymes. A lysosomal N-acetyl-α-galactosaminidase has been found to hydrolyze the O-glycosidic linkage between GalNAc and Ser (102). Beta-Aspartylacetylglucosaminidase that split GlcNAc-Asn linkage has also been demonstrated

carbohydrate linked Asn, which could not be substituted. But the carbohydrate could be either GlcNAc or an oligosaccharide with GlcNAc at the reducing end (104). The glucosylgalactose units that are bound to the Hyl of collagen by a β-o-glycosidic bond may be degraded by the sequential actions of lysosomal α-glucosidase and β-galactosidase (105).

ANIMAL LECTINS

Lectins are a group of di-or polyvalent carbohydrate binding proteins of non-immune origin, grouped together because they all agglutinate erythrocytes. Saccharide specificity of individual lectins reveals a wide range of variation with respect to configurations and substitution patterns at different carbon atoms of the monosaccharide. Lectin-carbohydrate interaction is used as a tool in various biochemical and clinical studies (108).

Vertebrate lectins can be subdivided on the basis of whether or not they are integrated into membranes: (a) integral membrane lectins that require detergents for their extraction and calcium ion for saccharide-binding activity and (b) soluble lectins (109,110). This subdivision probably reflects a fundamental difference in the general functions of these classes. The first group consists of lectins that differ in their saccharide specificities and physicochemical properties (111). Among the best characterized lectins of this class are the Gal and Man/GlcNAc specific

of cells (68). Recently, an endogenous lectin specific for thiodigalactoside and GalNAc has been isolated from microsomal fractions of baby hamster kidney cells (114), and a Gal specific lectin has been purified from extracts of metastatic B_{16} melanoma cells (115).

The first purified soluble vertebrate lectin was the β-galactoside-specific lectin from the electric organ of the eel Electrophorus electricus by Teichberg et al (116). Subsequently, many lectins with the same specificity were also found in many tissues of animals and in embryos at the very early stages of their development (109). Isolectins were also found (117). Most of the soluble lectins isolated from vertebrate tissues bind β-galactosides. The soluble β-galactoside-specific lectins are of similar molecular size, each consisting of two subunits of Mr.=13,500-16,000 (109). They exhibit immunological cross reactivity, which can be correlated to the phylogenetic distance separating the species from which the lectins are derived (118). All soluble β-galactoside-specific lectins require a reducing agent to maintain their carbohydrate binding activity. Significant sequence homology was demonstrated between the soluble β-galactoside-binding lectins from different vertebrate species (119).

Vertebrates also contain another group of soluble β-galactoside-binding proteins that have been isolated as monomers (Mr.=13,000-14,000). The monomeric lactose-

lectin-II) has been well characterized (120). It differs from the dimeric chicken lactose lectin-I in subunit molecular weight, isoelectric point and peptide map as well as immunologically. Since, chicken lactose lectin-II agglutinates erythrocytes, it must either have two carbohydrate binding sites per monomer or form dimers or oligomers when interacting with cells. A monomeric β-galactoside-binding lectin isolated from rabbit bone marrow has been named erythroid developmental agglutinin (121).

Not all soluble vertebrate lectins are β-galactoside specific. Those from the serum of the eel Anguilla rostrata are specific for L-Fuc and consists of 12 subunits (122). A soluble lectin from Xenopus laevis oocytes and embryos also contain 12 subunits and specific for both α-and β-galactosides (123).

Biological role of lectins

Integral membrane lectins are thought to mediate the binding of soluble extracellular and intracellular glycoproteins as well as cells. The classical example is the lectin of mammalian liver that binds Gal and is believed to function as a cell surface receptor for circulating asialoglycoproteins. The Man/GlcNAc specific lectins of avian hepatocytes binds to asialo-agalacto glycoproteins (113). Another example is the pinocytosis of glycoproteins with terminal non-reducing Man/GlcNAc residues by macrophages, mediated by a macrophage surface lectin specific for these

targetting of hydrolytic enzymes to lysosomes. Galactose-specific lectins present on various human and murine tumors were suggested to influence the pathogenesis of cancer metastasis by promoting the formation of tumor cell aggregates in the circulation and their adhesion to the endothelial layer of capillaries (125,126).

Soluble vertebrate lectins appears to be secreted. In some cases there is a shift from an intracellular to an extracellular location with differentiation. For example, chicken lactose lectin-I, which is concentrated intracellularly in developing muscle becomes extracellular with maturation (127). Like wise a rat β-galactoside-binding lectin in lung is concentrated in elastic fibers (128). There is also evidence that chicken heparin lectin is secreted by muscle cells differentiating in tissue culture. Lectin secretion into extracellular matrix suggests that it may function by interacting with extracellular glycoconjugates; it is likely that such binding occurs in nature and is biologically significant.

Since vertebrate lectins are ontogenetically regulated, evidence for a possible mechanistic role of lectins in the development of vertebrate tissue has evolved. The striking increase in chicken muscle lectin correlates temporarily with fusion of these cells to form polynucleated myotubes, indicating that the lectin plays a role in this process. Thiodigalactoside a potent inhibitor of the lectin blocks

To determine the functions of lectins it is necessary to isolate the tissue glycoconjugates which they normally bind. The natural ligands are abundant in tissues rich in the lectin and also localized in the same compartment. The endogenous receptor of the β-galactoside-binding lectin from chick embryo skin may have a polylactosaminoglycan structure (130), which has recently been suggested to be a differentiation marker in developmental tissues (131). The significant sequence homology demonstrated between the β-galactoside-specific lectins from phylogenetically distant vertebrate species, is likely to reflect an important physiological role for these lectins in ontogenic events and differentiation steps (119). Human placenta lectin stimulates the production of tumor killing factor by murine macrophages and human monocytes (132). These results suggest the possible involvement of this family of lectins in the immune system.

...

AIMS AND OBJECTIVES

Mammalian β-galactosidase (EC 3.2.1.23) exists in multiple forms and kinetic studies with synthetic and natural substrates have been reported in purified preparations (133-137). The acidic form is believed to be of lysosomal origin (138). The wide-spread occurrence and broad substrate specificity of the acid β-galactosidase in mammalian organs is probably related to the multiple physiological functions of the enzyme. Occurrence of an inborn error of metabolism, GM_1 gangliosidosis (139) and its clinical variants, characterized by the accumulation of gangliosides and glycosaminoglycans due to the deficiency of acid β-galactosidase had increased the interest in this field.

There are few reports in the literature on the structural studies of lysosomal enzymes. Detailed structural study of this enzyme would help in understanding its structure-function relationship. Evaluation of the oligosaccharide structure will add information towards the role of glycosylation in glycoproteins.

The presence of β-galactoside-binding lectins in the human placental tissue is an important observation. The structural comparison of the binding sites of the enzyme and the lectin acting on β-galactoside deserves attention. The exact biological function of lectins are not clearly known. Much effort has been invested in search of applications of lectins. As a result, lectins have

diverse fields. They continue to be the focus of intense interest in their own right. Recently, it was suggested that the galactoside-binding lectin is involved in the immune system (225). To gain an insight into the physiological function and relationship with other β-galactoside-binding lectins a detailed study has been undertaken.

The objective of the present study is to undertake structural studies on human placental acid β-galactosidase and β-galactoside-binding protein in the following directions: (a) Modification of specific amino acid to obtain an insight into the residues involved at the active site, (b) elucidation of structural features of oligosaccharide chains by lectin-Sepharose affinity chromatography and their effect on the activity, (c) inhibition by various saccharides, (d) N-terminal amino acid sequence determination, (e) binding characteristics of the lectin with fluorescence and isotope-labeled saccharide derivatives and (f) immunological studies of the enzyme and lectin.

...

CHAPTER - II

MATERIALS AND GENERAL METHODS

MATERIALS

Galactopyranosylamine, thiodigalactoside, galactosamine, dulcitol, melibiose, raffinose, stachyose, 1-0-methyl-α-and β-D-galactopyranoside, galactose, p-aminophenyl thiodigalactoside, N-acetylglucosamine, arabinogalactan, p-nitrophenol, p-nitrophenyl derivatives of α-and β--D-galactopyranoside, trinitrobenzene sulphonic acid, p-hydroxymercuribenzoate, dithionitrobenzoic acid, fetuin, N-ethylmaleimide, N-acetylimidazole, N-bromosuccinimide, bovine serum albumin, ovalbumin, soybean trypsin inhibitor, acrylamide, N,N'-methylene-bis-acrylamide, N,N,N',N',-tetramethylenediamine, Coomassie brilliant blue G, dansylchloride, dansyl derivatives of amino acids, galactose oxidase, horse radish peroxidase, trifluoroacetic acid, 2-mercaptoethanol, 4-methylumbelliferyl-β-D-galactopyranoside and lysostaphin were purchased from Sigma Chemical Co. St. Louis, U.S.A. Phenylisothiocyanate, 2-hydroxy-5-nitrobenzyl bromide, 2-methoxy-5-nitrobenzyl bromide, diethyl-pyrocarbonate and 1,2-cyclohexanedione were obtained from Fluka, Buchs, Switzerland. Sephadex, Sepharose and blue dextran 2000 were products of Pharmacia Fine Chemicals, Uppsala, Sweden. The molecular weight marker proteins and Coomassie brilliant blue R-250 were purchased from Pierce Chemical Co. Rockford, U.S.A. Biogel P-60 and P-100 were obtained from Biorad Laboratories, Richmond, U.S.A. 1-0-methyl α-D-mannopyranoside, 1-0-methyl-α-D-glucopyranoside, N-ethyl-

Colnbrook, England. SDS is a product of BDH chemicals, Ltd., England. Dowex 50x8 and dowex 1x4 were purchased from Serva Feinbiochemica, Hidelberg, Germany. Galacto-1-deoxynojirimycin was a generous gift from Prof. Legler of University of Cologne. Low and high methylated polymer of galacturonic acid was a gift from Dr. Richard Joseph, Central Food Technological Research Institute, Mysore. NaB^3H_4 was from Amersham International plc. England. All other reagents were of analytical grades and obtained from local sources.

METHODS

The protein concentration was estimated according to the method of Lowry et al (140) with crystalline bovine serum albumin as standard and/or Bradford's protein assay (141) with crystalline ovalbumin as standard.

The total carbohydrate content was estimated by phenol sulphuric acid method of Dubois et al. (142) with Gal as standard.

Preparation of affinity matrices

Concanavalin A was isolated from *Canavalia gladiata* according to the method of Surolia et al (143). It was coupled to Sepharose 4B by the proceedure of Axen et al (144). 50ml of thoroughly washed Sepharose 4B was suspended in 50ml of water and 100ml of 2M sodium carbonate. The temperature of the slurry was maintained at 10°C and 1.5gm of cyanogen

was filtered through a sintered glass filter and washed thoroughly with 0.1M sodium bicarbonate buffer, pH 8.5. The gel was transferred to Con A solution (500mg Con A dissolved in 50ml 0.1M sodium bicarbonate, pH 8.5) and stirred slowly at 4°C for 18 hr. The unreacted active groups were blocked by mixing for 1 hr with 0.5ml ethanolamine. The gel was washed successively with 0.1M sodium bicarbonate, pH 8.5, 0.1M sodium chloride, distilled water, 0.02M Tris-HCl, pH 7.4 containing 1mm Mg^{2+}, Ca^{2+}, Mn^{2+} and finally with 0.02M Tris-HCl, pH 7.4.

Wheat germ agglutinin was purified from wheat germ on chitin column according to Bloch and Burger (145). Castor bean (Ricinus cummunis) was the source of RCA and was purified by affinity chromatography on cross-linked guar gum according to Appukuttan et al (146). Jack seed agglutinin was purified from Jack fruit seeds (Artocarpus integrifolia) according to the method reported from this laboratory (147). Winged bean agglutinin was purified from Psophocarpus tetragonolobus on sepharose-6-aminocaproyl-D-galactosamine column according to Appukuttan and Basu (148).

Asialofetuin-Sepharose 4B was prepared according to deWaard et al (149). 200mg of fetuin type III was desialilated in 0.1N H_2SO_4 at 80°C for 1 hr. The desialylated protein was dialyzed against 0.2M sodium bicarbonate with 3 changes at 4°C for 18 hr. It was coupled to 20ml of packed activated Sepharose 4B according to Cuatrecasas (150).

Protein A was extracted from Staphylococcus aureus (Cowan strain I) by digestion with lysostaphin. It was purified by affinity chromatography on Immunoglobulin-Sepharose 4B coloumn according to Hjelm et al (151). The purified protein A was immobilised to Sepharose 4B by cyanogen bromide method as mentioned earlier.

Purification of β-galactosidase A and B

Two forms of acid β-galactosidase were separated and purified to homogeneity from human placenta by the method of Sarasija and Basu (152), using a four step procedure involving: (1) Con A-Sepharose 4B affinity chromatography, (2) gel filtration on Sephadex G-200, (3) ion exchange chromatography on DEAE-Sephadex A-50 and (4) affinity chromatography on mercurial-Sepharose CL-4B. The yield of the enzyme was low. The method was modified by inducting a second Con A-Sepharose affinity chromatography, after DEAE-Sephadex A-50 chromatography. Normal human full term placental tissues were collected from Trivandrum Medical College on ice. The tissue freed from cords and adhering tissue material was thoroughly washed in cold distilled water and kept frozen at $-20^{o}C$ until use. The trophoblasts were separated from fresh tissues and kept overnight at $0^{o}C$. All preparations were carried out at $0-4^{o}C$ unless otherwise mentioned.

Frozen placental tissue (500g) was homogenized

20,000g for 20 min in SORVAL RC-5B. The residue was discarded.

Solid ammonium sulphate (400gm/1) was added to the supernatent with constant stirring and pH was maintained at 7.0 with dilute NH_4OH. The suspension was stirred for 30 min and centrifuged as before. The pellet was dissolved in minimum volume of 0.05M phosphate buffer, pH 7.0 containing 0.5M NaCl. The solution was dialyzed against 20 volumes of the same buffer with two changes for 16 hr.

The dialyzed enzyme was centrifuged and passed through Con A-Sepharose 4B column (2.5x15cm). The column was washed with 0.05M phosphate buffer, pH 7.0 containing 0.5M NaCl at 25°C until the effluent had an absorbance of less than 0.05 at 280 nm. The enzyme was eluted at 25°C with the same buffer containing 0.5M α-methyl-D-glucopyranoside and 10ml fractions were collected. Active fractions were pooled and dialyzed against 0.02M phosphate buffer, pH 7.0 with 3 changes. The dialyzed solution was lyophilized. The residue was dissolved in minimum volume of 0.02M phosphate buffer, pH 7.0 containing 0.1M NaCl and dialyzed against the same buffer with 3 changes for 16 hr.

The dialyzed enzyme (5ml) was passed through a Sephadex G-200 column (2x78cm; Vo-104ml). The column was equilibrated and eluted with 0.02M phosphate buffer, pH 7.0, containing 0.1M NaCl at a flow rate of 12ml/hr.

The first peak, which appeared just after the void volume was β-galactosidase A and the second peak at inner volume of the column as β-galactosidase B.

Beta-galactosidase A and B-forms were dialyzed for 12 hr with 3 changes against 0.02M phosphate buffer pH 6.0, and applied to DEAE-Sephadex A-50 columns (1.8x10cm) equilibrated with the same buffer. The columns were washed with 100ml of the same buffer, followed by 100ml of the same buffer containing 0.1M NaCl. The enzyme was eluted with 0.25M NaCl in the equilibrating buffer.

The pooled active fractions of both A and B forms were adsorbed to second Con A-Sepharose column (1.8x7cm) after increasing the NaCl concentration to 0.5M. The column was washed with 0.05M phosphate buffer, pH 7.0 containing 0.5M NaCl until the effluent had an A_{280} below 0.05. The enzyme was eluted at 25°C with the same buffer containing 0.5M α-methyl-D-glucopyranoside and 5ml fractions were collected. Active fractions were pooled, dialyzed against 0.02M phosphate buffer, pH 7.0, containing 100mM NaCl with 3 changes for 15 hr and concentrated by ultrafiltration through Amicon PM-10 membrane.

Purification of β-galactoside-binding protein

Frozen placenta or trophoblasts were used for the isolation of β-galactoside-binding protein. The buffer used for all the experiments was 20mm phosphate-150mM

Galactoside-binding protein was isolated according to Hirabayashi and Kasai (153) with modification. One hundred gram of tissue was homogenized with 500ml of PBS, pH 7.2 in SORVAL Omni mixer at 25% of its maximum speed for 2 min. The homogenate was centrifuged at 6000g for 20 min. The pellet was extracted twice with 100ml of PBS, pH 7.2 containing 100mM lactose for 30 min. After each extraction the supernatent was collected by centrifugation as above.

The protein was precipitated with solid ammonium sulphate (56g/100ml) from the pooled lactose extract. The precipitate obtained by centrifugation at 10,000gx20 min was dissolved in minimum volume of PBS. The lectin solution was dialyzed for 18 hr against the same buffer.

The dialyzed solution was passed through an asialofetuin-Sepharose column (2x18cm). The column was equilibrated and washed with PBS, pH 7.2, until the effluent had A 280 below 0.02. The galactoside-binding protein was eluted with the same buffer containing 20mM lactose and 3ml fractions were collected. The hemagglutinating activities of the dialyzed fractions were assayed with trypsinized rabbit or human B+ve erythrocytes. The active fractions were pooled, dialyzed against PBS, pH 7.2 for 18 hr and concentrated by ultrafiltration through Amicon PM-10 membrane.

The purity of the proteins were examined by poly-

to Davis (154). 20-40μg proteins were used in each tube at 23°C. The protein was fixed on the gel by keeping in 12.5% trichloroacetic acid for 1 hr. The gels were stained with coomassie brilliant blue R-250 and destained with methanol:acetic acid:water 1:1.5:17.5 (V/V).

The molecular weight of the native protein was determined by gel filtration on Biogel P-100 according to Andrews (155). The column was (2x78cm) equilibrated with PBS, pH 7.2, containing 2.5mM mercaptoethanol at 25°C. 2.5ml fractions were collected at a flow rate of 12ml/hr. The column was caliberated with the standard proteins, cytrochome C (12,500), soybean trypsin inhibitor (21,500), jack seed agglutinin (40,000) and ovalbumin (45,000).

The subunit molecular weight was determined by SDS-polyacrylamide gel electrophoresis according to Laemmli (156). Electrophoresis was carried out in small vertical slab gel unit (Hoefer Scientific Instruments, San Fransisco, California) on 1.5mm thick slab gel (10% W/V) at 15 mA current at 23°C. The standard marker proteins used were jack seed agglutinin (10,000), aldolase (34,500), ovalbumin (45,000) and bovine serum albumin (67,000). All the proteins were heated in boiling water bath for 3 min with 2% SDS and 10mM 2-mercaptoethanol, prior to application on the gel. Each well contained 5-10 μg of protein. The protein was stained with Coomassie

Chemical modification of amino acids

The amino groups were modified by treatment with trinitrobenzene sulphonate according to Fields (158). The protein in 0.1M borate buffer, pH 8.5 was treated with 10μl of 1.1M TNBS for 5 min. at 23°C. The reaction was stopped by adding 0.3ml of 0.1M NaH_2PO_4.

The protein (112μg) was citraconylated as described by Atassi and Habeeb (159), using 50μl reagent in water pH adjusted to 8.2. The pH was maintained at 8.2 during the addition of citraconic anhydride using 5N NaOH and the reaction mixture was incubated at 23°C for 2 hr.

Maleylation was carried out as described by Butler et al (160). The protein (112μg) in 0.2M borate buffer, pH 9.0 was treated with 100μl maleic anhydride (3mg/ml) at 23°C for 5 hr.

Sulphydryl groups of the proteins were estimated and modified with DTNB according to Habeeb (161). The proteins in 0.08 M phosphate buffer, pH 8.0 were treated with 0.02ml DTNB (4mg/ml) for 15 min at 23°C to modify the -SH group.

Sulphydryl groups were also modified with pHMB and N-ethylmaleimide according to Riordan and Vallee(162). The proteins were incubated with pHMB (10^{-4}M) or N-ethylmaleimide (10^{-3}M) for 1 hr at 23°C. Modification of -SH group by iodoacetic acid (10^{-2}M) was carried out at

Histidine residues were modified by carbethoxylation with diethylpyrocarbonate according to Miles (164). The proteins in 0.1M potassium phosphate buffer, pH 6.5 were incubated with 25μl of 10mM diethylpyrocarbonate in ethanol at 23°C for 1 hr.

Arginine residues are specifically modified by the diketone phenylglyoxal hydrate by the method of Takashi (165). The reaction mixture prepared by mixing 1% solution of phenylglyoxal hydrate dissolved in 0.02M N-ethylmorpholine-acetate buffer, pH 8.0 to equal volume of proteins dissolved in the same buffer. The reaction was carried out for 16 hr. at 23°C.

Arginine residues were also modified by treating with 1,2-cyclohexanedione according to Smith (166). The enzyme proteins were treated with 15M excess of reagent in 0.25M borate buffer, pH 8.0 at 37°C for 2 hr. The reaction mixture was dialyzed in 0.2M borate buffer, pH 7.0, since arginine regenerates slowly when borate is removed by dialysis.

Tryptophan was modified with 2-hydroxy- and 2-methoxy-5-nitrobenzyl bromide according to Horton and Koshland (167). 3mg of these benzyl halides in 50μl acetone were added to the enzyme proteins in 0.02M phosphate buffer, pH 7.0 and lectin in PBS, pH 7.2. 2-Hydroxy-5-nitrobenzyl bromide was allowed to react for

Spande and Witkop (168). The enzyme proteins in 0.1 M-formate-buffer, pH 4.1 was treated with 50 μl of 20 mM N-bromosuccinimide for 30 min at 23°C.

The tryptophan residues were determined by measuring the absorbance at 250nm and 280nm before and after oxidation to oxindole chromophore with a mixture of dimethylsulphoxide, concentrated HCl and glacial acetic acid as described by Savige and Fontana (169).

Tyrosine residues were estimated and modified by acetylation with N-acetylimidazole as described by Riordan and Vallee (162). The proteins in 20mM sodium barbitone buffer, pH 7.5 was acetylated by treating with 400mM excess N-acetylimidazole at 23°C for 1 hr. The number of O-acetylated tyrosyl groups in the protein was calculated from the absorbance change at 278 nm after the addition of 1M NH_2OH to a solution of the acetylated enzyme.

The modified enzyme protein solutions were dialyzed in 20mM phosphate buffer, pH 7.0 containing 100mM NaCl with 3 changes for 8 hr before the assay of residual enzyme activity. Modified β-galactoside-binding protein was dialyzed with 3 changes against PBS, pH 7.2 for 8 hr prior to hemagglutination assay. The same proteins without modifying reagent in the reaction mixture were used in all modification assay systems as controls.

Modification of oligosaccharide chains

Spiro (170). Beta-galactoside-binding protein in 0.1M acetate buffer, pH 5.0 was incubated with 10^{-3}M sodium metaperiodate at 4°C in the dark for 16 hr. The reaction was stopped by the addition of ethylene glycol. The sample was dialyzed against PBS, pH 7.2 and hemagglutination activity was assayed.

Oligosaccharide chains were also modified by a mixture of sodium metaperiodate and sodium cyanoborohydride as described by Thorpe et al. (171). 80mM solution of $NaCNBH_3$ in 0.2M sodium acetate, pH 3.5 was prepared and stored in ice for 1 hr. It was mixed with an equal volume of freshly prepared 40 mM sodium periodate in the same buffer. The mixture was then added in equal volumes to the proteins in sodium acetate buffer, pH 3.5 and incubated on ice in the dark for various periods of time. One hundred microlitres of 20% (V/V) glycerol was added to stop the reaction and the samples were dialyzed and assayed for residual enzyme or hemagglutination activity.

N-terminal amino acid sequence analysis

Separation of various DNS-amino acids were standardized using Lichrosorb RP 18 5μM (4x250mm) column connected to LKB HPLC system according to Marquez et al (172) with modification of the gradient system. The gradient was programmed between the solvents, A-0.6% acetic acid and 0.008% triethylamine in water, B-methanol.

sequence of few aminoacids were performed using dansyl-Edman degradation as described by Gray (174).

Beta-galactosidases and β-galactoside-binding protein were dried under nitrogen at 40°C. The residues were dissolved in 200μl of water and 20μl was taken for N-terminal amino acid determination. 200μl of 5% (V/V) phenylisothiocyanate in pyridine was added to the remainder. The tube was flushed with nitrogen and incubated at 45°C for 1 hr. The reaction mixture was dried in vacuum desicator over NaOH pellets at 30°C. Trifluoroacetic acid (200μl) was added and incubated for 30 min at 45.C and dried. The sample was dissolved in 200μl water and extracted twice with N-butylacetate and the residue was dried as described earlier. The dried residue was dissolved in 200μl water and 20μl aliquot was removed for dansylation. The cycle was repeated.

The aliquots removed at each cycle were adjusted to pH 9.5 using 40 mM Li_2CO_3, pH 9.5. 10μl of 2.5mg/ml dansyl chloride in acetone was added and incubated at 37°C for 1 hr. The reaction mixture was dried and transferred to a hydrolysis vial using 200μl of 6N HCl. The vial was filled with nitrogen and sealed. The sample was hydrolyzed at 105°C for 18 hr. The hydrolysate was dried and dissolved in 50μl water and centrifuged at 15,600g for 1 min in Eppendorf cerntrifuge. 10μl was injected in to HPLC.

Preparation of antibody

Control serum from rabbit was collected before immunization. 500μg β-galactosidase A or 320μg β-galactoside-binding protein in 1 ml was emulsified with 1 ml of Freund's complete adjuvant and injected intramuscularly at multiple sites. Three booster doses with antigens in Freund's incomplete adjuvant were given at 10 days interval. 15 days after the last injection blood was collected from the marginal vein of the ear lobe and the serum was separated by certifugation, and stored frozen with azide. The antibody response was measured by Ouchterlony immunodiffusion technique.

Immunodiffusion

1% Agarose in PBS, pH 7.4 containing 0.1% sodium azide was used to prepare immunodiffusion plates. 10μl antibody in the centre and test samples were added to be peripheral wells. The immunodiffusion plate was kept in moist atmosphere at 23°C. The precipitin bands were observed after 24 hr.

The Class of immunoglobulins

The antibody was purified by ammonium sulphate precipitation (50%) and dialyzed in PBS, pH 7.4. The antibody was applied on JSA-Sepharose column (0.5x4cm) at 4°C, equilibrated with PBS, pH 6.5. The column was washed with the same buffer and eluted with the buffer

containing 0.1M Gal. The breakthrough and washing were directly loaded on Protein A-Sepharose column (0.5x4cm), equilibrated with 0.1M phosphate buffer, pH 7.0. The column was washed with the same buffer and eluted with 0.1M glycine-HCl, pH 3.0. The pH of the eluted fractions were immediately adjusted to 7.0 with 2M Tris.

...

CHAPTER - III

RESULTS AND DISCUSSION

β-GALACTOSIDASE OF HUMAN PLACENTA

INTRODUCTION

Mammalian β-galactosidase catalyzes the hydrolysis of terminal β-galactoside linkage of glycoconjugates. There are three types of enzymes in human liver capable of hydrolysing synthetic chromogenic derivatives of β-galactoside (174-176). The acid β-galactosidase which are optimally active in the pH range of 4.0-5.0, is believed to be of lysosomal origin. The neutral β-galactosidase shows pH optimum close to 6.0. Little is known of the alkaline β-galactosidase, which appears to have relatively low activity with an optimum pH in the region of 8.5. Acid β-galactosidase hydrolyses GM_1 ganglioside in the presence of an activator protein (137,93). The absence or reduced activity of the acid β-galactosidases has been associated with an inherited autosomal recessive metabolic disorder, GM_1 gangliosidosis, in which Gal containing glycolipids and glycoproteins accumulate (139). At present, the molecular basis for the variety of clinical phenotypes, ranging from generalized from to the adult form is not fully understood.

The acid β-galactosidase has been purified to apparent homogeneity from various animal and human tissues (177-182). In human liver and brain most of the enzyme activity occurs as two forms, Mr.=65,000-75,000 and 600,000-800,000 (134, 178). Both forms of the β-galactosidase were thermolabile, and

(Mr.=160,000-170,000) was reported to be present in purified preparations (95,184). Both of the low molecular weight forms can be generated from the high molecular weight form of acid β-galactosidase (95). The low molecular weight forms of acid β-galactosidase undergo pH or salt dependent aggregation (183). Mutoh et al reported the elution of human liver acid β-galactosidase from TSK G 4000 SW column as three symmetrical peaks, Mr.≠800,000, 140,000 and 65,000 (184). The dimeric form was absent in a patient with adult form of GM_1 gangliosidosis and the mutant enzyme had altered enzyme properties.

Metal ions did not have marked effect on the enzyme activity (185). The low molecular weight form of acid β-galactosidase showed single band on SDS-Polyacrylamide gel electrophoresis (134,95). The high molecular weight form showed additional protein bands (98). A common subunit was described for both high and low molecular weight forms of acid β-galactosidase (97,98). The high molecular weight component may consist of the low molecular weight forms bound to membrane fragments (95).

Polyclonal antibody raised against the purified low molecular weight form of the enzyme cross-reacted with high molecular weight form but not with neutral β-galactosidase (134). Antiserum to the purified mouse β-galactosidase cross reacted with β-galactosidase activity from mouse

against defective forms of β-galactosidase was found to inactivate normal enzyme (186). The antibody mediated inactivation may be attributed to the conformational change induced or stem from a steric blockage of catalytic sites of the enzyme by the inactivating antibody.

Two forms of lysosomal acid β-galactosidase from human placenta has been purified from our laboratory (152). A-form had a molecular weight of 500,000, while that of B-form is 100,000. No significant difference was found in the enzyme properties of both A and B-forms. γ-D-galactonolactone was a competitive inhibitor of the enzyme. The enzyme was found to be a glycoprotein containing 7.5% carbohydrate for A-form and 6% for B-form. Man, Gal, GlcNAc, GalNAc were identified as monosaccharide constituent Sodium chloride stabilized and enhanced the enzyme activity of A and B-forms upto 0.1M but was inhibitory above this concentration. The low molecular weight form undergoes pH dependent aggregation. The enzyme contained 10 moles of -SH groups per mole of A-form, while that of B-form was 8 moles. The amino groups were indespensable for the biological activity of both A and B-forms. Periodic acid treatment of both forms of the enzyme resulted in complete loss of the enzyme activity. The present section describes further structural studies of human placental acid β-galactosidase.

The chemical modification is defined as a chemical reaction which results in the quantitative, covalent derivatization of the functional groups of a single unique amino acid residue in a protein without any demonstrable effect on either any other functional groups or the conformation of the molecules (187). This is an ideal pursuit to analyze the relationship between structure and function in a complex heteropolymer. The specific chemical modification of amino acid residues in enzyme has provided considerable information regarding the participation of these residues at the active site. Another major use of chemical modification has been in the demonstration of the primary structure of proteins. Chemical modification is also used for the quantitation of various amino acid residues as well as determination of the conformational changes in proteins. There has also been considerable interest in the chemical modification of amino acid residues to introduce spectral probes into proteins.

DTNB reacts with protein thiols by an exchange reaction to form mixed disulphide of the protein and 1 mole of 2-nitro-5-thiobenzoate per mole of protein -SH group (160). pHMB react with thiols to form mercaptides. Sulfhydryl groups of protein combine with NEM containing an activated double bond to form stable thiol either (162). Oxidation of proteins in acidic medium with carefully controlled

acid provides a usual procedure for the modification of Trp to oxyndolyl alanine in proteins (169). One of the most useful modification procedure for Trp in proteins involve the use of Koshland's reagent 2-hydroxy-5-nitrobenzyl bromide and its various derivatives (167). N-acetylimidazole reacts with Tyr residues in proteins to form O-acyl derivative (162). The function of Arg residues in proteins stem from the work of Takahashi on the use of the diketone phenylglyoxal hydrate as a reagent for the specific modification of Arg (165). A selective modification of Arg residues in protein can also be achieved with 1,2-cyclohexanedione in sodium borate buffer (166). Diethylpyrocarbonate reacts stoichiometrically with His residues in proteins to yield N-carbethoxyhistidyl derivative (164).

Periodate oxidation is generally used for the structural studies of oligosaccharides in glycoproteins (170). Treatment with a mixture of sodium metaperiodate and sodium cyanoborohydride results in the oxidative cleavage of the oligosaccharides and the reduction of the aldehyde groups to primary alcohols. The reduction minimizes the schiff's base formation between the aldehyde groups and amino groups in the protein and the nonspecific oxidation of amino aids (171).

The oligosaccharide chains in glycoproteins influence many biological interactions. Lectins with different

utilization as reagents for the study of simple and complex oligosaccharides. Lectins considered 'identical' in terms of monosaccharide specificity, possess the ability to recognize fine differences in more complex structures (188,189). The high resolving power of lectins permits rapid, sensitive and specific separation of closely related variants of glycoproteins that differ in their glycosylation pattern or glycopeptides and oligosaccharides with small differences in their structure.

Con A-Sepharose interacts with high affinity with N-linked oligosaccharides in which at least two outer Man residues are unsubstituted or are substituted only at position C-2 by another sugar. Thus, it can bind complex biantennary N-linked oligosaccharides, hybrid oligosaccarides and high mannose oligosaccharides (Fig.1). The lectin has very low affinity for tri- and tetraantennary complex N-linked oligosaccharides and most known O-linked oligosaccharides (190). Krusius et al showed that complex biantennary glycopeptides can be eluted from Con A-Sepharose by low concentration of hapten sugar, where as elution of the high mannose glycopeptides requires higher concentration of hapten (191). By differential elution with α-methyl mannoside, these two types of oligosaccharides can be separated from each other. Con A-Sepharose can also bind phosphorylated high mannose oligosaccharides found in lysosomal acid hydrolases (192) Bisected biantennary

Con A-Sepharose column and elution does not require haptenic sugars (193). The presence of a core Fuc in complex biantermary N-linked oligosaccharides does not interfere with their binding to Con A-Sepharose.

WGA-Sepharose interacts specifically with GlcNAc and NeuNAc (194,195). WGA has very high affinity for a sequence of three $\beta 1\rightarrow 4$ linked GlcNAc (chitotriose) and the affinity was found to increase with increasing number of GlcNAc residues (196). Alpha-Fucose residue in the core inhibits the interaction of the glycopeptide with WGA-agarose (197). Glycoconjugates with high density of terminal nonreducing NeuNAc residues will interact specifical with WGA (198), and the interaction involves a charge effect called 'avidity' (199).

Immobilized RCA interacts with high affinity with bi-and triantennary Asn-linked oligosaccharides that contain terminal Gal residues (200). The affinity of RCA with glycopeptides depends on the number of terminal β-linked Gal residues and decreases with the number of terminal sialic acids (201). JSA possess high affinity for terminal α-anomer of D-Gal (146).

The determination of amino acid sequence used the degradation scheme developed by Edman (202). Phenyliso-thiocyanate reacts with the amino terminal amino acid of a polypeptide at basic pH to form phenylthiocarbamyl

cleaves of the first amino acid as its 2-anilino-5-thiazolinone derivative and then exposes the amino group of the second amino acid. The derivatized amino acid is removed by extraction, the remaining polypeptide is dried and the cycle is repeated. Intensely fluorescent derivatives result from the reaction of DNS-Cl with free amino groups. DNS-amino acid derivatives are stable to acid hydrolysis and exhibit yellow fluorescence (203). The amino terminal of the polypeptide at each cycle of degradation, can be identified by reacting it with DNS-Cl, hydrolysing the polypeptide and separating the fluorescent derivative on HPLC system.

Protein A of S.aureus reacts specifically with Fc part of lgG (150). Protein A coupled to Sepharose can be used as an immunosorbent for the isolation of lgG from the serum of different species. JSA binds specifically to both monomeric and polymeric form of lgA, but not lgG or lgM (204).

METHODS

Enzyme assay

The standard assay system for β-galactosidase contained 200μmoles of citrate phosphate buffer, pH 4.5, 100μg of bovine serum albumin, 500nmoles of p-nitrophenyl-β-D-galactopyranoside and suitable aliquot of enzyme in

the tubes at 100°C for 30 sec. The tubes after cooling were mixed with 2.5ml 0.4M glycine-NaOH, pH 10.5 and centrifuged for 5 min at 2000g. The yellow colour formed was measured at 405 nm. One unit of enzyme was defined as the amount of enzyme required to liberate one nmole of p-nitrophenol per min at 37°C.

Inhibition studies

The inhibitors in varying concentration were preincubated with the enzyme for 1hr at 23°C and assayed under standard conditions. The nature of inhibition and inhibition constants were determined from the Dixon plot 1/V against I (20

Affinity chromatography on immobilized lectins

Con A-Sepharose affinity chromatography was carried out by applying β-galactosidase A or B to a column of 0.5x4cm. The column was washed with 0.05M phosphate buffer, pH 7.0 containing 500mM NaCl. The adsorbed enzyme was eluted with 10mM α-methyl mannopyranoside in the same buffer followed by 500mM α-methyl mannopyranoside in the buffer. One ml fractions were collected and assayed for enzyme activity.

Another immobilized lectin used to study the oligosaccharides of β-galactosidase is WGA-Sepharose. The β-galactosidase A or B was loaded to WGA-Sepharose column (0.6x3cm) equilibrated with 20mM phosphate buffer,

was washed with the same buffer. The bound enzyme was eluted with the same buffer containing 0.1M GlcNAc. One ml fractions were collected and assayed for enzyme activity.

The enzyme was applied to RCA-Sepharose column (0.6x3cm) equilibrated with PBS, pH 7.4. The column was washed with the same buffer and eluted with 0.1M Gal in the buffer. The 1ml fractions collected were dialyzed in 20mM phosphate, pH 7.0 containing 100mM NaCl before the assay.

Affinity chromatography on JSA-Sepharose was carried out on a column (0.6x3cm) equilibrated with PBS, pH 6.5. The column was washed with the same buffer and eluted with the buffer containing 0.1M Gal. The dialyzed 1ml fractions were assayed for enzyme activity.

The antibody and the enzyme was incubated at 23°C for 1hr. The complex formed was separated by centrifugation at 15,600xg in Eppendorf centrifuge for 1 min. The complex was resuspended in 20mM phosphate, pH 7.0 containing 100mM NaCl and assayed for enzyme activity.

The materials and other relevant methods are described in chapter II.

RESULTS

The purification of β-galactosidase A and B from human placenta is summarized in Table 2. Experimental details of

TABLE - 2

PURIFICATION OF β-GALACTOSIDASE FROM HUMAN PLACENTA

Enzyme fraction	Total Protein (mg)	Total activity (units)	Specific activity
Supernatent	35,400	27,450	0.75
Ammonium Sulphate	23,510	24,190	1.00
Con A-Sepharose	248	19,860	80
Sephadex G-200			
A	58	5,500	95
B	52	10,640	205
β-Galactosidase A			
DEAE-Sephadex	20	2,350	118
Con A-Sepharose	9	2,010	223
β-Galactosidase B			
DEAE-Sephadex	18	5,870	326
Con A-Sepharose	12	4,660	338

Experimental details are given under and General Methods.

with 72% recovery. Neutral β-galactosidase activity was completely removed by this step. The dialysis to remove α-methyl glucopyranoside and subsequent lyophilization of the enzyme had no deleterious effect on its biological activity. The enzyme was separated into two forms by gel filtration on Sephadex G-200. The activity peak eluted immediately after the void volume of the column was called A-form and the peak eluted at the inner volume was denoted as B-form. A partial purification of the enzyme was achieved by DEAE-Sephadex A-50 ion exchange chromatography. β-galactosidase A and B were completely retained on DEAE-Sephadex A-50 equilibrated at pH 6.0 and could be eluted with 250mM NaCl. The enzyme preparation was free of other glycosidases except β-hexosaminidase at this stage. The sulfhydryl nature of the enzyme was utilized to purify the enzyme by employing a mercurial-Sepharose column. The yield of the enzyme was low. A second Con A-Sepharose affinity chromatography after DEAE-Sephadex ion exchange chromatography of both forms resulted in higher yield. The final preparation of purified enzyme was free of all other glycosidase activities.

Purified enzymes A and B were subjected to polyacrylamide gel electrophoresis in 7.5% gels at pH 8.3. Both forms of the enzyme gave single band with B-form having higher electrophorestic mobility (Fig.5) SDS-polyacrylamide gel electrophoresis of the purified enzyme was carried out on 10% acrylamide gels according to Laemmli (155). The enzyme

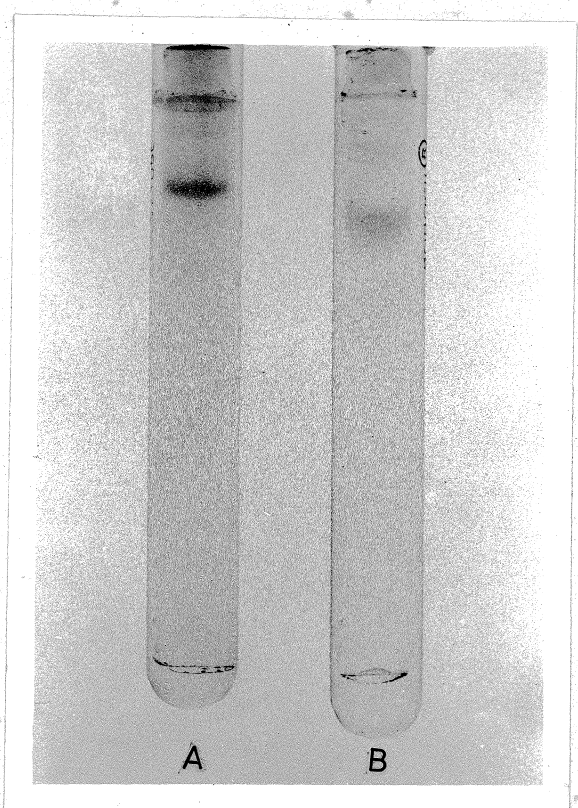


Fig.5. Polyacrylamide gel electrophoresis of β-galactosidase A and B at pH 8.3. Experimental details are given under General Methods.

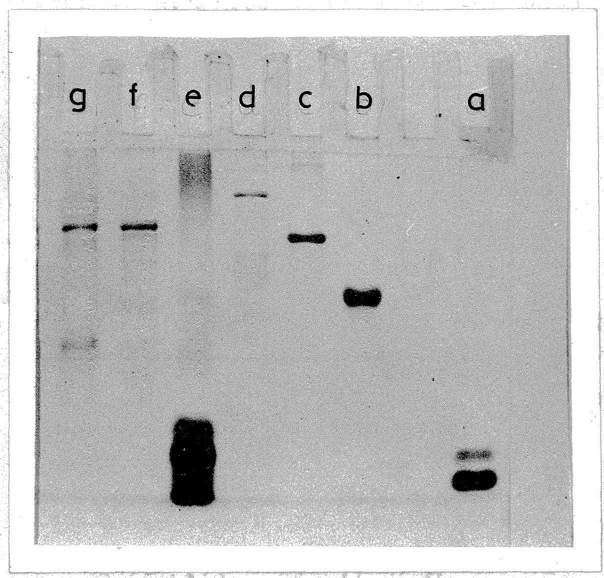


Fig.6. SDS-polyacrylamide slab gel eletrophoresis of β-galactosidase A and B.

Details of the experiment are given under General Methods.

a. Jack fruit seed agglutinin
b. Ovalbumin
c. Bovine serum albumin
d. E.Coli β-galactosidase
e. Ferritin
f. β-Galactosidase B
g. β-Galactosidase A.

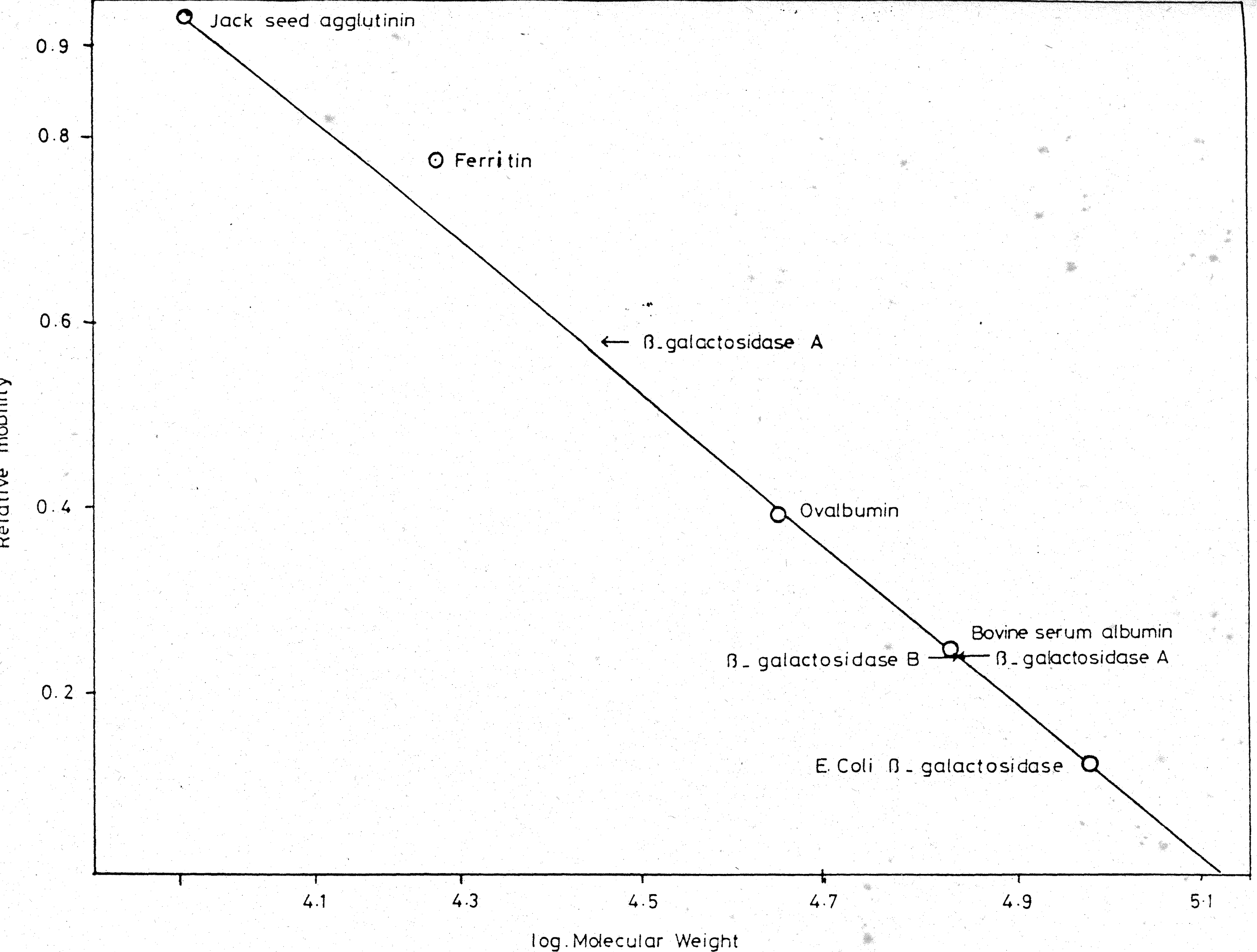


Fig. 7. Subunit molecular weight determination of ß-galactosidase A and B by SDS-polyacrylamide gel electrophoresis.

(Fig.6). The molecular weight of the band was approximately 68,000. A-form was dissociated into two bands under the same conditions (Fig.6). The major protein band had an apparent Mr.=68,000 while the minor band corresponded to Mr.=27,000 (Fig.7). It may be concluded that both A and B forms contained one common subunit.

The inhibition of β-galactosidase A and B by various substrate analogues is presented in Table 3. Inhibition of β-galactosidase by γ-D-galactonolactone is already reported (157). Galactose at a final concentration of 10^{-1}M completely inhibited the enzyme activity. Galacto-pyranosylamine at a final concentration of 10^{-3}M inhibited 38% and 30% activity of both A and B-forms respectively. Low and high methylated polymer of galacturonic acid was not found to be an inhibitor of placental β-galactosidases. Galacto-1-deoxynojirimycin at a final concentration of 10^{-5}M completely inhibited β-galactosidase A and B. No significant difference was observed between the effect of substrate analogues on β-galactosidase A and B. Dixon plot for the determination of Ki for galacto-1-deoxynojirimycin of β-galactosidase A and B revealed competitive nature of the inhibition. The inhibition constant Ki for B-form was 1.4×10^{-5}M (Fig.8) and 1.25×10^{-5}M for A-form (Fig.9) with p-nitrophenyl-β-D-galactopyranoside as substrate.

Tryptophan residues of the enzyme was quantitated by their oxidation according to Savige and Fontana (169). The

TABLE - 3

EFFECT OF SUBSTRATE ANALOGUES ON β-GALACTOSIDASES ACTIVITY

Inhibotor	Final Concentration (M)	% B	Inhibition A
Galacto-1-deoxynojirimycin	10^{-5}	81	83
Galactose	10^{-5}	92	91
Galactopyranosylamine	10^{-3}	38	30
Thiodigalactoside	10^{-3}	6	6
Lactitol	10^{-2}	0	0
Arabinogalactan	10^{-2}	0	0
High methylated polymer of galacturonic acid (2%)		0	0
Low methylated polymer of galacturonic acid (2%)		0	0

The details of the experiments are described in the text.

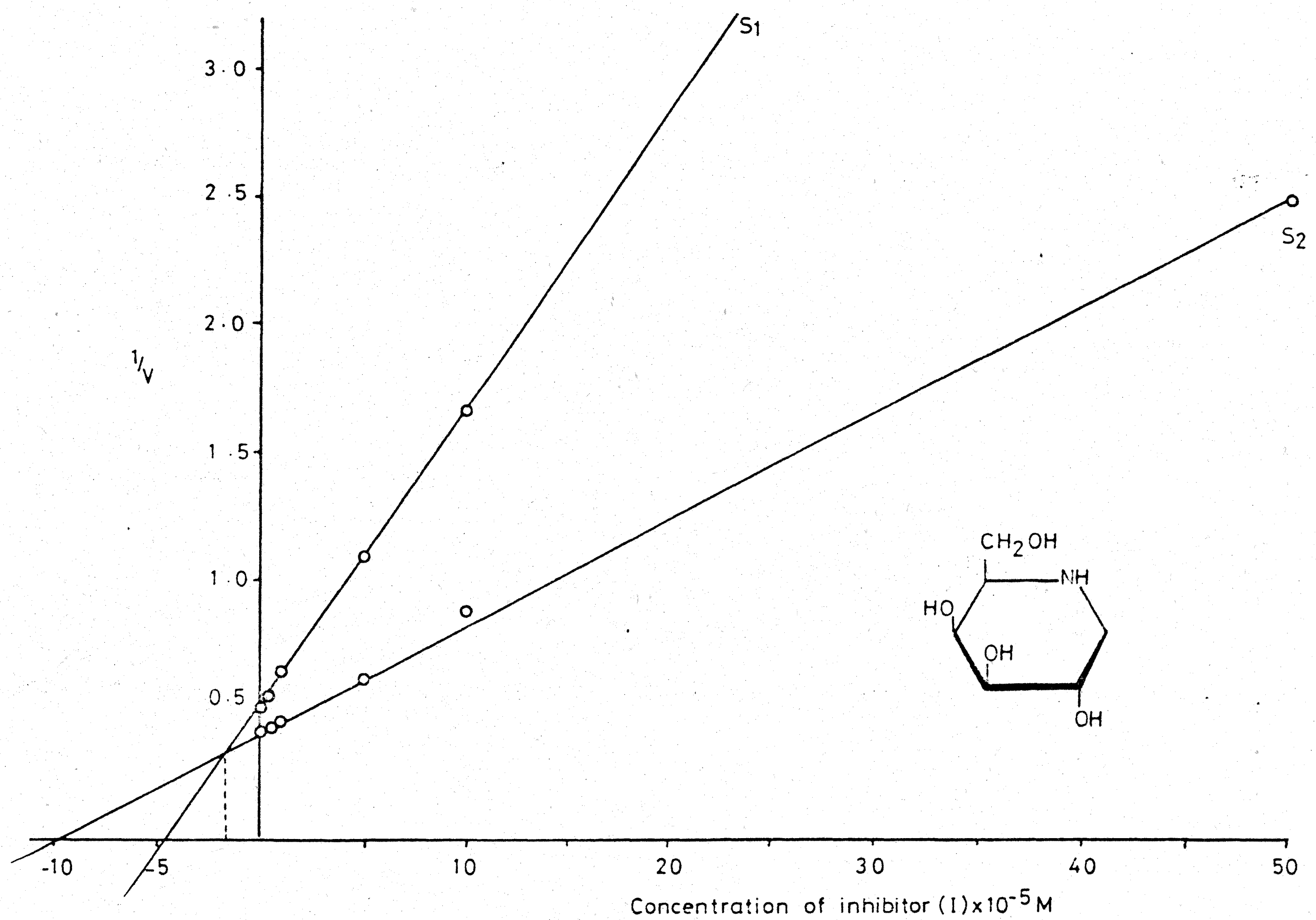


Fig.8. Dixon plot for the determination of Ki for galacto-1-deoxynojirimycin of β-galactosidase B.

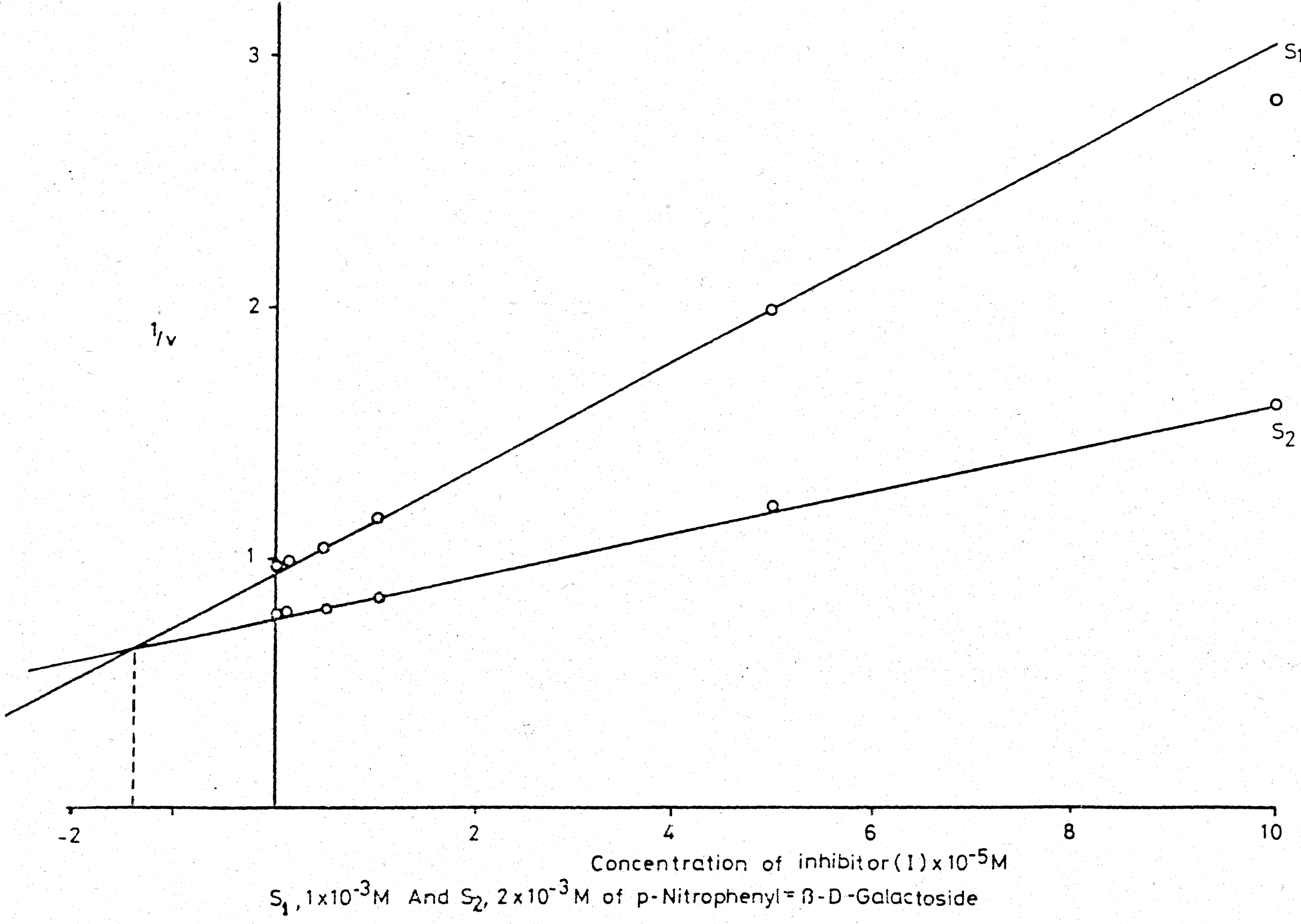


Fig.9. Dixon plot for the determination of Ki for galacto-1-deoxynojirimycin of ß-galactosidase A.

The total number of Tyr residues in the enzyme estimated with N-acetylimidazole (162) showed 48 and 11 residues in A and B-forms of the enzyme respectively.

In order to get some insight into the active site aminoacids, chemical modification of various amino acids were carried out. The results of chemical modification of various amino acids and saccharide chains of β-galactosidase A and B are summarized in Table 4. Sulfhydryl group modification with DTNB resulted in 33% loss of activity of A-form and 30% activity of B-form. Modification of -SH groups by pHMB (10^{-4}M) inhibited 47% activity of A-form and 63% activity of B-form. Modification of -SH groups with 10^{-3}M N-ethylmaleimide inhibited 40% and 29% activity of β-galactosidase A and B respectively.

Modification of the Trp residues by treatment of the enzyme with 2-hydroxy-5-nitrobenzyl bromide completely abolished the activity of both A and B-forms of β-galactosidase. The enzyme was treated with benzyl halide, 2-methoxy-5-nitrobenzyl bromide for the modification of Trp residues. Both forms of the modified enzyme lost their activity completely. Beta-galactosidase A and B also lost their activity when the Trp residues were modified by N-bromosuccinimide.

Specific modification of Tyr residues of β-galactosidase A and B with N-acetylimidazole had no deleterious effect on

TABLE - 4

CHEMICAL MODOFICATION OF AMINO ACID GROUPS AND OLIGOSACCHARIDE CHAINS OF β-GALACTOSIDASES

Modifying reagent	Amino acid group or oligosaccharide modified	% A	Residual activity B
Dithionitrobenzoate	-SH	67	63
P-hydroxymercuribenzoate	-SH	53	37
N-Ethylmaleimide	-SH	60	71
2-Hydroxynitrobenzyl bromide	Trp	7	8
2-Methoxynitrobenzyl bromide	Trp	11	13
N-Bromosuccinimide	Trp	8	10
N-Acetylimidazole	Tyr	89	86
Phenylglyoxal hydrate	Arg	13	10
1,2-Cyclohexanedione	Arg	46	45
Diethylpyrocarbonate	His	78	95
$NaIO_4$ / $NaCNBH_3$	Oligosaccharides	8	14

Experimental details are given under General Methods.

specifically by phenylglyoxal hydrate. Arginine modification with 1,2-cyclohexanedione resulted in 54% and 55% decrease in the activity of β-galactosidase A and B respectively. The enzyme activity was unaltered when the His residues of β-galactosidase A and B were modified with diethylpyrocarbonate.

The enzyme on oxidation with periodic acid, lost their activities (152). Treatment of β-galactosidase A and B with a mixture of sodium metaperiodate and sodium cyanoborohydride, resulted in the oxidative cleavage of the oligosaccharides and the reduction of the aldehyde groups to primary alcohols. By conducting the procedure at acid pH, the possibility of Schiff's base formation between the aldehyde groups and amino groups in the protein and the non-specific oxidation of amino acids were minimised. Both forms of the modified enzyme completely lost their activity.

Affinity chromatography using various immobilized lectins were employed for analyzing the Asn_linked oligosaccharides of β-galactosidase A and B. Both forms of the enzyme were fully retained on Con A-Sepharose. Affinity chromatography of β-galactosidase A and B on Con A-Sepharose is shown in Figure.10. A portion of the bound enzyme could be eluted with 10mM α-methyl mannopyranoside. However, major portion of the bound enzyme was eluted with 500mM α-methyl mannopyranoside. Separation of β-galactosidase A and B into two components was observed on application to WGA-Sepharose column (Fig.11). Twenty to thirty percent of the applied

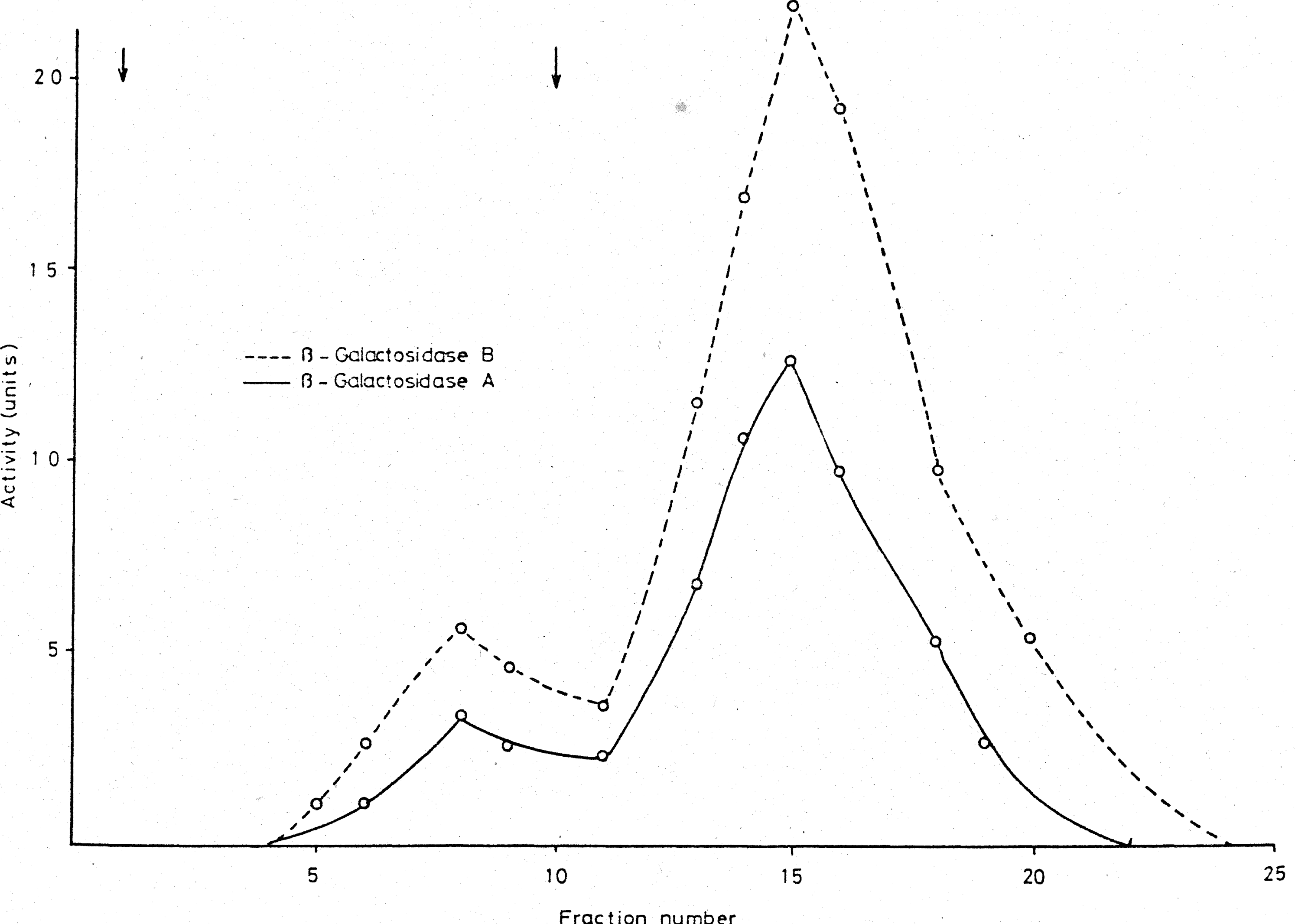


Fig. 10. Affinity chromatography of ß-galactosidase A and B on Con A-Sepharose column.

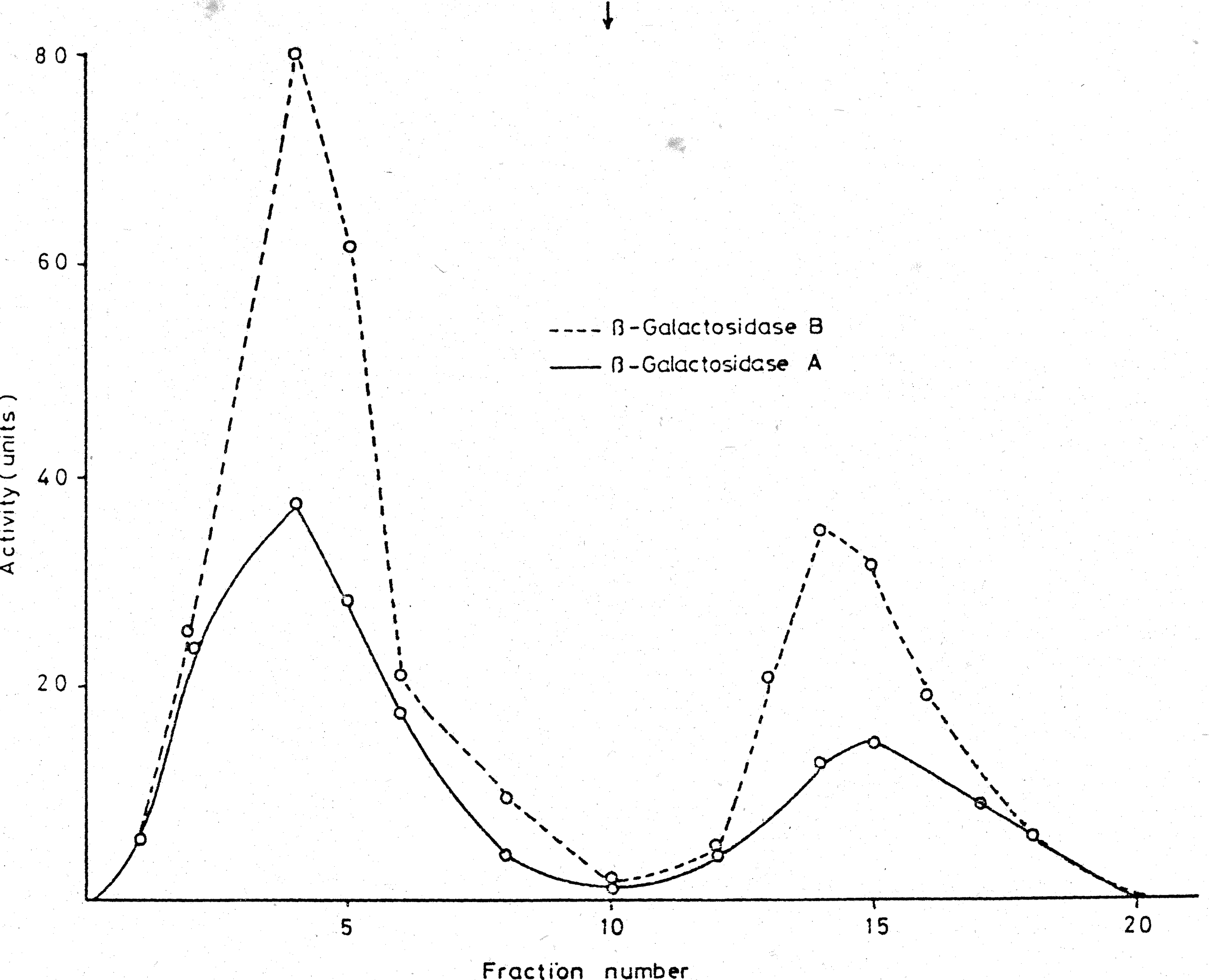


Fig.11. Affinity chromatography of ß-galactosidase A and B on WGA-Sepharose column.

Details of the experiment are described in the text.

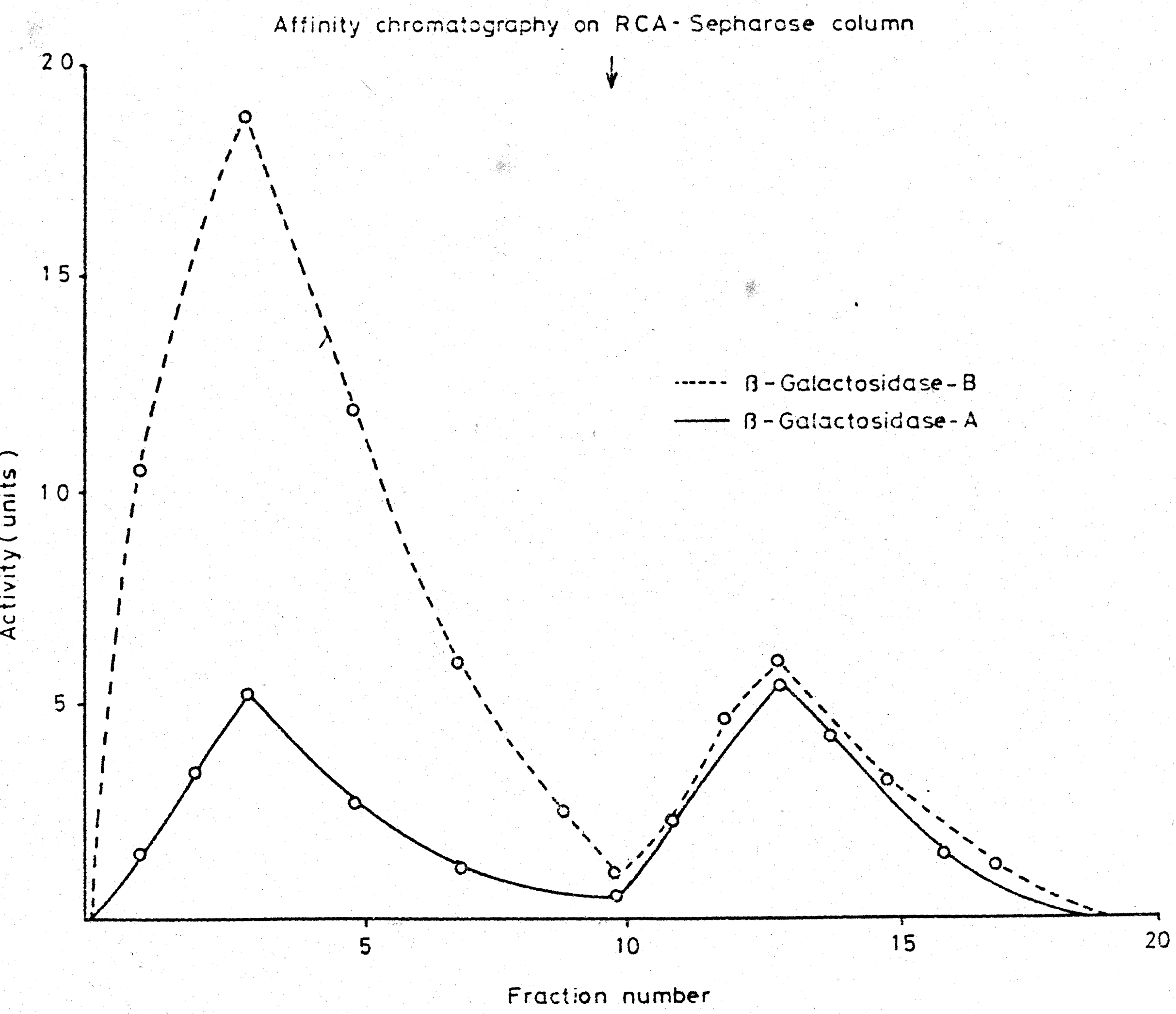


Fig.12. Affinity chromatography of ß-galactosidase A and B on RCA-Sepharose column.

Details are described in the text.

and unbound from of the enzyme showed similar electrophorectic mobilities. Affinity chromatography of the enzyme on RCA-Sepharose is shown in Figure 12. The enzyme bound to the column could be specifically eluted with Gal. Twenty percent of B-form and 50% of A-form were bound to RCA-Sepharose. The enzyme did not bind to JSA-Sepharose.

N-terminal amino acid sequence of β-galactosidase A and B was analyzed by dansyl-Edman degradation (173). The N-terminal amino acid of β-galactosidase B was found to be Leucine. The N-terminal amino acid sequence of B-form was found to be 'Leu-Ser-Ser-Lys-Val-His-'. N-terminal amino acid sequence analysis of β-galactosidase A showed two peaks corresponding to its two subunits. The subunits of β-galactosidase A were isolated by SDS-polyacrylamide gel electrophoresis (56). N-terminal amino acid sequence analysis of the isolated subunit indicated the sequence 'Leu-Ala-' at the N-terminal of 68 kDa subunit and that of 27 kDa subunit was 'Tyr-Met-'.

The polyclonal antibody was raised in rabbits against β-galactosidase A. The antiserum was found to cross-react with β-galactosidase A. The antiserum was chromatographed on JSA-Sepharose followed by protein A-Sepharose to determine the immunoglobulin class. The antiserum eluted from lgA specific JSA-Sepharose column cross-reacted with β-galactosidase A (Fig.13). The antibody and enzyme complex retained enzymic activity. The antibody cross-reacted with B-form as

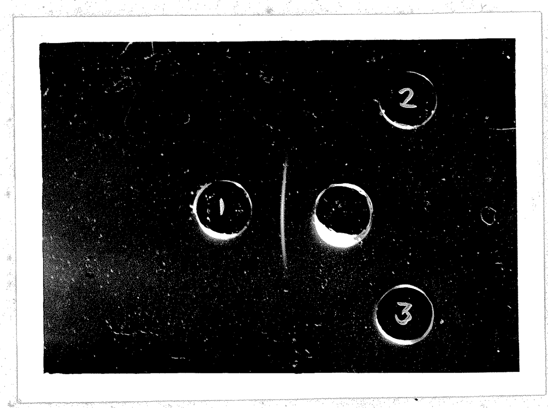


Fig.13. The class of immunoglobulins of ß-galactosidase A antibody.

Details are described in the text.

Central Well: ß-Galactosidase A

1 : JSA-Sepharose eluate

2 : Protein A-Sepharose eluate

3 : Unbound antiserum.

DISCUSSION

Two forms of acid β-galactosidase had been separated and purified to homogeneity from human placenta by Sarasija and Basu (152) with low recovery and stability. The modified method of purification described in the present work involved a second Con A-Sepharose affinity chromatography after DEAE-Sephadex A-50 ion exchange chromatography of both forms, resulting in higher yield and stability.

The purified enzyme was homogeneous as revealed by polyacrylamide gel electrophoresis at pH 8.3. Molecular weight of β-galactosidase determined by gel filtration at neutral pH was 500,000 for A-form and 100,000 for B-form. Acid β-galactosidase of human liver and brain was shown to occur as two forms with Mr. of 600,000-800,000 and 60,000-80,000 (134,178). Recently, in addition to the two forms a dimeric form of acid β-galactosidase (Mr.=140,000-170,000) was reported to be present in purified preparations (95,184). Two molecular weight species of feline liver acid β-galactosidase were observed on gel filtration of the native enzyme (180).

In SDS-polyacrylamide gel electrophoresis, A-form dissociated into two bands with Mr.=68 kDa and 27 kDa, whereas B-form showed a single subunit of Mr.=68 kDa. Both forms of the enzyme may have a common subunit. Additional bands for the high molecular weight form have also been observed by others ([illegible]).

inhibitor of β-galactosidase A and B. Dixon plot showed competitive nature of the inhibition with an inhibition constant, $Ki=1.25 \times 10^{-5}$M for A-form and 1.4×10^{-5}M for B-form. Competitive nature of the inhibition of placental β-galactosidase, with a higher Ki value, by γ-D-galactonolactone has been already reported (152). The competitive inhibition of the enzyme by galacto-1-deoxynojirimycin is significant because most of the available substrate analogues were found to be weak inhibitors of placental β-galactosidase. Galactose at a concentration of 10^{-1}M also inhibits β-galactosidase A and B. Amino group substitution at the carbon-1 of Gal increased its inhibitory capacity.

Beta-galactosidase A and B had 15 and 14 Tryptophan residues respectively when estimated according to Savige and Fontana (169). Tyrosine quantitated with N-acetylimidazole (162) showed 48 and 11 residues in A and B-forms respectively.

There are few reports on the amino acid, oligosaccharide chain modification and biological activity of the enzyme. The modification of the amino groups of β-galactosidase suggested their indespensability (152). In the present study an attempt has been made to determine the identity of the amino acids at the catalytic site. The chemical modification studies indicated the presence of Trp and Arg residues at the catalytic site as the specific modification of these residues completely

enzyme activity. No significant difference was observed on the effect of chemical modification of both A and B forms.

Both forms of the enzyme modified by sodium periodate and sodium cyanoborohydride at acid pH completely lost their biological activities. Thus a role for oligosaccharide chains on the biological activity may be presumed.

Affinity chromatography of the enzyme on various immobilized lectins indicated the presence of high mannose, hybrid and complex-type oligosaccharides in both A and B forms of β-galactosidase. Enzyme bound to immobilized Con A could be eluted with 10mM hapten sugar revealed the presence of complex biantennary oligosaccharides. Major portion of the enzyme activity was eluted with 50mM α-methyl mannopyranoside indicating the presence of high mannose and hybrid-type of oligosaccharides. WGA-Sepharose-bound form predominantly contained hybrid and complex-type oligosaccharides, whereas the unbound form was of high mannose-type. The results on the binding of human placental β-galactosidase to WGA-Sepharose are not similar to those obtained with human liver β-galactosidase (206). These authors reported that WGA-bound and unbound fractions of human liver β-galactosidase have different electophoretic mobilities. Affinity chromatography on RCA-Sepharose also reveals the presence of complex and hybrid-type oligosaccharides in the bound form. Fiddler et al also suggested the presence of two forms of acid β-galactosidase in human liver by affinity chromatography

nonbinding of placental β-galactosidase to JSA-Sepharose, since both RCA and JSA were specific for D-galactose residues.

The N-terminal amino acid sequence of β-galactosidase B was 'Leu-Ser-Ser-Val-Lys-His-'. The N-terminal amino acid analysis of A-form showed two peaks. The subunits isolated by SDS-polyacrylamide gel electrophoresis indicated the sequence 'Leu-Ala-' at the N-terminal of 68 kDa subunit and that of 27 kDa subunit was 'Tyr-Met'-.

The polyclonal antibody raised in rabbit against β-galactosidase A was chromatographed sequentially on lgA-specific JSA-Sepharose and lgG-specific protein A-Sepharose to determine the class of immunoglobulins. JSA-Sepharose eluate cross-reacted with β-galactosidase A indicating that the antibody belonged to lgA class of immunoglobulins. The enzyme-antibody complex retained enzymic activity demonstrating that the antigenic and catalytic sites were situated at different regions of the polypeptide. Roth and Rotman observed the inactivation of the enzyme by the antibodies elicited to certain mutant forms of β-galactosidase (186). A and B-forms of the enzyme were found to be immunologically identical. Immunological identity has also been reported for the two forms of human liver acid β-galactosidase (178). The antibody cross-reacted immunologically with similar enzymes from various human fetal and adult organs.

β-GALACTOSIDE-BINDING PROTEIN OF HUMAN PLACENTA

INTRODUCTION

The role of carbohydrates in cellular recognition is well documented. It has become increasingly apparent that cell surface carbohydrates are intimately involved in lymphocyte 'homing', tumour invasiveness, trophoblast implantation and intercellular adhesion (208). The recently emerged field of carbohydrate specific binding proteins particularly, the galactose-binding in hepatic tissue has been reviewed by Ashwell and Harford (10). Barondes has reviewed soluble β-galactoside-binding protein from several animal tissues (109). Some β-galactoside-binding protein of certain animal species are developmentally regulated. The soluble galactoside-binding proteins are extractable with lactose and are specifically inhibited by β-galactoside containing saccharides. They are distinct from membrane bound lectins, which require detergents for solubilization and Ca^{2+} for saccharide-binding activity (109,110). These β-galactoside-binding proteins are operationally defined as lectins and usually assayed by their ability to agglutinate erythrocytes. These lectins are usually been isolated in the mono-,di- or oligomeric state (209). The biological function of these soluble β-galactoside-binding lectins has not been clearly established.

Despite their diverse origin, the soluble β-galactoside-binding proteins share many common properties. They contain

activity is independent of divalent cations but dependent on the presence of exogenous thiols. Several soluble β-galactoside-binding lectins from phylogenetically distinct species cross-react immunologically, indicating that these proteins possess common structural determinants that have been maintained during evolution (210-213).

Electrolectin, a β-galactoside-binding lectin of electric eel was a dimer having 2.2% carbohydrate and is composed of presumably identical subunits of Mr.=16.5 kDa (116). Electrolectin is inactivated in the absence of exogenous thiols, eventhough the lectin contains no Cys residues. The loss of activity was traced to the oxidation of the single Trp residue at the saccharide-binding site by atmospheric oxygen which prevented the binding of saccharides. The reducing agents were required to reduce the molecular oxygen normally present in solution and prevent it from oxidizing the Trp.

A soluble β-galactoside-binding lectin from calf heart and lung has been purified by deWaard et al., was found to be a dimer of Mr.=9000(149). Carding et al raised a monoclonal antibody against the soluble galactoside-binding lectin of bovine heart muscle and showed an array of antigenically active components in addition to 13 and 26 kDa protein in bovine tissues ranging from 36 kDa to more than 200 kDa (209). Galactose-binding activity was demonstrable in 13,26 and 36 kDa components suggesting that

The soluble β-galactoside-binding lectins from the epidermis and dermis of chick embryo skin had an apparent subunit Mr.=14 kDa (214). The biochemical properties revealed that though dermis and epidermis develop from different origins, they contain the same lectin. These authors also reported the possible occurence of several oligomeric states which are in dynamic equilibrium. Such a situation may be important for cellular regulation, because the apparent binding strength changes depending on the number of binding sites of the molecule. Its high content and considerable change during development suggest a function in tissue differentiation. Two β-galactoside-binding lectins from the whole chick embryos were distinguis by the same authors, in SDS-polyacrylamide gel electrophores with an appropriate Mr. of 16 and 14 kDa. Beyer et al also isolated two β-galactoside-binding lectins, one from chicken embryonic liver composed subunits of Mr.=15.9 kDa and another from adult intestine, subunit Mr.=14.2 kDa (120) These two lectins are immunologically distinct and have different cellular localization (215). A 15 kDa membrane bound β-galactoside-binding lectin from embryonic chick muscle was purified by Den and Malinzak (216). Soluble extracts of chick pectoral muscle and myoblast clone L6 also contains a β-galactoside-binding lectin (217). The muscle lectins are involved in myoblast fusion and the proce is completely inhibited by the potent inhibitor thiodi-

Both immature and adult rat lungs contain three prominant soluble β-galactoside-binding proteins with subunit Mr.=14.5, 18 and 29 kDa (220). They are readily resolved by ion exchange chromatography and reported to be distinct proteins. Neither the 14.5 kDa nor the 18 kDa lectin are apparently derived by the proteolytic processing of the 29 kDa lectin. Powell also described the purification of soluble β-galactoside-binding lectin from rat lung (221). Rat brain β-galactoside-binding lectin was isolated and characterized by Caron et al (222).

Whitney et al demonstrated that iodoacetamide treated rat lung β-galactoside-binding lectin was stable to atmospher oxygen in the absence of thiol reducing agents (223). All the Cys residues in native rat lung galactoside-binding lecti are in the reduced form with no evidence of any disulphide linkage. Oxidation of the protein thiol groups by atmospheri oxygen does not result in intersubunit disulphide bond formation or an increase in molecular weight. Though the physiological significance of this phenomena is not known, it may be possible that the lectin is regulated by the oxidative state of its environment (224).

Primary structure of the 14 kDa soluble β-galacto-side-binding lectin of chick embryo was reported by Hirabayashi et al (225). Recently, Ohyama et al. determined the nucleotide sequence of chick 14 kDa β-galactoside-

N-terminal amino acid, serine, was blocked by acetylation, while the first and second residues of the coding region of cDNA were 'Met-Ser'-. It seems that translation of the chick 14 kDa lectin is initiated at the initial 'Met' code and after the completion of the translation, Met is removed and the resultant N-terminal serine is blocked by acetylation. Marked internal sequence homologies in chick 14 kDa lectin was found. This suggests that chick 14 kDa gene may have evolved via several gene duplications. Drickamer et al have reported the amino acid sequences of asialoglycoprotein receptor of chick and rat (227,228) and Spiess et al reported the nucleotide sequence of that of human (229).

The comparison between the partial sequences of the four lectins of human origin shows significant sequence homologies but also systematic differences, indicating that at least four different genes encoding β-galactoside-binding lectins may be present in the human genome (119). However, the homologies between human lectins and those from the eel and chicken suggest that the lectin encoding genes have been derived from a common ancestor gene.

Recently, Hirabayashi and Kasai have reported a β-galactoside-binding lectin from human placenta (153). This section describes the detailed physicochemical properties, structural and immunological studies of the

METHODS

Blood was collected with anticoagulant acid citrate-dextrose solution. Erythrocytes were separated from the fresh blood by centrifugation at 200 rpm for 5 min at 30°C. They were washed 3-4 times with PBS, pH 7.4. Five percent washed erythrocyte suspension in PBS, pH 7.4 was mixed with 0.1% trypsin and the mixture was incubated at 37°C for 1 hr. The trypsinized erythrocytes were then washed 5 times with PBS, pH 7.4. The cells were suspended in PBS, pH 7.4 to obtain a 5% trypsinized erythrocyte suspension.

Hemagglutination assay

Serial two fold dilutions of the protein samples were made in a final volume of 0.2 ml of PBS, pH 7.4 in test tubes. To each tube 0.05 ml native or trypsinized 5% erythrocyte suspension was added and mixed. The agglutination was scored after 2 hr at 30°C. Hemagglutination titre is defined as total number of lectin units each of which agglutinated 1% V/V trypsinized erythrocytes from 0.25 ml suspension in PBS, pH 7.4.

Saccharide inhibition of hemagglutination

Aliquots of 0.2ml PBS, pH 7.4 containing double the minimum amount of β-galactoside-binding protein required for hemagglutination, with or without serial dilution of the

added. The mixture was kept at 23°C with ocassional shaking and agglutination was noted after 2 hr.

UV spectral measurement

The UV spectrum of the lectin was measured on Shimadzu spectrophotometer UV-240 in PBS, pH 7.2 at a concentration of 1.0mg/ml.

Equilibrium dialysis

^{3}(H) Lactose the ligand for equilibrium dialysis was prepared by sequential exposure to galactose oxidase in the presence of horse raddish peroxidase and tritiated borohydride according to Morell and Ashwell (229). Equilibrium dialysis was carried out in 0.9ml cells separated by a semipermeable membrane using 1.07mg β-galactoside-binding protein and varying concentrations of ^{3}(H) lactose in PBS, pH 7.2. Equilibrium was allowed for 48 hr at 4°C and free ^{3}(H) lactose was measured by counting the radioactivity in LKB Rackbeta counter. The data were plotted according to Scatchard (230).

Fluorescence quenching studies

The binding of MeUmb-β-Gal to galactoside-binding protein was monitored by measuring the quenching of fluorescence of the saccharide derivative in Shimadzu Spectrofluorophotometer at pH 7.2. To 2ml of 3μM fluorescent saccharide derivative in 1x1x4cm cuvette were added varying quantities of β-galactoside-binding protein. The fluoresence

experiment to a fixed amount of protein (2ml, 3.7μM) were added various aliquots of MeUmb-β-Gal. The fluorescence maximum was measured after exciting at 315nm. The data were plotted according to Scatchard (230).

Amino acid analysis

Amino acid analysis of β-galactoside-binding protein was carried out using Amberlite IRA-120 ion exchange column (4.6x200mm) mounted on Beckman amino acid analyzer according to Spackman (231). The protein was hydrolyzed with 6N HCl at $105 \pm 1^{\circ}C$, in an evacuated tube for 24 hr. The hydrolysate was dried in a desicator over phosphorus pentoxide and NaOH pellets. The sample was dissolved in citrate buffer pH 2.2 and 50μl was injected to the analyzer. The flow rate of the buffer was maintained at 35 ml/hr, while that of ninhydrin was 25ml/hr.

Mitogenic studies of the β-galactoside-binding lectin on human lymphocytes were performed with PHA as standard according to Osawa and Toyoshima (232).

Affinity chromatography on immobilized lectins

The β-galactoside-binding protein was applied to Con A-Sepharose column (0.6x4cm). The column was washed with 0.05M phosphate buffer, pH 7.0 containing 500mM NaCl and eluted with the buffer containing 10mM α-methylmanno-pyranoside, followed by 500mM α-methylmannopyranoside.

The β-galactoside-binding protein was passed through a WGA-Sepharose column (0.6x3cm) equilibrated with 0.02M phosphate buffer pH 7.0 containing 100mM NaCl. The unadsorbe glycoprotein was washed with the same buffer. The bound protein was eluted with the same buffer containing 0.1M GlcNAc.

The protein was applied on RCA-Sepharose 4B column (0.6x3cm) equilibrated with PBS, pH 7.4. The column was washed with the same buffer and eluted with the buffer containing 0.1M Gal.

The β-galactoside-binding lectin was applied on JSA-Sepharose column (0.6x3cm) equilibrated with PBS, pH 6.5. The column was washed with the same buffer and eluted with 0.1M Gal in the buffer. Affinity chromatography on WBA-Sepharose column (0.6x3cm) was carried out in PBS, pH 7.4. The column was washed with the same buffer and eluted with 0.2M lactose in the buffer.

1ml fractions collected from the above lectin-Sepharose columns were dialyzed in PBS, pH 7.2 before hemagglutination assay.

Human adult and fetal organs obtained from Trivandrum Medical College Hospital were stored at -20°C until use. Sample of β-galactoside-binding lectin from various human adult and fetal organs were prepared by homogenizing the tissues in PBS, pH 7.2 containing 0.1M lactose. The

Bovine heart β-galctoside-binding lectin was purified on asialofetuin-Sepharose column according to deWaard et al. The β-galactosidase A and B were purified from human placenta as mentioned early.

Materials and other relevant methods are described in chapter II.

RESULTS

The β-galactoside-binding protein was purified to homogeneity from human placenta (Table.5). The major steps of purification were specific extraction with lactose and affinity chromatography on asialofetuin-Sepharose (Fig.14). The purified β-galactoside-binding protein showed a single band on polyacrylamide gel electrophoresis at pH 8.3 (Fig.15). The molecular weight of the native protein determined by gel filtration on Biogel P-100 column was found to be 26.9 kDa (Fig.16). SDS-polyacrylamide gel electrophoresis of the purified β-galactoside-binding protein showed a single band corresponding to Mr.=13.4 kDa (Fig.17). High molecular weight forms were not observed by gel filtration experiments. The lectin was devoid of any lysosomal enzymic activities.

The placental β-galactoside-binding lectin agglutinated native calf, pig, rat, mice and rabbit erythrocytes, but not those of sheep, goat and dog. The

TABLE - 5

PURIFICATION OF β-GALACROSIDE-BINDING PROTEIN FROM HUMAN PLACENTA

Fraction	Total protein (mg)	Total activity (titer.ml)	Specific activity (titer.ml/mg)
Lactose extract	640	64,000	100
Ammonium sulphate	426	51,200	120
asialofetuin-Sepharose	1.2	15,360	12,800

Experimental details are given under General Method.

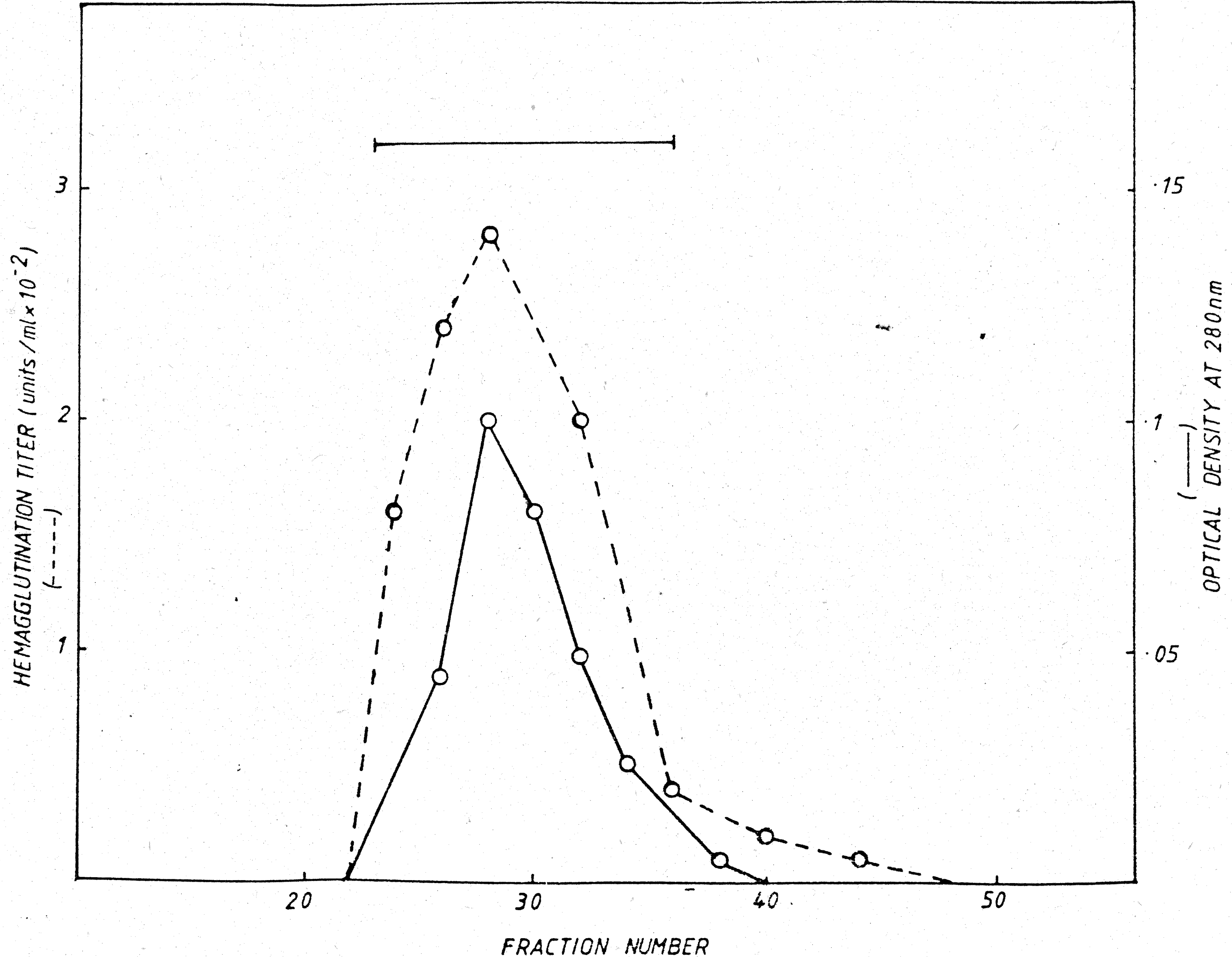


Fig.14. Affinity chromatography of the lactose extract

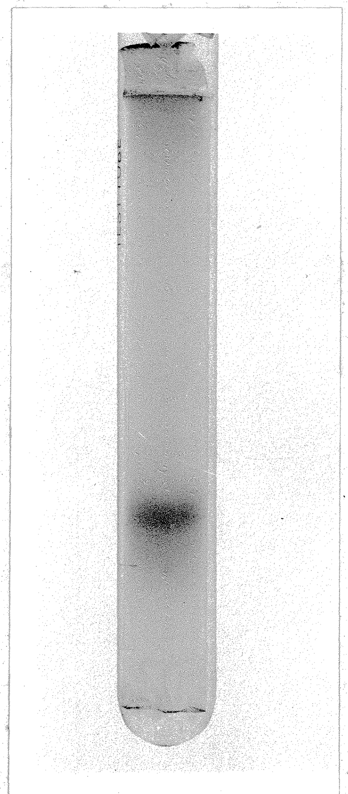


Fig.15. Polyacrylamide gel electrophoresis of purified ß-galactoside-binding protein at pH 8.3.

Experimental details are given under General Methods.

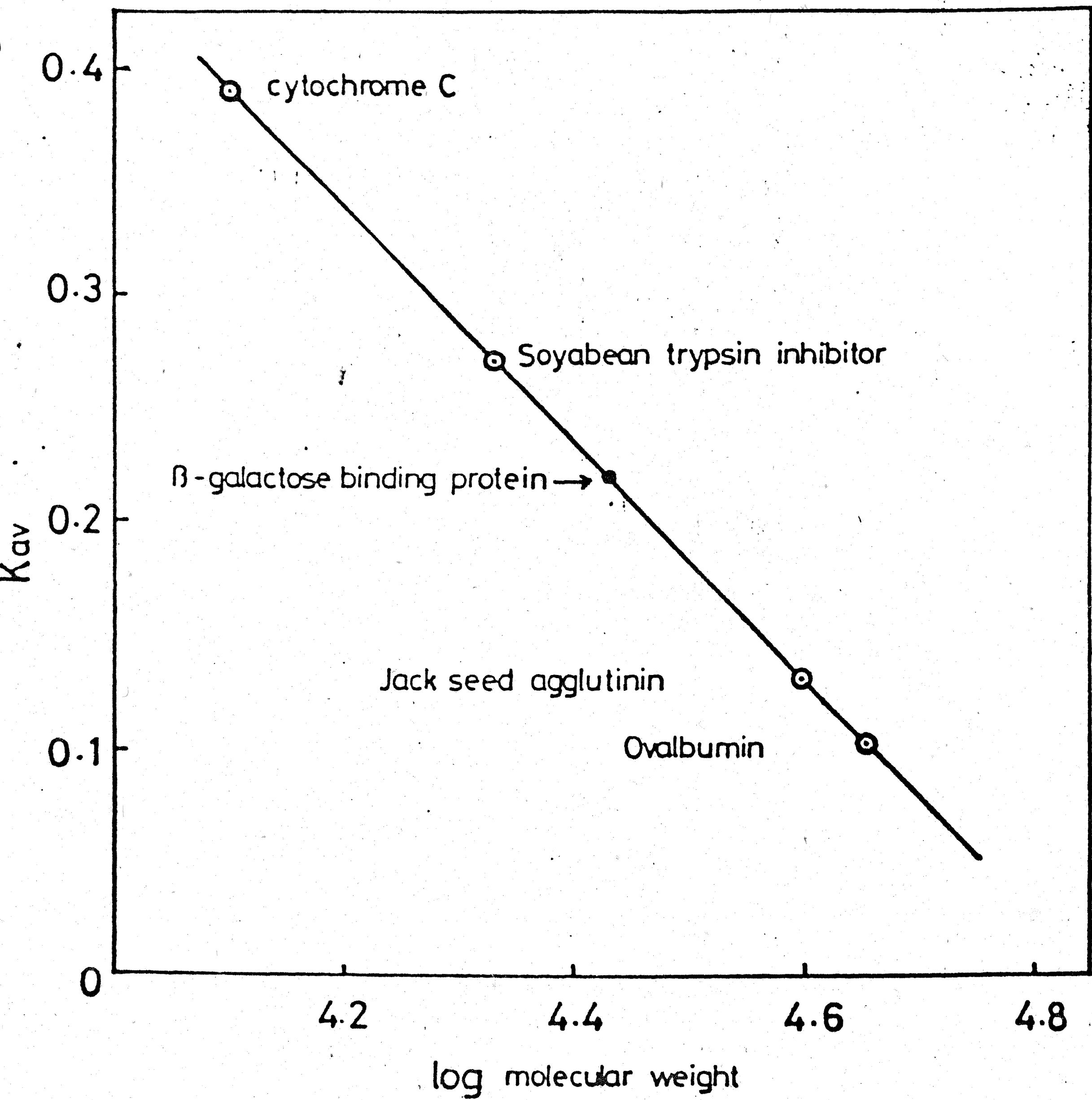


Fig.16. Molecular weight determination of β-galactoside-binding protein by gel filtration on Biogel P-100.

Details of the experiment are given under General Methods.

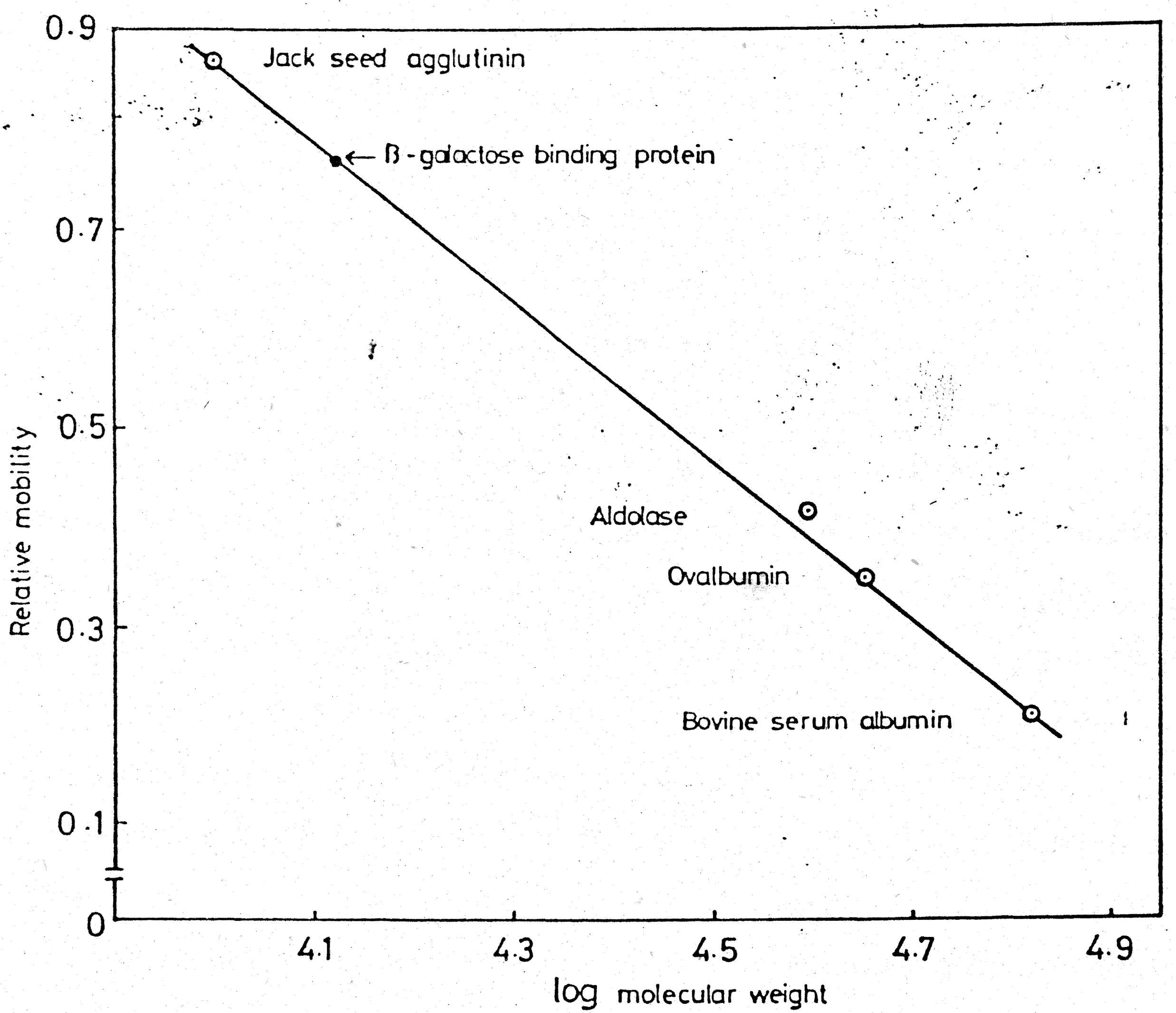


Fig.17. Subunit molecular weight determination of ß-galactoside-binding protein by SDS-polyacrylamide gel electrophoresis.

Experimental details are given under General Methods.

were agglutinated but not that of goat. Trypsinized erythrocytes of all human blood groups were also agglutinated.

The saccharide inhibition of hemagglutination were carried out with trypsinized rabbit and human B+ve erythrocytes. The results of saccharide inhibition studies are shown in Table 6. Thiodigalactoside was found to be the best inhibitor followed by lactose. Agglutination of trypsinized human cells was inhibited by 8-times lower concentration of thiodigalactoside as compared to rabbit cells. Galactopryanosylamine was a better inhibitor compared to Gal and galactosamine. Saccharide concentration required for inhibition by Gal, galactosamine and GalNAc was of the similar order. Neither methyl-α-D-glucopyranoside nor methyl-α-D-mannopyranoside was an inhibitor. Fucose did not inhibit agglutination with both the cell types. Beta-galactosides were found to be more effective inhibitors than their α-anomers, although methyl-β-D-galactopyranoside and p-nitrophenyl-β-D-galactoside were slightly less affective than their respective α-anomers. The agglutination of trypsinized human erythrocytes required 6-fold higher concentration of lectin as compared to rabbit cells. The saccharide concentration required for inhibition of hemagglutination were also lower in case of human cells. The lectin retained its activity when kept in the presence of 1mm 2-mercaptoethanol in PBS, pH 7.2 for several weeks at a concentration of 1mg/ml at $0^{o}C$. Protein concentration higher than 1mg/ml resulted

TABLE - 6

INHIBITION CAPACITY OF COMMON SACCHARIDES ON AGGLUTINATION OF ERYTHROCYTES BY GALACTOSE-BINDING LECTIN OF HUMAN PLACENTA

Saccharides	Minimum concentration (mM) required to inhibit twice the haemagglutinating amount of lectin	
	Trypsinised human B+ve cells	Trypsinised rabbit cells
Thiodigalactoside	0.019	0.16
Lactose	0.20	0.78
Lactitol	4	4
D-Galactose	25	50
Melibiose	12.5	50
Faffinose	25	100
Stachyose	12.5	25
D-Galactosamine	25	50
D-Galactopyranosylamine	0.39	6.25
N-Acetyl-D-galactosamine	25	100
1-O-Methyl-α-D-galactose	3.13	25
1-O-Methyl-β-D-galactose	12.5	50
P-Nitrophenyl-α-D-galactose	1.5	2.5
P-Nitrophenyl-β-D-galactose	3.13	5.0
L-Fucose	NI	NI
1-O-Methyl-α-D-glucose	200	NI
1-O-Methyl-α-D-mannose	200	NI

The experimental details are described in the text.

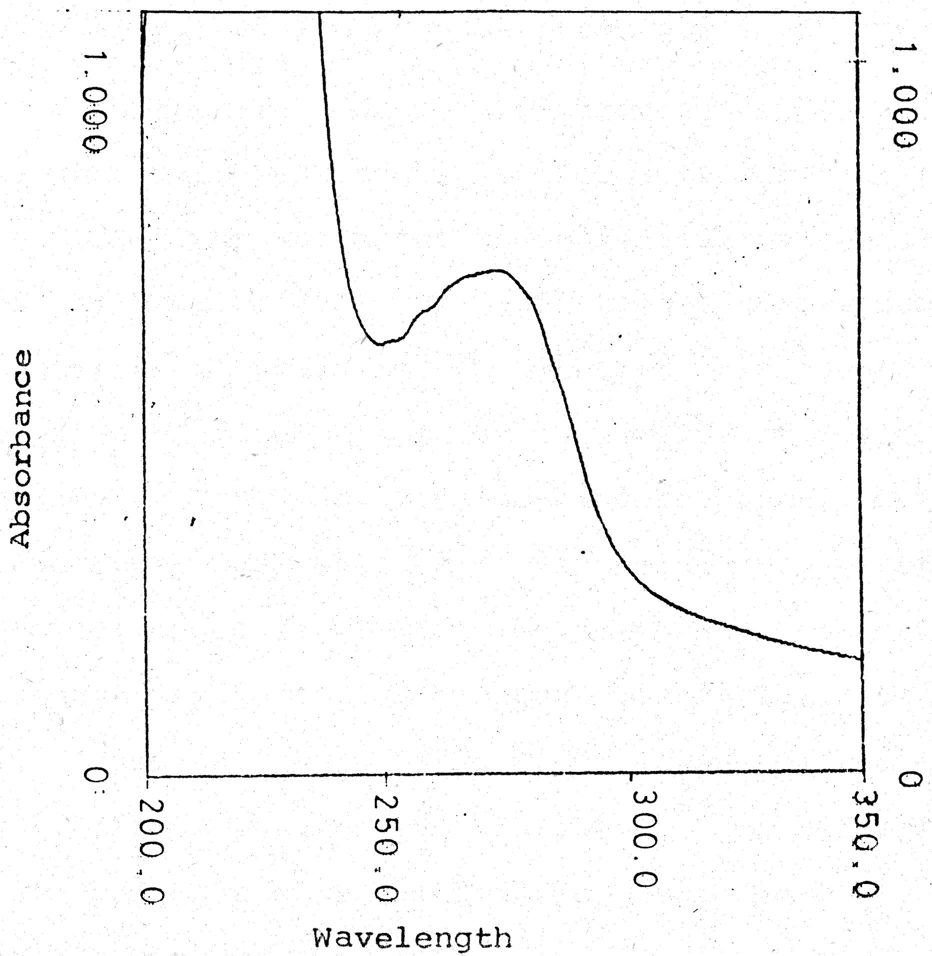


Fig.18. UV spectrum of human placental β-galactoside-binding protein.

Details are described in the text.

The UV spectrum was measured at pH 7.2 (Fig.18). Multiple shoulders are observed in the range between 250 and 270nm. Such a spectrum was also reported for electric eel lectin (116). These shoulders should be due to the presence of a large amount of Phe residues relative to Tyr residues.

The protein was found to contain one free -SH group when titrated with DTNB by direct Ellman's method (162). The total -SH group estimated under denaturing condition according to Habeeb (161) was found to be one per subunit. Tryptophan residue could not be detected in placental β-galactoside-binding protein by the method of Savige and Fontana (169). The β-galactoside-binding protein was found to contain three Tyr residues as determined by O-acetylation with N-acetylimidazole (162). The amino acid composition of the placental β-galactoside-binding protein is listed in Table 7. N-terminal amino acid was analyzed by the method of Gray (173) was found to be histidine. The N-terminal amino acid sequence of the lectin was found to be 'His-Pro-Ala-Pro-Tyr-Phe-Ser-'.

The total carbohydrate content was estimated by phenol sulphuric acid method with Gal as standard was found to be 16.7%.

The results of chemical modification of amino acids and saccharide chains are summarized in Table 8. Tryptophan modification by 2-hydroxy-5-nitrobenzyl bromide and 2-methoxy-

TABLE - 7

AMINO ACID COMPOSITION OF HUMAN PLACENTAL β-GALACTOSIDE-BINDING PROTEIN

AMINO ACID	MOLE/MOLE OF PROTEINS
K	11.36
H	3.08
R	6.60
B	19.72
T	9.49
S	12.24
Z	20.60
P	9.07
G	18.31
A	20.86
C[b]	2.0
V	9.51
M	1.41
I	6.25
L	13.12
Y	2.51
F	12.85
W[c]	0.0

Amino acid residues are denoted by single letter.

a - minimum molecular weight Mr = 26,900

b - Cysteine was determined by DTNB method

c - Tryptophan was estimated by dimethyl sulphoxide/HCl method.

	Amino acid Group or Oligosaccharide Modified	Minimum concentration of protein (ng) required for agglutination			
		Trypsinised rabbit cells		Trypsinised human cells (B+ve)	
		Control*	Test	Control*	Test
None	-	60	60	350	350
Trinitrobenzene sulphonate	$-NH_2$	830	NA	3300	NA
Citraconic anhydride	$-NH_2$	560	NA	2240	NA
Maleic anhydride	$-NH_2$	540	NA	2150	NA
Dithionitrobenzene	-SH	260	NA	1050	NA
P-hydroxymercuribenzoate	-SH	142	NA	1050	NA
N-Acetylimidazole	Try	580	580	2300	2300
Diethylpyrocarbonate	His	500	NA	2000	NA
Phenylglyoxal hydrate	Arg	1700	NA	4300	NA
2-hydroxynitrobenzyl bromide	Trp	580	580	2300	2300
2-methoxynitrobenzyl bromide	Trp	580	580	4600	4600
Sodium metaperiodate, pH 4.5	Oligosaccharides	500	NA	2200	NA
Sodium metaperiodate (40mM) & Sodium cyanobrohydride (80mM) pH 3.5	Oligosaccharides	600	NA	ND	ND

Control* - Identical conditions as required for test without the modifying reagent
N.A. - No agglutination
N.D. - Not done.

TABLE - 8

CHEMICAL MODIFICATION OF AMINO ACID GROUPS AND OLIGOSACCHARIDE CHAINS OF β-GALACTOSIDE-BINDING PROTEIN.

Details of the experiment are given under General Methods.

activity. Hemagglutination activity was not altered by modification of Tyr residues. On the other hand Arg residue modification resulted in complete loss of hemagglutination activity. Specific modification of His residues by diethylpyrocarbonate completely inhibited the hemagglutination activity. The hemagglutination activity of the lectin was completely destroyed when the amino groups were modified by TNBS, citraconic anhydride and maleic anhydride. Sulfhydryl group modification by DTNB and pHMB resulted in total loss of hemagglutination activity.

The oxidation of saccharide chains by periodic acid at $0^{o}C$ for 16 hr, completely abolished the hemagglutination activity. Treatment of the β-galactoside-binding protein with a mixture of sodium metaperiodate and sodium cyanoborohydride also resulted in complete loss of hemagglutination activity.

Native placental β-galactoside-binding protein did not bind to immobilized Con A, WGA, RCA and WBA. But the protein could bind to JSA-Sepharose.

Scatchard plot of the equilibrium dialysis data using ^{3}(H) lactose showed two sugar binding sites per 27 kDa lectin, with an association constant, $ka=9.4x10^{3}M^{-1}$ (Fig.19).

The fluorescence of MeUmb-β-D-Gal was progressively quenched with successive additions of β-galactoside-binding protein (Fig.20). The addition of excess amount of protein

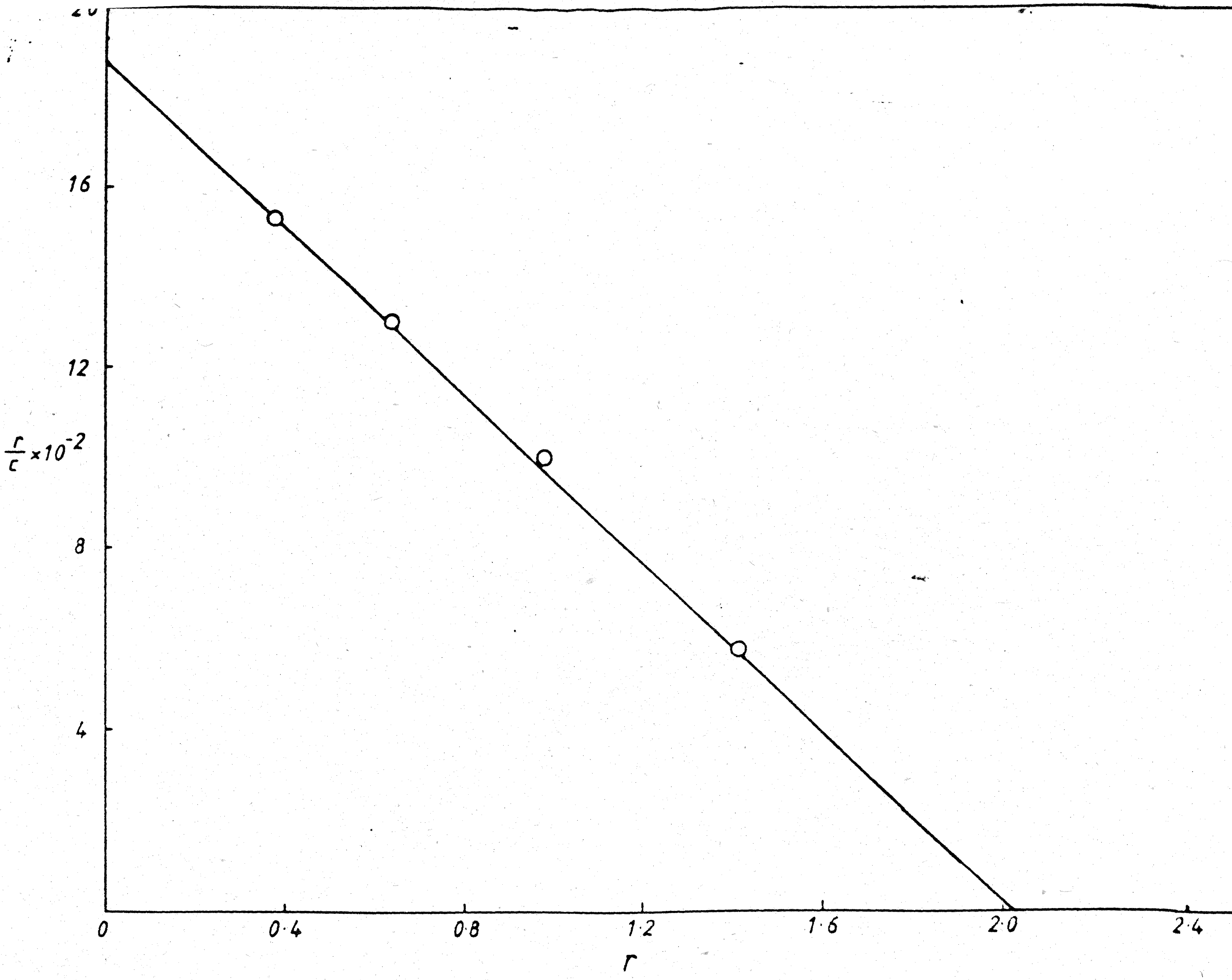


Fig.19. Scatchard plot of the binding of ^{3}(H) lactose to β-galactoside-binding protein.

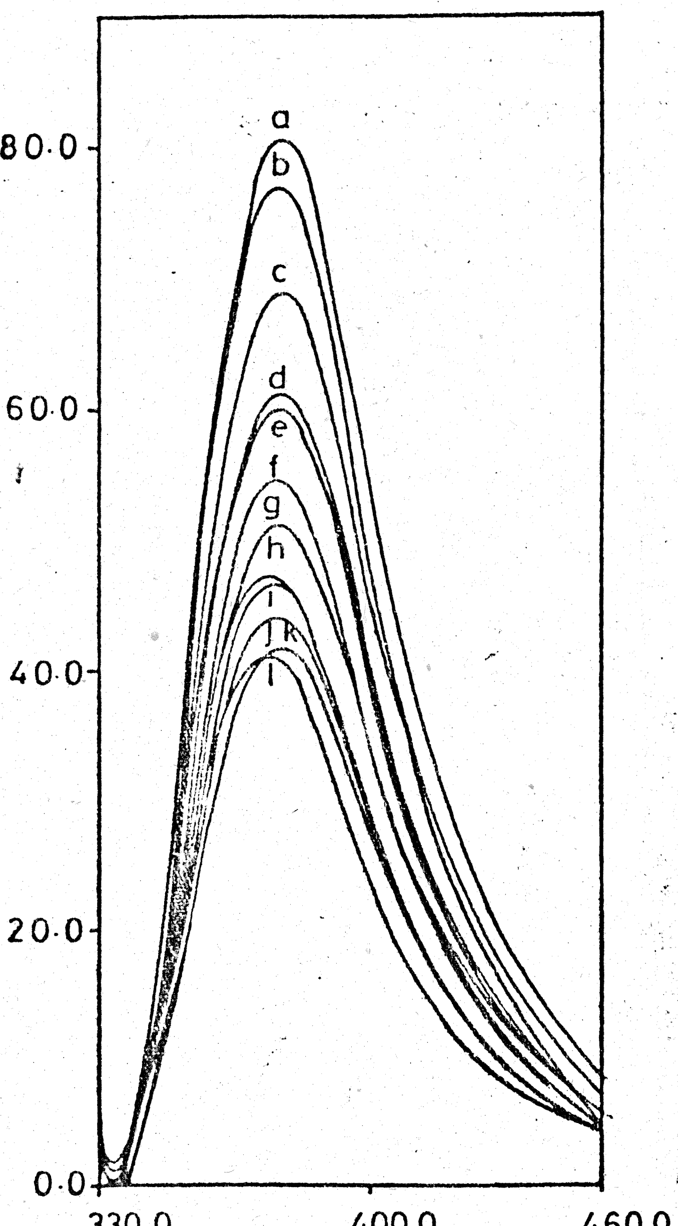


Fig.20. Quenching of the fluorescence of MeUmb-ß-Gal on additions of increasing quantities of ß-galactoside-binding protein. To 2 ml of 3M MeUmb-ß-Gal in a 1x1x4cm cuvette the following aliquots of 40mM ß-galactoside-binding protein were added (a) 0 µl, (b) 10 µl, (c) 20 µl, (d) 30 1, (e) 40 1, (f) 50 1, (g) 60 1, (h) 70 1, (i) 80 1, (j) 90 1, (k) 100 1 and (l) 120 1.

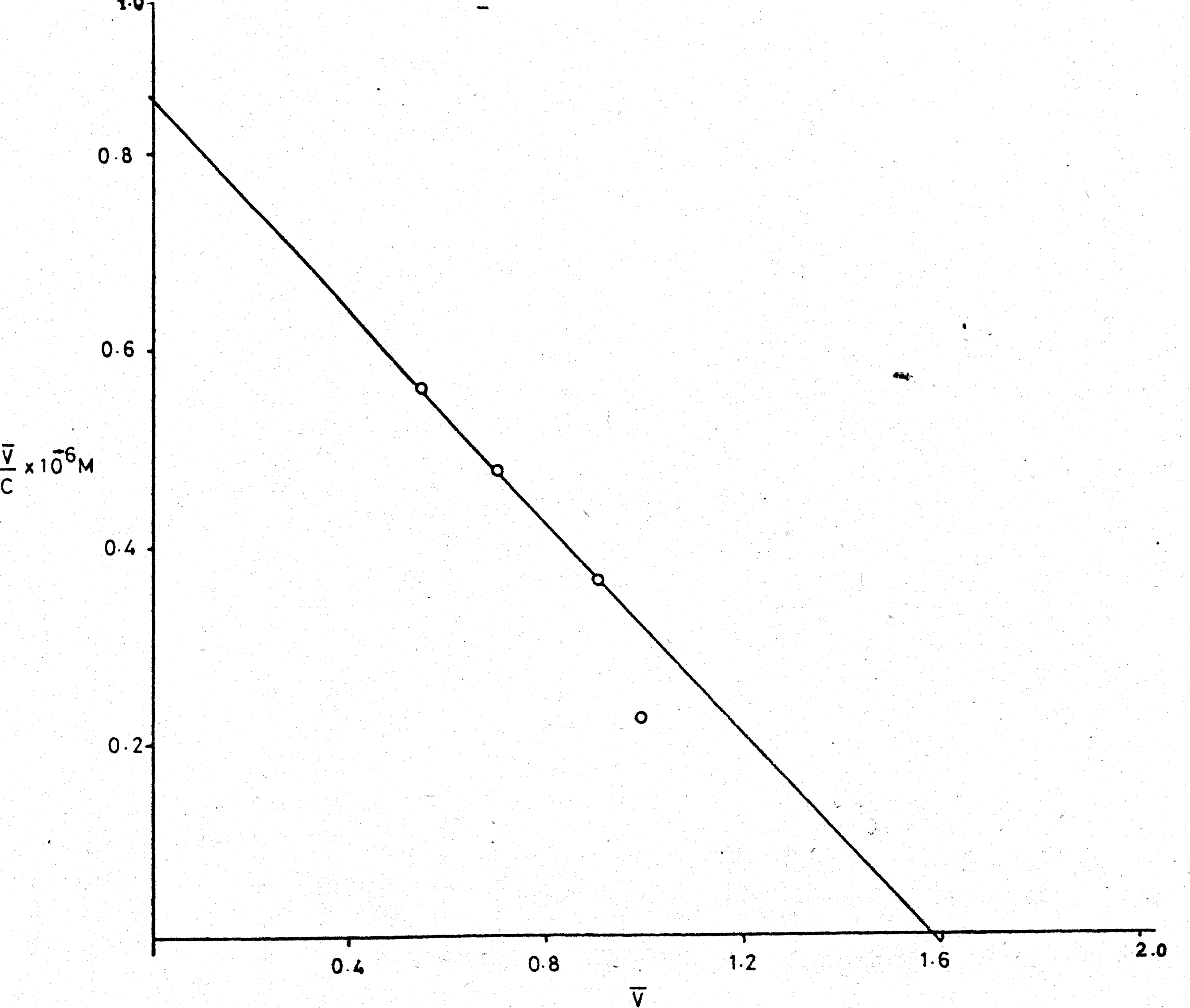


Fig.21. Scatchard plot for the binding of MeUmb-ß-Gal to ß-galactoside-binding protein.

Experimental details

fluorescence observed after successive additions of MeUmb-β-D-Gal to a fixed amount of lectin was a measure of the free ligand. Scatchard plot of the results of the fluorescence quenching of MeUmb-β-Gal showed an association constant, $Ka=4.3x10^{7}M^{-1}$. (Fig.21). The number of binding sites per 27 kDa β-galactoside-binding protein was found to be two.

Polyclonal antibody to the β-galactoside-binding lectin was raised in rabbit. Immunoglobulin class of the antibody was determined by sequential chromatography on lgA-specific JSA-Sepharose and lgG-specific protein A-Sepharose. The antibody eluted from lgA-specific JSA-Sepharose column precipitated with β-galactoside-binding protein (Fig.22). The lgG-specific protein A-Sepharose eluate and the unbound antiserum containing remaining class of immunoglobulins did not cross react with β-galactoside-binding protein. Hemagglutination activity was exhibited by the antibody-lectin complex. The antibody cross-reacted with purified bovine heart β-galactoside-binding lectin (Fig.23). Immunological cross reaction was also observed against both A and B-forms of human placental β-galactosidase (Fig.23). The antibody cross-reacted immunologically with various human fetal and adult organs like heart, liver, brain, kidney and muscle (Fig.24 and 25).

DISCUSSION

A soluble β-galactoside-binding protein has been

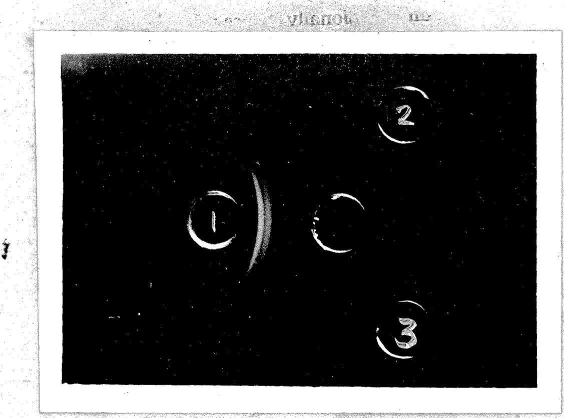


Fig.22. The class of immunoglobulins of β-galactoside-binding protein antibody.

Details are described in the text.

Central Well : β-Galactoside-binding protein

1 : JSA-Sepharose eluate
2 : Protein A-Sepharose eluate
3 : Unbound antiserum.

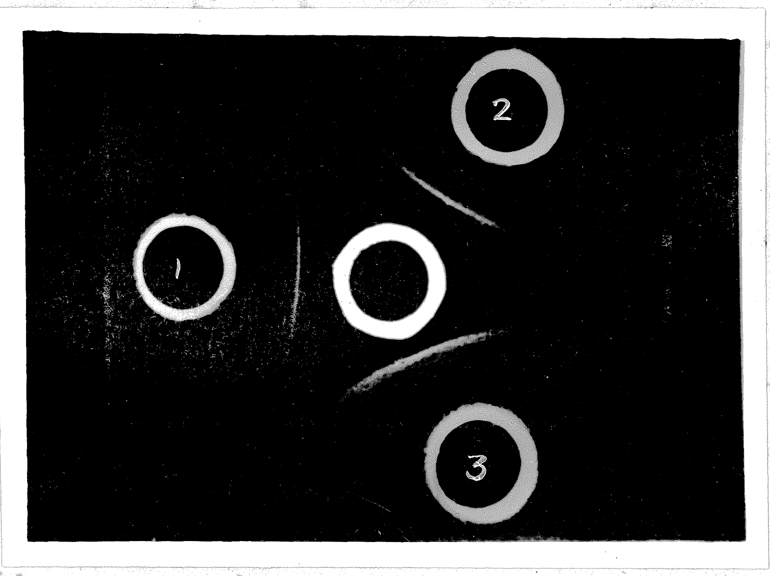


Fig.23. Immunological cross reactivity of β-galactoside-binding protein antibody.

Central Well : β-Galactoside-binding protein antibody.

1 : β-Galactosidase A

2 : β-Galactosidase B

3 : Bovine heart β-galactoside-binding protein.

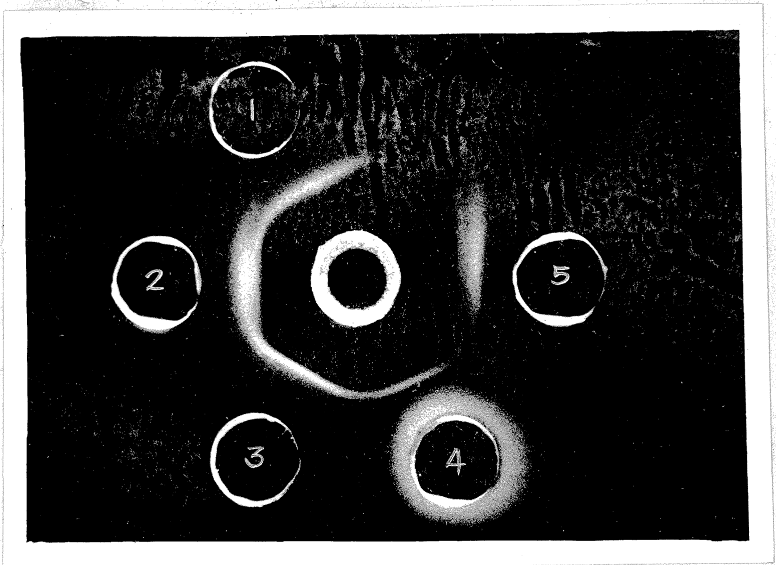


Fig.24. Immunological cross reactivity of β-galactoside-binding protein from various human fetal organs.

Details are described in the text.

Central Well: β-Galactoside-binding protein antibody.

1 : Placenta
2 : Skeletal muscle
3 : Brain
4 : Liver
5 : Heart

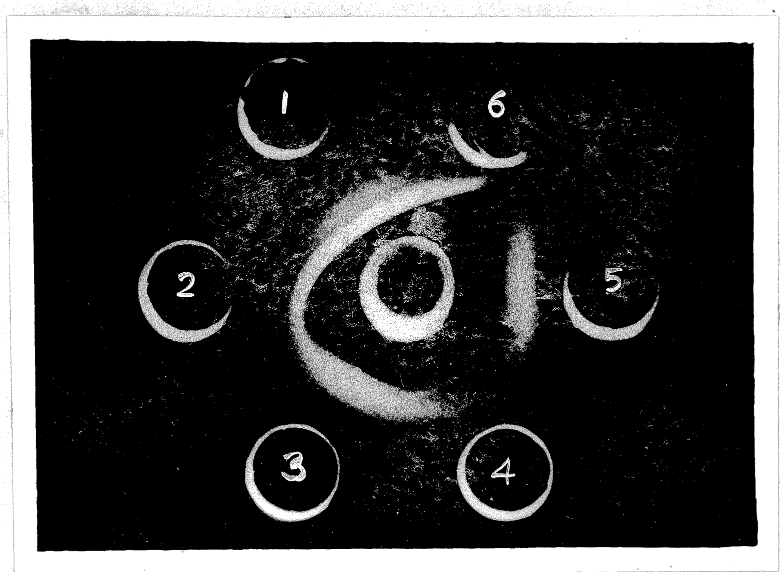


Fig.25. Immunological cross reactivity of β-galactoside-binding protein from various human adult organs.

Details are described in the text.

Central Well : β-Galactoside-binding protein antibody.

1 : Placenta
2 : Kidney
3 : Brain
4 : Liver
5 : Heart
6 : PBS, pH 7.4.

Kasai (153) with modifications. Specific extraction with lactose and affinity chromatography on asialofetuin-Sepharose resulted in homogeneous protein. The finding that the lectin was not extractable with buffer alone but required lactose. It could be presumed that the lectin may remain attached to the oligosaccharide chains of the glycoconjugates on the membrane and lactose replaced them during extraction process.

The protein migrates on SDS-polyacrylamide gels as a monomer with an apparent molecular weight of 13.4 kDa. The molecular weight of the native lectin determined by gel filtration on Biogel P-100 was found to be 26.9 kDa. The placental β-galactoside-binding protein may be a dimer. The subunit molecular weight of the protein was quite similar to that of other animal β-galactoside-binding proteins which exhibit subunit molecular weight in the range of 13-16 kDa (109,110). Higher molecular weight forms were not observed in the present study. In this respect it is different from bovine heart and brain lectin, which were reported to have higher molecular weight forms than 27 kDa (209).

The lectin agglutinated native calf, pig, rat, mice and rabbit erythrocytes. The trypsinized calf, pig, sheep, dog, rat, mice and rabbit erythrocytes were agglutinated but not that of goat. Trypsinized erythrocytes of all human blood groups were also agglutinated. Calf heart

trypsinized human erythrocytes and native rat erythrocytes (148).

No glycohydrolase activity was associated with the lectin. Human and rat lung lectins were also devoid of glycosidase and glycosyltransferase activity (221). The lectin was non mitogenic towards human lymphocytes. However, a prophylactic and therapeutic affect of a purified β-galactoside-binding lectin from electric eel on experimental autoimmune disease, myasthenia gravis was reported (234). Kajikawa et al showed that human placenta lectin stimulates the production of tumor killing factor by murine macrophages (132), suggesting the possible involvement of this family of lectins in the immune system.

The hemagglutination inhibition by a wide range of saccharides offered some in sight into the saccharide specificity of the lectin. As in most of the soluble β-galactoside-binding lectins, thiodigalactoside was the best inhibitor followed by lactose. Galactopyranosylamine was a better inhibitor than Gal indicating that the amino group substitution at C-1 of Gal is important in saccharide-binding. Substitution at C-1 of Gal with p-nitrophenol or methyl group also increased the affinity towards β-galactoside-binding protein than free Gal. The concentration of inhibitory saccharide required for inhibition by Gal, galactosamine and GalNAc were of the similar order. This indicates that amino group substitution at C-2 of Gal is

neither Glc, nor Man was an inhibitor. Melibiose and stachyose containing a common sequence Galα1→6Gal were found to inhibit β-galactoside-binding lectin at equal concentration.

Fucose, which differs from Gal by the absence of C-6 OH group did not inhibit agglutination indicating the importance of C-6 hydroxyl group on Gal for binding. Hemagglutination inhibition studies reveals that placental β-galactoside-binding protein is specific for saccharides bearing terminal β-galactosides. Methyl β-D-galactopyranoside was slightly less effective inhibitor than its α-anomer. Similar observation was also reported from several other soluble β-galactoside-binding lectins (120,217,123). PNP-β-D-galactopyranoside was also a less effective inhibitor than its α-anomer. The lectin had no affinity towards N-acetyl neuraminic acid. Similar pattern of saccharide inhibition of hemagglutination was observed in most of the soluble, animal β-galactoside-binding lectin (120,217,123). The agglutination of trypsinized human erythrocytes require six-fold higher concentration of lectin as compared to that of rabbit cells. It may be due to the lower affinity of the lectin to human cells relative to rabbit cells.

Placental β-galactoside-binding protein was found to contain one free -SH group. The lectin was stable in the presence of exogenous thiols indicating that the free -SH

thiol groups must remain in their reduced form to maintain the activity of rat lung β-galactoside-binding lectin. The loss of activity is probably due to conformational changes resulting in a more compact and not due to a direct critical function of a Cys residue at the carbohydrate binding site (223).

Amino acid composition of human placenta lectin is quite similar with that of the β-galactoside-binding lectins from various other animal sources (116,214,221), but differed from that of rat lung lectins (220). Amino acid analysis showed high content of Ala and Gly. Tryptophan residue could not be detected in placental β-galactoside-binding protein. Similar observation has been made by Hirabayashi and Kasai (153). Most of the soluble β-galactoside-binding lectins reported to contain at least one Trp residue. The presence of three Tyr residues in placental lectin is confirmed by amino acid analysis. The amino group of the N-terminal amino acid His was not blocked. The amino group of the N-terminal amino acid of chick 14 kDa lectin was blocked (225). Drickamer have reported the presence of blocked N-terminal amino acid in asialoglycoprotein receptor of chick (227). N-terminal amino acid sequence of placental β-galactoside-binding protein was found to be 'His-Pro-Ala-Pro-Tyr-Phe-'.

Total carbohydrate content estimated by phenol sulphuric acid method with Gal as standard was found to be 16.7%. Powell reported the presence of carbohydrate in

The electrolectin was found to contain 2.2% carbohydrate (116). Purified Xenopus laevis contains about 20% saccharides (123). The carbohydrate composition of human hepatic lectin resembles that of A subunit of the rabbit lectin (235,236).

Chemical modification of various amino acids were undertaken to differentiate those involved at the binding site. The results of chemical modification shows the presence of Lys. His and Arg at the saccharide-binding site. Modification in the presence of specific sugar, lactose, protected the lectin from inactivation. Cysteine residue also contributed for the activity as the selective modification resulted in complete loss of hemagglutination activity. Complete loss of hemagglutination activity was observed when Cys residue was modified with pHMB (10^{-4}M) and NEM (10^{-2}M). A difference in sensitivity to pHMB and NEM has also been observed with chick 14 kDa and rat lung soluble β-galactoside-binding lectin (214,223). Alkylation of native rat lung lectin with iodoacetate causes substantial loss of activity. In contrast, alkylation with iodoacetamide yields, fully active derivative, stable to atmospheric oxygen in the absence of disulphide reducing agents (223).

Oxidation of the oligosaccharide chains with periodate had complete inhibitory effect. Treatment of the lectin with a mixture of sodium metaperiodate and sodium cyanoborohydride results in the oxidation of carbohydrate chains and the reduction of the aldehyde to primary alcohols.

and amino groups in the protein and non-specific oxidation of amino acids. The modified lectin had lost hemagglutination activity, indicating the importance of saccharide chains for the activity.

Affinity chromatography on various immobilized lectins were used to elucidate the nature of the oligosaccharide chains of the β-galactoside-binding protein. The results of lectin-Sepharose affinity chromatography does not necessarily suggest the presence of terminal α-linked Gal residues in placental lectin.

The association constant calculated for lactose by equilibrium dialysis was $Ka=9.4x10^{3}M^{-1}$. The association constant, $Ka=4.3x10^{7}M^{-1}$ for MeUmb-β-Gal was obtained by fluorescence quenching studies. However, the results of equilibrium dialysis as well as fluorescence quenching clearly showed only two sugar binding siter per 27 kDa lectin.

Polyclonal antibody was raised against β-galactoside-binding protein in rabbits. Chromatography of the antibody on IgA-specific JSA-Sepharose followed by IgG-specific Protein A-Sepharose were carried out to determine the class of immunoglobulins. JSA-Sepharose eluate only cross reacted with β-galactoside-binding protein indicating that the antibody belonged to IgA class of immunoglobulins. No inhibition of hemagglutination activity was observed with lectin-antibody complex, indicating that the antigenic

hemagglutination activity of chick liver and embryonic muscle lectin in the presence of antiserum raised against the pure liver lectin (212). The antibody cross-reacted immunologically with purified bovine heart β-galactoside-binding lectin indicating the presence of common structural determinants in these lectins. Immunological cross-reaction of antiserum raised against purified bovine heart β-galactoside-binding lectin with 14.5 and 29 kDa rat lectins was reported by Cerra et al (220). Several other β-galactoside-binding lectins from phylogenetically distinct species cross-react immunologically. The finding of conservation of an antigenic structure through evolution together with the common features displayed by soluble β-galactoside-binding lectins can possibly be viewed as manifestations of the important physiological function(s) of these proteins.

The antibody cross-reacted with placental β-galactosidase A and B, indicating the presence of homologous sequence regions in both enzyme and lectin. The antibody also cross-reacted immunologically with similar lectins from various human fetal and adult organs indicating the close relationship among these groups of lectins.

...

CHAPTER - IV

GENERAL DISCUSSION AND SUMMARY

GENERAL DISCUSSION

Acid β-galactosidase is a lysosomal hydrolase which is involved in the catabolism of galactoglycoconjugates. The absence or deficiency of acid β-galactosidase leads to the lysosomal storage disease GM_1 gangliosidosis (139). The enzyme has been purified to apparent homogeneity from various animal sources and has been characterized with regard to substrate specificity, number of forms and their physicochemical characteristics (133-137). The wide spread occurrence and broad substrate specificity of acid β-galactosidases in mammalian organs is probably related to the multiple physiological functions of the enzyme. There are scanty reports on the structural studies of lysosomal acid hydrolases. Structural comparison of the binding-sites of the enzyme and lectin acting on β-galactosides deserves attention. The exact biological functions of the lectins is not known. Lectins have become invaluable tool in biological and medical research in diverse fields. The present study involved detailed structural and immunological studies on β-galactosidase and β-galactoside-binding protein of human placenta, an useful hospital waste material.

Two forms of acid β-galactosidase were separated and purified to homogeneity from human placenta by Saraija and Basu using a four step procedure involving (i) Con A-Sepharose affinity chromatography (ii) gel filtration on

Sephadex A-50 and (iv) affinity chromatography on mercurial-Sepharose CL-4B (152). This method of purification has been modified considering the low recovery and stability. A second Con A-Sepharose affinity chromatography after DEAE-Sephadex A-50 ion exchange chromatography of both forms resulted in purified enzyme with higher yield.

The purified enzyme was homogenous as revealed by polyacrylamide gel electrophoresis at pH 8.3. Molecular weight of β-galactosidase determined by gel filtration was 500,000 for A-form and 100,000 for B-form. Two forms of acid β-galactosidase have been reported from other organs (178,180). Recently, in addition to the two forms, a dimeric form of acid β-galactosidase was reported to be present in purified preparations (95,184). In SDS-polyacrylamide gel electrophoresis, A-form was dissociated into two major bands with apparent molecular weight of Mr.=68 kDa and 27 kDa, whereas B-form showed a single subunit of Mr.=68 kDa (Fig.6,7). The data suggested that both A and B-forms of the enzyme may have a common subunit.

Galacto-1-deoxynojirimycin was found to be a good inhibitor of β-galactosidase A and B. Dixon plot revealed competitive nature of the inhibition with an inhibition constant, $Ki=1.25x10^{-5}M$ for A-form and $1.4x10^{-5}M$ for B-form (Fig.8,9). The inhibition of the enzyme by galacto-1-deoxynojirimycin is significant because most of the available

Galactose (10^{-1}M) also inhibited both forms of the enzyme. Amino group substitution at carbon-1 of galactose increased its inhibitory capacity.

Beta-galactosidase A and B had 15 and 14 tryptophan residues respectively according to the method of Savige and Fontana (169). Tyrosine quantitated with N-acetylimidazole (162) showed 48 and 11 residues in A and B-forms respectively.

In the present study an attempt has been made to identify the amino acids at the catalytic site of placental acid β-galactosidase. The chemical modification studies indicated the presence of tryptophan and arginine at the catalytic site as the specific modification of these residues completely inactivated the enzyme. Cysteine residues had contributed for enzymic activity as the selective modification decreased the activity. Amino group modification suggested their indespensability. No significant difference was observed between the effect of chemical modification of A and B-forms of the enzyme.

Treatment of the enzyme with a mixture of sodium metaperiodate and sodium cyanoborohydride resulted in the oxidative cleavage of the oligosaccharides and the reduction of the aldehyde groups to primary alcohols, minimizing the Schiff's base formation between the aldehyde groups and the amino groups in the protein and non-specific

completely lost their activity indicating the importance of the oligosaccharides in the biological activity.

Affinity chromatography of β-galactosidase A and B on various immobilized lectins indicated the presence of high mannose, hybrid and complex-type of oligosaccharides. Enzyme bound to Con A-Sepharose could be eluted with 10mM hapten sugar revealing the presence of complex biantennary oligosaccharides. Major portion of the enzymic activity was esluted with 500mM hapten indicating the presence of high mannose and hybrid-type oligosaccharides. Twenty to thirty percent of the applied β-galactosidase A and B activities were bound to WGA-Sepharose. The bound form predominantly contained hybrid and complex-type where as the unbound form was of high mannose-type. Polyacrylamide gel electrophoresis at pH 8.3 of the bound and unbound form of the enzyme showed similar electrophoretic mobilities. Different electrophoretic mobilities of WGA-bound and unbound fractions of human liver acid β-galactosidase was reported by Heyworth and Wynn (206). 20% B and 50% A-forms were bound to RCA-Sepharose. Affinity chromatography on RCA-Sepharose also revealed the presence of hybrid and complex-type of oligosaccharides in the bound form. Human liver β-galactosidas was also separated into two forms by affinity chromatography on immobilized RCAI & WGA (207). Either form of the enzyme did not bind to JSA-Sepharose.

amino acid sequence analysis of A-form showed two peaks. The subunits isolated by SDS-PAGE indicated the N-terminal sequence 'Leu-Ala-' for kDa subunit and 'Tyr-Met'- for the 27 kDa subunit.

Polyclonal antibody was raised in rabbit against β-galactosidase A. The antiserum was chromatographed on IgA-specific JSA-Sepharose followed by lgG-specific protein A-Sepharose to determine the immunoglobulin class. The antiserum was retained only on JSA-Sepharose column indicating that the antibody belonged to lgA class of immunoglobulins. The enzyme-antibody complex retained enzymic activity demonstrating that the antigenic and catalytic sites were situated at different regions of the polypeptide. A and B-forms of the enzyme were found to be immunologically identical. Immunological identity has also been reported for the two forms of human liver acid β-galactosidase (134). The antibody cross-reacted with similar enzymes from various human fetal and adult organs.

A soluble β-galactoside-binding protein has been purified from human placenta according to Hirabayashi and Kasai with modifications (153). Major steps of purification involved specific extraction with lactose and affinity chromatograph on asialofetuin-Sepharose column and resulted in homogenous protein. The finding that the lectin was not extractable with buffer alone but required lactose suggests

replaces it during the extraction process.

The protein migrates on SDS-polyacrylamide gels as a monomer with an apparent molecular weight, Mr.=13.4 kDa. The molecular weight of the native lectin determined by gel filtration was found to be 26.9 kDa. Higher molecular weight forms than 27 kDa was not observed for the placental β-galactoside-binding protein. In this respect it is different from bovine heart and brain lectin which are reported to have higher molecular weight forms than 27 kDa (209).

The lectin agglutinated native calf, pig, rat, mice and rabbit erythrocytes. Trypsinized calf, pig, sheep, dog, rat, mice and rabbit erythrocytes were agglutinated but not that of goat. Trypsinized erythrocytes of all human blood groups were also agglutinated. Calf heart β-galactoside-binding lectin was not bound to normal or trypsinized human erythrocytes and normal rat erythrocytes (148).

No glycohydrolase activity was associated with the lectin. Human and rat lung lectins were also devoid of glycosidase and glycosyltransferase activity (221). The lectin was non-mitogenic towards human lymphocytes. However, a prophylactic and therapeutic effect of a purified β-galactoside-binding lectin from electric eel on experimental autoimmune disease myasthenia gravis was reported (234). Kajikawa et al showed that human placenta lectin stimulates the production of tumour killing factors by murine macrophages

Hemagglutination inhibition by a wide range of saccharides offered some insight into the saccharide specificity of the lectin (Table 6). Thiodigalactoside was the best inhibitor followed by lactose. Galactopyranosylamine was a better inhibitor than galactose indicating that the amino group substitution at C-1 of galactose is important in saccharide-binding. Substitution at C-1 of galactose with p-nitrophenol or methyl group also increased the affinity towards β-galactoside-binding protein than free galactose. The concentration of inhibitory saccharide required for the inhibition by galactose, galactosamine and N-acetyl-D-galactosamine were of similar order. This indicated that amino group substitution at C-2 of galactose is less stringent for binding. Axial hydroxyl group on C-4 of galactose is a stereospecific requirement for binding, since neither, glucose nor mannose was an inhibitor.

Fucose, 6-deoxygalactose, did not inhibit agglutination indicating the importance of C-6 OH group on galactose for binding. Hemagglutination inhibition studies reveals that placental β-galactoside-binding protein is specific for saccharides bearing terminal β-galactosides. Similar pattern of saccharide inhibition of hemagglutination was also reported from several other solube β-galactoside-binding lectins (120,217,221). The agglutination of trypsinized human erythrocytes required six fold higher concentration of protein as compared to that of rabbit cells. It may be due

Placental β-galactoside-binding protein was found to contain one free-SH group. The lectin was stable in the presence of exogenous thiols, absence of exogenous thiols leads to gradual loss of hemagglutination activity indicating that the free-SH group is essential for its activity. Absence of exogenous thils leads to gradual loss of hemagglutination activity. Whiteny et al suggested that some of the thiol groups must remain in their reduced form to maintain the activity of rat lung β-galactoside-binding lectin. The loss of activity is probably a result of conformation changes, resulting in more compact structure and not due to a direct critical function of cysteine residue at the carbohydrate binding-site (223).

Amino acid composition of human placental lectin is quite similar with that of the β-galactoside-binding lectins from various other animal sources (116,214,221). Amino acid analysis showed high content of alanine and glycine. Tryptophan residue could not be detected. Similar observation has been made by Hirabayashi and Kasai (153). The presence of 3 tyrosine residues in placental lectin was confirmed by amino acid analysis. The amino group of the N-terminal amino acid histidine was not blocked. The amino group of the N-terminal amino acid of chick lectin was blocked (225). Drickamer has reported the presence of blocked N-terminal amino acid in asialoglycoprotein receptor of chick (227). N-terminal amino acid sequence of placental β-galactoside

Total carbohydrate content estimated by phenol sulphuric acid method with galactose as standard was found to be 16.7%. Powell reported the presence of carbohydrate in human and rat lung soluble β-galactoside-binding lectins (221). Electrolectin was found to contain 2.2% carbohydrate (116). Purified _Xenopus laevis_ lectin contains about 20% saccharides (123).

Chemical modification of various amino acids were undertaken to differentiate those involved at the active site. The result of chemical modification showed the presence of lysine, histidine and arginine at the saccharide-binding site. Cysteine residue also contributed for the activity as its selective modification resulted in complete loss of hemagglutination activity.

Oxidation of the oligosaccharide chains with periodate had complete inhibitory effect. Treatment of the lectin with a mixture of sodium metaperiodate and sodium cyanoborohydride also resulted in complete loss of hemagglutination activity indicating the importance of oligosaccharide chains in the glycoprotein lectin for binding to specific sugars.

Native lectin did not bind to immobilized ConA, WGA, RCA and WBA. But it could bind to JSA-Sepharose. The result does not necessarily suggest the presence of terminal α-linked galactose residues in placental lectin.

The association constant calculated for lactose by

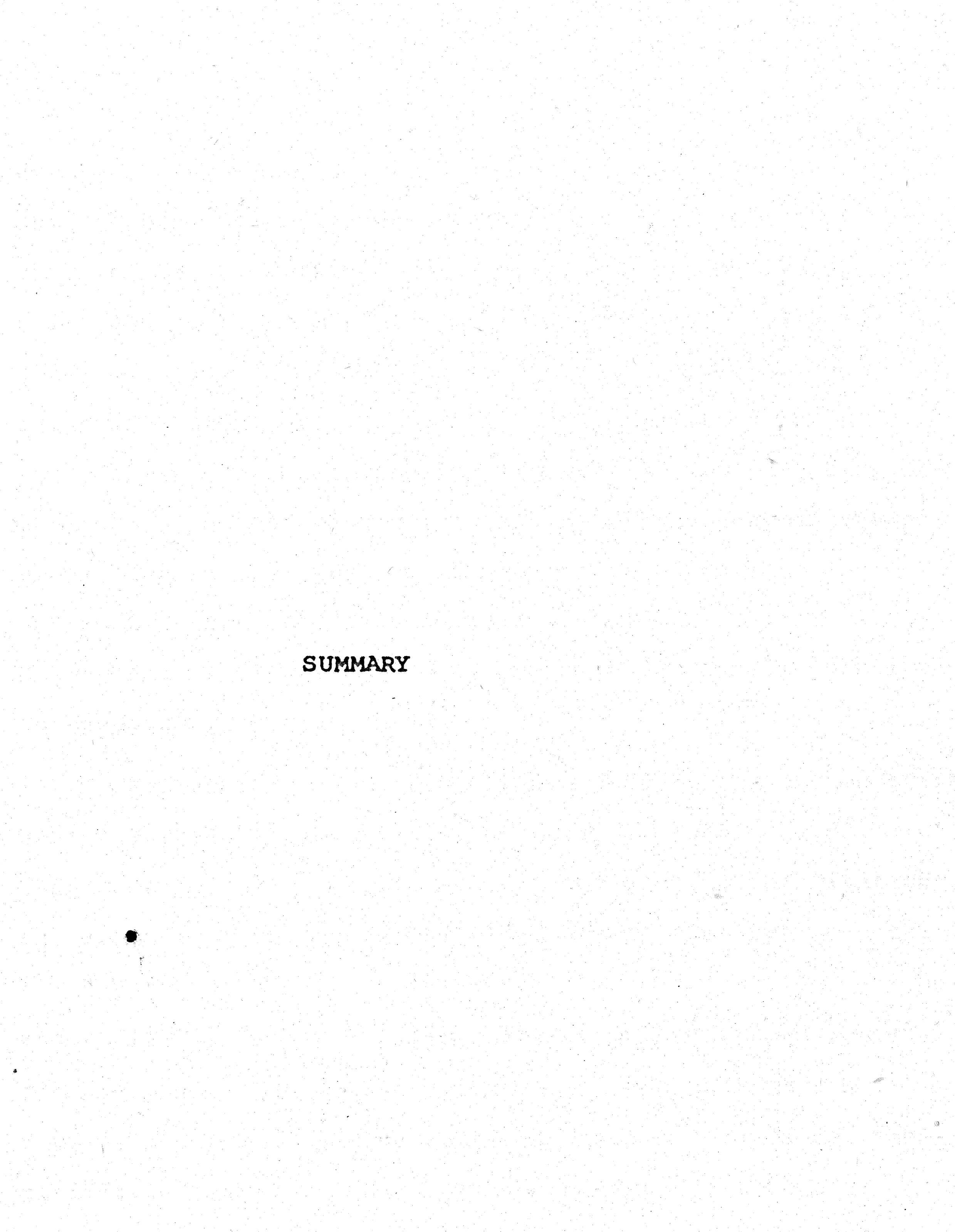
fluorescence of MeUmb-β-D-Gal was progressively quenched by the successive additions of β-galactoside-binding protein (Fig.20). The association constant, $Ka=4.3x10^{7}M^{-1}$ for MeUmb-β-D-Gal was obtained by fluorescence quenching studies (Fig.21). However, the results of equilibrium dialysis as well as fluorescence quenching clearly showed only two sugar binding sites per 27 kDa lectin.

Polyclonal antibody was raised against β-galactoside-binding protein in rabbits. The immunoglobulin class of the antiserum was determined by sequential chromatography on lgA-specific JSA-Sepharose and lgG-specific protein A-Sepharose. The antiserum eluted from JSA-Sepharose only cross reacted with β-galactoside-binding protein indicating that the antibody belonged to lgA class of immunoglobulins. No inhibition of hemagglutination activity was observed with lectin-antibody complex indicating that the antigenic and binding sites were situated at different regions of the polypeptide. Beyer et al reported the inhibition of hemagglutination activity of chick liver and embryonic muscle lectin in the presence of antiserum raised against the pure liver lectin (212). The antibody cross reacted immunologically with purified bovine heart β-galactoside-binding lectin indicating the presence of common structural determinants in these lectins. Several other β-galactoside-binding lectins from phylogenetically distinct species cross-react immuno-

features displayed by soluble β-galactoside-binding lectins can possibly be viewed as manifestations of the important physiological function(s) of these proteins.

The antibody cross-reacted with placental β-galactosidase A and B indicating the presence of homologous sequence regions in both enzyme and lectin. The antibody also cross-reacted immunologically with similar lectins from various human fetal and adult organs indicating the close relationship among these groups of lectins.

• • •



SUMMARY

SUMMARY

Two forms of acid β-galactosidase were separated and purified to homogeneity from human placenta using a modified four step procedure involving (i) Con A-Sepharose affinity chromatography, (ii) gel filtration on Sephadex G-200, (iii) ion exchange chromatography on DEAE-Sephadex A-50 and (iv) second Con A-Sepharose affinity chromatography after DEAE-Sephadex A-50 ion exchange chromatography of both forms. This resulted in higher yield and stability of both the forms of β-galactosidase. Purified enzymes showed single band on polyacrylamide gel electrophoresis. In SDS-PAGE A-form was dissociated into two bands with Mr.=68 and 27 kDa, where as B-form showed a single subunit corresponding to Mr.=68 kDa.

Galacto-1-deoxynojirimycin was found to be a competitive inhibitor with an inhibition constant, $Ki=1.4 \times 10^{-5}$M for B-form and 1.25×10^{-5}M for A-form. Amino group substitution at carbon-1 of galactose increased its inhibitory capacity. A and B-forms had 15,14 tryptophan and 48,11 tyrosine residues respectively. The chemical modification studies indicated the presence of lysine, tryptophan and arginine at the catalytic site. Cysteine residues had contributed for the activity. Both the forms lost their activity when the oligosaccharide chains were modified. Both forms of the

mannopyranoside showing the presence of complex biantennary oligosaccharides. Elution of the major portion of the enzyme with 500mM-α-methyl mannopyranoside revealed the presence of high mannose and hybrid-type oligosaccharides. 20% to 30% of the applied β-galactosidase A and B activities were bound to WGA-Sepharose. 20% of B-form and 50% of A-form were bound to RCA-Sepharose. Affinity chromatography on WGA and RCA showed that the bound form predominantly contained hybrid and complex-type oligosaccharides, while the unbound forms were of high mannose-type.

The N-terminal amino acid sequence of β-galactosidase B-was found to be 'Leu-Ser-Ser-Lys-Val-His'-. The two subunits of A-form isolated by SDS-PAGE contained the sequences 'Leu-Ala'- and 'Tyr-Met-' at the N-terminal in the 68 kDa and 27 kDa subunits respectively.

Polyclonal antibody was raised in rabbit against β-galactosidase A, belonged to lgA class. The antigenic and catalytic sites were situated at different regions of the polypeptides. The antibody cross-reacted with B-form as do similar enzymes from other adult and developing human organs.

A soluble β-galactoside-binding protein was isolated from human placenta. Major steps of purification involved specific extraction with lactose and affinity chromatography on asialofetuin-Sepharose. The purified β-galactoside-binding-protein showed a single band on PAGE. It was found to be

human erythrocytes required higher concentration of lectin than that of rabbit cells. No glycohydrolase activity was associated with the lectin. This lectin is non mitogenic to human lymphocytes. Thiodigalactoside was the best inhibitor followed by lactose. Galactose aminated at 1-position was a better inhibitor than galactose. Axial hydroxyl group on C-4 of galactose is a stereospecific requirement for binding as neither glucose nor mannose was an inhibitor. Fucose did not inhibit agglutination indicating the importance of C-6 OH group on galactose for binding.

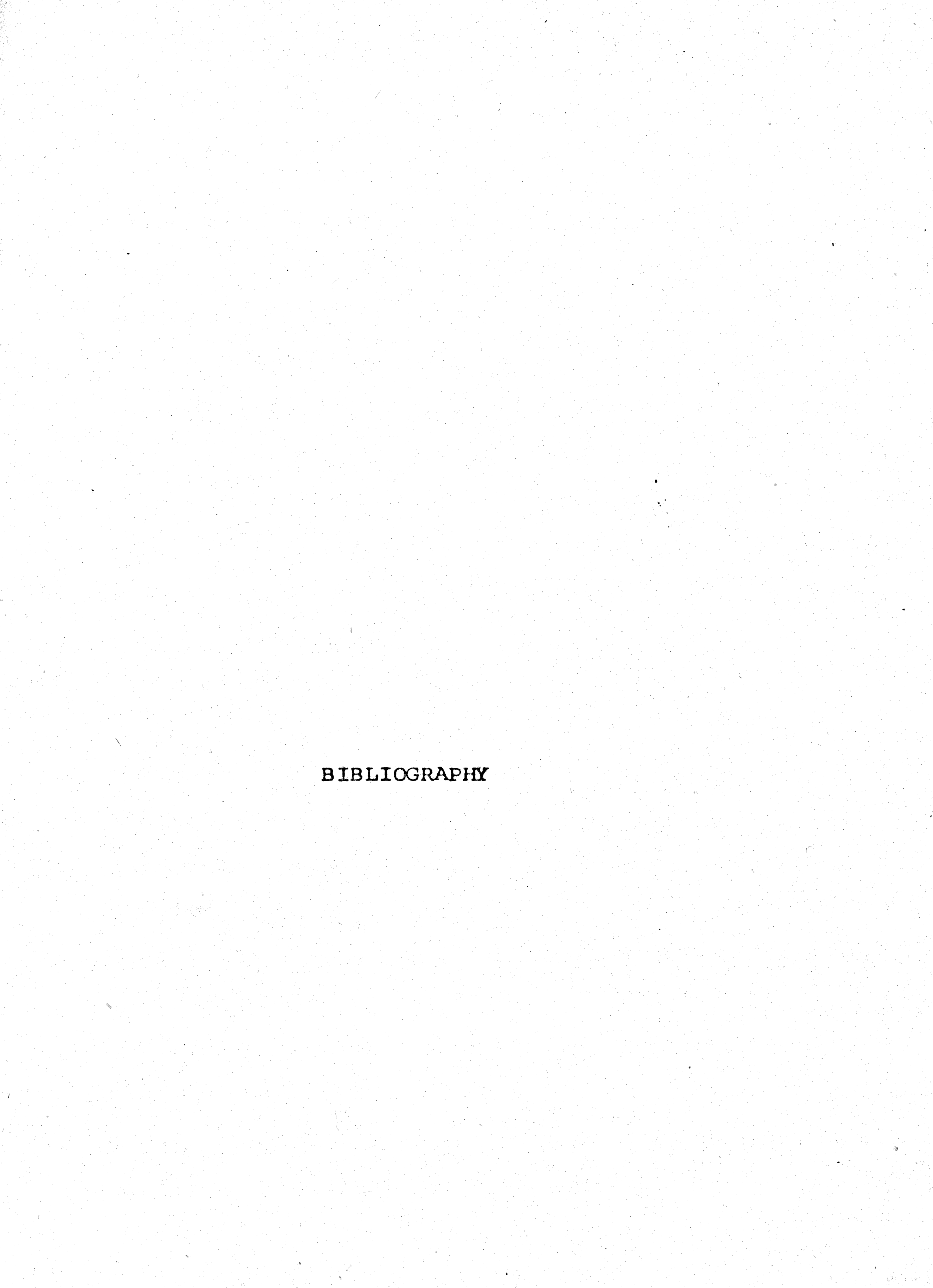
The lectin was found to contain one free -SH group. Amino acid analysis showed high content of alanine and glycine. Tryptophan residue could not be detected. N-terminal amino acid sequence was found to be 'His-Pro-Ala-Pro-Tyr-Phe'-Chemical modification studies indicated the presence of lysine, histidine and arginine at the saccharide binding site. Cysteine residue also contributed for the affinity of saccharides. The carbohydrate content was found to be 16.7% Oxidation of the oligosaccharide chains with periodate and with a mixture of sodium metaperiodate and sodium cyanoborohydride resulted in complete loss of hemagglutination activity. The lectin did not bind to immobilized Con A, WGA, RCA, and WBA, but it did bind to JSA.

The association constant for lactose by equilibrium

of MeUmb-β-D-Gal was progressively quenched by the successive additions of the lectin. The association constant, $Ka=4.3x10^{7}M^{-1}$ for MeUmb-β-D-Gal was obtained by fluorescence quenching studies. However, both the methods showed only two sugar binding sites per 27 kDa lectin.

Polyclonal antibody raised in rabbits against the lectin also belonged to lgA class of immunoglobulins. Hemagglutination activity was exhibited by lectin-antibody complex. The antibody cross-reacted with purified bovine heart β-galactoside-binding lectin. Immunological cross-reaction was also observed against both the forms of placental β-galactosidase. The antibody cross-reacted immunologically with similar lectins from various human adult and fetal organs.

...



BIBLIOGRAPHY

BIBLIOGRAPHY

1. Aronson, N.N. (1972) in 'Glycoproteins' (Gottschalk, A.ed.) Vol.5, pp-1211, Elsevier Publishing Company, Amsterdam.

2. Goldstone, A. and Koenig, H. (1970) Life. Sci. 9, 1341.

3. Sharon, N. and Lis, H. (1982) In 'The Proteins' (Neurath, H. and Hill, R.L. ed.) Vol.5, pp-1, Academic Press, New York.

4. Yosizawa, Z. (1972) In 'Glycoproteins'. (Gottschalk, A.ed.) Vol.5, pp-1000, Elsevier Publishing Company, Amsterdam.

5. Shier, W.T., Lin, Y. and DeVries, A.L. (1972) Biochem. Biophys. Acta. 263, 406.

6. Butler, W.T. and Cunningham, L.W. (1966) J. Biol. Chem. 241, 3882.

7. Gottschalk, A. and Thomas, M.A.W. (1961) Biochem. Biophys. Acta. 46, 91.

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

9. Chu, F.K., Trimble, R.B. and Maley, F. (1978) J. Biol. Chem. 253, 8691.

10. Ashwell, G. and Harford, J. (1982) Ann. Rev. Biochem. 51, 531.

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

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

13. Aubert, J.P., Biserte, G. and Loucheux-Lefebvre, M.H. (1976) Arch. Biochem. Biophys. 175, 410.

14. Bientema, J.J., Gaastra. W., Sheffer, A.J. and Welling, G.W. (1976) Eur. J. Biochem. 63, 441.

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

16. Tulsiani, D.R.P., Opheim, D.J. and Touster, O. (1977) J. Biol. Chem. 252, 1017.

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

18. Roth, J. (1987) Biochem. Biophys. Acta. 906, 405.

19. Schachter, H. (1978) In 'The Glycoconjugates'. (Horowitz, M.I. and Pigman, W. ed.) Vol.2, pp-87, Academic Press, New York.

20. Kivirikko, K.I. and Myllyla, R.C. (1979) Int. Rev. Connect. tissue. 8, 23.

21. Thrujllo, J.L. and Gan, J.C. (1973) Biochem. Biophys. Acta. 304, 32.

22. Salo, W.L. and Fletcher, H.G. Jr. (1970) Biochemistry. 9, 882.

23. Evans, W.H. (1974) Nature. 250, 391.

24. Ko, G.K.W. and Raghupathy, E. (1971) Biochem. Biophys. Acta. 244, 396.

25. Struck, K.D. and Lennarz, W.J. (1981) In 'The Biochemistry of Glycoproteins and Proteoglycans' (Lennarz, W.J. ed.) pp-59, Plenum Press, New York

27. Williams, D.B. and Lennarz, W.J. (1984) J. Biol. Chem. 259, 5109.

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

29. Liscum, L., Cummings, R.D., Anderson, R.G.W., DeMartino, G.N., Goldstein, J.L. and Brown, M.S. (1983) Proc. Natl. Acad. Sci. USA, 80, 7165.

30. Pollack, A and Atkinson, P.H. (1983) J. Cell. Biol. 97, 293.

31. Tkacz, J.S. and Lamben, J.O. (1975) Biochem. Biophys. Res. Commun. 65, 248.

32. Hickman, S., Kulczycki, A. Jr., Lynch, R.G. and Kornfeld, S. (1977) J. Biol. Chem. 252, 955.

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

34. Spencer, J.R., Kang, M.S. and Elbein, A.D. (1978) Arch. Biochem. Biophys. 190, 829.

35. Villemez, C.L. and Carlo, P.L. (1980) J. Biol. Chem. 255, 8174.

36. Muller, T., Bause, E. and Jaenicke, L. (1981) FEBS Lett. 128, 208.

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

38. Schwarz, R.T. and Datema, R. (1982) Carbohydr. Chem. Biochem. 40, 287.

39. Datema, R. and Schwarz, R.T. (1979) Biochem. J. 184, 113.

40. Pan, Y.T. and Elbein, A.D. (1985) Arch. Biochem. Biophys. 242, 447.

41. Elbein, A.D. (1987) Ann. Rev. Biochem. 56, 497.

42. Dorling, P.R., Huxtable, C.R. and Colegate, S.M. (1980) Biochem. J. 191, 6491.

43. Szumilo, T., Kaushal, G.P. and Elbein, A.D. (1986) Arch. Biochem. Biophys. 247, 261.

44. Frommer W., Jung, B., Muller, L., Schmidt, D. and Truscheit, E. (1979) Planta. Med. 35, 195.

45. Legler, G. and Julich, E. (1984) Carbohrdr. Res. 128, 61.

46. Van Diggelen, O.P., Galjaard, H., Sinnot, M.L. and Smith, P.J. (1980) Biochem. J. 188, 337.

47. Hortin, G. and Boime, I. (1980) J. Biol. Chem. 255, 8007.

48. Stern, A.M., Foxman, B.M., Tashjian, A.H. Jr. and Abeles, R.H. (1982) J. Med. Chem. 25, 544.

49. Goldstein, J.L., Brown, M.S., Anderson, R.G.W., Russel, D.W., and Schneider, W.J. (1985) Ann. Rev. Cell. Biol. 1, 1.

50. Mills, J.T. and Adamany, A.M. (1978) J. Biol. Chem. 253, 5270.

51. Cunningham, L.W. (1971) In "Glycoproteins of Blood Cells and Plasma" (Jamieson, G.A. and Greenwalt, T.J. ed.) J.B. Lippincot. Co., pp-16, Philadelphia and Toronto.

52. Huang, C.C., Mayer, H.E. Jr. and Montgomery, R. (1970)

53. Sharon, N. (1975) In "Complex Carbohydrates". pp-99. Addison-Wesley Publishing Company, Massachusetts.

54. Montgomery, R. (1972) In "Glycoproteins" (Gottschalk, A.ed.) Vol.5, pp-518, Elsevier Publishing Company, Amsterdam.

55. de Duve, C., Pressman, R.C., Gianetto, R., Wattiaux, R. and Applemans, F. (1955) Biochem. J. 60, 604.

56. de Duve, C. (1983) Eur. J. Biochem. 137, 391.

57. Nicholas, B.A., Bainton, D.F. and Farquhar, M.G. (1971) J. Cell. Biol. 50, 498.

58. Novikoff, P.M., Novikoff, A.B., Quintana, N. and Hauw, J.J. (1971) J. Cell. Biol. 50, 859.

59. de Duve, C. and Wattiaux, R. (1966) Ann. Rev. Physiol. 28, 435.

60. Smith, R.E., and Farquhar, M.G. (1966) J. Cell. Biol. 31, 319.

61. Dingle, J.T. (1969) in "Lysosomes in Biology and Pathology" (Dingle, J.T. and Fell, H.B. ed.) Vol.2, pp-421, North-Holland Publishers, Amsterdam.

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

63. Ohkuma, S. and Poole, B. (1978) Proc. Natl. Acad. Sci. USA, 75, 3327.

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

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

66. Mellman, I., Fuchs, R. and Helenius, A. (1988) Ann.

67. Helenius, A., Mellman, I., Wall, D. and Hubbard, A. (1983) Trends. Biochem. Sci. 8, 245.

68. Von Figura, K. and Hasilik, A. (1986) Ann. Rev. Biochem. 55, 663.

69. Iodice, A.A. (1967) Arch. Biochem. Biophys. 121, 241.

70. Keilova, H. and Keil, B. (1969) FEBS Lett. 4, 295.

71. McDonald, J.K., Zeitman, B.D., Reilly, T.J. and Ellis, S. (1969) J. Biol. Chem. 244, 2693.

72. Barret, A.J. (1969) In "Lysosomes in Biology and Pathology". (Dingle, J.T., and Bell, H.E. ed.) Vol.2, pp-245, North-Holland Publishers, Amsterdam.

73. Coffey, J.W. and deDuve, C. (1968) J. Biol. Chem. 243, 3255.

74. Mahadevan, S. and Tappel, A. (1968) J. Biol. Chem. 243, 2849.

75. Franson, R., Waite, M., and Lavia, M. (1971) Biochemistry 10, 1942.

76. Barenholz, Y., Roitman, A. and Gatt, S. (1966) J. Biol. Chem. 241, 3731.

77. Fowler, S. (1969) Biochem. Biophys. Acta. 191, 481.

78. Gatt, S. (1966) J. Biol. Chem. 241, 3724.

79. Bown, D.M. and Radin, S. (1968) Biochem. Biophys. Acta. 152, 599.

80. Ohman, R., Rosenberg, A. and Svennerholm, L. (1970) Biochemistry. 9, 3774.

81. Gatt, S. (1967) Biochem. Biophys. Acta. 137, 192.

83. Lejeune, N., Thines-Sempoux, D. and Hers, H.J. (1963) Biochem. J. 86, 16.

84. Hers, H.J. (1963) Biochem. J. 86, 11.

85. Bollet, A.J., Bonner, W.M. and Nance, J.L. (1963) J. Biol. Chem. 238, 3522.

86. Hutterer, F. (1966) Biochem. Biophys. Acta. 115, 312.

87. Aronson, N.N. and Davidson, E.A. (1967) J. Biol. Chem. 242, 441.

88. Sellinger, O.Z., Bufay, H., Jacques, P., Doyen, A. and de Duve, C. (1960) Biochem. J. 74, 450.

89. Grelling, H. Stahlsatz, H.W. and Kisters, R. (1970) In "Chemistry and Molecular Biology of Intercellular Matrix". (Balazs, E.A. ed.) Vol.2 pp-873, Academic Press, New York.

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

91. Gottschalk, A. (1966) In "Glycoproteins".(Gottschalk, A. ed.) pp-20, Elsevier Publishing Company, Amsterdam.

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

93. Li, S.C. Nakamura, T. Ogama, A. and Li, Y.T. (1979) J. Biol. Chem. 254, 10592.

94. Van Hoof, F. (1973) In "Lysosomes and Storage Diseases" (Hers, H.G. and Van Hoof, F. ed.) pp-305, Academic Press, New York.

95. Cheetham, P.S.J. and Dance, N.E. (1976) Biochem. J.

96. Rittmann, L.S. and O'Brien, J.S. (1981) in "Isozymes". (Rattazzi, M.C., Scandalios, J.G. and Whitt, G.S. ed.) Vol.5, pp-77, Alan R. Liss, Inc, New York.

97. Yamamoto, Y. Fujie, M.I., and Nishimura, K. (1982) J. Biochem. 92, 13.

98. Lo, J.T., Mukerji, K., Awasthi, Y.C., Hanada, E., Suzuki, K. and Srivastava, S.K. (1979) J. Biol. Chem. 254, 6170.

99. Weissmann, B., Hadjiioannou, S., and Tornheim, J. (1964) J. Biol. Chem. 239, 59.

100. Robinson, D. and Stirling, J. (1968) Biochem. J. 107, 321.

101. Snaith, S.M. and Levy, G.A. (1973) Adv. Carbohyd. Chem. Biochem. 28, 401.

102. Sukeno, T., Tarento, A., Plummer, T. Jr. and Maley, F. (1972) Biochemistry. 11, 1493.

103. Van Hoof, F. (1973) in "Lysosomes and Storage Diseases". (Hers, H.G. and Van Hoof, F. ed.) pp-277. Academic Press, New York.

104. Conchie, J and Hay, A.J. (1963) Biochem. J. 87, 354.

105. Mahadevan, S. and Tappel, A. (1968) Arch. Biochem. Biophys. 128, 129.

106. Ogushi, T. and Yamashina, I, (1979) Biochem. Biophys. Acta. 156, 417.

107. Tarentino, A.L. and Maley, F. (1969) Arch. Biochem. Biophys. 130, 295.

108. Lectins in Biology, Biochemistry and clinical Biochemistry (1981-85) (Bøg Hansen, T.C. ed.) Vol. 1-4, Walter de Gruyter, Berlin.

109. Barondes, S.H. (1984) Science. 223, 1259.

110. Lis, H. and Sharon, N. (1986) Ann. Rev. Biochem. 55, 35.

111. Monsigny, M., Kieda, C. and Roche, A.C. (1983) Biol. Cell. 47, 95.

112. Kawasaki, T., Etoh, R. and Yamashina, I. (1978) Biochem. Biophys. Res. Commun. 81, 1018.

113. Kawasaki, T. and Ashwell, G. (1977) J. Biol. Chem. 252, 6536.

114. Stojanovic, D. and Huges, R.C. (1984) Biol. Cell. 51, 197.

115. Raz, A. and Lotan, R. (1981) Cancer, Res. 41, 3642.

116. Levi, G. and Teichberg, V.I. (1981) J. Biol. Chem. 256, 5735.

117. Fitzgerald, J.E., Catt, J.W. and Harrison, F.L. (1984) Eur. J. Biochem. 140, 137.

118. Levi, G. and Teichberg, V.I. (1982) FEBS Lett. 148, 145.

119. Paroutaud, P., Levi, G., Teichberg, V.I. and Strosberg A.D. (1987) Proc. Natl. Acad. Sci. USA. 84, 6345.

120. Beyer, E.C., Zweig, S.E. and Barondes, S.H. (1980) J. Biol. Chem. 255, 4236.

121. Harrison, F.L. and Chesterton, C.J. (1980) Nature. 286, 502.

122. Springer, G.F. and Desai, P.R. (1971) Biochemistry. 10, 3749.

123. Roberson, M.M. and Barondes, S.H. (1982) J. Biol. Chem. 257, 7520.

124. Gartner, T.K. and Podleski, T.R. (1975) Biochem. Biophy. Res. Commun. 67, 972.

125. Lotan, R., Lotan, D. and Raz, A. (1985) Cancer Res. 45, 4349.

126. Gabius, H.J. Engelhardt, R., Cramer, F., Batage, R. and Nagel, G.A. (1985) Cancer Res. 45, 253.

127. Barondes, S.H. and Haywood-Reid P.L. (1981) J. Cell. Biol. 91, 568.

128. Cerra, R.F., Haywood-Reid, P.L. and Barondes, S.H. (1984) J. Cell. Biol. 98, 1580.

129. Den, H. and Chin, J.H. (1981) J. Biol. Chem. 256, 8069.

130. Oda, Y. and Kasai, K. (1984) Biochem. Biophys. Res. Commun. 123, 1215.

131. Muramatzu, H., Ishihara, H., Miyauchi, T., Gachelin, G. Fujisaki, T., Tejma, S. and Muramatzu, T. (1983) J. Biochem. 94, 71.

132. Kajikawa, T., Nakajima, Y., Hirabayashi, J., Kasai, K. and Yamazaki, M. (1986) Life Sci. 39, 1177.

133. Wallenfels, K. and Weil, R. (1972) 'The Enzymes' (Boyer, P.D. ed.) Vol.7, pp-617, Academic Press,

134. Norden, A.G.W., Tennant, L. and O'Brien, J.S. (1974) J. Biol. Chem. 249, 7969.

135. Forst, R.G., Holmes, E.W., Norden, A.G.W. and O'Brein, J.S. (1978) Biochem. J. 175, 181.

136. Alam, T. and Balasubramanian, A.S. (1978) J. Neurochem 30, 1199.

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

138. Alpers, D.H. (1969) J. Biol. Chem. 244, 1238.

139. Okada, S. and O'Brien, J.S. (1968) Science. 160, 1002.

140. Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randal, R.J. (1951) J. Biochem. 193, 265.

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

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

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

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

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

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

147. Sureshkumar, G., Appukuttan, P.S. and Basu, D. (1982) J. Biosci. 4, 257.

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

149. de Waard, A., Hickman, S. and Kornfeld, S. (1976) J. Biol. Chem. 251, 7581.

150. Cuatrecasus, P. (1970) J. Biol. Chem. 245, 3059.

151. Hjelm. H., Hjelm, K. and Sjoquist, J. (1972) FEBS Lett. 28, 73.

152. Sarasija, P.N. and Basu, D. (1984) Indian. J. Biochem. Biophys. 21, 39.

153. Hirabayashi, J. and Kasai, K.I. (1984) Biochem. Biophys. Res. Commun. 122, 938.

154. Davis, B.D. (1964) Ann. N.Y. Acad. Sci. 121, 404.

155. Andrews, P (1965) Biochem. J. 96, 595.

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

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

158. Fields, R. (1972) Methods Enzymol. 25, 464.

159. Atassi, M.Z. and Hebeeb, A.F.S.A. (1972) Methods Enzymol. 25, 456.

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

161. Habeeb, A.F.S.A. (1972) Methods Enzymol. 25, 457.

162. Riordan, J.F. and Vallee, B.L. (1972) Methods Enzymol. 25, 449, 500.

163. Ellman, G.L. (1959) Arch, Biochem. Bhophys. 82, 70.

164. Miler, E.W. (1977) Methods Enzymol. 47, 413.

165. Takashashi, K. (1968) J. Biol. Chem. 243, 6171.

166. Smith, E.L. (1977) Methods Enzymol. 47, 156.

167. Horton, H.R. and Koshland, D.E. Jr. (1972) Methods Enzymol. 25, 442.

168. Spande, T.F. and Witkop, B. (1976) Methods Enzymol. 11, 498.

169. Savige, W.E. and Fontana, A. (1977) Methods Enzymol. 47, 442.

170. Spiro, R.G. (1966) Methods Enzymol. 8, 26.

171. Thorpe, P.E., Detre, S.I., Foxwell, B.M.J., Brown, A.N.I Skilleter, D.N., Wilson, G., Forrester, J.A. and Stripe, F. (1985) Eur. J. Biochem. 147, 197.

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

173. Gray, W.R. (1972) Methods Enzymol. 25, 143.

174. Ockerman, P.A. and Hultberg, B. (1968) Scand. J. Clin. Lab. Invest. 22, 119.

175. Van Hoof, F. and Hers, G. (1968) Eur. J. Biochem. 7, 34.

176. Ho, M.W. and O'Brein, J.S. (1969) Science 165, 611.

177. Jungalwala, F.B. and Robins, E. (1968) Anal. Biochem. 55, 301.

178. Norden, A.G.W. and O'Brien, J.S. (1973) Arch. Biochem. Biophys. 159, 383.

179. Tomino, S. and Meisler, M. (1975) J. Biol. Chem.

180. Holmes, E.W. and O'Brien J.S. (1979) Biochemistry. 18, 152.

181. Suzuki, Y. and Suzuki, K. (1984) J. Biol. Chem. 249, 2098.

182. Meisler, M. (1972) Methods Enzymol. 28, 820.

183. Heyworth, C.M., Neumann, E.F. and Wynn, C.H. (1981) Biochem. J. 193, 773.

184. Mutoh, T., Naoi, M., Nagatsu, T., Takahashi, A., Matsuoka, Y., Hashizume, Y. and Fujie, N. (1988) Biochem. Biophys. Acta. 964, 244.

185. Kuo, C.H. and Wells, W.W. (1978) J. Biol. Chem. 253, 3550.

186. Roth, R.A. and Rotman, B. (1975) J. Biol. Chem. 250, 7759.

187. Lundblad, R.L. and Noyes, C.M. (1984) In "Chemical Reagents for Protein Modification". Vol.1, pp-1, CRC Press, Bocar Raton, Florida.

188. Debray, H., Decout, D., Strecker, G., Spik, G. and Montreuil (1981) Eur. J. Biochem. 117, 47.

189. Cummings, R.D. and Kornfeld, S. (1982) J. Biol. Chem. 257, 11230, 11235.

190. Baenziger, J.U. and Fiete, D. (1979) J. Biol. Chem. 256, 4894.

191. Krusius, T., Finne, J. and Ravuvala, H. (1976) FEBS Lett. 71, 117.

192. Gabel, C.A. and Kornfeld, S. (1982) J. Biol. Chem.

193. Harpaz, N. and Schachter, H. (1980) J. Biol. Chem. 256. 4894.

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

195. Burger, M.M. and Goldberg, A.R. (1967) Proc. Natl. Acad. Sci, USA. 57, 359.

196. Goldstein, I.J. Hammerston, S. and Sundblad, G. (1979) Biochem. Biophys. Acta. 405, 53.

197. Yamamato, K., Tsuji. T., Matsumoto, I. and Osawa, T. (1981). Biochemistry. 20, 5894.

198. Bhavananden, V.P., Umemoto, J. and Flashner, M. (1979) Biochemistry. 18, 5505.

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

200. Kornfeld, K., Reitman, M.L. and Kornfeld, R. (1981) J. Biol. Chem. 256, 6633.

201. Narasimhan, S., Freed, J.C. and Schachter, H. (1981) Biochemistry. 24, 1694.

202. Edman, P. (1956) Acta. Chem. Scand. 10, 761.

203. Gray, W.R. (1967) Methods Enzymol. 11, 139.

204. Roque-Barriera, M.C. and Campos-Neto, A. (1985) J. immunol. 13, 1740.

205. Dixon, M. (1953) Biochem. J. 55, 170.

206. Heyworth, C.M. and Wynn, C.H. (1982) Biochem. J. 201, 615.

207. Fiddler, M.B., Ben-Yoseph, Y. and Nadler, H.L. (1979)

208. Ashwell, G. (1977) Mammalian Cell Membranes. 4, 57.

209. Carding, S.R., Childs, R.A., Thorpe, R., Spitz, M. and Feizi, T. (1985) Biochem. J. 228, 147.

210. Kobiler, D., Beyer, E.C. and Barondes, S.H. (1978) 64, 265.

211. Childs, R.A. and Feizi, T. (1979) Biochem. J. 183, 755.

212. Beyer, E.C. Tokuyasu, K.T. and Barondes, S.H. (1979). J. Cell. Biol. 82, 565.

213. Levi, G. and Teichberg, V. I. (1982) FEBS Lett. 82, 565.

214 Oda, Y. and Kasai, K. (1983) Biochem. Biophys. Acta. 761, 237.

215. Beyer, E.C. and Barondes, S.H. (1982) J. Cell. Biol. 92, 23, 28.

216. Den, H. and Malinzak, D.A. (1977) J. Biol. Chem. 252, 5444.

217. Nowak, T.P., Haywood, P.L. and Barondes, S.H. (1976) Biochem. Biophys. Res. Commun. 67, 650.

218. Gartner, T.M. and Podleski, T.R. (1975) Biochem. Biophys. Res. Commun. 69, 621.

219. Eisenbarth, G.S., Ruffolo, R.R., Walsh, F.S. and Nirenberg, M. (1978) Biochem. Biophys. Res. Commun. 83, 1246.

220. Cerra, R.F., Gitt, M.A. and Barondes, S.H. (1985) J. Biol. Chem. 260, 10474.

222. Caron, M., Joubert, R. and Bladier, D. (1987) Biochem. Biophys. Acta. 925, 290.

223. Whitney, P.L., Powell, J.T. and Sanford, G.L. (1986) Biochem. J. 238, 683.

224. Hirabayashi, J., Kawasaki, H. Suzuki, K. and Kasai, K. (1987) J. Biochem. 101, 987.

225. Hirabayashi, J., Kawasaki, H., Suzuki, K. and Kasai, K. (1987) J. Biochem. 101, 775.

226. Ohyama, Y., Hirabayashi, J., Oda, Y., Ohno, S., Kawasaki, H., Suzuki, K. and Kasai, K. (1986) Biochem. Biophys. Res. Commun. 134, 51.

227. Drickamer, K. (1981) J. Biol. Chem. 256, 5827.

228. Drickamer, K., Menon, J.F., Binns, G. and Leung, J.O. (1984) J. Biol. Chem. 259, 770.

229. Spies, M., Schwartz, A.L. and Lodish, H.F. (1985) J. Biol. Chem. 260, 1979.

230. Morell, A.G. and Ashwell, G. (1972) Methods Enzymol. 28, 205.

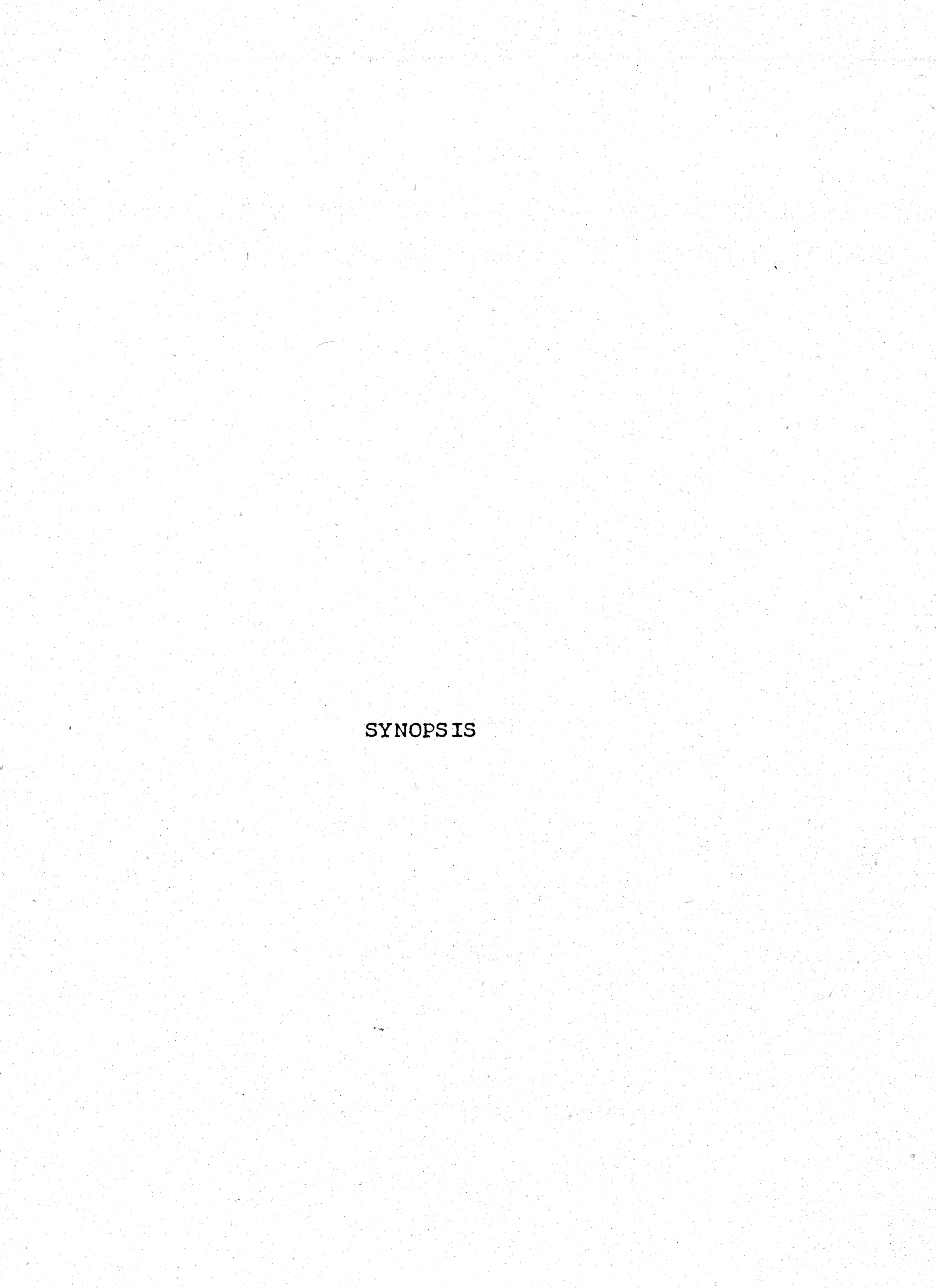
231. Scatchard (1949) Ann. N.Y. Acad. Sci. 51, 660.

232. Spackman, D.H. (1967) Methods Enzymol. 11, 1.

233. Osawa, T. and Toyoshima, S. (1972) Methods Enzymol. 28, 328.

234. Levi, G., Tarrab-Hazadai, R., and Teichberg, V.I. (1983) Eur. J. Immunol. 13, 500.

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



SYNOPSIS

STRUCTURE AND PROPERTIES OF β-GALACTOSIDASE AND β-GALACTOSIDE-BINDING PROTEIN OF HUMAN PLACENTA

SYNOPSIS

by

P. MADHUSOODANA NAMBIAR

for Ph.D Degree

of

BIOCHEMISTRY

SREE CHITRA TIRUNAL INSTITUTE
FOR
MEDICAL SCIENCES AND TECHNOLOGY
TRIVANDRUM

INTRODUCTION :

The process of degradation of cellular constituents is one of the main features in maintenance of the 'steady state'. Enzymes capable of cleaving various linkages found in glycoproteins are primarily localized in lysosomes (1). They exhibi optimum activity in acidic medium. Lysosomal storage diseases are inborn errors of metabolism due to deficiencies of one or more of these hydrolytic enzymes (2). Lysosomal acid hydrolases are glycoproteins. The glycoproteins contain carbohydrate residues covalently linked by either N- or O-glycosidic bond to the polypeptide chains. The former type involve N-acetylglucosamine linked to asparagine. They contain a commo pentasaccharide core to which additional saccharides are attached to form high mannose, hybrid and complex-type units. Oligosaccharide chains in O-glycosidic linkage are attached through N-accetylgalactosamine to the hydroxyl group of serine or threonine. Other less common type of O-glycosidic linkages are also distributed in many polymers (3). N-glycosidic linked oligosaccharides are synthesized by the sequential addition of sugars to a carrier lipid, dolichol phosphate. Lipid-linked oligosaccharide intermediate is transferred en bloc to a nascen polypeptide chain attached to ribosomes. Processing of the oligosaccharides occurs by trimming reactions in concert with the addition of peripheral sugars. The synthesis of O-glycosidi chains appears to occur almost entirely by a sequence of classi glycosyltransfer reactions namely via sugar nucleotide pathway.

microheterogeneity. The degradation of glycoproteins may be initiated by the action of either proteolytic enzymes or glycosidases in the lysosomes. Mammalian β-galactosidase (EC.3.2.1.23) catalyze the hydrolysis of terminal β-galactoside linkages of glycoconjugates. The acid β-galactosidase is believed to be of lysosomal origin. Acid β-galactosidase hydrolyzed GM_1 ganglioside in the presence of an activator protein (4). The wide spread occurence and broad substrate specificity of acid β-galactosidase is probably related to the multiple physiological functions of the enzyme.

Lectins are divalent or multivalent carbohydrate binding proteins of non-immune origin, grouped together because they all agglutinate erythrocytes. Specificity requirement of individual lectin reveals a wide range of variations with respect to configurations and substitution patterns at different carbon atoms of the monosaccharides. Vertebrate lectins are subdivided into two classes (a) integral membrane lectins, which require detergents for solubilization and Ca^{2+} for the activity and (b) soluble lectins (5). The soluble lectins are extractable with lactose and are specifically inhibited by β-galactoside containing saccharides. Despite their diverse origin they consist of dimer or oligomers of Mr.=13-16 kDa subunits with similar saccharide specificity and extensive sequence homologies. They cross react immunologically and require a reducing agent to maintain their activity. The biological function of these soluble β-galactoside-binding lectins has not been established.

AIMS AND OBJECTIVES

Mammalian β-galactosidase (EC 3.2.1.23) existed in multiple forms and kinetic studies with synthetic and natural substrates have been reported (7,8). Occurrence of an inborn error of metabolism, GM_1-gangliosidosis and its clinical variants due to the deficiency of the acid β-galactosidase had increased the interest in this field (9). Detailed structural studies of this protein would help in understanding its structure-function relationship. Evaluation of the oligosacch ride structure will add information towards the role of glycosylation in glycoproteins. The structural comparison of the binding sites of the enzyme and lectin acting on β-galacto sides deserves attention. Much effort has been invested in search for applications of lectins. As a result, lectins have become invaluable tool in biological and medical research in diverse fields. They continue to be the focus of intense interest in their own right. Recently, it was suggested that the galactoside-binding lectins are involved in the immune system (10). The objective of the present study is to undertake structural studies on acid β-galactosidase and β-galactos binding protein.of human placenta, an useful hospital waste material.

RESULTS AND DISCUSSION

Two forms of acid β-galactosidase were separated and purified to homogeneity from human placenta using a four step

(2) gel filtration on Sephadex G-200, (3) ion exchange chromatography on DEAE-Sephadex A-50 and (4) affinity chromatography on mercurial – Sepharose CL-4B(11). This method of purification was modified considering the low recovery and stability. A second ConA-Sepharose affinity chromatography after DEAE-Sephadex A-50 ion exchange chromatography of both forms resulted in purified enzyme with higher yield. The purified enzyme was homogeneous as revealed by PAGE at pH 8.3. In SDS-PAGE A-form was dissociated in to two bands with Mr.=68 kDa and 27 kDa where as B-form showed a single subunit corresponding to Mr.=68 kDa.

Galacto-1-deoxynojirimycin was found to be a competitive inhibitor with an inhibition constant (Ki) of $1.4x10^{-5}$M and $1.25x10^{-5}$M for B and A-forms respectively. This is of significance because a wide range of substrate analogues were weak inhibitors of β-galactosidase. Amino group substitution at carbon-1 of galactose increased its inhibitory capacity. The chemical modification of amino acids were utilized for those involved at the catalytic site (12-15). It has been observed that tryptophan and arginine were involved. Cysteine residues were necessary for the activity. Both the forms lost their activity when the oligosaccharide chains were modified. Beta-galactosidase A and B are glycoproteins containing 7.5% and 6% carbohydrates respectively. Immobilized lectins permit rapid, sensitive and specific separation of closely related variants of glycoproteins differing in their glycosylation

of β-galactosidase A and B. Enzyme bound to ConA-Sepharose could be eluted with 10mM α-methyl mannopyranoside revealed the presence of complex biantennary oligosaccharides. Elution of the major portion of the enzyme protein with 500mM α-methyl mannopyranoside revealed the presence of high mannose & hybrid-type oligosaccharides. 20 to 30% of the applied β-galactosidase A and B activities were bound to WGA-Sepharose. The bound form predominantly contained hybrid and complex-type oligosacchares where as the unbound form was of high mannose-type. 20% B and 50% A-forms were bound to RCA-Sepharose, indicated the presence of hybrid and complex-type oligosaccharides in the bound form.

N-terminal amino acid sequence was analyzed by dansyl-Edman degradation (18). The N-terminal amino acid sequence of B-form was 'Leu-Ser-Ser-Lys-Val-His-'. N-terminal amino acid sequence analysis of A-form showed two peaks. The subunits isolated by SDS-PAGE indicated the sequence 'Leu-Ala-' at the N-terminal of 68 kDa subunit and that of 27 kDa subunit was 'Tyr-Met'.

Polyclonal antibody was raised in rabbit for β-galactosidase A. The antiserum was retained on IgA specific JSA-Sepharose indicating that the antibody belonged to IgA class (19). The antigenic and catalytic sites were situated at different sites of the polypeptide chains. The antibody cross reacted with B-form as well as the similar enzymes from other

placenta according to Hirabayashi and Kasai (20) with modifications. Major steps of purification involved specific extraction with lactose and affinity chromatography on asialofetuin-Sepharose. The purified β-galactoside-binding protein showed a single band on PAGE at pH 8.3. It was found to be a dimer of 13.5 kDa subunits. The agglutination of trypsinized human erythrocytes required higher concentration of lectin when compared to that of rabbit cells. No glyco-hydrolase activity was associated with the lectin. This lectin is non-mitogenic to human lymphocytes. Hemagglutination inhibition by a wide range of saccharides offered some insight into the saccharide specificity of the lectin. Thiodigalactoside was the best inhibitor followed by lactose. Galactopyranosylamine was a better inhibitor than galactosamine and galactose. This indicated the importance of C-1 amino group substitution for binding. Axial hydroxyl group on C-4 is a stereospecific requirement for binding as glucose or mannose did not inhibit. Fucose did not inhibit agglutination indicating the requirement of C-6 hydroxyl group in binding.

The lectin was found to contain one free SH-group. Amino acid analysis showed high content of alanine and glycine. Tryptophan residue could not be detected. The carbohydrate content was found to be 16.7%. The chemical modification of various amino acids indicated that arginine, lysine and histidine were involved at the saccharide binding sites. Cysteine residue also contributed towards the affinity

did not bind to ConA, WGA and RCA, but it did bind to JSA. The result does not necessarily suggest the presence of terminal α-linked galactose residues in placental lectin. Association constant, $Ka=9.4x10^{3}M^{-1}$ of lactose was obtained by equilibrium dialysis. The association constant calculated by fluorescence quenching studies with MeUmb-B-gal was $Ka=4.3x10^{7}M^{-1}$. However, both the methods showed only two sugar binding sites per 27 kDa lectin. The N-terminal amino acid sequence was found to be 'His-Pro-Ala-Pro-Tyr-Phe-'.

Polyclonal antibody to the galactose-binding lectin raised in rabbits belonged to IgA class. Hemagglutination activity was exhibited by lectin-antibody complex. The antibody cross reacted with bovine heart galactose-binding lectin. Immunological cross reaction was also observed against both the forms of placental β-galactosidase. The antibody cross reacted immunologically with similar lectin from various human adult and fetal organs.

REFERENCES

1. Aronson, N.N. (1972) in "Glycoproteins" (Gottschalk, A.ed.) Vol.5, pp-1211, Elsevier Publishing Company, Amsterdam.
2. Hers, H.G. (1973) in "Lysosomes and Storage Diseases" (Hers, H.G. and Van Hoof, F., ed) pp.148, Academic Press, New York.
3. Sharon, N. and Lis, H. (1982) The Proteins (Neurath, H. and Hill, R.L. ed.) vol.5, pp.1, Academic Press, New York.
4. Li, S.C., Nakamura, T., Ogama, A., and Li, Y.T. (1979) J. Biol. Chem., 254, 10592.
5. Barondes, S.H. (1984) Science., 223, 1259.
6. Lis, H. and Sharon, N. (1986) Ann. Rev. Biochem., 55, 35.

7. Norden, A.G.W., Tennant, L. and O'Brien, J.S. (1974) J. Biol. Chem., 249, 7969.

8. Alam, T.Y. and Balasubramanian, A.S. (1978) J. Neurochem. 30, 119.

9. Okada, S. and O'Brien, J.S. (1968) Science., 160, 1002.

10. Kajikawa, T., Nakajima, Y., Hirabayashi, J., Kasai, K and Yamazaki, M, (1986) Life Sci., 39, 1177.

11. Sarasija, P.N. and Basu, D. (1984) Indian. J. Biochem, Biophys., 21, 39.

12. Riordan, J.F. and Vallee, B.L. (1972) Methods Enzymol., 25, 449, 500.

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

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

15. Miles, E.W. (1977) Methods Enzymol., 47, 431.

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

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

18. Gray, W.R. (1972) methods Enzymol., 25, 333.

19. Roque-Barreira, M.C. and Campos-Neto, A. (1985) J. Immunol., 13, 1740.

20. Hirabayashi, T. and Kasai, K.I. (1984) Biochem. Biophys. Res. Commun., 122, 938.