Studies on some biologically important proteins from the invertebrate *Unio*

Thesis submitted for the Degree of

DOCTOR OF PHILOSOPHY

By

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CERTIFICATE

This is to certify that this thesis entitled **"Studies on some biologically important proteins from the invertebrate *unio"*** submitted to the **University of Hyderabad by Ms. Radha Yalamarthy** for the degree of **Doctor of Philosophy,** is based on the studies carried out by her under my supervision. 1 declare to the best of my knowledge that this work has not been submitted earlier for the award of degree or diploma from any other University or Institution.

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DECLARATION

I, Radha Yalamarthy, hereby declare that the work presented in my thesis is entirely original and was carried out by me in the Department of Biochemistry, University of Hyderabad, under the supervision of Dr. N. Siva **Kumar.** I further declare that this work has not been submitted earlier for the award of degree or diploma from any other University or Institution.

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CONTENTS

ABBREVIATIONS

$Ba(OH)_2$	Barium hydroxide
BC1P	4-bromo 5-chloro-3-indolyI phosphate
BSA	Bovine serum albumin
Con A	Concanavalin A
DABITC	4-N,N-dimethylaminoazobenzene-4'-isothiocyanate
DEPC	Diethyl pyrocarbonate
EDTA	Ethylenediaminetetraacetic acid
FPLC	Fast protein liquid chromatography
H2SO4	Sulphuric acid
HEPES	N-[2-Hydroxyethyl]Piperazine-N'[2-ethanesulfonic acid]
kDa	Kilo Dalton
MPR	Mannose 6-phosphate receptor
nM	Nano meter
Na_2CO_3	Sodium carbonate
$NaHCO_3$	Sodium bicarbonate
$(NH_4)_2SO_4$	Ammonium Sulfate
NH_4HCO_3	Ammonium bicarbonate
NBT	Nitrobluetetrazolium
PAGE	Polyacrylamide gel electrophoresis
PITC	Phenylisothiocyanate
PM	Phosphomannan
PMP	Pentamannosyl phosphate
Rf	Relative front
rpm	Rotations per minute
SDS	Sodium dodecyl Sulfate
TCA	Trichloroacetic acid
TEMED	N,N,N',N'-Tetramethylethylenediamine
TFA	Trifluoro acetic acid
TLC	Thin layer chromatography
TNBS	Trinitro benzene sulfonic acid

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CHAPTER I

INTRODUCTION

INTRODUCTION

The finding of hemagglutinating properties of a protein from seed extracts of castor beans by Stillmark in 1888 is usually considered as the beginning of the actual lectin discovery. Initially they were referred to as hemagglutinins but later Boyd and Shapleigh introduced the term lectin [Boyd and Shapleigh, 1954]. Initially the biological activities of the unpurified lectins were studied in extracts of plant and animal tissues or body fluids. Erythroagglutination is the most frequently followed activity. Later with the use of affinity chromatography it was possible to purify the lectins. Both extensive and intensive research of these proteins has been carried out on a molecular level.

Lectins are 'nonimmune' in origin. They are different from antibodies since they are structurally different from the proteins with which they interact. Agglutinating property of the lectins might be because of the multimeric nature of the lectins. Lectins were defined as carbohydrate binding proteins of nonimmune origin that agglutinate cells or precipitate polysaccharides or glycoconjugates [Goldstein *et al.*, 1980]. Lectins are a group of proteins/glycoproteins different from immunoglobulins having binding sites for carbohydrates and do not induce any chemical changes on them. Lectins interact with glycoproteins and glycolipids by binding to specific carbohydrate residues and having more than one sugar-binding site. The carbohydrate for which they have the highest affinity defines their specificity. Con A is a non-glycoprotein while soybean lectin was found to be a glycoprotein [Lis *el al.*, 1964].

Many lectins agglutinate animal as well as human erythrocytes and some of them exhibit blood group specificity. In 1960, Nowell observed the mitogenic properties of a lectin

from *Phaseolus vulgaris* [Nowell, 1960]. He observed that the lectin triggered the proliferation of quiescent, nondividing lymphocytes *in vitro.* Lectins with tumor inhibitory property were found in lipase containing extracts from wheat germ [Ambrose *et al.,* 1961]. This lectin agglutinated the transformed cell lines and did not affect the normal cells.

Several lectins with enzymatic properties have been identified. Ricin, the well-studied lectin, is actually the enzyme RNA-N-glycosidase. Another lectin, charcot-leyden crystal protein (galectin-10) is a lysophospholipase and I-type lectins such as sialoadhesin are members of the immunoglobulin super family.

Lectins are ubiquitous in distribution and are present in plants, animals, fungi, bacteria, viruses etc.

Viral lectins

Adhesion of Chicken plague vims to erythrocytes was first observed by Landsteiner and Russ individually in 1906. Viral hemagglutination was inhibited by the removal of N-acetylneuraminic acid of the cell surface of the chicken erythrocytes. This observation indicated the importance of this carbohydrate in the viral infection. Influenza virus is most intensively studied [Rini, 1995]. Viral lectins contain multiple domains but only one domain has carbohydrate-binding pocket [Lis and Sharon, 1998]. Studies on the interactions of the viral lectins with antiviral antibodies and specific carbohydrate or glycoproteins have contributed significantly to the developments in the fields of immunology and biochemistry.

Bacterial lectins

Kraus and Ludwig were first to demonstrate bacterial agglutinins in *Staphylococcus aureus* [Kraus and Ludwig, 1902]. A mannose binding lectin has been characterized from the cell surface of *Escherichia coli*. Another bacterial lectin that is specific to L-fucose has been identified from the surface of *Vibrio cholerae* [reviewed in Sharon, 1986]. Binding of the bacteria to the host cell surface sugars initiates the bacterial infection [Van Driessche and Beeckmans, 1993; Van Driessche *et al.*, 1993]. Bacterial lectins resemble plant toxins such as ricin and both of them have A and B chains but the carbohydrate recognition domain is restricted to the B chain alone [Rini, 1995].

Fungal lectins

Kobert reported the presence of a hemolytic agent in *Amanitaphalloides* in 1893 but Ford confirmed the presence of hemagglutinins in mushrooms [Kobert, 1893; Ford, 1907]. Fungal lectins have been associated with various biological functions like host-cell recognition, developmental regulation and mating [Cooper *et al.*, 1997].

Animal lectins

Several lectins have been purified from both vertebrate and invertebrate species. Lectins from the hemolymph of two crustaceans, the horseshoe crab and the American lobster, were reported in 1902. Later several lectins from various invertebrates have been purified. Based on their occurrence, animal lectins can be classified into soluble lectins and membrane bound lectins. Soluble lectins may play an important role in secretion or organization of extracellular glycoconjugates [Barondes, 1984] or some times they function at membranes in association with glycoconjugates. Membrane bound lectins may be involved in the clearance of glycoproteins from the circulatory system and in the

intracellular translocation of the glycoconjugates [Ashwell and Harford, 1982] and targeting of glycoproteins.

Plants lectins

Several plant lectins have been purified and many of them are well studied. Legume lectins are well studied owing to their dietary importance. Extensive studies have been carried out on the protein sequences and crystal structures of these lectins. Generally, lectins are classified based on their requirement for monosaccharide.

Classification of lectins based on the configuration at C-3 and C-4 of the pyranose [Makela, 1957].

1. Mannose/glucose binding lectins
2. N-acetylglucosamine binding lectins
3. N-acetylgalactosamine/galactose binding lectins
4. L-fucose binding lectins
5. Sialic acid binding lectins

Generally lectins interact with the reducing, terminal glycosyl groups of polysaccharide and glycoprotein chain-ends.

Mannose/glucose binding lectins

They are generally found in the family Leguminosae and Con A from jack bean seeds is the most studied lectin. Seeds of *Dolichos lablab* have been shown to contain glucose/mannose specific lectin [Rajasekhar and Siva kumar, 1997]. Based on their structure they are again classified into 2 groups

1. lectins composed of 4 identical subunits (e.g., Concanavalin A) and
2. lectins having 2cx and 2β chains or $\alpha_2\beta_2$ type (e.g., pea, lentil, fava bean, etc.).

The lectins belonging to this class show the requirement for metal ions like Mn^{+2} and Ca^{+2} for activity. They are rich in acidic and hydroxylic amino acids. Majority of these lectins are mitogenic to lymphocytes. Amino acid sequences of these lectins show extensive homology.

N-acetylglucosamine binding lectins

Lectins of this group are specific for N-acetylglucosamine or P-(1→4) linked oligomers or some times glucosamine. These lectins are generally present in Graminae (wheat, barley, rye and rice), Solanaceae (potato, tomato tobacco, etc.,) and Leguminosae (*Griffoniasimplicifolia* II, *Ulex europaeus*). Among the Graminae, wheat germ lectin is well characterized, Triticale, a hybrid of wheat and rye, has a N-acetylglucosamine specific lectin [Padma and Siva kumar, 1986]. If this sugar occurs in a-anomeric linkage at oligosaccharide chain-ends, mannose/glucose binding lectins also show weak interactions.

N-acetylgalactosamine/galactose binding **lectins**

Lectins belonging to this group share considerable sequence homology and they generally contain Ca^{+2} and Mn^{+2} (lectins from ricinus, bauhinia, etc., lack metal binding). Lectins that exhibit preferential binding to N-acetylgalactoseamine or galactose have been identified from the same species. These lectins show similar properties with respect to protein structure, polymorphism and carbohydrate binding specificity. Ricin, the earliest plant hemagglutinin studied, belongs to this group. Some of the lectins belonging to this group are *Psophocarpus tetragonolobus* and *Hum crepitans.* Seeds of *Dolichos lab/ab* have been found to contain an unusual galactose specific lectin in addition to the glucose/mannose specific lectin [Rajasekhar and Siva kumar, 2002].

L-fucose binding lectins

Lectins belonging to this group share very little structural similarity and they were found to be useful serological reagents e.g., *Ulex europaeus* I lectin, exhibits anti-blood group O activity [Renkonnen, 1948]. Lectins of this group show preferential reactivity with only a portion of the methyl pentose ring structure. Eel hemagglutinin, *Griffonia simplicifolia*-I are some of the lectins belonging to this group.

Sialic acid binding lectins

They are mainly present in hemolymph or sera of invertebrates. Lectins from horseshoe crabs, lobsters, tunicates fall into this category [for reviews Vasta and Marchalonis, 1983; Yeaton, 1981]. These lectins consist of large number of subunits as many as 24.

Classification based on the carbohydrate recognition domains

With the discovery of several lectins, which are not specific to monosaccharides and generally showed high affinity towards oligosaccaharides, a need for new classification has aroused. With the help of sophisticated techniques like genetic engineering and computer-assisted homology searches, animal lectins were classified at the molecular levels.

The basic units of legitimate classification of a protein as a lectin, carbohydrate recognition domains (CRDs), has enabled computer assisted homology searches to classify them based on their protein sequences.

Based on the structural alignments, animal lectins have been classified into C-type, 1-type, Galectins (S-type), Pentraxins and P-type [Drickamer, 1995; Powell and Varki, 1995; Rini, 1995] (shown in the Table 1).

Table 1

Current categories for classification of various animal lectin [Gabius, 1997]

Family	Structural motif	Carbohydrate ligand	Modular arrange-ment	Examples
C-type	Conserved CRD	Variable (mannose, galctose, fructose, heparin tetrasaccharide)	Yes	Selectins, collectins, etc.
I-type	Immunoglobulin like CRD	Variable (Man6GlcNAc2, HNK- 1 epitope, hyaluronic acid, α 2,3/α2,6-sialyllactose)	Yes	ICAM, N-CAM, sialo-adhesin, etc.
Galectins	Conserved CRD	β-galactosides	Variable	Galectin-1-8, Charcot-Leyden crystal protein
Pentraxins	Pentameric subunit arrangement	4,6-cyclic acetal of β-galactose, galactose, sulfated and phosphorylated monosaccharides	Yes	C-reactive protein, serum amyloid P component, etc.
P-type	Similar but not yet strictly defined	Mannose 6-phosphate containing ligands	Yes	MPR 300, MPR 46

C-type

The dependence of sugar binding on the presence of Ca^{+2} ions and the preservation of a common sequence motif of 14 invariable and 18 highly conserved amino acids have been the two prerequisites for defining a C-type lectin.

This family is further divided into many subgroups based on further similarities in the initially recognized CRD. Gene disruption of selectin I in mice results in the impairment of T-cell response, selectin P in defective mobilization of mononuclear leukocytes in chronic inflammation and Selectin E in reduced leukocyte rolling and extra vasation. Mutation in gene coding for Asialoglycoprotein in mice results in the loss of nuclear

clearance of asialoorosomucoid and no accumulation of desialyated glycoprotein/lipoprotein.

I-type

Lectins of this family have immunoglobulin like CRD and carbohydrate ligand is variable ($Man_6GlcNAc_2$, HNK-1 epitope, hyaluronic acid, (a2,3/a2,6-sialyllactose).

Effects of the mutation of 1CAM-I, N-CAM, Po glycoprotein and myelin-associated glycoprotein have been observed in the mice. Mutations in the gene coding for ICAM-I causes severely attenuated delayed hypersensitivity response, resistance to lethal effect of high dose endotoxin and to development of ischemic renal failure, N-CAM in size reduction of olfactory bulb by 36% and deficits in spatial learning and Po glycoprotein in severe hypomyelination of axons in peripheral nerves and secondary effects on schwann cell gene expression. Disruption in the genes coding for myelin associated glycoprotein results in the up regulation of N-CAM expression.

Galectins (S-type lectins)

This is a family of galactoside binding proteins comprising of bioactive molecules with powerful immunoregulatory functions. Members of this family have a conserved CRD and are defined by 2 properties. These lectins share characteristic amino acid sequences and show affinity for β-galactoside sugars. Different members of this family have been shown to modulate, positively or negatively, the multiple steps of inflammatory response, such as cell-matrix interactions, cell trafficking, cell survival, cell growth regulation, chemotaxis and proinflammatroy cytokine secretion.

Pentraxins

C-reactive protein (CRP) is the most studied lectin of this group. Lectins of this family have a pentameric subunit arrangement and carbohydrate ligands 4,6-cyclic acetal of a-galactose, galactose, sulfated and phosphorylated monosaccharides.

P-type

Lectins showing the Carbohydrate specificity for mannose 6-phosphate containing ligands fall into this category. These lectins have a similar but not yet strictly defined CRD. Mannose 6-phosphate receptor proteins, MPR 300 and MPR 46, belong to this family.

Properties of some of the invertebrate lectins

Helix pomatia

Lectins from this invertebrate belond to N-acetylgalactosamine/galactose binding lectins. A lectin that specifically agglutinates human blood group type A has been purified from the albumin gland of snail [Prokop *et al,* 1965a,b; Uhlenbruck and Prokop, 1966; Hammarstrom and Kabat, 1969]. Specific agglutinins from *Helix hortensis* [Prokop *et al,* 1965a], *Otala lacteal* [Boyd and Brown, 1965] and *Euphadra periomphala* [Ishiyama and Takatsu, 1970] have been identified. These lectins were purified by affinity chromatography on N-acetylgalactosamine-Sepharose or by adsorption to insolubilized human or hog blood group A substance.

Purified snail agglutinin has a molecular mass of 79 kDa consisting of 6 identical polypeptide chains (Mr 13 kDa). Each polypeptide chain contains one intrachain disulfide bond and a single carbohydrate-binding site as determined by equilibrium dialysis. Two subunits are linked by interchain disulfide bond and three dimers are held

together by noncovalent interactions [Hammarstrom *et al.,* 1972]. This lectin contains high content of acidic and hydroxylic amino acids and a large proportion of proline residues [Hammarstrom and Kabat, 1969]. It is used as a probe for the detection of terminal, nonreducing N-acetyl-α-galactosaminyl end-groups in biopolymers and cell surfaces [Prokop *et al.,* 1965b; Hammarstrom, 1973].

Carcinscorpius rotunda cauda (Indian horseshoe crab)

This species contains a sialic acid binding lectin (carcinoscorpin) and it can be affinity purified on an ovaine submaxillary-Sepharose gel [Bishayee and Dorai, 1980; Dorai *et al.,* 1981]. It has a molecular mass of 42 kDa and consists of 16 glycosylated subunits of two types with molecular masses 27 and 28 kDa. This lectin has high contents of acidic and hydrophobic residues and contains about 5.8% neutral sugar. End group analysis of this lectin showed leucine to be the sole N-terminal amino acid [Dorai *el al.,* 1981]. Ca^{+2} is essential for the carbohydrate binding activity of the lectin [Bishayee and Dorai, 1980]. It was shown to be useful in resolving glycoconjugates based on the differences in the sialic acid contents.

Limax flavus **(slug)**

It contains a lectin that specifically agglutinates human erythrocytes [Miller *et al.,* 1982]. Slug lectin belongs to sialic acid binding lectins and it can be affinity purified on immobilized bovine submaxillary mucin. It has a molecular mass of 44 kDa consisting of two noncovalently associated subunits (Mr 22kDa). This lectin also has high contents of acidic (glutamic and aspartic acids) amino acids.

Limulus polyphemus (Horseshoe crab)

A sialic acid binding lectin, limulin, has been identified from the hemolymph of horseshoe crab. It agglutinates erythrocytes irrespective of blood type [Noguchi, 1903]. This has been isolated by affinity chromatography on bovine submaxillary mucin [Oppenheim *et al.*, 1974; Roche *et al.*, 1975] and on formalin treated horse erythrocytes [Nowak and Barondes, 1975]. This lectin contains a phosphorylcholine-binding site [Robey and Liu, 1981].

Several workers reported the molecular mass of this lectin to be in the range of 340 to 500 kDa. It contains 4% carbohydrate (N-acetylglucosamine and neutral sugar) [Roche and Monsigny, 1974; Nowak and Barnodes, 1975; Robey and Liu, 1981] and high content of acidic amino acids [Roche and Monsigny, 1974; Robey and Liu, 1981].

Pomacea flagellata

A β-galactose specific lectin has been identified from this mollusc [Espinosa and Lozanno, 1997]. It is a glycoprotein consisting of two isoforms with molecular mass of 30 kDa and can be affinity purified on Sepharose 4B. It eluted specifically with 0.2M D-galactose. Its hemagglutinating activity is independent of metal ions. Both the isoforms are rich in hydrophobic amino acids

Major biological effects of the lectins

Agglutination is one of the earliest properties discovered for the lectins. Majority of these lectins show agglutinating activity although some of them are non-agglutinins. Lectins are involved in a variety of functions in living organisms. Plants lectins were studied in detail owing to their importance in the human diet. Plant products from raw red kidney beans, snowdrop bulb and elderberry tree bark have toxic effects on the

human system. Research on these plant products showed that the antinutrional effects of these lectins are the result of the consequence of their binding to the intestinal epithelium in mammals. By binding to the epithelium they may result in the release of a secondary messenger molecule that might act as a signal to elicit a response on the system. The antinutritional effects are proportional to their ability to stimulate the growth of the gut at the cost of the growth of the animal itself [Pusztai *et al,* 1991].

In plants lectins act as storage proteins and several lectins have been purified from their storage tissues. Like storage proteins lectins are also developmentally regulated [Dannenhoffer *et al,* 1997]. In *Dolichos biflorus* no lectin was detected in the developing seeds during the first 26 days and large quantitites of lectins appeared on the 27th day [Talbot and Etzler, 1978]. Results of the similar studies indicated that the lectins appear during the later stages of maturation of seeds prior to their dehydration. In Aracea species, the major storage protein is a lectin [Van Damme *et al,* 1995] and they may help in the defense against pathogenic invasions [Peumans and Van Damme, 1995a, 1995b].

Another animal lectin, chicken lactose lectin-I (CLL-I), also shows developmental regulation. This lectin becomes prominent only at a specific stage of the development of chick embryo muscle [Nowak *el al,* 1976; Den *et al,* 1976]. Rat β-galactoside lectin appears with differentiation of a myogenic cell line [Nowak *et al,* 1976; Gartner and Podleski, 1976] and embryonic lung [Powell and Whitney, 1980].

Lectins are also involved in the defense mechanisms of the plant. Plant defense is either active or passive. In the active defense, the cells in the vicinity of the affected area are triggered to synthesize specific pathogenesis-related proteins or low molecular weight

compounds like phyloalexins. In this process lectins may also be triggered. Physical barriers, biochemical adaptations and morphological adaptations contribute to the passive defense. Due to their specificity to oligosaccharides, lectins may play an important role in the defense mechanisms. Accumulation of the toxic low molecular weight compounds in the whole plant or susceptible tissues is a biochemical adaptation [Peumans and Van Damme, 1995a,b]. Some of these toxins could be lectins, lectin-like toxins or ribosome inactivating proteins (RIPs) whose target may be a specific group of organisms [Stripe *et al.*, 1992].

Lectins may also act as cryoprotective agents. Gal/GalNAc specific lectin from mistletoe leaves is an example [Hincha *et al.*, 1997].

Animal lectins perform various functions and immune response is one of the important function and lectins belonging to selectins generally perform this function. Selectins are adhesion proteins on the cell-surface and act as receptors for leukocytes. Initially P-selectin was detected in the platelets and E-selectin in the endothelium. L-selectin is present in the lymphocytes, neutrophils, monocytes and cosinophils. They act in response to an inflammation and help in the elimination of bacterial infections. Excess accumulation of these lectins can lead to other complications such as in rheumatoid arthritis.

Certain lectins show tumor specificity. They preferentially agglutinate tumor cells when compared to normal cells [Sastry *et al.*, 1986; Puri *et al.*, 1992]. Some of the lectins can be altered to increase their tumor specificity [Sharma *et al.*, 1996]. Several attempts were made to use the tumor specific lectins for targeted drug delivery in tumor therapy [Gabius and Gabius, 1991].

Some of the lectins show mitogenic activity towards quiescent non-dividing lymphoctes, either B-cells or T-cells or both [Lis and Sharon, 1998]. Con A and red kidney bean agglutinin are the commonly used mitogens. Endogenous membrane lectins have been implicated in the triggering of recognition of target cells by natural killer cells [Bezouska *et al.*, 1991].

Wheat germ agglutinin and Con A lectin shows insulinomimetic activity. They mimic the behavior of insulin on adipocytes. Since the receptor for insulin is a glycoprotein, these lectins have been shown to compete with insulin in binding fat cells and there is a speculation that perhaps the two have the same receptor [Czech *et al,* 1974; Katzen *et al,* 1981].

Snake venoms contain a wide range of components, many of which affect haemostasis by activation or by inhibition of platelets or coagulation factors. One of the first C-type lectins with a defined function, echicetin, was demonstrated to bind to platelet GPIb and it blocks several functions of this receptor [Clemetson *et al,* 2002].

Major uses oflectins

Binding of lectins to the carbohydrates on the cell surface results in a variety of biological effects. Because of the agglutinating property and their specificity to the different carbohydrate residues, lectins were widely used to distinguish different blood groups and also in the structural studies of the blood group substances [Watkins *et al,* 1981].

Lectins are widely used in the isolation, purification and structural studies of carbohydrate containing proteins [Blake and Goldstein, 1982] (shown in Table 2).

Table 2 Lectins used for the cell **seperation**

Source of lectin	Source of cells	Examples of cells separated
Dolichos biflorus	Human	A1 and O(H) erythrocytes
Griffonia simplicifolia 1 lectin	Murine	Stimulated and resident macrophages
Helix pomatia	Human	Peripheral B and T lymphocytes
	Murine	B and T splenocytes
	Rat	B and T splenocytes
Limulus polyphemus	Murine	Spleen T-helper cells
Lotus tetragonolobus	Human	Peripheral blood neutrophils, bone marrow hemopoietic progenitor cells
Peanut	Human	Cortical and medullary thymocytes, immature and mature cord blood lymphocytes
	Murine	Cortical and medullary thymocytes, suppressor spleen T cells
	Chicken	Suppressor lymphocytes
Pokeweed	Murine	Granulocyte-macrophage progenitor cells
Soybean	Human	Helper and suppressor lymphocytes, bone marrow stem cells
	Murine	B and T splenocytes, stem cells from spleen
	Hamster	B and T splenocytes
	Monkey	Bone marrow stem cells
Wheat germ	Murine	B and T splenocytes

They also allow the purification of membrane proteins since the lectins are often stable in the presence of low concentrations of the certain detergents and also in demonstrating

their glycoprotein nature [Gioannini *et al.,* 1982; Hedo *et al.,* 1981; Shirakawa *et al.,* 1983]. Lectins have been used to disprove claims that certain proteins are glycoproteins. Carbohydrate containing purified fractions of F_1-ATPase from *Micrococcus leuleus* on Con A-Sepharose removes the carbohydrate components and leaves the protein with impaired enzymatic activity [Lim and Salton, 1981].

The presence of lectin receptors on the cells is readily demonstrated with use of suitable lectin derivatives generally with the use of techniques involved in the study of cell-surface antigens [Lotan, 1979]. Radioactively labeled lectins may be used to measure the number of lectin receptor sites on the cell surface and the affinity of lectin-receptor interactions.

They are also widely used as the probes in the study of the complex carbohydrate structures on cell surfaces of animals, plants and microorganisms. They are also used as markers in the cell growth and differentiation. Populations of cells from various sources (animals, plants and microorganisms) may be sorted into subpopulations by interactions with lectins, provided the cells differ in their cell-surface sugars. This can be achieved without any damage to the cells and their viability. First application of lectins to cell separation (leukocytes from erythrocytes in human blood with the aid of PHA) was reported in 1949 [Li and Osgood, 1949]. *Helix pomatia* agglutinin can be employed in the identification and isolation of T cells in mouse, man, rat and cow [Hammarstorm *et al.,* 1978] only when the cells are treated with sialidase prior to fractionation by it. PNA is another widely used lectin in the separation of human thymocytes [Reisner *et al,* 1979]. Peanut (PNA) and *Amaranthus leucocarpus* lectins discriminate between memory and naïve/quiescent porcine lymphocytes [Hernandez *et al.,* 2002].

Several studies have been carried out on the extent and pattern of lectin binding to malignant cells as compared to the normal cells [Howard *et al.,* 1981; Boland *et al.,* 1982; Louis *et al.,* 1983]. Some low metastatic B16 melanoma sublines bind fewer Con A than highly metastatic ones, while the latter has fewer binding sites for soybean and wheat germ agglutinins [Raz *et al.,* 1980]. In mouse lymphosarcoma cells, a sequential loss of Con A receptors with increasing malignancy and metastatic potential was observed [Nicolson *et al.,* 1980].

Agglutination of a microorganism from a primary isolate with a particular lectin may constitute a confirmatory identification of the organism, making it possible to dispense with subsequent expensive and time-consuming culturing or serological testing. *Neisseria gonorrhoeae* can be differentiated from other *Neisseria* species and related bacteria by its agglutination with wheat germ agglutinin [Schaefer *et al.,* 1979]. *Bacillus anthracis* and *Bacillus mycoides* can be separated from the other strains of *Bacillus* since they show agglutination with soybean agglutinin. Again these two species can be separated by their differences in agglutination with *Helix pomatia* agglutinin [Cole *et al,* 1984]. Strains of *Staphylococcus aureus* can be separated based on their ability to agglutinate wheat germ agglutinin [Raychowdhury *et al.,* 1982]. Wheat germ agglutinins show anti-fungal properties [Mirelman *et al.,* 1975].

Lectins are also used in the study of lysosomal storage disorders that are involved in the accumulation of carbohydrates in the cells due to defective catabolism [Alory *et al.,* 1991]. They can also be used to modify the structure and function of the absorptive surface of the gut. When specific lectins are introduced into the diet, they can compete

with the harmful bacteria for specific binding sites or they may alter the surface of the receptors required for the adhesion of the harmful bacteria.

The ability of some of the lectins to show preferential interaction with certain transformed cells has led to attempts to use these compounds as carriers for chemotherapeutic agents. Lectin serves to direct the cytotoxic agent to appropriate target cells in chimeric toxins consisting of Con A and the a chains of diphtheria toxin [Gilliland *et al,* 1978] or ricin [Yamaguchi *et al.,* 1979].

SCOPE OF THE PRESENT INVESTIGATION

Lectins are ubiquitous proteins/glycoproteins with distinct sugar binding properties. Compared to the vast amount of literature available on the plant lectins, information on animal lectins is limited. Further among the animal kingdom, only a few animal lectins have been well characterized from the invertebrate species. The laboratory where the present work was undertaken has been working in the area of plant and animal lectins with an emphasis on developing new affinity methods to purify new lectins and to understand their functions.

Several new lectins with varied sugar specificities have been purified from plant and animals in the laboratory employing new affinity methods developed. One of the goals of the laboratory is to establish the evolution of mannose 6-phosphate receptor proteins that mediate transport of lysosomal enzymes to lysosomes in eucaryotes. Initial studies carried out established the appearance of putative receptors among reptiles, amphibians and fish. Further the putative mannose 6-phosphate receptor (MPR 300 and MPR 46) proteins have been identified from the invertebrate mollusk, *unio*. The proteins were purified from the membrane extracts of the *unio* by phosphomannan affinity chromatography and an antibody was raised. Biochemical and immunological properties of the proteins were studied.

While working with the *unio* extracts, we detected that the extracts contained a protein that agglutinated pronase treated rabbit erythrocytes. Only the disaccharide lactose inhibited the activity of the lectin. An affinity matrix was developed to purify the lectin and it was purified to homogeneity. Since this new lectin seems to be unusual in its agglutinating properties, a systematic study was undertaken to characterize its

biochemical and immunological properties. Further extensive chemical modification studies were carried out on the purified lectin in order to identify the role of amino acids in its activity.

Glycosidases such as a-mannosidase from *Canavalia ensiformis* seeds has been shown to specifically interact with the lectin, Con A, purified from same species. Glycosidase activities related to a-mannosidase and a-fucosidase have been purified identified in the *unio* extracts. In order to understand the possible function of the newly identified *unio* lectin, the a-mannosidase and a-fucosidase have been affinity purified on mannosamine and fucosamine gels respectively. The identity of the a-mannosidase was established by its specific immunoreactivity with an antibody to the bovine lysosomal a-mannosidase. For a-fucosidase, the identity was established by its specific reactivity with Con A labeled with biotin.

With the availability of the three proteins from *unio*, purified MPR 300, lectin and a-fucosidase, an attempt was made to understand their possible functions by designing *in vitro* experiments.

Figure 1

Unio (The fresh water mussel)

Classification

Phylum	Mollusca
Class	Pelecypoda
Order	Eulamellibranchiata
Genus	*Unio*

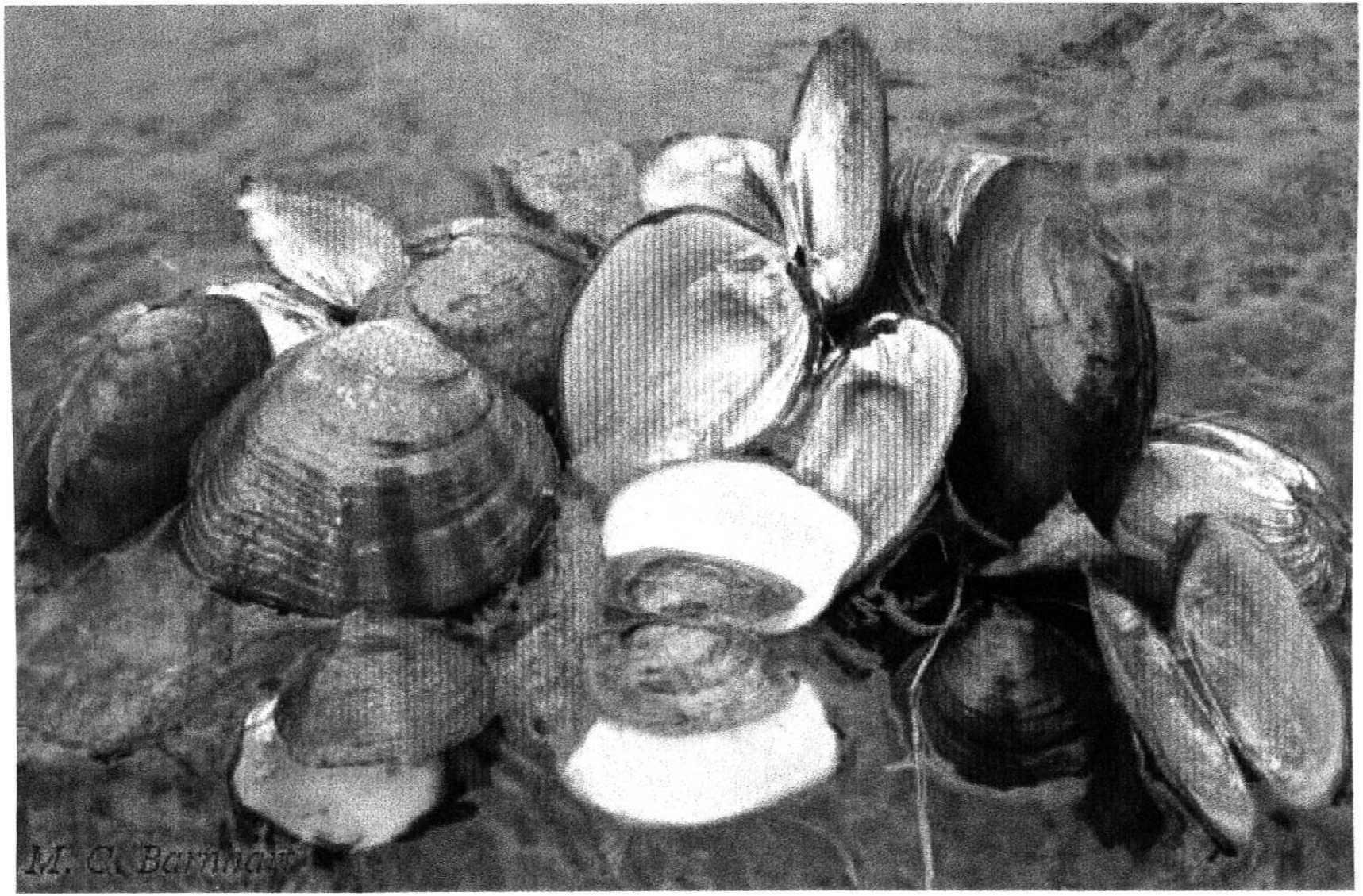


Figure 1

CHAPTER II

AFFINITY PURIFICATION AND CHARACTERIZATION OF A LACTOSE SPECIFIC LECTIN FROM *UNIO*

INTRODUCTION

Lectins are a group of proteins that interact with glycoproteins and glycolipids by binding to specific carbohydrate residues [Us and Sharon, 1973]. The carbohydrate for which they have the highest affinity defines their specificity.

Several invertebrate lectins with varied sugar specificity have been identified. *Helix pomatia* for N-acetyl-D-galactosamine [Hammarstrom and Kabat, 1971], *Tridacna maxima* for galactose [Baldo *et al.*, 1978], *Limax flaws* for sialic acid [Miller *et al.*, 1982], *Achatinina fulica* for O-acetyl-sialic acid [Basu *et al.*, 1986] and *Pomaceae flagellata* for galactose [Espinosa and Lozano, 1997]. Very limited information is available about the structures of these lectins.

Lectins are usually present in the body fluids of the invertebrates. Some of the observations show the enhancement of lectin production in body fluids when foreign substances are introduced into them. The binding of the lectins to foreign substances/cells activates phagocytosis. Many of these lectins agglutinate pathogenic bacteria [Slifkin and Doyle, 1990].

A marine invertebrate, sea urchin (*Toxpneustes pileolus*), has been shown to contain a D-galactose binding lectin in the venom of their spines that showed mitogenic stimulation on murine splenocytes [Nakagawa *et al.*, 1999]. The primary role of this lectin might be in the defense against a foreign body.

Some of the functions of other marine invertebrate lectins are matrix crystallization in sea urchin [Ameye *et al.*, 2001] and in controlling species-specific gamete interactions in sea urchin [Hirohashi and Lennarz, 2001].

A galactose specific lectin from sea cucumber (*Cucumaria echinata*), a marine invertebrate, binds to the specific carbohydrate chains on the erythrocyte surface and damages the cell membrane leading to its lysis [Kuwahara *et al*, 2002].

Probably lectins in invertebrates act as the body protection factors or as humoral factors in the defense mechanism similar to immunoglobulins in the vertebrates.

While working with the *unio* extracts on the mannose 6-phosphate receptors, we identified that the animal extracts additionally contained a lectin activity that was inhibited only by lactose. The objective of the present study was to purify this lectin by affinity chromatography and to analyze its various biological and physicochemical properties that could eventually help in understanding its functions. In the present study an affinity matrix, Sepharose-divinyl sulfone-lactose, was developed to purify this lectin.

MATERIALS AND METHODS

MATERIALS

Unio animals were purchased from local animal suppliers and the tissue was removed from the shells and stored frozen at -SOX. Standard proteins, divinyl sulfone, sugars, acrylamide, N,N, methylenebisacrylamide and TEMED were obtained from Sigma Chemical Company, St. Louis, MO, USA. Sepharose 6B was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. All the sugars and amino acids used in the study were purchased from Loba Chemie, Mumbai, India. All other chemicals used in the study were obtained from reputed local firms. Rabbit erythrocytes were collected from the University animal house. Biogel P-150 was purchased from Bio-Rad laboratories, USA. Secondary antidbody and BC1P/NBT were obtained from Bangalore genei.

METHODS

Extraction and purification of the lectin

300g of *unio* whole animal tissue was homogenized with 300ml of 50mM Tris-HCl pH 7.5 containing 50mM NaCl (TBS) and stirred overnight at 4°C. It was centrifuged at 10,000 rpm for 20 min at 4°C. To the clear supernatant, $(NH_4)_2SO_4$ was added to 80% saturation and stirred for 2hours. The suspension was centrifuged again at 10,000 rpm for 20 min. The pellet was dissolved and dialyzed against TBS. The dialyzed sample was clarified by centrifugation and used for hemagglutination and sugar inhibition assays.

Hemagglutination assay

This was carried out according to Siva Kumar and Rajagopal Rao [1986]. By ear vein puncture, Rabbit blood was collected into alsevier's solution. It was centrifuged at 3000 rpm for 10 min at 4°C. The sedimented erythrocyte pellet was washed thrice with 0.9% saline and the pellet was made to 4% suspension with saline. Processed erythrocytes were treated with trypsin, pronase and neuraminidase separately. Trypsin was added to a final concentration of 0.1% (w/v) and incubated at 37°C for 1hour. 100µg of pronase was added to 1 ml of erythrocytes and incubated at 37°C for 30 min. After incubation, erythrocytes were centrifuged at 3,000 rpm for 10 min at 4°C and then made up to the original volume. Neuraminidase (0.1U) was added to 0.5 ml of processed erythrocytes and incubated at 37°C for 30 minutes and used.

Aliquots were taken after each step of purification and assayed for hemagglutinating activity. To 200ul of 0-80% $(NH_4)_2SO_4$ sample, serially diluted in 200µl of saline, 200(il of the enzyme treated erythrocytes were added separately and incubated at 37°C for 1 hour and the hemagglutination was visually observed.

Sugar inhibition assay

The following sugars were tested for their inhibitory effect on the activity of the lectin in 0-80% $(NH_4)_2SO_4$ sample: glucose, mannose, galactose, lactose, N-acetyl-D-glucosamine and N-acetyl-D-galactosamine. Sugars in the range of 5 to 50mM concentrations were added to the wells of a hemagglutination plate and the volume was made up to 0.1ml with saline. To each of the wells 0.1 ml of 0-80% $(NH_4)_2SO_4$ sample containing 4 hemagglutination units was added and incubated for 1 hour at 37°C. To

each of the wells 0.2ml of pronase (100μg/ml) treated erythrocytes were added and the plate was visualized for agglutination after 1hour of incubation at 37°C.

Preparation of Sepharose-divinyl sulfone-lactose (affinity gel)

The affinity gel was prepared in 20ml batches. Sepharose 6B gel (20 ml) was washed thoroughly with double distilled water on a sintered glass funnel and the wet cake **was** suspended in 0.5M $Na_2CO_3/NaHCO_3$ buffer pH 11.0. 2 ml of divinyl sulfone was added and the suspension was shaken gently at room temperature for 70min followed by washing with double distilled water. The activated gel was washed with $Na_2CO_3/NaHCO_3$ buffer pH 10.0 and the wet cake was suspended in the same buffer containing 20% (w/v) of lactose. Coupling was allowed to proceed in cold for 24 hours and the gel was washed with distilled water. Finally it was suspended in 0.5M $Na_2CO_3/NaHCO_3$ buffer pH 8.5 containing 0.2ml of β-mercaptoethanol and mixed at room temperature for 3hours. The gel was washed with double distilled water and stored at 4°C in TBS until further use.

Chromatography on the affinity gel

The affinity gel (2.6 x 8 cm) was equilibrated with TBS at 4°C and the clear supernatant obtained from the above step was applied. The gel was washed extensively with TBS till A_{280} of the flow through was less than 0.05. Elution was carried out with 0.2M lactose in TBS. Protein containing fractions were pooled, dialyzed (against water), lyophilized and stored at $-20^{o}C$. The lyophilized protein (lectin) was used in all experiments.

Hemagglutination and sugar inhibition assay for the affinity purified lectin

As described earlier (for 0-80% $(NH_4)_2SO_4$ sample) hemagglutination and sugar inhibition assays were performed with the affinity-purified lectin.

Molecular weight determination

The native molecular weight of the lectin was determined by using a calibrated column of Biogel P-150 (1.4 x 86 cm) that was previously equilibrated with TBS buffer. The column was calibrated using the following standard proteins: Phosphorylase b (1,94,000 Da), BSA (66,000 Da), Ovalbumin (45,000 Da) and Trypsinogen (25,000 Da).

In order to confirm the homogeneity of the purified lectin, it was also applied on Superose-12 FPLC gel filtration column and the elution pattern was monitored.

Affinity chromatography on Con A -Sepharose gel.

Con A-Sepharose gel (0.5 ml) was equilibrated with TBS containing 5mM each of $MnCl_2$ and $CaCl_2$. Purified lectin in the same buffer was applied on the gel. The gel was washed till A_{280} was zero. Bound protein was eluted sequentially with 0.3M Methyl-α mannoside and 50mM sodium acetate buffer pH 4.0.

In a separate experiment, the lectin preincubated with 0.2M lactose for 16 hours was applied on Con A- Sepharose gel and the gel was washed till A_{280} was zero. Elution was carried out with 0.3M methyl a-mannoside followed by 50mM sodium acetate pH 4.0, Tris-HCl pH 10.0 and 0.1M acetic acid.

Protein estimation

Protein was determined by Lowry's method using crystalline BSA as standard [Lowry *et al.*, 1951].

Carbohydrate estimation

Carbohydrate content was determined by phenol-H_2SO_4 method [Dubios *et al,* 1956]. Glucose was used as the standard.

Effect of temperature and pH dependence on the binding ability of the lectin to the affinity gel

Lectin, 1 mg/ml, in TBS was incubated at 30, 40, 50, 60, 70 and 80°C for 20 min and applied to the affinity gel equilibrated with TBS at 4°C. The gel was washed with TBS till A_{280} reaches zero and the protein was eluted with 0.2M lactose in TBS.

Lectin, 1 mg/ml, was dissolved and dialyzed extensively in the buffers of different pH (4-10) and applied to the affinity gel equilibrated with the respective buffers. Buffers used for pH (4-6) are 50mM sodium acetate and pH (7-10) are 50mM Tris-HCl. Elution was carried out with 0.2M lactose in the same buffer.

Native and sodium dodecyl Sulfate polyacrylamide **gel electrophoresis**

Native PAGE was performed using Tris-Glycine buffer pH 8.3 on a 7.5% gel [Reisfeld *et al,* 1966]. 12% SDS-PAGE was carried out under reducing conditions [Laemmli, 1970] and the protein bands were detected either by silver or Coomassie staining. Periodic acid-Schiffs staining was used for analyzing the carbohydrate nature of the protein [Zacharius *et al,* 1969].

Carboxymethylation of the lectin

50mg of lectin was dissolved in 3ml of 6M guanidine-HCl (made in 0.6M Tris-HCl buffer pH 8.6). 30µl of β-mercaptoethanol was added and incubated under nitrogen for 3 hours at room temperature. 0.3ml of colorless iodoacetate (268mg/ml in 0.1M NaOH) was added and incubated in dark for 15 min. After incubation the sample was

dialyzed against 5mM NH_4HCO_3 in dark for 24 hours. It was denatured by incubating with 1% SDS for 24 hours at room temperature and applied on a P-60 gel filtration column (1.4 x 80 cm) equilibrated with 1% SDS. Fractions (2 ml) were collected and the absorbance was monitored at 280nm.

Preparation of the antisera to the lectin

250µg of the lectin in 0.5 ml of the TBS was emulsified with an equal volume of Freund's complete adjuvant and injected subcutaneously into a rabbit. Booster doses were given in Freund's incomplete adjuvant in the third and fifth week. Ten days after the third injection, rabbit was bled by ear vein puncture and the antisera was collected after centrifugation and stored at -20°C.

Immunodiffusion

Double diffusion was carried out on 1% agar (in PBS) plates for 24-48 hours at 4°C and visualized for precipitin arc [Ouchterlony, 1948]. Antiserum was placed in the central well with different concentrations of the lectin in the surrounding wells.

Western **blot analysis**

This was carried out according to Towbin *et al.* [1979]. Following SDS-PAGE of the lectin on 12% gel, proteins were transferred onto the nitrocellulose membrane. The membrane was incubated with 5% defatted milk powder in TBS followed by incubation for 1 hour at room temperature with primary antibody raised to the purified lectin at .1:1000 dilution. After incubation with the primary antibody, the membrane was washed twice with TBS and TBST (TBS containing 0.05% Tween-20) respectively. The membrane was then incubated at room temperature for 1 hour with secondary antibody (goat anti rabbit IgG) conjugated with alkaline phosphatase (1:500

dilution). The membrane was washed with TBS followed by TBST and developed using BC1P/NBT. The reaction was stopped by transferring the blot to distilled water.

N-terminal amino acid sequencing using DABITC reagent

This was carried out using DABITC (4-N,N-dimethylaminoazobenzene-4'-isothiocyanate) reagent [Chang, 1983].

Coupling: Lectin (5-10 nmoles) was dissolved in 80µl of 50% pyridine in a stoppered conical glass tube and 40µl (400nM) of DABITC reagent (2.8 mg/ml in pyridine) was added. The tube was purged with nitrogen. It was sealed and incubated at 54°C for 50 min. 5µl of PITC (phenyl isothiocyanate) was added and purged with nitrogen again. The tube was sealed and incubated at 54°C for 20 min.

Washings: 500µl of heptane:ethyl acetate (2:1) was added under nitrogen and stirred. The phases were separated by brief centrifugation for 1 min. The upper phase was aspirated and discarded. The lower phase was re-extracted with heptane:ethyl acetate four times and the upper phase was discarded each time. The lower (aqueous) phase was dried in a vacuum dessicator before proceeding to the cleavage step (It is most important that the samples are completely dry).

Cleavage: 50µl of 50% TFA (triflouroacetic acid) was added to the dried sample and purged with nitrogen. The tube was sealed and incubated at 54°C for 15 min. TFA was removed by drying under vacuum.

Extraction: 200µl of n-butyl acetate and 50µl of water was added to the dried sample. The content of the tube was mixed well and the phases were separated by brief centrifugation for 1 min. The upper (organic) phase was transferred into a conversion

tube and dried under vacuum. Aqueous phase was dried and subjected to further cycles.

Conversion: 50|il of 50% TFA was added to the dried sample in a conversion tube (thiazoline is converted to respective DABTH-amino acid derivative) and sealed. It was incubated at 54°C for 45 min and dried under vacuum. The residue obtained was dissolved again in 2-5μl of ethanol and suitable aliquot was taken for analysis on two-dimensional thin layer chromatography on polyamide sheets (3 x 3 cm).

2D TLC (Two dimensional thin layer chromatography)

Solvent used for carrying the first dimension TLC was acetic acid-water (1:2 v/v) and second dimension was toluene-n-hexane-acetic acid (2:1:1 v/v). After 2D TLC, the polyamide sheets were exposed to fumes of concentrated HCl and the pink spots of the amino acids were visualized. Standard DABITC amino acids were also prepared and separated on polyamide sheets (3 x 3 cm).

Amino acid analysis

Purified lectin (5mg) was hydrolyzed with 6N HCl for 24 hours at 110°C [Siva Kumar and Rajagopal Rao, 1986]. Following hydrolysis, the sample (140μg) was analyzed on a Beckman 119CL automatic amino acid analyzer. The analysis was performed according to the manufacturer's instructions.

RESULTS

Unio lectin agglutinated only pronase treated rabbit erythrocytes. It did not agglutinate untreated, trypsin and neuraminidase treated rabbit erythrocytes. When several sugars like glucose, galactose, mannose, lactose, N-acetyl-D-glucosamine and N-acetyl-D-galactosamine were used in sugar inhibition assays, both with the 0-80% $(NH_4)_2SO_4$ sample and purified lectin, only lactose was found to be inhibitory (Table 3).

When the *unio* extracts were processed as described under methods and passed through the affinity gel, the lectin was bound on the gel. It was specifically eluted using lactose as shown in Fig. 2. Neither glucose nor galactose used as eluant could desorb the bound protein (data not shown). From 300g of the tissue 100mg of the lectin could be obtained. Table 4 shows the purification of the lectin.

The purified lectin eluted as a single peak from a Biogel P- 150 gel filtration column with molecular mass corresponding to 105 kDa ± 5 kDa (Fig. 3). The protein eluted as a single peak from a FPLC column also (data not shown).

The lectin had 5% neutral carbohydrate as estimated by phenol-H_2SO_4 acid method. The glycoprotein nature of the lectin was further confirmed by its strong binding to Con A-Sepharose gel. When 0.3M methyl a- mannoside was used for elution, very little protein was eluted from Con A-Sepharose gel and it was completely desorbed at acid pH (Fig. 4A). When lectin preincubated with lactose was applied to Con A-Sepharose gel it could not be eluted when 0.3M methyl a-mannoside, 50mM sodium acetate pH 4.0 and Tris-HCl pH 10.0 buffers were used separately. It eluted only when 0.1M acetic acid was used indicating its strong affinity to Con A (Fig. 4B).

The lectin showed 100% binding to affinity gel upto 40°C. Only 89% of the lectin bound at 50°C and 78% at 60°C and 7.4% at 70°C. There was complete loss of binding at 80°C (Fig. 5B). At pH 7 and 8 the lectin showed 100% binding to the affinity gel (Fig 5B).

Lectin moved as a single band in native gel electrophoresis as shown in Fig. 6A. On SDS-PAGE it moved as three bands (with molecular masses 28, 23 and 16kDa respectively) as shown in Fig. 6B. All the bands stained positive for carbohydrate on periodic acid schiffs staining (Fig. 6C).

When the carboxymethylated lectin was applied on Biogel P-60 there was no separation of the subunits (Fig. 7).

The N-terminal analysis of the lectin by DABITC method gave two spots corresponding to valine and proline. The second cycle revealed histidine and lysine and third cycle showed arginine and histidine indicating that the lectin is possibly made of two or more subunits (Table 5).

Amino acid analysis of the lectin showed high content of acidic and hydrophobic amino acids (Table 6).

An antibody raised to the purified lectin showed precipitin arc with the protein in immunodiffusion experiment and it recognized all the three bands in western blot analysis (Fig 8A and 8B).

Table 3. Sugar inhibition assay of **the lectin.**

Sugar	**Haemagglutinating activity of 0-80% ammonium Sulfate sample and affinity purified lectin**
Glucose	Noninhibitory
Mannose	Noninhibitory
Galactose	Noninhibitory
Lactose	Inhibitory at 2.5mM concentration
N-Acetyl-D-glucosamine	Noninhibitory
N-Acetyl-D-galactosamine	Noninhibitory

Figure 2

Affinity purification of the *unio* lectin. The 0-80% ammonium Sulfate precipitated proteins after dialysis was applied to affinity gel at 4°C (2.6 x 8 cm) equilibrated with TBS at a flow rate of 20 ml/hour. 0.2M lactose was used for elution and 10ml fractions were collected.

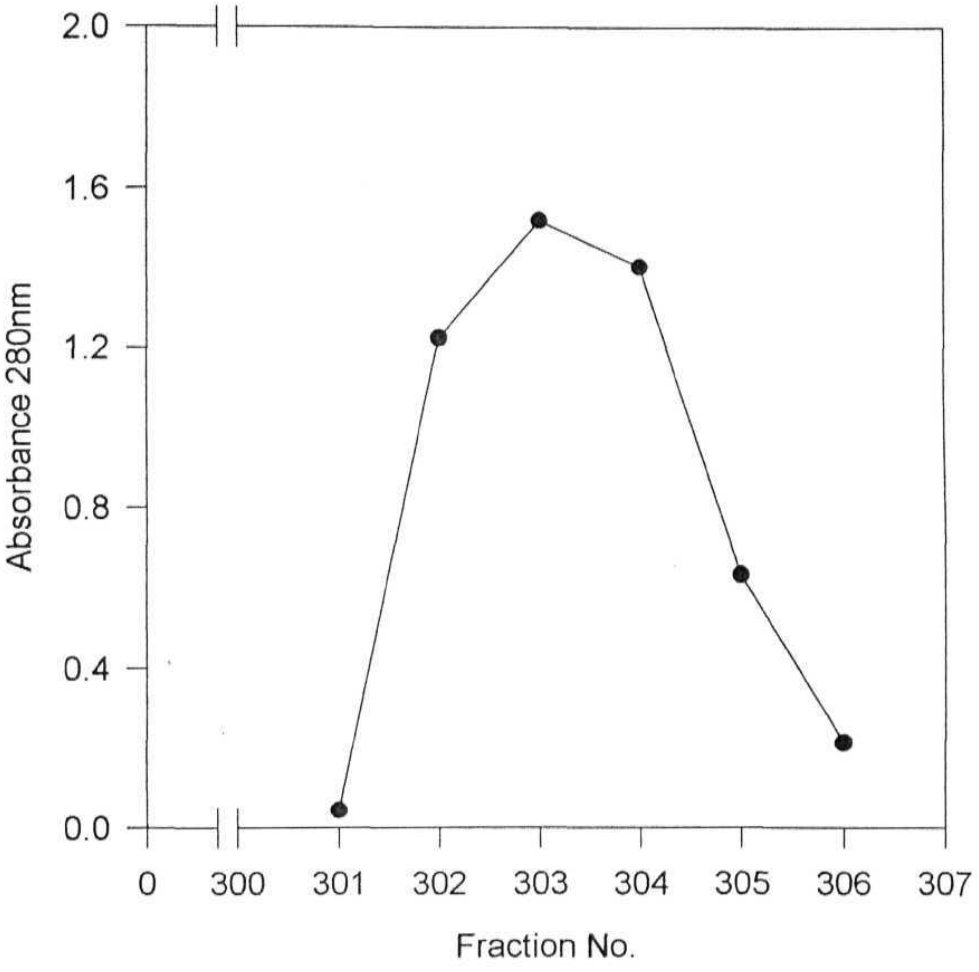

2.0
1.6
1.2
0.8
0.4
0.0
Absorbance 280nm
0
300
301
302
303
304
305
306
307
Fraction No.

Figure 2

Table 4. Purification of the *unio* lectin

Fraction	Total Volume (ml)	Protein (mg/ml)	Hemagglutination (units/ml)	Total Activity in HU	Specific Activity (units/mg protein)
Crude	560	42	—	—	—
Before $(NH_4)_2SO_4$ Precipitation	400	31	40	16000	1.29
After $(NH_4)_2SO_4$ precipitation	370	21.32	40	13000	1.92
Sepharose lactose eluate	40	2.5	160	6402	64

Figure 3

Elution profile of the lectin on Biogel P-150.

Biogel P 150 (1.4 x 86 cm) gel filtration column was equilibrated with TBS and 2ml fractions were collected at a flow rate of 8ml/hour.

Inset shows calibration curve of the column with standard proteins and ➔ indicates the molecular mass of the lectin.

Phosphorylase b	1,94,000 Da
BSA	66,000 Da
Ovalbumin	45,000 Da
Trypsinogen	25,000 Da
Uma lectin	1,05,000 Da

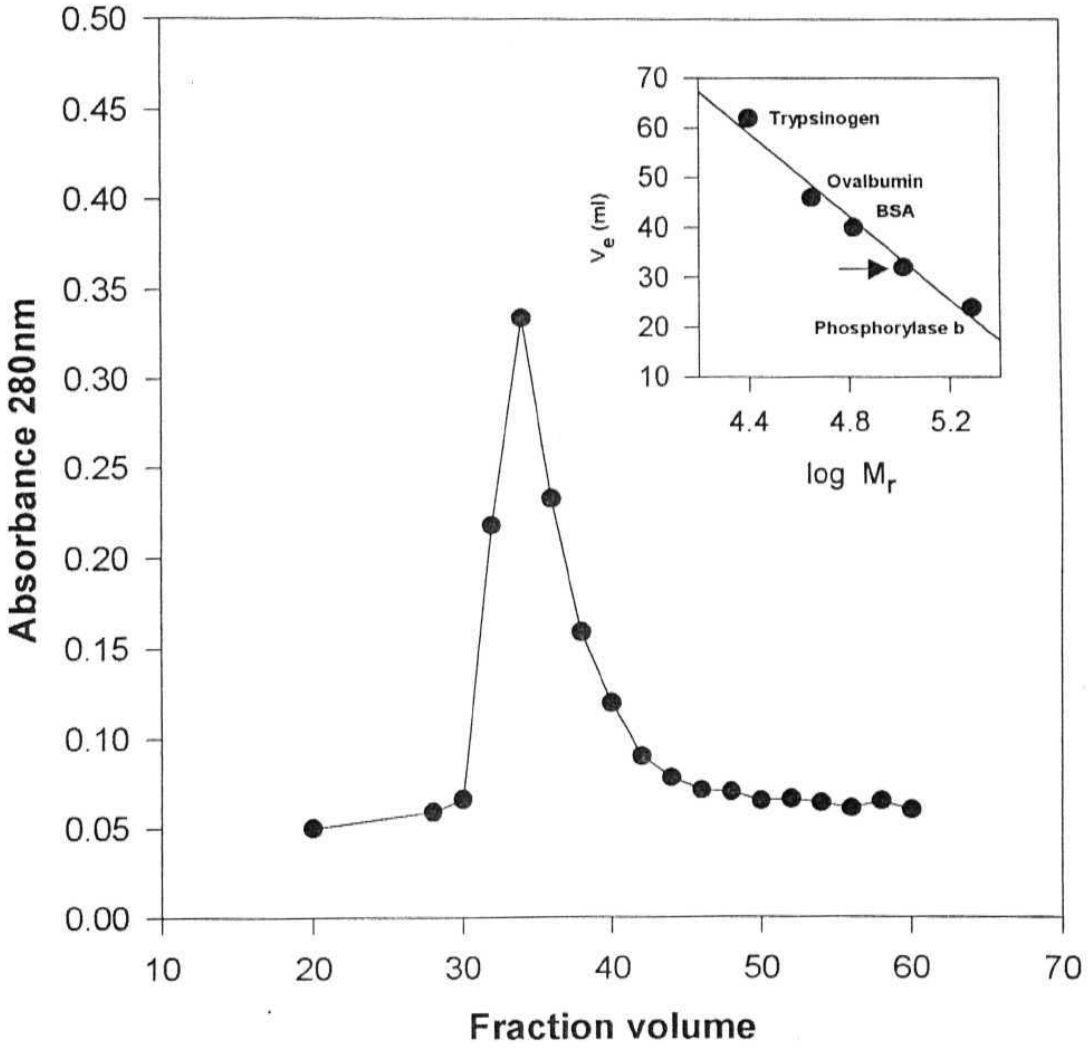

Absorbance 280nm
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
10
20
30
40
50
60
70
Fraction volume
V_e (ml)
70
60
50
40
30
20
10
4.4
4.8
5.2
log M_r
Trypsinogen
Ovalbumin
BSA
Phosphorylase b

Figure 3

Figure 4

A. Binding of the lectin on Con A-Sepharose gel. (Details are given under Methods).

B. Binding of the lectin preincubated with 0.2M lactose on Con A-Sepharose gel.
(Details are given under methods).

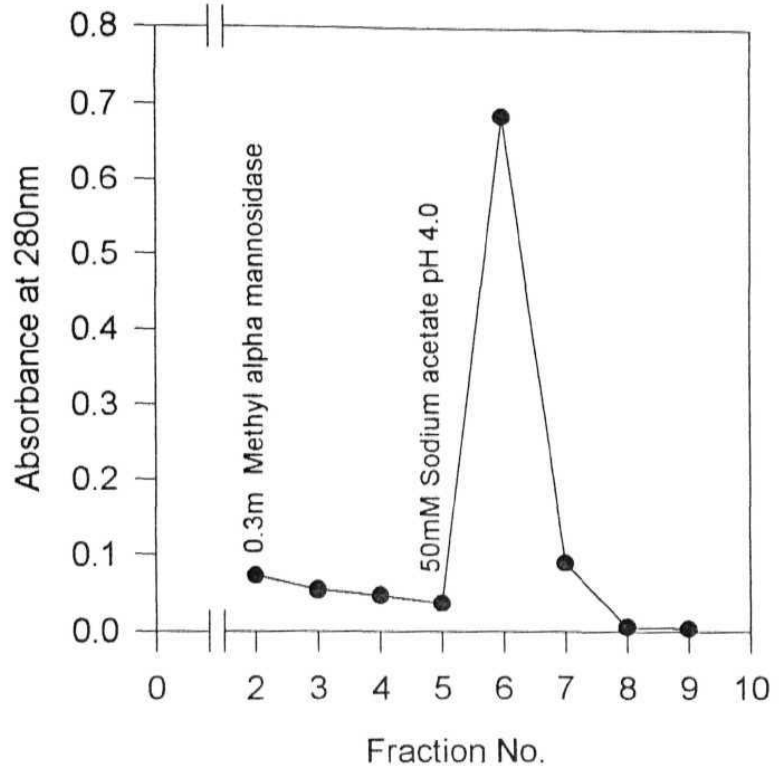


Figure 4A

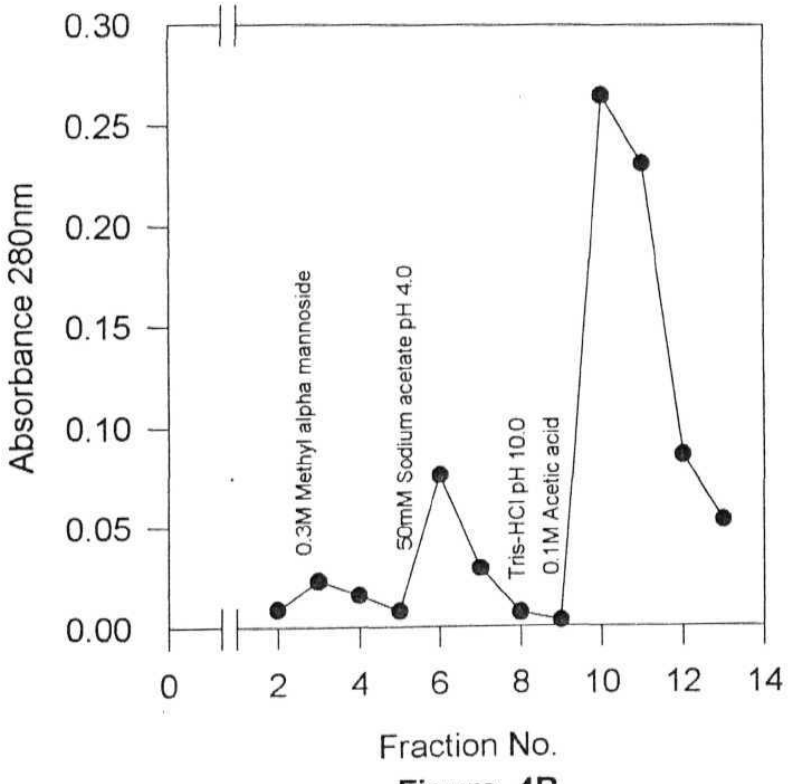


Figure 4B

Figure 5

A. Effect of temperature on the binding ability of the lectin to affinity gel.

B. Effect of pH on the binding ability of the lectin to affinity gel.

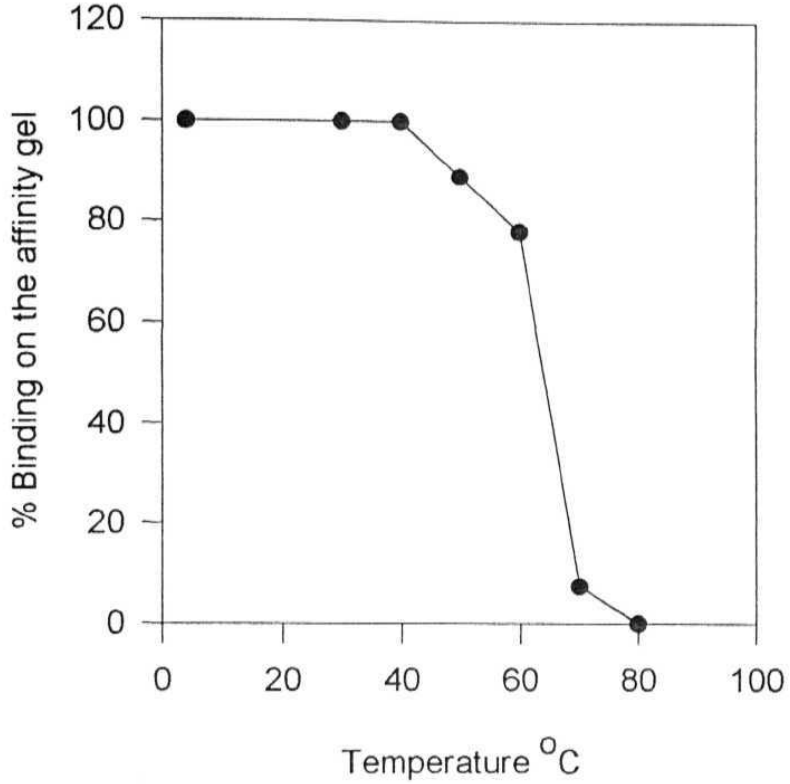


Figure 5A

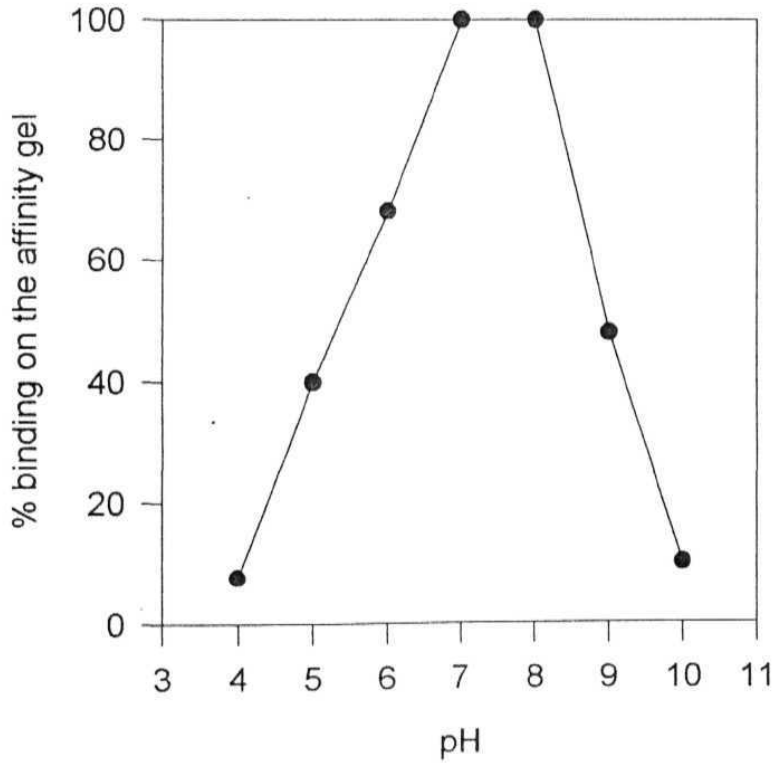


Figure 5B

Figure 6

A. Native PAGE pattern of the *unio* lectin

B. SDS-PAGE of the lectin

Lane 1 Standard high molecular weight proteins
Lane 2 Lectin purified on affinity gel
Lane 3 Affinity purified lectin eluted through Superose-12 FPLC gel filtration column

C. SDS-PAGE of the lectin and Periodic acid Schiff's staining.

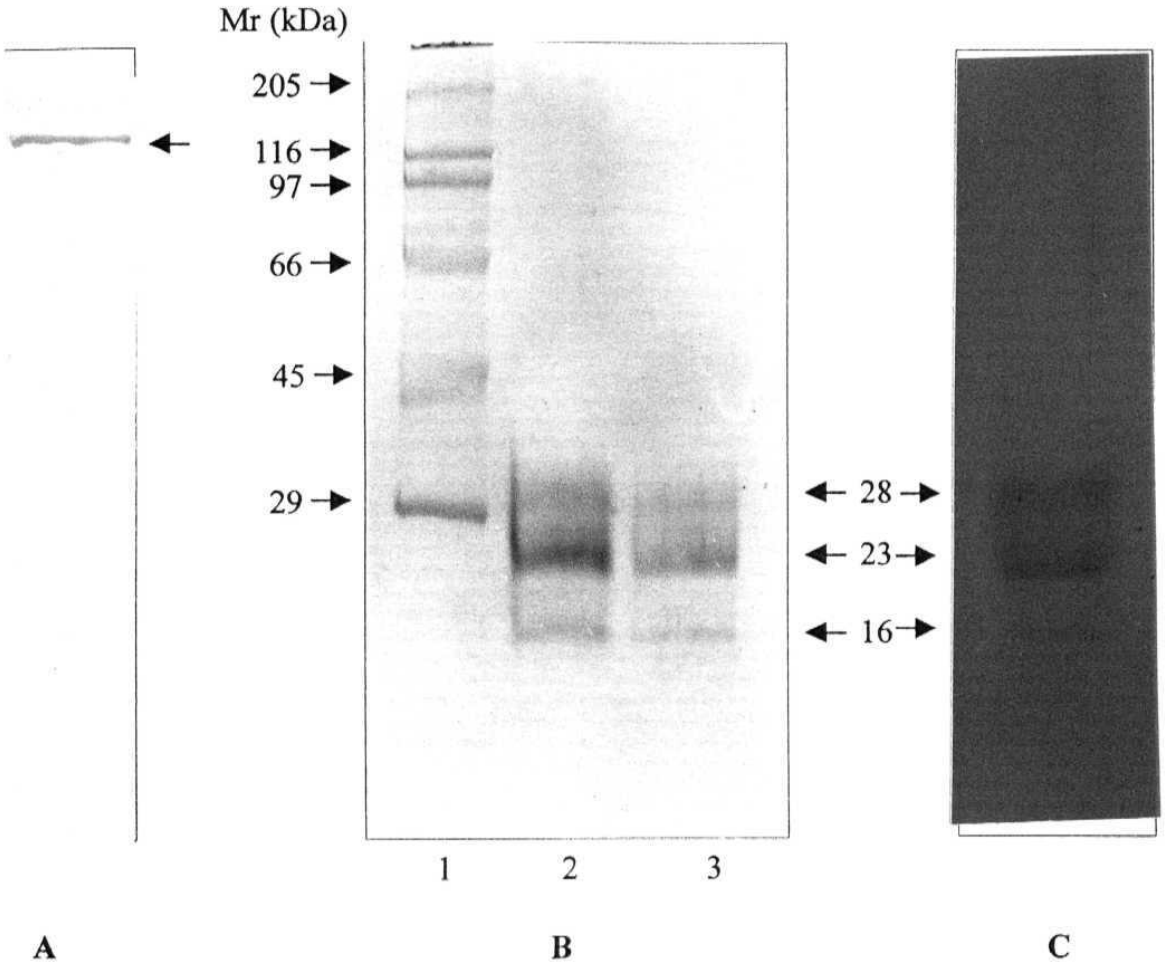


Figure 6

Figure 7

Carboxymethylation of the lectin (separation of subunits)

After carboxymethylation and denaturation, lectin was applied on Biogel P-60 gel filtration column (1.4 x 86 cm) equilibrated with 1% SDS. 2ml fractions were collected at a flow rate of 8 ml/hour.

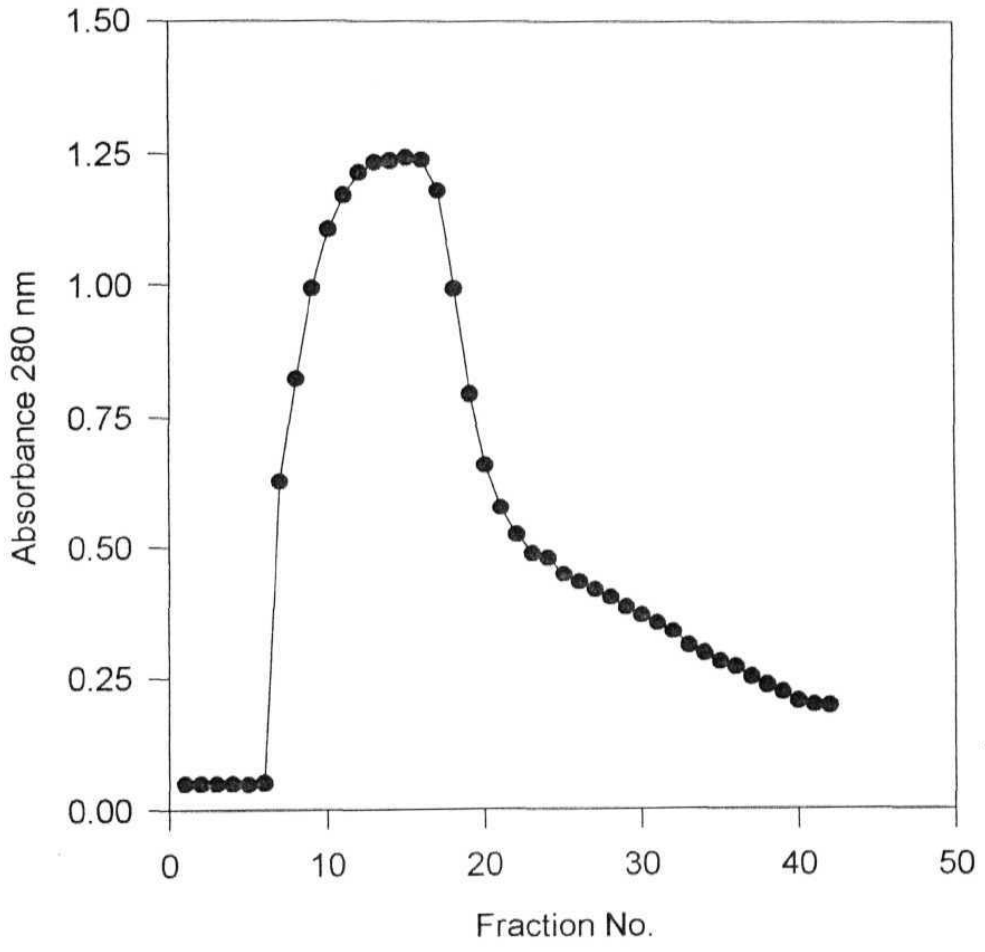

1.50
1.25
1.00
0.75
0.50
0.25
0.00
Absorbance 280 nm
0
10
20
30
40
50
Fraction No.

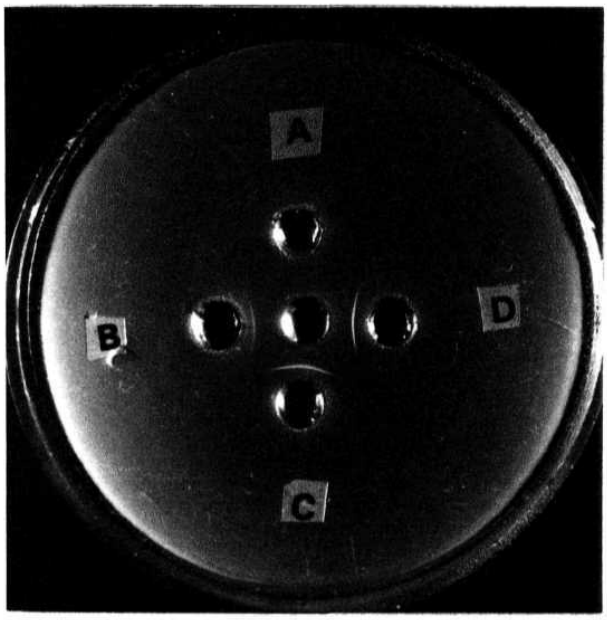
Figure 7

Figure 8

A. Immunodiffusion of the purified lectin

Central well	20µl antiserum
Well A	saline
Well B	4µg of lectin
Well C	6µg of lectin
Well D	8µg of lectin

B. Western blot analysis



A

B

Figure 8

Table 5. N-terminal sequence determination of the lectin

STAGE	RESIDUES
I cycle	Valine and proline
II cycle	Histidine and lysine
III cycle	Arginine and histidine

Table 6: Amino acid composition of the lectin

Residue	gAA/100g protein	No of residues/mole
Asparatic acid	12.9	118
Threonine	3.16	33
Serine	4.7	57
Glutamic acid	9.12	75
Proline	4.76	52
Glycine	1.67	31
Alanine	3.23	48
Valine	4.62	49
Isoleucine	2.21	21
Leucine	7.97	74
Tyrosine	1.28	8
Phenylalanine	2.12	15
Lysine	4.2	34
Histidine	2.7	21
Arginine	3.6	24

Cysteine and methionine were not detected.

Tryptophan was not determined.

Asparatic acid includes asparagine and glutamic acid includes glutamine.

Number of residues is converted to the nearest integer.

DISCUSSION

From the present work, it is clear that the fresh water mussel, *unio* contains a lectin that agglutinates only pronase treated rabbit erythrocytes whose activity is specifically inhibited by lactose. The protein was purified to homogeneity on the affinity gel. Affinity chromatography is the method of choice for the purification of several lectins from animals and in particular from invertebrates. Some of the sialic acid binding lectins from invertebrates such as *Carcinoscorpius rotunds cauda* [Bishayee and Dorai, 1980; Dorai *et al,* 1981], *Limax Flaws* [Miller *et al.,* 1982], *Limulus polyphemus* [Noguchi, 1903], etc., have been purified by affinity chromatography. Another lectin from the invertebrate, *Anguilla anguilla* (eel), has been purified to homogeneity on 1-*O*-α-fucosyl-polyacrylamide gels and eluted with L-fucose [Horejsi and Kocourek, 1978]. *Helix pomatia* (snail) lectin was affinity purified on a N-acetylgalactosamine-Sepharose gel and specifically eluted with N-acetylgalactosamine [Vretblad *et al,* 1979].

Affinity purified lectin eluted as a single peak on Biogel P-150 gel and also from FPLC gel filtration column. It has a native molecular mass of 105 kDa.

The lectin was found to contain about 5% neutral sugars. Strong binding of the lectin to Con A-Sepharose gel further substantiates its glycoprotein nature. All glycoprotein lectins have been shown to interact with Con A via multiple terminal non-reducing and 2-*O*-substituted α-mannopyranosyl units [Bessler and Goldstein, 1973, Goldstein *et al,* 1974].

When the lectin was analyzed on native PAGE only a single band was detected indicating its homogeneity. However, in SDS-PAGE it dissociates into three bands with molecular masses 28, 23 and 16 kDa.

Preliminary N-terminal amino acid analysis of the lectin by DAB1TC method indicated the possible presence of two amino acids at the amino terminal end of the lectin. Two amino acids were found in the second and third cycles also. This suggests that the lectin be possibly composed of two polypeptide chains although three bands were noticed in SDS-PAGE. Since the three bands were reactive with the lectin antibody it is suggested that the third band is also a part of the lectin. It is possible that the third subunit is either blocked or may be arising due to the anomalous behaviour of the lectin in SDS-PAGE (glycoproteins are known to have abnormal mobilities on SDS-PAGE). Inclusion of the protease inhibitor, phenylmethylsulfonyl fluoride, during the purification of the lectin did not alter the behavior of the lectin in SDS-PAGE and the subunits could not be separated after carboxymethylation and gel filtration on Biogel P-60.

N-terminal analysis of the eel lectin showed serine and alanine [Springer and Desai, 1971] indicating that this lectin has two dissimilar subunits. End group analysis of the Indian horseshoe crab lectin (limulin) revealed leucine to be the sole N-terminal amino acid [Dorai *et al.*, 1981]. The available evidences suggest that the lectin be possibly made of 2a and 2(3 chains as the amino terminal revealed only two amino acids in the first three cycles. The precise subunit nature of the *unio* lectin can be resolved after analyzing the peptide map analysis and sequence of the lectin, which is beyond the scope of the present investigation.

Amino acid analysis of the lectin showed high contents of acidic and hydrophobic amino acids. The lectin has neither cysteine nor methionine residues. The snail lectin also contains a high preponderance of acidic and hydroxylic amino acids and a large proportion of proline residues [Hammarstrom and Kabat, 1969; Ishiyama *et al.*, 1974]. *Limax flavus* lectin [Miller *et al*, 1982] and the Horseshoe crab lectins, Carcinoscorpin and limulin, also contain high contents of acidic amino acids [Dorai *et al.*, 1981a; Roche and Monsigny, 1974].

Four lines of evidence suggest that the protein purified in this study is a hemagglutinin. First, it agglutinates pronase treated erythrocytes, and second, its activity is inhibited by lactose and it can be affinity-purified on a biospecific adsorbent like Sepharose-lactose gel. Third, it is also a glycoprotein like other invertebrate lectins and fourth, it is made of more than two subunits.

There has been a report on the isolation and partial characterization of a galactose specific lectin from a mollusc *Pomacea flagellata* [Espinosa and Lozano, 1997] whose properties are different from the new lectin isolated by us.

CHAPTER III

CHEMICAL MODIFICATION STUDIES OF THE *UNIO* LECTIN

INTRODUCTION

Chemical modification of a protein is one of the widely used tools to modify the side chains of the amino acids in proteins. More often these studies are carried out with native proteins under non-denaturing conditions as it helps in analyzing the activity of the modified protein. Loss of biological activity of the protein upon modification of a specific amino acid reveals its importance. Precise localization of an amino acid residue in the active site of a protein can be analyzed by protein engineering. Combined use of both the techniques (chemical modifications and protein engineering) can be extremely powerful.

The extent of modification can be determined by characterizing a modified protein, if the modifying reagent has unique properties such as spectral absorption or radioactivity, 5,5'-dithiobis(2-nitrobenzoate), tetranitromethane, 2-hydroxy-5-nitrobenzyl bromide, ninhydrin, 2,4,6-trinitrobenzenesulfonic acid and diethylpyrocarbonate are some of the reagents (with spectral properties) used for establishing the stoichiometry of the reaction. In case of photo oxidation, the extent of modification can be determined by the loss of the amino acid modified (determined by amino acid analysis).

Chemical modifications of active-site amino acid side chains will alter the biological properties of the protein, if they contribute to it. Chemical modifications have been carried out on several plant lectins to identify the involvement of amino acids in lectin binding. Modification carried out in presence of the binding sugar and analysis of the results of such modifications would further substantiate the involvement of specific amino acid residues in the sugar-binding site of the lectins. Some of the modifications

cause **major** conformational changes in the protein and changes in biological activity are reflections of such changes. Certain chemical modifications are reversible which would specify the role of specific amino acids in the biological activity. For lectins, the affects of modification can be analyzed by the use of hemagglutination assay and affinity chromatography.

Citraconic anhydride, 2,4-dinitrofluorobenzene, acetic anhydride are some of the reagents used to modify available amino groups. When 2,4-dinitrofluorobenzene is used in the modification, α-NH_2 groups, histidines and cysteines are also modified [Bunning *et al,* 1990]. Citraconic anhydride modifies tyrosine apart from lysine amino groups [Shetty and Kinsella, 1980].

Arsanilic acid, N-acetylimidazole and tetranitromcthane are generally used to modify tyrosine residues. Use of arasanilic acid or N-acetylimidazole [Riordan and Vallee, 1972] results in the modification of lysines and histidines in addition to tyrosines.

N-Bromosuccinimide (NBS) or 2-hydroxy-5-nitrobenzyl bromide can be used to modify tryptophan residues but the use of NBS results in the modification of histidine, cysteine and methionine in addition to tryptophan [O'Gorman and Matthews, 1977]. Use of 2-hydroxy-5-nitrobenzyl bromide at low pH does not alter other amino acids [Horowitz and Heller, 1974].

Site-specific modification of arginine is difficult to achieve with the reagents available since it acts as a general anion recognition site in proteins [Riordan *et al.,* 1977]. It is relatively easy to obtain group specific modification. 1,2-cyclohexanedione [Suckau, 1992], phenylglyoxal [Krell *et al,* 1995] and 2,3-butanedione [Epperly and Dekker, 1989] can be used to modify arginine residues.

Modification of histidine residues can be achieved by using diethyl pyrocarbonate and other amino acids affected are tyrosine and lysine [Dumas and Raushel, 1990]. In case of photo oxidation, in addition to histidines, the other residues modified are cysteine, methionine, cystine and tryptophan [Funakoslii *et al.*, 1990].

MATERIALS AND METHODS

MATERIALS

Diethylpyrocarbonate, citraconic anhydride, 2,4,6-trinitrobenzenesulfonic acid, N-acetylimidazole, 2-hydroxy-5-nitrobenzyl bromide, 1,2-cyclohexandione were obtained from Sigma Chemical Company, St. Louis, MO, USA. Sephadex G-50 was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Lactose was obtained from Loba Chemie, Mumbai, India. Rabbit erythrocytes were collected from the University animal house. All other chemicals were purchased from reputed firms.

METHODS

Chemical modifications of the purified *unio* lectin

In order to identify the role of specific amino acids in lectin activity, purified *unio* lectin was subjected to chemical modifications using various group specific reagents.

Lysine modification

The side chain amino groups of lysine residues in the lectin were modified by the addition of citraconic anhydride [Dixon and Perham, 1968]. 4 mg/ml of lectin in 0.05M borate buffer pH 9.5 was allowed to react with a 400 fold molar excess of citraconic anhydride for 1 hour at 4°C. Modified lectin was separated from excess reagent by loading the reaction mixture on a Sephadex G-50 (1 x 25cm) gel filtration column pre-equilibrated with 0.05M borate buffer pH 9.5.

The extent of modification was estimated by using 2,4,6-trinitrobenzenesulfonic acid (TNBS) [Habeeb, 1966]. Modified lectin was tested for its agglutinating activity and

binding ability on the affinity gel. Control sample (native lectin) was processed under the same conditions as described above with out the addition of the modifying reagnet.

Tyrosine modification

Acetylation of tyrosine side chain phenoxy groups was done at room temperature by incubating the lectin (4mg/ml in 0.01 M Tris-HCl buiTer pH 7.5) with 60 fold molar excess N-acetylimidazole for 1 hour The modified lectin was separated from excess reagent by passing the mixture on a G-50 column equilibrated with the same buffer. Lectin containing fractions were analyzed for extent of modification. Agglutinating activity, binding ability on affinity gel and the number of residues modified were determined [Riordan *et al.*, 1965]. The modified lectin was incubated with hydroxylamine (0.5M) for 2 hours in order to reverse the *O*-acetylation of tyrosine residues and the increase in absorbance at 278nm was measured. Using $\Delta\epsilon 278=1160$ per mole (molar extinction coefficient of *O*-acetyltyrosine), the number of tyrosine residues modified was calculated.

Tryptophan modification

Tryptophan residues were modified using 2-hydroxy-5-nitrobenzyl bromide [Horton & Koshland, 1972J. To 4mg of lectin dissolved in 1 ml of 0.18M acetic acid pH 2.7, five 20µl portions of 2-hydroxy-5-nitrobenzyl bromide solution (50mg/ml in dry acetone) were added and incubated for 1 hour at room temperature. The pH was maintained below 3.0 by the addition of acetic acid. The modified lectin was separated from the reaction mixture by gel filtration on G-50 column The lectin containing fractions were pooled and dialyzed extensively against distilled water at 4°C and checked for the

extent of modification, agglutinating activity and binding percentage on the affinity gel.

Arginine modification

Arginine residues were modified using 1,2-cyclohexanedione [Pathy & Smith, 1975]. 4mg of lectin was dissolved in 200µl of 0.2M sodium borate buffer pH 9.0 containing 0.05M 1,2-Cyclohexanedione. The reaction vessel was flushed with N_2 and incubated at 37°C for 4hours in dark. The reaction was terminated by the addition of 1 ml of 5% (v/v) acetic acid. To remove the excess reagent, the mixture was dialyzed against 5% acetic acid followed by 5% acetic acid containing 0.5% NaCl. The modified lectin was checked for its binding ability on the affinity gel. Control was processed in the same manner except that the modifying reagent was not added.

Histidine modification

To modify the imidazolc side chains of histidine residues, 27µl of DEPC (10µl of DEPC diluted to 600µl with distilled ethanol) was added in aliquots at several time intervals to 1 mg/ml of the lectin in PBS pH 7.0 and incubated at room temperature for 120 min. The reaction was stopped by the addition of 20mM histidine (in PBS pH 7.0). Modified lectin was separated from the reaction mixture by applying on G-50 gel filtration column equilibrated with PBS pH 7.0 buffer. The absorbance was measured at 250nm and 280nm. The number of histidine residues modified was estimated by taking $\varepsilon 250$ as $1.6 \times 10^3\ M^{-1}.cm^{-1}$. Modification of histidine residues was carried out at various time intervals (20, 40 and 60 min). Hemagglutinating activity and binding ability were determined for the modified lectin.

Modification of the lectin preincubated with 0.1M lactose for 16 hours was also carried out to further substantiate the role of histidine residues in the lectin binding.

Modification was reversed by treating the modified lectin with 0.05M hydroxylamine at room temperature for 10 min or by incubating the sample at 37°C for 2 hours [Anderson and Ebner, 1979].

Agglutinating activity and binding ability on the affinity gel was tested for the modified lectin. Both the activities were also tested for the modified lectin after the reversal of the modification. Only binding ability was tested after modification of the lectin preincubated with 0.1M lactose. Additionally immuno-reactivity of the modified lectin (60 and 120 min) was tested by immunodiffusion.

RESULTS

Modification of the *unio* lectin with citraconic anhydride for 1 hour resulted in the modification of 12 amino groups per molecule. There was 45% binding of the lectin to affinity gel at 4°C and it did not alTect its hemagglutinating activity.

27 tyrosine residues were acetylated using N-acetylimidazole and modification did not affect the agglutinating activity and binding ability of the lectin to affinity gel at 4°C.

Only 5 tryptophan residues were modified using 2-hydroxy-5-nitrobenzyl bromide. There was no change in the hemagglutinating activity and binding ability of the modified lectin.

Arginine modified sample showed complete binding to the alTinity gel at 4°C (extent of modification not determined). As arginine modifications are stable only at low pH, binding ability to affinity gel was checked at acidic pH. It bound to the gel under acidic conditions and it could not be eluted with 0.2M lactose and eluted only on increasing the pH. Similar results were obtained when unmodified lectin (control) was processed under same conditions. These results are summarized in Table 7.

Since the modification of lysine, tyrosine, tryptophan and arginine residues did not alter the properties of the lectin, protection experiments for these residues have not been carried out.

Histidine modification resulted in the loss of activity of the protein and 29.24 residues were modified after 120min. After 20 min of incubation 4 histidine residues were modified, binding and hemaggiutinating activities were 55% and 58% respectively. After 40 and 60 min of modification the hemaggiutinating and binding abilities

dropped to nearly 25%. The lectin almost lost 95% of the both the activities at the end of the 120 min of incubation.

Hydroxylamine reversal of the 20, 40 and 60 min incubation resulted in regaining of only 60% of the activities. Hydroxylamine reversal of the 120 min modification resulted in regaining of 37% of binding ability to affinity gel and 27% of agglutinating activity.

Modifications performed on the lectin (preincubated with 0.1M lactose for 16 hours) for 20, 40 and 60 min resulted in the modification of 3.18, 5.8 and 6.8 histidine residues respectively. The binding ability of the modified lectin to the affinity gel was only 61%. The number of histidine residues modified after 120 min of incubation was 18 and binding ability was 5%. The results of the histidine modification and reversal of modification, binding ability and agglutinating activity of the modified lectin are shown in table 8. The number of histidine residues modified with and without lactose protection is shown in Fig. 9A.

After the hydroxylamine reversal of the 20, 40 and 60 min of histidine modification, the binding ability to affinity gel was 61%. 50% of the binding ability was regained after the reversal of 120 min modification.

The histidine modification of the lectin (modification performed at 60 and 120min in the absence of the lactose protection) however did not alter the immunological property of the lectin (Fig 9B).

Table 7. Chemical modifications of the *unio* lectin. Agglutinating activity and percentage binding of the modified lectin to the affinity gel

Group modified	Reagent used	Number of residues modified	Hemagglutinating activity	% Binding to affinity gel
Lysine	Citraconic anhydride	12	+	45
Tyrosine	N-Acetyl imidazole	27	+	100
Tryptophan	2-Hydroxy-5-nitrobenzyl bromide	5		100
Histidine	Diethyl pyrocarbonate	29.4	No activity	No binding
Arginine	Cyclohexane-1,2-dione	ND	ND	100

ND — NOT DETERMINED

Table 8. Time course of histidine modification of the lectin in the presence and absence of lactose protection. Hemagglutinating activity and binding ability of the modified lectin to the affinity gel.

Time of incubation in min	Number of residues modified	Hemagglutination activity		Binding ability to affinity gel	
		With out reversal	After reversal	With out reversal	After Reversal
20	4.0 (3.18)	58.19	67.59	55 (61)	54 (61)
40	11.48 (5.8)	42.7	59,7	22 (61)	61 (61)
60	17.70 (6.8)	22.5	45.45	25 (61)	45 (61)
120	29.24 (18)	5	27,54	5 (5)	37 (50)

Number in parenthesis indicate the results of the modification carried out in presence of lactose

Figure 9.

A. Number of histidine residues modified with and without lactose protection.

B. Immunodiffusion of the native and the histidine modified lectin tested with anti-*unio* lectin anti serum.

Central well	20 μl of anti sera
Well A	unmodified lectin
Well B	lectin after 60 min of histidine modification
Well C	lectin after 120 min of histidine modification

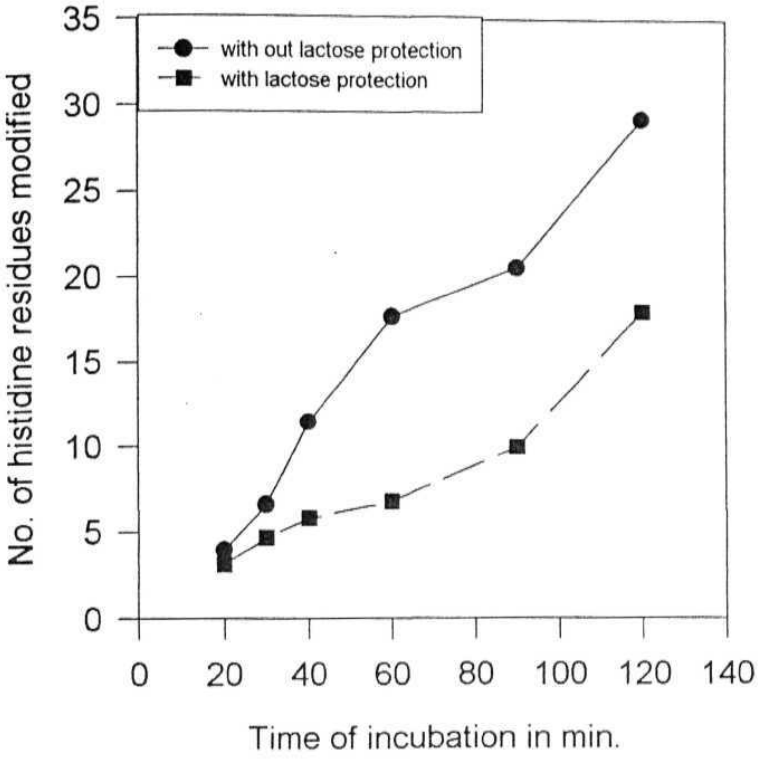


Figure 9A

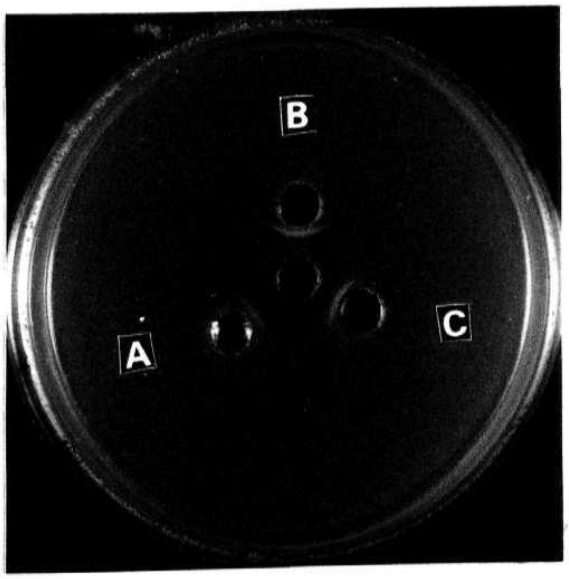


Figure 9B

DISCUSSION

Modification of several plant lectins using various group specific reagents showed the involvement of certain amino acids in the sugar binding, If the modification is carried out in the presence of the binding sugar and if a protection of modification is observed it is suggestive of the involvement of that amino acid in the sugar binding of the lectin, Certain modifications are also reversed by hydroxylamine treatment.

Modification of the lysine, tyrosine, tryptophan and arginine residues in the lectin using specific reagents did not alter its Agglutinating activity and binding ability to the affinity gel (except for lysine) indicating that these amino acids are not involved its sugar binding activity. Histidine residues, when modified resulted in the 95% loss of the activity of the lectin. Presence of the lactose resulted in partial protection of the binding ability of the lectin.

Reversal of the modification, both in the presence and absence of lactose, using either hydroxylamine or by incubation at 50°C resulted in the regaining of 60% activity (approximately). This indicates that the residues are not only involved in the sugar-binding site but also in the maintenance of the active conformation of the lectin.

Further immunodiffusion experiments of the histidine-modified lectin clearly indicated that it retained the ability to react with the antibodies raised to the native lectin. Though the histidine modification resulted in the loss of biological activity of the lectin, there is no alteration in its immunological property.

Histidine residues were found to be present in the sugar-binding site of snake gourd seed lectin (SGSL)[Komath *et al*, 1998]. Treatment of SGSL with diethyl pyrocarbonate for 2 hours resulted in the complete loss of agglutinating activity. In the

absence of the sugar, modification of 3 and 4 residues decreased the hemagglutinating activity of the SGSL by 35% and 56% respectively. In presence of the sugar (galactose), modification of 3 and 4 residues resulted in the loss of only of 6% and 35% of the activity respectively.

From literature it is evident that the lectins purified from a variety of sources showed the involvement of different amino acids in their sugar binding sites. Most of the lectins such as those purified from potato [Ashford *et al.*, 1981], wheat germ [Rice and Etzler, 1975], soyabean [Liener and Wada, 1956], lentil [Vancurova *et al.*, 1976], pea [Cermakova, 1976] and Indian lablab beans [Siva Kumar, 1999] have aromatic amino acids in their sugar binding sites. These results were further confirmed by sugar protection experiments.

Carboxy groups have been shown to be involved in sugar binding in Con A [Massing and Goldstein, 1972]. Activity of a galactose specific lectin from baker's yeast was lost when histidine side chains were modified. However, protection by oligosaccharide inhibitors was not observed, indicating the absence of histidyl residues in the active site of this lectin [Kundu *et al.*, 1987]. The involvement of histidine residues in the saccharide-binding site of B-chain of carboxy terminal region of ricin E [Yamasaki *et al*, 1989 and Hatakeyama, 1990] and in N-acetylneuraminyl-lactose-wheatgerm agglutinin [Wright, 1990] has been shown,

Results of the purification, characterization and chemical modifications of the lectins are accepted for publication in International Journal of Biochromatography [Radha and Siva kumar, 2002].

CHAPTER IV

ISOLATION AND AFFINITY PURIFICATION OF MANNOSE 6-PHOSPHATE RECEPTOR (MPR 300) PROTEIN FROM *UNIO*

INTRODUCTION

Lysosomal enzymes synthesized on the endoplasmic reticulum are transported through Golgi complex and endosomal membranes to lysosomes. These enzymes share a common pathway with other proteins of secretory pathway until their exit from Golgi complex, as other proteins do not require any specific sorting signals they are carried along the secretory pathway by bulk flow (pfeffer *et al.,* 1987).

UDP-N-acetylglucosamine 1-phospho transferase [Reitman and Kornfeld, 1981] and α-N-acetyl glucosaminidase [Varki and Kornfeld, 1980; Waheed *et al,* 1981] are the two enzymes involved in the generation of mannose 6-phosphate recogntion marker on the enzymes destined for lysosomes.

Two receptors, MPR 300 and MPR 46, are involved in the recognition and transport of the mannose 6-phosphate containing lysosomal enzymes to the lysosomes. These receptors belong to the category of P-type lectins and they show specificity towards mannose 6-phosphate containing ligands [Drickamer and Taylor, 1993]. Based on their molecular masses and requirement for cations for binding the ligands /// *vitro,* the receptors are designated as MPR 300/CI MPR (cation independent receptor with molecular mass 300 kDa) and MPR 46/CD MPR (cation dependent receptor with molecular mass 46 kDa). MPR 46 requires metal ions like Mg^{+2} or Mn^{+2} for binding the ligands *in vitro.* Both the receptors are immunologically distinct.

Two binding sites per polypeptide chain have been found for MPR 300 by equilibrium dialysis, whereas MPR 46 contains only one mannose 6-phosphate binding site per polypeptide chain. The ligand binding sites for mammalian MPR 46 have been identified initially by chemical modification studies [Stein *et al.,* 1987]. Subsequent

molecular biological studies have precisely identified the ligand binding sites for both MPR 46 and MPR 300 in mammals [Hille-Rehfeld, 1995].

Mammalian MPR 300 has been shown to be a multifunctional protein. It is also referred to as IGF-II receptor since it binds insulin-like growth factor-II in addition to mannose 6-phosphate containing ligands [Roth *et al.,* 1987; Tong *et al.,* 1988; Waheed *et al.,* 1988; Kiess *et al.,* 1988]. The binding of IGF-II exerts an allosleric effect or stearic hindrance on the binding of mannose 6-phosphate containing lysosomal enzymes to their independent binding sites and *vice versa.* It has been shown to **bind** several other ligands such as non-lysosomal enzyme human DNase I [Cacia *et al,* 1998J, leukemia inhibitory factor (L1F) [Blanchard *et al.,* 1999J, urokinase receptor [Nykjaer *et al.,* 1998], proliferin [Lee and Nathans, 1988] and retinoic acid [Rang *et al.,* 1997]. MPR 300 may be involved in the clearance and activation of polypeptides TGFβ1 and TGFβ2 [Dennis and Rifkin, 1991].

Functions of MPRs

MPRs are involved in the sorting and transport of the lysosomal enzymes. They are involved in intracellular sorting of newly synthesized lysosomal enzymes i.e., segregation from the secretory pathway and transport to the endosomal compartment. Export of minor fraction of newly synthesized lysosomal enzymes into the secretions of the cells is a specialized function of MPR 46 and the internalization of the exogenous mannose 6-phosphate containing ligands is an exclusive function of MPR 300. Both MPRs are involved in intracellular sorting of the ligands but MPR 300 is more efficient than MPR 46. The dominant role of MPR 300 in intracellular sorting of lysosomal enzymes had raised a question about the physiological relevance of MPR 46

in intracellular sorting *in vivo.* Generation of transgenic mice that express normal levels of MPR 46 and deficient for MPR 300 helped to understand its function.

Primary structure of Mannose 6-phosphate receptors

Both MPRs are type I integral membrane proteins which span the membrane once and expose their N-terminal, ligand binding domain into the lumen of membrane vesicles or to the cell surface [Hille-Rehfeld, 1995; Pohlmann, 1996].

cDNA cloning of MPR 300 showed 15 internal repeats within the extracytoplasmic domain (Morgan, *et al,* 1987). These repeats share 14-28% amino acid sequence homology. The 13th repeat (starting with no. 1 at the receptor's amino terminus) contains a unique insert which is homologous to the collagen binding domain of fibronectin type II [Hynes, 1985].

The extracytoplasmic domain of MPR 46 does not possess internal repeats, but resemble the consensus sequence of the repeated units of MPR 300 with 14-37% homology to individual repeats of MPR 300 [Dahms *et al,* 1987; Lobel *et al.,* 1988]. The position of cysteine residues that are most likely involved in disulfide bond formation is well conserved among the internal repeats of MPR 300 and also within the extracytoplasmic domain of MPR 46 [Lobel *et al.,* 1988]. This finding underlines the importance of disulfide bonds for the tertiary structure of both MPRs.

MPR 46 is a highly conserved protein with 93% over all homology from mouse to man and has completely identical amino acid sequence within the cytoplasmic domain in these species. MPR 300 has about 82% over all homology from mouse to man and its cytoplasmic domain shows considerable variability (80% homology from mouse to man), harbors functionally important stretches of highly conserved amino acids such as

the internalization signal, a putative G-protein binding region and two casein kinase-II phosphorylation sites [Mac Donald *et al,* 1988]. Isolation and characterization of a partial cDNA clone for fish MPR 300 suggested that the luminal domain has conserved repetitive cassette structures in the vertebrates [Udaya lakshmi *et al,* 2000].

Subcellular localization

The gross sub cellular distribution of mannose 6-phosphate receptors is in agreement with the model that they recycle between *trans*-Golgi network and endosomes for sorting of newly synthesized lysosomal enzymes [Dickson *et al.,* 1983; Brown and Farquhar, 1984] where as lysosomes are essentially devoid of MPRs. The membranes of the endoplasmic reticulum and the Golgi stack, where biosynthesis and maturation of the receptors occur, contain only small amounts of both MPRs. They are also present in clathrin-coated vesicles [Sahagian and Steer, 1985]. The plasma membrane of various cell types contains, on an average 10% of both MPRs as shown by immunocytochemistry and by binding of radiolabelled antibodies to cell surface.

Recent immunocytochemical studies have established MPR 300 as a marker to distinguish endosomes (MPR 300 positive) from lysosomes (MPR 300 negative) [Sahagian and Neufeld, 1983; Geuze *et al.,* 1985, Brown *et al,* 1986, Griffiths *et al,* 1988; Geuze *et al.,* 1988].

MATERIALS AND METHODS

MATERIALS

unto animals were purchased locally. (*O*)-phosphonomannan Y-2448 was a generous gift from Dr. M. E. Slodki, USDA, Peoria, Illinois, USA. Sepharose 6B was purchased from pharmacia fine chemicals, Uppasala, Sweden. Mannose 6-phosphate, glucose 6-phosphate, divinyl sulfone, sodium deoxycholate, high molecular weight markers, bicinchoninic acid and Triton X-100 were obtained from Sigma Chemical Company, St. Louis, USA. Secondary antibody (gaot anti-rabbit IgG conjugated to alkaline phosphatase), BCIP/NBT were purchased from Bangalore Genei. All other chemicals and reagents used in the study were purchased from reputed chemical firms.

METHODS

Hydrolysis of *O*-phosphonomannan Y-2448

This was carried out according to the method of Bretthauer *et al.* [1973]. 2.5g of *O*-phosphonomannan was suspended in 500ml of water in a screw cap bottle and left overnight in cold for swelling. 500mg of KC1 was added and the pH of the suspension was adjusted to 2.4 with acetic acid. The contents were then hydrolyzed in a boiling water bath at 100°C for 60 min. The suspension was cooled to room temperature and centrifuged at 10,000 rpm for 30min to remove any insoluble material. The clear supernatant was adjusted to pH 11 with saturated $Ba(OH)_2$. To this an equal volume of 95% ethanol was added and left overnight at 4°C. The precipitated phosphomannan (PM) core was dissolved in water and made gently acidic with acetic acid and dialyzed against water and lyophilized. To the supernatant which contains the PMP (pentamannosyl phosphate) an equal volume of ethanol was added and allowed to

stand for 1-2 hours on ice. The suspension was centrifuged at 10,000 rpm for 30 min and the pellet was redissolved in water with mild acidification and desalted by addition of Dowex 50 resin. The resin was removed and the solution lyophilized. About 1g of PM core and 300 mg of PMP were obtained from 2.5g of *O*-phosphonomannan.

Preparation of phosphomannan-Sepharose gel **(PM** gel)

Phosphomannan (PM) obtained by the hydrolysis of *O*-phosphonomannan was coupled to Sepharose 6B via divinyl sulfone. The methodology for activation and coupling are described in chapter 11 (in the place of lactose, 200mg of PM core was added to 10ml of gel).

Purification of MPR 300 protein

All operations were carried out at 4°C.

Preparation of acetone powder

100g of tissue *(unio* whole animal) was homogenized for 1 min in cold warring blender with 160ml of 0.5M $CaCl_2$ and lmM $NaHCO_3$. The homogenate was adjusted to pH 5.0 by the addition of cold 4N acetic acid and centrifuged at 10,000 rpm for 15min. Pellet obtained was resuspended in the same buffer and pH was adjusted to 5.0 by the addition of 4N acetic acid followed by centrifugation at 10,000 rpm for 15 min. Pellet was homogenized for 1 min in a cold warring blender with 600ml of chilled acetone (-20°C). The suspension was rapidly filtered through a buchner funnel using Whatmann 3mm paper. Reddish brown cake obtained was washed with 400ml of chilled ether (-20°C). The cake was dried under vacuum with occasional pulverization to remove ether. The dry powder obtained was stored at -70°C and used for the extraction of MPR300. At a given time about 1000g of tissue was processed.

Extraction of membrane proteins

100g of acetone powder was homogenized with 600ml of buffer A (50mM immidazole-HCl pH 7.0, 150mM NaCl, 0.5mM $CaCl_2$, 0.1 mM PMSF) and stirred overnight. Homogenate was centrifuged at 9,000 rpm for 20min. Pellet obtained was homogenized with 600ml of buflèr B (50mM sodium acetate pH 4.6 buffer containing 150mM NaCl and 0.5mM $CaCl_2$). The suspension was centrifuged at 10,000 rpm for 20 min and the pellet obtained was re-homogenized with buffer B followed by centrifugation. The pellet was homogenized with 600ml of buffer C (50mM immidazole-HCl pH 7.0, 5mM sodium β-glycerophosphate, 150mM NaCl buffer). Sodium deoxycholate and Triton X-100 were added to a final concentration of 0.1% and 1% respectively. Proteins were extracted from the membranes by stirring the suspension overnight. The suspension was centrifuged at 4000 rpm for 20min and the supernatant (membrane extract) contained MPRs. Volume of the supernatant was measured and EDTA was added to the final concentration of 2mM. The supernatant was stirred for 1hour and centrifuged at 9000 rpm for 45 min. The clear supernatant was used for *V.* purifying the receptor.

Affinity chromatography on PM gel

Clarified membrane protein extract was applied to the PM gel (2.6 x 6 cm) that was pre-equilibrated with buflèr C containing 0.05% Trition X-100 and 2mM EDTA (buffer D). The column was washed thoroughly with the same buffer. Bound protein was eluted with 20ml of buflèr D containing 5mM mannose 6-phosphate. Aliquots of the elutions were analyzed on SDS-PAGE.

SDS PAGE

Aliquots of the protein containing fractions were TCA precipitated (TCA was added to a final concentration of 10%) and the TCA pellet was neutralized with 10µl of 2M Tris. 7.5% gels were run under reducing conditions [Laemmli, 1970] and stained by silver staining method.

Protein estimation

Protein was estimated using bicinchoninic acid reagent and BSA was used as the standard.

Raising antibodies to the purified protein

Antibodies were raised to the purified MPR300 protein in a rabbit as described in chapter II.

Western blot analysis

Immuno reactivity of the antibodies raised to the purified receptor protein was checked by the western blot analysis. This was carried out according to Towbin *et al.* [1979]. Electrophoresis was carried out on a 7.5% SDS-PAGE and the proteins were transferred onto a nitrocellulose membrane. Primary antibody was used at a dilution of 1:500 and secondary antibody conujugated to alkaline phosphatase (1:500 dilution) was used. BCIP/NBT was used as the substrate. Details are given in chapter II.

RESULTS

In the present study MPR 300 was purified by affinity chromatography on PM-Sepharose from the membrane extracts of the *unio* whole animal tissue. It was purified to homogeneity from the whole animal extract in the presence of 2mM EDTA. From 100g of the acetone powder about 80µg of purified receptor was obtained. On 7.5% SDS-PAGE, a single protein band of molecular mass 300 kDa was obtained as detected by the silver staining method (Fig. 10A) The purified protein was immunologically detected by western blot analysis using antibodies raised against the purified protein (Fig. 10B).

Figure 10

A. SDS-PAGE of the purified *unio* MPR 300 protein

7.5% SDS-PAGE was carried out under reducing conditions and the gel was stained by silver staining method.

Lane 1. High molecular weight markers

Lane 2. Purified MPR 300 protein

B. Western blot analysis of the MPR 300 protein

Lane 1. High molecular weight markers

Lane 2. Purified MPR 300 protein

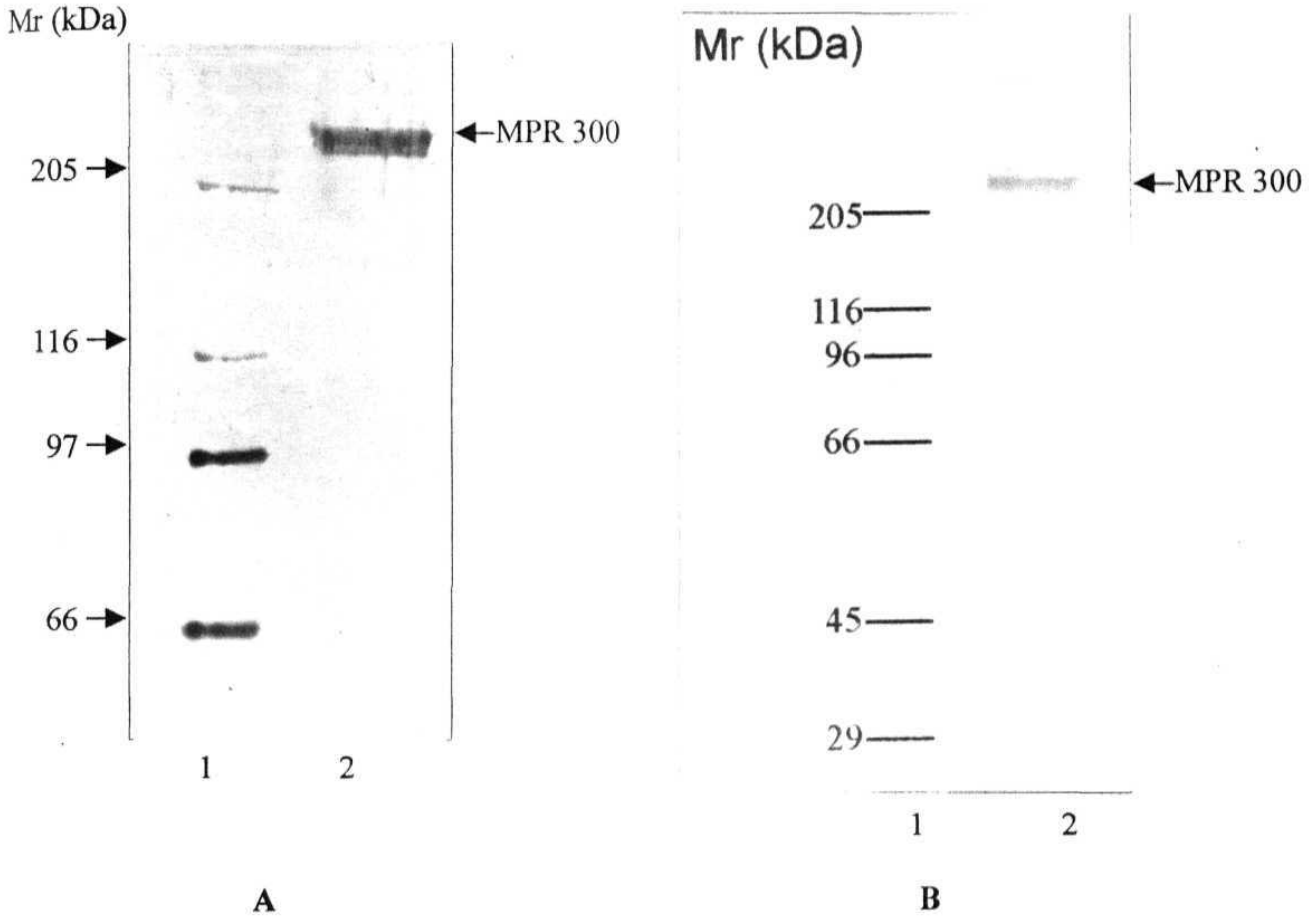

Mr (kDa)
205
116
97
66
MPR 300
1
2
A
Mr (kDa)
205
116
96
66
45
29
MPR 300
1
2
B

Figure 10

DISCUSSION

It is well established that two receptor proteins designated as MPR 300 and MPR 46 play a crucial role in the sorting and transport of lysosomal enzymes to lysosomes in vertebrates [Hille-Rehfeld, 1995]. Prior to 1995, very little information was available regarding the presence and evolution of these two receptors in the animal kingdom. By virtue of their affinity to phosphomannan, these receptors have largely been purified on phosphomannan coupled to cyanogen bromide activated Sepharose from bovine and human liver [Hoflack and Kornfeld, 1985]. Another simple affinity matrix consisiting of phosphomannan coupled to Sepharose activated by divinylsulfone was developed and used in the purification of both the receptor proteins from goat liver [Udaya lakshmi and Siva kumar, 1996].

The first biochemical and immunological evidence for the presence of both receptor proteins in other non-mammalian vertebrates such as the reptiles, amphibians and fish has also been obtained [Siva Kumar *et al.,* 1997, 1999]. Further the appearance of the MPR 300 in an invertebrate *unio* was established [Udaya lakshmi *et al.,* 1999].

Since these receptors show affinity to high mannose containing ligands they have been affinity purified largely employing PM gels. Only MPR 300 protein from mammals and frog has been shown to bind on another afTinity gel containing lysosomal secretions of *Dictyostelium discoideum* [Suresh and Siva Kumar 2001].

Antibodies raised to goat MPR 300 showed cross reactivity with *unio* MPR 300 and *vice versa* [Udaya lakshmi *et al.,* 1999]. This shows that the receptors among the mammals, non-mammalian vertebrates and in molluscs are immunologically related.

Three lines of evidence suggest that the receptor purified in the present study is indeed the putative MPR 300 protein. First, it can be readily purified by affinity chromatography on PM gel in the presence of EDTA. Second, it exhibits a typical molecular mass of 300 kDa similar to the receptors in other mammalian and non-mammalian vertebrates. Third, it reacts specifically with an antibody raised against the MPR 300 protein.

By virtue of its simpler structure it is hypothesized that MPR 46 protein is ancient when compared to MPR 300 in evolution. Recently, along with MPR 300 protein, the putative MPR 46 was also identified in molluscs [Siva kumar and von Figura, 2002]. Presence of these putative receptors remains to be established in other invertebrates such as arthropods and annelidae.

CHAPTER V

GLYCOSIDASES (a-MANNOSIDASE AND α-FUCOSIDASE)

INTRODUCTION

Lysosomes are acidic high-density intracellular organelles with unique membrane proteins. They contain enzymes such as proteases, glycosidases, nucleases, phosphotases and lipases that are responsible for the degradation of both internalized and endogenous macromolecules into simpler substances. In the transport of the lysosomal enzymes, mannose 6-phosphatc dependent pathway plays a major role but an independent pathway also exists [Neufeld and McKusick, 1983].

a-mannosidase, a-fucosidase and β-hexosaminidase are some of the lysosomal enzymes. a-mannosidase (EC 3.2.1.24) has been identified from different parts of the cell like endoplasmic reticulum, Golgi complex, lysosomes, sperm plasma membrane, etc. They show different specificities towards various substrates. Golgi mannosidase-I shows preference to α1,2-linked mannosyl residues and Golgi mannosidase-II shows little or no activity towards the oligosaccharide substrates containing α1,2-linked terminal mannosyl residues but cleaves α1,3-linked and α1,6-linked mannosyl residues. Lysosomal mannosidase shows greater activity towards linear than branched oligosaccharides. It is involved in the degradation of glycoproteins in lysosomes. a-mannosidase purified from rat sperm plasma membrane is thought to have a receptor like role in sperm-egg interaction during the fertilization process [Tulsiani *el al,* 1989]. a-fucosidase (EC 3.2.1.51), a lysosomal hydrolase, has been identified in tissues and body fluids in humans [Alhadeff et al., 1975]. α-Fucosidase is an enzyme involved in the metaboloism of several biologically active molecules containing L-fucose. In humans, the importance of this enzyme is mainly associated with neuro-visceral storage disease fucosidosis [Johnson and Alhadeff, 1991] characterized with mental

and motor retardation. This enzyme has also been found on the sperm membrane of tunicates and mammals [Aviles *el al.,* 1996]. In *unio* a nonglycosylated form of α-fucosidase (Mr 68 kDa) is present in the sperm plasma membrane. Another form of a-fucosidase that is glycosylated and having a molecular mass of 56 kDa has been purified from the seminal fluid [Focarelli *el al,* 1997]. Presence of this enzyme in the sperm membrane suggests that it may be involved in the sperm-egg interaction, the process that assures species-specificity in fertilization and triggers subsequent steps leading to successful fertilization. In the present study cx-mannosidase and a-fucosidase activities have been purified from *unio* and *in vitro* experiments were designed to study the interactions of α-fucosidase with the lectin and mannose 6-phosphate receptor.

MATERIALS AND METHODS

METHODS

Unio animals were purchased locally from animal suppliers. Mannosamine, L-fucose, Fucose derivatized agarose gel (fucosamine gel), p-nitrophenyl-α-mannopyranoside, p-nitrophenyl-α-fucopyranoside, p-nitrophenyl N-acetyl-β-D-glucosaminide and CNBr-Sepharose gel were purchased from Sigma Chemical Company, St. Louis, MO, USA. Sepharose 6B was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Biotin-labeled Con A was purchased from Bangalore Genei. Lactose was obtained from Loba Chemie, Mumbai, India. All other chemicals used in the study were obtained from reputed local firms. Affigel-10 was from Bio-rad laboratories, USA.

METHODS

Coupling of mannosamine to CNBr activated Sepharose gel

To 2.0 ml of CNBr-Sepharose gel, mannosamine (300mg/ml of the gel) was added and coupled according to the manufacturers instructions.

Assay for glycosidase activities

Aliquots of the extracts in 50mM sodium acetate buffer pH 5.0 in total volume of 500µl were incubated with 100µl of the substrate (p-nitrophenyl-α-D-mannopyranoside for a-mannosidase, p-nitrophenyl-α-D-fucopyranoside for a-fucosidase and p-nitrophenyl N-acetyl-β-D-glucosaminide for β-hexosaminidase). The reaction mixture was incubated at 37°C for 15 min. The reaction was terminated by the addition of 500µl of 0.2M Na_2CO_3 pH 9.0 bufTer and the absorbance was measured at

405nm. The activity of the enzyme was expressed as units/ml and calculated according to the formula given below

$$\frac{A_{405} \times 1}{15\text{min} \times V \times 18.5}$$

where A_{405} is the absorbance at 405nm, 15 is the time of incubation in min, V is the volume of the sample in ml and 18.5 is the extinction coefficient of p-nitrophenol.

1 unit of enzyme activity is defined as the amount of protein that liberated 1μmole of p-nitrophenol in 1 min. Specific activity is expressed as units/mg of protein.

PURIFICATION OF α-MANNOSIDASE

Unio whole animal tissue (50g) was homogenized with 5 volumes of 25mM Tris-HCl pH 8.0 (Tris buffer) and stirred for 3hours, followed by centrifugation at 10,000 rpm for 20 min. When the clear supernatant was assayed for glycosidases (α-mannosidase, a-fucosidase and β-hexosaminidase), all these enzyme activities were detected. It was applied on a DE-52 gel (2.6 x 10cm) pre-equilibrated with the Tris buffer. The gel was washed till the A_{280} reached zero. Elution was carried out with 0.2M NaCl in the same buffer and the absorbance was monitored at 280nrn. Aliquots of the eluted fractions were assayed for the enzyme activities. The active fractions were pooled and $(NH_4)_2SO_4$ was added to a final concentration of 1M and applied on a phenyl-Sepharose gel (2.5 x 3.5 cm) pre-equilibrated with Tris buffer containing 1M $(NH_4)_2SO_4$. Gel was washed till A_{280} was zero and the bound proteins were eluted with Tris buffer. Every alternate fraction was assayed for the enzyme activity.

Affinity chromatography of mannosnmine gel

Pooled phenyl-Sepharose eluates were dialyzed against 10mM NaH_2PO_4 pH 5,5 buffer and applied **to** mannosamine gel equilibrated with the same buffer. The **gel** was washed till A_{280} was zero and elution was carried out with 0.15 M mannose.

SDS-PAGE

10% SDS-PAGE was performed according to the method of Laemmli [1970] under reducing conditions and the protein was detected by silver staining.

Western blotting

Proteins were transferred onto nitrocellulose membrane according to Towbin *et al.* [1979] and the bovine lysosomal α-mannosidase antibody was used as the primary antibody. Details are given in chapter II

PURIFICATION OF α-FUCISODASE

Homogenization of the whole animal tissue

Unio whole animal tissue (50g) was homogenized with 5 volumes of Tris buffer and stirred for 3hours followed by centrifugation at 10,000 rpm for 20 min. The clear supernatant was assayed for a-fucosidase activity. Lectin was removed from **the** supernatant by passing the homogenate on Sepharose-lactose gel. To the flow through $(NH_4)_2SO_4$ was added to a final concentration of 1M and applied on phenyl-Sepharose gel.

Chromatography on Phenyl-Sepharose gel

Phenyl-Sepharose gel (2.5 x 3.5 cm) was pre-equilibrated with Tris buffer containing 1M $(NH_4)_2SO_4$. The column was washed till A_{280} was zero and the protein was

desorbed with the same buffer and assayed for enzyme activity. The active fractions were pooled, dialyzed against phosphate buffer pH 5.5.

Affinity chromatography on fucosamine gel

Phenyl-Sepharose eluates obtained above was applied to fucosamine gel equilibrated with the same buffer and the gel was washed till A_{280} was zero and the elution was carried out with 10mM fucose. Fractions were assayed for the enzyme activity and the homogeneity of the purified α-fucosidase was checked on SDS-PAGE.

Protein Estimation

Protein was determined according to Lowry's method using BSA as standard [Lowry *et al.*, 1951].

Electrotransfer and lectin blotting

Proteins separated on 10% SDS-PAGE were transferred to a nitrocellulose membrane according to the method of Towbin *et al.* [1979]. The membrane was blocked overnight in TBS (50 mM Tris-HCl pH 7.5, 150 mM NaCl) containing 2% BSA and washed thrice with TBS. For the specific detection of the glycan moieties, the membrane was treated with biotin-labeled Con A for 1 hour at a concentration **of** 4 ng/ml. After three washings in TBS the membrane was incubated with streptavidin conjugated with peroxidase for 1 hour at a concentration of 1μg/ml. Three washings were carried out with TBS and the membrane was incubated with the substrate solution consisting of 0.3% 4-chloro-1-naphthol in methanol and 0.01% H_2O_2 [Focarelli et al., 1993]. Transferring the membrane into distilled water stopped the reaction. Controls were run by omitting the lectin incubation step.

Temperature stability

Purified enzyme was incubated for 20 min at temperatures 4, 30, 40, 50, 60, 70, 80 and 90°C and the activity was assayed by the standard method. Crude extracts were also treated similarly and assayed for enzyme activity.

pH optimum

Purified enzyme was dialyzed extensively against the buffers Tris buffer and 10mM Na_2HPO_4 pH 5.5 separately and then assayed for the activity as described earlier.

Preparation of MPR 300-Affigel and *lectin*-Affigel

MPR 300 and lectin were coupled separately to the Affigel-10. Affigel-10 was washed consecutively with 4-5 bed volumes each of cold isopropanol, water and 0.1M HEPES buffer pH 7.5 and washings were completed in 20 minutes. Purified MPR 300 protein (1mg/ml) was added to the Affigel-10 in the presence of 5mM mannose 6-phosphate and lectin (7mg/ml) in presence of 0.1 M lactose. The column was closed and the gel suspension was rotated end over end for 24 hours at 4°C. At the end of the coupling the unbound fraction was acidified and the extent of coupling was determined by measuring the absorbance at 280nm. Unreacted sites in the gel were blocked using 0.1M ethanolamine-HCI pH 8.0 (200µl to 1ml of the gel) for 1hour at 4°C and the gel was washed thoroughly with PBS (phosphate buiTered saline pH 7.4) and stored at 4°C until use.

Chromatography on MPR 300-Affigel

Condition-I

Purified a-fucosidase was dialyzed extensively against the buffer A (50mM immidazole-HCl buffer pH 6.5, 0.15M NaCl, 5mM sodium β-glycerophosphate 2mM

EDTA, 10mM $MgCl_2$, 0.02% NaN_3) and applied on MPR 300-Affigel (0.2 ml) preequilibrated with the buffer A. Gel was washed till A_{280} was zero. Elution was carried out with 5mM glucose 6-phosphate followed by 5mM mannose 6-phosphate.

Condition-II

Purified α-fucosidase was applied on the MPR 300-Affigel equilibrated with buffer A and the gel was washed till A_{280} was zero. Elution was carried out by using 50mM sodium acetate pH 5.0 buffer. Fractions were assayed for the enzyme activity.

Chromatography on lectin-Affigel

Purified a-fucosidase was dialyzed extensively against 50mM sodium acetate buffer pH 5.0 and applied on the lectin-Affigel (I ml) equilibrated with the same buffer. Gel was washed till A_{280} was zero and the elution was carried out by increasing the pH (25mM Tris-HCl pH 8.0). Fractions were assayed for the enzyme activity.

SDS-PAGE and lectin blotting

Elutions from MPR 300 Affigel, condition-I and condition-II, and lectin-Affigel were checked on 10% SDS-PAGE under reducing conditions and by Con A-biotin blotting,

RESULTS

Several glycosidases like a-mannosidase, a-fucosidase, P-hexosaminidase are present in the crude extracts of *unio* whole animal tissue.

a-mannosidase purified from the whole animal extract, was purified to homogeneity from mannosamine gel (Fig. 11). These fractions did not show the activity when assayed for a-mannosidase but on SDS-PAGE it moved as 4 bands corresponding to molecular masses 118, 115, 97 and 66 kDa (Fig. 12A). All the four bands cross-reacted with the bovine lysosomal a-mannosidase antibody (Fig. 12B).

a-fucosidase was purified by passing the *unio* whole animal extracts through phenyl-Sepharose gel (Fig. 13) and fucosamine gel (Fig. 14). a-fucosidase purified from the whole animal extracts on fucosamine gel showed a single band corresponding to molecular mass of 56 kDa on SDS-PAGE (Fig. 15A). It is a glycoprotein since it reacts with Con A (Fig. 15B) Molecular weight was determined by plotting the relative fronts of the standard proteins and a-fucosidase against the log molecular weights respectively (Fig. 16). This enzyme is also stable over a range of temperature and retains about 68% of the activity at 80°C (Fig 17).

This enzyme was purified 17,222 folds to a specific activity of 93.22 units/mg. Purification is shown in Table 9.

About 50% of the MPR 300 protein and 60% of the lectin were coupled to the Affigel. Binding ability of a-fucosidase purified from fucosamine was checked on MPR 300-Affigel and lectin-Affigel. It binds to MPR 300-Affigel at pH 7.0 and was eluted specifically with mannose 6-phosphate but not with glucose 6-phosphate. In a separate experiment the enzyme bound at pH 7.0 and eluted at pH 5.0. a-fucosidase binds to

lectin-AiTigel at pH 5.0 and eluted at pH 8.0 and not vice versa. Elution profile of α-fucosidase is shown in figure 18. a-fucosidase did not bind to the lectin at pH 8.0. Elutions of MPR 300-Affigel and lectin-Affigel when checked on SDS-PAGE showed a single band corresponding to molecular mass of 56 kDa (Fig. 19A). It also reacted with Con A when Con A-biotin was used for blotting (19B).

Figure 11

Elution profile of the α-mannosidase on mannosamine gel

Active fractions from the phenyl-Sepharose gel were pooled and dialyzed extensively against phosphate buffer pH 5.5 and loaded on mannosamine gel (0.3ml). The gel was washed till the A_{280} reached zero. Elution was carried out with 0.1M mannose and 1ml fractions were collected. 50μl of every alternate fraction was assayed for the enzyme activity.

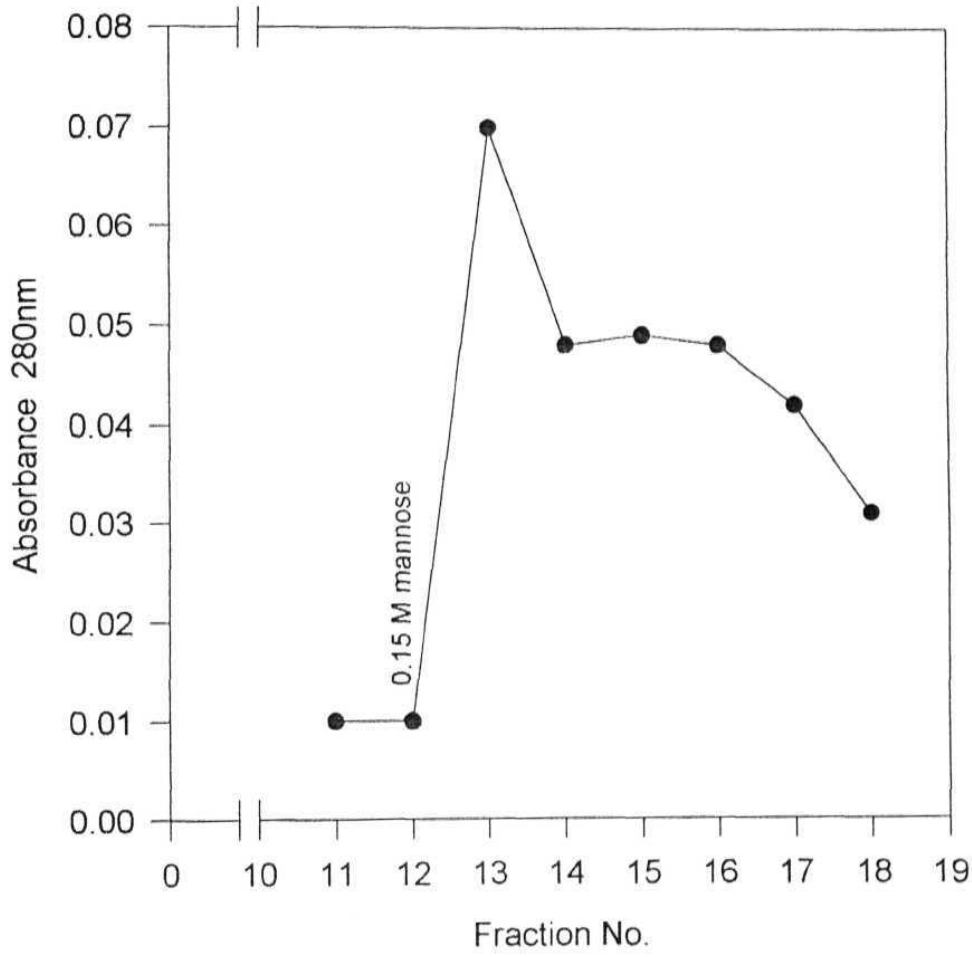

0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
Absorbance 280nm
0.15 M mannose
0
10
11
12
13
14
15
16
17
18
19
Fraction No.

Figure 11

Figure 12

A. SDS-PAGE of the purified α-mannosidase

10% SDS-PAGE was carried out under reducing conditions and the bands were detected by the silver staining method.

Lane 1. High molecular weight markers

Lane 2. DE-52 elutions

Lane 3. Phenyl-Sepharose elutions

Lane 4. Mannosamine elutions

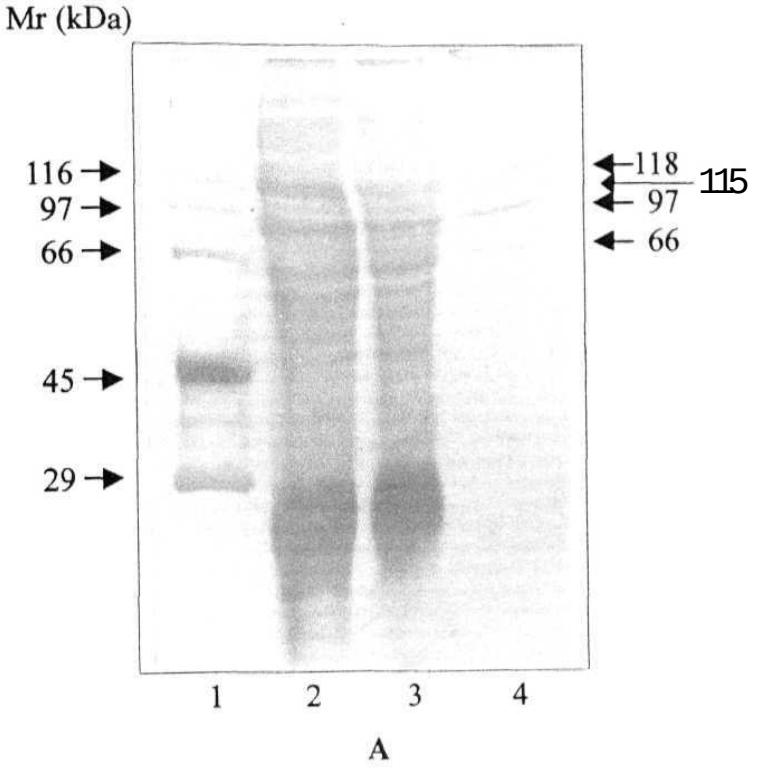
B. Western blot analysis of the purified α-mannosidase using bovine lysosomal α-mannosidase antibody

12% SDS-PAGE was carried and the proteins were transferred according to the method of Towbin et al.

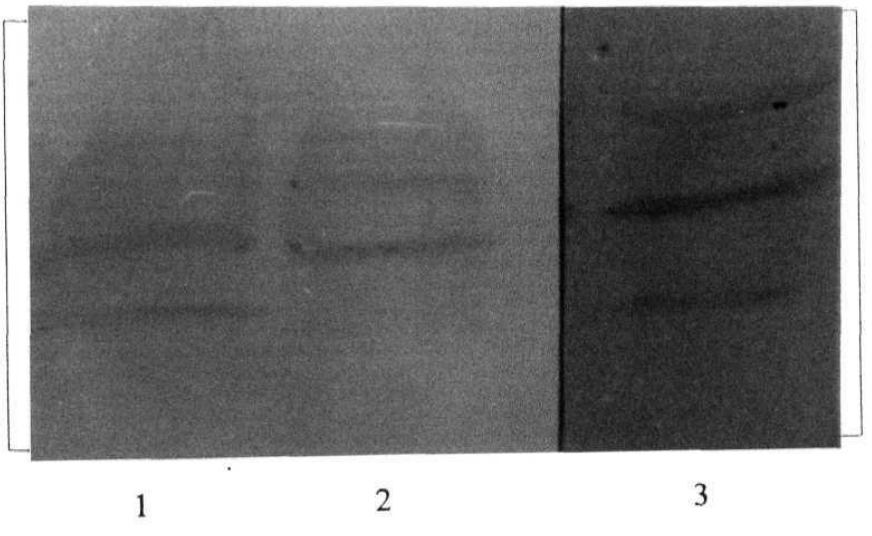
Lane 1. Phenyl-Sepharose elutions

Lane 2. Mannosamine elutions

Lane 3. silver stained gel



A



B

Figure 12

Figure 13

Elution profile of the α-fucosidase on phenyl-Sepharose gel

To the flow through of the Sepharose-lactose gel $(NH_4)_2SO_4$ was added to a final concentration of 1M and applied to phenyl-Sepharose gel (2.5 x 3.5 cm) equilibrated with Tris buffer containing 1M $(NH_4)_2SO_4$. The gel was washed till A_{280} reaches zero. Elution was carried out with Tris buffer and 2ml fractions were collected. 50µl of every alternate fraction was assayed for the enzyme activity.

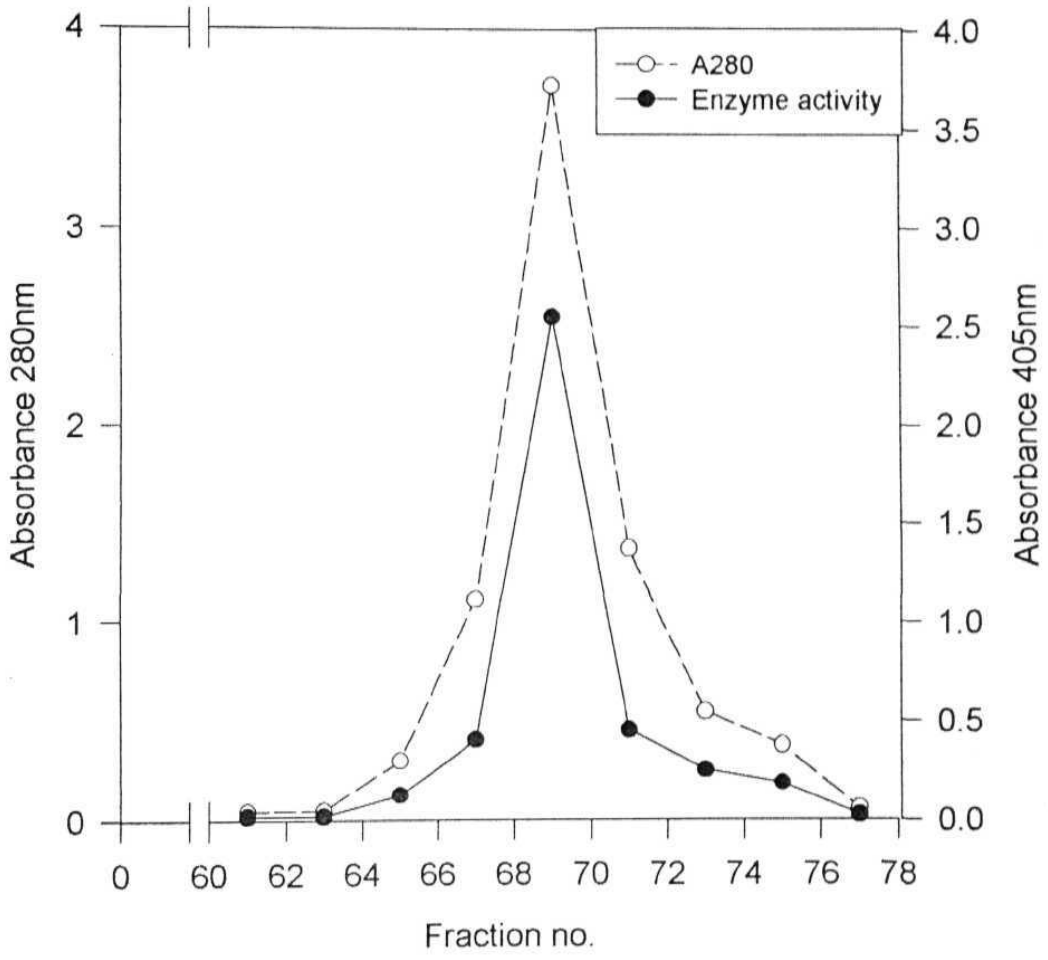

A280
Enzyme activity
Absorbance 280nm
Absorbance 405nm
Fraction no.
0
1
2
3
4
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
0
60
62
64
66
68
70
72
74
76
78

Figure 13

Figure 14

Elution profile of the α-fucosidase on fucosamine gel

Active fractions from the phenyl Sepharose gel were pooled and dialyzed extensively against 10mM NaH_2PO_4 buffer pH 5.5 and loaded on fucosamine gel (0.2ml) equilibrated with the same buffer. The gel was washed till the A_{280} reached zero. Elution was carried out with 0.15M mannose and 1ml fractions were collected. 50µl of every alternate fraction was assayed for the enzyme activity.

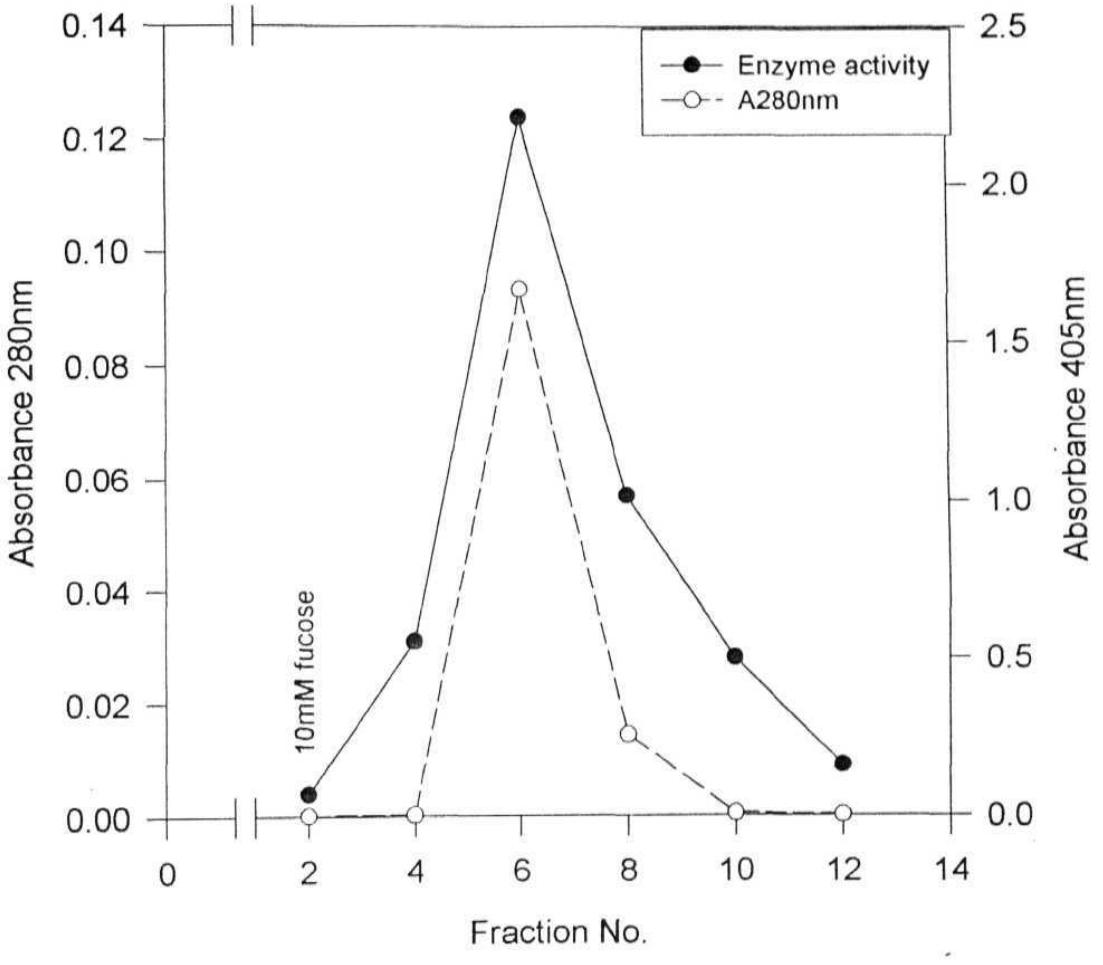

Enzyme activity
A280nm
Absorbance 280nm
Absorbance 405nm
10mM fucose
Fraction No.
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
2.5
2.0
1.5
1.0
0.5
0.0
0
2
4
6
8
10
12
14

Figure 14

Table 9. Purification of α-fucosidase from 10g *unio* whole animal tissue

Purification step	Total protein (mg)	Total enzyme (units)	Specific activity (units/mg)	Enzyme recovery (%)	Purification (fold)
Crude	500.0	2.7	0.0054	100	1
Phenyl-Sepharose elutions	74.9	0.94	12.7	34	2351
Fucosamine gelelutions	0.354	0.33	93.22	12	17,222

Figure 15

A. SDS-PAGE of the purified a-fucosidase

10% SDS-PAGE was carried out under reducing conditions and the bands were detected by the silver staining method.

Lane 1. Phenyl-Sepharose elutions

Lane 2. Fucosamine gel elutions

B. Con A biotiii blotting of the purified a-fucosidase

10% SDS-PAGE was carried out under reducing conditions and proteins were transferred on to a nitrocellulose membrane and processed as described under methods.

Lane 1. Fucosamine gel elutions

Lane 2. Phenyl-Sepharose elutions

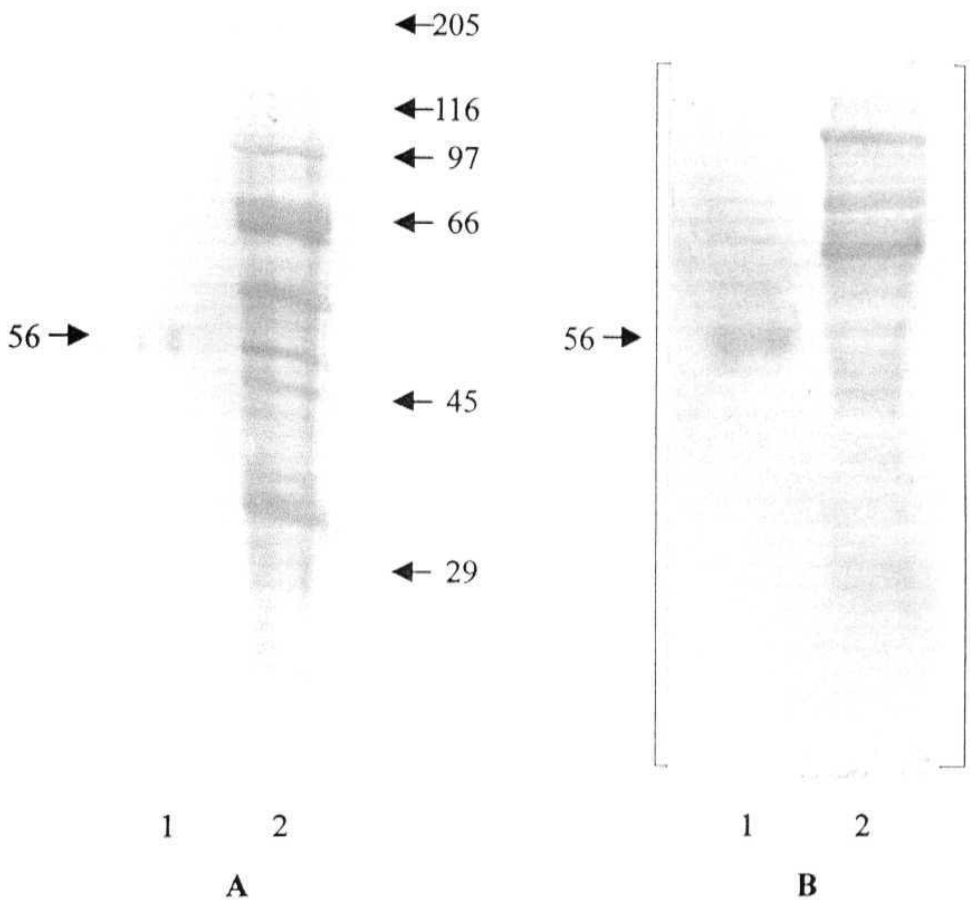


Figure 15

Figure 16

Molecular weight deterinination of the a-fucosidase

High molecular weight proteins were run on a 10% SDS-PAGE along with the purified a-fucosidase and the R_f values were calculated and plotted against the log molecular weight of the proteins.

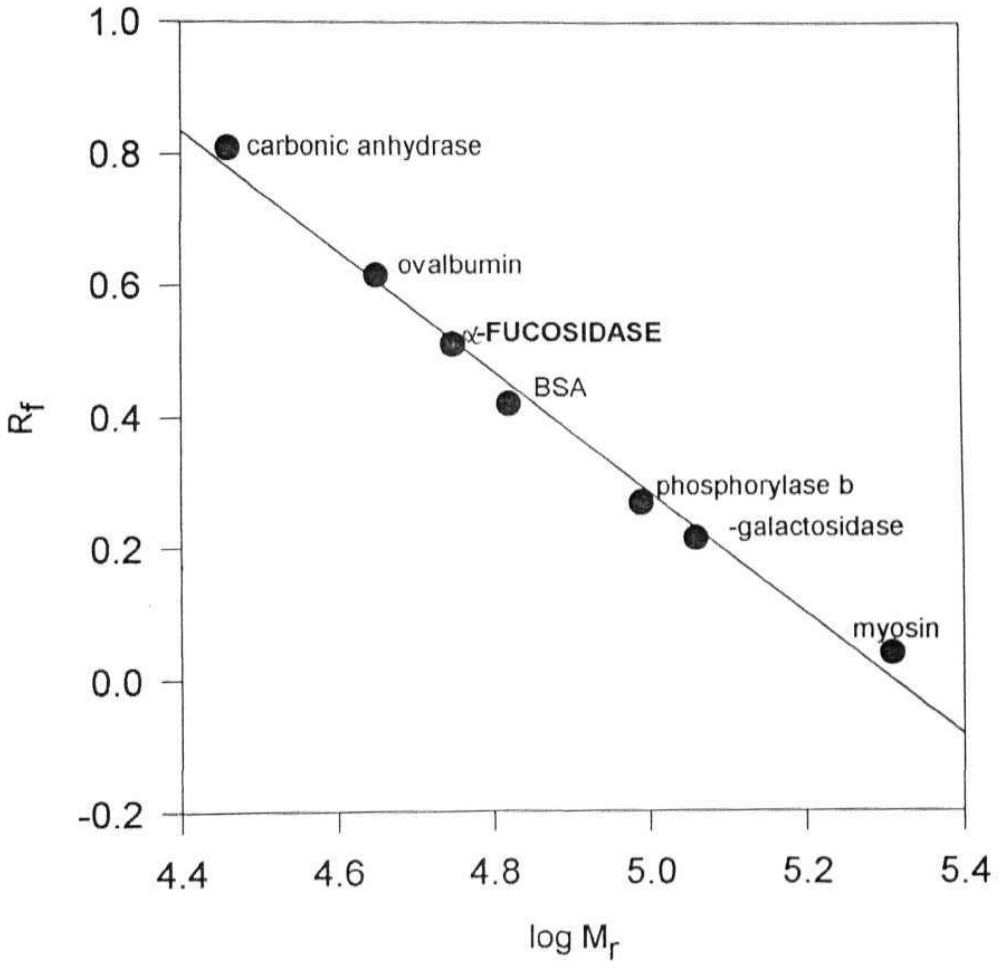

1.0
0.8
0.6
0.4
0.2
0.0
-0.2
R_f
carbonic anhydrase
ovalbumin
α-FUCOSIDASE
BSA
phosphorylase b
-galactosidase
myosin
4.4
4.6
4.8
5.0
5.2
5.4
log M_r

Figure 16

Figure 17

Effect of temperature on the enzyme activity of the purified α-fucosidase

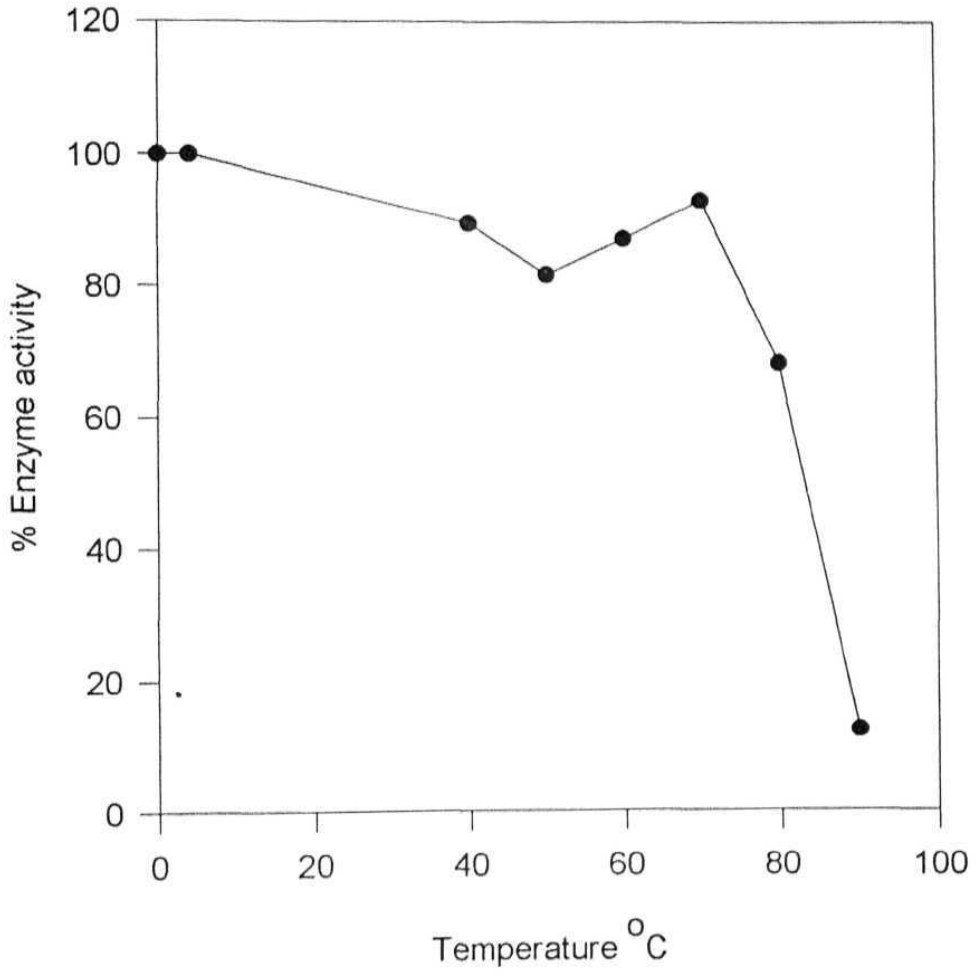

120
100
80
60
40
20
0
% Enzyme activity
0
20
40
60
80
100
Temperature °C

Figure 17

Figure 18

Elution profile of the a-fucosidase on the *unio* lectin-Affigel

Fucosidase purified on the fucosamine gel was loaded on the lectin-Affigel equilibrated with 50mM sodium acetate pH 5.0 buffer. The gel was washed till A 280 reaches zero. Elution was carried out Tris buffer and 1 ml fractions were collected. 50µl of the every alternate fraction was assayed for the enzyme activity.

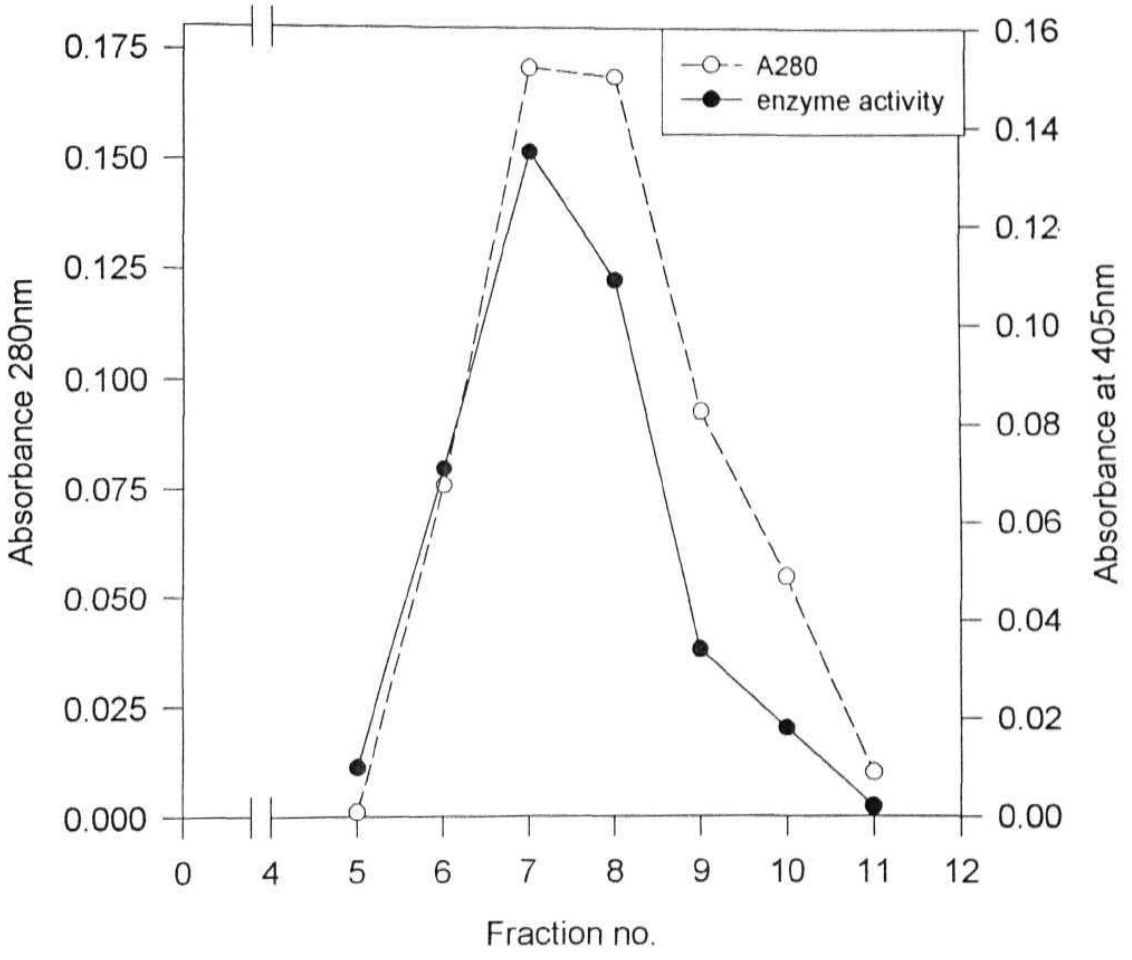

A280
enzyme activity
Absorbance 280nm
Absorbance at 405nm
Fraction no.
0.175
0.150
0.125
0.100
0.075
0.050
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Figure 18

Figure 19

A. SDS-PAGE of the elutions of the MPR 300-Affigel and lectin-Affigel

10% SDS-PAGE was carried out under reducing conditions and the gel was stained by the silver staining method.

Lane 1. High molecular weight markers

Lane 2. Fucosamine gel elutions (fucosidase purified from fucosamine gel)

Lane 3. Lectin-Affigel elutions

Lane 4. Glucose 6-phosphate elutions

Lane 5. Mannose 6-phosphate elutions

Lane 6. pH 5.0 elutions from MPR 300-Affigel

B. Con A-biotin blotting of the elutions of the MPR 300-Affigel and lectin-Affigel

Proteins were transferred onto a nitrocellulose sheet and the blot was processed as described under the methods.

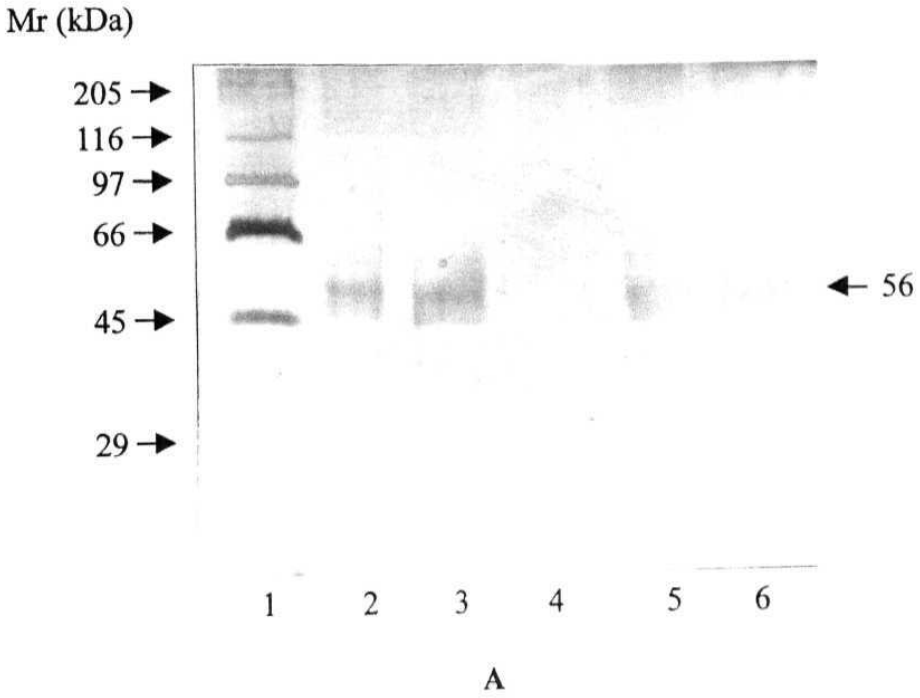
Lane 1. Fucosamine gel elutions (fucosidase purified from fucosamine gel)

Lane 2. Glucose 6-phosphate elutions

Lane 3. Mannose 6-phosphate elutions

Lane 4. pH 5.0 elutions from MPR 300-Affigel

Lane 5. Lectin-Affigel elutions



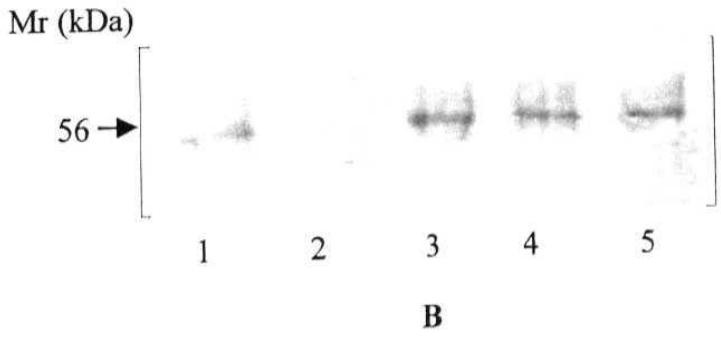


Figure 19

DISCUSSION

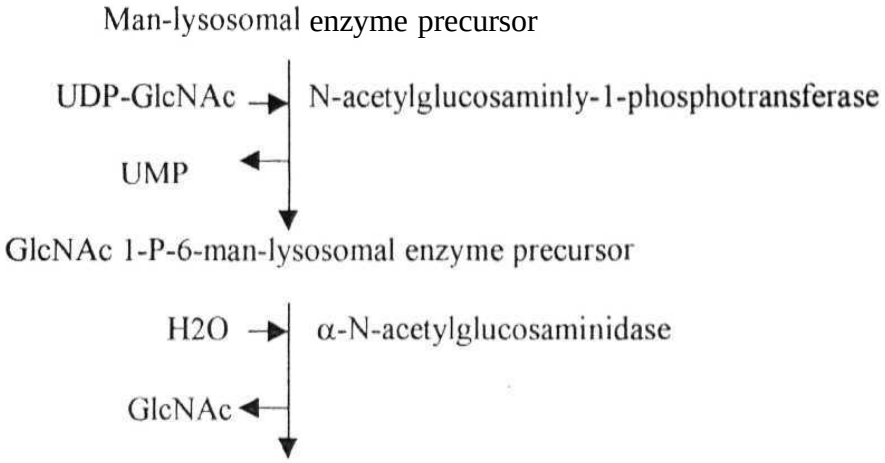
Interactions between cellular components, proteins and carbohydrate recognition signals present on the enzymes are involved in the targeting of lysosomal enzymes from their site of synthesis (RER) to their final destination in lysosomes [von Figura and Hasilik, 1986; kornfeld, 1987; Pfeffer, 1988J. During the early stages of biosynthesis these enzymes share a common pathway with secretory proteins. But sorting of these enzymes requires generation of phosphomannosyl residues on these enzymes.

Mannose **6-phosphate** dependent pathway

Specific sorting and transport of lysosomal enzymes is achieved in three steps (mannose 6-phosphate dependent pathway). In the first step the lysosomal enzymes are synthesized on the rough endoplasmic reticulum of the membrane bound ribosomes. They are inserted into the lumen of the RER by the cleavable signal Peptide present on them.

In the second step these enzymes undergo co-translational glycosylation when a preformed high mannose oligosaccharide [(Glc)3, $(Man)_9$, $(Glc\ NaC)_2$] is transferred from a dolichol intermediate onto selected asparagines residues and subsequent trimming of the glucose residues [Li *et al.*, 1978]. These newly synthesized glycoproteins are sequestered into membrane bound vesicles and the oligosaccharide chains may be processed further while they are transported to Golgi complex [Tabas and Kornfeld, 1980].

In the early region of the Golgi apparatus, two distinct enzymes act upon the enzymes destined to lysosomes and phosphorylate mannose residues [Pohlmann *et al.*, 1982; Pelham, 1988].



Mannose 6-phosphate containing lysosomal enzymes

N-acetylglycosaminyl-1-phosphotransferase transfers the GlcNAc-1-phosphate to 6-hydroxyl group of terminal mannose residues on the enzyme precursor resulting in the release of UMP [Reitman and Kornfeld, 1981].

α-N-acetylglucosaminidase cleaves the phosphodiester linkage of N-acetylglucosamine to mannose residues on the oligosaccharide chains releasing N-acetylglucosamine residue [Varki and Kornfeld, 1980; Waheed *et al.*, 1981].

In the third step, the lysososmal enzymes with mannose 6-phosphate recognition marker are identified by the MPRs present in the *trans*-Golgi network (TGN) and are released as clathrin coated vesicles [Brown and Farquhar, 1984], which fuse with the prelysosomal compartment. Due to the acidic nature of the lysosomes, the receptor-ligand complex dissociates releasing the ligand. The receptor may be recycled back to the TGN or to the plasma membrane.

Mannose 6-phosphate independent pathway

This pathway was proposed when Neufeld and Mc Kusick identified the activities of the lysosomal enzymes (α-glucocerebrosidase and Cathepsin D) in various tissues of the patients suffering from 1 cell disease (deficient in N-acetylglucosamine phosphotransferase) [Neufeld and McKusick, 1983]. Enzymes devoid of mannose 6-phosphate residues, lysosomal membrane glycoproteins (Lamp-1 and Lamp-2) and lysosomal acid phosphatase, are transported to lysosomes [Lippincott-Schwarz and Fambrough, 1986; Waheed *et al.,* 1988]. Transport may be mediated by the recognition of some sorting signals within the cytoplasmic domains of the enzymes [Guarnieri *et al.,* 1993]. These observations suggest the possibility of the existence of a mannose 6-phosphate independent transport of lysosomal enzymes.

Role of receptors in sorting and endocytosis

Various experiments on the cultured fibroblasts indicated that MPR 300/CIMPR is involved in the sorting of newly synthesized lysosomal enzymes and endocytosis of extra cellular phosphorylated lysosomal enzymes. Cells lacking endogenous MPR 300 [Gabel *et at.,* 1983] or by depletion of MPR 300 by treating with antisera to the protein [Nolan et al., 1987; Stein et al., 1987] showed 70% secretion of the newly synthesized lysosomal enzymes. The partial sorting of the enzymes in the absence of MPR 300 could be due to MPR 46. This defective sorting can be corrected when the cells lacking this protein are transfected with MPR 300 cDNA [Kyle *et al.,* 1988; Lobel *et al.,* 1989].

Lysosomal enzymes can be targeted to lysosomes by either direct intracellular route (biosynthetic pathway) or by endocytotic pathway.

In biosynthetic pathway, a major pathway, formation of phosphomannosyl monoesters occurs in the Golgi compartment [von Figura and Hasilik, 1986; Lazzarino and Gabel, 1988] and the possibility that MPR 300 binds to these enzymes in *cis*-Golgi is supported by immunocytochemical studies on some cells. A result of these studies showed that MPR 300 is concentrated in *cis*-Golgi and with very low levels in the *trans*-Golgi [Brown and Farquhar, 1987].

Extracellular lysosomal enzymes are delivered to the lysosome via the endocytotic pathway. These enzymes bind to the receptor near the cell surface and are internalized via clathrin coated pits and vesicles [Geuze *et al,* 1985; Willingham *et al,* 1981]. Studies using antibodies [Nolan *et al.,* 1987; Sahagian, 1984; Gartung *et al.,* 1985] or galactosyltransferase [Duncan and Kornfeld, 1988] to labeled receptors on the cell surface and the measurements of the number and half life of receptors and rates of internalization indicated that there is only one pool of receptors and single MPR 300 functions in the biosynthetic and endocytotic pathways [Sahagian, 1984].

Significant amounts of MPR 300 is present in the endosomal compartments and very low or undetectable levels in lysosomes [Sahagian and Neufeld, 1983; Geuze *et al.,* 1985; Griffiths *et al,* 1988; Geuze *et al,* 1988; Brown *et al,* 1986]. This has led to the concept that Golgi-derived vesicles containing lysosomal enzyme-receptor complexes are delivered to acidic prelysosomal/endosomal compartments rather than to lysosomes [Griffiths *et al,* 1988; Geuze *et al,* 1988; Brown *et al,* 1986; Sahagian, 1984]. The receptor binds to the lysosomal enzymes at neutral pH and releases at low/acidic pH (binding in the Golgi compartment and release in endosomal compartment).

a-Fucosidase purified from the whole animal extracts of *unio* showed a molecular mass of 56kDa. Its glycoprotein nature was confirmed by its interaction with Con A-biotin. Focarelli *et al.* purified a-Fucosidase with similar properties from seminal fluid of *unio* and the enzyme purified in the present study exhibits similar molecular mass [1997]. Purified a-fucosidase showed strong interaction with the immobilized lectin. When lactose, specific sugar with which the lectin shows the highest affinity, was used for the elution the bound a-fucosidase could not be eluted. The enzyme could not be desorbed from the Affigel even with a high ionic strength buffer. Bound enzyme was eluted only when buffer with alkaline pH was used (25mM Tris pH 8.0). It shows that the lectin-enzyme interaction is neither through the sugar binding site of the lectin nor the oligosaccharide chains of the enzyme. Glycosidascs from jack bean seeds have been shown to bind the lectin at pH 5.0 and desorbed using sugar [EinholT and Ruediger, 1986a,b]. Lectin from *Dolichos lablab* was shown to bind a-mannosidase at pH 5.0 and was released only when a buffer with high pH was used [Rajasekhar and Siva kumar, 1997]. In plants the binding or release of the glycosidases during the deposition and degradation of protein bodies may be enabled by the small changes in pH and ionic strength. The results obtained with the *unio* lectin suggests . strong *in vivo* interactions of the lectin and a-fucosidase.

CONCLUSIONS

CHAPTER II

- A lactose specific lectin has been purified from *unio* whole animal tissue by chromatography on the affinity gel (Sepharose-lactose gel).
- Purified lectin agglutinates only pronase treated rabbit erythrocytes. It did **not** agglutinate untreated, trypsin and neuraminidase treated erythrocytes.
- Lactose was the only inhibitory sugar.
- From 300g of tissue 100mg of lectin could be obtained.
- Purified lectin is a glycoprotein as it has 5% carbohydrate and binds **to Con** A-Sepharose gel.
- It was found to be homogeneous by native gel electrophoresis and exhibits **a** native molecular mass of 105 ± 5 kDa in gel filtration.
- In SDS-PAGE it dissociates into three bands with molecular masses 28, 23 **and** 16 kDa respectively.
- All the three bands stain positive (PAS staining) for carbohydrate
- Amino terminal analysis of the lectin revealed two amino acid residues corresponding to valine and proline in the first cycle. This suggests that the lectin is oligomeric.
- Antibodies raised to the purified lectin showed specific reactivity with the protein in immunodiffusion and western blot analysis.
- Purified lectin showed 100% binding to affinity gel upto 40°C. Beyond 60°C binding was less then 10%.
- At pH 7.0 and 8.0, the lectin showed complete binding on the affinity gel.
- The amino acid composition of this lectin shows high concentrations of acidic and hydrophobic amino acids. Cysteine and methionine could not be detected.

CHAPTER III

- Modification of the lysine residues did not alter the agglutination property **but** decreased the binding ability to the affinity gel (Sepharose-lactose gel) to 45%.

- Modification of tyrosine and tryptophan residues did not alter the agglutination property and binding ability of the lectin to affinity gel.
- Modification of arginine residues did not alter the binding ability to the affinity gel.
- Modification of histidine residues resulted in the loss of both agglutinating activity and binding ability to affinity gel.
- Histidine modification carried out in presence of lactose sugar, showed protection against modification, suggesting the possible involvement of histidine residues in the biological activity of the lectin.
- Reversal of the histidine modification has not resulted in regaining of the complete activity.
- However, histidine modification did not alter the immunological property of the lectin, indicating that loss of activity is not due to alterations in the gross changes in the overall structure of the protein.

CHAPTER-IV

- Mannose 6-phosphate receptor 300 was purified to homogeneity from membrane extracts *of unio* whole animal tissue by affinity chromatography on PM Sepharose gel.
- Antibodies raised to the purified MPR 300 protein reacted with the protein.

CHAPTER-V

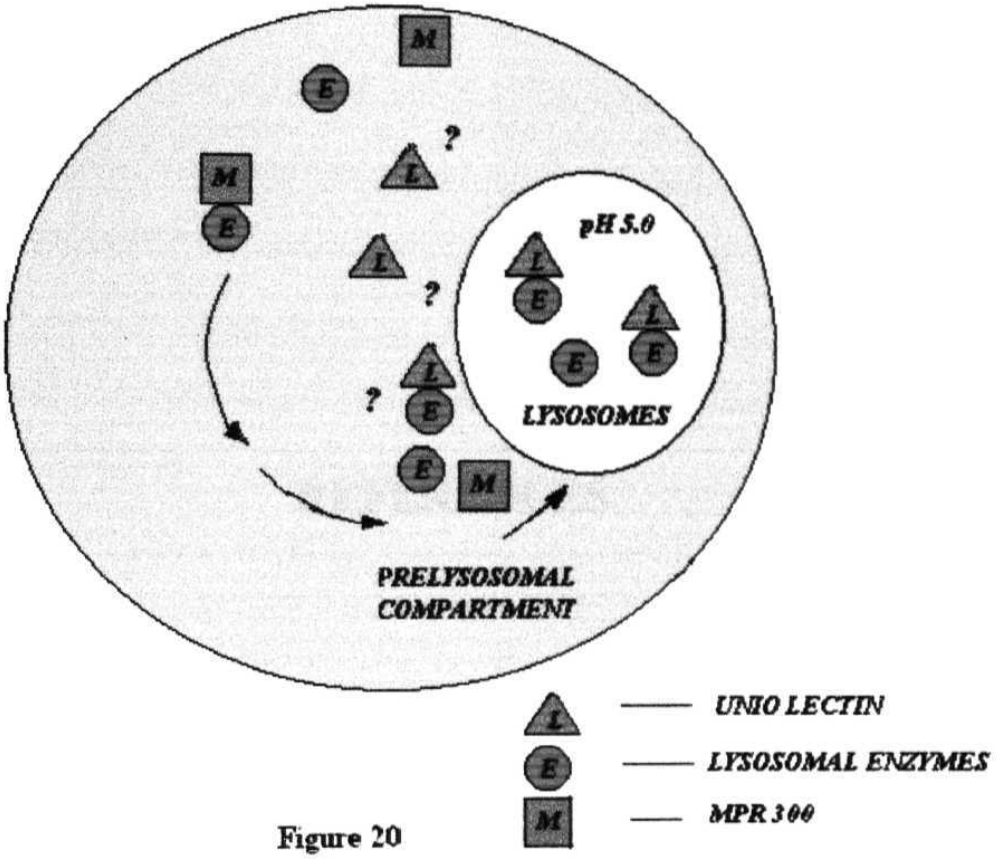
- *Unio* whole animal extract contains several glycosidase activities such as the α-mannosidase, a-fucosidase and (3-hexosaminidase.
- a-Mannosidase and a-fucosidase have been affinity purified on mannosamine and fucosamine gels respectively.
- a-Mannosidase shows four bands on 10% SDS-PAGE.

- All these 4 bands were recognized by bovine lysosomal α-mannosidase antibody.
- a-Fucosidase has a molecular mass of 56 kDa.
- a-Fucosidase retains upto 68% of the activity upto 80°C.
- a-Fucosidase is a glycoprotein as it is recognized by Con A.
- a-Fucosidase binds to *unio* MPR 300 Affigel at pH 7.0 but not at pH 5.0. It can be specifically eluted with mannose 6-phosphate but not with glucose 6-phosphate.
- a-Fucosidase also binds to *unio* lectin Affigel. It binds only at pH 5.0 but not at pH 8. It can be eluted from this gel al pH 8.0.
- Results of these initial studies demonstrate specific /// *vitro* interactions shown by the purified a-fucosidase enzyme both with MPR protein as well as with the lectin suggesting possible *in vivo* interactions of similar nature.

Figure 20

Possible *in vivo* interactions of the *unio* lectin, MPR 300 and lysosomal enzymes (α-fucosidase).

MPR 300, *in vitro,* does not bind the a-fucosidase at acidic pH but binds at pH 7.0 and releases it at acidic pH α-fucosidase does not bind the lectin at pH 8.0 but shows strong interaction with the lectin at pH 5.0 suggesting possible binding either in the prelysosomal compartment or in the lysosomes.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PRELYSOSOMAL
COMPARTMENT
L
UNIO LECTIN
E
LYSOSOMAL ENZYMES
M
MPR 300

Figure 20

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PUBLICATIONS

Publications

1. A new lactose specific lectin from the fresh water mussel unio. Role of histidine residues in the biological activity of the lectin.

Radha Yalamarthy and Siva kumar Nadimpalli

Accepted for publication in the **International Journal of Biochroniatograpliy.**

2. Identification of the putative mannose 6-phosphate Receptor 300 Protein (MPR 300) in the Invertebrate *unio*

Yerramalla Udaya Lakshmi, Yalamarthy Radha, Annette-Hille Rehfeld, Kurt von Figura and Nadimpalli Siva kumar

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Identification of the Putative Mannose 6-phosphate Receptor Protein (MPR 300) in the Invertebrate *unio*

Yerramalla Udaya Lakshmi,[1,2] Yalamarthy Radha,[1] Annette Hille-Rehfeld[2], Kurt von Figura,[2] and Nadimpalli Siva Kumar[1,3]

In mammals, Mannose 6-phosphate receptor proteins (MPR 300 and MPR 46) mediate transport of lysosomal enzymes to lysosomes. Both receptors have been found in non-mammalian vertebrates including fish. To investigate the presence of MPRs in invertebrates, MPR 300 protein was isolated from the mollusc *unio* by affinity chromatography. It was shown to exhibit biochemical and immunological properties similar to mammalian MPR 300.

KEY WORDS: Invertebrates; Mannose 6-phosphate receptor protein (MPR 300), Mollusc; phosphomannan Sepharose Chromatography; *unio*.

ABBREVIATIONS: MPR(s), Mannose 6-phosphate receptor(s).

INTRODUCTION

In mammals the two Mannose 6-phosphate receptor proteins (MPR 300 and MPR 46) mediate the transport of lysosomal enzymes to lysosomes (von Figura and Hasilik, 1986, and Kornfeld, 1992). MPR 300 is a multifunctional protein which, in addition to mannose 6-phosphate containing lysosomal enzymes binds IGF-II, thyroglobulin and retinoic acid (Hille-Rehfeld, 1995; Kang *et al.*, 1998). MPRs are type I integral membrane proteins. The luminal, ligand-binding domain of MPR 300 contains 15 internal repeats, which are homologous to the luminal domain of MPR 46. It is therefore of interest to study the evolution of the two MPRs. Our previous studies established the presence of both receptors in non-mammalian vertebrates such as birds (Matzner *et al.*, 1996) reptiles and amphibians (Siva Kumar *et al.*, 1997) and in fish as the earliest vertebrates (Siva Kumar *et al.*, 1999). In this study we report the first identification of MPR 300 in an invertebrate species by affinity purification of the receptor from the mollusc *unio*. The biochemical and immunological properties of the putative *unio* MPR 300 closely resemble those of the mammalian receptor.

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MATERIALS AND METHODS

Affinity Purification of *unio* MPR 300

Membrane proteins from *unio* (obtained from Hyderabad, India) whole animal acetone powder were extracted as described for goal liver MPR 300 (Udaya Lakshmi and Siva Kumar, 1996). The membrane extract was adjusted to 2 mM LDTA and clarified by centrifugation. The supernatant was passed through Phosphomannan (PM) Sepharose equilibrated with 50 mM imidazole-HCl buffer pH 7.0, 150 mM sodium chloride, 5 mM sodium β-glycerophosphate, 0.05% Triton X-100 and 2 mM EDTA (Buffer A). The mannose 6-phosphate eluates were concentrated by ultrafiltration (Amicon PM 10).

Radioiodination of the Purified MPR 300 and Rechromatography on PM Sepharose

Purified *unio* or goal MPR 300 was desalted by acetone precipitation and labeled with ^{125}I as described (Waheed *et al.,* 1990). Iodinaled receptor was rechromatographed on PM Sepharose (0.2 ml) equilibrated with buffer A. The column was washed with buffer A and sequentially eluted with 3 volumes each of 5 mM glucose 6-phosphale followed by 5 mM mannose 6-phosphale (both from Sigma, St. Louis, MO, U.S.A.) in buffer A. Radioactivity was measured by scintillation counting and the fractions precipitated with trichloroacetic acid for analysis by SDS-PAGL. To determine the pH optimum of ligand binding, analytical I'M Sepharosc chromatography was performed at pH4 7.5 as described (Siva Kumar *et al.*, 1999).

Immunoprecipitation of MPR 300

Purified *unio* MPR 300 was used for immunization of a rabbit as described for goat MPR 300 (Udaya Lakshmi and Siva Kumar. 1996). Radioiodinated MPR 300 was incubated will) 2 μl of anti-unio MPR 300 antibody al 4°C overnight in PBS containing 0.05% Tween-20 (PBS-Tween). The antigen-antibody complexes were then adsorbed to Pansorbin (40//I of a 10% suspension) (Calbiochem, Bad Soden, Germany). The Pansorbin pellets were washed live times with PBS-Tween and extracted with reducing SDS-sample buffer for SI)S-PA(iL (7,5% gel). Preimmune scrum was used as a control.

Analytical Methods

Protein was estimated with bicinchoninic acid (Sigma). SDS-PAGE was performed according to Laemmli (Laemmli, 1970). Where indicated, proteins were reduced by 10 mM PTT for 5 min at 95°C and alkylated with 50 mM iodoacetic acid for J5 min al 37°C. Radioiodinated MPR 300 was detected by fluorography (Bonner

and Laskey, 1974). Silver staining of proteins in polyacrylamide gels were performed as described (Ansorge, 1985).

RESULTS AND DISCUSSION

Our earlier studies clearly established the presence of both MPR proteins among the different mammalian vertebrates (Siva Kumar *et al.*, 1997, 1999). Here we investigated the presence of MPR proteins in inverlebrates in order to understand the evolution of MPRs. We chose *unio* (mollusc) for the purification of MPRs. The detergent extract of the whole animal acetone powder was passed through PM Sepharose, and the bound proteins were eluted using mannose 6-phosphate. When the eluates were subjected to SDS-PAGE and silver staining, a single polypeptide resembling by its apparent si/.e to MPR 300 was present, whereas MPR 46 like polypeptides were not detectable (Fig. 1). Similar results were obtained when 2 mM EDTA was replaced with 10 mM manganese chloride in the column buffer

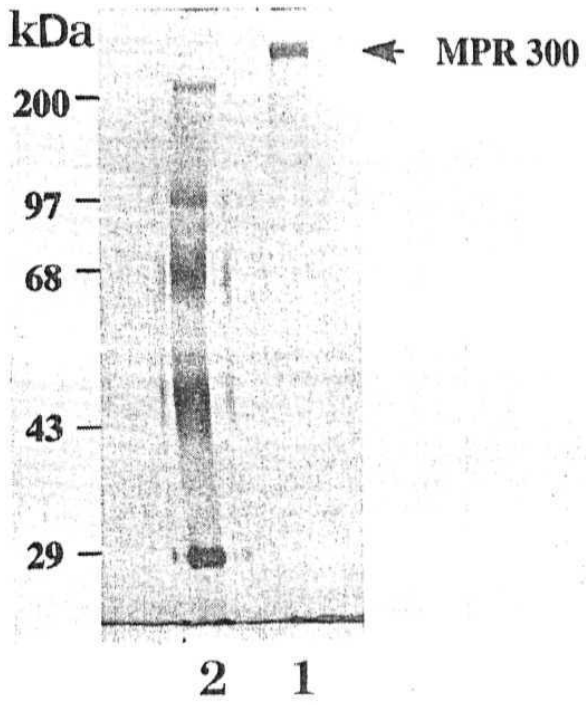

Fig. 1. Purity of affinity-purified *unio* MPR 300. The mannose 6-phosphate eluate of PM Sepharose was analyzed by SDS-PAGE (7.5%) and silver staining. Lane 1, affinity ñpurified *unio* MPR 300; lane 2, molecular mass standards.

(data not shown). The available data do not allow to decide whether the concentration of the MPR 46 is too low in *unio* to be detected or whether the *unio* MPR 46 failed to bind to the affinity matrix under the conditions used. From 100 g of the acetone powder 80 μg of the purified protein was obtained. For biochemical and immunological characterization, the affinity-purified *unio* MPR 300 was subjected to radioiodination. Radioiodinated *unio* MPR 300 protein was rebound to PM Sepharose and specifically eluted with excess of the free ligand mannose 6-phosphate but not with the phosphorylated epimer glucose 6-phosphate *(data not shown)*. The apparent molecular mass of the radioiodinated *unio* MPR 300 was similar to that of the goat MPR 300 (Fig. 2), the lower band in *unio* is more likely due to limited proteolysis. The mobility of MPRs from both species decreased lo the same extent under reducing conditions (Fig. 2). The pH optimum for PM Sepharose binding of *unio* MPR 300 was found to be between pH 5.5 and 7.0 (Fig. 3), whereas mammalian MPR 300 has been reported lo bind ligands poorly al pH 5.5, the optimum for ligand binding being between pH 6 and 7.4 (Hoflack *et al.*, 1987).

An antibody raised to the purified *unio* MPR protein specifically reacts with the purified iodinated MPR 300 protein and also cross-reacts with goat MPR 300 protein (Fig. 4), suggesting that MPR 300 from invertebrate species and mammals are

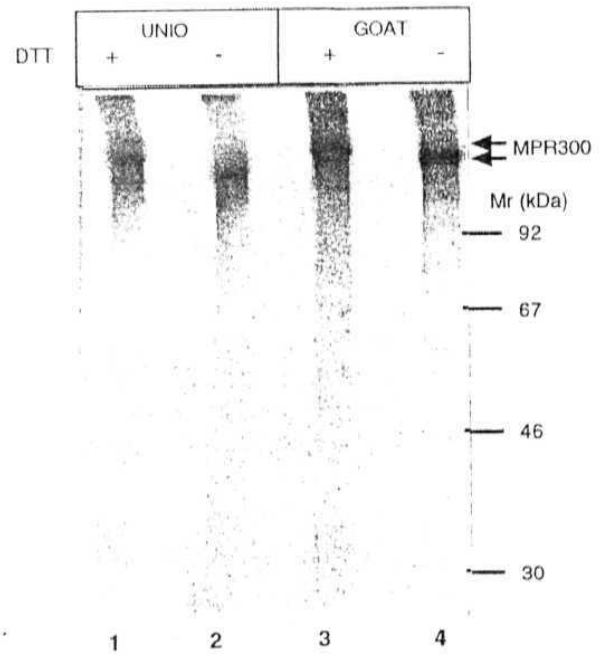

Fig. 2. Apparent molecular mass of radioiodinated MPR 300 from *unio* (lanes 1 and 2) and goat liver (lanes 3 and 4) on 7.5% SDS-gels under reducing (lanes 1 and 3) and non-reducing conditions (lanes 2 and 4). Arrows show the position of MPR 300.

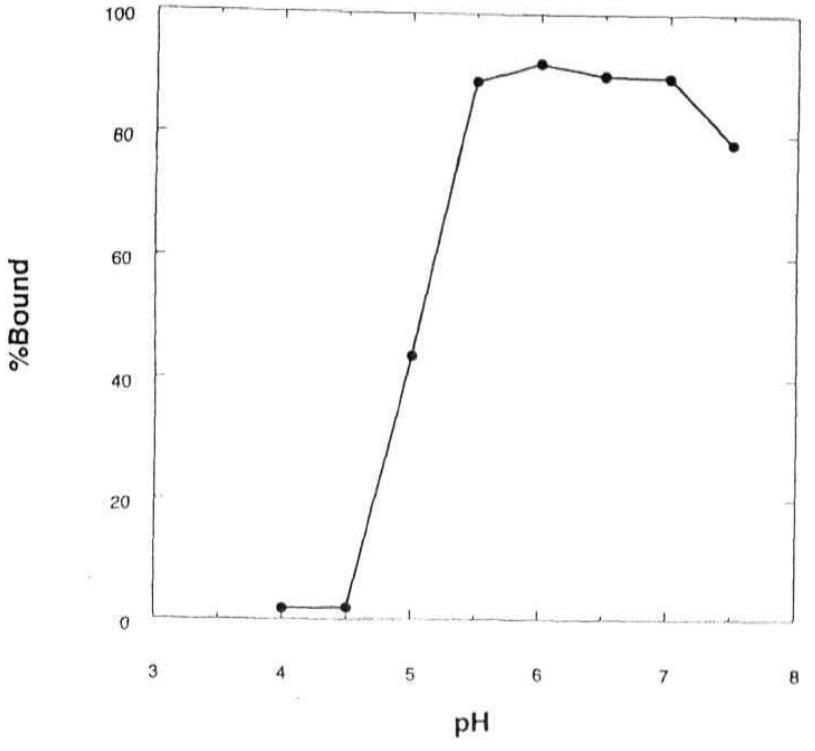

Fig. 3. pH-optimum for ligand binding of *unio* MPR 300. Radioiodinated *unio* MPR 300 was bound to analytical PM-Sepharose columns and specifically eluted with mannose 6-phosphate. Bound MPR 300 is plotted as percentage of total MPR 300 recovered from the column.

immunologically related. Taken together, our results present the first report on the existence of MPR 300 in the invertebrate *unio*. Mollusc MPR 300 was found to show biochemical and immunological properties similar to the mammalian counterparts. The aim of future studies is to determine the protein sequence of *unio* MPR 300 to investigate its structure and function as well as its homology to mammalian MPR 46 and MPR 300.

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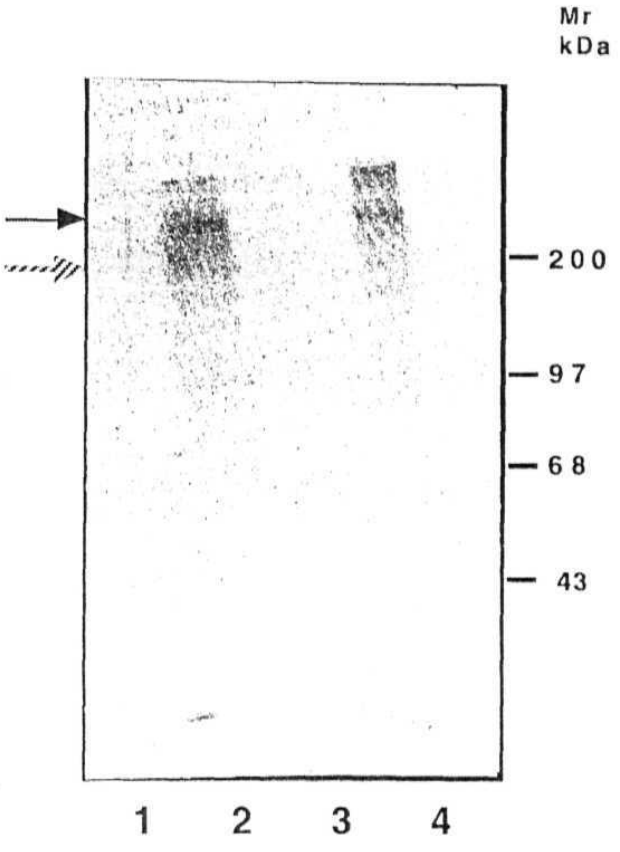

Fig. 4. Immunological cross-reaction of *mio* and goat MPR *MM.* Radioiodinated *mio* MPR 300 (lanes 1, 2) and goat MPR UK) (lanes 3 and 4) were subjected to immunoprecipitation with pre-immune serum (lanes 1, 3) or anti-*mio* MPR 300 serum (lanes 2, 4). Arrow indicates position of MPR 300. Slashed arrow shows proteolytic degradation product.

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