Biochemical characterization of cathepsin D and α-fucosidase from starfish: Mannose 6-phosphate receptors (MPR 300) from fish and starfish also interact with human IGF-II

Thesis submitted for the Degree of

Doctor of Philosophy

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CERTIFICATE

This is to certify that this thesis entitled "**Biochemical characterization of cathepsin D and α-fucosidase from starfish: Mannose 6-phosphate receptors (MPR 300) from fish and starfish also interact with human IGF-II**" submitted to the University of Hyderabad by **Miss. Merino Visa** for the degree of Doctor of Philosophy, is based on the studies carried out by her under my supervision. I 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 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 **Prof. 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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ACKNOWLEDGEMENTS

I give all the glory and honour to God, the Almighty for His undying love and guidance which has brought me this far. May his name be glorified through this work.

Taking this opportunity, I would like to thank my guide and mentor, Professor Nadimpalli Siva Kumar for being there for me right from the start. Sir, you are wonderful person and it has been a great experience working under your care. Thank you for trusting in me and giving me the opportunities to venture out of my 'pond'. Thank you for the suggestions and the feedbacks without which this research would have been incomplete and thank you for the assurance given to my mum-they have really helped to keep her comforted that I am in good hands.

A special thanks to Prof O.H.Setty, Head, Department of Biochemistry, Prof M.Ramanadham, Dean, School of Life Sciences and the former head Prof. K.V.A.Ramaiah for making this research work feasible with the infrastructure and laboratory facilities.

My sincere gratitude to my doctoral committee members Prof M.Ramanadham and Prof B.Senthilkumaran, for assessing my research work in between and for the useful discussions.

I would also like to thank all the faculty members and research scholars of Life Sciences for their constant support and motivation.

I am very grateful for the financial support rendered to me by the Deutscher Akademischer Austausch Dienst (DAAD) for an extension program at the University of Greifswald, Germany from October 2009 to September 2010.

It gives me immense pleasure to thank Prof Uwe Völker, University of Greifswald, Germany for the support and guidance during my stay in Germany. I am also thankful to Dr. Elke Hammer for helping me in analyzing my proteomics data. To all my friends who made my stay in Germany a memorable one.

I also extend my gratitude to the University of Hyderabad for providing the PURSE grant and enabling me to attend the international conference, Italy.

My special thanks to Dr.K.Suresh for his valuable suggestions and advice.

To all my former and present colleagues Dr.Nagender Rao Dr.Sivaramakrishna, Dr.Praveen Kumar, Dr Kiran Kumar, Mohammad, Venu, Gnanesh, Ismail Khan, Ajith, Praveen, Rohit, and lab attendant Ramana , I am very much grateful for your friendship . The help and support you have rendered to me are priceless. May God bless all of you with success and all the joys of life.

I am deeply grateful to Rajiv Gandhi National Fellowship (RGNF), India for the financial support through JRF and SRF. I thank DST-FIST, UGC-CAS, DBT, CREBB for the infrastructure provided at School of Life Sciences.

To my friends Khrienuo and Khruks: you guys are super. I cannot imagine my stay here without you. My research work has bœn fun and enjoyable with your presence. THANK YOU.

My seniors Daniel, Veni and Toshi, thank you for the help, the advices, the TREATS and not forgetting the rides. I am indebted to all of you. May God shower His blessings on you.

My loved ones, back at home have been the 'behind the scene' supporters. Thank you Azezhos, Amus, Anas, Aniis, Anos, cousins, relatives, nieces and nephews for your constant prayers without which my research would not have come this far.

My brothers and sisters, Alo, Angabo, Adi, Akra, BIL and nieces wen and chen, 'Thank You' cannot suffice for everything you have done but my prayer is that God, grant you with all the happiness of life.

My deepest and most sincere gratitude to my mum for her undying love, never failing support, never tiring words of encouragement and ceaseless prayers. Ave, you have been my pillar of strength and I cannot have imagined coming this far without you. Thank you for being there all the time. May God bless you with good health, happiness and a very, very long life ahead.

Merino Visa

I dedicate this Ph.D thesis to my father, Lt. Er. Hito Visa.

Apo, I have finally completed our 'Dream'
The 'Dream' we have planned together and prayed about
The 'Dream' we longed to see come alive
Today, when it did come true,
You are not with me physically
But I know you are rejoicing with me in Heaven
With the Lord and His angels.

Apo, I want to say 'Thank You' for your prayers, your advices and your encouragements.
Because of your hard work, honesty and strong principles, I have reached this far.
Your words of advices and encouragement will always be in my heart to guide me throughout.

Until the day we meet again……

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ABBREVIATIONS

ACN	Acetonitrile
ALP	Alkaline phosphatase
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
Bg	*Biomphalaria glabrata*
bp	base pair
BSA	Bovine serum albumin
Con A	Concanavalin A
cDNA	Complementary DNA
CID	Collision-induced dissociation
CD	Circular Dichroism
DAPI	4', 6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
DSS	Disuccinamidyl suberate
DTT	Dithiothreitol
DVS	Divinyl sulfone
DE-52	DiEthyl-52
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme-linked immunosorbent assay
et al	et alii (Latin: and others)
EtBr	Ethidium bromide
ESI	Electrospray Ionization
FBS	Foetal bovine serum
FITC	Fluorescein-isothiocyanate
HEK	Human Embryonic Kidney
HEPES	N-(2-Hydroxyethyl) piperazine-N'-2-ethane sulfonic acid
IGF-II or IGF2	Insulin like growth factor-II
IP	Immunoprecipitation
IgG	Immunoglobulin G
kb	Kilo base pair
K_d	Dissociation constant
kDa	Kilo Dalton

LC-MS	Liquid Chromatography-Mass Spectrometry
LSD	Lysosomal storage disorders
LTQ	Linear trap quadrupole
min	Minute
mL	Milli Litre
MS	Mass Spectrometry
MPR	Mannose 6-phosphate receptor
NBT	Nitroblue tetrazolium
NC	Nitrocellulose
nm	Nanometer
nM	Nano molar
NP-40	Nonidet-P40
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
Pen/Strep	Penicillin/ Streptomycin
pH	-log (H^+) concentration
PM	Phosphomannan
pmol	Picomole
pNP	*para* nitro phenol
PMSF	Phenyl methyl sulfonyl fluoride
PNGase F	Peptidyl N-glycosidase F
PVDF	Polyvinyl difluoride
rpm	Rotations per minute
RT	Reverse Transcription
SDS	Sodium dodecyl sulfate
SPIDER	Software Protein Identifier
Taq	*Thermophilus aquaticus*
TGN	*trans*-Golgi network
TMB	Tetramethyl benzidine

CHAPTER 1

INTRODUCTION

1. INTRODUCTION

1.1. Physiology of lysosome

Lysosomes were first described by de Duve over 50 years ago in eukaryotic cells and were termed “suicide bags” (de Duve, 1959). They are found in all mammalian cells except red blood cells. The lysosome was first recognized biochemically in rat liver as a vacuolar structure that contains various hydrolytic enzymes which function optimally at an acidic pH. Until the discovery of the ubiquitin-proteasome system, lysosomes were thought to be the sole major players in intracellular protein degradation or turnover (Chiechanover, 2005). However, this advance did not marginalize the study of lysosomes as varieties of biomacromolecules, such as glycoconjugates, lipids and nucleic acids are also degraded within the lysosome, followed by releasing their constituents (building blocks) and transporting the catabolic products back to the cytoplasm for reuse (Cuervo and Dice, 1998). The definition of the lysosome has been broadened over the years. This is because it has been recognized that the digestive process is dynamic and involves numerous stages of lysosomal maturation together with the digestion of both exogenous proteins (which are targeted to the lysosome through receptor-mediated endocytosis and pinocytosis) and exogenous particles (which are targeted through phagocytosis; the two processes are known as heterophagy), as well as digestion of endogenous proteins and cellular organelles (which are targeted by micro- and macro-autophagy; Fig 1.1). The degradative function of these organelles is carried out by around 50 acid hydrolases within their lumen, such as proteinases, lipases and glycosidases. The acidic nature of lysosomes is due to the activity of the vacuolar H^+-type ATP-ase which pumps protons into the lysosomal lumen and thereby maintains the pH at 4.5-5.0 (Mellman *et al.*, 1986). Lysosomes serve as the destination for

cytoplasmic constituents taken up via autophagy which are targeted for degradation and recycling. It also functions in the downregulation of cell surface receptors, inactivation of pathogenic organisms, and repair of the plasma membrane after wounding and loading of processed antigens onto MHC class II molecules *(*Major histocompatibility complex) (Storch and Braulke, 2005).

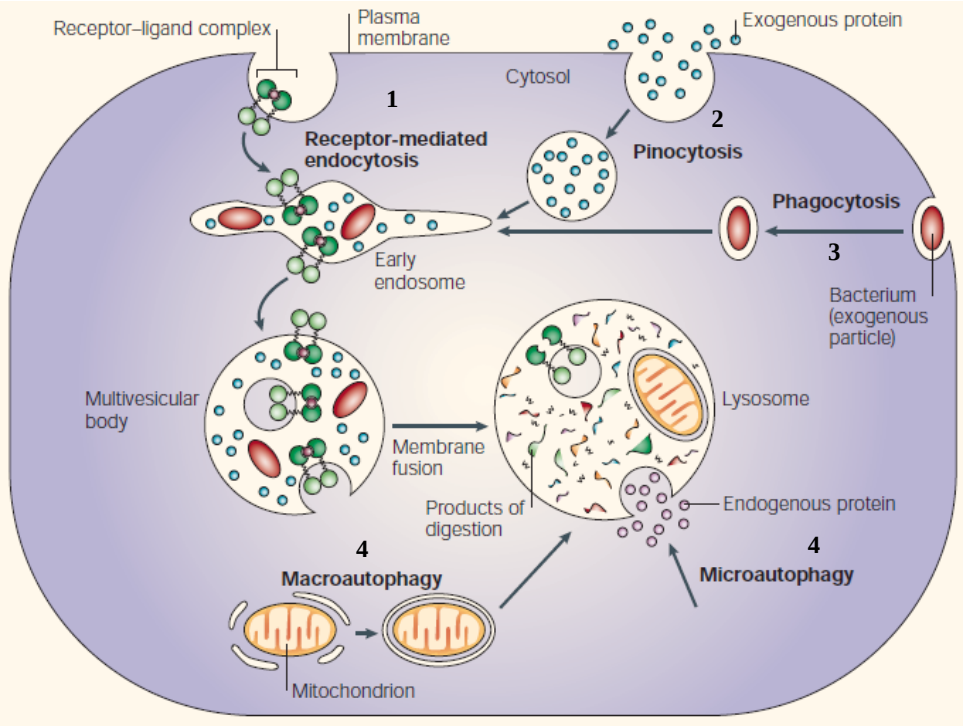


Figure 1.1. The four digestive processes mediated by the lysosome: 1) specific receptor-mediated endocytosis; 2) pinocytosis (nonspecific engulfment of cytosolic droplets containing extracellular fluid); 3) phagocytosis (of extracellular particles), and 4) autophagy (micro- and macroautophagy of intracellular proteins and organelles).

1.2. Lysosomal Proteins

Two classes of proteins are essential for the function of lysosomes: soluble lysosomal proteins (also referred to as acid hydrolases) and integral lysosomal membrane proteins (LMPs). Each of the 50 known lysosomal hydrolases targets

specific substrates for degradation, and their collective action is responsible for the total catabolic capacity of the lysosome.

1.2.1. Soluble Lysosomal Proteins

Degradative function of lysosomes are carried out by hydrolytic enzymes which according to their substrate specificity are classified as proteases, glycosidases, lipases, nucleases, phosphatases and sulfatases (Journet *et al.,* 2002; Kollmann *et al*., 2005; Sleat *et al*., 2005). Lysosomal proteases include three subgroups which represent their active site amino acids: cysteine, aspartic and serine proteases (Brix, 2005; Siintola, 2008). Although they are contained within the lumen of lysosomes and protected by the lysosomal membrane, the proteases are usually detected within all vesicles of the endocytic pathway, i.e. early endosomes, late endosomes, autophagic vacuoles and lysosomes. Several lysosomal proteases such as the aspartic proteinase cathepsin D or cysteine proteinases cathepsin B, H and L, were reported to be ubiquitiously expressed, while others tend to be tissue-specific (Brix, 2005). Among lysosomal proteases, lysosomal cysteine proteases have been well studied. These are also known as cathepsins that are optimally active in the acidic and reducing conditions found in lysosomes. A nuclear isoform of cathepsin B has been reported to be involved in the development of thyroid malignancies (Tedelind *et al*., 2010). It is the first cathepsin that was crystallized (Musil *et al*., 1991; Turk 2001) and whose gene has been silenced (Deussing *et al*., 1998, Foghsgaard *et al*., 2001; Brix, 2005). Cathepsin L represents one of the most potent lysosomal cysteine proteinase. It is 10 to 15 times more active than cathepsin B (Gordon *et al*., 1995). Its isoform has also been reported to be localized in the mammalian cell nucleus (Goulet *et al*., 2004). The majority of cathepsins are endopeptidases with the exception of cathepsin C - aminopeptidase (Barret *et al*., 1998), cathepsin X - carboxy mono- or dipeptidase

(Klemencic *et al*., 2000). Cathepsin B and H, are endopeptidases that also show carboxypeptidase and aminopeptidase activity, respectively (Turk *et al*., 2001). In addition to bulk degradation and pro-protein processing, lysosomal hydrolases are involved in antigen processing, degradation of the extracellular matrix and initiation of apoptosis (Conus and Simon 2008). Knockout mice lacking lysosomal enzymes have provided insights into the functions of lysosomal enzymes. They have also been useful in evaluating new therapies for lysosomal diseases.

1.2.2. Lysosomal Membrane Proteins

The mammalian lysosome contains ~25 LMPs (Schröder *et al.*, 2007; Lübke *et al*., 2009), which are highly glycosylated proteins decorating the luminal surface of lysosomal membranes. LMPs have diverse functions, including acidification of the lysosomal lumen, protein import from the cytosol, membrane fusion and transport of degradation products to the cytoplasm. The most abundant LMPs are lysosome associated membrane protein 1 and 2 (LAMP-1, 107 kDa and LAMP-2, 110 kDa) which are type I membrane glycoproteins and lysosomal integral membrane protein 1 and 2 (LIMP-1, 35-55 kDa and LIMP-2, 60-85 kDa) which are type III membrane glycoproteins. Lysosomes were initially thought to be non-specifically permeable to small molecules, allowing the degraded materials within the lysosomes to be transported out of lysosomes for further use. This view was changed after the identification of protein-mediated pathway for cystine across the lysosomal membrane (Gahl *et al*., 1982; Jonas *et al*., 1982). Further studies have also revealed the existence of transport pathways for amino acids, di- or tripeptides, sugars, inorganic ions and nutrients in purified lysosomal fractions (Pisoni and Thoene, 1991; Sagné and Gasnier, 2008). Thereafter, many studies have been carried out to unravel the composition and the dynamic nature of the lysosomal membrane.

1.2.3. Proteomic study of lysosomal membrane proteins

The greatest challenge in "whole cell" proteomic approaches to understand cellular function is the fact that protein identification techniques are greatly limited due to the large number of individual proteins, their isoforms and the wide range of their abundance. Proteomic analysis of organelles provides useful insights into the biological function of the individual proteins and the organelle in question. A number of proteomic studies (Kollmann *et al*., 2005, Czupalla *et al*., 2006) rely upon the fact that it is possible to distinguish soluble lysosomal proteins from other classes of proteins based on a specific post-translational modification that is required for lysosomal targeting. The proteomics study of lysosomal membrane protein has also been reported by Schröder *et al.,* 2007. In this study, lysosomal membrane proteins were isolated from human placenta and statistical analysis was introduced to significantly exclude co-purified proteins. A lysosome enriched fraction was initially produced by percoll gradient centrifugation, followed by selective disruption of lysosomes of the lysosome enriched fraction. LC-MS/MS data of the lysosomal membrane enriched fraction were compared. By using this strategy, researchers were able to exclude the majority of known mitochondrial, ER, plasma membrane and peroxisomal membrane proteins. 58 proteins previously known to reside at least partially in lysosomes were confirmed, and additional 86 proteins were significantly enriched in the lysosomal membrane fraction, 12 of which were novel proteins with unknown function (Schröder *et al*., 2007, reviewed in Lübke *et al*., 2009).

1.3. Diseases associated with lysosomes

1.3.1. Lysosomal transporter proteins

By the late seventies it became clear that the lysosomal membrane plays an important role in the disposal of metabolites produced by enzymatic degradation of

macromolecules inside the lysosomal compartment. Initially the lysosomal membrane was considered to be only a mechanical border separating the acid lysosomal environment from the neutral surrounding cytoplasm. Most of these transporters function as exporters and only a few as importers. Each of these lysosomal transporters has a high specificity for groups of amino acids, sugars, nucleosides, inorganic ions, and vitamins. Genetic defects due to these transporters cause a wide array of neurological and visceral diseases, ranging from developmental to degenerative disorders. With the sole exception in the Mucopolysaccharidosis IIIC disorder (MPS IIIC, or Sanfilippo C syndrome, Hrebicek *et al*., 2006), all of the known diseases related to the defect in the lysosomal membrane proteins are caused by defects in non-enzymatic membrane proteins, i.e. lysosomal transporter, channel or receptor proteins (Ruivo *et al*., 2009). The first characterized lysosomal storage disorder caused by failure of the transport of catabolism products across lysosomal membrane is cystinosis. In this disease, cystinosin, a 7-transmembrane-domain transporter, is defective that results in the failure to export cystine out of lysosomes. Clinical pathology of this disease is found in the kidney, eye, liver, muscles, pancreas, brain and white blood cells and is manifested in growth retardation and diabetes.

1.3.2. Lysosomal Storage Disorders (LSD)

The concept of "lysosomal storage disorder" was introduced by Hers in 1965 to explain how genetically determined absence of the lysosomal enzyme α-glucosidase could lead to the fatal disorder called Pompe disease. The undegraded substrate would gradually accumulate within lysosomes, causing progressive increase in the dimension and number of these organelles, the cellular pathology would lead the malfunction of the affected organs. This concept quickly led to the discovery of a number of additional lysosomal storage disorders. Lysosomal storage disorders (LSD)

represent a group over 40 distinct genetic diseases, each one resulting from a deficiency of a particular lysosomal protein or, in a few cases, from non-lysosomal proteins that are involved in lysosomal biogenesis. These groups of lysosomal storage disorders is usually caused by the lack of a hydrolase, its activator or a transporter causing accumulation of specific substrates in the lysosomes for each disorder type. Although individually rare the lysosomal storage disorders as a group has a frequency of about 1/8000 live births (Meikle *et al*., 1999, Poorthuis *et al*., 1999) making this disease groups a major challenge for the health care system.

Majority of the LSD's are inherited in an autosomal recessive manner with the exception of Fabry disease and Hunter syndrome {mucopolysaccharidosis type II (MPS)} which show X-linked recessive inheritance. The number of LSD's is increasing as new disorders are characterised both biochemically and genetically. A deficiency of cathepsin K has recently been described which results in LSD called pycnodysostosis (Gelb, 1996). Infantile neuronal ceroid lipofuscinosis (NCL) also known as Batten disease, has been shown to result from a deficiency of palmitoyl protein thioesterase (Vesa, 1995) and classical late-infantile NCL to result from a deficiency of a carboxypeptidase (Sleat, 1997), both lysosomal enzymes. Although each LSD results from mutations in a different gene and consequent deficiency of enzyme activity or protein function, all LSD's share one common biochemical characteristic in that the disorder results in an accumulation of normally degraded substrates within lysosomes. The particular substrates stored and the site(s) of storage vary, although the substrate type is used to group the LSD into broad categories including MPS, lipidoses, glycogenoses and the oligosaccharidoses (Hopwood and Brooks, 1997). These categories show many clinical similarities within groups as well as significant similarities between groups. Common features of many LSD include

bone abnormalities, organomegaly, CNS dysfunction and coarse hair. Most of the human disorders have animal models. Table 1.1. shows some of the major clinical symptoms of diseases associated with the degradation of each type of glycans. Many of these diseases share overlapping symptoms, and yet each disease has unique features that allow it to be specifically diagnosed by experienced clinicians. Usually, an infantile onset is the most severe and the juvenile or adult onsets have attenuated (milder) symptoms. The later onset forms may even affect organ systems different from those affected by early onset forms. Hundreds of mutations have been mapped in the different disorders. The severity usually depends on the combination of mutated alleles. Predicting the disease severity (prognosis) from the specific mutation is generally difficult, except for a combination of null alleles, which have severe outcomes.

TABLE 1.1. Genetic defects in lysosomal degradation of glycans.

Disease name	Enzyme or protein deficiency	Clinical symptoms
Tay-Sachs	β-hexosaminidase A	severe: neurodegeneration, death by 4 years less severe: slower onset of symptoms, variable symptoms all relating to parts of the nervous system
Fucosidosis	α-fucosidase	spectrum of severities includes psychomotor retardation, coarse facies, growth retardation
GM1 gangliosidosis	β-galactosidase	progressive neurological disease and skeletal dysplasia in severe infantile form
Sialidosis	sialidase	progressive, severe mucopolysaccharidosis-like features, mental retardation
Fabry	α-galactosidase	severe pain, angiokeratoma, corneal opacities, death from renal or cerebrovascular disease
Gaucher's	β-glucoceramidase	severe: childhood or infancy onset, hepatosplenomegaly, neurodegeneration mild: child/adult onset, no neurodegenerative course
Krabbe	β-galactoceramidase	early onset with progression to severe mental and motor deterioration
Metachromatic leukodystrophy	Arylsulfatase A (cerebroside sulfatase)	infantile, juvenile, and adult forms can include mental regression, peripheral neuropathy, seizures, dementia
Sly (MPS VII)	β-glucuronidase	wide spectrum of severity, including hydrops fetalis and neonatal form

1.4. Trafficking of lysosomal enzymes

1.4.1. Role of the M6P receptor and post-translational glycosylation

Lysosomal enzymes are enclosed in a membrane bound organelle that contains a set of highly glycosylated lysosomal membrane proteins. These are also components of cell type-specific compartments referred to as lysosome-related organelles which include melanosomes, lytic granules and MHC class II compartments. The biogenesis of new lysosomes or lysosome-related organelles requires a continuous substitution with newly synthesized components. The targeting of acid hydrolases depends on the presence of mannose 6-phosphate (M6P) residues on the enzymes that are recognized by specific receptors mediating the intracellular transport to an endosomal/prelysosomal compartment. The acidification of endosomes, lysosomes, and lysosome-related organelles facilitates not only the dissociation of the receptor-ligand complexes, but also the proteolytic processing required for the enzymatic activation of several hydrolases as well as the denaturation of proteins as prerequisite for lysosomal proteolysis. Most soluble lysosomal enzymes are synthesized as *N*-glycosylated precursors, and the initial steps of biosynthesis are shared with secretory proteins. A summary of lysosomal enzyme trafficking is depicted in Fig1.2. The diversion of the lysosomal enzymes from the secretory pathway is dependent on the acquisition of the M6P recognition marker (Ghosh *et al*., 2003; Storch, 2005). It is important to understand these two steps (Fig 1.3) because it is the acquisition of the M6P marker that separates glycoproteins that are destined for the lysosome from secretory glycoproteins. Failure of acquisition of this marker results in mistargeting of lysosomal enzymes as they will not enter the lysosome and substrate breakdown will not occur. This is precisely what happens in two of the mucolipidosis, I-cell disease and mucolipidosis III. The patients with these diseases lack the enzyme responsible

for the first step, i.e. the phosphotransferase. Consequently, all enzymes requiring the M6P marker fail to enter the lysosome; these patients have very high plasma levels of all such enzymes. In fact, it was this finding that led to the discovery of the M6P ligand and its receptor (Hickman and Neufeld, 1972).

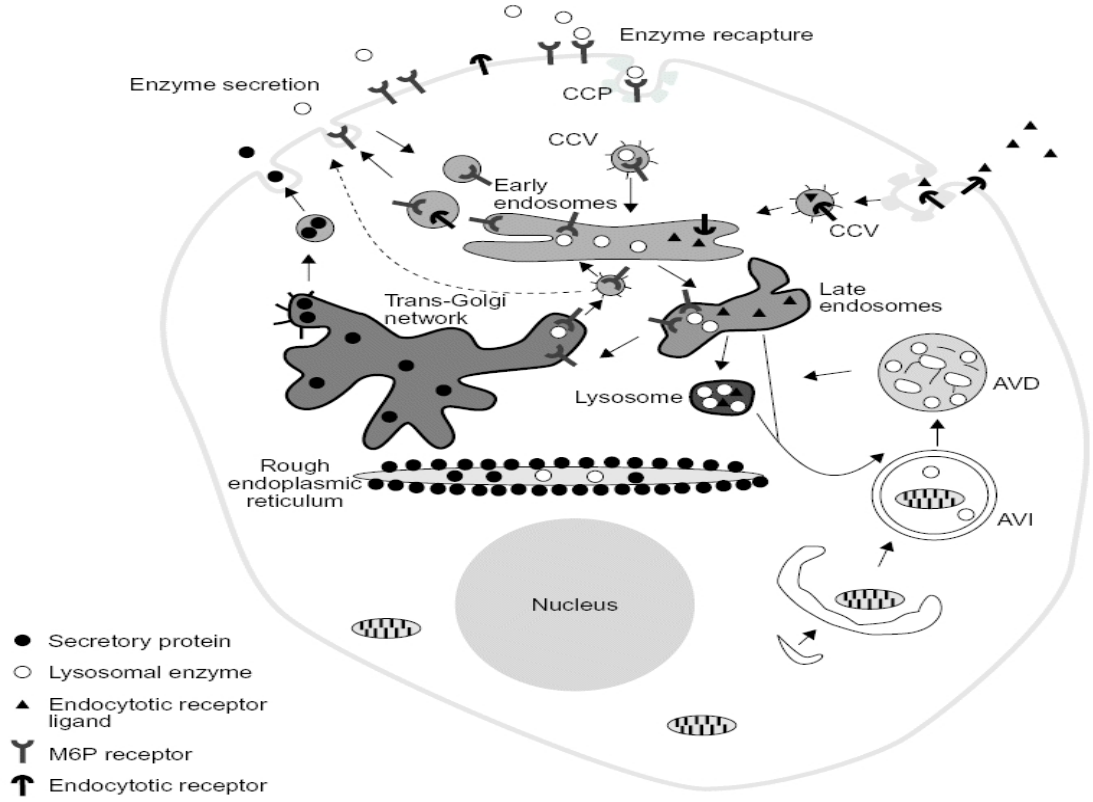


Figure 1.2. Overview of lysosomal enzyme trafficking and model of intracellular transport of the mannose 6-phosphate (M6P) receptor. After synthesis in the rough endoplasmic reticulum and modification in the Golgi apparatus (not shown), precursors of soluble lysosomal enzymes decorated with M6P residues meet the M6P receptor in the *trans*-Golgi network, are packaged into clathrin-coated vesicles (CCV) and transported to late endosomes either directly or indirectly via early endosomes. The process of enzyme transfer from the late endosome to the lysosome is not yet fully elucidated; possibly, the late endosome matures to become the lysosome, or the late endosome and lysosome fuse to form a transient hybrid organelle. The M6P receptor is recycled from the late endosome to the *trans*-Golgi network. The lysosome is devoid of M6P receptors. A minor portion of the enzyme precursor enters the secretory pathway (dotted line) and is recaptured into clathrin-coated pits (CCP) by M6P receptors, which may be transferred from the early endosomes to the plasma membrane. Thus, the enzyme precursors can reach the lysosome via the endocytic pathway, as do endocytic tracer molecules whose receptors are recycled from the tubular extensions of the early endosome. Late endosomes often resemble multivesicular bodies i.e. they display invaginations of their membrane and internal vesicles budded off the invaginations. AVI, early, immature autophagic vacuoles; AVD, mature, degradative autophagic vacuoles.
Taken from: *Lüllmann R. History and morphology of the lysosome.*

In the first step, GlcNAc-1-phosphate is added to the C6-hydroxyl group of selected mannose residues on high-mannose-type oligosaccharides in the lysosomal enzymes from UDP-*N*-acetylglucosamine by *N*-acetylglucosamine-1-phosphotransferase (phosphotransferase). In the second step, *N*-acetylglucosamine residues are removed by an uncovering enzyme (*N* -acetylglucosamine-1-phospho-diester α-*N*-acetylglucosaminidase) which exposes the M6P recognition marker. The human uncovering enzyme is a type I transmembrane glycoprotein of 515 amino acids with a transmembrane domain and a cytoplasmic tail of 41 amino acids. Following the uncovering of the M6P marker, lysosomal enzymes can be recognized by M6P receptors (Ghosh *et al*., 2003; Storch, 2005). Two MPRs - the 46 kDa CDMPR and the 300 kDa CIMR /IGF-IIR bind M6P-containing acid hydrolases in the Golgi apparatus and transport them to the endosomal/lysosomal system. This process involves binding of the hydrolases to the receptors, through their M6P-recognition moieties, and packaging of the ligand-receptor complexes into carriers that transport their cargo to target endosomes (Ghosh *et al*., 2003).

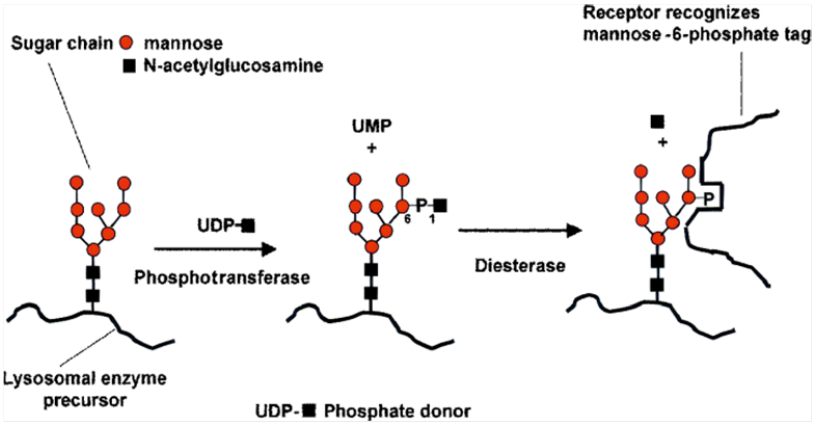


Figure 1.3. Formation of lysosomal recognition marker, mannose 6-phosphate.

1.4.2. Mannose 6-phosphate receptor independent targeting of lysosomal hydrolases to the lysosome

Most lysosomal hydrolases acquire a M6P tag during transport through the Golgi complex which is recognized by MPRs in the TGN (von Figura and Hasilik, 1986; Kornfeld and Mellman, 1989). It is the major pathway for the lysosomal enzyme targeting. Both MPRs are required to guarantee the targeting of all newly synthesized M6P-containing proteins to lysosomes (Ludwig *et al*., 1994). Re-expression studies, either with cation-dependent or cation-independent MPRs in MPR-deficient fibroblasts, have demonstrated that the two receptors exhibit complementary binding properties. Furthermore, each type of M6P receptor transports distinct subpopulations of lysosomal enzymes, presumably as a result of the heterogeneity of the M6P recognition marker (Kasper *et al*., 1996). A variable portion of newly synthesized lysosomal enzymes escape binding to MPRs in the Golgi apparatus and are secreted. These M6P-containing enzymes can be partially internalized and transported to the lysosomes through the cell surface CIMPR mediated endocytosis. About 3-10% of the total cellular MPRs are localized at the plasma membrane at steady state and exchange with MPRs cycling in the biosynthetic pathway. Studies of patients with I-cell disease and of mice lacking both MPRs, including several cell lines and primary cultured cells, have provided evidence for alternative MPR independent transport of newly synthesized lysosomal enzymes to lysosomes. The MPR independent pathways of lysosomal hydrolase transport are mostly unknown, with the exception of β-glucocerebrosidase (βGC) transport (Fig 1.4). Unlike most soluble lysosomal hydrolases, βGC does not obtain an M6P tag, and in I-cell diseased cells or in cells lacking MPR, βGC is normally transported to lysosomes. For a long time it was not understood how βGC, reaches lysosomes. In a

recent study, it was found that LIMP2 (lysosome integral membrane protein 2) was found to be a selective and specific binding partner for βGC (Reczek *et al.,* 2007). Binding between LIMP2 and βGC is pH dependent, thus enabling these proteins to associate in the ER that facilitates transport all the way to the lysosome, where they dissociate because of the acidic pH. In mice lacking LIMP2 (Gamp *et al.*, 2003), βGC is no longer sorted to lysosomes but is instead secreted. It is not yet known whether LIMP2 only carries βGC or if it also sorts any other lysosomal hydrolases. The observations that βGC and LIMP2 associate, that they co-localize in lysosomes and that the activity, levels and localization of βGC correlate with the expression of LIMP2 imply that MPR independent sorting of βGC requires βGC to bind to LIMP2. Furthermore, these data suggest that the transport of lysosomal membrane proteins (LMPs) can be linked to transport pathways of lysosomal hydrolases.

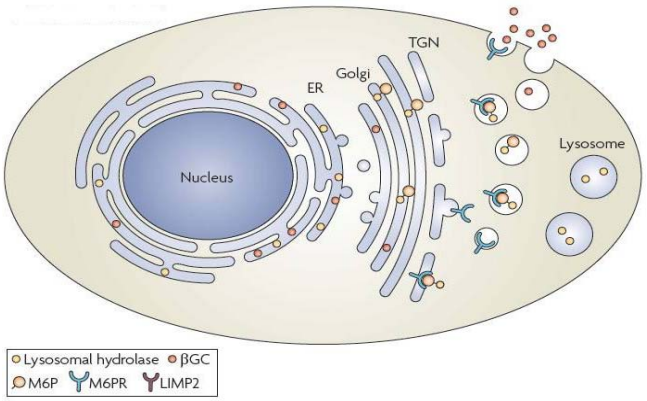


Figure 1.4. MPR independent targeting of lysosomal hydrolases to the lysosome. In cells that lack MPRs (or in I-cell diseased cells, which lack the phosphotransferase necessary to make the M6P tag), most lysosomal hydrolases are secreted, but βGC continues to be targeted to the lysosomes.

It has been shown that the sorting of LAMPs (lysosome associated membrane protein) and lysosomal acid phosphatase is independent of MPRs, but is dependent on short sequence motifs within the cytoplasmic tails, which are necessary and sufficient to target the protein to lysosomes. Newly synthesized LAMPs and LIMPs are transported from the *trans*-Golgi network to endosomes/lysosomes mainly via an intra-cellular route without appearing at the cell surface. LAMP-1 and LAMP-2

molecules in the *trans*-Golgi network are packed into vesicles that are distinct from the clathrin-coated vesicles containing MPRs and AP-1 adaptors. Lysosomal targeting depends on either a tyrosine-based (LAMP-1, LAMP-2, LIMP-1) or a dileucine-based (LIMP-2) sorting signal in the cytoplasmic tail. LAMP-1, LIMP-1/CD-63 and LIMP-2/LGP-85 are targeted from the *trans*-Golgi network to the lysosomes, dependent on the AP-3 adaptor complex (Mullins and Bonifacino, 2001; Eskelinen *et al*., 2003; Schu, 2005).

1.4.3. Ligand binding to M6P receptors is pH dependent

The binding of ligands to the extracellular domain of the CIMPR is pH dependent. This receptor binds to lysosomal enzymes optimally in the Golgi apparatus (pH 6.5), releases its ligands in the acidic environment of the endosomal compartment (pH < 6.0), but fails to interact with lysosomal enzymes at the plasma membrane (pH 7.4) (Ghosh *et al*., 2003). The lysosomal enzymes dissociate in the low pH of the endosomal compartment and are delivered to lysosomes, while the MPRs recycle back to the *trans*-Golgi network to mediate further transport. Small numbers of both types of MPR are localized at the plasma membrane, but only the CIMPR is capable of binding and internalizing M6P-containing lysosomal enzymes from the extracellular space. Besides soluble lysosomal enzymes, CIMPR binds other M6P-containing ligands such as latent transforming growth factor-β1 (TGF-β1), proliferin, granzyme B and thyroglubin. Of the non-M6P containing class of ligands to which the receptor binds, the most extensively studied is the polypeptide mitogen, insulin-like growth factor II (IGF-II). The other members of the non-M6P containing ligands are retinoic acid, urokinase-type plasminogen activator receptor (uPAR) and plasminogen (Braulke, 1999).

1.4.4. Localization of MPRs and signals for M6P receptor transport

The MPRs are localized in the *trans*-Golgi network, early endosomes, recycling endosomes, late endosomes and at the plasma membrane, but they are not found in lysosomes. Both receptors cycle constitutively between these compartments, directed by the specific amino acid signals located in their cytosolic tails (Braulke, 1999; Ghosh *et al*., 2003; Storch and Cheruku, 2005). The efficient sorting of lysosomal enzymes in the Golgi apparatus, mediated by the CIMPR, depends on an acidic cluster/dileucine-based sorting motif near the carboxyl terminus (Chen *et al*., 1997). The rapid internalization of the CIMPR from the plasma membrane requires a tyrosine-based sorting motif. This retrieval mechanism ensures that transiently secreted lysosomal proteins will also end up in the lysosomes, provided that they contain M6P residues. The sorting signals in the cytoplasmic domains of MPRs are recognized by cytosolic adaptor proteins (AP) in distinct subcellular compartments mediating the packaging of the receptors in transport vesicles. The anterograde transport of the MPRs is mediated by AP-1 and the Golgi localized, γ-ear-containing, ARF-binding proteins, whereas the retrograde transport from early and late endosomes requires AP-1/phosphofurin acidic cluster sorting protein-1 and TIP47/Rab 9. The internalization of MPRs from the plasma membrane depends on AP-2 (Schu, 2005).

1.5. Evolutionary Origins of the MPR system

Although the MPR clearly has a major role in lysosomal enzyme trafficking in vertebrate cells, its role in invertebrate systems is not prominent (Kornfeld, 1992; Ludwig *et al*., 1995; Siva Kumar and Praveen Kumar, 2010). Lysosomal enzymes are successfully targeted in lower eukaryotes such as *Saccharomyces*, *Trypanosoma*, and *Dictyostelium*, without the aid of identifiable MPRs. The slime mold *Dictyostelium*

discoideum produces a novel methylphosphomannose sequence on some of its lysosomal enzymes that can be recognized *in vitro* by the mammalian CIMPR (not the CDMPR) (Gabel *et al.*, 1984). However, despite the presence of a GlcNAc-phosphotransferase (GlcNAc-P-T) that recognizes α 1-2 linked mannose residues, no receptor for the phosphorylated mannose residues has been found in these organisms. Notably, although this phosphotransferase does not show the specific recognition of lysosomal hydrolases seen with the mammalian enzyme, it produces another transferase that selectively adds GlcNAc-1-P to serine residues. In contrast to this situation, the protozoan *Acanthameba* produces a GlcNAc-P-T that does show specific recognition of lysosomal enzymes. Although some of these organisms show evidence for an "uncovering" enzyme, no definable MPR has yet been found. The evolutionary divergence point at which the complete MPR system came into being is yet to be identified conclusively.

1.6. Mannose 6-Phosphate Receptors

The first candidate receptor for the phosphomannosyl recognition marker was isolated from bovine liver by Sahagian *et al.*, 1981; using affinity chromatography on immobilized yeast phosphomannan. Shortly thereafter, the same receptor was isolated from other sources by several other investigators, using a variety of affinity methods (Varki and Kornfeld, 1983; Hoflack and Kornfeld, 1985). This receptor (m.w. ~275,000) bound M6P containing ligands in the absence of cations and designated as CIMPR. The observation that certain cells deficient in this receptor still showed M6P-inhibitable binding of lysosomal enzymes led to the discovery of a second M6P receptor with an apparent molecular mass of about 46 kDa, which required divalent cations for optimal binding. The cation-independent M6P receptor (CIMPR) bound with highest affinity in a 1:1 stoichiometry to oligosaccharides carrying two

phosphomonoesters and poorly to molecules bearing GlcNAc-P-Man phosphodiesters. Genes encoding both the MPRs have subsequently been cloned and extensively characterized from several mammalian species such as the bovine, human, mouse. Both receptors are type-I membrane glycoproteins with large extracytoplasmic domains, single transmembrane hydrophobic regions, and relatively small carboxy-terminal intracytoplasmic domains (Kornfeld, 1992; Ludwig *et al*., 1995). The CIMPR has 15 unique contiguous repetitive units of about 145 amino acids each that have partial identity to one another. The cation-dependent receptor (CDMPR) has a single extracellular domain that has homology with some of the repeating domains of the CIMPR. Together with the conservation of certain intron-exon boundaries, this homology suggests that the two genes evolved from a common ancestor. On the basis of their sequence relationships and unique carbohydrate-binding properties, the two MPRs have been formally classified as P-type lectins. Some properties of the two mammalian MPRs are shown in Table 1.2. There are as yet no other members in this family.

The CDMPR exists mainly as a dimer, with each monomer component binding 1 mol M6P or 0.5 mol diphosphorylated oligosaccharides (Tong and Kornfeld, 1989). However, monomeric and tetrameric forms are also found, and the equilibrium between the forms is affected somewhat by temperature, pH, and the presence of ligands. The CIMPR seems to be primarily in a monomeric state. Surprisingly, this much larger molecule binds only two residues of M6P, utilizing just 2 of its 15 repeating units. Mutagenesis studies have identified specific residues of these receptors involved in M6P binding, and the crystal structure of a single extracytoplasmic domain of the CIMPR has recently been obtained, in complex with M6P. This domain crystallized as a dimer, and each monomer was found to fold into a

nine-stranded flattened β-barrel (Roberts, 1998), which has a striking resemblance to avidin. The distance between the two ligand-binding sites of the dimer provides a good explanation for the differences in binding affinity shown by the CDMPR towards various lysosomal enzymes.

The term phosphomannosyl recognition marker thus encompasses a family of M6P-bearing glycans, with varying degrees of affinity for the receptors, based on the position of the phosphate groups and the structure of the underlying oligosaccharide. The number and distribution of such glycans on each acid hydrolase could further alter the effective binding to the two receptors. Thus, whereas both MPRs have a preference for enzymes containing glycans with two phosphomonoesters, it appears that two appropriately spaced monophosphorylated oligosaccharides can together provide a high-affinity ligand. Taken together with factors such as the number, compartmental localization, and availability of the receptor molecules, the differences in the properties of the two receptors, and the concentration of cations, there is clearly much flexibility in this trafficking mechanism. Indeed, different cell types target different M6P-containing proteins to their lysosomes at different rates, with varying proportions being secreted.

Table 1.2. General properties of the two mammalian MPRs (P-type lectins).

Character	MPR 300	MPR 46
Topology	type I membrane glycoprotein	type I membrane glycoprotein
Subunit mass in SDS-PAGE	300 kDa	~46 kDa
Optimal pH for binding	6.0–7.0	6.0–6.5
Cation dependence for binding	no	yes
Domain structure	15 homologous repeating units of ~145 amino acids each	155 amino-acid unit homologous to each of the repeating units of CIMPR
Stoichiometry of M6P binding	two per monomer	one per monomer
K_d for N-glycan with two M6P units	2×10^{-9} M	2×10^{-7} M
Role in biosynthetic pathway	yes	yes
Role in endocytotic pathway	yes	no (except at high density)
Binding of other ligands:		
Methylphosphomannose	yes	no
IGF-II	yes (weak binding in chicken/Xenopus);CEF cells shows binding to IGF-II	no
Retinoic acid	yes	no
Urokinase-type plasminogen activator receptor (uPAR)	yes	no
Plasminogen	yes	no

1.6.1. Structural characterization of the two receptors from the vertebrates:

1.6.1.1. CIMPR

The bovine CIMPR comprises a short NH_2-terminal signal sequence, a 2269 residue extracytoplasmic domain, a single transmembrane domain and a 167 residue cytoplasmic domain. The extracytoplasmic domain contains 15 repeating segments of approximately 145 amino acids each sharing 16-38% of identical residues (Lobel *et al.*, 1988). The two M6P-binding sites have been mapped to luminal domains 3 and 9

and residues Arg 426 and Arg l325 respectively being essential for M6P binding (Dahms *et al*., 1993; Marron-Terada *et al*., 1998; Hancock *et al*., 2002). While domain 9 alone folds into a high affinity carbohydrate recognition site (K_d = 0.3 nM) domain 3 displays only low affinity (K_d ~500 nM) suggesting an importance of additional residues in adjacent domains 1 and/or 2. CIMPR also binds the nonglycosylated insulin-like growth factor II (IGF-11) (Braulke, 1999). The IGF-II binding site has been mapped to repeat 11 in the extracellular domain of the CIMPR (Dahms *et al*., 1994; Schmidt *et al*., 1995). Binding of IGF-II inhibits binding of lysosomal enzymes and their internalization, whereas the sorting and the transport of newly synthesized lysosomal enzymes are not affected by the overexpression of IGF-II (Braulke, 1999). The CIMPR forms dimers induced by the binding of multivalent M6P-ligands but not by IGF-II or M6P (Byrd and MacDonald, 2000; Byrd *et al*., 2000) resulting in an increased receptor internalization rate at the plasma membrane (York *et al*., 1999).

Complete genomic structure of the MPR 300/IGF-IIR is available for mouse and human in chromosome 17 (Laureys *et al*., 1988; Szebenyi and Rotwein, 1994) and chromosome 6 (Laureys *et al*., 1988; Killian and Jirtle, 1999) respectively distinct from the CDMPR and IGF-II genes. A partial cDNA clone (1.368 kb) for the MPR 300 protein from goat showed typical conserved cassette structure in the amino terminal domain similar to other known vertebrate MPR proteins with the conserved cysteine residues, the ligand binding arginine residue in the third domain and the carboxyl terminal border of each cassette defined by the conserved DLS (P/R/S)L motif, which strongly resembles the corresponding sequences found in mammalian counter parts (Suresh *et al*., 2004). The first evidence of MPRs in non-mammalian vertebrates chicken came from the studies of Zhou *et al*., 1995, the full length cDNA

clone of chicken MPR 300 showed 61% identity to the mammalian receptor. Later studies by Matzner *et al.,* 1996, reconfirmed the homologous nature of the chicken embryonic fibroblasts MPR 300 to the mammalian protein. The existence of both MPRs in the early vertebrate fish came from studies carried out in our laboratory (Siva Kumar *et al*., 1999). Trout liver MPR 300 protein was affinity purified from PM-Sepharose gel. The partial cDNA sequence of the fish (*Xiphophorus xiphidium*) MPR 300 displayed significant sequence similarity with chicken (52%) and mammalian MPR 300 (48–50%) (Udaya lakshmi *et al*., 2000). The amino terminal portion of fish MPR 300 is divided into cassettes that show similar length as the chicken and mammalian MPR 300. The border of each cassette was defined by the conserved DLS (P/R/S)L motif, as seen in mammalian and chicken MPRs (DLXXL). Fish MPR 300 contains a corresponding arginine (Arg 426) in cassette 3, which is surrounded by the highly conserved motif CSSGFQ**R**M (T:S)(I:V)INF(Q:E)C. Subsequently cloning and sequencing studies in our laboratory on the fugu fish MPR 300 protein also revealed that this protein is structurally related to other vertebrate MPR 300 proteins (Raju, 2004).

1.6.1.2. Multifunctional nature of cation-independent receptor

Although originally discovered as a receptor for lysosomal enzyme trafficking, the CIMPR turns out to be a remarkably multifunctional molecule (Morgan *et al*., 1987; Kornfeld, 1992; Ludwig *et al*., 1995). IGF-II was previously known to bind two receptors, one identical to the IGF-I receptor and another independent receptor. Molecular cloning of the latter receptor revealed the surprising fact that it is identical to the CIMPR (Kiess *et al*., 1988). Importantly, IGF-II does not carry M6P residues. Many studies have since explored potential interactions between these two different ligands for the CIMPR. Although the two ligands bind to distinct sites on the receptor,

there are conflicting reports regarding synergistic or antagonistic interactions between the two activities. It has also been suggested that the redistribution of the CIMPR upon IGF-II stimulation could explain some of the known metabolic effects of this hormone on protein degradation, by altering the trafficking of lysosomal enzymes. Morgan *et al*., (1987) provided the first evidence, which unequivocally showed that MPR 300 was also a receptor for the insulin-like growth factor II (IGF-II). A number of reports indicate that binding of IGF-II to the CIMPR regulates motility and growth in some cell types (O'Dell *et al*., 1998). It has also been found that the CIMPR binds retinoic acid with high affinity at a site that is distinct from those for M6P and IGF-II. This binding of retinoic acid seems to enhance the primary functions of the CIMPR receptor, and the biological consequence appears to be the suppression of cell proliferation and/or induction of apoptosis. The significance of this unexpected observation is still being explored. Other interesting ligands that the CIMPR binds include urokinase-type plasminogen activator receptor (uPAR) and plasminogen, whose binding sites have been mapped to a peptide region within domain 1 (Nykjaer *et al*., 1998). The proposed function for the interactions between the MPR300/IGF-IIR and these ligands is involvement in the complex responsible for the activation of TGF-β1 at the cell surface, as well as endocytosis and targeting of uPAR for degradation. There are also some unexplained changes in CIMPR expression in relation to malignancy (Sleat *et al*., 1995). Loss of heterozygosity at the CIMPR locus occurs in dysplastic liver lesions and in hepatocellular carcinomas associated with the high-risk factors of hepatitis virus infection and liver cirrhosis. Mutations in the remaining allele were detected in about 50% of these tumors, which also seem to frequently develop from clonal expansions of phenotypically normal, CIMPR-

mutated hepatocytes. Thus, the CIMPR fulfills many of the classic criteria to be classified as a liver "tumor-suppressor" gene.

1.6.1.3. CDMPR

Hoflack and Kornfeld in 1985 first discovered a cation-dependent mannose 6-phosphate receptor with an apparent molecular mass of 46 kDa. The function of the CDMPR in lysosomal enzyme transport was unclear until cells lacking this receptor were generated by gene disruption experiments in mice (Köster *et al*., 1993; Ludwig *et al*., 1993). This approach demonstrated that the CDMPR also functions in transport of hydrolases to lysosomes. The cDNA for the MPR 46 has been cloned and sequenced from several species such as human, bovine, mouse and goat (Suresh *et al*., 2004 and the references therein). Partial sequence for a cDNA from chicken (Matzner *et al*., 1996) and the full length cDNA sequence from chicken MPR 46 gene were obtained (Praveen and Siva Kumar, 2008). The early vertebrate Fugu and *Xiphophorous* fish (Raju, 2004) Zebra fish (Suresh *et al*., 2006) and the invertebrate starfish MPR 46 (Siva Ramakrishna and Siva Kumar; 2008) genes were also cloned. The human CDMPR gene has been mapped to chromosome 12 (Pohlmann *et al*., 1987) and is comprised of seven exons that span a 12 kb region. The genomic structure of the CDMPR has been analyzed for the human (Klier *et al*., 1991) and the mouse (Ludwig *et al*., 1992). MPR 46 is a highly conserved protein with 93-95% overall homology in different mammals from mouse to human and with completely identical amino acid sequence within the cytoplasmic domain of these species. The residues His 131 and Arg 137, shown to be important for M6P-recognition (Wendland *et al*., 1991), are located within the ligand-binding region. Conservative replacement of these 2 residues reduced the binding activity without affecting other properties that might change upon alterations of the receptor conformation. Crystal structures of the

extracellular domain of the bovine CDMPR complexed to pentamannosyl-phosphate revealed that the binding site of the receptor encompasses the phosphate group plus three of the five mannose rings of the ligand (Olson *et al*., 1999).

1.7. Lysosomal enzymes

Lysosomal enzymes play a very important role in cells and have been the subject of study ever since their early discovery. Although initially purified and characterized from mammals, subsequent studies using different vertebrates and invertebrates (animal models and specific cell types) revealed several lysosomal enzyme activities. To gain new information on these enzymes and to make a comparison with the well characterized mammalian enzymes, it becomes necessary to purify and characterize the enzymes from invertebrates.

1.7.1. Cathepsin D

Cathepsins are a class of globular lysosomal proteases, most of which contain an active-site cysteine residue. The main physiological role for cathepsins in general is degradation of proteins at acidic pH in the lysosomes, or extracellularly in the matrix. The possible involvement of cysteine and aspartic cathepsins in cancer has been the subject of more debate than that of serine proteases. The involvement of cathepsin D in several physiological functions such as protein degradation, apoptosis and autophagy (Benes *et al*., 2008) has been reported (Table 1.3). Additionally, it is also found to be associated with certain pathological conditions such as cancer (Vetvicka *et al*., 1994), Alzheimer's disease (Zhou W *et al*., 2006) and neuronal ceroid lipofuscinosis (Siintola *et al*., 2006). Cathepsin D is found in almost all mammalian cells and has a typical lysosomal localization (Sakai *et al*., 1989; Saku *et al*., 1991). It is one of the most widely studied proteinases of the muscle which participate in lysosomally mediated protein degradation *in vivo*. Its amino acid

sequence shows 40 to 48% homology to well known aspartyl proteinases such as rennin, pepsin and chymosin (Zeece and Katoh, 1989).

Table 1.3. General properties of cathepsin D.

S. No.	Characteristics	Cathepsin D	References
1	**Cellular distribution**	Widely distributed in almost all mammalian cells	Sakai, *et al* 1989; Saku, *et al* 1991; Yokota, *et al* 1983; Snyder, *et al* 1985
2	**Subcellular localization**	Lysosome	Sakai, *et al* 1989; Saku, *et al* 1991; Yokota, *et al* 1983; Snyder, *et al* 1985
3	**Specific inhibitors**	Pepstatin A	Byun, *et al* 2009; Fan, *et al* 2010
4	**Substrate Affinity**	Prefers aromatic amino acid at P1 and P1´ positions, Leucine is strongly favored at P1, the hydrophobic requirements are less strict at P2 and P1´. A charged preferably basic residue is found mostly at positions P2´and P5´; at least 1 basic residue appears to be required by cat D at either position.	Yasuda, 2005; van Noort, van der Drift, 1989
5	**pH Range**	2-5 (pH optimum is around pH 4.0).	Bazel, Alhadeff, 1999; Yasuda, *et al* 1999, 2005
6	**Purification**	Pepstatin A agarose gel column chromatography	Bazel, Alhadeff, 1999; Robert *et al* 2009; Merino, Siva Kumar, 2012
7	**Engineering**	The mutation is linked with Alzheimer's disease, the mutant is devoid of catalytic activity,	Vashishta *et al* 2009; Masson *et al* 2010
8	**Disease Association**		
i	Pathological condition developed in mice-deficient in cat D.	Massive intestinal necrosis, thromboembolia, lymphopenia, and neuronal ceroid lipofuscinosis.	Saftig, *et al* 1995; Koike, *et al* 2000
ii	Over expression of the enzyme observed	Prostrate, breast and ovarian cancer.	Hara, *et al* 2002; Duffy, *et al* 1991; Kozyreva, *et al* 1991
9	**Application**	A marker for the detection of cellular senescence, Pepstatin A on enzyme inhibits apoptosis, an independent marker of poor prognosis in breast cancer	Bidere, *et al* 2003; Byun, *et al*2009; Masson, *et al* 2010

1.7.2. α-L-Fucosidase

Alpha-L-fucosidases (EC 3.2.1.51) are exoglycosidases found in organisms ranging from bacteria (Cobucci-Ponzano *et al.*, 2005), to fungi (Wong-Madden and

Landry, 1995), molluscs (Focarelli *et al*., 1997; Berteau *et al*., 2002), ascidians (Matsumoto *et al*., 2002) and mammals (Alhadeff, 1998). α-L-fucosidases have been divided into two distinct glycoside hydrolase families: α-L-fucosidases that catalyze the hydrolysis using a retaining mechanism belong to the well known glycoside hydrolase family 29 (GH29) (Coutinho and Henrissat, 1999), while those that catalyze the hydrolysis using an inverting mechanism belong to the recently identified glycoside hydrolase family 95 (GH95) (Katayama *et al*., 2004). The GH29 family includes α-L-fucosidases from a variety of sources, from human to several pathogenic bacteria, whereas the α-L-fucosidases of GH95 family are from plants and various bacteria. Table: 1.4. summarizes the molecular properties of the α-L-fucosidase isolated and studied from different animal sources. Mammalian α-L-fucosidases are lysosomal enzymes involved in the hydrolytic degradation of fucose-containing molecules and their deficiency results in fucosidosis, a storage disease resulting in the lethal accumulation of fucosylated glycosphingolipids and glycoproteins in the central and peripheral nervous system (Michalski and Klein, 1999). Decreased α-L-fucosidase activity is found also in cystic fibrosis (Scanlin and Glick, 2000). Increased α-L-fucosidases activity in serum is a marker of the development of colorectal and hepatocellular carcinomas (Ayude *et al*., 2003; Zhou L *et al*., 2006). α-L-fucosidase has also received attention due to the ubiquity and biological significance of L-fucose and the importance of fucosylated glycoconjugates in numerous crucial biological processes (Becker and Lowe, 2003).

Table 1.4. Molecular properties of α-L-fucosidase characterized from some animal sources.

Species	Isoform	MW(kDa) subunit	pH	℃	Glycoprotein	Metal	Reference
Homo sapiens	I,II	1*100[a] 2*50[a]	I - 5.3 II -5.0	50	I :sialoglycoprotein II: mannose-rich	--	Chien *et al* 1980
Homo sapiens	--	50[b]	Very stable at neutral pH	--	--	--	Merino-Trigo *et al* 2002
Bos Taurus	I,II	330[a],200[a]	5.3	50	--	--	Jauhiainen *et al* 1986
Sus scrofa	--	4 *55[b]	5.1		+	--	Grove, Serif, 1981
Pectenmaximus	--	200[a], 4*50[b]	4	60	--	Zn^{2+}	Berteau *et al* 2002
Unio elongatulus	--	55.9[c],67.8[c]	5	50	+ for 55,--for 67	--	Focarelli *et al*1997
Chamelea gallina	--	200[a]	4	65		Zn^{2+},Ca^{2+}	Reglero, Cabezas,1976
Drosophila ananassae	--	256[a],48[b],55[b]	4	45	--	Ag^{+},Cd^{2+},Co^{2+}, Cu^{2+},Fe^{2+},Zn^{2+}	Cenni, Perotti, 2006
Penaeus monodon	--	233[a], 4*63[b]	5	65	+ sialylated	--	Chuang *et al* 1991
Bacillus circulans	--	285[a], 2 * 135[b]	5.5	50		Hg^{2+}	Tsuji *et al* 1990
Fusarium oxysporum	--	75[b]	5.5	60	+	Hg^{2+}	Yamamoto *et al* 1986
Dictyostelium discoideum	--	62[a]	3.7		+		Schopohl *et al* 1992
Trypanosoma cruzi	--	50[b]	7	37	+	--	Miletti *et al* 2003

[a] Molecular Weight (MW) determined by gel filtration, [b] MW determined by SDS-PAGE, [C] mass spectrometry, pH: Optimal pH, ℃: Optimal temperature & stability, Metal: Metal ion required.

1.8. Scope of the present investigation

The laboratory where this work has been carried out specializes in the field of protein biochemistry/glycobiology. The work involves development of new affinity methods for the large scale purification of biologically important proteins from plants and animal sources such as the lectins and glycosidases. Alternate affinity methods were developed to purify the mannose 6-phosphate receptor proteins [(MPR 300 (Mr 300 kDa) and MPR 46 (Mr 46 kDa)] from the goat liver. These proteins have been extensively characterized by other researches from different mammalian sources such as the human, bovine, mouse and have been found to be involved in the targeting of lysosomal enzymes to lysosomes. Mammalian MPR 300 is also the IGF-II receptor and has been implicated in internalization of the circulating IGF-II for degradation in the lysosomes. Antibodies to the purified goat receptors were developed which were used to quantify the receptors in different tissues of goat and chicken. Using the goat receptors as a model system, homologous receptors were affinity purified and biochemically characterized in the laboratory from different non-mammalian vertebrates such as the birds, reptiles, amphibians and fish. Several lines of evidences suggest evolutionary conservation of the receptors from fish to mammals. Of particular interest are the 15 repetitive cassette structures in the extracytoplasmic domain of the MPR 300 protein from fish to mammals which are conserved. In particular the critical arginine residues found in mammalian proteins responsible for the binding of lysosomal enzymes (mannose 6-phosphate) are conserved in 3rd and 9th domains of the protein from the fish to mammals (Siva Kumar and Praveen Kumar, 2010). Subsequent studies revealed that the invertebrates such as the echinodermates (starfish) and molluscs, (*unio*) also contain the mammalian homologues of both the receptors. Cloning and characterization of the starfish MPR 46 suggests high degree

of structural homology to the vertebrate receptors and explained its role in lysosomal enzyme sorting (Sivaramakrishna and Siva Kumar, 2008). Using *Biomphalaria glabrata* (snail cells) it has also been shown that the MPR 300 protein is able to endocytose lysosomal enzymes (Praveen Kumar *et al*., 2009). These data point to the fact that possibly in the invertebrates also the receptors are highly conserved and these animal species might have similar lysosomal biogenesis pathway as in mammals. However, no MPR 46 homologue could be detected in the *Drosophila melanogaster* and only a Lysosomal Enzyme Receptor Protein (LERP) which exhibits only 25 % sequence homology to the human MPR 300 protein and lacks the binding ability to phosphomannan-Sepharose gel was discovered (Dennes *et al*., 2005). These available informations suggest that possibly molluscs are the starting point for the evolution of lysosomal enzymes and their receptors and echinodermates and molluscs might have conserved lysosomal biogenesis pathway as in the vertebrates. To validate this hypothesis it becomes important to isolate as many lysosomal enzymes as possible from the highly evolved invertebrate starfish and the molluscs.

Therefore, the present study was initiated to gain more information on lysosomal enzymes from starfish, and to analyze the possible multifunctional nature of the early vertebrate fish and the starfish MPR 300 protein, with special emphasis on its IGF-II binding properties. Two lysosomal enzymes have been the subject of intense investigation. These are the cathepsin D and α-fucosidase. Each of them specifically is well characterized in humans. Cathepsin D enzymes from mammalian sources are well known and the enzyme plays a major role contributing to the lysosomal digestive activity. The importance of α-L-fucosidase has been fueled for the use of this enzyme as a tool for elucidating the structure and function of mammalian glycoproteins and glycolipids.

The structural relatedness of MPRs in the vertebrates and their role in lysosomal enzyme targeting and the identification of the putative MPRs in invertebrates, starfish, molluscs suggests that in invertebrates these receptors might have similar roles as in the vertebrates. Evidence that the starfish MPR 46 is the lysosomal sorting receptor has already been obtained (Sivaramakrishna and Siva Kumar, 2008). Defining the IGF-II binding property of different vertebrate receptors has been important in view of the finding that initially only mammalian MPR 300 proteins exhibited IGF-II binding and this binding is dependent on the isoleucine residue at 1572 position in the 11^{th} domain of the receptor. Chicken liver MPR 300 showed weak IGF-II binding and has a leucine residue at 1572 position. This suggested that possibly IGF-II binding property of the receptor is a late event in evolution. After the work of Mendez *et al*., (2001) who showed that the fish embryo MPR 300 binds IGF-II, chicken CIMPR as well as the chicken embryonic fibroblast cell receptor which falls above the fish in the evolutionary tree also shows IGF-II binding affinity (Suresh *et al*., 2006). Studies carried out by other workers on Zebra fish MPRs also revealed that the MPR 300 also exhibits conserved structural domains and has isoleucine residue as in mammals but whether or not it binds human IGF-II needs to be elucidated (Catherine *et al*., 2006). Also in fugu fish it has the threonine residue in place of isoleucine residue. Thus no such conclusive evidence has been shown so far for the IGF-II binding domains among the different vertebrate CIMPRs in fish. Putative receptors from the invertebrates were also reported (Udaya lakshmi *et al*., 1999; Siva Kumar and von Figura, 2002; Sivaramakrishna and Siva Kumar 2008). But no further investigation has been carried out regarding the IGF-II binding to the invertebrate CIMPR. Therefore, it is important to gain new insights into the IGF-II binding domain of the early vertebrate fish and invertebrate CIMPR to establish the evolution of the receptor

and functional significance of its domain structures in particular the IGF-II binding domain. Though some lysosomal enzyme activities have been identified from some invertebrate species, only a few enzymes have been purified and characterized. In an earlier study we affinity purified a lysosomal α-fucosidase from the mollusc (*unio*) (Siva Kumar *et al*., 2004) and characterized the same.

To gain new insights into the evolutionary conservation of lysosomal biogenesis pathway, the present study was carried out (i) isolation, affinity purification and biochemical characterization of two lysosomal enzymes cathepsin D and α-fucosidase from starfish and studied the interaction with starfish MPR 300 protein, (ii) cloning and sequencing of A_2 cell MPR 46, (iii) analyze the interaction between the human IGF-II and purified fish and starfish CIMPR protein and make a structural comparison of the sequences of the available IGF-II domains in the vertebrates.

CHAPTER 2

Isolation, affinity purification and biochemical characterization of a lysosomal cathepsin D from the deuterostome *Asterias rubens*

2.1. INTRODUCTION

Cathepsin D (EC 3.4.23.5) is one of the well characterized lysosomal enzymes which has been shown to be involved in protein degradation *in vivo*. This lysosomal enzyme is an aspartyl proteinase and a glycoprotein ubiquitously found in the cells and tissues of mammals and birds. Its amino acid sequence shows 40 to 48% homology to well known aspartyl proteinases such as rennin, pepsin and chymosin (Zeece and Katoh, 1989). Cathepsin D exists as several isoforms with pIs in the range of 5.7 to 6.8. The pH optimum of cathepsin D is in the range 3.0 to 4.5, which is typical for aspartyl enzymes, but it also exhibits a broad range of activity on muscle substrates in the pH range 3.0 to 6.0 (Schwartz and Bird, 1977). The native molecular weight of cathepsin D appears to vary depending on the tissue and species examined. Cathepsin D from skeletal muscle of the rat and rabbit, shows a molecular mass of 42 kDa and consists of a single polypeptide chain as evidenced by SDS-PAGE (Schwartz and Bird, 1977; Okitani *et al*., 1981). Cathepsin D isolated from canine cardiac muscle has a molecular mass of 42 kDa by gel filtration and dissociates to two subunits of 32 kDa and 14 kDa respectively when examined by SDS-PAGE (Edward *et al*., 1982).

Cathepsin D enzyme from mammalian sources are well known and the enzyme is probably one of the major factors contributing to the lysosomal digestive activity (Diment *et al.*, 1988; Metcalf and Fusek, 1993; Tsuji and Akasaki, 1994). It has been reported that it constitutes as much as 10% of the lysosomal proteins (Wittlin *et al*., 1998). In addition, cathepsin D may have other roles in cells such as activation of proteins destined for secretion and to process antigens for presentation to the immune system (Maric *et al*., 1994). Mammalian cathepsin D enzymes have been isolated and cloned from a wide range of sources such as bovine (Gubensek *et al*., 1976) and

human (Wright *et al*., 1997; Nogami *et al*., 2000). The enzyme has also been purified from some fish species such as tilapia (Jiang *et al*., 1991) and Antarctic icefish (Capasso *et al*., 1999). However in the lower invertebrates which lack a well developed digestive system, the enzyme is less extensively characterized (Barnard, 1973). In a recent study it was well established that the lysosomal enzyme targeting machinery is evolutionary conserved from fish to mammals (Siva Kumar and Praveen Kumar, 2010). In order to further understand the evolution of these receptors we extended our studies to the invertebrates and found the mammalian homologues of the putative receptors in the invertebrates such as the starfish and *unio*. Our recent studies using starfish as an animal model suggested evolutionary conservation of the MPR 46 protein (Sivaramakrishna and Siva Kumar, 2008), and that the MPR 300 is involved in specific binding of an affinity purified α-fucosidase enzyme (Merino Visa *et al*., 2012). In order to establish the evolutionary conservation of the lysosomal enzyme sorting machinery in the invertebrates, it becomes essential to purify and characterize different lysosomal enzymes and to understand their interaction with the receptor(s). The present study was carried out in order to gain new insights into these aspects with the following objectives:

i) Affinity purify the cathepsin D from starfish and biochemically characterize the same,

ii) Raise an antibody for the enzyme and determine its specificity as well as its utility to quantify the enzyme levels in different tissues of the animal,

iii) Study its specific interaction with the MPR 300 protein.

2.2. MATERIALS AND METHODS

2.2.1. Materials

Starfish animals were collected from North Sea, Germany and were stored frozen at -80℃ until use and were kindly provided by Prof. Dr. Sørge Kelm, University of Bremen, Germany. Affigel-10 was purchased from Bio-Rad laboratories (Hercules, CA, USA). Pepstatin A, Fruend's complete adjuvant, Freund's incomplete adjuvant and *p*-nitrophenyl phosphate were purchased from Sigma Chemical Co, (St.Louis, Missouri, USA). Nitrocellulose membrane was purchased from Millipore (Bedford, MA, USA). Mouse cathepsin D antiserum raised in a rabbit was a generous gift from Prof. Dr. Regina Pohlmann, University of Muenster, Germany. Other chemicals and buffer reagents were obtained from commercial sources and were of analytical grade.

2.2.2. Preparation of Pepstatin A affinity gel

10 mg of Pepstatin A was dissolved in 1 mL of absolute alcohol and kept on a 60℃ water bath for 10 min. The prepared Pepstatin A was then coupled to 2.0 mL Affigel-10 (Bio-Rad) following manufacturer's instructions. The gel was washed with 10 mM sodium-phosphate buffer, pH 7.4, containing 150 mM NaCl (PBS) and stored at 4℃ until use.

2.2.3. Extraction and affinity purification of cathepsin D from starfish

All operations were carried out at 4℃ unless otherwise stated. Fifty grams of the whole animal tissue was thawed on ice and homogenized with 4 volumes of 25 mM Tris-HCl buffer pH 7.4 (buffer A). The homogenate was allowed to stir overnight and centrifuged (26892 x g, 30 min). The pellet was discarded and the supernatant was recentrifuged for 15 min as described before. Ammonium sulfate was added to the supernatant to attain 80% saturation and the suspension stirred for 3 h. The

precipitated protein was collected by centrifugation (26892 x g, 30 min), dissolved in a small volume of 50 mM sodium acetate buffer, pH 3.5, containing 0.2 M NaCl (buffer B) and dialysed extensively against the same buffer. The dialysed sample was briefly centrifuged and the clear supernatant was applied to a 2 mL Pepstatin A affinity gel (1.5 x 9 cm) pre-equilibrated with buffer B. The unbound fraction was recycled several times through the column. The non-specific proteins were washed off with buffer B and elution was carried out with 50 mM Tris-HCl buffer pH 8.0 containing 0.2 M NaCl (buffer C) at a flow rate of 30 mL/h.

2.2.4. SDS-PAGE and western blotting

Protein concentrations in the extracted samples and column fractions were determined by the dye-binding method using bovine serum albumin (1.0 mg/mL) as the standard (Bradford, 1976). 10% SDS-PAGE analysis was carried out for column eluates according to Laemmli (1970) in the presence and absence of reducing agents, such as dithiothreitol (DTT). The proteins were boiled for five minutes in the SDS sample buffer, separated on a gel and were detected by coomassie staining. Native-PAGE was also performed for the purified enzyme and the protein detected by silver staining. Initially, the authenticity of the purified enzyme was analyzed in a western blot experiment using the mouse anti-cathepsin D antiserum (1:500). The enzyme band was detected by incubating the membrane with the secondary antibody (goat anti-rabbit IgG conjugated to alkaline phosphatase) followed by incubation with the substrate, BCIP/NBT (Bangalore Genei, India).

2.2.5. Immunological characterization of the purified cathepsin D

2.2.5.1. Production of antisera

A rabbit polyclonal antiserum was raised against the purified starfish cathepsin D. ~750 µg of purified enzyme in 0.5 mL PBS was mixed with 1.0 mL Freund's

complete adjuvant and injected subcutaneously into a rabbit. After three weeks, a booster injection was given with ~750 µg of the purified enzyme in 0.5 mL PBS mixed with 0.5 mL Freund's incomplete adjuvant. Two weeks after the booster injection, blood was collected by ear vein puncture from the rabbit into a falcon tube and allowed to clot. Antiserum was collected by centrifugation and stored at -20℃. Additional booster injections were given at two week intervals and the rabbit was bled 10 days after each booster injection. The final bleeding of the rabbit was performed after four weeks. The rabbit was housed and handled at the University of Hyderabad animal house.

2.2.5.2. Affinity purification of anti-cathepsin D antibodies and quantification of the purified enzyme by ELISA method

Starfish cathepsin D protein purified from Pepstatin A affigel was concentrated by centricon concentrator (MWCO-10) and 1 mg of the protein was coupled to 1.0 mL of Affigel-10 employing the conditions described by the manufacturer. Antiserum to purified protein was extensively dialyzed against 10 mM Tris-HCl buffer, pH 7.4, containing 150 mM sodium chloride (column buffer) and then applied to the cathepsin D affigel at 4℃ equilibrated with same buffer. After extensively washing the gel with column buffer, bound IgG was specifically eluted with three column volumes of 100 mM glycine-HCl buffer, pH 2.65. The eluted protein was immediately neutralized with 2 M Tris, and an aliquot was analyzed on a 7.5% SDS-PAGE under non-reducing conditions. This IgG was also used to identify the cathepsin D in the soluble extracts.

Affinity purified antibodies against cathepsin D proteins were adsorbed to microtiter wells of a 96 well ELISA plate for 4 h at 37℃ (250 ng of affinity-purified cathepsin D antibody in 50 µL of 25 mM Tris-HCl, pH 7.4). The wells were washed with 200

µL of 25 mM Tris-HCl buffer, pH 7.4, and incubated overnight at 4℃/ 1h at room temperature with 200 µL of buffer A (5% BSA in PBS). Fifty microliters of purified cathepsin D (0.5-10 ng), or soluble tissue extracts from whole animal tissue, digestive gland and gonads (0.5-5.0 µg) diluted in buffer A (quantitation of protein was done according to Bradford), were separately bound for 2.5 h at 37℃. The wells were washed four times with 200 µL of PBS (buffer B) followed by incubation with 200 µl of buffer A for 30 min at 37℃. Subsequently, 50 µL of diluted rabbit antiserum against cathepsin D (dilution in buffer A, 10^{-3}) was added and the plate incubated for 1 h at 37℃. After washing four times with buffer B, goat anti-rabbit IgG conjugated to alkaline phosphatase (Bangalore Genei) (dilution 1:2000 in buffer A) was added and incubated for 1 h at 37℃. The wells were washed four times with buffer B and one time with 200 µL of buffer C (0.1 M Tris-HCl pH 9.5, 0.1 M NaCl, 2 mM $MgCl_2$). The colour was developed with *p*-nitrophenyl phosphate (1.0 mg/mL in buffer C) for 10 to 20 min at room temperature and the absorbance was measured at 405 nm in a microplate ELISA reader.

2.2.5.3. Interaction of the purified enzyme with starfish MPR 300 analyzed by immunoprecipitation and ligand blot analysis

To confirm the specificity of the purified antibody, aliquot of the purified cathepsin D and receptor concentrated by centricon concentrator (MWCO-10) was taken in two separate tubes and incubated with preimmune serum (2 µL) and affinity-purified cathepsin D IgG (10 µg) respectively overnight at 4℃ with rotation, in PBS containing 0.05 % Tween 20 (PBST) in a total volume of 500 µL. The antigen-antibody complexes were adsorbed to protein A-agarose (40 µL of a 10% suspension; Bangalore genei, India). After incubation for 1 h, protein A-agarose was collected by

centrifugation at 3502 x g, and the supernatants were discarded carefully leaving a few µL to avoid pipetting of protein A-agarose beads. The pellet was washed four times with PBST. The immunoprecipitates were solubilized under reducing conditions and analyzed by 10% SDS-PAGE. The protein was transferred to a nitrocellulose membrane and the blot was incubated with secondary antibody, goat anti-rabbit HRP conjugate and developed using the Super Signal West Femto Maximum Sensitivity Substrate.

The interaction of cathepsin D with the starfish MPR 300 protein purified by phosphomannan-affinity chromatography (Sivaramakrishna and Siva Kumar, 2008) was analyzed by ligand blot analysis. The purified cathepsin D was separated on a 10% SDS-PAGE under reducing conditions and the proteins transferred to a nitrocellulose membrane. The membrane was incubated for 16 h with purified starfish MPR 300 (100 µg) in blocking buffer (PBST containing 1% BSA) and subsequently probed with *unio* MPR 300 antibody (10 µg) raised in a rabbit against purified *unio* protein. The band was visualized by incubating the membrane with secondary antibody goat-anti rabbit HRP conjugate and developed using the Super Signal West Femto Maximum Sensitivity Substrate.

2.2.6. Glycoprotein nature of the purified cathepsin D

Purified cathepsin D enzyme (2.0 mL) obtained from Pepstatin A affigel was applied on a Con A-Sepharose gel (1.0 mL) pre-equilibrated with 25 mM Tris-HCl buffer, pH 7.4, containing 5 mM $CaCl_2$ and 5 mM $MnCl_2$ (column buffer). After washing extensively with the column buffer, the bound protein was eluted with the column buffer containing 0.2 M methyl α-D-mannopyranoside. Protein was monitored at 280 nm and the extent of binding was determined. The carbohydrate content in the enzyme was determined (Dubois *et al.*, 1956). For detection of

glycoprotein nature of the enzyme, periodic acid Schiff's staining procedure was used (Zacharias *et al*., 1969).

Deglycosylation of the enzyme was performed as described (Keinanen *et al*., 1988). The enzyme was dialyzed against 0.1 M sodium-phosphate buffer pH 8.6, containing 0.2% SDS. To the dialyzed sample, NP-40 and β-mercaptoethanol were added to a final concentration of 1.2 and 1%, respectively. The sample was boiled at 95℃ for 5 min. To this PMSF, EDTA and iodoacetamide were added to a final concentration of 1, 1 and 5 mM, respectively. The sample was divided into two equal portions as control and experiment. Only experimental tube received 3 µL (3U) of PNGase F enzyme (Boehringer-Mannheim). Both the tubes were incubated at 37℃ for 16 h. The samples were separated on a 10% SDS-PAGE and proteins transferred to a nitrocellulose membrane. Protein bands were detected using 1:500 dilution of antibody raised against purified starfish cathepsin D.

2.2.7. Confocal microscopy

Human Embryonic Kidney 293 (HEK) cells were cultured in complete medium (DMEM with 10% (v/v) heat inactivated foetal bovine serum and 1% (v/v) penicillin/streptomycin at 37℃ in 5% CO_2, which are known to contain the MPR 300 protein. It is used in the study to analyze localization of the endocytosed FITC conjugated cathepsin D as described below.

2.2.7.1. Immunofluorescence

The HEK cells were analyzed for the presence of cathepsin D protein by immunofluorescence using affinity purified cathepsin D antibody. The cells were grown to 80% confluency on sterile glass cover slips in complete DMEM medium at 37℃. The medium was discarded and the cells were washed with sterile PBS three times, 5 min each. The cells were fixed in 4% paraformaldehyde in PBS buffer for 6

min at room temperature, washed and permeabilized with 0.2% saponin in PBS for 6 min. The cells were later washed and blocked with 5% BSA in PBS for 30 min at room temperature before incubation with purified cathepsin D specific IgG (1:100 dilutions) for 1 h. Subsequently the cells were washed 5-6 times with PBS, blocked with BSA as above and incubated with fluorescent tag secondary antibody for 1 h (in dark with FITC conjugated goat anti-rabbit IgG using 1000-fold dilution in PBST). Finally the slides were washed extensively, stained with DAPI and observed under confocal microscope. As a negative control in a separate experiment, the HEK cells were incubated directly with fluorescent tag secondary antibody without prior incubation with cathepsin D specific IgG. The cells were stained with DAPI and visualized microscopically. All the processing reactions were done at room temperature.

2.2.7.2. Localization of endocytosed FITC-conjugated cathepsin D

About 1 mg of purified cathepsin D was incubated with 550 µl borate buffer (50 mM, pH 9.0) and 100 µl fluorochrome solution of fluorescein-isothiocyanate (5 mg FITC dissolved in 1 mL of DMSO) was added to the enzyme sample, mixed well and the sample stored overnight at 4℃ in dark. Free FITC was removed by desalting using a Sephadex G-25 gel.

The endocytosis of FITC conjugated cathepsin D was studied by incubating with HEK cells grown to 80% confluency on sterile glass cover slips in complete DMEM medium at 37℃. Following incubation, cells were washed with PBS three times and then they were fixed in 4% paraformaldehyde for 20 min at room temperature. The fixed cells were washed, permeabilised with 0.02% Triton X-100 for 30 seconds and blocking with 1% BSA in PBS for 30 min at room temperature. The cells were incubated with goat MPR 300 antibody (This antibody was raised for purified goat

MPR 300 protein and has been shown in our laboratory to recognize the MPR 300 protein from molluscs to mammals) 1:200 dilution in 1% BSA in PBS. The cells were washed and incubated with fluorescent tagged anti-rabbit IgG-Alexa fluor 594 (molecular probes, Invitrogen) for 1 h at room temperature. Finally the cells were stained with DAPI and observed under confocal microscope.

2. 2.8. Specificity of the lysosomal enzyme (ligand) and MPR 300 (receptor) interaction

2.2.8.1. Chemical cross-linking of starfish cathepsin D with starfish MPR 300

Cross-linking studies were carried out as described (Causin *et al*., 1988). Thirty-seven microliters of starfish MPR 300 protein (2-3 µg) was dialyzed against 50 mM sodium phosphate buffer pH 7.4, containing 150 mM NaCl/0.1% Triton X- 100 and incubated for 3 h at 4℃ with 13 µL cathepsin D (5 µg) dialyzed against the same buffer. To this, DSS was added to a final concentration of 1 mM and the reaction was carried out for 15 min in ice. The reaction was stopped with 5 µL of 1 M Tris-HCl pH 7.4, 2.75 µL of 20% SDS and 3 µL of 100% glycerol. The reaction mixture was heated at 95℃ for 5 min and the samples were subjected to 7.5% SDS-PAGE under non-reducing conditions. The proteins were transferred to a nitrocellulose membrane and probed with starfish cathepsin D antibody (10 µg). The cross-linked product was visualized by incubating the blot with secondary antibody goat-anti rabbit HRP conjugate and developed using the Super Signal West Femto Maximum Sensitivity Substrate. In a separate experiment only affinity purified starfish cathepsin D and MPR 300 protein was separated and processed for western blot analysis as described above. The membrane was probed with cathepsin D antibody and *unio* MPR 300 antibody respectively.

2.2.8.2. Immobilized receptor-affinity chromatography

500 µg of affinity purified starfish receptor (Sivaramakrishna and Siva Kumar, 2008) was immobilized to 1.0 mL Affigel-10 following manufacturer's instructions. The gel was equilibrated with EDTA buffer (50 mM imidazole pH 7.0, containing 5 mM sodium β-glycerophosphate, 150 mM sodium chloride, 0.05% Triton X-100, 2 mM EDTA). The purified cathepsin D obtained by affinity chromatography was dialyzed against the EDTA buffer and applied onto the receptor-affigel column. The gel was washed with four gel volumes of the EDTA buffer and sequentially eluted with 2 mL each of 5 mM glucose 6-phosphate, 5 mM mannose 6-phosphate followed by 50 mM sodium acetate buffer pH 5.0. The eluates were completely TCA precipitated (final 10% TCA concentration), pellet collected by centrifugation and neutralized with 0.4 M Tris and analyzed by 10% SDS-PAGE. The protein bands were visualised by silver staining.

2.3. RESULTS

2.3.1. Purification, SDS-PAGE analysis and immunoblotting

The starfish tissue was processed as described under methods. The protein solution obtained after dialysis with buffer B was applied onto the pre-equilibrated Pepstatin A-affigel for purifying the enzyme. From 50 g of the tissue, 450 µg protein could be obtained in the final step. An aliquot of cathepsin D eluate was subjected to 10% SDS-PAGE in the presence and absence of reducing agent, which revealed a single protein band with an apparent molecular mass of 45 kDa (Fig 2.4.A, lane 2 and 3) indicating that the enzyme is a monomeric protein. The purified protein was detected by coomassie brilliant blue R-250. The protein purified also cross-reacted with the mouse cathepsin D antiserum (Fig 2.4.1B, lane 2) suggesting antigenic similarities among the invertebrate and the mammalian enzyme. Furthermore, the homogeneity of the affinity purified cathepsin D was also evaluated by using native-PAGE. From (Fig 2.4.1C) it is apparent that the enzyme from *Asterias rubens* migrated as a single protein band in the native-PAGE, confirming its homogeneity.

2.3.2. Immunological characterization of the purified cathepsin D

2.3.2.1. Affinity purification of anti-cathepsin D antibodies and quantification of the purified enzyme by ELISA method

In the present study, the purified starfish enzyme was used to generate a polyclonal antibody. Cathepsin D specific IgG was obtained from the antiserum developed by passing the antiserum on cathepsin D-affigel as described under methods. Elution profile of cathepsin D specific IgG from cathepsin D-affigel is shown in Fig 2.4.2A. To check the homogeneity of the cathepsin D specific IgG eluted from the gel, an aliquot of the eluate was subjected to 7.5% SDS-PAGE under non-reducing conditions (Fig 2.4.2B, lane 2). The specificity of the antibody obtained

was further tested in a western blot experiment using crude extracts containing the enzyme. From Fig 2.4.2C, it is apparent that the antibody recognizes the protein in the extracts. Using the affinity purified antibody and the antiserum to the cathepsin D protein, an ELISA method was developed to quantify the enzyme from different tissues of the starfish such as the whole body tissue, digestive gland and the gonads. In order to establish precisely the concentration of the protein that could be detectable by the antibody developed, initial ELISA was carried out employing purified cathepsin D protein. From (Fig 2.4.3A) it is clear that the level of detection of cathepsin D protein lies in the range of 1-10 ng. The enzyme concentration was also quantified by ELISA employing the total soluble extract obtained from whole body tissue (Fig 2.4.3B), digestive gland (Fig 2.4.3C) and gonads (Fig 2.4.3D). In these crude preparations, 1-5 µg concentration of the enzyme could be detectable in each of the tissues tested.

2.3.2.2. Interaction of the purified enzyme with starfish MPR 300 analyzed by immunoprecipitation and ligand blot analysis

Cathepsin D was immunoprecipitated using the cathepsin D antibody as described under methods. The precipitated protein was then subjected to SDS-PAGE and visualized by Western blotting. Under the same condition pre-immune serum did not show any reactivity with the purified enzyme suggesting the immunoprecipitation to be specific. The results of these are shown in Fig 2.4.4A, lane 1 and 2 respectively. To confirm the specificity of the interaction of the starfish enzyme with the starfish MPR 300 protein, ligand blot analysis was done. From the results obtained in this experiment (Fig 2.4.4B), it is clear that the enzyme binds to the purified starfish MPR 300 protein.

2.3.3. Glycoprotein nature of the purified Cathepsin D

Purified enzyme was found to be a glycoprotein with ~9% carbohydrate content as determined by phenol-sulfuric acid method. Con-A lectin has been widely used in ligand blot assays to detect glycoproteins and most N-linked glycoproteins have an affinity to bind to Con-A gel (Focarelli *et al*., 1997). When the purified enzyme was applied onto a Con-A gel, the enzyme was completely bound on the gel and could be specifically eluted using 0.2 M methyl α-D-mannopyranoside confirming the carbohydrate nature of the purified enzyme (Fig 2.4.5A, lane 2). A further confirmation to the glycoprotein nature of the purified enzyme came from periodic acid Schiff's staining (Fig 2.4.5B). Furthermore, the untreated and the PNGase F treated enzyme showed different mobilities on SDS-PAGE (Fig 2.4.5C, lane 1, 45 kDa and lane 2, ~40 kDa) respectively. Both protein bands have the ability to cross-react with the antibody raised in this study.

2.3.4. Confocal microscopy

2.3.4.1. Immunofluorescence

The presence of cathepsin D protein in mammalian cell line was shown by immunofluorescence (Fig 2.4.6.I Panel B). II Panel B shows the negative control where the cells were incubated directly with fluorescent tagged secondary antibody without prior incubation with cathepsin D specific IgG.

2.3.4.2. Localization of endocytosed FITC-conjugated cathepsin D

The ability of the FITC-conjugated cathepsin D to interact with the MPR 300 protein on the HEK cells was investigated using confocal microscopy. Most mammalian cells contain the MPR 300 protein on the cell surface which has been shown to be involved in the internalization of mannose 6-phosphate containing ligands. The endocytic function of the HEK cells MPR 300 was revealed by using

FITC-conjugated cathepsin D as described under materials and methods. The endocytosed FITC-cathepsin D can be seen in Fig 2.4.6.III panel B (green). In the same cells, MPR 300 protein was also visualized using the goat MPR 300 antibody and fluorescent tagged anti-rabbit IgG-Alexa fluor 594 antibody as shown in panel C (red). Extensive co-localization of endocytosed FITC-cathepsin D with the MPR 300 protein can be seen in panel C. These results suggest the labeled enzyme is internalized through the cell surface MPR300 protein.

2.3.5. Specificity of the lysosomal enzyme (ligand) and MPR 300 (receptor) interaction

2.3.5.1. Chemical cross-linking of starfish cathepsin D with starfish MPR 300

Chemical cross-linking agents such as DSS are used to understand the specific interaction of proteins with their ligands. Further the cross-linked product should exhibit higher molecular mass as compared to the native proteins prior to cross-linking. Purified starfish MPR 300 was used in cross-linking experiments. Cross-linking of the receptor with the purified enzyme resulted in a product that exhibited lower mobility and higher molecular mass on SDS gel (Fig 2.4.7A, lane 1). The molecular mass of the cross-linked product was roughly equal to the molecular mass of the receptor and cathepsin D enzyme together (300 and 45 kDa respectively). Figure 2.4.7A, lane 2 is the enzyme alone. The fact that this interaction is specific is supported by the reactivity of the cross-linked product with starfish cathepsin D antibody. Fig 2.4.7B shows the protein band of the starfish MPR 300 alone detected using *unio* MPR 300 antibody.

2.3.5.2. Immobilized protein-affinity chromatography

In order to gain further insight into the specificity of the interaction between the purified enzyme and the starfish MPR 300 protein, we analysed the binding of

cathepsin D to starfish MPR 300 protein immobilized to Affigel-10. At pH 7.0, (that favours binding) the purified enzyme bound strongly to the receptor-affigel. The bound enzyme could not be eluted using 5 mM glucose 6-phosphate (Fig 2.4.7C, lane 2) but could be eluted partially with 5 mM mannose 6-phosphate (Fig 2.4.7C, lane 3). When 50 mM sodium acetate buffer, pH 5.0 (that favours dissociation) was employed, some more protein was desorbed from the gel (Fig 2.4.7C, lane 3).

FIGURES 2.4

Figure 2.4.1. Electrophoresis of *Asterias rubens* cathepsin D.

(A) 10% SDS-PAGE analysis of the purified cathepsin D from starfish. Lane 2, under reducing conditions, Lane 3, under non reducing conditions. **(B)** Western blot analysis of the purified enzyme detected using mouse cathepsin D antiserum. **(C)** 7.5 % Native PAGE analysis of the purified cathepsin D. Arrow indicates position of the protein band.

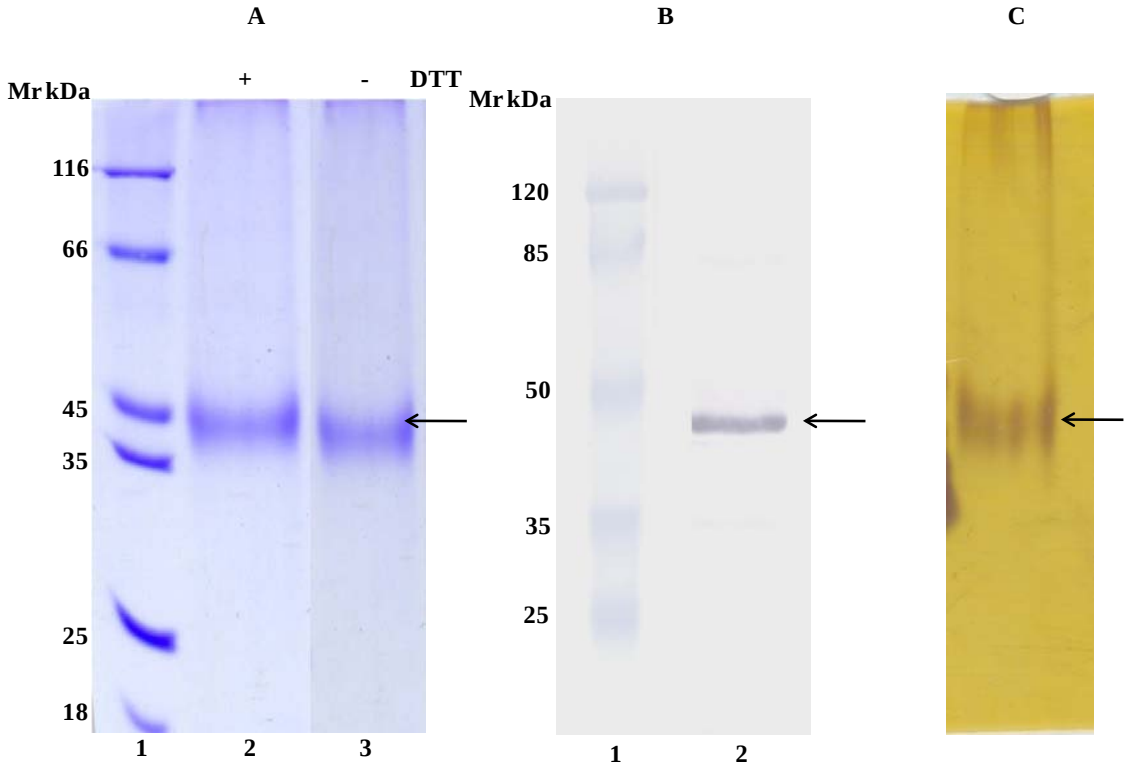


Figure 2.4.1

Figure 2.4.2. Purification of starfish cathepsin D specific IgG.

(A) Elution profile of cathepsin D specific IgG from cathepsin D-affigel. **(B)** 7.5% SDS-PAGE analysis (non-reducing conditions) of cathepsin D specific IgG eluted from cathepsin D-affigel column with 100 mM glycine-HCl buffer, pH 2.65. Arrow indicates IgG band (Mr 150 kDa). **(C)** Western blot analysis of the crude soluble extract detected using the purified cathepsin D specific IgG. Arrow indicates position of the protein band.

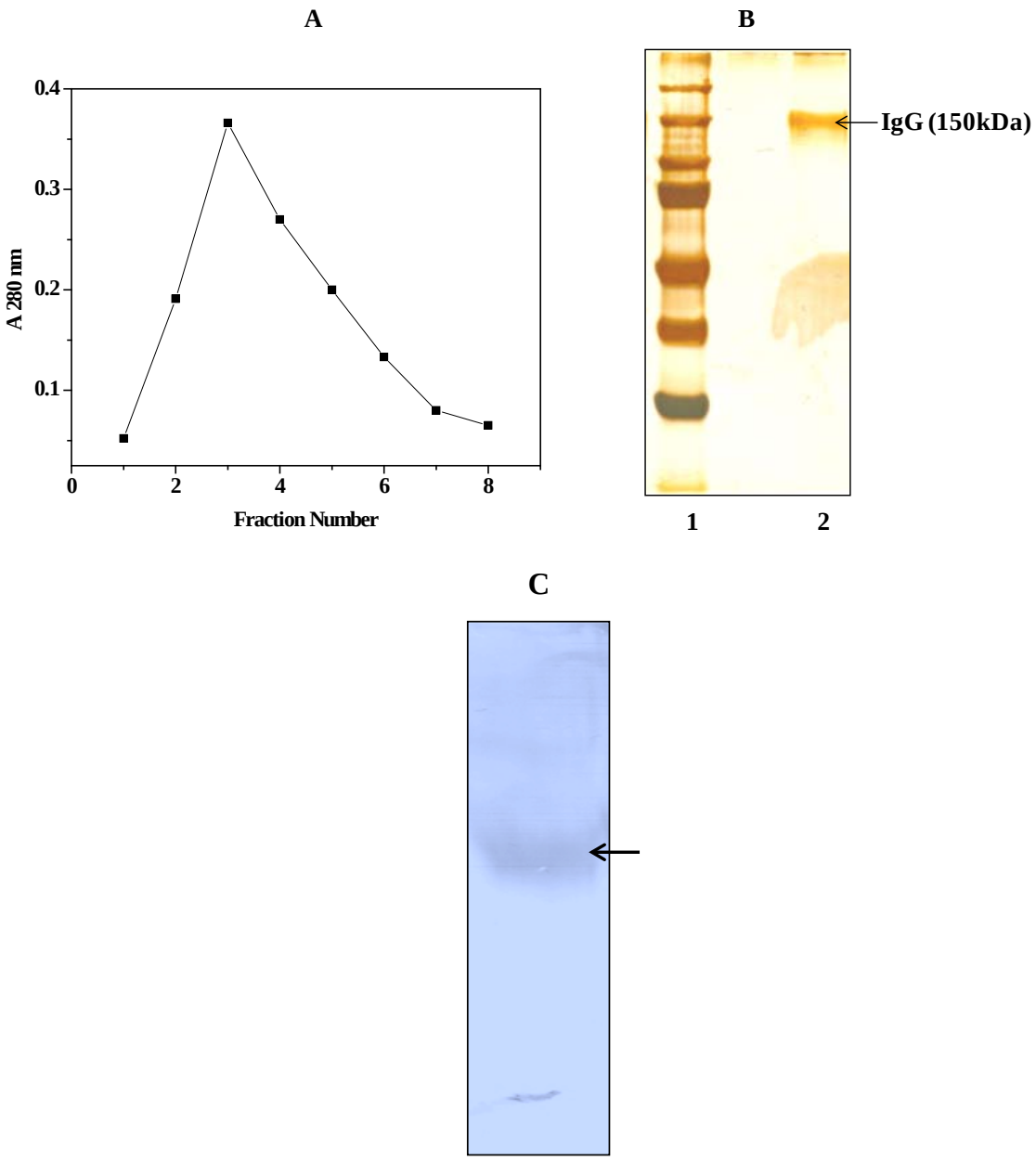


Figure 2.4.2

Figure 2.4.3. Quantification of the enzyme by ELISA. (A) represents purified cathepsin D. **(B)** represents soluble extract from whole animal tissue. **(C)** represents soluble extract from digestive gland and **(D)** represents soluble extract from gonad. The assays were done in triplicates and averages of the values were taken.

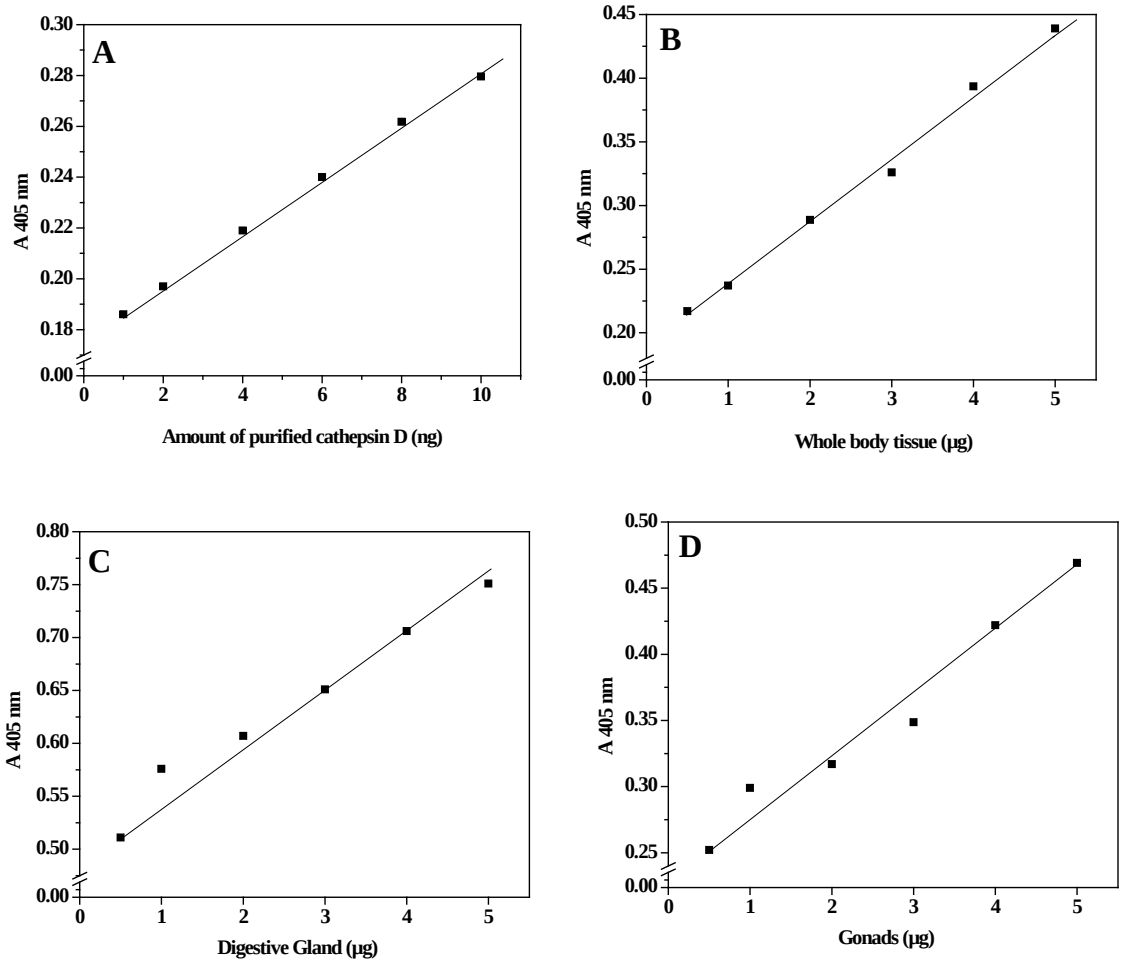

A
A 405 nm
0.30
0.28
0.26
0.24
0.22
0.20
0.18
0.00
0
2
4
6
8
10
Amount of purified cathepsin D (ng)
B
A 405 nm
0.45
0.40
0.35
0.30
0.25
0.20
0.00
0
1
2
3
4
5
Whole body tissue (µg)
C
A 405 nm
0.80
0.75
0.70
0.65
0.60
0.55
0.50
0.00
0
1
2
3
4
5
Digestive Gland (µg)
D
A 405 nm
0.50
0.45
0.40
0.35
0.30
0.25
0.00
0
1
2
3
4
5
Gonads (µg)

Figure 2.4.3

Figure 2.4.4. Interaction of the purified enzyme with purified starfish MPR 300. (A) Immunoprecipitation of the purified cathepsin D enzyme (10% SDS-PAGE). Lane 1, enzyme and pre-immune serum and Lane 2, enzyme and immune serum. **(B)** Ligand blot analysis of the purified cathepsin D enzyme in a 10% SDS-PAGE. Arrow indicates the recognition of enzyme-receptor complex by *unio* MPR 300 antibody.

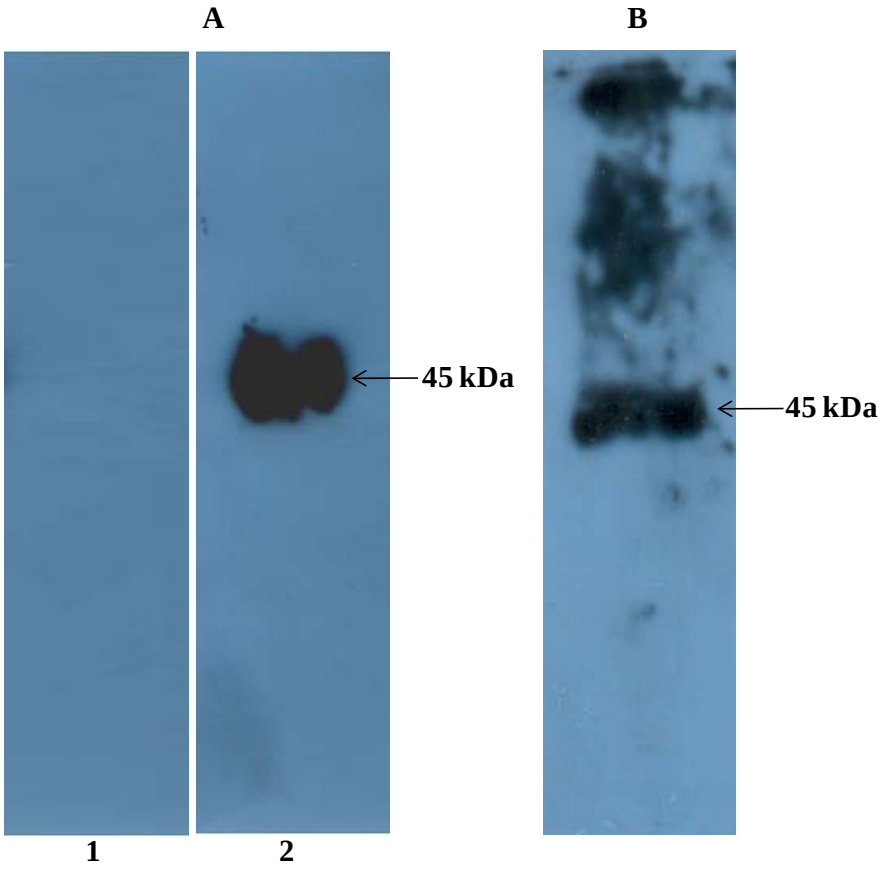


Figure 2.4.4

Figure 2.4.5. 10% SDS-PAGE analysis of the purified cathepsin D.

(A) Purified enzyme under reducing condition eluted from Con A-Sepharose gel and detected by silver staining. **(B)** Periodic acid schiffs (PAS) staining of the protein band. **(C)** Western blot analysis of the enzyme. Lane 1, native enzyme (- PNGase F) and lane 2, deglycosylated enzyme treated with (+ PNGase F) detected using starfish cathepsin D antibody.

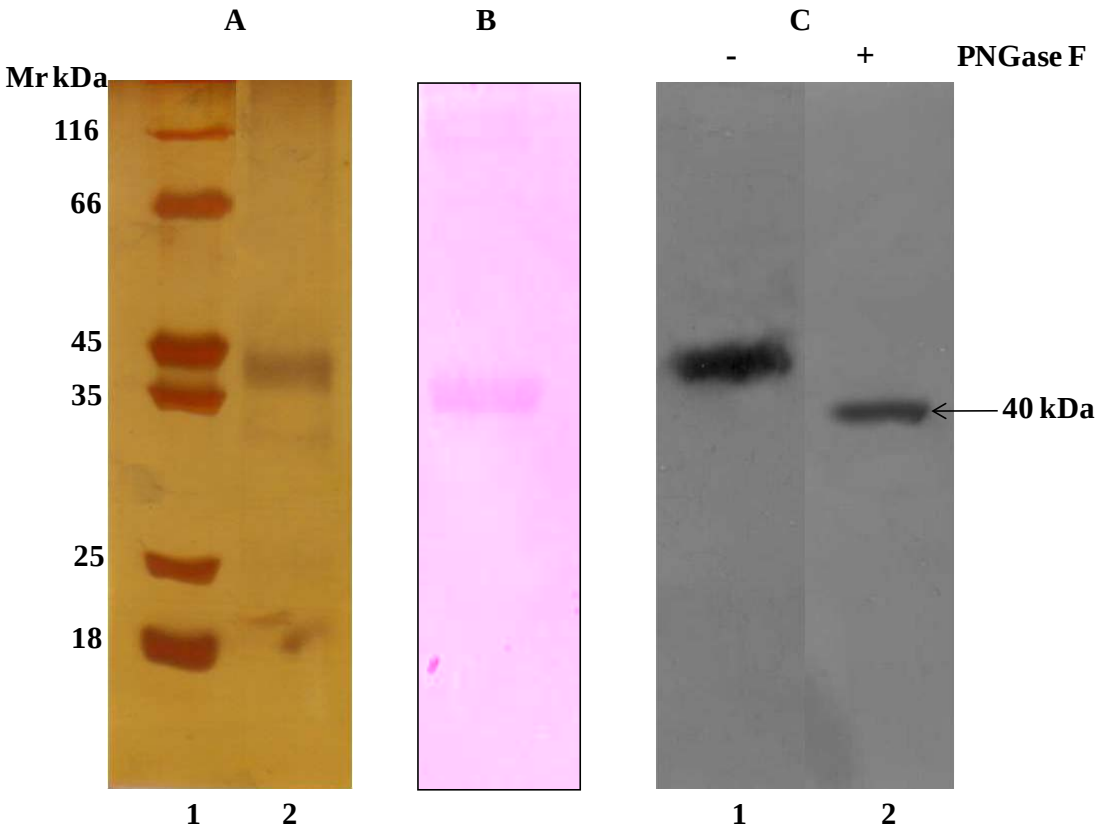


Figure 2.4.5

Figure 2.4.6. Immunofluorescence of cathepsin D.

(I). Cells incubated with specific IgG (A) DAPI stain, (B) probed with cathepsin D specific IgG (1:100 dilutions) followed by incubation with FITC conjugated secondary antibody (green) (1:1000 dilutions), (C) merged images of A & B, (D) the transmission image; **(II)** Cells without specific IgG (negative control). (A) DAPI stain, II (B) incubation with FITC conjugated secondary antibody (green) (1:1000 dilutions). (C) the transmission image. Bar in both the panel I & II is 25 µm. **(III) Localization of the endocytosed FITC-conjugated cathepsin D in HEK cells:** (A) staining with DAPI, (B) Incubation with FITC-conjugated cathepsin D (green) for 1 hour at 37℃, (C) Incubation with goat MPR 300 antibody followed by fluorescent tagged anti-rabbit IgG-Alexa fluor 594 (red) secondary antibody, (D) Merged images of A, B & C. (E) The transmission image. Bar in the panel is 10 µm.

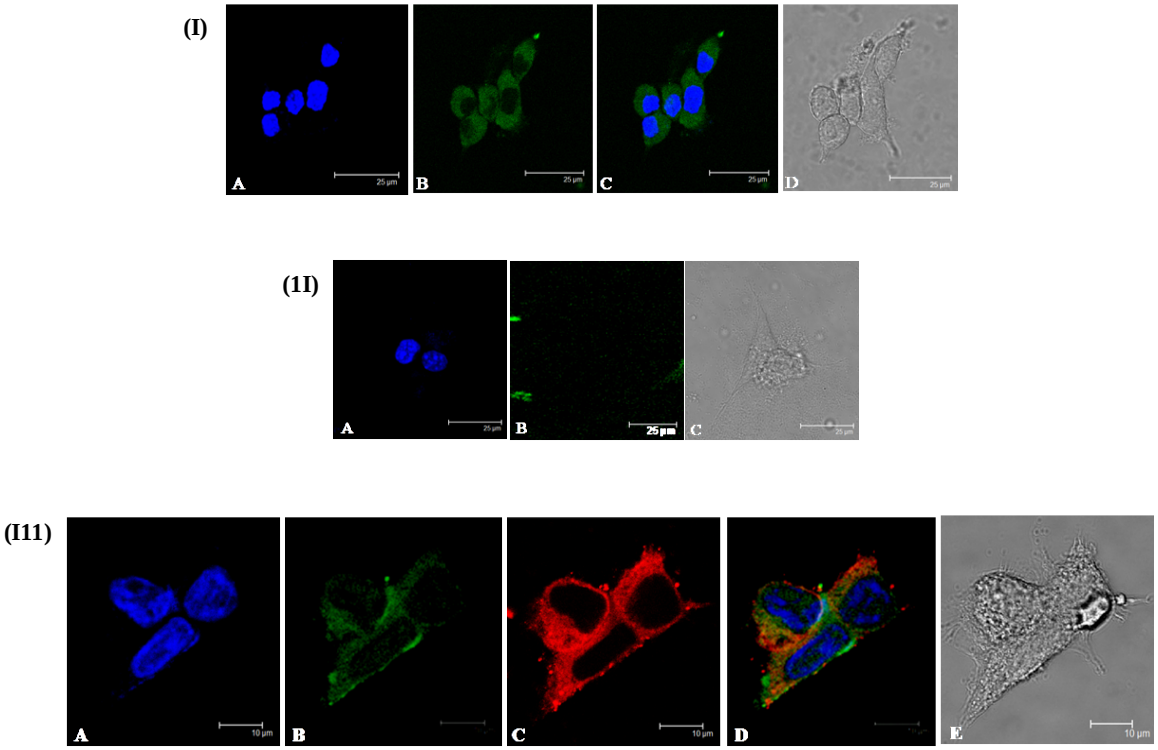


Figure 2.4.6

Figure 2.4.7(A) Cross-Linking of the ligand with the membrane receptor. Cathepsin D was affinity cross-linked to purified starfish MPR 300 receptor with DSS, as described in Materials and Methods. The complexes were visualized by western blotting after 7.5% SDS-PAGE under non-reducing conditions. **(A)** Lane 1, purified starfish MPR 300 protein cross-linked with purified cathepsin D using DSS. Lane 2, only purified cathepsin D. Both the cross-linked product and native purifed cathepsin D detected using starfish cathepsin D antibody. **(B)** Starfish MPR 300 (uncross-linked product) probed with *Unio* MPR 300 antibody. **(C) 10% SDS-PAGE analysis of the purified cathepsin D eluted from starfish MPR 300 affigel column**. Lane 2, 5 mM glucose 6-phosphate eluate, lane 3, 5 mM mannose 6-phosphate eluate and lane 4, 50 mM sodium acetate pH 5.0 eluate.

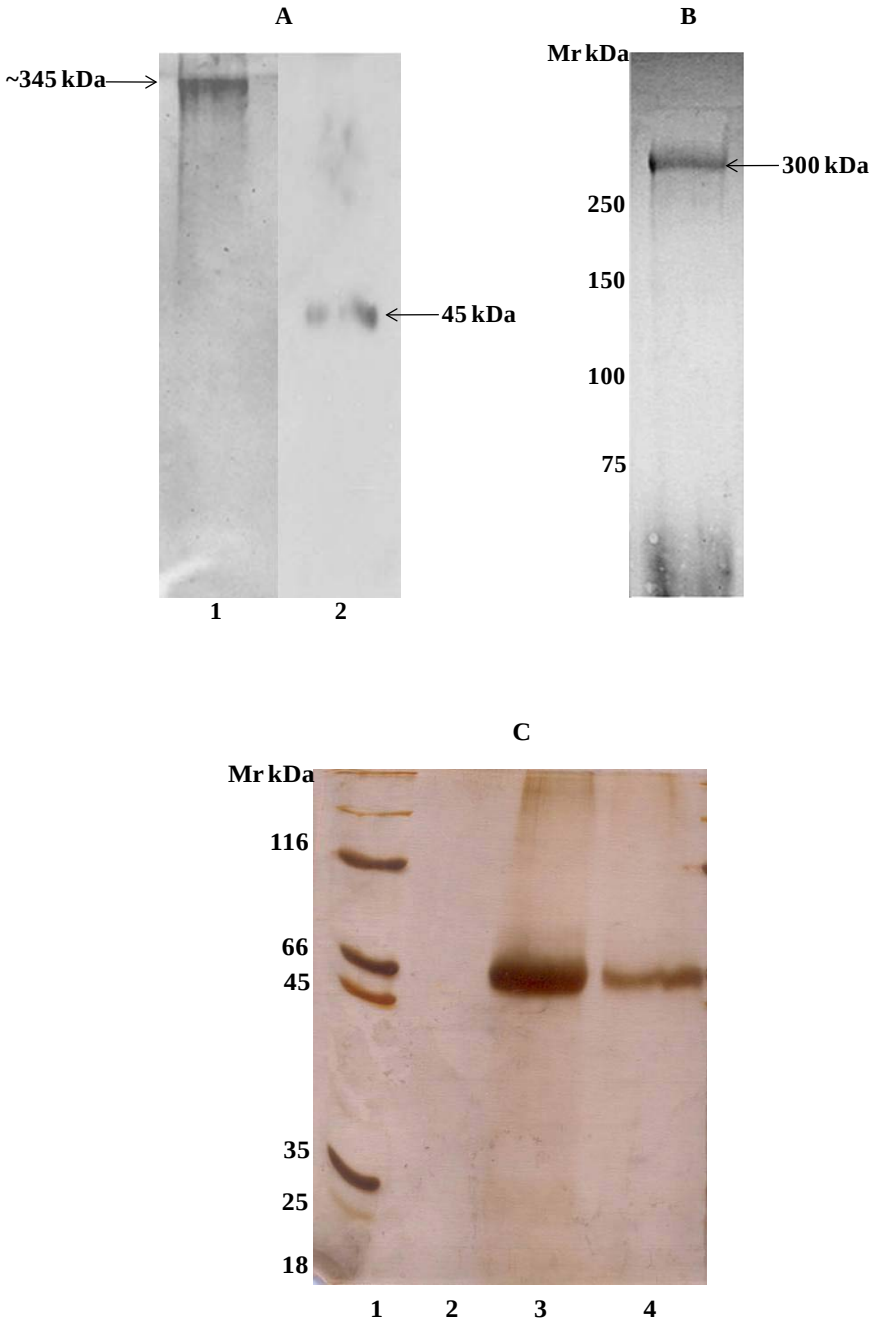

A
~345 kDa
45 kDa
1
2
B
Mr kDa
300 kDa
250
150
100
75
C
Mr kDa
116
66
45
35
25
18
1
2
3
4

Figure 2.4.7

2.5. DISCUSSION

The key function of mannose 6-phosphate receptors in the mammalian species is to mediate sorting of newly synthesized lysosomal acid hydrolases and the receptor recycles between TGN and late endosomes. It is well established from studies carried in the laboratory that both MPR proteins are conserved from fish to mammals among the vertebrates. We also affinity purified mammalian homologues of the receptors from the invertebrates starfish and molluscs (Siva Kumar and Praveen Kumar, 2010). The present study is a logical extension of the ongoing research project in the laboratory on establishing the evolutionary significance of the mannose 6-phosphate receptors, the lysosomal enzyme targeting proteins. It is shown from this study and also using mollusc cell lines that there is a specific interaction of the lysosomal enzymes with MPR 300 proteins suggesting that the invertebrates like the vertebrates also exhibit distinct and specific interactions between lysosomal enzymes and their receptors, which points towards the hypothesis that in the invertebrates, there might exist a similar lysosomal biogenesis pathway as in the mammals (Praveen Kumar *et al*., 2009). In *Drosophila* binding of mannose 6-phosphate receptor to phosphomannan could not be detected but identified only a lysosomal enzyme receptor protein (Dennes *et al*., 2005). These data suggest that possibly both functional lysosomal enzyme sorting receptors started appearing in evolution from the molluscs onwards. Therefore to gain new insights in establishing the lysosomal biogenesis pathway in the invertebrates and the evolutionary significance of the receptors, it is essential to purify as many lysosomal enzymes as possible, make a comparative analysis of their properties and establish their specific recognition by the lysosomal enzyme targeting receptors. The present study provides the first evidence on the affinity purification of the cathepsin D enzyme and its biochemical and

immunological characterization from starfish. The purity of the isolated enzyme and its molecular mass of approximately 45 kDa were confirmed by SDS- PAGE. The size and monomeric nature are close to what has been reported for cathepsin D from other species: Atlantic cod (*Gadus morhua*) (40 kDa) (Wang *et al*., 2007), Antarctic icefish (*Chionodraco hamatus*) (40 kDa) (Capasso *et al*., 1999). Cathepsin D enzymes isolated from some vertebrate and invertebrate species such as the common carp muscle, Atlantic cod and Japanese common squid are also single chain proteins (Goldman *et al*., 1995; Wang *et al*., 2007; Komai *et al*., 2004) respectively. Cathepsin D isolated from different species also exhibited different molecular mass, as exemplified by mackerel (*Scomber australasicus*) (51 kDa), milkfish (*Chanos chanos*) (54 kDa) (Jiang *et al*., 1993). Interestingly in *A. pectinifera* a different species of starfish, a protein size of 50 kDa has been reported (Ramaswamy *et al*., 2008). Rojo *et al*., (2010) recently described the isolation of a cathepsin D from the American lobster (arthropod) which exhibited a molecular mass of ~50 kDa. This difference in molecular masses could be due to the differences in their glycan nature of content.

Initial western blot experiment of the purified cathepsin D with a mouse antiserum suggested cross-reactivity between the invertebrate and the mammalian enzyme. In the present study we raised an antiserum for the purified enzyme with the following objectives. First to analyze its specific interaction with the purified enzyme, second, to develop an ELISA method to quantify the enzyme levels in different parts of the animal tissues and to investigate the specificity of interaction of the enzyme with the MPR 300 protein that may enable us to understand the function of the receptor in this species. Due to the high sensitivity of the ELISA, protein can be quantified at ng level. From the ELISA experiments, it is interesting to note that the expression levels

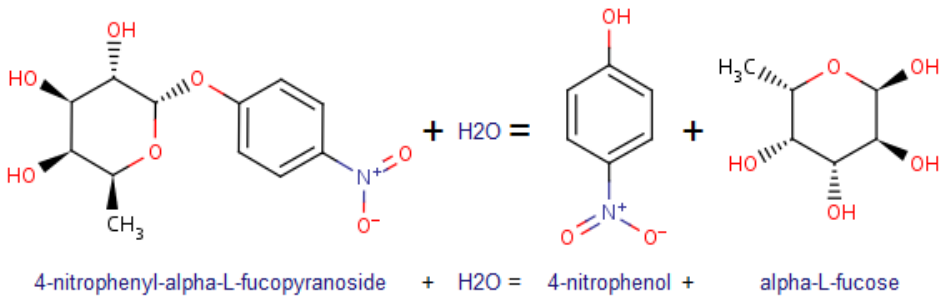
of the enzyme vary in different tissues and the enzyme level in the digestive gland is higher then in other tissues. In an earlier study antisera to purified MPRs were used to quantify the receptors in different tissues of goat and chicken (Suresh *et al*., 2002). In the present study, the results obtained clearly showed that the steady state concentrations of the enzyme (purified as well as crude soluble extracts from different organs) are independent of each other. Like the mammalian enzyme the invertebrate enzyme is also a glycoprotein as evidenced by its specific binding and elution from the Con A-Sepharose gel, periodic acid Schiff's staining of the purified enzyme. Furthermore, deglycosylation of enzyme revealed lower molecular mass of the protein and interestingly the protein retained its immunological reactivity. The specificity of the cathepsin D purified IgG was also analysed by confocal microscopy. The enzyme used in this study can bind and can be internalized by the mammalian cell type that harbors the MPR 300 protein. The specific interaction of the purified enzyme with the MPR 300 from starfish in a mannose 6-phosphate dependent manner further suggests that in starfish also there might exist a similar lysosomal biogenesis pathway as in the vertebrates. The interaction between the starfish enzyme and MPR 300 is also shown by cross-linking experiment as well as by the immuno-precipitation. In an earlier study it has been shown that the α-fucosidase from the mollusc shows specific interaction with the MPR 300 protein in a cross-linking experiment and recognition by the enzyme antibody suggesting also a possible *in vivo* interaction of the receptor and enzyme (Siva Kumar *et al*., 2004) thus establishing the receptors function. It is already well established that there is a specific interaction of the MPR 300 with cathepsin D not only in normal cells, but also in breast cancer cells. Furthermore, this receptor also binds IGF-II (Marc *et al*., 1990).

The study carried out on the cathepsin D from starfish suggest that the enzyme is a lysosomal enzyme and the MPR 300 receptor from the starfish as well as from mammalian cells shows specific interaction with cathepsin D. The results are one step forward towards understanding the evolution of lysosomal enzymes and receptors in the invertebrates.

CHAPTER 3

Purification and biochemical characterization of a lysosomal α-fucosidase from the deuterostomia *Asterias rubens*

3.1. INTRODUCTION



Reaction catalyzed by α-L-fucosidase (Taken from www.brenda-enzymes.org)

α-L-fucosidase (EC 3.2.1.51) is a ubiquitous lysosomal acid hydrolase involved in the metabolism of several biologically active molecules containing L-fucose (Aminoff and Furukawa, 1970). The importance of α-L-fucosidase in the degradation of fucosylglycoconjugates is evidenced by the fatal neurovisceral storage disease fucosidosis found in humans (Van Hoof, 1973) and English Springer Spaniels (Kelly *et al*., 1983) in which α-L-fucosidase activity is absent or grossly deficient. Interest in this enzyme has also been fueled for the use of this enzyme as a tool for elucidating the structure and function of mammalian glycoproteins and glycolipids. In general, α-L-fucosidase(s) cleaves L-fucose residues linked glycosidically α-1,2 to penultimate carbohydrate residues better than those linked glycosidically α-1,3, α-1,4 or α-1,6; low molecular weight substrates are hydrolyzed most efficiently (Chien and Dawson, 1980; Dicioccio *et al*., 1982; Bernard *et al*., 1983).

Like most lysosomal enzymes, human α-L-fucosidase is a glycoprotein and contains about eight percent carbohydrate by weight. It comprise of N-linked mannose-rich oligosaccharides (Beem *et al*., 1987; Argade *et al*., 1988), which are phosphorylated, suggesting that the intracellular trafficking of the enzyme to lysosomes is mediated by the mannose 6-phosphate receptor pathway. Alhadeff and Andrews-Smith (1978) have presented evidence that carbohydrate content affects the stability and kinetic

properties of this protein. Though α-fucosidase activities have been characterized in some mammalian species, only limited information is available about this enzyme in invertebrates. In the molluscs (*unio*), two forms of the α-fucosidase have been purified *viz*., a sperm plasma membrane bound non-glycosylated form (65 kDa) and a soluble glycosylated form (56 kDa) (Focarelli *e t al*., 1997). Some glycosidases are associated with the sperm plasma membrane and postulated to be directly involved in sperm-egg interaction in mammals (Tulsiani *et al*., 1989; Aviles *et al*., 1996) and in invertebrates. In *Unio*, a fucosidase isoform completely different from that present in the surrounding fluid, seems to be expressed and specifically bound to the sperm plasma membrane. Since the ligand for sperm-egg interaction in this species is known and available in sufficient quantities for biochemical studies, the *Unio* model is valuable for a detailed investigation of the mechanism of glycosidase substrate interaction during fertilization (Focarelli, 1985; Honegger, 1992).

A 56 kDa α-fucosidase has been purified to homogeneity from the whole body tissue of *Unio* using a series of chromatographic steps and antibodies to the purified enzyme was raised in a rabbit (Siva kumar *et al*., 2004). The enzyme has been shown to specifically interact with the mannose 6-phosphate receptor protein (MPR 300) purified from goat and *Unio*. Although these different biochemical properties of the enzyme have been studied and a possible function attributed to the mollusc enzyme little is known about its sequence and relatedness to the human enzyme.

The structural and functional domains of the lysosomal enzyme sorting receptors are highly conserved throughout the vertebrates (Siva Kumar *et al*., 1997; Siva Kumar *et al*., 1999; Suresh *et al*., 2004; Siva Kumar *et al*., ASCB 2005). However, in the starfish species, only MPR 46 protein has been extensively characterized so far and lysosomal enzymes is still lacking. Therefore, the present study was undertaken

- ✓ To clarify if a new enzyme class is catalyzing substrate transformation or if the activity is a property of an already known enzyme class.
- ✓ Investigate to what extent the enzymes are homologous
- ✓ Investigate how variable the active site of this enzyme type is and therefore to get a complete detailed analysis on the structure and function of the starfish enzyme the present study was undertaken with the following objectives,
  - i) to analyze different lysosomal enzyme activities from starfish tissue
  - ii) to affinity purify the α-fucosidase on fucosylamine gel and study its biochemical and immunological properties
  - iii) to study interactions between the purified enzyme and the starfish MPR 300 protein
  - iv) to obtain partial amino acid sequence of the enzyme using a proteomics approach.

3.2. MATERIALS AND METHODS

3.2.1. Materials

N-(ε-Aminocaproyl)-β-L-fucopyranosylamine (Carbosynth, Berkshire, UK), phenyl Sepharose (type CL-4B), enzyme substrates and sugars used in the study were purchased from Sigma Chemical Co, (St. Louis, Missouri, USA). DE-52 cellulose was purchased from Whatmann (Maidstone, UK). PVDF was purchased from Millipore (Bedford, MA, USA). Other chemicals used were obtained from Sisco Research Laboratory, Qualigens, Bangalore Genei, India.

3.2.2. Lysosomal enzyme assays

Enzyme assays were carried out as described earlier (Siva Kumar *et al*., 2004), using the following substrates: 4-nitrophenyl α-L-fucopyranoside for α-fucosidase, 4-nitrophenyl α-D-mannopyranoside for α-mannosidase, 4-nitrophenyl α-D-galactopyranoside for α-galactosidase, 4-nitrophenyl N-acetyl-β-D-glucosaminide for β-hexosaminidase, 4-nitrocatechol sulfate dipotassium salt for arylsulfatase A. An assay mixture (500 µL) consisting of a 50 mM sodium acetate buffer pH 5.0, 100 µL of a 5 mM substrate (1 mM final concentration) and the enzyme solution, was incubated at 37℃ for 30 min. The control contained all reactants except the enzyme. The reaction was stopped after 30 min of incubation by addition of 1 M Na_2CO_3 buffer pH 10.0 to the reaction mixture. The absorbance of the released *p*-nitrophenol was recorded at 405 nm. The activity of the enzyme (units/mL/min) was calculated according to the formula given below:

$$\text{Activity (units/mL/min)} = \frac{\text{Absorbance at 405 nm}}{\text{Time of incubation x 18.5 x Volume of enzyme}}$$

where 18.5 is the molar extinction coefficient of *p*-nitrophenol

Definition of enzyme unit: One unit of enzyme activity was defined as amount of enzyme hydrolyzing 1 μmoL of substrate per min. The specific activity of the enzyme was expressed as units per mg protein. (IU/mg). All assays were performed in triplicate and results were recorded as the mean of these experiments.

3.2.3. Extraction and isolation of α-fucosidase from starfish

All operations were carried out at 4℃ unless otherwise stated. The activity of the enzyme was monitored at each step of the purification process. Fifty grams of the whole animal tissue was thawed on ice and homogenized with 4 volumes (200 mL) of 25 mM Tris-HCl, pH 7.0 (buffer A). The homogenate was stirred overnight and centrifuged (26892 x g, 30 min; 4℃). The pellet was discarded and the supernatant (200 mL) was recentrifuged for 15 min as described above.

3.2.4. Anion exchange chromatography on DE-52

The clear supernatant was applied on a 20 mL DE-52 cellulose gel (2.5 x 20 cm), pre-equilibrated with buffer A. After washing extensively, the bound protein was eluted in 5 mL fractions at a flow rate of 60 mL/h with 25 mM Tris-HCl, pH 7.0, containing 0.2 M NaCl (buffer B) and assayed for the enzyme activity.

3.2.5. Hydrophobic interaction chromatography using phenyl-Sepharose CL-4B

The enzyme rich fractions containing protein with α-fucosidase activity were pooled and solid ammonium sulfate was added to a final concentration of 1M. The protein solution was applied on a 5 mL phenyl Sepharose CL-4B column (1.5 x 15 cm) pre-equilibrated with buffer C containing 25 mM Tris-HCl, pH 7.0 and 1M ammonium sulfate which is required for specific binding of glycosidases to the hydrophobic gel (Subha *et al*., 2002). The bound protein was eluted in 2.0 mL fractions at a flow rate of 1mL/ 5min with buffer A. Fractions containing α-fucosidase activity were pooled and dialyzed against 50 mM sodium acetate buffer,

pH 5.0 (buffer D). The dialyzed sample was briefly centrifuged and the clear supernatant was used for the purification of the enzyme by affinity chromatography.

3.2.6. Affinity purification and characterization of α -fucosidase on fucosylamine gel

0.66 g CNBr Sepharose 4B (GE Healthcare) was stirred in 1 mM HCl for 15 min in a glass beaker. The beads were rinsed on a sintered glass funnel with 200 mL of 1 mM HCl /1 g Sepharose. The gel (2.0 mL) was transferred into a falcon tube and 5 mg of N-(ε-Aminocaproyl)-β-L-fucopyranosylamine in 2.0 mL coupling buffer (0.1 M $NaHCO_3$ pH 8.3, 0.5 M NaCl) was mixed with the gel and incubated on an end-over-end rotator at 4℃ overnight. The gel was packed into a column and washed with ~ 5 mL coupling buffer, followed by 5 mL 0.1 M Tris / HCl, pH 8.0. The gel was incubated with 5 mL 0.1 M Tris / HCl pH 8.0 for 2 h at room temperature, washed three times with 5 mL 0.1 M sodium acetate pH 4, 0.5 M NaCl, and finally washed three times with 5 mL 0.1 M sodium acetate pH 8, 0.5 M NaCl. After dialysis, the enzyme containing fraction obtained from phenyl Sepharose chromatography was applied on a 2 mL fucosylamine affinity gel (1 x 10 cm) pre-equilibrated with buffer D and incubated overnight at 4℃. After washing extensively, specific elution was carried out with 60 mM fucose in 50 mM sodium acetate buffer, pH 5.0 (buffer E). Protein in the eluates was monitored by absorbance at 280 nm. Aliquots of the eluates were dialyzed against buffer D to remove fucose and assayed for fucosidase activity and other glycosidase activities as described above. The purified protein was pooled and concentrated using centricon concentrator (MWCO-10). This enzyme was used for all studies carried out in this chapter.

3.2.7. Substrate specificity

The relative substrate specificity of α-fucosidase towards various synthetic substrates was determined. Substrates were prepared at a final concentration of 5 mM

(stock solution). The reaction was carried out using fixed concentration of enzyme and substrate under standard assay conditions. Relative activity on various substrates is expressed as percentage of the activity calculated with *p*-nitrophenyl α-L-fucopyranoside as a substrate (100%) with which enzyme showed maximum activity.

3.2.8. Protein estimation

Protein concentrations in the column eluates were determined using Bradford reagent (Bio-Rad) using BSA as the standard (Bradford, 1976).

3.2.9. SDS-PAGE and Western blotting

Aliquots of the eluates were subjected to 10% SDS-PAGE under reducing conditions and the protein bands were detected by staining with silver nitrate. For the immunological detection, 2 µg of the purified protein was separated by SDS-PAGE and transferred to a PVDF membrane (Towbin *et al*., 1979). The membrane was incubated with the antiserum (1:500 dilution) raised against the purified *unio* α-fucosidase (Siva Kumar *et al*., 2004) for 1 h at room temperature or overnight (about 16 h) at 4℃. The membrane was washed with 10 mM phosphate buffer pH 7.4, 150 mM NaCl, 0.05% Tween 20 (PBST) and the protein band was detected by incubating the membrane with secondary antibody goat-anti rabbit HRP conjugate (Pierce, Rockford, USA). The blot was developed using the Super Signal West Femto Maximum Sensitivity Substrate (Thermo Scientific, Rockford, IL, USA).

3.2.10. Sample preparation for LC-MS/MS

The purified enzyme (2 µg) was separated on a 10% SDS-PAGE (7cm) and visualized by colloidal coomassie blue staining. Protein containing gel slices corresponding to a molecular mass of 56 kDa were destained by incubating two times with 100 µl of 200 mM ammonium bicarbonate (NH_4HCO_3)/ 50% ACN v/v for 15 min at 37℃ and dried with 100% ACN. In-gel digestion was performed overnight at

37℃ using trypsin (Promega, Mannheim, Germany) or chymotrypsin (Sigma, Taufkirchen, Germany) dissolved in 20 mM NH_4HCO_3 (pH 8.2). The resulting peptides were extracted in a water bath sonicator for 30 min with 0.1% acetic acid in 50% ACN and subsequently with 0.05% acetic acid in 80% ACN. The eluates of both extractions were pooled and concentrated in a vacuum centrifuge. Peptide extracts were desalted on µ-C18 ziptips (Millipore, Bedford, MA, USA) and finally dissolved in 10 µl 0.1% CH_3COOH/ 2% ACN for LC-MS/MS measurements.

3.2.11. Mass spectrometric analysis

Peptide solutions were analysed by ESI-LTQ-Orbitrap mass spectrometry (Thermo Electron, Bremen, Germany) after pre-fractionation by nano-HPLC (Proxeon, Odense, Denmark) on a reverse phase column (PepMap 75 µM, 150 mm, Dionex, Idstein, Germany) at 300 nl/min flow rate. The mass spectrometer was operated in data-dependent mode. Full scans were recorded at resolution of 60,000 and 300-2,000 m/z mass range at an average mass accuracy of 3 ppm. The five most intensive precursor ions were subjected to CID fragmentation in the ion trap for MS/MS acquisition.

3.2.12. Database search

Thermo RAW files were used for searching SwissProt 57 release 15 as well as MSDB database from NCBI (Version from December 2009; ftp://ftp.ncbi.nih.gov/repository/MSDB/) via PEAKS rel. 5.1 (www.bioinformaticssolutions.com) at a peptide mass tolerance of 10 ppm and fragment tolerance of 0.5 Da, allowing oxidation of methionine as variable modification. Peptides displaying scores > 70% were considered as significant and assigned to the corresponding protein. The search was performed covering the whole database without taxonomy restrictions. With this approach only proteins whose

sequence had been deposited could be identified. Precursor ions assigned to keratins and trypsin were excluded from further analyses.

3.2.13. De novo sequencing

Peak lists were extracted and subjected to *de novo* sequencing in PEAKS *de novo* (www.bioinformaticssolutions.com) using the following parameters: Spectra quality 0.6, mass filter 300-1600, fragment tolerance 0.2 Da, variable modification: oxidation of methionine. Only those peptide sequences independently identified from two different proteolytic digests with high confidence and Peak scores ≥ 50, as well as comprising at least six amino acids, were considered as *de novo* sequenced peptides. Obtained peptide sequences were subjected to error-tolerant search in the databases specified above via SPIDER algorithm, which is part of the PEAK software package. The result, while allowing for the ambiguity Leu versus Ile, was considered significant if the sequence matched partially to the one obtained in the database search and exceeded a protein score > 60.

3.2.14. Ligand blot analysis and chromatography on MPR 300 affinity gel

The interaction of α-fucosidase with the starfish MPR 300 protein (Sivaramakrishna and Siva Kumar, 2008) was analyzed by ligand blot analysis. The purified α-fucosidase was electrophoresed on a 10% SDS-PAGE and transferred to a PVDF membrane. The membrane was incubated for 16 h with purified starfish MPR 300 (100 µg) in blocking buffer (PBST containing 1% BSA) and subsequently probed with antiserum (1:500 dilutions) against purified goat MPR 300 protein raised in a rabbit (Suresh and Siva Kumar, 2003). The band was visualized by incubating the membrane with secondary antibody goat-anti rabbit HRP conjugate and developed using the Super Signal West Femto Maximum Sensitivity Substrate.

500 µg of purified starfish receptor in 500 µl of 0.1M HEPES buffer, pH 7.0 was coupled to 1.0 mL Affigel-10 following manufacturer's instructions. The gel was equilibrated with 50 mM imidazole (pH 7.0) containing 5 mM sodium β-glycerophosphate, 150 mM sodium chloride, 0.05% Triton X-100, 2 mM EDTA (EDTA buffer). The purified α-fucosidase obtained by affinity chromatography was dialyzed against EDTA buffer and applied onto the receptor-affigel column. The gel was washed with four gel volumes of EDTA buffer and sequentially eluted with 2 mL each of 5 mM glucose 6-phosphate, 5 mM mannose 6-phosphate followed by 50 mM sodium acetate buffer, pH 5.0. Protein in each of the three eluates was precipitated with TCA (final 10% concentration). The pellets collected by centrifugation were neutralized with 0.4 M Tris and analyzed by 10% SDS-PAGE. Protein bands were visualized by staining with silver nitrate. In a separate experiment, the partially purified enzyme preparation obtained from phenyl Sepharose gel chromatography was applied to a human MPR 300-affigel (kind gift from Prof. Dr. K. von Figura, University of Goettingen, Germany) in EDTA buffer. After washing the gel with EDTA buffer, the bound proteins were eluted with 5 mM mannose 6-phosphate. The eluates were processed as described above for electrophoresis and detection of protein bands.

3.2.15. Estimation of carbohydrate content

The sugar content of the purified enzyme preparation was determined by phenol sulfuric acid method using glucose as standard (Dubois *et al.,* 1956).

3.2.16. Glycoprotein nature of the purified alpha fucosidase

Deglycosylation of the enzyme was performed as described (Keinanen *et al*., 1988) and details as given under section 2.2.6.

3.2.17 Effect of metal ions, L-fucose and other chemical agents

Ethylenedinitrilotetraacetic acid (EDTA) and various metal ions (Hg^{2+}, Ag^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Ni^{2+} , Cu^{2+}, Ca^{2+}, K^{+}, Co^{2+}) were incubated with the purified enzyme to determine their effect on α-L-fucosidase activity according to Jack *et al*., 1974. It was tested both with and without removal of preexisting ions by EDTA treatment. After EDTA treatment the enzyme is dialysed against 50 mM NaOAc buffer pH 5.0 and tested by pre-incubating the enzyme in 50 mM NaOAc buffer pH 5.0 containing 0.1 mM concentration of each metal ion for 60 min prior to addition of the substrate and the residual enzyme activity was assayed by standard method. The enzyme activity without any metal ion is considered as control (100%). All incubations were carried out in duplicate at 37℃ for 30 min. The effect of product analogue (fucose), anionic detergent SDS and reducing agents: dithiothreitol (DTT), β-mercaptoethanol on enzyme activity was also determined as above.

3.2.18. Determination of pH optimum and pH stability

Buffers used in this study are sodium acetate (pH 2.5-6.0), sodium phosphate (pH 6-7) and Tris-HCl (pH 8). The pH-dependence of enzyme activity was performed at pH range 2.0-8.0 using 4-nitrophenyl α-L-fucopyranoside as substrate and incubated for 30 min. The stability of the enzyme at various pH values was investigated by a previous incubation of the enzyme in a series of buffers at pH 2.0-8.0 at 37℃ for 12 h. After incubation the residual enzyme activity was subsequently assayed under standard assay conditions. The control was a similar preparation run without the incubation. All incubations were carried out in duplicate.

3.2.19. Determination of temperature optimum and temperature Stability

Temperature optimum for the purified enzyme was performed as described above in the temperature range of 30℃ to 90℃. For thermostability experiments,

each 100 µl of purified α-L-fucosidase (in 50 mM acetate buffer, pH 5.0) was kept in buffer at 50℃, 60℃, 70℃, 80℃ for 60 min. At 10 min intervals, aliquot of the enzyme (30 µl) was removed and assayed for enzyme activity. The residual enzyme activity was determined by standard assay method.

3.2.20. Circular dichroism spectroscopy

Circular dichroism (CD) spectra were recorded at room temperature using a Jasco J-810 spectropolarimeter (Jasco International Co., Ltd., Tokyo, Japan, website: http://www.jascoint.co.jp) equipped with a Peltier thermostat supplied by the manufacturer. Samples were placed in a 2-mm pathlength rectangular quartz cell. α-fucosidase concentration was 0.3 mg/mL for measurements in the far UV region (260-190 nm). In order to study temperature-dependent structural changes, scans were performed between 30℃ and 90℃ at a scan rate of 1° per minute.

3.3. RESULTS

3.3.1 Lysosomal enzyme assays

When the soluble protein fractions obtained from extraction of whole tissue of starfish were analyzed for various lysosomal enzyme activities, α-fucosidase and α-mannosidase showed the highest activities although other enzyme activities could be detected as well (Fig 3.4.1). From the two most significant active enzymes we choose α-fucosidase for affinity purification and subsequently characterized this protein and studied the interaction of the enzyme with the mannose 6-phosphate receptor (MPR 300).

3.3.2. Extraction and isolation of α-fucosidase from starfish

The enzyme was first partially purified from the soluble extracts obtained by the methods described, using a combination of anion exchange chromatography and hydrophobic interaction chromatography (Fig 3.4.2 A & B respectively). From 50 g of the tissue, 11.2 units of enzyme activity were detected in the soluble extracts. After anion exchange chromatography, 8.0 units of enzyme activity were recovered. To further purify α-fucosidase, enzyme rich fractions were passed through a phenyl Sepharose gel in presence of high salt (binding condition). Desorption was initiated by application of buffer C without salt and 1.4 units of enzyme activity were recovered. At this stage, the enzyme preparation still contained other protein components when analyzed by SDS-PAGE (data not shown). Therefore, subsequently affinity chromatography on a fucosylamine gel as described below was used to achieve further purification.

3.3.3. Affinity purification and characterization of α -fucosidase

Fig 3.4.3A shows the reaction scheme for coupling the affinity ligand to CNBr Sepharose 4B activated gel. The enzyme containing fractions obtained above were bound on the affinity gel fucosylamine-Sepharose at pH 5.0. After extensive washing, the bound enzyme was eluted using 60 mM L-fucose in 50 mM sodium acetate buffer, pH 5.0. (Fig. 3.4.3B). From 50 g of the starting material, about 1 mg of purified enzyme was obtained (Table 3.1).

3.3.4. Substrate specificity

The ability of the α-fucosidase to hydrolyze different 4-nitrophenyl glycosides has been assayed spectrophotometerically. A variety of glycosides including 4-nitrophenyl α-L-fucopyranoside, 4-nitrophenyl α-D-mannopyranoside, 4-nitrophenyl α-D-galactopyranoside, 4-nitrophenyl N-acetyl-β-D-glucosaminide and 4-nitrocatechol sulfate dipotassium salt were used as substrates. An aliquot of the eluted sample was dialyzed against buffer D (see materials and methods) and analyzed for α-fucosidase as well as other glycosidase activities. The results clearly show enrichment of fucosidase activity and no substantial contamination by other glycosidases (Fig 3.4.4 A & B) under standard assay conditions and even if the incubation time was extended up to 90 min. SDS-PAGE analysis of the protein eluted from the affinity gel revealed a strong band corresponding to a molecular mass of about 56 kDa (Fig 3.4.5A lane 2). Western blot analysis of the purified enzyme with an antiserum raised against the mollusc α-fucosidase showed strong cross-reactivity to the 56 kDa protein (Fig 3.4.5B) suggesting antigenic similarities among both invertebrate enzymes. In addition, a faint band below the 56 kDa also was detected in SDS-PAGE as well as in western blot analysis, which likely represents a proteolytic degradative product of the enzyme.

3.3.5. Analysis of the amino acid sequence of purified enzyme by LC-MS/MS and de novo sequencing

Schematic representation of the PEAKS 5.1 workflow is shown in Figure 3.4.6. After SDS-PAGE analysis a colloidal coomassie blue stained band of 56 kDa corresponding to the enzyme was excised from the gel and used for the LC-MS/MS analysis. The protein band was subjected to in-gel proteolysis and the resulting peptide extracts were used for LC-MS/MS analysis. Sequence data was acquired by a combination of database searches, *de novo* sequencing and error tolerant searches of the resulting sequences. Database search of the mass spectrometric data revealed 12 peptides with sequences identical to those of other mammalian fucosidases (Table 3.2). Furthermore, 13 homologous peptides were observed in an error-tolerant search of the sequences resulting from *de novo* sequencing (Table 3.2). The sequence information obtained for the starfish enzyme was aligned with other mammalian enzyme sequences using CLUSTAL W 1.83. From the data shown in Fig 3.4.7, it is apparent that the starfish enzyme is highly homologous to the mammalian enzymes that have been sequenced. Of particular interest is the conservation of the cysteine residue at amino acid position 288 in the starfish enzyme.

3.3.6. Ligand blot analysis and specific interaction of the purified lysosomal α-fucosidase with the purified starfish MPR 300 protein

Ligand blotting is a sensitive assay method that has been widely used to analyze specific interactions of proteins with their ligands. We analyzed if the enzyme interacts with the purified starfish MPR 300 protein which has been shown to possess similar biochemical and immunological properties as that of the goat receptor (in the following chapter). The purified enzyme was separated on a 10% SDS-PAGE

and transferred to a PVDF membrane and processed as described under materials and methods. In control, the blot containing α-fucosidase enzyme was probed with goat MPR 300 antiserum, which has already been shown to specifically detect MPR 300 proteins from molluscs to mammals (Suresh and Siva Kumar, 2003) without prior incubation of the membrane with starfish MPR 300 protein. After processing the blot no band could be detected (Fig 3.4.8 lane 1). However, prior incubation of the blot with Starfish MPR 300 protein followed by goat MPR 300 antiserum resulted in a strong signal in the 56 kDa molecular mass region (lane 2) suggesting specific interaction of α-fucosidase and the starfish MPR 300 receptor. On the other hand, preincubation of the receptor with 5mM mannose 6-phosphate prevents the binding of the receptor to some extent (lane 3) but with 10 mM mannose 6-phosphate there was complete inhibition of the binding of the receptor to the enzyme suggesting that the binding of the receptor to ligand is sugar specific (lane 4).

In order to gain further insight into the specificity of the interaction between the purified enzyme and the starfish MPR 300 protein, the binding of α-fucosidase to starfish MPR 300 protein immobilized to Affigel-10 was analyzed. At pH 7.0 (which favors binding) the purified enzyme binds strongly to the receptor-affigel. The bound enzyme could not be eluted using 5 mM glucose 6-phosphate (Fig 3.4.9A lane 1) but could be eluted partially with 5 mM mannose 6-phosphate (lane 2). When 50 mM sodium acetate buffer, pH 5.0 (which favors dissociation of enzyme-receptor complex) was employed after elution with 5 mm mannose 6-phosphate, some more protein was desorbed from the gel (lane 3). α-fucosidase was also found to bind human MPR 300 coupled to affigel and could be eluted specifically with 5 mM mannose 6-phosphate (Fig 3.4.9B). The eluted protein migrated to the same extent as seen in Fig 3.4.9A lane 3. The additional bands seen below the fucosidase band might

possibly represent other receptor interacting proteins which were not characterized in this study. The purified α-fucosidase was found to be a glycosylated protein with ~3% carbohydrate as estimated by phenol sulphuric method. A further confirmation to the glycoprotein nature of the purified enzyme came from PNGase F treatment. The PNGase F treated enzyme showed different mobility on SDS-PAGE (Fig 3.4.9 lane 1, 56 kDa and lane 2, PNGase treated) respectively. Both protein bands have the ability to cross-react with the mollusc α-fucosidase antiserum.

3.3.7. Effect of various metal ions, sugar analogue and chemical agents

For the investigation of the effect of metal ions, various divalent metals (Hg^{2+}, Ag^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Ni^{2+} , Cu^{2+}, Ca^{2+}, K^{+}, Co^{2+}) and EDTA were added in an enzymatic assay. α-fucosidase activity was tested both with and without removal of pre-existing ions by EDTA treatment but has no affect on fucosidase activity. The results are summarized in Fig 3.4.10. No significant activity loss can be observed for the tested ions and reagents except for Hg^{2+} (10^{-4} M) and Ag^{2+} for which 97% and 72% respectively inhibition was obtained. Zn^{2+} had no significant effect on enzymatic activity. However, other metal ions such as Mg^{2+}, Mn^{2+}, Ni^{2+}, Cu^{2+}, Ca^{2+}, K^{+}, Co^{2+} did not show any significant loss of activity. Effect of product analogue (fucose), detergent (SDS) and reducing agents (DTT and β-mercaptoethanol) on the activity of the α-fucosidase was examined using purified enzyme. 11% of the original activity remained at 0.1 mM concentration of fucose, being a product analog it showed significant inhibition at higher then 5 mM concentration. The influence of the anionic detergent like SDS showed 91% inhibition on the enzyme activity at 0.1 mM concentration. DTT showed a decrease up to 53% in the enzyme activity at 1 mM concentration.

3.3.8. Biochemical properties of Asterias rubens α-fucosidase

The purified *A. rubens* α-fucosidase showed optimal enzyme activity at pH 5.0 as shown in Fig 3.4.11A. Purified α-fucosidase from *A. rubens* showed stability from pH 3-5 with more than 80% of the enzyme activity remained at this pH, after 12 h of incubation (Fig 3.4.11B). The α-fucosidase was incubated at various temperatures (30, 40, 50, 60, 70, 80, 90℃) at pH 5.0 and assayed at 37℃ under standard conditions where the temperature optimum is at 70℃ as shown in Fig 3.4.11C. Thermostability of enzyme was examined by incubation of the enzyme at various temperatures and the residual enzymatic activity was measured at every 10 minutes intervals over a period of 1 hour. As it can be observed from Fig 3.4.11D, it indicates that 70% of the activity is retained after incubation at 50℃ for 60 min. Enzyme completely lost its activity at 80℃ after 1h of incubation.

3.3.9. Circular dichroism spectroscopy

CD spectra of native α-fucosidase in the far UV region is shown in Fig 3.4.12A. In order to estimate the content of different types of secondary structures in α-fucosidase, the far UV CD spectrum of the native protein in the wavelength range 260–190 nm has been analyzed by the CDSSTR program using the routines available at DICHROWEB (www.cryst.bbk.ac.uk/cdweb/html) (Compton *et al*., 1986; Lobley *et al*., 2002). The values obtained for the different types of secondary structures are: α-helix (34%), β-sheet (25%), β-turns (20%) and unordered structures (21%).

To investigate the structure under thermal conditions, far UV CD spectra of the α-fucosidase were recorded at different temperatures. Spectra recorded at 30, 40, 50, 60, 70, 80 and 90℃ are shown in Fig 3.4.12B. Only moderate changes in the spectral features are seen in the spectra recorded at 30-90℃, including small decrease in the

spectral intensity. This indicates that α-fucosidase is a rather stable protein which was also observed in the results described above.

FIGURES 3.4

Figure 3.4.1. Lysosomal enzyme activities from the soluble extracts of the starfish tissue with the respective chromogenic substrates. *Para*-Nitro phenyl phosphate (*p*NP) derivatives were used as substrates to determine the specific activities of the enzymes. Assays were done in triplicates in three individual experiments and average values and standard deviations (error bars) were calculated in Sigma plot 9.0.

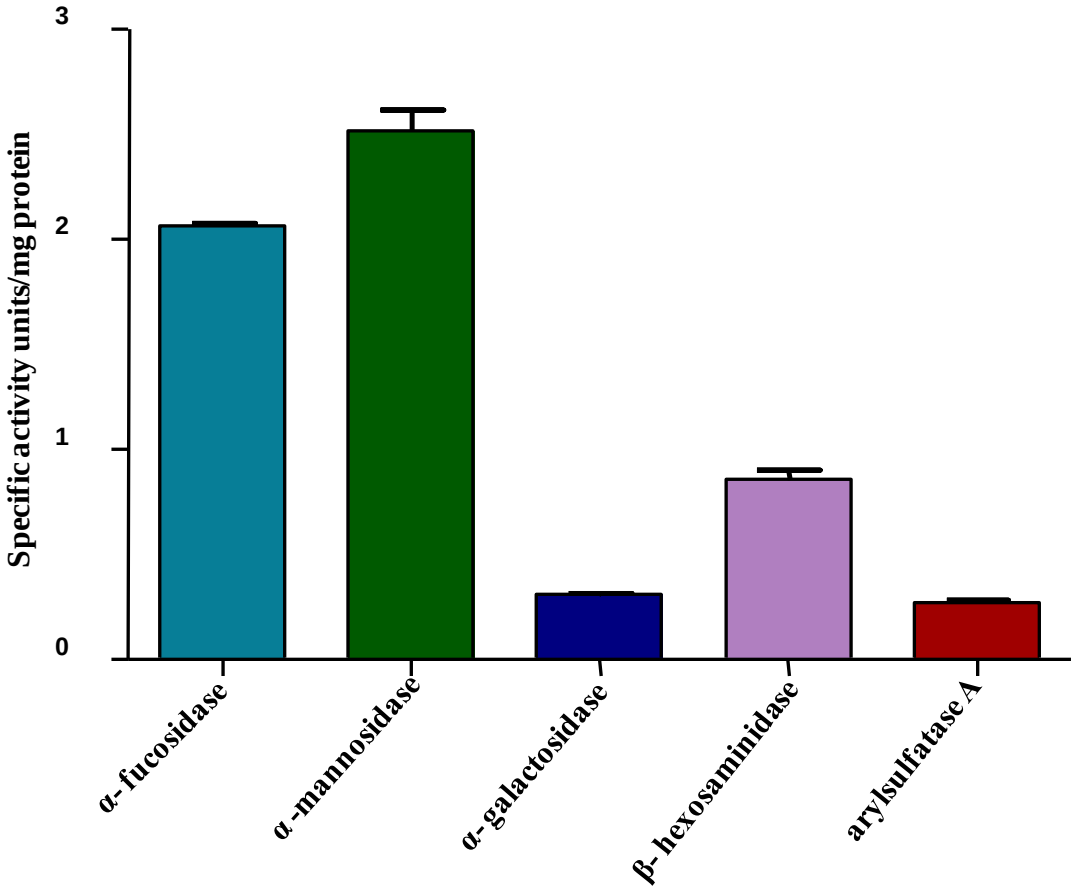
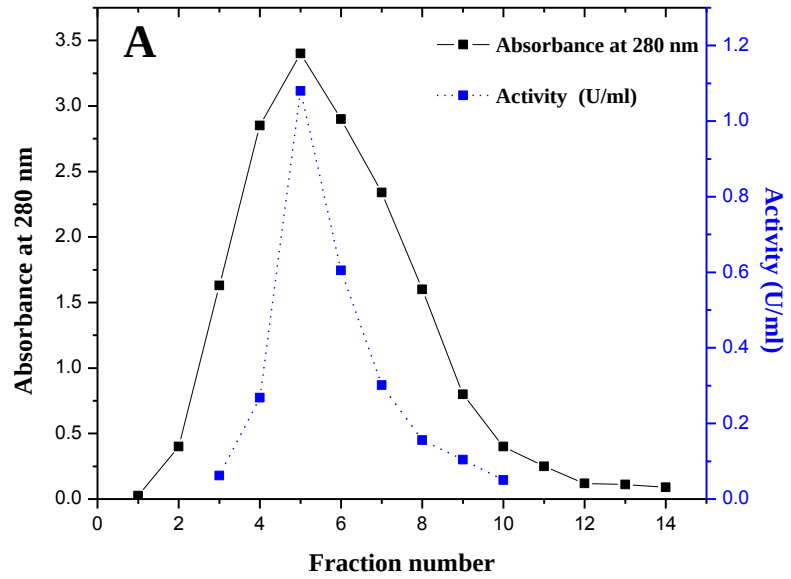

Specific activity units/mg protein
3
2
1
0
α- fucosidase
α -mannosidase
α- galactosidase
β- hexosaminidase
arylsulfatase A

Figure 3.4.1

Figure 3.4.2. Elution profile of *A. rubens* α-L-fucosidase from different chromatographic steps. (A) The crude enzyme fraction of *A. rubens* tissue after $(NH4)_2SO_4$ precipitation and dialysis was applied onto the DE-52 column (2.5 x 20 cm). The column was washed with 25 mM Tris-HCl buffer pH 7.4 (equilibration buffer), and the bound enzyme was eluted by 200 mM NaCl in the same buffer. Fractions of 5 mL were collected and the absorbance monitored at 280 nm. **(B)** The enzyme rich fractions from the DE-52 were concentrated and saturated to 1 M $(NH4)_2SO_4$ in equilibration buffer. After washing, the bound protein was eluted in 2.0 ml fractions at a flow rate of 1mL/ 5min with buffer in the absence of $(NH4)_2SO_4$.


A
Absorbance at 280 nm
Activity (U/ml)
Absorbance at 280 nm
Activity (U/ml)
Fraction number

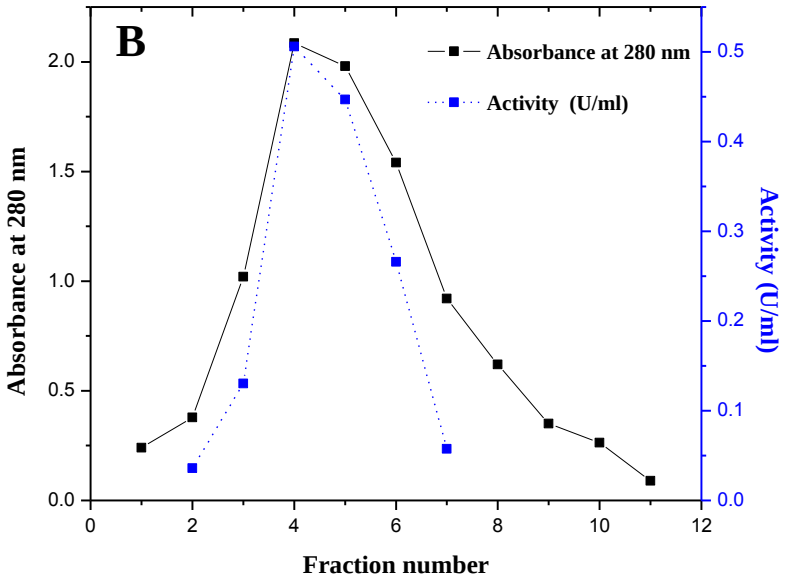
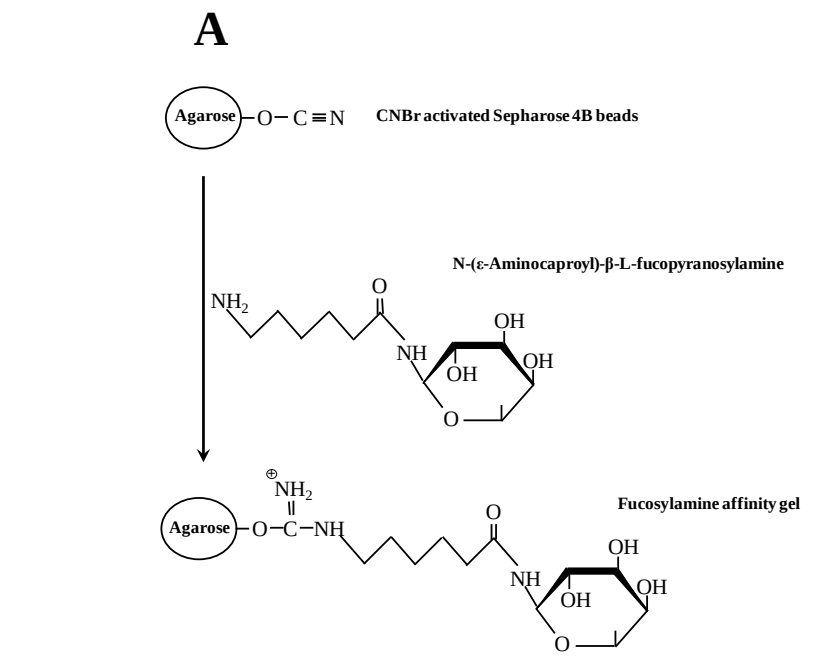

B
Absorbance at 280 nm
Activity (U/ml)
Absorbance at 280 nm
Activity (U/ml)
Fraction number

Figure 3.4.2

Figure 3.4.3.(A) Schematic reaction for coupling the affinity ligand to CNBr Sepharose 4B activated gel. **(B) Elution profile of *A. rubens* α-L-fucosidase from fucosylamine affinity gel:** Enzyme rich fractions from phenyl Sepharose chromatography were pooled and dialyzed against 50 mM NaOAc buffer, pH 5.0 and applied on fucosylamine affinity gel (1 x 10 cm) pre-equilibrated with 50 mM NaOAc buffer pH 5.0. After washing the gel with the same buffer, bound protein was specifically eluted with 60 mM L-fucose in 50 mM NaOAc buffer, pH 5.0


A
Agarose
O
C
N
CNBr activated Sepharose 4B beads
N-(ε-Aminocaproyl)-β-L-fucopyranosylamine
NH2
NH
OH
OH
OH
O
Fucosylamine affinity gel

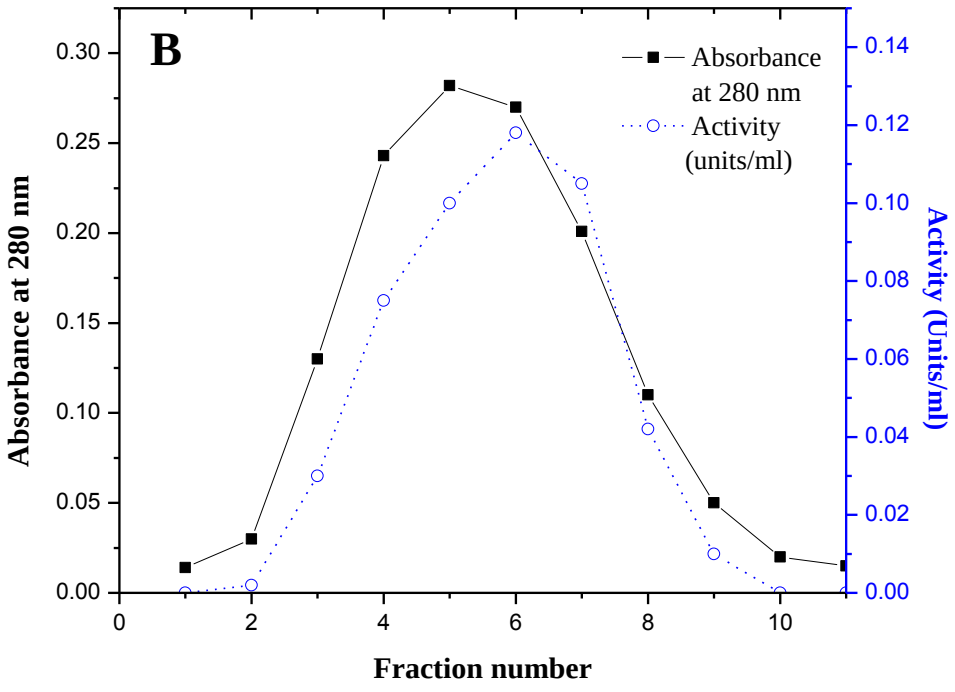
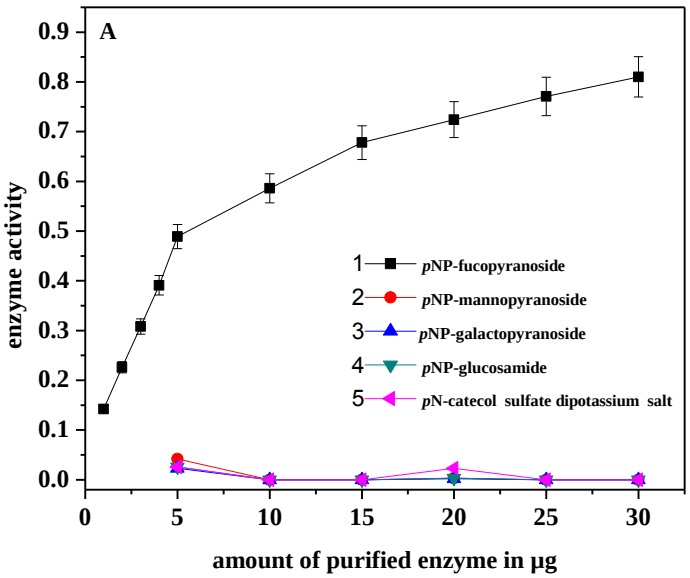

B
Absorbance at 280 nm
Activity (units/ml)
Absorbance at 280 nm
Activity (Units/ml)
Fraction number
0.00
0.05
0.10
0.15
0.20
0.25
0.30
0.00
0.02
0.04
0.06
0.08
0.10
0.12
0.14
0
2
4
6
8
10

Figure 3.4.3

Figure 3.4.4. (A) Substrate specificity of the purified α-fucosidase. 5 μg of the purified enzyme was assayed as described under materials and methods using five different synthetic substrates. **(B)** Relative activity of α-fucosidase with various synthetic substrates



B. Relative activity of α-fucosidase with various synthetic substrates

Substrate [a]	Relative activity (%) [b]
*p*NP-α-L-fucopyranoside	100
*p*NP-α-D-mannopyranoside	0.81
*p*NP-α-D-galactopyranoside	0.67
*p*NP-β-D-glucosamide	0.8
*p*N-catecol sulphate dipotassium salt	0.16

[a] All the synthetic substrate concentration used were 1 mM in the final mixture
[b] Relative activities were calculate in relation to *p*NP- α-fucopyranoside activity that was considered as 100%

Figure 3.4.4

Figure 3.4.5. 10% Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of the affinity purified α-fucosidase from starfish. (A) Detection of proteins by staining with silver nitrate. Lane 1, Molecular weight marker, lane 2 enzyme eluted from the affinity gel under reducing condition. **(B)** Western blot analysis of the purified enzyme using *unio* α-fucosidase antiserum. Arrow indicates the position of the enzyme band. The standard protein markers used are medium range Fermentas markers consisting of β-galactosidase (120.0 kDa), BSA (85.0 kDa), ovalbumin (50.0 kDa), Carbonic anhydrase (35.0 kDa), β-lactoglobulin (25 kDa), lysozyme (20.0kDa).

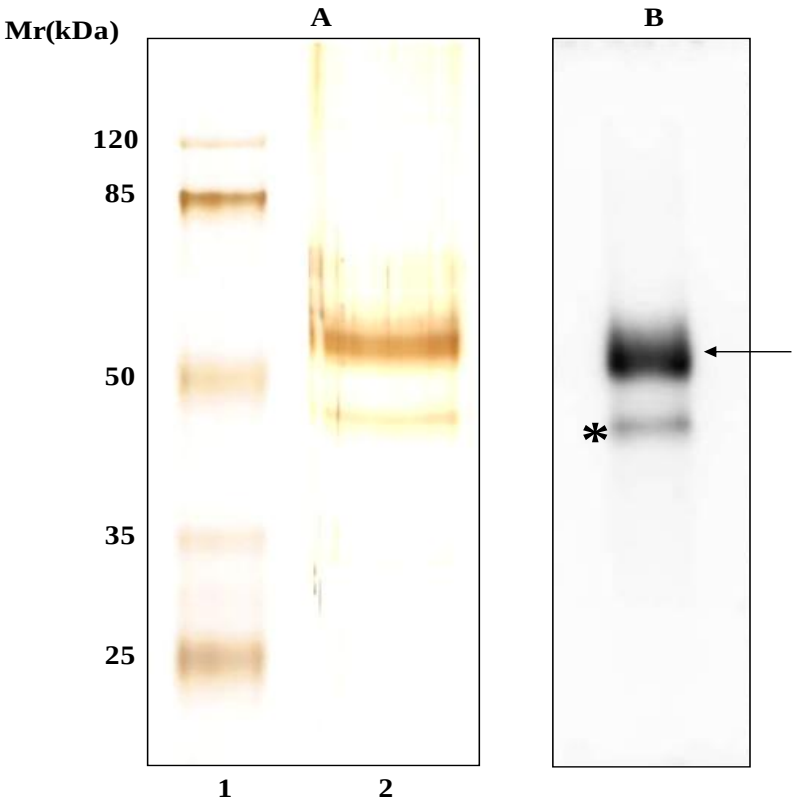


Figure 3.4.5

Figure 3.4.6. Schematic representation of the PEAKS 5.1 workflow

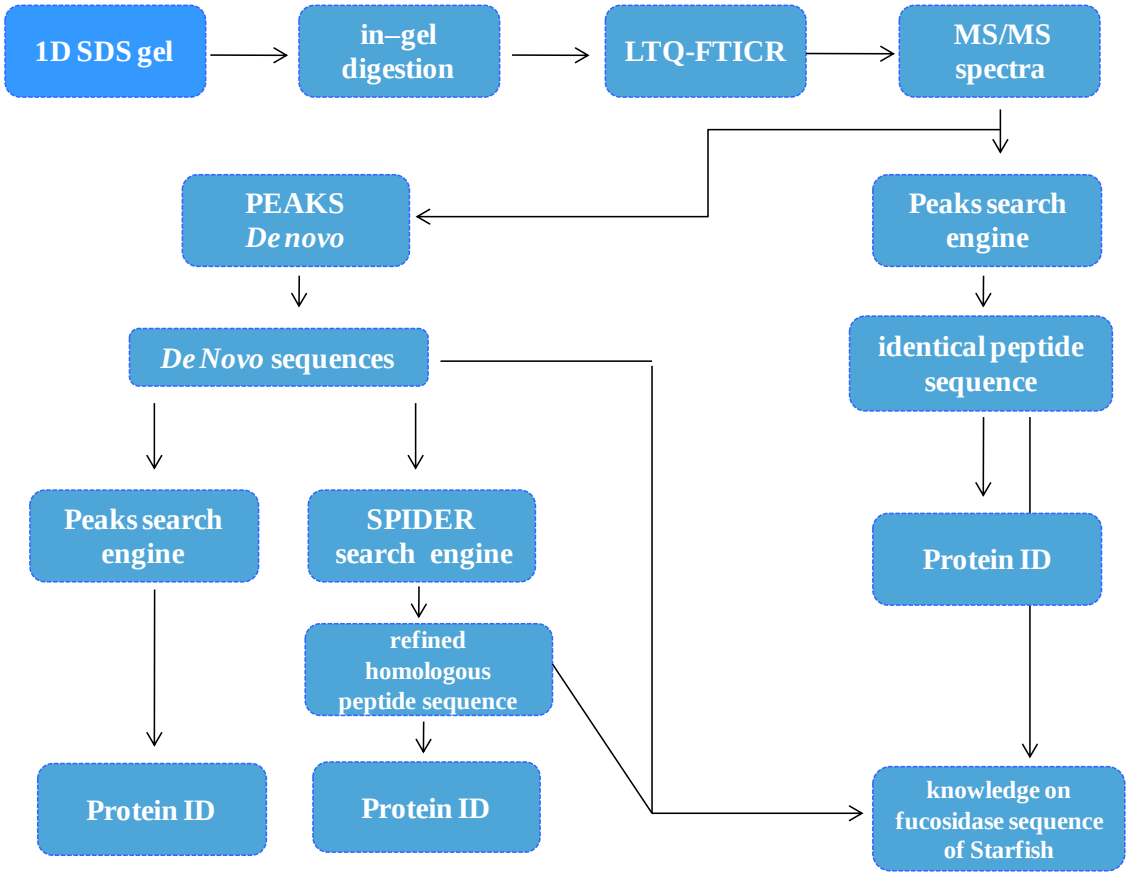

1D SDS gel
in–gel digestion
LTQ-FTICR
MS/MS spectra
PEAKS De novo
Peaks search engine
De Novo sequences
identical peptide sequence
Peaks search engine
SPIDER search engine
Protein ID
refined homologous peptide sequence
Protein ID
Protein ID
knowledge on fucosidase sequence of Starfish

Figure 3.4.6

Figure 3.4.7. Comparison of the amino acid sequence of starfish α-fucosidase with other known fucosidases reveals high sequence homology of this enzyme across different taxa. Fuc-Mouse, α-Fucosidase from *Mus musculus* (Q99KR8), Fuc-Rat, α-Fucosidase from *Rattus norvegicus* (Q6AYS4); Fuc-Human, α-fucosidase from *Homo sapiens* (Q9BTY2); Fuc-Bovine, α-Fucosidase from *Bos Taurus* (Q2KIM0); Fuc-Starfish, α-Fucosidase from *Asterias rubens*; ♦ indicates the residue potential for N-glycosylation site, **$** indicates the conserved amino residue responsible for catalysis.(*) marks identical amino acid residues; (:) related amino acid residues; (·) predominantly the same amino acid residue.


Fuc-Mouse ----------MRLGFLMLLPLLLLPLLRPWGVTRALSYDPTWESLDRRPLPAWFDQAK
Fuc-Rat ----------MRLGLLMFLPLLLLATR--YRAVTALSYDPTWESLDRRPLPAWFDQAK
Fuc-Human ------MRPQELPRLAFPLLLLLLLLLPPPPCPAHSATRFDPTWESLDARQLPAWFDQAK
Fuc-Bovine MRSWVVGARLLLLLQLVLVLGAVRLPPCTDPRHCTDPPRYTPDWPSLDSRPLPAWFDEAK
Fuc-Starfish ----------YEPNWPSLDSRPLPAWYDEAK
Fuc-Mouse FGIFIHWGVFSVPSFGSEWFWWYWQKEKKPQFVDFMNNNYAPGFKYEDFVVLFTAKYFNA
Fuc-Rat FGIFIHWGVFSVPSFGSEWFWWYWQKERRPKFVDFMDNNYPPGFKYEDFGVLFTAKYFNA
Fuc-Human FGIFIHWGVFSVPSFGSEWFWWYWQKEKIPKYVEFMKDNYPPSFKYEDFGPLFTAKFFNA
Fuc-Bovine FGVFVHWGVFSVPAWGSEWFWWHWQGEKLPQYESFMKENYPPDFSYADFGPRFTARFFNP
Fuc-Starfish F---IHWGVFSVPSFGSEW----------
Fuc-Mouse NQWADILQASGAKYVVFTSKHHEGFTMWGSDRSWNWNAVDEGPKRDIVKELEVAVRNRTG
Fuc-Rat NQWADLLQASGAKYVVLTSKHHEGFTLWGSAHSWNWNAVDEGPKRDIVKELEVAVRNRTD
Fuc-Human NQWADIFQASGAKYIVLTSKHHEGFTLWGSEYSWNWNAIDEGPKRDIVKELEVAIRNRTD
Fuc-Bovine DSWADLFKAAGAKYVVLTTKHHEGYTNWPSPVSWNWNSKDVGPHRDLVGELGTAIR-KRN
Fuc-Starfish --WAELFKASGAQYIVLTSKHHEGCCNWYS---WNWNSMDVGPK-DLVGELADAVR----
Fuc-Mouse LHFGLYYSLFEWFHPLFLEDQSSSFQKQRFPVSKTLPELYELVNRYQPEVLWSDGDGGAP
Fuc-Rat LHFGLYYSLFEWFHPLFLEDQSSAFQKQRFPVAKTLPELYELVTKYQPEVLWSDGDGGAP
Fuc-Human LRFGLYYSLFEWFHPLFLEDESSSFHKRQFPVSKTLPELYELVNNYQPEVLWSDGDGGAP
Fuc-Bovine IRYGLYHSLLEWFHPLYLRDKKNGFKTQYFVNAKTMPELYDLVNRYKPDLIWSDGEWECP
Fuc-Starfish ----------TMPELYELVNR----------
Fuc-Mouse DHYWNSTGFLAWLYNESPVRKTVVTNDRWGVGSICKHGGYYTCSDRYNPGYLLPHKWENC
Fuc-Rat DHYWNSTDFLAWLYNESPVRDTVVTNDRWGAGSICKHGGYYTCSDRYNPGHLLPHKWENC
Fuc-Human DQYWNSTGFLAWLYNESPVRGTVVTNDRWGAGSICKHGGFYTCSDRYNPGHLLPHKWENC
Fuc-Bovine DTYWNSTDFLAWLYNDSPVKDEVVVNDRWGQNCSCHHGGYYNCKDKFQPETLPDHKWEMC
Fuc-Starfish -------EYLAWLYNDSPVKDTVVTNDR--------HGGYYNMQDRYNPG-VLQKQWEQC
Fuc-Mouse MTIDKFSWGYRREAEISDYLTIEELVKKLVETVACGGNLLMNIGPTGDGTIPVIFEERLR
Fuc-Rat MTIDKFSWGYRREAEIGDYLTIEELVKQLVETVSCGGNLLMNIGPTLDGIIPVIFEERLR
Fuc-Human MTIDKLSWGYRREAGISDYLTIEELVKQLVETVSCGGNLLMNIGPTLDGTISVVFEERLR
Fuc-Bovine TSIDQRSWGYRRDMEMADITNESTIISELVQTVSLGGNYLLNVGPTKDGLIVPIFQERLL
Fuc-Starfish TTLDKASWGYRR----------ISRGGNMLLNVGPR-----IAPIFEER--
Fuc-Mouse QMGTWLKVNGEAIYETHTWRSQNDTVTPDVWYTSKPEKKLVYAIFLKWPISGKLFLGQPI
Fuc-Rat QMGTWLKVNGEAIYETHTWRSQNDTVTPDVWYTSKPEKKLVYAIFLKWPISGKLFLGQPI
Fuc-Human QMGSWLKVNGEAIYETHTWRSQNDTVTPDVWYTSKPKEKLVYAIFLKWPTSGQLFLGHPK
Fuc-Bovine AVGKWLSINGEAIYASKPWRVQSEKNS--VWYTSKG--LAVYAILLHWPEYGILSLISPI
Fuc-Starfish ----WLDVNGDAIYKAT----------NVWYTSK----------

Figure 3.4.7

Figure 3.4.8. Ligand blot analysis of the purified α-fucosidase enzyme on 10% SDS-PAGE. Lane 1, Direct probing of the membrane with goat MPR 300 antiserum without prior incubation with the receptor (negative control); lane 2, recognition of enzyme-receptor complex by goat MPR 300 antiserum; lane 3, receptor preincubated with 5mM mannose 6-phosphate and lane 4, receptor preincubated with 10 mM mannose 6-phosphate. At 10 mM concentration there is complete inhibition of the binding of the receptor to the enzyme. Arrow indicates the recognition of enzyme-receptor complex by goat MPR 300 antiserum.

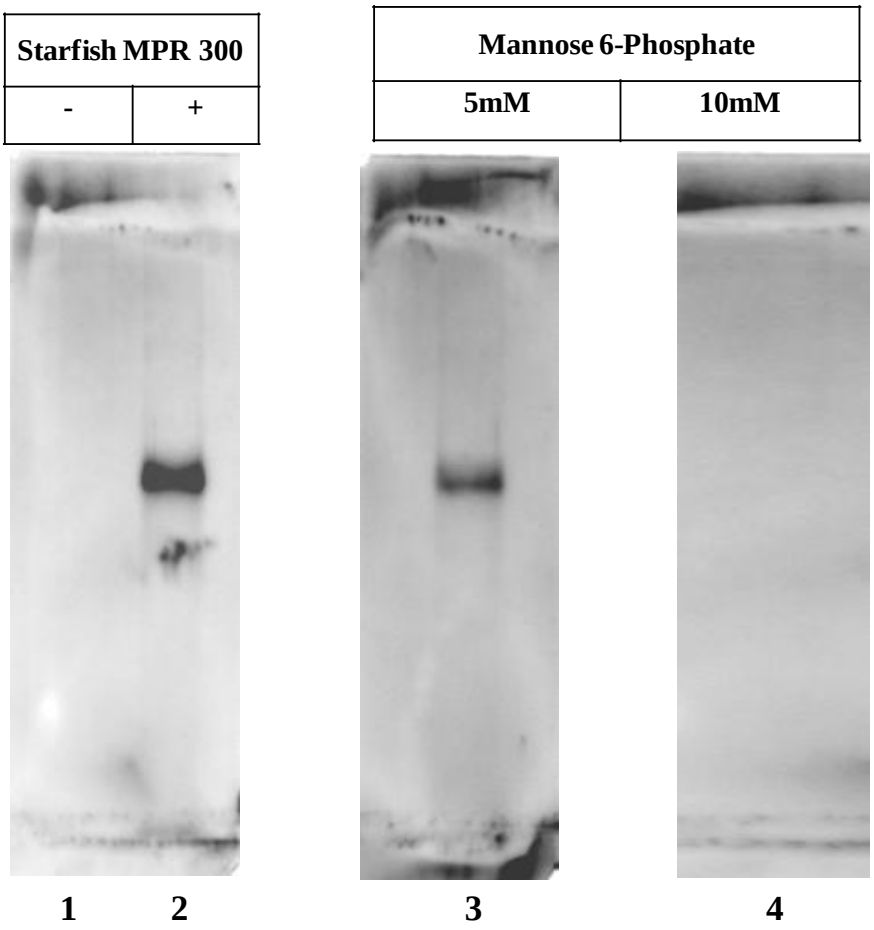


Figure 3.4.8

Figure 3.4.9. Analysis of α-fucosidase interaction with MPR 300 protein. (A) α-fucosidase specifically bound to starfish MPR 300-affigel can be partially desorbed using 5 mM mannose 6-phosphate. Lane 1, Molecular weight marker, lane 2, 5 mM glucose 6-phosphate eluate, lane 3, 5 mM mannose 6-phosphate eluate and lane 4, 50 mM sodium acetate eluate. (**B**) Eluates from phenyl Sepharose that contain the α-fucosidase enzyme were applied on human MPR 300-affigel and bound proteins specifically eluted with 5 mM mannose 6-phosphate. Arrow indicates the identity of the enzyme eluted from starfish receptor gel. *represent bands that might possibly represent other receptor interacting proteins which were not characterized in this study. **(C)** Western blot analysis of the purified α-fucosidase enzyme treated with PNGaseF as described in materials and methods. Lane 1, native enzyme (- PNGase F) and Lane 2, enzyme treated with (+ PNGase F) detected using *unio* α-fucosidase antiserum.

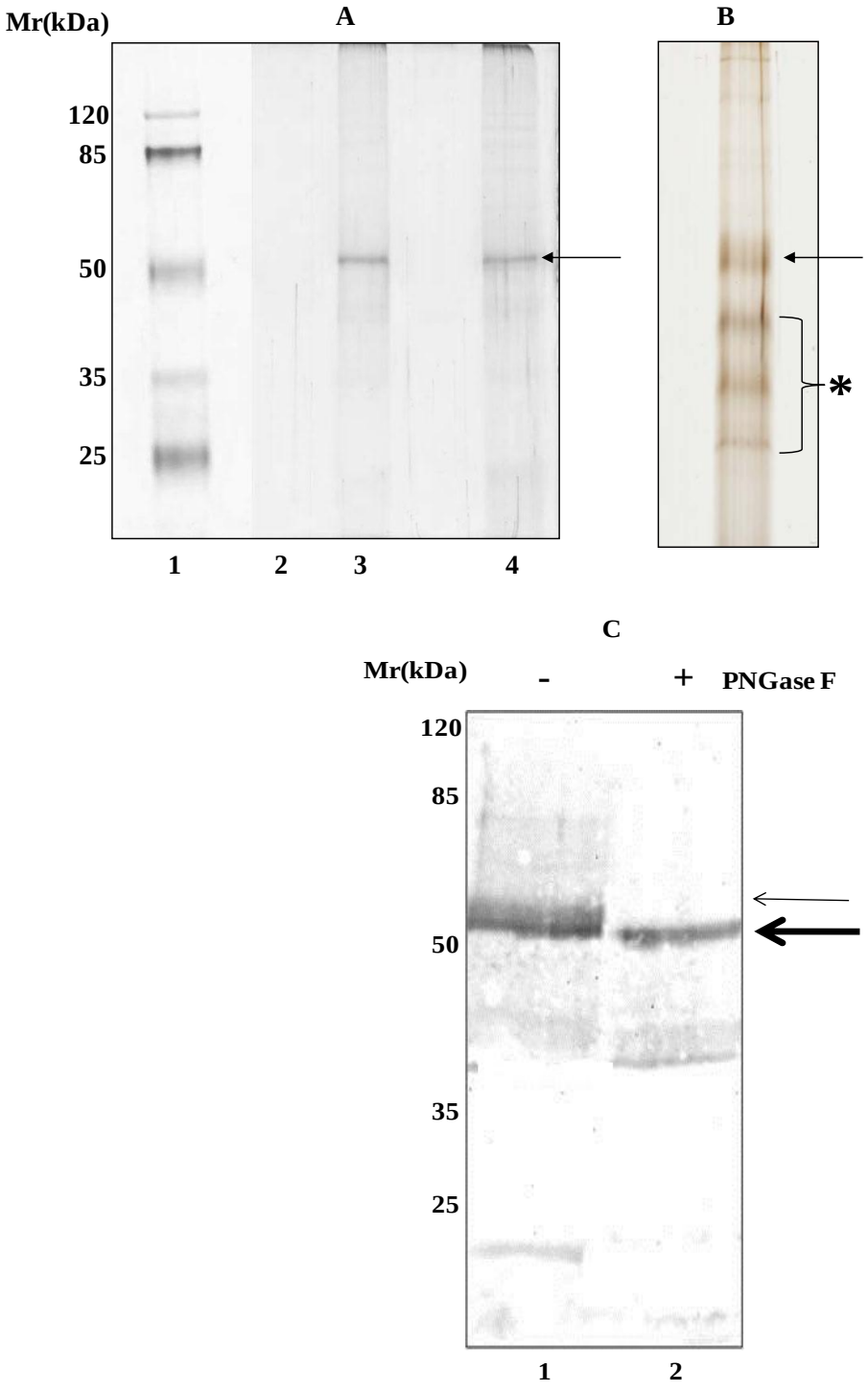


Figure 3.4.9

Figure 3.4.10. Effect of divalent metal ions, EDTA, sugar analogue and chemical agents on the *Asterias rubens* α-fucosidase activity.

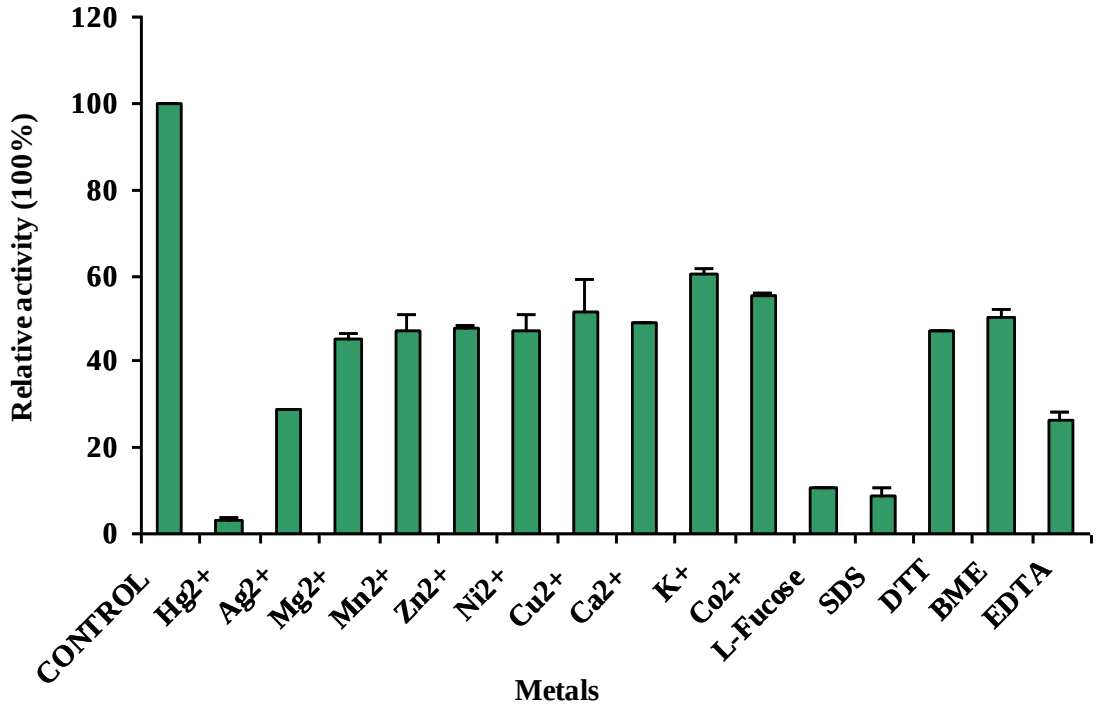

120
100
80
60
40
20
0
Relative activity (100%)
CONTROL
Hg2+
Ag2+
Mg2+
Mn2+
Zn2+
Ni2+
Cu2+
Ca2+
K+
Co2+
L-Fucose
SDS
DTT
BME
EDTA
Metals

Figure 3.4.10

Figure 3.4.11. Characterization of the enzymatic properties of purified α-L-fucosidase from *Asterias rubens*. (A) Effect of pH on the α-fucosidase activity of *A. rubens* was determined at 37℃ in buffers ranging from pH 2.0 to 8.0. The value obtained at pH 5.0 where α-fucosidase activity is maximum was taken as 100%. **(B)** pH stability of *A. rubens* α-fucosidase was determined by measuring its activity under standard assay conditions with *p*-nitrophenyl α-L-fucopyranoside as substrate after pre-incubation of the enzyme at 37℃ for 12 h in buffers ranging from pH 2.0 to 8.0. The activity of an untreated enzyme sample at pH 5.0 was taken as 100%. **(C)** Effect of temperature on α-fucosidase activity was determined in 50 mM NaOAc buffer (pH 5.0) at 30 - 90℃. The value obtained at 70℃ was taken as 100%. **(D)** Thermostability of α-fucosidase was determined by measuring α-fucosidase activity under standard assay conditions after pre-incubation of the enzyme in 50 mM NaOAc buffer (pH 5.0) at 50, 60, 70 and 80℃ for various periods. The activity of an unheated enzyme sample was taken as 100%. Data is the mean of duplicate experiments.

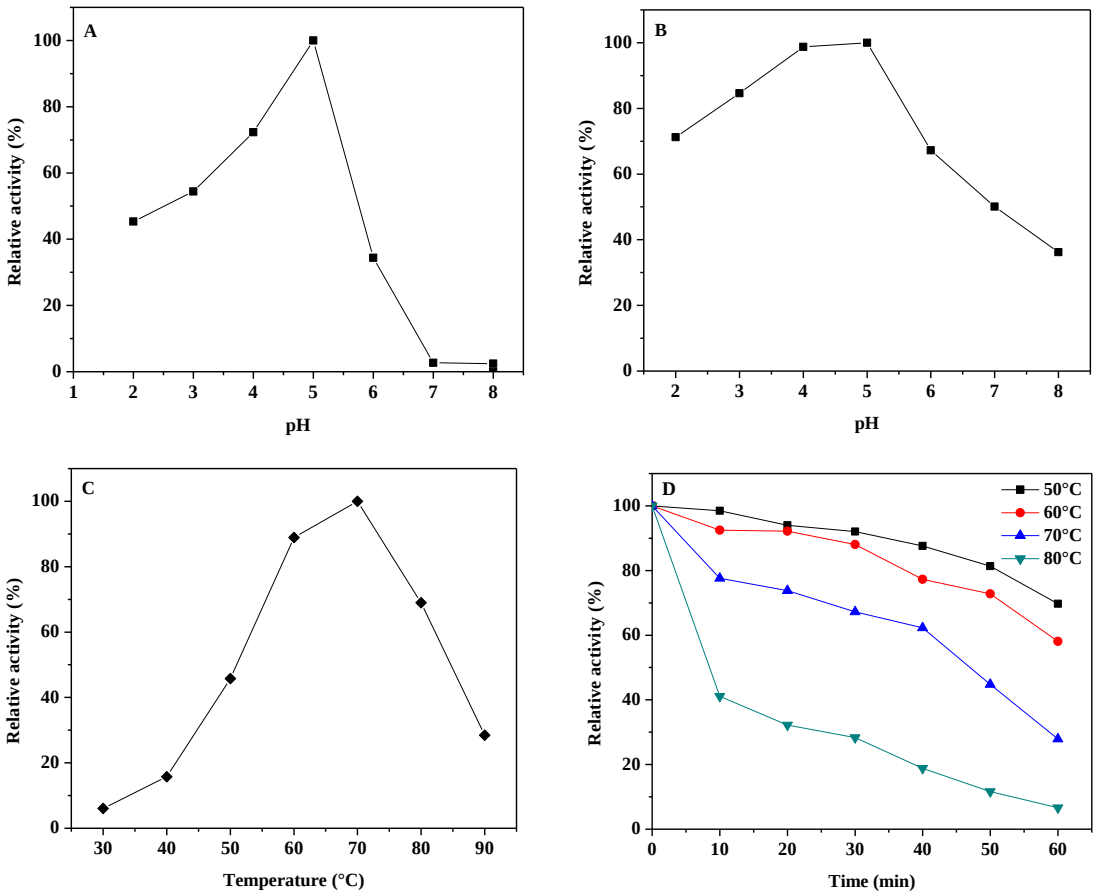
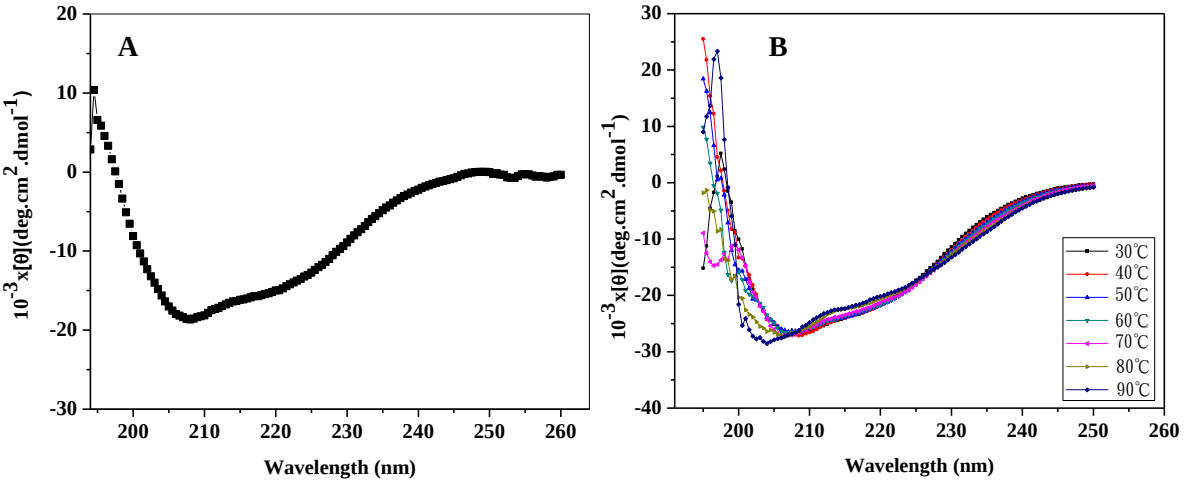

A
Relative activity (%)
pH
B
Relative activity (%)
pH
C
Relative activity (%)
Temperature (°C)
D
50°C
60°C
70°C
80°C
Relative activity (%)
Time (min)

Figure 3.4.11

Figure 3.4.12. (A) CD spectrum of the purified α-fucosidase enzyme. Far UV-CD spectrum (260-190 nm) of the α-fucosidase (0.3 mg/mL) in distilled water at 30℃. The structure parameters were analyzed by CDSSTR program (DICHROWEB). **(B) Effect of temperature on the CD spectrum of α-fucosidase.** Far UV CD spectra (260-190 nm) of α-fucosidase at different temperatures. Spectra were recorded at 30, 40, 50, 60, 70, 80 and 90℃.



Secondary structure	α-helix	β-sheets	β-turns	Unordered structures
α-fucosidase	34%	25%	20%	21%

Figure 3.4.12

Table 3.1. Purification of α-fucosidase from of starfish tissue

S. No.	Step	Total protein (mg)	Total enzyme [b] (Units)	Specific activity [c] (U/mg)	Purification fold	Yield (%)
1.	Crude [a]	1740	11.2	0.006	1	100
2.	DE-52 eluate	100	8.0	0.08	13.3	71
3.	Phenyl-Sepharose eluate	14.2	1.4	0.098	16.3	12
4.	Fucosylamine eluate	1.0	0.45	0.45	75	4

[a] 50 g of starfish tissue was used at a time and the purification in different chromatographic steps was carried out in batch wise. Proteins were measured in chromatographic fractions using A_{280}.
[b] One unit of enzyme activity was defined as amount of enzyme hydrolyzing 1 μmol of *p*-nitrophenol from PNPF per min at pH 5.0 and 37℃.
[c] The specific activity of the enzyme is expressed as units per mg protein. (U/mg of protein).

Table 3.2. Summary of results of database search as well as error tolerant search of amino acid sequences derived from *de novo* sequencing. Database searches were performed in SwissProt rel. 57.15 and MSDB from NCBI (rel. 2009). MS/MS data set were analysed with PEAKS DB software for direct database search and PEAKS Spider software for error tolerant search of *de novo* computed amino acid sequences. The table gives the details on peptides completely or partially identical to peptides of known fucosidases as computed by PEAKS 5.1. DB-database; Sp-SwissProt DB 57.15; m/z - precursor mass over charge; z-charge of the precursor ion; MH+ - protonated peptide mass; n.d.-MS/MS spectrum was assigned to an entry of a database - no denovo sequence computed.

Peptide Sequence	m/z	z	MH+	*de novo* Score ALC* (%)	DB	Protein accession number	Protein name	DB peptide sequence	Peptide Score** (%)	Protein Score*** (%)
Result of database search for tryptic peptides										
YNPGVLQK	459.76	2	917.4971	n.d.	NCBI	gi|170030992	plasma alpha-L-fucosidase [Culex quinquefasciatus]	YNPGVLQK	72.2	97.0
DTVVTNDR	460.23	2	918.4406	n.d.	NCBI	gi|170030992	plasma alpha-L-fucosidase [Culex quinquefasciatus]	DTVVTNDR	89.4	97.0
WENAMTIDK	554.26	2	1106.5066	n.d.	NCBI	gi|170030992	plasma alpha-L-fucosidase [Culex quinquefasciatus]	WENAMTIDK	98.5	97.0
WENAM[ox]TIDK	562.26	2	1122.5015	n.d.	NCBI	gi|170030992	plasma alpha-L-fucosidase [Culex quinquefasciatus]	WENAMTIDK	84.3	97.0
IAPIFEER	487.77	2	973.52325	n.d.	NCBI	gi|225707686	Plasma alpha-L-fucosidase precursor [Osmerus mordax]	IAPIFEER	75.8	85.3
DLVGELASAIR	572.32	2	1142.6294	n.d.	NCBI	gi|29840895	similar to alpha-L-fucosidase [Schistosoma japonicum]	DLVGELASAIR	89.4	82.0
DLVGELGTALR	572.32	2	1142.6294	n.d.	SP	P04066|FUCO_HUMAN	Tissue alpha-L-fucosidase	DLVGELGTALR	99.1	62.1
DTVVTNDR	460.23	2	918.4406	n.d.	SP	Q6AYS4|FUCO2_RAT	Plasma alpha-L-fucosidase	DTVVTNDR	99.1	80.0
TLPELYELVNR	673.87	2	1345.7241	n.d.	SP	Q99KR8|FUCO2_MOUSE	Plasma alpha-L-fucosidase	TLPELYELVNR	99.2	60.9
Result of database search for chymotryptic peptides										
FDEAKF	378.68	2	755.349	n.d.	SP	Q60HF8|FUCO_MACFA	Tissue alpha-L-fucosidase	FDEAKF	43.8	88.8
DSRPLPSWF	552.78	2	1103.5398	n.d.	SP	Q60HF8|FUCO_MACFA	Tissue alpha-L-fucosidase	DSRPLPSWF	72	88.8
IHWGVF	379.70	2	757.3911	n.d.	SP	Q99KR8|FUCO2_MOUSE	Plasma alpha-L-fucosidase	IHWGVF ****	92.7	98.3
SVPSFGSEW	498.23	2	994.43964	n.d.	SP	Q99KR8|FUCO2_MOUSE	Plasma alpha-L-fucosidase	SVPSFGSEW****	96.7	98.3
ELVNRY	397.21	2	792.413	n.d.	SP	Q99KR8|FUCO2_MOUSE	Plasma alpha-L-fucosidase	ELVNRY****	83.2	98.3
LKVNGEAIY	503.78	2	1005.5495	n.d.	SP	Q99KR8|FUCO2_MOUSE	Plasma alpha-L-fucosidase	LKVNGEAIY****	98.6	98.3

de novo Sequence	m/z	z	MH+	*de novo* Score ALC (%)	Reconstituted sequence	DB	Protein accession number	Protein name	DB peptide sequence	Peptide Score (%)	Protein Score (%)
Result of *de novo* sequencing and error tolerant database search for tryptic peptides											
EYPNWPSLDDK	682.32	2	1362.6091	39.8	YEPNWPSLDSR	SP	Q2KIM0|FUCO_BOVIN	Tissue alpha-L-fucosidase	YTPDWPSLDSR	15.5	117
LPPAWYDEAK	595.30	2	1188.5815	65.6	PLPAWYDEAK	SP	Q99KR8|FUCO2_MOUSE	Plasma alpha-L-fucosidase	PLPAWFDQAK	17.7	60
WAELFK	397.22	2	792.4170	63.4	WAELFK	SP	Q99LJ1|FUCO_MOUSE	Tissue alpha-L-fucosidase	WAELFQ	13.7	106
ASGAQYLVLTSK	619.34	2	1236.6714	69.1	ASGAQYIVLTSK	SP	Q6AYS4|FUCO2_RAT	Plasma alpha-L-fucosidase	ASGAKYVVLTSK	23.4	103
YTATNWNSFDVGPK	800.35	2	1598.7034	64.1	YSWNWNSM[ox]DVGPK	SP	Q6AYS4|FUCO2_RAT	Plasma alpha-L-fucosidase	HSWNWNAVDEGPK	24.0	103
NNVGELADAVR	579.31	2	1156.5835	74.2	DLVGELADAVR	SP	P17164|FUCO_RAT	Tissue alpha-L-fucosidase	DLVGELGAAVR	19.2	109
EYLAWLYNDSPVK	799.40	2	1596.7822	63.9	EYLAWLYNDSPVK	SP	P17164|FUCO_RAT	Tissue alpha-L-fucosidase	EFLAWLYNESPVK	30.1	109
HGGYYEYPDR	628.76	2	1255.5259	69.9	HGGYYNM[ox]QDR	SP	P48300|FUCO_CANFA	Tissue alpha-L-fucosidase	HGGYYNCQDK	18.2	122
EARGGNMLLNVGPR	742.39	2	1482.7725	65.8	ISRGGNMLLNVGPR	SP	P48300|FUCO_CANFA	Tissue alpha-L-fucosidase	VSLGGNYLLNLGPT	17.6	122
TQGVWYTSK	535.27	2	1068.5239	59.1	ATNVWYTSK	SP	P49713|FUCO_CAEEL	Putative alpha-L-fucosidase	ASNVWYTSK	13.8	92
QWENSMTLDK	626.31	2	1250.5600	51.8	QWEQCTTLDK	SP	P48300|FUCO_CANFA	Tissue alpha-L-fucosidase	KWEMCTSLDK	10.9	122
WLDVNGDALYK	647.33	2	1292.6401	61.9	WLDVNGDAIYK	NCBI	gi|198424065	PREDICTED: similar to Fucosidase, alpha-L- 2, plasma [Ciona intestinalis]	WLNVNGEAVYK	21.7	49
Result of *de novo* sequencing and error tolerant database search for chymotryptic peptides											
EDHQVHEGCCNW	728.78	2	1455.5295	41.5	LMARHHEGCCNW	NCBI	gi|29840895	similar to alpha-L-fucosidase [Schistosoma japonicum]	LTAKHHEGFCNW	10.9	30

*_De novo_ score ALC (%): Average local confidence (the confidence of the peptide sequence). It is calculated by adding the positional confidence for each amino acid in the peptide sequence and dividing by the total number of amino acids.

**Peptide score (%): A value from 1 to 99 representing the confidence of this peptide identification based on an LDF (linear discriminative function) score to measure the quality of the peptide-spectrum match. The LDF score uses not only the matching between the fragment ions and the peaks in the spectrum, but also many other factors such as the similarity between the de novo sequencing peptide and the database peptide.

*** Protein score (%): A value from 1 to 99 representing the confidence of this protein identification – calculated from the confidence on the ten best peptide hits for this protein, and normalized against the other identified proteins.

****also found in NCBI search for protein gi|31541791

3.5. DISCUSSION

Lysosomal enzyme transport in eukaryotic cells depends on their recognition by two homologous but distinct receptor proteins designated as MPR 300 and MPR 46, respectively (von Figura and Hasilik, 1986; Hille-Rehfeld, 1995; Udaya lakshmi *et al*., 1999; Siva Kumar and von Figura, 2002). The functional roles of these receptors in lysosomal enzyme transport have been established in different mammalian species and some non-mammalian vertebrates. In invertebrates, both the receptors from starfish and *unio* exhibit distinct phosphomannan binding abilities and the *unio* MPR 300 has been shown to interact with the lysosomal α-fucosidase from *unio* (Siva Kumar *et al*., 2004). Furthermore, the role of the MPR 300 protein in sorting lysosomal enzymes in the mollusc cells has also been recently established (Praveen Kumar *et al*., 2009). In *Drosphila melanogaster*, no putative receptors could be identified and only a lysosomal enzyme receptor protein was found (Dennes *et al*., 2005). Although some lysosomal enzyme activities could be detected in the invertebrate starfish as well, none of these were purified and only the MPR 46 was cloned, sequenced and found to transport lysosomal enzymes (Sivaramakrishna and Siva Kumar, 2008). As the information on detailed characterization of the α-fucosidase and its interaction with MPR 300 in this species is lacking, it is important to gain new insights on this to conclusively establish the functional roles of both MPR proteins in invertebrates. From these studies it can be hypothesized that a functional lysosomal enzyme targeting machinery started to appear from the mollusc onwards in the animal kingdom. This hypothesis can be supported and validated only by extensive biochemical immunological and functional characterization of different lysosomal enzymes and their receptors among the invertebrates. This would eventually provide additional information on the evolutionary conservation of the

receptors. Therefore, the present study was undertaken to affinity purify the α-fucosidase and to investigate the specific interaction of the purified lysosomal enzyme with the purified starfish MPR 300 protein. Initial assays of lysosomal enzyme activities from the starfish tissue revealed the presence of several enzyme activities, of which the fucosidase was prominent. This enzyme was chosen for further studies in order to enable a comparative analysis of invertebrate enzyme to the well characterized human enzyme analyze its sequence and to establish its specific interaction with the mannose 6-phosphate receptor protein (MPR 300).

The data obtained for the lysosomal enzyme from starfish provides evidence on the existence of α-fucosidase in this invertebrate. The enzyme was extracted from the soluble fraction of the tissue, bound to fucosylamine affinity gel and eluted specifically with fucose sugar. The molecular mass of around 56 kDa identified by SDS-PAGE, glycoprotein nature and the recognition by an antiserum raised against the mollusc enzyme indicate that the starfish enzyme is similar to other known fucosidases. The purified α-fucosidase was checked for other glycosidase activities which are present in the initial crude extract with *p*NP-substrates. It did not show significant activity with these substrates, indicating that the activity of this enzyme is restricted towards *p*NP α-L-fucopyranoside only and there is no other contaminating glycosidase. Partial identification of the amino acid sequence of the protein clearly demonstrated high sequence similarity of the starfish enzyme with other enzymes that have been characterized so far. The extent of similarity seen is as follows. Human (70%), mouse (72%), rat (73%), and bovine (70%). Furthermore, the cysteine residue important for activity was found to be conserved in all the species (Rui *et al*., 2009). All these facts support the notion that in the evolutionary tree, the α-fucosidase is conserved from the invertebrates to mammals. Indeed other results from the

laboratory indicate an evolutionary conservation of other lysosomal enzymes such as the hexosaminidase, cathepsin D. EDTA has no affect on fucosidase activity, indicating that fucosidase is unlikely to be a metalloenzyme, a similar study by Liu and Li, 2009. High inhibition of fucosidase was observed in Hg^{2+} and Ag^{2+} suggesting that α-L-fucosidase contains a sulfhydryl group which plays an important role in either the catalytic function or protein structure. The suggested presence of sulfhydryl-containing amino acids in fucosidase was confirmed by amino acid analysis revealing the presence of cysteine residue at position 288. Zn^{2+} had no significant effect on enzymatic activity unlike the large inhibition (90%) of rat epididymal α-L-fucosidase found with 5 mM Zn^{2+} by Carlsen and Pierce, 1972. The optimum pH of starfish α-fucosidase enzyme was 5.0, a similar condition studied by Piesecki *et al*., 1992 and stability from pH 3-5 with more than 80% of the enzyme activity remained at this pH, after 12 h of incubation. Koji *et al*., 1996 also reported a similar study where the α-fucosidase was active around pH 3.2-5.2. The temperature optimum is at 70℃ a similar observation seen by Antimo D'ANIELLO *et al*., 1982. Above this temperature, enzyme activity declined rapidly as the temperature increased. Circular dichroism spectroscopy of a purified protein gives valuable information with respect to secondary structure and the stability of the protein.

In summary, the present study describes the affinity purification and biochemical characterization of a lysosomal α-fucosidase enzyme from the highly evolved invertebrate starfish. Having already understood the role of MPR 46 in lysosomal enzyme targeting in starfish (Sivaramakrishna and Siva Kumar, 2008), the present study provides new information to understand the role of the starfish MPR 300 in lysosomal enzyme interaction. Our studies reveal that the starfish and molluscs contain both MPR proteins whose biochemical and immunological properties are

similar to the mammalian receptors. Furthermore, the interaction of the purified lysosomal enzyme with the MPR 300 protein points to the functional role of the receptor in targeting lysosomal enzymes in starfish. These findings suggest the presence of similar lysosomal enzyme targeting pathways in invertebrates as also in the vertebrates. Future studies on identifying other interacting proteins with the receptor/enzymes in this species would shed additional light on the lysosomal enzyme targeting pathway in this species. Additionally, cloning of fucosidase and other lysosomal enzyme genes and comparing their sequences to the known vertebrate enzymes would further strengthen the hypothesis on the evolutionary conservation of the lysosomal enzyme sorting machinery in the invertebrates.

CHAPTER 4

Cloning and sequence determination of the *Xiphophorus xiphidium* (A_2 cell) MPR 46

4.1. INTRODUCTION

In mammals, mannose 6-phosphate receptors (MPRs) play an important role in lysosome biogenesis by sorting newly synthesised acid hydrolases to lysosomes at the *trans*-Golgi network (TGN) (Dahms and Hancock, 2002; Ghosh *et al.*, 2003). The occurrence of both MPRs (MPR 300 and MPR 46) in mammalian and non-mammalian vertebrates has been well characterized (Siva Kumar and Praveen kumar, 2010). The MPR 46 mediates intracellular sorting of lysosomal enzyme but does not endocytose extracellular ligands. It has been shown to mediate the secretion of lysosomal enzymes besides endogenous lysosomal enzyme sorting (Chao *et al*., 1990). In view of the distinct functions exhibited by the two receptor proteins, it is of interest to understand how these proteins were evolved.

The first report showing the existence of both MPRs in the early vertebrate fish came from our laboratory (Siva Kumar *et al*., 1999). Purified trout liver MPR 300 showed electrophoretic mobility similar to the purified goat liver receptor and a pH optimum of 6.5-7.0 for binding to phosphomannan. However the MPR 46 exhibited a lower molecular mass as compared to mammalian protein, possibly due to differential glycosylation. The molecular weight of the polypeptide in chicken and *Xiphophorus* was same (Udaya Lakshmi *et al*., 1999). Zebrafish MPR 46 has also been shown to be homologous to the other vertebrate receptors (Suresh *et al*., 2006). In order to gain more insights into the MPR targeting system in the fish, the present study was taken up with the following objectives, (i) to identify the lysosomal enzyme activities in the A_2 cell extracts, (ii) check their cross-reactivity with the available lysosomal enzyme antibodies (iii) to prepare a full length cDNA for the fish MPR 46 receptor and to make a structural comparison of the sequences with other known receptors.

4.2. MATERIALS AND METHODS

4.2.1. Materials

Ham's F-12 Nutrient medium (Gibco), foetal bovine serum (FBS) was purchased from PAN Biotech, India. Chromogenic substrates used for enzyme assays were purchased from Sigma. The following antibodies were available in the laboratory; α-fucosidase antiserum (raised against purified protein from *unio*), β-hexosaminidase antiserum (raised against purified goat protein), arylsulfatase A antiserum (raised against human protein), affinity purified starfish cathepsin D IgG (raised against purified starfish cathepsin D protein); all antibodies used were polyclonal antibodies. Centrifugation steps in this study were done in Biofuge stratos centrifuge in 1.5 mL rotor unless otherwise mentioned.

4.2.2. Preparation of cell lysate and extraction of soluble proteins

Xiphophorous xiphidium (A_2) cells were cultured in complete medium (45 mL of Ham's F-12 Nutrient medium, penicillin/Streptomycin (5μg/mL), 15% heat inactivated foetal bovine serum) in standard culture flasks at 28℃ without CO_2. Confluent cultures are detached from the surface by gushing with PBS (10 mM sodium phosphate buffer pH 7.4, containing 150 mM NaCl); cell pellets were collected by centrifugation at 2991×g for 10 min. The pellet was suspended in 0.5 mL of 0.1 M sodium acetate buffer pH 6.0, containing 0.2 M NaCl, 1 mM PMSF, 5 mM iodoacetic acid, 1 mM EDTA, sonicated thrice for 35 sec each time with an interval of 1 min, incubated for 20 min on ice, and centrifuged in a Beckman centrifuge using a fixed angle 80Ti rotor at 161,280×g for 30 min. The supernatant obtained at this step is referred as acetate supernatant and was used for lysosomal enzyme assays. The lysosomal enzyme activities of α-fucosidase, α-galactosidase, β-hexosaminidase and α-mannosidase were assayed using *p*-nitrophenyl derivatives of the respective

substrates and arylsulfatase A was assayed using 4-Nitrocatechol sulfate dipotassium salt as described (section 3.2.2). The pellet was again resuspended with 0.5 mL of 50 mM imidazole buffer pH 7.0 containing 0.5% Triton X-100, 1mM PMSF, 5mM iodoacetic acid, 1mM EDTA to extract the membrane proteins as described above.

4.2.3. Immunoblotting

For immunological detection, 25 µg soluble protein was separated on a 10% SDS-PAGE and transferred onto a 0.45 mm pore diameter PVDF membrane (Immobilion-P, Millipore, Bedford, USA) as described (Towbin *et al.*, 1979). The membrane was placed in blocking solution containing 5% non-fat milk powder in phosphate buffer pH 7.4, containing 0.15 M NaCl and 0.2% Tween 20 (PBST) for 1.5 h. The blot was briefly washed with PBST and incubated overnight with the primary antibody in 1% milk with PBST. The following primary antibodies were used for the detection of the respective enzymes: Arylsulfatase A antiserum, dilution of 1:500; α-fucosidase antiserum, dilution of 1:300; β-hexosaminidase antiserum, dilution of 1:200; affinity purified starfish cathepsin D IgG, dilution of 1:500. The source of the antiserum used is described in section 4.2.1. The immunoreactive bands were visualized using a secondary antibody conjugated with HRP enzyme (Bangalore Genie) and detected by enhanced chemiluminescence using Super-Signals West Femto Maximum Sensitivity Substrate (Thermo Scientific, Rockford, IL, USA).

4.2.4. Isolation of total RNA

Total RNA was isolated from the cold cell pellet using an acid guanidinium thiocyanate–phenol-chloroform mixture commercially available as TRIzol (Invitrogen). The quality of the RNA was confirmed by using a Bioanalyzer 2100 (Agilent Technologies) to ensure samples with intact 18S and 28S ribosomal RNA

peaks, and the amount was determined with a NanoDrop spectrophotometer (Thermo Scientific).

4.2.5. ReverseTranscriptase - Polymarase Chain Reaction

From the RNA obtained above cDNA was synthesized using 0.5 ng of oligo (dT) primer and 200 units of reverse transcriptase enzyme in a total volume of 20 µL (according to the manufacturer's protocols; MBI Fermentas, India). The cDNA synthesized was used as template for amplification of the putative A_2 MPR 46 using the following primers.

Sense primer: 5'-CGGAATTCATGACGGTGCAC-3'.

Antisense primer 5'CCGCTCGAGTCACATCGGTAGCAA-3'. PCR was carried out in a total volume of 20 µL, 1µL (10 pM) each of primers, 2 µL of oligonucleotides, 4.5 µL of cDNA template, 10.5 µL nuclease free water and 1 µL of Taq DNA polymerase. After Taq DNA polymerase was activated by heating at 95℃ for 5 min, the PCR reaction proceeded for 30 cycles of 1 min at 94℃ (denaturation), 1 min at 55℃ (annealing), and 1 min at 72℃ (extension).

4.2.6. Cloning and transformation of A_2 cell MPR 46

The PCR product was analyzed on 1% agarose gel electrophoresis. The single band obtained was excised, gel purified using Qiagen PCR Purification kit and subjected to TA cloning into pTZ57R vector (MBI Fermentas). The product was transformed into DH5α competent cells. The positive clones were selected by blue-white selection and the plasmid DNA was isolated by mini kit (Qiagen, India). The size of the insert was confirmed by restriction digestion of the plasmid DNA with EcoR1 and Xba1 enzymes (MBI Fermentas).

4.2.7. DNA sequencing

DNA sequencing was done at Biosereve sequencing Pvt.Ltd. Sequence comparisons were performed with the CLUSTAL W method available online.

4.3. RESULTS

4.3.1. Enzyme assays and immunoblotting of lysosomal enzymes

To detect the lysosomal enzyme activities in A_2 cells, acetate supernatant from the cells was assayed using chromogenic substrates. Of the five lysosomal enzymes assayed viz., arylsulfatase A, α-fucosidase, α-galactosidase, β-hexosaminidase and α-mannosidase, β-hexosaminidase showed very high enzyme activity for the same amount of protein taken (Fig 4.4.1). In order to identify the respective lysosomal enzymes, the soluble proteins were subjected to 10% SDS-PAGE and probed using the respective enzyme antiserum as described in materials and methods. Arylsulfatase A and cathepsin D showed a single band (Fig 4.4.2 A & D), β-hexosaminidase showed a band corresponding to 60 kDa and additional two bands above it which might be a precursor form of the enzyme (Fig 4.4.2B). α-fucosidase showed a major band corresponding to 50 kDa. Two additional bands seen below the α-fucosidase protein in Fig 4.4.2C which could represent a cross-reactive protein or a proteolytic product of the enzyme.

4.3.2. RT-PCR and Sequence Analysis

The quality of the RNA was confirmed by using a Bioanalyzer 2100 (Agilent Technologies) as shown in Fig 4.4.3. The amount was determined with a NanoDrop spectrophotometer (Thermo Scientific). Using the primers mentioned under methods a putative A_2 MPR 46 gene was amplified (Fig 4.4.4A lane 2) which was then cloned into TA cloning vector. The presence of insert was confirmed by double digestion of the construct with EcoR1 and Xba1 (Fig 4.4.4B lane 3), lane 2 shows the undigested A_2 MPR 46 gene into vector. Sequence was confirmed by automated DNA sequencing. Nucleotide and deduced amino acid sequence of the A_2 fish CDMPR is presented in Fig 4.4.5. The deduced amino acid sequence of the proposed MPR 46

revealed that there is a 40% identity between the fish and mammalian CDMPRs (Human, bovine, goat and mouse). Interestingly, multiple sequence alignment of the fish CDMPR with other known mammalian CDMPR (Fig 4.4.6) further reveals that the amino acid residues, Q^{92}, H^{131}, R^{137}, E^{159}, R^{161} corresponding to the human CDMPR, which has been implicated for direct involvement in M6P ligand binding is also conserved. The six cysteine residues involved in disulfide bridge formation in the extracytoplasmic region of the known vertebrate MPR 46 proteins are also conserved in the *Xiphophorous xiphidium* protein. In addition the potential glycosylation sites and transmembrane domain are also partially conserved.

FIGURES 4.4

Figure 4.4.1. Lysosomal enzyme activities from the A_2 cell acetate supernatant using the respective chromogenic substrates: *Para*-nitro phenyl phosphate (*p*NP) derivatives. Aryl: Arylsulfatase A, Fuc: α-Fucosidase, Gal: α-Galactosidase, Hex: β-Hexosaminidase, Man: α-Mannosidase. Assays were done in triplicates in three individual experiments and the results obtained were plotted using Sigma plot 9.0 taking the averages and standard deviation for the error bars.

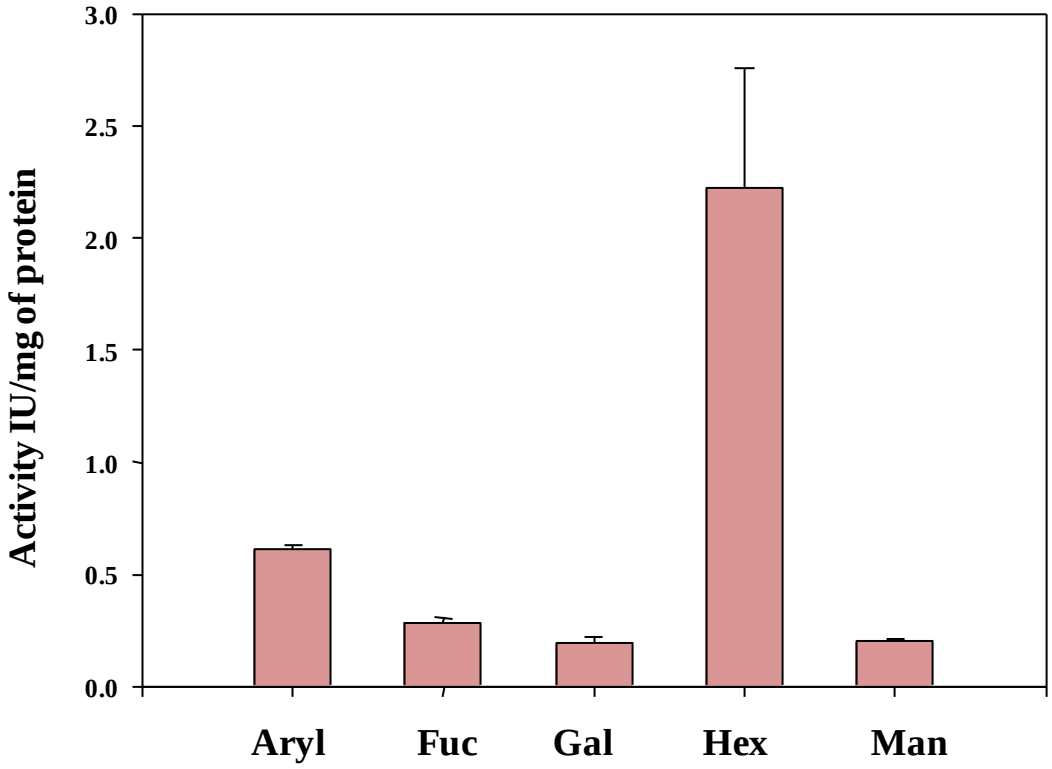

Activity IU/mg of protein
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Aryl
Fuc
Gal
Hex
Man

Figure 4.4.1

Figure 4.4.2. Immunoblotting of lysosomal enzymes from A_2 cells

Proteins were separated on 10% SDS-PAGE and transferred onto PVDF membrane. Antibody probing was done with the respective antibodies and detected by chemiluminiscence. **(A)** Immunoblot with arylsulfatase A antiserum, only one form of the enzyme is observed; **(B)** Immunoblot with β-hexosaminidase antiserum, additional bands might represent precursor form of the enzyme, **(C)** Immunoblot with α-fucosidase antiserum, **(D)** Immunoblot with Cathepsin D antibody, only one form is observed indicated by the arrow. (⟵) indicates the position of the enzyme.

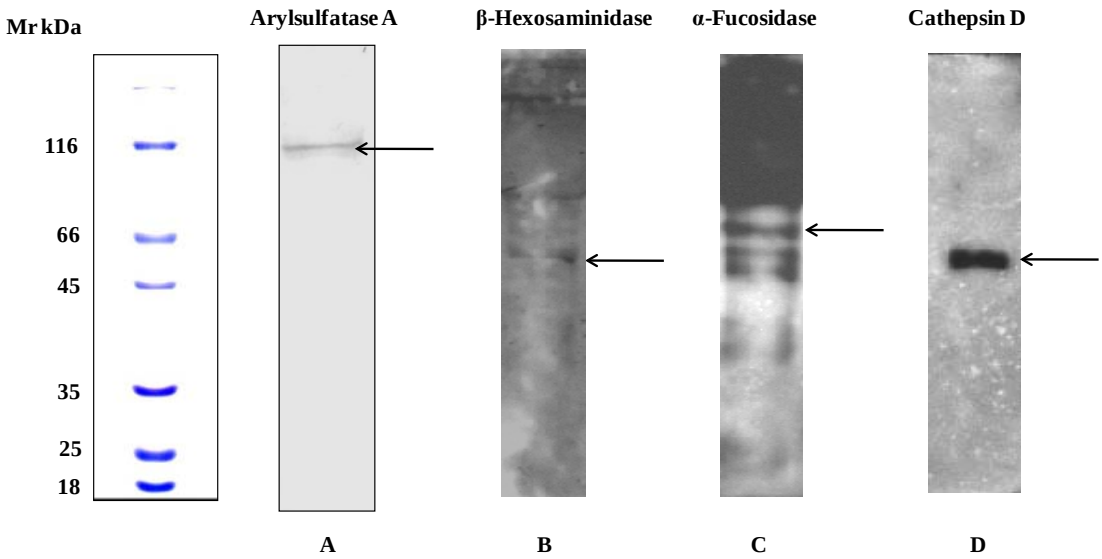

Mr kDa
Arylsulfatase A
β-Hexosaminidase
α-Fucosidase
Cathepsin D
116
66
45
35
25
18
A
B
C
D

Figure 4.4.2

Figure 4.4.3. Workflow of RNA isolation

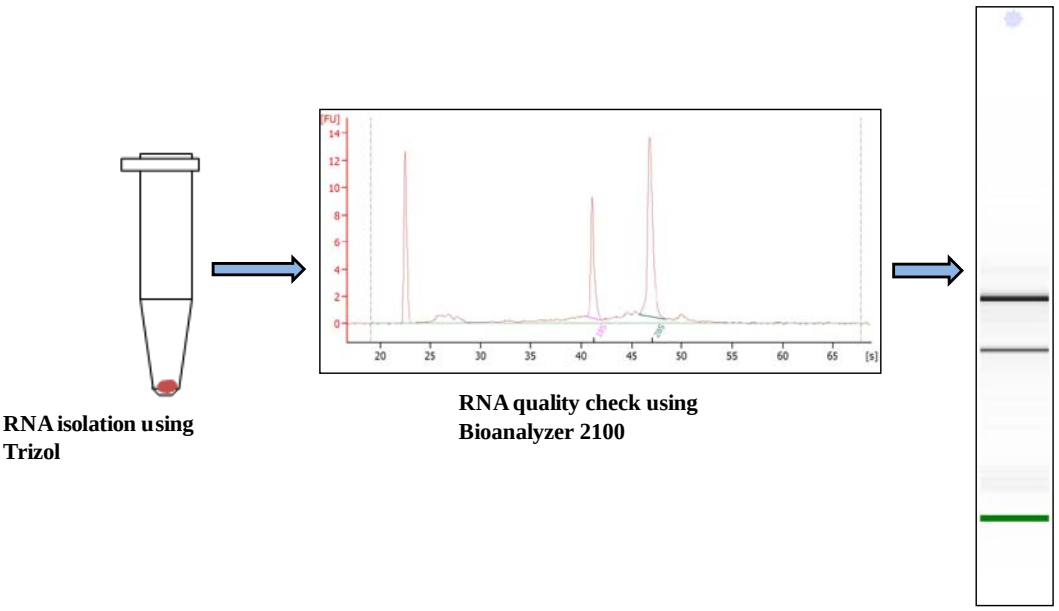

RNA isolation using
Trizol
RNA quality check using
Bioanalyzer 2100

Figure 4.4.3

Figure 4.4.4. Molecular cloning of *Xiphophorus xiphidium* (A_2 cells) MPR 46 (A) PCR amplification of A_2 MPR 46 gene using RT product as the template. Amplified product was subjected to 1% agarose gel electrophoresis. Lane 1, mixed DNA ladder (100 to 10,000 bp), lane 2, amplified product. **(B)** Restriction digestion analysis of the positive clone, lane 1, mixed DNA ladder (100 to 10,000 bp), lane 2, undigested A_2 MPR 46 gene into pTZ57R vector, lane 3, double digested A_2 MPR 46 into pTZ57R vector (with EcoRI and XbaI). (⟵) fragment released from the vector after digestion with EcoR1 and Xba1.

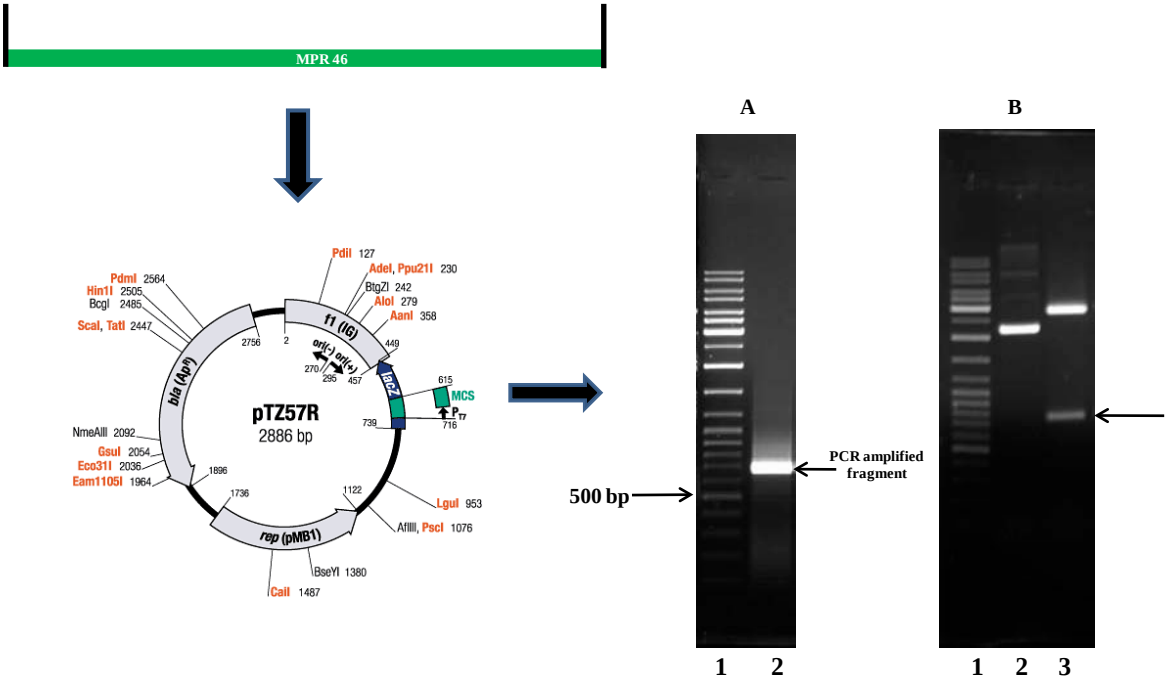

MPR 46
pTZ57R
2886 bp
f1 (IG)
bla (Ap^R)
rep (pMB1)
lacZ
MCS
PdiI 127
AdeI, Ppu21I 230
BtgZI 242
AloI 279
AanI 358
PdmI 2564
Hin1I 2505
BcgI 2485
ScaI, TatI 2447
NmeAIII 2092
GsuI 2054
Eco31I 2036
Eam1105I 1964
LguI 953
AflIII, PscI 1076
BseYI 1380
CaiI 1487
A
B
500 bp
PCR amplified fragment
1 2
1 2 3

Figure 4.4.4

Figure 4.4.5. Nucleotide and deduced amino acid sequence of the *Xiphophorus xiphidium* (A_2 cells) MPR 46 protein.

cDNA sequence

```
AAAACCTGGCTAGCGTTTAACTTAAGCTTGGTACCGAGCTCGGATCCACTAGT
CCAGTGTGGTGGAATTCATGACGGTGCACGGTAGCAGGATGCCATCACATTGC
CATACCTCTGCTGTGCTGGTAGTCTTTATGGCCCTTGCTGCTGGTGTGGGGGC
TGAACCGCTGTCGGAGAAGAGCTGTGATGTGGTTGGTGATGAGAGTACTGAGT
CCCAAATGGAAAAAGCCCTGCTGAAGAAACTAGAACCCCTGAGCCAAATAAGA
TTTAACACGACTGTGGAGATTGGCACAACTGGAAACTATGCCTACCATTTCAG
GGTATGCAGAGAGGTCAACAGCAGCTTGCATGATTTTGCTGGCCTAGTACAAA
TGGATAGGCAGTCTGGAAAGACTACAGTGATAGGAAGAATCAATGAAACCCAG
GTCTTCAATGGAAGTGACTGGATCATGCTGATTTATAAAGGAGGTGATTCATA
TGGTAGGCACTGCAGTGGTGAGAAAAGAAGAGCTGTGATAATGATTTCTTGCA
AGCGAGGAATTACAGCGAGTTCATTCAGCATTATTTCAGAAGAGAGGGAAAAG
GAGCAGACTGTTTCTACCTCTTTGAGATGGACAGCAGTGTGGCTTGTCCAG
```

Deduced Amino acid sequence

```
KPGRLTAWYRARIHSSVVEFMTVHGSRMPSHCHTSAVLVVFMALAAGV
GAEPLSEKSCDVVGDESTESQMEKALLKKLEPLSQIRFNTTVEIGTTG
NYAYHFRVCREVNSSLHDFAGLVQMDRQSGKTTVIGRINETQVFNGSD
WIMLIYKGGDSYGRHCSGEKRRAVIMISCKRGITASSFSIISEEREKE
QTVSTSLRWTAVWLVQX
```

Figure 4.4.5

Figure 4.4.6. Multiple sequence alignment of amino acids of MPR 46 protein from different species. (●) indicates the conserved residues essential for M6P-binding; (♦) indicates residues where mutation resulted in reduced binding; (▼) indicates a potential N -glycosylation site; (**$**) indicate the cysteines paired in disulfide bonds; the underlined sequence represents the transmembrane domain; (*) marks identical amino acid residues; (:) related amino acid residues; (·) predominantly the same amino acid residue. (BOS, bovine; GOT, Goat; HOM, Human; MUS, Mouse; XIP, *Xiphophorus*).

Zebrafish MPR 46 functions in lysosomal enzyme targeting. In killifish MPR 46 ligand binding region is conserved. In fugu fish the R^{137} residue in the ligand binding region is replaced by Ser^{137}.


CLUSTAL 2.1 multiple sequence alignment
BOS46 ----------MMSPLHSSWRTGLLLLLLFSVAVRESWQTE-EKTCDL
GOT46 ----------MSPLHSSWRTGLLLLLLFSMAVRESWQTE-EKTCDL
HOM46 ----------MFPFYSCWRTG-LLLLLLAVAVRESWQTE-EKTCDL
MUS46 ----------MFPFSGCWRTELLLLLLLAVAVRESWQIE-EKSCDL
XIP46 KPGRLTAWYRARIHSSVVEFMTVHGSRMPSHCHTSAVLVVFMALAAGVGAEPLSEKSCDV
BOS46 VGEKGKESEKELALLKRLTPLFNKSFESTVGQSPD-MYSYVFRVCREAGNHSSG-AGLVQ
GOT46 VGEKGKESEKELALLKRLTPLFNKSFESTVGQSPD-MYSYVFRVCREAGNHSSG-AGLVQ
HOM46 VGEKGKESEKELALVKRLKPLFNKSFESTVGQGSD-TYIYIFRVCREAGNHTSG-AGLVQ
MUS46 VGEKDKESKNEVALLERLRPLFNKSFESTVGQGSD-TYSYIFRVCREASNHSSG-AGLVQ
XIP46 VGDESTESQMEKALLKKLEPLSQIRFNTTVEIGTTGNYAYHFRVCREVNSSLHDFAGLVQ
BOS46 INKSNGKETVVGRFNETQIFNGSNWIMLIYKGGDEYDNHCGREQRRAVVMISCNRHTLAD
GOT46 INKSNGKETVVGRFNETQIFNGSNW-MLIYKGGDEYDNHCGREQRRAVVMISCNRHTLAD
HOM46 INKSNGKETVVGRLNETHIFNGSNWIMLIYKGGDEYDNHCGKEQRRAVVMISCNRHTLAD
MUS46 INKSNDKETVVGRINETHIFNGSNWIMLIYKGGDEYDNHCGKEQRRAVVMISCNRHTLAA
XIP46 MDRQSGKTTVIGRINETQVFNGSDWIMLIYKGGDSYGRHCSGEKRRAVIMISCKRGITAS
BOS46 NFNPVSEERGKVQDCFYLFEMDSSLACSPEISHLSVGSILLVTLASLVAVYIIGGFLYQR
GOT46 NFNPVSEERGKVQDCFYLFEMDSSLACSPEISHLSVGSILLVTFASLVAVYIIGGFLYQR
HOM46 NFNPVSEERGKVQDCFYLFEMDSSLACSPEISHLSVGSILLVTFASLVAVYVVGGFLYQR
MUS46 NFNPVSEERGKVQDCFYLFEMDSSLACSPEVSHLSVGSILLVIFASLVAVYIIGGFLYQR
XIP46 SFSIISEEREKEQ------TVSTSLRWT-------------------AVWLVQX-----

Figure 4.4.6

4.5. DISCUSSION

In the present study, lysosomal enzyme activities have been identified in A_2 cells by enzyme assays and the same were quantified. Although different lysosomal enzyme activities could be identified in the soluble extracts, their relative concentrations seemed different. Due to the limited availability of antibodies in our laboratory, we concentrated only on four lysosomal enzymes *viz.,* arylsulfatase A, α-fucosidase, β-hexosaminidase and cathepsin D. The authenticity of the isolated enzymes was confirmed by their specific immuno-reactivity using the available antibodies in the laboratory as described under methods. These initial results suggest there might be some antigenic similarities among the enzymes from different species. In the vertebrate species tested, it is already known that these enzymes are specifically transported to lysosomes by the two distinct but homologous receptors MPR 300 and 46 which have also been identified in the fish in our laboratory.

The presence of both the receptors with the biochemical properties similar to the mammalian proteins has been established by earlier work using *Xiphophorus xiphidium* A_2 cells (Siva Kumar *et al*., 1999). Subsequent studies on the partial sequence of the A_2 cell MPR 300 protein revealed high degree of structural homology of the A_2 cell protein to that of the other vertebrate MPR 300 proteins. The study also confirmed the presence of repetitive cassette structures in the extracytoplasmic domain and the 3rd domain containing the critical arginine residue for ligand binding (Udaya lakshmi *et al*., 2000). Preliminary study was carried out in our laboratory to obtain amino acid sequence for the MPR 46 protein from A_2 cells (Raju, 2004). To obtain more relevant information on the A_2 cell MPR 46 protein in particular its structural similarity to other vertebrate and fish receptor sequences known, in the

present study a cDNA clone was isolated for the A_2 cell MPR 46 protein and was characterized. This was essentially carried out to also compare the sequences of the A_2 cell MPR 46 protein to the other known vertebrate MPR 46 sequences.

From the available literature information and our present study on the A_2 cell receptor, it is logical to conclude that the early vertebrate fish has distinct lysosomal enzymes as well as their targeting receptors and the mechanism of binding the enzymes to receptors seems to be similar from fish to mammals as the functional domains of the receptors are highly conserved across the species. These results support that different fish species have homologous MPR 46 proteins which might have similar functions as the well studied Zebra fish protein. It has been seen in the preceding chapters, the lysosomal enzymes and their receptors seem to be conserved also among the invertebrates and our results so far conclusively establish their conserved nature among the starfish, the highly evolved invertebrate.

CHAPTER 5

Characterization of IGF-II binding property of MPR 300 from early vertebrate fish and highly evolved invertebrate starfish

5.1. INTRODUCTION

It is well established that the mammalian MPR 300 is a multifunctional receptor with high affinity binding site for mannose 6-phosphate in domain 3 and 9 (Westlund *et al.*, 1991; Dahms *et al.*, 1993) and an additional high affinity binding site for human IGF-II in domain 11 (Morgan *et al.*, 1987). Binding and endocytosis of IGF-II by MPR 300 contributes to the regulation of embryonic growth and development in mice. A critical isoleucine residue located at position 1572 in the 11^{th} domain of several mammalian CIMPR proteins has been suggested to be important for IGF-II binding. In contrast, cloning and sequencing of chicken MPR 300 (Zhou *et al*., 1995) revealed there is a leucine residue in domain 11 instead of isoleucine found in mammalian proteins. Chicken and *Xenopus* MPR 300 showed weak binding to IGF-II (Clairmont *et al.*, 1989; Canfield *et al.*, 1989). Therefore it was proposed that the acquisition of an IGF-II binding site by the CIMPR is a late event in evolution. Subsequent studies showed that the chicken liver MPR 300 as well as the chicken embryonic fibroblast MPR 300 and reptilian MPR 300 can bind human IGF-II (Suresh *et al*., 2006; Sivaramakrishna *et al*., 2009). There has been one report that the fish embryo MPR 300 binds human IGF-II although there has been no sequence information for the IGF-II binding domain (Mendez *et al*., 2001). Since M6P binding domains of the different vertebrate MPR 300 proteins are characterized and showed that they are evolutionarily conserved from fish to mammals, also the chicken and reptilian MPR 300 bind human IGF-II, it became necessary to further characterize the IGF-II binding domain in different fish species to establish the evolutionary conservation of the IGF-II binding among the vertebrates. The first report that the *Xiphophorus xiphidium* A_2 cell MPR 300 protein (whose partial amino acid sequence has

been determined from a cDNA clone) revealed the conserved M6P binding region in the third domain (Udaya lakshmi *et al*., 2000). In a later independent study we also obtained different clones for the fugu fish MPR 300 protein. Sequence analysis of these clones revealed that the fugu MPR 300 protein is also made of 15 repetitive cassette structures in the extracellular domain and the M6P ligand binding region has been confirmed to domain 3 and 9. Careful examination of the sequences in domain 11 suggested that in place of isoleucine seen in mammalian proteins that is responsible for IGF-II binding fugu fish has a threonine residue. It is also not known yet whether or not the fugu fish MPR 300 binds human IGF-II (Raju, 2004). In Zebrafish MPR 300 the M6P binding regions in domain 3 and 9 are highly conserved as in other vertebrates. Interestingly in the 11^{th} domain there is an isoleucine residue at the position as seen in mammals. Whether or not the Zebrafish MPR 300 binds human IGF-II is still not established (Catherine *et al*., 2006).

In our efforts to identify and characterize the lysosomal biogenesis pathways in the early vertebrate fish and the invertebrate starfish, in the preceding chapters we have extensively characterized two lysosomal enzymes from starfish and showed their specific interaction with the MPR 300 protein (Merino and Siva Kumar, 2012; Merino *et al*., 2012). To understand more about the IGF-II binding properties of different fish species as well as of the invertebrate starfish MPR 300 protein, we undertook the present study:

(i) Sequencing of the 11^{th} domain of *Xiphophorus* A_2 cells using total RNA and make a structural comparison of the sequences of the available IGF-II domains in the vertebrates.

(ii) To analyze the interaction between the human IGF-II and CIMPR from the striped snakehead fish and A_2 fish cell lysate by ligand blotting.

(iii) Analyze the affinity binding of starfish CIMPR to human IGF-II affigel.

(iv) To determine dissociation constant (Kd) for the binding of IGF-II.

5.2. MATERIALS AND METHODS

5.2.1. Materials

O-phosphonomannan was a generous gift from Dr.M.E.Slodki, USDA, Peoria, IL, USA. Mannose 6-phosphate (M6P), bovine serum albumin, Triton X-100, human IGF-II, DSS (disuccinamidyl suberate) was purchased from Sigma. Biotinylated IGF-II and antibody to human IGF-II were procured from IBT, Reutlingen, Germany. Fresh fish liver tissue was collected from the local market and carried on ice to the laboratory and used to purify the MPR 300 protein. Affinity purified antibody to the goat MPR 300 protein was obtained as described earlier (Suresh K *et al*., 2002). All other reagents and chemicals used in the study were of high quality and were purchased from reputed firms locally.

5.2.2. Isolation of total RNA from Xiphophorous xiphidium (A_2) cells

Total RNA was isolated as described in section 4.2.4.

5.2.3. ReverseTranscriptase - Polymarase Chain Reaction

From the RNA obtained above cDNA was synthesized using 0.5 ng of oligo (dT) primer and 200 units of Reverse transcriptase enzyme in a total volume of 20 µL (according to the manufacturer's protocols; MBI Fermentas, India). The cDNA synthesized was used as template for amplification of the putative CIMPR/IGF-II binding region (11^{th} domain) using the following primers. Sense primer (5'-GC*GAATTCACCCAGCAGGACGA-3') and antisense primer (5'-AC*TCTAGATTTGCAGTCTGTTCGCAGAG-3') flanking *EcoR1 and Xba1 restriction sites. The A_2 11^{th} domain was amplified by PCR using the following conditions (95℃x5', 94℃x1',53℃x30",72℃x45", for 30 cycles). After

electrophoresis and ethidium bromide staining, the 441 bp fragment was excised from the agarose gel and purified using Qiagen PCR DNA Purification Kit. The sequence and reading frame was verified using automated DNA sequencing (Biosereve sequencing Pvt. Ltd, India). The membrane proteins (as described in section 4.2.2.) was subjected to ligand blot analysis. Additionally, the MPR 300 protein from the membrane extracts (obtained as described under section 4.2.2.) of A_2 cells was detected using a ligand blot assay (monobiotinylated human IGF-II).

5.2.4. Purification of MPR 300 from Channa striata (Local name: Korra-matta)

Fresh liver tissue was washed with PBS and freed from connective tissue and fats. The total membrane extraction and purification of MPR 300 was carried as described in section 5.2.7. Protein concentrations were determined using Bradford method according to the manufacturer's instructions. PM gel eluate were analyzed by 7.5% SDS-PAGE under reducing conditions according to Laemmli, 1970 and the protein bands detected by silver staining. Purified goat liver MPR 300 served as a positive control.

5.2.5. Binding of biotinylated IGF-II to the purified fish MPR 300

Ligand blot analysis for fish CIMPR 300 and biotinylated IGF-II was done as described (Mendez *et al*., 2001). About 1 µg of purified fish liver receptor (MPR 300) as well as the purified goat liver MPR 300 protein were electrophoresed on 7.5% SDS-PAGE. The proteins were transferred on to a PVDF membrane overnight at 4℃ and the membrane was blocked for 1 h at room temperature in PBS containing 0.05% Tween 20 (PBST) and 1% BSA and incubated overnight at 4℃ with human monobiotinylated human IGF-II (20 ng/mL) in PBST containing 1% BSA. The membrane was washed

consecutively with PBST (4x5 min) and incubated with streptavidin coupled with horseradish peroxidase (HRP) for 1 hour at room temperature. Finally, the membrane was washed consecutively with PBST (4x5 min). The protein band was developed using the Super Signal West Femto Maximum Sensitivity Substrate.

5.2.6. Preparation of the total membrane extracts from starfish

The whole animal tissue was used to prepare the acetone powder as described earlier (Siva Kumar *et al*., 1997). All operations were carried out at 4℃. 50 g of the acetone powder prepared was homogenized with 300 mL of 50 mM imidazole buffer pH 7.0, 150 mM sodium chloride containing 0.1 mM PMSF and the homogenate stirred overnight. The suspension was clarified by centrifugation at 13583 x g for 15 minutes, and the supernatant discarded. The pellet was homogenized with 300 mL of 50 mM sodium acetate buffer pH 5.0, containing 150 mM sodium chloride, and centrifuged as described above. The pellet obtained was finally homogenized with 300 mL of 50 mM imidazole-HCl buffer pH 7.0, containing 5 mM sodium β-glycerophosphate, 150 mM sodium chloride. To this Triton X-100 and sodium deoxycholate were added to a final concentration of 1 and 0.1 % respectively and the suspension stirred overnight. This was then centrifuged at 670 x g for 15 minutes, and the supernatant was recentrifuged at 32869 x g for 30 minutes. The clear membrane extract was used as the source of the receptors.

5.2.7. Affinity chromatography on phosphomannan - Sepharose gel

Phosphomannan–Sepharose (PM) gel was prepared as described earlier (Siva Kumar *et al*., 1997). To purify MPR 300, EDTA was added to the membrane extract to a final concentration of 2 mM, and applied on a PM gel at 4℃ equilibrated with column

buffer (50 mM imidazole buffer (pH 7.0), containing 5 mM sodium β-glycerophosphate, 150 mM sodium chloride, 0.05 % Triton X-100, 2 mM EDTA (EDTA buffer). The membrane extracts were passed through the column at a flow rate of 30 mL/h, the gel was washed extensively with the same buffer. The bound protein was eluted using 5 mM mannose 6-phosphate in column buffer. Aliquots were further analyzed by SDS-PAGE as described below. The MPR 300 protein obtained was used in all subsequent studies.

5.2.8. SDS-PAGE and Western blot analysis

Starfish mannose 6-phosphate eluate was pooled and concentrated by acetone precipitation and analysed by SDS-PAGE under non-reducing and reducing conditions as described (Laemmli, 1970). Gels were stained using the colloidal coomassie blue. For immunological detection of MPR proteins, aliquots of the purified proteins were electrophoresed, and the proteins transferred to PVDF membrane as described (Towbin *et al*., 1979). The membrane was placed in blocking solution containing 5 % non-fat milk powder in phosphate buffer pH 7.4, containing 0.15 M NaCl and 0.05% Tween 20 (PBST). 10 μg of affinity purified goat MPR 300 IgG was used as the primary antibody. The protein band was detected by incubating the membrane with secondary antibody goat-anti rabbit HRP conjugate (Pierce, Rockford, USA) and developed using the Super Signal West Femto Maximum Sensitivity Substrate (Thermo Scientific, Rockford, IL, USA). The bands visualized and photographed. Purified goat liver MPR 300 was used as a positive control.

5.2.9. Ligand blot assay

This was carried out as described above (section 5.2.5.)

5.2.10. Chromatography on IGF-II Affinity gel

30 µg of human IGF-II in 0.1M HEPES buffer, pH 7.0 was coupled to 0.5 mL Affigel-10 (Bio-Rad) following manufacturer's instructions. The gel was washed with 1 mM sodium-phosphate buffer, pH 7.4, containing 150 mM NaCl (PBS) and stored at 4℃. The gel was equilibrated with EDTA buffer. The purified starfish MPR 300 protein obtained by affinity chromatography as described in section 5.2.7. was dialyzed against EDTA buffer and applied onto the receptor-affigel column. The unbound fractions were collected. The gel was washed with four gel volumes of EDTA buffer and eluted in three fractions each 200 µL with 50 mM sodium acetate buffer, pH 5.0. Protein in each of the three eluates was completely precipitated with TCA (final 10% concentration). The pellets collected by centrifugation were neutralized with 0.4 M Tris and analyzed by 7.5% SDS-PAGE. Protein bands were visualized by staining with colloidal coomassie blue.

5.2.11. Binding of biotinylated IGF-II

A novel ELISA based immunoassay (Heuer *et al*., 1996) was used to measure the binding affinity of CIMPR protein to HIGF-II. ELISA assays were performed with purified CIMPR directly in Nunc MaxiSorp 96-well polystyrene sterile ELISA plates. Purified CIMPR (100 ng), in 100 µL of interaction buffer (20 mM Tris pH 8.3, 150 mM NaCl, 0.2% Tween 20), was adsorbed to the wells of an ELISA plate by overnight incubation at 4℃. Subsequently, the wells were washed thrice with interaction buffer. The wells were then blocked by incubation with the blocking buffer (interaction buffer containing 5% bovine serum albumin) for 1h. Then the blocking buffer was removed, and the wells were added with 5-Fold serially diluted biotinylated HIGF-II protein in 100 µL

of interaction buffer plus 0.1% bovine serum albumin and proteins were allowed to interact with plate-bound purified starfish CIMPR for 1 h at room temperature. Following the interaction period, unbound IGF-II protein was removed, and the well were washed with interaction buffer. Finally the detecting reagent, streptavidin coupled with horseradish peroxidase (HRP) (Pierce, Rockford, USA), was added to the wells for 1 h, and unbound HRP-conjugated streptavidin was removed, by washing the wells with wash buffer (PBS with 0.1% BSA), and the receptor-ligand complex in the wells was detected using the chromogenic substrate TMB/H_20_2. The colour development was stopped after 15 min by adding 1 N HCl, and the absorbance was measured at 450 nm using a Bio-Rad model 450 micro plate reader.

5.3. RESULTS

5.3.1. RT-PCR and Polymerase Chain Reaction

Total RNA isolated from A_2 cells was amplified using an RT-PCR approach as described under *materials and methods*, to give a 441 bp fragment (Fig 5.4.1A lane2) that was purified, and sequenced. Fig 5.4.2 shows the comparison of IGF-II binding sequences of some known vertebrate receptors. The deduced amino acid sequence of the proposed IGF-II binding domain residues in *Xiphophorous xiphidium*, revealed that there is a 40 % identity between the A_2 fish and mammalian CIMPRs (Human, bovine, rat and mouse), 36% identity with chicken receptors and 80-90% identity to other fish species. Interestingly the Ile^{1572} of the human CIMPR, which has been implicated for direct involvement in ligand binding, is replaced by Thr in A_2 fish. Recent study by Oliver *et al*., 2006 indicates that there are other regions in 11^{th} domain of the mammalian CIMPR that also contribute to the binding of the IGF-II. We then separated the A_2 cell fish lysate in SDS-PAGE and a ligand blot analysis was done using biotinylated human IGF-II. Fig 5.4.1B showed that the receptor recognizes the human IGF-II and hence the fish receptor can be classed as CIMPR/IGF-IIR, like the vertebrate protein.

5.3.2. Fish MPR 300 purification and ligand blot analysis

MPR 300 from the fish liver membrane extracts was affinity purified using phosphomannan-Sepharose gel. The bound protein was eluted from the gel with 5 mM mannose 6-phosphate, concentrated by acetone precipitation. In SDS-PAGE a single band at 300 kDa was observed corresponding to molecular mass similar to the purified MPR 300 from goat liver tissue as shown in Fig 5.4.3A lane 1 represents molecular weight marker, lane 2 and 3 represents purified goat liver MPR 300 and fish liver MPR

300 respectively. These purified receptors were used in ligand blot analysis. Fig 5.4.3B is purified fish MPR 300 (lane 5), lane 4 represents purified goat liver MPR 300 (positive control). The ability of the purified fish receptor to bind human IGF-II in a ligand blot analysis revealed that indeed the receptor binds human IGF-II. Similar results were obtained with the fish embryo receptor (Mendez *et al*., 2001).

5.3.3. *Affinity purification of starfish MPR 300*

The membrane extracts from starfish whole animal tissue and goat liver were subjected to affinity chromatography on PM-Sepharose gel in the presence of 2 mM EDTA for the purification of MPR 300 alone. After washing the gel extensively, the bound protein was eluted in the column buffer supplemented with 5 mM mannose 6-phosphate. An aliquot of the eluted fractions were electrophoresed on 7.5 % SDS-PAGE. In Fig 5.4.4A lane 2 is goat liver MPR 300 and lanes 3 & 4 are starfish MPR 300 from whole tissue and from gonads respectively. Consistent with our earlier findings (Siva Kumar *et al*., 1996) in all the species (mammalian, non-mammalian and in highly evolved invertebrates) the purified MPR 300 migrated as a single protein band corresponding to molecular mass of about 300 kDa. In addition to this the apparent molecular mass of the non-reduced MPR 300 from both the species (goat and starfish) was nearly identical. The protein was separated on a 7.5 % SDS-PAGE under reducing (+DTT) and non-reducing (-DTT) conditions where the protein showed a typical decrease in the electrophoretic mobility in the presence of DTT due to the opening of the disulfide bridges as observed for the goat receptor (Fig 5.4.4B). The glycoprotein nature of the purified receptor was established by periodic acid Schiff's staining (Fig 5.4.4C).

Western blot analysis was performed for the starfish purified CIMPR as described under materials and methods. Affinity purified goat MPR 300 antibody can specifically detect starfish MPR 300 protein and goat MPR 300 protein (positive control) as shown in Fig 5.4.4D. It is evident that the antibody to goat MPR 300 protein shows immuno-reactivity with purified starfish receptor.

5.3.4. Ligand blot assay of purified starfish MPR 300

Specific interaction of the receptor to IGF-II was analyzed in a ligand blot assay. The CIMPR was separated on a 7.5 % SDS-PAGE and transferred to PVDF membrane. The membrane was probed with biotinylated human and chicken IGF-II (both these are structurally related) followed by incubation with strepatavidin conjugated HRP, washed and developed with ECL reagent. Protein bands were visualized showing a molecular mass of 300 kDa. From Fig 5.4.5A it is apparent that the band represents purified goat (control, lane 1) and starfish MPR 300 showed binding to IGF-II (lane 2). In addition the starfish receptor also binds chicken IGF-II (Fig 5.4.5B). This suggests that there is an interaction between the receptor and the IGF-II. The specific interaction of starfish MPR 300 with cold IGF-II followed by incubation with an antibody to the IGF-II also confirms its interaction as shown in Fig 5.4.5C. Arrow indicates the position of the receptor recognized.

5.3.5. Chromatography on IGF-II Affinity gel

In order to gain further insight into the specificity of the interaction between the purified starfish MPR 300 protein and IGF-II, we analyzed the binding of starfish MPR 300 protein and IGF-II immobilized to Affigel-10. At pH 7.0, (that favors binding) the

purified receptor bound strongly to the ligand-affigel. The bound protein could be completely desorbed from the gel using 50 mM sodium acetate buffer, pH 5.0 (that favors dissociation) as shown in Fig 5.4.6 lane 3, lane 1 standard marker and lane 2 is the unbound fraction.

5.3.6. Determination of Kd value for IGF-II

To determine the overall binding affinity of CIMPR to IGF-II, an ELISA-based immunoassay (Heuer *et al*., 1996) was used, where purified receptor was coated onto the wells of ELISA plates and allowed to interact with different concentrations of biotinylated human IGF-II (5-60 nM). After extensive washing, the amount of IGF-II bound to CIMPR was assessed using strepatavidin conjugated HRP. Bound HRP was detected and quantitated using the chromogenic substrate TMB (where colour development was directly proportional to IGF-II bound to CIMPR/IGF-IIR and was used to generate the binding curve (Fig 5.4.7A). Kd values for IGF-II and binding to CIMPR were derived by Scatchard plot analysis of the binding curves (Fig 5.4.7B). The calculated Kd value for IGF-II binding to CIMPR was ~ 8.5 nM (moderate affinity compare to mammals)

FIGURES 5.4

Figure 5.4.1. Polymarase Chain Reaction and ligand blot

(A). 1% Agarose gel electrophoresis of PCR amplified fragments (details given under materials and methods). Lane 1, standard DNA ladder (100 to 10,000 bp), Lane 2, PCR amplified fragment (441bp). **(B). Ligand blot analysis of the cell lysate from A_2 cells.** The arrow indicates the position of the MPR 300 protein.

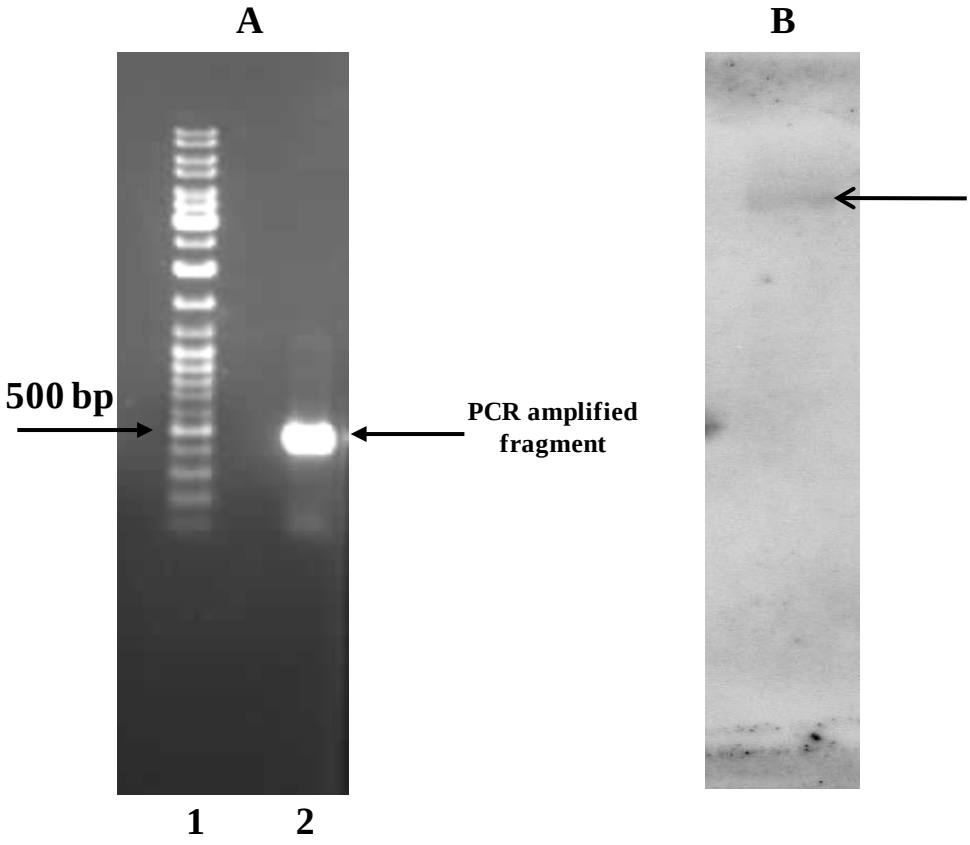


Figure 5.4.1

Figure 5.4.2. Multiple sequence alignment of amino acid sequences of the 11th domain of CIMPR from different animal species. ($) indicates the conserved IGF-II ligand binding residues; (★) indicates the N-glycosylation site; (*) marks identical amino acid residues; (:) related amino acid residues; (.) Predominantly the same amino acid residues; residues in blue colour indicates **AB loop**; in green color indicates **CD loop** and in pink indicates **FG loop** as indicated in the figure; Mus (*Mus musculus*), Rat (*Rattus norvegicus*), Hom (*Homo sapiens*), Bov (*Bos taurus*), ES (Elephant shark), XIP (*Xiphophorous xiphidium)*

Dr. Byrappa Venkatesh, ICMB, Singapore provided the elephant shark fish cDNA.

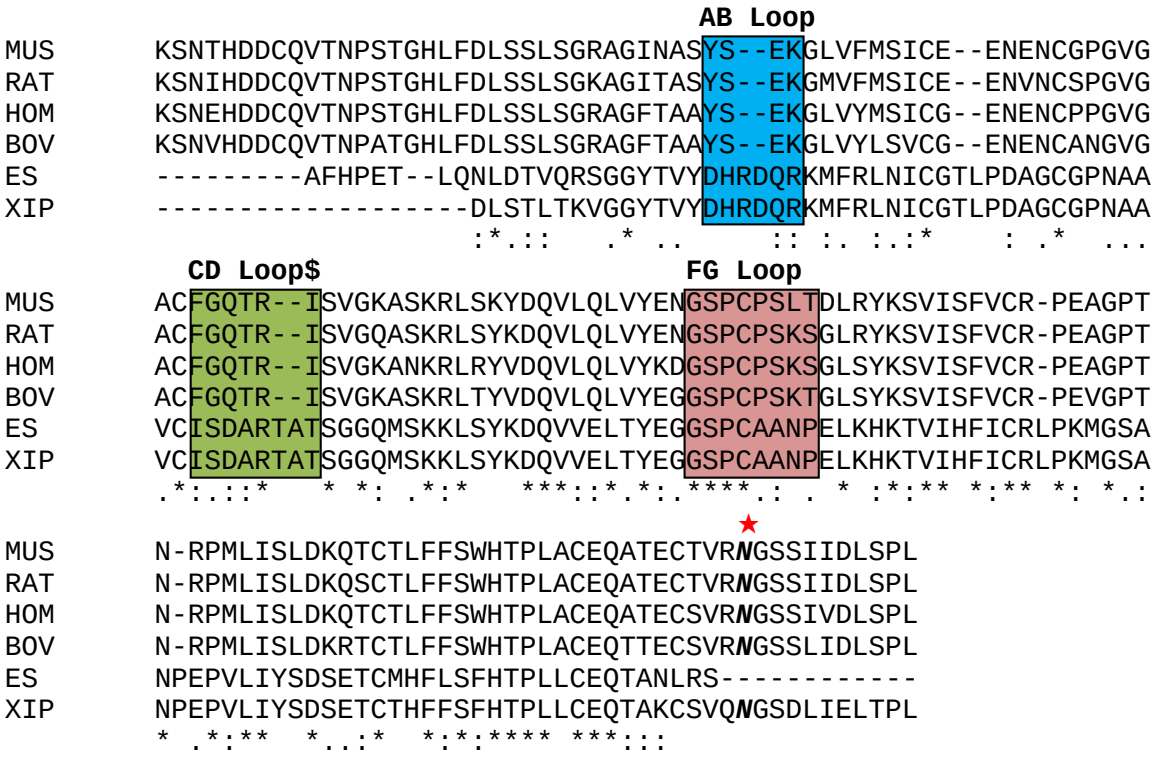

CLUSTAL 2.1 multiple sequence alignment
AB Loop
CD Loop$
FG Loop
MUS KSNTHDDCQVTNPSTGHLFDLSSLSGRAGINASYS--EKGLVFMSICE--ENENCGPGVG
RAT KSNIHDDCQVTNPSTGHLFDLSSLSGKAGITASYS--EKGMVFMSICE--ENVNCSPGVG
HOM KSNEHDDCQVTNPSTGHLFDLSSLSGRAGFTAAYS--EKGLVYMSICG--ENENCPPGVG
BOV KSNVHDDCQVTNPATGHLFDLSSLSGRAGFTAAYS--EKGLVYLSVCG--ENENCANGVG
ES ---------AFHPET--LQNLDTVQRSGGYTVYDHRDQRKMFRLNICGTLPDAGCGPNAA
XIP ------------------DLSTLTKVGGYTVYDHRDQRKMFRLNICGTLPDAGCGPNAA
MUS ACFGQTR--ISVGKASKRLSKYDQVLQLVYENGSPCPSLTDLRYKSVISFVCR-PEAGPT
RAT ACFGQTR--ISVGQASKRLSYKDQVLQLVYENGSPCPSKSGLRYKSVISFVCR-PEAGPT
HOM ACFGQTR--ISVGKANKRLRYVDQVLQLVYKDGSPCPSKSGLSYKSVISFVCR-PEAGPT
BOV ACFGQTR--ISVGKASKRLTYVDQVLQLVYEGGSPCPSKTGLSYKSVISFVCR-PEVGPT
ES VCISDARTATSGGQMSKKLSYKDQVVELTYEGGSPCAANPELKHKTVIHFICRLPKMGSA
XIP VCISDARTATSGGQMSKKLSYKDQVVELTYEGGSPCAANPELKHKTVIHFICRLPKMGSA
MUS N-RPMLISLDKQTCTLFFSWHTPLACEQATECTVRNGSSIIDLSPL
RAT N-RPMLISLDKQSCTLFFSWHTPLACEQATECTVRNGSSIIDLSPL
HOM N-RPMLISLDKQTCTLFFSWHTPLACEQATECSVRNGSSIVDLSPL
BOV N-RPMLISLDKRTCTLFFSWHTPLACEQTTECSVRNGSSLIDLSPL
ES NPEPVLIYSDSETCMHFLSFHTPLLCEQTANLRS-----------
XIP NPEPVLIYSDSETCTHFFSFHTPLLCEQTAKCSVQNGSDLIELTPL

Figure 5.4.2

Figure 5.4.3. Affinity chromatography on purification of *Channa striata* MPR 300 protein on PM gel and ligand blot analysis. (A). Proteins were separated on 7.5% SDS-PAGE and detection of proteins by staining with silver nitrate. Lane 1 Molecular weight markers, lane 2 goat MPR 300 and lane 3 fish MPR 300. **(B)**. Purified proteins were separated on 7.5% SDS-PAGE, transferred to PVDF membrane and incubated with biotinylated human IGF-II. Lane 4 goat MPR 300 (positive control) and lane 5 fish MPR 300. The arrow indicates the position of the MPR 300 protein.

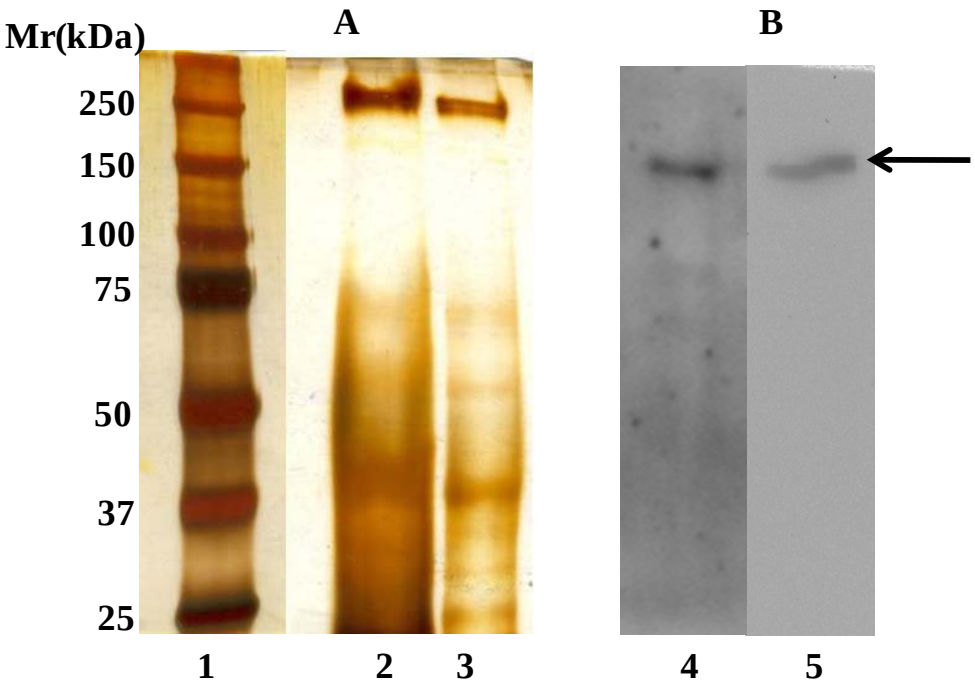


Figure 5.4.3

Figure 5.4.4. SDS-PAGE analysis. **(A)** 7.5% SDS-PAGE analysis of the purified starfish MPR 300 protein on PM Sepharose at pH 7.0. eluted with 5 mM mannose 6-phosphate. Lane 1, Molecular weight marker, lane 2 goat MPR 300 (positive control), lanes 3 and 4 represent starfish MPR 300 proteins from whole tissue and from gonads respectively and visualized by colloidal coomassie blue staining. **(B)** Apparent molecular mass of MPR 300 protein from goat (lanes 1 and 2) and starfish (lanes 3 and 4). Under non-reducing conditions (lanes 1 and 3) and under reducing conditions (lanes 2 and 4) by DTT. **(C)** Periodic acid schiffs (PAS) staining of the starfish MPR 300 protein band. **(D)** Western blot analysis of starfish MPR 300 protein (7.5% SDS-PAGE under reduced conditions). Lane 1 represent purified goat MPR 300 protein (positive control), lanes 2 starfish MPR 300 from whole tissue, detected using affinity purified goat MPR 300 antibody. The arrow indicates the position of the MPR 300 protein.

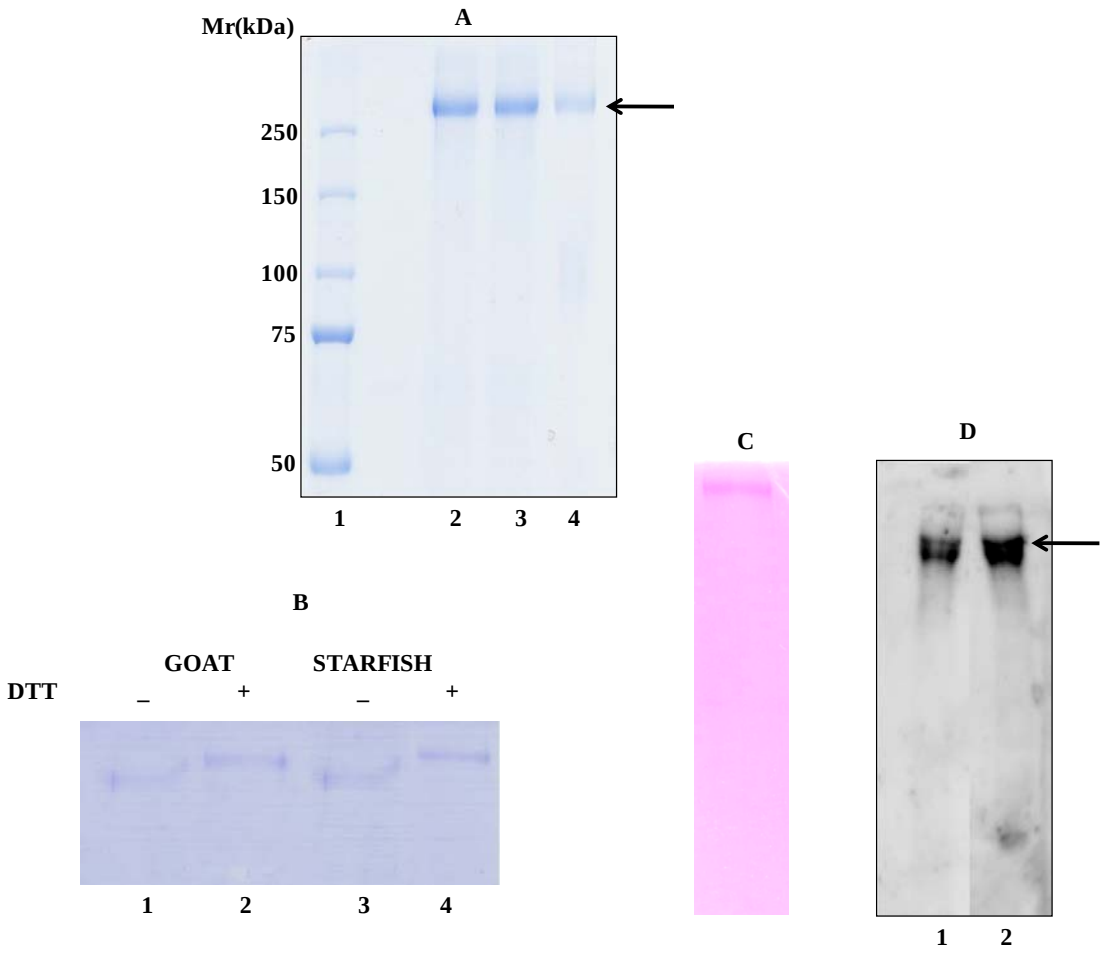


Figure 5.4.4

Figure 5.4.5. Interaction of purified MPR 300 proteins with biotinylated human IGF-II. Purified receptor after separation by SDS-PAGE was transferred to a PVDF membrane and incubated with 20 ng/ mL of biotinylated HIGF-II, followed by incubation with streptavidin-HRP conjugate and developed using ECL reagents. **(A)** lane 1, goat liver receptor (control), lane 2, starfish receptor. **(B)** Starfish MPR 300 also shows binding to biotinylated chicken IGF-II. **(C)** Ligand blot of starfish MPR 300 with cold HIGF-II, followed by incubation with antibody to HIGF-II. Arrow indicates the position of the MPR 300 protein.

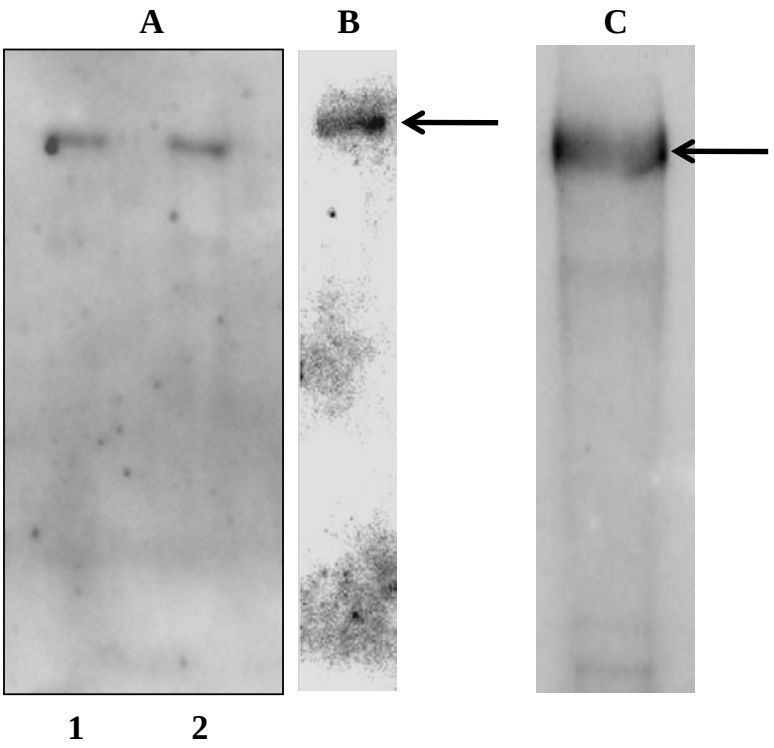


Figure 5.4.5

Figure 5.4.6. 7.5% SDS-PAGE analysis of the binding starfish MPR 300 on IGF-II coupled Affigel-10. Lane 1: Standard protein marker, Lane 2: unbound fraction, Lane 3: elution of the bound receptor on the IGF-II affigel with 50 mM sodium acetate buffer pH.5.0

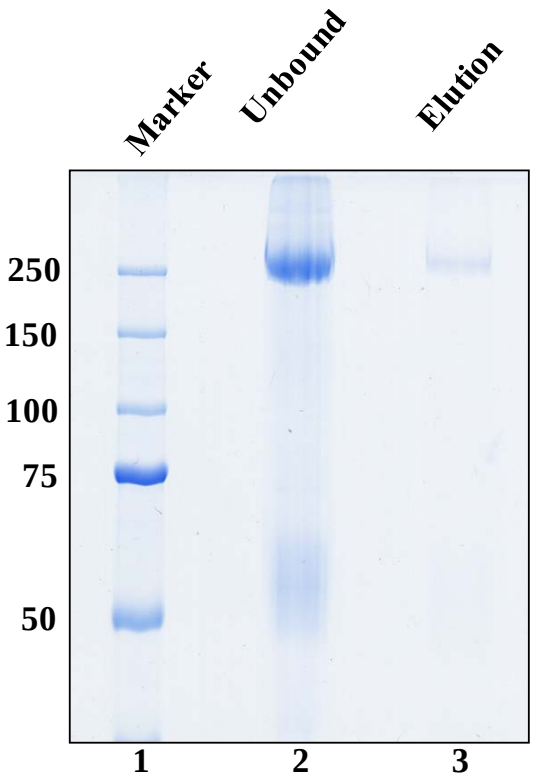
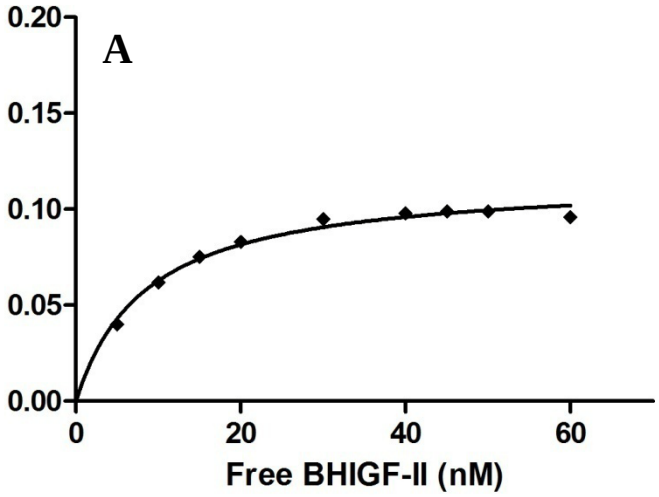


Figure 5.4.6

Figure 5.4.7. Determination of dissociation constants (Kd) for CIMPR interaction with HIGF-II. (A) Binding curves were obtained for CIMPR interaction with HIGF-II using the ELISA binding assay as described under materials and methods. Each data point on the curve represents the least mean square values obtained from three independent experiments done in duplicate. The CIMPR and HIGF-II binding curves were obtained using 100 ng of starfish purified MPR 300 protein coated onto the wells of the ELISA plates. **(B)** Binding curve was used for Scatchard analysis, where the slope was used to calculate the inverse of the dissociation constant (Kd) values.


A
0.20
0.15
0.10
0.05
0.00
0
20
40
60
Free BHIGF-II (nM)

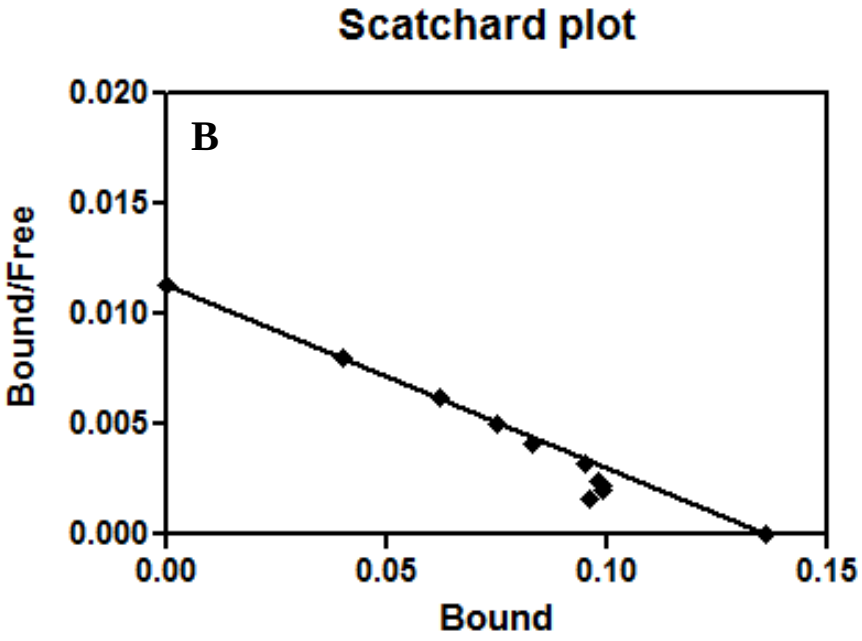

Scatchard plot
B
0.020
0.015
0.010
0.005
0.000
Bound/Free
0.00
0.05
0.10
0.15
Bound

Figure 5.4.7

Table 5.1. Domain structures of the known Mannose 6-phosphate/IGF-II receptors in the animal kingdom

Phylum and Species	Number of domains in MPR 300 *(M6P binding domain)*	Sequence known for domain 11 (IGF-II binding domain)	Reactivity with human IGF-II	Ref
Mammalia:				
Bovine	15 **(3,9)**	CFGQTR**I**SV	Yes	1
Human	15**(3,9)**	CFGQTR**I**SV	Yes	2
Kangaroo	ND	CFGLSK**I**NA	Low affinity	3
Non-mammalian vertebrates: Birds				
Chicken	15**(3,9)**	ITDGPKT**L**NA	Yes	4
CEF cells	15**(3,9)**		Yes	5,6
Reptilia: Garden lizard	ND	CFGDTR**I**NV	Yes	7
Amphibia: *Xenopus*	ND	ND	ND	--
Pisces: Fugu fish	15**(3,9)**	SDARTA**T**SG	ND	8
Zebra fish	15**(3,9)**	IKDNQMA**I**	ND	9
Brown trout	ND	ND	Yes	10
Xiphophorous Xiphidium	Partial 5**(3)**	ISDARTA**T**	Yes	Present study
Elephant shark fish	ND	SDARTAT	ND	Present study
Invertebrates: Echinodermata: *Asterias rubens*	ND	ND	Yes	Present study
Mollusca: *Unio*	ND	ND	ND	--
Bg cells	ND	ND	ND	--
Arthopoda: Prawn	ND	ND	ND	--
Drosophila melanogaster	5	QNSTT**I**	ND	11

(1) Morgan *et al*., (1987) *Nature* 329; 301-307. (2) Tong & Kornfeld. (1989) *J. Biol. Chem*. 264; 7970-7975. (3) Yandell *et al*., (1999) *J. Biol. Chem*. 274; 27076-27082. (4) Zhou *et al*., (1995) *Proc. Natl. Acad. Sci. USA*. 92; 9762-9766. (5) Matzner *et al*., (1996) *Dev. Dyn*. 207; 11-24. (6) Suresh *et al*., (2006) (7) Sivaramakrishna *et al*., (2009) *Int. J. Biol. Macromol*. 44; 435-440. (8) Raju (2004) Thesis, University of Hyderabad. (9) Catherine *et al*., (2006) *Dev. Genes. Evol*. 216; 144-151. (10) Mendez *et al*., (2001) 142; 1090-1097. (11) Dennes *et al*., (2005) *J. Biol. Chem*. 280; 12849-12857.

5.5. DISCUSSION

The cation independent mannose 6-phosphate receptor (CIMPR) is distinct from the cation dependent receptor (CDMPR) protein in its properties as described in the introduction chapter of this thesis. Table 5.1. summarizes the current knowledge of the IGF-II binding to MPR 300 protein in the animal kingdom. The information available for mammals is important for determining where in evolution the CIMPR acquired the ability to bind IGF-II. This knowledge, in turn, may lead to a greater understanding of the physiological role of this receptor with respect to its IGF-II binding in species where it is less understood.

Until the year 2000, studies on the IGF-II binding property of the CIMPR protein were restricted to different mammals (Siva Kumar and Praveen Kumar, 2010). There is one report that fish embryo MPR 300 (CHSE-214 and EPC cell lines) binds IGF-II revealing the presence of highly specific IGF-II binding and the absence of insulin binding but its sequence is not known (Mendez *et al*., 2001). Chicken and reptilian CIMPR have also been show to bind human IGF-II (Suresh *et al*., 2006; Siva Ramakrishna *et al*., 2009). In order to understand about the IGF-II binding property of other fish receptors, in the present study *Xiphophorus xiphidium* A_2 cell extracts as well as the affinity purified *Channa striata* CIMPR was used in a ligand blot experiment which revealed that the receptors indeed bind IGF-II. These studies so far suggest that possibly the IGF-II binding property of the MPR 300 protein is also conserved from fish to mammals. A recent study from our laboratory showed that the highly evolved invertebrate starfish contains both the putative MPR proteins that show distinct binding to phosphomannan-Sepharose gel. Mammalian antibodies goat MPR 300 antibody and MSC1 antibody

(raised against a synthetic peptide in the region of cytoplasmic tail of human MPR 46) can recognize the MPR 300 and 46 proteins respectively from the starfish. Similar receptors were also identified in the mollusc *unio* (Siva kumar and von Figura, 2002). However the ligand binding studies of CIMPRs from starfish and *unio* with IGF-II were not characterized. To examine whether the starfish CIMPR can bind IGF-II, initially starfish CIMPR was affinity purified as described earlier (Sivaramakrishna and Siva Kumar, 2008). The interaction of the starfish CIMPR with IGF-II has been examined by ligand blotting and IGF-II affinity chromatography. The high reliability and sensitivity of the biotinylated IGF-II was exploited in this study to investigate the binding of starfish MPR 300 to IGF-II. It is clear that there is specific interaction of the HIGF-II with the starfish receptor which was confirmed by the reactivity of the receptor with biotinylated HIGF-II. Additionally, in an ELISA binding assay purified starfish MPR 300 protein showed binding to the biotinylated human IGF-II. From these, a Kd value of 8.5 nM for the starfish CIMPR were obtained. The Kd values of human, rat, bovine and opossum were reported to be 1, 1-2, 0.2 and 14.5 nM, respectively (Dahms *et al.*, 1993). From previous studies, the Kd values for goat, chicken and fish receptors was found to be 12.5, 11.1 and 0.12 nM respectively. In conclusion, we have detected that in other fish species studied, the CIMPR exhibits IGF-II binding. Further studies are needed to establish the physiological role of IGF-II binding to the CIMPR/IGF-IIR in fish. In conclusion the purified starfish CIMPR i) binds biotinylated human IGF-II, ii) it binds on IGF-II-affigel at pH 7.0 and can be eluted using 50 mM sodium acetate buffer pH 5.0. These studies are the first observations that an invertebrate CIMPR also binds human IGF-II. However, the precise relevance of this

binding and the physiological significance can be only correlated by additional studies, on isolating IGF-II or related peptides from the starfish, identification of IGF binding proteins in starfish, which is beyond the scope of this present investigation.

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