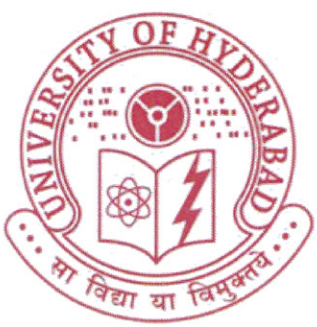
Biochemical characterization of lysosomal enzymes from the invertebrates *Lamellidens corrianus* and *Hydra vulgaris*

SUBMITTED FOR THE DEGREE THE OF

DOCTOR OF PHILOSOPHY

BY

ROHIT SAI REDDY KONADA



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CERTIFICATE

This is to certify that this thesis entitled "**Biochemical characterization of lysosomal enzymes from invertebrates *Lamellidens corrianus* and *Hydra vulgaris***" submitted by **Mr. Rohit Sai Reddy Konada** bearing registration number **13LBPH01** in partial fulfilment for the award of Doctor of Philosophy in the Department of Biochemistry, School of Life Sciences is a bonafide work carried out by him under my supervision and guidance.

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BC 804	Analytical Techniques	Pass	3
BC 805	Lab Work	Pass	4

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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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Rohit Sai Reddy Konada

Abbreviations

AP	Acid phosphatase
AP1	Adaptor protein1
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
Bg	*Biomphalaria glabrata*
bp	base pair
BSA	Bovine serum albumin
CCV	Clatharin Coated Vesicles
CDMPR	Cation dependent mannose 6-phosphate receptor
cDNA	Complementary DNA
CNBr	Cyanogen bromide
Con A	Concanavalin A
DIG	Digoxigenin
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
DVS	Divinyl sulfone
ECL	Enhanced chemiluminescence
EDTA	Ethylene diamine tetra acetic acid
EDC	1-ethyl-3(3-dimethylaminopropyl cabodiimide
et al	et alii (Latin: and others)
EtBr	Ethidium bromide
GGA	Golgi-localized, γ-ear-containing, ADP- ribosylation factor binding protein family
Hex A	Hexosaminidase A

Hex B	Hexosaminidase B
HRP	Horseradish peroxidase
IGF-II	Insulin like growth factor-II
kb	Kilo base pair
kDa	Kilo Dalton
LAMP-1	Lysosomal associated membrane protein1
LAP	1-ethyl-3(3-dimethylaminopropyl cabodiimide
LSD	Lysosomal storage disorders
min	Minute
MPR	Mannose 6-phosphate receptor
NBT	Nitroblue tetrazolium
NC	Nitrocellulose
NHS	N-hydroxy-succinimide
nm	Nanometer
nM	Nano molar
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
pH	-log (H+) concentration
PMSF	Phenyl methyl sulfonyl fluoride
rpm	Rotations per minute
SDS	Sodium dodecyl sulfate
SD	Standard deviation
SPR	Surface Plasmon Resonance
TBS	Tris-buffered saline
TGN	*trans*-Golgi network
Tris	Tris-(Hydroxymethyl) aminoethane

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Chapter 1

General introduction

1.1. Lysosomes

Eukaryotic cells are highly complex in nature with a wide variety of intracellular organelles that are responsible for various functionalities and also to maintain cellular homeostasis. One of the important components are the membrane bound lysosomes which constitute about 5% of cell volume and exhibit heterogeneity in size and morphology. Lysosomes were first discovered by Christian de Duve while studying the distribution of enzymes inside the cell using differential centrifugation (de Duve, 2005). These organelles are a part of the endosomal-lysosomal system, constituting early endosome, late endosome, and lysosomes. Together, this system is responsible for the digestion of endocytosed materials. Lysosomes are distinct from endosomes by lacking mannose-6- phosphate receptors (MPRs) (Appelqvist et al., 2013), smaller in size, and are enriched with hydrolases. They are involved in maintaining cellular homeostasis by involving in many cellular processes like degradation of macromolecules (major function), plasma membrane repair, cholesterol homeostasis, and cell death. They help in digesting proteins, carbohydrates, and lipids into their respective monomeric units and thus act as cell degradation center (Xu and Ren, 2015). This degradative function is carried out by more than 60 hydrolases, requiring an acidic environment for their activity. The lumen of lysosomes is acidic in nature, which is maintained at a pH of 4.6-5.0 by vacuolar proton pump (ATPases) (Mellman et al., 1986), and inability in maintaining this environment will impair the functions of lysosomal hydrolases, which is lethal to living cells. In addition to the proton pump, lysosome membrane contains transmembrane proteins that shuffle materials between lumen and cytosol. Lysosomal membrane is filled with several integral membrane proteins like lysosome-associated membrane proteins (LAMP) and lysosomal integral membrane

2proteins (LIMP) which make up to more than 50% of the membrane proteins (Winchester, 2001). Though the functions of these membrane proteins is not well understood, few reports suggest that these proteins help mostly in transport of the enzymes into lysosome and also help in phagocytosis. The luminal domains of membrane proteins are highly glycosylated forming a glycocalyx, which protects the membrane of lysosomes from the action of acid hydrolases (Granger et al., 1990).

1.2. Lysosomal storage disorders

The study of lysosomes and the hydrolases present within lysosomes gained importance due to the identification of various lysosomal storage disorders (LSDs) in humans. LSDs are one of the major subgroups of inborn errors of metabolism. They constitute about 50 genetic diseases, occurring due to the deficiency of specific lysosomal enzymes or proteins involved in lysosomal biogenesis (Fuller M, 2006). Although the majority of these disorders are inherited in an autosomal recessive manner (Hunter's disease, Fabry's disease and Danon disease), and the frequency of occurrence is rare, together the chances of individuals affected by LSDs is more. According to Fuller et al (2006), 1 in every 5000 births is affected with LSDs. LSDs are gene specific and all of them exhibit a common biochemical property by accumulating substrates inside lysosomes. Since the lysosomes are the final destination for the degradation of macromolecules, a defect in this function causes the accumulation of these compounds inside lysosomes. Initial studies on characterizing stored macromolecules, as in the case of Pompe's disease (Hers, 1963) implied that defective lysosomal hydrolases are causatives of LSDs. Degradation of macromolecules requires their transport to lysosomes by endocytic pathway; Depending upon the nature of the molecule, extracellular components enter by endocytosis and

phagocytosis where specific receptors present on cell surface are involved in receptor mediated endocytosis of important extracellular materials (Goldstein et al., 1985). The internalized materials are delivered to lysosomes via early endosomes and late endosomes and finally undergo degradation. Microorganisms and other debris are delivered to phagosomes by phagocytosis, which will fuse with primary lysosomes forming secondary lysosomes and are often destined for degradation. Intracellular macromolecules and materials undergo degradation by autophagy (Vellodi, 2005). Since the lysosomal hydrolases require acid milieu for their activity, bulk of the degradation occurs in lysosomes but not in early and late endosomes. Although there is a great diversity in the type of substrate accumulated in LSDs, the phenotypes of these diseases are similar and suggest the presence of a common mechanism for pathogenesis (Futerman and van Meer, 2004). Neurodegeneration is the common pathology associated with most of the LSDs. The reason for common pathology of LSDs can be due to the interference of accumulated undegraded substrates with autophagosome and lysosome fusion thereby blocking the autophagic pathway (Bajaj et al., 2019).

1.2.1. Classification of LSDs

Based on the type of macromolecules stored LSDs are grouped into broad categories like the mucopolysaccharidoses (glycosaminoglycan accumulation), the oligosaccharidoses (oligosaccharide accumulation), the lipidoses (lipid accumulation), and the glycogenosis (glycogen accumulation). Around eleven lysosomal enzymes (exoglycosidases, sulphatases, etc.,) are involved in glycosaminoglycan degradation and hence defect in any one of these enzymes causes mucopolysaccharidoses. Defect in the degradation of one macromolecule can accumulate more types of substrates. For example, glycoproteins,

proteoglycans and glycolipids can accumulate due to a failure in oligosaccharide degradation. Some of the examples of LSDs caused by above mentioned categories are summarized in Table.1. Some of the LSDs occur due to the defective lysosomal integral membrane proteins. Most of these proteins are transporters and help in exporting soluble metabolites. Salla disease (sialic acid storage disease), and cystinosis are two examples of LSDs with defective lysosomal membrane proteins sialin and cystinosin respectively (Verheijen et al., 1999) (Town et al., 1998). In addition to the classical LSDs occurring due to defects in lysosomal hydrolases, there are also some LSDs caused by defective lysosomal proteins. Sphingolipidoses are caused by mutations in the activator proteins that are required for the complete degradation of sphingolipids. These activator proteins are nonhydrolytic glycoproteins which includes GM2 activator protein and saposins. N-acetyl-glucosaminyl-1-phosphotransferase a non-hydrolase catalyzes the transfer of mannose-6-phosphate (M6P) to lysosomal enzymes in the Golgi apparatus. M6P acts as a recognition marker (Natowicz et al., 1979) for mannose-6-phosphate receptors which helps in targeting many hydrolases to lysosomes. The deficiency of this enzyme leads to I-cell disease, which was classified under mucolipidoses in which the lysosomal enzymes are misrouted and secreted, rather than being targeted to lysosomes. In I-cell disease not all cells lack lysosomal enzymes, this observation has led to the discovery of M6P independent lysosomal enzyme targeting and hence by studying LSDs normal cellular processes can be revealed (Dittmer et al., 1999). Table 1 summarizes some of the lysosomal storage diseases known in literature.

Table. 1. Lysosomal storage disorders

Lysosomal storage disease	Defective hydrolase	Stored substrate
Mucopolysaccharidoses (MPS)		
Hurler's disease	α-Iduronidase	Dermatan sulphate, heparan sulphate
Hunter's disease	Iduronate-2-sulphatase	Dermatan sulphate, heparin sulphate
MPS IIIA	Heparan sulfamidase	
MPS IIIB	N-acetyl glucosaminidase	
MPS IIIC	Heparan-α-glucosaminide N-acetyltransferase	
MPS IIID	N-acetylglucosamine 6-sulfatase	Heparan sulphate
MPS IVA	Galactose-6-sulfate sulfatase	Keratan sulfate, Chondroitin 6-sulfate
MPS IVB	β-galactosidase	Keratan sulfate
MPS VI	N-acetylgalactosamine-4-sulfatase	Dermatan sulphate
Sly syndrome	B-glucuronidase	Heparan sulphate, dermatan sulphate, chondroitin-4- and -6-sulphates
MPS IX	Hyaluronidase	Hyaluronic acid
Sphingolipodosis		
Fabry	α-galactosidase A	Ceramide trihexoside
Gaucher	Glucocerebrosidase	Glucocerebrosides
Krabbe	Galactocerebrosidase	Galactocerebrosides
Tay–Sachs	β-hexosaminidase A	GM2 gangliosides
Sandhoff	β-hexosaminidase A and B	GM2 gangliosides
Niemann-Pick	Sphingomyelinase	Sphingomyelin
Metachromatic leukodystrophy	Arylsulfatase A	Sulfatides
Oligosaccharidoses		
Fucosidosis	α-fucosidase	Fucose, glycolipids
Pompe	α-glucosidase	Glycogen
α-Mannosidosis	α-mannosidase	Mannosides
Others		
I cell disease	N-acetylglucosaminyl-phosphotransferase	Mucopolysaccharides, lipids, oligosaccharides
Cystinosis	Cystinosin	Cysteine
Danon disease	LAMP 2	Glycogen and cytoplasm debri
Salla disease	Sialin	Sialic acid

1.3. Lysosomal biogenesis

The formation of lysosome involves transport of lysosomal proteins formed at rough endoplasmic reticulum (RER) to the late endosomes via *trans* Golgi network (TGN). The

endosomal sorting occurs through a series of transport events which include 1. Transport of naïve lysosomal proteins from RER to *cis* Golgi network (CGN) with the help of clathrin coated vesicles (CCV) 2. Acquisition of recognition marker for the lysosomal proteins based on their specific location on the lysosome. 3,4 and 5 Sorting of the lysosomal proteins to lysosome (Kornfeld and Mellman, 1989), 6. Enzymes delivered into lysosomes. Figure 1.1 below shows a diagrammatic representation of the transport of lysosomal proteins in cells.

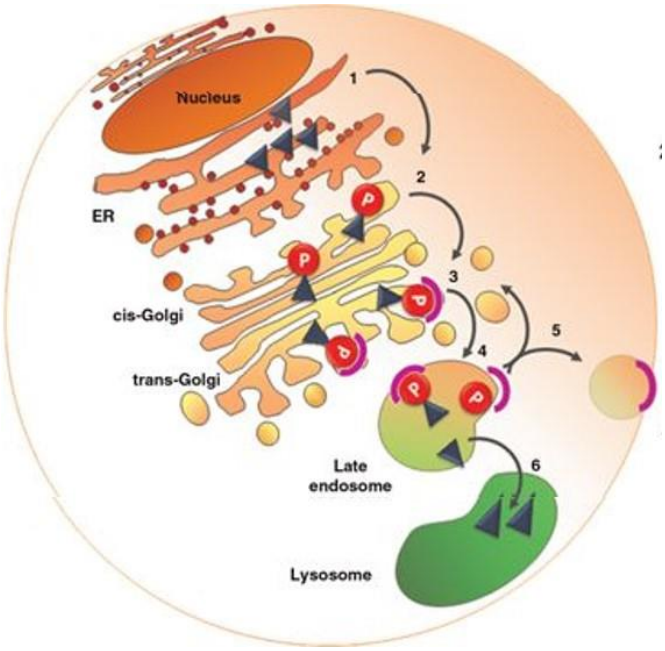


Fig 1.1. Biogenesis of Lysosomes

1.3.1. Lysosomal protein transport from ER to CGN

All the soluble proteins formed at the RER undergo processing for specific transport based on their destined location. Post translation modification of the lysosomal proteins occur in ER which are then delivered to CGN by CCV mediated vesicular transport. Several soluble

proteins follow the same mechanism, it is at the CGN the differentiation of proteins based on target happens (von Figura and Hasilik, 1986).

1.3.2. Acquisition of recognition marker

Lysosomal hydrolases attain a mannose 6-phosphate (M6P) recognition marker at GN with the help of two important enzymes UDP-N-acetylglucosamine-1-phospho-transferase and N-acetylglucosamine-1-phosphodiester α-N-acetyl glucosaminidase in a two-step manner. The occurrence of phosphate at the terminal mannose of the N-glycan serves as recognition marker for most of the lysosomal hydrolases. This event is an important step as failure of attainment of the M6P moiety leads to the secretion of the lysosomal enzyme which leads to malfunctioning of the lysosomes further turning into a lysosomal disorder. The other lysosomal proteins are transported across the CGN and TGN through vesicles which undergo varied processing to be sorted to the endosome (Kornfeld, 1987; Kornfeld and Mellman, 1989; Rohrer and Kornfeld, 2001; von Figura and Hasilik, 1986).

1.3.3. Sorting of the lysosomal proteins to lysosome

The sorting of the lysosomal proteins to lysosome occurs with the help of the receptors which locate the specific recognition marker. M6P on lysosomal enzymes (LE) are recognized by mannose 6-phosphate receptors (MPR) which sort them to the lysosomal lumen. The LE-MPR complex is recognized by a series of cargo proteins which target them to lysosome. Similar mechanism was reported for the other lysosomal proteins which share same sorting motif with that of MPRs. The details are presented in later sections (Braulke and Bonifacino, 2009; Kornfeld, 1987; Kornfeld and Mellman, 1989; Rohrer and Kornfeld, 2001; von Figura and Hasilik, 1986).

1.4. Mannose 6-phosphate receptors

Two distinct receptors have been identified which are involved in lysosomal enzyme targeting. These receptors recognize the M6P on the modified lysosomal hydrolase at the TGN and then transport it to lysosome-endosome complex. Hence, these are called mannose 6-phosphate receptors (MPRs). These are the sole members of P-type lectin family. Based on their structure, they are classified as MPR46 and MPR300 (Castonguay et al., 2011).

1.4.1. MPR 46

MPR 46 (~46 kDa) is also known as cation dependent MPR (CD MPR) based on the requirement of a divalent cation for its binding. It exists as dimer in the membrane and belongs to type 1 tarnsmembrane proteins. Each unit of MPR46 consists of a cytoplasmic tail, a single transmembrane domain and an extracytoplasmic domain which harbors the Mannose 6-phosphate receptor homology (MRH) domain that involves in M6P recognition. The important residues, glutamine (Q), arginine (R), glutamic acid (E) and tyrosine (Y), responsible for carbohydrate recognition have been identified among vertebrates and recently were also seen conserved among invertebrates. The cytoplasmic tail has an acidic dileucine motif DXXLL which is involved in the retrograde transport of LE-MPR complex to endosome-lysosome complex (Ghosh et al., 2003).

1.4.2. MPR 300

MPR 300 (~300 kDa) is also known as cation independent MPR (CI MPR) since it does not require divalent metal ions for its binding. MPR300 also belongs to type 1 transmembrane protein family and also occurs as a dimer. MPR300 is a complex structure with 15 repetitive domains in its extracytoplasmic tail, a single transmembrane domain and

a cytoplasmic tail domain. Each repetitive cassette is equivalent to the MRH domain of MPR46. Of all the 15 cassettes, domains 3, 5, 9 and 15 are the mannose 6-phosphate binding domains so far reported. Each M6P binding domain harbours residues Q, E, R and Y which are involved in M6P recognition (Castonguay et al., 2011; Ghosh et al., 2003). These residues are seen conserved across the mammalian and non-mammalian vertebrates. Among invertebrates, the complete domain topology of 15 cassettes is seen missing with exceptions in echinoderms and molluscs (Nadimpalli and Amancha, 2010a; Siva Kumar and Bhamidimarri, 2015).

Owing to its structural complexity, MPR300 perform diverse functions. Apart from lysosomal enzyme targeting, MPR300 is known to involve in binding for fibronectin, uPAR etc. MPR300 is known to bind and endocytose additionally a non-glycosylated insulin-like growth factor II (IGF-II) and hence called MPR300/IGF II receptor.

The cytosolic dileucine motif DXXLL is also seen in CIMPR and a hydrophobic tyrosine motif Yxxϕ which is also known as sorting motif is seen in vertebrates. Most of the non enzymatic lysosomal proteins have these two motifs in their cytosolic domain which helps them to follow the similar mechanism of transport to lysosomes as that of LE-MPR complex (Braulke and Bonifacino, 2009).

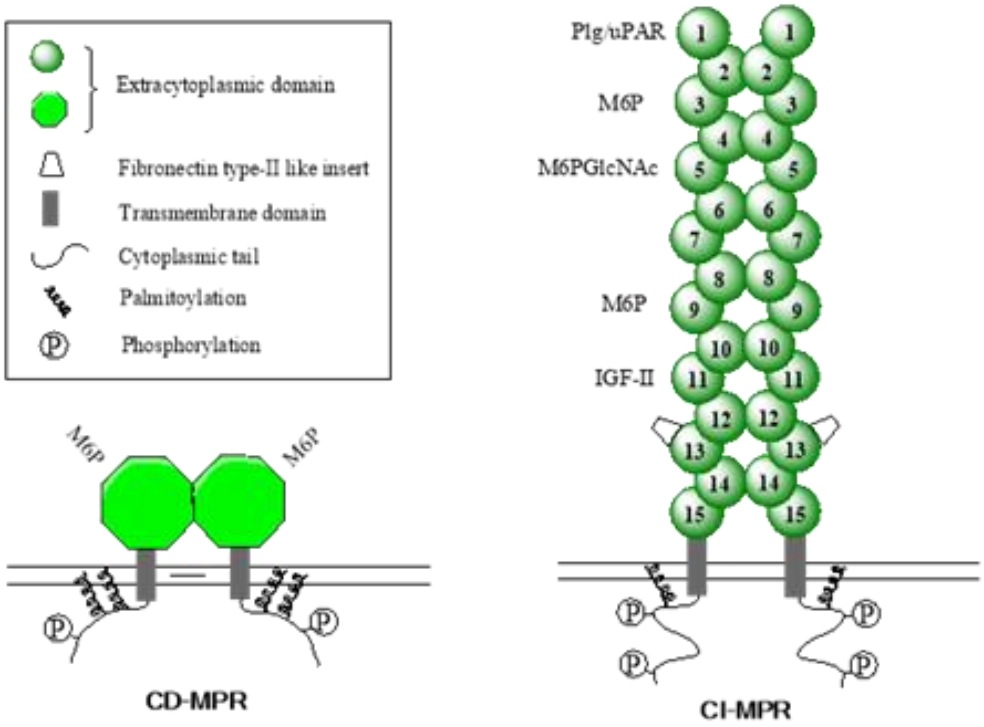


Fig 1.2. Mannose 6-phosphate receptors showing domain structures

1.5. Sorting Machinery

All the newly formed lysosomal proteins once reached TGN are transported to endosomes with the help of adaptor proteins. The cytosolic dileucine motif DXXLL in MPRs is detected by Golgi-localised γ - ear- containing ARF- binding (GGA) adaptor proteins in TGN which are associated with clathrin coated vesicles. MPRs are continuously recycled from TGN to endosomes and plasma membrane. Clathrin coated vesicles with GGA binding proteins help in this retrograde transport. The VHS domain in GGA protein interacts with dileucine motif and transported to endosome-lysosome complex. Apart from GGA binding DXXLL motif, the cytosolic tails of MPR have a YXXØ motif (where Ø is any hydrophobic residue) which interacts with another clathrin-associated adaptor protein 1 (AP-1). The acidic pH in the endosomes triggers the MPR to release the lysosomal

hydrolase. MPRs do not reach lysosomes, but recycle from endosomal compartments back to the TGN to repeat the sorting process (Braulke and Bonifacino, 2009).

The other lysosomal proteins which share the same dileucine motif for transport include membrane proteins like LIMP2 and LAMP.

1.6. MPR independent pathway

Studies on MPR-independent pathways have started when lysosomal enzyme were efficiently rescued in I-cell disease patients lacking M6P on their acid hydrolases. It was then understood that a MPR-independent, more specifically M6P independent process occurs in the cells. MPR dependent pathway is well studied among vertebrates and among invertebrates, only echinoderms and molluscs show a complete homology with the MPRs. In *Drosophila,* a lysosomal enzyme receptor protein (LERP) was identified which showed homology with MPRs but transports the lysosomal enzymes to lysosomes by protein-protein interaction rather than M6P recognition. Their MRH domain also lacks the Arginine residue responsible for the M6P interaction. This drew attention that a M6P independent and more specifically an MPR independent pathway might be prominent among invertebrates (Dennes et al., 2005).

The other proteins that were identified to involve in endosomal sorting are sortilin and LIMP2, is a lysosomal integral membrane protein that sorts β-Glucocerebrosidase (GCase) to endosome/lysosome compartment in a pH-dependent manner. Histidine residue present in a coiled coil motif of the LIMP2 is involved in binding and transport of GCase and another Histidine residue present in this motif acts as a pH sensor (Zachos et al., 2012). In *Xenopus* and chicken, Histidine at the active centre replaced by arginine and hence cannot

bind to the ligand. The role of such modified LIMP2 proteins is to be studied (Coutinho et al., 2012).

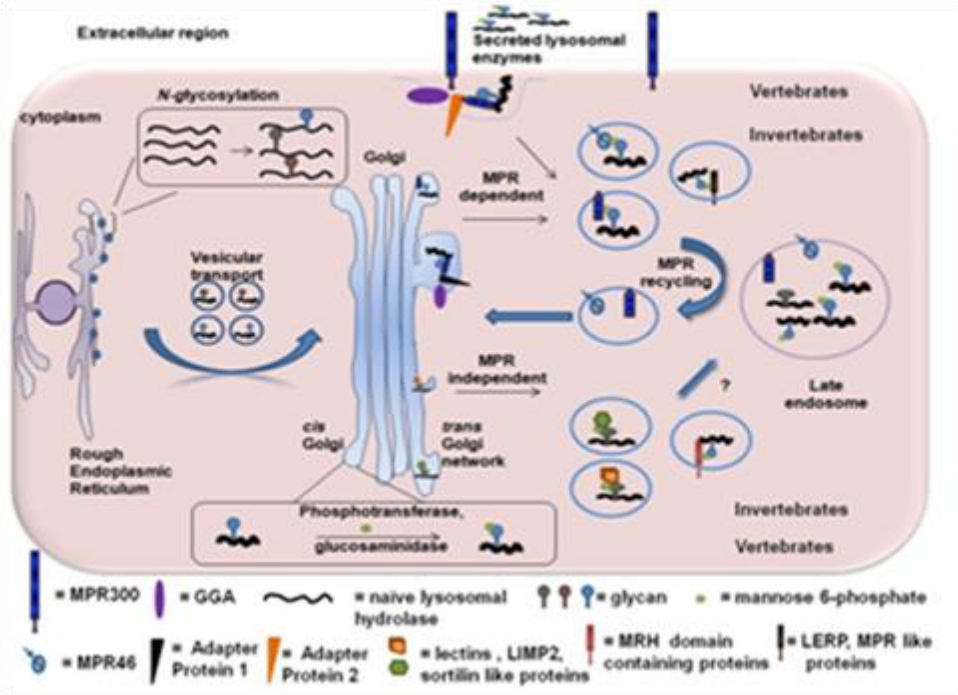


Fig 1.3. Lysosomal protein transport among vertebrates and invertebrates

Another receptor-like protein, which binds diverse ligands is sortilin, which is homologous to many known receptors and has diverse functions. Sortilin is predominately present in TGN and in early endosomes like MPRs. The extensive co-localization of sortilin protein with CIMPR led to a study where sortilin-CIMPR chimera constructs (cytosolic domain of sortilin and luminal domain of CIPMR) expressed in CIMPR knockout cells were found to efficiently transport newly synthesized lysosomal enzymes β- glucuronidase and β-hexosaminidase to lysosomes (Nielsen et al., 2001). Other studies demonstrated the direct binding of sortilin to GGA2 and its function in lysosomal trafficking of both enzymatic and non-enzymatic proteins (Lefrancois et al., 2003). Sortilin also targets cathepsins D and

H to lysosomes (Canuel et al., 2008b; Coutinho et al., 2012). Few reports suggested that sortilin sorting pathway is an evolutionarily ancient mechanism than MPR pathway and that the structural homology of sortilin in the cytoplasmic tail region is the reason for the similar retrograde trafficking pathway. Vascular protein sorting 10 protein (Vps10p) of Yeast, an analogous protein to MPR shares more homology with sortilin structurally that implicate their evolutionary relation. Both Vps10p and sortilin, function through protein-protein interaction with their ligands whereas MPR binds to M6P on N-glycans (Canuel et al., 2008a), hence these proteins are called MPR independent sorting proteins.

1.7. Lysosomal enzymes

The ability of lysosomes to degrade macromolecules such as proteins, carbohydrates and nucleic acids is due to the collective action of various lysosomal enzymes within them. Most of these enzymes are acid hydrolases and glycoproteins in nature. In fact lysosomes were discovered as separate cell organelles while studying the latency of acid phosphatase in subcellular fractionation (de Duve, 2005). These acid hydrolases like acid phosphatase are used as markers to distinguish lysosomes from other cell organelles. They require acidic environment for their activity and hence inactive in cytosol, which gives protection from cell damage due to membrane leakage. Most of the genes encoding for lysosomal proteins contains GTCACGTGAC sequence (CLEAR sequence) near the start site of transcription (Sardiello et al., 2009). The induction of transcription of lysosomal enzymes occurs when the CLEAR sequence elements are bound by transcription factor EB (TFEB) (Settembre et al., 2011). All of these enzymes are synthesized in cytoplasm and contain a signal sequence (common to plasma membrane, nuclear, Golgi complex and endoplasmic reticulum proteins) which directs the lysosomal proteins to endoplasmic reticulum. Glycosylation

and trimming of glycans present on these enzymes takes place to attain a recognition marker (M6P). This unique marker helps targeting lysosomal enzymes to lysosomes by MPRs. The hydrolases present in lysosomes include proteases, glycosidases, lipases, phosphatases and sulphatases.

1.7.1. Lysosomal proteases

Cathepsins are the best known proteases present in lysosomes. Based on the amino acids present in active site, cathepsins are classified into many types. Serine containing cathepsins are cathepsin A and G, cysteine containing cathepsins B, C, H, and L and aspartic acid containing cathepsins include cathepsin D and E. Amino acid sequencing of cathepsin D from porcine was used to determine the presence of signal sequence responsible for endoplasmic reticulum targeting (Erickson and Blobel, 1979).

1.7.2. Lysosomal glycosidases

Glycosidases are glycoside hydrolases which catalyze the breakdown of glycoside bonds in polysaccharides. They cleave O-glycosidic and N-glycosidic bonds present in carbohydrates and their derivatives. Carbohydrates being the most prolific macromolecules present in nature, glycosidases play an important role in maintaining homeostasis and cycling of these compounds. In humans they help in the degradation of glycoconjugates. Many of the inherited diseases like Gaucher's disease, Fabry disease, Tay-Sachs disease and Krabee diseases occur due to the deficiency of specific glycosidases (Kuo et al., 2018). Based on the type of reaction mechanisms, glycosidases are classified into retaining glycosidases and inverting glycosidases. Hexosaminidases and sialidases are the examples of retaining glycosidases, whereas β-amylases and glucomylases are the examples of

inverting glycosidases (Kallemeijn et al., 2014). Other lysosomal enzymes includes phosphatases, lipases and nucleases.

1.7.3. Lysosomal enzymes from invertebrates

Invertebrate model organisms like *Drosophila* and *Caenorhabditis elegans* have been used to understand the pathology of LSDs (De Voer et al., 2008). For example, *Drosophila* model was used to understand the pathology of Niemann-Pick disease (Wang et al., 2011). The digestive glands present in molluscs serves as a model organ to study the responsive biomarkers and environmental stress factors (*V dimitriadis*). Marine invertebrates are rich sources of lysosomal enzymes having many biotechnological applications. Enzyme replacement therapy used for treating LSDs utilize the lysosomal enzymes isolated from these sources (Siva Kumar and M Bhamidimarri, 2015). The role of these enzymes in physiology of vertebrates was extensively studied. Lysosomal enzymes from invertebrates also showed a potential role in maintaining the physiology of organism. In domestic fly lysosomal protease like cathepsins are involved in the digestion of food in the midgut region similar to that of vertebrates (Padilha et al., 2009). β-hexosaminidases present on the surface of the sperm mediates the fusion of the gametes to undergo fertilization in vertebrates and invertebrates. *Drosophila* contains two forms of hexosaminidases which helps in fertilization (Cattaneo et al., 2006). Arylsulphatases from sea urchins are reported to play a role in gastrulation by binding to sulfated polysaccharides present in the extracellular matrix (Mitsunaga-Nakatsubo et al., 2009). Lysosomal hydrolases are also used in defensive mechanisms in some invertebrates. Aspartylglucosaminidase was found to be active in the venom extracts of the wasp *Asobara tabida* (Moreau et al., 2004).

Table. 2. Lysosomal enzymes and their biochemical nature in invertebrates

Lysosomal Enzyme	Source	Molecular mass kDa	Reactivity with MPRs	Purification method	Glycoprotein nature
Cathepsin D	*Asterias rubens*	45	+	Affinity	+
	Lamellidens corrianus	43	+	Affinity	+
	Chlamys fareri	38	Not Known	EST	Not known
	Todarepsin pacificus	37	,,	Conventional	,,
	Bombyx mori	42	,,	Cloned	,,
	American lobster	50	,,	Conventional	,,
	Dictyostelium discoideum	44	,,	Cloned	,,
Fucosidase	*Asterias rubens*	56	+	Affinity	+
	Unio	56	+	Affinity	+
	Venus mercinaria	50	Not Known	Conventional	Not known
	Pecten maximus	45	,,	Conventional	,,
	Drosophila melanogaster	58	,,	Cloned	,,
	Dictyostelium discoideum	62	,,	Conventional	,,
		50	,,	Conventional	,,
Hexosaminidase	*Lamellidens marginalis*	Hex A (75, 30)	+	Conventional	+
	Penaeus japonicas	Hex B (40)			
	Bombyx mori	2X64(110)	+	Conventional	+
	Trichinella spiralis	2X61 2X50,			
	Trypanosoma cruzi		+	Conventional	+
		2X55 (200)	Not known	,,	+
			,,	,,	+

These enzymes also play a key role in innate immunity by destroying bacteria and viruses (Austin and Paynter, 1995). Some of the lysosomal enzymes are used as a biomarkers for assessing environmental stress and pollution. Size of the lysosomes and the permeability

of their membrane changes in response to environmental stress (Marigomez et al., 2005). Heavy metal toxicity damages lysosomal membranes and causes the leakage of hydrolases into the cytosol. Therefore, several lysosomal enzymes are used as markers for heavy metal pollution. The lysosomal enzymes used for assessing are β-hexosaminidase (Hex), β-glucuronidase (β-Gluc) and acid phosphatase (AP) (Moore et al., 2004). The histochemical localization of hex, β-Gluc and AP are used for monitoring the membrane stability of lysosomes (Dimitriadis et al., 2012). Especially alterations in the activities of acid phosphatases induced by heavy metal toxicity were detected in freshwater snails, and mussels (Vlahović et al., 2013).

1.8. Scope of the present investigation

In the earlier pages of this thesis a detailed description has been given on the lysosomal biogenesis pathway in general and in the vertebrates in particular. Two Mannose 6-phosphate receptors namely, MPR46 , Mr 46 kDa and MPR300, Mr 300 kDa are the key proteins in lysosomal enzyme targeting in eukaryotes has been well established in mammals (Kornfeld and Mellman, 1989). The existence of two structurally and functionally homologous but distinct proteins that mediate transport of lysosomal enzymes raised a question as to where in evolution the receptors appeared first and which of the two receptors is ancient? The laboratory where this work has been carried out has been working towards establishing the evolution of the lysosomal biogenesis pathway in the animal kingdom. Earlier studies clearly established that the lysosomal enzymes and their sorting receptors MPR46, and MPR300 are the key players involved in transport of newly synthesised lysosomal enzymes. Additionally the IGF-II binding property of the MPR300

which is also an endocytosis receptor has been shown to be conserved from fish to mammals (Ajith kumar and Nadimpalli, 2018).

In the last five years work carried out has logically concluded that indeed in higher invertebrates such as the echinodermates (starfish) and mollusc (unio) both lysosomal enzymes and their sorting receptors homologous to the vertebrate counter parts are present. However only a few lysosomal enzymes have been characterized from unio (Venugopal et al., 2017) and there has not been extensive quantitative studies on the receptor-ligand interactions in this organism. Interestingly *Drosophila* lacks the MPR46 completely and has a truncated MPR300 that does not bind to the phosphomannan gel used for the purification of other receptors (Dennes et al., 2005).

Therefore the question remained where in evolution the receptors appeared first and having established growth conditions of *Hydra vulgaris* in our laboratory, we explored this animal model to look for lysosomal enzymes and their receptors. Hydra is widely used as a model organism in stem cell biology, regeneration and axial patterning. They are diploblastic animals with two distinct layers outer ectoderm and inner endoderm separated by acellular mesoglea. Both of these layers contain stem cells which help in regeneration. Special type of multipotent cells called interstitial cells present in the ectoderm of hydra are responsible for the regeneration capacities.

The activities of lysosomal enzymes were identified in the soluble extracts of Hydra. We further discovered the MPR46 protein, cloned the gene for the same and found that it is structurally related to the human receptor. In particular, the cysteine residues, the ligand binding regions as well as the extra cytoplasmic domain, transmembrane domain and the cytosiolic tail of Hydra MPR46 are highly related to the human MPR46. In the same study

it was also found that Hydra contains a protein that exhibits some similarity to the human MPR30 protein but this protein has only 4 cassette structures unlike the 15 repetitive cassette structures of the human MPR300. Interestingly the mannose 6-phosphate binding regions are reflected in the second cassette of Hydra MPR300 while they are confined to third and ninth cassette in humans and other known MPR300s (Bhamidimarri et al., 2018a). This study thus provided evidence that MPR46 is the most ancient protein in evolution and possibly MPR300 arrived slowly in evolution by gene duplication. In view of these recent findings, it became important to examine in detail about the different lysosomal enzymes in Hydra, how they are related to the already known vertebrate enzymes in terms of their biochemical nature, sequence analysis and their relatedness with other well-known lysosomal enzymes.

Based on the background mentioned above, the present study was performed with the following objectives.

- Purification and biochemical characterization of β-glucuronidase from *Lamellidens corrianus*
- Ligand binding studies of β-hexosaminidases and MPRs from *Lamellidens corrianus*
- Identification and localization studies of lysosomal enzymes (acid phosphatase and hexosaminidase) from *Hydra vulgaris* Ind-pune
- Comparative analysis of lysosomal enzymes (Enzyme profiling- acid phosphatase and hexosaminidase) from *Hydra vulgaris* Ind-pune, *H. vulgaris* Naukuchiatil, *H. magnipapillata* sf-1 and Purification of hexosaminidase from *H. vulgaris*

Chapter 2

Purification and biochemical characterization of β-glucuronidase from the freshwater mussel *L. corrianus*

2.1. Introduction

Lysosomal β-glucuronidase [EC 3.2.1.31] is a glycosidase involved in processing of glycosaminoglycans at their glucuronide site (Paigen et al., 1975). β-glucuronidases from plants, vertebrates and bacteria have been studied for their functional importance and are used in bioassays like phytoestrogen assay, steroid hydrolysis assay (Chilke, 2010; Graef et al., 1977; Shibasaki et al., 2001). β-glucuronidase in plasma is used as a biomarker to evaluate the exposure of humans to a low-level organophosphate insecticide (Ueyama et al., 2010). Apart from its utilization in carbohydrate processing, β-glucuronidase has been employed in antibody dependent enzyme prodrug therapy (Compain et al., 2018). In humans, deficiency in β-glucuronidase causes an autosomal recessive disorder mucoploysaccharidosis-VII also known as Sly syndrome. Hence, an enzyme replacement therapy using a cloned, expressed and a purified β-glucuronidase protein was developed (Grubb et al., 2008). The β-glucuronidase gene (GUSB) is used as a reporter in plant biotechnology applications (Eudes et al., 2008; Jefferson et al., 1987). Among invertebrates, β-glucuronidase from mollusc species *Helix pomatia* and *Ampullaria* have been used in steroid hydrolysis to assess the urinary conjugate of cortisol (Grace and Teale, 2006; Graef et al., 1977). The recent reports on utilization of lysosomal hydrolases as markers of pollution among the species dwelling in water bodies suggests that there is a need to study the effects of environmental pollutants on lysosomal enzymes (Nguyen et al., 2015; Raftopoulou and Dimitriadis, 2012; Sforzini et al., 2018; Vlahovic et al., 2013).

Several studies on β-glucuronidase were based on its functions and applications and very few studies were performed to understand its biochemical nature. The crystal structure of β-glucuronidase from humans has been deciphered where the site specific for lysosomal

transport was identified confirming the mannose 6-phosphate receptor (MPR) dependent targeting to lysosomes (Jain et al., 1996).

Our laboratory has been studying the lysosomal biogenesis especially the mannose 6-phosphate dependent targeting of lysosomal enzymes among invertebrates (Kumar and Bhamidimarri, 2015; Nadimpalli and Amancha, 2010b; Vegiraju et al., 2012) and as a part of the study we could identify and characterize the lysosomal enzymes from mollusc *Lamellidens corrianus*, a commonly available species among freshwater bodies. The lysosomal hydrolases like α-fucosidase (Nadimpalli et al., 2004), β-N-acetyl hexosaminidase (Venugopal and Sivakumar, 2013a), α-mannosidase and cathepsin D (Venugopal and Siva Kumar, 2014) were reported earlier from the same species. Although occurrence of β-glucuronidase among invertebrates were reported earlier, clear biochemical analysis and structural studies were not done.

In this study, a comprehensive biochemical analysis of β-glucuronidase purified from *L. corrianus* was done and the effect of pH, temperature and certain chemical reagents on the activity was assessed. This is the first report to ascertain the biochemical features of β-glucuronidase from an invertebrate species which could be employed in developing a biological indicator in toxicology research.

2.2. Materials and methods

2.2.1. Materials

L. corrianus animals were procured from Chandrakala zoological dissection material supplier, Osmania University, Hyderabad. De-shelled soft tissue of *L. corrianus* were stored immediately at -80°C until use. Substrates used for lysosomal enzyme assays, phenyl Sepharose CL-4B, Con A-Sepharose, and Sephacryl S-200 gels used for the

purification of β-glucuronidase and standard molecular weight markers (Thyroglobulin, β-Amylase, Alcohol dehydrogenase, BSA, Carbonic Anhydrase, Cytochrome C) were obtained from Sigma Aldrich (St. Louis, MO, USA). 4-methyl umbelliferyl glucuronide was obtained from Carbosynth (Berkshire, UK) and DEAE-Sepharose was procured from GE Healthcare. All the other chemicals and reagents used in the present study were of the highest purity available and obtained from local suppliers.

2.2.2. Methods

2.2.2.1. Extraction of soluble proteins and enzyme assay

About 50 g of tissue was homogenized with 10 volumes (500 mL) of 50 mM sodium acetate-acetate buffer, pH 5.0 and the soluble extract was obtained as described in (Venugopal and Sivakumar, 2013a). Activities of the lysosomal enzymes from *L. corrianus* crude soluble extract were assayed as detailed in (Nadimpalli et al., 2004) with the respective substrates. "One unit of the enzyme activity is defined as the absorbance equivalent of 1 μmol *p*-nitrophenol released per minute per mL of the enzyme solution under the experimental conditions. Specific activity was expressed as the absorbance equivalent of 1 μmol *p*-nitrophenol released by 1 mg of protein per min". All the solutions were equilibrated thermally at 37˚C to perform enzyme assays and for determining kinetic data.

2.2.2.2. Hydrophobic chromatography using phenyl-Sepharose column

About 20 mL of the prepacked phenyl-Sepharose gel was equilibrated with buffer A (50 mM sodium acetate buffer pH 5.0, containing 1 M ammonium sulfate. To the obtained soluble extract 1M ammonium sulfate was added and the resulting solution was passed through the matrix and the unbound proteins were washed from the gel with buffer A. The

bound proteins were eluted with sodium acetate buffer, pH 5.0, without ammonium sulphate. The fractions collected were monitored for protein at 280 nm and the enzyme assay was performed as described above. The active fractions were pooled, dialyzed against 25 mM Tris-HCl, pH 7.4 (buffer B), and subjected to ion exchange chromatography on DEAE-Sepharose matrix.

2.2.2.3. Ion exchange chromatography using DEAE-Sepharose column

About 20 mL of the DEAE-Sepharose gel was packed into a glass column and equilibrated with buffer B. The dialyzed sample from the phenyl-Sepharose step was passed on the ion exchange matrix, and the unbound proteins from the gel were washed with buffer B. The bound proteins were eluted with increasing concentrations of NaCl (50 mM, 100 mM and 150 mM) in buffer B in a stepwise manner. The fractions were collected and the enzyme assay was carried out as described above. The active fractions were pooled, dialyzed against 25 mM Tris-HCl, pH 7.4 containing 0.5 M NaCl, 0.5 mM $MnCl_2$, and 1 mM $CaCl_2$ (buffer C), and subjected to affinity chromatography on Con A-Sepharose matrix.

2.2.2.4. Affinity chromatography on Con A-Sepharose

About 2 mL of the Con A-Sepharose gel was packed in to a glass column and equilibrated with buffer C. The fractions pooled and dialyzed from ion exchange column were passed on the affinity column and the unbound proteins were removed with 25 mM Tris-HCl, pH 7.4 containing 0.5 M NaCl (Buffer D). The bound proteins were sequentially eluted with 100 mM, 200 mM and 300 mM of methyl α-D-mannopyranoside in buffer D. The respective fractions were collected and checked for the enzyme activity. The active fractions were pooled and subjected to gel filtration chromatography on Sephacryl S-200.

2.2.2.5. Gel filtration chromatography on S-200 gel

About 80 mL of the Sephacryl S-200 gel was packed into a glass column and equilibrated with 10 mM Tris-HCl, pH 7.4. The active fractions obtained from the Con A-Sepharose gel were concentrated and applied on to the S-200 gel. The fractions of 500 µL were collected and assayed for the protein and enzyme activity. The fractions with β-glucuronidase activity were pooled, concentrated and analyzed by SDS-PAGE analysis.

2.2.2.6. Electrophoretic characterization and activity staining

The concentrated sample of the active fractions obtained from S-200 gel filtration chromatography was analyzed by 10% SDS-PAGE under both the reducing and non-reducing conditions, and by 10% native PAGE. The purified enzyme was verified by activity staining as described (Venugopal and Sivakumar, 2013a). Briefly, the purified protein was resolved in 10 % native PAGE. The gel was washed with 50 mM sodium acetate pH 5.0 and incubated with 0.1 mM 4-methyl umbelliferyl β-D glucuronide for 10 min at 37°C. After the incubation, the gel was illuminated under UV light to visualize the band.

2.2.2.7. Western blot analysis

The immune-reactivity of the purified β-glucuronidase protein with rabbit anti-human β-glucuronidase antibody was checked. The purified enzyme was resolved on 10 % SDS-PAGE and the protein was transferred onto the PVDF (polyvinylidene difluoride) membrane. The membrane was incubated with the blocking buffer [50 mM Tris-HCl, pH 7.0 with 0.05% Tween 20 (TBST) containing 5 % BSA] for 1 hour at room temperature and was further incubated with anti-human β-glucuronidase antibody (1:1000 in blocking buffer) at 4 ˚C overnight. The membrane was washed thrice with TBST and incubated with

HRP conjugated anti-rabbit IgG (1: 5000 dilution) for 1 hour at room temperature. The membrane was washed with TBST thrice and developed using ECL® reagent (G-Biosciences, St. Louis, USA).

2.2.3. Biochemical characterization

2.2.3.1. pH and temperature optima

The pH dependent activity of the β-glucuronidase was investigated by performing the enzyme assay at various pH ranging from 2.0 to 10.0. The optimum temperature was investigated by performing the enzyme assay at different temperatures ranging from 10 °C to 100 °C.

2.2.3.2. pH and temperature stability

The effect of the temperature on the stability of the β-glucuronidase was investigated by incubating the enzyme at different temperatures ranging from 10°C to 100°C for 30 min before the enzyme activity. The residual activities were measured as described above by performing the enzyme assay at 37°C. The pH dependent stability of the protein was studied by incubating the enzyme separately at different pH ranging from 2.0 to 10.0 for 12 hr. The residual activities of the enzymes were recorded as describe above by assaying at 37°C.

2.2.3.3. Effect of chemical agents and metal ions

The effects of various chemical agents like DTT, Acrylamide, β-mercaptoethanol, EDTA, sodium chloride, SDS and metal ions $NiCl_2$, $ZnSO_4$, CsCl, KI, $ZnCl_2$, $HgCl_2$, $MgCl_2$, $MnCl_2$, $CoCl_2$, KCl and Cu_2SO_4 on the activity of glucuronidase was investigated by incubating the purified enzyme separately with 1 mM of each of the chemical agent and metal ion at 37°C for 5 minutes, and the residual activities were measured by assaying the

enzyme activity at 37°C with p-nitrophenyl β-D-glucuronide as substrate. The enzyme assayed without any metal ion was considered as control and expressed as 100%.

2.2.3.4. Kinetic characterization

The effect of substrate on the activity of purified protein was investigated by incubating the enzyme with increasing concentrations of p-nitrophenyl β-D-glucuronide (0.5 mM to 5 mM). Kinetic parameters K_M and V_{max} were calculated from Michaelis-Menten and Lineweaver-Burk plots by using GraphPad prism 5 software.

2.2.3.5. Circular Dichroism (CD) spectroscopy

The secondary structure of purified glucuronidase was determined by CD spectroscopy. CD experiments were performed with a concentration of 0.1 mg/mL purified protein in the far UV range (190-250 nm) using J-1500 JASCO spectropolarimeter model equipped with a thermostat. Thermal unfolding of the protein was measured by increasing temperature from 25˚C to 90˚C with a scan speed of 20 nm/min. Each spectrum result was taken from the average of 3 scans from which buffer scans were subtracted. The secondary structure parameters of the purified enzyme were calculated by using dichroweb server.

2.3. Results

2.3.1. Purification of β-glucuronidase

The crude extract obtained from *Lamellidens corrianus* when analyzed for lysosomal enzyme activities, revealed the presence of glycosidases like β-N acetyl hexosaminidase, α-fucosidase, β-glucuronidase, α-glucosidase, α-mannosidase and α-galactosidase mentioned in the decreasing order of their respective activity [Figure 1].

The soluble extract with relatively good β-glucuronidase was passed on phenyl Sepharose gel and the proteins bound to the column were eluted with 50 mM sodium acetate buffer,

pH 5.0. The fractions collected were analyzed for protein concentration and β-glucuronidase activity as mentioned in the methods. The fractions (4-15) with high β-glucuronidase activity and good protein concentration were pooled [Figure 2A] and dialyzed against 25 mM Tris HCl buffer, pH 7.4. The pooled and dialyzed sample was then subjected to ion exchange chromatography on DEAE-Sepharose column and the protein was eluted sequentially by using different concentrations of sodium chloride 50 mM, 100 mM and 150 mM in 25 mM Tris HCl buffer, pH 7.4. Fractions collected at 50 mM NaCl showed maximum activity for β-glucuronidase [Figure 2B]. These fractions were pooled and further subjected to Con A Sepharose affinity chromatography where the bound protein eluted with 0.2 mM methyl mannopyranoside showed good β-glucuronidase activity. These active fractions were passed on size exclusion chromatography matrix Sephacryl S-200. The elution profile was monitored by the absorbance of proteins at 280 nm and enzyme activity was checked at 405 nm. The fractions 27 to 31 with β-glucuronidase activity [Figure 3A] were analyzed on SDS-PAGE for protein profile and homogeneity of the purified protein. The intact molecular mass of native protein was analyzed using standard molecular weight markers. The graph plot revealed that the eluted protein is showing an apparent molecular mass of ~ 250 kDa in size [Figure 3B]. The protein profile showed that β-glucuronidase occurs as heterotetramer with the molecular masses around 90, 75, 65, and 50 kDa respectively for each subunit [Figure 3C].

The purified protein when analyzed on native PAGE showed a single band under the non-denatured and non-reducing conditions [Figure 4A], which was confirmed as β-glucuronidase by activity staining with the specific fluorescent substrate 4-methyl umbelliferyl β-D glucuronide [Figure 4B]. When analyzed under denatured and reducing

condition, the purified protein showed four polypeptides indicating that the purified protein exists as a heterotetramer [Figure 4C]. Western blot analysis of the purified protein with rabbit anti-human β-glucuronidase antibody generated signals corresponding to the bands at 90 kDa and 50 kDa on SDS-PAGE [Figure 4D] suggesting that the antibody recognizes specifically two bands of the protein and the protein purified represents the β-glucuronidase. Since the regular SDS-PAGE revealed 4 bands with different molecular masses the native protein is likely to be a heterotetramer.

The fraction obtained from size exclusion chromatography was analyzed for the presence of other enzyme activities suggesting that the protein obtained is relatively pure with negligible traces of galactosidase activity [Figure 5]. The purification Table showed that the yield is 6.76% with 97.1 fold purification in the final step. The specific activity of the enzyme β-glucuronidase increased from crude to final purified sample [Table 1].

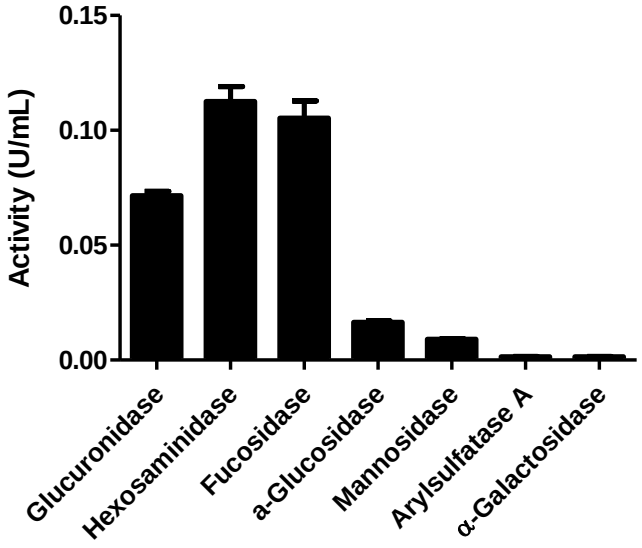
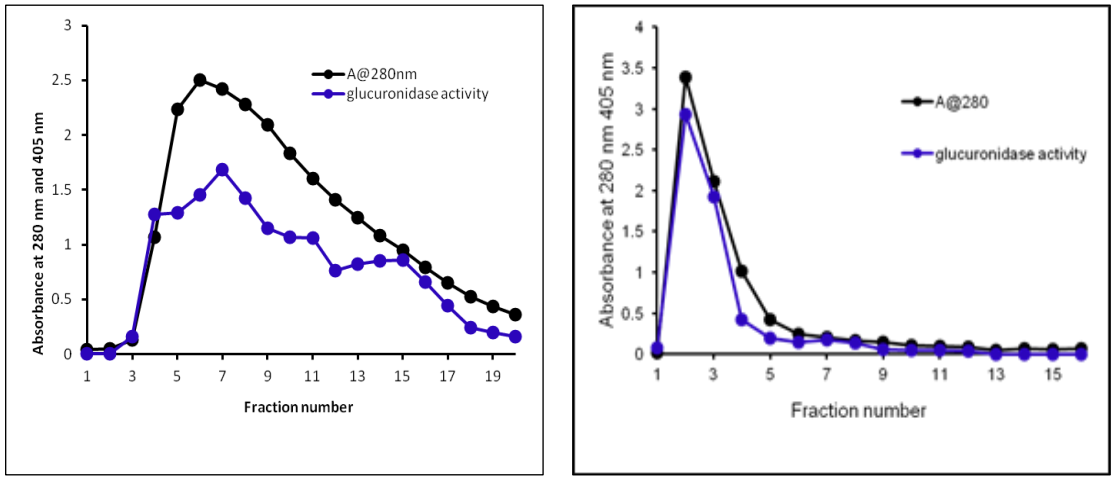
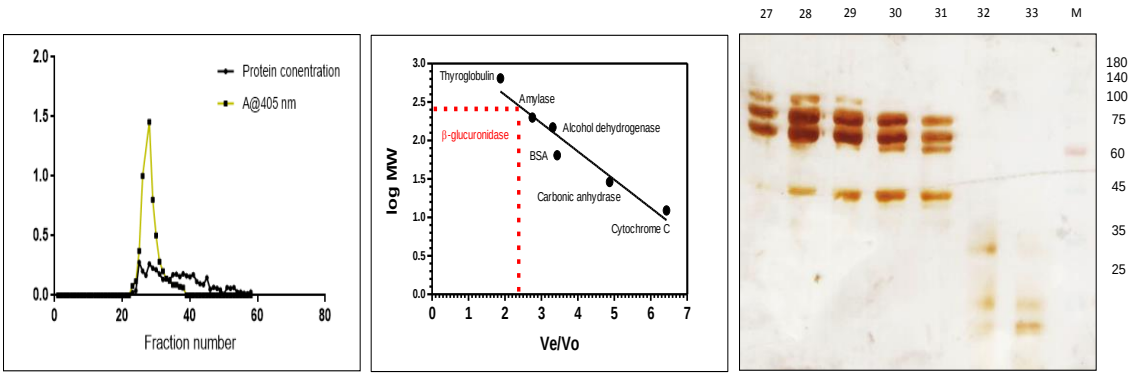


Figure. 1. Lysosomal hydrolase activity for crude soluble extract. The 4-nitrophenyl substrates were used to assay for the presence of lysosomal hydrolases in the soluble extract of *Lamellidens corrianus*. The assay was done in triplicates and plotted as mean ± SD. The activity was plotted as Units/ml.



A B

Figure. 2. (A). Elution profile of the enzyme from phenyl Sepharose gel. **(B)**. Elution profile of the enzyme from the DEAE-Sepharose gel**.**



A B C

Figure. 3. (A). Elution profile of the enzyme by Gel filtration chromatography (S-200**)**. The activity of enzyme was found in fractions 27-30. **(B).** The standard plot for molecular weight determination glucuronidase. The approximate molecular weight of glucuronidase was estimated to be 250 kDa. (**C).** The protein profile for the fractions 27 to 30 with glucuronidase activity was analyzed on a 10 % SDS-PAGE. The profile showed 4 bands in each lane.

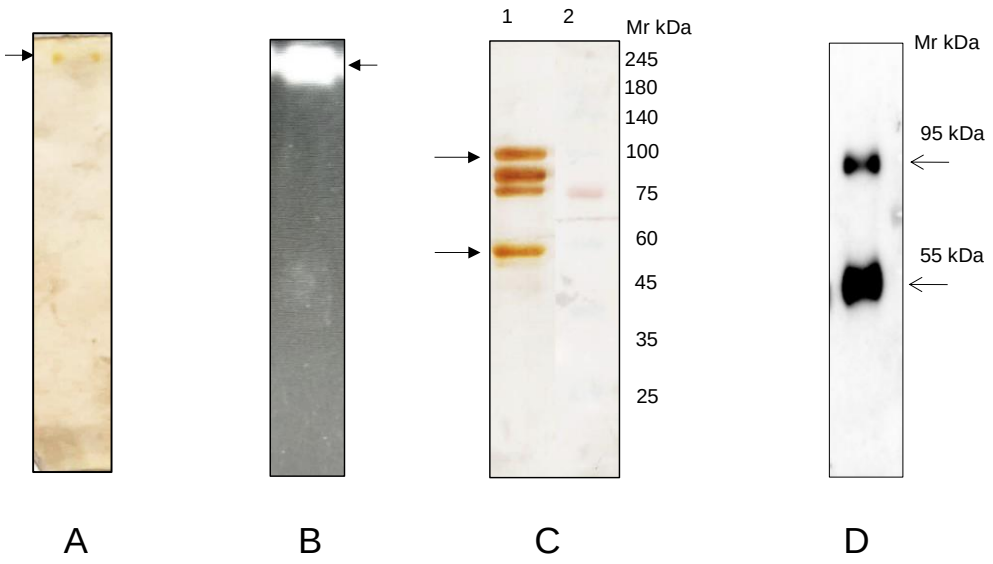


Figure. 4. (A). 10% Native PAGE of the purified glucuronidase, indicated by an arrow. (**B).** Activity staining using 4-methyl umbelliferyl-β-D-glucuronide substrate for the native PAGE. (**C).** 10% SDS-PAGE analysis of the purified glucuronidase (Lane 1: purified β-glucuronidase, lane 2: marker). (**D).** Western blot analysis for the purified β-glucuronidase with rabbit anti-human β-glucuronidase antibody. Arrow in each frame indicates the enzyme bands.

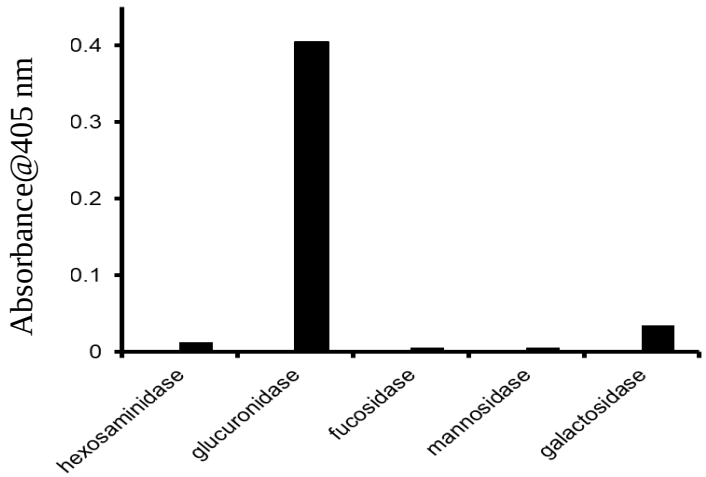


Figure. 5. Analysis of homogeneity of the purified enzyme. The purity of the enzyme was checked by determining activities of major glycosidases.

Table. 1. Purification Table for Lamellidens corrianus lysosomal β-glucuronidase**.**

S. No.	Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification fold	Yield (%)
1	Crude extract	920	3400	3.695	-	-
2	Phenyl Sepharose	178	1460	12.37	3.35	49.94
3	DEAE-Sepharose	8.6	730	84.88	22.97	21.47
4	Con A	1.6	284	177.5	48.037	8.35
5	S-200	0.64	230	359	97.158	6.76

2.3.2. Biochemical Characterization

Lysosomal β-glucuronidase protein purified from *L. corrianus* tissue was studied further to understand the biochemical properties and the structural features.

2.3.3. Assessing the effects of temperature and pH

The effect of temperature on the activity of β-glucuronidase was determined using 4-nitrophenol substrate and incubating at various temperatures from 10 °C to 100 °C. The enzyme showed increasing activity with increase in temperature until 70 °C marking its optimum [Figure 6A]. The activity was lost after 80 °C. The optimum pH value was evaluated by incubating the enzyme and the substrate in the buffers with varied pH in the range of 2 to 10. Lysosomal β-glucuronidase showed maximum activity at pH 5.0 which is a typical lysosomal hydrolase attribute [Figure 6B]. In the thermal stability experiments, the enzyme was incubated at different temperatures for 10 min and then kept on ice for 5 min. Post the incubation, substrate was added and the enzyme assay was performed both at 37˚C and 70˚C. In both the cases, β-glucuronidase was found to be stable up to 70°C [Figure 6 C and D]. The stability of the enzyme at different pH was examined by pre-

incubating the enzyme in the buffer for overnight. The assay was performed at two different temperatures 37 °C (standard temperature) and 70 °C (optimum temperature). Both the assays revealed that the protein was almost stable in the buffers with pH ranging from 3 to 8 [Figure 7 A and B]. Similarly, time dependent thermal stability experiment was performed by incubating the enzyme at respective temperatures (50, 60, 70, 80, and 90˚C) with different time points ranging from 10 min to 60 min. β-glucuronidase was found to be stable at 70˚C until 60 min when assayed at both 70˚C and 37˚C respectively [Figure 7 C and D].

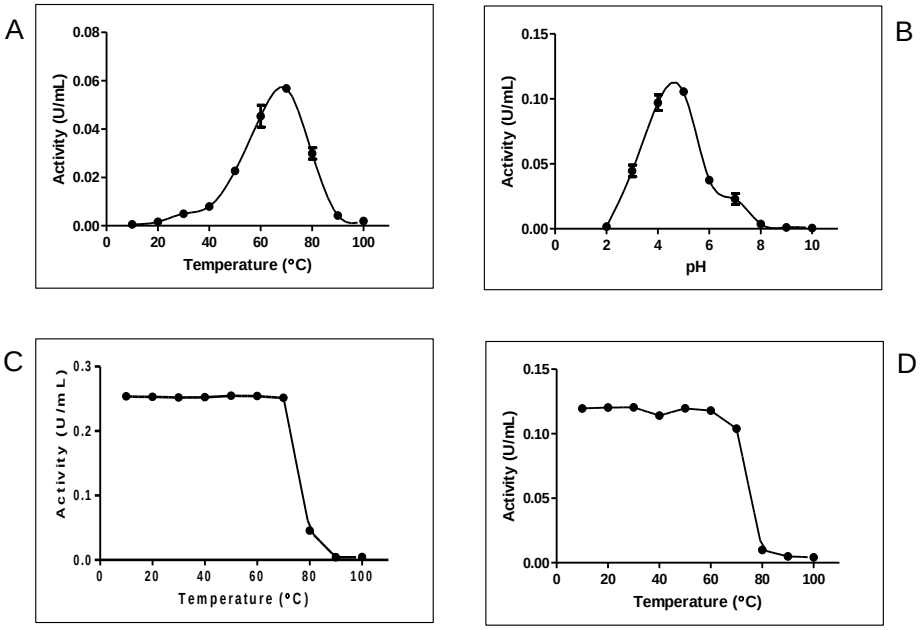


Figure. 6. (A). Effect of temperature on the activity of the enzyme **(B)**. Effect of pH on the activity of enzyme **(C).** Thermal stability of the purified enzyme assayed at 37˚C. **(D).** Thermal stability of the purified enzyme assayed at 70˚C.

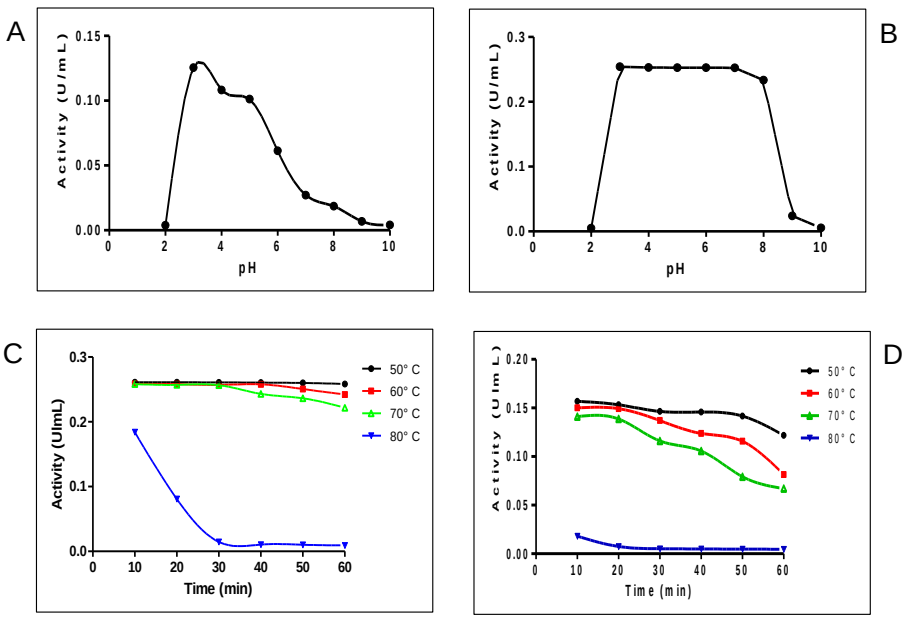


Figure. 7. (A). pH stability of β-glucuronidase (pH 2-10) assayed at 37˚C. **(B)**. pH stability of β-glucuronidase (pH 2-10) assayed at 70˚C. **(C).** Thermal stability at various time points (10-60 min) assayed at 70˚C. **(D).** Thermal stability at various time points (10-60 min) assayed at 37˚C.

2.3.4. Effect of metal ions and chemical reagents on the activity

The effect of various chemical reagents and metal ions on the activity of the purified β-glucuronidase was checked as mentioned in the methods. The sample with no addition of any reagent was considered as control and the activity obtained from it was considered as 100%. The routine laboratory reagents used in the study were Dithiotreitol (DTT) [used as a reducing agent], acrylamide (a flocculating agent), β-mercaptoethanol (reducing agent), EDTA (chelating agent) which showed very little effect on the activity of the enzyme. Sodium chloride interfered little in the activity resulting in nearly 20% reduction. These results indicate that the β-glucuronidase active site might not have amino acids that get affected by reduction and ionization agents. The denaturing agent, SDS showed complete inhibition in the activity as expected as the enzyme might have been denatured and lost its function (Figure 8).

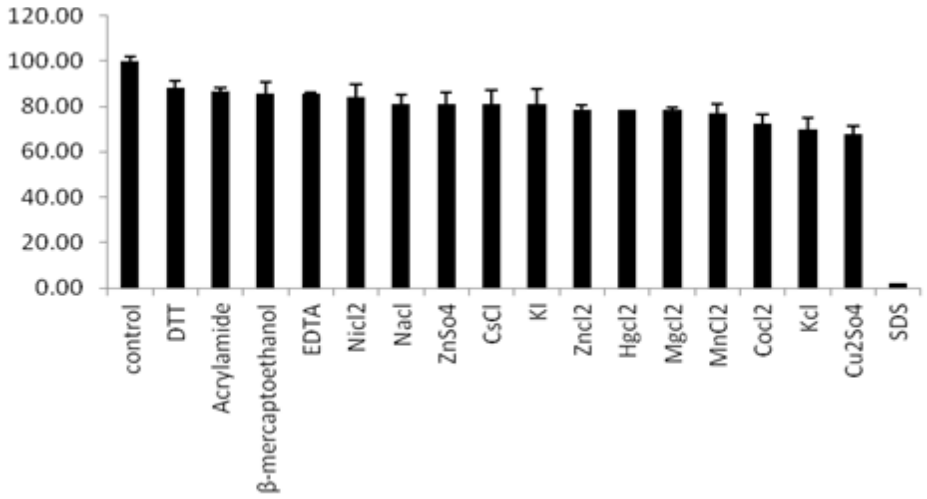


Figure. 8. The graph showing the effect of various chemical reagents and metals on the activity of the enzyme.

Table. 2. The effect of chemical reagents and the metals on the enzyme activity are with their respective percentage of activity

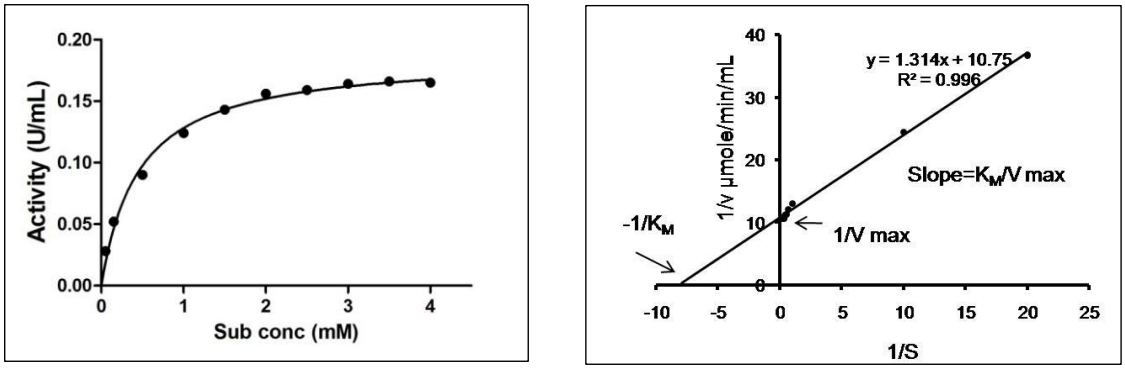
Reagent	**% of activity**
Control	100.00
DTT	88.25
Acrylamide	86.61
β-mercaptoethanol	85.79
EDTA	85.52
$NiCl_2$	84.15
NaCl	81.15
$ZnSO_4$	81.14
CsCl	81.14
KI	80.87
$ZnCl_2$	78.41
$HgCl_2$	78.37
$MgCl_2$	78.35
$MnCl_2$	76.77
$CoCl_2$	72.40
KCl	69.94
$Cu2SO_4$	67.76
SDS	1.91

Metals $NiCl_2$, $ZnSO_4$, CsCl, KI, $ZnCl_2$, $HgCl_2$, $MgCl_2$, $MnCl_2$, $CoCl_2$, KCl and Cu_2SO_4 showed 20-30 % inhibition in the activity of the enzyme mentioned in the increasing order of their effect (Table 2).

The metals like Ni^{+2}, Zn^{+2}, Hg^{+2}, Mn^{+2}, Co^{+2} and Cu^{+2} are considered as heavy metal contaminants which are regularly used to estimate the environmental pollution. Further analysis with varying concentrations of these metals and enzyme might help to estimate the minimum inhibitory concentrations.

2.3.5. Enzyme Kinetics

The kinetic parameters for purified lysosomal β-glucuronidase were determined using different substrate concentrations as mentioned in methods. The Michaelis-Menten plot showed the saturation point for substrate [Figure 8A], and from the Lineweaver-Burk plot, V_{max} and K_M values were calculated to be 0.457 mM and 0.11867 $\mu mol^{-1}min^{-1}mL^{-1}$ respectively [Figure 8B].



A B

Figure. 8. Graph representing the enzyme kinetic analysis. A: Michaelis–Menten kinetics graph showing the saturation of enzyme-substrate complex (Vmax). K_M value at ½ Vmax was calculated. **B**: Lineweaver-Burk plot showing the linear regression curve was analyzed for both the K_M and Vmax values. The X-intercept showed $-1/K_M$ value and Y-intercept showed 1/Vmax value.

These values are similar to the known vertebrate enzymes (Chilke, 2010; Lin et al., 1975). The K_M value suggests that the mollusc β-glucuronidase shows more affinity towards the synthetic substrate used in the assay than the previously studied enzymes.

2.3.6. Secondary Structure analysis

The secondary structural analysis using CD spectroscopy at far UV revealed that the β-glucuronidase from *L. corrianus* is dominated by α-helices and random coils with ~16% beta sheets [Figure 9]. Human β-glucuronidase structure is known to have domains with barrel like structures (Jain et al., 1996). Thermal folding experiments showed that the enzyme was found to be stable till 70˚C supporting the biochemical data.

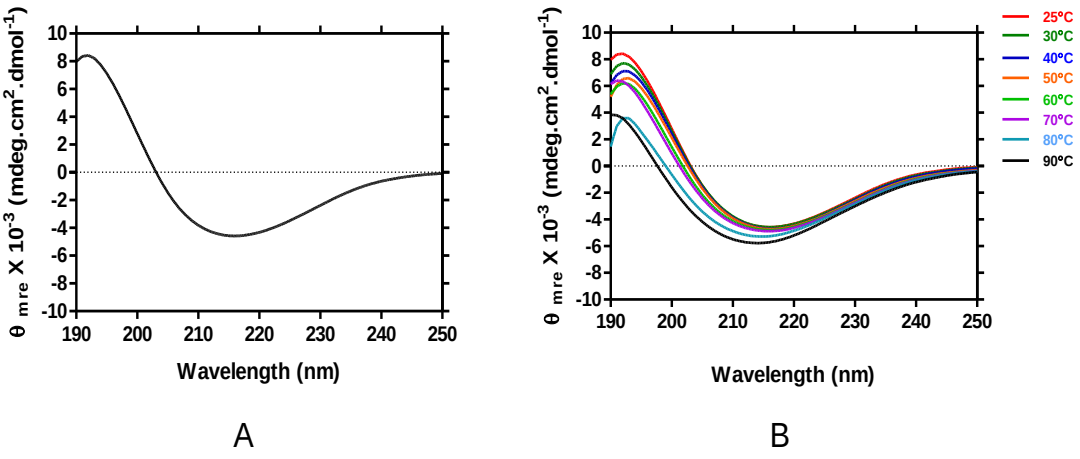


Figure. 9. Secondary structure of β-glucuronidase. **(A).** Far UV-CD spectrum of β-glucuronidase (0.1 mg/mL) at 25˚C. **(B).** Effect of temperature on the secondary structure, far UV-CD spectra were recorded at 25, 30, 40, 50, 60, 70, 80, 90˚C.

Table. 3. The percentages of the secondary structure forms of the enzyme.

Helix	Antiparallel	Parallel	Beta turn	Random coils	Total
34.2	7.2	8.6	16.6	33.4	100

2.4. Discussion

A lysosomal β-glucuronidase was purified from *L. corrianus* to homogenity in this study. The crude soluble extract identified with the enzyme activity was subjected to chromatographic separation in a step wise manner. The fractions with the β-glucuronidase activity from phenyl Sepharose elutions were pooled and passed on DEAE Sepharose column where the elution with 50 mM NaCl allowed the removal of the β-glucuronidase protein bound to the column. The final separation of β-glucuronidase from all the other contaminants was achieved by a size exclusion chromatography. The fractions obtained in this step were analyzed for protein profile on SDS-PAGE under reducing conditions which resulted in identification of four polypeptides. The same sample when separated on a native PAGE moved as single band under non-denaturing and non-reducing conditions indicating the purified protein to be a hetero tetramer. Most of the known β-glucuronidases till date however occur as homo tetramers (Gupta and Singh, 1983; Ikegami et al., 1995; Kim et al., 1995). The β-glucuronidase protein from the vertebrates mostly occurs as homotetramer with an overall molecular mass of 280-300 kDa with a single subunit of ~70 kDa (Jain et al., 1996). The enzyme purified and characterized from mouse kidney (Lin et al., 1975) and *Labeo rohita* (Chilke, 2010) showed that β-glucuronidase is heat stable and K_M values were found to be 1.18×10^{-4} M and 2.907 mM respectively.

The purified protein was analyzed for its activity on a gel based assay where a fluorescent substrate reacted with the enzyme separated on a native PAGE. The signal proved that the purified protein is indeed a β-glucuronidase. When an immunoblot analysis with a rabbit anti human β-glucuronidase antibody was performed with the protein separated on SDS-PAGE, two signals corresponding to the bands around 95 kDa and 55 kDa were visualized,

indicating the cross reactivity of an invertebrate protein with antibody against mammalian protein.

The purified β-glucuronidase was further used for biochemical analysis. The optimum pH and temperature were calculated to be 5.0 and 70 °C respectively which is a characteristic feature of lysosomal enzymes studied so far. In a separate experiment, the effect of pH on the stability of the enzyme was determined and the residual activity was checked both at standard (37 °C) and optimum (70 °C) temperatures. In both the assays, the enzyme was stable until pH 8 and then the activity was lost. Similarly, thermal stability was assessed for the purified protein where the enzyme showed good stability until 70 °C, which is comparable with earlier findings. In another assay, time dependent thermal stability was analyzed and the enzyme was found to be stable at 70 °C for one hour. The secondary structural analysis by CD spectroscopy revealed that the purified β-glucuronidase from *L.corrianus* occurs mostly as an alpha helical structure. Thermal unfolding of glucuronidase at different temperatures revealed that the enzyme was stable up to 70˚C which strongly supports the biochemical data of the thermal stability of the enzyme. The kinetic parameters for the enzyme were estimated and the K_M and Vmax values obtained were 0.457 mM and 0.11867 $\mu mol^{-1}min^{-1}mL^{-1}$ respectively.

Hence, a thermally stable lysosomal β-glucuronidase was purified (97.15 fold) from the freshwater invertebrate *Lamellidens corrianus* having more affinity towards the substrate 4-nitrophenyl-β-D-glucuronide with a 6.76% yield. Several lines of evidence strongly suggest that the enzyme purified in this study is the lysosomal β-glucuronidase. i. It can bind strongly on phenyl Sepharose gel like the other known lysosomal hydrolases as studied from our lab earlier (Venugopal and Siva Kumar, 2013), ii. Its large molecular

mass and its multi subunit nature, iii. Immune-reactivity of two subunits with an antibody to human enzyme, iv. pH optima of 5.0 and v. exhibiting largely alpha helical content in its secondary structure.

Chapter 3

Ligand binding studies of β-hexosaminidases and MPRs from *L. corrianus*

3.1. Introduction

The cation-independent mannose 6-phosphate receptor (MPR 300) and cation-dependent mannose 6-phosphate receptors (MPR 46) play an important role in the function of lysosomes by targeting newly synthesized lysosomal enzymes to lysosomes. This function of MPRs is accomplished by their ability to bind the mannose 6-phosphate (M6P) residues present on the lysosomal hydrolases. Along with secretory proteins and other glycoproteins, newly synthesized lysosomal hydrolases are glycosylated and modified in endosomal system. The N-glycans of the lysosomal hydrolases are trimmed and modified with phosphomannosyl residues in the Golgi. Two enzymes, GlcNAc phosphotransferase and N-acetylglucosamine-1-phosphodiester α-N-acetylglucosaminidase in Golgi compartments are responsible for this modification (Bohnsack et al., 2009). MPR 300 is a multifunctional protein and other than binding to mannose 6-phosphate present on lysosomal hydrolases, it also binds to retinoic acid, insulin like growth factor (IGF-II), and urokinasetype-1 plasminogen activator receptor (Kang et al., 1997; Nykjær et al., 1998). MPR 300 contains 15 homologous extracellular domains called as Man-6-P Receptor Homology (MRH) domains having similar size (Munro, 2001). The domains 3, 5, and 9 are responsible for M6P binding. MPR 46 is a homodimer and contains only one binding site for M6P per polypeptide. MPR 46, unlike MPR 300 is involved only in binding to phosphomannosyl residues present on acid hydrolases and facilitate their transport intracellularly. Earlier reports based on structural and mutagenic studies revealed that four amino acid residues (Glutamine, Arginine, Glutamic acid and Tyrosine) are important for M6P binding on these domains (Castonguay et al., 2011).

β-N-acetylhexosaminidases (EC 3.2.1.52) are the members of glycosyl hydrolases (GH) family involved in the catalysis of hydrolysis reaction removing the terminal N-acetylhexosamine residues from the non-reducing ends of oligosaccharides and glycoconjugates (Lv et al., 2018). Three different isoforms are known to be present in humans. β- hexosaminidase A (hex A) is a heterodimer consisting of covalently linked α and β subunits. β- hexosaminidase B and β- hexosaminidase S are homodimers with covalently linked β and α subunits respectively (Wendeler and Sandhoff, 2009). In humans β-hexosaminidases plays an important role in the catabolism of glycosphingolipids. Defects in these enzymes causes various lysosomal storage disorders. Deficiency in hex A causes Tay-Sachs disease (Gray-Edwards et al., 2018), a neurological disorder and deficiency of both hex A and hex B results in a lysosomal lipid storage disorder called Sandhoff's disease (Lecommandeur et al., 2017).

MPR 300 and MPR 46 are found to be expressed in mammals, vertebrates and some invertebrates. The M6P binding properties of MPRs have been studied extensively in mammals and other vertebrates (Castonguay et al., 2012). In invertebrates the binding properties of these receptors is under explored. Our lab has studied lysosome biogenesis in vertebrates and some invertebrates by identifying and characterizing MPRs from these organisms. Although it is well characterized, the information regarding lysosomal enzymes and their sorting in invertebrates is scarce so far. In invertebrates we have isolated and purified MPRs and lysosomal enzymes from mollusc, starfish, hydra and the ligand binding of lysosomal enzymes to MPRs has been determined qualitatively by ligand blot analysis (Kumar and Kumar, 2018; Venugopal and Kumar, 2014; Visa et al., 2012).

In order to study the ligand binding properties of lysosomal enzymes and receptors quantitatively, the present study was undertaken where the interaction of MPRs with lysosomal isoforms of β-hexosaminidases was determined quantitatively using surface plasmon resonance (SPR). β-hexosaminidases (hex A and hex B) as representive glycosidases from *Lamellidens corrianus* were used to determine the equilibrium constants for MPR 300 and 46 receptors from goat.

3.2. Materials and methods

3.2.1 Materials

L. corrianus animals used for the purification of two isoforms of β-hexosaminidase were supplied by UV Scientifics, Hyderabad, India. Mussel tissues were collected by de-shelling the animals and soft tissue was immediately frozen at -80˚C. Goat liver used for the purification of MPRs was obtained from local slaughter house. Phenyl Sepharose CL-4B, DE-52 cellulose, and Con A-Sepharose matrix were obtained from Sigma Aldrich, St. Louis, MO, USA. CM 5 censor chip used for surface plasmon resonance (SPR) was purchased from GE healthcare. All the sugars used for SPR analysis and reagents used in the present study were procured from SRL chemicals, Mumbai, India.

3.2.2. Methods

3.2.2.1. Protein extraction from Lamellidens corrianus

Fifty grams of de-shelled soft tissue of *L. corrianus* was taken for soluble protein extraction. This tissue was homogenized with 10 volumes (500 mL) of 50 mM sodium acetate buffer pH 5.0. The blended homogenate was stirred overnight at 4˚C. The homogenate was clarified by centrifugation at 9,000 rpm for 30 min and from the clear supernatant obtained, proteins were salted out by adding solid ammonium sulphate (80%)

[$(NH4)_2SO_4$] while stirring in cold. The suspension was centrifuged at 10000 rpm, for 30 min) and the pellet obtained protein pellet was dissolved in 50 mM sodium acetate buffer pH 5.0 containing 1 M $(NH4)_2SO_4$ (buffer A).

3.2.2.2. Purification of β-hexosaminidase A (hex A) from soluble extract of *Lamellidens corrianus*

β-hexosaminidase was purified from the soluble extract of *L. corrianus* as described previously (Venugopal and Sivakumar, 2013b). Briefly, the protein pellet dissolved in buffer A was applied to a phenyl Sepharose gel (5 mL) packed in a glass column. After extensive washing with buffer A, the bound proteins were eluted using buffer A without $(NH4)_2SO_4$. The eluted fractions containing the activity of hex A were pooled and dialyzed against 25 mM Tris-HCl buffer pH 7.4 (buffer B). After elution, the gel was saved for further use as explained below. The dialyzed sample was subjected to DE-52 cellulose chromatography where the gel was pre-equilibrated with buffer B. After washing the gel extensively with buffer B, it was eluted using step wise gradient of NaCl in buffer B (50 mM, 100 mM, 150 mM and 200 mM). The active fractions of hex A present in 100 mM elutes were pooled and subjected to Con A chromatography. The gel was pre-equilibrated with buffer B containing 0.5 mM $CaCl_2$, 0.5 mM $MnCl_2$, and 0.5 M NaCl. After washing the gel, bound proteins were eluted using 0.1 M, 0.2 M, and 0.3 M methyl-α-D-mannopyranoside in column buffer. The activity of hex A was found in 0.1 M methyl-α-D-mannopyranoside elutes, which were pooled and used for further analysis.

3.2.2.3. Purification of β-hexosaminidase B (hex B) from soluble extract of Lamellidens corrianus

The phenyl Sepharose gel used above and saved, was washed with buffer A. Hex B which was bound still bound to this column was eluted using buffer B pH 5.0. The activity of β-hexosaminidase was checked in the eluted protein fractions. The active fractions were pooled and dialyzed against buffer B. The sample obtained was further processed for the purification of hex B by employing DE-52 and Con A chromatography, as described above for hex A.

3.2.2.4. Extraction of total membrane proteins from Goat liver

Five hundred grams of goat liver was used to prepare acetone powder as described earlier (Kumar, 1996). All the operations for the extraction of membrane proteins were performed at 4˚C. About 50 g of goat liver acetone powder was homogenized and stirred overnight with 6 volumes of 50 mM imidazole-HCl buffer pH 7.0, containing 150 mM NaCl and 0.1 mM PMSF. The pellet obtained after the centrifugation of homogenate at 9000 rpm for 20 min was again homogenized with 6 volumes of 50 mM sodium acetate buffer pH 5.0, 150 mM NaCl. The suspension obtained was again centrifuged at 9000 rpm for 20 min. A final homogenization of the pellet was done with 6 volumes of 50 mM imidazole-HCl buffer pH 7.0, 150mM NaCl containing 5 mM sodium β-glycerophosphate. To this suspension 1% Triton X-100 and 0.1% sodium deoxycholate were added and stirred overnight. This mixture was then centrifuged at 4000 rpm for 15 min and the supernatant obtained was again centrifuged at 9000 rpm for 45 min to get a clear supernatant containing membrane proteins. This membrane extract was used for the purification of MPRs.

3.2.2.5. Purification of MPR 300 from goat liver membrane extract

Phosphomannan (PM) coupled to Sepharose gel was used for the purification of both MPR 300 and 46 as described earlier (Yadavalli and Nadimpalli, 2008). Briefly, for the purification of MPR 300, 2 mM EDTA was added to the membrane extract obtained from the above step. PM gel was equilibrated with 50 mM imidazole buffer pH 7.0, containing, 150 mM NaCl, 5 mM sodium β-glycerophosphate, 0.05% Triton X-100, and 2 mM EDTA (column buffer). The membrane extract was allowed to pass on equilibrated PM gel several times. After extensive washing with column buffer, MPR 300 bound to PM gel was eluted using 5 mM M6P. The purified MPR 300 was analyzed on 10% SDS PAGE.

3.2.2.6. Purification of MPR 46 from goat liver membrane extract

MPR 46 from goat liver membrane extract was purified in the same way as described above except that 10 mM MnCl2, CaCl2, and MgCl2, were added to the membrane extract and applied to PM gel equilibrated with column buffer containing the three divalent metal ions without EDTA. Bound MPR 46 was eluted using 5 mM M6P and the purified receptors were analyzed on 10%SDS PAGE.

3.2.2.7. Electrophoresis analysis

The homogeneity of purified hex A and B were analyzed on 10% Native and SDS PAGE. Resolved proteins were visualized by both Coomassie and silver staining methods. The homogeneity of purified receptors (MPR 300 and 46) were checked on 7.5% SDS PAGE and resolved receptors were visualized by silver staining method.

3.2.2.8. Surface plasmon resonance (SPR) analysis

To study the affinity of goat MPRs to hex A and hex B, SPR technique was employed. Biacore T 200 (GE Healthcare) instrument was used for this study. Purified hex A and B

were immobilized on CM-5 sensor chip according to the manufacturer's instructions. Briefly, the surface of the sensor chip was activated using amine coupling method. In this method 1:1 mix of 0.4 M 1-ethyl-3(3-dimethylaminopropyl cabodiimide (EDC) and 0.1 M N-hydroxy-succinimide (NHS) was added to activate the surface of the chip. One and three flow cells of the chip were used as blanks and flow cells 2 and 4 were used to immobilize hex A and B respectively. 20µg/mL concentration of hex A and B in 10 mM sodium acetate buffer, pH 4.5 were injected to the activated chip with a flow rate of 30 µL/min. Finally, after immobilization the uncoupled sites present on the surface were blocked with 1 M ethanolamine.

To determine the affinities, MPR 300 and 46 were prepared in 10 mM PBS, pH 7.4, containing 0.05% P20 (running buffer). These analytes were injected to the sensor chip in a volume of 80 µL with a flow rate of 30 µL/min. A contact time of 60 seconds and a dissociation time of 120 seconds was given for the analysis. To reuse the sensor chip for other analytes regeneration with 10 mM glycine HCl, pH 2.0 was performed with a contact time of 30 seconds.

In addition to MPRs, various sugars were used as analytes to determine their affinities towards hex A and B. The sugars used were glucosamine, galactosamine, N-acetyl-glucosamine, N-acetyl-galactosamine, mannose, methyl-α-D-mannopyranoside, and mannosamine. A concentration ranging from 5 mM to 60 mM was used for determining the affinities. All the SPR experiments were performed at 25˚C and each interaction study was performed at least two times.

3.3. Results

3.3.1. Purification of β-hexosaminidase A (hex A) from soluble extract of *Lamellidens corrianus*

The proteins from the soluble extract of *Lamellidens corrianus* tissue showing β-hexosaminidase activity were precipitated by adding 80% ammonium sulphate. Hydrophobic affinity chromatography using phenyl-Sepharose gel was performed and the bound proteins were eluted using 50 mM sodium acetate buffer pH 5.0. The fractions containing the activity of β-hexosaminidase were pooled, dialyzed and subjected to ion exchange chromatography using DE-52 as a matrix. The bound proteins were eluted using different concentrations of NaCl. 100 mM NaCl eluted proteins showed maximum activity of β-hexosaminidase and these fractions were passed on Con A affinity chromatography. After washing the gel, the bound proteins were eluted with increasing concentrations of methyl α-D- mannopyranoside; 100 mM, 200 mM, and 300 mM respectively. The activity of β-hexosaminidase was present only in 100 mM elutions. The purified hex A eluted with 100 mM methyl α-D-mannopyranoside migrated as two bands under both reducing and non-reducing conditions on 10% SDS-PAGE, with molecular masses of about 75 kDa and 30 kDa respectively (Figure. 1A). When analyzed on native PAGE, hex A migrated as a single protein, as detected by Coomassie staining (Figure. 1B).

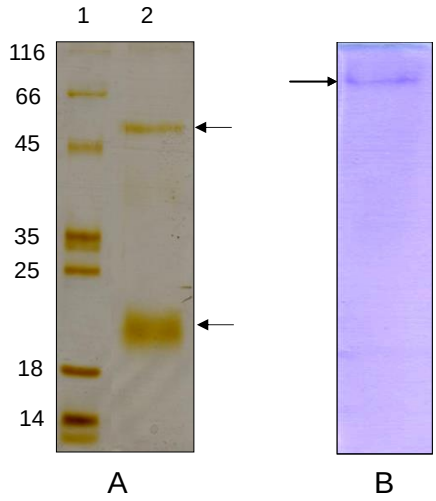


Figure.1. PAGE analysis of β-hexosaminidase A. **(A).** 10% SDS-PAGE for purified β-hexosaminidase A (Lane 1: protein ladder; lane 2: Purified hex A **(B)**. 7.5% Native PAGE analysis: Purified hex A. Arrows indicate the purified enzyme.

3.3.2. Purification of β-hexosaminidase B (hex B) from soluble extract of *Lamellidens corrianus*

β-hexosaminidase B was purified using methodology described under material and methods. The same phenyl Sepharose gel from which Hexosaiminidase A was eluted was utilized for eluting bound hex B using 25 mM Tris-HCl buffer pH 7.4. The eluted proteins also exhibited hexosaminidase activity, and hence this enzyme was termed as hex B. Con A chromatography was followed to also purify hex B. The purified enzyme migrated as a single band on native PAGE (Figure. 2B). The enzyme however migrated as a single band in 10% SDS-PAGE under reducing and non-reducing conditions with an apparent molecule mass of 40 kDa (Figure. 2A).

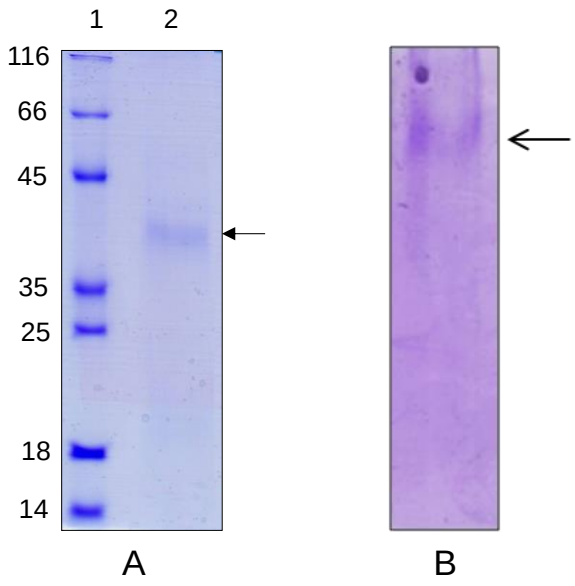


Figure. 2. PAGE analysis of β-hexosaminidase B. **(A).** 10% SDS-PAGE for purified β-hexosaminidase B (Lane 1: protein ladder; lane 2: Purified hex B). **(B). 7.5%** Native PAGE analysis of purified hex B. Arrows indicate the purified enzyme.

3.3.3. Purification of MPR 300 and MPR 46 from membrane extract goat liver

The total membrane extract obtained from the goat liver acetone powder was used to purify the two receptors MPR 300 and 46. Two separate phosphomannan-Sepharose gels (PM gel) were used for the purification of the receptors. For the purification of MPR 300, EDTA was added to the membrane extract at a final concentration of 2 mM. Same concentration of EDTA was included in column buffer which was used for the equilibration of PM gel that is used for MPR 300 purification. Since MPR 300 is a cation independent receptor and does not require divalent metal ions for its binding to ligands, EDTA was included to avoid MPR 46 binding. After washing the gel with column buffer, the bound MPR 300 was eluted from PM column using column buffer containing 5 mM mannose-6-phosphate. M6P is a strong ligand for MPRs and the concentration used for elution ensures complete desorption

of the receptor from the PM gel. For the purification of MPR 46 divalent metal ions ($MnCl_2$) was added to the membrane extract processed separately at a final concentration of 10 mM. The metal ion was also included in the column buffer used for the equilibration of PM gel utilized in MPR 46 purification. Since MPR 46 is a cation dependent receptor and requires divalent metal ions for its binding to ligands. 5 mM M6P was used to elute bound MPR 46 from the PM gel. Both the eluates from the two columns were checked for their homogeneity by 10% SDS PAGE. The results shown in Figure. 3 indicate a single protein band corresponding to MPR 300 (Lane 1) and a single protein band corresponding to MPR 46 (Lane 2). Silver staining method was employed to visualize the resolved receptors.

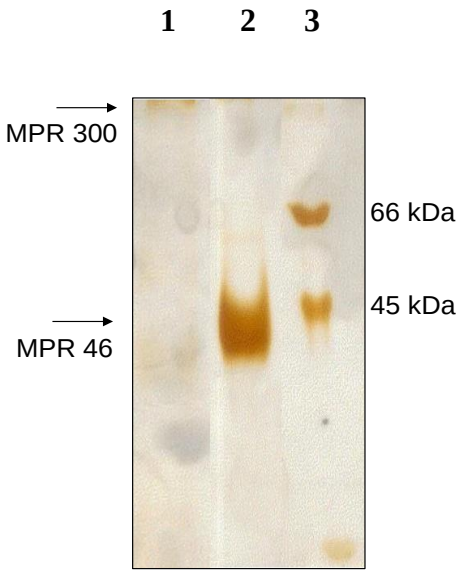
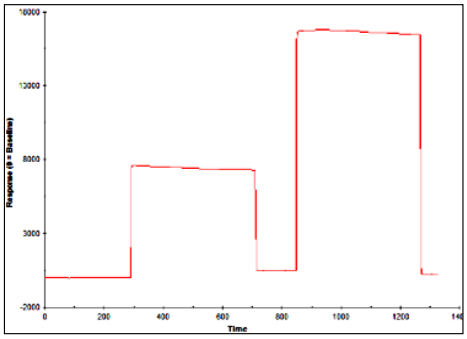


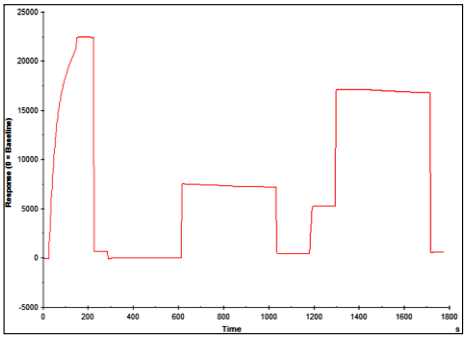
Figure. 3. SDS-PAGE analysis of MPR 300 and 46. Purified goat receptors by two different PM Sepharose gels were resolved on 10%SDS PAGE and detected using silver staining. Lane 1: Purified Goat MPR 300; lane 2: Purified Goat MPR 46; lane 3: Molecular weight markers.

3.3.4. Ligand binding analysis of β-hexosaminidases with MPRs (SPR analysis)

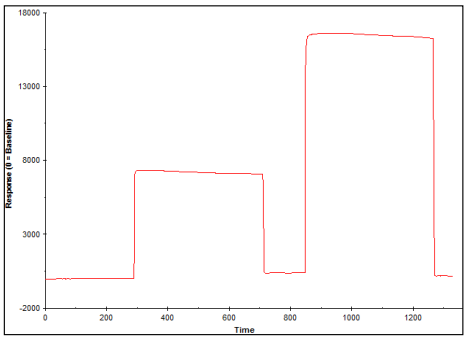
To analyze the interaction of hex A and hex B with goat MPRs surface plasmon resonance technique was employed. Lysosomal enzymes consist of mannose-6-phosphate on their glycans, which has high-affinity for the MPRs. For studying this affinity, purified hex A and B were immobilized on CM5 sensor chip. Amine coupling method was used for immobilization where the amine groups present on proteins gets immobilized on CM-dextran matrix. The sensorgrams for immobilization reached 628.8 and 435.5 response units (RU) for hex A and B respectively (Figure. 4).



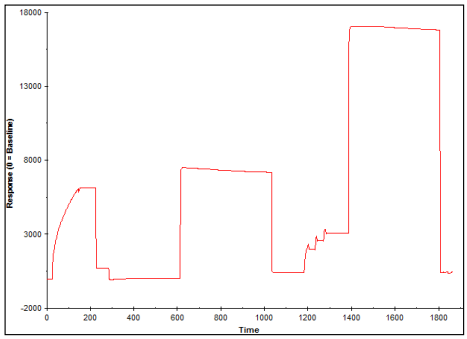
Blank 1, RU coated: 205.5



Hex A, RU coated: 628.8



Blank 2, RU coated: 173.6



Hex B, RU coated: 435.5

Figure. 4. Immobilization of hex A and hex B on the surface of CM 5 sensor chip. Hex A and hex B were immobilized on the surface of CM 5 sensor chip by employing amine coupling method. Flow cell 1 and 3 were used as blanks, 2 and 4 cells were used for immobilizing hex A and hex B respectively. A final response of 628.8 and 435.5 RU were coated for hex A and hex B respectively.

To determine the affinity of MPR 300 with hex A and B different concentrations of MPR 300 (30, 60, 120, 180, 240, 300 µg/mL) were injected to sensor chip. Figure 5 A, and C represents the sensorgram showing the interaction of MPR 300 with hex A and with hex B respectively. The K_d (equilibrium constant) values obtained for the interactions of MPR 300 with hex A and B are 145 nM and 25.3 mM respectively (Figure 5 B, and D). To determine the affinity of MPR 46 with hex A and B different concentrations of MPR 46 (1.25, 2.5, 5, 7.5, 10 µg/mL) were injected to sensor chip. Figure.6 A, and C represents the sensorgram showing the interaction of MPR 46 with hex A and B respectively. The K_d values obtained for the interactions of MPR 46 with hex A and B are 46 nM and 87.3 nM respectively (Figure. 6 B, and D)

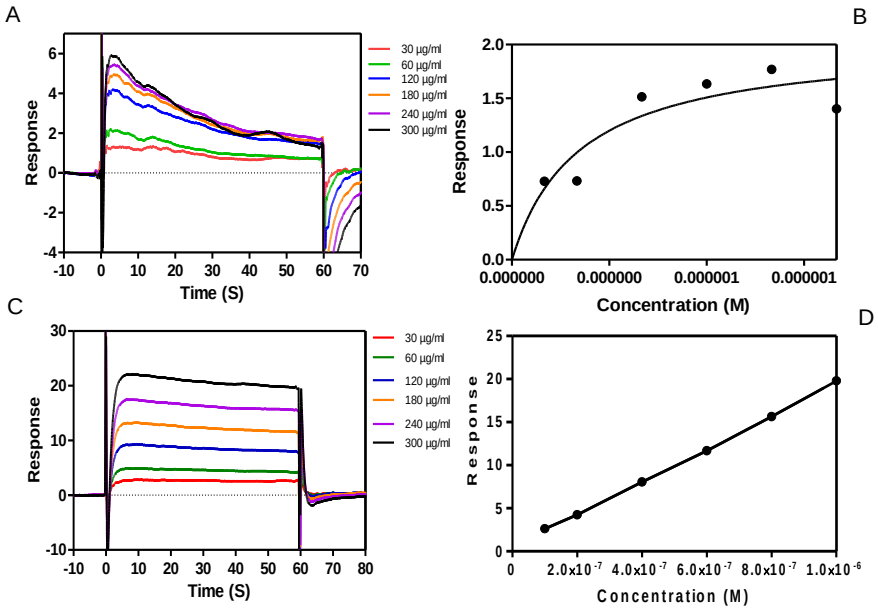


Figure. 5. Interaction of hex A and hex B with goat MPR 300**. (A).** Sensorgram showing the interaction of hex A with different concentration of MPR 300 (30, 60, 120, 180, 240, 300 µg/mL). **(B).** Affinity curve of hex A with MPR 300. The K_d value obtained was found to be 145 nM **(C).** Sensorgram showing the interaction of hex B with different concentration of MPR 300 (30, 60, 120, 180, 240, 300 µg/mL). **(D).** Affinity curve of hex A with MPR 300. The K_d value obtained was found to be 25.3 mM.

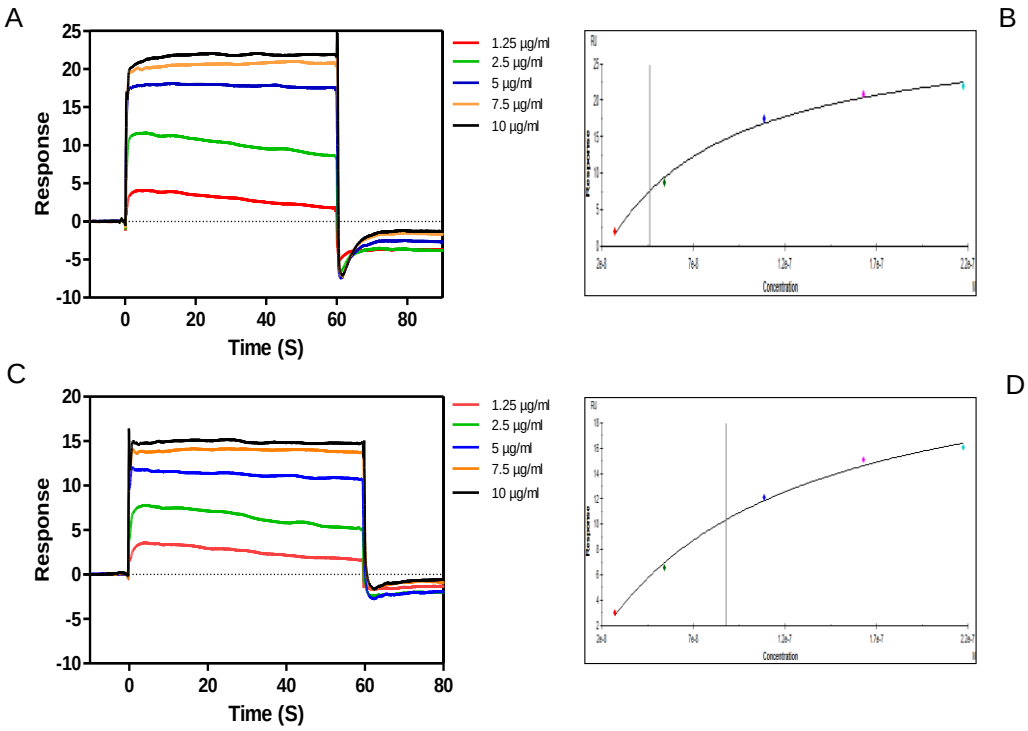


Figure. 6. Interaction of hex A and hex B with goat MPR 46. **(A).** Sensorgram showing the interaction of hex A with different concentration of MPR 46 (1.25, 2.5, 5, 7.5, 10µg/mL). **(B).** Affinity curve of hex A with MPR 46. The K_d value obtained was found to be 46 nM. **(C).** Sensorgram showing the interaction of hex B with different concentration of MPR 46 (1.25, 2.5, 5, 7.5, 10µg/mL). **(D).** Affinity curve of hex B with MPR 46. The K_d value obtained was found to be 87.8 nM.

3.3.5. Ligand binding analysis of β-hexosaminidases with sugars (SPR analysis)

The affinity of hex A and hex B with various sugars were also tested. The sugars tested for interaction studies are glucosamine, galactosamine, mannosamine (amino sugars), N-acetyl-glucosamine, N-acetyl-galactosamine, mannose, and methyl-α-D-mannopyranoside. These sugars were chosen because of their inhibitory property on the activities of β-hexosaminidase. The concentration of the sugars taken for interaction studies were 5, 10,

20, 30, 40, 50, and 60 mM. For each sugar a contact time of 60 sec and a dissociation time of 60 sec with a flow rate of 30 µL/min condition was given. After each study the chip was regenerated with 10mM Gly-HCl pH 2.0. Interestingly, hex A has not shown any significant binding with the sugars tested (Figure. 7). Hex B has shown significant binding with all the sugars tested, which can be observed from the sensorgrams obtained (Figure. 8-10).

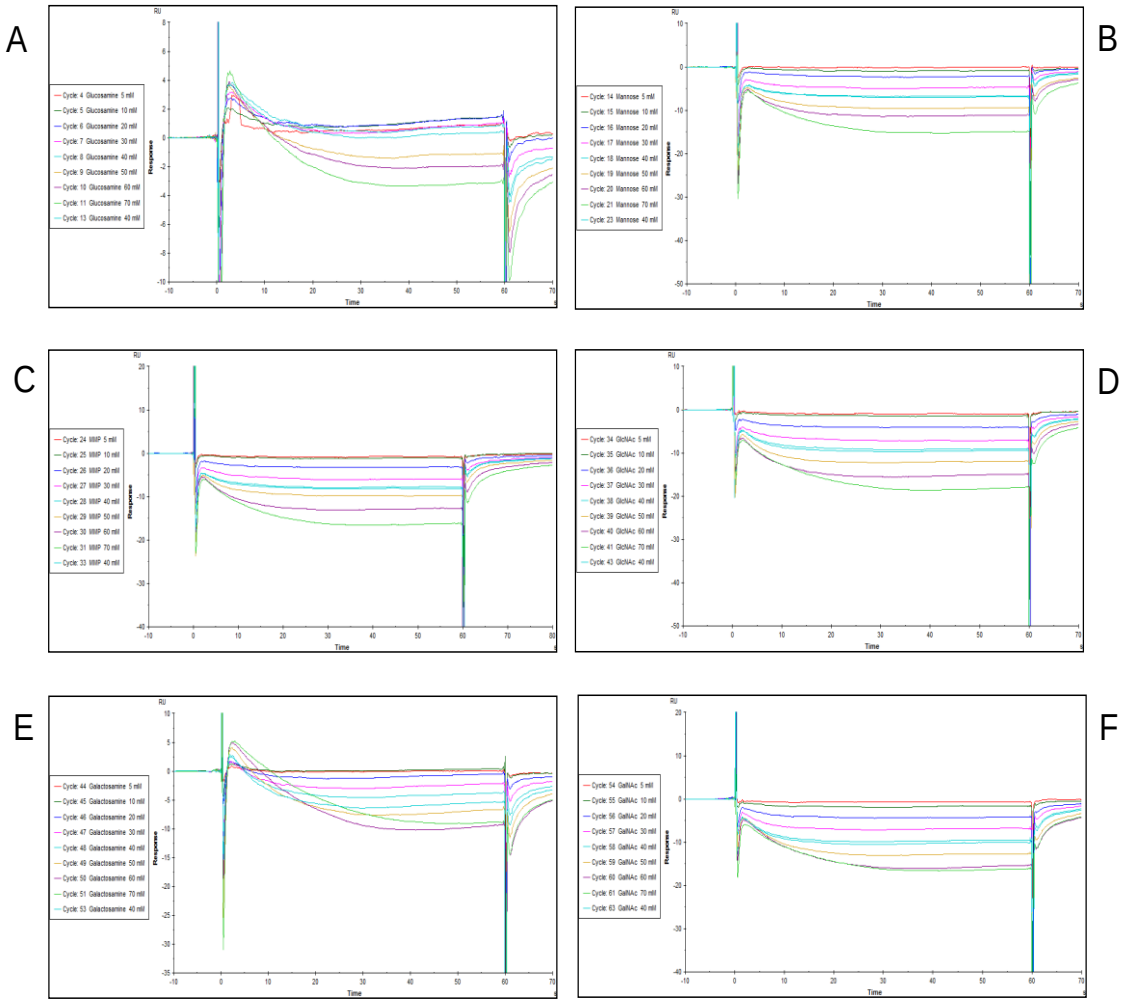


Figure. 7. Interaction of hex A with different sugars. Sensorgrams showing the interaction of hex A with different sugars; **(A)** glucosamine, **(B)** mannose, **(C)** methyl-α-D-mannopyranoside, **(D)** N-acetyl-glucosamine, **(E)** Galactosamine, **(F)** N-acetyl-galactosamine. From the sensorgram it is clear that there is no significant binding of sugars with hex A.

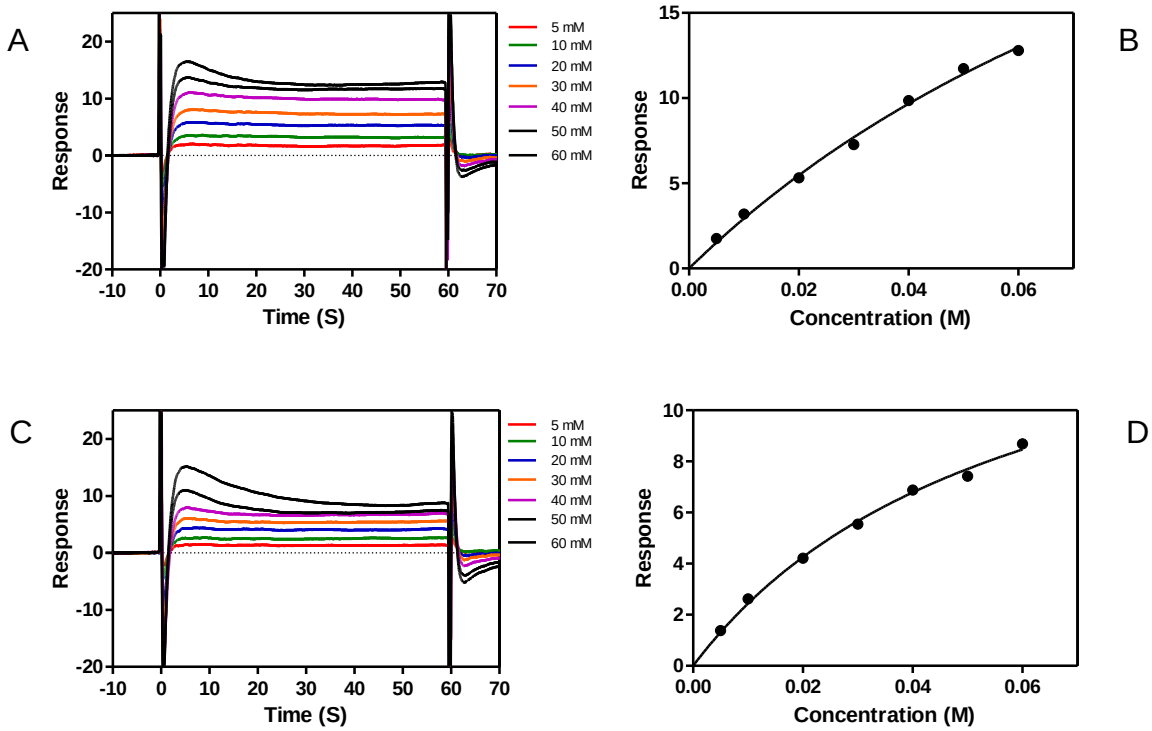


Figure. 8. Interaction of hex B with different sugar**s. (A).** Sensorgrams showing the interaction of hex B with N-acetyl-glucosamine at different concentrations ranging from 5 mM to 60 mM. **(B).** Affinity curve of hex B with N-acetyl-glucosamine **(C).** Sensorgrams showing the interaction of hex B with N-cetyl-galactosamine at different concentartions ranging from 5 mM to 60 mM. **(D).** Affinity curve of hex B with N-acetyl-galactosamine. E constant values were summarized in table.1.

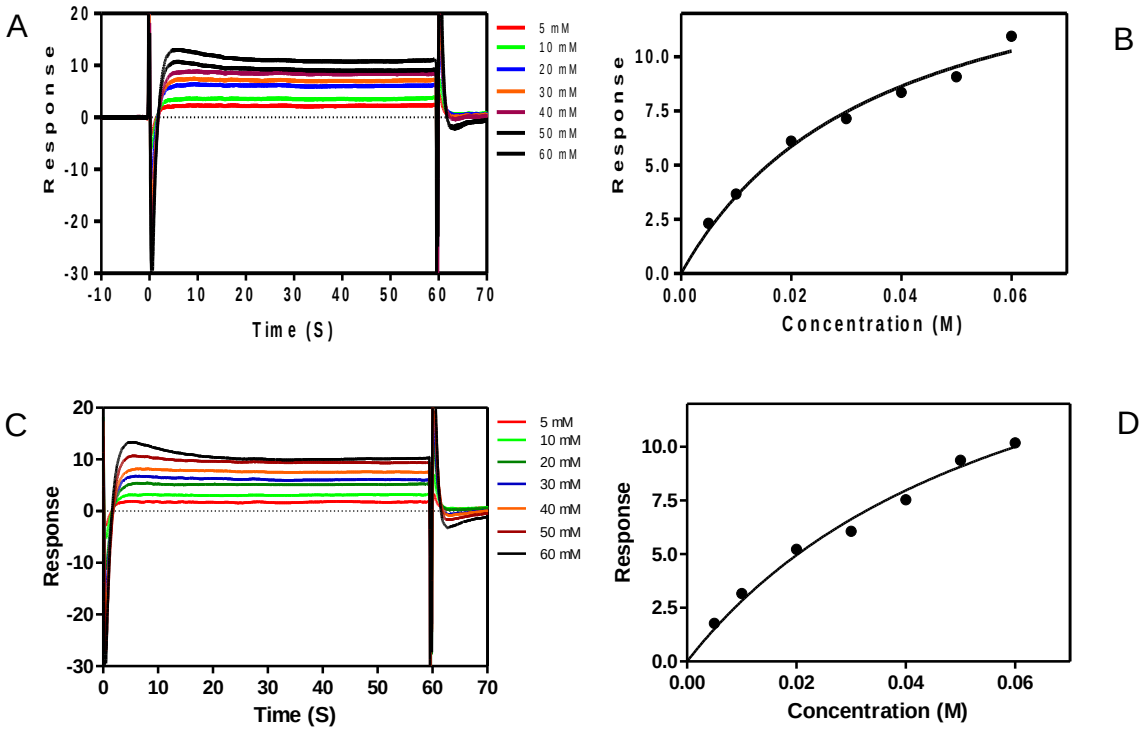


Figure. 9. Interaction of hex B with different sugar**s. (A).** Sensorgrams showing the interaction of hex B with mannose at different concentrations ranging from 5 mM to 60 mM. **(B).** Affinity curve of hex B with mannose **(C).** Sensorgrams showing the interaction of hex B with methyl-α-D mannopyranoside at different concentartions ranging from 5 mM to 60 mM. **(D).** Affinity curve of hex B with methyl-α-D mannopyranoside.

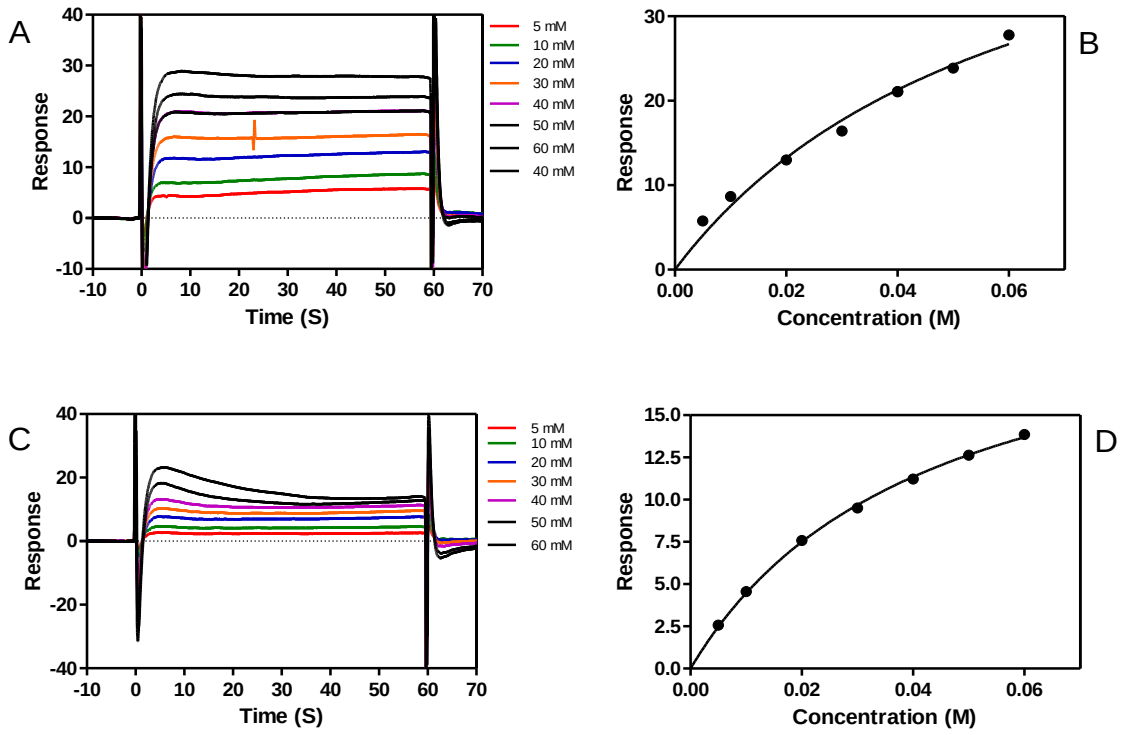


Figure. 10. Interaction of hex B with different sugar**s. (A).** Sensorgrams showing the interaction of hex B with glucosamine at different concentrations ranging from 5 mM to 60 mM. **(B).** Affinity curve of hex B with glucosamine **(C).** Sensorgrams showing the interaction of hex B with galactosamine at different concentartions ranging from 5 mM to 60 mM. **(D).** Affinity curve of hex B with galactosamine.

Table.1. The equilibrium constant values obtained from SPR analysis. Hex A is having higher affinity towards MPRs compared to hex B. Hex A has not shown significant binding with the sugars tested.

Analyte	K_d (affinity constant)	
	Hex A	Hex B
GlcNAc	x	18.7 mM
GalNAc	x	74.6 mM
Glucosamine	x	229 mM
Galactosamine	x	48.8 mM
Mannose	x	64.4 mM
Methyl mannopyranoside	x	84.7 mM
MPR 300	145 nM	25.3 mM
MPR 46	46 nM	87.8 nM

3.4. Discussion

The MPRs show high affinity towards phosphomannosyl residues present on the glycans of lysosomal enzymes. This property of MPRs enables them to play a key role in the biogenesis of lysosomes by targeting lysosomal enzymes to lysosomes for their various degradative functions. Studies using different mammalian models and other non-mammalian vertebrates revealed the evolutionary conservation of the functional domains

of MPRs. In the invertebrates MPRs and lysosomal enzymes have been identified, purified and characterized from starfish (echinodermate), *Lamellidens corrianus* (mollusc), and *Hydra vulgaris* (cnidarian) (Bhamidimarri et al., 2018b; Siva Kumar and Bhamidimarri, 2015). Although these lysosomal enzymes from invertebrates are shown to bind to MPRs qualitatively, the affinity of these enzymes towards MPRs quantitatively is not known. Therefore we chose to study this using mollusc lysosomal enzymes and therefore in the present study two isoforms of β-hexosaminidases from *Lamellidens corrianus* have been purified and their quantitative binding to the purified goat receptors have been determined using surface plasmon resonance.

The two isoforms of molluscs hexosaminidases, hex A and hex B like other glycosidases, these two forms were able to bind with phenyl Sepharose gel. The different biochemical properties of these isoforms was exploited to separate them in the initial chromatographic step using phenyl Sepharose gel. Hex A was eluted from phenyl Sepharose column by adding pH 5.0 buffer, whereas hex B was eluted using pH 7.4 buffer. After the separation of these two forms in phenyl Sepharose chromatography, ion exchange chromatography using DE-52 as a matrix and Con A chromatography techniques were used for further purification. The purity of these enzymes were confirmed by electrophoresis and then used for immobilization on CM 5 sensor chip. Purified goat MPRs as described in methods were injected to sensor chip at different concentrations to determine their affinities for β-hexosaminidases.

Interestingly from the SPR analysis we found that the affinity of MPR 300 towards hex A is higher when compared with its affinity towards hex B. The affinity constant (K_d) for MPR 300 towards hex A is 145 nM, whereas K_d value obtained towards hex B is 25.3

mM. This data suggests that the affinity of MPR 300 towards hex A is 1000 fold higher when compared with hex B.

MPR 300 contains 15 repetitive extracellular domains, which are homologous to each other. Out of these domains, 3, 5, and 9 domains are responsible for mannose-6-phosphate binding. Due to the sequence similarity of these 15 domains with MPR 46, it was proposed that MPR 300 evolved by gene duplication (Lobel et al., 1988). Earlier work on the affinity of bovine MPR 300 domains towards β-glucuronidase (Bohnsack et al., 2009) determined a significant difference in their interaction (Domain 3: K_d = 1 nM; domain 9: K_d = 70 nM). The affinity of β-glucuronidase was found to be different of other sources of MPRs due to its heterogeneous structure of the N-glycan. The size of the glycan, presence of phosphomonoester and phosphodiester residues, number of M6P residues in the glycan and their location determines the affinity of lysosomal enzymes to MPRs (Castonguay et al., 2012). The difference in the affinity of MPR 300 towards the two isoforms can be explained may be due to their difference in glycan composition. The glycans present on hex A may contain more M6P residues and more accessible than on the glycans present on hex B. The K_d values for MPR 46 towards hex A and hex B was found to be 46 mM and 87.8 nM respectively. The affinity of MPR 46 towards hex A was found to be twice the affinity of hex B. The K_d values of hex A and hex B for various sugars were tested. Surprisingly, we found that hex A was not having any significant binding and hex B was able to show significant binding with the sugars tested. The K_d values of hex B for the sugars tested were shown in Table.3.

This is the first study towards quantitative determination of the specific interaction of the invertebrate lysosomal enzymes with the mannose 6-phosphate receptors. The affinity

constants for the two isoforms of hexosaminidase with goat MPRs were determined. Goat receptors were chosen in this study due to their similarity with mollusc receptor i. ability to bind on phosphomannan –Sepharose gel, specific elution from the gels with 5 mM mannose 6-phosphate, ii. apparent molecular mass of the mollusc receptors and goat receptors being same, and iii. the ability of the mollusc receptors to cross-react with an antibody to the goat receptors suggesting antigenic similarities. Furthermore, in an independent study earlier from our laboratory it was shown that the mollusc cell lines were shown to contain several lysosomal enzymes and the Mannose 6-phosphat receptors (Nadimpalli and Amancha, 2010a) and also due to their availability in higher concentration. Cloning and expression of the mollusc receptor genes and obtaining the proteins in high concentrations should further provide additional evidences on the quantitative data obtained in this study. However, this part of the work is beyond the scope of the present investigation.

Chapter 4

Identification and localization studies of lysosomal enzymes (acid phosphatase and hexosaminidases) from *Hydra vulgaris* Ind-pune

4.1. Introduction

Hydra vulgaris species are fresh-water organisms belonging to the phylum Cnidaria. The length of the body of Hydra ranges from 10- 30mm under constant temperature and controlled feeding. They exhibit radial symmetry and the body is divided into oral and aboral axis. The body is divide into oral, body column and foot region. They are sessile organisms and hence attached to substratum with the help of foot. The body of these organisms are organized into tube like polyps with 5 to 7 tentacles at the oral region. Since they are sedentary, these organisms feed on any moving zooplankton by capturing with the help of tentacles. Tentacles are rich in cnidocytes, which can stun prey by neurotoxic chemicals. They are diploblastic animals with two distinct layers outer ectoderm and inner endoderm separated by acellular mesoglea. Both of these layers contain stem cells which help in regeneration. Special type of multi potent cells called interstitial cells present in the ectoderm of hydra are responsible for the regeneration capacities of Hydra (Bossert and Galliot, 2012). Hydra is widely used as a model organism in stem cell biology (David and Murphy, 1977), regeneration (Holstein et al., 2003) and axial patterning.

Acid phosphatases in the vertebrates are a distinct family of enzymes that cause hydrolysis of phosphomonoesters at an acidic pH of 3.0-5.0. Different types of acid phosphatases have been identified till date, based on their structure, catalytic and immunological properties, cellular localization and tissue distribution (Suter et al., 2001). Lysosomal acid phosphatase (EC 3.1.3.2, LAP), a ubiquitously expressed enzyme is often considered as a key biochemical marker enzyme for lysosomes (De Duve, 1983). Synthesized as a membrane-bound precursor with 7-8 N-linked oligosaccharides, a transmembrane domain and a cytoplasmic tail, it recycles from the early endosomes to the plasma membrane (Braun et

al., 1989). Once the precursor enzyme reaches lysosomes, it is proteolytically processed in the lysosomal matrix and a mature LAP is released (GOTTSCHALK et al., 1989). Though LAP is majorly localized to lysosomes, other forms of soluble acid phosphatase, specifically expressed and secreted in the prostate gland have also been identified. Both LAP and prostate acid phosphatases are glycoproteins and contain mannose and glucosamine in the carbohydrate moiety, show identical subunits of molecular weight of 48-52 kDa, and are sensitive to L-tartrate inhibition (Lemansky et al., 1985). Subsequently, a tartrate resistant type-5 acid phosphatase (Acp5), an orthophosphoric monoesterase has also been identified in lysosomal compartments of mononuclear phagocytes and osteoclasts (Bevilacqua et al., 1991; Hayman et al., 2000). Absence of either of these acid phosphatases, LAP/Acp5 leads to mild phenotypes, suggesting partial compensatory mechanisms by the other phosphatase. However, deficiency of both phosphatases (LAP/Acp5) leads to abnormal lysosomal storage in soft and mineralized tissues.

Another equally important lysosomal enzyme includes β-N-acetylhexosaminidase (EC 3.2.1.52, Hex), which catalyzes the hydrolysis of terminal N-acetylhexosamine residues from the non-reducing ends of glycoconjugates (Venugopal and Sivakumar, 2013b). Three isozymes of β-N-acetylhexosaminidase composed of two subunits, α and β are commonly identified: Hex A (α-β), Hex B (β-β), and Hex S (α-α). Hex A and Hex B are functionally more significant, while Hex S is a minor form and shows less activity (Hepbildikler et al., 2002). Both Hex A and Hex B are synthesized as precursors and are transported to lysosomes in a mannose 6-phosphate dependent manner by the mannose phosphate receptors. Deficiency of Hex A leads to Tay-Sachs disease, while deficiency of both isozymes, Hex A and B causes Sandhoff disease. In addition to these three isozymes, a

relatively less known β-hexosaminidase, Hex D, encoded by *Hex DC* gene also exists (Gutternigg et al., 2009). However, the function of this nuceocytoplasmic localized enzyme is not clearly understood (Alteen et al., 2016).

Our laboratory mainly focuses on delineation of lysosomal biogenesis pathway in vertebrates and invertebrates. Previous reports from our laboratory have established the evolutionary conservation of lysosomal enzymes as well as their sorting receptors from molluscs to vertebrates (Siva Kumar and Bhamidimarri, 2015). Recently we have also identified lysosomal hydrolases and mannose-6 phosphate receptor dependent lysosomal targeting system for the first time in a simple diploblastic Cnidarian, 'Hydra' (Bhamidimarri et al., 2018b). In order to understand the evolutionary conservation of lysosomal enzymes and their biogenesis pathway, particularly in the Cnidarians, it is important to carry out a systematic study on the enzymes and study their biochemical properties. These studies would eventually allow us to establish the evolutionary conservation of lysosomal biogenesis in the animal kingdom. In the current chapter lysosomal enzymes were identified from the soluble protein extract of *Hydra vulgaris* and the genes coding for acid phosphatase, β-hexosaminidase and β-glucuronidase were identified from the total mRNA and localization of these genes in the animal were also determined. An attempt was made for the heterologous expression of acid phosphatase and β-hexosaminidase.

4.2. Materials and methods

4.2.1. Materials

Lysosomal enzyme substrates and Trizol reagent were purchased from Sigma Aldrich, St. Louis, MO, USA. pGEMT easy vector was purchased from Promega, Madison, WI, USA.

In vitro transcription and *in situ* hybridization reagents were procured from Roche chemicals. Codon optimized synthetic genes V84636, V84630 were obtained from GenScript. The primary and secondary antibodies used for western blot analysis were purchased from AbCam, USA. All other chemicals used in the study were of highest purity and obtained from local suppliers.

4.2.2. Methods

4.2.2.1. Hydra culture maintenance

Clonal cultures of three hydra strains, *Hydra vulgaris* Ind-Pune (Reddy et al., 2011), were maintained in hydra medium at a constant temperature of 18 ± 1°C with 12 h light/dark cycle (Sugiyama and Fujisawa, 1977). Polyps were fed with freshly hatched *Artemia salina* nauplii on alternate days.

4.2.2.2. Extraction of soluble proteins from hydra

Hydra vulgaris Ind-Pune, animals were mass cultured as previously described. Starved polyps were collected and used for preparing the soluble extracts. About 1000 whole polyps after collection were washed separately in 0.9 % saline and centrifuged briefly to collect the polyps. Soluble proteins were extracted by standard protocol as described previously (Bhamidimarri et al., 2018a). Briefly, polyps were lysed in lysis buffer (0.5 M sodium acetate-acetic acid buffer pH 5.0, containing 0.2 M NaCl, 1 mM EDTA, 5 mM iodoacetic acid and 1 mM PMSF), in a probe sonicator with 30/60 s on/off pulse cycle for three times. The lysed homogenate was centrifuged at 26,892 X g for 30 min and the clear supernatant containing soluble proteins was collected, stored and used for further studies.

4.2.2.3. Lysosomal enzyme assays

Soluble extract obtained from the above procedure were assayed for the presence of lysosomal enzymes, and about 500 ng of protein was taken for the enzyme assays. The 4-nitrophenyl substrates of various lysosomal enzymes (acid phosphatase, β-hexosaminidase, β-glucuronidase, α-fucosidase, α-mannosidase, α-galactosidase and aryl sulphatase) are used for the enzyme assay. Soluble extract was incubated in a final substrate concentration of 1 mM, at 37°C for 30 min. The reaction was stopped by adding equal volume of 0.2 M Na_2CO_3 and the absorbance of released 4-nitrophenyl was measured at 405 nm. One unit of enzyme activity is defined as the absorbance equivalent to 1 μmol of paranitrophenol released per minute per mL of the enzyme under experimental conditions.

4.2.2.4. Isolation and cloning of β-hexosaminidase, acid phosphatase and β-glucuronidase from hydra and sequence analysis

Prior to RNA isolation, the polyps were starved for 48 h, and the RNA was isolated from the 30-40 polyps by Trizol method according to manufacturer's instructions (Sigma). About 1 μg of the RNA was used to synthesize cDNA by first-stand cDNA synthesis kit according to manufacturer's instructions (Thermo Fisher Scientific, USA). Gene specific primers (Table-1) for complete coding sequence for acid phosphatase, hexosaminidase and glucuronidase were designed based on *H. magnipapillata* genome, and the genes were amplified from *H. vulgaris* Ind-Pune. In order to prepare riboprobes for *in situ* hybridization, partial coding sequences were amplified using a second set of primers (Table-1). The conditions used for PCR were as follows: initial denaturation at 95°C for 5 min, denaturation at 94°C for 30 s, annealing at 52°C for 30 s, extension at 72°C for 1 min (35 cycles), and the final extension was at 72°C for 10 min. The amplified products were

cloned into pGEMT easy vector (Promega, Madison, WI, USA) and confirmed by sequencing. Phylogenetic trees were constructed by ML, NJ and MP methods using MEGAX. Bootstrap analysis with 1000 replicates was carried out for each tree with random addition of sequences for 10 replicates.

Table.1. List of primer sequences used for amplification of full and partial sequences of β-hexosaminidase, acid phosphatase, and β-glucuronidase

Gene name	**Sequence 5'---3'**
β-hexosaminidase Fw	ATGATTAGATTTGACTTGCGA
β-hexosaminidase Rev	TTAAAAACTGTCTAATATACGTGAAAC
Acid phosphatase Fw	ATGTTTAATTGTATATTTTTGTC
Acid phosphatase Rev	TTAATATAAATTAGTTTCATCTTTT
β-glucuronidase FW	ATGATCTTTTTCTTTTCTTTGGTTTTTC
β-glucuronidase Rev	TCATGTTGCAGGGTCACTTTC
β-hexosaminidase IVT Fw	TGAAATGGTTGAGCCTGTGA
β-hexosaminidase IVT Rev	GAAATGGACGTGTCCAACCT
Acid phosphatase IVT Fw	GATCTTTGCGTACGCCTTGT
Acid phosphatase IVT Rev	TGGAGAACTGCGATTTTGTG
β glucuronidase IVT FW	TGTTGGTTGGGTGTGGTATG
β glucuronidase IVT Rev	CCAGCACCCAAGAGTGAAAT

4.2.2.5. Whole mount in situ hybridization

The expression patterns of acid phosphatase, β-hexosaminidase and β-glucuronidase in hydra were detected by in situ hybridization using digoxygenin (DIG) labeled riboprobes. The plasmid vectors containing the inserts of β-hexosaminidase and acid phosphatase, digoxygenin labeled nucleotides, T7 and SP6 RNA polymerases (Roche, Germany) were used to synthesize DIG labeled sense and antisense riboprobes by in vitro transcription. A standard protocol was employed for in situ hybridization (Krishnapati and Ghaskadbi,

2014). Briefly, starved polyps (36-48 h) were relaxed in 2% urethane and fixed overnight in 4% paraformaldehyde at 4°C. Fixed polyps were permeabilized with proteinase K (10 mg/ml) at room temperature for 10 min. DIG labeled probes, both sense (control) and antisense were incubated with polyps for 48 h at 60°C followed by stringency washes (0.5X SSC + 0.1% CHAPS). Anti-digoxigenin antibody conjugated with alkaline phosphatase was added and incubated at 4°C overnight. Maleic acid buffer (100 mM maleic acid pH 7.5, 150 mM NaCl and 0.1% Tween 20) was used to remove unbound antibody. Staining of the polyps was done by nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate p-toluidine salt (NBT/BCIP) (Roche, Germany) substrates. Upon color development, polyps were transferred to methanol followed by imaging using Olympus SZX16 microscope.

4.2.2.6. Heterologous expression of Acid phosphatase and β-hexosaminidase

pGEMT vectors containing the insert of acid phosphatase was used for amplification with the primers designed for acid phosphatase (Forward: 'ATGTTTAATTGTATATTTTTGTC'; Reverse: 'TTAATATAAATTAGTTTCATCTTTT'). The amplified products were cloned into pET 28a expression vector using BamH1 and Xho1 restriction sites. The ligation of acid phosphatase into expression vector was confirmed by colony PCR and transformed into Rosetta *E. coli* cells for expression. 0.2 mM IPTG was used for induction and the recombinant protein was extracted from inclusion bodies using 0.2 % sarkosyl. The expressed protein was purified using Ni-affinity beads and checked on 10% SDS PAGE and western blot. The purified protein was also confirmed as acid phosphatase by MS MS analysis. Codon optimized β-hexosaminidase (V84636) and acid phosphatase (V84630)

synthetic genes were cloned into PGLAP5 mammalian expression vector. PGLAP5 vector containing the inserts of codon optimized genes were transfected using lipofectamine 2000 (Invitrogen) into HEK 293 T cells. The successful transfection and expression of recombinant proteins was confirmed as western blot analysis.

4.3. Results

4.3.1. Lysosomal enzyme assays

The soluble extracts of *Hydra vulgaris* Ind-Pune, showed different lysosomal enzyme activities when assayed with respective synthetic substrates. Among the enzymes assayed, the activities of β-hexosaminidase and acid phosphatase were found to be very high, which was consistent with the earlier report (Bhamidimarri et al., 2018b). The other enzymes detected were by α-fucosidase, α-mannosidase and β-glucuronidase. Whereas the activities of, α-galactosidase and aryl sulfatase were found to be minimum (Figure.1).

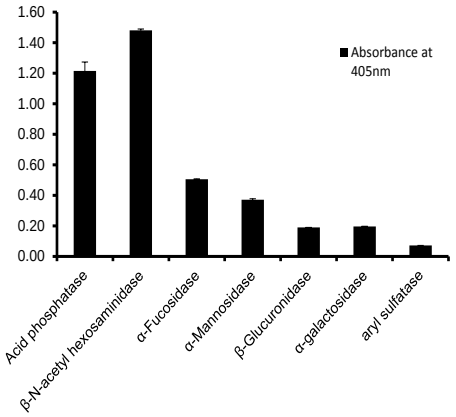


Figure. 1. Lysosomal enzyme activities in the soluble extracts of *Hydra vulgaris* Ind-Pune. 4-nitrophenyl derivatives specific for each lysosomal enzyme was used as substrate to measure the lysosomal enzyme activities. β-N-acetyl hexosaminidase enzyme activity was high, followed by acid phosphatase, α-fucosidase, and α-mannosidase. β-glucuronidase and α-galactosidase activities were comparable, while aryl sulfatase activity was minimum.

Since the activities of β-hexosaminidase and acid phosphatase were found to be high, localization and characterization of these two enzymes along with β-glucuronidase was attempted using *in situ* hybridization and heterologous expression respectively.

4.3.2. Identification of β-hexosaminidase, acid phosphatase and β-glucuronidase homologues in hydra

In an attempt to identify and clone the genes from hydra, the NCBI database was searched to identify the putative gene sequences coding for β-hexosaminidase, acid phosphatase and β-glucuronidase. Primers were synthesized based on the available *H. vulgaris* predicted and assembled sequences XM_012704317.1, HAAD01005185.1 and XM_012707391.1 respectively. The complete coding sequences of all these genes were cloned from *H. vulgaris* Ind-Pune (Fig. 2A-C) and confirmed by sequencing.

4.3.3. Structural conservation of β-hexosaminidase, acid phosphatase and β-glucuronidase and phylogenetic analysis

In silico analysis of translated amino acid sequences of β-hexosaminidase, acid phosphatase and β-glucuronidase revealed structural conservation of respective characteristic domains. Analysis by PSI BAST and Domain enhanced lookup time accelerated BLAST (Delta BLAST) showed high similarity with homology proteins across different phyla. Presence of characteristic domains, a GH20 catalytic domain, histidine phosphatase domain and a glycosyl hydrolase signature domain has been identified using PROSITE, in β-hexosaminidase, acid phosphatase and β-glucuronidase respectively. Presence of a signal peptide of 15 amino acid length was observed in both acid phosphatase

and β-glucuronidase, as predicted by SignalP 5.0 program, while no signal peptide was seen in β-hexosaminidase. Transmembrane domain prediction by Protter program showed presence of a single transmembrane domain in β-hexosaminidase, while no transmembrane domains were seen in acid phosphatase and β-glucuronidase (Figure. 3A-C).

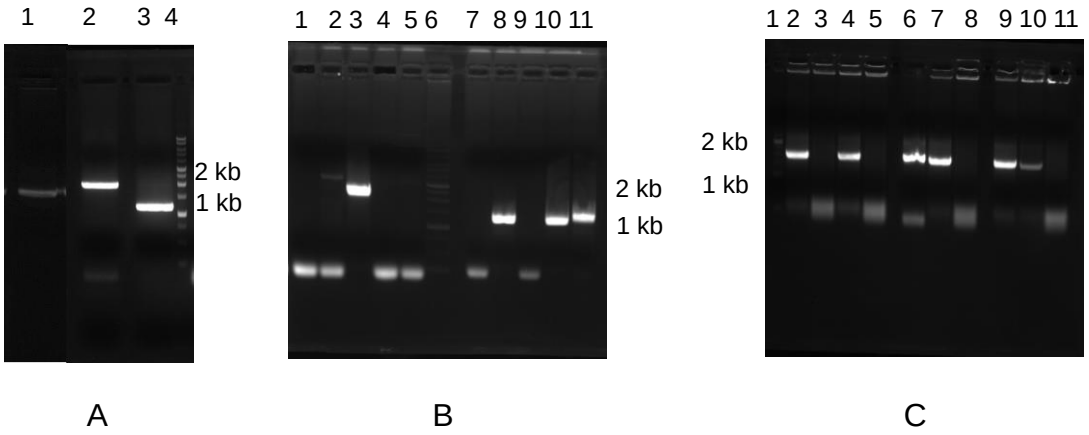


Figure. 2. (A) 1% agarose gel electrophoresis for PCR amplification of coding sequences of β-glucuronidase, β-hexosaminidase, and acid phosphatase (lanes 1-3, respectively); Lane 4 represent DNA ladder. **(B)** 1% agarose gel electrophoresis of colony PCR amplified products of β-hexosaminidase (lanes 1-5) and acid phosphatase (lanes 7-11); Lane 6 represents DNA ladder. **(C)** 1% agarose gel electrophoresis of colony PCR amplified products of β-glucuronidase (lanes 2-11); Lane 1 represents DNA ladder.

Figure. 3. Translated amino acids of β-hexosaminidase **(A)**, acid phosphatase **(B)** and β-glucuronidase **(C)** from *H. vulgaris* Ind-Pune. Sequence coding for a transmembrane helix is shown in blue in β-hexosaminidase (A). Aspartic acid that helps in binding the substrate is seen at 148th position followed by a Lysine residue (Shown in pink). Amino acids coding for a secretory signal sequence is located at the N-terminus (shown in red) in acid phosphatase (B) and β-glucuronidase (C). Presence of histidine signature is seen in acid phosphatase (B, highlighted in blue), while conserved glutamic acid residue signature, highlighted in blue is seen in β-glucuronidase (C).

Evolutionary analysis of the three genes was performed by constructing phylogenetic trees, using maximum likelihood (ML), neighbor joining (NJ) and maximum parsimony (MP) methods. The original trees were constructed by maximum likelihood (ML) method and

JTT matrix-based model was used for amino acid substitution using MEGAX software (Kumar et al., 2018). Bootstrap replicates, less than 50% are collapsed in the branches corresponding to partitions and bootstrap consensus trees inferred from 1000 replicates were taken to represent the evolutionary history of the taxa analyzed A separate analysis by neighbor joining (NJ) and maximum parsimony (MP) was also performed. For NJ method, the evolutionary distance was computed using Poisson correction method and for MP method, the trees were obtained using the close-neighbor-interchange algorithm in which the initial trees were obtained with the random addition of sequences for 10 replicates (Kumar et al., 2018). The analysis revealed that hydra enzymes cluster together with vertebrate counterparts suggesting their close similarity towards (Figure. 4A-C).

4.3.4. Localization of β-hexosaminidase, acid phosphatase and β-glucuronidase in *H. vulgaris* Ind-Pune

Whole mount *in situ* hybridization using digoxygenin labeled riboprobes was carried out to localize the transcripts of β-hexosaminidase (Figure. 5A'), acid phosphatase (Figure. 5B') and β-glucuronidase (Figure. 5C'). The expression of all three enzymes was predominantly localized in the endoderm region of upper body column of hydra, while hypostome and basal disc are clean without any signal. Probes hybridized with sense probes are clean and did not show any signal (Figure. 5A, B, C). This suggests that all three enzymes are present in the endodermal epithelial cells and/or in the gland cells and may have possible role in digestion.

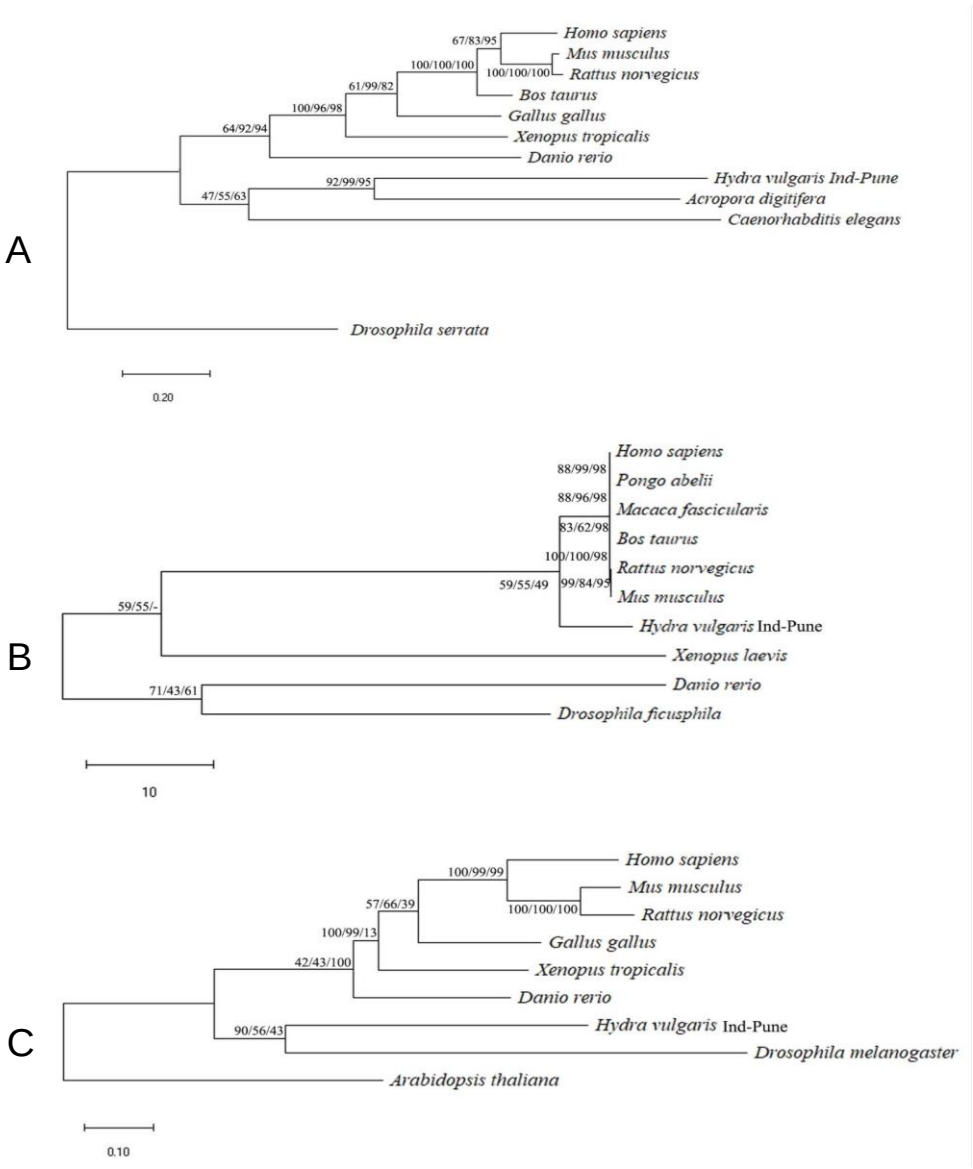


Figure. 4. Phylogenetic trees computed for β-hexosaminidase **(A)**, acid phosphatase **(B)** and β-glucuronidase **(C)**. The trees were inferred by maximum likelihood (ML) method in MEGAX. Original trees were represented with bootstrap values indicated at the branch points inferred from NJ, MP and ML methods (left to right). The bootstrap consensus tree was inferred from 1000 replicates to represent the evolutionary history of the taxa analyzed. Scale bar indicates amino acid substitutions per site.

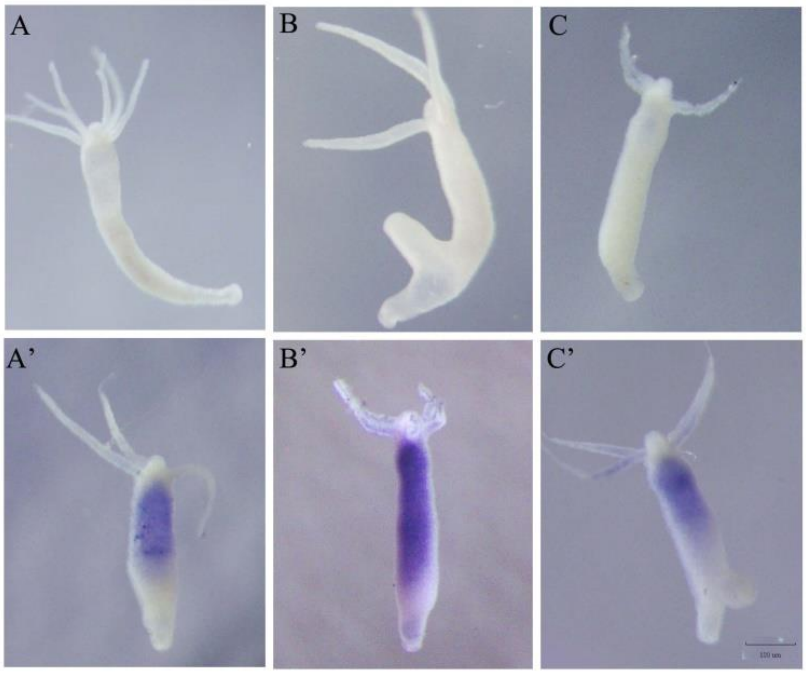
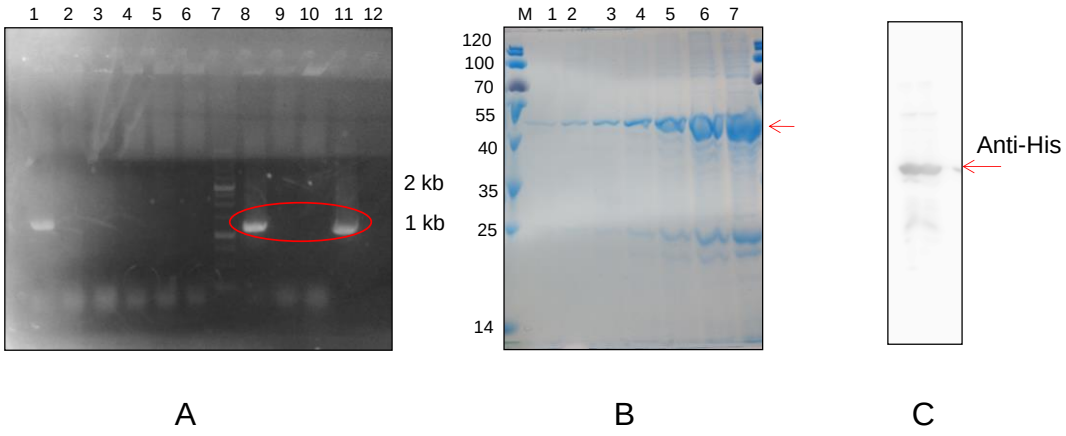


Figure. 5. Localization of β-hexosaminidase (A'), acid phosphatase (B') and β-glucuronidase (C') in hydra by whole mount *in situ* hybridization. Whole mount *in situ* hybridization with DIG-labeled anti sense riboprobes for each gene shows predominant expression in the endoderm of upper body column with clean hypostome and basal disc. Scale bar-100 µm. (A-C). sense probes for *in situ* hybridization.

4.3.5. Heterologous expression of Acid phosphatase and β-hexosaminidase

Heterologous expression of acid phosphatase and β-hexosaminidase proteins was attempted for the biochemical characterization studies. Acid phosphatase gene was cloned into bacterial expression vector pET 28a. Successful ligation was confirmed by colony PCR (Figure. 6A) and the positive clones were used to isolate plasmids containing the inserts of acid phosphatase gene. The isolated plasmids were transformed into *E.coli* Rosetta strain and induced overnight at 18˚C with 0.2 mM IPTG. After cell lysis, both the soluble fraction and pellet fraction were checked for the expressed recombinant protein. Since most of the protein was found to be present in pellet fraction, 0.2% sarcosyl was used

to extract proteins from inclusion bodies. These proteins were subjected to Ni-affinity chromatography for the purification of the expressed protein. 300 mM imidazole was used to elute the bound proteins from the Ni-affinity column. The authenticity of the purified protein was checked on SDS PAGE, with a molecular mass of 40 kDa. Western blot and MS MS analysis (Fig. 6B-D).



D

Results List

1. XP_012560499.1 **Mass:** 24034 **Score:** 127 **Expect:** 2.8e-05 **Matches:** 18
PREDICTED: testicular acid phosphatase homolog isoform X2 [Hydra vulgaris]

Observed	Mr(expt)	Mr(calc)	ppm	Start	End	Miss	Peptide
630.3039	629.2967	629.2955	1.80	146	150	0	R.TPCPR.L
647.3470	646.3397	646.3286	17.2	168	172	1	K.EKENK.M

le:///C:/Users/User/Documents/20180403/Protein%20Summary%20Report%20(1).mht

/3/2018 Protein Summary Report (1)

655.3151	654.3078	654.3047	4.81	162 - 166	0	K.VDYMK.K
693.3991	692.3918	692.3857	8.85	87 - 91	0	K.EIYIR.S
775.4283	774.4211	774.4235	-3.17	167 - 172	2	K.KEKENK.M
783.4185	782.4112	782.3996	14.8	162 - 167	1	K.VDYMKK.E
788.4501	787.4428	787.4439	-1.47	153 - 159	0	R.ELLSAQK.K
911.5079	910.5006	910.4946	6.62	161 - 167	2	K.KVDYMKK.E
916.5397	915.5325	915.5389	-7.02	153 - 160	1	R.ELLSAQKK.K
927.5222	926.5149	926.4895	27.4	161 - 167	2	K.KVDYMKK.E + Oxidation (M)
1057.6378	1056.6305	1056.6291	1.32	151 - 159	1	R.LRELLSAQK.K
1185.7284	1184.7211	1184.7241	-2.50	151 - 160	2	R.LRELLSAQKK.K
1298.5862	1297.5789	1297.5795	-0.42	56 - 66	0	K.VGMNMEYELGR.F
1314.5805	1313.5732	1313.5744	-0.89	56 - 66	0	K.VGMNMEYELGR.F + Oxidation (M)
1330.5748	1329.5676	1329.5693	-1.32	56 - 66	0	K.VGMNMEYELGR.F + 2 Oxidation (M)
1372.6408	1371.6335	1371.6493	-11.51	44 - 55	0	K.NDWPVDPGMLTK.V
1682.8160	1681.8088	1681.9199	-66.06	18 - 31	2	K.TLKMVHVVYRHGAR.S + Oxidation (M)
1775.8584	1774.8511	1774.8923	-23.25	98 - 113	0	R.CLQSAETQLAGLYPPK.G

No match to: 605.3403, 612.3885, 615.2911, 616.3325, 620.3543, 631.3562, 644.3328, 658.3762, 675.3691, 688.3926, 689.3659, 691.3413, 701.3729, 702.3754, 708.3754, 730.4008, 735.4172, 757.3693, 781.4359, 817.3986, 837.4964, 859.4367, 863.5244, 959.5413, 1027.5847, 1039.5957, 1063.5798, 1093.5526, 1114.4843, 1250.5983, 1305.6668, 1321.6472, 1487.6816, 1535.6557, 1551.6449, 1567.6429, 1825.8389, 1896.8736, 1898.8542, 1946.9000

Figure. 6. (A). Transformation of Acid phosphatase containing pET 28a into Rosetta strain. Lane 1-6, 8-11 colonies checked for acid phosphatase insert; Lane 7 represents DNA ladder. **(B).** 10 % SDS PAGE showing the elution profile (lanes 2-8) of Ni-affinity chromatography using 300 mM imidazole, lane 1 represents protein ladder. **(C).** Western blot with anti-His antibody to detect the target protein in the 0.2 % sarcosyl extract. **(D).** MS MS analysis of purified protein to confirm its authenticity as acid phosphatase.

Initial experiments to express acid phosphatase and β-hexosaminidase in mammalian cells was not successful. To optimize the expression of these genes in mammalian cells codon optimization of β-hexosaminidase and acid phosphatase genes was done. These synthetic genes were cloned into PGLAP5 mammalian expression vector (Figure. 7A, B) and transfected into HEK 293 T cells for expression. The expression of recombinant proteins in HEK 293 T cells was confirmed as western blot analysis (Fig. 7C).

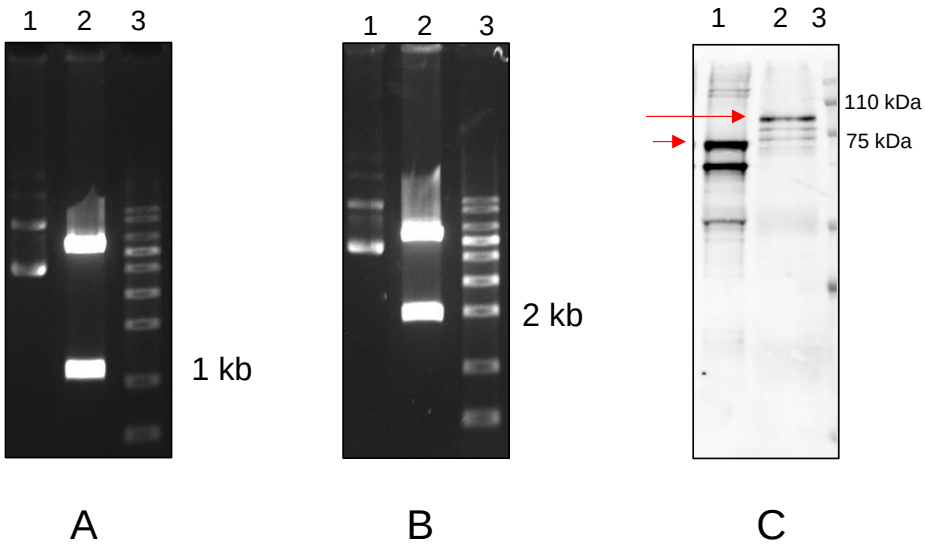


Figure. 7. (A). 1% agarose gel electrophoresis of acid phosphatase digested from PGLAP5 plasmid containing codon optimized acid phosphatase; lane1-undigested plasmid, lane2-insert out of acidphosphatase, lane 3 represents DNA ladder. **(B).** 1% agarose gel electrophoresis of β-hexosaminidase digested from PGLAP5 plasmid containing codon optimized β-hexosaminidase; lane1-undigested plasmid, lane2-insert out of β-hexosaminidase, lane 3 represents DNA ladder. **(C).** western blot analysis of the cell lysates HEK 293 T cells transfected with the codon optimized vectors using anti-S antibody; Lane 1, 2 represents cell lysates of HEK 293 T cells transfected with AP PGLAP5 and HexPGLAP5 plasmids respectively.

4.4. Discussion

Lysosomal enzymes have been well characterized in mammals including humans, and less characterized in non-mammalian vertebrates. However it is now well established that the lysosomal biogenesis pathway is highly conserved from fish to mammals. Ongoing studies reveal the existence of lysosomal enzymes and their sorting receptors in several invertebrate species, including starfish, Unio and Hydra. However, studies on lysosomal enzymes in lower invertebrates such as hydra are scarce and evidence on the evolutionary conservation of lysosomal biogenesis in them has not been clearly demonstrated. Research in our laboratory focuses on understanding the importance of lysosomal enzymes and their sorting receptors in invertebrates with a major focus on 'Hydra'. In view of this, we have recently provided the first biochemical evidence on the presence of lysosomal enzyme activities and MPR46 protein in hydra (Bhamidimarri et al., 2018b).

As a logical extension of this work, the present work was carried out to identify, localize and characterize the lysosomal enzymes present in Hydra. Initial lysosomal enzyme analysis in the soluble extracts of H. vulgaris Ind-Pune has showed predominant levels of β-hexosaminidase, acid phosphatase and β-glucuronidase as compared to other lysosomal enzymes. Lysosomal acid phosphatases are a distinct family of enzymes that cause hydrolysis of phosphomonoesters at an acidic pH. They act as biochemical marker for lysosomes and contain characteristic histidine phosphatase domain. *In silico* analysis of the translated amino acid sequence of hydra acid phosphatase by PROSITE revealed the presence of a phosphohistidine signature (19-33) at the N-terminus and an active site signature (269-285) at the C-terminus, which shows a conserved 'Histidine' residue at the center of each signature, responsible for the enzyme catalytic mechanism. This confirms

that the hydra acid phosphatase belongs to the histidine acid phosphatase super family. Similarly, hydra β-glucuronidase showed a highly conserved glutamic acid residue that acts as a signature pattern (433-447) for family 2 Glycosyl hydrolases confirming the conservation of key motifs in the active site responsible for enzyme catalysis. In case of hydra hexosaminidase, conservation of sequence-related family 20 glycoside hydrolases (GH20) catalytic domain was observed suggesting that the identified gene is indeed a β-hexosaminidase. However, a further analysis by PSI-BLAST and Delta BLAST revealed that hydra β-hexosaminidase could possibly is a HexD. Previous reports showed the presence of conserved active site amino acids, Aspartic Acid (Asp/D) and Glutamic acid (Glu/E) at positions 148 and 149 respectively in HexD, and are responsible for binding the substrates (Alteen et al., 2016). Analysis of Hydra peptide sequence also showed the presence of 'Asp' at 148th position suggesting the conservation of active site in hydra β-hexosaminidase. However, the enzyme lacks 'Glu' at position 149 and is replaced by a basic amino acid, 'Lys'.

Whole mount *in situ* hybridization studies revealed expression of β-hexosaminidase, acid phosphatase and β-glucuronidase in the endoderm layer. In hydra, the endoderm, also called gastrodermis lines the surface of the gastric cavity and is mainly made up of flagellated endodermal epithelial cells. Current localization studies have revealed the expression of all three enzymes in the endoderm of hydra. Previous studies in *Caenorhabditis elegans* have shown localization of acid phosphatase in the intestinal epithelium and its role in gut differentiation (Beh et al., 1991). Reports show that G20 glycoside hydrolases can function as chitinases due to their ability to cleave β-(1,4)-linked *N*-acetyl-D-glucosamine (GlcNAc) residues directly from the non-reducing ends of chitin

polymers and chitin oligomers (LeCleir et al., 2007; Scigelova and Crout, 1999). High expression of *N*-acetyl-D-glucosaminidase in intestinal mucosal tissues with active turnover has been reported (Pascolini et al., 1981). Hydra are often fed with crustacean larvae, artemia under laboratory conditions, while in natural environment, they feed on small aquatic insects such as daphnia, cyclops, etc. both of which are crustaceans. Since chitin acts as an important structural component of cell wall of many insects, it is highly expected that chitin degrading enzymes are expressed in the gastroderm of hydra. Similarly, β-glucuronidase cleaves β-D-glucuronic acid residues from the non-reducing ends of glycosaminoglycans. Function of β-glucuronidase in xenobiotic metabolism in the digestive tract of humans has been demonstrated. Similarly, role in digestion in invertebrate species has been demonstrated. Expression of β-glucuronidase in the gastroderm of hydra thus suggests its possible role in digestion. Our current localization results thus show the presence of both β-hexosaminidase and β-glucuronidase in the gastroderm suggesting their possible role in degrading the GlcNAc residues during digestion.

For the biochemical characterization of acid phosphatase and β-hexosaminidase heterologous expression approach by cloning these genes into bacterial and mammalian expression vectors was performed. Since purifying these enzymes from natural source is difficult because of the requirement of more amount soluble extract, this approach of heterologous expression was taken. Acid phosphatase was successfully cloned into pET 28a expression vector. However, most of the expressed protein was found in inclusion bodies after overnight induction with IPTG at 18˚C. Sarcosyl was used for solubilization and purification of these insoluble protein. The authenticity of the purified protein was confirmed as acid phosphatase both by western blot and MS MS analysis. Since the protein

was not active for biochemical characterization, heterologous expression in mammalian cells was attempted for acid phosphatase and β-hexosaminidase. Codon optimization was performed for successful expression of these proteins in mammalian cells as earlier experiments with unoptimized genes failed to express in mammalian cells. The expressed proteins were confirmed by western blot analysis using anti-S tag antibody. An increase in the molecular weight by 29 kDa for both acid phosphatase and hexosaminidase is due to the GFP fusion tag present in the vector. Further mass culture and expression using mannose 6-phosphate deficient cell lines should allow to determine their function, biochemical and biophysical properties of these two important proteins.

Chapter 5

Comparative analysis of lysosomal enzymes (Enzyme profiling-acid phosphatase and hexosaminidases) from *Hydra vulgaris* Ind-Pune, *H. vulgaris Naukuchiatal, H. magnipapillata sf-1* and purification of hexosaminidase from *H. vulgaris*

5.1. Introduction

The degradative functions of the cell are carried out by specialized organelles called lysosomes. Lysosomal hydrolases degrade extra cellular molecules; pathogens internalized by endocytosis or phagocytosis, and also aid in the turnover of intracellular proteins and maintain the cellular homeostasis and differentiation (de Marcos Lousa and Denecke, 2016; Stoka et al., 2016). Further, lysosomal enzymes also show important roles in tissue remodeling, membrane repair, cell adhesion, immune function, pigmentation, and signaling. Specially, lysosomal acid phosphatases, β-glucuronidase, acid sphingomyelinase, and proteases such as cathepsins, participate in tissue repair, dedifferentiation, aging, immune response, and other processes.

Several reports have demonstrated the role of lysosomal acid phosphatase (LAP) and β-hexosaminidase (Hex) during regeneration. Coward and his group (Coward et al., 1974) have demonstrated an increase in LAP activity during planarian regeneration. LAP activity was also found to be high during tail regeneration in lizards (Alibardi, 1998) and in tail regression during metamorphosis in *Xenopus* tadpoles (Robinson, 1970). Role of LAP in dedifferentiation, a condition prerequisite for limb regeneration was also detected in the adult urodele (Miller and Wolfe, 1968). Biochemical and immunohistochemical investigations also have demonstrated a prominent increase of LAP activity during retinoic acid mediated limb regeneration and dedifferentiation in regenerating salamander larvae (Ju and Kim, 2000; Ju and Kim, 1994). Similarly, role of hexosaminidase during regeneration has been well demonstrated. Role in chitin degradation by hydrolyzing glycosidic bonds of 2-acetamido 2-deoxy β-D-glycosides, along with chitinases in invertebrates has been identified (Cohen, 2009). Role of N-acetyl β-D-hexosaminidase (N-

acetylglucusoaminidase and N-acetylgalactosaminidase) during dedifferentiation in the first 24 h of regenerating planarians has also been demonstrated (Pascolini et al., 1981). These results point towards the involvement of lysosomal hydrolases in regeneration, tissue remodeling and differentiation.

Hydra, a fresh water Cnidarian has been used as a powerful model system in biology to dissect molecular mechanisms underlying different processes such as wound healing, regeneration, immune response and autophagy. Presence of unique features, such as, remarkable power of regeneration, absence of cellular senescence and maintenance of axial polarity, has resulted in using hydra as a favorite model to study morphogenesis and pattern formation. Since lysosomes participate in physiological and stressed conditions, hydra can act as a powerful experimental model to study their involvement during tissue remodeling and regeneration. The ready availability of different strains of 'Hydra' in our laboratory also has prompted us to look more closely into the details of various lysosomal enzyme activities in each strain. In the present chapter we characterized two lysosomal hydrolases, acid phosphatase and β-N-acetylhexosaminidase, biochemically and carried out comparative analysis among three different strains of Hydra, *Hydra vulgaris* Ind-Pune, *H. vulgaris* Naukuchiatal and *H. magnipapillata* sf-1. H. vulgaris Ind-Pune and H. vulgaris Naukuchiatal are distinct Indian strains belonging to the 'vulgaris' group of hydra. Though, both strains belong to same 'vulgaris' species, they show significant morphological and taxonomical variations (Londhe et al., 2017). *H. magnipapillata* sf-1 is a temperature sensitive mutant strain of Japanese *H. magnipapillata* that grows normally at 18°C, while at restrictive temperatures, at 28°C or more, the polyps loses interstitial stem cells when maintained for 8-10 days (SUGIYAMA and FUJISAWA, 1977). In this chapter, we report

isolation and quantification of the expression levels of lysosomal enzymes in these three strains. Comparative analysis of β-N-acetylhexosaminidase and acid phosphatase among these three hydra strains are presented, which showed significant differences in their biochemical properties. Purification of β-hexosaminidase from the soluble extract of Hydra vulgaris Ind-pune was attempted using gel filtration chromatography. This suggests possible differences in their cellular and tissue makeup and may thus point towards their differences in physiological adaptations to the external environment.

5.2. Materials and methods

5.2.1. Materials

4-nitrophenyl substrates used for lysosomal enzyme assays, Sephacryl 200 (S200) used for the purification of β-hexosaminidase, Thyroglobulin, β-Amylase, Alcohol dehydrogenase, BSA, Carbonic Anhydrase, Cytochrome C, and Bradford reagent were purchased from Sigma Aldrich, St. Louis, MO, USA. All other chemicals used in the study were of highest purity and obtained from local suppliers.

5.2.2. Methods

5.2.2.1. Hydra culture maintenance

Clonal cultures of three hydra strains, *Hydra vulgaris* Ind-Pune (Marimuthu et al., 2011), *H. vulgaris* Naukuchiatal (Londhe et al., 2017), and a mutant strain of *H. magnipapillata* sf-1 (SUGIYAMA and FUJISAWA, 1977) were maintained in hydra medium at a constant temperature of 18 ± 1°C with 12 h light/dark cycle (SUGIYAMA and FUJISAWA, 1977). Polyps were fed with freshly hatched *Artemia salina* nauplii on alternate days.

5.2.2.2. Extraction of soluble proteins from hydra

The three strains, *Hydra vulgaris* Ind-Pune, *H. vulgaris* Naukuchiatal and *H. magnipapillata* sf-1 were mass cultured as previously described. Starved polyps of each strain was collected and used for preparing the soluble extracts. About 1000 whole polyps of each hydra strain were washed separately in 0.9 % saline and centrifuged briefly to collect the polyps. Soluble proteins were extracted by standard protocol as described previously (Bhamidimarri et al., 2018a). Briefly, polyps were lysed in lysis buffer (0.5 M sodium acetate-acetic acid buffer pH 5.0, containing 0.2 M NaCl, 1 mM EDTA, 5 mM iodoacetic acid and 1 mM PMSF), in a probe sonicator with 30/60 s on/off pulse cycle for three times. The lysed homogenate was centrifuged at 26,892 x g for 30 min and the clear supernatant containing soluble proteins was collected, stored and used for comparison studies.

5.2.2.3. Lysosomal enzyme assays

Soluble extract obtained from three strains of hydra were assayed for the presence of lysosomal enzymes, and about 500 ng of protein from each extract was used for the enzyme assay. Acid phosphatase and β-hexosaminidase were incubated separately with respective substrates (4-nitrophenyl-phosphate and 4-nitrophenyl N-acetyl-β-D-glucosaminide), in a final substrate concentration of 1 mM, at 37°C for 30 min. The reaction was stopped by adding equal volume of 0.2 M Na_2CO_3 and the absorbance of released 4-nitrophenyl was measured at 405 nm. One unit of enzyme activity is defined as the absorbance equivalent to 1 μmol of paranitrophenol released per minute per mL of the enzyme under experimental conditions. Data are presented as mean ± S.D, and the statistical significance was measured by multiple comparison method, ordinary one-way ANOVA.

5.2.2.4. Optimum pH and temperature determination

The effect of pH and temperature on the activities of β-hexosaminidase and acid phosphatase from three strains of hydra was investigated separately with respective substrates. To determine the optimum pH, soluble extracts and the substrates were incubated in different pH buffers from pH 2.0 to 10.0, and the enzyme assays were performed as described above. Similarly the optimum temperature for β-hexosaminidase and acid phosphatase was investigated by performing the assay at different temperatures ranging from 10°C to 100°C as described above.

5.2.2.5. Enzyme kinetics

To study the rate of hydrolysis of 4-nitrophenyl N-acetyl-β-glucosaminide and 4-nitrophenyl phosphate by β-hexosaminidase and acid phosphatase in different strains, soluble extracts were incubated with increasing concentrations (0.1 mM to 5 mM) of respective substrates separately, and performed enzyme assay. Kinetic parameters K_M and Vmax values were calculated by Michaelis-Menten plots using Graph Pad Prism7 software.

5.2.2.6. Purification of β-hexosaminidase

The soluble extract of Hydra vulgaris was used for the purification of β-hexosaminidase. Gel filtration chromatography using Sephacryl 200 (S-200) was used for purification. The fractions collected were monitored for the presence of β-hexosaminidase activity and the protein profile of active fractions were analyzed on SDS-PAGE.

5.3. Results

5.3.1 Lysosomal enzyme assays

Since the activities of β-hexosaminidase and acid phosphatase were high, the activities of these two enzymes were compared in three different strains of Hydra, *H. vulgaris* Ind-Pune,

H. vulgaris Naukuchiatal and *H. magnipapillata* sf-1, and were biochemically characterized. An equal amount of protein from the soluble extract of each strain was used to measure the activities of β-hexosaminidase and acid phosphatase. The activity of hexosaminidase was observed to be high in *H. vulgaris* Naukuchiatal and *H. magnipapillata* sf-1, whereas in *Hydra vulgaris* Ind-Pune strain, comparatively less activity was observed. (Figure. 1A). Similarly, the activity of acid phosphatase was high in, *H. vulgaris* Naukuchiatal and *H. magnipapillata* sf-1 as compared to *H. vulgaris* Ind-Pune (Figure. 1B).

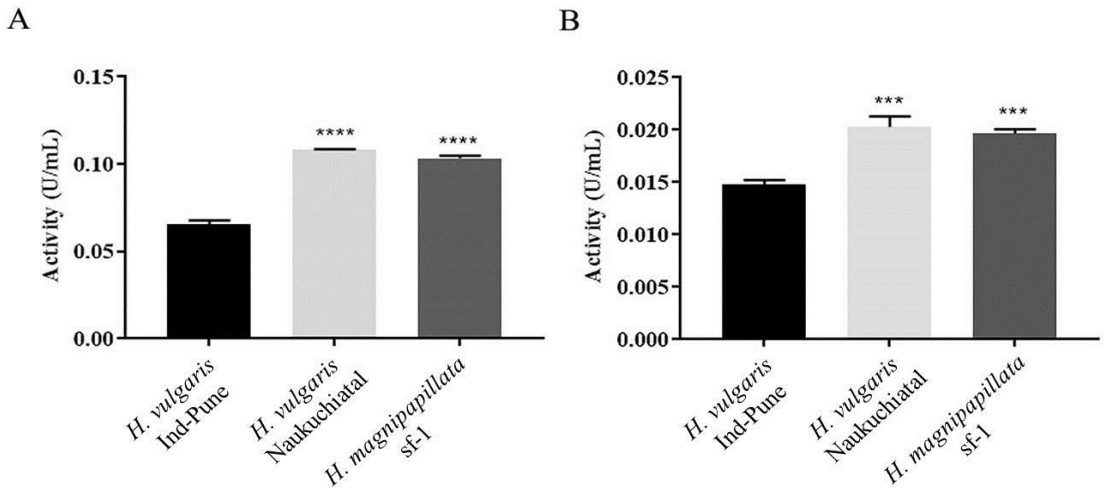


Figure. 1. Comparison of β-hexosaminidase and acid phosphatase enzyme activities. Histograms representing relative enzyme activities of β-hexosaminidase **(A)** and acid phosphatase **(B)** in three strains of hydra. Activities of both enzymes were high in *H. vulgaris* Naukuchiatal and *H. magnipapillata* sf-1 than *Hydra vulgaris* Ind-Pune. **** and *** show statistical significance, $p < 0.0001$ and $p = 0.0001$, respectively.

5.3.2 Effect of pH on the activities of β-hexosaminidase and acid phosphatase

The pH optima of β-hexosaminidase (Fig. 2A) and acid phosphatase (Fig. 2B) in three strains were determined by using 4-nitrophenyl N-acetyl-β-D-glucosaminide and 4-nitrophenyl-phosphate substrates respectively. The pH optimum for β-hexosaminidase was

found to be 6.0 for *H. vulgaris* Ind-Pune (Fig. 2Aa) and *H. vulgaris* Naukuchiatal (Figure. 2Ab), whereas the pH optima for *H. magnipapillata* sf-1 was found to be 5.0 (Figure. 2Ac). The pH optima for acid phosphatase in all three strains was found to be 3.0 (Figure. 2B).

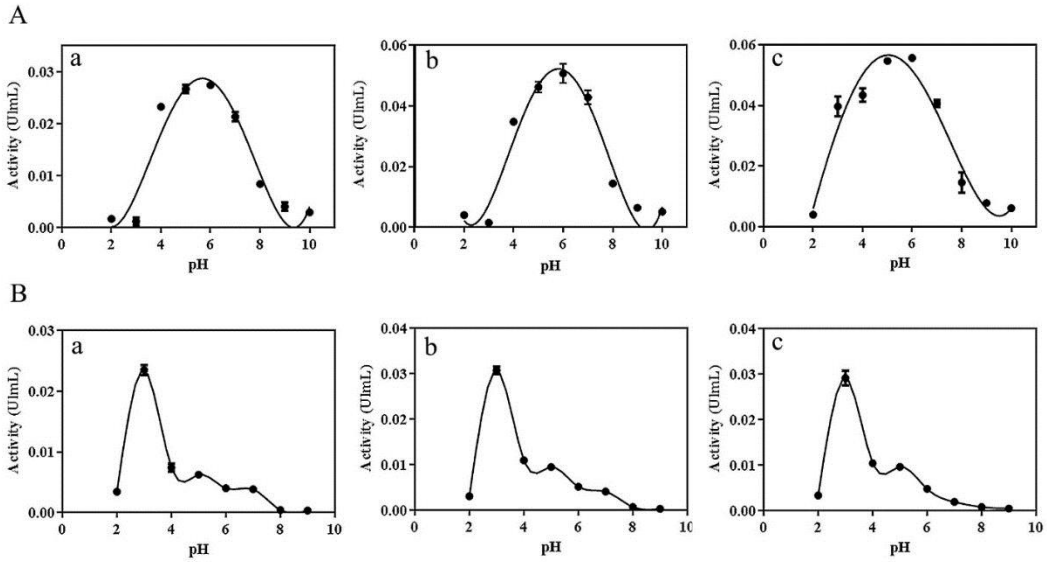


Figure. 2. The effect of pH on the activity of β-hexosaminidase **(A)** and acid phosphatase **(B)** in *Hydra vulgaris* Ind-Pune (a), *H. vulgaris* Naukuchiatal (b) and *H. magnipapillata* sf-1 (c). Values represent means of 2 replicas.

5.3.3 Effect of temperature on the activities of β-hexosaminidase and acid phosphatase

Temperature dependent activity of both the enzymes was determined by performing the enzyme assays at various temperatures ranging from 10°C to 100°C. The optimum temperature of *H. vulgaris* Ind-Pune β-hexosaminidase was found to be 60°C (Fig. 3Aa), whereas in *H. vulgaris* Naukuchiatal (Figure. 3Ab) and *H. magnipapillata* sf-1 (Figure. 3Ac), the optimum temperature for hexosaminidase was found to be 50°C. Interestingly, the temperature optima for acid phosphatase in all the three stains remained same at 40°C (Figure. 3B).

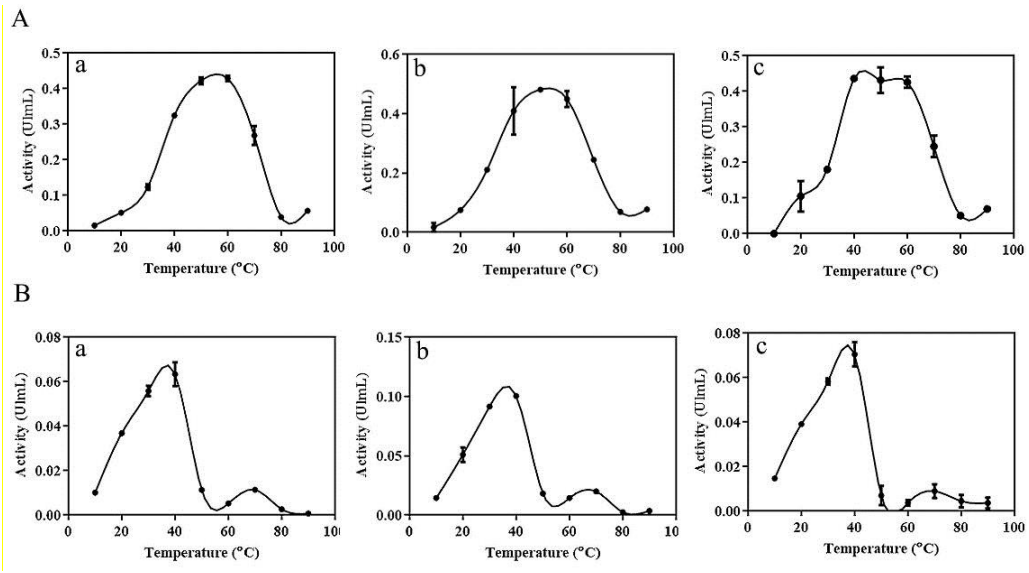


Figure. 3. The effect of temperature on the activity of β-hexosaminidase **(A)** and acid phosphatase **(B)** in the soluble extracts of *Hydra vulgaris* Ind-Pune (a), *H. vulgaris* Naukuchiatal (b) and *H. magnipapillata* sf-1 (c). Values represent means of 2 replicas.

5.3.4 Enzyme kinetics of β-hexosaminidase and acid phosphatase

The rate of hydrolysis of 4-nitrophenyl N-acetyl-β-D-glucosaminide by β-hexosaminidase and 4-nitrophenyl-phosphate by acid phosphatase was determined by respective substrates by Michaelis-Menten plots. Both the enzymes showed different affinities towards the substrates in three strains of hydra. The affinity of *H. magnipapillata* sf-1 hexosaminidase towards 4-nitrophenyl N-acetyl-β-D-glucosaminide was observed to be more as compared to other two strains with a K_M value of 0.8 mM, whereas the affinity of hexosaminidase towards 4-nitrophenyl N-acetyl-β-D-glucosaminide (Figure. 4A) from *H. vulgaris* Ind-Pune and *H. vulgaris* Naukuchiatal were almost similar with K_M values, 1.3 mM and 1.1 mM respectively. Interestingly, acid phosphatase from *H. vulgaris* Naukuchiatal showed less affinity towards 4-nitrophenylphosphate with a K_M value of 1.2 mM, whereas the affinity of acid phosphatase (Figure. 4B) from *H. vulgaris* Ind-Pune and *H. magnipapillata*

sf-1 showed same affinity towards 4-nitrophenylphosphate and more affinity than *H. vulgaris* Naukuchiatal with a K_M value of 0.38 mM and 0.52 mM.

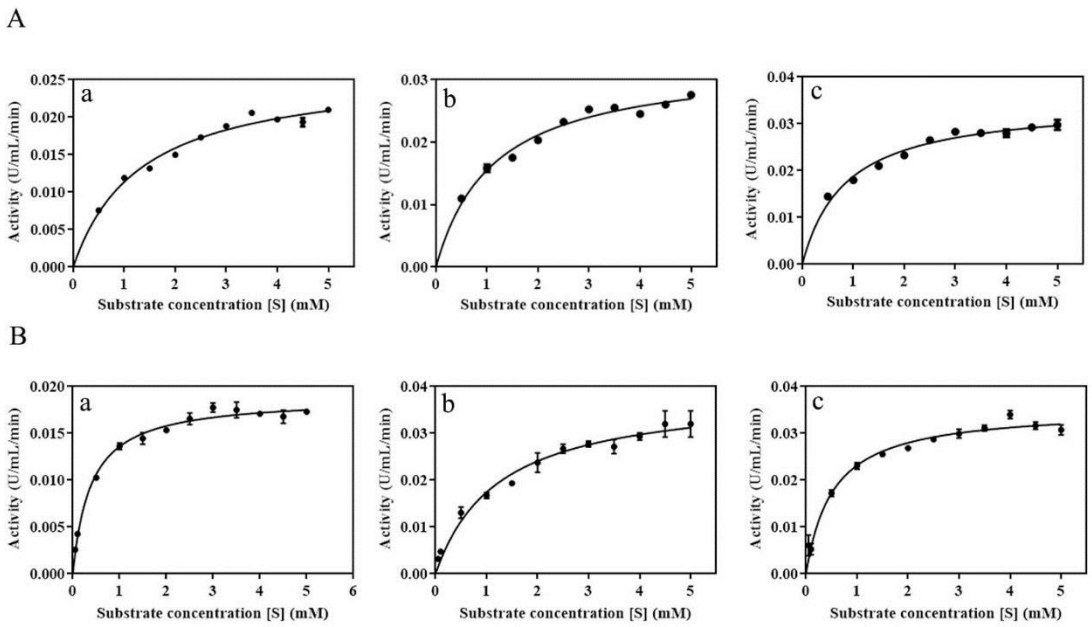
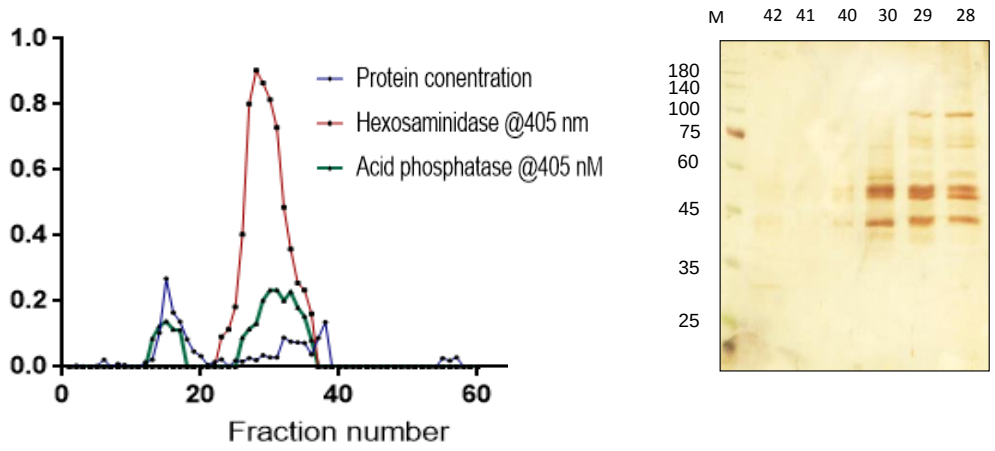


Figure. 4. Michaelis-Menten plots for the hydrolysis of 4-nitrophenyl N-acetyl glucosaminide by β-hexosaminidase from three strains of hydra showing K_M and Vmax values (A). Michaelis-Menten plots for the hydrolysis of 4-nitrophenyl phosphate by acid phosphatase from three strains of hydra showing K_M and Vmax values (B).

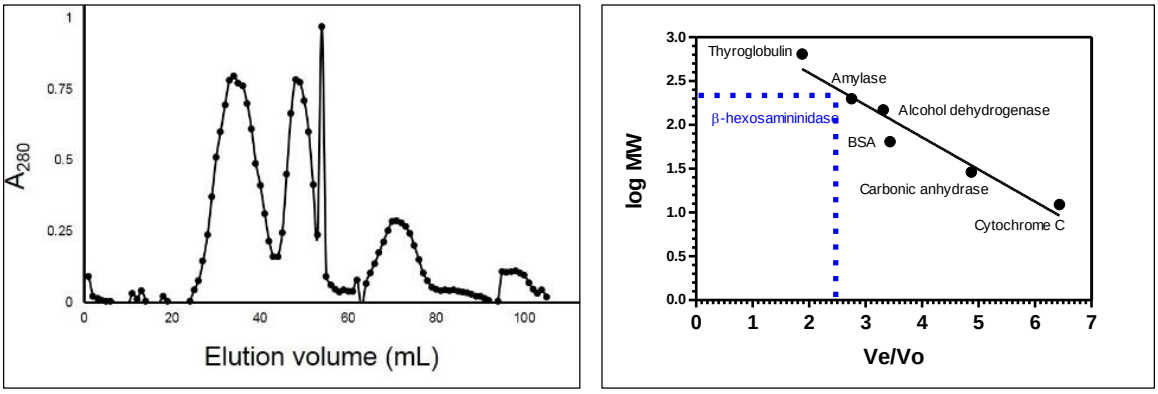
5.3.5 Purification of β-hexosaminidase

About 80 mL of the Sephacryl S-200 gel packed column equilibrated with 10 mM Tris-HCl, pH 7.4 was used for purification. The soluble extract (1 mL) was applied on to the S-200 gel. The fractions of 500 µL were collected and assayed for the protein and enzyme activity (Figure. 5A). The fractions with β-hexosaminidase activity (28, 29, 30) were analyzed by SDS-PAGE analysis (Figure. 5B). The molecular weight was determined by running standard proteins on S 200 column (Figure. 6 A and B).



A B

Figure. 5. The elution profile for gel filtration chromatography is presented here. Absorbance at 280 nm was showed in blue line, and the enzyme activity was plotted as absorbance at 405 nm **(A)**. The fractions containing hex activity (28, 29, 30) were analyzed on 10%SDS PAGE **(B)**.



A B

Figure. 6. The elution profile for gel filtration chromatography is presented here. The approximate molecular weight of hexosaminidase was estimated to be 200 kDa.

5.4. Discussion

Hydra polyps have been found to be sensitive to various environmental pollutants. Due to its simple, bilayered nature, hydra can be used as a biological indicator to evaluate the presence of contaminants in the medium, where the cells are in continuous contact with the medium, making it a sensitive biological indicator (Holdway et al., 2001; Patwardhan and Ghaskadbi, 2013). Due to the differences in the morphology and habitat, many species of hydra respond differently to a wide range of environmental pollutants (Karntanut and Pascoe, 2002). For example, the two hydra species, *H. vulgaris* and *H. viridissima*, showed differences in their sensitivities towards zinc and cadmium; both species are more sensitive to cadmium than to zinc, and the green hydra were more sensitive than *Hydra vulgaris* (Holdway et al., 2001).

At the cellular level, lysosomes are the major organelles that undergo changes due to the toxic effects of many contaminants, and the alterations in the lysosomes. They include changes in lysosomal contents and swelling of lysosomes and an increase in lysosomal hydrolase activities can be used as reliable tools to assess environmental contaminants (Dailianis, 2010 2011; Jing et al., 2006a; Moore, 2004; P Jayakumar, 2007). Further, the stability of lysosomes can also be assessed by the latent lysosomal activity of the lysosomal enzymes, N-acetyl-β-hexosaminidase, β- glucuronidase and acid phosphatase (Moore, 2004; P Jayakumar, 2007). With an aim to use these hydra strains as promising biological indicators in freshwater contamination assessment, the activities of two marker enzymes, acid phosphatase and hexosaminidase were compared in these three strains of hydra. From the figure 2A, it is interesting to see the differences in the hexosaminidase activity with respect to pH, where optimum activity was observed in *H. vulgaris* Ind-Pune and *H.*

vulgaris Naukuchiatal at pH 6.0, while in *H. magnipapillata* sf-1, it was observed to be at 5.0. Being an acid hydrolase, hexosaminidase showed maximum activity at an acidic pH, and many of the hexosaminidases shows maximum activity at an acidic pH (Venugopal and Sivakumar, 2013a). Surprisingly, no difference was observed in the pH optima of acid phosphatase in all three strains, though some of the acid phosphatases showed maximum activity in the pH range of 3-5 (Jing et al., 2006a; Mazorra et al., 2002). When temperature optima were investigated, hexosaminidase exhibited maximum activity at 60°C in *H. vulgaris* Ind-Pune, whereas in *H. vulgaris* Naukuchiatal and H. magnipapillata sf-1, 50°C showed maximum activity. It has been previously reported that glycosidases show maximum activity at higher temperatures in invertebrates (Venugopal and Siva Kumar, 2014; Venugopal and Sivakumar, 2013a; Venugopal et al., 2017). On the other hand it is interesting to notice that acid phosphatase showed no difference for optimum pH and temperature for activity in all three strains of hydra. Acid phosphatase, an inducible lysosomal enzyme, can be altered by the presence of xenobiotics and heavy metals, and is widely used as a biomarker, a potential indicator for assessing the impact of heavy metal pollutants (Jing et al., 2006a; Jing et al., 2006b; Mazorra et al., 2002; P Jayakumar, 2007), and increased activity was always associated with the damage of lysosomes (Jing et al., 2006b). Hence both these enzymes (hexosaminidase and acid phosphatase) can be used as tools in environmental pollution assessment.

Both enzymes showed different affinities towards the 4-nitrophenyl-conjugated synthetic substrate in three strains of hydra. The affinity (0.8 mM) of *H. magnipapillata* hexosaminidase was more as compared to other two strains, whereas the affinity of hexosaminidase from *H. vulgaris* Ind-Pune and *H. vulgaris* Naukuchiatal were almost

similar with K_M values 1.3 mM and 1.1 mM respectively. Interestingly, acid phosphatase from *H. vulgaris* Naukuchiatal showed less affinity towards 4-nitrophenylphosphate with a K_M value of 1.2 mM, whereas the affinity of acid phosphatase from *H. vulgaris* Ind-Pune and *H. magnipapillata* sf-1 showed similar affinity towards 4-nitrophenylphosphate with a respective K_M values of 0.38 mM and 0.52 mM. This differential affinity of both enzymes from three different hydra strains could be attributed to differential expression of the enzyme in these strains, where the expression of enzymes is further dependent on the ecological habitat of the animals. Since the activity of β-hexosaminidase was high in the soluble extract of *Hydra vulgaris*, an attempt was made to purify this enzyme using gel filtration chromatography. From the standard plot the molecular weight of β-hexosaminidase was found to be around 200 kDa.

In summary, a comparative analysis of three important lysosomal enzymes, from three different strains of 'Hydra' has been reported for the first time. Results obtained in this study are in agreement with our recent findings on the lysosomal activities from *H. vulgaris* Ind-Pune. A significant difference in the expression levels of acid phosphatase, hexosaminidase and glucuronidase across the three strains of Hydra was observed. Among the three enzymes, a significant difference was observed in the properties of hexosaminidase in all the three strains. In addition, a differential affinity of hexosaminidase and acid phosphatase towards the chromogenic substrates was observed in all the three strains of Hydra.

Summary

In summary, from all the work described in chapters 2-5, the following conclusions can be listed.

- Glucuronidase from the *L. corrianus* was purified to homogeneity. Purified enzyme is a hetero tetramer with an estimated native molecular mass of 250 kDa as determined by gel filtration chromatography. The optimum pH was 5.0, and the optimum temperature is 70°C which is typical for lysosomal enzymes. The enzyme was found to be active and stable up to 70°C for one hour.

- Michaelis-Menten constant K_M is 0.46mM, Vmax is 0.12 µmol/min. The secondary structure of β-glucuronidase contains equal proportions of alpha helix, beta sheets and random coils.

- The binding of mannose 6-phosphate receptors to hexosaminidase A and hexosaminidase B were quantitatively determined. The affinity of MPR 300 towards hexosaminidase A is higher than for hexosaminidase B. The binding affinity of MPR 46 towards Hex A was found to be twice the affinity of Hex B.

- Only hexosaminidase B has binding affinity for the sugars tested, and GlcNAc showed the higher affinity towards hexosaminidase B among the sugars tested.

- Lysosomal enzymes hexosaminidase and acid phosphatase were identified from the soluble extracts of *Hydra vulgaris*. The *in situ* studies revealed that the acid phosphatase is localized to ectoderm region and the hexosaminidase to the endoderm region.

- Codon optimization of acid phosphatase and hexosaminidase genes helped in their expression in mammalian cells.

- The optimum temperature for β-hexosaminidase was 60°C for *H. vulgaris* Ind-Pune, while 50°C was observed for *H. vulgaris* Naukuchiatal and sf-1 strains. The optimum pH for β-hexosaminidase was found to be 6.0 for *H. vulgaris* Ind-Pune and *H. vulgaris* Naukuchiatal, and 5.0 for sf-1. The optimum temperature and pH of acid phosphatase was similar in all three strains, viz., 40°C and 3.0, respectively. The native molecular weight of hexosaminidase was estimated to be around 200 kDa

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Publications:

1. Rohit Sai Reddy Konada, Lakshmi Surekha Krishnapati, Ashapogu Venugopal, Chung-Hung Lin, Siva Kumar Nadimpalli 2019, Comparative analysis of acid phosphatase and β-hexosaminidase from Hydra vulgaris Ind-Pune, H. vulgaris Naukuchiatal and H. magnipapillata sf-1: localization studies of acid phosphatase and β-hexosaminidase from H. vulgaris Ind-Pune. (communicated, to **Comparative Biochemistry and Physiology**, June 2019-Under review)
2. Rohit Sai Reddy Konada, Venugopal A, Siva Kumar Nadimpalli 2019, Purification, biochemical and biophysical characterization of lysosomal β-D-glucuronidase from an edible freshwater mussel *Lamellidens corrianus*. (will be communicated during July chapter-3)
3. Rohit Sai Reddy Konada, Venugopal A, Siva Kumar Nadimpalli 2019, Ligand binding studies of lysosomal β-hexosaminidases and Mannose 6-phosphate receptors from *Lamellidens corrianus*. (will be communicated during July chapter-3)

Poster Presentations:

1. Rohit Sai Reddy K, Poorna Manasa B, and Siva Kumar N. Lysosomal glycosidases from invertebrates. Poster presented in 'AS-UoH Joint Workshop on Frontiers in Life Sciences' workshop conducted by University of Hyderabad, Hyderabad, India, September 2016.
2. Nadimpalli Siva Kumar, Poorna Manasa Bhamidimarri, Lakshmi Surekha Krishnapati, Rohit Sai Reddy Konada, 'New insights into the lysosomal enzymes and their sorting receptors in Hydra' 29th Joint Glycobiology Meeting 2018, 21-23rd October 2018 in Ghent, Belgium.

Biochemical characterization of lysosomal enzymes from the invertebrates Lamellidens corrianus and Hydra vulgaris

by Rohit Sai Reddy Konada

Biochemical characterization of lysosomal enzymes from the invertebrates Lamellidens corrianus and Hydra vulgaris

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4	Poorna Manasa Bhamidimarri, Lakshmi Surekha Krishnapati, Surendra Ghaskadbi, Siva Kumar Nadimpalli. "Mannose 6-phosphate-dependent lysosomal enzyme targeting in hydra: a biochemical, immunological and structural elucidation", FEBS Letters, 2018 Publication	1%

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6	Sivaramakrishna Yadavalli. "Mannose-6-phosphate receptors (MPR 300 and 46) from the highly evolved invertebrate Asterias rubens (Echinodermate): biochemical and functional characterization of MPR 46 protein", Glycoconjugate Journal, 12/2008 Publication	1%
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