CHAPTER 2

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

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

Cathepsin D enzyme from mammalian sources are well known and the enzyme is probably one of the major factors contributing to the lysosomal digestive activity (Diment et al., 1988; Metcalf and Fusek, 1993; Tsuji and Akasaki, 1994). It has been reported that it constitutes as much as 10% of the lysosomal proteins (Wittlin et al., 1998). In addition, cathepsin D may have other roles in cells such as activation of proteins destined for secretion and to process antigens for presentation to the immune system (Maric et al., 1994). Mammalian cathepsin D enzymes have been isolated and cloned from a wide range of sources such as bovine (Gubensek et al., 1976) and
human (Wright et al., 1997; Nogami et al., 2000). The enzyme has also been purified from some fish species such as tilapia (Jiang et al., 1991) and Antarctic icefish (Capasso et al., 1999). However in the lower invertebrates which lack a well developed digestive system, the enzyme is less extensively characterized (Barnard, 1973). In a recent study it was well established that the lysosomal enzyme targeting machinery is evolutionary conserved from fish to mammals (Siva Kumar and Praveen Kumar, 2010). In order to further understand the evolution of these receptors we extended our studies to the invertebrates and found the mammalian homologues of the putative receptors in the invertebrates such as the starfish and unio. Our recent studies using starfish as an animal model suggested evolutionary conservation of the MPR 46 protein (Sivaramakrishna and Siva Kumar, 2008), and that the MPR 300 is involved in specific binding of an affinity purified α-fucosidase enzyme (Merino Visa et al., 2012). In order to establish the evolutionary conservation of the lysosomal enzyme sorting machinery in the invertebrates, it becomes essential to purify and characterize different lysosomal enzymes and to understand their interaction with the receptor(s).

The present study was carried out in order to gain new insights into these aspects with the following objectives:

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

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

iii) Study its specific interaction with the MPR 300 protein.
2.2. MATERIALS AND METHODS

2.2.1. Materials

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

2.2.2. Preparation of Pepstatin A affinity gel

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

2.2.3. Extraction and affinity purification of cathepsin D from starfish

All operations were carried out at 4°C unless otherwise stated. Fifty grams of the whole animal tissue was thawed on ice and homogenized with 4 volumes of 25 mM Tris-HCl buffer pH 7.4 (buffer A). The homogenate was allowed to stir overnight and centrifuged (26892 x g, 30 min). The pellet was discarded and the supernatant was recentrifuged for 15 min as described before. Ammonium sulfate was added to the supernatant to attain 80% saturation and the suspension stirred for 3 h. The
precipitated protein was collected by centrifugation (26892 x g, 30 min), dissolved in a small volume of 50 mM sodium acetate buffer, pH 3.5, containing 0.2 M NaCl (buffer B) and dialysed extensively against the same buffer. The dialysed sample was briefly centrifuged and the clear supernatant was applied to a 2 mL Pepstatin A affinity gel (1.5 x 9 cm) pre-equilibrated with buffer B. The unbound fraction was recycled several times through the column. The non-specific proteins were washed off with buffer B and elution was carried out with 50 mM Tris-HCl buffer pH 8.0 containing 0.2 M NaCl (buffer C) at a flow rate of 30 mL/h.

2.2.4. SDS-PAGE and western blotting

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

2.2.5. Immunological characterization of the purified cathepsin D

2.2.5.1. Production of antisera

A rabbit polyclonal antiserum was raised against the purified starfish cathepsin D. ~750 µg of purified enzyme in 0.5 mL PBS was mixed with 1.0 mL Freund's
complete adjuvant and injected subcutaneously into a rabbit. After three weeks, a booster injection was given with ~750 µg of the purified enzyme in 0.5 mL PBS mixed with 0.5 mL Freund's incomplete adjuvant. Two weeks after the booster injection, blood was collected by ear vein puncture from the rabbit into a falcon tube and allowed to clot. Antiserum was collected by centrifugation and stored at -20°C. Additional booster injections were given at two week intervals and the rabbit was bled 10 days after each booster injection. The final bleeding of the rabbit was performed after four weeks. The rabbit was housed and handled at the University of Hyderabad animal house.

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

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

Affinity purified antibodies against cathepsin D proteins were adsorbed to microtiter wells of a 96 well ELISA plate for 4 h at 37°C (250 ng of affinity-purified cathepsin D antibody in 50 µL of 25 mM Tris-HCl, pH 7.4). The wells were washed with 200
µL of 25 mM Tris-HCl buffer, pH 7.4, and incubated overnight at 4°C/1 h at room temperature with 200 µL of buffer A (5% BSA in PBS). Fifty microliters of purified cathepsin D (0.5-10 ng), or soluble tissue extracts from whole animal tissue, digestive gland and gonads (0.5-5.0 µg) diluted in buffer A (quantitation of protein was done according to Bradford), were separately bound for 2.5 h at 37°C. The wells were washed four times with 200 µL of PBS (buffer B) followed by incubation with 200 µl of buffer A for 30 min at 37°C. Subsequently, 50 µL of diluted rabbit antiserum against cathepsin D (dilution in buffer A, 10⁻³) was added and the plate incubated for 1 h at 37°C. After washing four times with buffer B, goat anti-rabbit IgG conjugated to alkaline phosphatase (Bangalore Genei) (dilution 1:2000 in buffer A) was added and incubated for 1 h at 37°C. The wells were washed four times with buffer B and one time with 200 µL of buffer C (0.1 M Tris-HCl pH 9.5, 0.1 M NaCl, 2 mM MgCl₂). The colour was developed with p-nitrophenyl phosphate (1.0 mg/mL in buffer C) for 10 to 20 min at room temperature and the absorbance was measured at 405 nm in a microplate ELISA reader.

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

To confirm the specificity of the purified antibody, aliquot of the purified cathepsin D and receptor concentrated by centricon concentrator (MWCO-10) was taken in two separate tubes and incubated with preimmune serum (2 µL) and affinity-purified cathepsin D IgG (10 µg) respectively overnight at 4°C with rotation, in PBS containing 0.05 % Tween 20 (PBST) in a total volume of 500 µL. The antigen-antibody complexes were adsorbed to protein A-agarose (40 µL of a 10% suspension; Bangalore genei, India). After incubation for 1 h, protein A-agarose was collected by
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Centrifugation at 3502 x g, and the supernatants were discarded carefully leaving a few µL to avoid pipetting of protein A-agarose beads. The pellet was washed four times with PBST. The immunoprecipitates were solubilized under reducing conditions and analyzed by 10% SDS-PAGE. The protein was transferred to a nitrocellulose membrane and the blot was incubated with secondary antibody, goat anti-rabbit HRP conjugate and developed using the Super Signal West Femto Maximum Sensitivity Substrate.

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

2.2.6. Glycoprotein nature of the purified cathepsin D

Purified cathepsin D enzyme (2.0 mL) obtained from Pepstatin A affigel was applied on a Con A-Sepharose gel (1.0 mL) pre-equilibrated with 25 mM Tris-HCl buffer, pH 7.4, containing 5 mM CaCl₂ and 5 mM MnCl₂ (column buffer). After washing extensively with the column buffer, the bound protein was eluted with the column buffer containing 0.2 M methyl α-D-mannopyranoside. Protein was monitored at 280 nm and the extent of binding was determined. The carbohydrate content in the enzyme was determined (Dubois et al., 1956). For detection of
glycoprotein nature of the enzyme, periodic acid Schiff’s staining procedure was used (Zacharias et al., 1969).

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

2.2.7. Confocal microscopy

Human Embryonic Kidney 293 (HEK) cells were cultured in complete medium (DMEM with 10% (v/v) heat inactivated foetal bovine serum and 1% (v/v) penicillin/streptomycin at 37°C in 5% CO₂, which are known to contain the MPR 300 protein. It is used in the study to analyze localization of the endocytosed FITC conjugated cathepsin D as described below.

2.2.7.1. Immunofluorescence

The HEK cells were analyzed for the presence of cathepsin D protein by immunofluorescence using affinity purified cathepsin D antibody. The cells were grown to 80% confluency on sterile glass cover slips in complete DMEM medium at 37°C. The medium was discarded and the cells were washed with sterile PBS three times, 5 min each. The cells were fixed in 4% paraformaldehyde in PBS buffer for 6
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About 1 mg of purified cathepsin D was incubated with 550 μl borate buffer (50 mM, pH 9.0) and 100 μl fluorochrome solution of fluorescein-isothiocyanate (5 mg FITC dissolved in 1 mL of DMSO) was added to the enzyme sample, mixed well and the sample stored overnight at 4℃ in dark. Free FITC was removed by desalting using a Sephadex G-25 gel. The endocytosis of FITC conjugated cathepsin D was studied by incubating with HEK cells grown to 80% confluency on sterile glass cover slips in complete DMEM medium at 37℃. Following incubation, cells were washed with PBS three times and then they were fixed in 4% paraformaldehyde for 20 min at room temperature. The fixed cells were washed, permeabilised with 0.02% Triton X-100 for 30 seconds and blocking with 1% BSA in PBS for 30 min at room temperature. The cells were incubated with goat MPR 300 antibody (This antibody was raised for purified goat
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MPR 300 protein and has been shown in our laboratory to recognize the MPR 300 protein from molluscs to mammals) 1:200 dilution in 1% BSA in PBS. The cells were washed and incubated with fluorescent tagged anti-rabbit IgG-Alexa fluor 594 (molecular probes, Invitrogen) for 1 h at room temperature. Finally the cells were stained with DAPI and observed under confocal microscope.

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

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

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

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

2.3.1. Purification, SDS-PAGE analysis and immunoblotting

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

2.3.2. Immunological characterization of the purified cathepsin D

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

In the present study, the purified starfish enzyme was used to generate a polyclonal antibody. Cathepsin D specific IgG was obtained from the antiserum developed by passing the antiserum on cathepsin D-affigel as described under methods. Elution profile of cathepsin D specific IgG from cathepsin D-affigel is shown in Fig 2.4.2A. To check the homogeneity of the cathepsin D specific IgG eluted from the gel, an aliquot of the eluate was subjected to 7.5% SDS-PAGE under non-reducing conditions (Fig 2.4.2B, lane 2). The specificity of the antibody obtained
was further tested in a western blot experiment using crude extracts containing the enzyme. From Fig 2.4.2C, it is apparent that the antibody recognizes the protein in the extracts. Using the affinity purified antibody and the antiserum to the cathepsin D protein, an ELISA method was developed to quantify the enzyme from different tissues of the starfish such as the whole body tissue, digestive gland and the gonads. In order to establish precisely the concentration of the protein that could be detectable by the antibody developed, initial ELISA was carried out employing purified cathepsin D protein. From (Fig 2.4.3A) it is clear that the level of detection of cathepsin D protein lies in the range of 1-10 ng. The enzyme concentration was also quantified by ELISA employing the total soluble extract obtained from whole body tissue (Fig 2.4.3B), digestive gland (Fig 2.4.3C) and gonads (Fig 2.4.3D). In these crude preparations, 1-5 µg concentration of the enzyme could be detectable in each of the tissues tested.

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

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

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

2.3.4. Confocal microscopy

2.3.4.1. Immunofluorescence

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

2.3.4.2. Localization of endocytosed FITC-conjugated cathepsin D

The ability of the FITC-conjugated cathepsin D to interact with the MPR 300 protein on the HEK cells was investigated using confocal microscopy. Most mammalian cells contain the MPR 300 protein on the cell surface which has been shown to be involved in the internalization of mannose 6-phosphate containing ligands. The endocytic function of the HEK cells MPR 300 was revealed by using
FITC-conjugated cathepsin D as described under materials and methods. The endocytosed FITC-cathepsin D can be seen in Fig 2.4.6.III panel B (green). In the same cells, MPR 300 protein was also visualized using the goat MPR 300 antibody and fluorescent tagged anti-rabbit IgG-Alexa fluor 594 antibody as shown in panel C (red). Extensive co-localization of endocytosed FITC-cathepsin D with the MPR 300 protein can be seen in panel C. These results suggest the labeled enzyme is internalized through the cell surface MPR300 protein.

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

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

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

2.3.5.2. Immobilized protein-affinity chromatography

In order to gain further insight into the specificity of the interaction between the purified enzyme and the starfish MPR 300 protein, we analysed the binding of
cathepsin D to starfish MPR 300 protein immobilized to Affigel-10. At pH 7.0, (that favours binding) the purified enzyme bound strongly to the receptor-affigel. The bound enzyme could not be eluted using 5 mM glucose 6-phosphate (Fig 2.4.7C, lane 2) but could be eluted partially with 5 mM mannose 6-phosphate (Fig 2.4.7C, lane 3). When 50 mM sodium acetate buffer, pH 5.0 (that favours dissociation) was employed, some more protein was desorbed from the gel (Fig 2.4.7C, lane 3).
Figure 2.4.1. Electrophoresis of *Asterias rubens* cathepsin D.

(A) 10% SDS-PAGE analysis of the purified cathepsin D from starfish. Lane 2, under reducing conditions, Lane 3, under non reducing conditions. (B) Western blot analysis of the purified enzyme detected using mouse cathepsin D antiserum. (C) 7.5 % Native PAGE analysis of the purified cathepsin D. Arrow indicates position of the protein band.
Figure 2.4.1
Figure 2.4.2. Purification of starfish cathepsin D specific IgG.

(A) Elution profile of cathepsin D specific IgG from cathepsin D-affigel. 

(B) 7.5% SDS-PAGE analysis (non-reducing conditions) of cathepsin D specific IgG eluted from cathepsin D-affigel column with 100 mM glycine-HCl buffer, pH 2.65. Arrow indicates IgG band (Mr 150 kDa). 

(C) Western blot analysis of the crude soluble extract detected using the purified cathepsin D specific IgG. Arrow indicates position of the protein band.
Figure 2.4.2

A

Fraction Number

0 2 4 6 8

0.1

0.2

0.3

0.4

A280 nm

B

IgG (150kDa)

C

Figure 2.4.2
Figure 2.4.3. Quantification of the enzyme by ELISA. (A) represents purified cathepsin D. (B) represents soluble extract from whole animal tissue. (C) represents soluble extract from digestive gland and (D) represents soluble extract from gonad. The assays were done in triplicates and averages of the values were taken.
Figure 2.4.3
Figure 2.4.4. Interaction of the purified enzyme with purified starfish MPR 300. (A) Immunoprecipitation of the purified cathepsin D enzyme (10% SDS-PAGE). Lane 1, enzyme and pre-immune serum and Lane 2, enzyme and immune serum. (B) Ligand blot analysis of the purified cathepsin D enzyme in a 10% SDS-PAGE. Arrow indicates the recognition of enzyme-receptor complex by *unio* MPR 300 antibody.
Figure 2.4.4
Figure 2.4.5. 10% SDS-PAGE analysis of the purified cathepsin D.  
(A) Purified enzyme under reducing condition eluted from Con A-Sepharose gel and detected by silver staining. (B) Periodic acid schiffs (PAS) staining of the protein band. (C) Western blot analysis of the enzyme. Lane 1, native enzyme (- PNGase F) and lane 2, deglycosylated enzyme treated with (+ PNGase F) detected using starfish cathepsin D antibody.
Figure 2.4.5
Figure 2.4.6. Immunofluorescence of cathepsin D.

(I). Cells incubated with specific IgG (A) DAPI stain, (B) probed with cathepsin D specific IgG (1:100 dilutions) followed by incubation with FITC conjugated secondary antibody (green) (1:1000 dilutions), (C) merged images of A & B, (D) the transmission image; (II) Cells without specific IgG (negative control). (A) DAPI stain, II (B) incubation with FITC conjugated secondary antibody (green) (1:1000 dilutions). (C) the transmission image. Bar in both the panel I & II is 25 μm. (III) Localization of the endocytosed FITC-conjugated cathepsin D in HEK cells: (A) staining with DAPI, (B) Incubation with FITC-conjugated cathepsin D (green) for 1 hour at 37°C, (C) Incubation with goat MPR 300 antibody followed by fluorescent tagged anti-rabbit IgG-Alexa fluor 594 (red) secondary antibody, (D) Merged images of A, B & C. (E) The transmission image. Bar in the panel is 10 μm.
Figure 2.4.6
Figure 2.4.7(A) Cross-Linking of the ligand with the membrane receptor. Cathepsin D was affinity cross-linked to purified starfish MPR 300 receptor with DSS, as described in Materials and Methods. The complexes were visualized by western blotting after 7.5% SDS-PAGE under non-reducing conditions. (A) Lane 1, purified starfish MPR 300 protein cross-linked with purified cathepsin D using DSS. Lane 2, only purified cathepsin D. Both the cross-linked product and native purified cathepsin D detected using starfish cathepsin D antibody. (B) Starfish MPR 300 (uncross-linked product) probed with Unio MPR 300 antibody. (C) 10% SDS-PAGE analysis of the purified cathepsin D eluted from starfish MPR 300 affigel column. Lane 2, 5 mM glucose 6-phosphate eluate, lane 3, 5 mM mannose 6-phosphate eluate and lane 4, 50 mM sodium acetate pH 5.0 eluate.
Figure 2.4.7
2.5. DISCUSSION

The key function of mannose 6-phosphate receptors in the mammalian species is to mediate sorting of newly synthesized lysosomal acid hydrolases and the receptor recycles between TGN and late endosomes. It is well established from studies carried in the laboratory that both MPR proteins are conserved from fish to mammals among the vertebrates. We also affinity purified mammalian homologues of the receptors from the invertebrates starfish and molluscs (Siva Kumar and Praveen Kumar, 2010). The present study is a logical extension of the ongoing research project in the laboratory on establishing the evolutionary significance of the mannose 6-phosphate receptors, the lysosomal enzyme targeting proteins. It is shown from this study and also using mollusc cell lines that there is a specific interaction of the lysosomal enzymes with MPR 300 proteins suggesting that the invertebrates like the vertebrates also exhibit distinct and specific interactions between lysosomal enzymes and their receptors, which points towards the hypothesis that in the invertebrates, there might exist a similar lysosomal biogenesis pathway as in the mammals (Praveen Kumar et al., 2009). In *Drosophila* binding of mannose 6-phosphate receptor to phosphomannan could not be detected but identified only a lysosomal enzyme receptor protein (Dennes et al., 2005). These data suggest that possibly both functional lysosomal enzyme sorting receptors started appearing in evolution from the molluscs onwards. Therefore to gain new insights in establishing the lysosomal biogenesis pathway in the invertebrates and the evolutionary significance of the receptors, it is essential to purify as many lysosomal enzymes as possible, make a comparative analysis of their properties and establish their specific recognition by the lysosomal enzyme targeting receptors. The present study provides the first evidence on the affinity purification of the cathepsin D enzyme and its biochemical and
immunological characterization from starfish. The purity of the isolated enzyme and its molecular mass of approximately 45 kDa were confirmed by SDS-PAGE. The size and monomeric nature are close to what has been reported for cathepsin D from other species: Atlantic cod (*Gadus morhua*) (40 kDa) (Wang *et al*., 2007), Antarctic icefish (*Chionodraco hamatus*) (40 kDa) (Capasso *et al*., 1999). Cathepsin D enzymes isolated from some vertebrate and invertebrate species such as the common carp muscle, Atlantic cod and Japanese common squid are also single chain proteins (Goldman *et al*., 1995; Wang *et al*., 2007; Komai *et al*., 2004) respectively. Cathepsin D isolated from different species also exhibited different molecular mass, as exemplified by mackerel (*Scomber australasicus*) (51 kDa), milkfish (*Chanos chanos*) (54 kDa) (Jiang *et al*., 1993). Interestingly in *A. pectinifera* a different species of starfish, a protein size of 50 kDa has been reported (Ramaswamy *et al*., 2008). Rojo *et al*., (2010) recently described the isolation of a cathepsin D from the American lobster (arthropod) which exhibited a molecular mass of ~50 kDa. This difference in molecular masses could be due to the differences in their glycan nature of content.

Initial western blot experiment of the purified cathepsin D with a mouse antiserum suggested cross-reactivity between the invertebrate and the mammalian enzyme. In the present study we raised an antiserum for the purified enzyme with the following objectives. First to analyze its specific interaction with the purified enzyme, second, to develop an ELISA method to quantify the enzyme levels in different parts of the animal tissues and to investigate the specificity of interaction of the enzyme with the MPR 300 protein that may enable us to understand the function of the receptor in this species. Due to the high sensitivity of the ELISA, protein can be quantified at ng level. From the ELISA experiments, it is interesting to note that the expression levels
of the enzyme vary in different tissues and the enzyme level in the digestive gland is higher than in other tissues. In an earlier study antisera to purified MPRs were used to quantify the receptors in different tissues of goat and chicken (Suresh et al., 2002). In the present study, the results obtained clearly showed that the steady state concentrations of the enzyme (purified as well as crude soluble extracts from different organs) are independent of each other. Like the mammalian enzyme the invertebrate enzyme is also a glycoprotein as evidenced by its specific binding and elution from the Con A-Sepharose gel, periodic acid Schiff’s staining of the purified enzyme. Furthermore, deglycosylation of enzyme revealed lower molecular mass of the protein and interestingly the protein retained its immunological reactivity. The specificity of the cathepsin D purified IgG was also analysed by confocal microscopy. The enzyme used in this study can bind and can be internalized by the mammalian cell type that harbors the MPR 300 protein. The specific interaction of the purified enzyme with the MPR 300 from starfish in a mannose 6-phosphate dependent manner further suggests that in starfish also there might exist a similar lysosomal biogenesis pathway as in the vertebrates. The interaction between the starfish enzyme and MPR 300 is also shown by cross-linking experiment as well as by the immuno-precipitation. In an earlier study it has been shown that the α-fucosidase from the mollusc shows specific interaction with the MPR 300 protein in a cross-linking experiment and recognition by the enzyme antibody suggesting also a possible in vivo interaction of the receptor and enzyme (Siva Kumar et al., 2004) thus establishing the receptors function. It is already well established that there is a specific interaction of the MPR 300 with cathepsin D not only in normal cells, but also in breast cancer cells. Furthermore, this receptor also binds IGF-II (Marc et al., 1990).
The study carried out on the cathepsin D from starfish suggest that the enzyme is a lysosomal enzyme and the MPR 300 receptor from the starfish as well as from mammalian cells shows specific interaction with cathepsin D. The results are one step forward towards understanding the evolution of lysosomal enzymes and receptors in the invertebrates.