Comparative Biochemistry and Physiology, Part B 169 (2014) 25–30

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part B

journal homepage: www.elsevier.com/locate/cbpb

Biochemical characterization of cathepsin D from the mussel *Lamellidens corrianus*

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ARTICLE INFO

Article history:
Received 19 April 2013
Received in revised form 12 December 2013
Accepted 14 December 2013
Available online 21 December 2013

Keywords:
Cathepsin D
Invertebrate
Lamellidens corrianus
Mannose 6-phosphate receptor
Zymogram

ABSTRACT

A lysosomal cathepsin D (EC 3.4.23.5) was purified to homogeneity from the soft tissues of the fresh water mussel (*Lamellidens corrianus*) by pepstatin A affinity chromatography. The purified enzyme is a glycoprotein and migrates as a single protein species in native PAGE and shows a single band in SDS-PAGE corresponding to a molecular mass of ~43 kDa. Under both these conditions cathepsin D hydrolyzes hemoglobin as shown by zymogram analysis. The purified enzyme cross-reacts with an antiserum to purified starfish (*Asterias rubens*) cathepsin D. Additionally, the enzyme was recognized by the starfish lysosomal enzyme targeting receptors (mannose 6-phosphate receptors: MPR 300 and 46) in ligand blot analysis. The K_M value of the purified enzyme with hemoglobin is 1.5 mM. pH and temperature optimum for the enzyme are 3.5 and 60 °C respectively.

1. Introduction

Cathepsin D (EC 3.4.23.5) is a soluble lysosomal aspartic proteinase that is ubiquitous in nature (Davies, 1990) and contributes lysosomal degradation (Balti et al., 2010). This enzyme is well studied in mammals and in different species (Benes et al., 2008) and has been shown to be involved in regulation of programmed cell death (Blanco-Labra et al., 1996; Gui et al., 2006) and digestion of food proteins in invertebrates (Blanco-Labra et al., 1996; Williamson et al., 2002; Padilha et al., 2009; Rojo et al., 2010). It is synthesized in the rough endoplasmic reticulum as preprocathepsin D, modified in the Golgi complex and is transported to the lysosomes by two mannose 6 phosphate receptors [MPR 300 (Mr 300 kDa) and MPR 46 (Mr 46 kDa)] (Hasilik and Neufeld, 1980; Kornfeld, 1990; Benes et al., 2008; Minarowska et al., 2008; Zaidi et al., 2008). Mammalian enzymes have been isolated and cloned from a wide range of sources, including human (Wright et al., 1997), bovine (Hayes et al., 2001) and porcine tissues (Canduri et al., 1998). The enzyme has also been purified from some fish species such as carp (Goldman-Levkovitz et al., 1995), herring (Nielsen and Nielsen, 2001) and Antarctic ice fish (Capasso et al., 1999). This enzyme plays a significant role in intracellular degradation of proteins, polypeptide hormones (Parathyroid Hormone), growth factors such as insulin, glucagon and fibroblast growth factor (Benes et al., 2008) and is also involved in the in vitro activation of enzymatic precursor cathepsins B and L that were isolated from rat liver and kidney (Nishimura et al., 1988). In addition to its protease activity, cathepsin D also acts as a mitogen. Its over expression in rat embryonic tumor cells stimulates cell proliferation. However, the exact mechanism behind the mitogenic activity of cathepsin D is poorly understood (Glondu et al., 2001). Mutations in cathepsin D genes, deficiency or abnormal activity of the enzyme is known to lead to inherited neurodegenerative disorder, congenital neuronal ceroid lipofuscinosis in humans that is characterized by neurodegeneration, developmental regression, visual loss and epilepsy (Siintola et al., 2006; Steinfeld et al., 2006). Cathepsin D involvement in the tumor cell death through apoptosis is reported (Jia and Zhang, 2009). In invertebrates, cathepsin D is involved in the metamorphosis of insects (Gui et al., 2006). Lysosomal enzyme targeting among mammals and non-mammalian vertebrates is mediated by both MPRs that are evolutionarily conserved (Nadimpalli and Amancha, 2010). Although both receptors have been identified in echinodermate (*Asterias rubens*) and mollusc (*Lamellidens corrianus*), the role of these receptors in targeting lysosomal enzymes in invertebrates is not well characterized. Our long term goal is to understand the lysosomal biogenesis pathway in invertebrates so that the evolution of receptors in the animal kingdom can be precisely established. It is therefore necessary to purify and biochemically characterize as many lysosomal enzymes as possible from these species. In a recent study we have affinity purified and characterized two lysosomal enzymes, α-fucosidase and cathepsin D from starfish and found that these enzymes show specific interaction with MPR proteins (Merino and Siva Kumar, 2012; Visa et al., 2012). To further extend studies to molluscs we purified and biochemically characterized two forms of hexosaminidases from the *L. corrianus* (Venugopal and Sivakumar, 2013). An α-

Abbreviations: BCIP, 5-bromo-4-chloro-3′-indolyl phosphate; ECL, enhanced chemiluminescence; Hb, hemoglobin; IU, international unit; MPR, mannose 6-phosphate receptor; NBT, nitro blue tetrazolium chloride; PBST, phosphate-buffered saline containing Tween 20; TCA, trichloroacetic acid.

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http://dx.doi.org/10.1016/j.cbpb.2013.12.003

fucosidase from this animal was earlier purified and biochemically characterized (Nadimpalli et al., 2004). In order to gain new insights into the presence of other lysosomal enzymes in this species the present work was carried out with the following objectives i) to affinity purify cathepsin D, ii) to biochemically characterize the enzyme and compare its properties to starfish enzyme and iii) to study the specific interaction between the enzyme and MPRs.

2. Materials and methods

Pepstatin A and hemoglobin (Methemoglobin) substrate were from Sigma Aldrich (St. Louis, MO, USA). Affigel-10 was from Bio-Rad Laboratories (Hercules, CA, USA). ECL reagent was from Pierce, Rockford, IL, USA. PNGase F was from Roche (Germany). Mussels *L. corrianus* (Kingdom Animalia; Phylum Mollusca; Class—Bivalvia; Order—Unionoida; Family Unionidae; Genus—Lamellidens; Species corrianus) were supplied by UV Scientifics, Hyderabad, India. De-shelled mussel tissues were frozen immediately at −80 °C. All other reagents and chemicals were of high purity and procured from Sisco Research Laboratories, India.

2.1. Extraction of soluble proteins

All operations were carried out at 4 °C unless otherwise stated. Twenty grams of whole animal tissue was homogenized with 200 mL of 50 mM sodium acetate buffer pH 3.5, and extracted overnight at 4 °C. The homogenate was centrifuged at 26,892 *g* for 20 min. The supernatant was brought to 80% ammonium sulfate saturation, by addition of solid ammonium sulfate, stirred for 3 h, and centrifuged as described above; pellet obtained after centrifugation was dissolved in 50 mM sodium acetate buffer pH 3.5, assayed for the enzyme activity and was used for the purification of cathepsin D. The enzyme was purified in more than two batches and presented here as data represented by one of the three independent purifications.

2.2. Enzyme assay

Enzyme assay was carried out according to Barrett (1970) with minor modification where 50 μL of 2.5% hemoglobin solution was mixed with 730 μL of the 100 mM sodium acetate buffer pH 3.5 and equilibrated for 2 min at 37 °C, to which 20 μL of the enzyme (~500 ng) was added and incubated for 30 min at 37 °C. Then 500 μL of chilled TCA was added and incubated for 10 min at 37 °C and centrifuged. The TCA soluble peptide supernatant was collected and absorbance was measured at 280 nm against blank. One unit was defined as an increment in absorbance at 280 nm by 1.0 in 1 min at pH 3.5 at 37 °C measured as TCA soluble products released using acid denatured hemoglobin as substrate. All enzyme assays were analyzed in triplicates, the average values were taken and standard deviations for the error are presented.

2.3. Purification of cathepsin D

Preparation of pepstatin A affigel and purification of cathepsin D were carried as described earlier (Merino and Siva Kumar, 2012). About 2 mL of pepstatin A affigel was packed into a small column, and equilibrated with 50 mM sodium acetate pH 3.5 containing 0.2 M NaCl. The soluble extract obtained above was applied onto this gel. The unbound proteins were removed with the equilibrating buffer, until A_{280} reached ≤0.05. The bound enzyme was eluted with 25 mM Tris–HCl buffer pH 8.5 containing 0.2 M NaCl. The protein eluted was assayed for activity and molecular mass determination.

2.4. Activity staining/zymogram and SDS-PAGE

For activity staining, 10% SDS-PAGE and native PAGE were performed (Li et al., 2010), with minor modification, by co-polymerizing the gel with 0.2% hemoglobin substrate. After the electrophoresis, the gel was incubated in 100 mM Tris–HCl buffer pH 7.4 with 20% glycerol for 10 min and this was repeated thrice. The gel was incubated in activity buffer (0.1 M sodium acetate buffer pH 3.5 containing 1 mM EDTA and 2 mM DTT) for 30 min at room temperature. Fresh activity buffer was added to the gel and incubated overnight at 37 °C. Finally the gel was washed twice with deionized distilled water, and stained with Coomassie blue for 1 h at room temperature. The clear activity zones of cathepsin D were visualized by destaining the gel. The purity of the enzyme was analyzed by performing 10% SDS-PAGE under reducing conditions (Laemmli, 1970) and the proteins were visualized by Coomassie staining method.

2.5. Deglycosylation with PNGase F

Deglycosylation of the *L. corrianus* cathepsin D was carried out as described previously (Merino and Siva Kumar, 2012) by dialyzing the purified enzyme against 0.1 M sodium phosphate buffer pH 8.6, containing 0.2% SDS. After the dialysis, NP-40 and β-mercaptoethanol were added to a final concentration of 1.2 and 1% respectively, and the sample was boiled at 95 °C for 5 min. To this, PMSF, EDTA and iodoacetamide were added in a final concentration of 1, 1 and 5 mM respectively. The sample was divided equally into two tubes; experiment and control. To the experiment tube 3 units of the PNGase F was added and the control tube received no enzyme. Both the tubes were incubated at 37 °C for 16 h. After the incubation, samples were subjected to 10% SDS-PAGE and the protein bands were visualized by Coomassie staining.

2.6. Western and ligand blot analyses

Purified enzyme was separated on a SDS-PAGE gel and transferred to a nitrocellulose membrane and processed for western and ligand blot analyses. Western blot analysis was carried out as described earlier (Nadimpalli et al., 2004) by incubating the membrane with the starfish cathepsin D primary antibody raised in a rabbit, followed by incubation with ALP conjugated antirabbit secondary antibody, finally the blot was developed by using BCIP/NBT substrate. Ligand blot for cathepsin D was carried out as described earlier (Venugopal and Sivakumar, 2013) by incubating the membrane with starfish MPR 300 and 46 proteins as ligands (BSA was used as ligand in negative controls for both MPR 300 and 46 ligand blots). After washing, the membranes were probed with *L. corrianus* MPR 300 and goat MPR 46 antibodies raised in a rabbit. Subsequently membranes were washed and incubated with HRP conjugated antirabbit secondary antibodies and developed by ECL substrate.

2.7. Kinetic analysis

The kinetic behavior of the purified enzyme was studied, by incubating the enzyme with hemoglobin as described above in the concentration range of 0.1 mM 4 mM. Lineweaver and Burk method was used to determine the K_M and V_{max} with the help of reciprocal plot. Enzyme activity was analyzed in triplicates in three individual experiments taking average values and standard deviation for the error.

2.8. pH and temperature optimum for cathepsin D

To determine the pH optimum of cathepsin D, purified enzyme was incubated with hemoglobin as described above in different buffers of the pH range 1.0–10, and the activities were measured as described above. Temperature optimum was determined by incubating the enzyme with hemoglobin at temperatures ranging from 10 °C–100 °C and activities of the enzyme were measured as described above.