Biochimie 94 (2012) 1199–1205

Contents lists available at SciVerse ScienceDirect

Biochimie

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

Research paper

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

Merino Visa [a], Elke Hammer [b], Uwe Völker [b], Hendrik Koliwer-Brandl [c], Sørge Kelm [c], Siva Kumar Nadimpalli [a,*]

[a] Protein Biochemistry and Molecular Biology Laboratory, Department of Biochemistry, University of Hyderabad, Hyderabad 500 046, India
[b] Interfaculty Institute for Genetics and Functional Genomics, Ernst-Moritz-Arndt-University Greifswald, Friedrich-Ludwig-Jahn-Str. 15A, 17487 Greifswald, Germany
[c] Centre for Biomolecular Interactions Bremen, Department for Biology and Chemistry, University of Bremen, Leobener Str. NW2, 28359 Bremen, Germany

ARTICLE INFO

Article history:
Received 21 June 2011
Accepted 6 February 2012
Available online 17 February 2012

Keywords:
α-Fucosidase
Invertebrate
LC–MS/MS
De novo sequencing
MPR300

ABSTRACT

In vertebrates, mannose 6-phosphate receptors [MPR300 (Mr 300 kDa) and MPR46 (Mr 46 kDa)] are highly conserved transmembrane glycoproteins that mediate transport of lysosomal enzymes to lysosomes. Our studies have revealed the appearance of these putative receptors in invertebrates such as the molluscs and deuterostomes. Starfish tissue extracts contain several lysosomal enzyme activities and here we describe the affinity purification of α-fucosidase. The purified enzyme is a glycoprotein that exhibited a molecular mass of ~56 kDa in SDS-PAGE under reducing conditions. It has also cross-reacted with an antiserum to the mollusc enzyme suggesting antigenic similarities among the two invertebrate enzymes. LC–MS/MS analysis of the proteolytic peptides of the purified enzyme in combination with *de novo* sequencing allowed us to do partial amino acid sequence determination of the enzyme. These data suggest that this invertebrate enzyme is homologous to the known mammalian enzyme. The purified enzyme exhibited a mannose 6-phosphate dependent interaction with the immobilized starfish MPR300 protein. Our results demonstrate that the lysosomal enzyme targeting pathway is conserved even among the invertebrates.

1. Introduction

It is well established that vertebrate lysosomal enzymes undergo post-translational modifications in the *trans*-Golgi network and acquire mannose 6-phosphate residues. These enzymes are then recognized by two distinct but homologous receptor proteins designated as mannose 6-phosphate receptor MPR300 (Mr300 kDa) and MPR46 (Mr46 kDa), which mediate their transport into the lysosomes. Although these receptors were initially characterized extensively in mammals, more recent studies have clearly established their existence and specific roles in lysosomal enzyme targeting among non-mammalian vertebrates including the fish [1–3]. Vertebrate MPR300 binds to the ligands in a cation independent manner while MPR46 requires divalent cations for ligand binding [4–7]. In vertebrates, MPR300 is a multifunctional protein, which in addition to binding mannose 6-phosphate containing ligands, also binds human IGF-II [5,8–10]. Previous studies on lysosomal enzymes and their receptors have demonstrated the evolutionary conservation of lysosomal enzyme targeting in vertebrates [3].

Putative mannose 6-phosphate receptors were identified in deuterostomes (starfish) and the MPR46 protein has been shown to be involved in lysosomal enzyme transport [17]. However, the functional role of the starfish MPR300 has not been characterized yet. Mammalian homologs of these receptors have also been identified in molluscs (*unio* and the snail-*Biomphalaria glabrata*). In *B. glabrata*, the MPR300 protein also mediates the transport of lysosomal enzymes [18]. All these studies suggest a possible evolutionary conservation of the lysosomal enzyme biogenesis pathway in the animal kingdom. Therefore, to strengthen this hypothesis, it is necessary to undertake a detailed study of these enzymes and their receptors in these species.

α-Fucosidase (EC 3.2.1.51) is a glycosidase involved in the metabolism of several biologically active molecules containing L-fucose [11]. It is well documented that in humans the absence of this enzyme leads to a severe lysosomal disorder, Fucosidosis [3,12,13]. Though α-fucosidase activities have been characterized in some mammalian species, only limited information is available

Abbreviations: SPIDER, Software Protein Identifier; ACN, Acetonitrile; ESIation, Electrospray Ionization; MS, Mass Spectrometry.

* Corresponding author. Tel.: +91 40 23134569; fax: +91 40 23010120.
E-mail address: drnsk7@yahoo.co.in (S.K. Nadimpalli).

0300-9084/$ – see front matter © 2012 Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.biochi.2012.02.007

about this enzyme in invertebrates. In the molluscs (*unio*), two forms of the α-fucosidase have been purified *viz*., a sperm plasma membrane bound non-glycosylated form (65 kDa) and a soluble glycosylated form (56 kDa) [14]. The structural and functional domains of the lysosomal enzyme sorting receptors are highly conserved throughout the vertebrates [1,2,15,16]. However, in the starfish species, only MPR46 protein has been shown to interact with lysosomal enzymes but a detailed characterization of these lysosomal enzymes is still lacking. Therefore, the present study was undertaken with the following objectives, (i) to analyze different lysosomal enzyme activities from starfish tissue (ii) to affinity purify the α-fucosidase on fucosylamine gel and study its biochemical and immunological properties (iii) to study interactions between the purified enzyme and the starfish MPR300 protein.

2. Materials and methods

2.1. Materials

Starfish animals were collected from the North Sea, Germany and were stored frozen at −80 °C until use. *N*-(ε-Aminocaproyl)-β-L-fucopyranosylamine (Carbosynth, Berkshire, UK), phenyl Sepharose (type CL-4B), enzyme substrates and sugars used in the study were purchased from Sigma Chemical Co., (St. Louis, Missouri, USA). Affigel-10 was purchased from Bio-Rad laboratories (Hercules, CA, USA). DE-52 cellulose was purchased from Whatmann (Maidstone, UK). PVDF was purchased from Millipore (Bedford, MA, USA). Other chemicals used were obtained from Sisco Research Laboratory, Qualigens, Bangalore Genei, India.

2.2. Lysosomal enzyme assays

Enzyme assays were carried out as described earlier [19], using the following substrates: 4-nitrophenyl α-L-fucopyranoside for α-fucosidase, 4-nitrophenyl α-D-mannopyranoside for α-mannosidase, 4-nitrophenyl α-D-galactopyranoside for α-galactosidase, 4-nitrophenyl *N*-acetyl-β-D-glucosaminide for β-hexosaminidase, 4-nitrocatechol sulfate dipotassium salt for arylsulfatase A (Sigma, USA). The absorbance of the released *p*-nitrophenol was recorded at 405 nm. One unit of enzyme activity was defined as the amount which liberates 1 μmol of *p*-nitrophenol per min. Specific activity was expressed as units per milligram of protein.

2.3. Extraction and isolation of α-fucosidase from starfish

All operations were carried out at 4 °C unless otherwise stated. The activity of the enzyme was monitored at each step of the purification process. Fifty grams of the whole animal tissue was thawed on ice and homogenized with 4 volumes (200 ml) of 25 mM Tris–HCl, pH 7.0 (buffer A). The homogenate was stirred overnight and centrifuged (26892× *g*, 30 min; 4 °C). The pellet was discarded and the supernatant (200 ml) was recentrifuged for 15 min as described above. The clear supernatant was applied on a 20 ml DE-52 cellulose gel (2.5 × 20 cm), pre-equilibrated with buffer A. The column was washed with the same buffer until A_{280} of the flow through was 0.01. The bound protein was eluted in 5 ml fractions with 25 mM Tris–HCl, pH 7.0, containing 0.2 M NaCl (buffer B). The fractions containing protein with α-fucosidase activity were pooled and solid ammonium sulfate was added to a final concentration of 1 M. The protein solution was applied on a 5 ml phenyl Sepharose CL-4B column (1.5 × 15 cm) pre-equilibrated with buffer C containing 25 mM Tris–HCl, pH 7.0 and 1 M ammonium sulfate which is required for specific binding of glycosidases to the hydrophobic gel [20]. The column was washed extensively with buffer C and the bound protein was eluted in 2.0 ml fractions with buffer A. Fractions containing α-fucosidase activity were pooled and dialyzed against 50 mM sodium acetate buffer, pH 5.0 (buffer D). The dialyzed sample was briefly centrifuged and the clear supernatant was used for the purification of the enzyme by affinity chromatography.

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

0.66 g CNBr Sepharose 4B (GE Healthcare) was stirred in 1 mM HCl for 15 min in a glass beaker. The beads were rinsed on a sintered glass funnel with 200 ml of 1 mM HCl/1 g Sepharose. The gel (2.0 ml) was transferred into a falcon tube and 5 mg of *N*-(ε-Aminocaproyl)-β-L-fucopyranosylamine in 2.0 ml coupling buffer (0.1 M $NaHCO_3$ pH 8.3, 0.5 M NaCl) was mixed with the gel and incubated on an end-over-end rotator at 4 °C overnight. The gel was packed into a column and washed with ~5 ml coupling buffer, followed by 5 ml 0.1 M Tris–HCl, pH 8.0. The gel was incubated with 5 ml 0.1 M Tris–HCl pH 8.0 for 2 h at room temperature, washed three times with 5 ml 0.1 M sodium acetate pH 4, 0.5 M NaCl, and finally washed three times with 5 ml 0.1 M sodium acetate pH 8, 0.5 M NaCl.

After dialysis, the enzyme containing fraction obtained from phenyl Sepharose chromatography was applied on a 2 ml fucosylamine affinity gel (1 × 10 cm) pre-equilibrated with buffer D and incubated overnight at 4 °C. After washing the gel extensively with the same buffer to remove the unbound proteins, specific elution was carried out with 60 mM L-fucose in 50 mM sodium acetate buffer, pH 5.0 (buffer E). Eluting protein was monitored by absorbance at 280 nm. Aliquots of the eluates were dialyzed against buffer D to remove fucose and assayed for fucosidase activity and other glycosidase activities as described above.

Protein concentrations in the column eluates were determined using Bradford reagent (Bio-Rad). Aliquots of the eluates were subjected to 10% SDS-PAGE under reducing conditions [21], and the protein bands were detected by colloidal coomassie blue as well as by staining with silver nitrate.

For the immunological detection, 2 μg of the purified protein was separated by SDS-PAGE and transferred to a PVDF membrane [22]. The membrane was incubated with the antiserum (1:500 dilution) raised against the purified *unio* α-fucosidase [19] for 1 h at room temperature or overnight (about 16 h) at 4 °C. The membrane was washed with 10 mM phosphate buffer pH 7.4, 150 mM NaCl, 0.05% Tween 20 (PBST) and the protein band was detected by incubating the membrane with secondary antibody goat-anti rabbit HRP conjugate (Pierce, Rockford, USA). The blot was developed using the Super Signal West Femto Maximum Sensitivity Substrate (Thermo Scientific, Rockford, IL, USA).

2.5. Sample preparation for LC–MS/MS

The purified enzyme (2 μg) was separated on a 10% SDS-PAGE (7 cm) and visualized by colloidal coomassie blue staining. Protein containing gel slices corresponding to a molecular mass of 56 kDa were destained by incubating two times with 100 μl of 200 mM ammonium bicarbonate (NH_4HCO_3)/50% ACN v/v for 15 min at 37 °C and dried with 100% ACN. In-gel digestion was performed overnight at 37 °C using trypsin (Promega, Mannheim, Germany) or chymotrypsin (Sigma, Taufkirchen, Germany) dissolved in 20 mM NH_4HCO_3 (pH 8.2). The resulting peptides were extracted in a water bath sonicator for 30 min with 0.1% acetic acid in 50% ACN and subsequently with 0.05% acetic acid in 80% ACN. The eluates of both extractions were pooled and concentrated in a vacuum centrifuge. Peptide extracts were desalted on μ-C18