Biochimie 93 (2011) 141–148

Contents lists available at ScienceDirect

Biochimie

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

Research paper

Novel cathepsin L-like protease from dermestid beetle *Dermestes frischii* maggot

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ARTICLE INFO

Article history:
Received 5 April 2010
Accepted 30 August 2010
Available online 22 September 2010

Keywords:
Dermestid beetle
Cysteine protease
Cathepsin
Primary structure
Enzymatic properties

ABSTRACT

A novel cathepsin L-like protease from dermestid beetle *Dermestes frischii* maggot guts was obtained and investigated. The protease was isolated through affinity chromatography at arginine-diasorb followed by FPLC gel-filtration at Superdex 75. Protease is active against chromogenic peptide substrates, containing Arg or Leu in P1 position and a hydrophobic residue in P2 position. PH optimum is about 4,5 and temperature optimum at 40 °C. Enzyme is inhibited completely by $HgCl_2$ and leupeptin that prove it's belonging to cysteine proteases of papain family.

cDNA analysis of cathepsin L-like protease showed that protein sequence consists of 339 amino acid residues. Mature cysteine protease contains 219 amino acid residues corresponding to molecular mass 24027.20 Da. Residues of the active site were identified: Gln^{140}, Cys^{146}, His^{285}, Asn^{306} and Trp^{308}. Calculated pI is 4,73. The amino acid sequence of the cystein protease from dermestid beetle displays high structural homology with cathepsin L of other insects.

1. Introduction

Members of the cathepsin family are known as principal components of lysosomal system and play an important role in cell protein metabolism [1]. Cathepsins from invertebrates responsible for severe diseases are extensively investigated in present times [2]. Insect cysteine cathepsin L-like proteases are poorely studied [3–8] though homologous to human and animal cathepsin L-like cysteine proteases genes in various insect genomes can be found (http://www.ncbi.nlm.nih.gov/Genbank/).

Previously a high-molecular collagenase D from *Dermestes frischii* maggot guts was obtained in our laboratory. Collagenase D is able to hydrolyse collagen as well as proline bonds in peptide substrates. This collagenase was tested as cysteine protease and studied for the purpose to use it in medical preparations to heal burns and cicatricial keloid [9].

The aim of this study is to research a novel low-molecular cysteine protease from *D. frischii*, determine acid sequence and enzymatic properties.

2. Materials and methods

Protein molecular mass markers were from Helicon (Russia), azocollagen, oxidized insulin chains A and B and melittin Sigma (USA). Chromogenic substrates of serine and cysteine proteases were obtained from the State Institute for Genetics and Selection of Industrial Microorganisms (Moscow) and Sigma (USA). Affinity sorbent arginine diasorb was synthesized at BioKhimMac (Russia). Isolation and sequencing of cathepsin-like protease cDNA from dermestid beetle *D. frischii* was performed by Eurogen (Russia) by standard method. Other agents of chemically pure grade were produced in Russia.

2.1. Isolation of cathepsin-like protease

Intestinal tracts of dermestid beetle were extracted from 60 maggots and homogenized in porcelain jar in 0,01 M Tris–HCl buffer pH 8.0, with 5 mM Cys (buffer 1). After filtration through paper filter 10 ml of extract containing 189 mg of protein was obtained. Bowel extract was applied to 40 ml arginine-diasorb column equilibrated with buffer 1 having washed out the unbound proteins with the same buffer, elution of active fractions was performed sequentially by buffer 2 (0.01 M Tris–HCl,

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doi:10.1016/j.biochi.2010.08.014

5 mM Cys, 1 M NaCl, pH = 8.0) and buffer 3 (0.01 M Tris–HCl, 5 mM Cys, 1 M NaCl, 20% isopropanol, pH = 8.0), fraction volume 5 ml. The most active fractions towards Z-Ala-Phe-Arg-pNA (peak 2) were pooled, concentrated by ultrafiltration through PM-10 membrane and applied to Superdex 75 column (60 × 1 cm) equilibrated with buffer 2 (fraction volume 1 ml). Purity degree of preparation obtained and their molecular mass was determined by 12,5% PAGE in the presence of 0,1% Ds-Na. Phosphorylase B (97 kDa), bovine serum albumin (66,2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21,5 kDa) and lysozyme (14 kDa) were used as markers.

2.2. MALDI analysis

After Ds-Na-PAGE the band corresponding to the cysteine protease was excised and distained. After in-gel reduction of Cys residues by DTT and their alkylation by iodacetamide, in-gel enzymatic hydrolysis of the enzyme by trypsin and glutamyl endopeptidase [10] was performed. MALDI analysis of the digest was performed in M.M. Shemyakin and Yu.A. Ovchinnikov Institute of Bioorganic Chemistry (Moscow, Russia) on a Ultraflex II MALDI TOF/TOF MS Bruker mass-spectrometer (Germany).

2.3. Activity measurements

Activity of the enzyme was determined towards Z-Ala-Phe-Arg-pNA [11] was determined by measuring A_{410} according to the method [12]. The substrates were initially dissolved in dimethyl formamide. The assay used 0.05 M Tris–HCl buffer pH 8.0, with 5 mM Cys. Enzyme/substrate proportion was 1:200. The reaction mixture was incubated at 37 °C. Reaction was terminated by addition of acetic acid to 5% final concentration. A unit of enzyme activity was defined as the amount of the enzyme which hydrolyses 1 μM of substrate for 1 min.

2.4. Inhibition effect

The enzyme was incubated with $HgCl_2$, E-64, iodacetamide, *N*-ethylmaleimide and other agents, during 1 h at 20 °C. Z-Ala-Phe-Arg-pNA was added and residual activity was measured at standard conditions.

2.5. Substrate specificity

Enzyme specificity towards native substrates was measured using oxidized insulin chains A and B and melittin. Aliquote of enzyme preparation 10 μl (1 mg/ml) in 0.02 M $NaHCO_3$, pH 8.0 was added to substrate solution (1 mg/ml in the same buffer) and incubated 4 h at 37 °C. Enzyme/substrate proportion 1:100. Masses of obtained peptides were determined by MALDI-MS method. Collagenolytic activity was identified by hydrolysis of azocollagen at 37 °C 60 min according to the method [13]. 300 μl of azocollagen suspension (10 mg/ml) in 0,05 M Tris–HCl buffer pH 8.0 was incubated with protease sample (0,3 μM). Then the mixture was centrifugated and A_{540} measured. A unit of enzyme activity was defined as the amount of the enzyme which increases the absorption at 540 nm per one AU under described conditions.

2.6. cDNA cloning

The sample of total RNA was obtained from intestinal tract of dermestid beetle (*D. frischii*) maggot. Double-stranded DNA was synthesized using total RNA as a template by SMART method and was amplified by PCR. Oligonucleotide primers for RACE were selected upon the analysis of homological genomes from those insect species whose similar genes of cathepsin-like proteases were described before [14]. Primers for 5'RACE were chosen based on data analysis received after 3'RACE procedure. Specific fragments of cDNA were isolated by RACE method and cloned to the vector pAL16. Amplified cDNA of cysteine protease was cloned to a vector

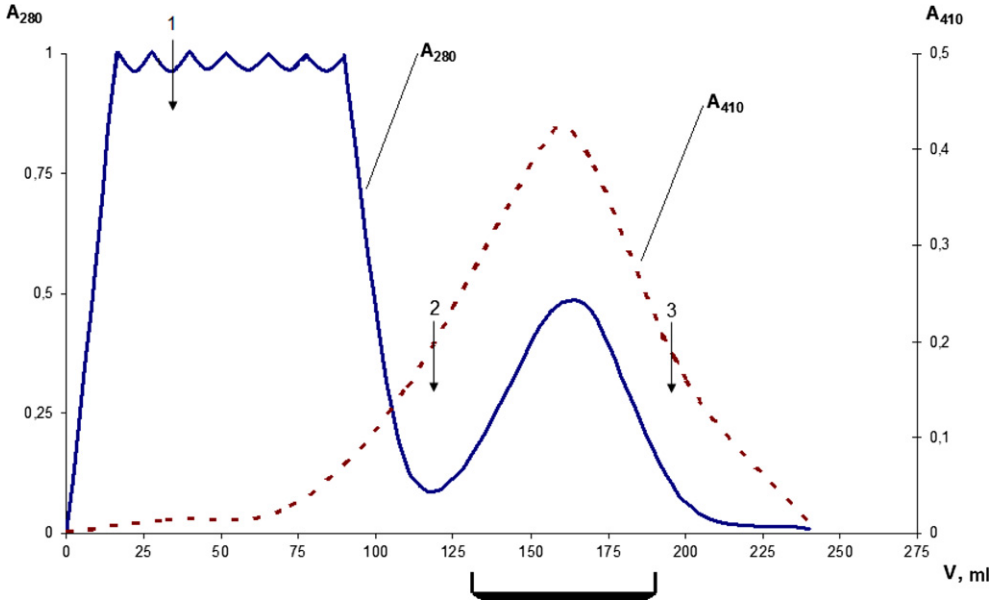

Fig. 1. Affinity chromatography at arginine-diasorb of *Dermestes frishcii* maggot bowels homogenate extract. 1–0.01 M Tris–HCl, 2 mM Ca^{2+}, 5 mM cysteine, pH 8,0; 2–0.01 M Tris–HCl, 2 mM Ca^{2+}, 5 mM cysteine, 1 M NaCl, pH 8,0; 3–80% 0,01 M Tris–HCl, 2 mM Ca^{2+}, 5 mM cysteine, 1 M NaCl, pH 8,0 + 20% iPrOH. The fractions which were collected for further purificationare marked by a bracket. ___ A_{280}; –– activity of cysteine protease towards Z-Ala-Phe-Arg-pNA.