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American lobster Cathepsin D, an aspartic peptidase resistant to proteolysis and active in organic solvents, non-ionic detergents and salts

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ABSTRACT

Suitable peptidases for biotechnological applications are those active at low temperature, in organic solvents, detergents or proteolytic additives. American lobster cathepsin D1 (CD1) is an enzyme highly efficient at 5–50 °C and at pH 2.5–5.5. We assessed the effect of common industrial additives on CD1 activity. CD1 was isolated from lobster gastric fluid by chromatography. The proteolytic activity was measured using a fluorogenic specific substrate and the conformation by intrinsic fluorescence. Non-ionic detergents Tween-20 and Triton X-100 stabilize the peptidase activity. Ethanol, methanol and isopropanol [5–15% (v/v)] increased the enzyme activity up to 80%. The enzyme is active until 2.5 M urea and is resistant to proteolysis by papain and renin. In this work, a crustacean peptidase that remains active when exposed to different chemical and proteolytic additives is reported, evincing that crustaceans are a good model for discovery of novel stable peptidases for future pharmaceutical, cosmetic and alimentary applications.

1. Introduction

The unique ability to catalyze reactions in a diversity of environments has allowed the application of enzymes in a variety of biocatalytic processes in the industry [1], during the manipulation, formulation, storage and use, enzymes face combined conditions that would hardly encounter in the nature, such as, extreme temperatures, ionic strength, solvents, as well as proteolytic enzymes. These conditions may affect the intramolecular interactions that maintain the active conformation of the proteins, leading to loss of activity [2].

Peptidases are the most important enzymes in biotechnology. They hydrolyze the peptide bonds of proteins and based on their catalytic mechanism, peptidases are classified in serine-, cysteine-, aspartic-, metallo-, threonine- and glutamic-peptidases. In biological systems, peptidases have different molecular structures, conformations and catalytic specificities fulfilling a variety of functions even under harsh environmental conditions; making peptidases the most used enzyme in industry, reaching 60% of the worldwide enzyme production [3]. While, microbial organisms are the source of most commercialized peptidases [1], marine eukaryotic organisms represent a significant stock of valuable peptidases with attractive characteristics that can potentially be applied and commercialized [4,5]. Numerous peptidases from marine animals had been characterized showing stability at extreme temperatures, and in the presence of organic solvents [6], metal ions [7] and proteolytic agents [8], however, structure-function relationship when exposed to those conditions have not been studied.

Aspartic peptidases (EC 3.4.23.X) catalyze reactions at acid pH, this family of endopeptidases is sensitive towards inhibition by Pepstatin A. The active site is characterized by the presence of two aspartic acid residues Asp32 and Asp215 that are located between two lobes [9]. In the industry, aspartic peptidases are used in food processing, cosmetic and health sector. Aspartic peptidases had been described in a wide variety of taxa and extensively studied in mammals, parasites and insects. In crustaceans, only the aspartic peptidase Cathepsin D1 (CD1) extracted from the digestive tract of American lobster (*Homarus americanus*), has been identified and

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characterized [10–13]. Identity of lobster CD1 was proved by N-Terminal sequencing, mass spectrometry and complementary DNA sequencing, furthermore, the enzyme has activity at a broad range of pH (3.0–5.5) and temperature (5–50 °C) [10,11,13]. At 5–35 °C the catalytic efficiency of this enzyme is 20-fold higher than the bovine and porcine homologue enzymes, also a significantly lower activation enthalpy (ΔH#) was observed when compared to bovine cathepsin D. For those reasons the lobster aspartic peptidase is denominated as a cold adapted enzyme [10].

The ocean is a potential source of enzymes that catalyze reactions in conditions at which they could be exposed during production, storage and application in biotechnology processes. In this work the conditions in which the lobster enzyme maintains activity are elucidated, the long-term stability, endurance to proteolysis, NaCl and chaotropic environment, as well as the stabilization by additives on the catalytic properties and the conformation of lobster CD1 is described to explore its potential as biocatalyst in the industry.

2. Materials and methods

2.1. Samples

Adult specimens of *Homarus americanus* lobster were purchased, transported, and maintained in the laboratory under controlled conditions of salinity, temperature and food supply. Weekly samplings were done by withdrawn of gastric fluid from live lobsters introducing a flexible teflon probe (5 cm long, 3 mm diameter) attached to a 5 mL syringe through oral cavity. The gastric fluid was immediately transferred into microcentrifuge tubes and centrifuged for 10 min at 4 °C at 10 000 × *g* to remove solids. The supernatant was stored in aliquots at −20 °C until further analysis. The protein concentration of the gastric fluid was quantified by the Bradford method (1976) [14] using bovine serum albumin (BSA) as the standard.

2.2. General technics

2.2.1. Electrophoretic analysis

To follow the CD1 isolation and to evaluate lobster CD1 endurance to proteolysis, Sodium-dodecyl-sulfate polyacrylamide-gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (1970) [15]. For analysis in denaturing conditions, samples were mixed with 4 x DTT-Sample Buffer and heated at 100 °C for 10 min, then immediately loaded into 12% polyacrylamide gel. Zymograms were utilized to evaluate the proteolytic activity of the isolated fractions and the long-term stability of CD1, by the Substrate-SDS-PAGE (S-SDS-PAGE) methodology described by García-Carreño et al. (1993) [16]. Both SDS-PAGE and zymograms were documented with Gel-Doc EZ System (BIO-RAD) and analyzed with the Image Lab software (BIO-RAD).

2.2.2. Activity assays

The proteolytic activity of lobster CD1 was measured by the hydrolysis of the specific substrate 7-methoxycoumarin-4-acetyl-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys(DNP)-D-Arg-amide (BML-P145-0001, EnzoLife Sciences), following the methodology of Yasuda et al. (1999) [17]. Cathepsin D activity is expressed as pmol of MCA liberated per minute per μg of protein. Activity assays were carried out in triplicate.

2.2.3. Fluorescence assays

Tryptophan emission fluorescence was recorded using a microplate reader Synergy 4 (BioTek) at excitation wavelength of 290 nm, and the emission spectra was recorded between 315 and 400 nm with a 5 nm emission steps. The protein concentration used for all fluorescence spectra was 0.05 mg/mL in 100 mM sodium phosphate, pH 4.5. Temperature was controlled to 25 °C. All fluorescence spectra were normalized to buffer background intensity.

2.3. Lobster CD1 purification

Before chromatography, lobster gastric fluid was centrifuged at 10 000 x *g* for 15 min at 4 °C to eliminate solids, then supernatant was collected and equilibrated with 50 mM sodium acetate pH 4.5 using a NAP-5 Sephadex G25 column (Amersham Biosciences). Purification of CD1 was accomplished in two steps using a Bio-Logic Duo Flow (BIO-RAD) system. As first purification step, affinity chromatography was performed in a 2 mL column of Pepstatin A-agarose (P2032, Sigma-Aldrich) equilibrated with 50 mM sodium acetate pH 4.5. Briefly, 1 mL of gastric fluid (20.3 mg protein) was loaded at 1 mL/min and washed with 15 mL of the same buffer. Then, unspecific proteins bonded to the matrix were desorbed with 20 mL sodium citrate pH 3.5 containing 1 M NaCl, followed by 20 mL 50 mM sodium citrate pH 3.5 and 25 mL of 100 mM Tris-HCl pH 7.0 containing 1 M NaCl. CD1 was eluted using 15 mL of 100 mM Tris-HCl, pH 7.5 containing 1 M NaCl. One mL fractions were collected and assessed for protein composition by SDS-PAGE under reducing conditions and revealed with silver stain. Fractions with highest purity were pooled, desalted and equilibrated with 100 mM Tris-HCl, pH 7.0 in a NAP-5 Sephadex G25 column. Sample was concentrated to 1 mL using an Amicon Ultra-15 Centrifugal filter 10 kDa cutoff (UFC901024, Millipore) at 4 000 x *g* and 4 °C. As second purification step, anionic exchange chromatography was performed using an UNO Q1 column (720-0001, BIO-RAD), equilibrated with 100 mM Tris-HCl pH 7.0. In brief, 1 mL of partially purified CD1 was loaded into the column at a flow rate of 1 mL/min. The elution of CD1 was reached by a linear gradient of 0–0.5 M NaCl in the same buffer at 1 mL/min flow rate. Two mL fractions were collected and assessed for purity of isolated CD1 by 12% SDS-PAGE silver stained and S-SDS-PAGE assays. Fractions with the pure CD1 were pooled, concentrated, subsampled and stored at −20 °C until use. During each step of purification, specific activity of the CD1 was measured by the methodology described in the Section 2.2.2.

2.4. Lobster CD1 long-term stability

To evaluate the long-term stability of lobster CD1, the activity of the enzyme was quantified at two enzyme concentrations. For low concentration, CD1 (0.5 mg/mL) was diluted 10 000 times to 0.05 μg/mL final concentration in 50 mM sodium acetate buffer pH 4.5, and incubated for 24 h at 25 °C. To quantify the activity, 90 μL of enzyme solution were pipetted into a microplate containing 10 μL of cathepsin D specific synthetic substrate (reaching a final concentration of 2 μM), the activity was quantified using the parameters described in Section 2.2.2. The activity was recorded at 1, 2, 4, 8 and 24 h incubation and was expressed in units of relative activity, considering the enzyme activity before incubation as positive control.

The activity at high enzyme concentration was carried out by diluting 100 times the purified enzyme (0.5 mg/mL) to a concentration of 5 μg/mL in 50 mM sodium acetate buffer pH 4.5, and incubated for 7 days at 25 °C. For optimal activity assay conditions, the enzyme was diluted anew 1:100 in the same buffer to a final protein concentration of 0.05 μg/mL just before mixing with the fluorogenic substrate. Lobster CD1 activity was measured immediately after the second dilution using the parameters described above. The activity was monitored daily for seven days and was expressed in units of relative activity, considering the enzyme activity before incubation as positive control. A qualitative evaluation of peptidase activity over time was carried out using S-SDS-PAGE. Purified CD1 was diluted to a final concentration of