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Characterization of arazyme, an exocellular metalloprotease isolated from *Serratia proteamaculans* culture medium

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Abstract

We investigated the biochemical properties of a 51.5 kDa metalloprotease (arazyme, 3.4.24.40) secreted into the culture medium by *Aranicola proteolyticus*, a symbiotic bacterium of the spider *Nephila clavata*. The enzyme was purified to apparent homogeneity by ion exchange chromatography in a Resource Q column (FPLC system). The substrate specificity requirements of purified arazyme were examined using fluorescence resonance energy transfer (FRET) peptides derived from the lead sequence Abz-KLRFSKQ-EDDnp (Abz = *ortho*-aminobenzoic acid; EDDnp = ethylenediaminedinitrophenyl). Three series of peptides were assayed to map the S_2, S_1 and S'_1 subsites: Abz-KXRFSKQ-EDDnp, Abz-KLXFSKQ-EDDnp and Abz-KLRXSKQ-EDDnp (X are natural amino acids). The results indicated that S_1 subsite has a broad specificity, being Gly the preferred amino acid for this subsite followed by positively charged residues (Arg and His). The S_2 and S'_1 subsites accommodated better hydrophobic residues with aliphatic or aromatic side chains (Leu, Phe). The pH effect on hydrolysis of Abz-KLFFSKQ-EDDnp indicated that optimal hydrolysis occurred at pH 8.0 or higher. The effect of NaCl on the arazyme activity depends on the substrate, but in general the activity was reduced with this salt. The temperature did not affect the enzyme from 10 to 45 °C, after which activity decreased sharply. Arazyme presented high hydrolytic activity on substance P and peptides related to bradykinin. In addition, arazyme activity was resistant to the treatment by pepsin, trypsin and chymotrypsin. In conclusion, arazyme has a broad hydrolytic profile and works in very aggressive conditions, which justify its potential use in therapeutics and biotechnological applications.

Keywords: Serralysin; *Serratia proteamaculans*; Fluorescent peptides

1. Introduction

Proteases are enzymes of physiological, pathological, but also of commercial importance. Microorganisms represent an excellent source of proteases owing to their broad biochemical diversity and their susceptibility to genetic manipulation. These enzymes are preferred to those from plants and animal sources due to the possibility to obtain in large scale, and they have almost all the characteristics desired for biotechnological applications. They have been used mainly as additive of detergents, in food industries, in leather treatment and in several bioremediation processes for the developing of environmentally friendly technologies. In addition, proteases are used in the pharmaceutical industry for preparation of medicines such as ointments for debridement of wounds [1].

Arazyme is a 51.5 kDa metalloprotease that is secreted into the culture medium by *Aranicola proteolyticus*, at present called as *Serratia proteamaculans*, a symbiotic bacterium of the spider *Nephila clavata* [2]. Preliminary studies of activ-

Abbreviations: Abz, *ortho*-aminobenzoic acid; Cbz, carbobenzyl; DMSO, dimethyl sulphoxide; EDDnp, *N*-(2,4-dinitrophenyl)ethylenediamine; EDTA, ethylenediaminetetraacetic acid; E-64, *trans*-epoxy-succinyl-L-leucylamido-(4-guanido)-butene; FPLC, fast protein liquid chromatography; FRET, fluorescence resonance energy transfer; MALDI-TOF, matrix assisted laser desorption ionization time of flight; PMSF, phenylmethylsulphonyl fluoride; *p*-HMB, sodium 7-hydroxymercuribenzoate; R_t, retention time; TLCK, tosyl lysyl chloromethyl ketone; TPCK, tosyl phenyl alanyl chloromethyl ketone

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ities of this enzyme show that it is very close to serralysin (EC 3.4.24.40), which belongs to the clan MA(M) and the family M10B [3]. Members of this subfamily are the alkaline proteinases from *Pseudomonas aeruginosa*, *Serratia marcescens*, *Proteus mirabilis* and *Escherichia freundii* [4]. Serralysin, a metallopeptidase with approximately 55 kDa was isolated from *Serratia*, as well as from some other bacteria (*Pseudomomas* or Erwinia chrysanthemi) is considered as one of the virulence factors produced during *Serratia* or *Pseudomonas* infection, though its importance seems to be less than that of other toxins. The physiological function of serralysins is not clear, but the enzyme seems to play a role in nutrient digestion/uptake by the bacteria [5].

Arazyme is produced in large scale and presents biotechnological applications as detergent additive, leather treatment, food processing. Similar to serralysin [6] arazyme has been observed to have beneficial effects in inflammatory processes particularly in bovine mastitis (data not published).

In the present paper, we investigated the specificity of arazyme subsites S_1, S_2 and S_1' using fluorescence resonance energy transfer (FRET) peptides derived from the peptide Abz-KLRFSKQ-EDDnp (Abz = *ortho*-aminobenzoic acid; EDDnp = *N*-[2,4 dinitrophenyl]-ethylenediamine; Abz/EDDnp = donor/acceptor fluorescent pair), which were earlier used to investigate cysteinyl proteases with high arginyl hydrolase activity [7]. We also examined the inhibition profile of arazyme, the salt and the pH effects on its hydrolytic activity on FRET peptides and casein. In addition, we reported the arazyme activity on biological active peptides.

2. Materials and methods

2.1. Peptides

Fluorescence resonance energy transfer (FRET) peptides were synthesized by the solid-phase synthesis method as described elsewhere [8] in an automated solid-phase peptide synthesizer, Shimadzu Model PSSM-8. All peptides obtained were purified by semi preparative HPLC and their molecular weight and purity were checked by amino acid analysis and by molecular mass determination with MALDI-TOF mass spectrometry, using a TofSpecE from Micromass. The stock solutions of these peptides were prepared in DMSO and the concentrations were measured spectrophotometrically using the molar extinction coefficient $\varepsilon_{365\,nm} = 17{,}300\ M^{-1}\ cm^{-1}$.

2.2. Enzyme purification

The supernatant of culture medium of *S. proteamaculans* was subjected to membrane filtration and separated from the biomass. The supernatant was concentrated 3–10 times through 10 kDa membrane. The purification of the concentrated solution was carried out by anion-exchange chromatography in a Resource Q column (FPLC-system; 1 mL) equilibrated with 20 mM Tris–HCl buffer, pH 8.0, and eluted with a NaCl gradient (0–0.5 M) in starting buffer. The protein elution profile was monitored by UV absorbance (280 nm). Fractions of 1 mL were collected at a flow rate of 1 mL/min and their activities were assayed using Abz-KLRFSKQ-EDDnp as substrate. The assays were performed in 50 mM Tris–HCl buffer, pH 8.0, at 37 °C. The fluorescence was measured continuously at $\lambda_{ex} = 320$ nm and $\lambda_{em} = 420$ nm (1.0 mL final volume) in a Hitachi F-2000 spectrofluorometer.

2.3. Optimum pH determination

The pH dependence was studied using Abz-KLFFSKQ-EDDnp and azocasein as substrates over a pH range of 3.5–10. The buffers used were 50 mM sodium acetate ($3.5 < pH < 5.5$) and 50 mM Tris–HCl ($6.0 < pH < 10.0$). Enzymatic activity upon the FRET peptide Abz-KLFFSKQ-EDDnp was followed using the continuous fluorimetric assay, at 37 °C. For each pH, the apparent second-order rate constant ($*k_{cat}/K_m$) was determined at low substrate concentrations where the reactions followed first-order conditions ($[S] \ll K_m$). The activity of arazyme on azocasein was determined in a spectrophotometer at 436 nm. The enzyme was mixed with 150 μL of the substrate and buffer in a total volume of 500 μL. After incubation at 37 °C for 5 min, 50 μL of 50% trichloroacetic acid was added, and after 5 min, the precipitate was separated by centrifugation. The supernatant was mixed with the same volume of 1 M NaOH and absorbance was measured [9].

2.4. Salt influence on catalytic activity

The influence of NaCl concentration (0–1000 mM) on catalytic activity of arazyme upon the peptides Abz-KLFFSKQ-EDDnp, Abz-KLRFSKQ-EDDnp and azocasein was investigated at 37 °C, in 50 mM Tris–HCl buffer, pH 8.0. Hydrolysis of the FRET peptides and azocasein was followed by continuous fluorometric and spectrophotometric assays, respectively, as described above.

2.5. Inhibitors

The effect of protease inhibitors on arazyme activity was studied at 37 °C using Abz-KLFFSKQ-EDDnp as substrate in 50 mM Tris–HCl buffer, pH 8.0. The enzyme was pre-incubated with the inhibitors for 5 min. The results were expressed as the percentage of residual activity relative to control reaction run in the absence of the inhibitor.

2.6. Determination of kinetic parameters

The fluorescence resonance energy transfer (FRET) peptides were assayed in a Hitachi F-2000 spectrofluorometer.