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Purification of a novel cysteine protease, procerain B, from *Calotropis procera* with distinct characteristics compared to procerain

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ABSTRACT

Proteases have applications in food, detergent and pharmaceutical industries. A novel protease has been purified from the latex of *Calotropis procera* and characterized. As another cysteine protease, procerain, is reported from the same source, the newly purified enzyme was named as procerain B. The enzyme shows distinct properties compared to procerain, in terms of cleavage recognition site, immunological properties and other physical properties like molecular weight, isoelectric point, etc. The newly purified enzyme shows a broad optimum pH (6.5–8.5) as well as broad optimum temperature (40–60 °C). Additionally, the enzyme retains its activity where most of other proteases are not active. Moreover, the enzyme appeared to be very efficient in hydrolysis of blood stain and may have potential application in detergent industries. Simple and economic purification of procerain B, together with easy availability of latex, makes the large-scale production of procerain B possible, thus enables to explore various industrial as well as biotechnological applications.

1. Introduction

Bioinformatics analysis of the human and mouse genomes indicates at least 2% genes of the genome codes for proteases [1] which indicates physiological role of proteases. Proteases have therapeutic and industrial applications as well. The annual sales of proteases accounts 60% of the total world enzyme market and estimated to reach 220 billion US$ by the year 2009 [2]. Plant cysteine proteases are used in industry owing to their high temperature stability and broad substrate specificity [3]. Plant genomes encode hundreds of proteases, but little is known about what roles they play in the life of a plant. Proteases are thought to be involved in a range of biological processes, including senescence, implicated in perception, signaling and execution leading to plant defense [4]. Few other cysteine proteases from the plant sources have been purified and characterized. As usefulness of the proteases depends on its unique cleavage site, stability as well as optimum activity condition search of a novel protease with unique properties is always on.

Calotropis procera (family Asclepiadaceae) is a tropical plant that has been widely used in Indian traditional medicinal system for the treatment of various diseases namely leprosy, ulcers, tumors, piles and diseases of the spleen, liver and abdomen [5]. Various parts of the plant show anti-microbial, anti-inflammatory, antipyretic and anti-malarial activities [6,7]. The latex of the plant shows antidiabetic, hepatoprotective, antiarthritic, cytotoxic and anticancerous properties [8]. Preliminary screening of the latex of the plant showed very high proteolytic activity. We have earlier reported purification and characterization of a novel protease procerain, from the latex of the plant [9]. Here we report purification of another protease from the plant, which we named as procerain B, with distinct properties and cleavage site.

2. Materials and methods

2.1. Materials

Superficial incisions on the *C. procera* yielded milk like latex. Fresh latex of the plant was collected. CM-Sepharose FF was purchased from GE Healthcare. Azocasein, DTNB (5,5′-dithiobis-[2-nitro benzoic acid]), DTT (dithiothreitol), GuHCl (guanidine hydrochloride), urea, o-phenanthroline, EDTA (ethylenediaminetetraacetic acid), EGTA (glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid), leupeptin, SBTI (soybean trypsin inhibitor), NEM (*N*-ethylmaleimide), β-mercaptoethanol, PMSF (phenylmethanesulfonylfluoride), acrylamide,*N,N*-methylene bisacrylamide, Coomassie brilliant blue R-250, E-64 (l-trans-epoxysuccinylleucylamide (4-guanidino) butane-*N*-[*N*-(L-3-trans-carboxyirane-2-carbonyl)-L-leucyl] agimatine), are obtained from Sigma Chemical Co., USA. Sodium tetrathionate ($Na_2S_4O_6{\cdot}2H_2O$) was

Abbreviations: BAPA, NR-benzoylarginine *p*-nitroanilide; DFP, diisopropylfluorophosphate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid; ELISA, enzyme-linked immunosorbent assay; GuHCl, guanidine hydrochloride; NEM, *N*-ethyl-maleimide; PCMB, *p*-chloromercurybenzoate; PMSF, phenyl-methanesulfonyl fluoride; SBTI, soybean trypsin inhibitor; TCA, trichloroacetic acid; TFA, trifluoroacetic acid; TEMED, *N,N,N,N*-tetramethylethylenediamines.

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synthesized by the method of Gilman et al. [10]. All other chemicals were of highest purity commercially available. Procerain was purified by the method of Dubey and Jagannadham [9].

2.2. Methods

2.2.1. Purification

All purification steps were carried out in cold to minimize any complication due to possible autodigestion or unfolding of the enzyme at higher temperature. Most of the purification steps were carried out at 4 °C unless stated. Latex was collected from the young stems of the plant in 0.01 M acetate buffer pH 4.5 containing 0.01 M sodium tetrathionate, a reversible inhibitor of cysteine protease, and was stored at −20 °C. Frozen latex was thawed to room temperature and centrifuged at 24,000 × *g* for 10 min to remove any insoluble materials. The supernatant was then subjected to 50% ammonium sulfate fractionation. The supernatant after 50% ammonium sulfate fractionation found to have more enzyme activity which is dialyzed extensively and used for further purification. The ion exchange chromatography of the dialyzed supernatant of 50% ammonium sulfate fractionation was performed at 4 °C. The protein was loaded on CM-Sepharose cation exchange column pre-equilibrated with 0.01 M acetate buffer pH 4.5. The column was washed with the same buffer until no protein was detected in elute and the bound proteins were eluted with a linear gradient of 0–0.8 M NaCl at a flow rate of 3 ml/min. Fractions of 6 ml were collected; the absorbance at 280 nm as well as caseinolytic activities of the protein in all fractions was checked using casein as a substrate. The partially purified fractions of the ion exchange chromatography were further purified by size exclusion chromatography using sephacryl S-300 column (1.6 cm × 40 cm). The loaded protein was eluted by 0.01 M acetate buffer pH 4.5 containing 250 mM NaCl.

2.2.2. Protein concentration

Protein concentration at different stages of purification was determined by absorbance at 280 nm as well as by the method of Bradford [11] using BSA as a standard.

2.2.3. Protease activity

The hydrolyzing activity of the protease was determined using denatured natural substrates casein or azocasein using the method of Dubey and Jagannadham [9]. Enzyme solution (5 μg) was incubated in final volume of 500 μl of 50 mM Tris-Cl buffer pH 7.5 at 37 °C for 10 min. Casein solution (1%) (w/v) was prepared at the same pH and added to the enzyme solution making the final reaction volume to 1 ml and the reaction mixture was incubated for the 30 min at 37 °C. The reaction was stopped by adding 0.5 ml of 10% TCA, incubated further for 10 min at room temperature and centrifuged (10,000 rpm for 10 min). The absorbance of the soluble peptides in the supernatant was measured at 280 nm. In the case of azocasein, as substrate, 0.5 ml of supernatant after TCA precipitation was mixed with equal volume of 0.5 M NaOH and incubated for 15 min. The development of colour was measured spectrophotometrically by taking absorbance at 440 nm. A control assay, without the enzyme was done and used as blank in all spectrophotometric measurements. One unit of enzyme activity was defined as the amount of enzyme, under given assay conditions that give rise to an increase of 0.001 unit of absorbance at 280 nm or an increase of 0.001 unit of absorbance at 440 nm per minute of digestion. Number of units of activity per milligram of protein was taken as the specific activity of the enzyme.

2.2.4. Electrophoresis

Homogeneity and intactness of the enzyme, during purification as well as molecular mass determination of the purified enzyme, was assayed by using SDS-PAGE [12] with little modification. The purified enzyme was inactivated to avoid autolysis by treatment with cocktail inhibitor mixture. The gel was stained with 0.1% Coomassie brilliant blue R-250. Molecular weight markers used were phosphorylase b (97.4 kDa), BSA (66.0 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (29.0 kDa), soybean tyrosine inhibitor (20.1 kDa), and lysozyme (14.3 kDa) and a plot of log molecular weight *vs* mobility was generated to extrapolate the molecular weight of the purified enzyme.

2.2.5. Isoelectric point

The isoelectric point of the purified enzyme was determined by isoelectric focussing in tube gels as described for procerain [9]. Ampholines, in the pH range 3.5–10.0 were used to generate the pH gradient. A 5% polyacrylamide gel containing 2% desired ampholine was cast in tube gels. Anodic and cathodic chambers buffer were 0.01 M-iminodiacetic acids and 0.01 M ethylenediamine, respectively. The buffers were flushed with nitrogen gas before electrophoresis. The gel was subjected to a pre-run at a constant current of 1 mA per rod for 2 h to develop the pH gradient. A protein sample (100 μg) containing 10% (v/v) ampholine and 25% glycerol was loaded on the gel and electrophoresed at constant current of 2 mA per rod for 4 h. Protein bands were stained with 0.04% (w/v) Coomassie brilliant G-250 dissolved in 6% (w/v) perchloric acid [13].

2.2.6. Carbohydrate content

Carbohydrate content of procerain B was determined by phenol sulphuric acid method [14,15].

2.2.7. Tryptophan and tyrosine content

Total numbers of tryptophan and tyrosine residues in the purified protein was determined by the method of Goodwin and Morton [16]. An absorbance spectrum of the purified enzyme in 0.1 M NaOH was recorded between 300 and 220 nm using a Beckman DU 640B spectrophotometer. Absorbance values at 280 nm and 294.4 nm were obtained from the spectra. For calculations, the formula, $w = (A_{280} - x\varepsilon_y)/(\varepsilon_w - \varepsilon_y)$, was used where, w is the estimated tryptophan content in moles per liter; A_{280} is the absorbance at 280 nm for one molar protein; ε_w and ε_y are molar extinction coefficients of tryptophan and tyrosine in 0.1 M NaOH at 280 nm (ε_y = 1576 and ε_w = 5225), respectively. The total tyrosine and tryptophan content in the protein, x was calculated using $\varepsilon_{294.4}$ = 2375. The number of a particular amino acid residue per molecule of the protein was calculated from the ratio of the molar concentrations of the amino acid residues to that of the total protein.

2.2.8. Measurement of free and total sulfhydryl content

Free and total cysteine residues of procerain B were determined by using DTNB method of Ellman [17]. For free cysteine content determination, the enzyme was activated with 0.05 M β-ME in 50 mM phosphate buffer pH 7.5 at 37 °C for 30 min and dialyzed extensively for 24 h against 500 ml of 0.1 M acetic acid with four changes of the dialyzate. After dialysis, 50 μl of the dialyzed enzyme sample was taken in 700 μl of 0.1 M Tris–HCl, pH 7.3 and the sample was allowed to stand for 10 min to attain the pH. Subsequently, 50 μl of 5 mM DTNB solution was added and the reaction mixture was thoroughly mixed. The liberated TNB anion after reaction of sulfhydryl group with DTNB was monitored spectrophotometrically. The numbers of free cysteine residues are assessed using extinction coefficient of TNB of 14,150 M^{-1} cm^{-1} at 412 nm [18]. For the estimation of the total number of cysteine residues, the enzyme was denatured in 6 M GuHCl and reduced with 0.05 M DTT in 0.05 M Tris–HCl, pH 8.0. The excess DTT in the reaction mixture was removed by dialysis against 500 ml of 0.1 M acetic acid with four changes of the dialyzate [19]. The liberated thiol groups were estimated as described in the case of free cysteine estimation. The numbers of disulfide bonds, in the protein, were deduced by the comparison of the number of free and total cysteine residues. To validate the measurements, similar contents of papain, ribonuclease A, and lysozyme were also determined.

2.2.9. Extinction coefficient

The extinction coefficient of procerain B was determined using spectrophotometric method [20]. The extinction coefficient was determined using the formula, $\varepsilon^{1\%}_{280\,nm} = 10(5690n_w + 1280n_y + 120n_c)/M$, where n_w, n_y, and n_c are the number of tryptophan, tyrosine, and cysteine residues in the protein; M is the molecular mass of the protein and 5690, 1280, and 120 are the respective extinction coefficients of tryptophan, tyrosine and cysteine residues. The total numbers of tryptophan, tyrosine and cysteine residues in the protein are determined as described below.

2.2.10. pH and temperature optima

The activity of the purified enzyme is measured as function of varying pH to determine the pH optima of the enzyme. 10 μg of enzyme was used for activity measurement. The buffers used were: 0.05 M KCl–HCl (pH 1.0–1.5); 0.05 M Glycine–HCl (pH 2.0–3.5); 0.05 M Na-acetate (pH 4.0–5.5); 0.05 M Na-phosphate (pH 6.0–7.0); 0.05 M Tris–HCl (pH 7.5–10.5) and 0.05 M sodium carbonate (pH 11–12.5) Substrate solution of azoalbumin or hemoglobin was prepared in the respective buffers. Procerain B was equilibrated in 0.5 ml of the buffer at a given pH for 15 min and added to the substrate solution of the same pH. The assay procedure is same as described above. Due to the insolubility of azocasein below pH 4.0, hemoglobin was used as substrate for activity measurements at lower pH [21].

The effect of temperature on the activity of procerain B was also studied using azocasein as substrate. 10 μg of enzyme was incubated at desired temperature in the range of 10–95 °C for 15 min in 50 mM phosphate buffer pH 7.5 and an aliquot was used for the activity measurement at the same temperature. Prior to the assays, substrate solution was also equilibrated at the corresponding temperature in the same buffer. At each temperature, a control assay was carried out without the enzyme was used as a blank.

2.2.11. Stability

The ability of purified enzyme to retain its activity under various conditions such as extreme pH, temperatures, strong denaturants and organic solvents were studied. The 15 μg of enzyme was incubated at different pH in the range of 0.5–12.0 for 24 h at room temperature and residual activity was measured as described earlier using azocasein as substrate. Similarly, enzyme samples were incubated at temperatures from 10 °C to 95 °C for 15 min and assayed for residual activity. The enzyme was also incubated for 24 h in the presence of chemical denaturants like GuHCl, urea and different solvents such as methanol, acetonitrile, and dioxan. The activity was measured after 24 h incubation as previously described.