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Biochemical characterization of a cysteine proteinase from *Bauhinia forficata* leaves and its kininogenase activity

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ABSTRACT

In this work, the proteinase activity detect in the acetone precipitate (80%, v/v) of *B. forficata* leaves, trivially known as cow paw, and popularly used in folk medicine for treatment of diabetes mellitus, was purified by chromatography on Sephadex G-25, Canecystatin-Sepharose, and on Con A-Sepharose. The molecular weight 30 kDa was estimated by SDS-PAGE and zymography, and the N-terminal sequence and CD spectra indicated a relationship with the papain family of cysteine proteinases. Denominated baupain, the enzyme was activated by dithiotreitol and inhibited by E-64 and iodoacetamide, but not by benzamidine, TLCK, TPCK and EDTA. The S2 and S1 substrate specificity of baupain, assayed with two series of fluorescence resonance energy transfer (FRET) peptide substrates derived from Abz-KLRSSK-Q-EDDnp, indicates a preference for Phe and Tyr at P2 position over Leu found in papain. Baupain releases bradykinin from HMWK (human high molecular weight kininogen) though its proteolytic activity is blocked by the sequence motif QVVA of kininogen ($K_{iapp} = 1.9 \times 10^{-8}$ M). Canecystatin, from sugar cane, which also lodges the QVVA sequence, inhibits baupain ($K_{iapp} = 0.18 \times 10^{-9}$ M).

1. Introduction

Within the members of cysteine proteinases expressed in viruses, bacteria, animals and plants [1], papain is the archetypical member of these endopeptidyl hydrolases (EC 3.4.22). This group also comprise the mammalian lysosomal cathepsins, the cytosolic calpains (*i.e.*, calcium activated cysteine proteinases) as well as several parasitic proteinases [2,3].

The action of these enzymes can be controlled by members of a family known as cystatins super family, which comprises three families, on the basis of sub-cellular localization, molecular weight, disulfide bonds and sequence similarity including the QXVXG motif. Specifically from plant, this group is known as plant cystatins or phytocystatins (PhyCys) whose primary sequences share a high degree of similarity within the cystatin family, including the sequence motif QXVXG [4,5].

In plants, cysteine proteinases are widely distributed in various tissues, and are involved in physiological events such as germination, senescence and environmental stress responses. Papain-like cysteine proteinases are often found in senescing organs particularly leaves [6–8], flowers [9], legume nodules [10] as well as in germinating seeds [10–13]. Cysteine proteinases have been intensively studied with various expression patterns, reported for different stages of plant development [14–16]. Equally studied is cysteine proteinases role in processing and degradation of seed storage proteins [17,18], in legume nodule development [19], in response to stresses such as wounding, cold, and drought [20] as well as in programmed cell death [21,22].

In the present work, the purification and the functional characterization of a new cysteine proteinase from *B. forficata* leaves are described. *B. forficata* is a Leguminosae known as cow paw, due to the characteristic bilobed aspect of its leaves from which the homemade extract is prepared and used in popular therapy for diabetes mellitus. The studies reported that the beneficial of leaf extracts (aqueous and alcoholic) in the prevention of diabetes complications is associated with oxidative stress *since B. forficata* and other plant extracts have significant antioxidant activity [23,24].

Abbreviations: FRET, fluorescence resonance energy transfer; Q-EDD, npglutaminyl-[N-(2,4-dinitrophenyl)-ethylenediamine]; Abz, ortho-aminobenzoic acid; HPLC, high performance liquid chromatography; LC/MS, liquid chromatography/mass spectrometry.

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Although this plant has been the subject of several studies [24–26], but a characterization of the enzyme activity is not yet described.

The substrate specificity of the enzyme, designated as baupain was assayed using peptides derived from the leading sequence Abz-KLRSSKQEDDnp [27–31] and the series Abz-KXRSSKQ-EDDnp and Abz-KLXSSKQ-EDDnp (X = different amino acids) used for mapping, respectively, the S2 and S1 substrate specificity [29–31]. To further compare the hydrolytic properties of baupain with papain and human cathepsin L, we explored in vitro the ability of baupain to release kinin from HMWK.

2. Materials and methods

2.1. Enzyme purification

Leaves (50 g) collected from wild *Bauhinia forficata* trees were homogenized in a blender with 0.15 M NaCl (700 ml). The proteins in the crude extract were precipitated by 80% (v/v) acetone at 4 °C. The sediment separated by centrifugation was dried at room temperature, and dissolved in 0.1 M sodium phosphate buffer, pH 6.3. The enzyme fraction (1.5 ml) was applied on a size exclusion chromatography (Sephadex G-25) equilibrated with 0.1 M sodium phosphate buffer, pH 6.3. The enzyme activity was followed using Z–Phe–Arg–MCA as substrate. The fractions containing enzyme activity were pooled and subsequently chromatographed on a canecystatin-Sepharose, equilibrated with the same phosphate buffer. The active material, eluted by 2.0 mM L-cysteine, was chromatographed on Con A Sepharose, equilibrated with 0.1 M sodium phosphate buffer, pH 6.3, used for removal of pigments. The whole purification procedure was carried out at 4 °C. The unbound fractions containing enzyme activity were separated and subsequently purified by HPLC on a μ-Bondapak C_{18} reverse phase column. The separation was achieved by an acetonitrile gradient (0–100%) in 0.1% TFA (v/v) during 75 min, at 1 ml/min flow rate and room temperature. Proteins were estimated spectrophotometrically (A280) as well as by Bradford [32] assay using bovine serum albumin as the standard.

2.2. N-terminal amino acid sequence

Purified baupain was denatured and reduced by addition of 200 μl 50 mM Tris/HCl buffer, pH 8.5, containing 6.0 M guadinium HCl, 1.0 mM EDTA, and 5.0 mM dithiothreitol for 3 h, at 37 °C. *S*-pyridylethylation of cysteines was achieved by addition of (5 μl) 4-vinylpyrimidine for 3 h at 37 °C in the dark and nitrogen atmosphere. The excess reagents were removed by reversed-phase HPLC on a C_{18} column using the same gradient conditions already described. N-terminal amino acid sequences were determined by Edman degradation using a PPSQ-23 Model Protein Sequencer (Shimadzu, Tokyo, Japan). Phenylthiohydantoin derivates of amino acids were identified.

2.3. Electrophoresis and gelatin zymography

The homogeneity and the molecular weight of baupain were assessed by SDS-PAGE under reducing and non-reducing conditions according to Laemmli [33], using 12% acrylamide gel. HMWK (10 μg) limited proteolysis cleavage by baupain (0.23 μM) was demonstrated by SDS-polyacrylamide gel electrophoresis [34]. Baupain and HMWK were incubated in 0.1 M sodium phosphate buffer, pH 6.3 for 10 and 60 min at 37 °C. The proteins were stained with Coomassie brilliant blue R-250. A broad range of molecular weight protein markers, from 25 to 175 kDa and 20 to 94 kDa New England BioLabs Inc. (Ipswich, MA, USA), was used.

The zymography experiment was performance under non-reducing conditions according to Becker et al. [35]. Baupain activity was detected using 10% (w/v) acrylamide (Gelatin-PAGE), with 0.04% (w/v) copolymerized gelatin included in the gel as substrate for the proteinase. After electrophoresis, the gels were incubated in renaturing buffer for 1 h at room temperature, followed with incubation in developing buffer at 37 °C overnight. We used Mini-Protean® II Cell Bio-Rad (Hercules, CA, USA).

2.4. Enzyme activity

Proteinase activity was measured on Z–Phe–Arg–MCA (Calbiochem Ltda, Darmstadt, Germany) and Bz-Arg-pNan (BAPA) (Sigma–Aldrich Company, St. Louis, USA) as substrates. Baupain was incubated at 37 °C in a microtiter plate in 250 μl final volume of assay buffer [0.1 M sodium phosphate buffer, pH 6.3 containing 0.4 M NaCl, 10 mM EDTA, and 2.0 mM DTT (dithiothreitol)]. The reaction was followed for 10–30 min and the reaction was stopped by the addition of 50 μl acetic acid 30% (v/v). The fluorescence release was measured on a FluoroCount Packard™, spectrofluorometer set at 355 nm for excitation and 460 nm for emission.

In the case of BAPA (0.8 mM) as substrate, the reaction was followed by measuring the absorbance of released *p*-nitroaniline at 405 nm in a spectrophotometer Packard™ with a 50 mM Tris/HCl buffer, pH 8.0 [36].

2.5. Effects of activators on enzyme activity

Baupain (0.23 μM) was preincubated with DTT, β-mercaptoethanol, and L-cysteine (2 mM) in 0.1 M sodium phosphate buffer, pH 6.3 containing 0.4 M NaCl, 10 mM EDTA, at 37 °C for 30 min. The enzyme activity was determined as described, using Z–Phe–Arg–MCA (0.4 mM) as substrate.

2.6. Effect of pH on the enzyme activity

Prior to the addition of the substrate Z–Phe–Arg–MCA 40 μl of baupain (0.23 μM) was pre-incubated at 37 °C for 30 min with 100 μl of the following buffer systems: 0.2 M sodium citrate, pH 4.0; 0.2 M sodium acetate, pH 5.0; 0.2 M sodium phosphate, pH 6.0; 0.2 M Tris/HCl, pH 7.0 and pH 8.0 and 0.2 M sodium bicarbonate, pH 9.0 and pH 10.0 in a final volume of 250 μl by adding 90 μl of distillated water. The enzymatic activity was measured using 20 μl of Z–Phe–Arg–MCA (5 mM) as substrate.

2.7. Proteinase inhibition studies

Effect of low molecular weight inhibitors: Baupain (0.23 μM) was incubated with 1.0 μM E-64 (L-trans-epoxysuccinyl-leucylamido [4-guanidino] butane), 1.0 mM benzamidine, 2.0 mM phenylmethanesulfonyl fluoride (PMSF), 2.0 mM ortho-phenantroline, 5.0 mM ethylenediamine tetraacetic acid (EDTA), 1.0 mM N-tosyl-L-phenylalanylchloromethyl ketone (TPCK), and 1.0 mM N-tosyl-L-lysyl chloromethyl ketone (TLCK), for 10 min at 37 °C, before the addition of the substrate Z–Phe–Arg–MCA (0.4 mM). The assay concentration of each inhibitor was chosen based in suppliers information and inhibitor mode of action [37]. Enzyme activity was expressed in percent of residual activity on Z–Phe–Arg–MCA compared to the control.

Effect of high molecular weight (proteinaceus) inhibitors: baupain (0.23 μM) was pre-incubated for 10 min in assay buffer with the serine proteinase inhibitors SbTI (Soy beans trypsin inhibitor) [38] 1.39, 2.78, 5.56, 8.35 μM; EcTI (*Enterolobium contortisiliquum* trypsin inhibitor) [39] 0.2, 0.7, 1.50, 2.0, 2.7 μM and with the cysteine proteinase inhibitors HMWK (High Molecular Weight Kininogen) 0.08, 0.16, 0.24, and 0.32 μM; canecystatin [5] 0.06, 0.12, 0.18, 0.24 and 0.34 nM and BbCI (*Bauhinia bauhinioides* cruzain inhibitor) [40] 1.2, 1.8, 2.4, 3.6, and 4.8 μM. Enzyme activity was expressed in percent of residual activity on Z–Phe–Arg–MCA compared to the control.

2.8. Determination of baupain substrate specificity

The hydrolysis of two series of FRET peptides derived from Abz-KLRSSK-Q-EDDnp (Q-EDDnp is the fluorescence acceptor and Abz is the fluorescence donor that corresponds to glutamine-[N-(2,4-dinitrophenyl)-ethylenediamine] and ortho-aminobenzoic acid, respectively), in which the residues L and R and S were substituted by natural amino acids were quantified in a Hitachi F-2500 spectrofluorimeter at 37 °C. Baupain concentration was fixed as 11 nM and the substrates as 4 μM. Fluorescence changes were monitored continuously at λ_{ex} = 320 nm and λ_{em} = 420 nm. The enzyme concentrations were chosen so that less than 5% of the substrate was hydrolyzed over the course of the assay. The reaction rate was converted into nanomoles of substrate hydrolyzed per second based on a calibration curve obtained from the complete hydrolysis of each peptide. The scissile bond of hydrolyzed peptides were identified by isolation of the fragments using analytical HPLC followed by determination of their molecular mass by LC/MS using an LCMS-2010 equipped with an ESI-probe (Shimadzu, Japan).

2.9. CD experiments

Circular Dichroism (CD) measurements were taken on a Jasco J-810 spectropolarimeter (Jasco, Japan). Far UV-CD spectra were recorded at 25 °C in a cuvette of 1 mm pathlength with a 10 μM protein solution in the presence of 10 mM sodium phosphate buffer, pH 6.3. The spectra were recorded in the 190–250 nm wavelength range. The CD intensities were expressed as mean residue ellipiticities $[\theta]$ (deg cm^2 dmol^{-1}) using the formula $[\theta] = \theta_e/10.C.l.N$, where θ_e is the experimental ellipticity in millidegrees, MRW is the mean residue weight, *C* is the concentration of the protein in molar, *l* is the cuvette pathlength in centimeters, and *N* being the average number of residues adopted as 110 residues baupain. For the analysis of baupain CD spectrum the CDPro program was used. CDPro software package consists of three programs for analyzing the protein CD spectra for determining the secondary structure fractions (SELCON3, CDSSTR and CONTIN) and a program for determining tertiary structure class (CLUSTER) [41,42]. The estimation of baupain secondary structure was performance using 43 proteins in the reference set.

2.10. Fluorescence experiments

Steady-state fluorescence was recorded on Hitachi F-2500 spectrofluorimeter. Intrinsic tryptophan fluorescence spectrum was recorded by exciting the protein sample at 290 nm at pH 6.3, 10 mM sodium phosphate buffer. The emission spectrum was recorded in the range of 305–450 nm with excitation and emission slit widths