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Proteolytic properties of *Funastrum clausum* latex

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Abstract

As part of a screening of latex endopeptidases from plants growing in Argentina, the presence of proteolytic activity in the latex of *Funastrum clausum* stems is reported. The proteases present in the crude extract showed the main characteristics of the cysteine proteolytic class, i.e. optimum pH at alkaline range, isoelectric point (pI) higher than 9.0, and inhibition of proteolytic activity by thiol blocking reagents. A remarkable thermal stability was also evident in the crude extract. Endosterolytic preference tried on *p*-nitrophenyl esters of *N*- α -carbobenzoxy-L-amino acids was higher for the alanine, asparagine and tyrosine derivatives. Preliminary peptidase purification by two-step ionic exchange showed the presence of two proteolytic fractions with molecular masses of approximately 24.0 kDa according to SDS-PAGE.

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Keywords: *Funastrum clausum*; Latex; Thiol proteases

1. Introduction

Latex is a milky fluid composed by a liquid serum holding, in suspension or in solution, a complex mixture of constituents. It may contain a variety of cellular components, like nuclei, mitochondria, ribosome-like particles, and lysosome analogues. Agglomerative low density materials, such as various enzymes, terpenes,

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alkaloids, vitamins, carbohydrates, lipids and free amino acids have been identified among the components. Latex has been reported to occur in 12 000 plant species belonging to 900 genera. In a given family, the fact that not all genera are latex bearing complicates the definition of a role for latex in plants [1]. The presence of certain enzymes in latex vacuoles like chitinases and proteases suggests that it may help plants defenses against pathogens, parasites and herbivores by attacking the invader once the plant cell is lysed [2].

Besides the significant physiological roles played by endopeptidases, their commercial applications are of utmost importance, since they are one of the three largest groups of industrial enzymes, accounting for approximately 60% of the total worldwide sale of enzymes. Use of proteases in development of environmentally friendly technologies include leather treatment and several bioremediation processes [3], as well as their application in pharmaceutical industry for preparation of medicines such as ointments for debridement of wounds, and their assistance in the hydrolysis of large polypeptides in smaller peptides and aminoacids, facilitating the digestion and absorption of proteins [4].

A common feature that can be found in the latex of the *Asclepiadaceae* (also known as milkweed family, since these plants ooze a sticky, white latex when cut) is the presence of proteolytic activity. The study of the proteolytic characteristics of latex of *Funastrum clausum* is reported in this paper as part of a project on systematic screening of regional plant endopeptidases carried out in our laboratory. The species is widely spread from USA to Argentina, and used in popular medicine to kill screw-worm larvae in human flesh by application of a poultice of the leaves, as well as its latex is used to remove warts [5].

2. Experimental

2.1. Abbreviations used

AMPSO, 3-[(1,1-dimethyl-2-hydroxyethyl)amino]-2-hydroxy-propanesulfonic acid; BSA, Bovine Serum Albumin; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; CBZ, carbobenzoxy; DEAE-Sepharose, diethylaminoethyl-Sepharose; DTT, dithiothreitol; E-64, trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane; EDTA, ethylenediaminetetraacetic acid; IEF, Isoelectric focusing; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; SP-Sepharose, sulfopropyl-Sepharose; TAPS, *N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid; TCA, trichloroacetic acid.

2.2. Chemicals

Casein (Hammarsten type) was obtained from Research Organics Inc., Cleveland, Ohio. AMPSO, CAPS, cysteine, DTT, E-64, EDTA, iodoacetic acid, MOPS, *p*-nitrophenyl esters of *N*- α -carbobenzoxy-L-amino acids, pepstatine A, PMSF, TAPS, Tris, glycine isoelectric point markers (IEF MIX 3.6–9.3), and Biolyte 3–10 carrier ampholytes were purchased from Sigma Chemical Company, St. Louis. Coomassie

Brilliant Blue R-250, acrylamide, bisacrylamide and low molecular weight markers were obtained from Bio-Rad, Hercules, California. DEAE-Sepharose Fast Flow, SP-Sepharose Fast Flow, and Pharmalyte 3–10 were purchased from Pharmacia Biotech, Uppsala. All other chemicals were obtained from commercial sources and were of the highest purity available.

2.3. Plant material

F. clausum (Jacq.) Schlechter [Latin synonym: *Sarcostemma clausum* (Jacq.) Roem. and Schult.] (*Asclepiadaceae*), stems obtained from plants grown in Rosario, province of Santa Fe, Argentina (Argentinean folk names: *tasi*, *doca*, English common name: white twinevine). The plant is a vine, with leaves narrowly linear to broadly elliptic and white to greenish cream flowers [6]. Voucher specimen (UNR 1492) is deposited at the UNR herbarium, Facultad de Ciencias Agrarias, Universidad Nacional de Rosario, Argentina.

2.4. Crude enzyme extract preparation

Latex, obtained by superficial incisions of stems in late summer, was collected on 0.1 M phosphate buffer (pH 7.5) containing 5 mM EDTA and cysteine, in order to avoid phenoloxidase activity and oxidation, respectively. This suspension was first centrifuged at $16\,000\times g$ for 30 min at 4 °C in order to discard gums and other insoluble materials, and the supernatant was ultracentrifuged at $100\,000\times g$ for 60 min at 4 °C. The resulting supernatant containing soluble proteins was called 'crude extract' fractionated and conserved at -20 °C for further studies.

2.5. Proteolytic activity determination

Casein was used as non-specific substrate for proteolytic activity determination of the crude extract and in the preliminary purified fractions when possible. The reaction mixture contained 0.1 ml of crude extract and 1.1 ml of 1% casein with 12 mM cysteine in a 0.1 M Tris–HCl buffer (pH 8.0). The reaction was carried out at 45 °C and stopped 20 min later by adding 1.8 ml of 5% trichloroacetic acid (TCA). Each test tube was centrifuged at $3000\times g$ for 30 min and the absorbance of the supernatant was measured at 280 nm [7]. One caseinolytic unit (U_{cas}) was defined as the amount of protease that produces an increment of one absorbance unit per minute in the assay conditions [8].

During the purification steps, proteolytic assays were performed as follows. In the case of fractions eluted from the anionic exchange column, caseinolytic activity was determined as mentioned above. Due to its higher sensibility, azocasein was used to measure the proteolytic activity of those fractions obtained from the cationic exchange column [9]. For this determination, 0.25 ml of 2% azocasein in 0.1 M glycine–NaOH buffer (pH 9.5) containing 20 mM cysteine was added to 0.15 ml of enzyme sample and incubated at 45 °C for 30 min. The reaction was stopped by adding 1 ml of 10% TCA. After centrifugation at $4000\times g$ for 15 min, 0.9 ml of

the supernatant obtained was added to 1 ml of 1 M NaOH, and the absorbance was measured at 440 nm. In this case, one unit of proteolytic activity (U_{azocas}) was defined as the amount of enzyme, which produced an absorbance increase of one unit per minute under the assay conditions.

2.6. Protein and carbohydrate content determination

Proteins present in the crude extract and in partial purified fractions were measured according to Bradford's method [10] using BSA as standard. The protein content of chromatography eluates was estimated by absorbance at 280 nm during separation.

Measurement of sugar content of the proteolytic extract was achieved according to Dubois [11] using glucose as standard.

2.7. Optimum pH

Crude extract caseinolytic activity was measured at 45 °C using 10 mM sodium salts of the following 'Good' buffers: MES, MOPS, TAPS, AMPSO and CAPS, in the pH range of 6.5–10.5 [12].

2.8. Heat stability

For testing heat stability, crude extract was incubated at different temperatures ranging from 25 to 70 °C for 2, 5, 10, 20, 40, 60 and 120 min. The reaction was stopped in ice-water and the residual caseinolytic activity was measured under standard assay conditions.

2.9. Effect of inhibitors and activators

To elucidate the mechanistic nature of crude extract proteolytic activity, a set of inhibitors for different types of proteases was proved [13]. The crude extract was preincubated for 10 and 30 min at room temperature in the presence of the following inhibitors: 30 mM iodoacetate; 1 mM PMSF; 0.1 mM E-64; and 10 μ M pepstatine, in separate aliquots. In the case of PMSF, reversion was assayed by adding cysteine 12 mM to the mixture. The remaining caseinolytic activity was tried according to the previously described method.

To prove the effect of activators, caseinolytic activity was also assayed in the presence of 12 mM cysteine, and 1.5 and 5 mM dithiothreitol.

2.10. Isoelectric focusing (IEF) and zymogram

IEF was carried out in a Mini IEF Cell (Model 111, Bio-Rad). The sample was precipitated with five volumes of cold acetone and redissolved in deionized water twice. The sample and pI markers (Sigma IEF MIX 3.6–9.3) were loaded onto 5% polyacrylamide gel with a pH gradient in the range from 3 to 10 (BioLyte 3–10 carrier ampholytes, Bio-Rad, Hercules, CA), and the focusing of proteases present

in crude extract was performed according to these conditions: 100 V for 15 min, 200 V for the following 15 min, and 450 V for the last 60 min. One of the gels was fixed and stained with Coomassie Brilliant Blue R-250, while the other, unstained, was contacted for 20 min at 56 °C with an agarose gel imbibed in 1% casein solution, in order to detect the bands with proteolytic activity [14]. After incubation, the agarose gel was dehydrated and stained with Coomassie Brilliant Blue R-250.

2.11. Endoesterolytic preference determination on synthetic substrates

Enzymatic hydrolysis of *N*- α -carbobenzoxy-*p*-nitrophenyl esters of different amino acids was carried out in 0.1 M Tris–HCl buffer (pH 9.0) containing 2 mM EDTA and 10 mM cysteine at 45 °C. To 50 μ l of crude extract solution, 50 μ l of 1 mM substrate acetonitrile stock solution and 2.9 ml of the mentioned buffer solution were added [15]. Absorbance was followed spectrophotometrically at 405 nm every 10 s for 2 min. The esterolytic activity was expressed using an arbitrary enzyme unit (U_{cbz}), defined as the amount of peptidase that released 1 μ mol/min of *p*-nitrophenolate in the assay conditions. A standard curve of *p*-nitrophenol was performed to determine the micromoles produced during the reaction.

2.12. Crude extract chromatographic profile

Crude extract (3.0 ml) was applied to a Pharmacia XK 16/40 column, packed with DEAE-Sepharose Fast Flow. The column was equilibrated with 55 mM

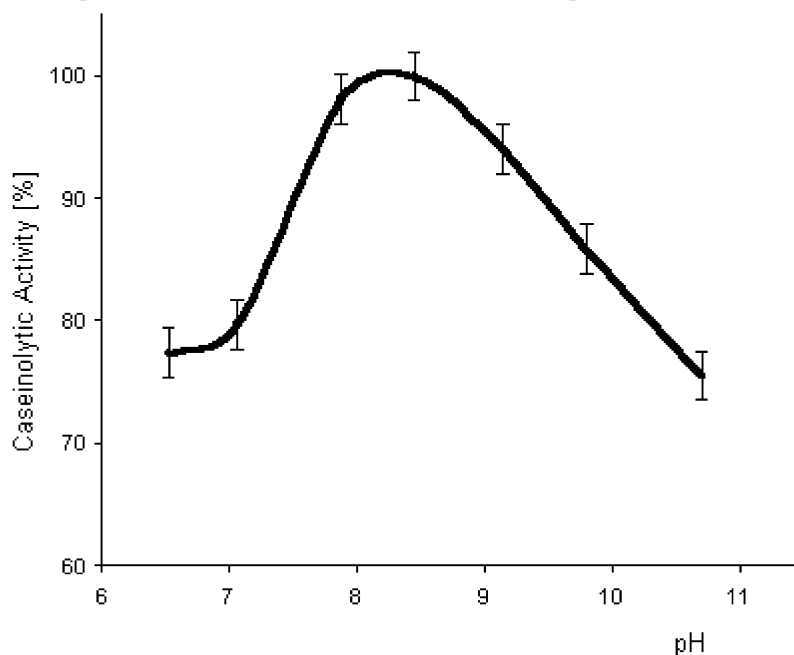


Fig. 1. Effect of pH on proteolytic activity of *F. clausum* latex crude enzymatic extract.

phosphate buffer pH 7.5, and the chromatography was developed in a FPLC equipment (Pharmacia) by washing with 17 ml of the equilibrating buffer and the bound material eluted with a linear gradient of sodium chloride (0–0.3 M) in the same buffer. The non-retained fraction was rechromatographed on SP-Sepharose Fast Flow equilibrated with citric-phosphate buffer 55 mM pH 6.5, and resolved by washing the column with 22 ml of the mentioned buffer and applying a NaCl gradient (0–0.4 M) in the same buffer.

2.13. Molecular mass determination by SDS-PAGE

SDS-polyacrylamide gel electrophoresis with tricine cathodic buffer was carried out in 10% polyacrylamide gels [16]. Samples were loaded together with molecular weight markers. Potential was kept constant at 40 mV for the stacking gel and at 150 mV for the resolution gel. The gels were stained with Coomassie Brilliant Blue R-250 and scanned for the estimation of molecular masses of the proteolytic fractions eluted from both columns by using the Scion Image software [17].

2.14. Caseinolytic activity detection in electrophoresis gels

Electrophoresis in polyacrylamide gels (12% acrylamide) was performed under non-denaturing conditions [18]. After the development of electrophoresis, SDS was

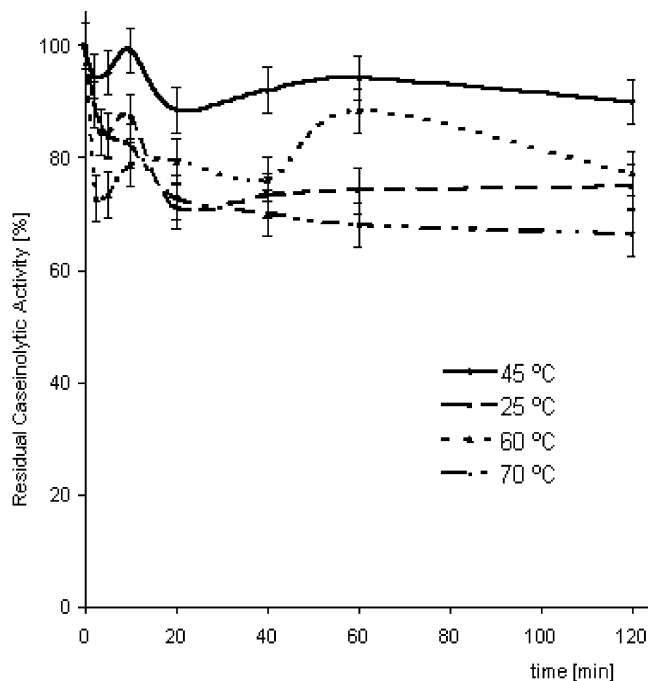


Fig. 2. Heat stability of *F. clausum* latex crude extract.

removed by gently shaking the gels at room temperature for 30 min in Triton X-100 2.5% in water and then soaked with deionized water three times during 10 min each. The resulting gels were incubated into 1% casein solution in buffer 0.1 M Tris–HCl pH 8 for 30 min at 45 °C. Proteolysis was stopped by staining with Coomassie Brilliant Blue R-250, after a brief water wash. Control electrophoresis was carried out under the same conditions, and simultaneously, was fixed and stained with silver to identify those bands with caseinolytic activity [19].

3. Results and discussion

3.1. Proteolytic activity of crude extract

Proteolytic activity presence was analyzed in the crude extract. The optimum pH range obtained was 8.0–10.0 (Fig. 1). The proteolytic extract showed a remarkable stability at different temperatures, including those as high as 60–70 °C (Fig. 2). Other crude extracts with proteolytic activity obtained from latices of *Asclepiadaceae*, such as *Morrenia brachystephana* [7] and *M. odorata* [20] showed a similar behaviour. Nevertheless, crude extract from *F. clausum* displayed an unusually high remaining activity at 70 °C (more than 60% after 2 h of incubation), whereas those belonging to the studied *Morrenia* genus were 45% for the former and 22% for the latter.

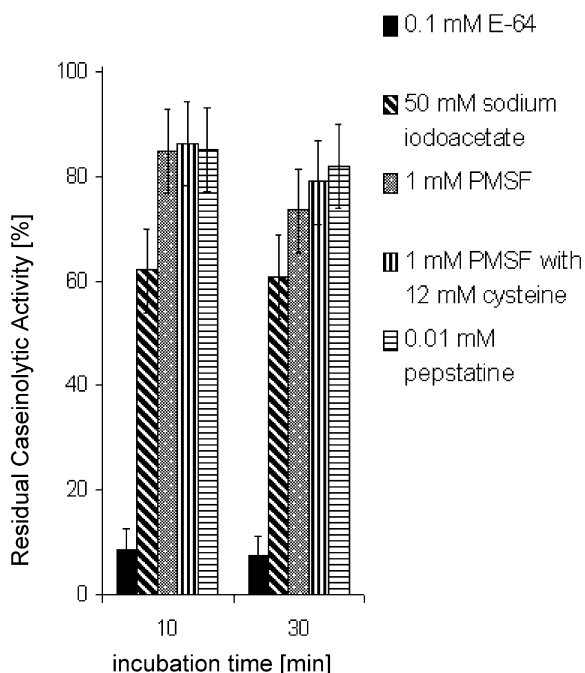


Fig. 3. Effect of different protease inhibitors on *F. clausum* latex crude extract activity.

3.2. Effect of inhibitors and activators

Proteolytic activity of the crude extract almost disappeared after incubation for 10 and 30 min with 0.1 mM E-64 (Fig. 3), an irreversible inhibitor for cysteine proteases belonging to the papain family, as well as it was enhanced by activators such as 12 mM cysteine (1.5-fold) and dithiotreitol (1.2-fold for 1.5 mM DTT and 1.5-fold for 5 mM DTT). Sodium iodoacetate provoked a decrease of 40% in the caseinolytic activity. A slight recovery of caseinolytic activity after incubating with cysteine, the sample inhibited by PMSF was observed, because of its reversible inhibitor nature in the case of cysteine proteinases. Pepstatin had little effect on activity. All these results suggested that the proteases present in latex of *F. clausum* belong to the cysteine group, as all the proteases already mentioned.

3.3. Esterolytic activity

Hydrolysis of several *N*- α -CBZ-amino acids *p*-nitrophenyl esters by crude extract revealed a major preference for the alanine (Ala) derivative, followed by those of asparagine (Asn) and tyrosine (Tyr), but in a very lower extent. The lowest preference was shown for the valine (Val), isoleucine (Ile) and proline (Pro)

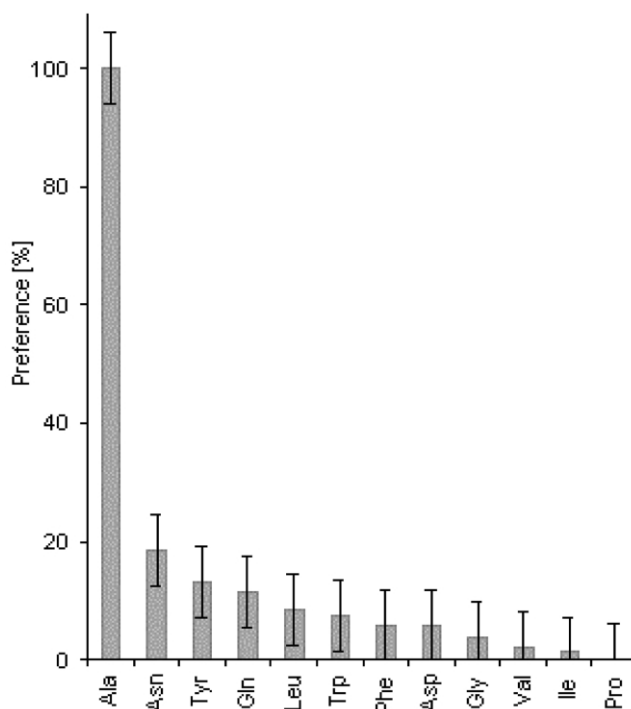


Fig. 4. Preference percentages of *F. clausum* latex crude extract on different *N*- α -CBZ-amino acids-*p*-nitrophenyl esters.

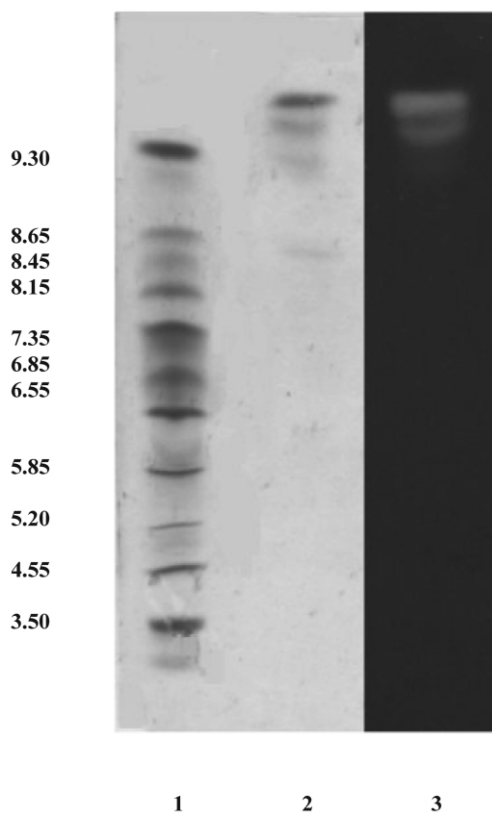


Fig. 5. IEF and zymogram. *Lane 1*: Sigma IEF MIX markers: Amyloglucosidase from *Aspergillus niger*, 3.6; Trypsin inhibitor from soybean, 4.6; β -Lactoglobulin A from bovine milk, 5.1; Carbonic anhydrase II from bovine erythrocytes, 5.9; Carbonic anhydrase I from human erythrocytes, 6.6; Myoglobin from horse heart, 6.8,7.2; Lectin from *Lens culinaris*, 8.2, 8.6, 8.8; Trypsinogen from bovine pancreas, 9.3. *Lane 2*: *F. clausum* latex crude extract. *Lane 3*: corresponding zymogram to *F. clausum* latex crude extract.

derivatives (Fig. 4). The high affinity for the *N*- α -CBZ-Ala *p*-nitrophenyl ester is a common feature for the proteases studied in our laboratory [21–23]. The low affinities for the Val, Ile and Pro derivatives are in good agreement with those obtained for *Araujia hortorum* proteases [24].

3.4. Chromatographic profile

Since the pI of the proteolytic fractions was above 9.3 (Fig. 5), according to the results observed in the isoelectric focusing, an anion exchange was chosen to separate the proteins without endopeptidasic activity (Fig. 6a). The protease purified in this step was collected under the name of funastrain c I, in agreement with previous nomenclature recommendations [25,26]. As the main fraction showing

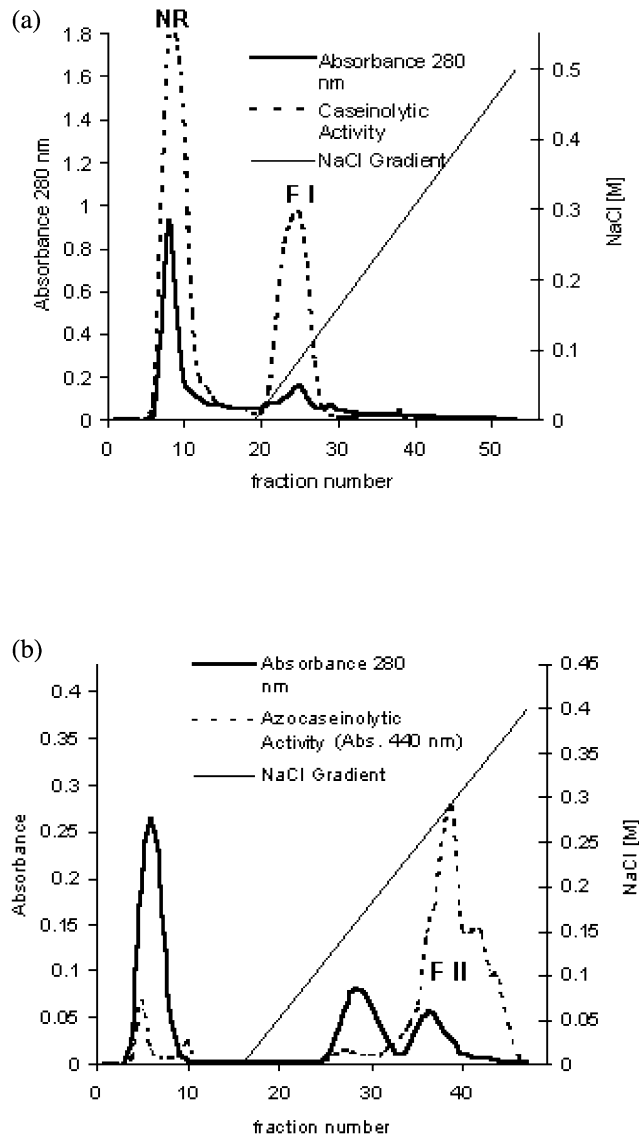


Fig. 6. (a) Anion exchange chromatography (DEAE-Sepharose Fast Flow Pharmacia XK 16/40 column, FPLC system). Elution buffer: 55 mM phosphate pH 7.5. Gradient: sodium chloride 0–0.5 M. Wash flow rate: 0.75 ml min^{-1} ; gradient flow rate: 0.50 ml min^{-1} . (b) Cation exchange rechromatography of NR (non-retained fraction) on SP-Sepharose Fast Flow Pharmacia XK 16/40 column, FPLC system. Elution buffer: 55 mM citric-phosphate pH 6.5. Gradient: sodium chloride 0–0.4 M. Wash flow rate: 0.75 ml min^{-1} ; gradient flow rate: 0.50 ml min^{-1} .

Table 1
Properties of *F. clausum* latex crude extract, funastrain c I and funastrain c II

| Sample | Volume (ml) | Protein content (mg/ml) | Carbohydrate content (mg/ml) | Total proteins (mg) | UCAS/ml | Total UCAS | Specific activity (UCAS/mg) | Purification (fold) | Yield (%) |
|-----------------|-------------|-------------------------|------------------------------|---------------------|---------|------------|-----------------------------|---------------------|-----------|
| Crude extract | 3.0 | 0.30 | 0.18 | 0.9 | 0.70 | 2.1 | 2.4 | – | – |
| NR ^a | 7.0 | 0.07 | – | 0.5 | 0.26 | 1.4 | 3.5 | 1.5 | 88.5 |
| Funastrain c I | 6.5 | 0.03 | – | 0.2 | 0.14 | 0.9 | 4.6 | 2 | 45 |
| Funastrain c II | 12.5 | 0.02 | – | 0.3 | 0.01 | 0.2 | 0.6 | 0.3 | 8.5 |

^a Non-retained fraction eluted from the anion exchange chromatography.

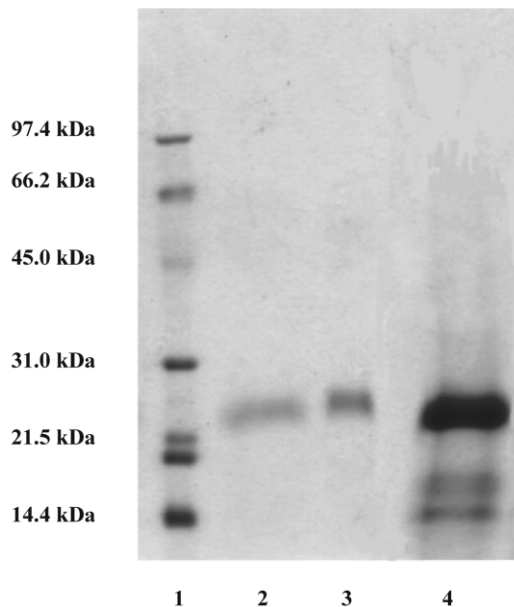


Fig. 7. SDS-PAGE. *Lane 1*: Molecular weight Bio Rad markers: Phosphorylase b, 97.4 kDa; Serum albumin, 66.2 kDa; Ovalbumin, 45.0 kDa; Carbonic anhydrase, 31.0 kDa; Trypsin inhibitor, 21.5 kDa; and Lysozyme, 14.4 kDa. *Lane 2*: Funastrain c I. *Lane 3*: funastrain c II. *Lane 4*: *F. clausum* latex crude extract.

caseinolytic activity was found in the non-retained peak, cation chromatography was applied to this fraction (Fig. 6b), allowing the separation of a second protease (funastrain c II). As can be seen in the purification table (Table 1), funastrain c I comprised 45% of the whole proteolytic activity, as its purification factor was two-fold. The specific activity of funastrain c II was much lower than the one expected from the results obtained for the non-retained fraction: its poor yield (8.5%) and the extreme low purification degree (0.2) would indicate that most biological activity of funastrain c II was lost during the purification process, and a new strategy to purify this fraction should be performed.

3.5. Electrophoresis

SDS-PAGE electrophoresis patterns of the crude extract and the purified fractions are shown in Fig. 7. Relative molecular masses of the two proteases preliminary purified were 24.4 for funastrain c I and 24.0 kDa for funastrain c II, in concordance with those of the purified proteases from other species belonging to the *Asclepiadaceae*, as *Asclepias glaucescens* [25,26], *A. syriaca* [27], *Calotropis gigantea* [28–30], *M. brachystephana* [7], *M. odorata* [20], *Araujia hortorum* [31,32] and *A. fruticosa* [23], all ranging from 23 to 27 kDa. Native electrophoresis (Fig. 8) and IEF zymogram (Fig. 5) confirmed the peptidasic activity of those fractions.

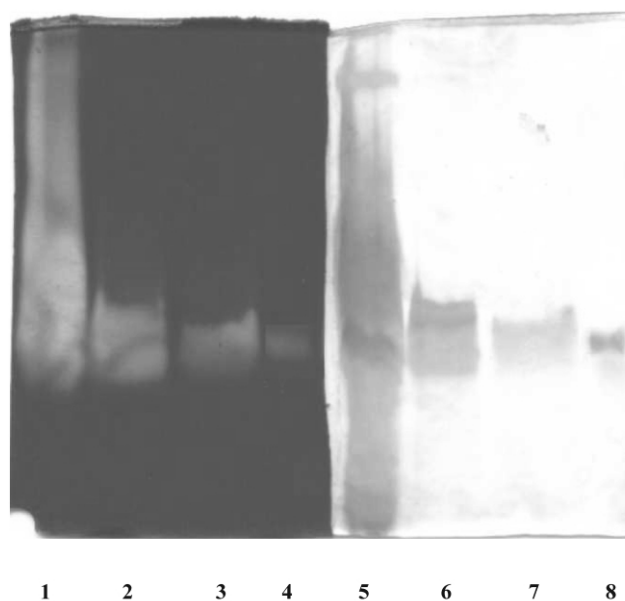


Fig. 8. Native electrophoresis and zymogram. Zymogram: in situ caseinolytic activity. *Lane 1*: *F. clausum* latex crude extract. *Lane 2*: Anion exchange non-retained fraction. *Lane 3*: Funastrain c I. *Lane 4*: Funastrain c II. Control native electrophoresis. *Lane 5*: *F. clausum* latex crude extract. *Lane 6*: Anion exchange non-retained fraction. *Lane 7*: Funastrain c I. *Lane 8*: Funastrain c II.

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