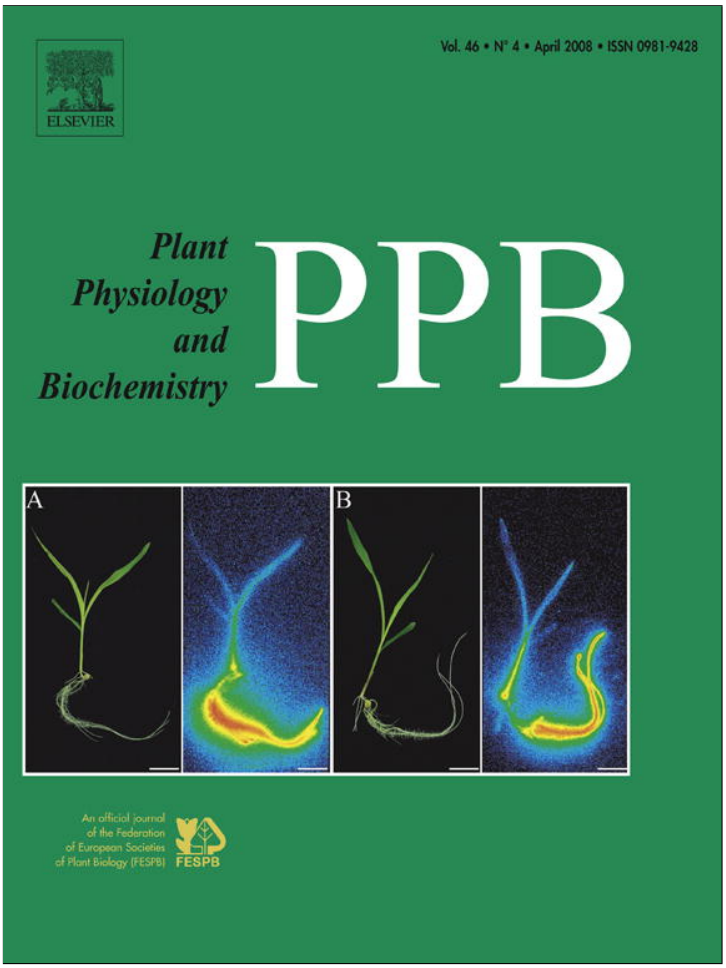

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Plant Physiology and Biochemistry 46 (2008) 403–413
Plant Physiology and Biochemistry
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Research article

Isolation and characterization of a dual function protein from *Allium sativum* bulbs which exhibits proteolytic and hemagglutinating activities

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Received 30 September 2006
Available online 26 December 2007

Abstract

A dual function protein was isolated from *Allium sativum* bulbs and was characterized. The protein had a molecular mass of 25–26 kDa under non-reducing conditions, whereas two polypeptide chains of 12.5 ± 0.5 kDa were observed under reducing conditions. E-64 and leupeptin inhibited the proteolytic activity of the protein, which exhibited characteristics similar to cysteine peptidase. The enzyme exhibited substrate specificity and hydrolyzed natural substrates such as α-casein (K_m: 23.0 μM), azocasein, haemoglobin and gelatin. It also showed a high affinity for synthetic peptides such as Cbz-Ala-Arg-Arg-OMe-β-Nam (K_m: 55.24 μM, k_{cat}: 0.92 s^{-1}). The cysteine peptidase activity showed a remarkable stability after incubation at moderate temperatures (40–50 °C) over a pH range of 5.5–6.5. The N-terminus of the protein displayed a 100% sequence similarity to the sequences of a mannose-binding lectin isolated from garlic bulbs. Moreover, the purified protein was retained in the chromatographic column when Con-A Sepharose affinity chromatography was performed and the protein was able to agglutinate trypsin-treated rabbit red cells. Therefore, our results indicate the presence of an additional cysteine peptidase activity on a lectin previously described.

Keywords: Proteolytic activity; *Allium sativum*; Sativain; Garlic bulbs; Mannose-binding lectin; Cysteine peptidase; Hemagglutinating activity

1. Introduction

Cysteine peptidases (EC 3.4.22) are one of the main four classes of proteinases widely distributed in the microbial, plant, and animal kingdoms [1]. CPs have been found in latex, fruits, and seeds [2–5], although a few reports on cysteine peptidases isolated from the underground parts of the plant have been published. Among them, a cysteine protease from *Zingiber officinale* Roscoe rhizomes [6] and from *Phytolacca americana* roots [7] has been studied. In addition, there were reports on the cysteine peptidase activity exhibited by a protein isolated from garlic bulbs [8–10].

For long, it has been believed that the plant proteolytic machinery has only a housekeeping role, serving to remove non-functional proteins and to release amino acids necessary for recycling [11]. Recently, the role of plant proteases in the regulation of biological processes such as the recognition of effective defence responses has attracted the interest of several researchers [12].

Plants, in contrast to vertebrates, have no immune system and have developed different methods to defend themselves

Abbreviations: ASA II, *Allium sativum* agglutinin II; BAPNA, benzoyl-arginine-*p*-nitroanilide; Brij-35, polyoxyethylene-23-lauryl ether; CPs, cysteine peptidases; DEAE, diethylaminoethyl; DTT, dithiothreitol; E-64, *trans*-epoxysuccinyl-L-leucylamide-(4-guanidino)-butane; EDTA, ethylenediaminetetracetic acid; β-ME, β-mercaptoethanol; MES, 2-[*N*-morpholino]ethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffer saline; PIs, proteinase inhibitors; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid.

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doi:10.1016/j.plaphy.2007.11.003

against pathogens. Different types of antimicrobial proteins isolated from plants have been studied including thionins, chitinases, ribosome-inactivating proteins, defensins, glucanases, proteinases, proteinase inhibitors, and lectins [13]. While PIs are generally believed to actively contribute to plant defence mechanisms, Konno et al. [14] recently provided evidence that the cysteine proteinases and not the PIs stored in the laticifers of papaya were the active compounds participating in its defence mechanism against herbivorous insects. Experimental evidence has shown that latex generally contributes to the protection of the plant against predators. Moreover, in maize genotypes, a defence mechanism against insects was resolved by induction of cysteine peptidases without involvement of cysteine proteinase inhibitors [15]. Associations between the induction of protease genes and defence have also been found for genes that encode metallo-, aspartic-, and cysteine-peptidases. Certain cysteine peptidases called the caspases (clan CD, family C14) play a key role in the plant hypersensitive response, a defence mechanism that involves programmed cell death [1]. In addition, a cysteine endopeptidase secreted by the pathogenic bacterium *Porphyromonas gingivalis* (clan CD, family C25) named gingipain and a metalloprotease-hemagglutinin secreted by *Vibrio cholerae* (clan MA) have been considered as possible pathogenic factors [1,16].

Besides plant lectins, a widespread group of carbohydrate-binding proteins that bind to glycoproteins in the peritrophic matrix linking the insect midgut and disrupt digestive processes and nutrient assimilation have been associated with defence-related phenomena [17,18].

Hemagglutinating activity is a well-known characteristic of lectins, although in the past it was reported that certain microbial and plant proteases have a red-cell agglutinating activity in addition to proteolytic activity [1,7,16].

Garlic is one of the edible plants which has generated considerable interest throughout human history because of its pharmaceutical properties. A wide range of microorganisms including bacteria, fungi, protozoa, and viruses has been shown to be sensitive to crushed garlic preparations [19]. Moreover, it has been reported that garlic reduces blood lipids and has anticancer effects [20,21].

The original aim of this work was the isolation and biochemical characterization of a cysteine peptidase from garlic bulbs. After protein purification and sequencing, the purified protein displayed a 100% sequence similarity to the sequences of a mannose-binding lectin previously described. Therefore, hemagglutination assays and sugar specificity tests were performed to determine whether the proteolytic and the hemagglutinating activities were associated with the same protein.

2. Results

2.1. Purification of the proteolytic enzyme isolated from Allium sativum bulbs

Previous studies have shown that proteolytic activity was found in storage tissues and embryos of garlic bulbs [10]. In this study, the main objective is to characterize the protein responsible for this activity.

The crude extract of *A. sativum* bulbs containing 2.0 mg mL^{-1} of proteins showed a high caseinolytic activity (27.3 Ucas mL^{-1}) at acidic pH. The enzymatic activity of the crude extract was activated by thiol-reducing reagents and inhibited by E-64 and leupeptin, suggesting that it belongs to the cysteine-type proteases (Table 1). It is well-known that garlic cloves contain very high concentrations of sulfur-containing compounds; therefore, a reducing environment is needed to maintain the activity of the cysteine residues involved in the catalytic mechanism [22].

In an attempt to purify the protein, the crude extract was applied onto a DEAE-Sepharose Fast Flow column and eluted with 50 mM phosphate buffer at pH 7.4 with a linear saline gradient (0–0.5 M KCl). Three main peaks related to caseinolytic activity were resolved following this chromatography (Fig. 1A). Peak B (P_B) corresponded to the bulk of the proteolytic activity, whereas peaks A (eluted proteins) and C corresponded to slight enzymatic activities. SDS-PAGE analysis of the P_B obtained after ion-exchange chromatography revealed the presence of only one contaminating polypeptide next to the protein with the highest proteolytic activity (Fig. 1A, inset lane B). Therefore, after concentration by ultra filtration, a size-exclusion chromatography on a Sephadex G-75 column was performed to remove the undesired proteins. Only one active fraction was eluted in this chromatographic step and when the chromatography of this active fraction was performed again, under the same conditions, a single protein was observed (Fig. 1B). The protein displaying caseinolytic activity was named sativain and was purified to homogeneity with a 13.8-fold purification and a 2% recovery over the initial crude extract (Table 2). Sativain migrated as a single band with a molecular mass of approximately 26 kDa on SDS-PAGE under non-reducing conditions (Fig. 2, lane 2), whereas in the presence of β-ME at high temperatures, the protein

Table 1
Effect of different compounds on caseinolytic activity of crude extract prepared from *Allium sativum* bulbs

Modulators	Concentration[a]	Caseinolytic activity[b] (in %)
Control (without modulators)	–	100
Pepstatin[d] (μg mL^{-1})	100	90 ± 5
1,10-Phenanthroline[d] (mM)	10	98 ± 3
PMS[c,d] (mM)	10	50 ± 5
E-64[d] (μM)	1	30 ± 6
$HgCl_2$[d] (mM)	10	25 ± 5
Iodoacetic acid[d] (μM)	100	60 ± 5
Leupeptin[d] (mM)	1	10 ± 5
Cysteine[e] (mM)	5	121 ± 2
Cysteine plus β-ME[e] (mM)	5/10	130 ± 4

[a] Concentrations refer to those present in the reaction mixture.
[b] Each value is an average of three replicates of at least three extractions.
[c] Inhibition was fully restored by the addition of 12 mM cysteine. Activities are given as a percentage of the control.
[d] Samples were preincubated with the inhibitors for 30 min at 30 °C, then the reaction was initiated by adding α-casein as protein substrate.
[e] These effectors were added in the incubation mixture.

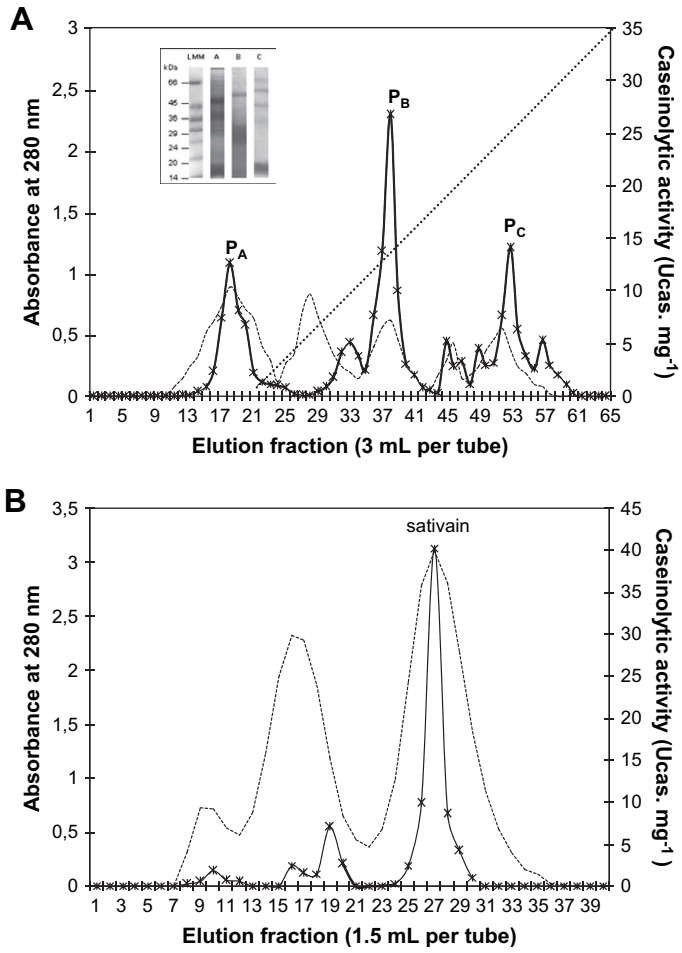


Fig. 1. (A) Elution profile of the proteolytic enzyme from *Allium sativum* bulbs on DEAE-Sepharose Fast Flow column equilibrated with 50 mM potassium phosphate buffer at pH 7.4. The column was eluted with a linear gradient of 0–0.5 M potassium chloride salt (·····) at the same pH. Fractions of 3 mL were collected and assayed for caseinolytic activity with 0.2% (w/v) α-casein as protein substrate. Inset – 14% non-reducing SDS-PAGE of fractions A–C eluted from the anion-exchange chromatography. Sigma Low Molecular Markers were taken as standard (lane 1). (B) Size-exclusion chromatography of fraction B (eluted from the DEAE-Sepharose column) onto a Sephadex G-75 column. The active protein was eluted in 50 mM potassium phosphate buffer (pH 7.4) at a flow rate of 20 mL h^{-1}. Caseinolytic activity was measured as indicated previously and the protein profile was estimated by the absorbance at 280 nm (-----).

gave two bands of 12.5 ± 0.5 kDa (Fig. 2, lane 3). In addition, after cutting the 26 kDa band from the gel and homogenizing it in sample buffer with the addition of β-ME and boiling the sample for 3 min at 80 °C, it was observed that the protein exhibits an apparent molecular mass of 12 ± 0.5 kDa (Fig. 2, lane 4). Moreover, from Western blot analysis, it was found that mice anti-sativain antibodies against the 12 ± 0.5 kDa band recognized the monomer as well as the dimeric form in soluble and membrane fractions (Fig. 3). The native Mr of the enzyme estimated by the size-exclusion method was consistent with the SDS-PAGE results, indicating the dimeric nature of the protein (data not shown).

Proteolytic activity was also investigated in 0.1% gelatin copolymerized gels. Fig. 2, lane 5 showed a clear band corresponding to substrate digestion in the gel, with a mobility similar to the dimeric form of the protein. Whereas the monomeric form did not show gelatinolytic activity (Fig. 2, lane 6). The gelatinolytic activity of the dimeric enzyme was completely destroyed by E-64 and leupeptin during gel incubation after electrophoresis (data not shown); however, PMSF (without the subsequent addition of 12 mM cysteine) as well as 1,10-phenanthroline, EDTA, or pepstatin did not affect the enzyme activity. Therefore, the inhibition patterns of caseinolytic activity of the protein present in crude extracts (Table 1) as well as that present in the pure fraction were indicative of characteristics of cysteine peptidases.

The results of isoelectric focusing and the corresponding IEF-zymogram showed the acidic characteristics and homogeneity of the protein, with a p*I* value of 4.9 (Fig. 4, lanes 2 and 3, respectively).

2.2. Biochemical characterization of sativain

2.2.1. Thermal and pH stability

The optimum pH for proteolytic activity was achieved between pH 5.0 and 6.5 for hydrolysis of denatured natural substrates, whereas a 50% loss of activity was observed below pH 4.5 and over pH 8.0 (Fig. 5A). Since temperature is used to inactivate the protease activity, thermostability was examined by measuring the residual caseinolytic activity of the enzyme incubated at different temperatures. As can be seen in Fig. 5C, the thermostability of sativain proved to be higher than 80% when it was incubated for 20 min at moderate temperatures (40 °C); however, a 90% decrease was observed at 70 °C, showing a rapid thermal inactivation at high temperatures. Stability of the enzyme over the pH range of maximal caseinolytic activity was assessed by incubation at 40 °C. It was found that 80–90% of its activity was retained at 60 min of incubation time and only 50% residual caseinolytic activity was conserved after 120 min of

Table 2
Purification chart of the proteolytic enzyme isolated from garlic bulbs

Purification step	Protein (mg mL^{-1})	Total protein (mg)	Total activity (Ucas)	Specific activity (Ucas mg^{-1})	Fold purification	Yield (%)
Soluble fraction	2.00	40.00	546.0	13.65	1.00	100.0
DEAE-Sepharose	0.90	6.75	180.0	26.70	1.96	33.0
Sephadex G-75	0.43	1.29	51.9	40.20	2.95	9.5
Sativain	0.089	0.089	16.3	187.30	13.72	2.0

One unit of α-caseinolytic activity is defined as the amount of enzyme required to cause a unit increase in absorbance per minute at 280 nm across a 1-cm path length, under the conditions of the assay (40 °C).

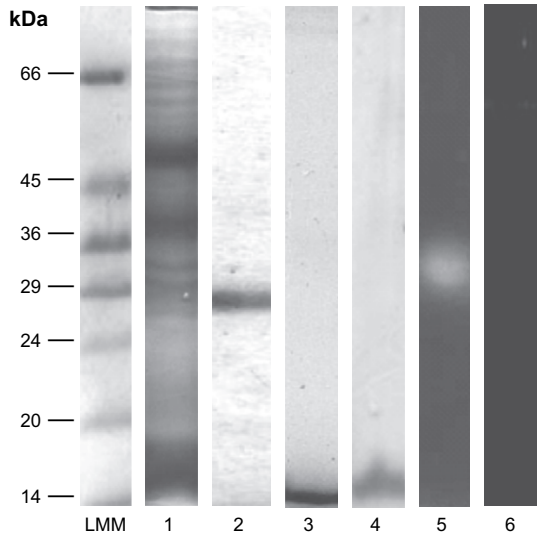


Fig. 2. SDS-PAGE (14%) under non-reducing and reducing conditions. Non-reducing SDS-PAGE of crude extract (lane 1) and purified fraction (lane 2). Reducing SDS-PAGE of the 26 kDa band (lane 3) and dimeric band cut from the non-reducing gel, boiled with cracking buffer containing β-Me and re-electrophoresed (lane 4). Denaturing and non-reducing 12% PAGE containing 0.1% gelatin from the dimeric fraction purified by Sephadex G-75 (pure sativain) (lane 5) and monomeric form obtained after reducing treatment of the homogeneous protein (lane 6). Sigma Low Molecular Markers were used as standards.

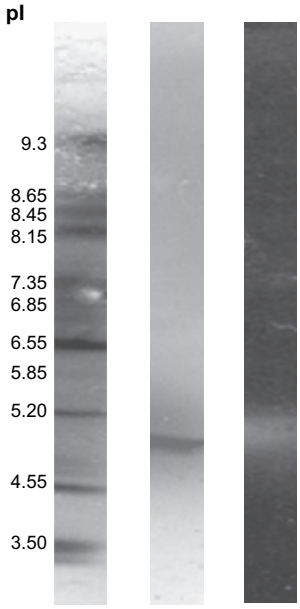


Fig. 4. IEF of the purified protein eluted from Sephadex G-75 column (lane 2) and IEF-zymogram (lane 3). Lane 1: carrier ampholytes (3.5–10).

incubation time (Fig. 5D). Finally, the thermostability was analyzed by self-digestion of the enzyme under activated conditions at room temperature, since generally, peptidases are prone to undergo autolysis. A 25% loss of activity was found after 24 h incubation (Fig. 5C) in contrast to data obtained for several cysteine endopeptidases from plant origin such as ervatamin C and papain, which under the same experimental conditions, retained 35% and 10% of residual activities, respectively, after 6 h of incubation [23].

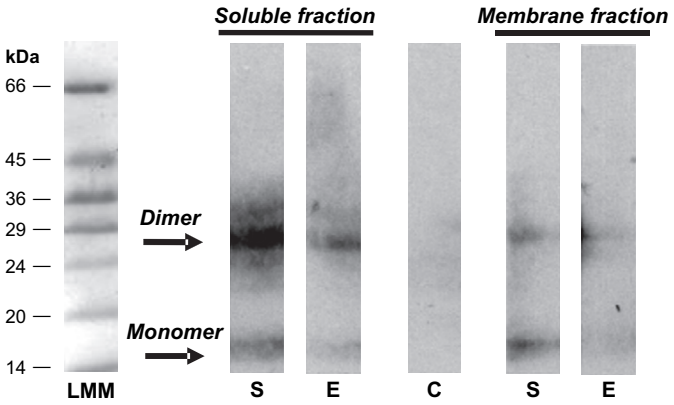


Fig. 3. Western blot analysis of sativain. Different tissues of garlic bulbs (20 μg protein) were subjected to SDS-PAGE, transferred to nitrocellulose, and probed with anti-sativain polyclonal antibodies (1/2000, v/v). Lanes represent different tissues tested in soluble and membrane fractions. E, embryos; S, storage tissue and C, bovine serum albumin as negative control.

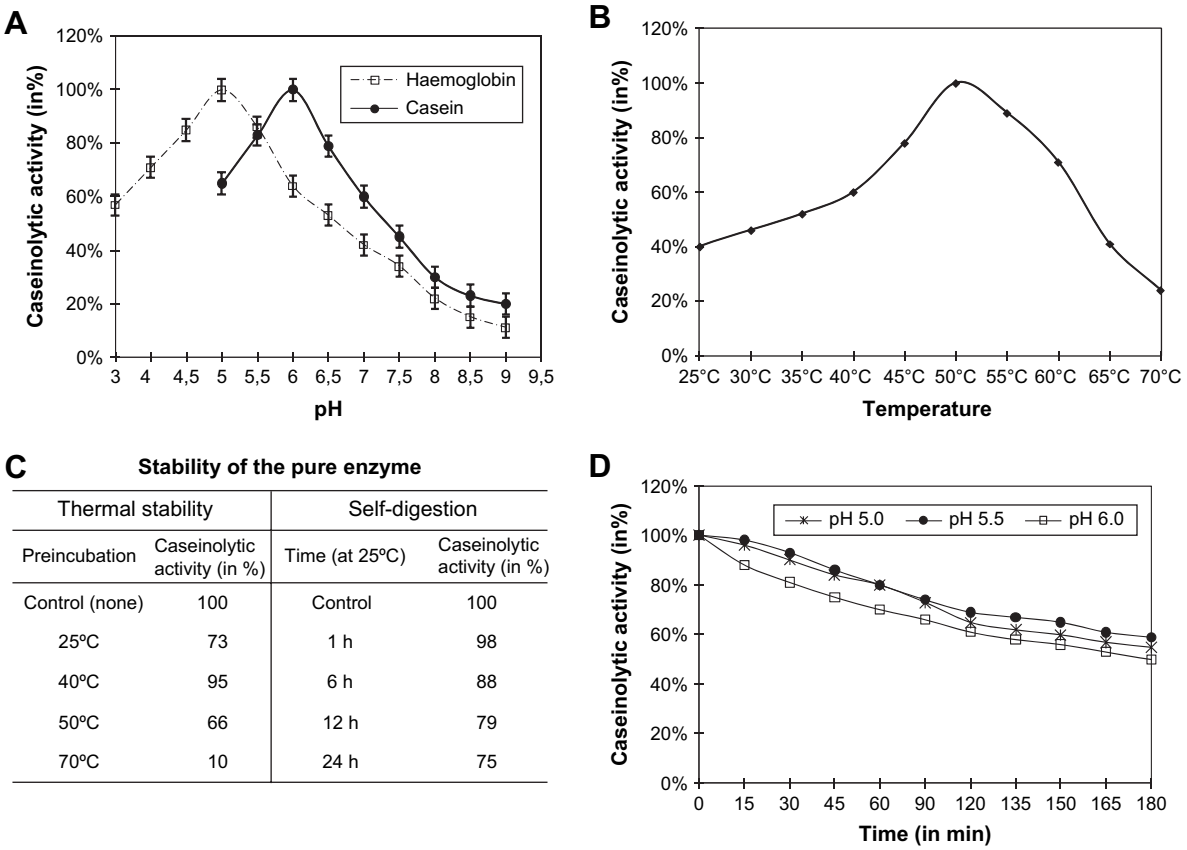
Other modulators, such as metallic cations (calcium, magnesium, zinc, manganese, sodium, and potassium), did not significantly modify the enzymatic activity, suggesting that metallic cofactors were not necessary to enhance the enzymatic activity.

All these observations indicated that sativain presented several biochemical properties, which makes it a potential enzyme to explore up on for biotechnological applications.

2.2.2. Substrate specificity and kinetic analysis

A high specific activity of sativain toward denatured natural substrates was observed (Table 3). Specific activities of 236 U mg^{-1} and 188 U mg^{-1} were found with α-casein and azocasein, respectively. In addition, sativain showed amidolytic activity toward different synthetic chromogenic peptides such as β-naphthylamides and *p*-nitroanilides. The enzyme cleaves CBZ-Ala-Arg-Arg-OMe-β-naphthylamide and CBZ-Phe-Arg-OMe-β-naphthylamide, with similar specific activity, as well as Bz-Arg-*p*-nitroanilide, which is an ideal substrate for papain, ficin, and some other plant peptidases [24,25]. Nevertheless, sativain did not show esterolytic activity at the optimum pH range of the enzyme when benzoyl-arginine-ethyl ester was used as synthetic peptide (data not shown).

The enzyme exhibited Michaelis–Menten behavior with denatured natural substrates as well as synthetic chromogenic peptides. The K_m values obtained from the Lineweaver–Burk plots were 23.0 μM for α-casein and 55.24 μM for Cbz-Ala-Arg-Arg-OMe-β-Nam (Fig. 6). These values were very close to the K_m values reported for procerain and ervatamin using casein as protein substrate and were greater than the values obtained for cruzipain. When BAPNA was used, K_m value for sativain was 100-fold higher than that for papain and 10-fold lower than that for ervatamin and procerain and



Thermal stability		Self-digestion	
Preincubation	Caseinolytic activity (in %)	Time (at 25ºC)	Caseinolytic activity (in %)
Control (none)	100	Control	100
25ºC	73	1 h	98
40ºC	95	6 h	88
50ºC	66	12 h	79
70ºC	10	24 h	75

Fig. 5. Stability of the enzyme as a function of pH and temperature. (A) Effect of the pH on the hydrolysis of α-casein (–) and haemoglobin (···). (B) Effect of temperature on the reaction velocity. The assays were performed at the specified temperatures in the range of 25–70 °C. (C) Thermal stability of sativain. The enzyme was incubated at 25, 40, 50, and 70 °C for 20 min in 50 mM potassium phosphate buffer pH 6.0, after which the residual activity against α-casein was assayed. Self-digestion of the enzyme was carried out during 1, 6, 12, and 24 h at 25 °C. (D) Stability of the enzyme was measured at the pH range of maximum proteolytic activity. Caseinolytic activity values (in %) were calculated considering 100% to the highest activity obtained in each case.

other cysteine plant endopeptidases that belong to the papain superfamily (Table 4) [3,23]. The activity of the enzyme toward all the substrates was inhibited by preincubation of the enzyme with E-64.

Electrophoretically homogeneous enzyme was titrated with E-64 as described by Barrett and Kirschke [26]. E-64 inhibited sativain with a k_2 value of 16,000 s^{-1} M^{-1} and the enzyme concentration determined by active-site titration was 2.3 μM (Fig. 7).

2.3. N-terminal sequence

The amino terminal sequence obtained from the first 10 amino acids of the 12.5 kDa monomer was determined and was compared with those of other plant cysteine proteinases. Using the NCBI-Blast data bank, it was found that sativain showed a 100% sequence similarity to the sequences of a mannose-binding lectin (ASA II) [27]. In addition, it showed a high sequence similarity (80% and 70%) to the sequences of other lectins isolated from *Allium ursinum* and *Allium ascalonicum* [28]. A minor percentage of homology to cDNA internal deduced sequences of cysteine peptidases from *Gossypium hirsutum* and *Oryza sativa* (40–50% homology) was observed (Table 5). Similar results were observed after the dimeric form of the protein was sequenced.

2.4. Affinity chromatography on Con-A Sepharose

The results obtained above suggested that both activities, peptidase and lectin, were present in the same protein.

Table 3
Substrate specificity of the proteolytic enzyme isolated from *Allium sativum*

Substrate	Specific activity
(1) β-Naphtylamides (100 μM, 50 mM buffer MES)	
CBZ-Ala-Arg-Arg-OMe-β-Nam (μmol mg^{-1})	17.0
CBZ-Phe-Arg-OMe-β-Nam (μmol mg^{-1})	13.9
(2) *p*-Nitroanilides (500 mM, 50 mM buffer MES)	
Bz-Arg-pNA (μmol mg^{-1})	10.7
(3) Denatured protein (0.2%, 50 mM potassium phosphate pH 6.0)	
α-Casein (μmol mg^{-1})	236
Haemoglobin (μmol mg^{-1})	104
Azocasein (μmol mg^{-1})	188
Azocollagen (μmol mg^{-1})	56

Specific activity is defined as (1) the number of micromoles of β-naphthylamine liberated per min of substrate digestion per mg of enzyme, (2) the number of micromoles of *p*-nitroaniline released per minute of substrate digestion per mg of enzyme and (3) the number of enzyme units produced per mg of protein (3).

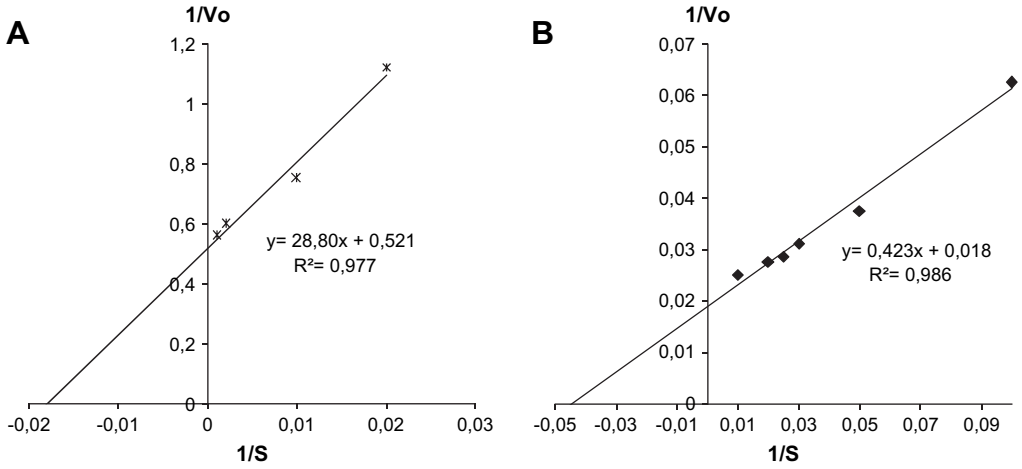


Fig. 6. Lineweaver–Burk plots using Cbz-Ala-Arg-Arg-OMe-β-Nam (A) or α-casein (B) as substrates. The enzyme activity was determined in the reaction mixture previously described in 50 mM MES buffer at pH 6.0 for 15 min at 37 °C (A) and 50 mM potassium phosphate buffer at pH 6.0 for 30 min at 40 °C (B). The substrate concentrations (μM) are expressed as $1/(S)$ on the abscissa and the reaction rate, V, as μM min^{-1} mg^{-1}. The data shown are the average of triplicate assays.

Therefore, several experiments were performed to investigate this feature.

First, the purified sativain eluted from Sephadex G-75 column was subjected to affinity chromatography on Con-A Sepharose resin. Fig. 8 shows a single active peak bound to the matrix displaying a caseinolytic activity of 451 U mg^{-1} with a 33-fold purification and 1.5% recovery.

2.5. Hemagglutinating activity of sativain

Finally, we tested whether the pure enzyme was able to agglutinate erythrocytes by means of an agglutination test with trypsin-treated rabbit erythrocytes. Table 6 shows that the purified protein exerted a specific hemagglutinating activity of 320 HAU mg^{-1}. In addition, the inhibition of the lectin activity was carried out using different saccharides and it was observed that sativain displayed the same inhibition pattern as ASA II [29].

Table 4
Comparative analysis of the kinetic parameters of different cysteine peptidases

Substrate	Enzyme	K_m (μM)	k_{cat} (s^{-1})
CBZ-Ala-Arg-Arg-OMe-β-Nam	Sativain	55.2	0.9
	Cathepsin B	4.3	25.0
	Cruzipain	20.0	0.9
CBZ-Phe-Arg-OMe-β-Nam	Sativain	76.9	2.3
	Cruzipain	62.0	14.5
BAPNA	Sativain	400.0	241.5
	Papain	2.9	0.7
	Procerain	4100.0	
	Ervatamin	6600.0	
	Cruzipain	35.0	9.9
α-Casein	Sativain	23.0	–
	Procerain	22.0	–
	Ervatamin	25.0	–

3. Discussion and conclusions

A protein with proteolytic activity, named sativain, was isolated from *A. sativum* bulbs and was purified to homogeneity. The estimated molecular mass of the protein was 26 kDa by size-exclusion chromatography and SDS-PAGE under non-reducing conditions, whereas two subunits of 12.5 ± 0.5 kDa were found under reducing conditions. The molecular mass of the protein was in the range of the molecular masses reported for other cysteine peptidases, although it showed a dimeric nature. The latter fact was unexpected, since almost all the cysteine peptidases reported from plant origin were monomeric [30].

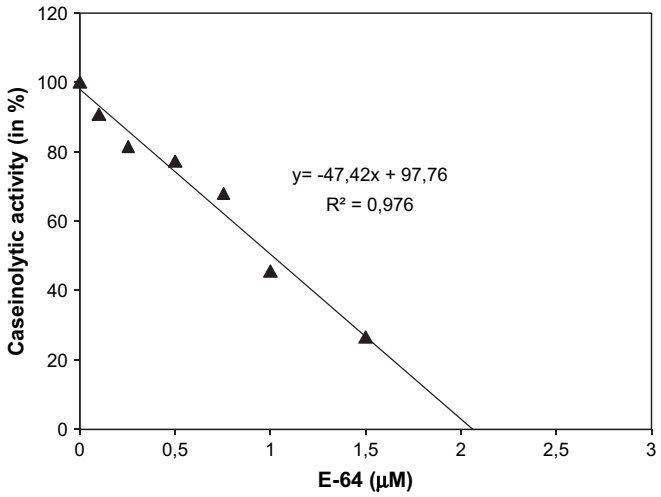


Fig. 7. Active-site titration of the cysteine peptidase from *Allium sativum* bulbs with E-64. Enzyme solution (100 μL) was mixed with 10 μL of E-64 solutions at different concentrations and with 100 μL of 50 mM potassium phosphate buffer at pH 6.0. After incubation for 30 min at room temperature, the aliquots were assayed spectrophotometrically to detect the remaining enzyme activity using α-casein as substrate. The active-site titration yielded 2.3 μM. The results are means of three independent experiments.

Table 5
Alignment of sativain amino terminal sequence with other plant sequences

Amino terminal alignment

Allium sativum peptidase (100%)	1	RNILMNDEGL	10
Allium sativum agglutinin (100%)	1	RNILMNDEGL YAGQSLDVEP YHLIMQEDCN LVLYDHSTAV ASNTDIPGK	50
Allium ursinum mannose-specific lectin precursor (80%)	29	RNILLNGEGL YAGQSLEEGS YKLIMQDDCN LVLFEYSTQV WASNTGVSGR	78
Allium ascalonicum agglutinin (70%)	1	RNVLVNNEGL YAGQSLVEEQ YTFIMQDDDN LVLYEYST	38

cDNA deduced internal fragment sequences

Allium sativum peptidase (100%)	1	RNILMNDEGL	10
P2 Lotus japonicum cysteine peptidase (40%)	35	DDEGL	39
Oryza sativa putative cysteine peptidase (50%)	251	NDEGL	254
Gossypium hirsutum cysteine peptidase (40%)	5	RNIL	8

The amino acid composition of sativain was compared with the composition determined from other plant lectins or inferred from the nucleotide sequence. The percentages of homology are indicated by numbers in parentheses.

Sativain showed a broad spectrum of activity between pH 5.0 and 7.0, and the same result was reported for actinidin [31]. The protein showed stability through the storage time and a low level of auto-degradation at room temperature. In addition, the enzyme hydrolyzed natural substrates and, like other cysteine peptidases, showed amidolytic activity. The kinetics constants for different protein and synthetic substrates showed that β-naphthylamide derivatives presented high affinity and the highest k_{cat}/K_m ratio was obtained for CBZ-Phe-Arg-OMe-β-Nam. This substrate exhibited the maximum response for cruzipain, a cysteine peptidase isolated from epimastigotes of *Trypanosoma cruzi* [30]. The inhibition pattern by E-64 and other specific inhibitors indicated characteristics similar to those of cysteine peptidases.

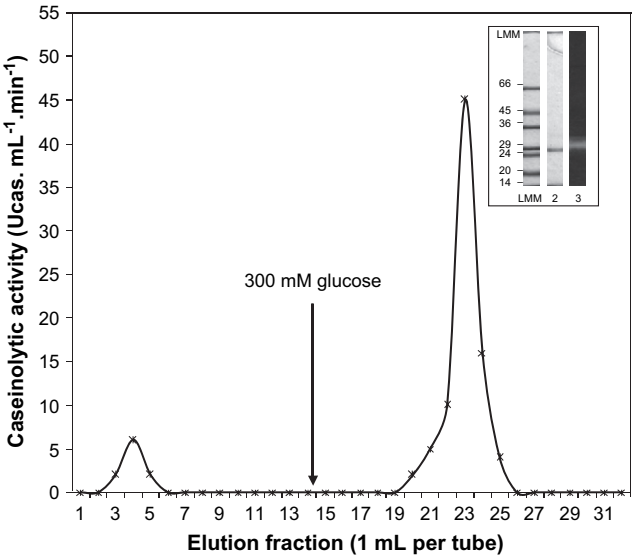


Fig. 8. Elution profile of sativain on Con-A affinity chromatography. The column was equilibrated with 50 mM phosphate buffer at pH 7.4 and eluted with the same buffer with the addition of 300 mM glucose. Caseinolytic activity was determined using 0.2% (w/v) α-casein as protein substrate. *Inset*: 14% non-reducing SDS-PAGE of the purified protein eluted from Con-A affinity column. Low molecular markers as controls (lane 1), affinity fraction with proteolytic and agglutinating activities eluted from Con-A column (lane 2), and zymogram with 0.1% (w/v) gelatin (lane 3). Each lane contains 2 μg of protein.

Surprisingly, the proteolytic enzyme isolated from *A. sativum* bulbs showed the same amino terminal sequence reported for ASA II, one of the lectins isolated from garlic bulbs [27]. Subsequently, we found hemagglutinating properties associated with the pure protein [32], so a plausible proposal was that both activities were associated with the same protein.

In the past few years, several plant proteases with multiple activities have been described as ‘‘moonlighting proteins’’ [33]. Phytolacain, a proteolytic enzyme isolated from *P. americana* roots exhibiting lectin-like activity and mitogenic properties was reported [7]. A protein with a dual behavior functioning as a lectin and as a trypsin inhibitor was isolated from *Peltophorum dubium* seeds [34]. Bioinformatics studies of the active site of legumain (family C13), a plant cysteine endopeptidase, revealed homology to three other families of cysteine proteases: clostripain from *Clostridium histolyticum* (family C11), caspases (family C14), and gingipains from *P. gingivalis* (family C25), leading to the grouping of these proteases into the cysteine protease clan CD [35]. They all have similar biochemical properties; however, little is known about the biological meaning of the dual function proteins. In this sense, gingipain R, a dimeric cysteine protease of microbial origin containing a peptidase catalytic

Table 6
Inhibitory effect of sugars on the hemagglutinating activity of sativain

Sugars	IC_{50} (mM)
D-Glucose	50.00
D-Sucrose	>200.00
D-Mannose	6.75
α-Methylglucopyranose	100.00
α-Methylmannoside	3.38

IC_{50} is the concentration required to inhibit hemagglutinating activity by 50%. The amount of sativain used in these assays was 5 μg.

domain and also a C-terminal hemagglutinin unit, has been proposed to act as virulence factors [36,37].

Up to date, the bulk of cysteine peptidases reported from plants belong to the papain family (family C1, clan CA), which showed an evolutive divergence [1]. Therefore, sativain, which presents a dimeric nature with proteolytic and hemagglutinating activities, is probably more related to the microbial cysteine peptidases from C25 family (clan CD) than the peptidases of plant origin.

Concerning the catalytic mechanism of proteolysis, gingipain contains a Cys25 residue, which provides the free thiol group for the active site; His159, which participates in general acid/base catalysis; G19, which forms the oxyanion hole and the Asn75, which orients the imidazolium ring of His159 in optimal positions for various steps in the catalytic mechanism [1,38]. The sequence of sativain also shows a Cys residue in position 29 and a His residue in position 74, possibly involved in the proteolytic mechanisms of action.

With regard to the role of lectins, several evidences suggested that lectins play a role in the defence mechanism against predators [39]. The toxicity of plant lectins toward higher animals has been well documented [17,37–39] and the possible health risk arising from their use in food and feed production has been assessed. In addition, the epithelial cells of insects are covered with membrane glycoproteins and highly glycosylated mucins, which are target sites for interactions with dietary plant lectins. Although most of the research on toxic lectins has been performed using PHA (*Phaseolus vulgaris* agglutinin), other lectins provoke similar effects [39].

Interestingly, two lectins (LI and LII) stripped from the surface of *Bacillus polymyxa* cells were found to possess proteolytic activity, which was associated with their hemagglutinating activity. The inhibition of hemagglutinating activity by glucuronic acid or fructose 1,6-diphosphate decreased the proteolytic activities of both lectins, whereas the blocking of this activity with D-glucosamine or D-galactosamine enhanced the proteolytic activity, suggesting that both lectins contain two active centers responsible for hemagglutinating and proteolytic activities [40]. These results open relevant aspects about the co-regulation of the two activities present in the same protein.

Summing up, the proteolytic activity associated with the mannose-binding lectin molecule might add a new dimension to our understanding of the functions of lectins and might elucidate an important role in plant defence responses.

4. Materials and methods

4.1. Materials

All chemicals used were of analytical reagent grade or were purchased from Sigma Chemical Co (St. Louis, MO, USA).

Cloves of garlic (*A. sativum* L. cv. Blanco Inco 283, virus-free stock) were cultured in the National Institute of Agricultural Technology (INTA La Consulta, Mendoza, Argentina) and supplied by Engineer José Luis Burba.

4.2. Enzyme preparation

Storage tissues from garlic cloves were homogenized in 50 mM potassium phosphate buffer (pH 7.5) with 5 mM EDTA, 10 mM β-ME, and 20 mM cysteine, filtered through cheese cloth and centrifuged at 20,000 × *g* for 30 min at 4 °C using a SS-34 Sorvall rotor. The supernatant was ultracentrifuged at 100,000 × *g* for 1 h in a Ti 50 rotor (4 °C), and the crude extract obtained was concentrated by ultra filtration using Diaflo Ultrafilters membranes YM10 (Amicon, Inc. MA, USA) under vacuum and was used for protease assays.

4.3. Enzyme purification

Crude extracts (40 mg protein content) were loaded onto a DEAE-Sepharose Fast Flow column previously equilibrated with 50 mM potassium phosphate buffer (pH 7.4) and 5 mM EDTA. After washing the column, the retained proteins were eluted with a linear saline gradient (0–0.5 M KCl) in the starting buffer. Fractions of 3 mL were collected at a flow rate of 1 mL min^{-1} and the major active peak was concentrated by ultra filtration and subjected to size-exclusion chromatography on Sephadex G-75 (fractions of 1.5 mL were collected at a flow rate of 20 mL h^{-1}). The active fraction (sativain) was then re-chromatographed under the same conditions.

4.4. Electrophoresis

4.4.1. SDS-PAGE

SDS-PAGE was performed in 4.5% and 14% gels according to Laemmli [41] under non-reducing and reducing conditions (in this case, the protein samples were reduced with β-ME and boiled for 3 min at 80 °C). Proteolytic activities were analyzed under non-reducing conditions (12% SDS-PAGE) by adding gelatin (0.1% w/v) to acrylamide solution prior to polymerization of the gel mixture [42]. After the electrophoretic run, the gels were washed with 5% Triton X-100 to eliminate SDS and incubated at 37 °C overnight in phosphate buffer (pH 6.0) to digest gelatin. The gels were stained with Coomassie Brilliant Blue R-250 and destained with methanol/acetic acid/distilled water (25/10/65, v/v/v). At low protein concentration, the gels were silver stained [38]. Bovine serum albumin (66 kDa), chicken egg ovoalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen from bovine pancreas (24 kDa), soybean trypsin inhibitor (20 kDa), α-lactalbumin (14.2 kDa), and

bovine lung aprotinin (6.5 kDa) were used as molecular mass standards (Low Molecular Weight Range Sigma Marker, Product N° M3913).

4.4.2. Isoelectric focusing and zymogram

Isoelectric point was performed in a Mini IEF Cell (Model 111, Bio-Rad Lab., Inc., Hercules, CA, USA). The deionized sample was loaded onto a 5% polyacrylamide gel containing ampholytes functioning over a broad pH range (Ampholine, pH 3.5–10, carrier ampholytes, Sigma Chemical Co, St. Louis, MO, USA), and the focusing of the pure enzyme was carried out according to the 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 and the other one was contacted with an agarose gel saturated in 0.5% casein solution for 30 min to develop the zymogram-IEF. Bands showing caseinolytic activity were detected after staining the agarose gel with Coomassie Brilliant Blue R-250.

4.4.3. Immunoblot analysis

The crude extract was subjected to SDS-PAGE and transferred onto a nitrocellulose sheet and soaked for 2 h with a solution containing 100 mM PBS and 1% (w/v) BSA. The membrane was washed four times with PBS containing 0.3% (v/v) Tween 20 (PBST) and then incubated for 2 h with mice anti-sativain polyclonal antibodies (1/2000, v/v). After four washes with PBST solution, the blot was allowed to react for 2 h with a goat anti-mice antibody (1/2000, v/v) and the bound antibodies were detected with an Amersham ECL™ Western Blotting kit (Amersham Biosciences, Uppsala, Sweden) according to procedures recommended by the manufacturer.

4.5. Enzymatic assays

Caseinolytic activity was measured with α-casein as protein substrate in the presence or absence of different modulators. In α-casein assay, 150 μL of enzyme preparation (100 μg mL^{-1} protein content) was added to 50 mM phosphate buffer (pH 6.0) containing 5 mM EDTA. The reaction was started by the addition of 500 μL of 0.2% (w/v) α-casein, and the reaction was stopped after 30 min of incubation at 40 °C with the addition of 1 mL of 10% (w/v) TCA. TCA-soluble products were determined in 1 mL of the supernatant by measuring the absorbance at 280 nm.

One unit of proteolytic activity is defined as the amount of enzyme required to cause a unit increase in absorbance per minute at 280 nm across a 1-cm path length, under the conditions of the assay (40 °C).

Proteolytic activities were also evaluated using denatured natural substrates such as haemoglobin [43], azocasein [44], and azocollagen [45]. When *p*-nitroanilides (BAPNA) were used as substrate, the reaction was followed directly using a spectrophotometer at 410 nm. An extinction coefficient of 8800 M^{-1} for the *p*-nitroaniline liberated was used for activity evaluation [24].

For the β-naphthylamide (CBZ-Ala-Arg-Arg-OMe-β-Nam and CBZ-Phe-Arg-OMe-β-Nam) assay, the enzyme (25 μL) was activated for 10 min with 25 μL of 50 mM MES buffer and incubated with 100 μL of the substrate (100 μM in 50 mM MES buffer). After 15 min incubation at 37 °C, the enzymatic reaction was stopped by the addition of Fast Garnet Base diazonium salt mixed with 20 mM $NaNO_2$ and 0.4% (w/v) Brij-35. The β-naphthylamine liberated was measured spectrophotometrically at 520 nm [25].

One unit of enzyme is defined as the amount of enzyme that releases 1 μmol of product per min under the reaction conditions.

4.6. Protein measurement

Protein concentrations were estimated according to the method described by Bradford [46] using bovine serum albumin as standard.

4.7. Effect of different compounds on proteolytic activity

Thiol-specific reagents such as β-ME and cysteine were used to study possible enzyme activation. The crude extract was incubated with the reducing reagent for 15 min and the enzymatic activity was evaluated using the α-casein method described above. To determine class and enzyme specificity, inhibition assays were performed using leupeptin, E-64, mercuric chloride, pepstatin, PMSF, and *o*-phenanthroline. The enzyme was incubated for 30 min at 30 °C with the inhibitors and the residual caseinolytic activity was measured as indicated previously [1,47]. A control without inhibitors was taken as 100%. The inhibitors were also tested using zymogram on 12% polyacrylamide gel copolymerized with 0.1% gelatin.

4.8. Effects of temperature and pH on enzyme activity and stability

Effect of pH on the activity of the purified enzyme was measured on 0.2% (w/v) α-casein and haemoglobin solutions within the pH range from 4.0 to 10.0 using 50 mM sodium acetate (pH 4.0–5.5), 50 mM sodium phosphate (pH 6.0–7.5), 50 mM Tris–HCl (pH 8.0–9.0), and 50 mM sodium carbonate (pH 10.0). Sativain was equilibrated for 10 min in 100 μL of the buffer at a given pH, added to the substrate solutions at the same pH, and assayed as described in Section 4.5. Haemoglobin was used as substrate for activity measurements at low pH.

For pH stability, the enzyme was evaluated by incubation with the optimum pH buffers for 60 min at 37 °C. Residual activities were assayed at the end of the incubation period.

Similarly, the effect of temperature on enzyme activity was studied using α-casein as substrate. The enzyme was activated at 25–70 °C for 20 min at pH 6.0 and then an aliquot was used for the activity estimation at the same temperature. Before the assays were performed, substrate solutions were equilibrated at the respective temperatures and at same pH. The samples

and a blank without enzyme were placed in an ice bath until the caseinolytic activity was determined.

Thermal stability of sativain was tested by incubating the enzymatic preparation in 50 mM potassium phosphate buffer pH 6.0 at different temperatures (25–70 °C) for 20 min. Enzyme aliquots were withdrawn at the end of specified time intervals and assayed for activity at 37 °C.

Self-digestion of the activated enzyme was followed by incubation of the sample at room temperature for 24 h.

4.9. Kinetic studies

The effect of substrate concentration on the reaction rate of enzyme hydrolysis at pH 6.0 (37 °C) was studied using both natural and synthetic amidolytic substrates. For denatured natural substrates, 10 μg of the enzyme and substrate concentrations (α-casein) over the range of 10–200 μM were used. Similarly, for synthetic substrate, 5 μg of enzyme was used in the assay, with a concentration ranging from 10 to 500 μM CBZ-Ala-Arg-Arg-OMe-β-naphthylamide as described above. Blank determinations were carried out simultaneously at the specific substrate concentrations without the enzyme. In each case, Lineweaver–Burk plots were constructed and Michaelis–Menten constants (K_m and V_{max}) were calculated.

Enzyme concentration was determined by performing active-site titration with E-64 as described by Barrett and Kirschke [26]. In order to express the V_{max} values as k_{cat}, a molecular weight of 26 kDa was used. The value of the second-order rate constant, k_2, for the reaction of the enzyme with this inhibitor was calculated as $k_{app}/(I)$, where k_{app} is the apparent first-order rate constant for the exponential decline of enzyme activity in mixtures of enzyme, substrate, and inhibitor.

4.10. Protein sequencing

Monomeric and dimeric (native) forms of the protein were sequenced. In the first procedure and after separation by 14% SDS-PAGE, the monomeric protein was electroblotted onto polyvinylidene difluoride membrane according to Matsudaira [48] in a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad Lab., Inc., Hercules, CA, USA). The analysis of the N-terminus of the transferred protein was carried out on an Applied Biosystems Procise 494 (Nebraska Health Institute, Nebraska, USA). The second analysis of the dimeric protein in solution was performed on an Applied Biosystems 477a (LANAIS PRO, Universidad de Buenos Aires, Buenos Aires, Argentina). Both sequences were subjected to automatic alignments using the NCBI-Blast search system.

4.11. Affinity chromatography on Con-A Sepharose

Affinity chromatography of the pure protein was performed on a Con-A column and eluted with 50 mM phosphate buffer (pH 7.4) with the addition of 1 mM $MgCl_2$, 1 mM $CaCl_2$, 150 mM NaCl, and 300 mM glucose. Fractions (1 mL per tube) were collected at a flow rate of 20 mL h^{-1} and caseinolytic activity was determined using 0.2% (w/v) α-casein as previously described.

4.12. Hemagglutinating assay

The determination of hemagglutinating activity was performed using trypsin-treated rabbit erythrocytes in a U-bottomed 96-well microtiter plates in a final volume of 50 μL containing 25 μL of a 2% (w/v) suspension of rabbit erythrocytes previously trypsinized [49]. Agglutination was monitored visually after 1 h at room temperature. To test the inhibitory action of different sugars on hemagglutinating reaction, 25 μL samples of serial twofold dilutions of the protein in PBS were mixed with the same volume of different sugars (glucose, mannose, glucopyranosides, and α-methylmannosides) in the same buffer in each well and left for 30 min at room temperature. The same volume of rabbit erythrocyte solution was added and the extent of hemagglutination was examined after 1 h of incubation at room temperature.

Acknowledgments

This study is a part of a PhD degree thesis of Mónica G. Parisi, carried out at the Universidad Nacional de Luján. We acknowledge the financial support of the Departamento de Ciencias Básicas, Universidad Nacional de Luján. Silvia Moreno is a Career Investigator of the CONICET, Buenos Aires, Argentina.

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