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RESEARCH PAPER

A comparative study of the expression of serine proteinases in quiescent seeds and in developing *Canavalia ensiformis* plants

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Abstract

An alkaline proteinase activity is present in quiescent seeds and up to the 24th day of development of *Canavalia ensiformis* DC (L.) plants. By a simple protocol consisting of cation exchange chromatography, followed by an anion exchange column, a serine proteinase (Q-SP) was purified to homogeneity from quiescent seeds. Q-SP consists of a 33 kDa chain with an optimum pH between 8.0 and 9.0. Arginine residues at P1 and P2 subsites favour binding to the substrate, as shown by the K_M assay with *N*-α-benzoyl-DL-arginine-4-nitroanilide-hydrochloride and *N*-benzoylcarboxyl-L-arginyl-L-arginine-7-amido-4-methylcoumarin. The same protocol was used for partial purification of benzamidine-sensitive enzymes from the developing plant. On the 7th day, a new benzamidine-sensitive enzyme is synthesized in the seedling, seen as the second active peak appearing in anion exchange chromatography. A benzamidine-sensitive enzyme purified from cotyledons presented a similar gel filtration profile as Q-SP, although it was eluted at different salt concentrations in the anion exchange chromatography. None of the enzymes was inhibited by PMSF, APMSF, or SBTI, but they were inactivated by benzamidine, TLCK, and leupeptin. Q-SP did not cleave *in vitro C. ensiformis* urease, concanavalin A, or its main storage protein, canavalin. In conclusion, a ubiquitous benzamidine-sensitive proteolytic activity was found in *C. ensiformis* from quiescent seeds up to 24 d of growth, which apparently is not involved in the hydrolysis of storage proteins and might participate in an as yet unidentified limited proteolysis event.

Key words: Benzamidine, *Canavalia ensiformis*, characterization, cotyledons, germination, proteinase, purification, roots, seeds, serine proteinase.

Introduction

Germination and growth are important phases of plant development. Massive consumption of endogenous proteins, carbohydrates, and other sources of energy occurs at these stages. Protein degradation is also important as a source of reduced nitrogen for further steps of development (Gruis *et al*., 2004). This phase of endogenous

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Abbreviations: 5D, plant material processed at the 5th day of imbibition; 7D, plant material processed at the 7th day of imbibition; APMSF, 4-amidinophenyl-methane-sulphonyl fluoride; BIS-TRIS, 2-[*bis*-(2-hydroxyethyl)-amino]-2-(hydroxymethyl)-propane-1,3-diol; BSE, benzamidine-sensitive enzyme; buffer A (assay buffer), 50 mM TRIS-HCl at pH 8.0; buffer E (extraction buffer), 20 mM Na-phosphate buffer at pH 6.0; C, cotyledons; CE, crude extract; CM, fraction derived from CM Sepharose™ fast flow chromatography; Con A, concanavalin-A; DL-BApNA, *N*-α-benzoyl-DL-arginine-4-nitroanilide-hydrochloride; DMF, *N,N*-dimethylformamide; DMSO, dimethylsulphoxide; DTT, dithiothreitol; E-64, L-*trans*-epoxysuccinyl-leucylamido-butane; EDTA, ethylene-diaminetetraacetic acid; HQ, fraction derived from HiTrap™ HP chromatography; L, leaf; NR, non-retained fraction from a chromatographic step; PMSF, phenylmethylsulphonyl fluoride; Q, quiescent seed; Q-SP, serine proteinase from quiescent seeds; R, root; SBTI, trypsin inhibitor from soybean; TLCK, *N*-α-tosyl-L-lysine chloromethyl ketone; Z-Arg-Arg-AMC, *N*-benzoylcarboxyl-L-arginyl-L-arginine-7-amido-4-methylcoumarin.

protein degradation has been extensively studied using several approaches, including immunohistochemistry (Famiani *et al*., 2000) and proteomics (Sheoran *et al*., 2005).

Protein mobilization during germination was reviewed in detail (Muntz *et al*., 2001). A number of reports on the role of cysteine proteinases (Tiedemann *et al*., 2001; Grudkowska and Zagdanska, 2004) and aspartic proteinases (Simões and Faro, 2004) in the development of seeds has been published. Although serine proteinases (EC 3.4.21) (Barret *et al*., 1998) are not as common in plants as cysteine proteases (Muntz *et al*., 2001) a number of them have been purified in the past 30 years, as recently reviewed by Antao and Malcata (2005). They are finely controlled by serpins which are abundant in plants (Fontes *et al*., 1997; Gettins, 2002). Most plant serine proteinases purified to date are members of the subtilisin-like subfamily of endopeptidases (Siezen and Leunissen, 1997). Cucumisin, a subtilisin-like enzyme purified from the melon *Cucumis melo* fruit by Uchikoba *et al*. (1995) remains the best characterized plant serine proteinase to date. Several cucumisin-like enzymes were isolated from other Curcubitaceae and from rice, maize, wheat, and barley (Antao and Malcata, 2005). Most of these enzymes show M_r values in the range 60–70 kDa and exhibit broad specificity, preferring hydrophobic amino acid residues in the P1 position (Arima *et al*., 2000). Mature cucumisin (54 kDa) shows optimum pH in the range 8–10 and is stable at 60 °C and over a broad pH range (4–11). Cucumisin is not affected by trypsin inhibitor from soybean (SBTI), ovomucoid, cysteine proteinase inhibitors, or ethylenediaminetetraacetic acid (EDTA), but is strongly inhibited by chloromethyl ketone derivatives of peptide substrates, phenylmethylsulphonyl fluoride (PMSF), and diisopropyl fluorophosphate (Kaneda and Tominaga, 1975; Uchikoba *et al*., 1995).

Canavalia ensiformis (jack bean) is the source of some proteins that have been known and studied for a very long time. They include urease, the first ever enzyme to be crystallized (Sumner, 1926), as well as the lectin concanavalin A (Con A) (Sumner and Howell, 1936). Jack bean seeds contain a potent insecticidal protein called canatoxin (Carlini and Guimarães, 1981), which is a urease isoform (Follmer *et al*., 2001; Carlini and Grossi-de-Sá, 2002). Canatoxin must be proteolytically activated to produce its entomotoxic effect, and the enzymes responsible for its activation were identified as cathepsins from the insect digestive system (Ferreira-DaSilva *et al*., 2000).

Canavalia ensiformis seeds are rich in acidic enzymes, which are probably involved in endogenous protein degradation during germination and growth. Oshikawa *et al*. (2000) reported isolation of a trypsin-like peptidase from quiescent seeds of *C. ensiformis*. In this study, while attempting to isolate a cysteine proteinase activity that would hydrolyse canatoxin in the seed, a strong activity was found in alkaline pH, which is present in quiescent seeds and in different tissues of the developing plant. It was decided to purify this enzyme (or possibly different enzymes) in order to provide material for comparative studies.

Materials and methods

Materials

Jack bean (*Canavalia ensiformis*) seeds were purchased from Casa Naterra & Produtores Associados, Wolf Seeds do Brasil, Ribeirão Preto, SP, Brazil. The seeds used for all experiments came from the same lot. Jack bean urease, Con A, and canavalin were all purified from *C. ensiformis* seeds in the authors' laboratory. CM-Sepharose™ fast flow, HiTrap™ Q HP, Sephadex™ G-50, Superdex™ 75 HR 10/30 were from GE Healthcare, Amersham Biosciences. YM-10 membranes with 10 kDa cut-off Centriprep™ were purchased from Amicon. Dialysis membranes were obtained from SpectraPor™. *N*-α-Benzoyl-DL-arginine-4-nitroanilide-hydrochloride (DL-BApNA), *N*-benzoylcarboxyl-L-arginyl-L-arginine-7-amido-4-methyl-coumarin (Z-Arg-Arg-AMC), L-*trans*-epoxysuccinyl-leucylamido-butane (E-64), benzamidine, dithiothreitol (DTT), dimethylsulphoxide (DMSO), dimethylformamide (DMF), SBTI, bovine serum albumin (BSA), PMSF, 1,10-orthophenanthroline, leupeptin, EDTA, bovine trypsin, albumin, and haemoglobin were obtained from Sigma (St Louis, MO, USA). *N*-α-Tosyl-L-lysine chloromethyl ketone (TLCK) and 4-amidino-phenyl-methane-sulphonyl fluoride (APMSF) were kindly provided by Dr Russolina Zingali, Universidade Federal do Rio de Janeiro. All other chemicals were obtained from commercial sources and were of the best quality available.

Plant material, germination, and growth conditions

Germination starts with water uptake by the seed and finishes when the radicle breaks through the seed coat (Bewley and Black, 1986). Jack bean seeds usually swell within 2 h in the presence of water, and germination is complete by the 2nd day after imbibition, according to the previous definition of germination. To avoid confusion between germination of the seeds and seedling growth, it was decided to indicate days after water imbibition.

Jack bean seeds were disinfected in solutions of 1% sodium hypochlorite and 70% ethanol, for 1 min in each, washed, and treated with distilled water at 45 °C for 30 s. They were allowed to germinate on moist filter paper and watered with distilled water once a day. On the 5th day after imbibition the radicle is about 1.0–1.5 cm long. Seeds on the 5th day (5D) and 7th day (7D) after imbibition were removed, frozen in liquid nitrogen, freeze-dried, fine-ground, and stored at – 80 °C. The remaining seeds were transferred to pots containing sand, watered once a day, and allowed to grow for 24 d, when the plant is about 30 cm tall and shows fully expanded primary leaves. On the 24th day, leaves (L), cotyledons (C), and roots (R) were separated, washed with distilled water, frozen in liquid nitrogen, and processed as 5D and 7D materials. Epicotyls and hypocotyls were discarded. Quiescent seeds (Q) were only disinfected prior to processing. All plant materials (other than quiescent seeds) were maintained throughout the experimental period (24 d) at 28±5 °C and 60±15% relative humidity with an 18 h light/6 h dark cycle.

Crude extract

Purification steps and handling of protein fractions were conducted at 4 °C. Crude extracts were prepared as follows: frozen freeze-dried and fine-ground plant materials were mixed with 20 mM Na-phosphate buffer at pH 6.0 (buffer E), in different weight/volume

proportions. The mixture was stirred for 2 h, centrifuged at 12 000 *g* for 1 h, and then the supernatants were exhaustively dialysed against buffer E, using a 12 kDa cut-off membrane. Dialysed materials were centrifuged (12 000 *g* for 20 min) and the supernatants were designated as crude extracts.

Protein determination

The RC DC™ protein assay kit (Bio-Rad, Co.), which is based on Lowry protocol (Lowry *et al*., 1951), was used to measure protein concentration, using bovine serum albumin as standard.

Chromatography

Unless otherwise specified, all chromatographic procedures were performed in an Äkta Purifier apparatus (GE Healthcare, Amersham Biosciences) at 4 °C. Protein was monitored at 280 nm.

Cation exchange chromatography

For cation exchange chromatography (CM chromatography), 60 ml of CM Sepharose™ fast-flow resin was packed into a 40 cm× 2.5 cm column and equilibrated with buffer E, at a flow rate of 0.75 ml min^{-1}. The column was washed with buffer E to remove non-retained proteins and then eluted with 120 ml of buffer E containing 1 M NaCl. Non-retained and eluted materials were collected in 15 ml fractions. Enzymatically active fractions were pooled and dialysed against 50 mM TRIS-HCl at pH 8.0 (buffer A), in a 12 kDa cut-off membrane.

Anion exchange chromatography

Anion exchange chromatography (HQ chromatography) was performed using two connected HiTrap™ Q HP columns, 5 ml each, equilibrated with buffer A, at a flow rate of 0.5 ml min^{-1}. Samples previously dialysed against buffer A and concentrated in YM-10 (Millipore) membranes, were loaded onto the columns. Non-bound proteins were collected in 5 ml fractions and, after washing the column, elution was performed with a NaCl linear gradient (0–1 M in 100 ml of buffer A). Active fractions (1 ml) were pooled and dialysed against buffer A, in a 12 kDa cut-off membrane.

Removal of Con A by affinity chromatography

To remove Con A from the sample, batch adsorption was performed using 20 ml of Sephadex™ G-50, equilibrated in buffer A containing 500 mM NaCl, at 4 °C. The sample was mixed with the resin, and submitted to 2 h shaking at 4 °C. The mixture was packed into a column and the non-retained, Con A-free, enzymatically active fraction was collected.

Gel filtration chromatography

Gel filtration was performed using a Superdex™ 75 HR 10/30 column, equilibrated in buffer A containing 150 mM NaCl, at 0.5 ml min^{-1}. Samples of 200 µl (amount of protein described in the figures), previously concentrated in 10 kDa cut-off Centriprep™, were applied to the column. Protein peaks, monitored at 280 nm, were collected individually and assayed for enzymatic activity. For molecular mass determination, the column was previously calibrated with protein markers from a gel filtration LMW Calibration Kit (GE Healthcare, Amersham Biosciences).

Electrophoresis

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed using NuPAGE™ Novex 4–12% 2-[*bis*-(2-hydroxyethyl)-amino]-2-(hydroxymethyl)-propane-1,3-diol (BIS-TRIS) gel (Invitrogen), under denaturating conditions. Samples were dissolved in NuPage™ LDS 4X sample buffer (Invitrogen). Gels were stained with Simply Blue™ Safe Stain (Invitrogen). Aliquots of 20 µl (about 50 µg protein) were denaturated by boiling at 100 °C for 5 min prior to loading. Molecular weight markers were from LMW-SDS Marker Kit™ (GE Healthcare, Amersham Biosciences) or Bench Mark Pre-stained Protein Ladder™ (Invitrogen).

Enzymatic assays

Two substrates were used to assay crude extracts and purification fractions. The reactions started with addition of substrate. The fluorogenic substrate Z-Arg-Arg-AMC (Sentandreu *et al*., 2003) was used for samples derived from quiescent seeds. Stock solutions were prepared at 100 µM in 1% DMSO. The hydrolysis of Z-Arg-Arg-AMC at the final concentration of 10 µM, in buffer A, or 100 mM Na-acetate pH 5.6, was measured at 37 °C by the increase of emitted fluorescence at 470 nm after excitation at 360 nm. The total reaction and sample volumes used were 100 µl and 50 µl, respectively. Fluorometric assay was performed in a *f* max™ microplate reader (Molecular Devices) for 10 min, with readings every 20 s. Fractions from all other plant materials were assayed using the chromogenic substrate DL-BApNA. In these assays, 100 µl of samples was incubated with DL-BApNA (0.625 mM final concentration, from a 5 mM stock solution in 100% DMSO) to make 200 µl final volume, using the same buffer, time, and temperatures as for the Z-Arg-Arg-AMC assay. The *p*-nitroaniline released was measured at 405 nm, using a SpectraMax™ microplate reader (Molecular Devices), with readings every 20 s. One unit of enzyme activity using Z-Arg-Arg-AMC was defined as the amount of enzyme able to perform a variation of 1000 AFU min^{-1} (AFU=arbitrary fluorescence units), under the conditions described. One unit of enzyme activity using DL-BApNA was defined as the amount of enzyme able to perform a variation of 0.1 A_{405} nm min^{-1} in the assay conditions.

Inhibition of enzymatic activities

Aliquots of samples, either 50 µl or 10 µl, were assayed using DL-BApNA (0.625 mM final concentration) or Z-Arg-Arg-AMC (10 µM final concentration) in final volumes of 200 µl and 100 µl of buffer A, respectively. Reactions were performed at 37 °C for 15 min. Samples and inhibitors were pre-incubated for 20 min, 3 h, or 24 h with samples at room temperature (22 °C) or for 3 h and 24 h at 4 °C before the reaction started. The inhibitors tested and their stock solutions were: PMSF (50 mM in 100% DMSO), APMSF (10 mM in DMF), TLCK (1 mM in water), benzamidine (500 mM in water), SBTI (5 mg ml^{-1}, in 100 mM TRIS-HCl at pH 8.0), leupeptin (5 mM in water), zinc chloride (10 mM in water), 1,10-orthophenanthroline (10 mM in 100% ethanol), EDTA (250 mM in water), and E-64 (1 mM in water). The results are represented as a percentage of residual activity compared with controls, which had no inhibitor added. Bovine trypsin (0.01 µg) assayed in the same conditions was used as the positive control.

Thermal stability, optimal temperature, and pH assays

Enzymatic activity upon DL-BApNA or CBZ-Arg-Arg-AMC was tested in 100 mM Na-acetate (pH 5.0), 100 mM BIS-TRIS (pH 6.0), and 100 mM TRIS-HCl (pH 7.0, 8.0, and 9.0). After optimum pH determination, the thermal stability and optimum temperature assay were performed at that pH. In this case, sample and substrate were incubated separately for 10 min at the temperature tested, and the reaction started with addition of the substrate into the plate well was followed for 10 min. There was no previous incubation of the samples for determination of optimum pH.

Michaelis–Menten constant (K_M) determination

Determination of K_M was done using Z-Arg-Arg-AMC at final concentrations of 0.1, 0.25, 0.5, 0.75, 1.5, 10, and 20 μM. When DL-BApNA was used for K_M determination, the concentrations were 0.01, 0.02, 0.05, 0.10, 0.15, 0.20, 0.30, 0.50, 0.60, 1.0, and 2.0 mM. Reactions of substrate and enzyme (about 1.5 μg) in buffer A were performed for 5 min at 37 °C, being within the linear phase for all points tested. Data were processed using Grafit Version 3.0 from Erithacus Software.

Hydrolysis of protein substrates

Bovine serum albumin, bovine haemoglobin, *C. ensiformis* urease, Con A, and canavalin were tested for their susceptibility to hydrolysis by the purified serine proteinase isolated from dry seeds. The protein substrates (50 μg each) were incubated with the enzyme (Q-SP, 5 μg) in 80 μl of buffer A at 37 °C, for 16 h. The reaction was stopped by adding 20 μl of SDS-PAGE sample buffer 5X, and boiling for 2 min. Aliquots of 25 μl of reaction mixtures were loaded onto a 12% SDS-PAGE. The gel was stained after completion of the separation with Simply Blue™ Safe Stain (Invitrogen).

Auto-proteolysis assay

The auto-proteolysis analysis was performed taking 50 μg of freshly purified protein, and incubating it for 18 h at 37 °C in buffer A, with a final volume of 1 ml. Upon completion of incubation the sample was freeze-dried and suspended in 50 μl of water. About 10 μl was used for mass-spectrometry analysis and the remaining material was analysed by SDS-PAGE (4–12%). A control was analysed in the same way, but without pre-incubation of the protein sample. The same assays were performed with a 1-month-old purified protein sample.

Mass spectrometry analysis

The mass spectrometry analysis was performed in an Agilent 1100 series LC/MSD with a UV detector (280 nm) and electrospray quadrupole mass spectrometer. Previous to MS-analysis, protein solutions were desalted in a Zorbax 300 reverse-phase column. Afterwards, mass spectral deconvolution was performed according to the manufacturer's protocols.

Results

Serine proteinase from quiescent seeds

An enzyme with the characteristics of a serine proteinase, designated as Q-SP, was purified to homogeneity from quiescent jack bean seeds. For that purpose, 40 g of seed meal (about 30 seeds) was extracted in 300 ml of buffer E to yield 210 ml of crude extract (CE) of quiescent seeds (Q-CE), containing 6.66 mg protein ml^{-1}. Since cysteine proteinases were expected to be present (Sentandreu *et al.*, 2003), Q-CE was assayed for enzymatic activity upon Z-Arg-Arg-AMC (10 μM) at pH 5.6 in the presence of either DTT or E-64. Although Q-CE was able to partially hydrolyse the substrate at pH 5.6, no activation by DTT or inhibition by E-64 were observed (data not shown). On the other hand, total hydrolysis of the substrate occurred within 10 min at pH 8.0, suggesting the presence of enzyme(s) with a more alkaline optimum pH. It was decided to purify this protein (or proteins) and, from that point on, all the enzymatic assays were performed at pH 8.0.

Q-CE proteins (193 mg) were separated by cation exchange chromatography at pH 6.0 and the activity upon Z-Arg-Arg-AMC was recovered in the non-retained fraction (Q-NRCM; 70.3 mg). Q-NRCM (23.7 mg) was then applied into a HiTrap™ Q-HP column. A single active peak (Q-HQ) was eluted within a narrow region of the salt gradient, between 300 mM and 350 mM NaCl (Fig. 1A).

The last purification step consisted of affinity chromatography in Sephadex™ G-50 to remove any remaining contaminant Con A (Carlini and Guimarães, 1981). Q-HQ (1.83 mg) was mixed with the resin and the active material was recovered in the pass-through fraction (1.48 mg protein) consisting of a single band in SDS-PAGE, under either non-reducing (Fig. 1A inset, Q-SP lane) or reducing conditions (Fig. 1B insets). Inset A in Fig. 1B shows an SDS-PAGE (4–12%), under the reducing condition of all active fractions throughout the purification steps. The molecular mass of the purified enzyme (Q-SP) was determined as 28 kDa by SDS-PAGE and 33 kDa by mass-spectrometry (not shown). As seen in Fig. 1B, gel-filtration of Q-HQ gave two peaks that were active upon Z-Arg-Arg-AMC, interpreted as the enzyme's monomer (23 kDa, peak 3), and a more active oligomer (88 kDa, peak 1). The oligomeric form was not seen in the SDS-PAGE, even when the silver staining procedure was used (data not shown), suggesting it is formed by non-covalent linkage of the monomeric species. Table 1 shows the purification index and the yield of Q-SP.

Q-SP was stable for 3 months at 4 °C when stored in buffer A in the presence of 0.02% w/v sodium azide. After the third month, the enzyme loses activity rapidly. Other studies have shown that Q-SP is not capable of autolysis (Fig 1B, inset B). Mass spectrometry analysis confirmed both the purity and stability of the enzyme as a single molecular species of 33 kDa (not shown), found either in freshly purified protein without incubation or incubated for 18 h at 37 °C (Fig. 1B, inset B).

Inhibitors were tested with the aim of determining the catalytic mechanism of Q-SP. Two pre-incubation conditions of 3 h and 24 h, both at 4 °C, were used. Bovine trypsin was tested in parallel. The results summarized in Table 2 show that benzamidine, leupeptin, and TLCK blocked significantly the activity of Q-SP, allowing it to be classified as a serine proteinase. TLCK, a lysine analogue of the transition state, was effective in low concentrations. Benzamidine, an arginine analogue, inhibited about 65% of the enzymatic activity at 10 mM concentration and almost complete inhibition was achieved at 50 mM, independent of incubation time (Table 2). In spite of this inhibition, several attempts to purify the enzyme using benzamidine–agarose chromatography failed, probably due to the low pH used for the elution step. PMSF and APMSF, a hydrophilic derivative of PMSF, had

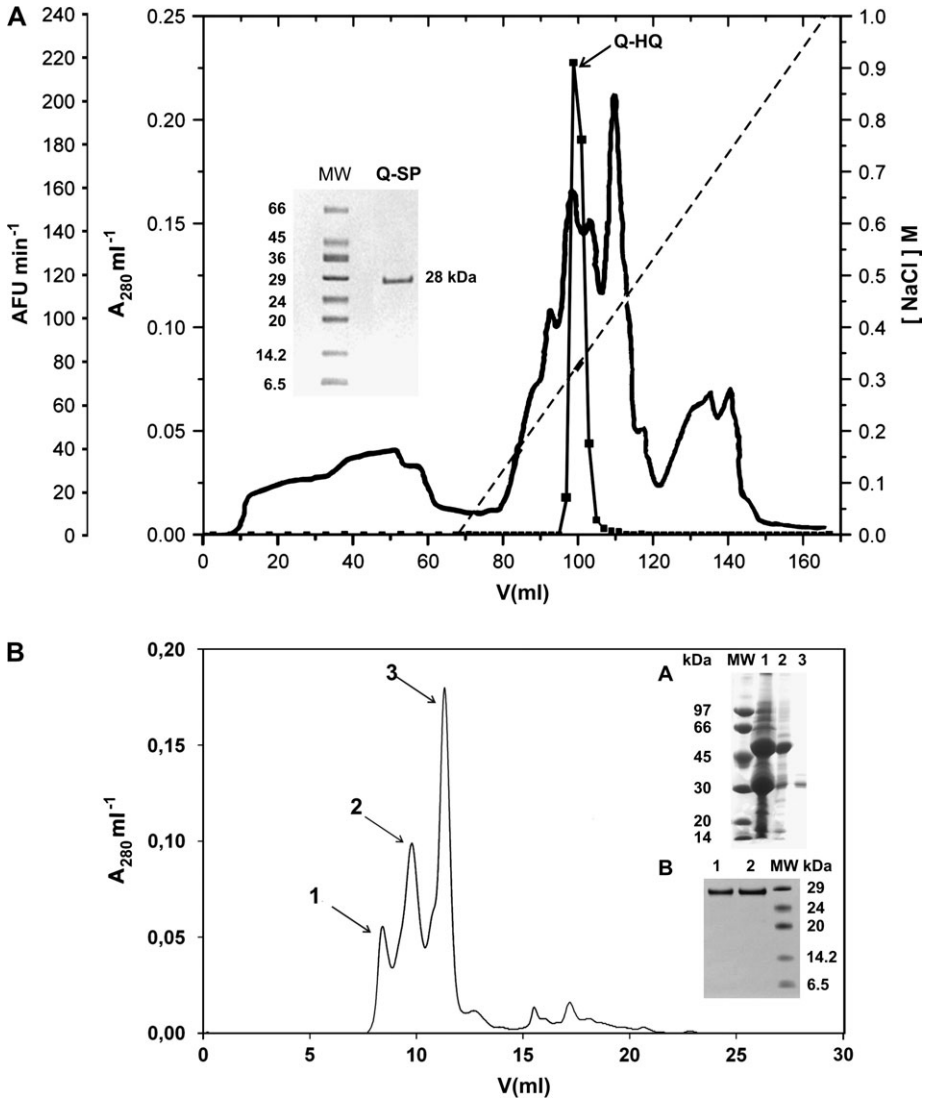


Fig. 1. Purification of a serine proteinase from quiescent seeds. (A) HQ chromatography of partially purified enzyme (Q-NRCM) from quiescent seeds. Activity of fractions (closed squares) upon Z-Arg-Arg-AMC at pH 8.0 is expressed in arbitrary fluorescence units per minute (AFU min^{-1}). Pooled active fractions were designated Q-HQ. Continuous line, absorbance at 280 nm; dashed line, NaCl gradient. Inset: a non-reducing SDS-PAGE of Q-HQ (10 μg) in which MW markers were run in parallel. (B) Gel filtration of Q-HQ. The sample (183 μg protein) was gel-filtered in a Superdex TM 75 HR 10/30 at a flow rate of 0.5 ml min^{-1}. Protein peaks were assayed with Z-Arg-Arg-AMC. Peaks 1 and 3 were enzymatically active, corresponding to 82 kDa and 23 kDa, respectively. Peak 2 was not active. Inset A: a non-reducing SDS-PAGE (4–12%) of purification fractions. Molecular mass markers (MW) are indicated. Lane 1, crude extract of quiescent seeds (Q-CE); lane 2, non-retained fraction of CM chromatography (Q-NRCM); lane 3, active peak eluted from HQ chromatography (Q-HQ). About 50 μg of protein was applied in lanes 1 and 2. In lane 3, 20 μg was used. Inset B: SDS-PAGE (4–12%) of Q-SP and auto-proteolysis assay. Lane 1, freshly purified Q-SP (20 μg); lane 2, freshly purified Q-SP (20 μg) incubated at pH 8.0 for 18 h at 37 °C; Lane 3, molecular mass markers. The gels were stained with Simply Blue™ Safe Stain kit.

a very weak effect on Q-SP, even at high concentrations, contrasting with the rapid and complete inhibition of bovine trypsin under the same conditions (Table 2). No inhibitory effects were seen for DTT and E-64 (not shown), indicating the absence of relevant cysteine residues. SBTI did not inhibit Q-SP, although bovine trypsin was inhibited effectively at 0.1 mg ml^{-1}. Typical metalloenzyme inhibitors, EDTA and 1,10-orthophenanthroline, were also tested. Inhibition by EDTA (about 65–70%) was observed only for bovine trypsin after 24 h pre-incubation (Table 2). On the other hand, higher concentrations of 1,10-orthophenanthroline only inhibited Q-SP at about 40% after 24 h of pre-incubation.

The optimum pH range of Q-SP was 7.0–8.0, which is typical for most trypsin-like serine proteinases. No activity was detected above pH 9.0 or below pH 5.0 (data not shown). The K_M determination (Fig. 2) confirmed the high affinity of Q-SP for arginine, as suggested by the inhibition by benzamidine (Table 2). A high affinity for lysine analogues is expected from the TLCK inhibition.

The K_M of the enzyme is 0.98 μM for the Z-Arg-Arg-AMC substrate (Fig. 2A) and 53 μM for DL-BApNA (Fig. 2B). Since the DL-BApNA is a racemic mixture, which contains about 50% of L-BApNA and D-BApNA, the K_M value was estimated as 27 μM for L-BApNA. The difference in K_M observed for the two substrates suggested that the presence of an Arg residue at the P2 subsite is important for the binding of the enzyme to its substrate.

The ability of Q-SP to hydrolyse protein substrates was tested using bovine serum albumin, haemoglobin, and proteins endogenous to *C. ensiformis* seeds such as urease, Con A, and canavalin, the major seed storage protein (Sammour *et al*., 1984). As shown in Fig. 3, at a ratio of 1:10 enzyme:substrate Q-SP did not cleave any of these proteins.

Table 1. *Purification table of Q-SP*

The enzymatic activity of the samples was assayed upon 10 μM Z-Arg-Arg-AMC at 37 °C for 10 min, in 100 mM TRIS-HCl at pH 8.0. Q-CE, Quiescent crude extract; Q-NRCM, non-retained fraction from CM-chromatography performed with Q-CE; Q-HQ, active pool from HQ-chromatography, performed with Q-NRCM; Q-SP, pure enzyme eluted from S-50 chromatography preformed with Q-HQ. One unit of enzyme activity was defined as the amount of enzyme able to perform a variation of 1000 AFU min^{-1} (AFU=arbitrary fluorescence units), in the conditions described.

Sample	Protein content (mg)	Specific activity (U mg^{-1})	Purification factor	Total activity (units)	Yield (%)
Q-CE	193	626	1.0	121026	100
Q-NRCM	70.3	2456	3.9	62720	51
Q-HQ	23.7	5808	9.3	10664	9
Q-SP	1.48	14054	22.3	20800	17

Benzamidine-sensitive enzymes (BSE) from 5D and 7D plant material

Crude extracts of 5D and 7D material (5D-CE and 7D-CE, respectively) showed high levels of enzymatic activity upon DL-BApNA at pH 8.0. The same protocol used for Q-SP purification was applied to isolate BSEs from growing seedlings. After CM chromatography, activity was recovered in the non-retained fractions of both extracts: 5D-NRCM from 5D-CE; 7D-NRCM from 7D-CE. HQ chromatography of 5D-NRCM and 7D-NRCM fractions produced different peak patterns, as shown in Fig. 4A, B. In both cases, a narrow active peak eluting around 300 mM NaCl (5D-HQ and 7D-HQ_1) was observed. Moreover, the 7D material showed a second activity peak (7D-HQ_2) eluting around 450 mM, suggesting *de novo* synthesis of a new enzyme at this time in the developmental process. The appearance of this second active peak in the ion exchange chromatography was not seen for plant materials processed before the 7th day of seedling growth.

The proteolytic activities of 5D-HQ and 7D-HQ_1 were inhibited by 85% with 1 mM benzamidine for 20 min, whereas 10 mM benzamidine abolished their activities completely. E-64, DTT, PMSF, and SBTI did not exhibit

Table 2. *Screening of inhibitors performed with Q-SP*

The assay was performed in 100 mM TRIS-HCl at pH 8.0. In 80 μl buffered volume, 50 μl of Q-SP (7 μg) was pre-incubated with the tested inhibitors at the indicated final concentrations, either for 3 h or for 24 h at 4 °C. The reaction started with addition of 20 μl of Z-Arg-Arg-MCA (10 μM final concentration) and followed at 37 °C for 10 min. The results (means ±standard deviation; n=5 experiments) are expressed as a percentage of residual activity as compared with controls, with no addition of inhibitor. Bovine trypsin (0.01 μg) was used as the positive control.

Inhibitor tested	Concentration	Residual activity			
		Bovine trypsin (pre-incubation time)		Q-SP (pre-incubation time)	
		3 h	24 h	3 h	24 h
$ZnCl_2$	1 mM	87.3±5.1	100.4±2.0	103.1±1.4	85.9±2.9
	10 mM	73.6±3.3	93.0±4.3	62.1±1.5	59.4±0.4
1,10-orthophenanthroline	5 mM	86.1±1.7	102.8±1.2	75.2±3.1	67.0±2.9
	10 mM	103.3±3.2	103.5±4.9	56.8±0.8	48.0±0.6
EDTA	2 mM	78.2±3.3	33.8±5.3	87.3±5.1	85.6±8.1
	20 mM	73.4±2.8	43.0±1.5	93.2±4.6	88.8±4.3
Leupeptin	10 μM	18.0±2.1	75.9±4.3	13.4±1.2	9.5±0.2
	100 μM	3.6±0.9	14.9±6.9	5.5±1.3	5.7±0.1
Benzamidine	10 mM	1.3±0.2	14.3±6.1	36.3±0.6	38.1±0.8
	50 mM	0±0	3.2±1.8	8.7±1.3	9.6±0.3
SBTI	0.1 mg ml^{-1}	4.1±1.9	0.7±0.3	118.1±1.7	104.4±3.1
PMSF	1 mM	13.8±3.5	4.5±2.2	91.8±0.6	75.5±2.5
	10 mM	0.0±0.0	0.0±0.0	71.2±1.8	70.0±0.6
AMPSF	100 μM	93.3±1.0	ND[a]	93.7±0.9	ND
	1 mM	0.4±0.1		88.8±1.1	
TLCK	10 μM	34.3±0.6	ND	0.8±0.3	ND
	200 μM	0.8±0.2		2.4±0.7	

[a] ND, Not determined.

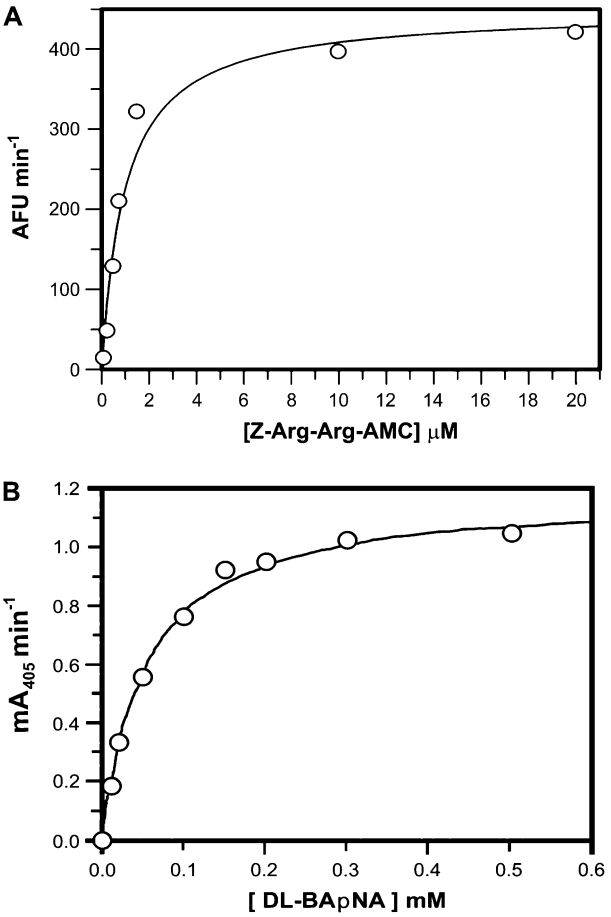


Fig. 2. Determination of the Michaelis–Menten constant for Q-SP. The substrates used were Z-Arg-Arg-AMC (A) and DL-BApNA (B). Assays were performed for 5 min, 37 °C, at pH 8.0. Reactions started with addition of 10 μl (1.5 μg) of Q-SP. The graphs are representative of several experiments. For Z-Arg-Arg-AMC, velocity was expressed as arbitrary fluorescent units per minute (AFU min^{-1}). For DL-BApNA, activity is expressed as milli absorbance units at 405 nm per minute (mA_{405} min^{-1}). The figure was prepared with Grafit Version 3.0.

any effect upon 5D-HQ, 7D-HQ_1 or 7D-HQ_2 (data not shown).

Further steps aimed at purification of 7D-HQ_1 or 7D-HQ_2, including benzamidine–agarose chromatography and S-50 gel filtration, were not successful due to inactivation of the enzymes.

BSEs from roots, leaves, and cotyledons

BSEs were found in residual cotyledons (C), roots (R), and leaves (L) of plantlets on the 24th day after imbibition. For comparison to the serine proteinase isolated from quiescent seeds (Q-SP), the same chromatographic steps were applied to these plant materials. When applied to a CM column, BSE activity was recovered in the non-retained fractions of root (R-NRCM), leaf (L-NRCM), and cotyledon (C-NRCM) extracts. HQ chromatography of each of these non-retained fractions resulted in a single sharp BSE peak. While the BSE from leaves (L-HQ,

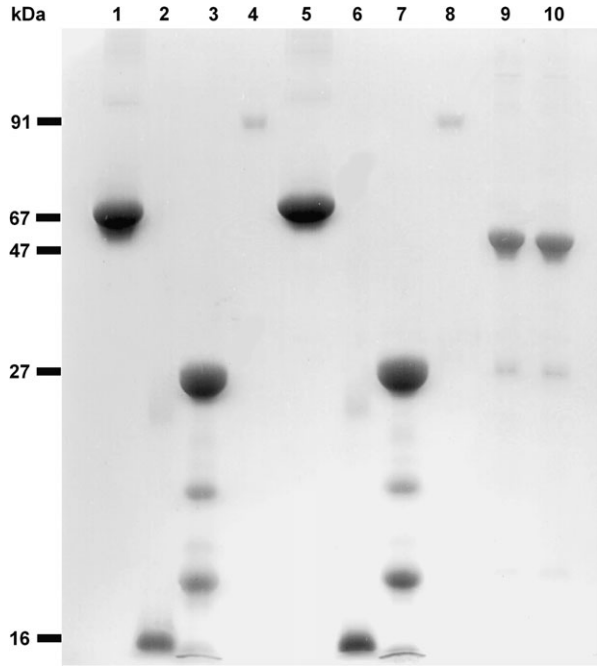
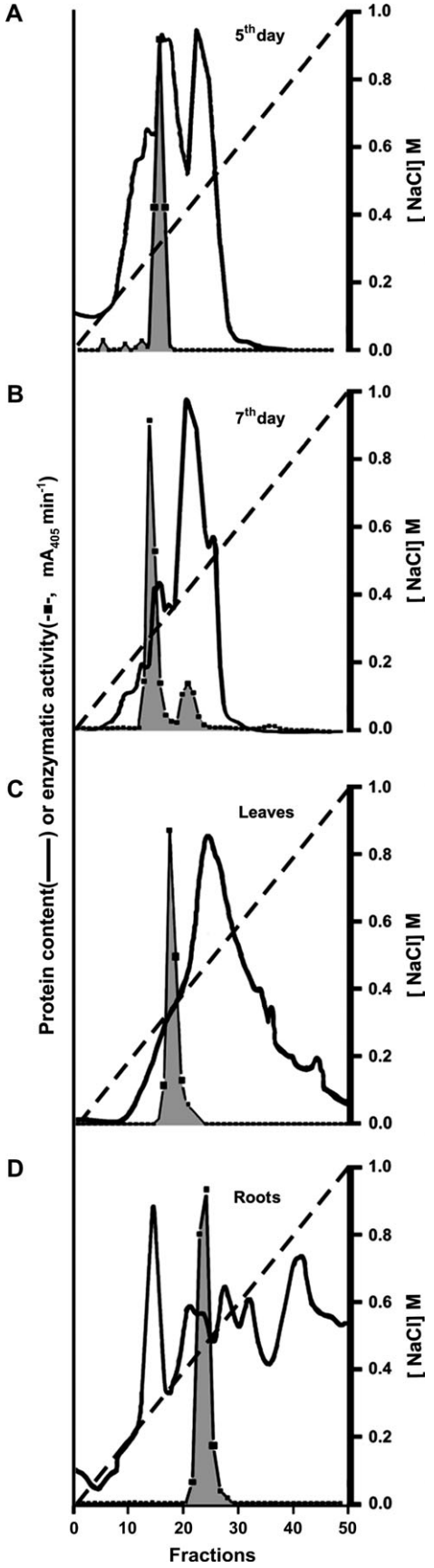


Fig. 3. Hydrolysis of protein substrates by the serine proteinase Q-SP isolated from *Canavalia ensiformis* quiescent seeds. Protein substrates (50 μg each) were incubated with the purified serine proteinase (Q-SP, 5 μg) in 80 μl of buffer A at 37 °C for 16 h. Aliquots of 25 μl of reaction mixtures were loaded onto a 12% SDS-PAGE. The gel was stained with Simply Blue™ Safe Stain (Invitrogen). Lanes 1 and 5, bovine serum albumin (67 kDa); lanes 2 and 6, bovine haemoglobin (subunits, 16 kDa); lanes 3 and 7, concanavalin A (subunit, 27 kDa); lanes 4 and 8, *C. ensiformis* urease (subunit, 90.7 kDa); lanes 9 and 10, canavalin (subunit, 47 kDa). Lanes 1, 2, 3, 4, and 9, controls (no enzyme added); lanes, 5, 6, 7, 8, and 10, after incubation with Q-SP for 16 h.

Fig. 4C) eluted at 350 mM NaCl, similar to Q-HQ and 7D-HQ_1, the BSEs from roots (R-HQ; Fig. 4D) and cotyledons (C-HQ; Fig. 5) were eluted at 450 mM NaCl, the same ionic strength which eluted the second peak of activity (7D-HQ_2; Fig. 4B) found in seeds on the 7th day after imbibition. Although BSE of the cotyledons (C-HQ) could be purified to homogeneity after a gel-filtration step (Fig. 5A, inset), it was not possible to purify the enzymes from roots or leaves further, as they were inactivated after both gel filtration and benzamidine–agarose chromatography.

Gel filtration of C-HQ in a Superdex 75 HR 10/30 column (Fig. 5B, dashed line) indicated the presence of two active peaks, probably corresponding to monomer and oligomer forms, as previously observed for Q-SP. Although Q-SP and C-HQ showed different apparent molecular masses in SDS-PAGE, with C-HQ appearing heavier (*c*. 40 kDa) than Q-SP, the elution pattern of their active monomeric and oligomeric forms coincided in the gel-filtration chromatography, as shown in Fig. 5B. Q-HQ eluted at a lower NaCl concentration (350 mM) as compared with C-HQ (450 mM). It seems that the more highly charged 7D-HQ_2 and C-HQ are probably the same enzyme, representing a different isoform of Q-SP, or an



entirely new enzyme, synthesized after the 7th day of imbibition.

Incubation of L-HQ, R-HQ, or C-HQ with 1 mM benzamidine for 20 min resulted in *c*. 85% inhibition of their proteolytic activity, and 10 mM benzamidine abolished their activity completely. There was no effect of E-64, DTT, PMSF, or SBTI upon the enzymatic activities of L-HQ, R-HQ, or C-HQ (data not shown).

The optimum temperature assay helped to differentiate the BSEs isolated from different plant tissues or developmental stages (Fig. 6). Q-SP demonstrated an optimum activity temperature of 45 °C, with activity increasing by about a factor of four from 35 °C to 45 °C (Fig. 6, inset). The activity drops very rapidly with the increase of the temperature to 50 °C or higher. The enzymes from quiescent seeds (Q-SP) and from roots (R-HQ) showed the highest activities at 45 °C. All the enzymes were inactivated at 50 °C. Interestingly, only C-HQ, whose optimal temperature was 40 °C, was completely denatured after exposure for 10 min at 45 °C. Between 30 °C and 40 °C, the enzymes showed different increments in their activities: a 4-fold increase for Q-SP, a 2.5-fold increase for C-HQ, and a 1.25–2.0-fold increase for 7D-HQ_1, R-HQ, 5D-HQ, and L-HQ.

Discussion

Only a few serine proteinases have been described so far in Leguminoseae plants. C1 protease (70 kDa), isolated from leaves and cotyledons of soybean *Glycine max* seedlings, was shown to be involved in the processing of the storage protein β-conglycinin. It is optimal at pH 3.5–4.5, is susceptible to PMSF, and cleaves preferentially at Glu or Gln residues (TanWilson *et al*., 1996; Boyd *et al*., 2002). KLSP enzyme (72 kDa) was isolated from leaves of the common bean *Phaseolus vulgaris*, is optimal at pH 9.9, and prefers Arg residues in the P1 and P2 positions of the substrate (Popovic *et al*., 2002).

As far as is known, only two proteolytic enzymes have been described so far in *C. ensiformis*. An asparagine-specific cysteine endopeptidase (37 kDa), with optimal activity in acidic medium and unstable at pH above 7.5, is

Fig. 4. Comparison of chromatographic behaviour of benzamidine-sensitive enzymes (BSE) isolated from growing seedlings of *Canavalia ensiformis*. Non-retained fractions of CM chromatography of plant material extracted at different stages of development were submitted to HQ chromatography as described under Materials and methods. Chromatographic fractions (50 µl) were assayed with 0.625 mM of DL-BApNA, in 100 mM TRIS-HCl pH 8.0, at 37 °C for 10 min. Proteolytic activity (closed squares, shaded peaks) is expressed as milli absorbance units at 405 nm per minute (mA_{405} min^{-1}). Maximal activity of each peak is given below. Continuous line, absorbance at 280 nm; dashed line, sodium chloride gradient. (A) Fifth day after imbibition (maximal activity 110 mA_{405} min^{-1}); (B) 7th day after imbibition (73 mA_{405} min^{-1}); (C) leaves (53 mA_{405} min^{-1}); (D) roots (150 mA_{405} min^{-1}).

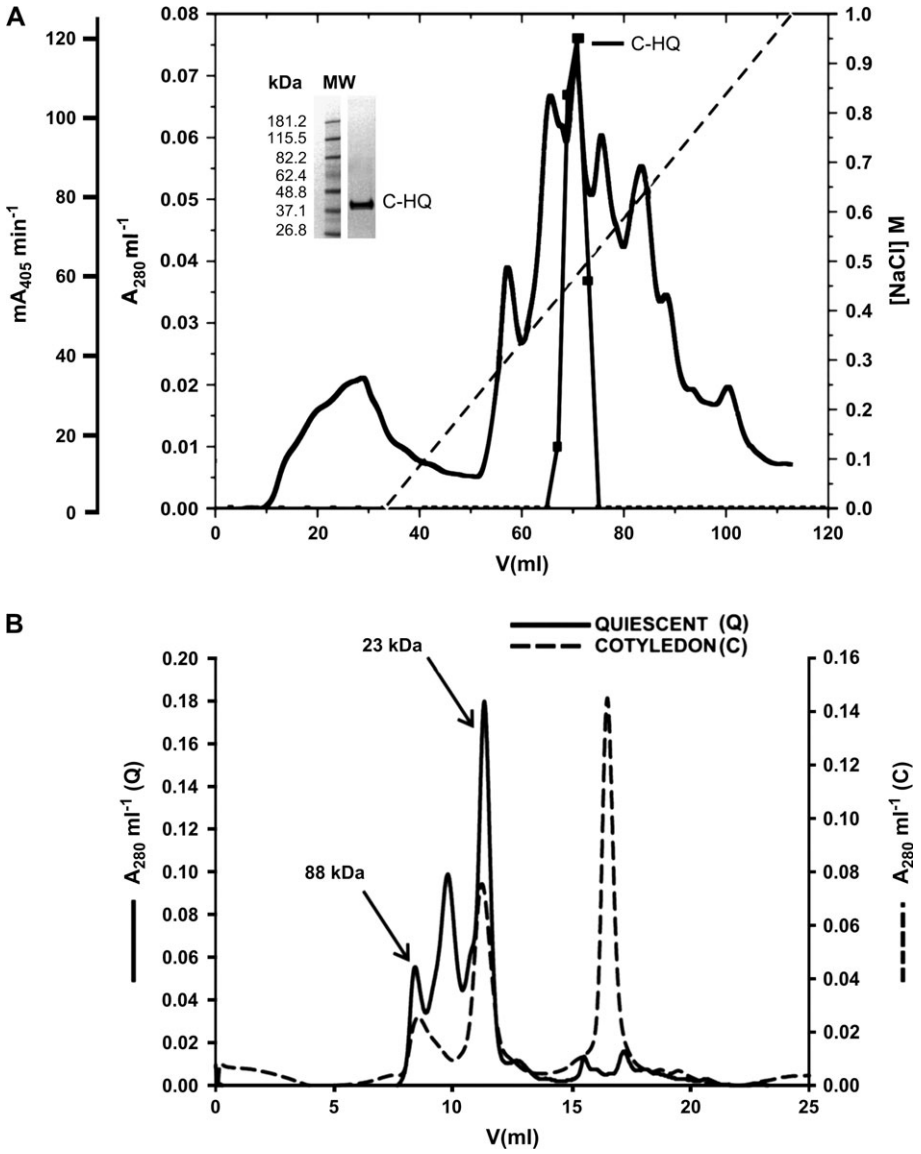


Fig. 5. (A) HQ chromatography of cotyledon material (C-NRMC). The active pool (tested upon DL-BApNA at pH 8.0) was named C-HQ. A non-reducing SDS-PAGE of C-HQ is shown in the inset. (B) Superposition of gel filtration patterns of Q-BSE (continuous line) and C-HQ (dashed line). Arrows indicate the active oligomeric and monomeric forms of Q-BSE. No activity was recovered after gel-filtration of C-HQ.

responsible for the post-translational proteolysis and trans-peptidation (ligation) of the precursor of the seed lectin, Con A (Carrington *et al*., 1985; Abe *et al*., 1993). A trypsin-like peptidase from quiescent seeds of *C. ensiformis* was previously isolated and characterized by Oshikawa *et al*. (2000). The enzyme showed an M_r of 41 000 and maximal activity at pH 9 and 60 °C. The enzyme hydrolysed synthetic substrates at Arg-X and Lys-X bonds, did not cleave protein substrates, and was inhibited by diisopropylfluorophosphate (but not by PMSF), TLCK, leupeptin, benzamidine, and by $ZnCl_2$.

In the present work, a confirmed serine proteinase from quiescent seed (Q-SP), and other enzymes that were strongly inhibited by benzamidine and found in roots and leaves of developing *C. ensiformis* plants were described. A purification protocol was developed that resulted in a homogenous enzyme from the quiescent seed (Q-SP). This protocol was useful for comparing Q-SP and BSEs found in the other plant tissues. Q-SP, with 33 kDa, was classified as a trypsin-like serine proteinase due to the strong inhibition by benzamidine, leupeptin, and TLCK, the later two being classical inhibitors of serine proteinases (Kourteva *et al*., 1987; Shaw, 1988). Q-SP was not affected by the transition state analogues PMSF (Polgar, 2005) or APMSF, a hydrophilic derivate of PMSF (Laura *et al*., 1980). Although serine proteinases are structurally conserved enzymes, their catalytic triad may vary slightly and such a variation might account for the observed lack

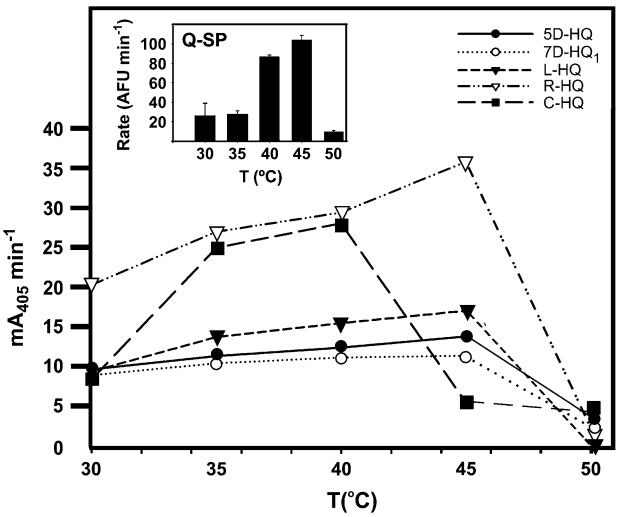


Fig. 6. Optimum temperature assay. The inset bar graph shows the optimum temperature assay performed with Q-SP in the presence of 10 μM CBZ-Arg-Arg-AMC. HQ fractions assayed with 0.625 mM of DL-BApNA are shown as a line graph. In both cases, samples (50 μl) were pre-incubated for 10 min at the desired temperature in 100 mM TRIS-HCl at pH 8.0, before addition of substrate, and the assays were carried out for 10 min. Samples tested were: 5D-HQ (closed circles); 7D-HQ_1 (open circles); L-HQ (closed triangles); R-HQ (open triangles); C-HQ (closed squares). Representative results are shown.

of inhibition of Q-SP by PMSF. As described for most plant serine proteinases (Antao and Malcata, 2005), Q-SP was not inhibited by the serpin SBTI. Some trypsin-like enzymes need a calcium ion to maintain their stability, although the presence of this metal is not necessary for catalysis. The chelating agent 1,10-orthophenanthroline partially inhibited Q-SP, suggesting the participation of a divalent metal cation in maintaining its structure.

As compared with cucumisin-like proteinases which show affinity in the millimolar range for hydrophobic residues (Antao and Malcata, 2005), Q-SP displays high affinity for arginine in the P1 position, as indicated by the K_M in the low micromolar range (Fig. 2). In addition, arginine in the P2 subsite is important for positioning the substrate in the active cleft, demonstrated by the higher affinity of the enzyme for Z-Arg-Arg-MCA than for DL-BApNA. Although differing in optimal pH and molecular mass, Q-SP resembles KLSP protease from common bean (*P. vulgaris*), which also displays micromolar affinity for substrates with Arg residues in P1 and P2 subsites (Popovic *et al*., 2002). Apparently, Q-SP shows only narrow specificity for protein substrates as it did not hydrolyse bovine serum albumin, haemoglobin, or some proteins found in *C. ensiformis* seeds, such as urease, concanavalin-A, or canavalin, the main storage protein of jack bean seeds (Sammour *et al*., 1984).

Apparently, Q-SP is a protein different from the enzyme previously described in jack bean seeds by Oshikawa *et al*. (2000). That enzyme was shown to have its optimal pH 9.0 (pH 7–8 for Q-SP, being inactive at pH 9.0), optimal temperature 60 °C (45 °C for Q-SP), molecular mass of 41 kDa (33 kDa for monomeric Q-SP), with no reported tendency for oligomerization, higher thermal stability (50% residual activity after 1 h at 50 °C versus 5% for Q-SP after 20 min at 50 °C), higher sensitivity to inhibition by benzamidine [46% residual activity (1 mM) versus 37% (10 mM) for Q-SP] and by zinc chloride [0% residual activity (1 mM) versus 90% for Q-SP], and lower sensitivity to inhibition by leupeptin [17% residual activity (60 μM) versus 10% (10 μM) for Q-SP] (Oshikawa *et al*., 2000). The enzyme isolated by Oshikawa's group, which did not hydrolyse casein, lysozyme, or insulin B, was not tested upon proteins from *C. ensiformis* seeds.

With the aim of investigating the fate of the Q-SP enzyme during early stages of *C. ensiformis* development, the same purification protocol was applied to identify BSE in seeds at 5 d and 7 d after imbibition. These enzymes might also be serine proteinases, although they were not unambiguously identified as such in this study. A new enzyme is synthesized on the 7th day and is seen as a second active peak in the anion exchange chromatography (Fig. 4B). This is a critical moment of the developmental process when several nutrients have already been consumed (Muntz *et al*., 2001) and new enzymes might be necessary to accomplish functions different from those performed by maternal enzymes stored in the seed during earlier stages of development (Schlereth *et al*., 2000; Muntz *et al*., 2001; Grudkowska and Zagdanska, 2004). BSEs were also found in roots (R-HQ), leaves (L-HQ), and cotyledons (C-HQ) of 24-d-developed plants. Although no activity was recovered after C-HQ gel filtration, two peaks were seen at the same elution volumes of the active peaks obtained for gel-filtered Q-SP (Fig. 5A). However, SDS-PAGE of Q-SP and C-HQ indicated different molecular masses for the monomeric state of these enzymes (28 kDa and 40 kDa, respectively). That these two proteins are not the same was also indicated by their behaviour in the ion exchange chromatography. The enzyme derived from quiescent seeds (Q-HQ) eluted with 350 mM NaCl (Fig. 1), whereas the more charged fraction isolated from cotyledons of 24-d-developed plants (C-HQ) was eluted with 450 mM (Fig. 5A). The second peak of activity present in seeds 7 d after imbibition (7D-HQ_2), also eluted with 450 mM, is most likely to be the same enzyme (C-HQ) that was isolated from cotyledons of 24-d-developed plants. Although C-HQ and R-HQ eluted in the same ionic strength (450 mM NaCl), they are clearly different proteins as indicated by their thermal stability at 45 °C (Fig. 6). On the other hand, BSE from leaves (L-HQ) paralleled the behaviour of the enzymes present in quiescent (Q-SP) and 7 d seedlings (7D-HQ_1) regarding the elution with 350 mM NaCl in the ion exchange step, and in its similar optimal temperature and thermal stability. These facts most likely indicate different members of an

alkaline serine protease family and a developmentally regulated expression of the members of the corresponding gene family. At this point, expression of the same gene in different organs cannot be excluded. Thus L-HQ may represent a developmental regulation of the quiescent enzyme gene with expression in dry seeds and leaf tissues. Q-SP shared with the apparently newly synthesized enzymes R-HQ and C-HQ the optimal pH 8.0, the affinity for Arg residues, inhibition by benzamidine, and no sensitivity to SBTI or PMSF.

The physiological role of Q-SP and other BSEs found in the developing *C. ensiformis* plants cannot be assigned yet. It is known that seed storage protein breakdown takes place in acidic vacuoles and is mediated by acidic proteases. Although subcellular localization of Q-SP was not performed in this work, this alkaline protease was incapable of degrading the main seed storage protein canavalin as well as other proteins endogenous to the seed. Thus a putative role of this enzyme in protein mobilization can be ruled out. In conclusion, Q-SP and other members of this alkaline protease family might participate in some specific limited proteolysis event(s) yet to be characterized.

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