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A SERINE PROTEASE FROM SUSPENSION-CULTURED SOYBEAN CELLS*

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Abstract—A serine protease was purified from suspension-cultured soybean cells, by a combination of anion exchange, hydrophobic interaction and affinity chromatography. *A* 90 000 M_r subunit, which could be photoaffinity labelled with ^{3}H-diisopropylfluorophosphate (DFP), was identified by SDS-polyacrylamide gel electrophoresis. The enzyme had a broad pH optimum from 5.5 to 8.5, and was strongly inhibited by antipain, leupeptin, aminoethylbenzenesulphonyl fluoride (AEBSF) and DFP, but not by soybean trypsin inhibitor. It cleaved several peptide 4-methylcoumaryl-7-amide derivatives after arginine or lysine residues. Mass spectroscopic analysis of oligopeptide digestion products indicated that the preferred cleavage positions were between paired arginine residues, or C-terminal to single arginine residues, depending on the oligopeptide substrate. Partial amino acid sequences from the purified protein showed sequence identity to bacterial protease II and prolyl peptidase, although the enzyme lacked prolyl endopeptidase activity. We discuss the possible involvement of the protease in plant defense responses.

INTRODUCTION

Proteolysis is ubiquitous in biological systems, providing a means for cells to change their protein content during development and adaptation to altered environmental conditions. In plants, significant progress has been made in understanding the mechanisms by which short-lived proteins are targeted and then degraded during their normal cellular turnover [1]. Furthermore, a number of proteases have been characterized and ascribed functions in the degradation of storage proteins during seed germination [2–5]. Most of these endopeptidases have been shown to be thiol or aspartic proteases, and less is known about serine proteases in plants.

In mammalian cells, many physiologically active peptides are produced from their precursors by specific cleavage [6, 7]. However, evidence for 'peptide hormones' in plants is scarce, with the notable exception of the wound signal systemin, an 18-amino-acid peptide that is released from a larger precursor protein by proteolytic processing [8]. A protease with similarity to the yeast and mammalian Kex2 family of subtilisin serine proteases, which cleave after dibasic sites and are involved in the processing of a wide range of biologically active peptides [6], has been identified in tomato leaf plasma membranes, and may be involved in the processing or degradation of systemin [9]. The demonstration of the correct processing of the KP6 killer toxin of virus-inoculated *Ustilago maydis* in transgenic tobacco has provided further evidence for the presence of Kex2-like processing activity in plants [10]. In addition, a subtilisin-like protease is expressed in actinorhizal root nodules, where it may be involved in early nodule development [11], and the juice of melon fruits contains an alkaline serine protease related to the subtilisin family [12]. Taken together, these results suggest that serine proteases may be of more widespread occurrence in plants than previously thought.

Recently, a number of proteases have been shown to be induced during responses of plants to environmental cues. These include leucine aminopeptidases and an aspartic protease that accumulate during the response of tomato or potato plants to wounding [13–15], and a protease activity of undefined specificity that accumulates in tobacco leaves inoculated with *Pseudomonas tabacina* [16]. Bestatin, an inhibitor of mammalian and plant aminopeptidases, can induce normally wound-induced defense response genes in tomato mutants deficient in the octadecanoid sig-

* This paper is dedicated to Clarence (Bud) Ryan on the occasion of his sixty-fifth birthday.

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nalling pathway [17] suggesting that some plant proteases may be involved in the regulation of defense responses at or near the transcriptional level. A protease from tomato showing homology to subtilisin-like endoproteases is induced following viroid infection [18]. Plant proteases have also been implicated in the release of elicitor-active molecules upstream in the defense gene signal transduction pathway [19, 20].

We have been studying the response of cultured soybean cells to avirulent bacteria and isolated microbial elicitors. Typical responses include the production of active oxygen species known as the oxidative burst [21, 22] and the transcriptional activation of phytoalexin biosynthetic genes [23]. During these studies, we have observed that serine protease inhibitors strongly potentiate both the oxidative burst and phytoalexin production in response to avirulent bacteria or elicitor, but are inactive when applied to cells on their own (Z.-J. Guo and R. A. Dixon, unpublished results). This prompted us to investigate the nature of the serine protease(s) present in suspension cultured soybean cells. We have purified a novel serine protease from this source, and here report its isolation and properties.

RESULTS

Purification of protease

Initial studies using crude extracts from soybean cell suspension cultures revealed no protease activity against two general serine protease substrates, BSA-fluorescein isothiocyanate (FITC) and aza-casein. However, activity against the model peptide *N-t*-Boc-Gly-Arg-Arg-MCA was readily detected. This protease activity was not induced following exposure of the cells to yeast elicitor (data not shown).

Using the above model peptide as substrate to monitor protease activity during purification, a protease was extensively purified from suspension cultured soybean cells as summarized in Table 1. The specific activity increased over 5-fold following $(NH_4)_2SO_4$ and low pH precipitation steps. Anion exchange chromatography effected a further 10-fold

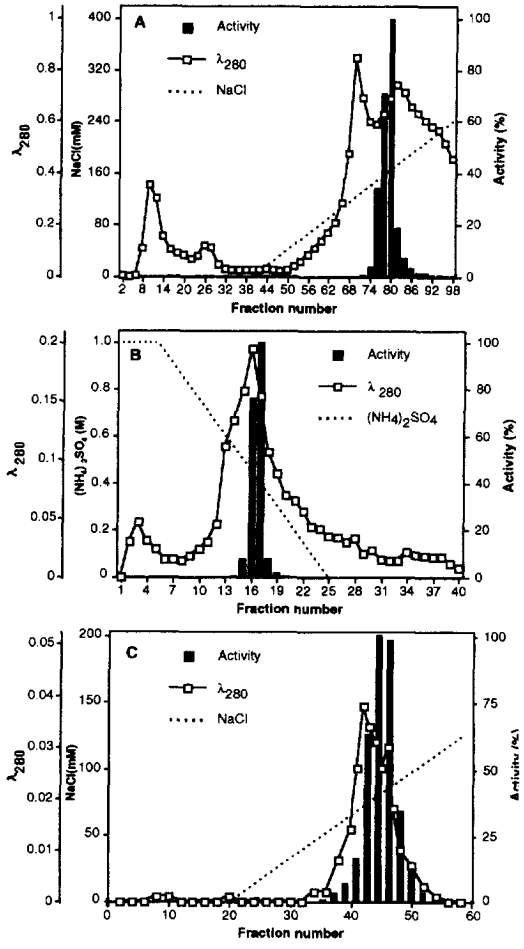

Fig. 1. Purification of serine protease from suspension cultured soybean cells. Chromatography on: A, DEAE-Sepharose fast flow; B, phenyl-Superose; C, arginine-Sepharose 4B (second round). Enzyme activity is normalized. Absolute activities in the pooled active fractions at each stage are given in Table 1.

increase in specific activity [Fig. 1(A)], and a final purification of 1217-fold was obtained following hydrophobic interaction chromatography [Fig. 1(B)] and two rounds of affinity chromatography on arginine-Sepharose [Figure 1(C), Table 1].

The purified enzyme preparation migrated as a sin-

Table 1. Purification of serine protease from suspension-cultured soybean cells

Step	Total protein (mg)	Total activity (unit)*	Specific activity (unit mg^{-1})*	Purification (fold)	Yield (%)
Crude extract	2840	9940	3.5	1	100
$(NH_4)_2SO_4$ ppt.; low pH ppt.	332	5876	17.7	5.1	59
Q-FF	16.6	3270	197	56.3	33
Phenyl-Superose; Arg-Sepharose	0.56	791	1413	404	8
Microcon 50 K; Arg-Sepharose	0.12	511	4260	1217	5

* One unit of enzymatic activity is defined as the amount of protein releasing 1 nmol of 7-amino-4-methylcoumarin min^{-1} 0.2 ml^{-1} at 25°.

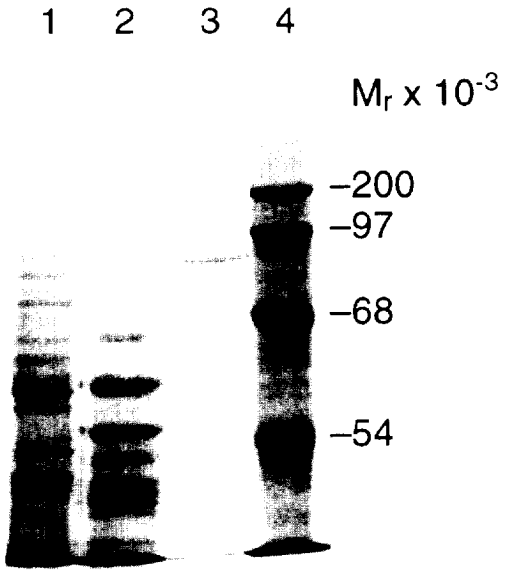

Fig. 2. SDS-PAGE analysis of fractions during the purification of soybean serine protease. Lane 1, crude extract; lane 2, material after DEAE-Sepharose; lane, 3, material after second arginine-Sepharose step; lane 4, mol weight markers.

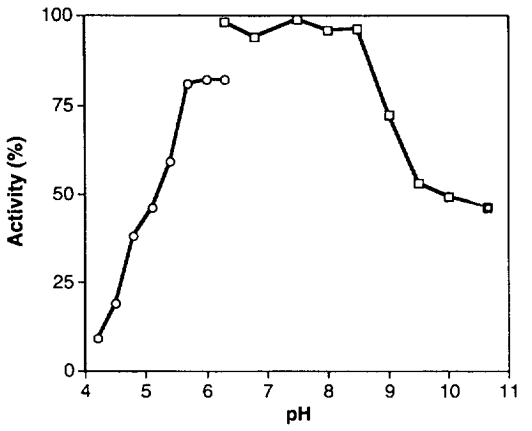

Fig. 3. pH profile for the activity of purified soybean serine protease. The buffers used were 100 mM Tris–HCl (squares) and 100 mM NaOAc–HOAc (circles).

Table 2. Activity of purified soybean serine protease against synthetic peptides

Substrate	Relative activity (%)
N-*t*-Boc-Gly-Arg-Arg-MCA	100
N-*t*-Boc-Gln-Ala-Arg-MCA	127
N-*t*-Boc-*r*-Benzyl-Glu-Ala-Arg-MCA	112
N-*t*-Boc-Leu-Ser-Tyr-Arg-MCA	40
N-CBZ-Phe-Arg-MCA	110
Arg-MCA	4
N-*t*-Boc-Glu-Lys-Lys-MCA	42
N-*t*-Boc-Val-Leu-Lys-MCA	37
N-Succinyl-Ala-Phe-Lys-MCA	31
N-CBZ-Lys-MCA	1
N-Succinyl-Gly-Pro-Leu-Gly-Pro-MCA	0
Gly-Pro-MCA	0
N-Succinyl-Ala-Ala-Ala-MCA	0
N-Succinyl-Ala-Ala-Pro-Phe-MCA	0
N-Succinyl-Leu-Leu-Val-Tyr-MCA	0

gle band of M_r 90 000 on SDS-PAGE (Fig. 2), and a single band of this M_r was observed following SDS-PAGE analysis of polypeptides from the anion exchange chromatography step labelled with [^{3}H]-DFP (data not shown), indicating that the enzyme is a serine protease. Furthermore, using native PAGE of soybean cell extract fractions after anion exchange chromatography or after the final affinity chromatography step, a single band was observed that showed activity toward *N*-*t*-Boc-Gly-Arg-Arg-MCA (data not shown). These results, and the single peak of activity observed at all stages in Fig. 1, suggest that a single protease with activity against the model peptide substrate is present in soybean cells.

Effect of pH on proteolysis

The pH dependence of activity of the purified soybean serine protease toward *N*-*t*-Boc-Gly-Arg-Arg-MCA, using NaOAc and Tris buffer systems, is shown in Fig. 3. The enzyme exhibited a broad optimum pH, from 5.5 to 8.5.

Substrate and product specificity of the soybean serine protease

The relative activity of the protease toward a range of peptide-MCA substrates is shown in Table 2. Peptides with Arg at the P1 position were the best substrates, followed by peptides with Lys at this position. Model substrates for chymotrypsin and prolyl endopeptidase were not digested. Likewise, the purified enzyme exhibited no activity against the high molecular weight protein substrates BSA-FITC and azacasein.

In order to confirm the position of peptide cleavage, three Arg-containing peptides that would be predicted to be substrates for the protease were subjected to digestion by the enzyme, and the products separated by HPLC and identified by mass spectrometric analysis. In the case of YGGFLRRIRPKLK, the digested product gave two peaks on reverse-phase HPLC (Fig. 4). Under the conditions of the experiment, cleavage proceeded slowly and was only 30% complete after 20 hr (Fig. 4). The products were identified by MS analysis as YGGFLR and RIRPKLK, indicating that the R–R linkage was preferentially cleaved, and that single Arg or Lys did not constitute a preferred cleavage site in this peptide. These conclusions were confirmed from the hydrolysis pattern of RRLIEDNEYTARG, which was rapidly cleaved (90% digestion within 5 hr) and from which a single peptide was identified resulting from cleavage of the R–R linkage.

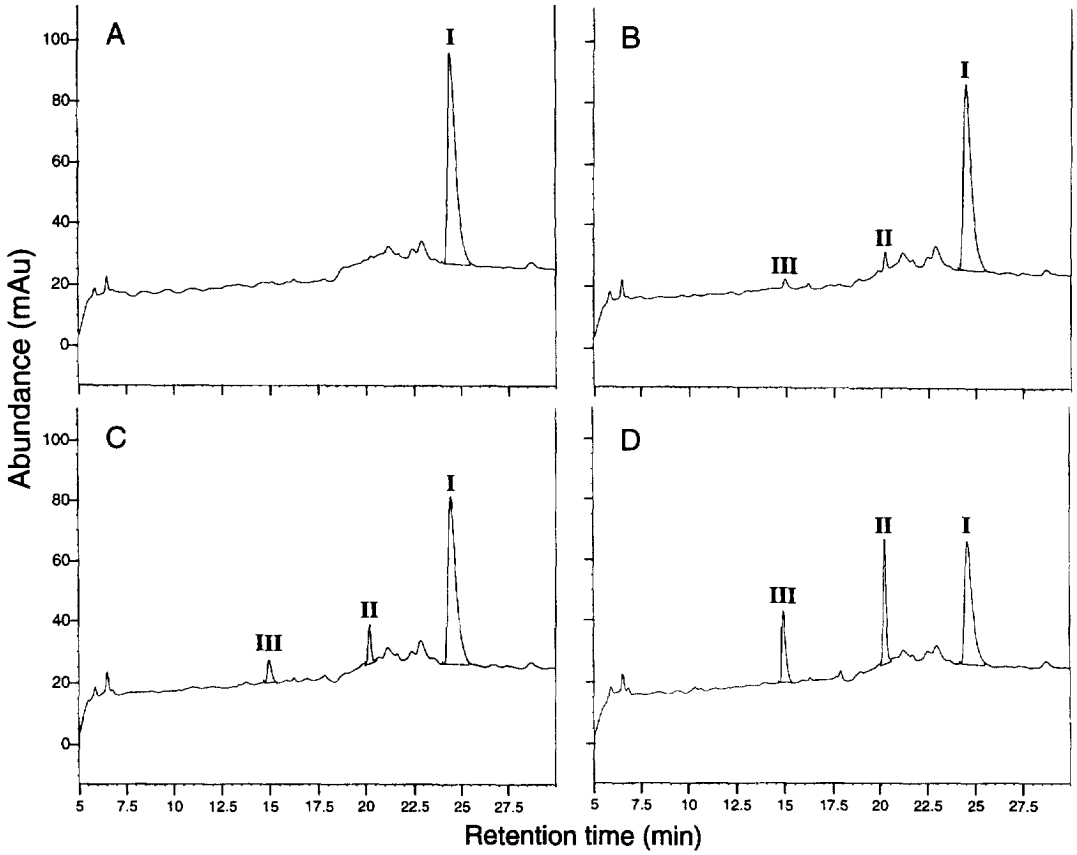

Fig. 4. Digestion kinetics of the model peptide YGGFLRRIRPKLK by purified soybean protease. Substrate and products were analyzed by HPLC. Incubations were for zero time (A), 2 hr (B), 5 hr (C) and 20 hr (D). The peaks correspond to the substrate (I) and the products YGGFLR (III) and RIRPKLK (II), as confirmed by MS analysis.

However, cleavage C-terminal to a single Arg residue was observed in peptide GRGDTP (data not shown).

Effect of inhibitors on the protease activity

As shown in Table 3, the purified soybean protease was strongly inhibited by typical serine protease inhibitors such as diisopropylfluorophosphate (DFP), 3,4-dichloroisocoumarin (DCI), antipain, and leupeptin. Phenylmethylsulphonylfluoride (PMSF) was only a weak inhibitor at 1 mM, but its analog aminoethylbenzenesulphonyl fluoride (AEBSF) gave nearly complete inhibition at this concentration. Other protease inhibitors such as bestatin (an inhibitor of leucine aminopeptidase), pepstatin A (an inhibitor of acidic aspartyl protease), tosyl-L-lysine chloromethyl ketone (TLCK, and inhibitor of trypsin-like serine proteases) and tosyl-L-phenylalanine chloromethyl ketone (TPKC, an inhibitor of chymotrypsin-like proteases) had little or no inhibitory activity. Thiol reagents such as DTT and iodoacetate had little effect on the protease activity, which was also unaffected by addition of zinc ions (required for leucine aminopeptidase) or removal of calcium ions (required for the activity of subtilisin-like proteases). Significantly, trypsin inhibitor from soybean had little effect on the enzyme activity.

Table 3. Effect of inhibitors and effectors on the activity of purified soybean serine protease.

Reagent	Concentration	Activity (%)
AEBSF	1 mM	4
Antipain	20 μM	0
APMSF	1 mM	64
Bestatin	0.2 mM	97
DCI	1 mM	26
DFP	1 mM	2
DTT	5 mM	85
Iodoacetate	1 mM	106
Leupeptin	15 μM	0
Pepstain A	0.24 mM	96
PMSF	1 mM	81
TLCK	1 mM	61
TPCK	1 mM	47
Trypsin inhibitor Sigma (Type I-S from soybean)	0.1 mg ml^{-1}	86
Zn^{2+}	1 mM	101
$-Ca^{2+}$		102

Amino acid sequence of the soybean protease

Four tryptic peptide fragments from the purified protease were subjected to automated Edman degra-

Table 4. Internal peptide sequences of soybean serine protease (SSP) and alignment with previously reported sequences from pig or human prolyl endopeptidase (PE) and *E. coli* protease II (PII)

Peptide 1.	SSP.	QWAPSSDGTLIPISIVYR
	PE.	IFYPSKDGTKIPMFIVHK
	PII.	LWIVARDGVEVPVSLVYH
Peptide 2.	SSP.	LESGQAVSFVDPVYSAES
Peptide 3.	SSP.	LDGSDPLLLYGYGSYEICIDPS
	PE.	LDGSHPAFLYGYGGFNISITPN
	PII.	RKGHNPLLVYGYGSYGASIDAD
	PII.	LDACDALLKLGYGSPSLCYAMG
Peptide 4.	SSP.	QENAYTDSIMSGTK

dation sequencing, and the results are shown in Table 4. Searching the EMBL protein sequence database with the BLAST program indicated that peptides 1 and 3 exhibited significant sequence identity to *Escherichia coli* protease II and human or porcine prolyl endopeptidases [24–26]. The other two fragments did not appear to have sequence similarity to other known proteins.

DISCUSSION

Using the synthetic peptide *N-t*-Boc-Gly-Arg-Arg-MCA as substrate, we have purified a protease over 1200-fold, to apparent homogeneity, from extracts of suspension cultured soybean cells. The enzyme had a subunit M_r of 90 000, and the subunit could be labelled with ^{3}H-DFP, indicating that the enzyme is a serine protease. This was confirmed by the inhibition of the enzyme by a variety of serine protease inhibitors such as DFP, DCI, APMSF, antipain, and leupeptin.

Cleavage specificity studies using synthetic peptide substrates showed that the enzyme preferentially hydrolyses at the carboxyl side of Arg and, to a lesser extent, Lys residues. Cleavage occurs C-terminal to both monobasic and dibasic sites, and cleavage between paired arginine residues is preferred in some substrates. These properties suggest that the enzyme is a trypsin-like serine protease, distinct from Kex-2-like proteins of the subtilisin family that have been implicated in the processing of biologically active peptides and require a dibasic cleavage site and cut C-terminal to arginine [6]. However, the lack of inhibition of the soybean enzyme by soybean trypsin inhibitor, its inability to digest BSA or casein, and its broad pH optimum that extends to the acid side of neutral, clearly distinguish it from other plant trypsin-like proteins.

Two serine proteases have been previously reported from soybean seeds [5, 27]. The enzyme of Nishikata *et al.* [27] has several properties in common with the enzyme we have purified, but, like trypsin, is most active above pH 8.0. The other enzyme is strongly inhibited by soybean trypsin inhibitor, is active against large proteins, including the α subunit of conglycinin and the A3 polypeptide of the glycinin G5 subunit [28], and will not cut C-terminal to Lys residues [5]. These two proteins are probably involved in the mobilization of storage proteins during seed germination.

Sequence comparisons of proteolytic fragments indicated that the new soybean protease had amino acid identity to regions of prolyl endopeptidases and protease II. Protease II is the only endopeptidase from *E. coli* with substrate specificity for basic amino acids and, on the basis of sequence comparisons, may be related to the prolyl endopeptidases [24]. It resembles trypsin, but differs in acting preferentially on low molecular mass peptides. Because the purified soybean protein did not cleave the Pro-MCA linkage, or cleave next to Pro in a model oligopeptide, it is clearly not a prolyl endopeptidase.

We are now interested in determining the possible function of the soybean serine protease. Preliminary experiments have indicated that treatment of cultured soybean cells with various serine protease inhibitors strongly accelerates and potentiates the oxidative burst in response to avirulent bacteria or yeast elicitor (Z.-J. Guo and R. A. Dixon, unpublished results). Precedences exist in the literature for the involvement of proteolytic steps at various stages in the signal transduction pathways leading to activation of plant defense genes [8, 17, 19, 20]. However, the soybean protease does not appear to be induced following elicitation. Using the sequence information reported in this paper, we have recently obtained partial cDNA clones encoding the soybean protease, as a prelude to functional analysis by reverse genetic strategies.

EXPERIMENTAL

Materials. Arginine-Sepharose, FPLC phenyl-Superose, Sephadex G-25, and DEAE-Sephadex Fast Flow (Q-FF) were from Pharmacia Biotech Inc. Bestatin was purchased from CalBiochem and DFP was from ICN. Other chemicals were purchased from Sigma.

Plant material. Soybean cell suspension cultures were maintained as described [21]. Cells collected for enzyme purification were from cultures four days after subculture.

Enzyme purification procedures. The complete purification procedure was performed at 4°, using *N-t*-

Boc-Gly-Arg-Arg-MCA as substrate. Soybean cells (125 g) were ground in liquid N_2 with 125 ml 0.1 M Tris–HCl buffer, pH 7.5, containing 0.1% Triton X-100. After centrifugation at 12000 *g* for 15 min, $(NH_4)_2SO_4$ was added to the supernatant to 40% satn. Insoluble material was removed by centrifugation, $(NH_4)_2SO_4$ was added to the supernatant to 60% satn, and the soln left to stand overnight. The ppt collected by centrifugation was dissolved in 20 ml Tris–HCl (20 mM, pH 7.5). The pH of the soln was adjusted to *ca* 4.0 by addition of 2 ml of 1 M NaOAc, pH 4.0, followed by centrifugation to remove the ppt. The pH was then adjusted back to 7.5 with 1 M KOH, and the soln centrifuged to remove insoluble material. Proteins were pptd by addition of $(NH_4)_2SO_4$ to the supernatant to 70% satn, collected by centrifugation, and dissolved in a minimum vol. of 20 mM Tris–HCl (pH 7.5). After de-salting through a Sephadex G-25 column, the resulting soln was applied to a DEAE-Sephadex Fast Flow column (2.5 × 15 cm) equilibrated with 20 mM Tris–HCl (pH 7.5). The column was eluted with the same buffer until the *A* at 280 nm returned to baseline, then with a gradient of increasing NaCl concn from 0 to 0.4 M in the same buffer. Active frs were pooled and concd by Centricon-3 (Amicon) filtration. The concd soln was loaded onto a phenyl-Superose FPLC column, equilibrated with NaP buffer (pH 7.0) containing 1 M $(NH_4)_2SO_4$, and proteins eluted with a reversed gradient of $(NH_4)_2SO_4$ concn to zero. After concn using Centricon-3 membranes, the enzyme soln was passed through an arginine-Sepharose 4B column (1.4 × 6 cm). The protein was eluted by a gradient of NaCl from 0 to 0.2 M in Tris–HCl buffer, pH 7.5. Active frs were pooled and concd using a Microcon 50 membrane (Amicon). The enzyme soln was passed through the arginine column again, and the active frs pooled and concd as before. The enzyme prepn was stored at −80°.

DFP labelling. Purified soybean protease (5 μg) was incubated with [^{3}H]-DFP (final concn 30 μM) for 2 hr at 25°. The reaction mixt. was dialysed against 0.01% SDS at 4° to remove unreacted label, lyophilised, and the protein subjected to gel electrophoretic analysis.

Enzyme assays and inhibitor studies. The enzyme assays using highly purified enzyme and peptide-MCA substrates were performed as described by Morita *et al.* [5] with some modifications. Reactions were performed in 0.1 M Tris–HCl, pH 7.5, containing 10 mM $CaCl_2$, with incubations carried out in 96 well microtiter plates with a reaction vol. of 200 μl per well. Fluorescence was measured at 460 nm with excitation at 380 nm, using a Perkin–Elmer LS50B luminescence spectrophotometer. One unit of activity is defined as the amount that releases 1 nmol of 7-amino-4-methylcoumarin min^{-1} 0.2 ml^{-1} at 25°. Inhibitors were incubated with the enzyme for 30 min prior to addition of *N-t*-Boc-Gly-Arg-Arg-MCA. For assay against BSA, BSA–FITC (400 ng) in 200 μl of 0.1 M Tris–HCl, pH 7.5, was added to the purified soybean protease (0.2 units), and the fluorescence of the solution determined at 525 nm (excitation wavelength 495 nm) after 10 and 30 min.

Cleavage of oligopeptides. Oligopeptides (50 nmol) were digested with purified protease (1 unit) in 200 μl of 50 mM Tris–HCl buffer, pH 7.5, at 25°. Aliquots (25 μl) were removed at various time periods, to which were added 5 μl 6 M HCl to stop the reactions. The digested products were sepd on a C_{18} HPLC column (250 × 4.6 mm) at a flow rate of 0.8 ml min^{-1}, and the elution was monitored at 215 nm. Different linear gradients utilizing 0.1% aq TFA and CH_3CN containing 0.1% TFA were used depending on the products. Peptides were analysed by MALDI mass spectrometry.

Electrophoresis. SDS-PAGE was performed by the method of Laemmli [29] using 7.5% gels. After electrophoresis, proteins were stained with Coomassie Brilliant Blue R-250. Native PAGE was run in the absence of SDS. The proteolytic activity was detected fluorimetrically under UV light after spraying the gel with *N-t*-Boc-Gly-Arg-Arg-MCA.

Peptide sequencing. After SDS-PAGE, the purified protein (M_r 90000 band) was electroblotted onto PVDF membranes (Bio-Rad, Hercules, CA) and subjected to amino acid sequence analysis by automated Edman degradation.

Protein determination. Protein was determined by the dye-binding method of Bradford [30] using BSA as standard.

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