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Isolation and characterization of a serine protease from the sprouts of *Pleioblastus hindsii* Nakai

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Abstract

An endopeptidase has been purified from sprouts of bamboo (*Pleioblastus hindsii* Nakai) to electrophoretic homogeneity by four purification steps. Its M_r was estimated to be 82 kDa by SDS–PAGE. Enzyme activity was inhibited strongly by diisopropyl fluorophosphate, and weakly by *p*-chloromercuriphenylsulfonic acid, but not at all by EDTA or pepstatin, indicating that it was a serine protease. The preferential cleavage sites for this protease were found to be large hydrophobic and amide residues at the P_1 position. The specificity of the bamboo serine protease differed from that of cucumisin [EC 3.4.21.25], which cleaved the charged amino acid residues at the P_1 position.

Keywords: *Pleioblastus hindsii*; Gramineae; Bamboo sprout; Cucumisin; Serine protease; Plant protease

1. Introduction

Although many proteases have been isolated from plant latex, fruits and seeds (Boller, 1986), little is known about endopeptidases from plant sprouts. Earlier, we found a caseinolytic activity in sprouting stems of asparagus, *Asparagus officinalis* L. var. *altilis* L. (Yonezawa et al., 1998), as well as in bamboo sprouts (Uchikoba et al., 1991). We, however, are generally interested in the character and function of endopeptidases from the growing parts of plants.

Most plant proteases have been classified as cysteine proteases or, rarely, as aspartic proteases (Boller, 1986). Cucumisin [EC 3.4.21.25], a serine protease from *Cucumis melo* var. *Prince*, has been well characterized (Kaneda and Tominaga, 1975; Yamagata et al., 1994; Uchikoba et al., 1995). Recently, cucumisin-like proteases have been found in the fruits of the Cucurbitaceae (Kaneda and Uchikoba, 1994; Uchikoba et al., 1998), and other species. These include PR-P69 (P69A) and P69B, pathogen-induced in *Lycopersicon esculentum* (Tornero et al., 1996, 1997; Jordá et al., 1999; Meicgtry et al., 1999), ag12, expressed in the early stages of actinorhizal nodule formation in *Alnus glutinosa* (Rebeiro et al., 1995), a serine protease induced in meiotic prophase in lily microsporocytes (Kobayashi et al., 1994; Taylor et al., 1998), and an *Arabidopsis thaliana* protease, specifically found in the roots (EMBL accession number X85974). It is interesting to note that the primary structures of proteases in the cucumisin family are homologous to those of proprotein-processing proteases, such as Kex2 from yeast and human furin (Rebeiro et al., 1995). However, there are unclassified serine proteases from plant sources, e.g. soybeans (Nishikata, 1984; Morita et al., 1996; Guo et al., 1998).

In this paper, we describe the isolation, general properties, and substrate specificity of a new serine protease from the growing region of bamboo sprouts, and compare its enzymatic characteristics and structural properties to those of other cucumisin-like proteases.

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Nomenclature

AMC	7-amido-4-methylcoumarin	*p*NA	*p*-nitroanilide
DFP	diisopropyl fluorophosphate	TCA	trichloroacetic acid
Glt	glutaryl	Tos-Lys-CH_2Cl	*N*-Tosyl-L-lysine chloromethylketone
MCA	4-methy-coumaryl-7-amide		
PCMB	*p*-chloromercuribenzoic acid	Tos-Phe-CH_2Cl	*N*-Tosyl-L-phenylalanine chloromethylketone
PCMPS	*p*-chloromercuriphenylsulfonic acid		

2. Results

Hydrolytic activity using Suc-Ala-Ala-Pro-Phe-*p*NA as a substrate was detected in the homogenate of bamboo sprouts, but not in the leaves or stems (data not shown). The elution profile from CM-Sepharose column chromatography showed a single active peak (Fig. 1A). The active fractions were combined, and the enzyme was further purified by Sephacryl S-200 gel filtration (Fig. 1B).

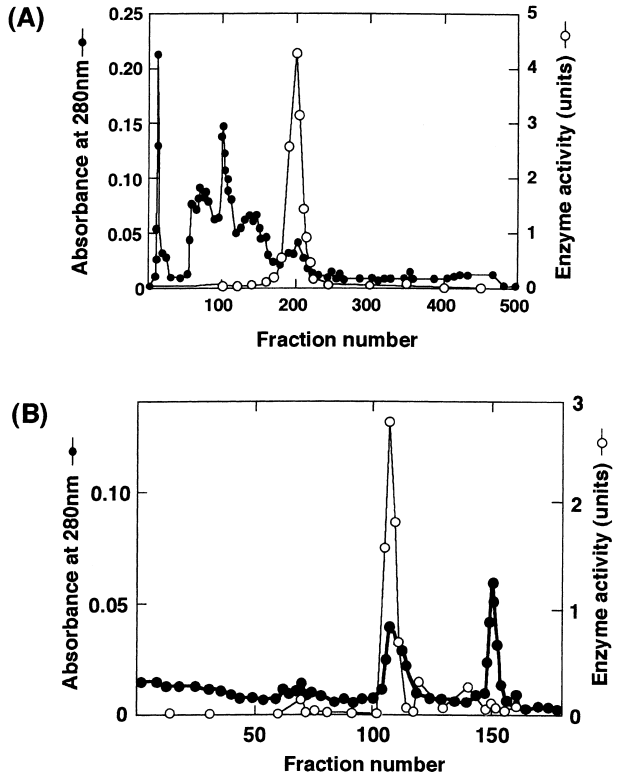

Fig. 1. (A) Elution profiles of bamboo serine protease on CM-Sepharose column chromatography. CM-Sepharose column chromatography of the proteases from the active fractions of a CM-cellulose chromatography. *A* at 280 nm for each fraction is shown by closed circles. Protease activity was assayed with Suc-Ala-Ala-Pro-Phe-*p*NA (open circles) as a substrate under the conditions given in Section 4. Fractions (9 ml) were collected at the flow rate of 1.8 ml/min. (B) Elution profiles of bamboo serine protease following Sephacryl S-200 gel-filtration of the active fractions from CM-Sepharose chromatography.

A protease from bamboo sprouts (2 kg) was purified 2360-fold with 38% (0.4 mg of the purified enzyme) recovery by a four-step procedure (see Table 1 for a summary). The purified protein migrated as a single band of M_r ca. 82 kDa on SDS–PAGE (Fig. 2). The M_r of this protease is greater than that of cucumisin [EC 3.4.21.25] (67 kDa) isolated from melon (*C. melo*) (Uchikoba et al., 1998). This protease (82 kDa) showed limited autolysis to produce a 66 kDa protein, which also had protease activity (Fig. 2).

The effect of pH on the stability of the protease from bamboo sprouts was determined (Fig. 3A). At least 80% of the activity remained for 2 h when incubated between pH 6.5 and 10.5. The thermal stability of the enzyme was examined by incubating at various temperatures for 10 min prior to assay at pH 7.0. Ninety percent of the initial activity was maintained at 40°C (Fig. 3B).

The effects of various inhibitors on the enzymatic activity using Suc-Ala-Ala-Pro-Phe-*p*NA as a substrate are shown in Table 2. The enzyme was completely inactivated by incubation with 2.0 mM DFP for 1 h at

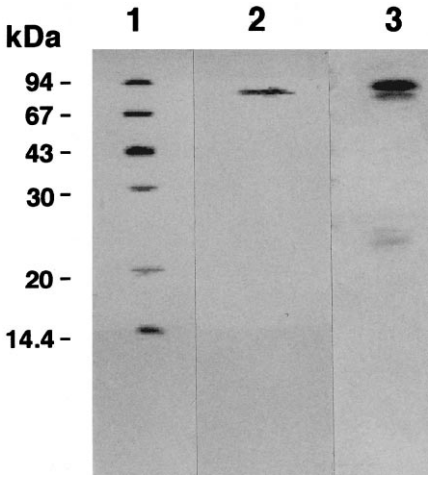

Fig. 2. SDS–PAGE analysis of bamboo serine protease. The gel was stained in Coomassie Brilliant Blue R-250 for 15 min and then destained. Lane 1: protein size markers; Lane 2: 5 μg of bamboo serine protease purified by Sephacryl S-200 gel-filtration. Lane 3: purified bamboo serine protease (10 μg) was incubated in 0.2 M Na, K–Pi buffer, pH 7.0, at 30°C for 30 min, and then subjected to SDS–PAGE analysis.

Table 1
Purification of bamboo sprout serine protease

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification factor (fold)	Recovery (%)
Crude extract from $(NH_4)_2SO_4$ ppt.	2450	6770	2.76	1	100
CM-cellulose	419	5200	12.4	4.5	77
CM-Sepharose	27	4140	153	55	61
Sephacryl S-200	0.39	2540	6510	2360	38

30°C. PMSF, PCMPS, and chymostatin inhibited enzyme activity, but inhibition by pefabloc SC was weak. Aprotinin, *p*-APMSF, and STI, which are serine protease inhibitors, had no effect on the enzymatic activity, nor did MIA, E-64, TLCK, TPCK, 1,10-phenanthroline, EDTA, and PCMPS. The results indicated that the bamboo protease is a serine protease having (a) cysteine residue(s) around the active site of the enzyme. The inhibitory effects observed for the enzyme (hereinafter referred to as 'bamboo serine protease') are consistent with those reported for cucumisin (Uchikoba et al., 1995).

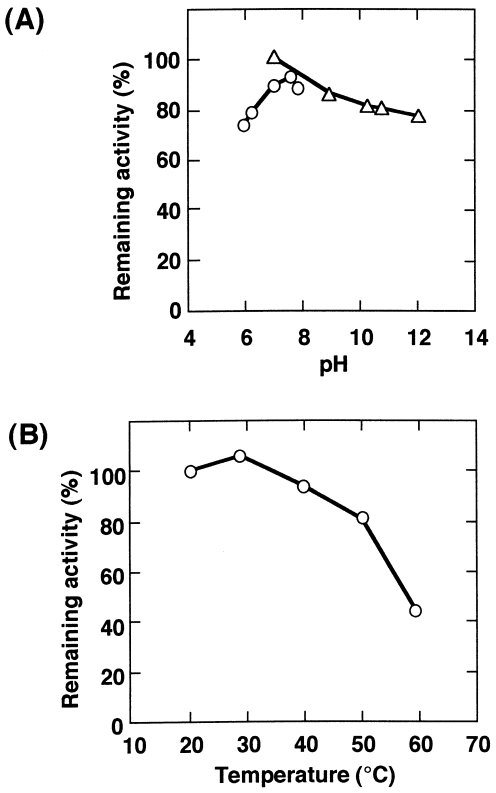

Fig. 3. Effect of pH and temperature on the stability of the protease. (A) The pH stability of the enzyme was determined followings incubation at various pH for 2 h. Buffers: 0.2 M Na, K–Pi (open circles, pH 6–8.5). 0.2 M sodium carbonate buffer (open triangles, pH 8.5–10). (B) Thermal stability of the enzyme. The protease solution was incubated at various temperatures for 10 min, and the residual activity was assayed.

The activity of bamboo serine protease toward several peptidyl-MCA substrates was detected (Table 3). The best substrate for the protease was Suc-Ala-Ala-Pro-Phe-MCA, which is also a good substrate for chymotrypsin. Suc-Leu-Leu-Val-Tyr-MCA was also hydrolyzed by this protease. However, Glt-Ala-Ala-Phe-MCA, a substrate for chymotrypsin, was not hydrolyzed by this protease. Ac-Tyr-Val-Ala-Asp-MCA, the substrate most favored by cucumisin, was only slightly hydrolyzed by bamboo serine protease. Suc-Ala-Pro-Ala-MCA, a substrate for elastase, was not hydrolyzed by this protease, nor were substrates that contained a charged residue, such as Arg or Glu at the P_1 position. Thus, the specificity of bamboo serine protease for peptidyl-MCA substrates differs from that of cucumisin.

Oxidised insulin B-chain was digested with bamboo serine protease at 30°C for 5 h, after which we determined the amino acid compositions of the resulting peptides. The sites of cleavage of the oligopeptides by bamboo serine protease were thus determined, and are summarized in Fig. 4. Nine cleavage sites on the oligo-

Table 2
Effect of various compounds on the caseinolytic activity of bamboo protease

Compound	Concentration[a] (mM)	Relative activity (%)
None	–	100[b]
DFP	2.0	0
PMSF	2.0	8
p-APMSF	2.0	104
Pefabloc-SC	2.0	89
TLCK	0.5	99
TPCK	0.5	102
Aprotinin	0.01	112
Chymostatin	0.1	1
Leupeptin	0.2	71
STI	0.01	91
PCMPS	2.0	34
MIA	2.0	102
Antipain	0.1	83
E-64	0.6	100
EDTA	2.0	96
1,10-Phenanthroline	2.0	98

[a] Final concentration.
[b] Results are for 1 h incubation at 30°C.

Table 3
Amidolytic activity of bamboo serine protease and cucumisin

Substrate	Relative activity (%)	
	Bamboo serine protease	Cucumisin
Suc-Ala-Ala-Pro-Phe-MCA	100[a]	69[b]
Suc-Leu-Leu-Val-Tyr-MCA	12	11
Ac-Tyr-Val-Ala-Asp-MCA	5	100
Boc-Asp(Obzl)-Pro-Arg-MCA	0	6
Boc-Val-Pro-Arg-MCA	0	0
Suc-Ala-Glu-MCA	0	6
Glt-Ala-Ala-Phe-MCA	0	2
Suc-Ala-Pro-Ala-MCA	0	0

[a] Activity with Suc-Ala-Ala-Pro-Phe-MCA was taken as 100%.
[b] Activity with Ac-Tyr-Val-Ala-Asp-MCA was taken as 100%.

peptide were detected. The residue at the P_1 position of five of these cleavage sites was a large hydrophobic residue, such as Tyr, Phe, Val, or Leu. The residue at the P_1 position of the other cleavage sites was an amide residue, such as Gln. The specificity of the bamboo serine protease differed from that of cucumisin, which cleaved at sites with charged amino acid residue and hydrophobic residue at the P_1 position.

The N-terminal sequence of the first 20 residues of bamboo serine protease was determined, and was compared to those of seven plant serine proteases: cucumisin, expressed in prince melon fruit (Yamagata et al., 1994); white gourd protease (Uchikoba et al., 1998); PR-P69 (Tornero et al., 1996) (also called P69A) and P69B (Tornero et al., 1997), pathogen-induced in tomato; ag12, expressed in the early stages of actinorhizal nodule formation in *A. glutinosa* (Rebeiro et al., 1995); *A. thaliana* protease (EMBL accession number X85974), and lily protease LIM9, induced in meiotic prophase (Kobayashi et al., 1994; Taylor et al., 1998) (Fig. 5). Five of the 10 N-terminal residues of bamboo serine protease are identical to those of cucumisin.

3. Discussion

In this study, a new protease purified from bamboo sprouts was characterized as a serine protease. Previous studies have identified proteases in the latex of several *Euphorbia* species, which were also found to be serine proteases (Lynn and Clevette-Radford, 1988); however, as no structural study of these enzymes has been conducted, the relation between the proteases of *Euphorbia* species and those of other plants is not known. Bamboo serine protease displayed enzymatic properties similar to proteases from *Euphorbia* species obtained by Lynn and Clevette-Radford (1988).

The M_r of bamboo serine protease is similar to that of the serine protease hevain 1 (80 kDa), isolated from latex of *Hevea brasiliensis* (Lynn and Clevette-Radford, 1986), The M_r of the other latex proteases from the *Euphorbia* species ranges from 60 to 80 kDa (Lynn and Clevette-Radford, 1988); M_r's of well-known plant serine proteases of the cucumisin family range from 60 to 70 kDa (Uchikoba et al., 1995; Tornero et al., 1996, 1997; Jordá et al., 1999; Meichtry et al., 1999; Ribeiro et al., 1995; Kaneda et al., 1997; Kim et al., 1997).

The optimum pH of bamboo serine protease using Suc-Ala-Ala-Pro-Phe-*p*NA as a substrate was neutral (data not shown), similar to the latex proteases from *Euphorbia* species, which show optimal activity between pH 6 and 8 (Lynn and Clevette-Radford, 1988). In contrast, cucumisin-like proteases possess an optimum pH in the range 8–10 (Uchikoba et al., 1995; Yonezawa et al., 1997).

Bamboo serine protease is less stable than cucumisin, with initial activity decreasing upon incubation at 40°C for 20 min. Conversely, cucumisin is very stable up to 60°C, pH 7.1, for 60 min, and even at pH 11.1, it retains about 80% activity (Uchikoba et al., 1995). Other peptidases detected in extracts from bamboo sprout were also unstable (data not shown).

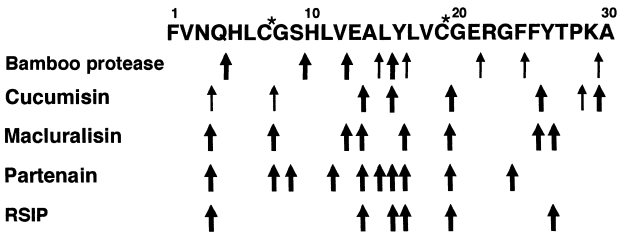

Fig. 4. Cleavage patterns of oxidised insulin B-chain by bamboo serine protease. The cleavage sites were identified from amino acid analysis of the digestive peptides as described in Section 4. C* indicates cysteic acid. The arrows show predicted split sites.

	1 10 20
Bamboo protease	**TTRTPSFLRL** SAV**G**RT**LPFV**
Cucumisin	**TTR**SWD**FL**GF PLTVPRRSQ**V** ···
White gourd	**TTR**SWD**FL**NF PQNIQRVNQ**V** ···
Lily LIM9	**TTHTP**DY**L**GI QTGVWPELGG ···
Arabidopsis	**TTRTP**L**FLGL** DEHTAD**L**FPE ···
Alnus ag12	**TTHTPRFLSL** NPT**G**GLW**P**AS ···
Tomato P69A	**TTHTSSFLGL** QQNMGVMKDS ···
Tomato P69B	**TTRSPTFLGL** EGRESRSFF**P** ···

Fig. 5. Comparison of the N-terminal amino acid sequences of various plant serine proteases: bamboo serine protease (this report), cucumisin (Yamagata et al., 1994), white gourd protease (Uchikoba et al., 1998), lily protease LIM 9 (Kobayashi et al., 1994; Taylor et al., 1998), *Arabidopsis* protease (EMBL accession number X85974), *Alnus* ag12 (Ribeiro et al., 1995), tomato PR-P69 (Tornero et al., 1996), and tomato P69B (Tornero et al., 1997). The eight sequences were aligned for maximum homology. The bold print shows those amino acid residues identical in the sequences. Numbering is according to that of bamboo serine protease.

Limited autolysis of bamboo serine protease occurred during incubation at 30°C for 30 min. Protein precursors of some serine proteases have both N- and C-terminal pro-sequences, e.g. cucumisin (Yamagata et al., 1994), aqualysin I (Kim et al., 1997), *Acromobacter* protease I (Ohara et al., 1989), and bacillopeptidase F (Wu et al., 1990). The C-terminal pro-sequence of aqualysin I is cleaved when the precursor (which has no N-terminal pro-sequence) is incubated at 65–80°C for several hours. The C-terminal pro-sequence of the proteases mentioned above might play an important role in the extracellular secretion of proteases (Kim et al., 1997). Cucumisin precursor (67 kDa) gradually generates the active form (54 kDa) during incubation at 60°C. It was shown that the active form (54 kDa) is derived from a limited autolysis by cucumisin itself (Yamagata et al., 1994). The mechanism of the limited autolysis of the bamboo serine protease requires further study.

The N-terminal sequence of bamboo serine protease showed some similarity to those of other plant serine proteases (Fig. 5). Proteases from the fruits of several Cucurbitaceae species were found to be serine proteases by Kaneda and Tominaga (1975) and Curotto et al. (1989). The amino acid residues common to cucumisin-like proteases (Uchikoba et al., 1998) were also conserved in the sequence of bamboo serine protease.

Sites of cleavage by bamboo serine protease in the oligopeptides were occupied by uncharged amino acid residues at the P_1 position. The cucumisin-like proteases preferentially cleaved the peptide bonds of the carboxyl side of the charged amino acids (Uchikoba et al., 1995). Cucumisin also hydrolyzed peptidyl-MCAs that had charged residues at the P_1 position as shown in Table 3. It did not appear that the specificity of bamboo serine protease for the oligopeptides was related to the residue on the carboxyl terminal side of the cleavage site. Leu11-Val12 and Leu17-Val18 of insulin B-chain which had hydrophobic residues at the site of P_1 position, were not hydrolyzed by the protease.

The enzyme activity in bamboo sprouts was greater in the tissue containing the vegetative shoot apex (upper side) than in opposing tissue (lower side) (data not shown). Cysteine-protease activity was also detected in the vegetative shoot apex (upper side) of asparagus spears (Yonezawa et al., 1998). Some proteases in the vacuole may help supply free amino acids during periods of rapid growth (Vierstra, 1996). It seems that bamboo serine protease may act along with other peptidases to supply a nitrogen source for a brief period of time when bamboo sprouts grow rapidly. The mechanism of efficient proteolysis in bamboo sprouts will be elucidated from the determination of the expression period, localization, and the amounts of these peptidases, with the view to further our understanding of bamboo sprout growth.

4. Experimental

4.1. Materials

Bamboo, *P. hindsii* Nakai, was cultured in Kagoshima, Japan. The sprouts of the plants were obtained within 3 days of germination. DEAE-cellulose was purchased from Whatman. CM-Sepharose was a product of Pharmacia. Casein was obtained from Merck. TLCK was a product of Sigma. TPCK was from Seikagaku Kogyo. Suc-Ala-Ala-Pro-Phe-*p*NA and peptidyl-MCAs were purchased from Peptide Institute, Osaka. Other materials and reagents were obtained from Wako Pure Chemical.

4.2. Assay of enzymatic activity

Enzyme solution, (0.2 ml) was added to 10 μl of 10 mM substrate Suc-Ala-Ala-Pro-Phe-*p*NA stock solution. in dimethylsulfoxide and 0.6 ml of 0.2 M Tris–HCl buffer (pH 7.0) at 25°C. The rate of enzymatic hydrolysis of substrates was followed spectrophotometrically with a Hitachi U-1100 spectrophotometer. An extinction coefficient of 8800 M^{-1} cm^{-1} at 410 nm was used to determine the yield of the hydrolysis product, *p*-NA. One unit of activity was defined as the activity giving 1 μmol of the product per minute under the above conditions.

The activity assay using peptidyl-MCAs was carried out as follows: buffer solution (67 mM K, Na–Pi, pH 7.0, 2.96 ml) was added to 10 μl of 10 mM substrate stock solution in dimethylsulfoxide at 30°C. Enzyme solution (30 μl) was then added to the substrate solution and the increase in fluorescence at 460 nm (excitation at 380 nm) was recorded in a Hitachi spectrofluorometer F-2000.

Proteolytic activity was measured with casein as a substrate by a method described previously (Yonezawa et al., 1998). A unit of activity is defined as giving 0.001*A* unit at 280 nm/min under the above conditions.

4.3. Electrophoresis and immunoblotting analysis

The SDS–PAGE method of Laemmli (1970) was followed, using 12.5% polyacrylamide gel. The purified enzyme was treated with 67 mM DFP for 30 min at 30°C to avoid autolysis. The diisopropyl-phosphorylated enzyme was analyzed by SDS–PAGE. The proteins were stained with Coomassie Brilliant Blue. The molecular standards used were phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43

kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa), and α-lactoalbumin (14.4 kDa) (Pharmacia).

4.4. Enzyme purification

All procedures for purification of the enzyme were performed at 7°C. Bamboo sprouts (2 kg) were homogenized with a domestic mixer in an appropriate volume of buffer A (20 mM Na, K–Pi buffer, pH 5.9). The homogenate was filtered through a cotton cloth, after which solid ammonium sulfate was added to the filtrate (2.5 l) to 60% saturation and kept for 24 h. After centrifugation (4000 × g for 30 min), the pellet was dissolved in the same volume of buffer A and dialyzed against the buffer. The dialysate was applied to a column of CM-cellulose (5 × 45 cm) equilibrated with buffer A, washed with buffer A (500 ml), and then eluted with buffer B (0.3 M Na, K–Pi buffer, pH 6.5). The active fractions eluted from the CM-cellulose column were collected and dialyzed against buffer A, and then chromatographed with a linear gradient to buffer B (0.16 M Na, K–Pi buffer, pH 6.5, 1 l), from buffer A (1 l) on a column of CM-Sepharose fast-flow (2.0 × 11 cm). The major peaks of proteolytic activity were combined, and then solid ammonium sulfate was added to the supernatant to 35% saturation. The enzyme solution from CM-Sepharose fast-flow was concentrated on a column of Butyl-Toyopearl (1.0 × 5 cm), equilibrated with 67 mM phosphate buffer (pH 7.0), containing ammonium sulfate (35% saturation), and eluted with buffer C (67 mM Na, K–Pi buffer, pH 7.0). The collected fractions from the Butyl-Toyopearl column were put on a Sephacryl S-200 gel-filtration column (2.4 × 132 cm), equilibrated with buffer C, and eluted. Proteolytic activity in each fraction was measured using Suc-Ala-Ala-Pro-Phe-*p*NA as a substrate. The active fractions were collected and kept at −20°C.

4.5. pH effects on the stability

To measure the pH stability of the enzyme, an incubation mixture consisting of 0.2 ml of enzyme solution (60 μg/ml) in various pH buffers was used. Following incubation at 25°C for 2 h, the residual activity of the enzyme was assayed after the solutions were made neutral with 0.5 M Tris–HCl buffer (pH 7.0).

4.6. Temperature effects on the stability

To examine the effects of temperature on enzyme stability, the residual activities were measured after the enzyme (0.5 ml) had been incubated in buffer C at various temperatures (20–65°C) for 10 min. The assay was carried out as described above.

4.7. Effects of compounds

The enzyme solution (0.5 ml, 1.8 μM) was added to 0.5 ml of inhibitor solution in buffer C and incubated at 30°C for 60 min. The remaining activity was measured as described above. A control assay was done under the same conditions without inhibitors.

4.8. N-Terminal sequencing analysis

Automated Edman degradation of the purified protease was performed with an Applied Biosystems 477A protein sequencer. The phenylthiohydantoin derivatives were identified with an Applied Biosystems 120A analyzer.

4.9. Hydrolysis of oligopeptides

Oxidised insulin B-chain (10 nmol, 25 μl) was digested with 0.1 nmol of bamboo protease in 25 μl of 0.1 M Tris–HCl buffer (pH 7.2) at 30°C. After incubation for 5 h, 10 μl of 0.1% TFA was added to the mixture to stop the reaction. The digested peptides were separated on a TSK-gel ODS-120T column (4.6 × 250 mm, Toso) with a linear gradient of 0–85% acetonitrile containing 0.1% TFA, using a Gilson HPLC system. The eluate was monitored by measuring the absorbance at 220 nm. For analysis of the amino acid composition, the purified peptides were hydrolyzed in 6 M HCl containing 0.1% phenol at 110°C for 24 h. The samples were derivatized with phenylisothiocyanate by using a Pico-Tag work station (Waters). Phenylthiocarbamyl derivatives of amino acids were analyzed with a pico-tag HPLC system. From the amino acid compositions of the resulting peptides, it was possible to locate each peptide in the primary structure of oxidised insulin B-chain.

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