Purification and Characterization of a Novel Cysteinyl-Specific Peptidase from Tainong 57 Sweetpotato Roots

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A cysteinyl-specific peptidase (CSPase) was successively purified from Tainong 57 sweetpotato roots by ammonium sulfate fractionation, two steps of column chromatography on DEAE-Sephadex, and one step of chromatofocusing column chromatography. The purified enzyme showed a single protein band on native disc-PAGE that was coincident with its activity. The molecular weight of the enzyme was estimated to e 233 000 by Sephacryl S-400 gel filtration and 59 000 by SDS–PAGE. The enzyme, accordingly, consisted of four identical subunits. Its p*I* was 4.3. The optimal pH and temperature of the CSPase for the activity assay were 6.0 and 37 °C, respectively. The enzyme was stable at pH 6.0–8.0 but was unstable when the pH was lower than 6.0. It was heat labile above 50 °C. The enzyme was completely inhibited by DFP and partially inhibited by PMSF, TLCK, iodoacetic acid, bestatin, and leupeptin. The CSPase could specifically hydrolyze cysteine-containing peptides. Oxytocin, a hormone peptide, was also hydrolyzed by the enzyme.

Keywords: *Cysteinyl-specific peptidase; sweetpotato; purification*

INTRODUCTION

Characterizing and understanding the biological functions of proteases are currently active fields of study. Roles of proteases are considered to be in posttranslational processing, protein turnover, activation and inactivation of specific proteins, nutrient supplementation, morphogenesis and differentiation in microorganisms, and pathogen resistance (North, 1982).

In plant tissues, most research works on proteolysis have centered on the role of proteases in the mobilization of reserve proteins and in the regulation of protein metabolism. Protein degradation in plants is important because it is linked with various physiological phenomena such as seed germination (Doi *et al.*, 1980; Gang and Virupakslia, 1980; Vodkin and Scandalios, 1980; Mikola, 1986; Mitsuhashi *et al.*, 1986), seedling development (Kaneda *et al.*, 1986; Kitamura and Maruyama, 1986), chloroplast development (Aducci *et al.*, 1986), leaf senescence (Lynn and Clevette-Radford, 1985), and hormonal regulation (Walker-Simmons *et al.*, 1984; Hammerton and Ho, 1986).

Sweetpotato is a major economic crop in Taiwan. One of the authors has reported the occurrence and some properties of trypsin inhibitors in sweetpotato roots (Lin and Chen, 1980; Lin *et al.*, 1988; Lin, 1989). To illustrate the physiological functions of the trypsin inhibitors, the proteolytic system of the sprouts and the storage roots of sweetpotato were also studied (Lin and Chu, 1988; Chen and Lin, 1989). The activities of endopeptidases (Lin and Chu, 1988; Lin and Chan, 1990), aminopeptidases (Chen and Lin, 1989), tripeptidases (Lin and Wang, 1990), and carboxylpeptidases (Lin and Tsai, 1991) have been investigated. These proteases are considered to participate in protein metabolism before and after sprouting of the stored roots of sweetpotato.

Among the detected proteases, a cysteinyl-specific peptidase (CSPase) which could specifically cleave *S*-benzylcysteinyl-*p*-nitroanilide (Cys-pNA) was further studied. According to our limited knowledge, peptidases that specifically recognize cysteinyl residues are rarely reported, except for oxytocinase (EC 3.4.11.3) (Lampelo and Vanha-Perttula, 1980; Gopalaswamy *et al.*, 1984; Krishna and Kanagasabapathy, 1989; Roy *et al.*, 1989). Therefore, we are interested in the existence and the physiological functions of CSPase in the stored roots of the Tainong 57 sweetpotato. This study aims to purify and characterize some basic properties of the enzyme.

MATERIALS AND METHODS

Materials. *S*-Benzyl-L-cysteinyl *p*-nitroanilide (Cys-pNA), alanyl *p*-nitroanilide (Ala-pNA), arginyl *p*-nitroanilide (Arg-pNA), glutamyl *p*-nitroanilide (Glu-pNA), benzoyltyrosinyl *p*-nitroanilide (Bz-Tyr-pNA), prolyl *p*-nitroanilide (Pro-pNA), cysteinyl methyl ester, *N*-carbobenzoxyl-*S*-benzylcysteine (*N*-CBZ-*S*-benzyl-Cys), cystinyl dimethyl ester, cystinyl diethyl ester, oxytocine, glutathion, oxidized insulin A and B chains, acrylamide, ammonium persulfate, bis(acrylamide), phenylmethanesulfonyl fluoride (PMSF), diisopropyl fluorophosphate (DFP), tosyllysine chloromethyl ketone (TLCK), tosylphenylalanine chloromethyl ketone (TPCK), bestatin, E-64, iodoacetic acid, *o*-phenanthroline, and leupeptin were bought from Sigma (St. Louis, MO). Cystinyl bis(*p*-nitroanilide) (2Cys-bpNA) was purchased from Merck (Darmstadt, Germany). The molecular weight calibration kits for gel filtration, isoelectrofocusing, and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) were obtained from Bio-Rad (Hercules, CA). DEAE-Sephadex, PBE 94 gel, and Polybuffer 74 for chromatofocusing, Sephacryl S-400, and the prepared gels and the buffer strips for Phastsystem were the products of Pharmacia Biotech (Uppsala, Sweden).

Storage roots of the sweetpotato [*Ipomoea batatas* (L.) Lam, cv. Tainong 57] were obtained from a local market in Taipei.

Enzyme Extraction. All operations were carried out at 4 °C. Peeled storage roots of the sweetpotato (250 g) were cut into pieces and homogenized in 1000 mL of sodium phosphate buffer (0.01 M, pH 7.0, containing 0.7 mM 2-mercaptoethanol;

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referred as buffer P hereinafter) with a commercial fruit blender and then with a Polytron homogenizer (Kinematic, Switzerland) at 7000 rpm for 3 min. The homogenate was filtered with four layers of cheesecloth, and the filtrate was centrifuged at 8000 rpm for 30 min (Beckman, J2-21M/E centrifuge, JA14 rotor). The supernatant was collected and used as crude enzyme extract.

Assay of CSPase Activity. The CSPase assay was carried out at pH 7.0 using Cys-pNA as a substrate. One gram of Cys-pNA was dissolved in 10 mL of 30% *N,N*-dimethylformamide solution (diluted with deionized water, v/v) to give a concentration of 10% (w/v) first and then diluted with deionized water to a final concentration of 1.0 mM. Added 0.2 mL of a suitable diluted crude extract (0.2 mL) and the substrate (0.3 mL) were added to 0.5 mL of buffer P, then mixed well, and incubated at 37 °C for 4.5 h. The reaction was stopped by adding 0.5 mL of acetic acid solution (5%). The released pNA was measured at 410 nm. In the control assay, the enzyme solution was replaced by an equal volume of buffer P. All of the assays were in triplicate. One unit of the enzyme activity was defined as the amount of the enzyme that can hydrolyze the substrate and release 1 millimole of pNA within 1 h under the experimental conditions. Substrates other than the pNA-containing ones were measured with the ninhydrin reagent (Moore and Stein, 1954).

Protein Determination. Protein concentration was determined according to the Lowry method (Lowry *et al.*, 1951), and bovine serum albumin was used as standard.

Purification of CSPase. All operations were carried out at 4 °C.

Step 1. Ammonium Sulfate Fractionation. The fine ground powder of ammonium sulfate was added to the crude enzyme extract to a concentration of 40% saturation with stirring. Any precipitate was removed by centrifugation at 8000 rpm for 30 min, and the fine ammonium sulfate powder was added again to the supernatant to a final concentration of 80% saturation with stirring. This fraction was left for at least 1 h, and the precipitate was recovered by centrifugation at 8000 rpm for 30 min. The precipitate was redissolved in a minimum volume of buffer P and then dialyzed for 24 h against the same buffer.

Step 2. First DEAE-Sephadex Column Chromatography. The dialyzed enzyme solution was applied to a DEAE-Sephadex column (2.4 by 30 cm) which had been equilibrated with buffer P. After the column was washed with 500 mL of the same buffer, the CSPase was eluted with a linear gradient of 0–1.0 M NaCl in the same phosphate buffer (500 mL in each reservior) at a flow rate of 20 mL/h. Fractions (5 mL) were collected and assayed for the CSPase activity. The fractions containing the major enzyme activity were pooled and dialyzed against buffer P.

Step 3. Second DEAE-Sephadex Column Chromatography. The desalted CSPase solution was applied to the second DEAE-Sephadex column (2.4 by 30 cm) which had been equilibrated with buffer P. After the column was washed with the same buffer, the CSPase activity was eluted with a gradient of 0.1–0.5 M NaCl in the same buffer (400 mL in each reservoir) at a flow rate of 20 mL/h. Fractions (4 mL) were collected and assayed for the CSPase activity. Fractions containing the enzyme activity were pooled and dialyzed against buffer P for further purification.

Step 4. Chromatofocusing Column Chromatography. The dialyzed enzyme solution was equilibrated with piperazine–HCl buffer (pH 5.5) and applied to a chromatofocusing column (1.5 by 20 cm) which had been packed with PBE 94 gel and equilibrated with piperazine–HCl buffer, pH 5.5. The enzyme was eluted with a diluted Polybuffer 74 (1:8 v/v) at a linear pH gradient from 5.5 to 4.0. The flow rate was 15 mL/h. Fractions (4 mL) were collected and assayed for the CSPase activity. The enzyme activity eluted from the column was precipitated by adding the fine ammonium sulfate powder to give a 100% saturation. The resultant precipitate was collected by centrifugation, then redissolved in a minimum volume buffer P, and centrifuged again to remove the insoluble materials. The enzyme fraction was dialyzed against the same buffer for 24 h.

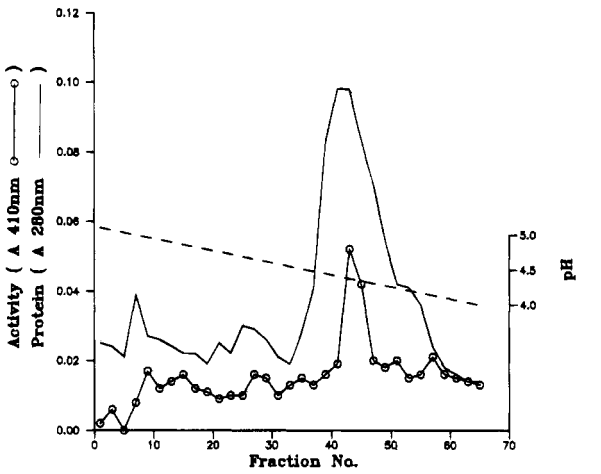

Figure 1. Chromatofocusing of CSPase. The pooled CSPase activity fractions, obtianed from the second DEAE-Sephadex chromatography, were applied to a column of PBE 94 (1 by 20 cm). CSPase was eluted at a flow rate of 15 mL/h using a pH gradient (– –) by 8 bed volumes of Polybuffer (pH 5.5 to 4.0). Fractions of 4.0 mL were collected. Protein (—) was monitored at 280 nm. (○) Activity of CSPase.

Electrophoresis. Discontinuous polyacrylamide gel electrophoresis (disc-PAGE) was carried out with 7.5% gel at pH 8.3 and 4 °C according to the method of Davis (1964). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed in 7.5% gel (Weber and Osborn, 1968).

Purity Identification. Purity of the obtained CSPase was tested by a native disc-PAGE at 7.5% polyacrylamide gel (pH 8.3) (Davis, 1964). After electrophoresis, one gel was stained with Coomassie brilliant blue R-250, while the other was cut into 2-mm slices, and each gel slice was extracted with buffer P. The enzyme activity of each extract was determined.

Estimation of Molecular Weight. The molecular weight of the purified CSPase was determined by Sephacryl S-400 gel filtration (Andrews, 1964) and SDS–PAGE (Weber and Osborn, 1969). The Sephacryl S-400 gel filtration column (2.0 by 90 cm) was equilibrated and eluted with buffer P at a flow rate of 20 mL/h. Fractions of 4.0 mL were collected and assayed for CSPase activity. Ferritin (molecular weight 450 000), catalase (molecular weight 233 000), and aldolase (molecular weight 160 000) were used as standard markers. The molecular weight of the enzyme subunits was estimated by SDS–PAGE and calibrated with bovine serum albumin (molecular weight 67 000), ovalbumin (molecular weight 45 000), and myoglobin (molecular weight 17 800) as standard proteins.

RESULTS AND DISCUSSION

Purification. The CSPase was purified from the storage roots of the sweetpotato cultivar Tainong 57 using ammonium sulfate fractionation, two steps of DEAE-Sephadex, and chromatofocusing chromatographies. From the activity and protein profiles of the chromatofocusing chromatography, the enzyme activity peak was coincident with the protein peak, indicating that the enzyme has been purified (Figure 1). Homogeneity of the purified CSPase was identified by native disc-PAGE. Only a single protein band was detected after electrophoresis (Figure 2A). According to the enzyme activity assay of the sliced gels, the CSPase activity was coincident with the position of the single protein band (Figure 2B). The purified preparation gave a single protein band on SDS–PAGE (Figure 3, inset A). These results indicate the homogeneity of the purified enzyme. The purification of this enzyme is summarized in Table 1. The enzyme was purified about 20-fold relative to the crude extract. From 250 g of storage roots of Tainong 57 sweetpotato, which was

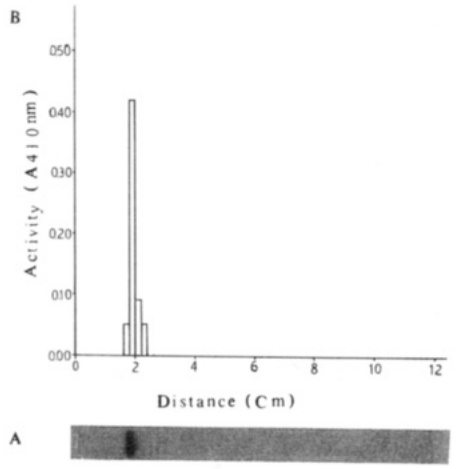

Figure 2. Disc-PAGE of CSPase under nondenaturing conditions. Purified CSPase (20 μg) was applied to the top of the gel, and electrophoresis was carried out as described under Materials and Methods. (A) One gel was stained for protein. (B) A second gel was cut into 2-mm slices, and each sliced gel was assayed for CSPase activity.

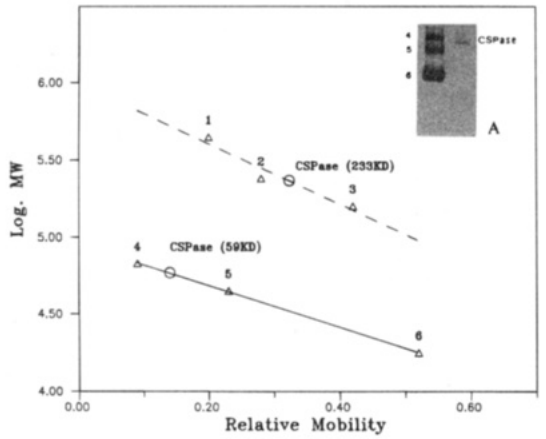

Figure 3. Molecular weight determination of the purified CSPase on Sephacryl S-400 gel filtration (– –) and SDS–PAGE (—, and inset A). 1, ferritin (molecular weight 450 000); 2, catalase (molecular weight 233 000); 3, aldolase (molecular weight 160 000); 4, bovine serum albumin (molecular weight 67 000); 5, ovalbumin (molecular weight 45 000); 6, myoglobin (molecular weight 17 800). (○) CSPase.

Table 1. Purification of CSPase from Storage Roots of Tainong 57 Sweetpotato

purification step	total act. (U)[a]	total protein (mg)	specific act.[b]	yield (%)	purification (fold)
crude extract[c]	3310	3462	0.956	100	1.0
ammonium sulfate fraction (40–80%)	2103	1195	1.76	64	1.8
1st DEAE-Sephadex	1674	593	2.82	51	2.9
2nd DEAE-Sephadex	1296	233	5.56	39	5.8
chromatofocusing	1158	62	18.7	35	19.6

[a] Unit activity was calculated as μmole of pNA released per h. [b] Specific activity was calculated as unit per mg of protein. [c] The crude extract was obtained from 250 g of resting roots of the Tainong 57 sweetpotato which had been extracted with 1000 mL of the sodium phosphate buffer (0.01 M, pH 7.0 containing 0.7 mM 2-mercaptoethanol).

extracted with phosphate buffer, 62 mg of the CSPase was obtained. The final yield of the CSPase was about 35%.

Substrate Specificity. The relative rates of hydrolysis toward various substrates are summarized in Table 2. As can be seen from this table, the enzyme has a fairly high substrate specificity toward the cysteinyl- and cystinyl-containing substrates. Among the substrates tested, Cys-pNA was the most favorable. Although 2Cys-bpNA offers two susceptible cleavage sites for the CSPase, the relative rate of the 2Cys-bpNA is only half that of the Cys-pNA. A similar result is also observed with the substrates of cysteine methyl ester and cystinyl dimethyl ester. The relative activity of the latter is only a fourth that of the former. Besides, the CSPase did not hydrolyze Ala-pNA, *N*-CBZ-*S*-benzyl-Cys, glutathione, Glu-pNA, Pro-pNA, Gly-Pro-pNA, Ala-Ala-pNA, Z-Leu-pNA, and insulin oxidized A chain and B chain, indicating that the enzyme had a highly specific affinity for the N-terminal free cysteinyl residues. An aminopeptidase has been isolated from the sprouts of the Tainong 64 sweetpotato (Chen and Lin, 1989). The enzyme had a broad substrate specificity which is different from the CSPase. Since some unique substrates of the oxytocinase (EC 3.4.11.3) such as Cys-pNA and oxytocine were also hydrolyzed by the CSPase, the enzyme may be considered as an oxytocinase-like enzyme. However, the molecular weight, subunit composition, and some enzymatic properties of the CSPase are different from those reported for oxytocinases from mammalian tissues (Krishna and Kanagasabapathy, 1989; Roy *et al.*, 1989; Gopalaswamy *et al.*, 1984; Lampelo and Vanha-Perttula, 1980). The physiological functions of the enzyme in the roots of sweetpotato are not clear and are being studied in our laboratory.

Table 2. Substrate Specificity of CSPase[a]

substrate (1.0 mM)	rel act. (%)	substrate (1.0 mM)	rel act. (%)
Cys-pNA	100	Cys-methyl ester	91
Arg-pNA	0	*N*-Cbz-*S*-benzyl-Cys	0
Ala-pNA	0	cystine dimethyl ester	23
Glu-pNA	0	cystine diethyl ester	21
Pro-pNA	0	gluthathione	0
Bz-Tyr-pNA	0	oxytocin	60
Gly-Pro-pNA	0	insulin chain A (oxidized)	0
2Cys-bpNA	50	insulin chain B (oxidized)	0

[a] The purified CSPase (0.6 μg) was incubated with each substrate (1.0 mM) in 0.01 mM sodium phosphate buffer containing 0.7 mM 2-mercaptoethanol, pH 7.0, at 37 °C for 4.5 h. For the *p*-nitroanilide-containing substrates, the released pNA was measured at 410 nm; and for the non-pNA-containing substrates, the released free amino acid was measured at 570 nm with the ninhydrin reagent. The assay methods were described under Materials and Methods.

Molecular Weight, Subunit Composition, and p*I*. According to the calibration curve for the molecular weight determination of the purified CSPase on Sephacryl S-400 gel filtration and SDS–PAGE, the molecular weight of the CSPase was estimated to be 233 000 and 59 000, respectively (Figure 3). Consequently, the enzyme is considered to be a tetramer consisting of four identical subunits. A high molecular weight tripeptidyl peptidase has been isolated from the sweetpotato roots (Lin and Wang, 1990); however, the enzyme was a monomer of 840 000, which is different from the purified CSPase. The aminopeptidase of the Tainong 64 sweetpotato was also a monomer of 63 000 (Chen and Lin, 1989). From the activity profile of the chromatofocusing chromatography (Figure 1), the p*I* of the CSPase was estimated to be 4.3, which was coincident with the result of isoelectrofocusing gel electrophoresis (data not shown). The p*I* value is lower than those reported for proteases of potato and sweetpotato (Kitamura and Maruyama, 1986; Chen and Lin, 1989; Lin and Wang, 1990).

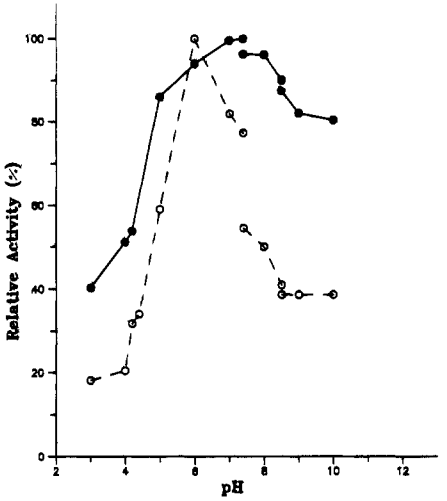

Figure 4. Effect of pH on the activity and stability of CSPase. The optimal pH of CSPase (○) was measured with the following buffer systems: pH 2.6–7.6, 10 mM McIlvaine buffer; pH 7.0–9.0, 10 mM Tris-HCl buffer; and pH 8.0–10.6, 10 mM glycine–NaOH buffer. The stability of CSPase (●) was measured by preincubating the enzyme at the pH range at 4 °C for 24 h and then, after each solution was adjusted to pH 7.0, adding the substrate Cys-pNA to assay the remaining activity as described under Materials and Methods.

Optimal pH and Stability. The effect of pH on the CSPase activity was determined at pH values ranging from 3.0 to 10.4. The optimal pH of the enzyme was at pH 6.0 when using Cys-pNA as the substrate (Figure 4). The effect of pH on the enzyme stability was measured by incubating the CSPase at 4 °C under various pH conditions for 24 h and assaying the remaining enzyme activity after the samples were adjusted to pH 7.0. The results showed that the CSPase was stable in the pH range 6.0–8.0 but lost its activity rapidly at pH below 6.0 (Figure 4). The optimal pH and pH stability of the CSPase are similar to those of the peptidases of sweetpotatoes (Lin and Chu, 1988; Chen and Lin, 1989; Lin and Wang, 1990), which may suggest that these proteases are participating in protein metabolism under physiological conditions. However, the protease of potato had a lower optimal pH (5.0–6.0) and was stable at pH 4.5–6.5 (Kitamura and Muruyama, 1986), different from the enzymes of sweetpotatoes.

Optimal Temperature and Thermal Stability. The optimal temperature of the CSPase was observed at 37 °C (Figure 5). The enzyme was incubated at various temperatures for 30 min and then quickly cooled at 4 °C. The remaining activity was assayed at the standard conditions. The results showed that the enzyme was stable at temperatures below 40 °C but lost its activity rapidly at temperatures higher than 45 °C and was completely inactivated at temperatures higher than 50 °C (Figure 5). It is revealed that the enzyme is liable to heat. The optimal temperature and thermal stability of the CSPase are similar to those of the aminopeptidase and the tripeptidyl peptidase of the sweetpotato tubers (Chen and Lin, 1989; Lin and Wang, 1990). This heat-labile property of the purified CSPase may result from the dissociation or denaturation of the subunits during incubation.

Effect of Metal Ions. The effect of some metal ions on the activity of the CSPase was studied by adding the cations in the standard assay conditions at a final concentration of 1.0 mM each. The results are shown in Table 3. The enzyme was partially inhibited by Hg^{2+} and slightly inhibited by Mg^{2+}, Cd^{2+}, Zn^{2+}, and Ca^{2+}.

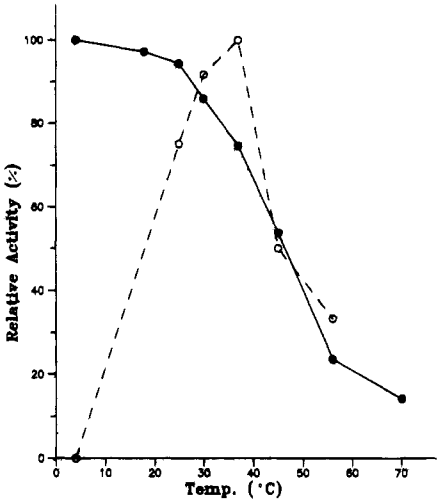

Figure 5. Effect of temperature on the activity and stability of CSPase. The optimal temperature of CSPase (○) was measured by incubating the enzyme at the temperature range. The stability of CSPase (●) was measured by preincubating the enzyme at the temperature for 30 min and adding the substrate Cys-pNA to assay the remaining activity as described under Materials and Methods.

Table 3. Effects of Metal Ions on CSPase Activity[a]

metal ion (1.0 mM)	rel act. (%)	metal ion (1.0 mM)	rel act. (%)
none	100	Zn^{2+}	75
Ca^{2+}	85	Cu^{2+}	104
Cd^{2+}	73	Hg^{2+}	51
Mg^{2+}	69		

[a] The purified CSPase (0.6 μg) was incubated with each metal ion (1.0 mM) which was used as chloride salt in 0.01 M sodium phosphate buffer containing 0.7 mM 2-mercaptoethanol, pH 7.0, at 37 °C for 4.5 h. The remaining activity was measured as described under Materials and Methods.

Table 4. Effects of Various Reagents on CSPase Activity[a]

reagent (1.0 mM)	rel act. (%)	reagent (1.0 mM)	rel act. (%)
none	100	leupeptin	40
EDTA	109	bestatin	41
o-phenanthroline	71	DFP	0
iodoacetic acid	46	PMSF	19
2-mercaptoethanol	103	TLCK	46
cysteine	72	TPCK	78
E-64	104		

[a] The purified CSPase (0.6 μg) was incubated with each effector (1.0 mM) in 0.01 M sodium phosphate buffer containing 0.7 mM 2-mercaptoethanol, pH 7.0, at 37 °C for 4.5 h. The remaining activity was measured as described under Materials and Methods.

Cu^{2+} did not affect the activity of the purified CSPase. None of the tested metal ions could activate the enzyme activity. These results support the inhibitory results of chelating agents EDTA and *o*-phenanthroline, which had no or only slight effect on the CSPase activity. Obviously the enzyme does not require metal ions as cofactor. The inhibition of those metal ions may be due to the damage of the active site of the enzyme. The tripeptidyl peptidase of sweetpotato was greatly inhibited by Ag^{+} and Cu^{2+} (Lin and Wang, 1990), and the cysteine proteinase of the sprouting potato tubers was strongly inhibited by Zn^{2+} and some heavy metal ions (Kitamura and Maruyama, 1986).

Effect of Inhibitors. The effect of some protease-specific inhibitors on the enzyme activity was studied by adding the inhibitors in the standard assay condi-

tions at final concentrations of 1.0 mM each. The results are shown in Table 4. Considering the effect of the protease-specific inhibitors on the purified CSPase, we found the enzyme was not inhibited by EDTA, 2-mercaptoethanol, and E-64; slightly inhibited by *o*-phenanthroline, cysteine, TPCK; greatly inhibited by iodoacetic acid, leupeptin, bestatin, TLCK, and PMSF; and completely inhibited by DFP. EDTA and *o*-phenanthroline, chelating agents, had no or slight inhibition on the CSPase activity, indicating that the enzyme is not a metalloprotease. E-64, a sulfhydryl group inhibitor, showed no effect on the CSPase; however, iodoacetic acid, leupeptin, and cysteine showed significant inhibition. Since E-64 is an effective irreversible inhibitor for cysteine proteases without affecting cysteine residues in other enzymes, the CSPase is not a cysteine protease and the other sulfhydryl inhibitors may inhibit the CSPase via disturbing the active site or the structure of the enzyme. Bestatin, an inhibitor of cysteine-type alanyl aminopeptidase, gave 60% inhibition on the CSPase activity, suggesting that the CSPase is an aminopeptidase. The serine residue blocking agents such as DFP, PMSF, and TLCK could effectively inhibit the enzyme activity, revealing that the CSPase is a serine protease. Because the inhibition by TLCK was stronger than that by TPCK, the CSPase is probably a trypsin-type rather than a chymotrypsin-type protease. According to the inhibition study and the substrate specificity, the purified CSPase is a trypsin-type serine aminopeptidase with a novel substrate specificity for cysteine-containing substrates.

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