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# A serine endopeptidase from cucumber leaves is inhibited by L-arginine, guanidino compounds and divalent cations

Yasuo Yamauchi[a,*], Toshio Sugimoto[b], Kuni Sueyoshi[b,1], Yoshikiyo Oji[b], Kiyoshi Tanaka[a]

[a]*Laboratory of Plant Biotechnology, Faculty of Agriculture, Tottori University, Koyama, Tottori 680-8553, Japan*
[b]*Department of Biological and Environmental Sciences, Faculty of Agriculture, Kobe University, Rokkodai 1-1, Nada-ku, Kobe 657-8501, Japan*

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**Abstract**

An endopeptidase was purified and characterized from green leaves of cucumber (*Cucumis sativus* L. suyo). The purified enzyme, a basic amino acid-specific endopeptidase with a pI of 5.0, was a monomeric protein of 80 kDa whose pH optimum was 9.5. Inhibitor analysis suggested that it was a serine endopeptidase and contained sulfhydryl groups essential for catalytic activity. Analysis of internal amino acid sequences of the endopeptidase showed no significant similarity to other proteins. Its activity was inhibited by L-Arg and guanidino compounds having high hydrophobicity, as well as divalent cations such as $Mg^{2+}$ and $Ca^{2+}$. The $K_i$ values of L-Arg and $Mg^{2+}$, which are also likely in vivo inhibitors, were 3.5 and 10 mM, respectively. Inhibition by L-Arg and $Mg^{2+}$ was additive, and more than 70% of the activity was reversibly inhibited under their physiologically significant concentrations. These results suggest that the enzyme is possibly regulated by L-Arg and/or guanidino compounds, and by divalent cations in vivo. 

*Keywords:* *Cucumis sativus*; Cucurbitaceae; Serine endopeptidase; Trypsin-like; Inhibition; Arginine; Guanidino compound; Magnesium

## 1. Introduction

In all organisms, protein turnover is a highly regulated, selective process. It is well known that the mammalian cell contains highly regulated protein degradation systems such as ubiquitin-proteasome in the cytosol (Hershko and Ciechanover, 1992) and the $Ca^{2+}$, calpain, and calpastatin systems (Sorimachi and Suzuki, 1998). Many reports also imply the existence of highly regulated proteolytic systems in plants, but only a few have described the role played by plant endopeptidases in regulation of protein turnover (Vierstra, 1993; von Kampen et al., 1996).

It is difficult to investigate endopeptidases since they are both present at low levels and occur in multiple isoforms. To overcome this problem, we have developed both polyacrylamide gel electrophoretic and activity staining methods to separate and detect low proteolytic activities, as well as a method for detection of measurable endopeptidase activities using artificial substrates (Yamauchi et al., 1995). These techniques were used to detect two major endopeptidases that degraded benzoyl (Bz)-Arg-naphthylamide (NA) and carbobenzoxy (CBZ)-Leu-Leu-Glu-NA, respectively (Yamauchi et al., 1996). This report describes the purification and characterization of the Bz-Arg-NA degrading endopeptidase (CEP 5.0). The enzyme was a basic amino acid-specific endopeptidase that is plausibly regulated by L-Arg and/or guanidino compounds and divalent cations whose cellular concentrations may change with environmental conditions.

## 2. Results and discussion

The Bz-Arg-NA degrading endopeptidase was purified to apparent homogenets by successive chromatography on DEAE-Toyopearl, hydroxyapatite, phenyl-

*Abbreviations:* Bz, benzoyl; CBZ, carbobenzoxy; CEP, cucumber endopeptidase; 2-ME, 2-mercaptoethanol; PB, phosphate buffer; NA, naphthylamide

* Corresponding author. Tel.: +81-857-31-6702; fax: +81-857-31-6702.

*E-mail address:* yamauchi@muses.tottori-u.ac.jp (Y. Yamauchi).

[1] Present address: Department of Applied Biological Chemistry, Faculty of Agriculture, Niigata University, Niigata 950-2181, Japan.

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sepharose, Arg-sepharose, and mono Q columns (Table 1). The endopeptidase was named CEP 5.0 because the purified enzyme migrated as a single band (pI = 5.0) when submitted to nondenaturing isoelectric focusing (Fig. 1A). The molecular mass of CEP 5.0 was 80 kDa as estimated by both SDS-PAGE and gel filtration (Fig. 1B and C), suggesting that CEP 5.0 was an 80 kDa monomeric protein. The enzyme, with an optimum pH of around 9.5, showed half-maximal activities at pH7.4 and 10.5. CEP 5.0 was most active at 50 °C and was completely denatured when incubated at 70 °C (data not shown).

As shown in Table 2, diisopropyl fluorophosphate and phenylmethylsulfonyl fluoride reduced the activity of CEP 5.0 as did leupeptin, suggesting that CEP 5.0 is a serine endopeptidase. Enzyme inhibitors such as *p*-chloromercuribenzoic acid and *N*-ethylmaleimide also inhibited CEP 5.0. This suggests that CEP 5.0 contained sulfhydryl groups essential to its catalytic activity.

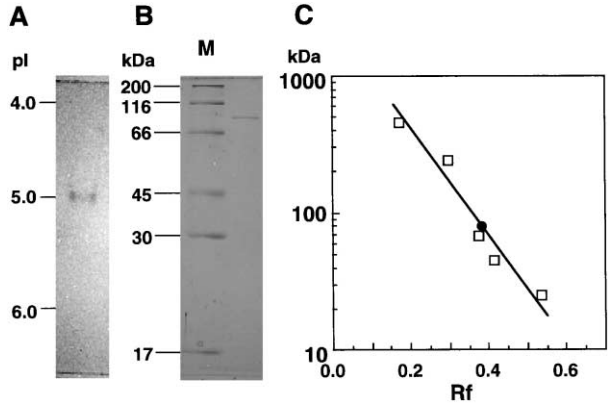


Fig. 1. Estimation of pI and molecular mass of CEP 5.0: (A) 1 μg of purified CEP 5.0 was subjected to isoelectric focusing on a 7.5% (w/v) acrylamide, 2% SERVALYT (pH 4.0–6.0) gel; (B) 1 μg of purified CEP 5.0 was subjected to SDS-PAGE on a 13% (w/v) acrylamide gel under reducing conditions. The molecular mass of protein markers are given in the lane M. (C) Plot of the $R_f$ values against log molecular mass for CEP 5.0 (●) and marker proteins (□) on Superose 12 column. Marker proteins were ferritin (450 kDa), catalase (240 kDa), bovine serum albumin (67 kDa), egg albumin (45 kDa), and chymotrypsinogen A (25 kDa).

The substrate specificity of CEP 5.0 was determined to be trypsin-like by using various artificial substrates. The enzyme cleaved artificial substrates such as Bz-Arg-NA and Bz-Lys-*p*-nitroanilide, but did not cleave those diagnostic for aminopeptidase, caspase, cathepsin D, chymotrypsin, elastase, glutamyl endopeptidase and subtilisin (data not shown). Plant serine endopeptidases of 80–82 kDa whose pH optimum are at alkaline pH, have been isolated from ragweed, *Euphorbia supina* and bamboo (Bagarozzi et al., 1996; Arima et al., 2000a,b); however, their substrate specificities are quite different from CEP 5.0, i.e. they have chymotyrpsin-like or subtilisin-like activities. CEP 5.0 degraded gelatin, but did not degrade azocasein, carboxymethyl-BSA, acid-denatured haemoglobin and Rubisco (data not shown).

Since amino acid sequencing of the N-terminus of CEP 5.0 showed that it was blocked, internal amino acid sequences were analyzed after CNBr cleavage treatment of purified CEP 5.0 and separation of the cleaved peptides by SDS-PAGE. The N-terminal amino acids sequences of a 25 and a 20 kDa polypeptide were "FSLDLEAQE" and "PTGPQAQQE", respectively. Homology search of the Swissplot and translated EMBL databases revealed

Table 2
Effects of inhibitors on CEP 5.0 activity

| Inhibitor | Concentration | Relative activity (%) |
|---|---|---|
| Control | – | 100[a] |
| Diisopropyl fluorophosphate | 1 mM | 6.8 |
| | 10 mM | 0 |
| Phenylmethylsulfonyl fluoride | 1 mM | 79.3 |
| | 10 mM | 22.4 |
| *p*-Chloromercuri benzoic acid | 0.5 mM | 42.9 |
| | 2.5 mM | 25.3 |
| *N*-Ethylmaleimide | 1 mM | 21.3 |
| | 10 mM | 11.6 |
| Leupeptin | 10 μM | 0.9 |
| Pepstatin A | 10 μM | 78.4 |
| Soybean trypsin inhibitor | 0.1% | 127 |
| SDS | 0.1% | 0 |

[a] Activity in the absence of inhibitor is defined as 100% (0.28 units of Bz-Arg-NA cleaving activity).

Table 1
Summary of purification of CEP 5.0

| Step | Total protein (mg) | Total activity (Units)[a] | Specific activity (Units/mg) | Purification-fold | Yield (%) |
|---|---|---|---|---|---|
| Crude extract | 4430 | 89 090 | 22.15 | 1.0 | 100 |
| 45–55% Ammonium sulfate | 653.6 | 82 600 | 130 | 5.87 | 84.2 |
| DEAE-Toyopearl | 20.16 | 25 980 | 1289 | 58.2 | 26.5 |
| Hydroxyapatite | 7.26 | 21 500 | 2960 | 133.7 | 21.9 |
| Phenyl-sepharose | 1.46 | 10 600 | 7274 | 328.5 | 10.8 |
| Arg-sepharose | 0.917 | 12 880 | 14 050 | 634.6 | 13.1 |
| MonoQ | 0.0245 | 1627 | 66 400 | 2998 | 1.66 |

[a] Bz-Arg-NA was used as a substrate, and one unit was defined as the formation of 1 nmol of naphthylamine per minute.

no significant similarity of these sequences to other proteins.

Although substrate inhibition at low substrate levels (Fig. 2A) prevented the determination of the Km for Bz-Arg-NA, the Km for Bz-Lys-*p*-nitroanilide was 50 μM. Thus, Bz-Lys-*p*-nitroanilide was used as the substrate in subsequent kinetic experiments. To determine what portion of Bz-Arg-NA inhibited CEP 5.0, we first examined the effects of amino acids on CEP 5.0 activity. Only L-Arg significantly inhibited CEP 5.0 (Fig. 2B), whereas other amino acids, Ala, Asn, Glu, Gln, His, Ile, Lys, Met, L-ornithine, Phe, Pro, Ser, Val, Tyr showed little or no inhibitory effects. The Ki value of L-Arg was 3.5 mM and about 90% of activity was inhibited in the presence of 20 mM L-Arg (Fig. 2B).

The increased inhibition with Bz-Arg-NA compared to L-Arg (Fig. 2A and B) suggests that modification of L-Arg caused the increased inhibition. The effect of modification of L-Arg on inhibitory activity using various L-Arg-derivatives was further examined (Fig. 3A and Table 3). $N^{\alpha}$-amino group modified L-Arg-derivatives such as acetyl-L-Arg slightly changed its inhibitory activity, compared with the large inhibition observed with Bz-L-Arg (Fig. 2B). Modification of the carboxyl group of L-Arg increased inhibitory activity drastically (Fig. 2C). Methylester and ethylester derivatives became stronger inhibitors, and L-Arg modified by naphthylamine and nitroaniline almost completely inhibited CEP 5.0 at much lower range. $N^{\gamma}$-methyl-L-Arg which is a guanidino group-methylated L-Arg-derivative, showed an increase of inhibition. These results indicate that strong inhibition is due to hydrophobicity of L-Arg-derivatives, and that Bz-Arg-NA showed substrate-inhibition because it had hydrophobic bases on both the amino- and carboxyl-groups of L-Arg. Inhibition by L-Arg-derivatives

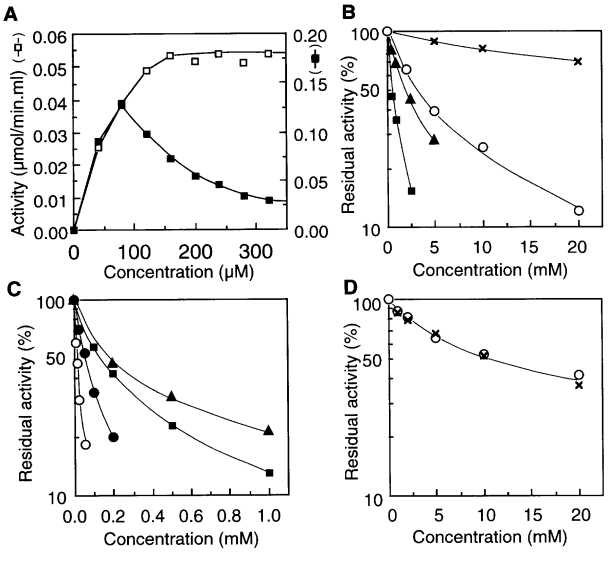


Fig. 2. Inhibition of CEP 5.0 by various Arg-derivatives and divalent cations: (A) CEP 5.0 was incubated with various concentrations of Bz-Lys-*p*-nitroanilide (□) or Bz-Arg-NA (■) as the substrates; (B) CEP 5.0 activity was measured with various concentrations of L-Arg (○), L-Lys (x) or $N^{\alpha}$-modified L-Arg-derivatives; acetyl-L-Arg (▲) or Bz-L-Arg (■). (C) CEP 5.0 activity was measured with various concentrations of C-modified L-Arg-derivatives; L-arginine methylester (▲), L-arginine ethylester (■), L-Arg-*p*-nitroanilide (●) or L-Arg-NA (○). (D) CEP 5.0 activity was measured with various concentrations of $Mg^{2+}$ (x) or $Ca^{2+}$ (○). Activity in the absence of effector was defined as 100%. The values represent the average of at least three independent measurements using 0.27 units of Bz-Lys-*p*-nitroanilide cleaving activity of CEP 5.0 and the range of the experimental error was within ±3%.

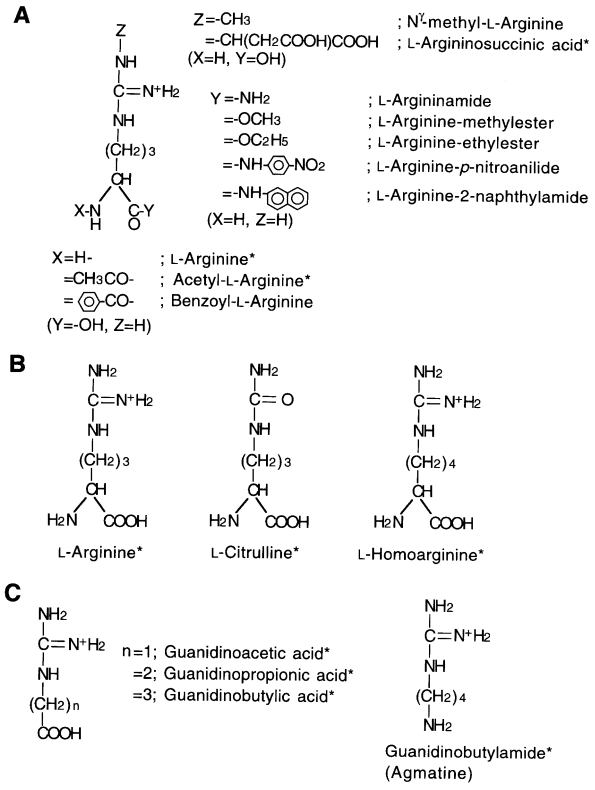


Fig. 3. Structures of L-Arg-derivatives and guanidino compounds using in this study: (A) L-Arg-derivatives modified by various chemical groups; (B) L-Arg and amino acids whose structures are similar to L-Arg; (C) guanidino compounds observed in plants. Asterisk shows substance observed in plants.

Table 3
Summary of kinetic analysis of L-Arg-derivatives against CEP 5.0 activity

| Site modified | L-Arg-derivatives | $K_i$ (mM) | Inhibitory fashion |
|---|---|---|---|
| Intact | L-Arg | 3.5 | Competitive |
| $N^{\alpha}$-modified | Acetyl-L-Arg | 2.5 | Competitive |
| | Bz-L-Arg | 0.39 | Competitive |
| C-modified | L-Argininamide | 0.19 | Competitive |
| | L-Arginine-methylester | $94 \times 10^{-3}$ | Competitive |
| | L-Arginine-ethylester | $66 \times 10^{-3}$ | Competitive |
| | L-Arg-*p*-nitroanilide | $58 \times 10^{-3}$ | Non-competitive |
| | L-Arg-NA | $14 \times 10^{-3}$ | Non-competitive |
| $N^{\gamma}$-modified | $N^{\gamma}$-Methyl-L-Arg | 1.5 | Competitive |

0.27 units of Bz-Lys-*p*-nitroanilide cleaving activity was used.

was competitive except for non-competitive inhibition by L-Arg-*p*-nitroanilide and L-Arg-NA (Fig. 4).

Neither L-citrulline, which lacks a positively charged guanidino group (structure is referred to in Fig. 3B), nor L-Lys and L-ornithine that have a positive charge at physiological pH, inhibited the reaction (data not shown). L-Argininosuccinic acid, in spite of having positively charged guanidino group, also did not inhibit activity, indicating steric hindrance by the large succinic group. These results suggest that an intact positively charged guanidino group at physiological pH is essential for inhibition.

We examined whether other guanidino compounds observed in plant tissues also inhibit CEP 5.0 (structures are referred to Fig. 3B and C, Table 4). Guanidino butylic acid showed almost equal inhibition to L-Arg; however, guanidino propionic acid showed less inhibition and guanidino acetic acid showed no inhibitory activity, suggesting that shortening of the hydrocarbon chains of guanidino compounds decreased inhibitory activity. Homoarginine, which has a longer hydrocarbon chain than L-Arg, and guanidinobutylamine (agmatine) showed strong inhibition at much lower range than L-Arg. Although these highly hydrophobic guanidino compounds only occur at low levels (Kato et al., 1986), it is possible that they and other unidentified guanidino compounds might regulate CEP 5.0 activity in vivo.

Arg is thought to be an endogenous inhibitor of CEP 5.0 because plants can contain Arg up to 5 mM (Winter et al., 1993). As Arg is the most economic utilizer of nitrogen because of its high N/C ratio (4N:6C), plants usually use Arg for storage and transport of N. Generally Arg contents in plants are affected by nutrient conditions and aging. For example, Arg content in leaves of young tulips cultivated in a medium containing nitrogen was over 70 times higher than those in leaves

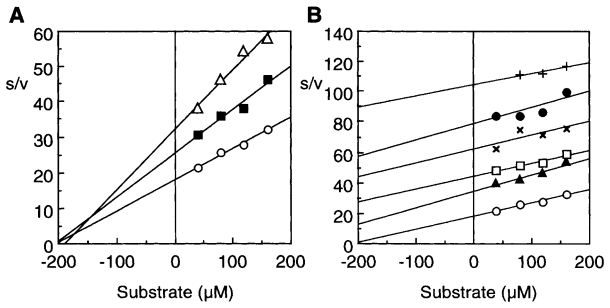


Fig. 4. Kinetic analysis of the inhibition of CEP 5.0 on Hanes–Woolf plots: (A) CEP 5.0 was incubated without (○) or with 5 μM (■) or 10 μM (△) L-Arg-NA; (B) CEP 5.0 was incubated without (○) or with L-Arg-derivatives. Symbols are 10 mM Bz-L-Arg (+), 0.2 mM $N^{\gamma}$-methyl-L-Arg (●), 4 mM L-Arg (**x**), 0.1 mM L-arginine methylester (□), or 5 mM $Mg^{2+}$ (▲), respectively. The values represent the average of at least three independent measurements using 0.27 units of Bz-Lys-*p*-nitroanilide cleaving activity of CEP 5.0 and the range of the experimental error was within ±3%.

Table 4
Summary of kinetic analysis of guanidino compounds detected in plants against CEP 5.0 activity

| Guanidino compounds | $K_i$ (mM) | Inhibitory fashion |
|---|---|---|
| Guanidinoacetic acid | –[a] | – |
| Guanidinopropionic acid | >20 | Competitive |
| Guanidinobutyric acid | 2.8 | Competitive |
| Guanidinobutylamide (agmatine) | 0.5 | Competitive |
| Homoarginine | $30 \times 10^{-3}$ | Competitive |

0.27 units of Bz-Lys-*p*-nitroanilide cleaving activity was used.
[a] Not inhibited.

after flowering and in nitrogen starved conditions (Ohyama et al., 1985). Therefore, CEP 5.0 may be regulated in response to nutrient and developmental changes in Arg content. A few endopeptidases inhibited by L-Arg have been reported in animals; however, the inhibition was less effective than the case for CEP 5.0 (Hatton, 1973; Polakoski and McRorie, 1973), and no plant endopeptidase regulated by Arg has been reported.

CEP 5.0 was also competitively inhibited by divalent cations such as $Mg^{2+}$ and $Ca^{2+}$ (Figs. 2D and 4B). This indicates that $Mg^{2+}$ and $Ca^{2+}$ interferes with binding of the substrate, either due to binding of themselves to the catalytic site or to a closely related site. The $K_i$ value of inhibition by divalent cations was about 10 mM.

Divalent cations such as $Ca^{2+}$ and $Mg^{2+}$ are well known regulators of plant endopeptidases (Liu and Jagendorf, 1986; Bushnell et al., 1993; Anastassiou and Argyroudi-Akoyunoglou, 1995), and may play important roles in protein degradation of plants. The most likely divalent cation for regulation of CEP 5.0 in vivo is $Mg^{2+}$ because it is the most abundant soluble divalent cation while intracellular free $Ca^{2+}$ is usually kept to low levels (Kreimer et al., 1988). The concentration of $Mg^{2+}$ is assumed to be 13–17 mM in the vacuole and 2–10 mM in cytosol and chloroplasts (Leigh and Wyn Jones, 1986; Stelzer et al., 1990; Marschner, 1995). These concentrations are high enough to inhibit CEP 5.0.

Moreover, we examined the inhibition of CEP 5.0 in the presence of various physiological concentration of L-Arg and $Mg^{2+}$, which are the most likely inhibitors in vivo (Fig. 5). Inhibition by L-Arg and $Mg^{2+}$ was additive and reversible (data not shown), and 5 mM of L-Arg and $Mg^{2+}$ inhibited the enzyme activity more than 70% compared to the control activity. This suggests the plausible regulation of CEP 5.0 by physiological concentration of L-Arg and $Mg^{2+}$ in vivo.

In conclusion, CEP 5.0 is possibly regulated by L-Arg and/or guanidino compounds and $Mg^{2+}$ whose cellular concentrations may change with environmental conditions. To clarify the physiological role of CEP 5.0, we will investigate in future the native substrate and localization of CEP 5.0.

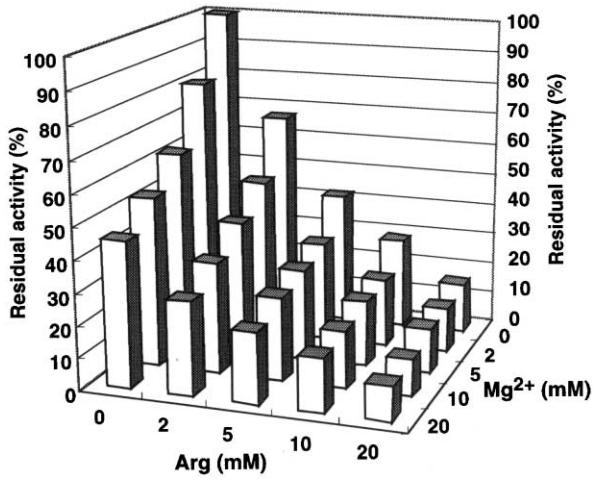


Fig. 5. Inhibition of CEP 5.0 by L-Arg and $Mg^{2+}$ is additive. CEP 5.0 (0.27 units of Bz-Lys-*p*-nitroanilide cleaving activity) was incubated with various combination of L-Arg and $Mg^{2+}$ concentration. Activity in the absence both of L-Arg and $Mg^{2+}$ was defined as 100%.

## 3. Experimental

### 3.1. Materials

All of the Arg-derivatives and Fast Garnet GBC were purchased from Sigma Co. Ltd. (St. Louis, MO, USA). Leupeptin and pepstatin A were purchased from Peptide Institute, Inc. (Osaka, Japan). All other reagents were purchased from Wako Pure Chemical Co. Ltd. (Osaka, Japan). Cucumber seeds (*Cucumis sativus* L. suyo) purchased from Takii Seed Co. Ltd. (Kyoto, Japan), were germinated in the dark at 25 °C for 6 days then transferred to a greenhouse. Harvested leaves were frozen in liquid $N_2$ and stored below −20 °C until use.

### 3.2. Enzyme activities

To monitor benzoyl (Bz)-Arg-naphthylamide (NA) degrading activity, assays using 60 μM of Bz-Arg-NA as a substrate by the method described previously were employed (Yamauchi et al., 1996). For kinetic analysis of the endopeptidase, 160 μM of Bz-Lys-*p*-nitroanilide was used as a substrate in 50 mM Hepes-KOH, pH 8.0, to a final volume of 1 ml. After incubation at 37 °C, the reaction was stopped by addition of 0.5 ml of 30% (v/v) acetic acid and absorbance at 410 nm measured. One unit was defined as the formation of 1 nmol of nitroaniline or naphthylamine per minute.

### 3.3. Purification of cucumber endopeptidase with a pI of 5.0 (CEP 5.0)

Leaves (300 g) were extracted with 5 volumes of 50 mM potassium phosphate buffer (K-PB), pH 7.0, containing 2 mM 2-mercaptoethanol (2-ME) and 0.1 mM EDTA. Debris was removed by filtration using two layers of cheesecloth. After centrifugation at 20,000 *g* for 20 min, the supernatant was fractionated by the addition of solid ammonium sulfate. The fraction precipitating between 45 and 55% ammonium sulfate saturation was dissolved in a minimum volume of 25 mM K-PB, pH 7.0, containing 1 mM 2-ME (buffer A) and dialyzed against buffer A. The dialysate was applied to a DEAE-Toyopearl column (4×20 cm, Tosoh Corp., Japan) equilibrated with buffer A, and a linear gradient of 0–0.2 M NaCl (total 1.2 l) was applied to elute the enzymes. Active fractions of CEP 5.0 after DEAE-Toyopearl chromatography were combined and dialyzed against 10 mM Na-PB, pH 6.8, containing 1 mM 2-ME. The dialysate was applied to a hydroxyapatite column (2×15 cm, Bio-Rad, CA, USA) and eluted by a linear gradient of 0–0.2 M Na-PB (total 200 ml). After active fractions were combined, an equal volume of 60% saturated ammonium sulfate in 50 mM K-PB, pH 7.0, containing 1 mM 2-ME was added. This solution was applied to a Phenyl-Sepharose column (1.5×8 cm, Amersham Pharmacia Biotech, Sweden) equilibrated with 30% ammonium sulfate saturated in 50 mM K-PB, pH 7.0, containing 1 mM 2-ME and the proteins were eluted by a linear gradient of 30–0% ammonium sulfate (total 100 ml). Active fractions were concentrated by a DEAE-mini column (1 ml), then applied to an Arg-Sepharose (1.5×8 cm, Amersham Pharmacia Biotech, Sweden) column equilibrated with buffer A containing 0.1 mM EDTA. A linear gradient of 0–0.1 M NaCl was applied to elute the enzyme (total 100 ml). The active fractions were dialyzed against buffer A, then applied to a Mono Q column (0.32×3 cm, SMART system, Amersham Pharmacia Biotech, Sweden). The column was eluted with a linear gradient of 0–0.3 M NaCl in buffer A (total 8 ml). All (purification) steps were performed at temperatures from 0 to 4 °C.

### 3.4. Characterization of CEP 5.0

To investigate the effect of inhibitors, CEP 5.0 was preincubated with each inhibitor in the reaction mixture without substrate for 30 min at 4 °C. The substrate was then added to the mixture and residual activity was measured. Native molecular weights were estimated on the basis of comparison of the retention time of marker proteins on a Superose 12 column (0.32×30 cm, SMART system, Amersham Pharmacia Biotech, Sweden) equilibrated with 25 mM K-PB, pH 7.0, containing 0.1 M NaCl and 1 mM 2-ME. In the experiment on the effect of L-Arg-derivatives on CEP 5.0, L-Arg-derivatives were used as inhibitors because CEP 5.0 could not cleave them even though their structure resemble the Bz-Arg-NA substrate.

### 3.5. Protein analysis

Isoelectric focusing was performed with a 7.5% (w/v) polyacrylamide gel containing SERVALYT of pH

range 4.0–6.0 (Serva Electrophoresis GmbH, Germany) under non-denaturing conditions. Cathode and anode buffers were 40 mM Glu and 0.2 M His, respectively. SDS-PAGE and Tricine/SDS-PAGE were performed by the method of Laemmli (1970) and Schägger and von Jagow (1987), respectively. Gels were stained for proteins with Coomassie Brilliant Blue R-250. Protein concentration was determined by the method of Bradford with bovine serum albumin as the standard (Bradford, 1976).

### 3.6. Sequencing of internal peptides of CEP 5.0

CEP 5.0 (2 μg) was dissolved in 1 ml of 70% formic acid containing 1 mg of CNBr, and allowed to stand overnight in the dark. After evaporation using a SpeedVac concentrator (model A160, SAVANT Instruments Inc., NY, USA), the cleaved peptides were dissolved in 50 μl of SDS sample buffer and separated by Tricine/SDS-PAGE using a 15% polyacrylamide gel. After electroblotting to a poly (vinylidene difluoride) membrane, peptides were stained by CBB R-250 and portions of major bands were excised and sequenced by an automated pulsed liquid protein sequencer (model 491, Applied Biosystems, Foster City, CA, USA).

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