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A High Molecular Weight Glutamyl Endopeptidase and Its Endogenous Inhibitors from Cucumber Leaves

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We purified a glutamyl endopeptidase that is a major foliar endopeptidase in cucumber. The endopeptidase had a molecular mass of 400 kDa, consisted of four subunits of 97 kDa, and was inactivated by SH-modifying reagents. Its optimum pH and optimum temperature were 8.0 and 30–37°C, respectively. An internal amino acid sequence of the endopeptidase was highly homologous to a partial sequence of unidentified proteins deduced from genetic information for *Arabidopsis thaliana*, soybean and rice, but not to the sequences of bacterial glutamyl endopeptidases or animal proteases. Therefore, the unidentified proteins might be glutamyl endopeptidases and be widely distributed only among plant species. The activity of the cucumber glutamyl endopeptidase was inhibited by at least three inhibitors existing in cucumber leaves. One of the inhibitors was a competitive inhibitor of 25 kDa, which did not significantly inhibit commercial endopeptidases derived from animals and microorganisms. This suggests that the cucumber glutamyl endopeptidase might be controlled by endogenous inhibitors *in vivo*.

Key words: cucumber, glutamyl endopeptidase, inhibitor.

Glutamyl endopeptidase (GEP) is a general term for enzymes cleaving the peptide bonds on the carboxyl sides of Glu residues (*1*). For the detection of GEPs, artificial substrates such as carbobenzoxy (CBZ)-Leu-Leu-Glu-naphthylamide (NA) and CBZ-Glu-phenylester are often used. Bacterial GEPs and the GEP-subunits of proteasomes in various organisms have been well studied (*1, 2*)

Previously, we revealed the existence of a GEP with a pI of 4.5 as the main endopeptidase in cucumber leaves (*3*). Then, Laing and Christeller partially purified GEP from spinach chloroplasts (*4*). This endopeptidase had a much higher molecular weight, 350–380 kDa, than those of bacterial GEPs, 25–30 kDa, and did not need SDS for the cleavage of CBZ-Leu-Leu-Glu-NA, unlike proteasomes (*4*).

In this study, we purified a cucumber GEP (CGEP), and compared its biochemical properties, such as molecular weight, optimum pH, and internal amino acid sequences, with those of bacterial GEPs, proteasomes and spinach. Furthermore, we investigated the regulation mechanism for CGEP.

MATERIALS AND METHODS

Materials—Artificial substrates for endopeptidases and Fast Garnet GBC were purchased from Sigma (St. Louis, Mo, USA). Leupeptin and pepstatin A were purchased from Peptide Institute (Osaka). Other reagents were purchased from Wako Pure Chemical (Osaka). Cucumber seeds (*Cucumis sativus* L. suyo) purchased from Takii Seed (Kyoto) were sown and grown in a field. Harvested leaves were frozen in liquid N_2 and stored at –20°C.

Enzyme Activity—Measurement of CBZ-Leu-Leu-Glu-NA cleaving activity was performed by the method described previously (*3*) except that the reaction buffer was Hepes-KOH, pH 8.0, the optimum pH of CGEP. One unit was defined as the formation of 1 μmol of naphthylamine per minute.

Inhibitory activities were estimated from the residual activity of the purified CGEP in the mixture using the standard assay as described above. One unit of inhibitor was taken as the amount that decreased the reaction rate by one CGEP unit. Degradation of Inhibitor2 was detected on SDS-PAGE after incubation of protease and Inhibitor2 in 50 mM Hepes-KOH, pH 8.0, for 16 h at 37°C.

Purification of CGEP—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. After centrifugation at 20,000 ×*g* for 20 min, the supernatant was fractionated by the addition of solid ammonium sulfate. Proteins precipitating between 45 and 55% ammonium sulfate saturation were dissolved in a minimum volume of 25 mM K-PB, pH 7.0, containing 1 mM 2-ME (buffer A), and then dialyzed against buffer A. The dialysate was applied to a DEAE-Toyopearl column (4 × 20 cm; Tosoh, Tokyo) equilibrated with buffer A, and then a linear gradient of 0 to 0.2 M NaCl was applied to elute the enzymes (total, 1,200 ml). The active fractions were

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Abbreviations: Bz, benzoyl, CBZ, carbobenzoxy; CGEP, cucumber glutamyl endopeptidase, CHES, 2-(cyclohexylamino) ethanesulgonic acid, GEP, glutamyl endopeptidase, 2-ME, 2-mercaptoethanol, NA, naphthylamide; PB, phosphate buffer

combined and dialyzed against 10 mM sodium phosphate buffer (Na-PB), pH 6.8, containing 1 mM 2-ME. The dialysate was applied to a hydroxyapatite column (2 × 15 cm; Bio-Rad, CA) and eluted with a linear gradient of 0 to 0.2 M Na-PB, pH 6.8 (total, 200 ml). An equal volume of 60% ammonium sulfate–saturated 50 mM K-PB, pH 7.0, containing 1 mM 2-ME was added to the active fraction. Next, the solution was applied to a Phenyl-Sepharose column (1.5 × 8 cm, Amersham Pharmacia Biotech, Sweden) equilibrated with 30% ammonium sulfate-saturated 50 mM K-PB, pH 7.0, containing 1 mM 2-ME, and eluted with a linear gradient of 30 to 0% ammonium sulfate (total, 100 ml). The active fractions were concentrated on a DEAE-mini column (bed volume, 1 ml), and then applied to a gel filtration column (1.5 × 60 cm, HW-55F; Tosoh) equilibrated with buffer A containing 0.3 M NaCl. The active fractions were dialyzed against buffer A, and 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 to 0.3 M NaCl in buffer A (total, 8 ml). All purification steps were performed at 0 to 4°C.

Purification of CGEP Inhibitors—Leaves (100 g) were extracted with 5 volumes of 50 mM K-PB, pH 7.0, containing 2 mM 2-ME, 0.1 mM EDTA, and 10% (v/v) glycerol (buffer B). After centrifugation at 20,000 ×*g* for 20 min, the supernatant was directly loaded onto a DEAE-Toyopearl column (4 × 20 cm) equilibrated with buffer B. After the unbound proteins had been precipitated by 80% saturation with ammonium sulfate, the precipitate was dissolved in a minimum volume of 25 mM K-PB, pH 7.0, containing 1 mM 2-ME and 10% (v/v) glycerol, and then applied to a gel filtration column (1.5 × 60 cm, HW-55F) equilibrated with the same buffer. The fractions containing inhibitory activity were combined and an equal volume of 60% ammonium sulfate–saturated 50 mM K-PB, pH 7.0, containing 1 mM 2-ME was added. The solution was applied to a Phenyl-Sepharose column (1.5 × 8 cm) equilibrated with the same buffer saturated with 30% ammonium sulfate and eluted with a linear gradient from 30 to 0% ammonium sulfate saturation (total, 100 ml). The fractions containing inhibitory activity were again combined and dialyzed against 20 mM Hepes-KOH, pH 7.0, containing 1 mM 2-ME and 10% (v/v) glycerol The dialysate was applied to a hydroxyapatite column (1.5 × 8 cm) and eluted with a linear gradient of 0 to 150 mM Na-PB, pH 6.8 (total, 100 ml).

Purification of one of the inhibitors, a heat-stable competitive inhibitor named Inhibitor2, was performed by the following procedure. Leaves were homogenized in 5 volumes of buffer B. After centrifugation at 20,000 ×*g* for 10 min, the supernatant was incubated in a water bath at 80°C for 10 min. After centrifugation, solid ammonium sulfate was added to the supernatant to obtain 80% saturation. The precipitated proteins were collected by centrifugation, and then resuspended in 1 ml of 200 mM Na-acetate buffer, pH 4.0. After centrifugation, the supernatant was dialyzed against 25 mM K-PB, pH 7.0. Then the dialysate was applied to a DEAE-Toyopearl column (1.5 × 8 cm) equilibrated with the same buffer. The flow through fraction containing Inhibitor2 was applied to a hydroxyapatite column (1.5 × 8 cm) equilibrated with the same buffer, and then Inhibitor2 adsorbed to the resin was eluted with 100 mM Na-PB, pH 6.8.

Characterization of Endopeptidases and Inhibitors—In order to investigate the effects of inhibitors, CGEP was preincubated with each inhibitor in the reaction mixture without a substrate for 30 min at 4°C Then the substrate was added to the mixture, and the mixture was incubated at 37°C. The native molecular mass was estimated on the basis of comparison of the mobilities of marker proteins on gel filtration on a Superose 12 column (0.32 × 30 cm, SMART system) equilibrated with 25 mM K-PB, pH 7.0, containing 0.3 M NaCl, 1 mM 2-ME, and 10% (v/v) glycerol.

Protein Analysis—Polyacrylamide gel electrophoresis was performed on a 7.5% polyacrylamide gel at pH 8.3 by the method of Davis under non-denaturing conditions (*5*). SDS-PAGE was performed by the method of Laemmli (*6*) Protein concentrations were determined by the method of Bradford (*7*) with bovine serum albumin as the standard

Sequencing of Internal Peptides of CGEP—Two micrograms of CGEP was dissolved in 1 ml of 70% (v/v) formic acid containing 1 mg of CNBr, and then stood on overnight in the dark. After evaporation with a SpeedVac concentrator (model A160; SAVANT Instruments, New York), the cleaved peptides were dissolved in 50 μl of SDS sample buffer and then separated by SDS-PAGE on a 15% polyacrylamide gel. After electroblotting onto a poly (vinylidene difluoride) membrane, the peptides were stained with Coomassie Brilliant Blue R-250, and portions of the major bands were excised and sequenced with an automated pulsed liquid protein sequencer (model 491; Applied Biosystems, Foster City, CA). Homology searches were performed using DNA and protein sequence databases obtained from GenBank and SwissProt.

RESULTS AND DISCUSSTION

Purification and Characterization of CGEP—The purification of CGEP is summarized in Table I. Although low or no CGEP activity was detected in the crude extract, abundant CGEP activity was first detected in the precipitate on 45 to 55% ammonium sulfate saturation. This indicates that inhibitory activities co-exist in the crude extract, and

TABLE I **Summary of the purification of CGEP.**

Purification step	Total protein (mg)	Total activity (Units)[a]	Specific activity (Units/mg)	Purification (-fold)[b]	Yield (%)[b]
Crude extract	6,600	–[c]	–	–	–
Ammonium sulfate (45-55%)	907	12 6	0 014	1 0	100
DEAE-Toyopearl	85 2	12 8	0 15	10.8	102
Hydroxyapatite	30 5	4 71	0 16	11.1	37 4
Gel filtration	6 24	8 05	1 29	92.8	63 9
Phenyl-Sepharose	1.37	4 74	3 46	249	37.6
Mono Q	0.033	0 56	17.0	1,220	4 44

[a]One unit was defined as the formation of 1 μmol of naphthylamine per minute. [b]These values were calculated on the basis of total activity and specific activity on 45–55% ammonium precipitation [c]Not accurately detected Details are given in the text

that the CGEP and inhibitory activities were separated in the ammonium precipitation step.

The purified CGEP gave only one band on PAGE under both denaturing and non-denaturing conditions (Fig. 1A) The molecular mass of CGEP was estimated to be 97 kDa on SDS-PAGE (Fig. 1A) and 400 kDa on gel filtration (Fig. 1B). These results suggest that CGEP is a tetramer of 400 kDa consisting of identical subunits of 97 kDa. CGEP exhibited an optimum pH of 8.0 and an optimum temperature of 30 to 37°C (Fig. 2). CGEP was inhibited by SH-modifying reagents, *p*-chloromercuribenzoic acid and *N*-ethylmaleimide, but not by inhibitors of other classes (Table II), indicating that CGEP had cysteine residues critical for its enzymatic activity. CGEP did not cleave other artificial substrates, CBZ-Arg-Arg-NA, benzoyl (Bz)-Arg-NA, CBZ-Ala-Ala-Lys-NA, CBZ-Phe-Arg-NA, Bz-Arg-Gly-Phe-Phe-Leu-NA, CBZ-Gly-Gly-Leu-NA, Bz-Phe-NA, glutaryl-Gly-Gly-Phe-NA, acetyl-Ala-Ala-Pro-Ala-NA, CBZ-Pro-Ala-Gly-Pro-NA, CBZ-Pro-His-Leu-Leu-Val-Tyr-Ser-NA, and acetyl-Tyr-Val-Ala-Asp-*p*-nitroanilide. This suggests that CGEP preferentially cleaves peptide bonds at the C sites of Glu residues. These properties of CGEP were similar to those of a chloroplast GEP found in spinach, which has a native molecular weight of about 350 to 380 kDa, an optimum pH of 8 0 and the same substrate specificity (*4*).

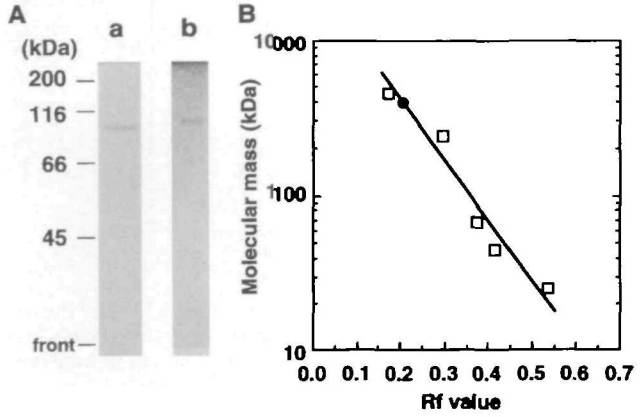

Fig 1 **Electrophoretic profile and estimation of the molecular weight of CGEP.** (A) Purified CGEP (3 μg) was subjected to SDS-PAGE with 8% acrylamide (a), and the purified CGEP (0 8 μg) was subjected to nondenaturing PAGE with 7 5% acrylamide (b) Proteins were stained with Coomassie Brilliant Blue R-250 (B) Plot of mobility against molecular mass on a logarithmic scale for CGEP (●) and marker proteins (□) on gel filtration (Superose 12) The marker proteins were apoferritin (443 kDa), catalase (240 kDa), BSA (67 kDa), albumin egg (45 kDa), and chymotrypsinogen A (25 kDa)

Amino Acid Sequence and Identification of CGEP—Internal amino acid sequences of CGEP were determined after CNBr cleavage treatment, since the N-terminus of CGEP was blocked The determined sequences of two obtained peptides were "IRYERKDGVQLTATLYL" and "PDDSLGPEKE". A database search revealed that sequence "(M)-IRYERKDGVQLTATLYL" was homologous to sequences of unidentified proteins of three plant species, soybean (*8*), *Arabidopsis thaliana* (*9*), and rice (*10*) (Fig. 3A). The unidentified protein of *A. thaliana* contained almost homologous sequences to cucumber sequences (Fig. 3, A and B). These findings suggest that the high molecular weight GEP might be widely distributed among plant species. The above two sequences of CGEP showed no similarity to those of bacterial GEPs. In addition, these bacterial GEPs are serine- or metallo-endopeptidases and their molecular masses are 25–30 kDa (*11–13*). CGEP was not inhibited by a serine-modifying reagent and a metal-chelating reagent. Therefore CGEP is evidently different from bacterial GEPs

Proteasome with a molecular mass of over 850 kDa, consisting of at least 10 subunits of 21–32 kDa, is found in plants (*14, 15*). It also cleaves CBZ-Leu-Leu-Glu-NA only in

TABLE II **Effects of chemical inhibitors on the activity of CGEP.** After CGEP had been preincubated with each chemical inhibitor in the reaction mixture without a substrate for 30 min at 4°C, residual activity was measured after addition of the substrate and incubation at 37°C

Inhibitor	Concentration	Relative activity
None	–	100%[a]
Diisopropyl fluorophosphate	1 mM	97 0
	10 mM	67 2
p-Chloromercuribenzoic acid	0 5 mM	34 7
N-Ethylmaleimide	1 mM	96 0
	10 mM	28 8
EDTA	10 mM	109
Leupeptin	10 μM	91 4
	100 μM	92 5
Pepstatin A	10 μM	90 5
SDS	0 1% (w/v)	0

[a]Activity with no inhibitor is defined as 100%

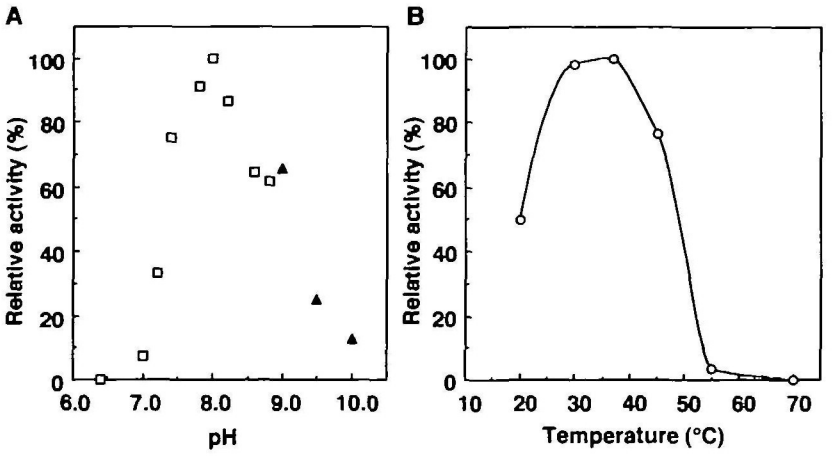

Fig. 2 **Optimum pH and temperature of the CGEP activity.** (A) CGEP was incubated with 50 mM Hepes-KOH (pH 6.4–8.6, □) or 50 mM 2-(cyclohexylamino) ethanesulgonic acid (CHES)–KOH (9 0–10 0, ▲) at 37°C (B) CGEP was incubated with 50 mM Hepes-KOH, pH 8 0, at various temperatures

the presence of SDS (*14*). In contrast to that of proteasome, CGEP activity was completely inhibited by SDS (Table II). Moreover, CGEP consisted of four subunits of a molecular mass of 97 kDa, and its amino acid sequences did not exhibit similarity to the sequences of any proteasomes, suggesting that CGEP is also different from proteasomes.

CGEP Inhibitors—In the crude extract, CGEP activity was almost completely masked. Potential CGEP and inhibitory activities in the crude extract were estimated after separating them on a DEAE-Toyopearl column since CGEP bound to the column but the inhibitors did not As a result, inhibitory activity was found in the crude extract at an excess level compared to CGEP, *i.e.* the potential activities of CGEP and inhibitors were 44.6 ± 3.12 and 120 ± 39 munits per gram fresh weight of leaves, respectively.

Three CGEP inhibitors were partially purified by DEAE-Toyopearl, gel filtration, Phenyl-Sepharose, and then hydroxyapatite column chromatography. Inhibitor1 and 2 were separated from Inhibitor3 at the Phenyl-Sepharose column chromatography step (Fig. 4A). Inhibitor1 was separated from Inhibitor2 at the hydroxyapatite column chromatography step (Fig. 4B). The molecular masses of Inhibitor1 and 2 were estimated to be 20–22 kDa, and that of Inhibitor3 to be 8–8.5 kDa on gel filtration (data not shown). Inhibitor1 was entirely inactivated on heat treat-

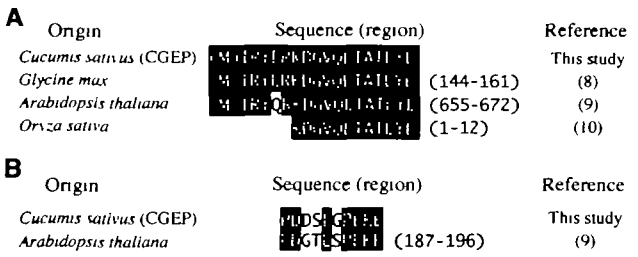

Fig. 3 **Internal amino acid sequence aliginment of CGEP and homologs of different plant species.** The amino acid sequences of 30 kDa (A) and 20 kDa (B) peptides, and homologous proteins are shown The amino acid sequences of homologous proteins are translated from the DNA sequences of accession numbers AF091304 (*Glycine max*), AC002337 (*Arabidopsis thaliana*), and AU063885 (*Oryza sativa*) Identical amino acid residues in all species are shown by filled boxes

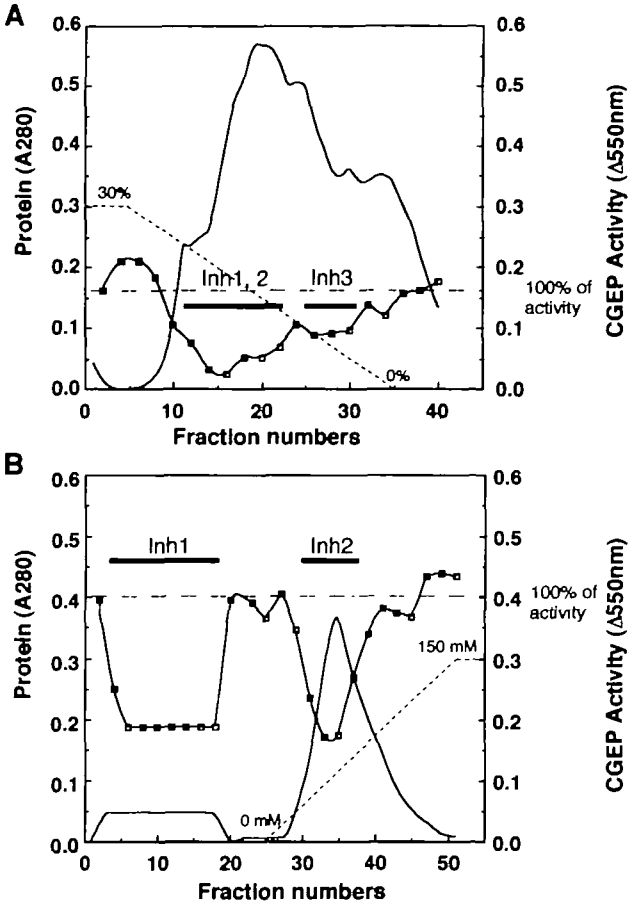

Fig 4 **Partial purification of CGEP inhibitors.** (A) The fraction containing inhibitors obtained on gel filtration was subjected to Phenyl-Sepharose chromatography (B) Fractions containing Inhibitor1 and 2 obtained on Phenyl-Sepharose chromatography were subjected to hydroxyapatite column chromatography Inhibitory activity was represented as reduction of CGEP activity (□). CGEP activity in the absence of an inhibitor represents 100% activity Bars show the pooled fractions containing each Inhibitor The solid line shows the absorbance at 280 nm

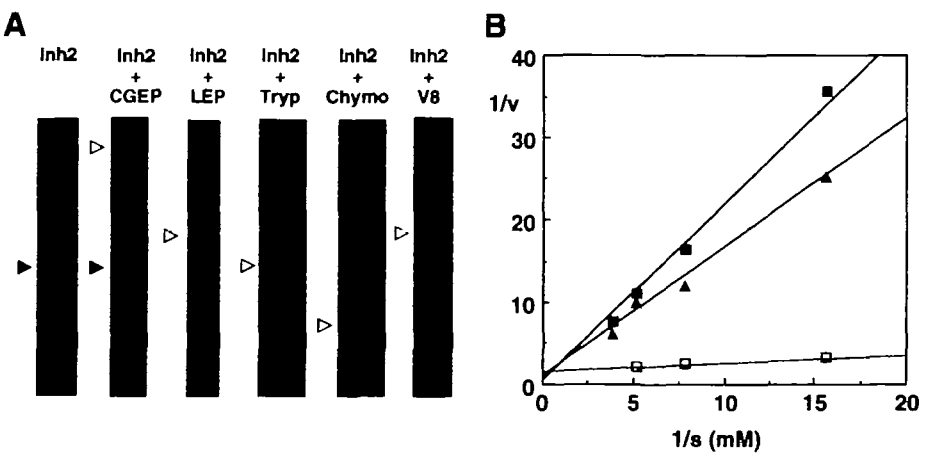

Fig 5 **Properties of Inhibitor2.** (A) Inhibitor2 and its degradation by proteases After incubation of Inhibitor2 (0 3 μg) without (lane 1) or with CGEP (0 3 μg, lane 2), lysyl endopeptidase (0 5 μg, lane 3), trypsin (0 5 μg, lane 4), α-chymotrypsin (0 5 μg, lane 5), or V8 protease (0 5 μg, lane 6) for 16 h at 37°C, the degradation of Inhibitor2 was detected by SDS-PAGE with 12% polyacrylamide. Closed and open arrowheads indicate the bands of Inhibitor2 and the respective protease, respectively. (B) Kinetic analysis of the inhibition of CGEP by Inhibitor2 Data are shown as double reciprocal plots (Lineweaver-Burk plots). Forty munits of CGEP was incubated without (□), or with 11.2 nM (▲) and 28 nM (■) of Inhibitor2

ment for 10 min at 80°C, but Inhibitor2 and 3 were not inactivated (data not shown).

We purified Inhibitor2 to homogeneity by 80°C treatment, ammonium sulfate precipitation, pH 4.0 treatment, and then DEAE-Toyopearl and hydroxyapatite column chromatographies. Thirty five micrograms (1.64 U) of Inhibitor2 was recovered from 30 g of fresh cucumber leaves. Inhibitor2 showed a molecular mass of 25 kDa on SDS-PAGE (Fig. 5A, lane 1), suggesting that Inhibitor2 was monomeric. This molecular mass was larger than those of other plant inhibitors of acidic amino acid–specific endopeptidases (*16, 17*) Double reciprocal plots (Lineweaver-Burk plots) indicated that inhibition of CGEP by Inhibitor2 was competitive (Fig 5B), and K_i was about 2 nM. The physiological roles of almost all plant inhibitors are thought to be the prevention of invasion through inhibition of the proteases of enemies (*16*). Inhibitor2 was rather degraded by commercial endopeptidases derived from animals and microorganisms, including bacterial GEP (V8 protease) (Fig. 5A). Therefore, Inhibitor2 did not significantly inhibit commercial proteases (data not shown). On the other hand, Inhibitor2 inhibited CGEP without degradation (Fig. 5A). Thus, the physiological role of Inhibitor2 is likely to be control of the activity of CGEP not protection against enemies.

The wide distribution among various plant species of CGEP-like proteins implies that they might commonly play an essential role in the fundamental metabolism of plants In cucumber leaves, CGEP is a most active protease and is thought to be involved in protein turnover in photosynthesizing tissues during the developing stage (*3*). The inhibitors found in this study might regulate this protein turnover. In the present study, to further clarify the physiological role of CGEP-like proteins, we have cloned a cDNA of a CGEP homolog of *A. thaliana*, and the cDNA has been introduced into *A. thaliana* to produce transgenic plants with increased and decreased levels of CGEP-like protein.

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