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Identification of Oligopeptidase B in Higher Plants. Purification and Characterization of Oligopeptidase B from Quiescent Wheat Embryo, *Triticum aestivum*

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Proteolytic enzymes in general, and cysteine proteases in particular, play key roles in seed germination and early seedling growth. However, the precise mechanism by which the serine proteases are regulated remains unclear. Trypsin-like activity was detected in wheat germ (quiescent embryo) and this activity increased in the germinating embryo. In this work, a trypsin-like serine protease expressed in wheat germ was purified to homogeneity by chromatography through DEAE-cellulose, phenyl-Sepharose, Ultrogel AcA-34 and Blue-Sepharose. The molecular mass of the enzyme was estimated to be 81 kDa by SDS-PAGE under reducing conditions. Amino acid analysis of the peptides generated following digestion of the enzyme with lysyl endopeptidase indicated that the enzyme is a plant homologue of *Escherichia. coli* oligopeptidase B. The subsite specificity of the enzymes differ, although both enzymes hydrolyze synthetic substrates and model peptides at the carboxyl side of basic amino acids. The wheat enzyme is more sensitive to leupeptin and antipain than the *E. coli* emzyme. These results provide the basis for characterizing plant oligopeptidase B and contribute to our understanding of its role in the early development of seedlings.

Key words: germination, oligopeptidase B, plant, serine protease, wheat.

Abbreviations: ACTH, adrenocorticotropic hormone; Boc, *t*-butyloxycarbonyl; DFP, diisopropyl fluorophosphate; E-64, *trans*-epoxysuccinyl-L-leucylamide-(4-guanidino)butane; OPB, oligopeptidase B; PMSF, phenylmethanesulfonyl fluoride; *pyr*, L-pyroglutamyl; STI, soybean trypsin inhibitor; *Suc*, succinyl; MCA, 4-methylcoumaryl 7-amide; TLCK, *Nα-p*-tosyl-L-lysine chrolomethyl ketone; TPCK, L-1-tosylamido-2-phenylethyl chrolomethyl ketone; WCP; wheat cysteine protease; Z, carbobenzoxy.

The germination of seeds is an important event in the life cycle of plants and initiates numerous physiological and biochemical activities when the apparent metabolic dormancy of desiccated seeds is disrupted by imbibition. During seed germination and early development of the embryo, the store reserve of proteins in the aleurone layer and endosperm are hydrolyzed by proteases and mobilized to supply amino acids that support the growth of the embryo (*1*). Wheat is a well-known crop that easily shows preharvest sprouting. This developmental disorder occurs when grains mature under cool, damp conditions. Preharvest sprouting causes devastating damage to the quality of wheat flour due to the associated degradation of seed starch and protein. Although it is known that genetic variability for sprouting resistance exists (*2*), the underlying biochemical mechanisms are poorly understood. Papain-like cysteine proteases have been studied extensively and have been implicated in the degradative processes in seed germination (*1*, *3–5*). When germination is initiated with the uptake of water by the seed, cysteine proteases are synthesized in the aleurone layer following stimulation by gibberellic acid, and are subsequently secreted into the endosperm where storage proteins undergo degradation. Previously, we cloned a novel wheat cysteine protease, WCP2, that is expressed in quiescent and germinating embryos (*6*). The expression of WCP2 protein significantly increased following imbibition. In contrast with this accumulated information concerning cysteine proteases, little is known about the serine proteases involved in seed germination, although recent genome analysis of *Arabidopsis thaliana* has indicated that higher plants possess various serine protease genes. Recently, a subtilisin-like serine protease has been shown to play a critical role in the early development of plants (*7–10*).

Oligopeptidase B belongs to a new prolyl oligopeptidase family of serine proteases and cleaves peptides consisting of no more than 30 amino acids at the carboxyl side of basic amino acids (*11*, *12*). Oligopeptidase B (previously called protease II) has been purified from *E. coli* (*13*) and was characterized as a trypsin-like endoprotease, although the role of this enzyme remains unknown. Oligopeptidase B is only found in gram-negative bacteria (*13*, *14*) and ancient eukaryotic unicellular organisms (*15–17*). Recently, we found trypsin-like protease activity in wheat quiescent embryos (wheat germ) and showed that this activity increased during germination, just as in the case of WCP2. In this work, we purified a major protease possessing trypsin-like activity from wheat germ. The protease was shown to be an oli-

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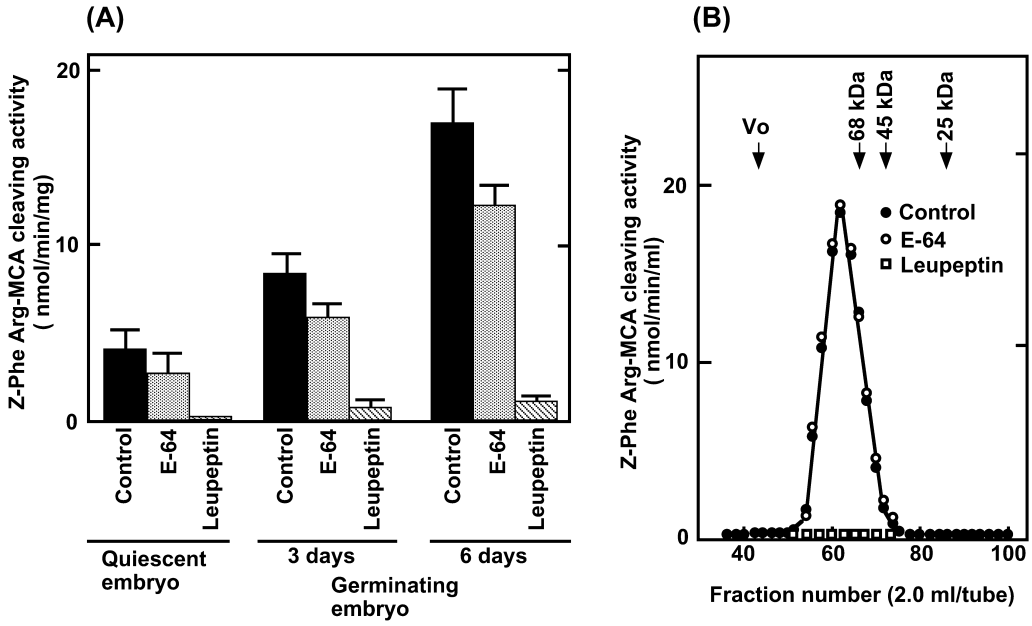

Fig. 1. **Trypsin-like activity in wheat quiescent and germinating embryos.** (A) Wheat quiescent embryos (wheat germ) and germinating embryos (shoot and radicle) following imbibition (3 and 6 days) were extracted with 20 volumes of 20 mM Tris-HCl buffer (pH 7.0). Z-Phe-Arg-MCA-hydrolyzing activity at pH 8.0 was assayed in the absence or presence of inhibitors (50 μg/ml E-64, leupeptin). (B) Sephadex G-100 gel filtration of Z-Phe-Arg-MCA cleaving activity from wheat quiescent embryo.

gopeptidase B by amino acid sequence and enzymatic analyses. This is the first report detailing the isolation and preliminary characterization of oligopeptidase B from a higher plant. Our results suggest that oligopeptidase B plays an important role in the early development of the wheat embryo.

EXPERIMENTAL PROCEDURES

Materials—Peptide 4-methylcoumaryl 7-amide (MCA) substrates, human dynorphin A, glucagon, adrenocorticotropic hormone (ACTH, 1–24), leupeptin, *trans*-epoxysuccinyl-L-leucylamide-(4-guanidino) butane (E-64), chymostatin and pepstatin were purchased from the Peptide Institute (Osaka). Somatostatin-28 was purchased from the American Peptide Co. (Sunnyvale, CA, USA). Phenyl-Sepharose (HiLoadTM16/10) and Blue-Sepharose were purchased from Pharmacia (Uppsala, Sweden). [1,3-^{3}H]Diisopropylfluorophosphate (DFP) was purchased from Dupont (Boston, MA, USA). Lysyl endopeptidase (from *Achromobacter lyticus*) was purchased from Wako Chemicals (Osaka). All other chemicals used were of analytical grade.

Wheat—Wheat germ was kindly provided by Showa Sangyo Co. (Tokyo, Japan). Wheat seeds (*Triticum aestivum* L. cv. Chikugoizumi) were allowed to imbibe water on filter paper at 20°C in the dark until collection.

Enzyme Assay—Protease activity was assayed using carbobenzoxy-Phe-Arg-4-methylcoumaryl 7-amide (Z-Phe-Arg-MCA) unless otherwise stated. The reaction mixture contained 0.1 M Tris-HCl (pH 8.0) and 50 μM Z-Phe-Arg-MCA. The reaction was initiated by addition of the enzyme solution. Following incubation at 37°C for 10–20 min, the reaction was terminated and the MCA liberated was determined fluorometrically as previously described (*18*). Protein was determined by the method of Bradford using BSA as a standard (*19*).

Purification of Z-Phe-Arg-MCA–Cleaving Activity from Wheat Germ—All purification procedures were performed at 4°C unless otherwise stated. Wheat germ (100 g) was homogenized in 500 ml of ice-cold 50 mM acetate buffer (pH 5.5) containing 1 mM EDTA and 1 mM β-mercaptoethanol (buffer A) using a Waring blender followed by a Polytron homogenizer. The homogenate was centrifuged at 12,000 × *g* for 10 min at 4°C. The precipitate was rehomogenized with 250 ml of buffer A and centrifuged. Both supernatants were combined and fractionated with ammonium sulfate (35–60% saturation). The precipitate was dissolved with buffer A and dialyzed against the same buffer. Following centrifugation at 17,000 × *g* for 30 min at 4°C, the resultant supernatant was applied to a DEAE-cellulose column (4.3 × 15 cm) pre-equilibrated with 20 mM sodium phosphate buffer (pH 7.0) and then washed with the same buffer. Z-Phe-Arg-MCA-cleaving activity was eluted using a linear gradient of NaCl (0–0.2 M) in the same buffer. The fraction possessing activity was eluted as a single peak and concentrated by ammonium sulfate precipitation (0–60% saturation). The precipitate was dissolved in 20 ml of 20 mM sodium phosphate buffer (pH 7.0) and dialyzed against 25 mM Tris-HCl buffer (pH 7.5) containing 1 M ammonium sulfate. Following centrifugation, the supernatant was applied to a HiLoad™ 16/10 phenyl-Sepharose column, and bound proteins were eluted using a linear gradient of ammonium sulfate (1–0 M) in the same buffer. The fraction possessing activity was pooled and concentrated by ultrafiltration. The concentrate was applied to an Ultrogel AcA-34 column (2.0 × 100 cm) and eluted with 20 mM sodium phosphate buffer (pH 7.5) containing 0.15 M NaCl. The fraction possessing activity was concentrated and dia-

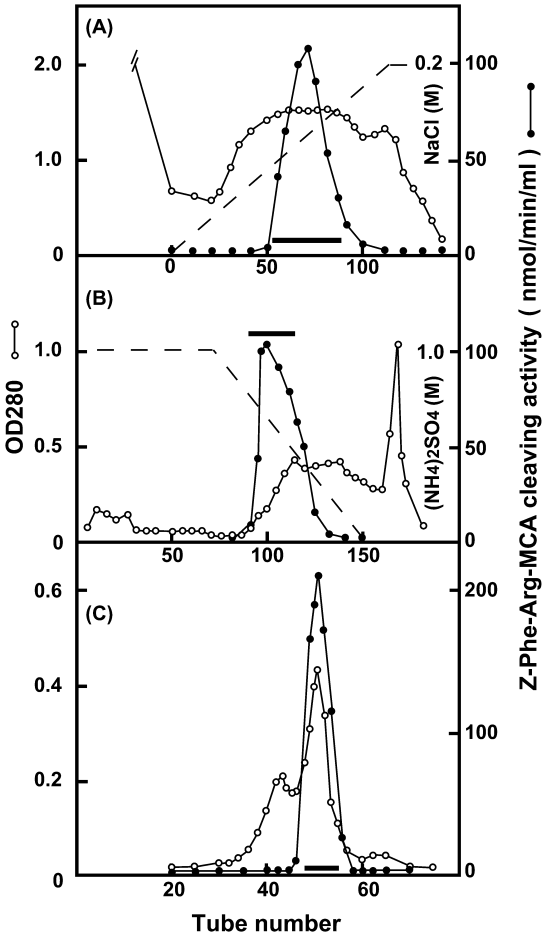

Fig. 2. **Purification of Z-Phe-Arg-MCA cleaving activity from wheat germ by a series of column chromatographic steps.** (A) 1st DEAE-cellulose column chromatography, (B) Phenyl-Sepharose chromatography and (C) Ultrogel AcA-34 gel filtration. Column chromatographies were carried out as described under "EXPERIMENTAL PROCEDURES." The fractions indicated by the horizontal bar were pooled.

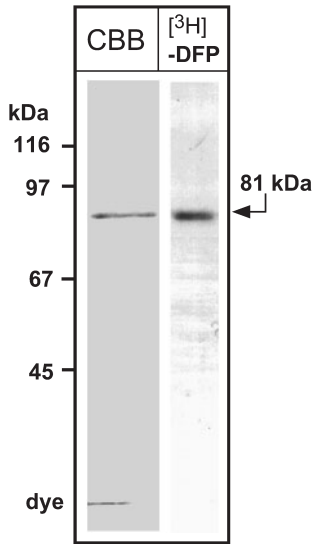

Fig. 3. **SDS-PAGE of the purified enzyme.** The purified enzyme (0.2 μg) was boiled in 2% SDS solution containing 10% β-mercaptoethanol and then resolved by electrophoresis through a 10% gel. Protein was detected using Coomassie Brilliant Blue (CBB). The purified enzyme was incubated with [^{3}H]DFP at 25°C for 30 min and then resolved by electrophoresis. The gel was soaked in Amplify (Amersham-Biosciences) and dried. Autoradiography was performed at –80°C using Konica X-ray film.

lyzed against 20 mM sodium phosphate buffer (pH 7.5). The dialysate was applied to a DEAE-cellulose column (0.9 × 4 cm) equilibrated with the same buffer and eluted using a linear gradient of NaCl (0–0.15 M). The fraction possessing activity was concentrated, dialyzed against 20 mM sodium phosphate buffer (pH 7.2) and applied to a Blue-Sepharose column (1.4 × 0.8 cm). The enzyme activity passed through the Blue-Sepharose column and was used as the final preparation with which to characterize the enzyme.

Sequence Analysis—The purified enzyme separated by SDS-PAGE (*20*) was electroblotted onto a PVDF membrane (Immobilon™, 0.45 μm; Millipore, Bedford, MA, USA) according to the manufacturer's instructions. The protein band was detected by staining with Ponsou 3R. The protein band (81 kDa) was cut into small pieces, reduced with dithiothreitol, alkylated with iodoacetic acid in the presence of 8 M guanidine-HCl and then digested with lysyl endopeptidase (*21*). Following incubation at 37°C overnight, the digestion mixture was lyophilized and dissolved in 0.1% trifluoroacetic acid. Samples were then subjected to reversed phase high performance liquid chromatography (RP-HPLC) on a μRPC C2/C18 pc3.2/3 column (Pharmacia, Sweden). Peptides were eluted at 0.24 ml/min with a linear gradient of acetonitrile (5–50% in 35 min) in 0.1% trufluoroacetic acid. The peak was collected, applied to glass fiber discs that had been coated with Polybrene and analyzed using an automated protein sequencer (Shimadzu PPSQ-10, Kyoto, Japan). For the cleavage specificity analysis, 2 nmol of peptide substrates (dynorphin A, glucagon, ACTH and somatostatin-28) were digested with purified oligopeptidase B (5 pmol) at 37°C. The mixture was then acidified with trifluoroacetic acid and separated by RP-HPLC as described above. Sequencing of the fragments was performed using a protein sequencer.

RESULTS

Trypsin-Like Protease Activity in Wheat Germ and Growing Embryo—Trypsin-like activity, reflected by the hydrolysis of Z-Phe-Arg-MCA at pH 8.0, was detected in the wheat germ extract (Fig. 1A). This activity, which was almost completely inhibited by leupeptin but not by E-64, eluted as a single peak at a position corresponding to a molecular mass of approximately 80 kDa on Sephadex G-100 (Fig. 1B). In an effort to investigate the temporal changes of this activity in germinated seeds, wheat (cv. Chikugoizumi) seeds were allowed to imbibe water for 6 days at 20°C. Germinating embryos (shoot and radicle) were dissected at 4°C from seedlings and the trypsin-like activity assayed. As shown in Fig. 1, the trypsin-like activity was also found in germinating embryos and increased following germination. As was the case with wheat germ (quiescent embryos), the activity in germinating embryos was inhibited by leupeptin but not by E-

Table 1. **Purification of trypsin-like enzyme from wheat quiescent embryos.**

Steps	Total protein	Activity		Yield	Purification
	(mg)	(μmol/min)	(μmol/min/mg)	(%)	(fold)
Crude extract	9,500	51.8	0.0054	100	1
$(NH_4)_2SO_4$ 35–60%	2,790	39.3	0.014	76	2.6
1st DEAE-cellulose	383	42.7	0.111	82	21
Phenyl Sepharose	22.9	29.9	1.30	58	241
Ultrogel AcA-34	9.6	24.2	2.50	47	463
2nd DEAE-cellulose	2.9	13.4	4.62	26	856
Blue-Sepharose	0.45	3.93	8.73	7.6	1,620

64. The trypsin-like activity also eluted as an 80 kDa protein when subjected to Sephadex G-100 gel filtration.

Purification of Trypsin-Like Activity from Wheat Embryo—Trypsin-like activity, reflected by the hydrolysis of Z-Phe-Arg-MCA, was purified from wheat germ as described in "EXPERIMENTAL PROCEDURES." The activity eluted as a single peak in all column chromatographic steps (1st DEAE-cellulose, phenyl-Sepharose and Ultrogel AcA34) as shown in Fig. 2. The final preparation yielded a single protein band corresponding to a molecular mass of 81 kDa on SDS-PAGE (Fig. 3). The 81-kDa band was successfully labeled with [^{3}H]DFP, suggesting that the enzyme is a serine protease (Fig. 3). Table 1 summarizes the results of a typical purification of the enzyme. From 100 g of wheat germ, we obtained 200 μg of purified enzyme.

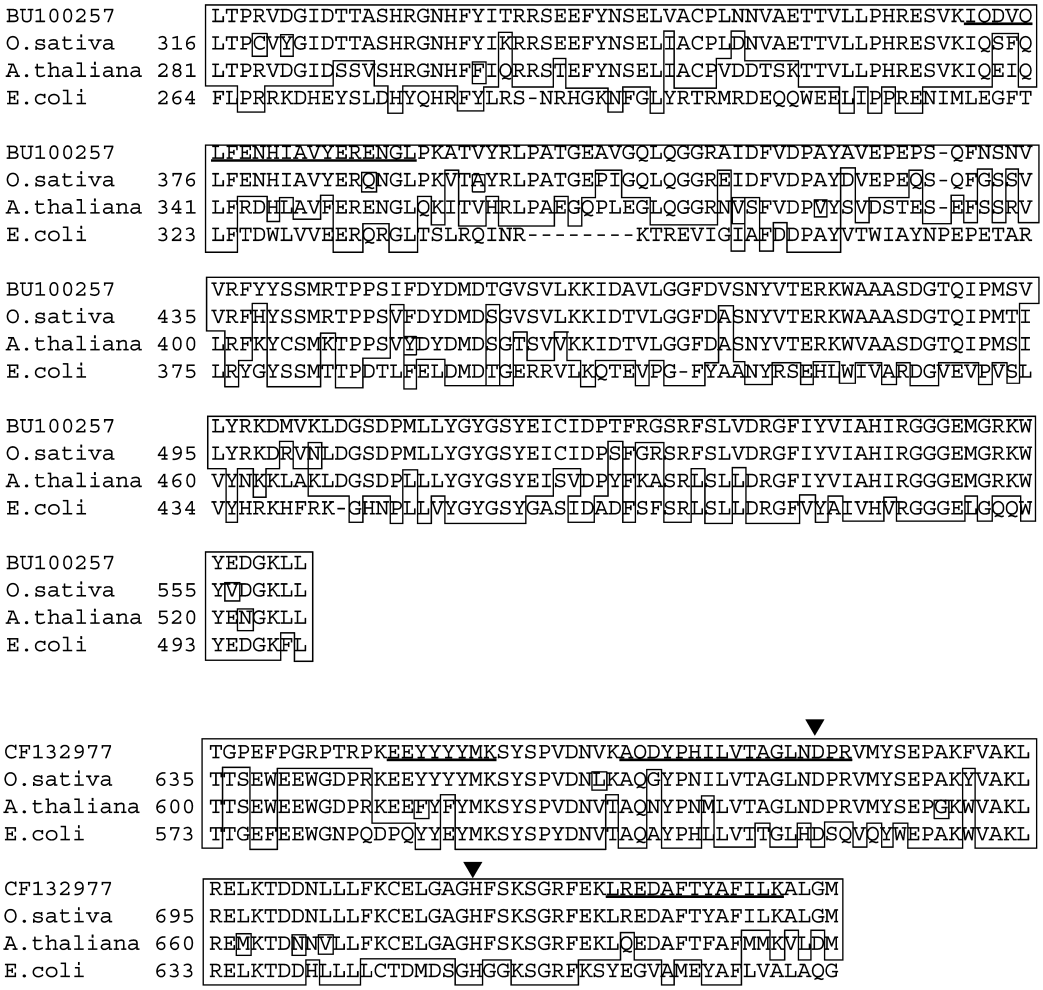

Fig. 4. **Alignment of the deduced wheat enzyme sequences with F14I3.4 from *Arabidopsis thaliana*, AK070316 from *Oriza sativa* and *E. coli* oligopeptidase B.** The wheat EST clones BU100257 and CF132977 were found by homology searches using amino acid sequences obtained for fragments generated by lysyl endopeptidase digestion of the purified enzyme. The translated sequence of BU100257 contains the peptide 35 sequence (underlined). CF132977 contains sequences corresponding to peptides 17, 29 and 39 (underlined). Identical amino acid residues among all aligned sequences are boxed. Arrowheads indicate active site residues, Asp and His.

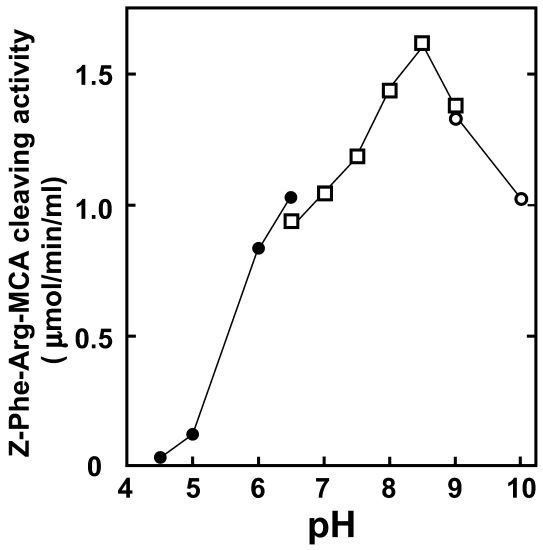

Fig. 5. **Effect of pH on the activity of the purified enzyme.** The pH optimum for the enzyme activity was determined in buffers of various pHs. Assays were performed under the standard conditions except for the buffers used, which were: acetate buffer (closed circles), Tris-HCl buffer (open squares) and Glycine-NaOH buffer (open circles).

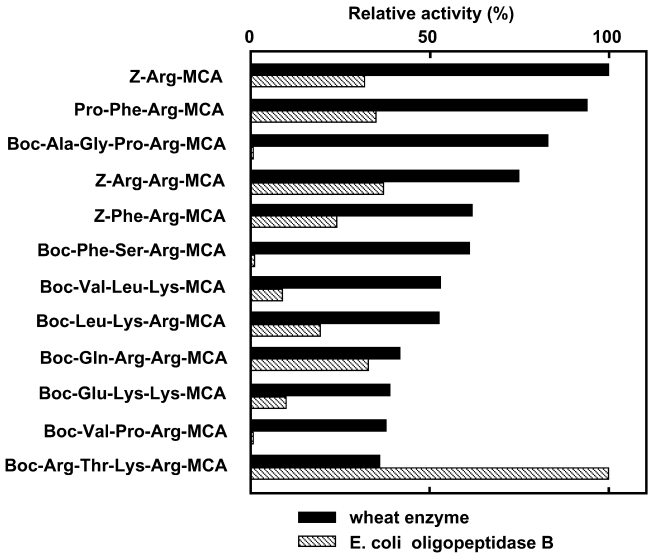

Fig. 6. **Cleavage specificity of the purified enzyme toward synthetic substrates.** Enzyme activity was determined in 0.1 M Tris-HCl buffer (pH 8.0) with various substrates at a concentration of 50 μM.

Sequence Analysis of the Purified Enzyme—In an effort to characterize the wheat trypsin-like serine protease at the amino acid level, the N-terminal sequence of the purified enzyme was examined. Approximately 100 pmol of the enzyme was applied to a protein sequencer, but no amino acid sequence was obtained, suggesting that the N-terminus of the enzyme was blocked. Consequently, the sequences of some of the peptides generated by lysyl endopeptidase digestion were determined. Seven peptides were purified by HPLC and sequenced. The sequence of peptide 12, 17, 21, 29, 35, 39 and 43 were GYYYYK, EEYYYYMK, AEGHDYYSIGAFK, AQDYPHILVTAGLNDPR, IQDVQLFENHIAVYERENGL, LREDAFTYAFILK and ALEDEIFAEIRGRIK, respectively. Investigations of homology using a protein database revealed that *E. coli* oligopeptidase B possesses homologous sequences to the sequences of peptide 17 (50% identity), peptide 29 (65%) and peptide 43 (53%). Similarly, use of TrEMBL revealed that the F14I3.4 protein, predicted from *Arabidopsis thaliana* cDNA nucleotide sequences (*22*), shows a high degree of homology to the collective enzyme peptide sequences. The amino acid identity in the region of F14I3.4 with amino acid number 84–93, 612–620, 140–152, 630–646, 336–355, 689–701 and 61–75 to the peptide sequences 12, 17, 21, 29, 35, 39 and 43 is 88%, 75%, 77%, 82%, 70%, 69% and 67%, respectively. The amino acid sequence predicted from the rice full-length cDNA (AK070316) (*23*) possesses even higher homology. The peptide sequences 17, 21 and 39 are identical to sequences 612–620, 175–187 and 724–736 of the rice protein, respectively. Peptides 29, 35 and 43 possess 94%, 85% and 80% identity, respectively. Screening of a wheat EST library revealed that translated sequences within clones BU100257 and CF132977 contained sequences corresponding to the peptides generated from the enzyme (Fig. 4). The peptide 35 sequence was found within the translated sequence of BU100257. The translated sequence of CF132977 possesses identical sequences to peptides 17, 29 and 39. The translated sequence of CF132977 is homologous to the C-terminal catalytic domain of *E. coli* oligopeptidase B. This fragment contained the aspartic acid and histidine active site residues of the serine protease. These sequence data strongly suggested that the wheat trypsin–like protease is an oligopeptidase B–related enzyme.

Cleavage Specificity toward Synthetic Substrate—The enzymatic properties of the wheat enzyme were examined in an effort to characterize the enzyme. When the effect of pH on the activity toward Z-Phe-Arg-MCA was examined, the enzyme displayed highest activity at pH 8.5 as shown in Fig. 5. The activity of the purified wheat enzyme was also examined against various synthetic substrates as shown in Fig. 6. The wheat enzyme hydrolyzed substrates possessing an arginine or lysine residue at the P1 position, indicative of trypsin-like proteases. The hydrolysis of a number of substrates followed Michaelis-Menten kinetics. However, plots of velocity *versus* substrate concentration for substrates possessing dibasic amino acids at P1 and P2 such as Z-Arg-Arg-MCA, Boc-Gln-Arg-Arg-MCA and Boc-Leu-Lys-Arg-MCA were not hyperbolic but exhibited pronounced substrate inhibition. This inhibition was apparent at 40 μM. The kinetic parameter k_{cat}/k_m (mM^{-1} s^{-1}) obtained for the hydrolysis of Boc-Gln-Arg-Arg-MCA, Z-Arg-Arg-MCA, Z-Arg-MCA and Z-Phe-Arg-MCA by the wheat enzyme were 1230, 443, 424 and 381, respectively. The wheat enzyme displayed broad subsite specificity for a number of substrates. The wheat enzyme failed to hydrolyze Arg-MCA, Gly-Pro-MCA, Suc-Gly-Pro-Leu-Gly-Pro-MCA, Suc-Leu-Leu-Val-Tyr-MCA and Suc-Ala-Ala-Asn-MCA. In contrast, *E. coli* oligopeptidase B did not display broad subsite specificity, although it cleaves at the carboxyl side of basic amino acids just like the wheat enzyme. Substrates possessing proline at P2 such as Boc-Ala-Gly-Pro-

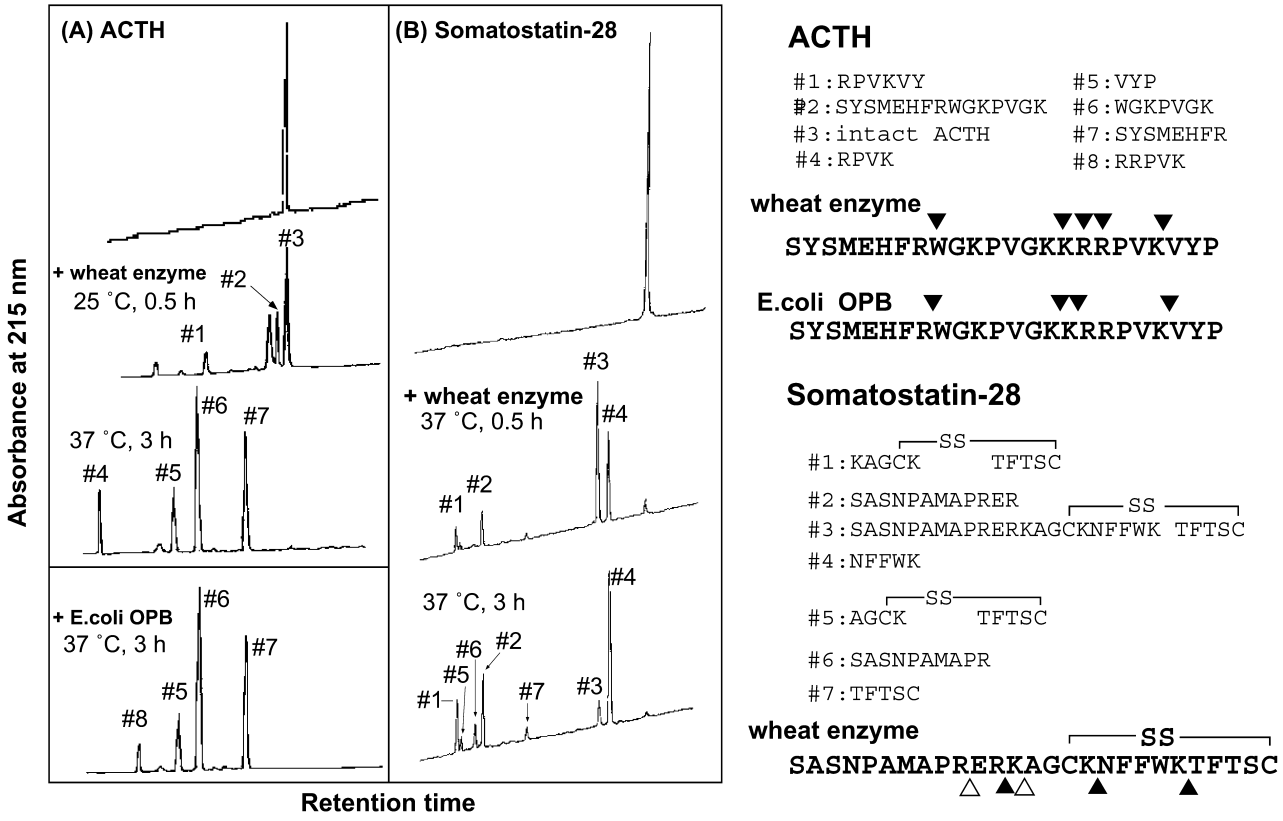

Fig. 7. **Cleavage of ACTH and somatostatin-28 by the purified enzyme and *E. coli* oligopeptidase B.** Left: HPLC profiles of the digested mixture of ACTH (A) and somatostatin-28 (B) with the purified enzyme and *E. coli* oligopeptidase B. Peaks were collected and sequenced. Right: The amino acid sequences (one-letter code) of the peptides and cleavage sites are shown. Closed and open arrowheads indicate major and minor cleavage sites, respectively.

Arg-MCA and Boc-Val-Pro-Arg-MCA were negligibly hydrolyzed by *E. coli* oligopeptidase B. Although Boc-Phe-Ser-Arg-MCA is a good substrate for the wheat enzyme, it was not efficiently hydrolyzed by *E. coli* oligopeptidase B.

Hydrolysis of Adrenocorticotropic Hormone (ACTH)—The ability of the wheat enzyme to hydrolyze model peptides was examined. Human ACTH was digested with the wheat enzyme at 25°C for 0.5 h and at 37°C for 3 h (Fig. 7A). Cleavage products were separated by reverse phase HPLC and the amino acid sequences determined. Partial digestion of ACTH at 25°C for 0.5 h generated SYSMEHFRWGKPVGK and RPVKVYP. These results indicated that dibasic amino acid sites (Lys^{15}–Lys^{16} and Arg^{17}–Arg^{18}) were preferentially hydrolyzed by the enzyme. Complete digestion of ACTH by the enzyme at 37°C for 3 h yielded the same amount of the four peptides. Cleavage occurred on the carboxyl side of Arg^{8}, Lys^{15}, Lys^{16}, Arg^{17} and Lys^{21}. However, Lys^{11}-Pro^{12} and Arg^{18}-Pro^{19} bonds were not hydrolyzed. The cleavage profile of ACTH by *E. coli* oligopeptidase B was also examined. As shown in Fig. 7A, cleavage of ACTH yielded four peptides. Peptide 8 was detected instead of peptide 4. The sequence of peptide 8 was Arg-Arg-Pro-Val-Lys, indicating that the Arg^{17}-Arg^{18} bond was not hydrolyzed by *E. coli* oligopeptidase B. Sequences of other peptides (5, 6, 7) were identical to the sequences of peptides generated by the wheat enzyme.

Hydrolysis of Somatostatin-28—Somatostatin-28 was digested with the wheat enzyme at 37°C for 0.5 h and 3 h (Fig. 7B). Cys^{17} and Cys^{28} of somatostatin-28 form a disulfide bridge, and somatostatin was used without reduction of the disulfide bond. Partial digestion of somatostatin for 0.5 h resulted in cleavage of the Arg^{13}-Lys^{14}, Lys^{18}-Asn^{19} and Lys^{23}-Thr^{24} bonds of somatostatin-28. Peptide 3 resulted from cleavage of somatostatin at the Lys^{23}-Thr^{24} bond. Arg^{11}-Glu^{12} and Lys^{14}-Ala^{15} of somatostatin-28 were hydrolyzed in addition to Arg^{13}-Lys^{14}, Lys^{18}-Asn^{19} and Lys^{23}-Thr^{24} following digestion for 3 h. These results indicated that the Lys^{23}-Thr^{24} bond of somatostatin-28 was the most sensitive site in the presence of the wheat enzyme.

Hydrolysis of Glucagon and Dynorphin A—Glucagon and dynorphin A were also digested with the wheat enzyme at 37°C for 3 h. The bonds cleaved were exclusively Arg-X and Lys-X as shown in Fig. 8. However, the Arg-Pro bond was not hydrolyzed. Although glucagon and dynorphin A both possess a dibasic sequence (Arg-Arg), their cleavage profiles differed. The wheat enzyme preferentially cleaved Arg^{6}-Arg^{7} in dynorphin A, while the Arg^{7}-Ile^{8} bond was not hydrolyzed. The wheat enzyme also cleaved Arg^{18}-Ala^{19} more efficiently than the Arg^{17}-Arg^{18} bond in glucagon.

Susceptibility to Inhibitors—The effect of various protease inhibitors on the enzyme was tested. The wheat

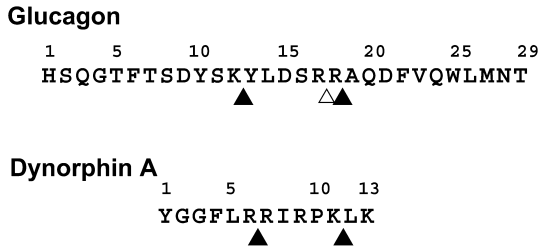

Fig. 8. **Hydrolysis of glucagon and dynorphin A by the purified enzyme.** The peptides (2 nmol) were digested at 37°C for 30 min and analyzed as described under "EXPERIMENTAL PROCEDURES." Closed and open arrowheads indicate major and minor cleavage sites, respectively.

Table 2. **Effect of various protease inhibitors on the activity of the purified enzyme.**

Protease inhibitor	Concentration	% Inhibition
DFP	5 mM	78
PMSF	1 mM	20
Benzamidine	5 mM	65
Aprotinin	100 μg/ml	16
STI	100 μg/ml	0
TPCK	0.5 mM	2
TLCK	0.5 mM	76
Leupeptin	5 μg/ml	96
E-64	5 μg/ml	2
EDTA	5 mM	0

enzyme was sensitive to potent inhibitors of trypsin-like protease. As shown in Table 2, the enzyme activity was strongly inhibited by leupeptin, TLCK, DFP and benzamidine. However, soybean trypsin inhibitor, TPCK, E-64 and EDTA had no significant inhibitory effect. The susceptibility to inhibition was very similar to that of *E. coli* oligopeptidase B, except in the case of leupeptin. About 30% of the activity of *E. coli* recombinant oligopeptidase B was inhibited by 1 mM leupeptin (420 μg/ml), although the activity was completely inhibited by 1 mM antipain (*11*). The difference in inhibitory effect of leupeptin against the wheat enzyme and *E. coli* oligopeptidase B is shown in Fig. 9A. The concentration of leupeptin required to achieve 50% inhibition (IC_{50}) of the wheat and *E. coli* enzymes was 0.05 μg/ml (0.12 μM) and 1.6 μg/ml (3.8 μM), respectively. The wheat enzyme was inhibited with a 32-fold lower concentration of leupeptin than the *E. coli* enzyme. The effect of antipain on enzyme activity was also compared (Fig. 9B). The wheat enzyme (IC_{50} 0.12 μg/ml) was inhibited with a 41-fold lower concentration of antipain (IC_{50} 4.9 μg/ml) than the *E. coli* enzyme.

Effect of NaCl—Kinetic analysis of *E. coli* oligopeptidase B showed that the enzyme–substrate interaction was disrupted by high ionic strength, suggesting the involvement of significant ionic interactions in the substrate binding (*24*). We compared the effect of NaCl concentration on the wheat and *E. coli* enzymes (Fig. 10). Both enzymes were strongly inhibited by high concentrations of NaCl. However, the wheat enzyme (IC_{50}: 0.8 M) was more sensitive to NaCl than the E.coli enzyme (IC_{50}: 1.4 M).

DISCUSSION

Until recently, investigations into oligopeptidase B in higher plants received little attention, since it was thought that this enzyme was present only in prokaryotes and ancient eukaryotic unicellular organisms. This study has provided the first identification of oligopeptidase B at the protein sequence level in plants. A trypsin-like serine protease was purified to homogeneity from wheat germ. The characteristics of this enzyme resembled those of serine peptidase oligopeptidase B more than those of trypsin. Further enzymatic analysis corroborated this initial finding. A trypsin-like serine-endoprotease has been found in wheat seedlings (*4*) and soybean (*25*), although a comparison with oligopeptidase B was not reported. Putative oligopeptidase B–encoding genes can be identified in *Arabidopsis* (F14I3.4) and *Oriza* (AK070316) databases, although no definitive evidence

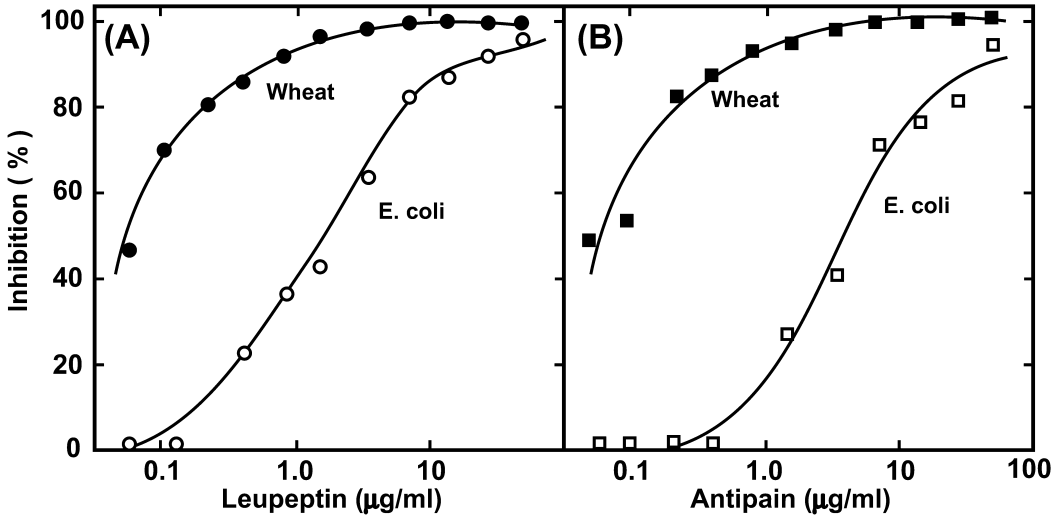

Fig. 9. **Comparison of inhibitor sensitivity of the wheat enzyme and *E. coli* oligopeptidase B.** Increasing amounts of leupeptin (A) or antipain (B) were added to the reaction mixture.

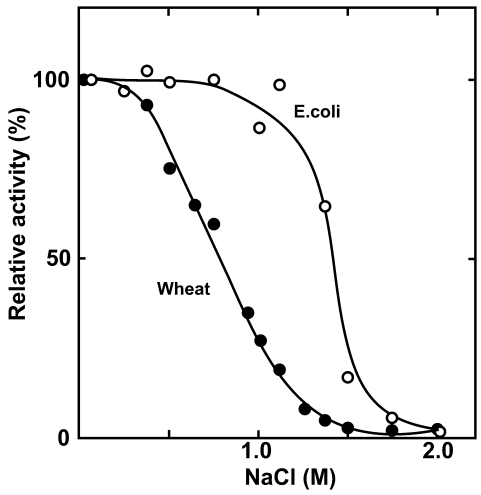

Fig. 10. **Comparison of the effect of NaCl concentration on the activity of wheat enzyme and *E. coli* oligopeptidase B.**

exists to confirm that these code for active proteases. The wheat EST clones BU100257 and CF132977 encode part of the N- and C-terminal catalytic domains of oligopeptidase B, respectively. These sequences are highly homologous to the corresponding region of *Arabidopsis* and rice oligopeptidase B. These results strongly support the notion that active oligopeptidase B is expressed in these plants. A comparison of the enzymatic properties of wheat and *E. coli* oligopeptidase B revealed differences in subsite specificity and sensitivity to various inhibitors. Wheat oligopeptidase B hydrolyzed the carboxyl side of Arg or Lys residues, whereas, unlike the *E. coli* enzyme, it did not hydrolyze Arg-MCA. On the other hand, wheat oligopeptidase B displayed broader subsite specificity than *E. coli* oligopeptidase B. Furthermore, the wheat enzyme was more sensitive to leupeptin and antipain than the *E. coli* enzyme. Thus, we were able to demonstrate for the first time the expression of active oligopeptidase B in higher plants and its unique enzymatic properties. Oligopeptidase B has been classified in the prolyl oligopeptidase family (*11*, *12*). Although oligopeptidase B and prolyl oligopeptidase consist of an N-terminal β-propeller domain and a C-terminal catalytic domain of serine peptidase (*26*), their cleavage specificities differ markedly. Prolyl oligopeptidase is expressed in mammals, whereas oligopeptidase B is not, while both enzymes seem to be expressed in plants. Prolyl oligopeptidase has been purified from carrot (*27*). The genes encoding prolyl oligopeptidase (F14O10.2 protein) and oligopeptidase B (F14I3.4 protein) have been identified in *Arabidopsis thaliana*. As shown in Fig. 1A, oligopeptidase B activity in wheat embryos increased following germination. These results strongly suggested a unique physiological role of plant oligopeptidase B in germination. Oligopeptidase B displayed no significant activity against protein substrates, which suggested that the enzyme does not play an important role in the initial degradation of reserve proteins. Although the localization of oligopeptidase B in germinating embryos suggested its role in the processing or turnover of peptides, rather than in the digestion of storage proteins present in the endosperm, further analysis of its cell-specific expression profile is necessary. Endoproteolytic cleavage, which usually occurs at sites possessing paired basic amino acids, is commonly observed in the post-translational activation of proteins such as peptide hormone precursors and differentiation factors (*28*). Subtilisin-like proprotein convertases possessing this type of activity have been identified in eukaryotes. In plants, subtilisin-like proteases have been shown to play a role in cell differentiation (*8*, *9*). Given the strict cleavage specificity of oligopeptidase B, it seems likely that the enzyme is involved in the limited hydrolysis of certain physiologically important peptides, although the precise physiological substrate(s) of plant oligopeptidase B remain unknown. Systemin is an 18-amino-acid peptide (AVQSKPPSKRDPPKMQTD) and a possible endogenous substrate of oligopeptidase B. Systemin has been isolated from wounded tomato leaves and shown to regulate the activation of over 20 defensive genes including proteinase inhibitors, signal pathway components, and proteinases in response to mechanical wounding and insect attack (*29*). Systemin is derived from prosystemin, a 200-amino-acid precursor. Like oligopeptidase B, prosystemin does not possess a putative signal peptide, suggesting that they are synthesized in free ribosomes in the cytosol. It is unlikely that oligopeptidase B would be involved in the maturation of prosystemin, since the processing sites of prosystemin, REDL^{178}↓A and MQTD^{196}↓N, are not coincident with the cleavage specificity of the enzyme. Systemin possesses three potential cleavage sites (Lys^{9}, Arg^{10} and Lys^{14}) for oligopeptidase B, suggesting the involvement of oligopeptidase B in the cytosolic degradation of systemin. Similarly, prolyl oligopeptidase has been shown to play a role in the degradation of various peptide hormones, such as substance P in animals (*30*). On the other hand, oligopeptidase B has been found in *Trypanosoma cruzi*, and the enzyme has been shown to play an important role in the generation of a signaling ligand for mammalian host cells that is involved in the mechanism of host cell invasion by this intracellular pathogen (*16*). *Tripanosoma* oligopeptidase B generates a calcium-signaling factor that is responsible for the mobilization of Ca^{2+} from intracellular calcium pools. Targeted deletion of the oligopeptidase B gene in *Tripanosoma cruzi* resulted in the generation of trypanosomes that were severely impaired with respect to mammalian cell Ca^{2+} signaling and invasion. Just as in animals, Ca^{2+} is known to play a key role in the early development of plant embryos. It would be of interest to determine whether plant oligopeptidase B is involved in Ca^{2+} signaling like oligopeptidase B in trypanosomatids. The generation and investigation of mutant plants lacking oligopeptidase B activity would assist us in delineating the *in vivo* function of this enzyme.

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