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Identification and Possible Roles of Three Types of Endopeptidase from Germinated Wheat Seeds[1]

Keita Sutoh, Hideki Kato,[2] and Takao Minamikawa

Department of Biological Sciences, Tokyo Metropolitan University, Minami-ohsawa, Hachioji, Tokyo 192-0397

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Little or no endopeptidase activity was detected in extracts of dry mature wheat seeds, but when they were allowed to imbibe water in darkness, the activity expressed per seedling increased notably after d 1, reached a maximum on d 3 and then decreased. Two major endopeptidases, named WEP-1 and WEP-2, were present in the 50-70% saturated ammonium sulfate fraction of d-3 seedlings, and could be separated by hydrophobic column chromatography. WEP-1 was further purified and identified as a 31-kDa polypeptide that was immunoreactive to antiserum raised against REP-1, a major rice cysteine endopeptidase. Experiments with proteinase inhibitors revealed that WEP-1 and WEP-2 are cysteine and serine endopeptidases, respectively. The two enzymes differed in substrate specificity, pH dependence, and the ability to digest major wheat seed proteins. Determination of its amino-terminal amino acid sequence indicated the similarity of WEP-1 to other cereal cysteine endopeptidases which are involved in the digestion of seed storage proteins. The expression of WEP-1 in de-embryonated seeds was induced in the presence of gibberellic acid and its effect was eliminated by abscisic acid. In addition to WEP-1 and WEP-2, a legumain-like asparaginyl endopeptidase was identified in the extract of seedlings on hydrophobic chromatography. The asparaginyl endopeptidase may function in the early step of mobilization of wheat storage proteins in germinated seeds.

Key words: asparaginyl endopeptidase, cysteine endopeptidase, gibberellic acid, serine endopeptidase, wheat seed protein.

Cereal grains such as rice, barley, and wheat store reserve proteins in the aleurone layer and the starchy endosperm. During seed germination and early seedling growth, the protein is hydrolyzed by proteases and mobilized to supply amino acids which support the growth of the embryo. Proteases in these seeds are synthesized primarily in the aleurone layer. Although it is generally accepted that reserve proteins are digested by a combination of various peptide hydrolases (*1*), a number of reports have shown that endopeptidases play a central role in the mobilization process (*2-5*). In our previous studies (*6*) we isolated and characterized a cysteine endopeptidase, designated SH-EP, from the cotyledons of *Vigna mungo* seedlings. This enzyme functions to degrade the storage globulin in combination with a serine endopeptidase. The amino acid sequence of SH-EP, as deduced from the nucleotide sequence of the cDNA, showed high similarity (60%) to that of EP-B, one of the secreted cysteine endopeptidases that are synthesized in the barley aleurone layer and hydrolyze the reserve proteins, hordeins (*3, 7*). Both SH-EP and EP-B are known to be synthesized *via* multiple post-translational processing events (*8, 9*). Another cysteine protease, called aleurain, was originally described as a cDNA from barley seedlings (*10*). This enzyme was later isolated as a non-secreted 32-kDa aminopeptidase functionally similar to mammalian cathepsin H (*11*). Using a cDNA for aleurain as a probe, three cDNA clones (oryzains α, β, and γ) were selected from a cDNA library of rice seedlings (*12*). The amino-terminal amino acid sequence of a 23-kDa protease isolated from rice seedlings was consistent with the corresponding sequence deduced from the nucleotide sequence of oryzain α cDNA (*13*). Two major cysteine endopeptidases, termed REP-1 and REP-2, have been isolated from extracts of rice seeds (*14*). REP-1 was shown to be the major enzyme that digests rice glutelin, and REP-2 to be an asparaginyl endopeptidase, but the relevance of these enzymes to oryzains remains uncertain.

The mature seed of wheat, one of the most important cereal crops, contains 12% protein on average, glutenin (wheat glutelin) and gliadin (wheat prolamin) accounting for 46 and 40% of the total storage proteins, respectively (*1*). Only a limited number of studies, however, have been performed on proteases acting to degrade reserve proteins in wheat seeds during germination, while information at the protein level is basically crucial to understand biochemical events occurring in germinating wheat seeds. In the present study we intended to identify and characterize proteolytic enzymes that play greater roles in the mobilization of wheat

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[2] To whom correspondence should be addressed. Tel: +81-426-77-2562, Fax: +81-426-77-2559, E-mail: kato-hideki@comp.metro-u.ac.jp
Abbreviations: ABA, abscisic acid; CBB, Coomassie Brilliant Blue R; DFP, diisopropyl fluorophosphate; E-64, *trans*-epoxysuccinyl-L-leucylamido(4-guanidinobutane); GA_3, gibberellic acid; MCA, 4-methylcoumaryl-7-amide; Z, carbobenzoxy.

seed proteins. We postulated that germination-specific cysteine endopeptidases corresponding to leguminous SH-EP, rice REP-1, and barley EP-B should occur in wheat seedlings, and should have an important function in the mobilization of seed storage proteins. We separated two major endopeptidases, termed WEP-1 and WEP-2, from extracts of wheat seedlings by hydrophobic chromatography and found that WEP-1 of 31 kDa, but not WEP-2, was immunoreactive to an antiserum raised against REP-1. Experiments with various proteinase inhibitors revealed that WEP-1 and WEP-2 are cysteine and serine endopeptidases, respectively. In addition, a legumain-like asparaginyl endopeptidase was separated from the extract. This enzyme may function in an early step of the seed protein digestion. As to proteases in wheat seeds, three isoenzymes of 33.5-kDa cysteine endopeptidase (proteinase A) were identified (*15*), and a 23-kDa enzyme was isolated from dormant seeds (*16*). Another cysteine endopeptidase acting on gliadin was separated from extracts of the durum wheat endopserm (*17*). However, no endopeptidases consistent with these enzymes were identified in the present study. The relevance of these enzymes to those identified in this study will be discussed.

EXPERIMENTAL PROCEDURES

Plant Materials—Wheat seeds (*Triticum aestivum* L. cv. Norin 61) were allowed to imbibe water on a layer of quartz sand at 25°C in darkness until collection.

Endopeptidase Assay—Endopeptidase activity was measured as described elsewhere using azoalbumin as a substrate (*7*). One unit of enzymatic activity is defined as the amount of enzyme that gives an increase in absorbance of 1.0 unit at 366 nm under the assay conditions. The activity was also measured with Z-Phe-Arg-MCA (Peptide Institute) or Z-Ala-Ala-Asn-MCA as a substrate (*18*), and one unit is defined as the amount of enzyme that releases 1 μmol 7-amino-4-methylcoumarin in 1 min under the standard assay conditions. Z-Ala-Ala-Asn-MCA was synthesized as described (*19*).

Chromatographic Isolation of Endopeptidases—All procedures were conducted at 0-4°C. D-3 whole seedlings (800 g) were powdered in liquid nitrogen, and the powder was homogenized with two volumes of 50 mM sodium acetate buffer, pH 6.0, containing 10 mM 2-mercaptoethanol (buffer A). The homogenate was squeezed through bleached cotton cloth, and centrifuged twice at 10,000 × *g* for 30 min to obtain a crude extract. Solid ammonium sulfate was added to the extract to give 50-70% saturation. After centrifugation at 10,000 × *g* for 30 min, the precipitate was dissolved in 50 mM Tris-Cl buffer, pH 6.5, containing 10 mM 2-mercaptoethanol (buffer B). The solution was dialyzed against the same buffer, and loaded onto a column (22 × 200 mm) of DEAE-Toyopearl (Tosoh) which had been equilibrated with the same buffer. The column was first washed with the same buffer, and the adsorbed proteins were eluted with a linear gradient (160 ml/160 ml) of 0 to 0.3 M potassium chloride in buffer B (*cf.* Fig. 2). The active fraction (tubes 28-33) was dialyzed against buffer A containing 1 M ammonium sulfate, and then applied onto a column (16 × 400 mm) of butyl-Cellulofine (Seikagaku Kogyo) which had been equilibrated with the same buffer. The column was first washed with the buffer, and then the adsorbed proteins were eluted with a linear gradient (180 ml/180 ml) of 1.0 to 0 M ammonium sulfate in buffer A. The endopeptidase activity was separated into two fractions by this chromatography: WEP-1 in tubes 27-32 and WEP-2 in tubes 33-38. The WEP-1 fraction, which showed immunoreactivity to rabbit antiserum raised against REP-1 (*14*), was further purified by gel filtration. Proteins in the fraction were precipitated by adding ammonium sulfate to 80% saturation. The precipitate was dissolved in a small volume of 50 mM sodium acetate buffer, pH 6.0, containing 10 mM 2-mercaptoethanol and 0.3 M potassium chloride, and dialyzed against the same buffer. The fraction was then loaded onto a column (16 × 900 mm) of Sephacryl S-200 (Amersham Pharmacia Biotech) which had been equilibrated with the buffer. The active fraction (tube 9), eluted with the same buffer, was desalted on a column of PD-10 (Amersham Pharmacia Biotech) and lyophilized. The amounts of protein were estimated by the method of Bradford (*20*) using BSA as a standard.

In experiments to separate asparaginyl endopepetidases, a 50-70% saturated ammonium sulfate fraction prepared from 300 g of d-3 seeds was applied onto a column of butyl-Cellulofine. Fractions obtained from the column were examined for endopepetidase activities with Z-Phe-Arg-MCA and Z-Ala-Ala-Asn-MCA as substrates, and also analyzed as to their immunoreactivities to antisera against REP-1 (*14*) and legumain (*21*).

Gel Electrophoresis and Immunoblotting—SDS-PAGE (*22*) was conducted on 12.5% gels, and the gels were stained with CBB. After SDS-PAGE, immunoblotting with antiserum raised against REP-1 or legumain was performed as described elsewhere (*8*). Protein bands were visualized with an ECL kit (Amersham Pharmacia Biotech). The antiserum against legumain was generously provided by Dr. Y. Miura-Izu, Biotechnology Research Laboratories, Takara Shuzo.

To study the change over time in the level of the WEP-1 protein in germinated seeds, wheat seeds were allow to imbibe water for 1 to 7 d. Seedlings (20 pieces) with shoots and roots removed at each indicated growth stage were homogenized in 5 ml of 50 mM sodium acetate, pH 6.0, containing 10 mM 2-mercaptoethanol. The homogenate was centrifuged at 20,000 × *g*, and the clarified supernatant was used as the enzyme source.

To examine hormonal effects on the level of the WEP-1 protein, de-embryonated dry seeds (1 g, *ca.* 40 pieces) were crushed into several pieces in a mortar. The broken seeds were sterilized in 0.1% sodium hypochlorite containing 0.05% Tween 20 for 30 min, rinsed thoroughly with sterile water, and then incubated at 25°C in 20 ml of sterile 20 mM sodium citrate buffer, pH 5.0, containing 20 mM calcium chloride and 10 μg/ml chloramphenicol in the presence and absence of plant hormones for the times indicated. After the incubation, the broken seeds together with the whole culture medium were homogenized in a mortar and pestle, and the homogenate was centrifuged at 8,000 × *g* for 10 min. The supernatant was mixed with three volumes of cold acetone, and the mixture was allowed to stand for 2 h at −80°C. Following the centrifugation, the precipitated protein was subjected to SDS-PAGE/immunoblotting. In some experiments, whole seeds (60 pieces each) were allowed to germinate for 3 d in the presence and absence of ABA or uniconazole, and protein fractions were prepared

and analyzed as above.

Preparation of Wheat Glutenin and Gliadin, and Their Digestion In Vitro—Wheat glutenin and gliadin were prepared essentially according to Rubin *et al.* (*23*). Wheat seeds (10 g) were ground liquid nitrogen, and then the powder was homogenized in ten volumes of 50 mM Tris-Cl, pH 6.5, containing 0.5 M sodium chloride. The homogenate was centrifuged twice at 10,000 × *g* for 10 min. The precipitate was extracted with 60% (v/v) 1-propanol for 2 h with shaking, and the extract was centrifuged at 15,000 × *g* for 1 h. The supernatant was dialyzed overnight against distilled water. The precipitate formed during dialysis was collected by centrifugation (gliadin fraction). The precipitate not dissolved in 60% (v/v) 1-propanol was suspended in 4 M urea-50 mM Tris-Cl, pH 6.5, and shaken for 10 h, and the suspension was centrifuged at 15,000 × *g* for 1 h. The supernatant was dialyzed against distilled water, and after centrifugation at 15,000 × *g* for 1 h the glutenin fraction was obtained as the precipitate. Both fractions were lyophilized to fine powders. SDS-PAGE analysis showed that the polypeptide compositions of the fractions were consistent with those reported for glutenin and gliadin (*23*).

One digestion mixture consisted of 1 mg glutenin, 50 mM sodium citrate buffer (pH 6.0) containing 10 mM 2-mercaptoethanol and a WEP-1 fraction (45 units) obtained from the Sephacryl S-200 column, in a total volume of 1.0 ml. The other digestion mixture consisted of 1 mg gliadin, 50 mM Tris-Cl buffer (pH 7.5) containing 10 mM 2-mercaptoethanol and a WEP-2 fraction (45 units) obtained from the butyl-Cellulofine column, in a total volume of 1.0 ml. After a mixture had been incubated at 30°C for the indicated times it was centrifuged, and the precipitate and supernatant were analyzed by SDS-PAGE/CBB. As control runs, the buffer solution was added instead of the enzyme solution.

Determination of the Amino-Terminal Amino Acid Sequence of WEP-1—The WEP-1 preparation obtained from the Sephacryl S-200 gel filtration column (Fig. 2C) was desalted on a PD-10 column (Amersham Pharmacia Biotech), and then lyophilized (68 μg). The protein was dissolved in 100 μl SDS-PAGE sample buffer, boiled for 3 min, and then subjected to SDS-PAGE. After the electrophoresis, polypeptides in the gel were electroblotted onto a PVDF membrane (Millipore) (*24*). The region containing WEP-1 was cut out from the membrane, and then the amino-terminal amino acid sequence was determined with an automated sequence analyzer (model 477A, Applied Biosystems) equipped with an on-line HPLC system for the quantitative identification of phenylthiohydantoin derivatives of amino acids.

RESULTS

Changes over Time in Endopeptidase Activity and the Amount of WEP-1 Protein in Wheat Seeds after Onset of Imbibition—Endopeptidase activity in extracts of dark-grown wheat seedlings was measured (Fig. 1). Virtually no endopeptidase activity was observed in extracts of dry (quiescent) seeds, but the activity, as expressed per seedling, increased notably after d 1, reached a maximum on d 3 and gradually decreased thereafter. The relative levels of the WEP-1 protein were determined by scanning immunoreactive bands on an immunoblot with a densitometer. No detectable amount of the WEP-1 protein was found in extracts of dry seeds, but the level expressed per seed increased notable after d 1, reached a maximum on d 3 and then decreased rather sharply (Fig. 1). Activity staining by the gelatin-plate method (*8*) revealed that WEP-1 was the major endopepetidase occurring in extracts of germinated seeds (data not shown). This enzyme exhibited high activity between d 3 and d 5, whereas WEP-2 showed lower activity, probably partly because gelatin was not a good substrate for the enzyme.

Purification of WEP-1—An ammonium sulfate fraction (50-70% saturation) of extracts of d-3 seedlings was separated by anion exchange chromatography on DEAE-Toyopearl, and the endopeptidase activity in the fractions was measured using Z-Phe-Arg-MCA as the substrate (Fig. 2A), since Arg at P_1 with Phe at P_2 is the most efficient cleavage site for proteinases of the papain family (*25, 26*), although the enzymes of this type are thought to have rather broad substrate specificity ranges. One major active peak (tubes 28-33) was eluted from the column. Immunoblot analysis showed that only these tubes contained the 31-kDa polypeptide that was immunoreactive to the antiserum against REP-1. The active fraction was then subjected to hydrophobic chromatography on butyl-Cellulofine. Two major fractions of the endopeptidase activity were eluted from the column, and designated WEP-1 (tubes 27-32) and WEP-2 (tubes 33-38). Immunoblotting showed that only WEP-1, *i.e.* not WEP-2, was immunoreactive to the antiserum. The WEP-1 fraction was used for further chromatographic purification on Sephacryl S-200 (Fig. 2C), while no further purification of the WEP-2 fraction was performed in this study. Tube 9 eluted from the gel filtration column gave one major distinguishable silver-stained band, which corresponded to the immunoreactive 31-kDa polypeptide (Fig. 3A). Thus, this region of this polypeptide was used to determine the amino-terminal amino acid sequence of WEP-1.

Six of the seven amino-terminal amino acid residues of

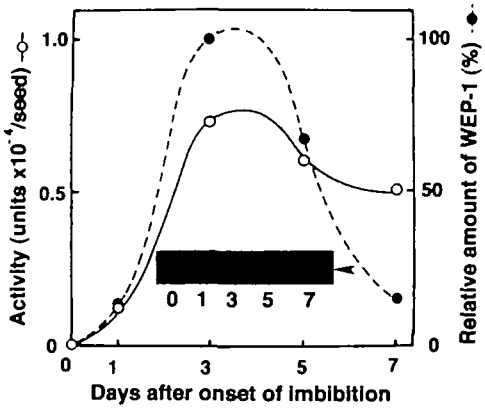


Fig. 1. **Changes with time of endopeptidase activity in wheat seedlings during germination and early growth.** Endopeptidase activity toward azoalbumin (open circles) was measured in extracts of seedlings at the growth stages indicated. The relative levels of the WEP-1 protein (closed circles) in the extracts were determined by SDS-PAGE/immunoblotting with rabbit antiserum against REP-1. The relative amounts of the WEP-1 protein per seed were analyzed by scanning the immunoreactive bands (Inset) with a densitometer, and the highest level was set as 1.0. The arrowhead indicates the 31-kDa polypeptide corresponding to WEP-1.

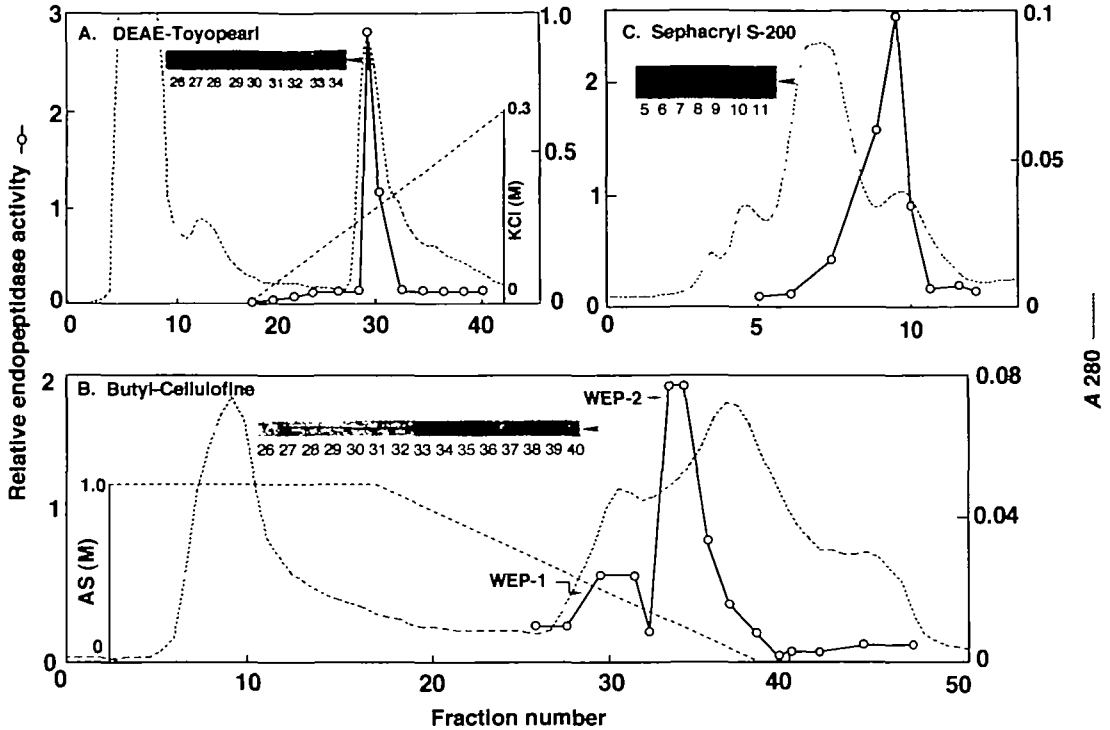


Fig. 2. **Elution profiles of WEP-1 and WEP-2 on column chromatography.** A: The 50-70% saturated ammonium sulfate fraction was chromatographed on a DEAE-Toyopearl column. The active fraction was immunoreactive to the anitiserum against REP-1 after SDS-PAGE (Inset). B: The active fraction obtained from the DEAE-Toyopearl column was applied onto a butyl-Cellulofine column. Endopeptidase was distributed in two peaks (designated WEP-1 and WEP-2). Only the WEP-1 fraction was immunoreactive to the antiserum against REP-1. C: The WEP-1 fraction obtained from the butyl-Cellulofine column was concentrated and loaded onto a Sephacryl S-200 column. The enzymatic activity was measured with Z-Phe-Arg-MCA as the substrate. For details, see "EXPERIMENTAL PROCEDURES."

WEP-1 were found to be identical to those of two well defined cereal cysteine endopeptidases, EP-B from barley seedlings (*3*) and REP-1 from rice seedlings (*14*) (Fig. 3B).

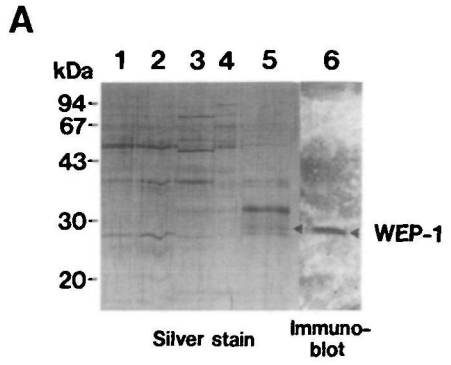
Separation of Asparaginyl Endopeptidases—When a 50-70% saturated ammonium sulfate fraction of d-3 seedlings was chromatographed on a column of butyl-Cellulofine, two major peaks (tubes 44-49 and tubes 55-56) of endopeptidase activity toward Z-Ala-Ala-Asn-MCA were detected (Fig. 4). The peaks of asparaginyl endopeptidase activity did not correspond to the fractions active toward Z-Phe-Arg-MCA. However, only one peak (tubes 55-56) showed clear immunoreactivity to the antiserum against legumain, the jackbean asparaginyl endopeptidase (*21*). The results indicate that there is at least one legumain-like asparaginyl endopeptidase with a molecular mass of 40 kDa in extracts of the seedlings. The activity toward Z-Phe-Arg-MCA was also separated into two peaks, but exhibited immunoreactivity to the antiserum against REP-1 in a wide range of fractions (tubes 38-47), indicating that this chromatographic procedure was unable to clearly separate WEP-1 and WEP-2.

Characterization of the Reactions—The WEP-1 fraction obtained from the Sephacryl S-200 column and the WEP-2 fraction from the butyl-Cellulofine column were mainly used to characterize the reactions. WEP-1 showed a sharp pH-activity curve with an optimum at pH 6.0 when Z-Phe-Arg-MCA was used as the substrate (Fig. 5), whereas WEP-2 showed a pH profile with a high activity range of pH 7.0-7.5.

The effects of various protease inhibitors on the enzymatic reactions are summarized in Table I. WEP-1 was sensitive to inhibition by a low concentration (0.01 mM) of E-64, a potent inhibitor of cysteine proteases, but insensitive to a high concentration (1 mM) of DFP, a typical inhibitor of serine proteases, or 0.02 mM pepstatin, an inhibitor of aspartate proteases. A high concentration of EDTA weakly inhibited the activity. DFP showed a strong inhibitory effect against WEP-2, but E-64 or EDTA showed virtually no inhibition of the WEP-2 activity, and EDTA caused very weak inhibition. The results indicate that WEP-1 is a cysteine endopeptidase and WEP-2 a serine endopeptidase.

WEP-1 exhibited high activity toward Z-Phe-Arg-MCA and weak activity toward Z-Ala-Ala-Asn-MCA, but essentially no activity toward four other substrates tested (Table II). In contrast, WEP-2 showed high activities toward a wide range of substrates with the exception that Z-Ala-Ala-Asn-MCA was not an efficient substrate. The K_m values of WEP-1 and WEP-2 for Z-Phe-Arg-MCA were determined to be 5.5 μM at pH 6.0 and 2.1 μM at pH 7.5, respectively.

Digestion of Wheat Seed Proteins by WEP-1 and WEP-2—The ability of WEP-1 and WEP-2 to hydrolyze two major storage proteins of wheat seeds was examined using enzyme preparations obtained from the butyl-Cellulofine column (Fig. 2B). The wheat glutenin preparation used consisted of 30- to 50-kDa low molecular weight and 70- to 100-kDa high molecular weight subunits. WEP-1 digested both glutenin subunits nearly completely within the initial



B

WEP-1	D L P P X V D X R
REP-1	D L P R A V D W R
EP-B	D L P P S V D W R
Proteinase A	S E Q L P K V F D A R

Fig. 3. **Partial purification (A) and amino-terminal amino acid sequence (B) of WEP-1.** A: The protein compositions of partially purified fractions of WEP-1 were examined by SDS-PAGE and immunoblotting. Lane 1, crude extracts; lane 2, 50-70% saturated ammonium sulfate fraction; lane 3, fraction eluted from a DEAE-Toyopearl column; lane 4, fraction eluted from a butyl-Cellulofine column; lanes 5 and 6, fraction from a Sephacryl S-200 column. Proteins (0.3 μg) were silver-stained (lanes 1-5) or immunoblotted with antiserum raised against REP-1 (lane 6). The arrowheads indicate the 31-kDa polypeptide corresponding to WEP-1. B: The amino-terminal seven residues of WEP-1 were sequenced and compared with those of rice REP-1 (*14*), barley EP-B (*3*), and wheat proteinase A (*15*). The wheat enzyme consists of three electrophoretic components with an identical amino-terminal sequence. The amino acid sequences are presented in the one-letter code, and the identical residues in the upper three enzymes are shaded. X, unidentified residue.

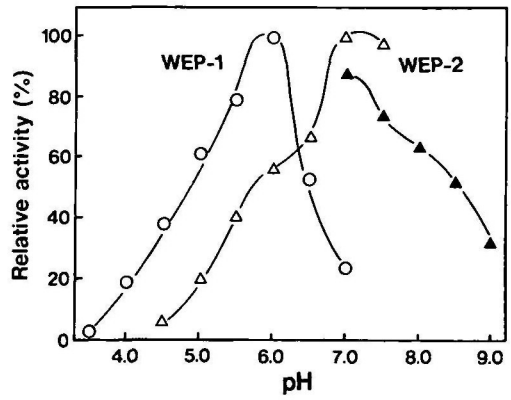


Fig. 5. **pH dependence of WEP-1 and WEP-2.** The reaction mixture consisted of 50 mM sodium citrate buffer (pH 3.5-7.5) or Tris-Cl (pH 7.0-9.0), 10 mM 2-mercaptoethanol, 25 mM Z-Phe-Arg-MCA as a substrate and 1.2 units of the WEP-1 fraction (open circles) obtained from the Sephacryl S-200 column or 3.3 units of the WEP-2 fraction (open and closed triangles) from the butyl-Cellulofine column, in a total volume of 2 ml. The reaction was conducted for 15 min under the standard conditions. The highest level was set as 100%.

TABLE I. **The effects of various inhibitors on the activities of WEP-1 and WEP-2.** The WEP-1 fraction (0.15 unit) obtained from a Sephacryl S-200 column or the WEP-2 fraction (0.23 unit) from a butyl-Cellulofine column was mixed with an inhibitor, followed by incubation for 30 min at 4°C. Then the enzymatic activity was measured under the standard conditions.

Inhibitor	mM	Activity (%)[a]	
		WEP-1	WEP-2
No addition	–	100	100
E-64	0.01	24	93
EDTA	10	81	99
Pepstatin	0.02	107	86
DFP	0.01	–[b]	54
	1	97	5

[a]The enzymatic activities are presented relative to that measured in the absence of an inhibitor. [b]n.d., not determined.

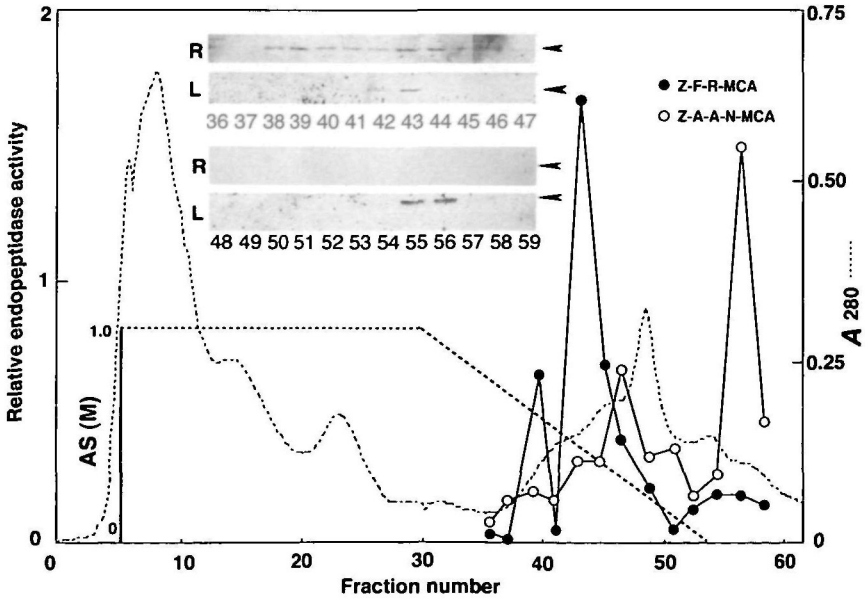


Fig. 4. **Separation of asparaginyl endopeptidases by butyl-Cellulofine chromatography.** An ammonium sulfate fraction (50-70% saturation) was chromatographed on a column of butyl-Cellulofine. Endopeptidase activities in the fractions obtained from the column were assayed using Z-Ala-Ala-Asn-MCA and Z-Phe-Arg-MCA. Each fraction was also subjected to immunoblotting with antisera against REP-1 (R) and legumain (L) (Inset).

TABLE II. **Substrate specificities of WEP-1 and WEP-2.** The enzymatic acitivity of the WEP-1 fraction (0.30 unit) obtained from a Sephacryl S-200 column or the WEP-2 fraction (0.23 unit) from a butyl-Cellulofine column was measured with various synthetic substrates under the standard assay conditions.

Substrate	Activity (%)[a]	
	WEP-1	WEP-2
Z-Phe-Arg-MCA	100	100
Z-Ala-Ala-Asn-MCA	14	2
Z-Pyr-Gly-Arg-MCA	n.d.	99
Z-Arg-Arg-MCA	n.d.	60
Phe-MCA	n.d.	56
Arg-MCA	n.d.	30

[a]The enzymatic activities presented are relative to that measured with Z-Phe-Arg-MCA as the substrate. n.d., not detected.

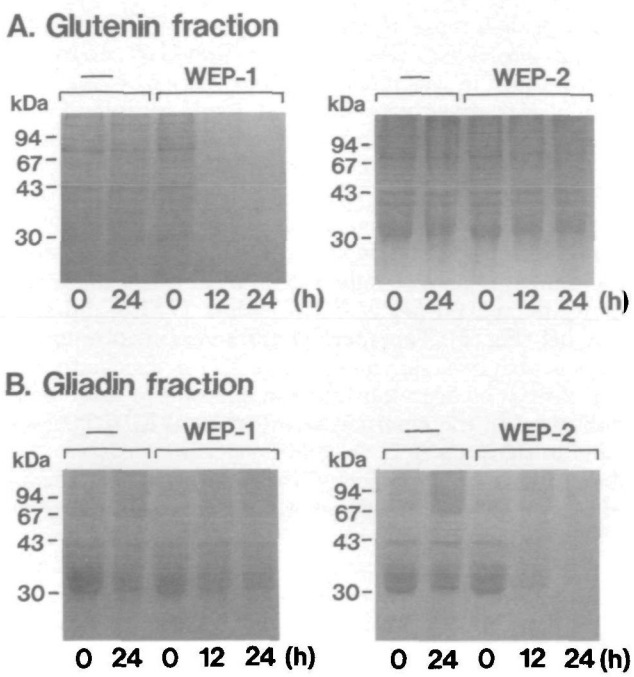


Fig. 6. **SDS-PAGE analysis after *in vitro* digestion of wheat glutenin (A) and gliadin (B).** Glutenin or gliadin (1 mg each) was incubated for 0, 12, and 24 h in the presence of the WEP-1 fraction (WEP-1; 45 units) obtained from the Sephacryl S-200 column or the WEP-2 fraction (WEP-2; 45 units) from the butyl-Cellulofine column (Fig. 2B) under the conditions given in "EXPERIMENTAL PROCEDURES." As control runs (−), buffer solution was added instead of the enzyme. After the incubation, each reaction mixture was centrifuged and the precipitate was analyzed by SDS-PAGE/CBB.

12 h under the *in vitro* conditions described (Fig. 6A). WEP-2 hydrolyzed glutenin to a much lesser extent as compared with WEP-1; there were some decreases in the amounts of the two subunits in the first 12 h, but little digestion occurred thereafter. WEP-1 exhibited low activity toward wheat gliadin, which consisted of 30- to 50-kDa polypeptides (Fig. 6B). In contrast, WEP-2 digested gliadin polypeptides efficiently within the initial 12 h and their amounts further decreased during an additional 12 h. After incubation for 12 and 24 h, the supernatants of the reaction mixtures of both WEP-1 and WEP-2 were examined by SDS-PAGE, but no limited digestion products of smaller molecular sizes were detected on the gels. This might be

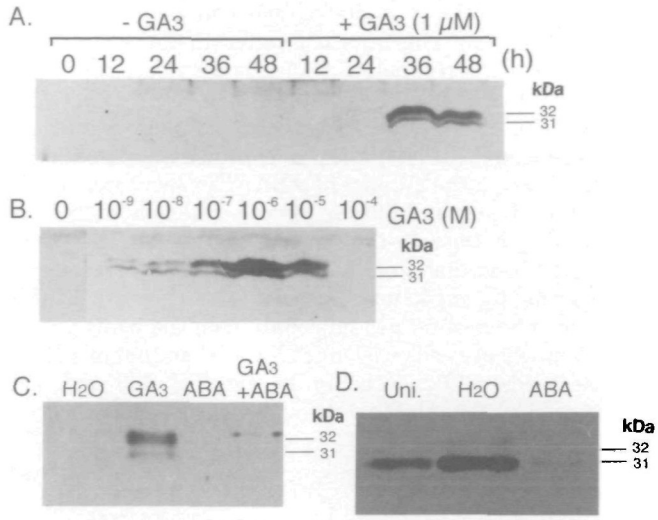


Fig. 7. **Effects of GA_3, ABA, and uniconazole on the expression of WEP-1 in seeds.** A: Changes over time in the amount of WEP-1 in de-embryonated wheat seeds in the presence and absence of GA_3. De-embryonated seeds were incubated for the times indicated in the presence (+) and absence (−) of 1 μM GA_3. B: De-embryonated seeds were incubated for 48 h in the presence of various concentrations of GA_3. C: De-embryonated seeds were incubated for 48 h in the absence (H_2O) and presence of 1 μM GA_3 (GA_3), 50 μM ABA (ABA), and 1 μM GA_3 plus 50 μM ABA (GA_3+ABA). D: Whole seeds were allowed to germinate for 3 d in the absence (H_2O) and presence of 50 μM ABA (ABA) and 50 μM uniconazole (Uni.). Extracts (10 μg protein per lane) of the seeds were analyzed by immunoblotting using the antiserum against REP-1. The 31- and 32-kDa polypeptides correspond to WEP-1 and its possible intermediate, respectively.

due to the action of other peptidases coexisting in the enzyme preparations used; the digestion products of the reaction were further degraded into amino acids and smaller peptides. The digestion pattern of glutenin or gliadin with a combination of WEP-1 and WEP-2 indicated that there were no notable synergistic effects of the two enzymes on the digestion of the storage proteins (data not shown).

Effects of GA_3 and ABA on the Expression of WEP-1— Since WEP-1 is immunoreactive to antiserum raised against REP-1, the hormonal response of the WEP-1 protein was examined by protein immunoblot analysis. When de-embryonated wheat seeds were allowed to imbibe water for 48 h in the absence of GA_3, no immunoreactive polypeptides were detected on SDS-PAGE/immunoblotting. In contrast, in the presence of 1 μM GA_3, intense bands were observed after 36- and 48-h incubation (Fig. 7A). The immunoreactive 32-kDa band detected above the band corresponding to 31-kDa WEP-1 might be an intermediate of the mature WEP-1 protein. This was the case with cysteine endopeptidases in the cotyledons of germinated French bean (*27*) and *Vigna mungo* (*28*) seeds. The bands of higher molecular size than the mature enzyme were thought to be intermediates generated during post-translational processing of the precursor polypeptide because they were only detected at early stages of germination.

GA_3 at a concentration of 1 μM was most effective in inducing the synthesis of WEP-1 48 h post-imbibition. Even in the presence of GA_3 at a concentration as low as 1 nM, the immunoreactive band of 31-kDa WEP-1 together

with a stronger band of its putative intermediate were detected (Fig. 7B). This increasing effect of GA_3 on the level of the 31- and 32-kDa polypeptides clearly decreased when 50 μM ABA was added to the GA_3 medium (Fig. 7C). Supplying 50 μM ABA to whole seeds had a strong effect in that it reduced the level of the polypeptides when compared with the level in the absence of ABA. This level was partly reduced d 3 post-imbibition by the addition of 50 μM uniconazole, a triazole-type growth retardant which inhibits the biosynthesis of gibberellins on the conversion of *ent*-kaurene to *ent*-kaurenoic acid (*29*) (Fig. 7D). This inhibitory effect of 50 μM uniconazole on the synthesis of WEP-1 in wheat seeds was much lower than that of REP-1 in rice seeds (*30*), probably because of less efficient penetration of the inhibitor through the seedcoat.

DISCUSSION

WEP-1—This endopeptidase was primarily detected in wheat seeds as a protein that is immunoreactive to antiserum raised against REP-1, a major seed endopeptidase of rice (*14*). The time-course change in the amount of the WEP-1 protein in wheat seeds after the onset of imbibition corresponded to that of the total endopeptidase activity measured with azoalbumin as a substrate (Fig. 1). Thus, we supposed that this enzyme is a major endopeptidase involved in the digestion of wheat seed storage proteins, and attempted to isolate it. On butyl-Cellulofine chromatography, the endopeptidase activity measured with Z-Phe-Arg-MCA as a substrate was found to be distributed in two peaks named WEP-1 and WEP-2. Only WEP-1 was immunoreactive to the antiserum (Fig. 2).

The purified WEP-1 efficiently hydrolyzed Z-Phe-Arg-MCA, a specific substrate for endopeptidases of the papain family. The amino-terminal amino acid sequence well corresponded to those of other cysteine endopeptidases from cereal seeds (Fig. 3B), indicating that WEP-1 is a typical plant cysteine endopeptidase. The enzyme had optimum activity at pH 6.0, suggesting that it acts in acidic cellular compartments such as the vacuole. WEP-1 digested both the high and low molecular weight subunits of the two major seed proteins of wheat, but it degraded glutenin much more efficiently than gliadin under *in vitro* conditions. For de-embryonated wheat seeds, the supplementation of 1 μM GA_3 was essential for inducing the synthesis of WEP-1, and the effect of GA_3 clearly decreased in the presense of 50 μM ABA in the medium (Fig. 7). These results indicate that WEP-1 isolated in the present study is a cysteine endopeptidase belonging to the same class of enzymes as barley EP-B (*3*) and rice REP-1 (*14*), both of which are synthesized in the presence of gibberellin, and participate in the digestion of major cereal storage proteins during germination and early seedling growth.

Recently, Jivotovskaya *et al.* (*15*) reported the occurrence in germinated seeds of three isoenzymes of 33.5-kDa wheat cathepsin B-like endopeptidase, called proteinase A, which correspond to products of 2529 cDNAs (*31*). The amino-terminal amino acid sequences of these isoenzymes are identical, but very different from those of WEP-1 and related cereal cysteine endopeptidases (Fig. 3). In the present study, this enzyme was not identified probably due to the different assay methods used. Kuroda *et al.* (*16*) purified a 23-kDa cysteine endopeptidase from dormant wheat seeds. Although no amino-terminal amino acid sequence was determined, this enzyme from dormant seeds could be distingushed from germination-specific cysteine endopeptidases such as WEP-1 and REP-1, since virtually no endopeptidase activity was detected in dry wheat seeds when assayed with azoalbumin as a substrate and no polypeptides were observed on immunoblotting with antiserum against REP-1 (Fig. 1).

WEP-2—Experiments with various protease inhibitors showed that WEP-2 is a serine endopeptidase (Table I). In contrast to WEP-1, this enzyme hydrolyzed a rather wide range of substrates including Z-Phe-Arg-MCA but had essentially no activity toward the carboxyl side of Asn residues (Table II). The WEP-2 fraction used also had aminopeptidase activity, probably due to coexisting aminopeptidases. WEP-2 digested the subunits of gliadin more efficiently than those of glutenin whereas WEP-1 digested glutenin effectively (Fig. 6). In a previous report, we showed that, in germinated *Vigna mungo* seeds, seed globulin was effectively digested by a combination of cysteine and serine endopeptidases (*6*). The present study has provided the first evidence of the involvement of a serine endopeptidase in the digestion of monocot seed proteins, although no cooperative action of WEP-1 and WEP-2 was observed in *in vitro* digestion experiments with glutenin and gliadin as substrates. WEP-2 had maximum activity at neutral pH (7.0–7.5), while WEP-1 did so at acidic pH (Fig. 5). This marked difference in optimum pH between the two enzymes allows us to speculate that WEP-2 may be secreted into the endosperm after being synthesized in the aleurone layer, while WEP-1 is transported to the vacuole in the aleurone cells.

Bottari *et al.* (*17*) reported the separation of a gliadin-hydrolyzing cysteine endopepetidase from durum wheat (*Triticum durum*). It may be of interest that there are two types of gliadin-hydrolyzing enzymes with very different pH optima in *Triticum* species; the durum enzyme is a cysteine type with an optimum at pH 4.25, whereas WEP-2 is a serine type with an optimum at pH 7.0–7.5.

Asparaginyl Endopeptidases—Asparaginyl endopeptidase (legumain) was first isolated as a unique processing enzyme for a precursor molecule of concanavalin A, a lectin of jackbean, and has strict substrate specificity toward the carboxyl side of Asn residues (*21*). Recently, this enzyme was isolated from germinated seeds of legumes such as *V. mungo* (*18, 32*), vetch (*33*), and kidney bean (*34*). The enzyme from *V. mungo* seedlings was shown to play a role in the processing of the SH-EP precursor (*26, 32*). The enzymatic activities in maturing and germinating durum seeds were also detected using crude extracts (ammonium sulfate precipitates at 80% saturation), but no further attempt was made to separate the enzyme (*35*). The occurrence of isoforms of this enzyme in rice seedlings is currently evident (Kato, H., unpublished data).

When an ammonium sulfate fraction of d-3 seeds was chromatographed on a butyl-Cellulofine column, the enzymatic activity toward Z-Ala-Ala-Asn-MCA was separated into two peaks, and these peaks were distinguishable from the peaks of activity toward Z-Phe-Arg-MCA (Fig. 4). One of the two asparaginyl endopeptidase fractions was immunoreactive to the antiserum raised against legumain, but the other was not immunoreactive. Senyuk *et al.* (*34*) reported that a legumain-like proteinase is likely to trigger

the proteolysis of native phaseolin, the major globulin of kidney bean. Thus, it may be postulated that, during germination and early seedling growth, insoluble glutenin and gliadin in the wheat seed are initially subjected to limited proteolysis by the two asparaginyl endopeptidases, and that the denatured seed proteins are then digested through the action of major endopeptidases, WEP-1 probably in vacuoles of the aleurone layer and WEP-2 in the endosperm, to soluble peptides, which are subsequently degraded to smaller mobile peptides and amino acids by exopeptidases, although it can not be ruled out that the asparaginyl endopeptidases function in post-translational processing of precursor molecules of other endopeptidases such as WEP-1 and WEP-2. The actual roles of these enzymes remain to be further elucidated.

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