Plant Physiol. (1990) 94, 251–258
0032-0889/90/94/0251/08/$01.00/0

Received for publication March 9, 1990
Accepted May 25, 1990

A Major Gibberellic Acid-Induced Barley Aleurone Cysteine Proteinase Which Digests Hordein[1]

Purification and Characterization

Susan M. Koehler[2] and Tuan-Hua David Ho*

Department of Biology, Plant Biology Program, Division of Biology and Biomedical Sciences, Washington University, St. Louis, Missouri 63130

ABSTRACT

We previously described the purification and characterization of a 37,000 M_r cysteine proteinase, designated EP-A, from gibberellic acid (GA_3)-induced barley (Hordeum *vulgare* L.) aleurone layers (S Koehler, T-HD Ho [1988] Plant Physiol 87: 95–103). A second, more abundant protease has now been purified from this tissue. This protease, designated EP-B, has an apparent M_r of 30,000 on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). It resolves into two bands during native isoelectric focusing with pI of 4.6 to 4.7. The analysis of hemoglobin digestion products by both gradient SDS-PAGE and Bio-Gel P2 chromatography, the inhibition of protease activity by E-64, leupeptin, iodoacetate, and *p*-hydroxymercuribenzoate, and N-terminal amino acid sequence analysis all indicate that EP-B is a cysteine proteinase. The first 22 amino acids at the N terminus of EP-B have been determined, and their sequence is 90% similar to that of EP-A. EP-B has properties similar to EP-A; however, EP-B is much more sensitive to high pH during gel electrophoresis and therefore is not detectable on native activity gels used to detect EP-A. Its pH optimum against azocasein and hemoglobin is 4.5 to 4.6. Both of these proteinases digest hordeins enriched for the B and D fractions into similar peptides of 25,000 to 2,000 M_r as determined by gradient SDS-PAGE.

Barley aleurone layers have been used as a model system to analyze the hormonal regulation of endosperm mobilization to support early seedling growth. Gibberellic acid (GA_3) induces the synthesis and/or secretion of several hydrolytic enzymes from isolated aleurone layers including α-amylase isoenzymes (14), nuclease (7), β-1,3;1,4-glucanase (29), and proteases (10). GA_3 increases the steady-state mRNA levels of β-1,3;1,4-glucanase (29), a putative cysteine proteinase designated aleurain (26), and α-amylase isoenzymes (8, 21, 25), and at least for α-amylase this is mainly due to an increase in the rate of α-amylase gene transcription (13). In most cases, ABA antagonizes most of the effects of GA_3 on the level of steady-state mRNA, protein synthesis, and secretion (12).

In contrast to α-amylase, β-amylase, which is also necessary for starch degradation, is present in the endosperm of dry seeds primarily in a protein-bound form (28). The hydrolysis of bound β-amylase into a more active, soluble form by endosperm extracts from germinating grains is inhibited by cysteine proteinase inhibitors. In this case, GA_3 may regulate β-amylase activity by inducing the synthesis and secretion of cysteine proteinases, which can account for up to 95% of proteinase activity in the starchy endosperm (28). The GA_3-induced cysteine proteinases are probably also responsible for the extensive proteolysis of seed storage proteins, hordeins, into small peptides (24). These can then be utilized by an active peptide transport system in the scutellar epithelium (11), or alternatively hydrolyzed by carboxypeptidase whose secretion into the endosperm is also GA_3-enhanced (10). Aleurain has not been observed to be secreted from the aleurone layer (B. Holwerda, JC Rogers, personal communication); therefore, other cysteine proteinases are candidates for these functions. Cysteine proteinases (29,000–30,000 M_r) recently purified from green malt of the barley cultivars Schooner and Clipper (22) and Morex (23) are capable of hydrolyzing hordeins.

In our quest to identify these secreted proteinases in the barley cultivar Himalaya, we purified to homogeneity a 37,000 M_r GA_3-induced cysteine proteinase designated EP-A (15). In the course of purification of this enzyme another protease activity even more abundant was observed. We now report the purification and characterization of this second GA_3-induced cysteine proteinase with an approximate M_r of 30,000 designated EP-B. The activity of both proteases against hordeins is also demonstrated.

MATERIALS AND METHODS

Materials

Barley (*Hordeum vulgare* L. cv Himalaya) seeds from the 1985 harvest were purchased from the Department of Agronomy and Soils, Washington State University, Pullman, WA. Fungizone, Thimerosal, azocasein, bovine hemoglobin, Bio-Rad low mol wt protein standards, Bio-Gel P-2, Sephadex G-

[1] Supported by National Science Foundation grant DCB-8702299.

[2] Current address: USDA/ARS, Soybean and Alfalfa Laboratory, Room 328, Building 001, BARC-West, Beltsville, MD 20705.

Table I. *Purification of EP-B from Incubation Media of Barley Half-Seeds*

The media from barley embryoless half-seeds incubated in the presence of 1 μM GA_3 according to "Materials and Methods" were collected and protease activity was followed during enzyme purification using azocasein as the substrate.

Purification Step	Total Activity	Total Protein	Specific Activity	Yield	Purification
	units[a]	*mg*	*units/mg*	%	*-fold*
1. Crude medium	6,300	243	25.9	100	1.0
2. 50% $(NH_4)_2SO_4$ pptn	2,737	101	27.0	43	1.0
3. Gel filtration (G-100)	1,373	28	49.0	22	1.9
4. SP-HPLC (pH 4.5)	901	1.00	900	14	34.7
5. DEAE-HPLC (pH 6.0)	364	0.36	1,020	5.8	39.4

[a] One unit for protease activity is one ΔA_{330}/3 h.

100, and the protease inhibitors *p*HMB[3], IAc, aprotinin, STI, pepstatin, 1,10-phenanthroline, E-64, leupeptin, and PMSF were purchased from suppliers as indicated previously (15). Acrylamide was either the ultrapure grade from Schwartz Mann Biotech, Cambridge, MA or electrophoresis grade from Fisher Biotech, Pittsburgh, PA. Antibiotic/Antimycotic 100× solution was purchased from Sigma Chemical Co., St. Louis, MO. Carrier ampholines were from LKB-Pharmacia, Piscataway, NJ.

Tissue Incubation

Batches of 1000 embryoless half-seeds were surface sterilized and incubated aseptically in medium containing 1 μM GA_3 as previously reported (15). To prevent media contamination, Antibiotic/Antimycotic diluted 1:100 was sometimes used instead of Fungizone and chloramphenicol.

Protease Assays

Assays with azocasein in 20 mM Na-succinate (pH 5.0) were used to follow protease activity during purification as previously described (15). Assays with hemoglobin as substrate were also used in some protease characterization studies. The assays were conducted as previously described (15) except the buffer pH was approximately 4.5, and the reactions were incubated at 40°C prior to TCA precipitation for determination of ninhydrin reactive α-NH_2, or for analysis by Bio-Gel P2 chromatography or urea SDS-PAGE (15).

Determination of Mol Wt and Enzyme Purity

Protein samples during purification were analyzed by SDS-PAGE followed by silver staining as previously described (15).

Ammonium Sulfate Fractionation

Medium harvested from nine flasks of half-seeds between 72 and 96 h of incubation was adjusted to 0.1% (v/v) βME and precipitated with $(NH_4)_2SO_4$ to 50% saturation on ice. Protein precipitated at 50% saturation was resuspended in H_2O, dialyzed extensively against 50 mM Na-acetate (pH 5.0), and centrifuged at 10,000*g* for 15 min to remove particulates.

Gel Filtration

The ammonium sulfate fraction was chromatographed on a G-100 Sephadex column, and fractions were assayed for protease activity against azocasein as previously described (15). The protein profile was estimated by the A_{280}. Fractions containing protease activity were combined, adjusted to 0.5 mM DTT, and precipitated with ammonium sulfate to 80% saturation on ice. The precipitate was resuspended and dialyzed extensively in 20 mM Tris acetate (pH 4.5), 0.25 mM DTT, and then centrifuged 10,000*g* for 15 min to remove particulates.

Cation Exchange HPLC

The G-100 fractions containing protease activity were filtered through a 0.45 μm nylon Acrodisc and chromatographed on a 7.5 cm × 7.5 mm Waters 5PW HPLC-SP cation exchange column at room temperature. The sample was

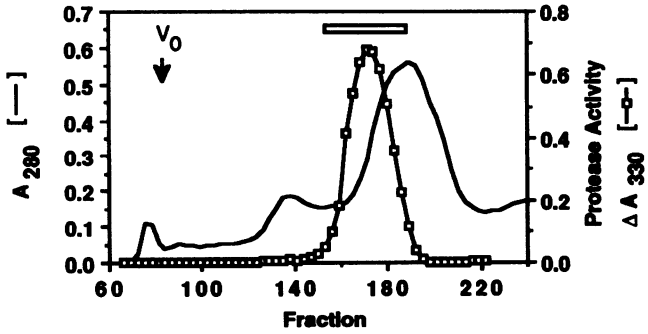

Figure 1. Gel filtration chromatography on Sephadex G-100. The 50% ammonium sulfate fraction (total volume 44 mL) was chromatographed on a 100 × 5 cm column of G-100 Sephadex and aliquots (40 μL) of every third fraction beginning at the void volume (V_0 = 556 mL) were assayed for protease activity against azocasein (□) as described previously (15). The protein profile was estimated by the A_{280} (——). Fractions 153–192 (total volume 325 mL), designated by the bar, were pooled for further protease purification.

[3] Abbreviations: *p*HMB, *p*-hydroxymercuribenzoate; IAc, iodoacetate; STI, soybean trypsin inhibitor; E-64, L-*trans*-epoxysuccinyl-leucylamido(4-guanidino)butane; βME, β-mercaptoethanol; NIEF, native isoelectric focusing; TEMED, *N'*,*N'*,*N'*,*N'*-tetramethylethylenediamine; PVDF, polyvinylidene difluoride; PTH, phenylthio-hydantoin; Nα-BZ-DL-Arg-βNap, Nα benzoyl-DL-arginine-β naphthylamide; UWGCG, University of Wisconsin, Genetics Computer Group.

injected at initial conditions of 20 mM Tris acetate (pH 4.5), 0.25 mM DTT with a flow rate of 1 mL/min. One mL fractions were collected. The protein profile was monitored by the A_{280}. When the A_{280} was near the base line again, a linear gradient of 20 to 900 mM Tris acetate (pH 4.5), 0.25 mM DTT over 30 min was used to elute proteins from the column. Fractions were assayed, and those containing protease activity were pooled.

Anion Exchange HPLC

The pooled cation exchange fractions were adjusted to 20 mM Tris acetate (pH 6.0), 0.25 mM DTT, and chromatographed on a Waters 5PW HPLC-DEAE anion exchange column equilibrated in the same buffer as previously described (15). The column was eluted following 10 min at the initial conditions by employing a more shallow, linear 20 to 900 mM Tris acetate gradient (pH 6.0) in the presence of 0.25 mM DTT for 1 h. Fractions were assayed, and those containing protease activity were pooled and concentrated using Centricon 10 microconcentrators according to the manufacturers instructions (Amicon Division, W. R. Grace and Co., Danvers, MA). Protease samples were adjusted to 10% glycerol and 0.5 mM DTT and stored in small aliquots at −20°C.

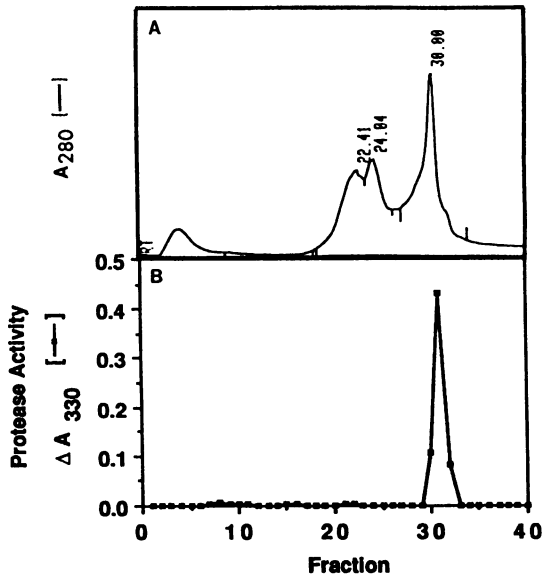

Figure 2. DEAE-anion exchange HPLC. A 17 mL portion of the pooled cation exchange fractions was injected on a Waters 5PW HPLC-DEAE anion exchange column and eluted at a flow rate of 1 mL/min with 20 mM Tris acetate (pH 6.0), 0.25 mM DTT for 10 min, followed by a linear 20 to 900 mM Tris acetate gradient (pH 6.0) in the presence of 0.25 mM DTT for 1 h. A, The A_{280} profile of this run was measured using a Spectroflow 757 Absorbance Detector, and the peaks were integrated using a Hewlett-Packard model 3392 Integrator. No peaks were detected past 40 min. B, Equal aliquots (3 μL) of fractions 1 to 40 were assayed for protease activity against azocasein as before (15). Fractions 30 to 32 contained purified protease.

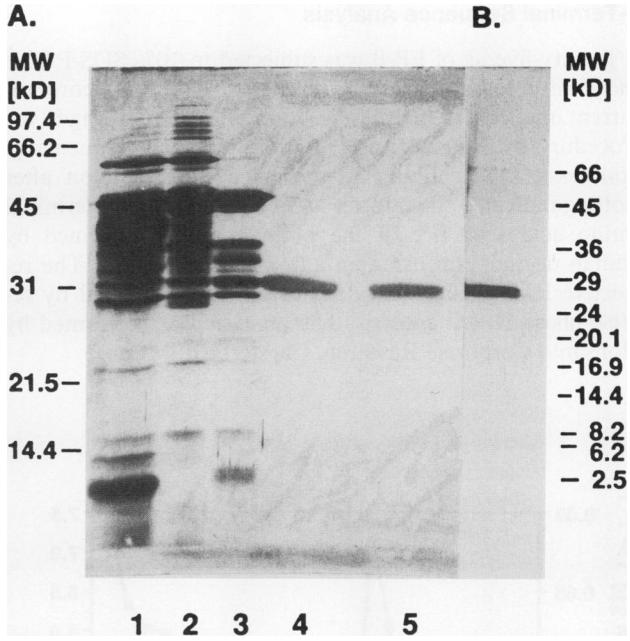

Figure 3. Electrophoretic analysis of samples from each step during EP-B purification. A, 12% SDS-PAGE. Lane numbers designate steps during purification (see Table I). Lane 5 contains 2.0 μg of purified EP-B. The migration of Bio-Rad low mol wt markers is indicated on the left. B, Ten to 20% linear gradient SDS-PAGE of 2.0 μg of purified EP-B. The migration of Sigma molecular mass standards from MW-SDS-70L and MW-SDS-17 kits are indicated on the right. The two bands between the 66 and 45 kD molecular mass are artifacts frequently observed in silver stained gels (7).

Native Isoelectric Focusing

NIEF was performed according to Barrett (3) except the concentration of the acrylamide stock was 30/0.8% acrylamide/bis-acrylamide and an equal portion of the carrier ampholines pH 3.5 to 5.0 and 4.0 to 6.0 were used. Purifed EP-B protease (20 μg) in 1.66 mL was mixed with 0.83 mL of gel solution and polymerized in 12 cm long glass tubes (5 mm i.d.) with riboflavin and TEMED using an 8 W fluorescent lamp for 30 to 45 min. The gels were run under conditions described by Wrigley (30), and 1 mA per gel was maintained by gradually increasing the voltage up to 350 V, and then the gels were maintained at 350 V. Total running time was 5 h.

Inhibitor Studies

All inhibitors, except *p*HMB, were preincubated with EP-B (0.26 μg) in 20 mM Na-succinate (pH 4.5), 10 mM βME in a final volume of 0.747 mL at 4°C for 1 h. Then, 53 μL of 2% (v/v) azocasein in the same preincubation buffer was added, and the assays were carried out at 40°C for 3 h. The inhibitor, *p*HMB, was preincubated and assayed in the absence of βME, and leupeptin was preincubated in the absence of βME; however, βME was added to 10 mM during substrate addition. PMSF was dissolved in isopropanol and E-64 was dissolved in DMSO.

N-Terminal Sequence Analysis

Twenty-five μg of EP-B was subjected to 10% SDS-PAGE and electroblotted for 3.5 to 5 h at 4°C at 400 mA constant current onto PVDF membrane (Immobilon) according to the procedure by Matsudaira (17) for N-terminal sequencing. Coomassie blue staining of the gel and Immobilon after blotting indicated the transfer was complete. The N-terminal amino acid sequence of the protease was determined by Edman degradation using an automated sequencer. The respective PTH-amino acid derivatives were identified by reverse phase HPLC analysis. The analysis was performed by Monsanto Corporate Research, Chesterfield, MO.

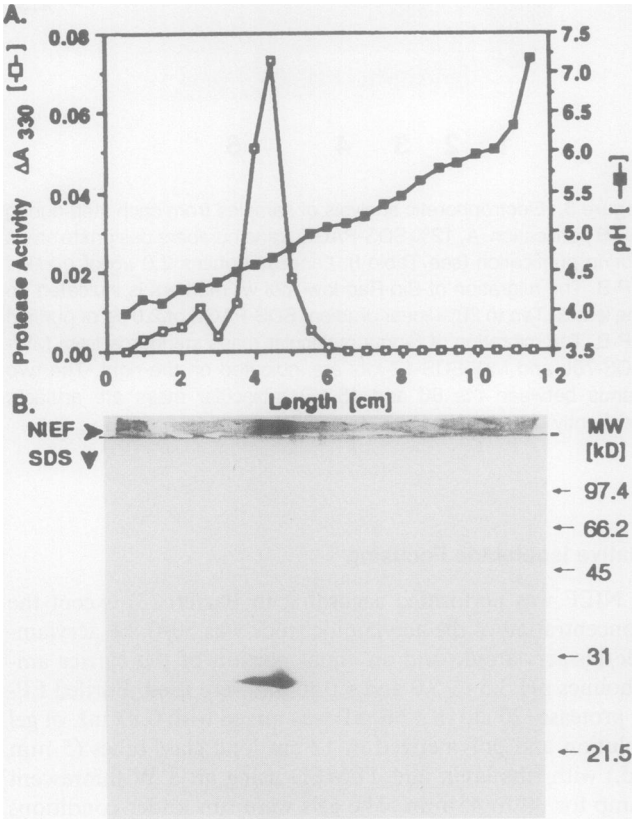

Figure 4. Native isoelectric focusing of purified EP-B. A, Protease activity and pH profile. Following NIEF, a control gel containing no protease was cut into 5 mm serial sections which were equilibrated in 3 mL of H_2O in order to determine the pH profile (■). A duplicate gel containing 20 μg of EP-B was similarly cut into 5 mm serial sections, each of which was equilibrated in 0.8 mL of 20 mM Na succinate (pH 5.0), 10 mM βME for 1.5 h at 4°C. This buffer was then decanted, 0.50 mL of fresh buffer was added, and the gel slices were pulverized with a glass rod. These were stored overnight at 4°C. Duplicate aliquots (150 μL) from each tube were assayed for protease activity against azocasein for 6 h (□). B, Two-dimensional NIEF/SDS-PAGE. A NIEF gel containing protease (8 μg) was cut in half lengthwise; one-half was silver stained (20) and is shown above with the basic end on the right and the acidic end on the left. The other half was subjected to 12% SDS-PAGE and then similarly silver stained. Bio-Rad mol wt markers were used to determine M_r.

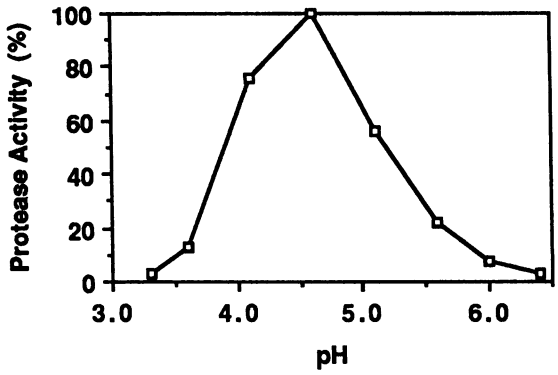

Figure 5. Effect of pH on EP-B activity against hemoglobin. Protease activity was assayed at different pH values in reaction mixtures that were 0.11% (w/v) hemoglobin, 33 mM Na-succinate, 10 mM βME. EP-B was added at 0.43 μg/mL and the reactions incubated at 40°C for 3 h. TCA-soluble, ninhydrin-reactive α-NH_2 released was determined. Activity is expressed as percent of maximum which was 238 μmol α-NH_2 released·mg enzyme·h^{-1}.

Hordein Extraction and Digestion

Hordeins were extracted using a protocol modified from Shewry *et al.* (27). Himalaya barley seeds were ground and passed through a 0.5 mm sieve. Twenty-five g of meal was extracted twice with 200 mL of 0.5 M NaCl for 1 h and centrifuged 20,000*g* for 30 min at room temperature. This pellet was then frozen at −20°C and lyophilized. The meal was then extracted twice with 15 mL/g of 50% propanol at 60°C on a shaker and centrifuged as above. The pellet was then extracted three times with 50% propanol as above, but in the presence of 2.0% βME. Supernatant from the propanol extractions plus and minus βME were dialyzed extensively against water to precipitate hordeins. The hordein fractions were centrifuged as above, lyophilized, ground to a fine powder, and stored at −20°C. Hordeins extracted in the presence of βME were resuspended at 200 μg/200 μL of 20 mM Na-succinate buffer, 10 mM βME, and digested for 2 h either at pH 4.5, 40°C in the presence of 1.0 μg of EP-B or at pH 5.0, 45°C in the presence of 1.5 μg of EP-A. Aliquots of 12 μL were removed at intermittent times and analyzed by 10 to 20% linear gradient SDS-PAGE described previously for the analysis of CNBr cleavage products of EP-A (15).

RESULTS

Protease Purification

During purification of the EP-A proteinase it became evident that a significant portion of the initial protease activity was due to another protease fraction which eluted prior to EP-A during DEAE-anion exchange HPLC (15). Our initial observations indicated that this activity was also sensitive to cysteine proteinase inhibitors. Ammonium sulfate fractionation experiments revealed that two- to threefold more of this activity could be recovered from protein precipitating at 50% saturation compared to 50 to 75% saturation. In addition, little if any EP-A precipitates at 50% ammonium sulfate saturation; therefore, this was used as the first step in the

purification of EP-B. The steps used in a typical purification of EP-B are summarized in Table I.

Gel filtration of Sephadex G-100 of the ammonium sulfate fraction resulted in a single peak of activity (Fig. 1). Generally 50 to 75% of the activity loaded on the column was recovered in the peak fractions after ammonium sulfate precipitation in preparation for the subsequent cation exchange HPLC (Table I, step 3).

During sulfopropyl cation exchange HPLC, the protease did not bind to the column at 20 mM Tris acetate (pH 4.5) and therefore eluted before the gradient began. This step resulted in the largest increase in specific activity. Approximately 66% of the activity injected on the cation exchange column was recovered (Table I, step 4). The proteins normally contaminating this fraction were approximately 17,000 M_r or less, and appear to have been either degraded or unretained when this sample was concentrated using a Centricon 10 microconcentrator for SDS-PAGE.

DEAE anion exchange HPLC at pH 6.0 was used as a final step to remove the remaining minor contaminants. A single peak of protease activity was detected and corresponded to the largest and final protein peak eluting from the column (Fig. 2). This peak consisted of protein which migrated as a single band with an M_r of 30,000 following 12% SDS-PAGE (Fig. 3A, lane 5) or with an M_r of approximately 27,000 following 10 to 20% linear gradient SDS-PAGE (Fig. 3B) performed as previously described (15). The specific activity of the purified EP-B was 1,020 units/mg protein and was purified at least 40-fold (Table I, step 5). The final yield was 5.8%. Apparently some protease inactivation occurs during DEAE HPLC or during the subsequent concentration step. Inactivation is a common problem when purifying cysteine proteinases. Barrett and Kirshke (4) found that their homogeneous preparation of cathepsin B contained 50% inactive protein based on active-site titration by E-64. Simply adjusting the SP HPLC protease fraction from pH 4.5 to 6.0 did not result in a loss of activity. Purified EP-B can be stored in 10% glycerol, 0.25 mM DTT, about 50 mM Tris acetate (pH 4.5 or 6.0) for up to 46 h at 4°C with no change in activity, and it was stable at pH 6.0 at −20°C following five freeze and thaw cycles.

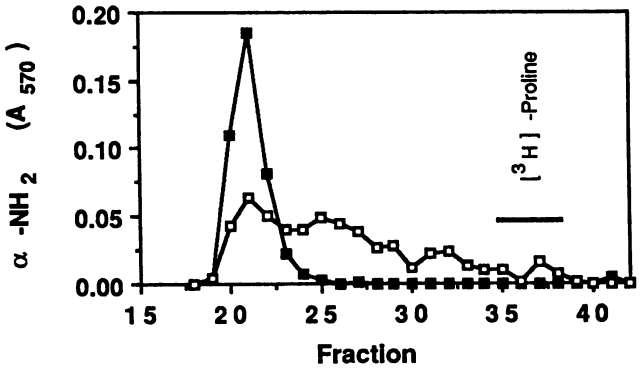

Figure 7. Separation by Bio-Gel P-2 chromatography of hemoglobin digested by EP-B. Hemoglobin was incubated in the absence (■) or presence of (□) EP-B as in Figure 6 for 12 h. One mL aliquots, together with 25 nCi of [^{3}H]-proline, were chromatographed on a Bio-Gel P-2 column and 100 μL aliquots of effluent were assayed for α-NH_2 by ninhydrin and for radioactivity as described previously (15). The bar indicates where the peak of [^{3}H]-proline eluted.

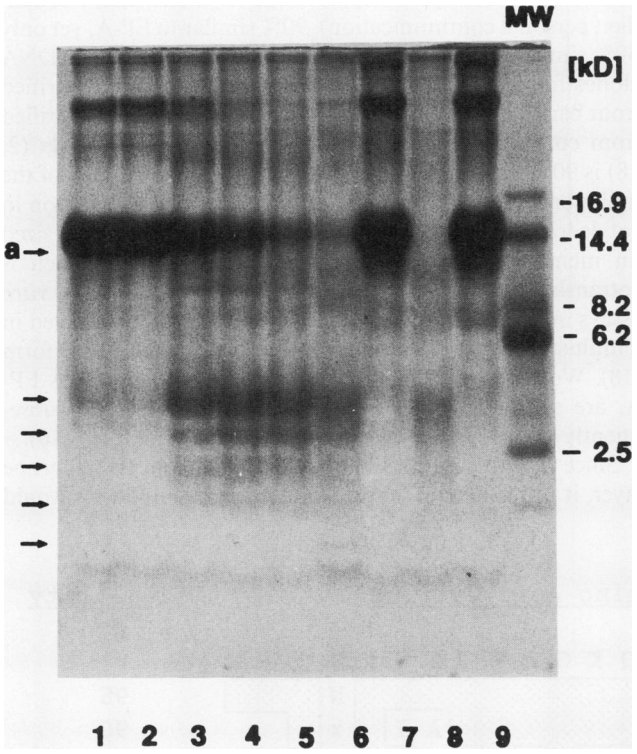

Figure 6. Separation by urea/SDS-PAGE of hemoglobin digested by EP-B. Hemoglobin was incubated as described in Figure 5 at pH 4.5 in the presence of 0.21 μg/mL EP-B and at 0, 2, 4, 6, 12, and 24 h (lanes 2–6, and 8, respectively), 10 μL aliquots of the digests were added to 10 μL of urea/SDS sample buffer, heated for 15 min at 60°C, and the proteins separated by urea/SDS-PAGE. Nondigested hemoglobin incubated in the absence of EP-B for 0, 12, and 24 h was also analyzed (lanes 1, 7, and 9, respectively). Band a represents hemoglobin monomers. New bands generated by hemoglobin digestion are indicated by arrows. Myoglobin fragments, last lane, were used to calibrate the gel, and the mol wt of each is indicated on the right.

Characterization

Purified EP-B had an apparent pI of approximately 4.6 to 4.7 based on native isoelectric focusing (Fig. 4A). The protease activity comigrated with two very tightly spaced bands, one approximately two- to threefold more abundant than the other. Both forms had the same relative mobility following SDS-PAGE (Fig. 4B). This could be due to the presence of two EP-B isozymes with very similar pIs and/or limited autoproteolysis.

The optimum temperature for EP-B activity against azocasein was 40°C (data not shown). The pH optimum against azocasein was determined at the optimum temperature (40°C), over a pH range from 4.1 to 6.6. The optimum pH was 4.5, EP-B was similarly assayed against hemoglobin over a pH range from 3.3 to 6.4, and the optimum pH was

Table II. *Effects of Inhibitors on EP-B Activity*

EP-B activity is expressed as the percent of controls (100%) in which no inhibitors were added. In the presence of βME, 100% activity was 1159 units·mg enzyme^{-1}·h^{-1} using azocasein as substrate at pH 4.5 and 40°C. All mixtures were preincubated for 1 h at 4°C prior to addition of the substrate, during which time the inhibitor concentration was 1.07 times higher than the final concentration listed below.

Treatment	Concentration	EP-B Activity
		%
E-64	10 μM	8
Iodoacetate	1 mM	1
*p*HMB	100 μM	0[a]
Leupeptin	10 μM	0
Deletion of βME		69
PMSF	5 mM	72
Aprotinin	320 μg/mL	57
STI	10 μg/mL	111
Pepstatin	1 μg/mL	90
1,10-phenanthroline	100 μM	89
EDTA	10 mM	90

[a] Activity was assayed in the absence of βME and compared to the control without βME.

approximately 4.6 (Fig. 5). This is slightly lower than the pH optimum of EP-A, which is 5.0 (15).

The time course of hemoglobin digestion by EP-B for up to 24 h was followed by the ninhydrin assay and urea/SDS-PAGE. The assay was linear up to at least 6 h with the release of 378 μmol α-NH_2 released·mg enzyme·h^{-1}. Hemoglobin incubated in the presence of EP-B was rapidly digested. Within 1 h, at least five new bands resulting from digestion were detected which increased then decreased in intensity with time (Fig. 6). By 24 h no more undigested hemoglobin subunits were detected.

To confirm the endoproteolytic activity of EP-B, hemoglobin digested for 12 h was subjected to Bio-Gel P2 chromatography, and the fractions were assayed for ninhydrin-reactive αNH_2 (Fig. 7). The products were distributed in a slope descending from the void volume, and very little product accumulated which coeluted with [^{3}H]-proline. EP-B was also capable of hydrolyzing the artificial endoprotease substrate Nα-Bz-DL-Arg-βNap at pH 5.0 using an assay described by Barret and Kirschke (4) (data not shown).

Inhibitors specific to each class of proteinase were assayed for their ability to inhibit EP-B activity against azocasein at the optimal pH and temperature (Table II). As for EP-A, only the cysteine proteinase inhibitors E-64, IAc, *p*HMB, and leupeptin inhibited EP-B activity by at least 90%. The exclusion of βME also reduced EP-B activity. These results together with the analysis of the products of hemoglobin digestion indicate that this protease belongs to the class of cysteine proteinases.

The N-terminal amino acid sequence of EP-B was determined up to 22 amino acids (Fig. 8). The sequence further confirms the classification of EP-B as a cysteine proteinase since this sequence is highly homologous to other plant cysteine proteinases. Compared to other GA_3-induced cysteine proteinase (or putative cysteine proteinase) sequences described in Himalaya barley, it is 95% similar to the deduced amino acid sequence of a cDNA (pHV14) encoding a GA_3-induced putative cysteine proteinase (L Huiet, and P Chandler, personal communication), 90% similar to EP-A, yet only 59% similar to aleurain. The proteins encoded by the cDNA clones for pHV14 and aleurain have not yet been purified from barley. A cysteine proteinase designated SH-EP purified from cotyledons of germinating *Vigna mungo* seedlings (2, 18) is 90% similar to the N terminus of EP-B. It is one of the major enzymes responsible for storage protein degradation in cotyledons of *V. mungo* seedlings (19). SH-EP is synthesized on membrane polysomes as a 45 kD precursor which is cotranslationally cleaved to a 43 kD intermediate. *In vitro* assays indicate that the 43 kD intermediate can be cleaved in a multistep fashion to produce the mature, active 33 kD form (18). We have evidence indicating that EP-B, as well as EP-A, are synthesized as larger proenzymes which are subsequently processed *in vivo* to produce the mature form (16).

Since both EP-B and EP-A are secreted from the aleurone layer, it is presumed that one of their major functions would

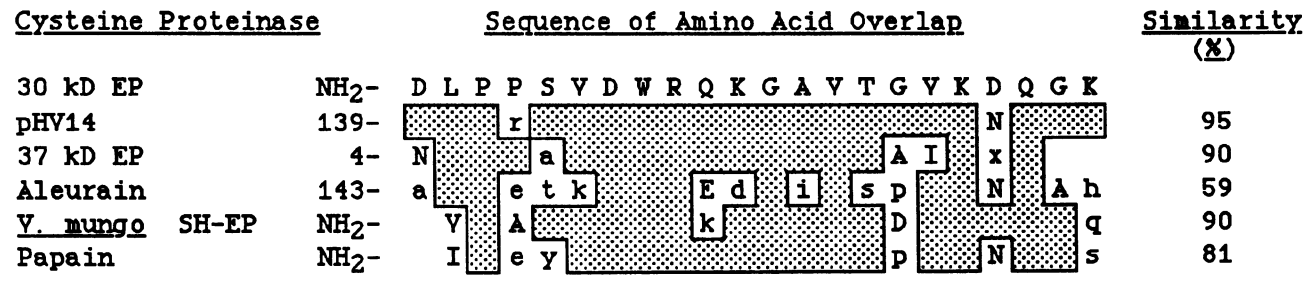

Figure 8. N-terminal amino acid sequence of EP-B compared to other cysteine proteinases. The N-terminal sequence of EP-B was optimally aligned to other plant cysteine proteinase sequences using the GAP program of the UWGCG Package (9; and as previously described, 15) to find the maximum similarity between any two sequences. Amino acids are shown as identical (shaded) or similar (capital letters) to EP-B based on comparison values ≥0.5, and the percent similarity is shown on the right. Amino acids that are not similar are shown in lower case. There were no gaps in the alignments. The sequences for pHV14 (P Chandler, personal communication) and aleurain (26) were deduced from cDNA clones and the N-termini of the mature proteins have not been determined. The N-terminal sequence for *V. mungo* SH-EP was determined by a combination of amino acid sequence analysis of purified protein and from a cDNA clone (18) The 'x' in the sequence of EP-A indicates this position was undetermined due to low yields (15).

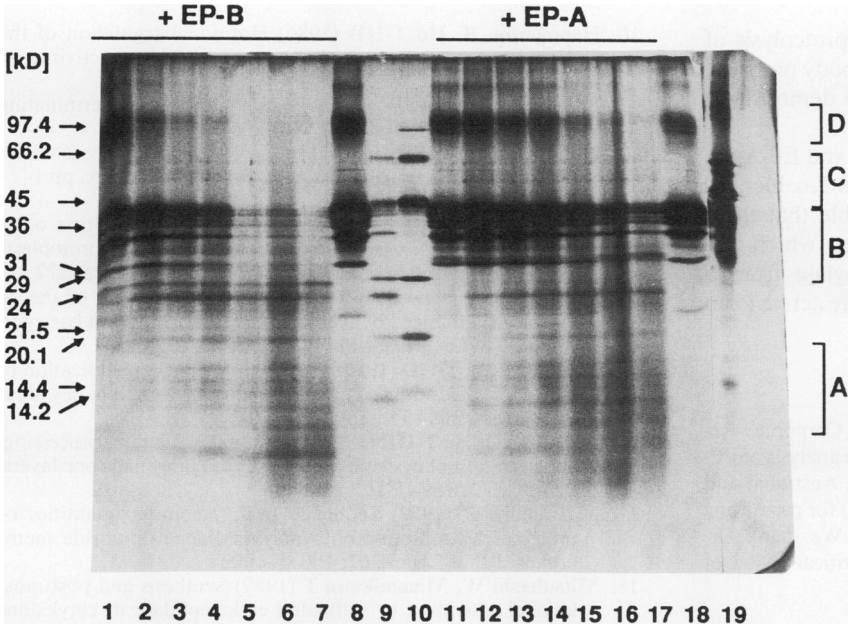

Figure 9. Hordein digestion by barley EP-B and EP-A analyzed by SDS-PAGE. Hordeins extracted with propanol in the presence of βME were incubated in the presence of EP-B (lanes 1–7) or EP-A (lanes 11–17) as described in "Materials and Methods." Equal aliquots were removed after 0 min (lanes 1 and 11), 5 min (lanes 2 and 12), 15 min (lanes 3 and 13), 30 min (lanes 4 and 14), 60 min (lanes 5 and 15), 90 min (lanes 6 and 16), and 120 min (lanes 7 and 17) and analyzed by 10 to 20% linear gradient SDS-PAGE. The same hordein extract incubated 2 h in the absence of protease at pH 4.5 (40°C) or pH 5.0 (45°C) is shown in lanes 8 and 18, respectively. Sigma low molecular mass protein standards (MW-SDS-70L) and Bio-Rad low mol wt protein standards are shown in lanes 9 and 10, respectively, with the molecular mass indicated on the left. Total undigested hordeins extracted with propanol alone are shown in the last lane, and hordein classifications (A–D) are indicated on the right.

be the degradation of hordeins in the endosperm. The hordeins we extracted with propanol plus βME were enriched for the B and D hordeins. Both proteinases were capable of digesting these hordein species into at least 10 distinct smaller peptides of 25,000 M_r to as low as approximately 2,000 M_r as visualized by gradient SDS-PAGE (Fig. 9). EP-B appeared to have a higher specific activity under these conditions than did EP-A, however, both produced similar sized fragments.

DISCUSSION

Of the proteases secreted between 72 to 96 h from barley embryoless half-seeds incubated in the presence of GA_3, EP-B accounts for the vast majority of the activity as assayed against azocasein (pH 5.0). This protease accounts for greater than 50% of the activity recovered from the 50 to 75% ammonium sulfate fraction during EP-A purification (15), and it is two- to threefold more abundant in the 50% ammonium sulfate fraction. This protease, however, is not active in the activity gel assay used to characterize EP-A. The specific activity of EP-B at pH 5.0 against azocasein (1,020 units/mg) is similar to that of EP-A (912 units/mg).

It is possible that EP-B exists as two closely related isozymes since two tightly spaced protein bands were resolved by NIEF, both of which comigrated with protease activity and had the same M_r of 30,000 following SDS-PAGE. N-terminal sequence analysis of this protein band electroblotted from the SDS gel resulted in a single signal. Based on periodic acid Schiff staining and endoglycosidase H digestion of EP-B (data not shown), the protein does not appear to be glycosylated. Characterization of cDNA clones which represent members of the EP-B family support this hypothesis, and none of the clones contain potential *N*-linked glycosylation sites in the mature protein coding region (16). The putative cysteine proteinase encoded by the cDNA clone, pHV14 (P Chandler, personal communication), might represent another isozyme of this EP-B family based on sequence similarity. The 29,000 M_r cysteine proteinase (MEP-1) purified from green malt of the cultivars Schooner and Clipper also exists in two forms of pI 4.2 and 4.3 based on isoelectric focusing (22).

The analysis of hemoglobin digestion products by both gradient SDS-PAGE and Bio-Gel P2 chromatography, the results of inhibitor studies, and N-terminal sequence analysis provide concrete evidence that EP-B, like EP-A, belongs to the cysteine endoprotease (proteinase) class (EC 3.4.22). Purified or partially purified cysteine proteinases have been implicated in the degradation of seed storage proteins in several species including rice (1), mung bean (5), *Phaseolus vulgaris* (6), and *Vigna mungo* (19). In general, their low mol wt (about 22,000–33,000 M_r), low pI, and relatively acidic pH optima are similar to both EP-B and EP-A of barley. A cysteine proteinase, also 30,000 M_r, purified from green malt of the barley cultivar Morex was capable of hydrolyzing hordein rapidly, especially B and D hordein, into peptides which were so small that they ran off a 13% acrylamide SDS gel (23). When this protease was used to analyze C and D hordein hydrolysis specifically, some 45,000 to 32,000 M_r peptides were seen, but again, most of the products migrated off the gel. We have been able to show that both the barley EP-B and EP-A proteinases were also capable of rapidly hydrolyzing B and D hordein. Similar size discrete peptides from 25,000 to 2,000 M_r were observed by gradient SDS-PAGE following digestion by both proteases. Since no N-terminal sequence was reported for either of the green malt cysteine proteinases, *i.e.* the 30,000 M_r protease described by Poulle and Jones (23) or the 29,000 M_r MEP-1 protease described by Phillips and Wallace (22) the relationship between these proteases and EP-B reported here is uncertain; however, the proteases do have either different pH optima against hemoglobin or different pIs. It is probable that EP-B and EP-A, as well as the green malt cysteine proteases, represent the GA_3-induced proteases suggested by Rastogi and

Oaks (24) to be responsible for the extensive proteolysis of hordein into peptides of 15,000 M_r or less. Antibody prepared against both EP-B and EP-A have been used to demonstrate their *de novo* GA_3-induced expression (16).

In addition to hordein hydrolysis, both EP-B and EP-A are capable of modifying β-amylase (B Svensson, S Koehler, T-HD Ho, unpublished observation). It is possible that these cysteine proteinases represent those proteases which are thought to be responsible for releasing β-amylase from a protein-bound, less active form to a soluble, more active form (28). This remains to be examined.

ACKNOWLEDGMENTS

We sincerely thank Christine Smith (Monsanto Corporate Research, Chesterfield, MO) for the N-terminal sequence analysis of EP-B. We thank Dr. Peter Chandler (CSIRO, Canberra, Australia) and Dr. Layne Huiet (University of California, Davis, CA) for permitting us to cite their unpublished sequence for pHV14. We thank Dr. Virginia Peschke, Greg Heck, and Qingxi Shen for critical review of the manuscript.

LITERATURE CITED

1. **Abe M, Arai S, Fujimaki M** (1977) Purification and characterization of a protease occurring in endosperm of germinating corn. Agric Biol Chem **41** (5): 893–899
2. **Akasofu H, Yamauchi D, Mitsuhashi W, Minamikawa T** (1989) Nucleotide sequence of cDNA for sulfhydryl-endopeptidase (SH-EP) from cotyledons of germinating *Vigna mungo* seeds. Nucleic Acids Res **17** (16): 6733
3. **Barrett AJ** (1970) Cathepsin D: Purification of isoenzymes from human and chicken liver. Biochem J **117:** 601–607
4. **Barrett AJ, Kirschke H** (1981) Cathepsin B, cathepsin H, and cathepsin L. Methods Enzymol **80:** 535–561
5. **Baumgartner B, Chrispeels MJ** (1977) Purification and characterization of vicilin peptidohydrolase, the major endopeptidase in the cotyledons of mung-bean seedlings. Eur J Biochem **77:** 223–233
6. **Boylan MT, Sussex IM** (1987) Purification of an endopeptidase involved with storage-protein degradation in *Phaseolus vulgaris* L. cotyledons. Planta **170:** 343–352
7. **Brown PH, Ho T-HD** (1986) Barley aleurone layers secrete a nuclease in response to gibberellic acid. Purification and partial characterization of the associated ribonuclease, deoxyribonuclease, and 3′-nucleotidase activities. Plant Physiol **82:** 801–806
8. **Chandler PM, Zwar JA, Jacobsen JA, Higgins TJV, Inglis AS** (1984) The effects of gibberellic acid and abscisic acid on α-amylase mRNA levels in barley aleurone layer studies using an α-amylase cDNA clone. Plant Mol Biol **3:** 407–418
9. **Devereaux J, Haeberli P, Smithies O** (1984) A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res **12** (1): 387–395
10. **Hammerton R, Ho T-HD** (1986) Hormonal regulation of the development of protease and carboxypeptidase activities in barley aleurone layers. Plant Physiol **80:** 692–697
11. **Higgins CF, Payne JW** (1977) Peptide transport by germinating barley embryos. Planta **134:** 205–206
12. **Ho T-HD** (1983) Biochemical mode of action of abscisic acid. *In* FA Addicott, ed, Abscisic Acid. Prager, New York, pp 147–170
13. **Jacobsen JV, Beach LR** (1985) Control of transcription of α-amylase and rRNA genes in barley aleurone layer protoplasts by gibberellic acid and abscisic acid. Nature **316:** 275–277
14. **Jacobsen JV, Higgins TJV** (1982) Characterization of the α-amylase synthesized by aleurone layers of Himalaya barley in response to GA_3. Plant Physiol **70:** 1647–1653
15. **Koehler S, Ho T-HD** (1988) Purification and characterization of gibberellic acid-induced cysteine endoproteases in barley aleurone layers. Plant Physiol **87:** 95–103
16. **Koehler SM, Ho T-HD** (1990) Hormonal regulation, processing, and secretion of cysteine proteinases in barley aleurone layers. Plant Cell **2:** 769–783
17. **Matsudaira P** (1987) Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. J Biol Chem **262:** 10035–10038
18. **Mitsuhashi W, Minamikawa T** (1989) Synthesis and posttranslational activation of sulfhydryl-endopeptidase in cotyledons of germinating *Vigna mungo* seeds. Plant Physiol **89:** 274–279
19. **Mitsuhashi W, Koshiba T, Minamikawa T** (1986) Separation and characterization of two endopeptidases from cotyledons of germinating *Vigna mungo* seeds. Plant Physiol **80:** 628–634
20. **Morrissey JH** (1981) Silver stain for proteins in polyacrylamide gels: a modified procedure with enhanced uniform sensitivity. Anal Biochem **117:** 307–310
21. **Nolan RC, Ho T-HD** (1988) Hormonal regulation of gene expression in barley aleurone layers. Planta **174:** 551–560
22. **Phillips HA, Wallace W** (1989) A cysteine endopeptidase from barley malt which degrades hordein. Phytochemistry **28** (12): 3285–3290
23. **Poulle M, Jones BL** (1988) A proteinase from germinating barley. Plant Physiol **88:** 1454–1460
24. **Rastogi V, Oaks A** (1986) Hydrolysis of storage proteins in barley endosperms. Plant Physiol **81:** 901–906
25. **Rogers JC** (1985) Two barley α-amylase gene families are regulated differently in aleurone cells. J Biol Chem **260** 3731–3738
26. **Rogers JC, Dean D, Heck GR** (1985) Aleurain: A barley thiol protease closely related to mammalian cathepsin H. Proc Natl Acad Sci U S A **82:** 6512–6516
27. **Shewry PR, Field JM, Kirkman MA, Faulks AJ, Miflin BJ** (1980) The extraction, solubility, and characterization of two groups of barley storage polypeptides. J Exp Bot **31:** 393–407
28. **Sopanen T, Lauriere C** (1989) Release and activity of bound β-amylase in a germinating barley grain. Plant Physiol **89:** 244–249
29. **Stuart IM, Loi L, Fincher GB** (1986) Development of (1-3, 1-4)-β-D-glucan endohydrolase in isolated scutella and aleurone layers of barley (*Hordeum vulgare*). Plant Physiol **80:** 310–314
30. **Wrigley CW** (1968) Analytical fractionation of plant and animal proteins by gel electrofocusing. J Chromatogr **36:** 362–365