Plant Physiol. (1988) 87, 95–103
0032-0889/88/87/0095/09/$01.00/0

Purification and Characterization of Gibberellic Acid-Induced Cysteine Endoproteases in Barley Aleurone Layers[1]

Received for publication July 20, 1987 and in revised form January 20, 1988

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ABSTRACT

Using in series ammonium sulfate precipitation, gel filtration, and DEAE anion exchange high performance liquid chromatography, we have purified to homogeneity a protease of M_r 37,000 secreted from barley (*Hordeum vulgare* L. cv Himalaya) embryoless half-seeds. This protease exists in three isozymic forms whose synthesis and secretion from barley aleurone layers was shown to be a gibberellic acid (GA_3)-dependent process (R Hammerton, T-HD Ho 1986 Plant Physiol 80: 692–697). This protease constitutes a major portion of the protease activity secreted from half-seeds between 72 to 96 hours of incubation in the presence of GA_3 as detected on activity gels containing hemoglobin as the substrate. Analysis of digestion products by urea/sodium dodecyl sulfate-polyacrylamide gel electrophoresis and gel filtration indicated that this protease is an endoprotease, therefore it is designated as barley endoprotease-A (EP-A). Inhibitor studies demonstrated that EP-A belongs to the cysteine class of endoproteases. The optimum pH for EP-A activity was 5.0, and the temperature optimum was 45°C. Comparison of cyanogen bromide generated peptide fragments and NH_2-terminal sequence analyses of the three individual EP-A isozymes demonstrates that they are very similar to each other. The NH_2-terminal sequence shows extensive sequence homology to the NH_2-terminal sequence of papain and several other cysteine proteinases. We also provide evidence that EP-A is not 'aleurain,' a putative cysteine proteinase encoded by a GA_3-induced barley cDNA clone (JC Rogers, D Dean, GR Heck 1985 Proc Natl Acad Sci USA 82:6512–6516).

In germinating barley, gibberellins produced by the embryo diffuse to the aleurone layer where they induce the synthesis and secretion of several hydrolytic enzymes including α-amylase (7), nuclease (6), β1,3-1,4 glucanase (28), and protease (13). Proteases, particularly those secreted into the endosperm, are necessary for the mobilization of seed storage proteins during seedling growth (24), and they may also be involved in the activation of latent, protein-bound enzymes such as β-amylase (14). Many types of proteases have been characterized in germinating barley, some of which have been purified. These include five carboxypeptidases, four neutral aminopeptidases (naphthylamidases), and three alkaline amino- or di-peptidases (for a review, see Mikola and Mikola [18]). Of these exo- or di-peptidase activities, only carboxypeptidase activity is secreted from aleurone layers and/or scutella into the starchy endosperm (17). Sundblom and Mikola (29) demonstrated that at least four different endoproteases are present in and secreted by GA_3-treated aleurone layers. Based on assays with gelatin as a substrate, a major enzyme with a pH optimum of 3.9 and two others with higher pH optima are active in the presence of βME.[2] The fourth enzyme was metal activated with a pH optimum of 7.0. Others have also described acid cysteine proteinases and metal-activated proteinases in barley seedlings (9). Most of of these studies dealt with crude or partially purified enzyme preparations, and purification and characterization of each of these proteases will be necessary before an understanding of the roles they play in germination can be elucidated.

As a start towards this goal, Hammerton and Ho (12) characterized GA_3-induced protease activity secreted from aleurone layers. Carboxypeptidase activity (assayed against Z-Ala-Phe) was shown to develop in the aleurone layers in the absence of added hormones, but its secretion was greatly enhanced by GA_3. In contrast, both the development of protease activity (as assayed against hemoglobin as a substrate) in the aleurone layer and its secretion into the medium were dependent on GA_3. This newly synthesized hemoglobinase activity migrated as three bands on an activity gel, but as a single band of M_r 37,000 on SDS-polyacrylamide gels. This activity was sensitive to the cysteine proteinase inhibitors leupeptin, bromate, and *p*HMB, and was enhanced by βME.

A cDNA clone, prepared with mRNA isolated from GA_3-treated barley aleurone layers, has been isolated whose coding region shares amino acid sequence homology with the mammalian cysteine proteinase cathepsin H (25). The predicted mol wt of the mature protein, designated 'aleurain' (25), is 37 kD. The level of mRNA hybridizing to this clone is enhanced sevenfold when aleurone layers are incubated 18 h in the presence of GA_3.

In this communication, we report the purification of the 37,000 M_r protease described by Hammerton and Ho (12) and provide evidence that it is of the cysteine proteinase (endoprotease) class. However, the NH_2-terminal amino acid sequence of this 37,000 M_r protease indicates that it is not aleurain but is another enzyme closely related to papain.

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 (a fungicide) was purchased from Gibco Laboratories, Madison, WI. GA_3, azocasein, *p*HMB, IAc, aprotinin, STI, pepstatin, 1,10-phenanthroline, E-64, Thimerosal (an antibacterial and antifungal agent), and bovine hemoglobin were

[1] Supported by National Science Foundation Grant DCB-8316319.

[2] Abbreviations: βME, β-mercaptoethanol; Z-Ala-Phe, *N*-carbobenzoxy-Ala-Phe; *p*HMB, *p*-hydroxymercuribenzoate; IAc, iodoacetate; STI, soybean trypsin inhibitor; E-64, L-*trans*-epoxysuccinyl-leucylamido(4-guanidino)butane; PMSF, phenylmethyl-sulfonylfluoride; PVDF, polyvinylidene difluoride; PTH, phenylthiohydantoin; NBRF, National Biomedical Research Foundation; UWGCG, University of Wisconsin Genetics Computer Group; EP-A, endoprotease A.

purchased from Sigma Chemical Co., St. Louis, MO. Ultrapure acrylamide and $(NH_4)_2SO_4$ were purchased from Schwartz Mann Biotech, Cambridge, MA. Low mol wt protein standards and Bio-Gel P-2 were purchased from BioRad, Richmond, CA. Leupeptin was purchased from Boehringer Mannheim Biochemicals, Indianopolis, IN. Sephadex G-100 was purchased from Pharmacia, Piscataway, NJ. PMSF was purchased from Calbiochem-Behring, La Jolla, CA.

Protease Assays. Assays with azocasein were used to follow protease activity during purification. Enzyme aliquots were added to 20 mM Na-succinate (pH 5.0) buffer to a final volume of 267 μl and preincubated in the presence of 10 mM βME for 30 min at 4°C. Then 533 μl of 0.2% (w/v) azocasein in preincubation buffer were added and the reaction mixtures incubated at 30°C for 3 h. Two hundred μl of 50% (w/v) TCA were added to terminate the reactions, and the mixtures were kept at 4°C for at least 10 min to precipitate TCA-insoluble peptides. Following centrifugation of the reaction mixtures for 10 min in a Beckman microfuge, TCA-soluble peptides were determined by reading the A_{330} of the supernatant. Assay blanks were prepared with 267 μl of Na-succinate buffer alone. One unit of activity is defined as 1.0 ΔA_{330}/3 h.

Assays with hemoglobin were used to characterize the purified 37,000 M_r protease isozymes (EP-A). Protease activity was assayed in a reaction mixture that was 33 mM Na-succinate (pH 4.0), 10 mM βME, and 0.11% (w/v) hemoglobin as previously described (12). EP-A was preincubated in the reaction mixture for at least 30 min at 4°C prior to addition of the substrate to allow activation by βME. Following addition of the substrate, the reaction mixtures were incubated at 30°C for 3 h. Temperature and pH optima studies were all conducted using 3 h incubations. The TCA (5% [w/v]) soluble peptides and amino acids were assayed by the ninhydrin reaction and compared to L-leucine standards. Activities were expressed as μmol α-NH_2 released·mg enzyme^{-1}·h^{-1}.

Protease activity during purification was also analyzed by nondenaturing (native) PAGE (12). Hemoglobin was added at a final concentration of 0.02% (w/v) to the stacking gel (3.75% [w/v] acrylamide) and resolving gel (8% [w/v] acrylamide) prior to polymerization. The gels were run at 4°C and then incubated as described in Figures 1, 2, and 5B. The gels were then stained with Coomassie blue stain (12) and destained in 7.5% (v/v) acetic acid.

Determination of Molecular Weight and Enzyme Purity. Samples from steps during protease purification were analyzed by SDS-PAGE using a 12.5% (w/v) acrylamide slab resolving gel according to the procedure of Laemmli (15). Proteins were visualized following silver-staining by the method of Morrissey (20). Protein concentration was determined by the Bradford microassay using BSA as a standard (5).

Tissue Incubation. Typically, up to 15 batches of 1,000 embryoless half-seeds or 1,000 whole seeds cracked in a Waring blender for 20 s at top speed were surface-sterilized in 20% (v/v) clorox for 30 min. Seeds were rinsed four times in sterile H_2O and incubated 1,000 seeds/1 L flask with 100 ml of medium containing 0.02 M Na-succinate (pH 5.0), 0.02 M $CaCl_2$, 1.0 μM GA_3, 1.5 ml of Fungizone (250 μg/ml Amphotericin B), and 1.5 mg chloramphenicol. The seeds were incubated at room temperature on an orbital shaker set at 200 rpm. Medium was collected and replaced every 24 h for 96 h with fresh medium as above but with 1 ml of Fungizone and 1 mg of chloramphenicol per 100 ml of medium. Medium was centrifuged at 10,000g for 15 min to remove cellular debris and stored at −20°C.

Ammonium Sulfate Fractionation. The medium collected from three flasks each of half-seeds and cracked seeds between 72 to 96 h after GA_3 addition was adjusted to 0.1% (v/v) βME and precipitated with $(NH_4)_2SO_4$ on ice. Protein precipitating between 50 to 75% saturation was resuspended in H_2O and dialyzed extensively in 50 mM Na-acetate, 1 mM cysteine, and 2 mM Mg-acetate (pH 6.0). The dialyzed medium was centrifuged at 10,000g for 10 min to remove undissolved protein and then adjusted to pH 5.0 with acetic acid. All steps were performed at 4°C.

Gel Filtration. The sample from the previous step was applied to a 100 × 5 cm column of G-100 Sephadex (particle size 40–120 μm) equilibrated with 0.05 M Na-acetate buffer (pH 5.0) and 1.0 mM cysteine at 4°C. The sample was eluted with column buffer at a flow rate of approximately 2 ml/min, and 7.0 ml fractions were collected. Fractions were assayed for protease activity, and protein was estimated by the A_{280}. Fractions containing protease activity which co-migrated with EP-A on protease activity gels were pooled. The pooled fractions were concentrated by lyophilization and dialyzed three times against 500 ml of 20 mM Tris acetate (pH 6.0), and 0.5 mM DTT over 6 h at 4°C.

HPLC-DEAE. Following dialysis, pooled gel filtration fractions were chromatographed on a 7.5 cm × 7.5 mm Waters 5PW HPLC-DEAE anion exchange column at room temperature. The column was eluted at a flow rate of 1 ml/min with 20 mM Tris acetate (pH 6.0), and 0.25 mM DTT for 10 min, followed by a linear gradient from 20 to 900 mM Tris-acetate (pH 6.0) in the presence of 0.25 mM DTT over the next 30 min. One ml fractions were collected and assayed for protease activity. The peak fractions of protease activity which co-migrated with EP-A on an activity gel were pooled and dialyzed at 4°C three times against 500 ml of 20 mM Tris acetate (pH 7.5), 0.25 mM DTT, and 10% glycerol for 6 h.

The peak fractions from the previous HPLC run were chromatographed again on the HPLC-DEAE column and eluted as above except at pH 7.5 and in the presence of 10% glycerol. Each fraction was assayed for protease activity, and peak fractions were combined and adjusted to approximately 300 mM Tris acetate (pH 7.5), and concentrated using Centricon 10 microconcentrators according to the manufacturers instructions (Amicon Division, W. R. Grace and Co., Danvers, MA). Aliquots were removed for protein determination and protease assays, and the purified EP-A was divided into 50 μl aliquots and frozen at −20°C. Purification of EP-A from incubation medium was performed several times with similar results.

Urea/SDS-PAGE and Bio-Gel P-2 Chromatography of Hemoglobin Digests. Digestion of hemoglobin by EP-A was performed as above for up to 24 h. Ten μl aliquots were removed at intermittent times and subjected to urea/SDS-PAGE according to the procedure for the separation of myoglobin fragments (MW-SDS-17 mol wt marker kit; SDS Molecular Weight Markers, Technical Bulletin No. MWS-877P, 1986 Sigma Chemical Co., St. Louis). This procedure is a modification of the method by Swank and Munkres (30). Vertical slab gels (13 × 13 cm) were used. A stacking gel of 6% (w/v) acrylamide/0.16% (w/v) bis-acrylamide in the same buffer as the resolving gel was poured for sample loading. Gels were run under constant current at 30 mamps until the dye front passed through the stacking gel, then at 60 mamps until the dye front reached the bottom. The gels were silver-stained according to Morrissey (20).

Aliquots of the reaction mixture were removed for determination of TCA-soluble ninhydrin-reactive products. Prior to TCA precipitation, 1.0 ml aliquots were removed from the reaction mixture at 0.5, 2, 6, and 12 h and kept at 4°C in the presence of 10 μM leupeptin to inhibit further digestion. These aliquots were chromatographed on a 110 × 0.6 cm column of Bio-Gel P-2 equilibrated and eluted with 95 mM Na acetate (pH 5.0) and 0.005% Thimerosal at 0.05 ml/min at room temperature. To calibrate each column run, 25 nCi of L-[5-^{3}H]proline was added to each aliquot prior to sample loading. Fractions of 0.75 ml were collected and assayed (without TCA precipitation) for ninhydrin-

reactive α-NH_2 by measuring A_{570}.

Inhibitor Studies. All inhibitors, except *p*HMB, were preincubated with protease as above for 1 h prior to addition of hemoglobin; *p*HMB was preincubated under the same conditions except βME was omitted. PMSF was dissolved in isopropanol and E-64 was dissolved in DMSO; therefore, 'plus enzyme' controls for these incubations without inhibitors included the equivalent concentrations of isopropanol and DMSO, respectively. The ΔA_{570} in the ninhydrin assay was calculated by subtracting the values of control incubations containing inhibitor alone from those of incubations containing both enzyme plus inhibitor. The assays were conducted at 45°C at pH 5.0. To check the effectiveness of some of the inhibitors, E-64 and IAc were tested with 0.80 μg/ml of papain; PMSF, aprotinin, and STI were tested with 10 μg/ml trypsin at pH 7.5 (Na-Hepes buffer) in the absence of βME. These assays were conducted at 37°C.

Cyanogen Bromide Cleavage. Purified EP-A isozymes were separated by native PAGE as previously described (12). The gel was stained briefly with Coomassie blue stain, rinsed in 50% (v/v) cold methanol, and then rinsed in water. Each band was cut into a narrow strip which was subsequently CNBr treated for 2 h at 37°C according to the method of Nikodem and Fresco (22). Following CNBr treatment, the rinse time in equilibration buffer (0.125 M Tris HCl[pH 6.8], 0.1% [w/v] SDS) was reduced to two 20 min washes. Gel slices were heated at 68°C for 4 min in Laemmli SDS sample loading buffer (15) in the presence of βME, then subjected to 10 to 20% (w/v) acrylamide, 0 to 15% (w/v) sucrose gradient SDS-PAGE. A modified Laemmli discontinuous buffer system (10) with high concentrations of Tris in the resolving gel (0.75 M) and in the running buffer (0.05 M) was employed to improve resolution. Electrophoresis was at 22 mamp constant current until the dye front was about 1 cm from the bottom. The gel was silver-stained as described (20).

NH_2-Terminal Sequence Analysis. EP-A isozymes (about 10 μg each) were again separated by native PAGE as above. The protein was electroblotted for 2 h at 4°C onto PVDF membrane according to the procedure of Matsudaira (16) for NH_2 terminal sequencing. Coomassie blue staining indicated that transfer of protein to the membrane was complete. The NH_2-terminal amino acid sequence of the PVDF blotted isozymes was determined by Edman degradation using an automated sequencer. The respective PTH-amino acid derivatives were identified by reverse phase HPLC analysis. The analyses were performed by Monsanto Corporate Research, Chesterfield, MO.

RESULTS

Protease Purification. Results from protease activity gels demonstrated the secretion of numerous electrophoretically distinct proteases in resonse to GA_3 (Fig. 1). We have arbitrarily divided these into four groups (A through D). Proteases in groups A and B were sensitive to the thiol-protease inhibitors, $KBrO_3$, leupeptin (Fig. 1), and *p*HMB (data not shown). Bromate has been used to inhibit thiol-protease activity in barley malt (4), presumably by oxidation of essential sulfhydryl groups. We will demonstrate that the three proteases in group A are the isozymic forms of the 37,000 M_r protease as previously described by Hammerton and Ho (12). These proteases (EP-A, see "Discussion" below) appear to comprise the major secreted protease activity visualized on the activity gel using hemoglobin as substrate.

The time course of EP-A secretion from barley half-seeds incubated in the presence of 1.0 μM GA_3 for 96 h was assessed on a protease activity gel (Fig. 2). Substantial secretion occurred between 72 and 96 h; therefore, incubation medium from the 96 h time point was used as the starting material for enzyme purification. Cracked seeds were used in addition to half-seeds in order to simplify the procedure. The secretion time course for EP-A from cracked seeds was similar to that of half-seeds. The

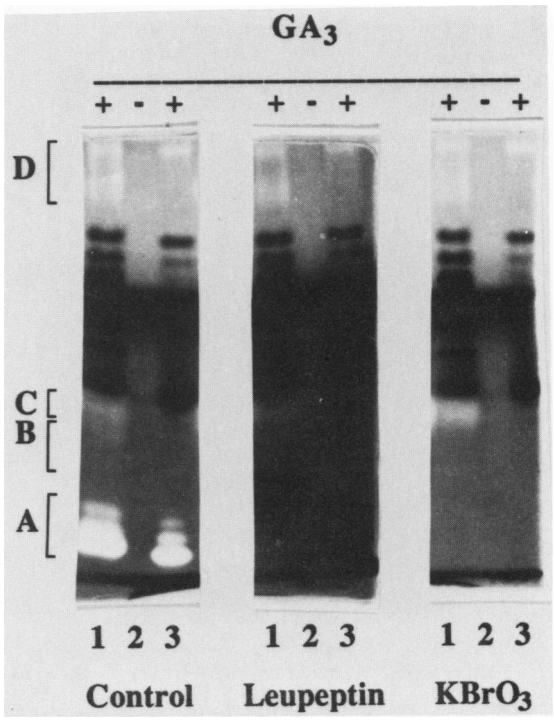

FIG. 1. Protease activity gel analysis of proteases secreted from barley half-seeds between 24 and 96 h of incubation. Half-seeds were incubated in the absence (−) or presence (+) of 1 μM GA_3 except that after 24 h of incubation, fresh medium was added and then harvested at 96 h. Fractionated medium was subjected to nondenaturing PAGE in a gel containing hemoglobin as described in "Materials and Methods." The gel was subsequently incubated in buffer (20 mM Na-succinate, pH 4.0) in the presence or absence of the inhibitors, leupeptin (10 μM) and $KBrO_3$ (10 mM), as indicated, for 30 min at 4°C and then further incubated for 3 h at 30°C. Four regions of protease activity (A–D) were visualized as clear areas in the gel following Coomassie blue staining and destaining. Lanes 1 and 2, equal volumes of medium were precipitated with 6 volumes of cold acetone, centrifuged at 10,000*g*, 15 min, then resuspended in 0.12 times the original volume in 20 mM Tris acetate (pH 6.0). Lane 3, medium was precipitated with 50 to 75% $(NH_4)_2SO_4$, centrifuged 10,000*g*, 15 min, dialyzed against 20 mM Tris acetate (pH 6.0), then resuspended as for the acetone precipitated medium. Protease activity (determined in a separate assay against azocasein) was 0.83 units for lane 1 and 0.55 units for lane 3.

steps used in a typical purification of EP-A are summarized in Table I. Fractionation of 72 to 96 h medium with $(NH_4)_2SO_4$ resulted in an enrichment of EP-A in the 50 to 75% $(NH_4)_2SO_4$ fraction; however, other protease activities were also present in this fraction. Approximately 30% of the protease activity recovered, predominantly proteases in groups B through D, was in the 0 to 50% $(NH_4)_2SO_4$ fraction. Therefore, the 50 to 75% $(NH_4)_2SO_4$ fraction was further processed.

Gel filtration on Sephadex G-100 of the $(NH_4)_2SO_4$ fraction resulted in three peaks of activity (Fig. 3). Protease activity gel analysis showed that a minor peak eluting at the void volume and a second minor peak with a K_{av} of 0.14 contained the group D proteases (data not shown). The third peak with a K_{av} of 0.42 contained group B and EP-A protease activities. The EP-A activity was enriched in the second half of the peak. Of the total activity loaded on the column, 56% was recovered in the peak fractions pooled which were enriched with EP-A activity as seen on the activity gel (Table I, step 3).

HPLC of the gel filtration fractions through a DEAE anion

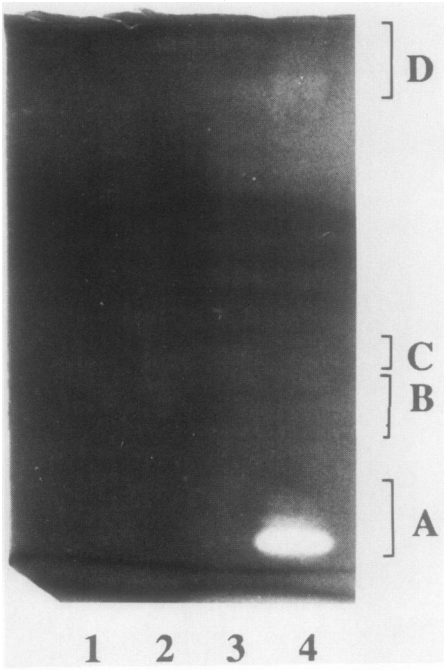

FIG. 2. Protease activity gel analysis of the time course of EP-A secretion from half-seeds. Embryoless half-seeds were incubated in the presence of 1 μM GA_3, and the incubation medium was analyzed on protease activity gels as described in "Materials and Methods." Fifty μl of incubation medium from each 24 h time-point up to 96 h were analyzed for secreted proteases following electrophoresis in a native gel containing 0.02% (w/v) hemoglobin. After electrophoresis, the gel was incubated in 150 ml of 20 mM Na-succinate (pH 4.0), and 10 mM βME, at 40°C for 3 h. Protease activity was seen as clear areas following Coomassie blue staining and destaining. Lanes 1 through 4 are media from d 1 through 4, respectively. The positions of proteases A through D are indicated.

exchange column at pH 6.0 resulted in two peaks of activity, a major peak eluting at fractions 25 and 26 and a smaller peak eluting at fractions 34 and 35. The latter peak constituted an average of 39% of the total activity recovered (Fig. 4). Activity gels indicated that EP-A activity is present only in the smaller peak (data not shown). Following dialysis, rechromatography of the pooled fractions enriched for EP-A on the HPLC DEAE anion exchange column at pH 7.5 resulted in a single peak of protease activity eluting at high salt (fractions 40 through 42). Analysis of the peak fractions showed a single band of M_r 37,000 following SDS-PAGE (Fig. 5A, lane 5) and three acidic bands corresponding to EP-A isozymes 1 through 3 (bottom to top, respectively) following activity gel electrophoresis (Fig. 5B). The purified EP-A had a specific activity of 912 units/mg protein and was purified at least 80-fold (Table I, step 5).

The major protease peak following HPLC on the DEAE column at pH 6.0 (fractions 25 and 26) is sensitive to cysteine proteinase inhibitors and has been purified to yield a protein of M_r 30,000 as determined by SDS-PAGE (manuscript in preparation).

Protease Characterization. Hemoglobin was used as a substrate during preliminary characterization of thiol-protease activity in crude medium (12), therefore hemoglobin protease assays were used to characterize purified EP-A. With this assay the increase in TCA-soluble α-NH_2 was linear at 52.4 μmol α-NH_2·mg enzyme^{-1}·h^{-1} between 2 to 12 h of digestion at 30°C and at pH 4.0. No activity was detected in the absence of EP-A. The time course of hemoglobin digestion up to 12.5 h was followed by urea/SDS-PAGE (Fig. 6). Band **a** represents undigested hemoglobin monomers. Discrete bands resulting from hemoglobin digestion (bands **b** and **d**) were seen as early as 0.5 h (lane 3). By 6 h (lane 7) approximately 50% of the hemoglobin subunits were digested with a corresponding increase in bands **b** and **d** as well as two additional bands **c** and **e**. By 12.5 h (lane 9) only traces of undigested hemoglobin subunits remained, and bands **b** through **e** were still evident. By 24 h (not shown) bands **b** through **e** were barely visible. This pattern of digestion indicated that EP-A is an endoprotease. Analysis of hemoglobin digests by Biol-Gel P-2 chromatography resulted in an elution profile also indicative of endoproteolytic activity. If EP-A were an exoprotease, free amino acids would be released at the initial stage of digestion of hemoglobin. However, even following 12 h of digestion (Fig. 7), ninhydrin-reactive products were distributed in a descending slope from the void volume, and there was no increase in ninhydrin-reactive products co-eluting with [^{3}H]proline.

To determine the temperature optimum of EP-A against hemoglobin, protease assays were conducted at 5°C intervals from 20 to 60°C. Activity increased to an optimum at 45°C with 86.4 μmol α-NH_2·mg enzyme^{-1}·h^{-1}. The protease was still active at 60°C with 20% of the maximum activity.

The pH optimum for EP-A, determined at the optimum temperature, 45°C, over a pH range from 3.4 to 6.4, was 5.0 (Fig. 8). The assay was linear for at least 3 h under these optimum conditions. Activity at pH 5.0 (169 μmol α-NH_2·mg enzyme^{-1}·h^{-1}) was at least 2.7 times greater than the activity at pH 4.0.

Inhibitors specific to each class of proteinases, *i.e.* E-64, IAc, and *p*HMB for cysteine proteinases; PMSF, aprotinin, and STI for serine proteinases; pepstatin for aspartic proteinases; and 1,10-phenanthroline and EDTA for metallo-proteinases (1), were assayed for their ability to inhibit EP-A in the hemoglobin assay under the optima temperature and pH. The inhibitor concentrations employed for E-64, IAc, PMSF, aprotinin, STI, and pepstatin achieved at least 96% inhibition when tested against known proteinases from each respective class. Only inhibitors of cysteine proteinases significantly inhibited EP-A (Table II). βME enhanced EP-A activity. These results indicate unequivocally

Table I. *Purification of EP-A from Media of Barley Half-Seeds and Cracked Seeds*

The media from barley half-seeds and cracked seeds incubated in the presence of 1 μM GA_3 according to the "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
	U[a]	*mg*	*U/mg*	*%*	*-fold*
1. Crude medium	2,161	190	11.39	100	1.0
2. $(NH_4)SO_4$ pptn	751	81.1	9.26	35	0.8
3. Gel filtration	422	11.0	38.33	19	3.4
4. HPLC-DEAE (pH 6.0)	76	0.08	950	3.52	83.4
5. HPLC-DEAE (pH 7.5)	73	0.08	912	3.38	80.1

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

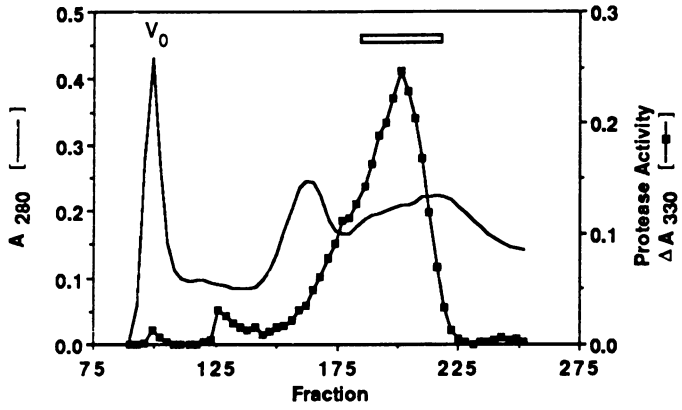

FIG. 3. Gel filtration chromatography on Sephadex G-100. The 50 to 75% $(NH_4)_2SO_4$ fraction (total volume 46 ml) was chromatographed on a 100 × 5 cm column of G-100 Sephadex as described under "Materials and Methods." Aliquots (80 μl) of every third fraction beginning at the void volume (V_0 = 696 ml) were assayed for protease activity against azocasein (■) as described under "Materials and Methods," and the protein was estimated by the A_{280} (——). Fractions 186 through 219, marked by the bar, were pooled for further protease purification. V_t = 2396.

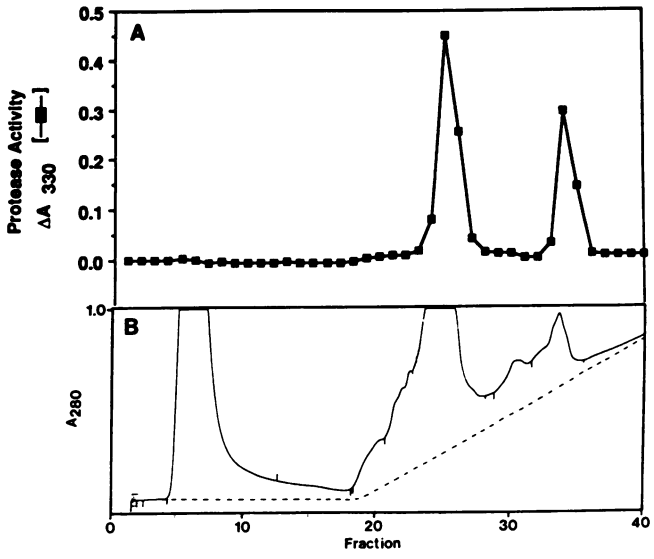

FIG. 4. DEAE-anion exchange HPLC (pH 6.0). A, A 9.0 ml portion of the pooled gel filtration fractions following dialysis was injected on a Waters 5PW HPLC-DEAE anion exchange column and eluted at a flow rate of 1 ml/min with 20 mM Tris acetate (pH 6.0) and 0.25 mM DTT for 10 min, followed by a linear gradient from 20 to 900 mM Tris acetate (pH 6.0) in the presence of 0.25 mM DTT for 30 min. One ml fractions were collected, and 12 μl aliquots of each fraction were assayed for protease activity (■) against azocasein as described under "Materials and Methods." Fractions 34 and 35 were pooled for further purification of EP-A. B, The A_{280} profile of a representative HPLC run following a 0.5 ml sample injection [——] was measured using a Spectroflow 757 Absorbance Detector, and the peaks were integrated using a Hewlett-Packard model 3392 Integrator. The last peak, which corresponds to fractions 34 and 35, was approximately 4% of the total area. The baseline position with no sample injected (– – –) shifts upward with increasing salt concentrations.

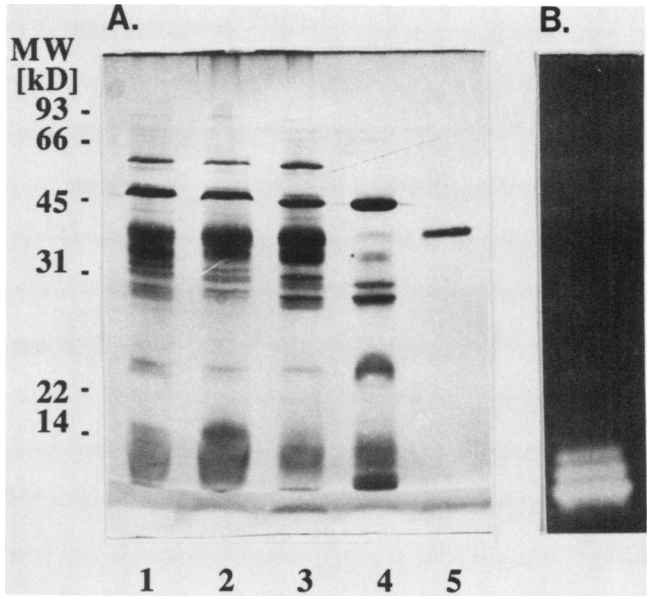

FIG. 5. Electrophoretic analysis of the purification of EP-A. A, Portions of the samples following the various purification steps listed in Table I were analyzed by SDS-PAGE on 12.5% gels, and polypeptides were visualized with silver stain as described under "Materials and Methods." Lanes 1 and 2, crude 72 to 96 h media from half-seeds and cracked seeds, respectively; lane 3, material from 50 to 75% $(NH_4)_2SO_4$ precipitation; lane 4, pooled fractions from G-100 gel filtration following dialysis; lane 5, purified EP-A following sequential DEAE-anion exchange HPLC at pH 6.0 and 7.5. Lanes 1 to 4 contain 12 μg of protein, and lane 5 contains 2.1 μg of protein. The migration of mol mt markers phosphorylase B (92.5 kD), BSA (66.2 kD), ovalbumin (45 kD), carbonic anhydrase (31 kD), soybean trypsin inhibitor (21.5 kD), and lysozyme (14.2 kD) is indicated on the left. B, Purified EP-A (0.80 units as determined by azocasein digestion) was analyzed on a protease activity gel as described under "Materials and Methods." The activity gel was incubated and processed as in Figure 2 except the incubation time was 3.5 h.

that EP-A is a cysteine proteinase. Moderate increases in activity were seen in the presence of the natural inhibitors aprotinin and pepstatin. This may be due to hydrolysis of these inhibitors themselves by the barley protease.

The complexity of the individual EP-A isozymes separated by native PAGE was assessed by CNBr cleavage. Peptides generated after 2 h of CNBr treatment were compared following SDS-PAGE (Fig. 9). The CNBr cleavage patterns for the three isozymes (lane c) were nearly identical; at least nine major fragments of similar mobility were obtained for each isozyme. Only a minor amount of acid-induced hydrolysis or autodegradation occurred if native gel slices containing the ioszymes were either incubated 2 h under identical conditions but in the absence of CNBr (lane b) or equilibrated immediately without incubation (lane a) prior to SDS-PAGE. These results indicate that similarity exists between the amino acid sequence of the isozymes.

The NH_2-terminal amino acid sequence, for between 19 and 21 residues, was determined for each isozyme (Fig. 10A). The only difference between the three sequences was in the position of the NH_2-terminus. Compared to isozyme 1, the NH_2-terminus of isozyme 2 starts at the third residue, whereas the N_2-terminus of isozyme 3 starts at the fifth residue. The amino acid sequence in the region of overlap between each isozyme was identical. A search of the NBRF protein sequence databank and translated GenBank database identified at least six cysteine proteinases

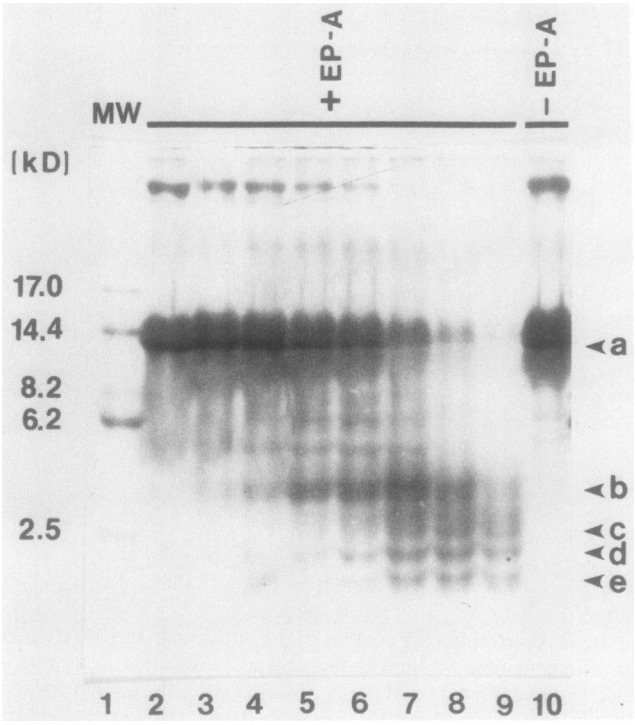

FIG. 6. Separation of hemoglobin digestion products by urea/SDS-PAGE. Hemoglobin digests were incubated at pH 4.0 and at 30°C in the presence of 0.05 μg EP-A/ml (lanes 2–9). At 0, 0.5, 1, 2, 3, 6, 9, and 12.5 h (lanes 2–9, respectively), 10 μl aliquots of the digests were added to 10 μl of urea/SDS sample buffer, heated for 15 min at 60°C, and the proteins then separated by urea/SDS PAGE as described in the "Materials and Methods." Nondigested hemoglobin, incubated in the absence of EP-A for 12.5 h, was also analyzed (lane 10). Band **a** represents hemoglobin monomers. Bands **b** through **e** are polypeptides generated by hemoglobin digestion. Myoglobin fragments, lane 1, were used to calibrate the gel, and the mol wt of each is indicated on the left.

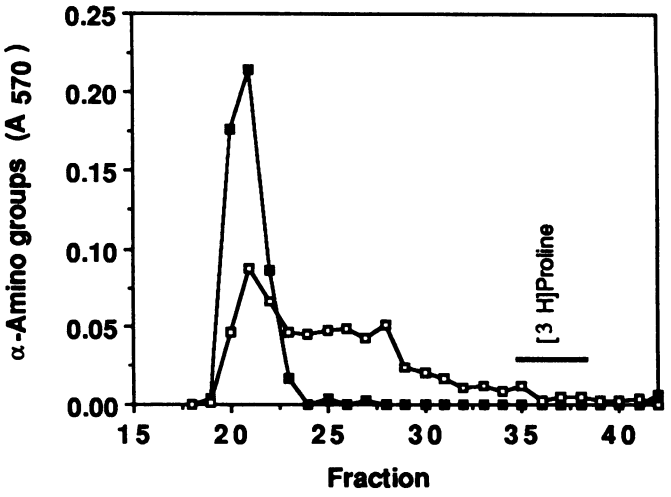

FIG. 7. Separation of nondenatured hemoglobin digestion products by Bio-Gel P-2 chromatography. Hemoglobin was incubated in the absence (■) or presence of (□) EP-A at 30°C (pH 4.0) for 12 h. One ml aliquots, together with 25 nCi of [^{3}H]proline, were chromatographed on a Bio-Gel P-2 column as described in "Materials and Methods." Aliquots (100 μl) of effluent were assayed for α-amino groups by ninhydrin and for radioactivity. The bar indicates where the peak of [^{3}H]proline eluted.

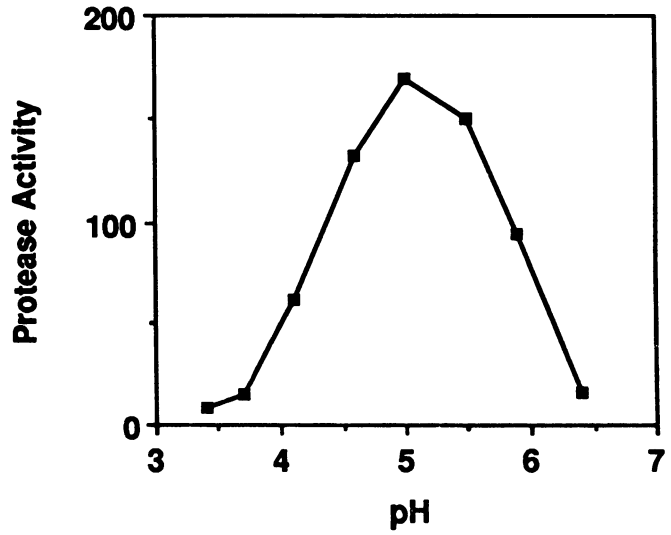

FIG. 8. Effect of pH on EP-A activity against hemoglobin. Protease activity was assayed at different pH values in reaction mixtures that were 0.11% (w/v) hemoglobin, 33 mM Na-succinate, 10 mM βME. EP-A was added at 0.05 μg/ml and the reactions incubated at 45°C for 3 h. TCA-soluble, ninhydrin-reactive α-NH_2 released was determined as described under "Materials and Methods." One unit of protease activity is equal to 1 μmol α-NH_2 released·mg enzyme^{-1}·h^{-1}.

having greater than 50% identity in their amino acid overlap with the EP-A NH_2-terminal amino acid consensus sequence extended at both ends to include the first four residues of isozyme 1 and last three residues of isozymes 2 and 3 (Fig 10B). Optimal alignment of these sequences to the extended EP-A consensus sequence using the GAP program (8) revealed that papain was the most similar (84%). The sequence for aleurain (25), also included in the comparison, was the least similar (56%). The extended EP-A consensus sequence contained sequences that were conserved between all of these cysteine proteinases.

DISCUSSION

Barley embryoless half-seeds secrete several proteases in response to the hormone GA_3. We have purified to homogeneity the three protease isozymes of M_r 37,000 whose synthesis and secretion from barley aleurone layers is a GA_3-dependent process. We have designated these proteases as barley endoprotease A (EP-A). Based on activity gels, EP-A is the most abundant protease secreted from barley embryoless half-seeds from 72 to 96 h of incubation in the presence of GA_3. The 3.38% recovery and 80-fold purification in the EP-A purification scheme were actually underestimates since several different proteases are present in the crude medium. Three different peaks of activity are seen in the Sephadex G-100 profile, and following DEAE-anion exchange HPLC at pH 6.0, at least 60% of the protease activity assayed against azocasein was due to a protease other than EP-A. The recovery of EP-A is simlar to that of other plant cysteine proteinases, *i.e.*, vicilin peptidohydrolase (8.0%) from mung bean seedlings (3) and EP-1 (1.6%) from barley seedlings (19).

Previous reports of putative endoprotease activity in germinating barley and barley malt were based on measurements of crude or partially purified enzyme preparations (4, 9, 23, 29). In some cases (4, 23) protease assays were based on the release of TCA-soluble products during hemoglobin digestion. Exoprotease and endoprotease activities cannot be differentiated in such

assays. Electrophoretic detection of smaller peptide products hydrolyzed from larger peptides or proteins is indicative of endoprotease activity. For example, the digestion of vicilin by purified vicilin peptidohydrolase resulted in the appearance and gradual disappearance of discrete lower mol wt peptides (3). Sephadex G-15 chromatography of vicilin digested by carboxypeptidase or vicilin peptidohydrolase demonstrated that for carboxypeptidase, ninhydrin-reactive products co-eluted with [^{14}C]proline, whereas for the proteinase, the products were predominantly distributed between the void volume and the elution volume of proline (3). The digestion of hemoglobin by barley EP-A displayed a similar pattern of proteinase activity, *i.e.* the appearance and gradual disappearance of four discrete bands between 1800 and 3500 M_r visualized by urea/SDS-PAGE and elution of ninhydrin-reactive products prior to [^{3}H]proline on a Bio-Gel P-2 column.

Table II. *Effects of Inhibitors on Protease Activity*

Protease activities are expressed as the percentage of the control (100% = 171 μmol α-NH_2 · mg enzyme^{-1} · h^{-1}) in which no inhibitors were added. All mixtures were preincubated for 1 h at 4°C prior to addition of the substrate, during which time the inhibitor concentration was 1.28 times higher than the final concentration listed below.

Treatment	Concentration	Activity, EP-A
		%
E-64	10 μM	9
Iodoacetate	1 mM	6
*p*HMB	100 μM	0[a]
Deletion of βME		12
PMSF	5 mM	101
Aprotinin	320 μg/ml	151
STI	10 μg/ml	135
Pepstatin	1 μg/ml	105
1,10-phenanthroline	100 μM	75
EDTA	10 mM	90

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

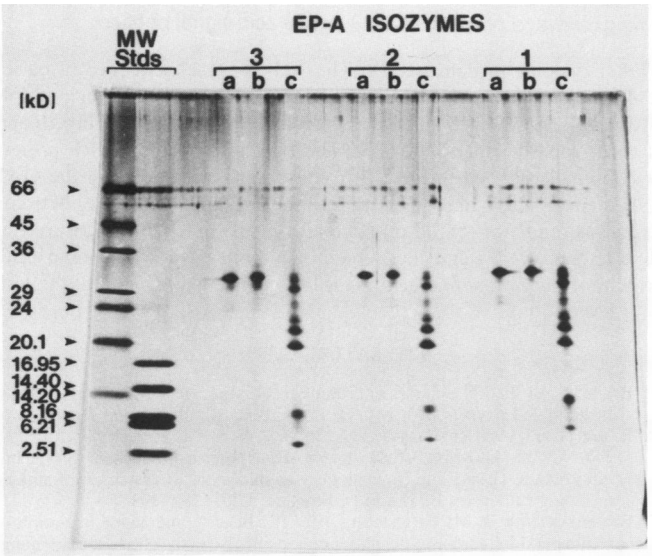

FIG. 9. Cyanogen bromide cleavage of EP-A isozymes. Separate native gel slices containing about 3 μg of EP-A isozyme 1, 2, or 3 were incubated 2 h in microfuge tubes containing about 50 mg of CNBr dissolved in 20 μl acetonitrile and 200 μl each of 0.125 M Tris HCl (pH 6.8) and 0.6 N HCl. The slices were then rinsed in equilibration buffer and heated in sample loading buffer prior to SDS-PAGE in an acrylamide-sucrose gradient gel as stated in "Materials and Methods" (lane c). In addition, separate native gel slices containing about 1 μg of isozyme 1, 2, or 3 were either incubated as above but in the absence of CNBr (lane b) or equilibrated immediately without incubation (lane a) prior to SDS-PAGE. The first two lanes on the left contain the mol wt markers ranging from 66 to 14.2 kD and from 16.95 to 2.51 kD (MW-SDS-70L and MW-SDS-17 kits, respectively, Sigma Chemical Co., St. Louis, MO) used to calibrate the gel. The two bands running the width of the gel between the 66 and 45 kD mol wt markers are artifacts frequently observed in silver-stained gels (6).

EP-A (and possibly the other major 30,000 M_r protease) may represent the major endoproteinase activity secreted from GA_3-treated half-seeds at 96 h of incubation that was previously described by Sundblom and Mikola (29). This activity (assayed against gelatin) had an optimum pH of 5.0 (identical to that for EP-A), whereas protease activity secreted between 24 and 72 h had a pH optimum of approximately 4.0 (29). Hammerton and Ho (12) reported that protease activity secreted from GA_3-treated aleurone layers between 24 and 48 h also had a pH optimum of 4.0. EP-A was the only protease activity detectable in their activity gels containing hemoglobin following electrophoresis of medium from this time point (12). Since purified EP-A has a pH optimum of 5.0, it is possible that the more acidic protease activity is due to yet another protease which was not detected by their activity gels.

Inhibition of EP-A by E-64 indicates it is a cysteine proteinase. E-64 is an active-site directed, irreversible inhibitor specific to cysteine proteinases (2). It has been shown to effectively inhibit several cysteine proteinases from plants (papain, ficin, and bromelains) and mammals (cathepsins B, H, and L, and calpain) but is inactive against several different serine-, aspartic-, and metallo-proteinases (2).

The NH_2-terminal sequence analysis of EP-A provides further support to the classification of EP-A as a cysteine proteinase. Information obtained from the NBRF protein sequence databank and translated GenBank database indicates that several cysteine proteinases are produced as zymogens (proenzymes). For example, papain is synthesized as a zymogen with a 133 amino acid NH_2-terminal propeptide that is not part of the active enzyme. Heterogeneous processing of a possible propeptide from the mature EP-A isozymes might explain the difference in the NH_2-termini of the isozymes. Previous speculation that aleurain was EP-A (25) depended on the cleavage of merely a putative signal peptide resulting in a mature protein with the predicted size of 37 kD. Since EP-A apparently is not aleurain, it is possible that aleurain actually exists as a smaller protein (about 24 kD) following cleavage of an additional 143 amino acids from the NH_2-terminal region which is nonhomologus to mature cysteine proteinases (25). Aleurain may be one of the other cysteine protease activities characterized in barley, for example the 30,000 M_r protease. Differences in the NH_2-termini of the EP-A isozymes, particularly the absence of the negatively charged amino acid, aspartic acid, in isozyme 3, could contribute to the slower mobility of isozymes 2 and 3 during native PAGE.

Cysteine proteinases may play a major role in protein turnover, degradation, and possibly even enzyme activation in germinating barley. Miller and Huffaker (19) have extensively purified and characterized a cysteine proteinase from 12 d old primary leaves of barley. This major vacuolar proteinase (EP-1) has a predicted mol wt of 28.3 kD, has a pH optimum of 5.5 to 5.7, and has been shown to hydrolyze both the large and small subunit of ribulose 1,5-bis-phosphate carboxylase into discrete polypeptides (19, 31). In addition to carboxypeptidase activity, the GA_3-induced cysteine proteinases may be responsible for mobilization of prolamine seed storage proteins (hordeins) during germination. Rastogi and Oaks (24) have shown that the initial stage of hydrolysis of the major high mol wt groups of hordein is independent of GA_3-induced protease activity. However, extensive

A) NH_2-Terminal Sequence of Individual EP-A Isozymes

EP-A Isozyme 1	NH_2- X A D N L P P A V D W R Q K G A V T A I
EP-A Isozyme 2	NH_2- D N L P P A V D W R Q K G A V T A I K D Q
EP-A Isozyme 3	NH_2- L P P A V D W X Q K G A V T A I K X Q

B) Comparison of the EP-A Consensus Sequence to other Cysteine Proteinases

Cysteine Proteinase	Sequence of Amino Acid Overlap	Similarity (%)
EP-A Consensus	NH_2- X A D N L P P A V D W R Q K G A V T A I K X Q	
Aleurain	140[a]- D A A A L P E T K D W R E D G I V S P V K N Q	56
Papain	NH_2- I P E Y V D W R Q K G A V T P V K N Q	84
Mouse[b]	NH_2- I P K S V D W R E K G C V T P V K N Q	79
Human Cathepsin L	NH_2- A P R S V D W R E K G Y V T P V K N Q	74
Slime mold #1[c]	95- F I W S I P T A F D W R T R G A V T P V K N Q	69
Actinidin	NH_2- L P S Y V D W R S A G A V V D I K S Q	63
Slime mold #2[d]	101- D L Q T N P K S I D W R T K N A V T P I K D Q	61

[a]Position of the first amino acid assuming the cleavage of a 22 amino acid signal peptide.
[b]Sequence translated from GenBank Database locus Muscpr.
[c]Sequence translated from GenBank Database locus Slmcyspro assuming cleavage of a 19 amino acid signal peptide.
[d]Sequence translated from Genbank Database locus Slmcpro2r assuming cleavage of a potential 18 amino acid signal peptide.

FIG. 10. NH_2-Terminal amino acid sequence of EP-A and homology to other cysteine proteinases. A, The NH_2-terminal sequence of barley EP-A isozymes determined as described in "Materials and Methods" is presented. An 'X' refers to an undesignated call due to either multiple signals present from free amino acids (isozyme 1, position 1) or low yields (isozyme 3, positions 8 and 18). B, Cysteine proteinases having greater than 50% identity in their amino acid overlap with the EP-A NH_2-terminal consensus sequence were identified by searching the NBRF protein sequence databank and translated GenBank database. These sequences were optimally aligned to the EP-A consensus sequence using the GAP program of the UWGCG Package (8) which uses the alignment method of Needleman and Wunsch (21) to find the maximum similarity between two sequences. Residues in the amino acid overlaps similar to the EP-A consensus sequence (based on comparison values ≥0.5 as measured by Dayhoff and normalized by Gribskov and Burgess [11]) are shown in outline letters (*e.g.* P) and the percent similarity is shown. Amino acids conserved among all the sequences are boxed. The position in the mature protein of the first amino acid in each sequence is indicated to the left.

proteolysis occurred in embryoless half-seeds incubated in the presence of GA_3, resulting initially in the production of smaller polypeptide fragments, and with time an increase in small peptides and free amino acids was observed. Due to the timing of its secretion, we anticipate that EP-A might be involved in the later stages of GA_3-induced hordein hydrolysis. A major cysteine proteinase partially purified from wheat seeds germinated for 4 d is capable of hydrolyzing wheat gluten (aggregates of prolamines and glutelins) into small peptides of a few amino acids (27). During germination, proteases might also be responsible for the activation of latent, protein-bound forms of β-amylase. Protein-bound forms of β-amylase exist in the mature endosperm primarily in the highly cross-linked glutelin fraction (26). Extraction of barley grain with papain resulted in the appearance of two β-amylase forms with pI identical to those of the predominant forms found in germinating barley (14).

With the purification of EP-A and the other major cysteine proteases, the physiological roles of these specific proteases in germinating barley and the regulation of their expression by GA_3 can be addressed.

Acknowledgments—We sincerely thank Christine Smith, Monsanto Corporate Research, Chesterfield, MO, for the NH_2-terminal sequence analysis of EP-A and NBRF protein sequence databank and GenBank database searches. We thank Dr. Peter Brown for technical advice and helpful criticism and Paul Ho for technical assistance.

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