Partial Purification and Characterization of Endoproteinases from Senescing Barley Leaves[1]

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ABSTRACT

Two major endoproteinases were purified from senescing primary barley leaves. The major enzyme (EP₁) appeared to be a thiol proteinase and accounted for about 85% of the total proteolytic activity measured *in vitro*. This proteinase was purified 5,800-fold and had a molecular weight of 28,300. It was highly unstable in the absence of dithiothreitol or at a pH greater than 7.5. Leupeptin, at a concentration of 10 micromolar, inhibited this enzyme 100%. A second proteinase (EP₂) was purified approximately 50-fold and had a molecular weight of 67,000. It was inhibited 20% by 1 millimolar dithiothreitol and 50% by 1 millimolar phenylmethyl sulfonyl-fluoride. EP₂ contributed about 15% of the total proteolytic activity measured *in vitro*. Both proteinases hydrolyzed a variety of artificial and protein substrates, and both had pH optima of 5.5 to 5.7 when either azocasein or [¹⁴C]ribulose-1,5-bisphosphate carboxylase ([¹⁴C]RuBPCase) was the substrate. The thiol endoproteinase hydrolyzed azocasein linearly but hydrolyzed [¹⁴C]RuBPCase biphasically. A third endoproteinase (EP₃), not detected by standard proteolytic assays, was observed when [¹⁴C]RuBPCase was the substrate.

The predominant EPs[2] found in many leaf tissues appear to be either acid- (4, 8, 10, 16, 19, 24) or sulfhydryl-stimulated (1, 8, 10, 20, 23, 26, 27). Neutral proteinases from corn (8) and *Lolium temulentum* (24) have been described, but not classified, as thiol, serine, or metallo proteinases. Proteinases ascribed primary importance in the rapid turnover of proteins during senescence are acid proteinases in corn (8) and wheat (4, 9), thiol proteinases in wheat (27), and a serine proteinase in oats (6, 16).

Leaf proteinases have usually been studied using crude extracts or slightly purified (several-fold increase in specific activity) proteinase preparations. In only a few cases have leaf proteinases been purified to homogeneity or near homogeneity. Ragster and Chrispeels (21) purified two enzymes from soybean leaves that were capable of hydrolyzing azocoll. Azocollase A had a mol wt of 17,500 and a pH optimum of 9.0, and it was inhibited by 3 mM EDTA. Azocollase B had a mol wt of 52,000 and a pH optimum of 9.0, and it was inhibited by pCMB. Ragster and Chrispeels suggest, however, that these enzymes may not play a major role in

the hydrolysis of leaf protein in crude extracts. Frith *et al.* (9) and Peoples *et al.* (19) purified from green wheat leaves several acid proteinases capable of hydrolyzing hemoglobin or RuBPCase. Two of the proteinases had mol wt of 89,000 and 135,000. A serine proteinase having an acidic pH optimum and a sulfhydryl-stimulated exoproteinase were purified from senescing oat leaves (6). Pike and Briggs (20) purified, from etiolated oat shoots, a proteinase that was stimulated by sulfhydryl reagents and was inhibited by PMSF.

This report presents information on the endoproteolytic system of barley leaves. Our results indicate that there are two predominant proteinases (EP₁ and EP₂). The major enzyme is a thiol proteinase (EP₁) which is highly unstable unless proper protective measures are taken and it has been purified 10-fold greater than leaf proteinases previously described (6, 9, 20). Finally, evidence is given for the existence of a third proteinase (EP₃) which appears to be closely associated with RuBPCase and is not readily detected by standard proteolytic assays.

MATERIALS AND METHODS

Reagents. Egg albumin and aldolase were obtained from Boehringer Mannheim, SDS from BDH Biochemicals (Gallard-Schlesinger), and all other reagents and standard proteins from Sigma.

Plant Material. Barley seeds (*Hordeum vulgare* v. Numar) were planted in 28- x 33-cm plastic pans containing vermiculite. Nutrient solution was supplied continuously by cotton wicks linked to a reservoir pan below. Plants were grown for 12 days in continuous light (550 μE/m²·s) at 27 C and 55% RH.

Hydrolysis of Azocasein. The reaction mixture contained 4 mg/ml azocasein, 50 mM K-phosphate (pH 5.7), 1 mM DTT, and 1 μg EP₁ (DEAE preparation). The assay for EP₂ had 5 to 15 μg EP₂ (DEAE preparation) and no DTT. The reaction was stopped after 1 to 3 h at 40 C by adding 1.0 ml 14% HClO₄, and the protein was pelleted by centrifugation. Hydrolysis was measured as the increase in A of the supernatant at 340 nm.

Hydrolysis of [¹⁴C]RuBPCase. The final reaction mixture was 90 mM K-phosphate (pH 5.7), 1 mM DTT, 2 to 4 mg/ml [¹⁴C]RuBPCase, and 1 μg EP₁ (DEAE preparation). Assay temperature was 40 C, and an aliquot was removed at designated times and added to an equal volume of 10% TCA. After 1 h on ice, the precipitated protein was pelleted by centrifugation, and the supernatant was used to determine TCA-soluble counts.

Ninhydrin Assay for α-amino Nitrogen. α-Amino nitrogen was determined as described by Moore (17). At various times in either the azocasein or [¹⁴C]RuBPCase assay described above, a 0.5-ml aliquot was taken, and 0.5 ml of 10% TCA was added to precipitate the high mol wt protein fragments and unhydrolyzed substrate. After centrifugation, 0.5 ml of supernatant was added to 1.0 ml ninhydrin reagent and heated for 10 min at 100 C. Four ml water was added to each sample, and the A at 570 nm was measured.

[2] Abbreviations: EP, endoproteinase; pCMB, *p*-chloromercuribenzoic acid; RuBPCase, ribulose-1,5-bisphosphate carboxylase; PMSF, phenylmethyl sulfonylfluoride; BAEE, α-*N*-benzoyl-L-arginine ethyl ester; BTEE, *N*-benzoyl-L-tyrosine ethyl ester; BANA, α-*N*-benzoyl-DL-arginine-β-naphthylamide; STI, soybean trypsin inhibitor; Pep A, pepstatin A; PMA, phenyl mercuric acetate; IAA, iodoacetamide.

The increase in free amino groups (isoleucine was used for the α-amino N standard) was compared with the increase in either A at 340 nm (azocasein) or cpm ([^{14}C]RuBPCase). The following relationships were obtained: (a) nmol α-N = 14.09 (A_{340}) − 0.069, r^2 = 0.99; and (b) nmol α-N = 2.63 × 10^{-3} cpm + 3.00; r^2 = 0.98.

Hydrolysis of Artificial Substrates. The final reaction mixture for the hydrolysis of the carbobenzyoxyamino acid-nitrophenyl ester substrates contained 80 mM K-phosphate (pH 6.0), 1 mM DTT (no DTT was included for the assay of EP$_2$), and 0.2 mM substrate. Each assay included one of the following: 1 μg EP$_1$, 4 μg EP$_2$, 2 μg trypsin, or 3 μg papain. After hydrolysis for 30 min at 26 C, the reaction mixture was adjusted to 5% TCA and centrifuged, and the A of the supernatant at 320 nm was measured. The final substrate concentration for the BAEE, BTEE, and BANA hydrolysis was 0.5 mM. The increases in A at 252 nm, 256 nm, and 335 nm were measured during the first 5 min of the reaction for BAEE, BTEE, and BANA, respectively.

Gelatin-Agarose Assay. Fifty ml 0.2 M K-phosphate buffer (pH 6.0) were added to 0.6 g agarose and 0.12 g gelatin. This solution was boiled to dissolve the agarose and gelatin. When the solution had cooled to 60 to 65 C, DTT was added to make a final concentration of 2 mM DTT. Solution was poured into a small black Plexiglas tray to make a layer 1 to 2 mm thick. After proteolytic activities were separated by native gel electrophoresis, the polyacrylamide tube gels were sliced longitudinally and placed with flat surface down onto the gelatin-agarose layer. The tray was incubated at 40 C for 1 to 3 h under N$_2$ atmosphere in a Desaga/Brinkmann (Brinkmann Instruments, Inc., NY) double chamber. The assay was terminated by removing the gel slice and developing the gelatin-agarose layer by covering the surface with a solution of 15% HgCl$_2$ and 20% HCl. This resulted in a milky white background and cleared zones that appeared as black bands. A method similar to that of Santarius and Ryan (22) was also used. Wells were made into an agarose layer containing gelatin, azocasein, or RuBPCase, and samples containing proteolytic activity were placed into these wells. These protein-agarose layers were incubated at 40 C for a given time and were then developed as described above. The diameter of the cleared zones is a semi-quantitative measure of proteolytic activity.

Purification of Endoproteinases. Twelve-day-old primary barley leaves were detached (330 g) and homogenized in a Waring Blendor with 0.1 M K-phosphate buffer (pH 6.0) containing 2 mM DTT and 1 mM EDTA. Three ml of buffer and 0.1 g of insoluble PVP was used for each g of tissue. The homogenate was filtered through eight layers of cheesecloth and centrifuged for 20 min at 27,000g. Solid ammonium sulfate was added to the resulting supernatant, and the 35 to 70% pellet was recovered. The pellet was resuspended in 50 mM K-phosphate (pH 6.0) containing 1 mM DTT and 1 mM EDTA and dialyzed for 20 h against the same buffer. Protein, which precipitated from the solution during dialysis, was removed by centrifugation. Recovery of endoproteolytic activity (against azocasein) was 80%. Approximately 44 ml of the dialyzed 70% ammonium sulfate fraction were eluted at 15 ml per h through a 3- x 50-cm Sephadex G-100 column equilibrated with 50 mM K-phosphate (pH 6.0) containing 1 mM DTT.

This step resolved the activity into two separate activity peaks. The two activities were further purified by chromatography on a 2- x 20-cm DEAE-cellulose column. EP$_2$ did not bind to a column equilibrated with 50 mM K-phosphate (pH 6.0) containing 1 mM DTT, but EP$_1$ did bind. EP$_1$ was eluted from the column with a 0.0- to 0.2-M NaCl gradient and dialyzed against 50 mM K-phosphate (pH 6.0) and 1 mM DTT. A major inactive protein was removed from the DEAE-cellulose-purified EP$_1$ by gel filtration on a Sephadex G-75 superfine column equilibrated with 50 mM K-phosphate (pH 6.0) and 1 mM DTT.

Purification of ^{14}C-Labeled RuBPCase. Barley seeds (*Hordeum vulgare* v. Numar) were planted in 15.2-cm pots containing ver-miculite. Nutrient solution was supplied continuously by cotton wicks that linked the pot to a reservoir jar below. Seedlings grew in darkness for 6 days and then were placed into a chamber under light ($\sim$550 μE/m^2·s) and allowed to green in the presence of ^{14}CO$_2$. [^{14}C]RuBPCase was purified using a modification of the method described by Kleinkopf et al. (13). Leaves were harvested after 48 h and homogenized in a Waring Blendor using 5 ml of buffer and 0.1 g insoluble PVP per g of tissue. Homogenization buffer was 0.2 M Tris-SO$_4$ (pH 8) containing 10 mM MgCl$_2$, 10 mM NaHCO$_3$, 2 mM DTT, and 1 mM EDTA. The crude homogenate was filtered through eight layers of cheesecloth and centrifuged at 27,000g for 20 min. Solid ammonium sulfate was added to the resulting supernatant, and the 35 to 65% pellet was recovered and resuspended in 50 mM Tris-SO$_4$ (pH 8.0), 2 mM DTT, and 1 mM EDTA. Fifteen ml of the dialyzed protein were chromatographed on a 3- x 50-cm Sephadex G-100 column equilibrated with homogenization buffer. Only the first one-half of the RuBPCase peak was kept to prevent possible contamination by the major leaf proteinases (B. L. Miller, unpublished). The pooled fractions, $\sim$45 ml, were applied to a 2- x 20-cm DEAE cellulose column equilibrated with 50 mM Tris-SO$_4$ (pH 8.0), 2 mM DTT, and 1 mM EDTA. After the column was washed with the same buffer, RuBPCase was eluted with a 0.0- to 0.2-M ammonium sulfate gradient and dialyzed against 50 mM Tris-SO$_4$ (pH 8.0), 2 mM DTT, and 1 mM EDTA. The RuBPCase had a specific radioactivity of 4.0911 × 10^6 cpm/mg protein. SDS-polyacrylamide electrophoresis indicated that it was homogeneous.

Electrophoresis. Disc-gel electrophoresis was carried out using methods of Davis and Ornstein (5, 18). The gels were 7.5% acrylamide with a monomer:bis ratio of 39:1. SDS electrophoresis was done in 10% polyacrylamide slabs (1 mm) using the buffer system of Laemmli (14), with a current of 15 mamp/slab. All gels were stained according to Fairbanks et al. (7).

Mol Wt Determination. Mol wt of EP$_1$ and EP$_2$ were determined using the method of Hedrick and Smith (11). After electrophoretic separation of the proteolytic enzymes at various acrylamide concentrations, the locations of the enzymes were determined by the gelatin-agarose assay described above. Standard proteins used were insulin; egg albumin; α-amylase; pepsin dimer; hexokinase; aldolase; and BSA monomer, dimer, and trimer.

Inhibitors and Activators. EP$_1$ and EP$_2$ were preincubated for 5 h at 4 C in the presence of the desired activator or inhibitor. The pretreatment and final reaction concentrations of these compounds were identical. The control reaction was 50 mM Mes buffer (pH 6.0), 1 mM DTT (except for EP$_2$), and 4 mg/ml azocasein or 2 mg/ml [^{14}C]RuBPCase.

Profiles of pH Activity and pH Stability. All pH measurements were taken directly from the reaction or from preincubation solutions. EP$_1$ and EP$_2$ were preincubated on ice for 17 h for the stability studies. All buffers were phosphate.

Determination of Protein. Protein was determined by the method of Lowry et al. (15) or by the micromethod of Bradford (3).

RESULTS AND DISCUSSION

Enzyme Purification. Proteinases were purified from 12-day-old senescing primary barley leaves. The leaves were yellow and had lost approximately 50% of the total soluble protein present before the onset of senescence. The two proteinases described here are also the major proteinases observed in crude extracts or 70% ammonium sulfate-fractionated preparations of 7-day-old mature green leaves or 20-day-old yellow leaves (late senescence stage). The proteinases were detected after electrophoresis using the gelatin-agarose assay (B. L. Miller and R. C. Huffaker, in preparation).

Attempts to purify barley proteinases using affinity chromatography on hemoglobin-Sepharose 4B prepared using the methods

of Drivdahl and Thimann (6) and Frith et al. (9) were completely unsuccessful. The proteolytic activity would bind to the hemoglobin-Sepharose, but no procedure was found that could elute the enzymes from the column. A combination of ammonium sulfate precipitation, gel filtration on Sephadex G-100, and DEAE-cellulose chromatography, however, did provide a 50-fold purification of EP₂ and a 620-fold purification of EP₁ (Table I).

Chromatography on Sephadex G-100 separated the proteolytic activity into a major (~85%) and a minor (~15%) fraction (Fig. 1). An aliquot of each fraction was tested using a method similar to that described by Santarius and Ryan (22) (see also, "Materials and Methods"), using gelatin, azocasein, or RuBPCase incorporated into buffered-agarose layers. Figure 1 shows that all substrates gave similar results and that no RuBPCase-specific proteinases were detected. No additional major proteinase was observed that would hydrolyze RuBPCase at pH 7.5 (data not shown). An aliquot of each G-100 fraction was also subjected to electrophoresis, and the proteinases were detected with the gelatin-agarose assay. Figure 2 (A and B) shows the major proteolytic activities present in the ammonium sulfate-precipitated proteins. The presence of EP₂ in the shoulder and EP₁ in the main activity peak of the column is confirmed in Figure 2 (C and D). The fractions from the column were checked for proteolytic activity that might be specific for RuBPCase as a substrate or that might hydrolyze azocasein or RuBPCase but not gelatin. Azocasein or RuBPCase was substituted for gelatin in the gelatin-agarose assay, and proteolytic activity in the column fractions was detected after electrophoresis. The substrates all gave similar enzyme patterns. However, gelatin was normally used in this assay because it required the least amount of assay time to see cleared zones.

After gel filtration, EP₁ and EP₂ could be separated readily by chromatography on DEAE-cellulose. EP₂ did not bind to a column equilibrated with 50 mM K-phosphate (pH 6.0) containing 1 mM DTT. EP₁, the major enzyme, did bind and could be eluted from the column with a 0.0- to 0.2-M NaCl gradient. At this stage of purification, native gel electrophoresis and gelatin-agarose detection of EP₂ activity indicated that the 50-fold purified enzyme still contained several contaminating inactive proteins but that it was homogeneous with regard to endoproteolytic activity. The mol wt of EP₂ was 67,000 ± 3,500, as determined by the gelatin-agarose assay in conjunction with the method of Hedrick and Smith (11). This enzyme was not purified further because of the very small quantities available. Native gel electrophoresis of the DEAE-purified EP₁ showed a single band of protein and only two very minor contaminating proteins. This indicated that EP₁ was essentially homogeneous. At this stage, EP₁ had been purified about 620-fold. This is comparable to the purification of about 530-fold and 630-fold, obtained, respectively, by Drivdahl and Thimann (6) and Frith et al. (9) and the 600-fold purification reported by Pike and Briggs (20). Determination of the location of EP₁ by the gelatin-agarose assay indicated that the DEAE preparation was homogeneous with regard to endoproteolytic activity. However, the major protein band and EP₁ were not identical (Fig. 3). Scanning of a polyacrylamide gel stained for protein (Fig. 3) showed that there was a protein present at the position of EP₁ but that it was only about 2% of the total protein present in the gel. Although the DEAE preparation appeared to be nearly homogeneous, this protein was not EP₁. It had a mol wt of about 65,000 ± 3,000, as determined by the method of Hedrick and Smith (11). The mol wt of EP₁ was 28,300 ± 2,000, determined by using the gelatin-agarose assay in conjuction with the method of Hedrick and Smith (11). On this basis, the major contaminating protein was removed by gel filtration on Sephadex G-75 superfine (Table I) while 80% of the endoproteolytic activity was recovered. EP₁ was purified about 5,800-fold. Because of the relatively low amounts of highly purified EP₁ available, many of the experiments performed in this study used the enzyme from the DEAE-cellulose stage of purification.

General Properties. EP₁ was very unstable for even short periods of time in the absence of reduced sulfhydryl reagents. These reagents were also required for enzymic activity. Maximum activity occurred at 0.25 mM DTT, with some inhibition above 1 mM DTT. Therefore, EP₁ was always stored in the presence of 1 mM DTT, and all reactions involving EP₁ included 1 mM DTT. EP₁

Table I. *Purification of EPs from 12-Day-Old Primary Barley Leaves*

Assay involved hydrolysis of azocasein at pH 5.7; 1 unit = 1.00 A_{340}/h.

Procedure	Units	Total Units	Protein	Units	Yield	Purification
	per ml		*mg/ml*	*mg protein*	%	*-fold*
Crude extract[a]	0.88	902.3	1.60	0.55	100	
70% (NH₄)₂SO₄ pellet after 20-h dialysis against 50 mM K-phosphate (pH 6.0), 2 mM DTT	13.68	636.1	2.89	4.73	71	9
G-100 chromatography with 50 mM K-phosphate (pH 6.0), 2 mM DTT						
EP₁	8.57	334.2	0.61	14.2	37	30[b]
EP₂	1.44	30.4	1.09	0.92	3	11[b]
DEAE chromatography with 50 mM K-phosphate (pH 6.0), 1 mM DTT wash; 0 to 0.2 M NaCl elution						
EP₁	12.3	246.40	0.043	286.5	27	615[b]
EP₂	0.66	14.2	0.176	3.75	2	46[b]
G-75 superfine chromatography with 50 mM K-phosphate (pH 6.0), 1 mM DTT						
EP₁	4.72		1.75[c]	2,697	1.6	5,800[b]

[a] K-phosphate (0.1 M, pH 6.0); 2 mM DTT, 1 mM EDTA; 0.1 g insoluble PVP/g fresh wt tissue.

[b] Based on analysis of G-100 chromatography, which suggests that EP₁ and EP₂ are roughly 85% and 15% of the total crude activity, respectively.
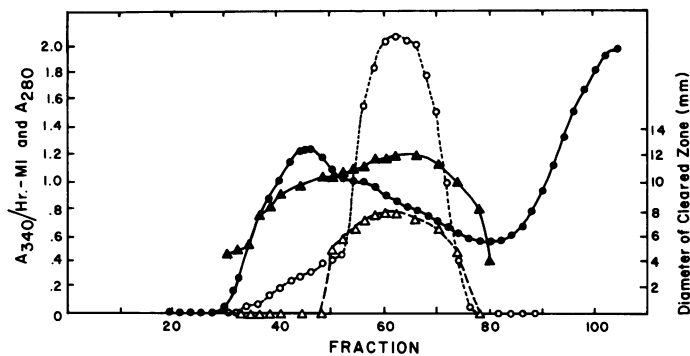
[c] Unit of measure, μg/ml.

FIG. 1. Chromatography of EPs on Sephadex G-100. EPs were separated by gel filtration on a Sephadex G-100 column. ●——●, A_{280}; O– – –O, azocasein, test-tube assay; ▲——▲, gelatin-agarose; △– – –△, RuBPCase-agarose, hydrolysis at pH 5.7.
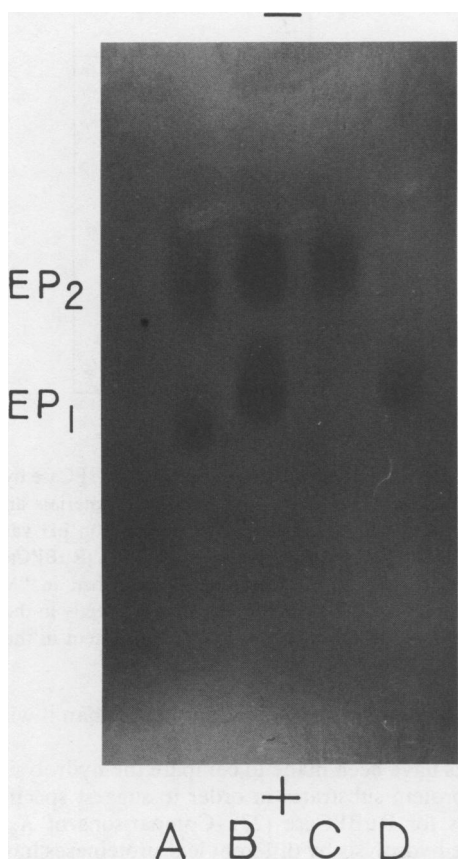

FIG. 2. Detection of electrophoretically separated EPs by the gelatin-agarose assay. Proteins were separated by Ornstein-Davis polyacrylamide disc electrophoresis. Tube gels were sliced longitudinally and placed on 1- to 2-mm layers of gelatin-agarose. A, 50 μl 70% $(NH_4)_2SO_4$ pellet after dialysis; B, 100 μl 70% $(NH_4)_2SO_4$ pellet after dialysis; C, 100 μl fraction No. 46 from G-100 chromatography showing EP_2; D, 100 μl fraction No. 64 from G-100 chromatography showing EP_1.

retained maximal stability at a pH between 5 and 7; instability began when the pH was approximately 7.5, and loss of activity was almost complete when the enzyme was stored at a pH below 4.0 or above 8.0. EP_1 was unstable also if preincubated at 40 C for longer than 10 min in the absence of an added protein substrate, perhaps because of autolysis.

EP_1 activity was unaffected by Ca^{2+} or Mg^{2+}, inhibited 50% by Mn^{2+}, and inhibited completely by Cu^{2+}, Zn^{2+}, or H^{2+} (Table II). Of the other compounds tested, EDTA, PMSF, STI, and Pep A
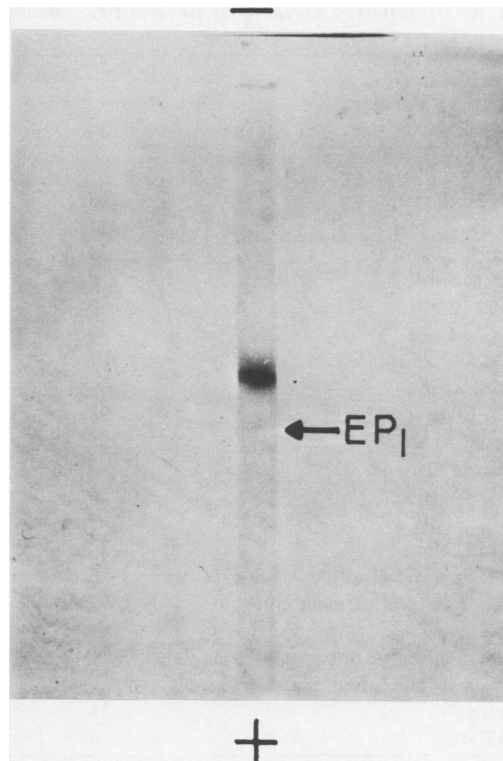

FIG. 3. Electrophoresis of EP_1 from the DEAE-cellulose chromatography stage. Proteins were separated by Ornstein-Davis polyacrylamide electrophoresis, and gels were stained for protein. Location of EP_1 was determined by the gelatin-agarose assay described in "Materials and Methods." This figure shows what appears to be a single homogeneous protein; it is actually a nonactive protein (not EP_1).

had no effect. Inhibition was 20% by pCMB and almost complete by PMA and IAA. Leupeptin, an inhibitor of thiol proteinases (2), completely destroyed EP_1 activity. These results suggest that EP_1 is a thiol proteinase.

Although EP_1 was quite labile in the absence of sulfhydryl reagents or when the pH was alkaline, pretreatment with 5 M urea did not affect its activity, and EP_1 still had 59% of the control activity if assayed in the presence of 5 M urea (Table II). Samples of EP_1 that had been made 4% SDS, boiled for 2 min, and assayed, retained 31% of the control activity (Table II).

EP_2 was the more stable of the two enzymes. It was stable if stored at a pH of 4 to 9 or in the absence of sulfhydryl reagents. This proteinase was stimulated 20 to 40% by EDTA, Ca^{2+}, and Mg^{2+} (Table II). Unlike EP_1, EP_2 was not inhibited by Cu^{2+} or Zn^{2+}. STI, Pep A, and leupeptin did not affect EP_2 activity, and DTT was not required for maximal EP_2 activity. In fact, DTT was slightly inhibitory at all concentrations used with 20% inhibition at 1 mM. Addition of 1 mM PMSF to the reaction mixture reduced EP_2 activity by 50%.

Profiles of pH Activity. Both EP_1 and EP_2 had pH optima of 5.5 to 5.7, whether the protein substrate was azocasein or [^{14}C]-RuBPCase (Fig. 4, A and B). There was a slight pH shift downward for EP_1 and a slight pH shift upward for EP_2 when RuBPCase, instead of azocasein, was the substrate. Shifts in the pH optima for different protein substrates have also been observed for proteinases of wheat leaves (19, 27).

Hydrolysis of Artificial Substrates. Various artificial substrates were hydrolyzed by both enzymes (Table III). Among carbobenzoxyamino acid-nitrophenyl esters, the tryptophan ester was hydrolyzed at the highest rate by EP_1, and the tyrosine ester was hydrolyzed at the highest rate by EP_2. EP_2, but not EP_1, rapidly hydrolyzed BTEE, and both enzymes had only low activity against

Table II. *Hydrolysis of Azocasein by EP_1 and EP_2: Inhibition and Activation*

Treatment[a]	Control[b]	
	EP$_1$	EP$_2$
	%	
CaCl$_2$, 10 mM	108	122
MgCl$_2$, 15 mM	111	136
MnCl$_2$, 10 mM	54	80
CuCl$_2$, 5 mM	0	116
ZnCl$_2$, 2 mM	0	106
HgCl$_2$, 1 mM	0	25
EDTA, 10 mM	107	130
PMSF, 1 mM	99	51
STI, 10 μM	98	96
Pep A, 10 μM	105	92
Leupeptin, 10 μM	0	94
IAA, 1 mM	20	39
PMA, 1 mM	2	
pCMB, 1 mM	80	
2,2'-Dipyridyl disulfide, 1.5 mM	0	
Urea, 5 M, (pretreatment only)	93	
Urea, 5 M	59	
SDS, 4%, (pretreatment only)	51	
SDS, 4%, (boil 2 min; pretreatment only)	31	
SDS, 4%	6	

[a] EP$_1$ and EP$_2$ were preincubated in the presence of the above concentrations of effectors for 5 h at 4 C.

[b] EP$_1$ control is 50 mM Mes (pH 6.0), 1 mM DTT, 4 mg/ml azocasein. No DTT is present in the assay for EP$_2$. Control activity is 286.5 A_{340}/h·mg for EP$_1$ and 3.75 A_{340}/h·mg for EP$_2$.

BAEE or BANA. The hydrolysis of these compounds by papain, another plant proteinase, and by trypsin, a nonplant proteinase, have been included for comparison.

Hydrolysis of Azocasein and RuBPCase. Both barley proteinases hydrolyzed casein, hemoglobin, myoglobin, azocasein, and RuBPCase (12). They also hydrolyzed BSA, but at very low rates. Casein, azocasein, myoglobin, and hemoglobin could be used as competitive inhibitors in assays in which RuBPCase was the substrate. However, the rate of [^{14}C]RuBPCase hydrolysis was not reduced if BSA was included, even at concentrations (mg/ml) as much as 2-fold greater than concentrations of [^{14}C]RuBPCase. BSA has often been added to extraction media to protect proteins of interest from attack by cellular proteinases. The above results indicate that BSA was not a good alternate substrate for barley leaf proteinases and may not provide its protective effect in this way.

The time course for hydrolysis of azocasein was linear for the first 1 to 2 h when either EP$_1$ (Fig. 5, A and B) or EP$_2$ was used. The assay was linear if hydrolysis was measured by the increase in A at 340 nm (Fig. 5A) or by the increase in α-amino nitrogen by the ninhydrin reaction (Fig. 5B). Both enzymes exhibited normal Michaelis-Menten kinetics and linear replots by Woolf-Augustinsson-Hofstee analysis (Fig. 6). EP$_1$ had a V_{max} of 325 A_{340}/h·mg, which was roughly 75 times greater than that of EP$_2$. The K_m values for azocasein were very similar: 0.47 mg/ml for EP$_1$ and 0.21 mg/ml for EP$_2$. Both enzymes were inhibited slightly when concentrations of azocasein were greater than 2 mg/ml.

At equal concentrations (mg/ml) of [^{14}C]RuBPCase and azocasein, RuBPCase was hydrolyzed by EP$_1$ or EP$_2$ 3 to 3.7 times faster than was azocasein, whereas, trypsin hydrolyzed the two substrates at nearly equal rates (Table III). These results, however, do not suggest any specificity of the barley leaf proteinases for RuBPCase, because papain, the papaya latex proteinase, will also
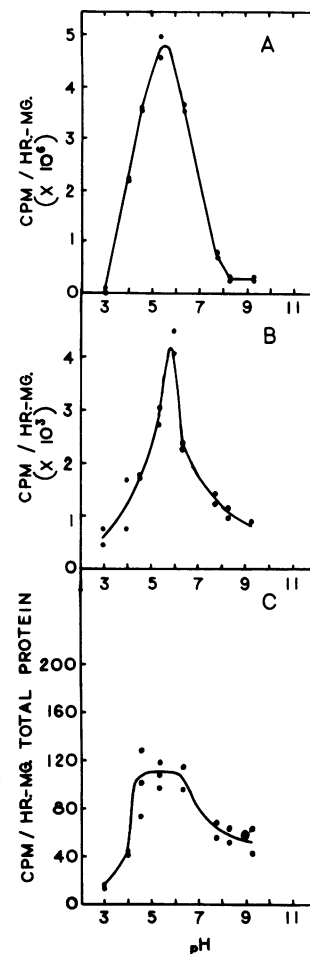

FIG. 4. pH Profile for the hydrolysis of [^{14}C]RuBPCase by leaf proteinases. The [^{14}C]RuBPCase assay, described in "Materials and Methods," was used for EP$_1$ and EP$_2$, except that the reaction pH varied. The pH profile for EP$_3$ was determined by incubating [^{14}C]RuBPCase at 40 C in the absence of any added proteinase, as described in "Materials and Methods." Reaction pH values were measured directly in the reaction test tubes. Rates for EP$_3$ are on the basis of total protein in the reaction. A, EP$_1$; B, EP$_2$; C, EP$_3$.

hydrolyze RuBPCase about 3.5 times faster than it will hydrolyze azocasein (Table III).

Attempts have been made to compare the hydrolysis kinetics of different protein substrates in order to suggest specificity of leaf proteinases for RuBPCase (27). Comparisons of K_m values for RuBPCase hydrolysis by different leaf proteinases have also been made (19). We attempted to make such comparisons with barley leaf proteinases. However, EP$_1$ and EP$_2$ behaved very differently when hydrolyzing [^{14}C]RuBPCase instead of azocasein. Repeated experiments showed that hydrolysis of RuBPCase by EP$_1$ was consistently biphasic (Fig. 7); both the ninhydrin and [^{14}C]RuBPCase assays gave the same results. The rate of hydrolysis of RuBPCase was nonlinear for the first 10 to 15 min, after which it was linear for the next 1 to 2 h. This biphasic nature was due not to EP$_1$, itself, but to a selective attack on the large subunit of RuBPCase (B. L. Miller and R. C. Huffaker, unpublished). As a result of this behavior, unusual kinetic analysis was obtained when the linear rates of the time course were used. A Michaelis-Menten plot of these linear rates showed no sign of saturation, even at levels of 4 to 5 mg/ml. These results appear quite different from those of Wittenbach (27) and Peoples *et al.* (19).

Thomas and Huffaker (25) recently reported that [^{14}C]RuBPCase, purified from either whole leaves or isolated chloroplasts,

Table III. *Hydrolysis of Various Substrates by EP_1 and EP_2*

All assays were run as described in "Materials and Methods."

Substrate	Assay[a]			
	EP_1	EP_2	Trypsin	Papain
	units/mg protein			
C-Tyr-N	197	141	1,246	362
C-Pro-N	47	9		
C-Try-N	663	10		
C-Phe-N	126	0		
BAEE	67	29	620	34
BTEE	0	363	24	0
BANA	83	9	21	126
Azocasein	4,037	53	9,095	3,210
^{14}C-RuBPCase	12,813	195	7,605	11,024
RuBPCase/azocasein	3.2	3.7	0.8	3.4

[a] Trypsin and papain are included for comparison. For carbobenzoxy-amino acyl-nitrophenyl esters, 1 unit = $1.00\ A_{320}$/h; for BAEE, 1 unit = $1.00\ A_{252}$/h; for BTEE, 1 unit = $1.00\ A_{256}$/h; for BANA, 1 unit = $1.00\ A_{335}$/h; for azocasein, 1 unit = 1.00 nmol α-N/h; and, for [^{14}C]RuBPCase, 1 unit = 1.00 nmol α-N/h.
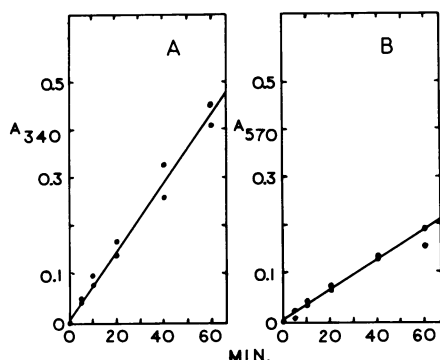

FIG. 5. Hydrolysis of azocasein by EP_1. The time course for the hydrolysis of azocasein was determined with the standard azocasein assay and compared with results obtained by measuring the increase in free amino groups using ninhydrin. A, Standard assay for azocasein; B, ninhydrin assay for azocasein.
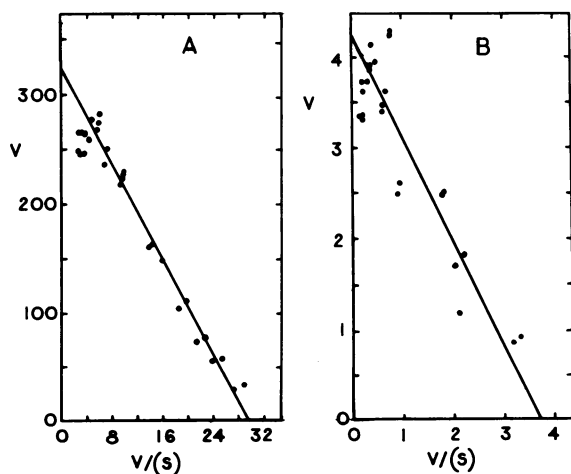

FIG. 6. Woolf-Augustinsson-Hofstee plots for the hydrolysis of azocasein by EP_1 and EP_2. The hydrolysis of azocasein was measured, as described in "Materials and Methods." Data were plotted as v *versus* v/(S). A, Hydrolysis by EP_1; V_{max} = 325, A_{340}/h·mg EP_1, K_m = 0.47 mg/ml. B, Hydrolysis by EP_2; V_{max} = 4.34, A_{340}/h·mg EP_2, K_m = 0.21 mg/ml.
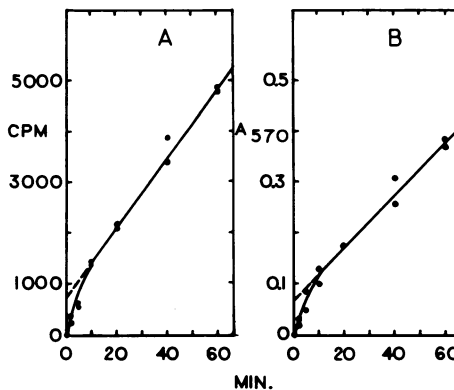

FIG. 7. Hydrolysis of [^{14}C]RuBPCase by EP_1. The time course for the hydrolysis of [^{14}C]RuBPCase was determined by measuring the increase in TCA-soluble radioactive counts or the increase in free amino groups using ninhydrin. A, Standard [^{14}C]RuBPCase assay; B, ninhydrin assay for [^{14}C]RuBPCase.

appeared to have an EP which remained closely associated with RuBPCase throughout purification. The present study shows that this enzyme is not one of the major proteinases, as measured by standard proteolytic assays. EP_3 was not detected during purification of leaf proteinases, because it releases TCA-soluble counts at very low rates. However, hydrolysis of [^{14}C]RuBPCase by EP_3 was quite significant when the reaction mixture was analyzed by SDS polyacrylamide electrophoresis. Most of the products formed during hydrolysis by EP_3 appeared to have mol wt larger than 5,000 and were, therefore, insoluble in 5% TCA (B. L. Miller and R. C. Huffaker, unpublished). This enzyme had a broad pH optimum that centered on 5.2 (Fig. 4C) and was not inhibited by leupeptin, PMSF, EDTA, or Pep A. This enzyme is being further characterized.

CONCLUSION

Three endoproteinases of senescing primary barley leaves have been described. A thiol proteinase contributes the majority of the *in vitro* activity measured. The second predominant proteinase is inhibited by PMSF but not by leupeptin or Pep A; its mechanism of action needs to be further defined. These same enzymes are the predominant proteinases found in both green and senescing leaves. It would be highly desirable to compare kinetic data for different substrates and use this information to demonstrate the role of an EP in normal cellular protein turnover or in the rapid loss of RuBPCase during senescence. However, caution must be used when trying to use information directly from the *in vitro* hydrolysis of protein substrates to ascertain the role of an EP *in vivo*. A protein may not behave as a uniform substrate, as evidenced by the hydrolysis of [^{14}C]RuBPCase by EP_1. Further, an EP that hydrolyzes a protein substrate *in vitro* may not do so *in vivo* because of differential compartmentation within the cell. Finally, as exemplified by EP_3, there may be endoproteinases present in the cell that hydrolyze proteins such as RuBPCase but that are not detected by standard proteolytic assays. Despite these difficulties, it will be of considerable interest to determine the roles that EP_1, EP_2, and EP_3 may play in the turnover of RuBPCase.

LITERATURE CITED

1. ANDERSON JW, KS ROWAN 1965 Activity of peptidase in tobacco-leaf tissue in relation to senescence. Biochem J 97: 741–746
2. BARRET AJ 1980 The many forms and functions of cellular proteinases. Fed Proc 39: 9–14
3. BARDFORD MM 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dye binding. Anal Biochem 72: 248–254
4. DALLING MJ, G BOLANTT, JH WILSON 1976 Relation between acid proteinase

activity and redistribution of nitrogen during grain development in wheat. Aust J Plant Physiol 3: 721–730

5. DAVIS BJ 1964 Disc electrophoresis. II. Method and application to human serum proteins. Ann NY Acad Sci 121: 404–427

6. DRIVDAHL RH, KV THIMANN 1978 The proteases of senescing oat leaves. II. Reaction to substrate and inhibitors. Purification and general properties. Plant Physiol 61: 501–505

7. FAIRBANKS G, JH STECK, DFB WALLACH 1971 Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. Biochemistry 10: 2606–2617

8. FELLER UK, J-SJ SOONG, RH HAGEMAN 1977 Proteolytic activities and leaf senescence during grain development of field grown corn (Zea mays L). Plant Physiol 59: 290–294

9. FRITH GJT, LB SWINDEN, MJ DALLING 1978 Proteolytic enzymes in green wheat leaves. II. Purification by affinity chromatography and some properties of some proteinases with acid pH optima. Plant Cell Physiol 19: 1029–1041

10. HAMPP R, DF DE FILIPPIS 1980 Plastid protease activity and prolamellar body transformation during greening. Plant Physiol 65: 663–668

11. HEDRICK JL, AJ SMITH 1968 Size and charge isomer separation and estimation of molecular weights by disc gel electrophoresis. Arch Biochem Biophys 126: 155–164

12. HUFFAKER RC, BL MILLER 1978 Reutilization of ribulose bisphosphate carboxylase. In HW Siegelmann, GH Hinds, eds, Photosynthetic Carbon Assimilation. Plenum Press, New York, pp 139–152

13. KLEINKOPF GE, RC HUFFAKER, A MATHESON 1970 A simplified purification and some properties of ribulose-1,5-diphosphate carboxylase from barley. Plant Physiol 46: 204–207

14. LAEMMLI UK 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T₄. Nature (Lond) 277: 680–685

15. LOWRY OH, NJ ROSEBROUGH, AL FARR, RJ RANDALL 1951 Protein measure-ment with the Folin phenol reagent. J Biol Chem 193: 265–275

16. MARTIN C, KV THIMANN 1972 The role of protein synthesis in the senescence of leaves. I. The formation of protease. Plant Physiol 49: 64–71

17. MOORE S 1968 Amino acid analysis: aqueous dimethyl sulfoxide as solvent for the ninhydrin reaction. J Biol Chem 243: 6281–6283

18. ORNSTEIN L 1964 Disc electrophoresis. I. Background and theory. Ann NY Acad Sci 121: 321–349

19. PEOPLES MB, HG FRITH, MG DALLING 1979 Proteolytic enzymes in green wheat leaves. IV. Degradation of ribulose bisphosphate carboxylase by acid proteases isolated by DEAE cellulose. Plant Cell Physiol 20: 253–258

20. PIKE CS, WR BRIGGS 1972 Partial purification and characterization of a phyto-chrome-degrading neutral protease from etiolated oat shoots. Plant Physiol 49: 521–530

21. RAGSTER L, MJ CHRISPEELS 1979 Azocoll-digesting proteinases in soybean leaves. Characteristics and changes during leaf maturation and senescence. Plant Physiol 74: 857–862

22. SANTARIUS K, C RYAN 1977 Radial diffusion as a sensitive method for screening endopeptidase activity in plant extracts. Anal Biochem 77: 1–9

23. STOREY R, L BEEVERS 1977 Proteolytic activity in relationship to senescence and cotyledonary development in Pisum sativum L. Planta 137: 37–41

24. THOMAS H 1978 Enzymes of nitrogen metabolism in detached leaves of Lolium temulentum during senescence. Planta 142: 161–169

25. THOMAS H, RC HUFFAKER 1981 Hydrolysis of labeled ribulose-1,5-bisphosphate carboxylase by an endopeptidase from the primary leaf of barley seedlings. Plant Sci Lett 20: 251–262

26. VAVREINOVA S, J TURKOVA 1975 SH-proteinases from bean Phaseolus vulgaris var Perlicka. Biochim Biophys Acta 403: 506–513

27. WITTENBACH VA 1978 Breakdown of ribulose bisphosphate carboxylase and changes in proteolytic activity during dark-induced senescence of wheat seed-lings. Plant Physiol 62: 604–608