Partial Purification and Characterization of Endoproteinases from Senescing Barley Leaves

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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 dithiorthreitol 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 dithiorthreitol and 50% by 1 millimolar phenylmethylsulfonyl fluoride. EP₂ contributed about 15% of the total proteolytic activity measured in vitro. Both proteinases hydrolyzed a variety of artificial and plant 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 EP₁s 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 sulphydryl-stimulated exoproteinase were purified from senescing oat leaves (6). Pike and Briggs (20) purified, from etiolated oat shoots, a proteinase that was stimulated by sulphydryl 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 EP, (DEAE preparation). The assay for EP₂ had 5 to 15 µg EP (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 EP, (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.

1Supported in part by National Science Foundation Grant NSF-AER 77-07301.
2Abbreviations: EP, endoproteinase; pCMB, p-chloromercuribenzoic acid; RuBPCase, ribulose-1,5-bisphosphate carboxylase; PMSF, phenylmethyl sulfonyl fluoride; 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, indoleacetic acid.
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\text{av}_0) - 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 carboxbenzoxamid acid-nitrophenyl ester substrates contained 80 mM K-phosphate (pH 6.0), 1 mM DTT (no DTT was included for the assay of EPs), and 0.2 mM substrate. Each assay included one of the following: 1 µg EP1, 4 µg EP3, 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 0.0- to 0.1 M solution of DTT, but EP1, and EP2 were incubated at 40°C for a given time and were then developed on a Sephadex G-75 column equilibrated with 50 mM K-phosphate (pH 6.0) 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-× 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, ~45 ml, were applied to a 2-× 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 4.091 × 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 Ormstein (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 EP1 and EP2 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. EP1 and EP2 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 EP4), 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. EP1 and EP2 were preincubated on ice for 17 h for the stability studies. All buffers were phosphate.

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 1).

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-purified proteins. The presence of EPs 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. EP1 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 EP1 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 conjunction 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.

Table 1. Purification of EPs from 12-Day-Old Primary Barley Leaves

<table>
<thead>
<tr>
<th>Assay involved hydrolysis of azocasein at pH 5.7; 1 unit = 1.00 A400/h.</th>
<th>Procedure</th>
<th>Units</th>
<th>Total</th>
<th>Protein</th>
<th>Protein</th>
<th>Yield</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>per ml</td>
<td>mg/ml</td>
<td>g protein</td>
<td>%</td>
<td>-fold</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crude extract*</td>
<td>0.88</td>
<td>902.3</td>
<td>1.60</td>
<td>0.55</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>70% (NH4)2SO4 pellet after 20h dialysis against 50 mM K-phosphate (pH 6.0), 2 mM DTT</td>
<td>13.68</td>
<td>636.1</td>
<td>2.89</td>
<td>4.73</td>
<td>71</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>G-100 chromatography with 50 mM K-phosphate (pH 6.0), 2 mM DTT</td>
<td>EP1</td>
<td>8.57</td>
<td>334.2</td>
<td>0.61</td>
<td>14.2</td>
<td>37</td>
<td>30a</td>
</tr>
<tr>
<td></td>
<td>EP2</td>
<td>1.44</td>
<td>30.4</td>
<td>1.09</td>
<td>0.92</td>
<td>3</td>
<td>11b</td>
</tr>
<tr>
<td>DEAE chromatography with 50 mM K-phosphate (pH 6.0), 1 mM DTT wash; 0 to 0.2 mM NaCl elution</td>
<td>EP1</td>
<td>12.3</td>
<td>246.40</td>
<td>0.043</td>
<td>286.5</td>
<td>27</td>
<td>615b</td>
</tr>
<tr>
<td></td>
<td>EP2</td>
<td>0.66</td>
<td>14.2</td>
<td>0.176</td>
<td>3.75</td>
<td>2</td>
<td>46b</td>
</tr>
<tr>
<td>G-75 superfine chromatography with 50 mM K-phosphate (pH 6.0), 1 mM DTT</td>
<td>EP1</td>
<td>4.72</td>
<td>1.75a</td>
<td>2,697</td>
<td>1.6</td>
<td>5,800b</td>
<td></td>
</tr>
</tbody>
</table>

* 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.
BARLEY LEAF ENDOPROTEINASES


Fig. 1. Chromatography of EPs on Sephadex G-100. EPs were separated by gel filtration on a Sephadex G-100 column. ○, $A_{280}$; □, $CQ$; △, 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% (NH₄)₂SO₄ pellet after dialysis; B, 100 μl 70% (NH₄)₂SO₄ pellet after dialysis; C, 100 μl fraction No. 46 from G-100 chromatography showing EP2; D, 100 μl fraction No. 64 from G-100 chromatography showing EP1.

Fig. 3. Electrophoresis of EP1 from the DEAE-cellulose chromatography stage. Proteins were separated by Ornstein-Davis polyacrylamide electrophoresis, and gels were stained for protein. Location of EP1 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 EP1).

had no effect. Inhibition was 20% by pCMB and almost complete by PMA and IAA. Leupeptin, an inhibitor of thiol proteinases (2), completely destroyed EP1 activity. These results suggest that EP1 is a thiol proteinase.

Although EP1 was quite labile in the absence of sulfhydryl reagents or when the pH was alkaline, pretreatment with 5 mM urea did not affect its activity, and EP1 still had 50% of the control activity if assayed in the presence of 5 mM urea (Table II). Samples of EP1 that had been made 4% SDS, boiled for 2 min, and assayed, retained 31% of the control activity (Table II).

EP2 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, Ca²⁺, and Mg²⁺ (Table II). Unlike EP1, EP2 was not inhibited by Cu²⁺ or Zn²⁺. STI, Pep A, and leupeptin did not affect EP2 activity, and DTT was not required for maximal EP2 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 EP2 activity by 50%.

Profiles of pH Activity. Both EP1 and EP2 had pH optima of 5.5 to 5.7, whether the protein substrate was azocasein or [¹⁴C]-RuBPCase (Fig. 4, A and B). There was a slight pH shift downward for EP1 and a slight pH shift upward for EP2 when RuBPCase, instead of azocasein, was the substrate. Shifts in the pH optima for different protein substrates have also been observed for proteasea of wheat leaves (19, 27).

Hydrolysis of Artificial Substrates. Various artificial substrates were hydrolyzed by both enzymes (Table III). Among carboxbenzoxamino acid-nitrophenyl esters, the tryptophan ester was hydrolyzed at the highest rate by EP1, and the tyrosine ester was hydrolyzed at the highest rate by EP2. EP3, but not EP1, rapidly hydrolyzed BTEE, and both enzymes had only low activity against...
Table II. Hydrolysis of Azocasein by EP₁ and EP₂: Inhibition and Activation

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>Controlb</th>
<th>EP₁</th>
<th>EP₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl₂, 10 mm</td>
<td>108</td>
<td>122</td>
<td></td>
</tr>
<tr>
<td>MgCl₂, 15 mm</td>
<td>111</td>
<td>136</td>
<td></td>
</tr>
<tr>
<td>MnCl₂, 10 mm</td>
<td>54</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>CuCl₂, 5 mm</td>
<td>0</td>
<td>116</td>
<td></td>
</tr>
<tr>
<td>ZnCl₂, 2 mm</td>
<td>0</td>
<td>106</td>
<td></td>
</tr>
<tr>
<td>HgCl₂, 1 mm</td>
<td>0</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>EDTA, 10 mm</td>
<td>107</td>
<td>130</td>
<td></td>
</tr>
<tr>
<td>PMSF, 1 mm</td>
<td>99</td>
<td>51</td>
<td></td>
</tr>
<tr>
<td>STI, 10 μM</td>
<td>98</td>
<td>96</td>
<td></td>
</tr>
<tr>
<td>Pep A, 10 μM</td>
<td>105</td>
<td>92</td>
<td></td>
</tr>
<tr>
<td>Leupeptin, 10 μM</td>
<td>0</td>
<td>94</td>
<td></td>
</tr>
<tr>
<td>IAA, 1 mM</td>
<td>20</td>
<td>39</td>
<td></td>
</tr>
<tr>
<td>PMA, 1 mM</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pCMB, 1 mM</td>
<td>80</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2,2'-Dipyridyl disulfide, 1.5 mm</td>
<td>0</td>
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</tr>
<tr>
<td>Urea, 5 mM (pretreatment only)</td>
<td>93</td>
<td></td>
<td></td>
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<tr>
<td>Urea, 5 mM</td>
<td>59</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SDS, 4%, (pretreatment only)</td>
<td>51</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SDS, 4%, (boil 2 min; pretreatment only)</td>
<td>31</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SDS, 4%</td>
<td>6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* EP₁ and EP₂ were preincubated in the presence of the above concentrations of effectors for 5 h at 4 °C.

b EP control is 50 mM MES (pH 6.0), 1 mM DTT, 4 mg/ml azocasein.

No DTT is present in the assay for EP₂. Control activity is 286.5 A₄₅₀/h·mg for EP₁ and 3.75 A₄₅₀/h·mg for EP₂.

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 [¹⁴C]RuBPCase hydrolysis was not reduced if BSA was included, even at concentrations (mg/ml) as much as 2-fold greater than concentrations of [¹⁴C]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 EP₁ (Fig. 5, A and B) or EP₂ 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 ninyhdrin reaction (Fig. 5B). Both enzymes exhibited normal Michaelis-Menten kinetics and linear replots by Woolf-Augustinsson-Hofstee analysis (Fig. 6). EP₁ had a Vₘₐₓ of 325 A₄₅₀/h·mg, which was roughly 75 times greater than that of EP₂. The Kₘ values for azocasein were very similar: 0.47 mg/ml for EP₁ and 0.21 mg/ml for EP₂. Both enzymes were inhibited slightly when concentrations of azocasein were greater than 2 mg/ml.

At equal concentrations (mg/ml) of [¹⁴C]RuBPCase and azocasein, RuBPCase was hydrolyzed by EP₁ or EP₂ 3 to 3.7 times faster than 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 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 Kₘ values for RuBPCase hydrolysis by different leaf proteinases have also been made (19). We attempted to make such comparisons with barley leaf proteinases. However, EP₁ and EP₂ behaved very differently when hydrolyzing [¹⁴C]RuBPCase instead of azocasein. Repeated experiments showed that hydrolysis of RuBPCase by EP₁ was consistently biphasic (Fig. 7); both the ninyhdrin and [¹⁴C]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 EP₁; 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 [¹⁴C]RuBPCase, purified from either whole leaves or isolated chloroplasts,
Table III. Hydrolysis of Various Substrates by EP<sub>1</sub> and EP<sub>2</sub>

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

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Assay*</th>
<th>Units/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EP&lt;sub&gt;1&lt;/sub&gt;</td>
<td>EP&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>C-Tyr-N</td>
<td>197</td>
<td>141</td>
</tr>
<tr>
<td>C-Pro-N</td>
<td>47</td>
<td>9</td>
</tr>
<tr>
<td>C-Try-N</td>
<td>663</td>
<td>10</td>
</tr>
<tr>
<td>C-Phe-N</td>
<td>126</td>
<td>0</td>
</tr>
<tr>
<td>BAEE</td>
<td>67</td>
<td>29</td>
</tr>
<tr>
<td>BTEE</td>
<td>0</td>
<td>363</td>
</tr>
<tr>
<td>BANA</td>
<td>83</td>
<td>9</td>
</tr>
<tr>
<td>Azocasein</td>
<td>4,037</td>
<td>53</td>
</tr>
<tr>
<td>14C-RuBPCase</td>
<td>12,813</td>
<td>195</td>
</tr>
<tr>
<td>RuBPCase/azocasein</td>
<td>3.2</td>
<td>3.7</td>
</tr>
</tbody>
</table>

* Trypsin and papain are included for comparison. For carboxbenzoyl-
amino acyl-nitrophenyl esters, 1 unit = 1.00 A<sub>280</sub>/h; for BAEE, 1 unit =
1.00 A<sub>405</sub>/h; for BTEE, 1 unit = 1.00 A<sub>405</sub>/h; for BANA, 1 unit = 1.00
A<sub>360</sub>/h; for azocasein, 1 unit = 1.00 nmol a-N/h; and, for 14C-RuBPCase,
1 unit = 1.00 nmol a-N/h.

Fig. 5. Hydrolysis of azocasein by EP<sub>1</sub>. 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, nin-
hydrin assay for azocasein.

Fig. 6. Woolf-Augustsson-Hofstee plots for the hydrolysis of azoca-
sein by EP<sub>1</sub> and EP<sub>2</sub>. The hydrolysis of azocasein was measured as
described in "Materials and Methods." Data were plotted as v versus v/
(S). A, Hydrolysis by EP<sub>1</sub>; V<sub>max</sub> = 325, A<sub>360</sub>h-mg EP<sub>1</sub>, K<sub>m</sub> = 0.47 mg/ml.
B, Hydrolysis by EP<sub>2</sub>; V<sub>max</sub> = 4.34, A<sub>360</sub>h-mg EP<sub>2</sub>, K<sub>m</sub> = 0.21 mg/ml.

Fig. 7. Hydrolysis of 14C[RuBPCase by EP<sub>1</sub>. The time course for the
hydrolysis of 14C[RuBPCase was determined by measuring the increase
in TCA-soluble radioactive counts or the increase in free amino groups
using ninhydrin. A, Standard 14C[RuBPCase assay; B, ninhydrin assay
for 14C[RuBPCase.

proteolytic assays. EP<sub>1</sub> was not detected during purifi-
cation of leaf proteinases, because it releases TCA-soluble counts
at very low rates. However, hydrolysis of 14C[RuBPCase by EP<sub>2</sub>
was quite significant when the reaction mixture was analyzed by
SDS polyacrylamide electrophoresis. Most of the products formed
during hydrolysis by EP<sub>2</sub> appeared to have mol wt larger than
7,600 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.

A protein may not behave as a uniform substrate, as evidenced by
the hydrolysis of 14C[RuBPCase by EP<sub>1</sub>. 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
eemplified by EP<sub>3</sub>, there may be endoproteinases present
in the cell that hydrolyze protein enzymes such as RuBPCase but that are
not detected by standard proteolytic assays. Despite these diffi-
culties, it will be of considerable interest to determine the roles
that EP<sub>1</sub>, EP<sub>2</sub>, and EP<sub>3</sub> may play in the turnover of RuBPCase.

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