Isolation and Partial Characterization of an Acid Endoprotease Present in Dormant Apple Shoot Bark

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ABSTRACT

A major protease present in dormant bark tissues of the apple (Malus domestica Borkh. cv. "Golden Delicious") was partially purified by hemoglobin-coupled Sepharose column chromatography. The protease active at pH 4.6 and at temperatures of 30 to 50°C was found to be sulfhydryl-dependent. Phenylmericarboxylate inhibited the enzyme approximately 50% at 2 mM, whereas phenylmethylsulfonyl fluoride inhibited less than 30% even at 10 mM. Substrate specificity and the separation of the enzyme reaction products indicated that the enzyme is likely to be an endoprotease. We suggest that the storage proteins in apple bark tissue undergo some modification prior to their eventual hydrolysis to amino acids, which requires a multi-enzyme system(s). Evidence is presented that the apple bark protein extracts enzymically release ninhydrin-positive compounds upon storage at 5°C. It is concluded that the activation of the sulfhydryl-dependent acid endoprotease is associated with the rapid metabolism of storage proteins which accompanies bud break upon regrowth.

It has recently been recognized that proteins in apple shoot bark are of prime importance in supplying nitrogen for early spring growth (17, 25, 26). However, the enzymatic breakdown of these proteins in the apple has not been documented. Tromp and Ovaa (25, 26) reported the spring mobilization of protein nitrogen in apple shoots, but did not study the enzymes involved. To understand the biochemical sequence of events involved in the annual cycling of nitrogenous compounds in the apple, we have reported the enzymic steps of nitrogen mobilization and conservation during annual senescence (11, 12). Here, we report the separation and a partial characterization of a sulfhydryl-dependent acid endoprotease present in dormant apple shoot bark.

MATERIALS AND METHODS

Plant Materials. One-year-old shoots were collected during the winter period of 1978 to 1979 from 6-year-old apple trees (Malus domestica Borkh. cv. "Golden Delicious" on M 26 rootstocks) grown in the orchard of the Department of Horticulture. All studies were conducted with the bark of the shoots. The shoot samples were used immediately after collection or stored at -20°C until used.

Preparation of the Affinity Column. The coupling of the denatured Hgb to CNBr-activated Sepharose 4B was based on the manufacturer's recommendations (20), with some modifications. Five g solid Sepharose 4B were extensively washed with 1 M HCl at room temperature. To the gel suspension was added the protein solution (0.2 g denatured Hgb dissolved in 20 ml of 0.1 M NaHCO₃ containing 0.5 M NaCl, pH 8.3). The mixture was allowed to stand overnight at 5°C with occasional stirring. The Hgb solution was then decanted. The residual active groups were blocked by suspending the gel in 200 ml of 1 M ethanolamine (pH 9.0), for 3 h at room temperature. The remaining uncoupled Hgb and the blocking agent were washed away with 0.1 M NaHCO₃ in a sintered glass funnel (medium porosity) with mild suction. The gel suspension was then washed alternately five times with 200 ml each 0.1 M NaHCO₃ (pH 8.3), and 0.1 M sodium acetate-acetic acid buffer (pH 4.0), both containing 0.5 M NaCl. After the final wash with the acetate buffer, the gel was thoroughly washed with 50 mM sodium phosphate buffer (pH 6.0). The gel was then packed into a column (1 x 14 cm) and washed until the wash showed no A at 280 nm. The column was carefully washed prior to each use.

Enzyme Isolation. The bark was separated from the shoot wood, cut up into small pieces with scissors, and weighed immediately. Twenty g bark were routinely used for enzyme extraction. The bark was extracted with 0.1 M sodium phosphate-citric acid buffer (pH 6.0), containing 0.4 M NaCl and 6 mM cysteine, using a 2:1 ratio of insoluble PVP to tissue. The PVP was hydrated overnight in the extraction medium prior to use. Extractions were carried out twice using a Brinkmann Polytron at a setting of 7 for 30 s until tissue disruption appeared to be complete. The homogenate was filtered through eight layers of cheesecloth and centrifuged at 17,000g for 30 min.

The supernatant was brought to 40% saturation with solid ammonium sulfate. The resultant suspension was allowed to stand for 1 to 2 h at 0°C. It was then centrifuged at 22,000g for 30 min. Preliminary experiments showed that less than 5% of the total protease activity was present in the pellet. The supernatant was brought to 75% saturation with solid ammonium sulfate, and the suspension was allowed to stand for 3 to 5 h at 0°C. The 40 to 75% fraction was resuspended in 7 ml of 50 mM sodium phosphate, (pH 6.0), and passed through a column of Sephacryl S-200 Superfine (2.5 x 60 cm) previously equilibrated with 25 mM sodium phosphate buffer (pH 6.0). The high mol wt effluent (55-65 ml) containing most of the protease activity was layered on the affinity column previously equilibrated with the same buffer as above. The bulk of the other proteins was eluted with the equilibrating buffer. When the A at 280 nm of the eluate had declined to a constant level, the reservoir buffer was replaced with 0.1 M acetic acid (pH 3.1) to release the proteases. The pH 3.1 eluate was quickly neutralized with 4 M sodium acetate and pooled.

Preparation of Substrates. Unless otherwise specified, 2 g denatured Hgb (Sigma) were dissolved in 100 ml water or 0.1 M sodium acetate buffer (pH 4.6), wherever appropriate. Azobivin (Sigma), RuBP carboxylase of Spinach (Sigma), casein (Miles Research), and BSA (General Biochem, fraction V) were dissolved.

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2 Abbreviations: Hgb, hemoglobin; RuBP, ribulose 1,5-bisphosphatase; Leu-NA, leucine nitrilamidase; Z-phe-al, carboxbenzoxycarbonylalanylala- nine; PMSF, phenylmethylsulfonyl fluoride; PMA, phenylmercuric acetate; Hgb-ase, hemoglobin-hydrolyzing enzyme; ABP-ase, apple bark protein-hydrolyzing enzyme.
in water to make a 2% solution and simply substituted for Hgb in the standard assays.

Leu-NA (28.8 mg) was dissolved in 1 ml dimethyl sulfoxide (3) and diluted to 50 ml with 25 mM sodium phosphate buffer, (pH 7.0). The final concentration was 2 mM. One ml of this solution was added to 3 ml reaction mixtures. Z-phe-alal was dissolved in 0.1 M potassium acetate buffer (pH 4.6) to a final concentration of 2 mM (27).

The apple bark protein was extracted as described above. To the supernatant was added concentrated NaOH for a final concentration of 2 M. The mixture was allowed to stand at room temperature for 2 to 3 h and then centrifuged at 17,000 g for 30 min. Concentrated trichloroacetic acid solution was added to the supernatant to give a final concentration of 20%. After centrifugation at 12,000 g for 20 min, the protein pellet was resuspended in a small volume of 0.1 M NaOH, containing 8 mM urea, and allowed to stand overnight at room temperature. The protein solution was then extensively dialyzed against water. The final concentration was adjusted to 10 mg/ml. No increase in the ninhydrin-positive compounds was observed when this protein solution alone was incubated for 3 h at 40 C.

**Enzyme assays.** Unless otherwise indicated, the standard reaction mixtures contained: 0.5 ml of 0.1 M sodium acetate buffer (pH 4.6), 0.2 ml of 2% Hgb dissolved in the assay buffer, and 0.2 ml enzyme preparation. Reactions were run at 40 C for 3 h and terminated by adding 0.1 ml of 50% trichloroacetic acid. Incubation mixtures containing only substrate or enzyme solution, or boiled enzyme (100 C for 5 min) in the same assay buffer, were similarly treated and analyzed to serve as controls. The protein precipitate was removed immediately by centrifugation in a Beckman Microfuge B for 3 min, and the ninhydrin-positive compounds were determined by the method of Moore and Stein (16).

To ensure measurement of protease activity under optimum conditions, assays were also conducted over different time intervals and with varying amounts of enzyme added to the same standard reaction mixtures.

Unless otherwise specified, protease activity is expressed as µg α-NH₂ released per h under the standard assay conditions as described above.

**Inhibitor Studies.** Appropriate amounts of PMSF and PMA were dissolved in 100% ethanol, keeping the final concentration of ethanol at 4.4%. Controls at this level of ethanol without the inhibitors showed no effect on protease activity. EDTA, 1.10 phenanthroline, and β-mercaptoethanol were dissolved in the assay buffer. The inhibitors were added to the enzyme solutions, and the mixtures were preincubated at 25 C for 30 min prior to the standard assays. Control preincubation mixtures contained equal amounts of the corresponding solvents.

**Breakdown of Apple Bark Proteins in Solution.** The crude protein extracts were stored at 5 C for 10 days, with or without prior boiling at 100 C for 5 min. Another batch of crude extracts was stored in the presence of 2 mM PMA added as an ethanolic solution. Changes in ninhydrin-positive compounds were measured after the indicated period of storage.

Immediately after extraction, an aliquot of the extracts was passed through a Sephacryl S-200 Superfine column (2.5 x 60 cm) equilibrated with 25 mM sodium phosphate buffer (pH 6.0). High mol wt proteins were easily separated from the ninhydrin-positive compounds. The proteins containing protease activity were stored at 5 C for 5 days and then rechromatographed on the same column under the same conditions. The reappearance of ninhydrin-positive compounds was taken to indicate the enzymic breakdown of apple bark proteins in these solutions.

**Protein Determination.** Two ml of crude extracts were mixed with 2 ml of 2 M NaOH, and allowed to stand for 1 to 2 h at room temperature. The insoluble material was removed by centrifugation. The NaOH solubilization step was omitted with the protein solutions containing little or no chromophores after the column chromatography. Three ml 50% trichloroacetic acid (w/v) were added to the supernatant. The protein pellet was resuspended in an appropriate volume of 0.1 M NaOH. Protein was then measured by the method of Lowry et al. (14) and/or by the dye-binding method of Bradford (1), using BSA as a standard.

The method of dye binding yielded significantly lower values than did the Lowry procedure. However, a consistent quantitative relationship existed between the values obtained by the two procedures. Therefore, the apparent protein content estimated by the dye-binding method was readily converted to equivalent values for the Lowry method.

**RESULTS AND DISCUSSION**

**Enzyme Purification.** The apple bark proteases were precipitated with 40 to 75% ammonium sulfate and partially purified by gel filtration and affinity chromatography (Table I). The loss of Hgb-ase activity occurred mainly in the ammonium sulfate fractionation step, which involved a 30% loss of proteolytic activity. After gel filtration, little loss of proteolytic activity was observed during the affinity column chromatography. A 40-fold purification was easily achieved with 65 to 70% recovery of the Hgb-ase activity. With the crude extracts, it was found that caution was necessary to measure the proteolytic activity because the background was high in ninhydrin assays, and the amount of ninhydrin-positive compounds gradually increased upon incubation with no substrate added.

**Figure 1** shows the elution profile of partially purified apple bark proteases on the affinity column. More than 90% of the protein was washed through the column with the equilibrating buffer at pH 6.0. The initial wash is termed the "void protein fraction". The proteases adsorbed onto the column were eluted by changing the elution buffer to 0.1 M acetic acid (pH 3.1). Less than 7% of the protein applied to the affinity column was collected in the protease fractions. The specific activity was constant among the fractions which contained Hgb-ase activity. Rapid neutralization was necessary to stabilize the enzymes. Disc gel electrophoretic separation of the protease-active proteins revealed at least six different protein species.

A small amount of Hgb-ase activity was observed in the void protein fractions (Fig. 1). This may be considered as proteases which have little or no biological activity with Hgb. No attempt was made to isolate the proteases detected in the void protein fractions. A similar phenomenon has been reported with germinated barley (2), oat leaves (4), and wheat leaves (8). Burger (2) reported that the amount of proteolytic activity detected in the void protein fraction was proportional to the amount of enzyme applied to the column.

**Characteristics of the Hgb-ase.** The Hgb-ase activity was directly proportional to the amount of enzyme added up to 100 µg protein in the standard reaction mixtures (data not shown). The enzyme exhibited a high temperature optimum of 30 to 50 C (data?)

**Table I. Purification of Proteases Extracted from Dormant Apple Shoot Bark**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume</th>
<th>Protein</th>
<th>Total Activity</th>
<th>Specific Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ml</td>
<td>mg/ml</td>
<td>units*</td>
<td>units/mg protein</td>
</tr>
<tr>
<td>Crude extract</td>
<td>283</td>
<td>1.1</td>
<td>338</td>
<td>11.1</td>
</tr>
<tr>
<td>40-75% (NH₄)₂SO₄</td>
<td>62</td>
<td>1.2</td>
<td>243</td>
<td>3.3</td>
</tr>
<tr>
<td>Affinity chromatography</td>
<td>28</td>
<td>0.2</td>
<td>222</td>
<td>39.6</td>
</tr>
</tbody>
</table>

* One unit of enzyme activity is defined as 1 µg α-NH₂ released/h under the standard assay conditions.
The proteolytic activity against denatured apple bark proteins (ABP-ase) showed maximal activity at pH 5.3, with a relatively broad range of pH from 4.6 to 5.4 (Fig. 2). The same pH profile was observed with the proteins denatured by boiling the crude extracts. Maximal proteolytic activity against native proteins has previously been shown to be around pH 5.0 (18, 19, 24).

In the pH profiles, two shoulders with Hgb-ase (pH values 4.0 and 5.6) and one shoulder with ABP-ase (pH 3.6) appeared to be present (Fig. 2). Similar results have been reported with the acid proteases of oat leaves (4) and wheat (18). The shoulder(s) and, especially with the ABP-ase, a relatively broad pH optimum below pH 5.6 may suggest the presence of more than one enzyme.

Substrate Specificity. The rate of proteolysis of Hgb, as measured by the release of ninhydrin-positive compounds, was always greater than that of the denatured bark proteins (Fig. 2). The differential sensitivity of the apple bark protease to different substrates is further shown in Table II. Casein, BSA, and azoalbumin were poorly attacked by the apple bark protease, whereas the rate of hydrolysis of RuBP carboxylase and denatured apple bark proteins was one-third that of Hgb. However, it is possible that the ABP-ase was not measured at its optimum conditions. This remains to be clarified.

The differential sensitivity of nonphysiological substrates has
Plantation of percent h. Inset rate assay. Hgb lII. been substrate loses teolytic Reports of wheat The plot. This be could Hgb the Although A between substrate relationship involved 2

\[
\text{FIG. 3. Relationship between substrate (Hgb) concentration and the rate of hydrolysis by the apple bark protease. Proteolytic activity was measured under the standard assay conditions, but at the indicated concentrations of Hgb. Reaction velocity is expressed as } \mu\text{g } \alpha\text{-NH}_2 \text{ released/h. Inset shows the double reciprocal plot of the saturation curve.}
\]

Table III. Effect of Various Compounds on the Activity of the Apple Bark Protease

<table>
<thead>
<tr>
<th>Compound</th>
<th>Residual Activity %</th>
<th>mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Na_2-EDTA (10)</td>
<td>100</td>
<td>10</td>
</tr>
<tr>
<td>1,10-Phenanathrine (5)</td>
<td>72</td>
<td>10</td>
</tr>
<tr>
<td>PMSF (10)</td>
<td>55</td>
<td>2</td>
</tr>
<tr>
<td>PMA (2)</td>
<td>138</td>
<td>1</td>
</tr>
</tbody>
</table>

been reported (18, 23, 24). If proteases possess protein specificity, as has been substantiated in some cases (21, 28), the choice of substrate may be critical for the detection of proteolytic activity in vitro. The best substrate might be a protein(s) which disappears (or loses activity) in the tissue during the time period in question. Reports on little correlation between protein depletion and proteolytic activity support this notion.

\[\text{Kinetics. Figure 3 shows the saturation curve of the Hgb-ase activity of apple bark extracts and includes a double reciprocal plot. The approximate } K_m \text{ value was 0.088% Hgb. Assuming the mol wt of Hgb is 68,000, 0.088% corresponds to 12.9 } \mu\text{M. The } K_m \text{ for Hgb of the apple bark protease is lower than that of oat leaf acid protease, 0.115% (5), but higher than that of the acid protease of wheat seedling, 0.026% (6). The } K_m \text{ for } N,N\text{-dimethyl Hgb of the acid protease from germinated barley was 0.064 to 0.067% (2). Although the pH profile (Fig. 2) indicated that the enzyme system could be heterogenous, the double reciprocal plot shows a linear relationship between substrate concentration and the reaction velocity. This suggests that any subspecies would have the same } K_m \text{. A study which is underway to clarify the number of enzymes involved in this system may solve this problem.}

\[\text{Evidence for an Endoprotease. An attempt was made to eluci-}

\[\text{date the action mechanism of the acid protease by employing Leu-
N} \text{A and Z-pha-ala as substrates for aminopeptidase and carbox-
ypeptidase, respectively. The reaction rates of the apple bark protease against these synthetic substrates were less than 10% of those against Hgb (data not shown). It is thus conceivable that the acid protease is an endoprotease. This conclusion is further sup-
ported by separating the enzymic reaction products against Hgb in a Sephacryl S-200 Superfine column. Heterogenous sizes of nitro-
genous compounds were produced by this enzyme reaction (data not shown). Therefore, it may be concluded that the hy-
drolysis of apple bark storage proteins to amino acids requires a multi-enzyme system(s). Once a substantial quantity of new ter-
mini has been generated by this major endoprotease, exoprote-
ase(s) may then become more important for the complete break-
down of storage proteins.}

\[\text{Effect of Metal Chelators and Inhibitors. The metal chelators}
EDTA and 1,10-phenanthroline did not affect the enzyme activity at 10 and 5 mM, respectively (Table III). Ten mM phenanthroline
created a 5% inhibition of the enzyme activity. It is thus unlikely
that the apple bark protease is a metalloprotease. The small inhibition may be attributable to an ionic strength effect. Aggre-
gation of proteins is often dependent on ionic strength (14), and
differences in enzyme activity might thus arise.

\[\text{PMSF inhibited the apple bark protease activity by 28% in the}
range from 5 to 10 mM. It is unlikely that the enzyme is a serine-
type. The concentration needed to cause less than 30% inhibition
appeared to be higher than would be expected. It was not possible
to obtain 50% inhibition within the limited range of solubility of
this compound. Furthermore, there is evidence that PMSF is not
as specific for serine protease as is sometimes supposed (5, 22, 29).
Instead, preincubation of the enzyme with PMA resulted in a 45%
inhibition of enzyme activity at 2 mM, indicating that free sulphy-
dryl groups participate in catalysis. The stimulation of the enzyme
activity in the presence of } \beta\text{-mercaptoethanol could support this conclusion.}

\[\text{Since there appeared to be more than one enzyme present, this}
would account for the partial inhibition observed in the presence

\[\text{FIG. 4. Evidence for proteolytic breakdown of apple bark proteins in}
solution. Crude extracts were stored at 5 C for 10 days, with (— —) or
without (—_—_) prior boiling at 100 C for 5 min. Another aliquot of
extract contained 2 mM PMA (—_—_). Ninhydrin-positive compounds
were measured at the indicated times. Results are expressed as percent
increase in } \mu\text{g } \alpha\text{-NH}_2 \text{ over day zero.}
of either PMSF or PMA. (The pH curve shows heterogeneity; it might be possible to detect differences in inhibitor sensitivity by repeating the experiment at different values.) The presence of substrate proteins during enzyme preparation, as well as conformational changes induced by substrate binding, could alter the accessibility of liable groups. The limited solubility of the mercurial compound in water could also limit its approach to the relatively hydrophilic centers of the proteins.

**Breakdown of Apple Bark Proteins in Solution.** Crude apple bark protein extracts containing active proteases produced increasing amounts of ninhydrin-positive compounds during storage at 5°C (Fig. 4). This increase in ninhydrin-positive compounds was found to be enzymic since it did not occur in boiled extracts. In nonboiled crude extracts in which proteases are in contact with their substrate proteins, a nearly linear increase was observed for 6 days, resulting in an approximately 60% increase in ninhydrin-positive compounds. The rate of increase leveled off after 2 weeks of storage. However, the addition of 2 mM PMA reduced the rate of increase by one-half. A 30% increase in ninhydrin-positive compounds was observed after 6 days of storage with no significant changes thereafter.

Further evidence for the enzymic breakdown of apple bark proteins in solution is presented in Figure 5. An aliquot of crude extract was chromatographed on a Sephacryl S-200 Superfine column. The Hgb-ase active protein and the fractions containing ninhydrin-positive compounds were separated (Fig 5A). The latter fractions were discarded. Only the protease-active protein fractions were pooled and stored for 5 days at 5°C. They were then rechromatographed on the same column (Fig. 5B). The protein content decreased nearly 35% during this storage period, with a concomitant decrease in proteolytic activity by about 40%. Ninhydrin-positive compounds reappeared after storage, the increase amounting to about 30% over the level at day zero. Since the total protein content apparently decreased in the crude extracts, and since the boiled extract did not exhibit an increase in ninhydrin-positive compounds, we concluded that the ninhydrin-positive compounds which increased upon storage were small peptides and amino acids enzymically generated from the proteins. These results indicate that the proteases degrade their native substrates in solution. It is also possible that the proteases may have undergone autolysis. Martin and Thimann (15) suggested that the rapid decrease in protease activity of oat leaves toward the end of senescence period resulted from self-digestion of the proteolytic enzymes. Drivdahl and Thimann (5) also presented evidence for *in vitro* autolysis of oat leaf proteases. If the autolytic breakdown of proteases occurred in the enzyme preparation, minor species of proteases could have been generated, thus affecting the pH dependence of the enzyme activity as previously discussed.

In addition to the problem of protease aggregation with other proteins, the enzymic breakdown of apple bark proteins in solution and the possible autolysis of proteases pose an important problem in protein purification and enzyme isolation. The entire purification procedure should be carried out as quickly as possible. In addition to handling extracts at low temperatures, the addition of protease inhibitors such as PMA to the extraction medium could prove helpful.

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**LITERATURE CITED**

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