Polyphenoloxidase in Higher Plants

IMMUNOLOGICAL DETECTION AND ANALYSIS OF IN VITRO TRANSLATION PRODUCTS

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WILLIAM H. FLURKEY
Department of Chemistry, Indiana State University, Terre Haute, Indiana 47809

ABSTRACT

Antibodies to broad bean polyphenoloxidase (PPO) were used to detect and demonstrate that the PPOs found in several different plants have many similarities in common. Crude extracts from leaves of broad bean, bush bean, lettuce, mung bean, pea, soybean, spinach, tobacco, and tomato contained enzyme which cross-reacted with polyclonal anti-PPO in Ouchterlony double diffusion analysis. The results suggested that plant polyphenoloxidase from a wide range of species may contain similar antigen determinants. Poly A+ mRNA was isolated from leaves of each plant species and translated in vitro using a rabbit reticulocyte translation system. An in vitro synthesized product corresponding to PPO from each species was identified after specific immunoprecipitation with anti-PPO. The molecular weight of this in vitro product was similar in all plants examined and found to be approximately 45 kilodaltons. Peptide maps of the in vitro synthesized product from all plant species were similar and showed at least three peptides in common. Plant PPOs may have more structural similarities than commonly thought in spite of the great variety in observed isoenzyme forms.

PPO (EC 1.14.18.1) has been studied extensively for many years with regard to its latency, substrate specificity, mol wt, subcellular location, and isoenzyme forms (16, 19, 26). Less information is available on the biosynthesis of this chloroplast-localized enzyme and on the immunological relationships among the isoenzyme forms found in many plant species. Isoenzyme forms have been suggested to arise from conformational changes (17), association-dissociation phenomena (13), covalent attachment of phenolic material (11), and possible attachment of carbohydrate (4, 7, 21). Many of the isoenzyme forms differ in mol wt but in recent years the subunit mol wt of PPO has been shown to be approximately 40 to 45 kD in spinach (10), pears (20), olives (3), Neurospora (16), Mucuna pruriens (27), and broad beans (9). Reports on the native mol wt of PPO vary considerably from source to source.

With the availability of antibodies to broad bean PPO (12) it became possible to identify a specific in vitro translation product corresponding to broad bean PPO. Flurkey (9) reported that the mol wt of the in vitro translation product corresponding to PPO was similar, if not identical, to the mol wt of the enzyme isolated from broad bean leaves. Vaughn and Duke (25, 26) have also reported that the mol wt of an unprocessed inactive PPO from tentoxin treated Vicia faba leaves was similar to the active enzyme found in the chloroplast. Thus, the subunit mol wt of broad bean PPO synthesized in vitro and in vivo appears to be similar and may be synthesized without a transit peptide. In this paper, we report that antibodies to broad bean PPO cross-react with PPO found in nine plant species and that an in vitro synthesized product corresponding to PPO can be identified from all plants tested. In addition, the mol wt of this in vitro product (45 kD) is approximately the same in all plant species examined. This is the first report comparing immunological cross-reactions and primary translation products of PPO found in higher plants.

MATERIALS AND METHODS

Plant Material. Broad bean (Vicia faba L. cv Long pod), bush beans (Phaseolus lunatus cv Blue Lake), lettuce (Lactuca sativa L. cv Salad bowl), mung bean (Vigna radiata L. Wilczek cv Berken), pea (Pisum sativum cv Snow peas), soybean (Glycine max L. Moensh), spinach (Spinacia oleracea L. cv Winter Bloomsdale), tobacco (Nicotiana tabacum L. cv Turkish), and tomato (Lycopersicum esculentum cv Big Boy) plants were grown in a local garden or under greenhouse conditions. A variety of leaf sizes, representing different leaf ages, was harvested for preparation of crude enzyme extracts and for isolation of RNA.

Extraction and Assay of PPO activity. Plant extracts were prepared by blending 3 g of leaf tissue in 25 ml of 20 mM sodium phosphate (pH 6.0) for 1 min at high speed in a Sorvall Omnimixer. The suspension was filtered through four layers of cheesecloth, assayed for PPO activity, and stored frozen at −20°C. For immunological assays, 5 ml of the crude homogenate was brought to 90% saturation with (NH₄)₂SO₄. After centrifugation, the precipitate was washed with cold acetone (−20°C) and resuspended in 0.5 to 1 ml of 20 mM sodium phosphate (pH 6.0). PPO activity in crude homogenates and precipitates was assayed using 2 ml of 50 mM sodium phosphate (pH 6.0) containing 10 mM catechol. Assays were performed in the presence or absence of 0.05% SDS (w/v) as described by Flurkey (9). One unit of enzyme activity was defined as a change of one absorbance unit per min at 410 nm and 25°C.

Electrophoresis. SDS PAGE was carried out as described by Angleton and Flurkey (1) using the method of Laemmli (14). All gels contained 10% sucrose (w/v) in the separating gel. Crude homogenate samples (10–50 µl) of the various plant extracts were mixed with glycerol and applied directly to polyacrylamide gels containing 0.1% SDS (w/v). Electrophoresis was carried out for 5 h at room temperature. Gels were stained for PPO activity in 100 ml of 20 mM sodium phosphate (pH 6.0) containing 2 mM L-β-3,4-dihydroxyphenylalanine (L-Dopa), 0.01% SDS (w/v), and 200 µl of catalase.

Ouchterlony Double Diffusion Analysis. Ouchterlony analysis was performed in 1% noble agar gels containing PBS on microscope slides. Anti-PPO (10 µl, 70 mg/ml) was placed in the center well and allowed to diffuse for 1 h before adding crude plant homogenates (10 µl) to outer wells. Anti-PPO was a gift from Dr. Bob Buchanan (Division of Plant Molecular Biology, University of California, Berkeley). The slides were incubated for 24 h at 25°C and then washed with repeated changes of PBS for 2 days.

1 Abbreviation: PPO, polyphenoloxidase.
and with water for 1 d. The agar gels were dried onto the microscope slides and stained for protein with 0.1% Coomassie blue R-250 in 10% acetic acid and 10% methanol. Gels were destained in 10% acetic acid and 10% methanol. Duplicate gels were stained for PPO activity as described above using t-Dopa.

**RNA Isolation and In Vitro Translations.** Total RNA from plant leaves was isolated by the method of Flurkey (9). Poly A+ mRNA was isolated following the method of Flurkey and Kolattukudy (8). Poly A+ mRNA (2 μg) from all plant species was translated in vitro using a rabbit reticulocyte lysate system (Promega Biotech, Madison, WI) following established procedures (8, 9). Each translation reaction mixture contained 50 μl total volume and 50 μCi of [35S]methionine (1000 Ci/ml). Specific translation products corresponding to PPO were immunoprecipitated with anti-PPO (7 μg) and Staphylococcus aureus cells as described previously by Flurkey (9). Control immunoprecipitations contained normal rabbit serum (BioRad Laboratories, Richmond, CA) in place of anti-PPO. After 90 min of incubation at 34°C, 1.5 μl of the translation mixture was removed for analysis of the total translation products. The remaining mixture was mixed with two volumes of 10% S. aureus cells for 1 h at room temperature. The suspension was centrifuged and the supernatant removed for immunoprecipitation of PPO. One-third of the supernatant was mixed with normal rabbit serum (2 μl) and two-thirds were mixed with anti-PPO. Each of the mixtures was incubated at 4°C overnight. On the following day, 15 μl of 10% S. aureus cells were added and the resulting suspension shaken for 1 h at room temperature. The immunoprecipitates were centrifuged and the pellets washed four times with phosphate buffer saline containing 1% Triton X-100 (v/v) and 1% sodium deoxycholate (w/v). All immunoprecipitates were boiled in a small volume of four-fold concentrated Laemmli sample buffer to dissociate antigen antibody complexes (9, 14). The in vitro translation products were analyzed by SDS-PAGE followed by autoradiography and fluorography (5, 9). Bands of radioactivity corresponding to in vitro synthesized PPO were excised from the gel, rehydrated in buffer, and subjected to limited proteolysis in 13% polyacrylamide gels as described by Cleveland et al. (6).

**RESULTS**

**PPO Activity in Plant Extracts.** PPO activity was detected in crude extracts of lettuce, mung bean, spinach, tobacco, and tomato when assayed in the absence of SDS (Table I). All plant extracts showed PPO activity when assayed in the presence of SDS although the levels of activity varied from source to source. Activation of latent PPO by SDS was detected in all sources except tomatoes. The level of activation by SDS varied among the species, but was greatest in broad bean extracts. Of the nine plant species the highest levels of PPO activity were found in broad bean, lettuce, and mung bean leaf extracts.

The number of isoenzyme forms of PPO in the various plant tissues was determined by electrophoresis in the presence of SDS (Fig. 1). Considerable variation was present in the intensity of enzyme stained bands, but the most intense enzyme staining was present in broad bean and lettuce extracts. Broad bean, lettuce, and soybean extracts showed one major isoenzyme form of PPO. Mung bean extracts were characterized by two bands of staining within a broad diffuse zone of enzyme staining. Bush bean extracts contained two bands of enzyme staining but the staining intensity was extremely weak. This may be due to low levels of PPO present in bush bean extracts (Table I). Extracts from spinach, tobacco, and tomato showed three to four isoenzyme forms with varying mobility. We have not been able to demonstrate PPO activity in pea extracts by this method, probably because of the very low levels of enzyme activity present in these extracts. Previous results have shown that the mobility of the active broad bean enzyme was approximately 45 kD under these conditions (9).

**Ouchterlony Analysis of Plant PPO.** Extracts from all nine plant species cross-reacted with anti-broad bean PPO in Ouchterlony double diffusion analysis (Fig. 2). Under a variety of conditions, PPO from bush beans, mung beans, soybeans, spinach, tobacco, and tomato showed stronger cross-reactions than PPO from peas or lettuce when gels were stained for protein. Fused arcs were present in all plant extracts except broad bean. Extracts from broad bean showed spurs and no fusion with extracts of bush beans, soybeans, pea, and tomato (Fig. 2, data not shown). Similar reactions were observed when the gels were stained for either protein or for enzyme activity (data not shown) and no cross-reactions were observed using normal rabbit serum in place of anti-PPO.

**Identification of in Vitro Synthesized PPO from Higher Plants.** To determine whether broad bean anti-PPO would recognize PPO synthesized in vitro, mRNA was isolated from all of the above plant species and translated in vitro. Figure 3A shows that a 45 kD protein was immunoprecipitated by anti-PPO from all plant species. This protein was not found in immunoprecipitations using a control antibody. The mol wt of this protein was approximately the same in all species except spinach and possibly tomato. Other proteins present were apparently due to nonspecific binding since they also appear in control immunoprecipitations with non-PPO antiserum. This same type of nonspecific binding was encountered in the identification of in vitro synthesized broad bean PPO (9). Anti-spinach Form X PPO (a gift from Bole Biehl, Botanical Institute, Technical University, Braunschweig, W. Germany) did not immunoprecipitate the in vitro spinach translation product as well as antibroad bean PPO.

At present we do not know the reason for this finding. Total translation products synthesized in vitro were analyzed to determine if one species produced significant levels of PPO (Fig. 3B). In all species examined, only very faint bands of radioactivity appear in the region of 45 kD which suggests that PPO does not account for a significant percentage of the total proteins translated.

Because Ouchterlony analysis suggested possible homology between the various plant PPO, the in vitro synthesized PPO from all plants was subjected to limited proteolysis (Fig. 4). Peptide maps of the in vitro synthesized products corresponding to PPO showed that all plant products contained at least three peptides in common. The mol wt of the three peptides was approximately 30, 18, and 15, respectively. The parent in vitro synthesized PPO product was also present and was approximately 45 kD. The in vitro synthesized spinach PPO appeared slightly larger in mol wt than the other plant PPO products. Very faint bands of radioactivity were present in bush bean, mung bean, and soybean samples but could not be photographed well. These faint bands were of similar mobility in the three species.

**DISCUSSION**

Considering the vast number of PPO isoenzyme forms reported in higher plants, it seems rather remarkable that all nine plants chosen in this study react with antibroad bean PPO.

Immunological comparisons among PPO in higher plants have not been reported although Lieberei et al. (18) have used anti-spinach PPO Form X to show that the spinach isoenzyme forms of PPO were immunologically related and not true isoenzymes. Vaughn and Duke (23-25) have also used antibroad bean PPO to localize PPO by immunocytochemistry and to show that tentoxin treated *Vicia faba* accumulates an inactive PPO at the chloroplast envelope. In the present study, Ouchterlony double diffusion analysis suggests that many plant species may have at least one antigenic determinant in common because of fused cross-reactions. A different antigenic site may be present in broad bean PPO because spurs were evident in cross-reactions between
bush bean (or soybean) and broad bean extracts. These results suggest that a highly conserved antigenic site(s) may be present in PPO found in a variety of plant species. This suggestion is supported by the findings of Lax et al. (15) who found a highly conserved isoenzyme form of PPO in species of tobacco which had an apparent mol wt of 48 kD.

Antibodies to broad bean PPO can be used to identify translation products corresponding to PPO from a variety of plant species. Although PPO appeared to account for only a small percentage of the total proteins translated, a specific product could be immunoprecipitated from the translation with anti-PPO. This product was observed in all plants examined and had a mol wt of approximately 45 kD. These results suggested that the subunit mol wt of PPO in higher plants was similar and approximated the reported subunit mol wt of PPO found in broad beans (9). Peptide maps of the in vitro synthesized PPO from the various plants were also similar. Staphylococcus aureus V₈ protease is specific for cleavage on the carboxyl side of aspartic and glutamic acid residues. Because the peptide cleavage patterns of in vitro synthesized PPO were similar, this suggested that specific regions in the protein structure of PPO may also be conserved in higher plant PPO. The cleavage patterns also indicated that methionine was present in only a few polypeptides after cleavage. Since plant PPO has been purified to homogeneity in only a few instances (3, 10, 20, 27), the in vitro synthesized PPO cannot be compared to the purified enzyme from each

Table 1. Polyphenoloxidase Activity in Plant Extracts

<table>
<thead>
<tr>
<th>Plant Source</th>
<th>Polyphenoloxidase Activity -SDS</th>
<th>+SDS</th>
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<tbody>
<tr>
<td></td>
<td>units/g fresh wt</td>
<td></td>
</tr>
<tr>
<td>Broad bean</td>
<td>0</td>
<td>69</td>
</tr>
<tr>
<td>Bush bean</td>
<td>0</td>
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<td>Mung bean</td>
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<td>180</td>
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<tr>
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<td>0.3</td>
</tr>
<tr>
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<td>2.0</td>
</tr>
<tr>
<td>Spinach</td>
<td>5.4</td>
<td>17</td>
</tr>
<tr>
<td>Tobacco</td>
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<td>2.0</td>
</tr>
<tr>
<td>Tomato</td>
<td>17</td>
<td>16</td>
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Fig. 1. Isoenzyme patterns of PPO separated by electrophoresis. Crude extracts (50 µl) from the various plant leaves were subjected to electrophoresis in the presence of 0.1% SDS in 8% polyacrylamide gels. The gels were stained for PPO activity using 2 mM L-Dopa in 20 mM phosphate buffer (pH 6.0) containing 0.02% SDS and 300 µl of catalase per 100 ml. Broad bean (V), bush bean (B), lettuce (L), mung bean (M), pea (P), soybean (So), spinach (Sp), tobacco (Tb), and tomato (Tm). Electrophoresis was from top to bottom. Arrows indicate very faint bands of enzyme staining.

Fig. 2. Ouchterlony double diffusion analysis of anti-PPO and PPO from crude plant extracts. Concentrated crude extracts (10 µl) from leaves of broad bean (V), bush beans (B), lettuce (L), mung bean (M), peas (P), soybeans (So), spinach (Sp), tobacco (Tb), and tomato (Tm) were placed in the outer wells. Antibroad bean PPO (10 µl; 70 mg/ml) was placed in the center well. Gels were incubated for 24 h at 25°C. The gels were dried and stained for protein in 0.1% Coomassie blue R:250, 10% acetic acid, and 10% methanol. Duplicate gels were stained for PPO activity using 2 mM L-Dopa in 20 mM phosphate (pH 6.0) containing 0.01% SDS.
Fig. 3. A, Immunoprecipitated products from translations primed with poly A+ from different plant species. Two µg of poly A+ mRNA was translated from leaves of broad bean (V), bush beans (B), lettuce (L), mung beans (M), peas (P), soybeans (So), spinach (Sp), tobacco (Tb), and tomatoes (Tm). Lanes a, c, e, g, i, k, m, p, and r contain translation products incubated with control rabbit serum (Bio-Rad Laboratories, Richmond, CA). Lanes b, d, f, h, j, l, n, q, and s contain translation products immunoprecipitated with broad bean anti-PPO (7 µg). Lane 0 contains translation products immunoprecipitated with anti-spinach PPO Form X (provided by B. Biehl, Botanical Institute, Technical University, Braunschweig, W. Germany). Translations immunoprecipitation, SDS PAGE, and autoradiography were carried out as described in “Materials and Methods.” X-ray films were exposed for 10 d. B, Autoradiogram of total translation products primed with poly A+ mRNA isolated from various plant leaves. Aliquots (1.5 µl) were removed from translations containing 2 µg of broad bean (V), bush bean (B), lettuce (L), mung bean (M), pea (P), soybean (So), spinach (Sp), tobacco (Tb), and tomato (Tm) poly A+ mRNA. The aliquots were boiled with 15 µl of 4X Laemmli sample buffer and subjected to SDS PAGE on 10% gels. Gels were treated with Amplify (Amersham Corporation, Arlington Heights, IL) dried, and x-ray films exposed for 7 d.

Fig. 4. Limited proteolysis of in vitro synthesized plant PPO. The 45 kD protein bands corresponding to various plant PPO in Figure 4 were excised from the gel and subjected to limited proteolysis using 0.1 µg V8 protease according to method of Cleveland (6). Samples from broad bean (V), bush bean (B), lettuce (L), mung bean (M), pea (P), soybean (So), spinach (Sp), tobacco (Tb), and tomato (Tm) were electrophoresed in 13% polyacrylamide gels. Autoradiography was carried out for 40 d. Arrows indicate presence of less intense radioactive bands.
plant source examined in this study. The mol wt of \emph{in vitro} synthesized spinach PPO reported in this study appeared to be slightly larger than the native mol wt of spinach PPO reported by Golbeck and Camarrata (10) and by Lieberei \emph{et al.} (18). Future studies are needed to confirm and establish the relationship between the mol wt of the \emph{in vitro} synthesized PPO and the isolated enzyme of each species.

The relationship between the mol wt of the \emph{in vitro} synthesized and isolated PPO needs to be established for several reasons. Vaughn and Duke (23, 25, 26) and Lax \emph{et al.} (15) have shown that the PPO is nuclear encoded and thus must be transported into the chloroplast. Many nuclear encoded chloroplast proteins have been shown to be synthesized as precursors and to contain a transit sequence. Flurkey (9) has reported that broad bean PPO may not contain a transit sequence since the mol wt of the \emph{in vitro} synthesized and isolated enzyme are similar (45 kD). In this study, the \emph{in vitro} synthesized PPO from all species was characterized with a mol wt of 45 kD and was similar to the \emph{in vitro} synthesized broad bean PPO. These results suggest, but do not prove, that PPO does not have a transit sequence in other plant species. If so, PPO would be one of the few examples of a nuclear encoded chloroplast protein synthesized without a transit sequence. Other considerations must include the possibility of PPO being modified posttranslationally. Broad bean, spinach, peach, and potato PPO have been reported to be associated with carbohydrates (2, 4, 7, 21). If PPO is glycosylated, then the apparent subunit mol wt of isolated PPO would appear to be larger than the unglycosylated subunit due to the added carbohydrate. Thus, the polypeptide portion subunit mol wt would be smaller than the \emph{in vitro} synthesized PPO.

The results presented in this report are difficult to reconcile with the report by Söderhäll \emph{et al.} (22) who isolated an inactive prophenoloxidase from plant cell cultures of \emph{Daucus carota}, \emph{Nicotiana tabacum}, and \emph{Haploppappus gracilis}. This proenzyme was converted to the active enzyme by high Ca\textsuperscript{2+} concentrations or by low concentrations of Ca\textsuperscript{2+} and trypsin addition. These authors also suggested that a plant protease may be responsible for activation of proPPO. Although the mol wt of the proenzyme and active enzyme was not determined, their results suggested that PPO might be synthesized as a larger preproenzyme and undergo processing events to yield the active enzyme. The phenoloxidase isolated by Söderhäll \emph{et al.} (22) may be another example of the latent PPO often found in higher plants. Because latent PPO can be activated without an apparent change in the mol wt, a precursor/product relationship would not exist as suggested by these authors. The difference between their results and those presented in this report, however, require future evaluation.

In conclusion, antibodies to broad bean PPO have been used to identify the enzyme found in higher plants. All plant species examined cross-reacted with anti-PPO and an \emph{in vitro} synthesized PPO from each species was identified. The mol wt of this \emph{in vitro} synthesized PPO was similar in size and in peptides obtained after limited proteolysis. PPOs in higher plants may have more structural similarities in common than previously thought.

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