Homology of Plant Peroxidases
AN IMMUNOCHEMICAL APPROACH

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ABSTRACT
Antisera specific for the basic peroxidase from horseradish (Armoracia rusticana) were used to examine homology among horseradish peroxidase isoenzymes and among basic peroxidases from root plants. The antisera cross-reacted with all tested isoperoxidases when measured by both agar diffusion and quantitative precipitin reactions. Precipitin analyses provided quantitative measurements of homology among these plant peroxidases. The basic radish (Raphanus sativus L. cv. Cherry Belle) peroxidase had a high degree of homology (73 to 81%) with the basic peroxidase from horseradish. Turnip (Brassica rapa L. cv. Purple White Top Globe) and carrot (Daucus carota L. cv. Danvers) basic peroxidases showed less cross-reaction (49 to 54% and 41 to 46%, respectively). However, the cross-reactions of antisera with basic peroxidases from different plants were greater than were those observed with acidic horseradish isoenzymes (30 to 35%). These experiments suggest that basic peroxidase isoenzymes are strongly preserved during evolution and may indicate that the basic peroxidases catalyze reactions involved in specialized cellular functions. Anticatalytic assays were poor indicators of homology. Even though homology among isoperoxidases was detected by other immunological methods, antibodies inhibited only the catalytic activity of the basic peroxidase from radish.

Plant peroxidases (EC 1.11.1.7) are important in diverse cellular functions such as lignin biosynthesis, hormone generation, and detoxification of hydrogen peroxide. These enzymes are glycoproteins composed of a single polypeptide chain, and they contain ferrirprotoporphyrin IX as a prosthetic group. Each plant has numerous peroxidase isoenzymes that differ in substrate specificity and localization within the plant. In addition, isoperoxidases within some species and those from different species exhibit size heterogeneity. Isoenzyme mol wt range from 30,000 to 50,000 daltons.

Comparative studies of root plant peroxidases have been performed with horseradish (12) and turnip (15) isoenzymes by peptide mapping. These studies provide only a qualitative estimate of homology between peroxidase isoenzymes. Primary structure comparisons have not been made, because only a basic isoenzyme from horseradish has been completely sequenced. Other studies of homology among peroxidases have focused on catalytic and physicochemical properties of the isoenzymes (11). These probes have not yielded quantitative estimates of homologies among root plant isoperoxidases.

The purpose of the present study was to determine the relationships among horseradish peroxidases and basic isoperoxidases from a number of root plants by immunological methods. Antibody specific for a basic isoenzyme of horseradish was used as a probe of homology among these plant peroxidases. Immunological methods were chosen, because these techniques provide quantitative measurements of homology and their application is simple.

MATERIALS AND METHODS
HORSEARDISH PEROXIDASES. Horseradish peroxidase isoenzymes were purchased from Sigma Chemical Co. Two acidic isoenzymes (types VII and VIII) and a single basic isoenzyme (type IX) were homogeneous when assessed by SDS-PAGE (14) and were used without further purification. Isoelectric points for each isoenzyme were determined by focusing in a polyacrylamide gel containing ampholytes that gave a pH 3 to pH 10 gradient (10). Enzyme concentrations of horseradish peroxidase solutions were determined spectrophotometrically. The A of a 1 mg/ml solution at 403 nm is 2.5 (13). Kinetic constants for horseradish peroxidases were determined by the method of Lineweaver and Burk (3).

PEROXIDASES FROM OTHER ROOT PLANTS. Basic peroxidase isoenzymes were purified from 1 kg each of turnip (Brassica rapa L., cv. Purple White Top Globe), radish (Raphanus sativus L., cv. Cherry Belle), and carrot (Daucus carota L., cv. Danvers). The roots were washed, sliced, and homogenized with 1 L of distilled H2O in a Waring Blender. Homogenates were then filtered through four thicknesses of cheesecloth and Whatman No. 3 filter paper. The filtered aqueous extracts were brought to 50% saturation with solid ammonium sulfate and centrifuged at 4,000g for 20 min. After the pellets were discarded, the supernatant fluids were brought to 85% saturation with ammonium sulfate and centrifuged for 20 min at 4,000g. The 4,000g pellets were suspended in 5 mm sodium phosphate (pH 8.0), dialyzed extensively against the same buffer, and placed on DEAE-cellulose columns (2.5 × 40 cm) equilibrated in the 5 mm sodium phosphate buffer. Flowthrough fractions were pooled, concentrated by ultrafiltration (Amicon PM-10 membrane), and placed on Sephadex G-100 columns (2 × 90 cm) equilibrated in 50 mm sodium phosphate (pH 7.5) containing 0.15 M NaCl. Fractions with peroxidase activity were pooled, concentrated by ultrafiltration, and used without further purification. The mol wt and homogeneity of the final products were assessed by SDS-PAGE.

MEASUREMENT OF ENZYME ACTIVITY. The reaction used to monitor the peroxidase activity of all isoenzymes was reduction of H2O2 (Merck) by the electron donor o-dianisidine (Sigma). Reaction velocities were determined spectrophotometrically by measuring the appearance of oxidized dianisidine at 460 nm (16). Activity measurements were done at room temperature in 0.1 M citrate buffer (pH 5.3).

ANTISERUM PRODUCTION. New Zealand White rabbits were immunized biweekly with 1 mg of the basic horseradish isoenzyme. For immunization, an aqueous solution of the basic isoenzyme (1

1 Abbreviation: SDS-PAGE, SDS-polyacrylamide gel electrophoresis.
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mg in 1 ml) was emulsified with an equal volume of Complete Freund's adjuvant (Difco Laboratories). Inoculations were made subcutaneously at several sites on the animal. Bleedings were taken from the marginal ear vein 10 days after each inoculation. Antisera are identified by animal number followed by the bleeding number.

Agar Diffusion Analyses. Double diffusion in agar analyses were performed as described by Ouchterlony (8). Peroxidases and antibody diffused toward one another at room temperature in a 1% agarose medium containing 0.15 M NaCl.

Precipitin Tests. Antiserum and varied amounts of plant peroxidases were incubated for 48 h at 47°C. The immune precipitates that formed were collected by centrifugation, washed twice with 0.9% (w/v) NaCl, dissolved by adding 0.1 ml 0.1 M NaOH, and brought to a final volume of 0.5 ml with water. The antibody content of the immune precipitates was determined spectrophotometrically using the E1%0 value of 13.6 for rabbit IgG (1) after correcting for the A of plant peroxidases at 280 nm. Determinations of the cross reactivity of the antiserum were made by comparing the maximum amount of antibody precipitated by a given isoenzyme to the greatest quantity of antibody precipitated by the basic horseradish isoenzyme.

Anticatalytic Assays. Inhibition of peroxidase activity by antibody was determined by the method of Marucci (5). Various amounts of antibody were incubated with isoenzymes (80 ng) for 16 h at 4°C. The residual peroxidase activity of the isoenzyme-antibody mixtures was then determined. Inhibition was calculated by comparing the catalytic activity of enzyme in the presence of antibody to a reference standard containing enzyme alone.

RESULTS

Characterization of Horseradish Peroxidase Isoenzymes. Some properties of the horseradish peroxidases used in this study are summarized in Table I. The basic isoperoxidase has greater affinity for hydrogen peroxide and o-dianisidine, as measured by apparent Michaelis constants. However, the acidic peroxidases exhibit maximum velocities 2.5 to 4 times greater than those of the basic peroxidase. On the basis of their isoelectric points and catalytic properties, types VII and VIII peroxidase correspond to group A or group I in the classification systems of a number of workers (2, 9, 12). In these same classification systems, the type IX peroxidase is a member of groups C, III, or IV.

Characterization of the Basic Peroxidases from Other Root Plants. The final yields of basic peroxidases from 1 kg each of radish, turnip, and carrot roots were 2.6 mg, 1.8 mg, and 0.3 mg, respectively. In SDS-PAGE, the final products contained only minor contaminants. Some physicochemical properties of the basic peroxidases are summarized in Table II. By mol wt comparison, the basic turnip peroxidase corresponds to isoenzyme P7 (6). By the same criterion, the radish enzyme was identified as isoperoxidase C (12). Both radish and turnip basic peroxidase preparations were more than 85% pure, as determined by comparison with published A ratio values (6, 7). Because carrot isoperoxidases have not been characterized, neither the identification nor the estimation of purity of the basic isoenzyme could be performed.

Relationships among Horseradish Peroxidases. Ouchterlony double diffusion in agar analysis (Fig. 1) revealed that antibody specific for the basic horseradish peroxidase cross-reacts with both acidic isoenzymes. Both acidic isoenzymes gave reactions of partial identity with the basic isoenzyme. This result indicates that the acidic isoenzymes share some, but not all, antigenic determinants of the basic horseradish peroxidase. In addition, the acidic isoenzymes gave a reaction of identity with one another, i.e. antibody specific for the basic isoenzyme can not distinguish the acidic horseradish peroxidases. The reaction of identity indicates that the cross-reacting sites are common to both acidic peroxidases.

Precipitin tests and anticatalytic assays were then performed to quantify the extent of cross-reaction. The results of precipitin analyses (Table III) show that 30 to 40% of the antibodies specific for the basic isoenzyme reacted with either acidic peroxidase. This result confirms that acidic peroxidases could not be distinguished by rabbit antisera specific for the basic isoenzyme. Anticatalytic assays, however, failed to show any homologies between horseradish peroxidases. While three antisera maximally inhibited basic isoenzyme activity 80%, 64%, and 40%, respectively, neither of the acidic peroxidase activities were affected by combination with antisera. Immunoprecipitation experiments were also performed to determine whether the antibodies inhibiting basic peroxidase activity could be removed from antisera by reaction with acidic peroxidases. The results indicated that antisera absorbed of antibodies reactive with acidic peroxidases retained the ability to inhibit the basic isoenzyme (not shown). The acidic isoenzymes do not have the antigenic determinants of the basic peroxidase that bind anticatalytic antibodies.

Relationships among Basic Isoperoxidases. Double diffusion in agar analyses (not shown) was used to establish that antisera to the basic horseradish peroxidase cross-react with basic peroxidases from radish, turnip, and carrot. Precipitin analyses were then performed to obtain a quantitative measure of the relationships among these basic isoperoxidases. The data from the precipitin analyses (Table IV) revealed that the radish enzyme was most closely related to the basic horseradish peroxidase. Turnip and carrot peroxidases, respectively, are more distant. However, in all cases, the cross-reactions observed among basic peroxidases from different plants were greater than the cross-reactions observed with acidic horseradish isoenzymes.

Anticatalytic assays were also performed to determine whether antibody decreased the enzymic activity of these peroxidases. The results of anticatalytic assays performed with antisera 4-4 are shown in Figure 2. Similar results were obtained with other antisera. Antisera 2-4, 3-4, and 4-4 maximally inhibited the basic horseradish isoperoxidase activity 64%, 40%, and 77%, respectively. The same antisera decreased the radish enzyme activity by 50%, 31%, and 49%. No inhibition of turnip or carrot peroxidases by any antisera was found.

Table I. Properties of Horseradish Peroxidase Isoenzymes

<table>
<thead>
<tr>
<th>Isoenzyme</th>
<th>pl</th>
<th>Km (o-dianisidine)</th>
<th>Km (H2O2)</th>
<th>Vmax</th>
</tr>
</thead>
<tbody>
<tr>
<td>VII</td>
<td>3.5</td>
<td>3.60</td>
<td>6.90</td>
<td>2.50</td>
</tr>
<tr>
<td>VIII</td>
<td>4.2</td>
<td>0.14</td>
<td>1.60</td>
<td>1.50</td>
</tr>
<tr>
<td>IX</td>
<td>9.0</td>
<td>0.04</td>
<td>0.30</td>
<td>0.65</td>
</tr>
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</table>

Table II. Physicochemical Properties of Basic Isoperoxidases

<table>
<thead>
<tr>
<th>Plant</th>
<th>A40S/A400</th>
<th>Mol Wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Radish</td>
<td>2.90</td>
<td>32,000</td>
</tr>
<tr>
<td>Turnip</td>
<td>2.64</td>
<td>35,000</td>
</tr>
<tr>
<td>Carrot</td>
<td>2.19</td>
<td>40,000</td>
</tr>
</tbody>
</table>

DISCUSSION

Precipitin analyses revealed that the extent of homologies among basic peroxidases from root plants parallels the taxonomic distance between these plants. The greatest cross-reactions of antibody specific for the basic horseradish peroxidase occurs with...
FIG. 1. Double diffusion in agar analysis of horseradish peroxidases. The outer wells contain 5 µg each of horseradish peroxidases. The center well contains antiserum (3-4) specific for type IX peroxidase.

Table III. Cross-Reactions among Horseradish Peroxidases
These data were determined by quantitative precipitin analyses.

<table>
<thead>
<tr>
<th>Isoenzyme</th>
<th>Cross-Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>VII</td>
</tr>
<tr>
<td>Antiserum</td>
<td></td>
</tr>
<tr>
<td>2-4</td>
<td>34.4</td>
</tr>
<tr>
<td>3-4</td>
<td>32.3</td>
</tr>
<tr>
<td>4-4</td>
<td>34.8</td>
</tr>
</tbody>
</table>

Table IV. Cross-Reactions among Basic Peroxidase Isoenzymes

<table>
<thead>
<tr>
<th>Plant</th>
<th>Cross-Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Horseradish</td>
</tr>
<tr>
<td>Antiserum</td>
<td></td>
</tr>
<tr>
<td>2-4</td>
<td>100</td>
</tr>
<tr>
<td>3-4</td>
<td>100</td>
</tr>
<tr>
<td>4-4</td>
<td>100</td>
</tr>
</tbody>
</table>

The enzyme from the radish (77%) and turnip (49%) enzymes. The enzyme from the taxonomically distant carrot cross-reacts to a lesser extent (41%). However, in all cases, greater homology was observed among basic peroxidases from different plants than among horseradish isoperoxidases. This unexpected result suggests strong evolutionary conservation of basic peroxidases, which may be related to a specialized function of these isoenzymes in the plant. Liu (4) has proposed that basic peroxidases are lignin polymerases because of their cell membrane association. An alternative interpretation of the homology of basic peroxidases from different species is that the immunological methods measure enzyme sites that are critical in cell membrane association.

Precipitin analyses provide a more accurate estimate of homology among plant peroxidases than do anticatalytic assays. Anticatalytic assays by themselves are poor indicators of homology, inasmuch as only the radish enzyme activity was inhibited after incubation with antisera. Despite the cross-reactions observed in precipitin analyses, neither the acidic horseradish peroxidases nor the basic isoenzymes from turnip or carrot were inhibited by antisera. Precipitin reactions give more reliable indications of homology, because these techniques detect antibodies binding to all antigenic sites of the isoenzyme. Anticatalytic assays, on the other hand, measure only those antibodies in the total antiperoxidase population that are capable of mediating inhibition by
binding to restricted enzyme sites.

Measurements of homology among horseradish peroxidases by immunological methods are in agreement with estimates obtained by other methods. By comparison of amino acid compositions and peptide maps, Shih et al. (12) concluded that acidic horseradish peroxidase isoenzymes were closely related. Our study confirms the similarities between the acidic isoenzymes, inasmuch as rabbit antiserum against the basic isoenzyme did not distinguish the acidic isoperoxidases. The immunological methods indicate that the acidic isoenzymes share common antigenic sites. However, the results do not constitute a determination of immunological identity between the acidic isoperoxidases. Each acidic isoenzyme may have additional unique antigenic sites that are not recognized by our antiserum. Shih et al. (12) also reported that the basic horseradish peroxidase differs from the acidic isoenzymes in amino acid composition and peptide maps. The decreased reactivity of antiserum prepared against the basic isoenzyme with the acidic horseradish peroxidases supports their conclusion.

Our data also provide indirect evidence that antibodies inhibit-

ing the catalytic activity of the basic horseradish peroxidase do not combine with the enzyme-active site. Inasmuch as basic peroxidases may catalyze specialized cellular functions, strong conservation of enzyme-active sites would be expected. If inhibitory antibody combined with the active site of the basic horseradish peroxidase, antiserum should decrease the catalytic activity of all basic peroxidases. The reduced inhibition of the radish peroxidase and the lack of inhibition of the turnip and carrot enzyme suggest that anticatalytic antibodies bind to a poorly conserved site, i.e., a site other than the catalytic center.

Further experiments to determine homologies among acidic peroxidase isoenzymes from a number of plants using antiserum prepared against an acidic horseradish peroxidase are now in progress. These studies may help in the understanding of the functions performed by peroxidase isoenzymes in plants.

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