Purification and Developmental Analysis of the Major Anionic Peroxidase from the Seed Coat of Glycine max

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ABSTRACT

We show that the majority of peroxidase activity in soybean (Glycine max var Williams 82) seeds is localized to the seed coat. A single isozyme is responsible for this activity and has been purified to electrophoretic homogeneity by successive chromatography on DEAE Sepharose Fast Flow, concanavalin A-Sepharose, and Sephadex G-75. The peroxidase exhibits a pI of 4.1, an apparent molecular mass of 37 kilodaltons, and has properties characteristic of a glycoprotein. The enzyme begins to accumulate approximately 21 days after anthesis and continues to do so throughout the maturation of the seed coat where it can represent at least 5% of the soluble protein in dry seed coats. Due to its localization in the seed, we propose that this isozyme may play a role in the hardening of the seed coat.

Peroxidases (donor: H2O2 oxidoreductase; EC 1.11.1.7), a ubiquitous class of plant proteins, are enzymes whose primary function is to oxidize a variety of hydrogen donors at the expense of hydrogen peroxide. Peroxidase can also use molecular oxygen as an electron acceptor in oxidasic reactions (4). This class of enzyme has been extensively studied during this century resulting in considerable knowledge about the chemistry and biochemistry of the molecule (9). Peroxidase has been implicated in a variety of physiological processes in plants. The literature abounds with reports of peroxidase involvement in lignin biosynthesis (10), extensin polymerization (8), auxin metabolism (9), disease resistance (11, 20), wound healing (6), and the response to air pollutant stress (17). Considering the vast number of publications in this field, the physiological function of individual members within this class of enzymes is only partially understood. The lack of knowledge is due, in part, to the complexity of peroxidase isozymes expressed within a given plant tissue. The presence of multiple isozymes, each of which may be capable of utilizing a variety of cellular substrates, makes the assignment of specific biological functions to individual isoforms difficult.

Our laboratory is interested in studying the physiological function of peroxidase isozymes in the soybean plant. To this end, we have chosen to isolate individual isozymes as a prerequisite to the assignment of in vivo function. This communication reports on the isolation to homogeneity of a soybean seed peroxidase activity. We show that the enzyme is an abundant protein within the seed coat, represents the dominant peroxidase isozyme, and accumulates during the maturation of this tissue.

MATERIALS AND METHODS

Plant Material

Seeds (Glycine max var Williams 82) used for the purification of peroxidase were obtained from Midwood, Inc., Bowling Green, OH. Soybean plants were grown in potting soil under glasshouse conditions.

Enzyme Assay and Protein Determination

Peroxidase activity toward guaiacol was quantitated by following the rate of conversion of this substrate to tetraguaiacol at 470 nm. The reaction mixture contained 8 mM guaiacol, 0.5 mM H2O2, 50 mM potassium acetate (pH 5.5), and was run at 25°C. A second assay of seed coat peroxidase activity employed the use of 4-chloro-1-naphthol as the electron donor. Nitrocellulose imprints and IEF2 gels were placed in a solution containing 8.5 mM 4-chloro-1-naphthol, 10 mM Na2HPO4 (pH 7.2), 75 mM NaCl, and 3.3 mM H2O2.

Protein concentration was determined by the Bio-Rad assay system (1) according to the manufacturer’s procedures. BSA was used as the standard.

Enzyme Purification

Seed coats were obtained by soaking whole seeds in distilled water for approximately 5 min or until the coats became wrinkled. The coats were then removed from the seed and homogenized with a Tekmar Tissuemizer in extraction buffer (25 mM KH2PO4 [pH 7.5]) at a ratio of 10 mL extraction buffer per gram tissue. All purification steps were performed at 4°C. The homogenate was filtered through two layers of Miracloth and the filtrate centrifuged (12,000g, 10 min). The supernatant fluid was applied to a DEAE Sepharose Fast Flow (Sigma Chemicals) column (2.5 × 20 cm) previously equilibrated with extraction buffer. Peroxidase activity was eluted with a linear salt gradient from 0 to 300 mM KCl in extraction buffer. Fractions containing peroxidase activity were made 1× in Con A-Sepharose (Sigma Chemicals) binding buffer by addition of one-fourth volume of a 5× stock solution (250

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2 Abbreviations: IEF, isoelectric focusing; Con A-Sepharose, concanavalin A-Sepharose; NMWL, nominal molecular weight limit; TMFS, trifluoromethanesulfonic acid; dda, days after anthesis.
were lightly soybean localization of peroxidase of cal peak of enzyme activity was determined by analytical flatbed isoelectric focusing polyacrylamide gels containing ampholines in the pH range of 3.5 to 9.5 (LKB, Bromma, Sweden). The gel was loaded with a seed coat extract in one lane and was mock loaded with H₂O in an adjacent lane. The gel was subjected to electrophoresis for 1.5 h at 0.125 W/cm² at 10°C (14). The lanes were then separated and the one containing the seed coat extract was stained with 4-chloro-1-napthol solution to determine the distance the peroxidase activity migrated relative to the anode. The mock loaded lane was sliced into 0.25 cm pieces. Gel slices were then added to 2 mL of H₂O and incubated at room temperature overnight with shaking. The pH of the solution containing each gel slice was determined and plotted as a function of distance from the anode. By comparing the distance from the anode that the activity migrated to the pH of the gel at that position, the pI of the enzyme was ascertained.

Determination of Peroxidase Isozyme Tissue-Specific Activity

Determination of isozyme activity levels in specific tissues was accomplished by densitometric tracing of peroxidase activity-stained gels. The cotyledons and seed coats were homogenized with a Tekmar Tissuemizer in distilled H₂O at a ratio of 10 mL per g of tissue. Samples were then centrifuged (12,000g, 10 min). Samples were electrophoresed on analytical IEF gels as described above. Gels were then placed in 4-chloro-1-napthol solution and allowed to develop for 60 s followed by a 5 min wash in distilled H₂O. Densitometric tracings of the activity stained gels were performed at 582 nm using a Shimadzu CS-930 Dual Wavelength TLC Scanner. Units of activity are arbitrary values calculated by measuring peak areas of peroxidase activity in the IEF gel. The specific activity of the SP4.1 seed coat peroxidase is defined as units/mg of soluble protein applied to IEF gels.

Localization of Peroxidase Activity

Visualization of peroxidase activity in immature seeds was accomplished by tissue imprinting (2). Nitrocellulose filters were soaked in 4-chloro-1-napthol solution (see above) and then lightly blotted to remove excess substrate. Immature soybean seeds were halved with a razor blade and allowed to dry for 1 min before blotting onto the moist filter. Seed halves were then pressed firmly onto the filter and color development was allowed to proceed for 15 s. The filters were immediately transferred to a 65°C oven and allowed to dry for 10 min.

Chemical Deglycosylation of SP4.1

Five hundred micrograms of purified SP4.1 was deglycosylated by the TFMS method previously described by Edge et al. (5). Following deglycosylation, the sample was dialysed overnight against 2 mM pyridine (pH 5.5), followed by dialysis against several changes of distilled H₂O for 1 d. The sample was then lyophilized to dryness and taken up in electrophoresis loading buffer and analyzed by denaturing gel electrophoresis.

Antibody Production

Antibodies to SP4.1 were raised in a rabbit with three subcutaneous injections of the purified peroxidase (200 μg/injection) at 2 week intervals. Partial purification of polyclonal antibodies was accomplished by ammonium sulfate precipitation followed by DEAE chromatography according to the methods of Livingston (15). The preparation was adjusted to the original serum volume with 10 mM Na₂HPO₄ (pH 6.8). Western blot analysis was performed using a 1:1500 dilution of the partially purified primary antibody.

SDS-PAGE and Western Blot

Denaturing gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (13) and silver staining was carried out according to the methods employed by Morrissey (16). Molecular mass markers used for gels were bovine serum albumin (66 kD), egg albumin (45 kD), glyceraldehyde-3-phosphate dehydrogenase (36 kD), carbonic anhydrase (29 kD), trypsinogen (24 kD), trypsin inhibitor (20.1 kD), and α-lactalbumin (14.2 kD). Immunoblots were performed as described by Towbin et al. (21).

RESULTS

Localization of Peroxidase Activity Within the Soybean Plant

Tissue imprinting experiments reveal that the majority of the peroxidase activity in soybean seeds directed toward 4-chloro-1-napthol is localized to the seed coat (Fig. 1A). Analysis of peroxidase activity (guaiacol) in dissected immature seeds shows that the activity in the seed coat is 99-fold higher than observed in the cotyledon on a dry weight basis (data not shown). Seed coats do show other minor electrophoretic forms on analytical IEF gels other than the major seed peroxidase. However, the detection of these minor species (<5%) requires 50 times the amount of protein loaded in Figure 1B. The isoelectric point (pI) of the peroxidase was determined using analytical IEF gels and was shown to be approximately 4.1. The major seed coat peroxidase isoform will be henceforth referred to as SP4.1. The observation that the greatest concentration of SP4.1 activity is localized to the seed coat and is the dominant isoform suggests that this tissue be used in the purification of the enzyme.
Accumulation of SP4.1 in Maturing Seed Coats

Before attempting the isolation of SP4.1, we determined the stage of seed coat maturation at which the enzyme reached its highest specific activity. Figure 2 shows a general increase in the total peroxidase specific activity as the seed coat matures. Peroxidase specific activity begins to increase 21 to 28 daa and continues throughout the maturation of the seed coat. Commercially obtained dry seed actually show higher peroxidase specific activity (1.2×) than that observed in 49 daa seeds still attached to the plant.

The increase in SP4.1 specific activity during the development of the seed coat correlates with the accumulation of a polypeptide with a molecular mass of 37 kD (Fig. 3). Figure 3 also shows that the complexity of the pattern of soluble seed coat polypeptides decreases dramatically during the maturation of the coat. Taken together, these data suggest that the increase in peroxidase specific activity is due, in part, to the accumulation of SP4.1. The increase in specific activity may also be due to a general increase in the degradation of other soluble proteins during seed coat maturation (cf. lanes 2 and 7, Fig. 3). Indeed, SP4.1 activity remains high in seeds at least 3 years after harvest suggesting that this activity is stable. Thus, we isolated the peroxidase activity from mature seed coats to determine if the 37 kD polypeptide is SP4.1.
Enzyme Purification

Purification of SP4.1 required 10 g of seed coats (wet weight). Figure 4A shows the elution profile of seed coat homogenates from a DEAE Sepharose Fast Flow ion exchange column. The activity eluted from DEAE Sepharose Fast Flow at 0.10 M KCl and was 5.5-fold purified after this step (see Table 1). The pooled peroxidase activity was applied to Con A-Sepharose and eluted at 0.75 to 1 M methyl α-D-mannopyranoside (see “Materials and Methods”) resulting in a 26.1-fold purification (see Table 1). The final step employed a Sephadex G75 gel filtration column (Fig. 4B), resulting in a 40.8-fold purification, a recovery of 14% (Table I), and a Reinheitzahl value of 2.8. Due to the abundance of this protein in the crude extract (Fig. 3, lane 7) a 40-fold purification is sufficient to purify the enzyme.

Analysis of the material from the Sephadex G75 column on both silver stained denaturing gels (Fig. 5A) and on analytical IEF gels (Fig. 6A) demonstrate the presence of a single polypeptide. Glycosylation of purified SP4.1 is suggested by its affinity for Con A; this was confirmed by treatment with TFMS. TFMS treatment resulted in a shift in the molecular mass of the polypeptide from 37 kD to 30 kD, thus the modifications represent a significant portion of the mass of this protein (Fig. 5). The polypeptide also stains with Schiff’s Reagent (24) on SDS-PAGE (data not shown).

Accumulation of SP4.1 during Maturation of the Seed Coat

Polyclonal antibodies generated against the purified peroxidase were used as additional confirmation that the 37 kD polypeptide accumulating during seed coat maturation is SP4.1. Western analysis shows an increase of a single polypeptide with a molecular mass of 37 kD beginning approxi-
DISCUSSION

The partial purification of the predominant peroxidase activity within soybean seeds has been previously reported by Sessa and Anderson (18) using whole seeds as the source of enzyme. Their preparation contained one major anionic peroxidase activity and at least two minor activities along with several other contaminating polypeptides. The study was concerned with the peroxidatic properties of the mixture and the possible involvement of peroxidase in generating off-flavors from endogenous seed constituents (18). A closer examination of the seed shows that the preponderance of the peroxidase activity is localized within the testa or seed coat (Fig. 1, Table II). By employing seed coats as the source of enzyme, we are able to purify the major peroxidatic activity within the seed to homogeneity by standard biochemical separation techniques.

The relative ease in isolation of this isozyme is due to the fact that mature soybean seed coats do not contain a complex assortment of soluble proteins. Moreover, the anionic perox-

Figure 6. Analysis of purified SP4.1 by analytical isoelectric focusing. A, Silver-stained analytical isoelectric focusing gel of purified SP4.1 (5 μg); B, activity stained analytical isoelectric focusing of purified SP4.1 (10 ng). The pH of the anodic and cathodic ends of the gel are shown in the left margin.

imately 28 daa (Fig. 7). This result correlates with the appearance of the 37 kD polypeptide observed with SDS-PAGE (Fig. 3) and the increase in the peroxidase specific activity (Fig. 2) during maturation of the seed coat.

Specific Activity of SP4.1 in Soybean Tissues

The presence of SP4.1 was determined in other regions of the soybean plant to ascertain the abundance of this isozyme. The seed pod contained the highest specific activity of SP4.1 in tissues other than the seed coat, but the activity was only 4% of the level in seed coats (Table II). Other tissues examined had specific activities of SP4.1 less than 1% of mature seed coats. These tissues contain multiple anionic and cationic forms of peroxidase activities. We were interested in confirming the IEF data with Western blot analysis, but found that the technique was not useful because multiple bands were observed in these tissues. The banding pattern was probably due to the presence of similar carbohydrate moieties shared by SP4.1 and other soybean proteins. In contrast, seed coat tissue yields only a single band by Western analysis. We have observed as many as 20 different isozymes among soybean plant tissues using analytical isoelectric focusing (data not shown). No tissue, except the seed coat, exhibits a peroxidase isozyme pattern where one form predominates.

Figure 7. Western blot of SDS-PAGE gel of soybean seed coat soluble protein. Samples are identical to those used in Figure 2. Lanes 1 to 6 represent 5 μg of extracts from seed coats 14, 21, 28, 35, 42, and 49 daa, respectively. Lane 7 is a seed coat extract from commercially obtained seeds. The position of molecular mass markers are shown in the left margin.
Table II. Specific Activity of SP4.1 from Various Soybean Plant Tissues

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Specific Activity</th>
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<tbody>
<tr>
<td>Mature seed</td>
<td>20,620</td>
</tr>
<tr>
<td>Seed coat</td>
<td>15,620</td>
</tr>
<tr>
<td>Cotyledon</td>
<td>&lt;100</td>
</tr>
<tr>
<td>Pod</td>
<td>807</td>
</tr>
<tr>
<td>Leaf</td>
<td>&lt;100</td>
</tr>
<tr>
<td>Stem</td>
<td>&lt;100</td>
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</table>

Table II suggests that there is sufficient activity present during early seed coat development to catalyze this reaction. As mentioned previously, the increase in specific activity of SP4.1 at later stages of seed development appears to be due to the inherent stability of this protein relative to other seed coat proteins (see Fig. 3).

The purification of the seed coat may also require the presence of SP4.1. The polymerization of cinnamyl alcohols is widely accepted as being a peroxidase catalyzed reaction (10). The hardening of the seed coat during later stages of maturation most certainly requires the presence of peroxidase activity. This may explain the abundance of this isozyme in mature soybean seed coats.

The suberized tissue of the mature seed coat represents a diffusion barrier sealing the seed coat from the environment (12). Previous studies have implicated an anionic peroxidase in the deposition of the aliphatic and aromatic components of suberin (19). Although we have not yet measured the hydroxyproline or lignin content in developing seed coat tissue, we do have preliminary transmission electron micrographs showing the deposition of this peroxidase within the cell walls of both hour glass and palisade cells of the mature soybean seed coat. Additional experimentation is in progress to elucidate the physiological function of this abundant seed coat protein.

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LITERATURE CITED

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