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Peroxidase Isozymes of First Internodes of *Sorghum*

TISSUE AND INTRACELLULAR LOCALIZATION AND MULTIPLE PEAKS OF ACTIVITY ISOLATED BY GEL FILTRATION CHROMATOGRAPHY[1]

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ABSTRACT

Electrophoretic analyses using Sepraphore III strips indicate the presence of a minimum of five bands of peroxidase activity detectable with *o*-dianisidine and H_2O_2 in extracts from first internodes of *Sorghum vulgare* var. Wheatland milo. Three of these isozymes were anodic and two were cathodic forms at pH 8.3. The relative amounts of these forms are compared in zero time and incubated excised internodes, stelar and cortical tissues of internodes, and in other parts of the plant. Localization of these isozymes with respect to walls and cytoplasm was characterized by differential centrifugation after grinding of the internodes and by an *in situ* extraction of walls by centrifugation after vacuum infiltration. Using the latter *in situ* method, 32% of the total activity of the fast moving cathodic form was exchanged from the wall after infiltration with 50 mM $CaCl_2$. Only trace amounts of the other isozymes were localized in the walls of the cortex. The isozymes were eluted as two peaks from columns of Sephadex G-100 and three peaks from Agarose A-15m. Although such groupings may be due to asymmetric molecules and ionic interactions as well as to molecular weight differences, they may indicate associations with complexes or membranes of different cytoplasmic constituents.

The varied forms and numerous catalytic activities of peroxidase (3, 6, 10, 20) are understandable physiologically only if the isozymes are associated with different cellular or tissue compartments. Since the first internode of *Sorghum* is capable of synthesizing an assortment of phenolic compounds (21), and the cortex and stele can be physically separated, it is a useful biological tool for the study of peroxidase function.

MATERIALS AND METHODS

Seeds of *Sorghum vulgare* var. Wheatland milo (United States Department of Agriculture Station, Woodward, Okla.) were surface sterilized in saturated calcium hypochlorite and grown on moist filter paper in the dark at 25 C for 3 to 4 days. Internodes were excised and assayed directly (zero time) or after incubation for 2 days in the dark or in bright light at 25 C (21). Steles could be stripped from the cortex by making a cut in the base of the cortex to expose the stele "thread" which could be pulled free.

Preparation of Extracts. Plant parts were ground in a chilled mortar with twice their weight of 50 mM K_2HPO_4-KH_2PO_4 buffer, pH 6, plus one-tenth their weight of Polyclar-AT powder and a small amount of clean washed sand. The homogenate was strained through four layers of cheesecloth, and centrifuged at 500*g* for 1 min to remove the sand and Polyclar, and then generally at 12,000*g* for 10 min. The almost clear supernatant fraction was decanted and used as a source of cytoplasmic enzymes. A typical extract contained about 0.6 mg of protein or 0.4 g of fresh weight per milliliter.

To extract peroxidases from the walls *in situ*, internodes were vacuum infiltrated with water or 50 mM $CaCl_2$, then placed basal end down in plastic syringe barrels (no needle or plunger) and centrifuged at 4 C at 3,000*g* for 10 min. The infiltration and centrifugation were repeated as indicated. The liquid collected in the centrifuge tube, about 0.5 ml, contained the *in situ* wall enzymes (1).

To extract peroxidase from isolated cell walls, the internodes were ground in either 50 mM phosphate buffer or 0.5% Nonidet P-40 in H_2O (2), without Polyclar or sand, and the homogenate was strained through cheesecloth. The residue remaining in the cheesecloth was then washed well with buffer or H_2O, respectively, and extracted with 200 mM $CaCl_2$ at 4 C for 30 min. The mixture was then filtered through cheesecloth and centrifuged as above. The supernatant was decanted and used as the Ca-soluble or exchangeable wall extract. The remaining residue was washed with buffer and constituted the Ca-insoluble wall enzyme. An estimate of this activity was made by resuspending part of the mat in the assay mixture and analyzing the solubilized product spectrophotometrically.

Electrophoresis. Crude extracts or fractions eluted from G-100 or Agarose columns were applied to 25 × 88 mm strips of Sepraphore III (Gelman) cellulose polyacetate electrophoresis strips. Electrophoresis was carried out for 2 hr at 1 ma per strip in an LKB paper electrophoresis apparatus (LKB3276B, Sorvall), modified by inserting the Sepraphore strip between two paper wicks (each 17 cm long). The buffer was 300 mM boric acid and 50 mM NaOH, pH 8.3. For visual estimation, peroxidase bands were located by immersing the strips for 15 to 30 min in modified Brewbaker's solution containing 4 mM *o*-dianisidine (6), 4.4 mM H_2O_2, 50 mM sodium acetate buffer, pH 5.4, and 70% ethanol (v/v). After development, strips were dehydrated in 100% methanol, cleared in 10% acetic acid in methanol for 30 sec, and dried on a glass plate at 60 C for 15 min.

Peroxidase Assays. A spot test to locate the activity in gel column fractions consisted of 1 drop of the enzyme fraction and 3 drops of a mixture containing 15 mM guaiacol, 5 mM H_2O_2, 50 mM phosphate buffer, pH 6.0.

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For a quantitative assay of extracts or column fractions, a 3-ml volume contained a freshly prepared mixture of 0.35 mM *o*-dianisidine (Sigma, purified grade), 5 mM H_2O_2 (from 30% H_2O_2), and 50 mM acetate buffer, pH 5.4. A 1 to 20 μl aliquot of enzyme was added, and readings were taken at 460 nm every 15 sec for 2 to 4 min, using a Coleman junior spectrophotometer. Activity was expressed as change in absorbance per minute per gram fresh weight. When quantitating electrophoresis strips, unstained bands were eluted overnight at 4 C in 1 ml of 50 mM sodium acetate, pH 5.4, with or without 200 mM $CaCl_2$. Then 2 ml of a mixture of the above constituents were added to give a final concentration of substrate equivalent to the standard test.

IAA Oxidase Activity. The assay for IAA oxidase in crude preparations, fractions from G-100 columns, or eluted electrophoresis strips was carried out with a Gilson Oxygraph and Clark O_2 electrode. The reaction mixture contained 1.9 ml of enzyme + phosphate buffer, 0.05 ml 10 mM $MnCl_2$, 0.05 ml 10 mM 2,4-dichlorophenol. IAA was added as a solid (about 250 μg) to start the reaction.

Gel Filtration Column Chromatography. Column chromatography was performed at 4 C on 35 to 40 cm × 1.3 cm columns of Sephadex G-100 or Agarose (Bio-Gel A-15m, 100–200 mesh) equilibrated with 50 mM phosphate buffer, pH 6. The void volume of the column was determined with a solution of Blue Dextran.

RESULTS

Electrophoretic Pattern and Analysis of Cytoplasmic Peroxidase Isozymes in Extracts of First Internodes. A minimum of five isozymes could be identified in extracts of excised internodes incubated for 2 days. Two of these are cathodic and 3 are anodic at pH 8.3 (Fig. 1). The best results have been obtained with *o*-dianisidine and 8 mM H_2O_2 as the staining agents. The amount of H_2O_2 used was critical in both electrophoretic stain and test tube assays. The problem was intensified with guaiacol as the phenolic substrate; only C_3 and A_1 bands were detectable. A comparison of activities with 1, 2, 4, 8, and 17 mM H_2O_2 with 0.35 mM *o*-dianisidine as the staining mixture gave sharp optima at 8 mM for the cathodic forms, and broader optima toward the lower range of 2 to 8 mM for the anodic isozymes. A_2 was the least sensitive to these concentration changes, but all were inhibited by 17 mM H_2O_2. Similar variations were obtained in the test tube assay methods (see Table VII). If electrophoresis was done in acetate buffer at pH 3.8, all bands were cathodic except A_3 which migrated slightly towards the anode.

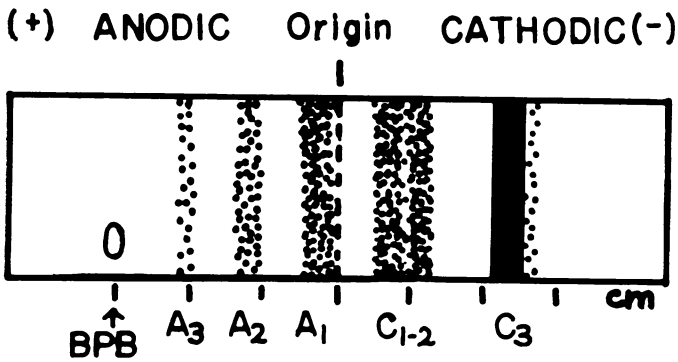

FIG. 1. Electrophoretic pattern of peroxidase isozymes in incubated internodes. Sepraphore III strips were stained with *o*-dianisidine (6) after electrophoresis for 2 hr in borate buffer at pH 8.3. BPB: bromophenol blue. Marks at centimeter intervals to indicate the R_F values in centimeters from the origin.

Table I. *Changes in Activities of Isozymes of 3- to 4-day-old First Internodes Incubated as Intact Excised Internodes or as 1-cm Segments*

Values are expressed as change in absorbance per minute per gram of fresh weight and as percentage of total in parentheses. They are based on electrophoretic separation and elution with 50 mM sodium acetate buffer, pH 5.4.

Treatment	A_3	A_2	A_1	C_{1-2}	C_3	Σ
Zero time internodes	2 (4)	4 (8)	3 (6)	7 (13)	39 (71)	55
2 Days incubated in dark as intact internodes	5 (3)	11 (7)	18 (11)	19 (11)	113 (68)	166
Incubated/zero time (ratio)	3	3	6	3	3	
2 Days incubated in dark as segments	8 (5)	20 (12)	54 (34)	25 (16)	55 (34)	162
Incubated as segments/incubated intact (ratio)	1.6	1.7	3	1.3	0.5	

Use of the inhibitor phenylmethylsulfonylfluoride in the extraction medium ruled out the possibility that protease activity was responsible for the multiple isozymes in *Sorghum*.

Rechromatography of the five bands never gave rise to any other band. The area at C_{1-2} frequently was detectable as two discrete bands, but rechromatography of the two parts gave no satisfactory evidence of more than one band in that area. Treatment with 6 M urea, 1 M NaCl, freeze-thawing, or 1% (w/v) Triton X-100 did not alter the relative position of the bands.

All of these isozymes were found in 3- to 4-day-old internodes (zero time), but only the fast moving cathodic form C_3 was detected in significant amounts (Table I). No C_3 was detected in the 2-mm tip of the internode (the meristematic area just below the coleoptilar node), while A_1 and A_2 were the dominant forms here and were present in concentrations greater than that of C_3 in the lower part. There was a gradient of increasing activity of C_3 towards the base. Upon incubation of the excised internodes in the dark or in light, the total activity increased approximately 3- to 6-fold. While the greatest absolute increase was in the activity of C_3 so that it remained the dominant form, the greatest percentage of increase was in the slow moving anodic form, A_1. The gradient towards the base of increasing activity of C_3 found in zero time internodes was maintained in incubated internodes. The C_{1-2} complex in incubated internodes differed from C_3 in that it was considerably stronger in the top one-third than in the basal two-thirds portion.

Although the total amount of C_3 was greater in the bottom one-third, the greatest increase per gram of fresh weight was in the top 2-mm section that initially had no detectable C_3 activity. This increase was not just due to damage to the tissue by cutting because the increase was much less in the basal 2-mm section. However, cutting the internode into 1 cm segments before incubation does sometimes increase the total activity and alters the activities of two of the isozymes. In three experiments the total activity was greatly increased in one case, but stayed approximately equal in the other two. In all cases, however, the activity of A_1 was much greater than in the whole excised internodes while that of C_3 was generally less (Table I).

Table II. *Relative Activities of Isozymes from Incubated Internodes as Peroxidases and as IAA Oxidases*

Bands were eluted in 50 mM sodium acetate buffer, pH 5.4, from Sepraphore III strips.

	A_3	A_2	A_1	C_{1-2}	C_3
Peroxidase[1]	8	16	29	22	117
IAA Oxidase[2]	0	30	131	60	210
IAA Oxidase/peroxidase		2	5	3	2

[1] Change in absorbance per minute per gram of fresh weight using the *o*-dianisidine assay.

[2] Slope per gram of fresh weight using the oxygen electrode. To convert to micromoles O_2/sec·g fresh weight in the 2-ml incubation mixture, multiply by 4.26×10^{-5}.

Table III. *Relative Amounts of Isozymes in Various Tissues of Sorghum*

Electrophoresis strips were stained and visually estimated.

	A_3	A_2	A_1	C_{1-2}	C_3
First internode[1]					
Zero time	trace	trace	trace	trace	+++
+ 2-day incubation	+	++	+++	+++	++++++++
Root, zero time	trace	+	trace	trace	trace
Coleoptile, zero time	0	+	++	++	0
Green leaves	+	++	++++	++	+++
Ungerminated seeds	0	0	trace	0	+

[1] See Table I for actual quantitative values.

Mixing of zero time internodes with incubated ones during grinding did not activate any new activity, and incubation of frozen and thawed internodes did not induce any new activity. No other experiments have been done to determine whether protein synthesis was actually involved in this new activity.

Four isozymes were active as IAA oxidases (Table II). The only possible variation was in the anodic form A_1 which showed the highest ratio of oxidative to peroxidative activity. The highest absolute activity, however, was associated with the dominant peroxidatic form C_3.

Comparison of Isozymes of First Internodes with Other Parts of the Plant. Semiquantitative approximations of the relative amounts of these same isozymes are indicated for other tissues of *Sorghum* (Table III). No major new band was detected. Although the ones indicated have the same R_F values as those of the first internodes, no study has been made to determine whether these are identical forms or are merely migrating to a similar position.

Cellular Localization of Cytoplasmic Peroxidase Activities. While 5 to 15% of the total cytoplasmic activity was associated with unwashed particulate fractions sedimented between 500 and 100,000*g*, resuspension of these pellets in the original medium removed all but less than 1% of the total activity recovered. No difference was observed when internodes were ground with 500 mM sucrose at pH 6. The isozymes identified in these washed particulate fractions contained only the slow moving forms C_{1-2} and A_1 or traces of A_2.

Cytoplasmic *versus* Wall Localized Isozymes. The above data are based on the activity extracted in 50 mM phosphate buffer, which consisted of cytoplasmic and easily removed wall isozymes. The difficulty in assessing the localization of enzymes by biochemical extraction techniques is well known. Artifacts can occur even in wall sites because of leaching of isozymes or as a result of adsorption during isolation of a cytoplasmic enzyme. Two methods have been used to circumvent these difficulties. One of these is the use of nonionic detergents such as Nonidet, reported to prevent inclusion of cytoplasmic enzymes in wall fractions (2); the other is the use of infiltration and centrifugation of whole tissues to isolate wall enzymes by *in situ* extraction without membrane destruction (1).

In the latter method, after vacuum infiltration, wall enzymes were isolated by centrifugation of the wall fluids. When distilled water was used as the infiltration solution, only traces of all isozymes but C_3 were removed (Table IV). The only other significant result was that A_3, normally present in the smallest amounts in the cytoplasm and not detectable in washed wall preparations (see Table VI), was slightly greater than the other components. Similar patterns were obtained if noninfiltrated internodes were centrifuged to remove endogenous aqueous components. Infiltration with 50 mM $CaCl_2$ removed the bulk of the wall localized activity by centrifugation. The values given in Table IV are the sum of three sequential infiltrations and centrifugations with similar amounts removed by the first two, a technique more effective than just recentrifugation without repeated infiltrations. Although traces of all isozymes were detected in this wall fluid obtained by centrifugation, by far the major constituent was C_3 (about 95%).

Subsequently, the infiltrated and centrifuged internodes were ground using the regular procedure except that sand and Polyclar were omitted to prevent contamination and the cheesecloth mat was thoroughly washed with 50 mM phosphate buffer. The remaining wall activity was then removed from the washed walls by cation exchange with 200 mM $CaCl_2$ (Table IV).

The washings of the wall mat in all cases contained only 8 to 19% of the total activity of the homogenate. All isozymes were detected in the cytoplasmic fractions of the *in situ* extracted walls of internodes, but the amount of C_3 in the $CaCl_2$ infiltrated internodes was noticeably less than that of the water infiltrated ones. The absolute amounts of total activity recovered are hard to compare since infiltration with a solution of $CaCl_2$ appears to cause a significant activation of the total

Table IV. *Relative Amounts of Peroxidase Activity in Walls*

The wall activities were first isolated by centrifugation *in situ* after infiltration three times with H_2O or 50 mM $CaCl_2$ and subsequently by cation exchange of washed walls. Values are expressed as percentage of total analyzed, with major isozymes identified electrophoretically in parentheses.

	Infiltration Medium		
	None	H_2O	50 mM $CaCl_2$
Total activity recovered (ΔA/min·g fresh wt)	365	417	540
Centrifugible from walls	trace	1 (A_3)	32 (C_3)
Exchanged from washed walls[1]	20 (C_3)	16 (C_3)	7 (C_3)
Cytoplasmic (+ washes)[2]	80 (C_{1-2} + C_3)	83 (C_{1-2} + C_3)	61 (C_{1-2})

[1] Solubilized by extraction with 200 mM $CaCl_2$.

[2] Solubilized by grinding tissues in 50 mM phosphate buffer, pH 6.

Table V. *Total Activities in Washed Walls after Extraction of Cytoplasmic Activities of Excised Internodes*

Internode	Incubated as Intact Internodes	Incubated as Segments
	ΔA/min·g fresh wt	
Washed Walls		
Ca^{2+} soluble[1]	125 (23%)	134 (16.5%)
Ca^{2+} insoluble	5 (1%)	13 (1.5%)
Cytoplasmic[2]	311 (57%)	506 (63%)
Washes of wall mat	104 (19%)	... (19%)[3]
Total	545	653

[1] Solubilized by extraction with 200 mM $CaCl_2$.

[2] Solubilized by grinding tissues in 50 mM phosphate buffer, pH 6.

[3] Not determined; percentage based on washing of intact internodes.

amount recovered from the walls (Table IV). (Ca^{2+} also increased the activity of crude cytoplasmic extracts when added during the assay.)

These *in situ* results were compared with an analysis of washed walls after grinding of tissues with and without 0.5% Nonidet P-40 (2). The initial washes contained a pattern of isozymes similar to that of the cytoplasmic extract. While the presence of Nonidet inhibited the activity of the cytoplasmic fraction, it had no detectable effect on the subsequent extraction of the wall activities. Data for tissues ground without Nonidet are shown in Table V. Only about 23% of the total activity was solubilized from washed walls by 200 mM $CaCl_2$ in contrast with the 40% by the infiltration-centrifugation *in situ* method (Table IV). Again, most of this activity of the washed wall was due to C_3, but C_{1-2} was relatively more detectable (Table VI). The results were similar when the internodes were incubated as 1-cm segments; the increase in cytoplasmic activity was always due to an increase in the A_1 component (and generally a decrease in C_3). (See also Table I.)

At the 200 mM level, K^+ salts were just as effective as Ca^{2+} in solubilizing the wall activities. The accompanying anions were unimportant in this wall extraction, as nitrates were just as effective as chlorides. This is in keeping with the interpretation that cation exchange was the mechanism involved in the solubilization.

Comparison of Isozymes of the Cortex and Stele. The cortical tissues could be separated from those of the central stele of the internode by a stripping technique; the endodermis appeared to remain with the stele. While the activities based on fresh weight were similar in the two tissues (Table VI), the steles contained only 20% of the total activity per internode.

The relative amounts of the isozymes in cortical tissue reflected that of the whole internodes (Table VI). The stelar tissue was characterized by the absence of A_3 and a relatively low C_3 component, and by C_{1-2} as the major component, followed by A_1 and A_2. The wall activities of the stele solubilized with Ca^{2+} were due mainly to cathodic C_{1-2} forms.

Separation of Cytoplasmic Activites by Column Gel Filtration. When extracts of incubated internodes were added to columns of Sephadex G-100, the percentage recovery with *o*-dianisidine and guaiacol as substrates was dependent on the concentration of H_2O_2 used in the assay (Fig. 2 and Table VII). In crude preparations using *o*-dianisidine, 5 mM H_2O_2 was optimal, while in dialyzed extracts 3 to 5 mM was optimal. A seemingly high percentage recovery observed in initial experiments was due to the use of too low a H_2O_2 concentration (Table VII). The apparent decrease in percentage total activity in peak II (due mainly to C_3) in the presence of guaiacol was probably due to a sharper optimum concentration for H_2O_2 in the case of guaiacol. (See also Fig. 2, where the peak with guaiacol approaches that with *o*-dianisidine at the 5 mM level of H_2O_2.)

Table VI. *Comparison of Isozyme Patterns of Cytoplasmic and Washed Wall Extracts of Incubated Whole Internodes[1] and of Steles and Cortex Tissue Separated from Incubated Internodes*

Values are based on electrophoretic separation, and elution of bands with 50 mM sodium acetate buffer, pH 5.4, plus 200 mM $CaCl_2$.

Isozymes Eluted	A_3	A_2	A_1	C_{1-2}	C_3	Σ
	ΔA/min·g fresh wt					
Whole internode						
Cytoplasmic[2]	17	40	68	52	221	398
Wall[3]	0	3	11	23	62	99
Cortex						
Cytoplasmic	20	29	116	66	218	449
Wall	0	0	trace	76	52	128
Stele						
Cytoplasmic	0	97	125	155	53	430
Wall	0	0	trace	56	0	56

[1] Same extracts as in Table V.

[2] Solubilized by extraction with 200 mM $CaCl_2$.

[3] Solubilized by grinding tissue in 50 mM phosphate buffer, pH 6.

Table VII. *Comparison of Activities of Crude Extracts and of Peak Fractions Eluted from Sephadex G-100 and Agarose A-15m Columns and from Sepraphore III*

	Crude Activity	Peaks I	Peaks II	Σ Peaks	Recovery
	ΔA/min·g fresh wt				%
Sephadex G-100:					
Zero time internode extract[1]	147	34 (17%)	165 (83%)	199	135
Incubated internode extract[1]	427	176 (30%)	417 (70%)	593	139
Same incubated internode extract[2]	242	271 (50%)	276 (50%)	546	226
Same incubated internode extract[3]	393	203 (46%)	241 (54%)	443	113
Sepraphore III:					
Incubated internode extract[4]	170	49 (29%)	118 (71%)	167	99
Agarose A-15m:					
Incubated internode extract[1,5]	571	23 (5%)[6] 92 (18%)[7]	412 (77%)	527	92

[1] Assay: 0.35 mM *o*-dianisidine, 5 mM H_2O_2.

[2] Assay: 15 mM guaiacol, 3 mM H_2O_2.

[3] Assay: 15 mM guaiacol, 15 mM H_2O_2.

[4] Assay: 0.35 mM *o*-dianisidine, 5 mM H_2O_2 after electrophoresis and elution with 50 mM sodium acetate buffer, pH 5.4. Values for peaks I and II calculated by summing values for A_1, A_2, C_{1-2}; and A_3, C_3, respectively.

[5] Data of Figure 3, top.

[6] Higher molecular weight form.

[7] Lower molecular weight form.

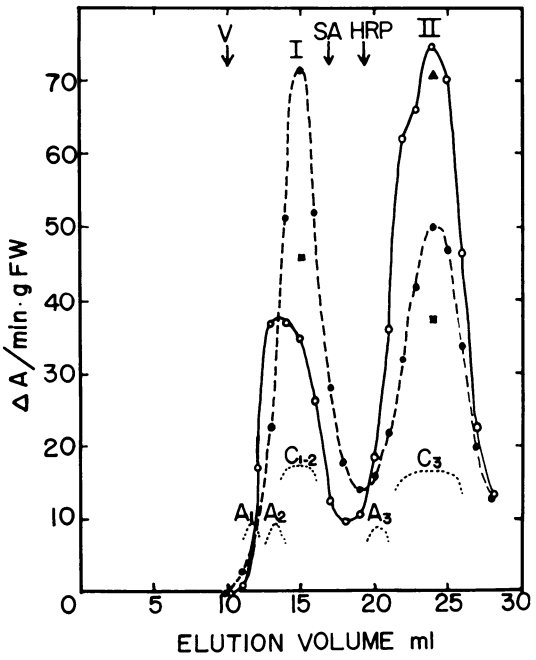

FIG. 2. Sephadex G-100 elution profiles of peroxidase activities in extracts of incubated internodes. Assays were made with 0.35 mM *o*-dianisidine + 5 mM H_2O_2 (○) or with 15 mM guaiacol + 3 mM H_2O_2 (●) (5 and 15 mM H_2O_2 in peak tubes are ▲ and ■ respectively). The dotted line profiles of peaks in the lower part of the graph indicate the positions of the various isozymes within peaks I and II. They were estimated visually after G-100 fractions were electrophoresed and stained with H_2O_2 and *o*-dianisidine. V: initial void volume; SA: bovine serum albumin; HRP: horseradish peroxidase.

When pooled fractions from either peak I or II were rechromatographed on Sephadex G-100, all of the original activity was recovered in fractions at the original elution volume.

Horseradish peroxidase (Nutritional Biochemicals Corp.), containing traces of bands similar to those of *Sorghum* but in which the one in the area of A_1 was by far the major form, gave a single peak of activity on Sephadex G-100 (Figs. 2 and 3). Mixing of horseradish preparations with those of *Sorghum* did not affect the position of the peaks, although the activity of horseradish peroxidase was inhibited.

Electrophoresis of key fractions showed that the isozymes were eluted from the Sephadex gel column in the following order: A_1, A_2, C_{1-2}, A_3, and C_3, with a distinct gap between C_{1-2} and A_3 to give the double peak. Visual estimates indicated that C_{1-2} and C_3 were of approximately equal intensity, with A_1, A_2, and A_3 falling into another group of considerably less intensity. On the other hand, when crude extracts were analyzed electrophoretically, C_3 was generally considerably stronger than C_{1-2}. But the percentage of activity in the two peaks, calculated by summing the individual bands, is strikingly similar.

Extracts of zero time internodes gave a small peak of activity in the presumed high molecular weight area, and a major one in the low molecular weight area. Electrophoresis of aliquots indicated that C_3 was the major component with only trace amounts of A_1, A_2, and C_{1-2} in the high molecular weight region.

Elution profiles from Agarose A-15m columns of whole incubated internodes and for steles stripped from similar internodes are shown in Figure 3. These demonstrated the complexity of the isozymes of the high molecular weight fraction from Sephadex peak I, with a small amount being eluted at the void volume, and the rest in an intermediate peak which was near the major band of horseradish peroxidase. The bulk of the C_3 isozyme formed a broad third peak, eluted after small molecular weight compounds.

The guaiacol spot test (with 15 mM H_2O_2) failed to give any positive test in the fractions of the whole internode extracts after an elution volume of about 40 ml. Use of a lower H_2O_2 content (5 mM) still did not produce any positive reaction. Subsequent analysis by the anisidine method, however, demonstrated that there was considerable activity in these fractions. The presence of large amounts of the C_3 band was corroborated by electrophoretic analysis. No such anomaly was observed in the case of stelar extracts, which contained only small amounts of C_3. Presumably, an inhibitor was present in the fractions separated on Agarose, but not on Sepraphore III or Sephadex G-100.

DISCUSSION

Peroxidase isozymes of horseradish have molecular weights of approximately 45,000 (9), but higher molecular weight forms have recently been detected in peas (4, 8), wheat embryos (11), and *Sorghum* seeds (19), and in tissue cultures which secreted complexes associated with hydroxyproline into the medium (14). There is no simple explanation of the stable, multiple peaks of activity of peroxidase in extracts from *Sorghum* eluted from either Sephadex or Agarose columns, but the causes probably are in common with green leaves since similar peaks are found (23). The elution profiles from Agarose columns indicate both a complexity of forms in Sephadex peak I (A_1, A_2, and C_{1-2}) and retardation of peak II (A_3 and C_3) beyond the inclusion volume. The association of the peroxidase isozymes with phenolic compounds could lead either to an in-

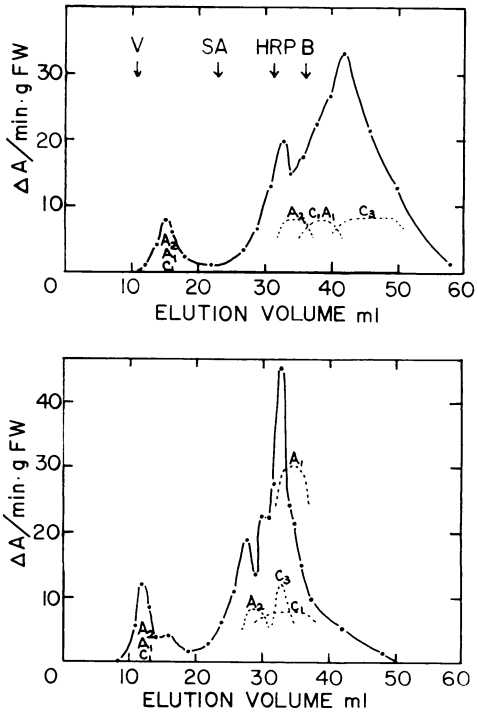

FIG. 3. Agarose A-15m elution profiles of peroxidase activity in extracts of incubated whole internodes (upper graph) and of steles from incubated internodes (lower graph). Dotted line peaks are visual estimates of isozyme constituents. Symbols as in Figure 2, plus B: bromcresol green.

creased rate of elution due to complexing into larger aggregates or to a delay in elution due to retention on the columns of associated aromatic compounds. The postulated ester linkages of cell wall polysaccharide to ferulic acid which is linked to protein by a hydroxyquinoid chelate bridge are of interest here (10). Attempts to separate ionic charge effects from molecular weight sieving during electrophoresis or the effects of molecular size, asymmetry, and ionic interactions during gel filtration have not been successful with these *Sorghum* extracts.

The intracellular localization of the cytoplasmic activities has not been satisfactorily answered by the techniques used involving varied grinding media and differential centrifugation. Small amounts of activity were associated with washed high speed particulate fractions obtained from media containing 50 mM phosphate buffer, pH 6, with and without 500 mM sucrose; the isozymes involved all were in the "high molecular weight" group or were slow moving electrophoretic bands. Similar amounts of activity in particulate fractions have been reported to be associated with mitochondria, plastids, cytosomes, or microsomes (11, 16, 17). Recent evidence indicates that peroxidase is both wall and membrane oriented (3, 7, 13, 18, 24). Present and past results would indicate the fragility of the membrane association. It is plausible that the tightly bound activity in particulate fractions as well as the high molecular weight fractions of the final supernatant solution are remnants of membrane associations or of multienzyme complexes.

The present data clearly show that a significant proportion of activity was associated with the cell walls (or intercellular spaces). The *in situ* wall extraction technique gave the best picture of the forms and amounts involved, at least in the cortex. The data indicated that the major anodic forms were actually missing from walls instead of being an artifact of a loss during the grinding procedure and that the cathodic forms were not merely adsorbed on the walls during isolation. Instead, about 40% of the total activity, mainly the isozyme C_3, was found in walls. Besides C_3, a significant proportion of A_3 may also be in the walls as it was equivalent to or even greater than that of the other minor forms according to the *in situ* method instead of being present in much smaller amounts. The data based on washed walls separated from the cytoplasm after grinding the tissues in buffer gave similar results, but some C_3 was preferentially removed or lost from the walls during the grinding and washing so that C_{1-2} was sometimes the dominant wall form. The elution of C_3 from wall sites *in situ* and both C_3 and C_{1-2} from washed cell walls solely by cation exchange emphasizes the importance of adsorption to negative charged sites in the wall *in vivo*. Calcium has been used before to remove wall bound activities either by the secretion of peroxidases into the surrounding medium (12) or by extraction of washed walls (5, 15, 25). Calcium in soils may control lignification in intact plants (15).

Besides being the major wall-localized isozyme, C_3 was further characterized by being almost completely limited to cortical cells rather than to those in the vascular stele, and by existing in a steep gradient of increasing concentration from the tip to the base of the internode (fresh weight basis). While it was not detectable in the top 2-mm section containing the meristem of zero time internodes, this area showed the greatest increase (on a fresh weight basis) upon incubation of the excised internodes. Since C_3, the fastest moving isozyme electrophoretically, was the major component of peak II from Sephadex G-100 and the retarded peak from Agarose columns, it is probably a highly positive charged protein of a relatively low molecular weight. Its *in vivo* function is certainly not lignification since it was absent from the wall of the stele and was present in only small amounts in the cytoplasm of the stele, the only tissue of the internode with positive histochemical lignin tests (21). Its function instead may be concerned with some aspect of differentiating but nongrowing walls, possibly related to effects of hydroxyproline or ethylene (10, 25). An isozyme of the C_{1-2} band (the main component of Sephadex peak I) would be the more likely candidate for the presumed peroxidatic function producing lignin in the stele.

While C_3 was the dominant form in nongrowing areas of the cortex, the "high molecular weight" forms, A_1 and A_2, were dominant in the top 2-mm segment of zero time internodes, possibly an indication of a function in growing tissues. The increase upon incubation of the excised internodes could be due to adventitious root formation (22). The higher IAA oxidase to peroxidase activity ratio of band A_1 may be of significance.

The two major wall isozymes, C_3 in the cortex and C_{1-2} in the stele, are also found in significant amounts in the cytoplasm. Such a double localization would be expected if vesicles containing peroxidase and its various substrates are being constantly budded off the Golgi and transported to wall sites (7, 18, 20, 24). The recent data indicating that peroxidases of the cytoplasm are localized on a variety of membrane surfaces (7, 13) may explain not only the ease of solubilization of this activity and the existence of higher molecular weight complexes, but may provide a clue to the significance of the multiple forms of peroxidase and the varied biochemical reactions they can catalyze.

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