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ISOPEROXIDASES FROM TOBACCO TISSUE CULTURES*

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Abstract—Two anodic isoperoxidases (A_1 and A_2) from tobacco tissue culture W-38 and two cathodic isoperoxidases (C_3 and C_4) from tobacco suspension culture WR-132 have been separated and characterized. Molecular weights for each of the isoperoxidases have been determined by two different methods. Only C_4 contained a carbohydrate component. The substrate specificity and the pH optima for the four enzymes with each of five substrates were determined.

INTRODUCTION

Multiple forms of peroxidase exist in a large number of different plants [1]. Many of these isoenzymes of peroxidase from several plants, notably horseradish, have been purified, and in several cases such physical properties as MW and amino acid and carbohydrate compositions have been determined [2,3].

Although the isoperoxidases so studied have been shown to differ structurally, relatively little specific information is known about the effect of naturally occurring phenolic compounds on their activity. Reigh *et al.* [4] have reported that an isoperoxidase from tobacco tissue cultures, called A_3, can utilize scopoletin as a substrate, and Pickering *et al.* [5] have shown that ferulic acid may act as a substrate for tobacco tissue culture isoperoxidases A_2 and C_4. The present paper reports the isolation of two additional isoperoxidases, A_1 from W-38 tobacco tissue cultures, and C_3 from WR-132 tobacco suspension cultures. The MWs of these isoperoxidases, as well as of A_2 and C_4 reported earlier [5], have been determined. Only one of the four, C_4, was found to be a glycoprotein. The substrate specificities of the four isoperoxidases were determined with the naturally occurring phenolic compounds scopoletin, ferulic acid, chlorogenic acid and esculetin.

RESULTS AND DISCUSSION

Isoperoxidase A_1 from W-38 and isoperoxidase C_3 from WR-132 have now been separated from all the other isoperoxidases originally present. This separation has been accomplished by modifying and refining our purification techniques reported previously [5]. In particular, for isoperoxidase A_1, DEAE–cellulose chromatography was followed by CM-cellulose batchwise techniques to achieve final purification, while for isoperoxidase C_3, the procedure reported earlier was modified only by increasing the Pi buffer concentration to 0·05 M (pH 6) during CM-cellulose chromatography.

Since we had earlier demonstrated [5] that certain phenolic compounds, such as ferulic acid and chlorogenic acid, have a pronounced effect on the guaiacol oxidizing activity of isoperoxidases A_2 and C_4, similar studies were carried out using A_1 and C_3. The latter two isoperoxidases displayed remarkably similar characteristics towards the various phenolic compounds tested, as did isoperoxidases A_2 and C_4 previously reported [5], and thus, the data are not presented here. Furthermore, using methods previously described [5],

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ferulic acid can be shown to serve as a substrate for A_1 and C_3. These results extend the work of Gelinas which showed that commercial horseradish peroxidase could oxidize ferulic acid in the presence of H_2O_2 [6]. Thus, four isoperoxidases have now been isolated which have the ability to oxidize ferulic acid in the presence of H_2O_2.

In the investigation of the specificity of these four isoperoxidases for naturally occurring compounds, numerous phenolic compounds which affected the guaiacol oxidizing ability of the enzymes were examined as possible substrates. Thus far, two of the compounds, chlorogenic acid and esculetin, have been shown to act as substrates. This finding was verified using TLC on Avicel SF with methylisobutylketone–HCO_2H–H_2O (14:3:2) as solvent. Both compounds were shown to have disappeared after incubation with enzyme and H_2O_2 with the concomitant appearance of several new products in both cases. In addition, visible spectrophotometry revealed a new A_{max} for both compounds. Under the same conditions [4] that led to rapid oxidation of scopoletin by H_2O_2 in the presence of isoperoxidase A_3, very little, if any, oxidation of scopoletin could be observed with A_1, A_2, C_3 or C_4.

The significance of multiple substrates for individual isoperoxidases is currently unknown. Even the physiological reasons for multiple isoperoxidases in these cultures are not clear. Recently, an attempt to clarify certain physiological effects, such as dehydration, upon isoperoxidase substrate specificities for nonphysiological compounds has been reported [7]. Also, activity ratios between some of these synthetic substrates have been used to try to clarify the role of isoperoxidases in plants [8]. It seemed to us, however, of importance to attempt to relate these effects in terms of probable substrates *in vivo*. In order to do this, assays have been developed for each of the four phenolic substrates which occur naturally (chlorogenic acid, scopoletin, esculetin and ferulic acid) and the synthetic substrate guaiacol. Table 1 illustrates the pH optima for isoperoxidases A_1, A_2, C_3 and C_4 for each of the five substrates. A comparison of the potential to be a substrate for each isoperoxidase is shown in Table 2. In order to control as many variables as possible, all assays were run with that amount of isoperoxidase (either A_1, A_2, C_3 or C_4) which gave the same initial velocity for guaiacol. The velocity obtained for the other four substrates was then compared to guaiacol. In other words, an activity ratio, natural substrate to guaiacol, is presented in Table 2. These isoperoxidases catalyzed the oxidation of scopoletin very slowly and oxidized ferulic acid quite rapidly.

Table 1. pH Optima for isoperoxidases A_1, A_2, C_3 and C_4 for each of five substrates

Substrate	A_1	A_2	C_3	C_4
Ferulic acid	4·5	5·0	5·5	5·5
Scopoletin	5·25	4·5	4·5	4·5
Esculetin	7·5	7·5	7·5	7·5
Chlorogenic acid	5·0	4·5	5·5	5·5
Guaiacol	6·0	6·0	6·0	6·0

Although the four isoperoxidases exhibit similar behavior in response to both effectors and substrates, Table 3 reveals that the physical properties of these isoperoxidases are different. MW's as determined by SDS polyacrylamide gel electrophoresis and gel filtration chromatography are in reasonable agreement except for isoperoxidase A_1. In this case, the MW obtained from G-150 chromatography is approximately twice that obtained from SDS electrophoresis. Since SDS is known to disrupt subunit structure in proteins, a possible dimeric structure for isoperoxidase A_1 is suggested. These results are in conflict with those reported by Rucker *et al.* [9] who indicated

Table 2. Substrate specificity comparison: velocities for each naturally occurring compound are presented relative to velocity using guaiacol as substrate. Ratios are presented for each isoperoxidase (A_1, A_2, C_3, C_4) with each substrate at the pH indicated

Substrate	pH optima				pH = 4·5				pH = 5·25				pH = 6·0				pH = 7·5			
	A_1	A_2	C_3	C_4	A_1	A_2	C_3	C_4	A_1	A_2	C_3	C_4	A_1	A_2	C_3	C_4	A_1	A_2	C_3	C_4
Guaiacol	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0
Esculetin	0·35	0·33	0·68	0·63	0·10	0·05	0·37	0·26	0·14	0·17	0·49	0·32	0·20	0·27	0·32	0·34		0·70	1·1	1·4
Scopoletin	0·03	0·02	0·02	0·01	0	0·02	0·03	0·02	0·03	0·01	0·05	0	0·03	0·02	0	0·04		0·05	0·06	0·03
Chlorogenic acid	0·63	0·95	0·53	0·46	1·1	1·2	1·0	0·86	0·77	0·57	0·68	0·66	0·58	0·39	0·60	0·38		0·90	0·67	0·61
Ferulic acid	2·8	2·0	2·1	2·2	5·2	1·9	2·7	2·9	2·9	2·1	2·7	3·2	2·8	1·7	1·9	1·9		3·2	1·7	2·4

Table 3. MW's of isoperoxidases A_1, A_2, C_3 and C_4

Enzyme	SDS electrophoresis	Method of determination: Gel filtration G-150	Gel filtration Guanidinium HCl	Presence of carbohydrate
A_1	49000	103000		No
A_2	89000	90000		No
C_3	68000		67000	No
C_4	44000		46000	Yes

that all peroxidases from tobacco cultures are of similar MW. Furthermore, as shown in Table 3, only one of the four isoperoxidases, C_4, contains a carbohydrate component. Thus, all four isoperoxidases (A_1, A_2, C_3 and C_4) have different physical properties, but they behave similarly in their ability to oxidize the phenolic substrates studied.

The isolation of several isoenzymic components of peroxidase from tobacco tissue culture has revealed significant differences, mainly in physiological properties and more subtly in substrate specificities. Because of the proposed involvement of many of the naturally occurring phenolic compounds tested as precursors of lignin in higher plants [10], the results presented in this paper indicate that peroxidase is an essential enzyme in the initial stages of this conversion.

EXPERIMENTAL

Growth of tobacco cultures and electrophoresis were accomplished as previously described [5]. Isolation of isoperoxidases was described above.

MW determinations. MW's for the 4 isoperoxidases were determined using the SDS-gel electrophoresis procedure of ref. [11] in gels 7 cm long at a final acrylamide concn of 7·5%. Protein standards had MW's ranging from 25700 to 130000. *Ca* 50 μg of protein was applied to each gel and electrophoresis was continued for about 4·5 hr at 8 mA per gel. Protein bands were detected using Coomassie Blue as described in ref. [11]. Gels were stained for glycoprotein using the general procedure described in ref. [12] except that the gels were incubated in Schiff's reagent for approximately 18 hr. Schiff's reagent was prepared according to the method of ref. [13]. MW's for isoperoxidases C_3 and C_4 were also determined by gel filtration in guanidinium chloride as described in ref. [14]. A 90 × 1·5 cm column equipped with a Mariotte flask was packed with 4% Agarose (Sepharose 4B). Samples (0·5 ml) applied to the column contained 2 mg of each protein sample. The eluting solvent was 6 M guanidinium chloride in 50 mM acetate buffer, pH 4·75. MW's for C_3 and C_4 were obtained from a standard plot of distribution coefficient, K_d, for protein standards vs the log of their MW.

A Sephadex G-150 column prepared by the method of ref [15] in a 58 × 1·5 cm Ace glass column was used for a second determination of the MW's of isoperoxidases A_1 and A_2. The equilibrating and eluting buffer was 5 mM NaPi, pH 6. Protein standards and unknowns (2 mg of each) were dissolved in 2 ml of eluting buffer. Sucrose was added to the soln to increase the density and the entire 2 ml sample was layered on the top of the column bed using a Pro pipettor. Flow rate was adjusted to *ca* 20 ml/hr and 2 ml fractions were collected. $A_{280\,nm}$ was measured to determine the presence of protein peaks. The peroxidase activity of appropriate fractions was measured to determine the elution vol. of isoperoxidases A_1 and A_2. The MW's of A_1 and A_2 were obtained from a plot of elution vol. vs log of protein standard MW.

Enzyme assays. Peroxidase activity with respect to guaiacol was determined as previously described [5]. Assays for chlorogenic acid, scopoletin and esculetin as substrates were based upon the formation of oxidation products for the 3 substrates absorbing at 400, 450 and 469 nm, respectively. The peroxidase catalyzed oxidation of ferulic acid was monitored by following the loss of $A_{310\,nm}$. Determinations of pH optima and the comparison study were run at saturating conditions with respect to each substrate. The final concns in the assay solns were 15 mM for guaiacol, 1·25 mM for scopoletin, 1mM for esculetin and chlorogenic acid, and 0·2 mM for ferulic acid. Either Pi or citrate buffers were used, and buffer concns were maintained at 50 mM. H_2O_2 was maintained at 5 mM throughout. Enzyme reactions were initiated by the addition of 0·1 ml of the enzyme preparation. Activity ratios were calculated as described above.

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