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# ISOLATION AND CHARACTERIZATION OF TWO ISOPEROXIDASES FROM TOBACCO TISSUE CULTURES

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**Key Word Index**—*Nicotiana tabacum*; Solanaceae; tobacco; isoperoxidase; tissue culture.

**Abstract**—Two isoperoxidases ($A_f$ and $C_n$) from the medium of tobacco tissue suspension culture WR-132 grown in darkness have been purified to apparent homogeneity and partially characterized. $C_n$ and $A_f$ have MWs of *ca* 30 000 and 54 000, respectively. $A_f$ has *ca* 5.1% carbohydrate, but none could be detected in $C_n$. Both isoperoxidases appear to follow simple Michaelis–Menten kinetics with respect to guaiacol as the substrate. The $K_m$s for guaiacol are 4 and 13.3 mM for $A_f$ and $C_n$, respectively, while both isoperoxidases have a pH optimum at 6.5. $C_n$ is dissimilar to other isoperoxidases from tobacco tissue cultures, but $A_f$ is very similar to isoperoxidase $A_3$ from W-38 tobacco tissue culture.

## INTRODUCTION

In a study of the effect of light on various components of *Cichorium intybus* L. leaf tissues cultured *in vitro*, Legrand and coworkers [1, 2] found that the isoperoxidase (peroxidase isoenzyme) pattern was the same in both illuminated and non-illuminated tissues. The total peroxidase level, however, was greater in the dark-grown *Cichorium* leaf than in the light-grown leaf. The opposite effect was observed for the total phenolic compound content. Also, a considerable reduction in growth was noted when these *Cichorium* tissues were cultured in darkness rather than under continuous illumination (1.6 klx). In contrast, Penel and Greppin [3] have reported that transfer of spinach plants, grown with a daily photoperiod of 8 hr for 4 to 8 weeks, to continuous light (4 klx) enhanced the activity of acidic peroxidase while lowering the basic peroxidase activity. There also appeared to be some alteration in the isoperoxidase composition. Leu and coworkers [4] in our laboratory, using WR-132, have shown that a complete lack of illumination has a definite influence, both qualitatively and quantitatively, on the isoperoxidases present in both the cells and in the culture media. Most striking was the appearance of a new cathodic isoperoxidase, designated $C_n$, in cells grown in the dark. Five anodic isoperoxidases, designated as $A_a$, $A_b$, $A_d$, $A_e$, and $A_f$, which were not seen in normal cells, could also now be detected.

Thus, there appears to exist more than a casual relationship among the degree of illumination, phenolic compounds, plant growth, and the production and/or activity of isoperoxidases. In the study reported here, two of these major isoperoxidases ($C_n$ and $A_f$) have been isolated from *Nicotiana tabacum* tissue culture WR-132 grown in total darkness, and some of their kinetic, physical, and chemical properties have been investigated.

## RESULTS AND DISCUSSION

Two isoperoxidases, $A_f$ and $C_n$, from the medium of WR-132 tobacco tissue culture grown in darkness were isolated and purified to apparent homogeneity, as described in Experimental. These preparations were used in all the studies presented in this paper. $A_f$ is a very fast, anodic-migrating isoperoxidase, while $C_n$ is a very rapidly-migrating cathodic isoperoxidase. As reported previously by Leu *et al.* [4], neither of these two isoperoxidases is detectable by electrophoresis until the tissue culture is grown in darkness.

### *MWs*

The gel filtration chromatography method of Andrews [5] and the SDS gel electrophoresis procedure of Weber *et al.* [6] yielded values of 28 000 and 30 000, respectively, for the MW of $C_n$, and 53 000 and 54 000, respectively, for $A_f$. Table 1 lists MWs of other isoperoxidases from tobacco tissue cultures, WR-132, W-38, and Hicks 2. $C_n$ has the smallest MW of those reported, while the MW of $A_f$ from WR-132 is identical to the MW of $A_3$ from W-38. A wide range of MWs has been reported for isoperoxidases from various sources. These include values of 38 700, 50 000, 51 000, and 51 600 for 4 turnip isoperoxidases [7]; 33 400, 45 000, and 57 000 for 3 ribosome-associated isoperoxidases from lentil roots [8]; 40 000 and 50 000 for red alga isoperoxidases [9]; 40 000 for 5 horseradish isoperoxidases [10]; 60 000 for 4 peanut isoperoxidases [11]; and 30 000, 40 500, 44 000, and 54 500 for Japanese-radish isoperoxidases [12, 13]. Thus, the MWs for the tobacco isoperoxidases fall within the range of the typical MWs reported for many isoperoxidases from numerous higher plant sources.

### *Carbohydrate content*

Many isoperoxidases from different plants have been shown to contain carbohydrate. Morita and

Table 1. MWs of isoperoxidases from tobacco tissue culture WR-132 and W-38

| Source | Isozyme | MW Gel filtration | MW SDS electrophoresis |
|---|---|---|---|
| WR-132 | *$C_3$ | 67 000 | 68 000 |
| | *$C_4$ | 46 000 | 44 000 |
| | $C_n$ | 28 000 | 30 000 |
| | $A_c$ | 80 000 | 80 000 |
| | $A_f$ | 54 000 | 53 000 |
| W-38 | *$A_1$ | 103 000 | 49 000 |
| | *$A_2$ | 90 000 | 89 000 |
| | †$A_3$ | 54 000 | 54 000 |
| Hicks 2 | ‡C-1, C-2, C-3 | 50 000 | — |

*Data taken from Powell *et al.* [14].
†Data taken from Reigh *et al.* [15].
‡Data taken from Shinshi and Noguchi [16].

Kameda [17] have reported that Japanese-radish peroxidase a contains mannose, xylose, arabinose, and hexosamine. Turnip isoperoxidases contain glucose, mannose, fucose, glucosamine, and galactosamine [7]. Acidic horseradish peroxidases $A_1$, $A_2$, and $A_3$ contain arabinose, galactose, and mannosamine; wheras xylose, fucose, mannose, mannosamine, and galactosamine were identified in the basic isoperoxidases B and C [10]. Isoperoxidase $C_4$ from WR-132 also was reported to contain carbohydrate, but none could be detected in the $C_3$ isoperoxidase [18]. In the studies reported here, isoperoxidases $A_c$ and $A_f$ were shown to contain carbohydrate, but carbohydrate could not be detected as a component in $C_n$. $A_c$ is an anodic-migrating isoperoxidase that is present in the media from normally-grown as well as dark-grown WR-132 cells.

The total sugar content in $A_f$ is *ca* 5.1% with mannose, xylose, and amino sugars (*ca* 0.7%) being verified as the major carbohydrate compontents. On the other hand, $A_c$ and $C_4$ were shown to contain 4.2 and 4.8% carbohydrate, respectively. Both contained *ca* 0.5% amino sugars with the major carbohydrate components in $A_c$ being the same as $A_f$, while the carbohydrates in $C_4$ appear to be mainly galactose and arabinose. The total carbohydrate content in $A_c$, $A_f$, and $C_4$ is small when compared to most other carbohydrate-containing isoperoxidases. For example, five horseradish isoperoxidases [10], turnip isoperoxidases [7], and Japanese-radish peroxidase a [17] have been reported to contain *ca* 18, 12–18 and *ca* 20% carbohydrate, respectively. However, Japanese-radish peroxidase c contains *ca* 5% carbohydrate [19], which is very similar to the carbohydrate content of the isoperoxidases $A_c$, $A_f$, and $C_4$ from WR-132.

*Kinetic studies*

Isoperoxidases $A_f$ and $C_n$ appear to have a different catalytic activity when guaiacol is used as the organic substrate. $A_f$ has a broad pH optimum, extending from *ca* 6.5 to at least 8, while $C_n$ has a very narrow and sharp pH optimum at pH 6.5. At pH 6.5 both isoperoxidases exhibit the typical Michaelis–Menten kinetics [20] and the linear Lineweaver–Burk plots [21]. They yield $K_m$ values of 4 and 13 mM for $A_f$ and $C_n$, respectively. A comparison of pH optima and $K_m$ values for guaiacol oxidation of $A_f$ and $C_n$ to other isoperoxidases from tobacco tissue cultures is illustrated in Table 2. All these isoperoxidases exhibit pH optima between pH 5.5 and 6.5, although some of the optima are fairly broad. Furthermore, in this study, with the exception of $C_n$, all the $K_m$ values are very similar. The $K_m$ for $C_n$ ranges from 2 to 3 times higher than most of the tobacco isoperoxidases studied. In contrast to the results obtained from tobacco tissue cultures, 4 isoperoxidases isolated from peanut suspension cultures showed 10 to 30 times higher $K_m$

Table 2. Summary of Michaelis constants and pH optima for some phenolic substrates of isoperoxidases

| Substrate | $K_m$ values (mM) | | | | | | pH optima | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $A_f$ | $C_n$ | $A_3$* | $A_1$† | $A_2$† | $C_4$‡ | $A_f$ | $C_n$ | $A_3$ | $A_1$† | $A_2$† | $C_4$‡ |
| Scopoletin | 0.22 | — | 0.6 | — | — | 0.53 | 5.0 | — | 5.5 | — | — | 4.5 |
| Esculetin | 0.31 | 1.25 | 0.27 | — | — | 0.45 | 7.5 | 7.5 | 7.5 | — | — | 7.5 |
| Ferulic acid | 0.29 | 0.19 | — | 0.4 | 0.4 | 0.3 | 5.5 | 5.0 | — | 4.5 | 5.0 | 5.5 |
| Chlorogenic acid | 0.26 | 1.10 | — | — | — | — | 5.0 | 5.0 | — | — | — | 5.5 |
| Guaiacol | 4.0 | 13.3 | 4.0 | 5.0 | 5.8 | 4.0 | 6.5 | 6.5 | 6.5 | 6.0 | 6.0 | 6.0 |

| | $G_I$§ | $G_{II}$ | $G_{III}$ | $G_I$ | $G_{II}$ | $G_{III}$ |
|---|---|---|---|---|---|---|
| Scopoletin | 0.235 | 0.95 | — | — | — | — |
| Ferulic acid | 0.125 | 0.11 | 0.057 | — | — | — |
| Chlorogenic acid | 0.625 | 0.8 | 0.15 | 5.8 | 5.5 | 5.5 |
| Caffeic acid | 0.51 | 0.43 | 0.31 | — | — | — |
| Guaiacol | 3.28 | 5.26 | 3.85 | 5.5 | 5.8 | 6.0 |

*W-38 strain: Data were supplied by Reigh [22].
†W-38 strain: Data were supplied by Powell [14].
‡WR-132 strain: Data were supplied by Pickering [18].
§*Nicotiana tabacum* var. Burley strain: Data were taken from Mader *et al.* [23].
—: No results reported.

values than the tobacco tissue culture isoperoxidases. In a search for physiological substrates for $A_f$ and $C_n$, we initiated studies similar to those previously done in our laboratory [14, 15, 18, 22, 24, 25]. We have found that numerous naturally occurring phenolic compounds will serve as substrates for these 2 isoperoxidases. These results in the form of $K_m$ values and pH optima are listed in Table 2 along with the data for other isoperoxidases. Interestingly, $A_f$ oxidizes scopoletin very rapidly, while $C_n$ has to be incubated several hours with scopoletin and $H_2O_2$ before any oxidation is detectable. The color changes, non-linear Lineweaver–Burk plot, and the $n$ greater than 1 for the Hill plot [26], which were observed by Reigh *et al.* [15] when $A_3$ caused the oxidation of scopoletin, are identical to those observed with $A_f$. Also, studies with esculetin and other naturally occurring substrates show marked similarity between $A_f$ and $A_3$. It therefore appears that isoperoxidase $A_3$ from W-38, which elaborates a wide range of isoperoxidases, is the same as isoperoxidase $A_f$, which is present in detectable quantities only when WR-132 is grown in total darkness. Furthermore, when grown in the light, WR-132 elaborates only a limited number of isoperoxidases. Also, $A_3$ and $A_f$ have the same electrophoretic mobility that has been reported many times for one of the isoperoxidases obtained from many different higher plant systems. On the other hand, isoperoxidase $C_n$ appears to have no counterpart in all the isoperoxidases studied so far.

## EXPERIMENTAL

*Growth of tobacco cultures.* Tobacco tissue WR-132 (*Nicotiana tabacum* L., var. Xanthi), obtained from Dr. A. C. Olson of USDA, Albany, Califorina, was grown in the dark by the method outlined in ref. [4]. Each passage of cells consisted of 10 days' growth.

*Preparation of isoperoxidases.* Since isoperoxidases $A_f$ and $C_n$ were found in much larger amounts in the medium than in the cell extracts in the dark-grown culture, the medium was used as the source for the isolation of $A_f$ and $C_n$. Typically, media from the third and fifth dark-grown passages were used in the prepn of $A_f$ and $C_n$, respectively. *Ca* 1 l. of medium was collected by filtration and then subjected to $(NH_4)_2SO_4$ precipitation. The protein which precipitated between 20 and 90% satn was collected by centrifugation (27 000 *g* for 20 min), redissolved in a minimum vol. of 5 mM Na-Pi buffer (pH 6), and this soln was dialysed against 100 vols. of $H_2O$ for 24 hr with 3 changes. Protein (15–20 mg) (*ca* 15 ml of the dialysed sample) was applied to a CM-cellulose column pre-equilibrated with the 5 mM Na-Pi buffer (pH 6). Initial elution with $H_2O$ yielded largely a mixture of isoperoxidases $A_c$ and $A_f$, which after concn by lyophilization, could be separated by chromatography on a DEAE-cellulose column. $A_c$ eluted with 10 mM Na-Pi buffer (pH 6), while $A_f$ could be eluted with 40 mM Na-Pi buffer (pH 6), if a stepwise elution is used. As judged by electrophoresis (both starch gel and disc gel), both isoperoxidases were free from contaminating proteins. $C_n$ was isolated from the CM-cellulose column when stepwise elution with Na-Pi buffer from 10 to 100 mM (pH 6) followed exhaustive washing with $H_2O$. The most active $C_n$ fractions were obtained from the 50 mM elutions. Occasionally, $C_3$ co-eluted with $C_n$, but these could be separated using Sephadex G-150 filtration.

*MW determination.* The MWs of $A_f$ and $C_n$ were estimated by (a) gel filtration chromatography using Sephadex G-150 according to the method of ref. [5], and (b) gel electrophoresis procedure of ref. [6]. Combinations of lipoxidase, creatine kinase, serum albumin, ovalbumin, α-chymotrypsinogen, β-galactosidase, and catalase were used as the standard proteins with known MWs.

*Glycoprotein determination.* To determine if the isoperoxidases contain carbohydrate, the procedure described in ref. [27] was used with the Schiff's reagent of ref. [28] being the visualizing agent.

*Gel electrophoresis.* Starch gel electrophoresis was performed, using a modified Smithies apparatus [29], as illustrated in ref. [4]. Anodic disc gel electrophoresis was performed using a Buchler Polyanalyst apparatus according to the method of ref. [30]. Gels were 7.5% acrylamide and 0.2% *N,N'*-methylene bisacrylamide. The running pH was 9.5 with bromophenol blue used as the tracking dye. Cathodic disc gel electrophoresis was performed in the same manner except that the electrical current was reversed. K persulfate was used as the gel polymerization catalyst. The running pH was 4.3 with methyl green as the tracking dye. In the disc gel electrophoresis peroxidase bands were visualized by placing gels in a mixture of 2 parts of 1% guaiacol, 2 parts 50 mM Na-Pi buffer (pH 6) and 1 part of 0.2% $H_2O_2$. Protein staining was accomplished using the method of ref. [6].

*Peroxidase assays.* When guaiacol was used as a substrate, the method of Lance, as modified in ref. [31], was followed. The methods of refs. [22, 25] were used when either scopoletin or esculetin were used as substrates, while the methods of refs. [14, 18] were used with ferulic acid and chlorogenic acid. Protein concns were determined according to ref. [32].

Total neutral sugars were determined on the intact protein with the anthrone reagent of ref. [33], using a mixture of galactose and mannose in a ratio of 1:1 as the standard. To determine the carbohydrate composition, neutral and amino sugars were released from the protein by hydrolysis at 100° for 20 hr in N $H_2SO_4$ *in vacuo*. The hydrolysate was column chromatographed according to the procedure of ref. [34]. The eluted amino sugars were detected by the Elson–Morgan reaction [34]. To identify the neutral sugars, PC was employed using aniline hydrogen phthalate as the visualizing agent [35].

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