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PURIFICATION AND UNUSUAL KINETIC PROPERTIES OF A TOBACCO ANIONIC PEROXIDASE*

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Key Word Index—*Nicotiana sylvestris*; Solanaceae; tobacco; peroxidase; purification; spectroscopic and molecular properties; substrate specificity; thermal stability.

Abstract—The tobacco anionic peroxidase has been isolated from the leaves of transgenic *Nicotiana sylvestris* plants overproducing this enzyme. The plant expression system and the purification protocol developed allow the preparation of greater than 60 mg of homogeneous enzyme (M_r 36 kDa, pI 3.5) from 1 kg of fresh leaves, which is an order of magnitude higher than for wild-type tobacco plants. The tobacco anionic peroxidase exhibits rather unusual catalytic properties in comparison with horseradish peroxidase (HRP C). Compound I is less active than Compound II in the tobacco enzyme. The enzyme is nearly inactive towards iodide, reflecting the peculiarities of its molecular structure. In particular, the presence of the negatively charged glutamate residue 141 at the entrance of the haeme-binding pocket seems to affect the stabilities of Compounds I, II and III, leading to a different enzyme substrate specificity than that of HRP C. Investigation of thermal stability towards a number of electron donors reveals the following 'order of stabilities': ferrocyanide > guaiacol > 2,2′-azino-bis(3-ethyl-6-benzothiazoline sulphonate) > iodide > *o*-dianisidine, which may indicate different binding sites and rate-limiting steps in the mechanism of the substrate oxidation.

INTRODUCTION

Peroxidase (EC 1.11.1.7) has attracted considerable interest for decades in part because of its activity towards a wide array of chromogenic substrates. The availability and abundance of horseradish peroxidase (HRP C) in horseradish root has made this peroxidase isoenzyme the subject for numerous biochemical studies. In contrast, the biochemical characterization of the tobacco anionic peroxidase, which is localized predominantly in the aboveground regions of the plant, has been slow because of the small quantities present [1]. Progress in the elucidation of structure–function relationships for haeme-containing plant peroxidases, which play important physiological roles, is closely related to the isolation and biochemical description of novel enzymes with known primary structures. However, further success depends on the amount of enzyme available for detailed studies including crystallography, NMR and Raman spectroscopy, rapid kinetics, etc. To obtain sufficient quantities of the tobacco anionic peroxidase for such studies we have used a genetically engineered tobacco plant which overexpresses this peroxidase isoenzyme.

The cloning [2] and expression [3] of the tobacco anionic peroxidase gene in transgenic tobacco plants has led to the overproduction of the wild-type enzyme. The present paper describes the purification procedure for the tobacco anionic peroxidase and its biochemical properties. The results obtained show that the tobacco peroxidase exhibits unusual kinetic properties affecting its substrate specificity.

*Part 1 in the series Tobacco Anionic Peroxidase Overexpressed in Transgenic Plants.

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RESULTS AND DISCUSSION

Purification of the tobacco anionic peroxidase

According to the data presented in Table 1, transgenic tobacco leaves can be considered as a prospective source of peroxidase. The yield of the homogeneous enzyme was *ca* 60 mg kg^{-1} of fresh leaves. The total peroxidase activity was an order of magnitude higher than for the wild-type tobacco plants [3]. One undesirable effect of peroxidase overproduction was an increase in the content of polyphenolic pigments [4], which appeared to inhibit peroxidase activity. However, no activity loss was observed during pigment removal and DEAE-chromatography, although 10–15% of the activity did not adsorb on DEAE-cellulose. Thus, total peroxidase activity showed an apparent increase by 10–15%, and total activity yield following gel filtration was *ca* 80%. The preparation exhibited a single band according to SDS-PAGE and had a M_r of 36 kDa and pI 3.5 as estimated earlier [2]. The specific activity of the tobacco anionic

Table 1. Tobacco peroxidase purification

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U mg^{-1})	Purification (-fold)	Yield (%)
Homogenate	350000	n.d.	n.d.	1	100
Pigment removal	350000	760	460	1	100
DEAE	350000	159	2200	4.8	100
Gel filtration	280000	60.9	4600	10	80

n.d. = not determined.

peroxidase towards 2,2′-azino-bis(3-ethyl-6-benzothiazoline sulphonate) (ABTS) in 0.05 M citrate buffer at pH 4.5, which has been shown to be optimal (Fig. 1), was 4600 U mg^{-1} of protein.

Spectroscopic properties

The RZ value, which is usually considered as a proof of the peroxidase quality, was 3.4. The spectrum of the native tobacco peroxidase is shown in Fig. 2 (curve a) with a Soret maximum at 403 nm, and 497 and 635 nm bands in the visible region. Compound I (Fig. 2, curve b) is easily produced at pH 4.5 by hydrogen peroxide addition at 3 to 300-fold excess to enzyme and is very stable. This results in a characteristic 350 nm shoulder in the Soret peak and absorbances at 554 and 655 nm in the visible region. The spectrum exhibits no changes after 20 min. This is in contrast to HRP which reacts with equimolar amounts of hydrogen peroxide to form a mixture of Compounds I and II independent of the peroxidase concentration [5].

An attempt to obtain the tobacco anionic peroxidase Compound II at pH 4.6–5.0 was unsuccessful. Compound I converted directly into Compound III (Fig. 2, curve c) at very high ratios of hydrogen peroxide to enzyme (1000-fold). Compound III revealed a Soret band at 414.5 nm and two characteristic peaks–544 and 578 nm—in the visible region. Compound III itself was also very stable (> 40 min), and did not show Compound II as a decomposition intermediate (Fig. 3). Inactivation proceeded via a decrease of the Soret band, and an appearance of 'so called' compound P-670 characteristic for HRP Compound III decomposition. Within 60 min the RZ dropped to 1.0 and the residual catalytic activity towards ABTS was less than 2% of the initial. The Compound II spectrum, with characteristic peaks in the visible region (527 and 557 nm), was only observed at pH 7.5 and 30–50 fold excess of hydrogen peroxide over the enzyme. However, the wide shape of the Soret band and its shift in the 411–415 nm range (Fig. 4) revealed the presence of the Compound I species.

Thus, the newly obtained tobacco anionic peroxidase exhibits very interesting kinetic properties–its Compound I is less active than Compound II. In this, it is different from most of the other plant peroxidases including the commercial horseradish enzyme. The spectral data on the reaction with hydrogen peroxide indicate that at neutral and alkaline pH the reactivity of the

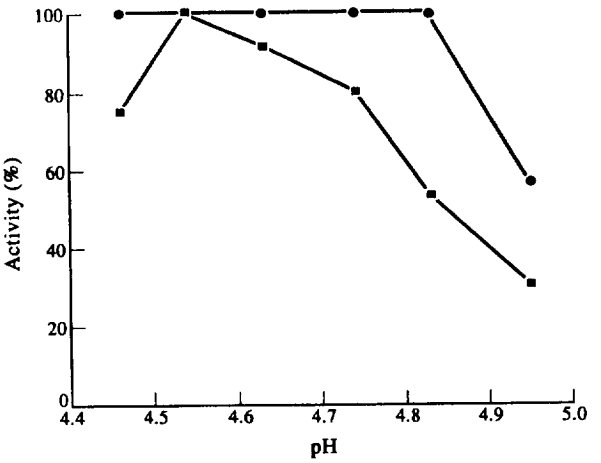

Fig. 1. pH-dependence of ABTS oxidation catalysed by tobacco (—■—) and horseradish (—●—) peroxidases (0.05 M Na citrate buffer, 10^{-8} M peroxidase, 25°).

tobacco peroxidase Compound II decreases and becomes close to that of Compound I. From this we would predict that the pH-dependence of the reactions catalysed by the tobacco peroxidase should exhibit sharp maxima. Actually, the pH-dependence of ABTS oxidation by the tobacco peroxidase showed the maximum at pH 4.55, whereas the HRP demonstrated a plateau in the pH range 4.45–4.85 (Fig. 1).

Catalytic properties

The comparative data on substrate specificity (Table 2) also indicate a low activity of Compound I. The tobacco peroxidase is nearly inactive towards potassium iodide, which is a two-electron donor interacting directly with Compound I, in contrast to the other one-electron donor substrates tested. The tobacco enzyme is very active towards ferrocyanide compared to the horseradish enzyme. This probably reflects the nature of the rate-limiting step in ferrocyanide oxidation, which seems to be the reduction of Compound II.

Figure 5 presents the thermal inactivation data on the horseradish and tobacco peroxidases, which demonstrate nearly the same stability at elevated temperature. Unfortunately, it was difficult to measure the activities of the tobacco peroxidase towards iodide and of horseradish peroxidase towards ferrocyanide, even with high enzyme

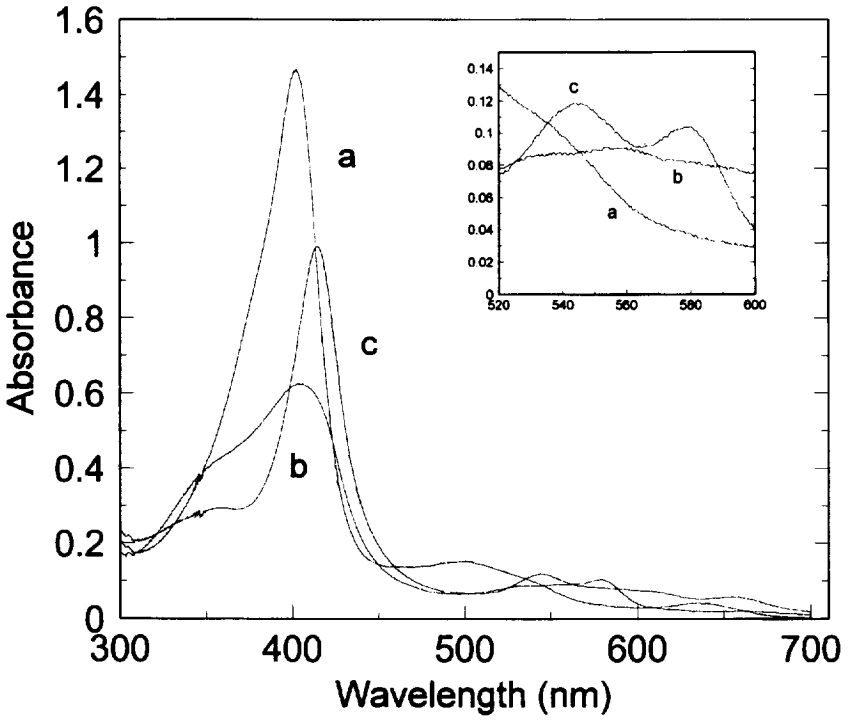

Fig. 2. The absorption spectra of the native tobacco peroxidase (a) and its Compound I (b) and Compound III (c) in 0.05 M Na citrate buffer, pH 4.5, in the presence of (b) 50- and (c) 1000-fold excess of hydrogen peroxide. The insert is an enlargement of the 520–600 nm region of the spectrum.

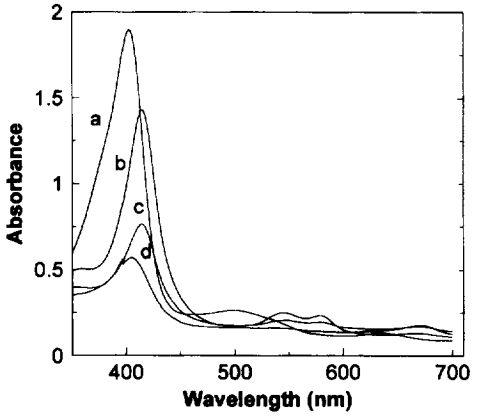

Fig. 3. Stability of the tobacco peroxidase Compound III: (a) the original spectrum of the native enzyme; (b) Compound III spectrum obtained in the presence of 500-fold excess of hydrogen peroxide; (c) and (d) the spectral changes observed 40 and 80 min later, respectively, during incubation at 25°.

concentrations. Nevertheless, it was the first experiment comparing peroxidase thermal stabilities in terms of their substrate specificity. The activity of peroxidase towards different substrates can help predict reaction mechanisms. The 'order of stabilities' of the tobacco enzyme for the activities towards different substrates was ferrocyanide > guaiacol > ABTS > iodide > *o*-dianisidine. Such an order identifies different mechanisms of the substrates' oxidation in terms of the substrate binding sites and rate-limiting steps. The conformational changes caused by elevated temperature affect primarily the activity towards organic substrates. Thus, the protein moiety provides the actual binding sites for organic substrates and/or the different arrangements of the electron transport chain from the haeme moiety to a donor substrate.

One possible explanation for the unique catalytic properties of the tobacco anionic peroxidase can be related to its primary structure. All plant peroxidases are characterized by the highly conserved peptides coordinating the haeme moiety, but exhibit variability in the sequences controlling the accessibility of the active centre—so called 'entrance to the haeme-binding pocket' [6]. The comparison of these sequences for tobacco and horseradish peroxidases show the presence of the negatively charged group—*Glu*-141 in the tobacco enzyme [2] corresponding to *Phe*-143 in the horseradish enzyme [7]—which may influence the enzyme reactivity towards donor substrates:

HRP 66 DAFGNAN ... helix C
TOP 65 DAPANVG ... helix C
HRP 138 LPAPFF ... helix E
TOP 136 IPSPFE ... helix E

Glu-141 could affect both the accessibility of the active centre for electron donors and the stability of Compound I. In the case of iodide oxidation both effects are

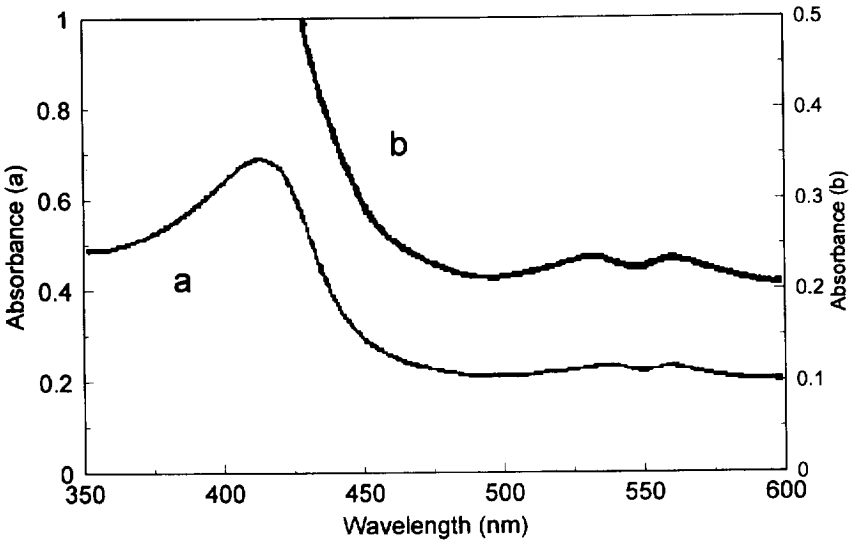

Fig. 4. Appearance of the Compound II spectrum (a) after 2 min incubation of 10 μM enzyme with 300 μM hydrogen peroxide in 0.1 M K-Pi buffer, pH 7.5 at 25°, and (b) magnification of (a) in the visible region.

Table 2. Molecular properties and substrate specificity of the tobacco anionic peroxidase (TOP) and horseradish peroxidase (HRP C) (Sigma)

Property	TOP	HRP C
M_r (kDa)	36	44
pI	3.5	8.9
RZ	3.4 ± 0.2	2.0
Specific activity		
ABTS ($U mg^{-1}$)	4600 ± 200	2700 ± 100
Relative activity (%)		
ABTS	100	100
Guaiacol	85 ± 2	170 ± 10
o-Dianisidine	130 ± 5	145 ± 5
Ferrocyanide	30 ± 2	5.5 ± 0.5
Iodide	0.07 ± 0.005	10 ± 0.5

Data presented as means ± S.E. ($n = 3$).

overlapping and lead to a very low enzymic activity towards this substrate. The replacement *Phe*-143 > *Glu* simulating the tobacco peroxidase was made recently for recombinant HRP expressed and reactivated from *Escherichia coli* inclusion bodies [8]. This had a significant effect on the enzymic properties of HRP. The appearance of the negatively charged *Glu* residue at the entrance of the HRP haeme-binding pocket led to the increased stability of the mutant against radicals and solvated electrons produced under conditions of low-dosage radiolysis [8]. These results coincide with the catalytic properties of the newly isolated tobacco peroxidase which has *Glu*-141 at the entrance of the active centre. Site-directed mutagenesis of the tobacco enzyme should confirm the role of *Glu*-141 in catalysis.

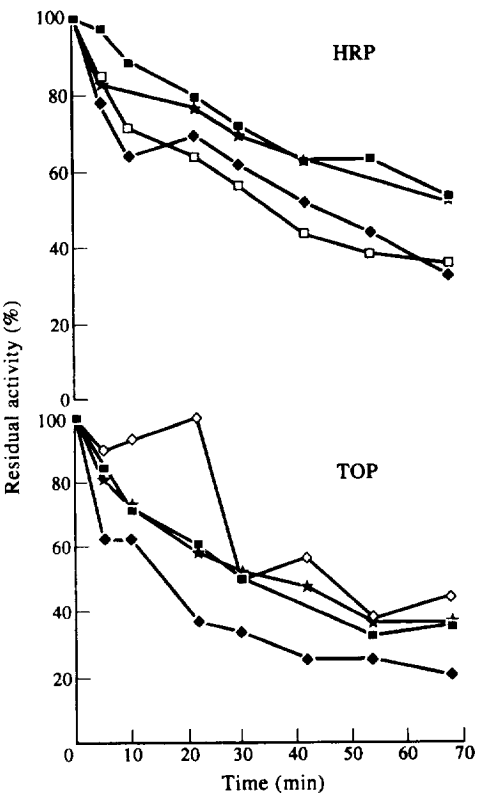

Fig. 5. Thermal inactivation profile for horseradish (HRP) and tobacco anionic peroxidase (TOP) using FeCN (—◇—), guaiacol (—■—), ABTS (—*—), KI (—□—) or *o*-dianisidine (—◆—) as substrate. Thermal inactivation was performed with 0.2 μM enzyme in 0.1 M Tris–HCl buffer, pH 6.0, at 65°.

CONCLUSIONS

The overproduction of the tobacco anionic peroxidase in transgenic plants allowed us to obtain sufficient enzyme for kinetic and spectroscopic studies. We have determined the physiological consequences of the specific over- or under-expression of the anionic isoenzyme in transformed plants [3], and are concluding an extensive study of the spatial and temporal pattern of expression of the anionic peroxidase gene. It is now critical that we have a firm understanding of the biochemical properties of this protein to tie together expression patterns to catalytic activity and finally the physiological consequences of enzyme function. For purposes of comparison we have chosen the benchmark peroxidase—HRP. Characteristics of the newly isolated tobacco anionic peroxidase distinguish it from commercial HRP in its catalytic properties and provide new insight into complicated reaction mechanisms. We have already revealed several significant differences between the two enzymes, and are now focusing our attention on developing a biochemical model which will describe the functioning of the tobacco anionic peroxidase in growth and development.

EXPERIMENTAL

Enzyme purification. Transgenic *Nicotiana sylvestris* plants were obtained as described previously [3]. Leaf tissue (1 kg) from immature plants was homogenized in 2 l 0.1 M Tris–HCl buffer, pH 6.0. The disrupted tissues were removed by filtration and centrifugation (10000 rpm, 20 min), and the homogenate obtained (3.5 l) was treated with DE-52 cellulose to remove the brown pigment. The filtrate was then diluted 10-fold and applied to a DE-52 column cellulose equilibrated with 0.01 M Tris–HCl buffer, pH 6.0. Enzyme was eluted by a continuous gradient of 0.1–0.15 M Tris–HCl buffer, pH 6.0. The active (40 ml) were collected and applied to a Sephacryl S-200 column (5 × 100 cm) in 0.01 M Tris–HCl buffer, pH 6.0. The active frs were collected, sterilized by filtration and stored at 4°.

Enzyme assays. Activity was expressed in U (μmol min^{-1}) using 0.36 mM ABTS (ammonium salt) (Boehringer Mannheim) and 5.88 mM H_2O_2 at 25° in 50 mM Na citrate buffer at the estimated pH optimum for the tobacco peroxidase (pH 4.5). An absorption coefficient at 405 nm of 36.8 mM^{-1} cm^{-1} was used [9]. The substrate specificity of the tobacco peroxidase was compared to that of the horseradish enzyme with the following substrates: 2.25 mM guaiacol and 5.88 mM H_2O_2 in 0.1 M K-Pi buffer, pH 6.0, absorption coefficient at 470 nm, 5.57 mM^{-1}cm^{-1} [10]; 0.54 mM ferrocyanide and 5.88 mM H_2O_2 in 0.1 M K-Pi buffer, pH 7.0, absorption coefficient at 420 nm, 1.04 mM^{-1}cm^{-1} [11]; 0.127 mM *o*-dianisidine and 5.88 mM H_2O_2 in 0.1 M K-Pi buffer, pH 7.0, absorption coefficient at 460 nm, 30.0 mM^{-1}cm^{-1} [12]; 1.68 mM KI and 5.88 mM H_2O_2 in 0.1 M Na acetate buffer, pH 5.0, absorption coefficient at 353 nm, 25.5 mM^{-1}cm^{-1} [13]. All kinetic and spectral measurements were performed at 25° using a Shimadzu UV 160 spectrophotometer.

Thermal stability was investigated by incubating 0.2 μM enzyme solns in 0.1 M Tris–HCl buffer, pH 6.0, at 65° for various times. After heating, the tubes were placed at 4° for 1 hr to restore the activity lost due to the reversible inactivation. Aliquots from each tube were used to measure the activity towards different substrates. The activities were expressed as % initial activity.

The spectral changes induced by H_2O_2 addition were studied in 50 mM Na citrate buffer, pH 4.5, at 25°.

Protein assay. The protein content was calculated by the formula using absorbance at 230 and 260 nm [14] and by the Bradford method [15] with BSA as standard. Both methods gave the same results for purified enzyme samples.

Electrophoresis. SDS-PAGE was performed according to ref. [16] followed by Coomassie Brilliant Blue and/or GELCODE silver staining (Pierce). Isoelectric focusing was performed using Pharmacia PAG plates (pH 3.5–10.5) on a Pharmacia FBE-3000 flat bed apparatus using a Bio-Rad Model 3000 Xi electrophoresis power supply. Electrophoresis gels were developed for peroxidase activity in the presence of 8.8 mM H_2O_2, 3,3′-diaminobenzidine (0.2 mg ml^{-1}) and $CoCl_2$ (0.04 mg ml^{-1}) in 50 mM K-Pi buffer, pH 7.6, containing 1 M NaCl.

Reagents. All reagents were purchased from Sigma unless otherwise stated.

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