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A LACCASE-TYPE POLYPHENOL OXIDASE FROM LIGNIFYING XYLEM OF TOBACCO

ANDREW RICHARDSON and GORDON J. MCDOUGALL*

Unit for Industrial Crops, Cell and Environmental Physiology Department, Scottish Crop Research Institute, Invergowrie, Dundee DD2 5DA, U.K.

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Key Word Index—*Nicotiana tabacum*; Solanaceae; tobacco; polyphenol oxidase; laccase; coniferyl alcohol; purification; lignification; cell wall; xylem.

Abstract—Lignifying xylem from tobacco (*Nicotiana tabacum*) expresses oxidase activity capable of oxidizing a range of chromogenic substrates by a non-peroxidative mechanism. These oxidases appear to be ionically bound to the cell wall and can be extracted using 1M NaCl. The extracted oxidases can oxidize the monolignol coniferyl alcohol. Extracts from xylem cell walls contain a number of different oxidase isoforms with isoelectric points in the neutral to mildly acidic range. Xylem from younger, apical tobacco stems yield a different set of oxidase isoforms than xylem from older, basal areas which suggests that oxidase isoforms may be differently expressed during xylem maturation. Non-denaturing SDS–PAGE confirms the presence of a band of oxidase activity (M_r *ca* 100 kDa) in these extracts that has properties similar to those of the laccase-type polyphenol oxidases (*p*-diphenol: O_2 oxidoreductase; EC 1.14.18.1) previously identified in the lignifying tissues of trees. Ion-exchange chromatography on DEAE–Sepharose retained this 100 kDa laccase-like activity and resulted in a ten-fold purification and a six-fold increase in the recovery of oxidase activity, probably as the result of the removal of inhibitors. In contrast, a subsequent hydrophobic interaction chromatography step was unsuccessful, probably as a result of the precipitation of the laccase-like oxidase in the concentrated ammonium sulphate buffers required for this procedure. Copyright © 1996 Elsevier Science Ltd

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INTRODUCTION

There has been a reawakening in the possibility, first suggested by Freudenberg [1], that cell wall-associated laccase-type polyphenol oxidases could participate in the final enzymic step of lignin formation, the oxidation and polymerization of monolignols. These enzymes appear to fulfil the prime criteria for lignin-specific enzymes: they are specifically expressed in the cell walls of lignifying tissues [2, 3] and can oxidize and polymerize monolignols to lignin-like polymers *in vitro* [2–5]. It has been proposed that these enzymes play a specific role in the earliest stages of lignin deposition, possibly in the cell walls of living cells, where use of oxygen as their co-substrate would give them advantages over peroxidases and cytotoxic hydrogen peroxide [5]. As this role would also involve these enzymes in forming the first lignin structures, they have been identified as a target for the genetic manipulation of the content, structure and cross-linking (and thereby extractibility) of lignin [6, 7]. Apart from a recent paper that correlates the presence of laccase-like polyphenol oxidases with the lignification of *Zinnia* stems [8] and previous studies from this laboratory [9, 10], comparatively little work has been done on the possibility that these enzymes may participate in lignin deposition in plants other than trees. This paper reports the extraction and partial purification of a laccase-like oxidase from the lignifying xylem of tobacco stems.

RESULTS

Localization of oxidase. Cytochemical studies located oxidase activity against ABTS, TMB and SYR exclusively in the outermost, actively lignifying xylem of tobacco stems (Fig. 1). The activity was exclusively present in the outermost, newly formed ring of xylem throughout the length of the stem. The activity was also unaffected by the presence of concentrations of catalase that interfered with the staining of peroxidase activity in similar sections (results not shown).

Oxidase isoforms in cell wall extracts. Oxidase activity against ABTS was greatly enriched in the 1M NaCl extracts and, by analogy to previous reports [3,

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*Author to whom correspondence should be addressed.

Fig. 1. Oxidation of ABTS by tobacco xylem. The cross-section is from the 5th internode of an 8-week old tobacco stem. The dark coloration exclusively present in the outer, newly formed layers of the xylem (noted by arrow) is due to the formation of the oxidized ABTS chromophore. ×24; bar = 0.4 mm.

11, 17], is probably associated with the cell wall. The presence of an appreciable, but unquantifiable, amount of activity that remains associated with the cell walls, even after extraction with 1M NaCl–Triton X-100 and then treatment with glycanases [9], confirms that these oxidases are cell wall enzymes and suggests that they may be attached to the wall in a manner similar to peroxidases [11].

A preparation of ionically bound cell wall proteins from xylem from the entire tobacco stem yielded three distinct isoforms upon ion-exchange chromatography (Fig. 2). The third and most acidic of these isoforms (estimated isoelectric point of 5–6; shaded area, Fig. 2) was pooled for further purification as it was the most abundant and had the lowest peroxidase activity.

Ionically bound cell wall proteins from xylem of the older, basal half of the tobacco stem contained only one main oxidase isoform (Fig. 3(a)), whereas the same fraction from the xylem of the younger, apical half of the stem contained four discernible oxidase isoforms (Fig. 3(b)). As equivalent amounts of protein were applied to the ion-exchange step from two tissues, this suggests that three of these oxidase isoforms are either not expressed or not expressed in an active form in older, more mature xylem.

Non-denaturing SDS–PAGE of oxidase activity. An oxidase band of estimated M_r 100 000 capable of oxidizing ABTS could be detected in samples of ionically bound xylem cell wall proteins separated by non-denaturing SDS–PAGE (Fig. 4, lane a). An oxidase band of similar M_r was also present in the most abundant isoform fraction from ion-exchange chromatography (Fig. 4, lane b). The intensity of staining of these bands was neither enhanced nor diminished by

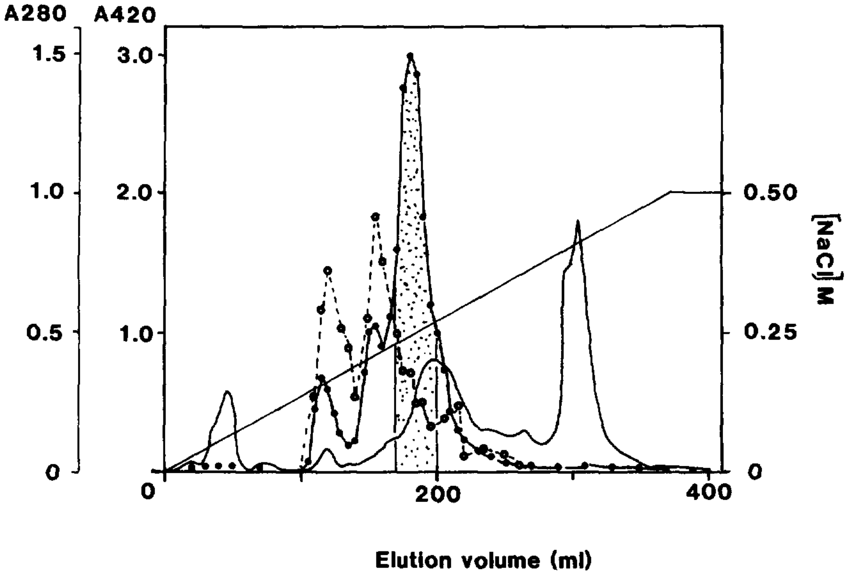

Fig. 2. Separation of oxidase isoforms from tobacco xylem cell walls by ion-exchange chromatography on DEAE–Sepharose. Ionically bound cell wall proteins were obtained from xylem from the entire stem. Protein content (A_{280}) is denoted by the solid line. Oxidase activity is shown by the closed circles, peroxidase activity by the open circles. The gradient of 0–0.5 M NaCl is also shown. The shaded portion represents the pooled fraction.

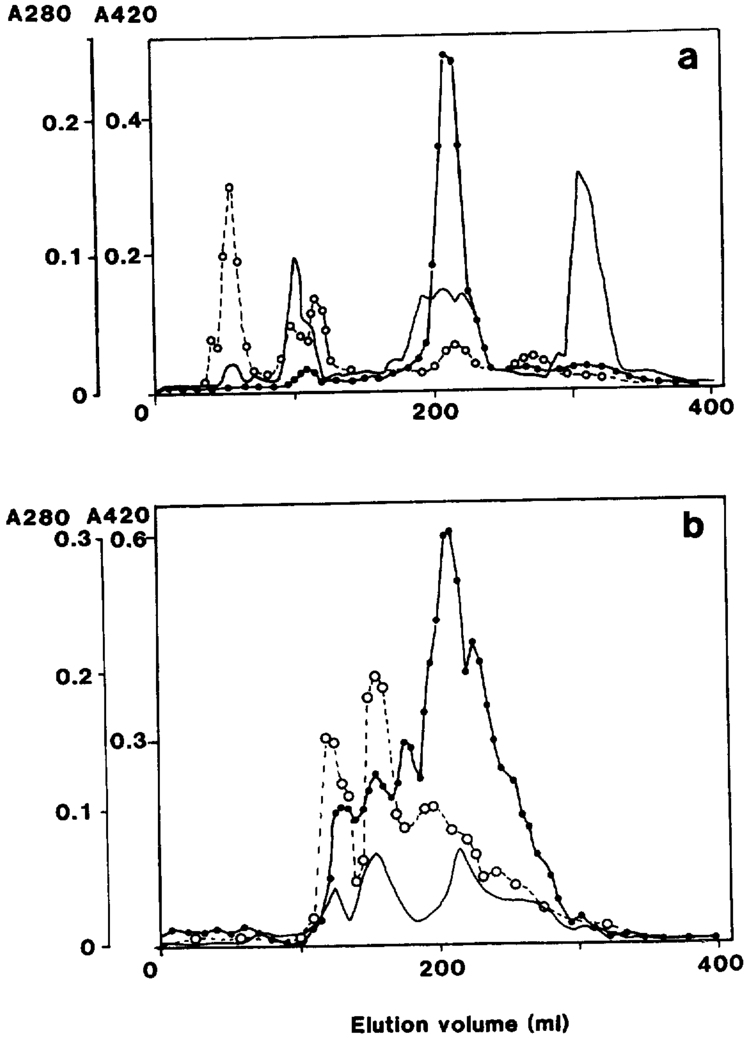

Fig. 3. Oxidase isoforms of xylem cell walls from apical and basal portions of tobacco stem. Ionically bound cell wall proteins from the xylem of basal (a) and apical (b) portions of the tobacco stem were separated by ion-exchange chromatography on DEAE–Sepharose. Approximately equal amounts of protein were applied to each. See the legend of Fig. 2 for description of other features. The salt gradients are omitted to avoid confusion.

the addition of 2.5 mM H_2O_2, although other peroxidase bands were identified.

Purification of oxidase isoforms. The major oxidase isoform prepared by ion-exchange chromatography was subjected to hydrophobic interaction chromatography (HIC). The oxidase isoform, which was apparently homogenous after ion-exchange, was eluted as three distinct peaks on the HIC column (Fig. 5). These three forms must have different hydrophobic properties and may differ in their glycosyl composition. Indeed, all of the oxidases from tobacco xylem bound tightly to Concanavalin-A Sepharose and are probably glycoproteins. Microheterogeneity in the glycosyl groups of laccases from *Acer pseudoplatanus* has been noted [12]. Although the pooled fractions from the HIC step had activity against ABTS (Table 1), the high concentration of $(NH_4)_2SO_4$ (0.1–0.2 M in the assay) interfered with the CA-oxidase assay, which is based on the reduction of A_{260}. As a result, CAO assays were carried out on pooled, desalted samples and these samples had no detectable activity against CA and much reduced activity (<5% of original) against ABTS. Further studies suggested that the oxidase is unstable in the concentrations of $(NH_4)_2SO_4$ used in the HIC procedure and likely to precipitate. Despite the quality of separation provided by this method, it is clear that other means of purification must be sought.

All of the oxidase isoforms separated by ion-exchange chromatography (Fig. 2) could oxidize coniferyl alcohol. This is illustrated by the increase in the specific activity against coniferyl alcohol noted during the purification procedure (Table 1). The ion-exchange step results in a great loss of contaminating protein and a ten-fold increase in specific activity. This step also

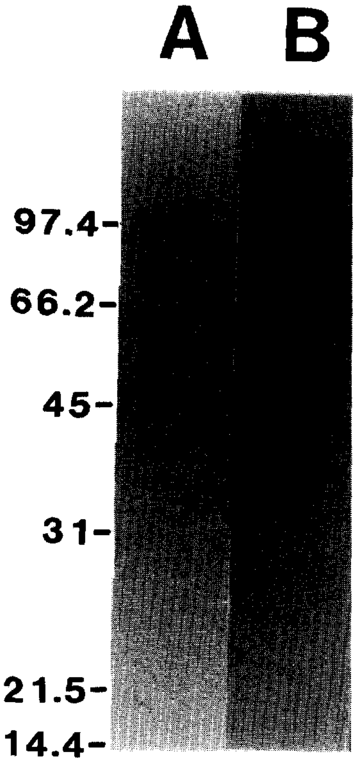

Fig. 4. Non-denaturing SDS–PAGE electrophoresis of extracts from tobacco xylem cell walls. Lane A contains 10 μg equiv. of the 1 M NaCl cell wall extract; lane B contains 1 μg equiv. of the main oxidase containing peak from the ion-exchange chromatography step. The positions of M_r marker proteins are shown.

increases the total recovery of oxidase activity suggesting either the removal of inhibitors or activation of latent activity [13].

DISCUSSION

A previous report from this laboratory [9] provided evidence for the presence of laccase-type polyphenol oxidases in the cell walls of tobacco xylem but was unable to detect oxidase activity in extracts, probably for two main reasons. Firstly, the extracts were obtained from the whole xylem cylinder rather than the outermost, oxidase-rich tissues. Secondly, the addition of DTT and $CaCl_2$ in the homogenization buffer and $CaCl_2$ to the extraction buffer improved the yield of oxidase from these tissues.

Calcium ions are known to stabilize or activate a number of polyphenol oxidases [13, 14] but no such role has yet been identified for laccase-type oxidases [6]. The presence of latent polyphenol oxidases capable of oxidizing coniferyl alcohol in lignifying tissues and their possible means of activation requires further study. DTT may stabilize catalytically important sulphhydryl groups [15] or prevent interfering redox reactions. Also, it is now apparent from the three-fold increase in total oxidase activity recovered after ion-exchange chromatography (Table 1) that the crude xylem cell wall extracts contain inhibitors which are removed by this step. The nature of these inhibitors is unknown but monophenolic compounds are known to inhibit fungal laccases [16], and initial studies also suggest that ascorbate is a potent inhibitor of these oxidases.

Lignifying tobacco xylem expresses a number of oxidase isoforms capable of oxidizing coniferyl alcohol. The most abundant coniferyl alcohol oxidase (CAO) isoform has properties (i.e. stability to SDS, independence towards H_2O_2 and an apparent M_r of *ca* 100 000) that are characteristic of the polyphenol oxidases associated with lignifying tissues of trees [3, 4], which have been described as laccase-like. Therefore, this finding confirms previous cytochemical [7] and biochemical [8] evidence for the presence of laccase-type polyphenol oxidases in lignifying tissues of plants other than trees. However, the other CAO isoforms have not been confirmed as being laccase-like and may be more akin to the polyphenol oxidase extracted from the cell walls of mung bean hypocotyls that can also oxidise coniferyl alcohol [17]. The nature of these CAOs will only be ascertained by stringent studies of the substrate specificity of the purified enzymes or, ultimately, by studies of sequence homology. The importance of this area is highlighted by the work of Udagama-Randeniya and Savidge [18] who found that a purified CAO from *Pinus strobus* had a substrate specificity more akin to catechol oxidases than laccase-type oxidases.

The major CAO isoform from tobacco xylem has an estimated pI of 5–6 which is in the same range as laccases from *Acer pseudoplatanus* suspension cultures (pIs of 4.0–5.2) [4] and the laccase from *Zinnia* stems (pI 6–7) [8]. However, the oxidases identified in extracts of *P. strobus* and a range of other conifers at pI 7.6 [19] whilst the laccase from *Pinus taeda* has a pI of 9.0 [3].

The presence of multiple oxidase isoforms, also reported previously from suspension cultured cells of *A. pseudoplatanus* [4], may result from the expression of different genes. It is, therefore, intriguing that maturation of tobacco xylem is accomapnied by the apparent down-regulation of three oxidase isoforms that are expressed in young xylem. This may be the first indication that the expression of CAO isoforms may be subject to differential developmental control. However, the possibility that these isoforms cannot be detected in extracts of the older xylem due to latency or the presence of inhibitors [13] cannot be discounted.

The CAOs reported in this paper fulfil two of the main criteria required of lignin-specific enzymes: they catalyse a reaction specific to lignin formation and their expression is limited to lignifying tissues. If these enzymes are involved in lignin formation, the reasons why there should be so many different oxidase isoforms must be addressed. A range of isoforms can have a range of kinetic properties [20] and isoforms with widely different isoelectric points can have different pH

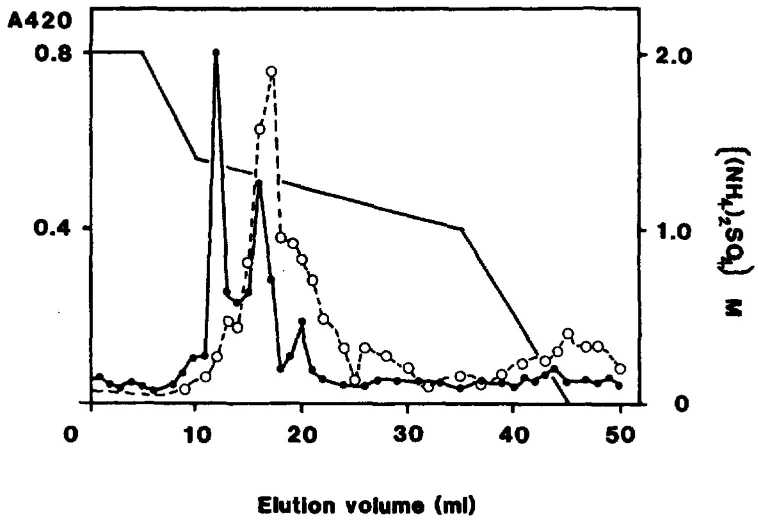

Fig. 5. Purification of oxidase isoforms by hydrophobic interaction chromatography. Oxidase activity against ABTS is denoted by the closed circles; peroxidase activity by the open circles. The reverse gradient of ammonium sulphate [2–0 M $(NH_4)_2SO_4$] is shown by the solid line.

optima and, in the case of peroxidases [21, 22], different redox potentials. As a result, the expression of a range of oxidase isoforms might allow the catalysis of diverse oxidation reactions. Therefore, the expression of a more diverse range of CAO isoforms may reflect the greater range of cross-linking reactions between the nascent lignin and cell wall components required in younger tobacco xylem. However, although these oxidases oxidize CA *in vitro*, tobacco xylem cell walls can oxidize a broad spectrum of substrates [9] and it is possible that monolignols are not their physiological substrates. The complete understanding of the putative roles of CAO/laccases in lignin deposition will probably require the production of transgenic plants with altered levels of expression of these enzymes.

EXPERIMENTAL

Plant material. Tobacco (*Nicotiana tabacum* var. Samsun) plants were grown under controlled greenhouse conditions as described previously [9]. Plants were harvested after 10 weeks, just as the plants were beginning to flower. All leaves were removed and the stem from the 2nd internode to the inflorescence (70–80 cm long) was excised and kept moist. For some studies, the stem was halved into apical and basal portions. The stem pieces were cut longitudinally down to the xylem and the xylem cores (and pith) peeled out. The cores were then scraped with razor blades to remove the outer layers of the xylem. In the most apical parts of the stem, the xylem was not strong enough to withstand scraping and these pieces were added to the scraped tissue after removal of the pith. Xylem tissue was stored at −20° prior to use.

Histochemistry. Transverse sections of tobacco stems were cut by hand and floated on water prior to use. Sections were transferred to 100 mM NaOAc buffer pH 5 containing 1.82 mM 2,2-azinobis-(3-ethylbenzothiazoline-6-sulphate) (ABTS), 0.28 mM syringaldazine (SYR) or 0.13 mM tetramethyl benzidine (TMB) and incubated with gentle stirring for 10 min (ABTS and TMB) to 24 hr (SYR). Catalase at 1000 units ml^{-1} was added to some incubations. Specimens were examined using a Zeiss Universal microscope.

Extraction of cell-wall-bound proteins and preparation of cell walls. When required, tobacco xylem scrapings (*ca* 30 g) were thawed and homogenized in ice-cold 50 mM Tris HCl buffer pH 7.5 containing 2.5 mM $CaCl_2$ and 1 mM dithiothreitol (DTT) using an Ultra-Turrax disintegrator (10 × 30 sec bursts, cooled on ice). The yield of oxidase from frozen tissue was not significantly different from fresh tissue. The insoluble material was then collected by filtration through coarse glass sinters and the procedure repeated until the filtrate was no longer coloured. After a final wash with buffer, the crude cell wall preparation was extracted with ice-cold 1 M NaCl containing 2.5 mM $CaCl_2$ for 1 hr, an adaptation of a commonly used procedure to obtain an extract enriched in ionically bound cell wall proteins (see refs [3, 11, 17]). The 1M NaCl extract routinely contained 40–50% of the total oxidase activity (measured against ABTS), whereas the initial tissue homogenate contained 40% of total oxidase activity but at a sp. act. 50–60-fold lower than the 1M NaCl extract. The insoluble cell walls were then purified by repeated homogenization in 50 mM Tris HCl pH 8.0 containing 1 M NaCl and 0.5% (v/v) Triton X-100 which removed the remaining soluble oxidase activity. Once the filtrates were clear, the cell walls were washed free of detergent, resuspended in H_2O and frozen.

Peroxidase, oxidase and coniferyl alcohol oxidase assays. For oxidase assays, ABTS was used at a final concn of 1.82 mM in 100 mM NaOAc pH 5.0. The A_{420} of the coloured product was measured. 2.5 mM H_2O_2

Table 1. Purification of oxidase activity from tobacco xylem cell walls

	OXIDASE ACTIVITY			
Sample	Total protein (μg)	Specific activity (units mg^{-1})*	Total activity	Yield
1 M NaCl extract	8700 [4870]‡	7.0±0.2†	60.9 [34.1]	100
Ion exchange peak III	3080 [265]	70.1±0.8	216 [18.5]	354
HIC peak I	28	158±1.2§	4.4	150

	CONIFERYL ALCOHOL OXIDASE ACTIVITY			
Sample	Total protein (μg)	Specific activity (nmol min^{-1} μg^{-1})	Total activity	Yield
1 M NaCl extract	8700 [4870]	0.182±0.002	1580 [887]	100
Ion exchange	3080 [265]	1.948±0.014	6000 [516]	379
HIC peak 1	28	N.D.‖	N.D.	N.D.

* A unit of activity is defined as causing a increase in $A_{420} = 0.1\ min^{-1}$.

† These figures are averages of triplicate assays±standard error.

‡ Figures in square brackets represent the amount of protein or activity taken on to the next step.

§ Activity against ABTS measured in presence of $(NH_4)_2SO_4$.

‖ Activity against CA could not be detected (N.D.), see Experimental for details.

was added for peroxidase assays. Coniferyl alcohol oxidase (CAO) activity was measured by a continuous spectrophotometric assay monitoring the decline in A_{260} using a final concn of 0.2 mM coniferyl alcohol (CA) in 100 mM NaOAc pH 5.0. The extinction coefficient of CA was estimated empirically.

Purification of cell-wall-associated oxidases. Ionically bound cell wall proteins were desalted by dialysis against three changes of 5 l of 10 mM Tris HCl pH 7.5 at 5° over 24 hr. The extracts were then concd to <20 ml by a combination of dialysis against polyethylene glycol compound (Product no. P2263; Sigma) and the use of centrifugal membrane concentrators (Centriplus-10 concentrators, Amicon). Up to 15 ml of this sample was applied to a 150 ml bed vol. column of DEAE–Sepharose pre-equilibrated in 50 mM Tris HCl pH 8.5 and eluted with a linear gradient of 0–0.5 M NaCl in the same buffer. Frs were collected and assayed for peroxidase and oxidase activity. The elution of the oxidase peaks was compared against the elution of standards with known isoelectric points (carbonic anhydrase, pI 6.6; myoglobin pI 6.8 and 7.4; methyl red, pI 3.8 (Sigma)). Oxidase-containing frs were pooled, concd using Centriplus-10 units then buffer exchanged into 100 mM Na–P_i pH 7 containing 2 M $(NH_4)_2SO_4$ using PD-10 units (Pharmacia) for hydrophobic interaction chromatography (HIC). Samples were injected onto a Hydropore-HIC HPLC column (4.6 × 100 mm, Rainin Instruments, Anachem) pre-equilibrated in 100 mM Na–P_i pH 7 containing 2 M $(NH_4)_2SO_4$ then eluted with a diminishing gradient to the same buffer without $(NH_4)_2SO_4$ over 45 min. The gradient was 2 M $(NH_4)_2SO_4$ at 0 min.; 2 M $(NH_4)_2SO_4$ at 5 min; 1.4 M $(NH_4)_2SO_4$ at 10 min; 1.0 M $(NH_4)_2SO_4$ at 35 min; 0 M $(NH_4)_2SO_4$ at 45 min. Elution was monitored at 280 nm and frs were immediately assayed for peroxidase and oxidase activities against ABTS. Protein was measured by the method of ref. [23].

Analytical denaturing SDS–PAGE. This technique was carried out using a Mini-Protean II slab gel system according to the makers instructions (Bio-Rad) using a slightly modified version of the buffer system of ref. [24]. Standard low M_r range protein markers for M_r estimation were obtained from Bio-Rad and gels were stained using the Bio-Rad silver stain method.

Non-denaturing SDS–PAGE for oxidase activity. This method was adapted from [8]. Enzyme samples were prepd as normal for SDS–PAGE except they were not boiled prior to loading. Marker proteins were boiled to ensure that they were completely denatured. Gels were made the day before use and stored at 8° overnight. Running buffer was chilled prior to use and the gels were run at 8° to minimize the possibility of enzyme inactivation. Proteins were separated by standard SDS–PAGE conditions. The gels were briefly prewashed in 100 mM NaOAc pH 5 and oxidase activity was detected by staining with 4.5 mM ABTS in 100 mM NaOAc pH 5. Staining was carried out at 25° for up to 30 min, although oxidase bands were usually visible after 10 min. H_2O_2 was added to a final concn of 2.5 mM when required to visualize peroxidase activity.

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