Thermostable extracellular peroxidases from *Streptomyces thermoviolaceus*

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***Streptomyces thermoviolaceus* is a thermophilic actinomycete that was found to produce relatively large amounts of extracellular peroxidase activity when grown on xylan as primary carbon source. The activity was due to multiple isoforms of peroxidase, of which two, designated P-3 and P-5, were predominant. The two proteins were purified to homogeneity by a combination of ultrafiltration, ammonium sulphate precipitation, anion-exchange chromatography, gel filtration and preparative gel electrophoresis. The peroxidases were found to be haemoproteins that catalysed the oxidation of a range of substrates in the presence of hydrogen peroxide. Both are monomeric acidic proteins (P-3: 82 kDa, pI 5.0; P-5: 60 kDa, pI 4.75) but with some differences in substrate specificity, P-3 exhibiting the broader substrate range. Peroxidase activity was optimal at pH values close to neutrality, and both enzymes were robust, exhibiting activity at elevated temperatures in the presence of denaturing agents such as SDS or 8 M urea. Peroxidase P-3 was stable at 50 °C for more than 24 h and had a half-life of 70 min at 70 °C. Polyclonal antibodies prepared against each isoform cross-reacted, indicating that the proteins were antigenically related. No cross-reactions were detected against horseradish peroxidase or crude peroxidase preparations from two other thermophilic streptomycetes.**

Keywords: *Streptomyces thermoviolaceus*, extracellular peroxidases, thermostability

INTRODUCTION

Peroxidases can catalyse oxidations on a number of substrates in the presence of hydrogen peroxide (H₂O₂). They are widely distributed in biological systems and display both structural diversity and a range of functions in cellular metabolism (see Everse *et al.*, 1990). Horseradish peroxidase is by far the best-characterized and has accordingly been exploited in several diagnostic applications, where it acts as a reporter of antibody or DNA-sequence binding. An additional function was credited to peroxidases relatively recently when they were implicated as the extracellular agents of lignin degradation by the white-rot fungi, and by *Phanerochaete chrysosporium* in particular (Tien & Kirk, 1983). This organism produces multiple forms of two classes of peroxidase, the lignin and the manganese-dependent peroxidases, both of which appear to be important in the degradative process (Farrell *et al.*, 1989; Gold *et al.*, 1989; Odier & Delattre,

1990). Extracellular peroxidase activity has subsequently been found amongst biodegradative actinomycetes (Ramachandra *et al.*, 1988; Ball *et al.*, 1990), where it appears to be relatively common (Winter *et al.*, 1991; Godden *et al.*, 1992). The activity is due to multiple, immunologically related peroxidase isoforms in *Streptomyces viridosporus* and *Streptomyces badius* (Adhi *et al.*, 1989; Magnuson *et al.*, 1991), and zymogram analysis suggests that this multiplicity may be true of actinomycetes in general (Godden *et al.*, 1992). In *S. viridosporus*, the major peroxidase isoform has been designated as a lignin-oxidizing enzyme, i.e. a lignin peroxidase (Ramachandra *et al.*, 1988), although a more recent study on substrate specificity of peroxidase preparations has questioned this classification (Spiker *et al.*, 1992). Further progress in our understanding of the function of actinomycete extracellular peroxidases and their properties will require biochemical analysis of highly purified enzymes, and this forms the rationale for the work described here.

Abbreviations: ABTS, 2,2'-azino-di-[3-ethyl-benzthiazoline-(6)-sulphonic acid], diammonium salt; 2,4-DCP, 2,4-dichlorophenol; L-DOPA, L-3,4-dihydroxyphenylalanine; FPLC, fast protein liquid chromatography.

Streptomyces thermoviolaceus is a thermophilic actinomycete originally isolated from rotting stable manure (Henssen

& Schnepf, 1967) and occasionally encountered as a component of the actinomycete population of compost and mouldy fodders. Its physiology has been well studied in relation to production of the pigmented antibiotic granaticin (James & Edwards, 1989), and *S. thermoviolaceus* is a good producer of extracellular enzymes such as xylanases (Tsujibo *et al.*, 1992) and proteases (James *et al.*, 1991). In the course of screening actinomycetes for extracellular peroxidases, we discovered that *S. thermoviolaceus* was also a particularly good source of this activity. Our selection of this organism for the purification of extracellular peroxidases and determination of their properties was further supported by the probability that *S. thermoviolaceus* enzymes would exhibit thermostability properties of relevance to biotechnological applications.

METHODS

Culture maintenance and peroxidase production. Stock cultures of *Streptomyces thermoviolaceus* (NCIMB 10076) were maintained at 4 °C and subcultured at 3–8 week intervals. Cultures were routinely incubated for growth and sporulation at 50 °C for 72 h on YEME agar, which contained yeast extract (Amersham, MC1) (4 g l⁻¹), malt extract (Amersham, MC23) (10 g l⁻¹), D-glucose (4 g l⁻¹) and agar (15 g l⁻¹) at pH 7.2. Distilled-water suspensions of growth from agar-slant cultures were used as inocula for all liquid cultures.

For peroxidase production, suspensions of *S. thermoviolaceus* spores and hyphae were inoculated directly into a sterile production medium (400 ml in a 2 l conical flask) based on that described by Ramachandra *et al.* (1988). This medium contained (g l⁻¹): oat spelt xylan (Sigma, X-0627), 6.0; yeast extract (Amersham, MC1), 6.0; (NH₄)₂SO₄, 0.1; NaCl, 0.3; MgSO₄·7H₂O, 0.1; CaCO₃, 0.02; trace-elements solution, 1 ml; pH 7.2. The trace-elements solution contained (g l⁻¹): FeSO₄·7H₂O, 1.0; ZnSO₄·7H₂O, 0.9; MnSO₄·7H₂O, 0.2; pH 7.2. Cultures were incubated at 50 °C with shaking at 150 r.p.m. for 50 h and harvested by centrifugation at 15000 g for 20 min. The culture supernatant was concentrated by ultrafiltration (YM10 membrane; Amicon) to produce a crude preparation for peroxidase purification and further analysis.

Enzyme assays. Peroxidase activity was assayed with 2,4-dichlorophenol (2,4-DCP) (Sigma) as substrate (Ishida *et al.*, 1987). A final volume of 1 ml of the reaction mixture contained 0.1 M potassium phosphate buffer, pH 7.0 (200 µl); 1 mM 4-aminoantipyrine (Sigma) (200 µl); 5 mM 2,4-DCP (200 µl); 50 mM H₂O₂ (200 µl); appropriately diluted enzyme preparation (200 µl). The reaction was initiated by addition of H₂O₂ and the increase in *A*₅₁₀ was monitored for 1 min. One unit of enzyme activity was defined as the amount required for an increase in absorbance of 1.0 unit min⁻¹. During the protein purification procedure, peroxidase activity was further confirmed against ABTS (2,2'-azino-di-[3-ethyl-benzthiazoline-(6)-sulphonic acid], diammonium salt) using the assay procedure described by Püetter & Bekker (1983).

In all of the experiments reported here, protein concentration was measured routinely by the method of Bradford (1976), using a commercial protein assay kit (Bio-Rad) calibrated with BSA.

Purification of peroxidases. The concentrated crude enzyme preparation was precipitated with ammonium sulphate to give 20% saturation. The precipitate formed was discarded after

centrifugation and ammonium sulphate was added to the resultant supernatant to 80% saturation. After centrifugation (15000 g for 40 min) the precipitate was collected, dissolved in 0.1 M sodium acetate buffer, pH 6.0 (buffer A), and dialysed against the same buffer at 4 °C.

The dialysed enzyme solution (2 ml per cycle) was applied to a fast protein liquid chromatography (FPLC) anion-exchange column (Mono Q, HR10/10; Pharmacia) equilibrated with the same buffer. The column was washed with the buffer, and the proteins were eluted by a NaCl gradient from 0 to 1 M in the same buffer (the gradient was held for 5 min at 30%, w/v, NaCl concentration). Partially purified peroxidase P-3 fractions from the ion-exchange column were pooled and concentrated to 2 ml by ultrafiltration (Amicon; YM10 membrane) and further purified by preparative column electrophoresis on 7.5% (w/v) non-denaturing polyacrylamide gels as described in the manufacturer's manual (Prep Cell model 491, Bio-Rad). The sample (2 ml) was mixed with the sample buffer (containing glycerol and bromophenol blue) and applied to the top of the column. The electrophoresis was carried out at 40 mA constant current, and the fractions were collected when the dye front began to elute at a flow rate of 0.7 ml min⁻¹.

The fractions from the Mono Q column that contained peroxidase P-5 activity were pooled and further purified to homogeneity by gel filtration column chromatography (Superdex-75, Pharmacia) equilibrated with 100 mM sodium phosphate buffer, pH 7.0.

Molecular masses of the purified peroxidases were determined by gel filtration in 0.1 M potassium phosphate buffer (pH 7.0) on a Superdex-75 (HR10/30, Pharmacia) column previously calibrated with the following reference proteins (molecular masses in parentheses): ribonuclease A (13700); chymotrypsinogen A (25000); ovalbumin (43000); albumin (67000); and blue dextran 2000 to determine the void volume of the column.

The protein concentration of all FPLC column eluates was monitored by measurement of *A*₂₈₀, and fractions containing peroxidase activity were stored at -20 °C.

Electrophoresis. Discontinuous SDS-PAGE and non-denaturing PAGE (Laemmli, 1970) were used to ascertain the degree of protein purity and to confirm molecular masses derived from the gel-filtration separations. Where appropriate, samples of proteins were boiled for 5 min prior to electrophoresis on 8% (w/v) polyacrylamide resolving gels using the Mini-Protein II Dual Slab Cell (Bio-Rad).

After electrophoresis, protein bands were visualized by staining with Coomassie blue using 0.1% Phast Gel Blue R (Pharmacia) in 30% (v/v) methanol and 10% (v/v) acetic acid, and destained with methanol/acetic acid/water (3:1:6 by vol.). The following proteins were used as molecular mass standards (subunit molecular masses in parentheses): trypsin inhibitor (21000); carbonic anhydrase (30000); ovalbumin (43000); albumin (67000); phosphorylase *b* (94000).

Polyacrylamide gels were also developed as zymograms to detect the presence of enzyme activity using Phast gels (12.5%, w/v, polyacrylamide) as described in the manufacturer's manual (Pharmacia; Phast System-unit). Prior to electrophoresis, samples were mixed with sample buffer, with or without SDS or urea as appropriate. Immediately after electrophoresis, the gels were incubated with a range of substrates. These included the following: L-3,4-dihydroxyphenylalanine (L-DOPA); caffeic acid; homoprotocatechuic acid; 2,4-DCP; N,N,N',N'-

tetramethyl-*p*-phenylenediamine containing 0.05 M 4-amino-antipyrine. Gels were flooded with a 10 mM solution of substrate plus 50 mM H_2O_2 in 0.1 M potassium phosphate buffer (pH 7.0) at room temperature. The gels were also developed with 3,3'-diaminobenzidine, *o*-dianisidine, ABTS and 4-chloro-1-naphthol containing 1 ml substrate solution (10% substrates dissolved in methanol), mixed with 1 ml 30 mM H_2O_2 and 8 ml 0.1 M sodium phosphate buffer (pH 6.0). All of the peroxidase substrates were obtained from Sigma, except ABTS (Boehringer). When the stained peroxidase bands appeared on the gels, they were photographed immediately or dried.

The pI values of peroxidases P-3 and P-5 were determined using commercially available isoelectric-focusing gels (Phast gels, pH 4–6.5; Pharmacia) on a Phast System-unit (Pharmacia). An isoelectric-focusing calibration kit (pI 3–10; Pharmacia) was used for pI determination.

Antibody preparations. Antibodies against peroxidases P-3 and P-5 were raised in rabbits by injection with antigen preparations that each comprised 100 μg (0.5 ml) purified enzyme in 10 mM potassium phosphate buffer (pH 7.0) mixed with equal volumes of Freund's complete adjuvant. Booster injections were administered with complete adjuvant after 3 weeks and with incomplete adjuvant after a further 3 weeks. Antibodies specific for the peroxidases were detectable by the Ouchterlony double diffusion assay (Ouchterlony & Nilsson, 1978) 9 weeks following the initial injection of antigen, and at this point serum was collected and stored at -20°C .

Western blot analysis. Peroxidases were separated by SDS-PAGE, and the protein bands were electrophoretically transferred, using the Mini Trans-blot system (Bio-Rad), to the nitrocellulose membrane in 10 mM CAPS buffer (Sigma) containing 10% (v/v) methanol (pH 11.0) at 50 V for 30 min. The membrane was then blocked with 5% (w/v) non-fat dried milk in Tris-buffered saline (TTBS: 20 mM Tris, 0.1 M NaCl, 0.05% Tween 20, pH 7.5) and incubated for 1 h at room temperature with gentle agitation. The membrane was then washed twice (10 min each) with TTBS. The first antibody solution was prepared in TTBS (1:1000 dilution) and incubated with the membrane for 1 h. The membrane was washed twice with TTBS (10 min each) and incubated for 1 h with biotinylated secondary antibody (goat anti-rabbit IgG; Bio-Rad), diluted 1:3000 in TTBS. Streptavidin and biotinylated alkaline phosphatase (1:3000 dilution each) (Bio-Rad; Immuno-Blot assay kit) were mixed in TTBS. The membrane was washed again with TTBS and the streptavidin–biotinylated alkaline phosphatase complex added and incubated for a further 1 h. Finally, the membrane was washed and bands visualized using an alkaline phosphatase colour development reagent kit (Bio-Rad).

Factors affecting peroxidase activity and stability. The activity of purified peroxidase P-3 activity was measured at pH values in the range pH 2–12 using 2,4-DCP as the substrate. The potassium phosphate buffer in the reaction mixture (see above) was replaced with Universal buffer which contained (g l^{-1}): citric acid, 6.008; boric acid, 1.769; KH_2PO_4 , 3.893; diethylbarbituric acid, 5.26. The pH was adjusted with 0.1 M NaOH or HCl. The effect of temperature on P-3 peroxidase activity was determined by carrying out the 2,4-DCP assay at different temperatures in the range 30–80 $^\circ\text{C}$ at pH 7.0. For thermostability determinations, enzyme preparations were pre-incubated without substrate under a range of time/temperature regimes prior to determination of peroxidase activity against 2,4-DCP under standard assay conditions.

The relationship between peroxidase activity on 2,4-DCP and H_2O_2 concentration was determined by varying the latter, in the range 1–20 mM, in the final reaction mixture of the standard assay procedure.

The stability of the peroxidases against detergents, such as SDS, and urea at elevated temperatures was examined by pre-incubating the partially purified enzyme preparation containing both P-3 and P-5 with 5% (w/v) SDS and 8 M urea at various temperatures (30–100 $^\circ\text{C}$) for 10 min. After heating, the samples were cooled on ice and the activity of the enzymes was visualized by electrophoresing enzyme preparations in SDS-PAGE gels containing 8 M urea and developed as zymograms with L-DOPA as substrate.

RESULTS

Culture supernatants containing peroxidase activity were produced by growing *S. thermophilaceus* in a liquid medium in which the main carbon source was xylan. Under these conditions, sufficient activity was produced to enable its detection by the 2,4-DCP assay without first concentrating the supernatant samples by ultrafiltration. In order to maximize the peroxidase content of enzyme preparations, the effect of xylan concentration on peroxidase production was investigated (Fig. 1a). The maximum level of peroxidase activity (1.1 U ml^{-1} with 2,4-DCP as substrate)

Fig. 1. Production of extracellular peroxidase activity by *S. thermophilaceus*. The cultures were grown in 250 ml flasks containing 50 ml medium. After inoculation, cultures were incubated with shaking (150 r.p.m.) at 50 $^\circ\text{C}$. (a) Effect of oat spelt xylan concentration. The cultures were harvested after 50 h incubation. (b) Effect of incubation time on peroxidase production in basal medium supplemented with 0.6% oat spelt xylan. Culture supernatants were assayed for peroxidase activity by the 2,4-DCP assay method. The results are the means of three replicates.

Fig. 2. Native-PAGE analysis of *S. thermoviolaceus* peroxidases. The concentrated crude samples were applied on the gels, except lanes 4 and 5, which were loaded with purified P-3 and P-5, respectively. Native Phast gels (homogeneous, 12.5%) were developed as a zymogram with various peroxidase substrates. Lanes: 1, L-DOPA; 2, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; 3, caffeic acid; 4 and 5, 3,3'-diaminobenzidine; 6, 4-chloro-1-naphthol; 7, 2,4-DCP; 8, *o*-dianisidine; 9, ABTS; 10, homoprotocatechuic acid.

was produced in media containing 0.6% (w/v) xylan. Incubation of cultures for 50 h in this medium was found to be optimal for the production of extracellular peroxidase preparations (Fig. 1b).

Zymogram analysis of peroxidase isoforms

Concentrated extracellular peroxidase preparations were produced by subjecting *S. thermoviolaceus* culture supernatants to ultrafiltration and ammonium sulphate fractionation as described in Methods. Proteins were separated by non-denaturing discontinuous PAGE (native-PAGE) and peroxidases were revealed as dark bands upon incubation with a range of substrates in the presence of H_2O_2 (Fig. 2). When H_2O_2 was omitted, no bands of activity appeared, thus confirming identification of the enzymes as true peroxidases. A total of five bands of peroxidase activity was observed on native gels stained with L-DOPA or *N,N,N',N'*-tetramethyl-*p*-phenylenediamine, but only two, designated as isoforms P-3 and P-5, were predominant (Fig. 2). The other three bands were faintly stained, and disappeared from the gels after only a few minutes. P-3 showed reactivity with all of the substrates used (see Methods) but P-5 only reacted with L-DOPA, caffeic acid, homoprotocatechuic acid, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine and 3,3'-diaminobenzidine. This reflects differences in the substrate specificity of the two isoforms, as these substrate ranges were confirmed with purified preparations of each (data not shown).

Purification of peroxidase isoforms

The two predominant peroxidase isoforms identified by zymogram analysis of crude extracellular enzyme preparations from *S. thermoviolaceus* were purified. Culture supernatant was concentrated 5–10-fold by ultrafiltration, by which 77% of the peroxidase activity was recovered. Most of this activity (71% of the original) was retained

after the ammonium sulphate precipitation step, and from this the major peroxidase isoform P-3 was purified by ion-exchange chromatography using FPLC, and finally by the application of preparative native gel electrophoresis (see Methods). The purification data for isoform P-3 are presented in Table 1; the enzyme was purified approximately 62-fold, and the final specific activity of the purified enzyme was $2.73 \text{ U (mg protein)}^{-1}$.

The application of FPLC ion-exchange chromatography enabled recovery of both isoforms P-3 and P-5, as indicated by peroxidase assays with 2,4-DCP as substrate (Fig. 3a); the former was eluted in fraction 19 and the latter in fractions 22–24. Fractions containing both P-3 and P-5 (20 and 21) were pooled and re-chromatographed on the same column. Fractions containing peroxidase activity were also analysed by native-PAGE with development as zymograms using L-DOPA or diaminobenzidine (see Methods) (Fig. 3b). This further confirmed the presence of peroxidase activity in these fractions and enabled identification of the isoforms present in particular fractions. Fraction 19 from the ion-exchange separation was concentrated by ultrafiltration and peroxidase was further purified by preparative electrophoresis (see Methods). It was necessary to use preparative electrophoresis for the purification of P-3 because the application of gel filtration always resulted in the co-elution of a minor contaminating protein. The enzyme activity recovered from this purification step gave only a single homogeneous band upon analysis by SDS-PAGE (Fig. 4a, lane 1), identified as peroxidase P-3. The ion-exchange chromatography fractions (22–24) containing peroxidase P-5 were pooled and the enzyme was further purified to homogeneity (Fig. 4a, lane 2) by gel filtration. The overall recovery of peroxidase P-5 was not monitored because this isoform is not sufficiently reactive with 2,4-DCP, ABTS or *o*-dianisidine, which were used to monitor peroxidase activity during the FPLC purification. Consequently, detailed purification data for the P-5 isoenzyme cannot be provided.

Table 1. Purification of extracellular peroxidase P-3 from *S. thermoviolaceus*

Units are defined as the amount of enzyme required for an increase of 1.0 absorbance unit min⁻¹ using 2,4-DCP as a substrate.

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U mg ⁻¹)	Purification factor (-fold)	Percentage recovery
Culture supernatant	2268	101.4	0.044	1	100
> 10000 kDa retentate	1442	78.5	0.054	1.2	77
(NH ₄) ₂ SO ₄ precipitation (20–80 %, w/v)*	800	72.3	0.09	2.1	71
Anion-exchange chromatography (Mono-Q, HR 10/10)	11.24	21.6	1.92	43.6	21
Preparative gel electrophoresis (Prep Cell)	3.8	10.4	2.73	62	10

* Followed by resolubilization and dialysis against 0.1 M acetate buffer (pH 6.0).

Fig. 3. Purification of *S. thermoviolaceus* peroxidases by FPLC. (a) Elution profiles of the ammonium-sulphate-precipitated and dialysed samples containing peroxidases by anion-exchange chromatography on Mono Q HR10/10. The peroxidases P-3 and P-5 were eluted at a 30% (w/v) NaCl gradient of buffer B at a flow rate of 3 ml per min. —, A_{280} ; ---, NaCl gradient; ●—●, peroxidase P-3 activity. Fractions (3 ml) were collected, and activity of peroxidase was determined with the 2,4-DCP assay method. (b) Column eluates of fractions 19–24 (20 μ l amounts) were applied on the native-PAGE gel and the zymogram developed with L-DOPA.

The molecular masses of the enzymes P-3 and P-5 were estimated by gel filtration. On SDS-PAGE, both enzymes were recovered as single bands after boiling in the presence of SDS and mercaptoethanol. Both gel filtration and SDS-PAGE estimated the molecular masses of the enzymes at approximately 82 kDa for P-3 and 60 kDa for P-5. The pI values of the enzymes were determined by

Fig. 4. Purified *S. thermoviolaceus* peroxidases analysed by (a) SDS-PAGE and (b) isoelectric focusing (IEF) gel electrophoresis (pI 4–6.5). Lanes: 1 and 4, P-3; 2 and 5, P-5; 3, molecular mass markers; 6, IEF standards.

isoelectric focusing gel electrophoresis. Both were found to be acidic proteins with pI values of 5.0 and 4.75 for P-3 and P-5, respectively (Fig. 4b).

Properties of the purified peroxidases

Both P-3 and P-5 exhibited distinct absorbance peaks at 405–409 nm (Soret maxima), indicating that they can be classified as haemoproteins. The addition of sodium

Fig. 5. Thermostability of *S. thermoviolaceus* peroxidase P-3. (a) Enzyme preparations were incubated at various temperatures for 10 min prior to assay. (b) Enzyme solutions were incubated for different times at 50 °C (■), 60 °C (●) or 70 °C (◆). Peroxidase activity was measured using the 2,4-DCP assay method.

azide to reaction mixtures containing crude enzyme preparations resulted in the complete inhibition of peroxidase activity, further confirming that the enzymes are haemoproteins. H_2O_2 was found to be required for the oxidation of substrates, and for P-3 > 80% activity in the 2,4-DCP assay was expressed across the range 4–16 mM H_2O_2 (data not shown). The optimum pH for activity against 2,4-DCP was in the region of 6.5–7.0, with a dramatic reduction in activity at pH values > 7.5 and

< 5.0. Good activity was exhibited in the temperature range 50–65 °C under the specified assay conditions.

Thermostability is a more meaningful parameter in considering the temperature relationships of an enzyme and, in the case of the extracellular peroxidase P-3, the thermostability properties (Fig. 5a) reflect the moderately thermophilic nature of *S. thermoviolaceus* itself. There was no loss of activity during 24 h at 50 °C or 10 min at 70 °C. The half-lives at 60 °C and 70 °C were found to be 15 h and 70 min, respectively (Fig. 5b). The relative stability of P-3, P-5 and horseradish peroxidase was examined by incubating enzyme preparations at different temperatures in the presence of SDS or urea as denaturing agents in polyacrylamide gels. The results of this experiment are presented in Fig. 6. The *S. thermoviolaceus* peroxidases were significantly more robust than horseradish peroxidase, which showed no detectable activity in SDS after incubation at temperatures > 40 °C (Fig. 6c). Both P-3 and P-5 were active after incubation at 60 °C in the presence of SDS (Fig. 6a), with P-5 appearing slightly more thermostable than P-3 in the presence of urea (Fig. 6b).

Relationships between peroxidases

Western blot analysis was used to examine the immunological relatedness of P-3 and P-5 to one another. Identical results were obtained with both antisera to P-3 and P-5, i.e. the purified peroxidases reacted with both homologous and heterologous antisera (Fig. 7). Furthermore, both bands were also detected in Western blots against a mixture of the proteins P-3 and P-5. All of these results were confirmed using the Ouchterlony double-diffusion technique for observing immunological cross-reactions, with the addition that neither P-3 nor P-5 antisera reacted with a preparation of horseradish peroxidase (Sigma) or crude peroxidase preparations from *Streptomyces thermovulgaris* and *Streptomyces* sp. EC22 (data not shown). The two predominant extracellular peroxidases of *S. thermoviolaceus* are therefore antigenically related to one another, but are distinct from horseradish peroxidase or peroxidases from other thermotolerant *Streptomyces* species.

Fig. 6. Effect of SDS and urea on the stability of *S. thermoviolaceus* and horseradish peroxidases at elevated temperatures. Concentrated culture supernatants of *S. thermoviolaceus* (a, b) and horseradish peroxidase (c) were incubated at various temperatures in the presence of 5% (w/v) SDS (a, c) and 8 M urea and 5% SDS (b), prior to electrophoresis. Lanes (a, b): 1–8, 30 °C; 40 °C; 50 °C; 60 °C; 70 °C; 80 °C; 90 °C; and 100 °C, respectively. Lanes (c): 1–6, 4 °C; 30 °C; 40 °C; 50 °C; 60 °C; and 70 °C. The gels were developed as a zymogram with L-DOPA (a, b) or with 3,3'-diaminobenzidine (c).

Fig. 7. Western blot analysis of *S. thermoviolaceus* peroxidases. (a) SDS-PAGE gel stained with Coomassie blue; (b) and (c), Western blots (see Methods). The primary antibodies used were (b) antiserum raised against peroxidase P-3 and (c) antiserum raised against peroxidase P-5. Lanes were loaded as follows: 1, 6 and 7, purified peroxidase P-3; 2, 5 and 8, purified peroxidase P-5; 4, partially purified samples containing P-3 and P-5; 3, molecular mass markers.

DISCUSSION

Bacteria produce a range of peroxidases with well-characterized functions (Hochman, 1993). Such enzymes are also produced by streptomycetes and include the non-haem haloperoxidases involved in antibiotic synthesis (Wang *et al.*, 1991) as well as the more common catalase-peroxidases (Mliki & Zimmermann, 1992). However, these are intracellular enzymes, and it is the secretion of extracellular peroxidases by actinomycetes that is the more unusual property. By analogy with the white-rot fungi, these enzymes have been investigated as possible agents of lignin degradation, with inconclusive results. The lignocellulolytic actinomycete *S. viridosporus* produces multiple peroxidases of which at least one can oxidize lignin substructure models (Ramachandra *et al.*, 1988). This must be considered against the observation that extracellular peroxidase activity is prevalent amongst actinomycetes (Winter *et al.*, 1991; Godden *et al.*, 1992), including those such as *S. thermoviolaceus* described here, for which there is no evidence of activity against lignin-related substrates. Furthermore, it is generally proposed that a lignin peroxidase is defined by the ability to oxidize nonphenolic substrates (Gold *et al.*, 1993), and this has yet to be demonstrated in actinomycetes (Spiker *et al.*, 1992). At this stage, a more cautious view would be that peroxidases are secreted along with hydrolytic enzymes by actinomycetes growing on plant biomass, and that they participate in the disorganization of the lignocarbhydrate complex. This is supported by the observation that xylanase activity is almost ubiquitously produced by biodegradative actinomycetes (McCarthy & Williams, 1992) and that high levels of extracellular peroxidase activity are produced by *S. thermoviolaceus* when grown on xylan as described here, a property also exhibited by *S. viridosporus* (Ramachandra *et al.*, 1988).

The extracellular peroxidase activity of *S. thermoviolaceus* was found to be due to the secretion of multiple peroxidase isoforms with different physico-chemical properties. The predominant form, P-3, exhibited the broadest substrate range, a significant feature of which may be its ability to oxidize 2,4-DCP. In *S. viridosporus*, only the major peroxidase was capable of oxidizing this compound

(Ramachandra *et al.*, 1988), and Winter *et al.* (1991), who reported that peroxidase activity was common amongst actinomycetes, conceded that only a few were active against 2,4-DCP. In this paper, we report the purification to homogeneity of two extracellular peroxidases, P-3 and P-5, from *S. thermoviolaceus*. Both are monomeric haemoproteins, and the molecular mass of P-3 (82 kDa) would support the possibility that it is a member of the plant peroxidase superfamily (Welinder, 1991). Bacterial catalase-peroxidases are classified in that group, but the monomeric structure of the extracellular *S. thermoviolaceus* peroxidases reported here would be more typical of the plant and fungal peroxidases. Resolution of the relationships of *S. thermoviolaceus* peroxidases to other members of this group will be dependent on the amino acid sequence information which we hope to produce in the near future.

The observation of immunological cross-reactivity between the isoforms P-3 and P-5 demonstrates that they share antigenic determinants. This is similar to the situation in *P. chrysosporium* (Datta *et al.*, 1991; Waldner *et al.*, 1988), where immunological cross-reactivity may be explained by the presence of multiple peroxidase genes that are related, in addition to gene-product modification (Leisola *et al.*, 1987). Gene and amino acid sequence information will be required before similar data can be provided for streptomycete peroxidases. In plants, peroxidase multiplicity is due to multigene families, and gene duplication and fusion events have been postulated as the evolutionary mechanism that has led to a superfamily comprising plant, fungal and bacterial peroxidases (Welinder & Gajhede, 1993). The extracellular peroxidases of actinomycetes have yet to be sufficiently characterized for their position in this scheme to be ascertained. We have been unable to detect any immunological cross-reactivity between *S. thermoviolaceus* peroxidases and those of two other thermophilic streptomycetes. However, Magnuson *et al.* (1991) reported antigenic relatedness between the major extracellular peroxidases of the mesophiles *S. viridosporus* and *S. badius*.

Purification of extracellular streptomycete peroxidase has

not previously been reported on this scale. Irrespective of the natural role and function of these enzymes, the high levels of production, specific activity and particularly robustness of the *S. thermoviolaceus* peroxidases suggests potential applications, such as those which currently use horseradish peroxidase. They exhibit significantly higher thermostability and resistance to denaturing agents than peroxidases from other bacterial, fungal or plant sources (Ferrer *et al.*, 1992; Tuisel *et al.*, 1990; Lodha *et al.*, 1991), and the major peroxidase, P-3, has an unusually high H₂O₂ requirement for optimal activity.

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