Purification, characterization and comparison of two non-haem bromoperoxidases from *Streptomyces aureofaciens* ATCC 10762

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(Received 15 March 1991; revised 8 July 1991; accepted 5 August 1991)

Two non-haem bromoperoxidases (BPO 1 and BPO 2) were purified from the 7-chlorotetracycline-producing strain *Streptomyces aureofaciens* ATCC 10762. Both enzymes showed azide-insensitive brominating activity, and bromide-dependent peroxidase activity. BPO 1 was a dimer (M_r 65000) with subunits of identical size (M_r 31000). The pI was estimated to be 4.5. The enzyme did not cross-react with antibodies raised against the non-haem bromoperoxidase (M_r 90000) from *S. aureofaciens* Tü24, a strain that also produces 7-chlorotetracycline. The M_r of BPO 2 was estimated to be 90000. The enzyme had three identical subunits (M_r 31000), and its isoelectric point was 3.5, identical with that of the bromoperoxidase from *S. aureofaciens* Tü24. Moreover, BPO 2 was immunologically identical with the bromoperoxidase from *S. aureofaciens* Tü24, although both it and BPO 1 could be distinguished electrophoretically from the latter bromoperoxidase.

Introduction

Halogenated organic products of natural origin are widely distributed. Bacteria produce many chlorinated compounds with antibiotic activity. For example, 7-chlorotetracycline is produced by *Streptomyces aureofaciens* (Duggar, 1948), chloramphenicol by *Streptomyces venezuelae* (Ehrlich *et al.*, 1947) and pyrrolnitrin by *Pseudomonas pyrocinia* (Arima *et al.*, 1964). Haloperoxidases are believed to catalyse the introduction of halogen into these organic compounds at certain steps during their biosynthesis. These enzymes accept chloride, bromide or iodide ions as substrates in the presence of hydrogen peroxide. Fluoride, however, cannot be oxidized by haloperoxidases (Neidleman & Geigert, 1986).

Some haloperoxidases of eukaryotes and prokaryotes contain haem as a prosthetic group (Olsen & Little, 1984; Hewson & Hager, 1980; van Pée & Lingens, 1985a). They have peroxidase as well as catalase activity. The reaction mechanism of these enzymes is well known (Neidleman & Geigert, 1986). Other haloperoxidases possess no haem, and hence are not inhibited by azide (Vilter, 1983; Wiesner *et al.*, 1986); they have no peroxidase activity in the absence of bromide or iodide. However, peroxidase activity is greatly enhanced by the

presence of bromide or iodide (Vilter, 1984; Krenn *et al.*, 1988). Whereas algal non-haem haloperoxidases need vanadium for halogenating activity (De Boer *et al.*, 1986), bacterial haloperoxidases do not require metal ions or any other cofactor (van Pée *et al.*, 1987; Wiesner *et al.*, 1988). Nothing is known about the reaction mechanism and active site of bacterial non-haem haloperoxidases. Investigation of their properties and comparison of their genes could give some information about the active site. However, bacterial non-haem haloperoxidases are difficult to isolate and are usually available only in very small quantities.

7-Chlorotetracycline is produced by *S. aureofaciens* strains ATCC 10762 and Tü24. From each species, a bromoperoxidase has been isolated but, surprisingly, the properties of the enzyme from ATCC 10762 described by Krenn *et al.* (1988) were different from those of the enzyme purified from *S. aureofaciens* Tü24 by van Pée *et al.* (1987). To check whether differences in the two bromoperoxidases were due to the fact that they were produced by different strains, we purified the ATCC 10762 enzyme and examined its immunological and molecular properties. During the purification a second bromoperoxidase, also mentioned by Krenn *et al.* (1988), was detected in cell extracts of this strain.

To be able to compare the molecular and catalytic properties, as well as the immunological relationship, of the two bromoperoxidases from *S. aureofaciens* ATCC 10762, and to obtain information which might help

Abbreviations: BPO 1, BPO 2, bromoperoxidases 1 and 2 from *Streptomyces aureofaciens* ATCC 10762.

towards cloning the corresponding genes, we purified and partially characterized both enzymes.

Methods

Chemicals. Monochlorodimedone, *p*-nitrophenyl-agarose, and protein-A-peroxidase were purchased from Sigma. Hydrogen peroxide (30%, v/v) and 3,3'-5,5'-tetramethylbenzidine were from Merck, and *o*-dianisidine was purchased from Bayer.

Bacterial strains, culture conditions and plasmids. The 7-chlorotetracycline-producing strain *S. aureofaciens* ATCC 10762, *S. aureofaciens* Tü24, and *S. lividans* TK64 containing the cloned bromoperoxidase gene from *S. aureofaciens* Tü24 on a plasmid (pHM621) (van Pée, 1988), were used. *S. aureofaciens* ATCC 10762 was grown in 100 ml flasks containing 25 ml of complex medium (per litre of deionized water: glucose, 4 g; yeast extract, 4 g; malt extract, 10 g; KCl, 0.5 g; FeSO₄·7H₂O, 20 mg) for 4 d at 30 °C on a rotary shaker. Complex medium (1 litre) was inoculated with this culture and incubated for 5 d at 30 °C. A fermenter containing 20 litres of complex medium was inoculated with this culture. After 96 h incubation, the contents of the fermenter were transferred to a 200 litre fermenter containing mineral salt medium (per litre of deionized water: glucose, 20 g; DL-isoleucine, 0.4 g; MgSO₄·7H₂O, 0.2 g; KH₂PO₄, 4.5 g; K₂HPO₄, 10.5 g; NaCl, 90 mg; CaCl₂·2H₂O, 90 mg; ZnSO₄·7H₂O, 4 mg; FeSO₄·7H₂O, 9 mg; CuSO₄·5H₂O, 0.18 mg; MgSO₄·H₂O, 0.03 mg; H₃BO₃, 0.02 mg; and (NH₄)₆Mo₇O₂₄·4H₂O, 0.02 mg; Chatterjee *et al.*, 1983) and incubated for 40 h at 30 °C. *S. aureofaciens* Tü24 was grown as described for *S. aureofaciens* ATCC 10762 or in medium containing 2% (w/v) soybean flour and 2% (w/v) mannitol for 5 d. Cultures of *S. lividans* TK64 harbouring recombinant plasmids were grown in soybean flour/mannitol medium containing thiostrepton (5 µg ml⁻¹; kindly donated by E. R. Squibb & Sons Inc., Princeton, NJ, USA).

Enzyme assays. Brominating activity was measured spectrophotometrically (Hewson & Hager, 1980) with monochlorodimedone (44 mM), H₂O₂ (7.8 mM), bromide (100 mM), sodium azide (10 mM) and a suitable amount of enzyme in 1 M-sodium acetate buffer (pH 5.5). All assays were started with enzyme, and absorbance changes were measured against appropriate blanks. One unit (U) of bromoperoxidase activity is defined as the amount catalysing the formation of 1 µmol monobromomonochlorodimedone min⁻¹. Protein was determined by the Lowry method using bovine serum albumin as a standard.

Electrophoresis. For electrophoresis of native enzymes, 7.5% (w/v) polyacrylamide gels (pH 7.5) were used (Maurer, 1964). PAGE in the presence of 0.1% SDS was done according to Schägger & von Jagow (1987) using a vertical apparatus with 1 mm gels (Desaga) or with 0.5 mm gels (Phase). Proteins from a low molecular mass calibration kit (Pharmacia-LKB) were used as standards.

Analytical isoelectric focusing was done using a horizontal apparatus (Pharmacia-LKB) with a 0.5 mm polyacrylamide gel (5%, w/v) and carrier ampholytes (Serva) in the pH range 2–11. The pI was determined by comparing the migration rate with standard proteins in the pI range 2.5–6 (Pharmacia-LKB). Gels were stained for protein with Serva Blue R and for peroxidase activity with *o*-dianisidine in the presence of 0.1 M-sodium bromide to form brown bands (Shannon *et al.*, 1966). Brominating activity was detected by conversion of phenol red to bromophenol blue (Loo *et al.*, 1964). The reagent contained 1 M-sodium acetate buffer (pH 5.5), 0.002% phenol red, 1 M-sodium bromide, 10 mM-sodium azide and 15.6 mM-H₂O₂.

Production of antisera. Purified bromoperoxidase (1 ml) from *S. aureofaciens* Tü24 at a concentration of 5 mg ml⁻¹ in PBS was mixed

1:1 with incomplete Freund's adjuvant and injected subcutaneously at seven points into a rabbit. After 4 weeks a booster injection was given with the same solution. After 2 weeks the same amount of bromoperoxidase was injected again twice. The rabbit was bled and the blood was collected in tubes and centrifuged.

Ouchterlony gel diffusion assay. Agarose (5%, w/v) with 0.05% (w/v) sodium azide in 0.1 M-potassium phosphate buffer (pH 7.5) was used to fill a plate with agarose bond film (Pharmacia-LKB) to a height of 0.5 cm. The assay was done as described by Ouchterlony (1948).

Electroblotting of proteins, sequencing and amino acid analysis. Proteins separated by native PAGE were transferred to a nitrocellulose filter (0.2 µm pore size; Schleicher and Schuell) with an LKB Multiphor NovaBlot apparatus. Electroblotting was done with the discontinuous buffer system for isoelectric focusing gels as described in the LKB manual. For amino acid sequencing, proteins were transferred to an Immobilon PVDF membrane (Millipore) at a constant current of 0.8 mA cm⁻² for 2.5 h. Protein bands were stained with 0.2% Coomassie brilliant blue in 45% (v/v) methanol and 10% (v/v) acetic acid for 10–15 min, then destained in 40% (v/v) methanol and 7% (v/v) acetic acid for 10–15 min. The NH₂-terminal amino acid sequence of proteins blotted on Immobilon PVDF was determined with a protein sequencer from Applied Biosystems. For amino acid analysis (Biotronic LC 6000 amino acid analyser), samples were hydrolysed in evacuated, sealed tubes in 6 M-HCl at 110 °C for 24, 48, 72 and 96 h (Spackman, 1967). Half-cysteine was determined as cysteic acid after performic acid oxidation (Hirs, 1967). Tryptophan was determined after hydrolysis with *p*-toluenesulphonic acid (Liu, 1972).

Partial purification of bromoperoxidases from *S. aureofaciens* ATCC 10762 and Tü24

Crude extracts. One vol. (1700 g wet wt) of cells was suspended in 2 vols of 0.2 M-Tris/H₂SO₄ buffer (pH 8.3) and disrupted with a Branson sonifier (J 17-A; twenty 30 s periods, 70 W, 4 °C). Cell debris was removed by centrifugation for 30 min at 22100 *g*.

Ammonium sulphate precipitation. Ammonium sulphate was added to 25% saturation and the mixture was stirred for 30 min at 0 °C. After centrifugation at 22100 *g* for 30 min, the supernatant solution was brought to 80% ammonium sulphate saturation. The precipitate was collected by centrifugation, redissolved in 0.1 M-sodium acetate buffer (pH 5.5) and dialysed against the same buffer for 15 h. After centrifugation the pellet was discarded and the supernatant solution was dialysed against 0.03 M-Tris/H₂SO₄ buffer (pH 8.3) for 15 h.

DEAE-Sephacel. The dialysed sample was clarified by centrifugation and applied to a DEAE-Sephacel column (Pharmacia-LKB) (5.6 × 10 cm) equilibrated with 0.03 M-Tris/H₂SO₄ buffer (pH 8.3). The column was washed with 0.1 M-NaCl in 0.03 M-Tris/H₂SO₄ buffer and proteins were eluted with a gradient of 0.1–0.7 M-NaCl in the same buffer (800 ml). Fractions (4 ml) were assayed for protein (A₂₈₀) and brominating activity as described above. Fractions containing bromoperoxidase activity were pooled separately as BPO 1 and BPO 2 and concentrated by ultrafiltration with a PM-10 membrane (Amicon).

Further purification of bromoperoxidase 1 (BPO 1)

Heat treatment. 'Pool 1' from the DEAE-Sephacel column was incubated at 75 °C for 10 min, and the precipitate obtained by centrifugation was discarded. The supernatant solution was dialysed against 0.1 M-sodium acetate buffer (pH 4.6) for 15 h and then centrifuged. The solution was immediately dialysed against 0.1 M-ammonium acetate buffer (pH 6.8) for 3 h and then against 10 mM-ammonium acetate buffer (pH 6.8) for 15 h. The dialysed extract was concentrated by ultrafiltration with a PM-10 membrane.

Sephacryl S 300 HR. The concentrated extract was applied to a Sephacryl S 300 HR column (90 × 2 cm) equilibrated with 0.1 M-ammonium acetate buffer (pH 6.8). After elution with the same buffer, fractions were assayed for brominating activity, pooled and concentrated as described above.

Phenyl-Superose HR 5/5. Ammonium sulphate was added to a concentration of 1 M to the solution obtained after Sephacryl S 300 chromatography. The Phenyl-Superose column was eluted with a descending gradient of 1.0 M-ammonium sulphate in 10 mM-ammonium acetate buffer (pH 6.8). Fractions in which brominating activity was detected were combined. After addition of ammonium sulphate to 1 M the pooled fractions were rechromatographed on Phenyl-Superose as described above.

Chelating Sepharose 6 B. After the two purification steps with Phenyl-Superose the pooled active fractions were dialysed against 20 mM-sodium phosphate buffer (pH 7.0) and applied to a Chelating Sepharose 6 B column (Pharmacia-LKB) saturated with Cu^{2+} and equilibrated with 20 mM-sodium phosphate buffer (pH 7.0). Proteins were eluted with the same buffer. Fractions with brominating activity were combined.

Further purification of bromoperoxidase 2 (BPO 2)

Heat treatment. The 'pool 2' fractions from DEAE-Sephacel chromatography were incubated at 70 °C for 10 min and then centrifuged at 22100 g. After dialysis against 10 mM-ammonium acetate buffer (pH 6.8) for 15 h and concentration by ultrafiltration with a PM-10 membrane, the supernatant solution was used for further purification.

Phenyl-Superose HR 5/5. Ammonium sulphate was added to the concentrated protein solution to 0.4 M. The solution was then applied to a Phenyl-Superose HR 5/5 column equilibrated with 0.4 M-ammonium sulphate in 10 mM-ammonium acetate buffer (pH 6.8). Protein was eluted with a descending gradient of 0.4–0 M-ammonium sulphate in 10 mM-ammonium acetate buffer (pH 6.8). Fractions showing brominating activity were pooled and dialysed against PBS (0.13 M-NaCl, 0.007 M- Na_2HPO_4 , 0.003 M- NaH_2PO_4).

Purification of bromoperoxidase from *S. aureofaciens* Tü24 cloned in *S. lividans* TK64. The enzyme was purified as described by van Pée (1988).

Immunoaffinity chromatography. Antibodies (IgG) against bromoperoxidase from *S. aureofaciens* Tü24 were purified by chromatography on a Protein-A-Sepharose CL-4B column (Pharmacia-LKB) according to the method of Goding (1976). The isolated antibodies were coupled to *p*-nitrophenyl-agarose as described by Wilchek & Miron (1982). The protein solution from the Phenyl-Superose column was applied to this immunoaffinity column (2 ml) at a flow rate of 8 ml h⁻¹. The cross-reactive BPO 2 was eluted with 0.05 M-glycine/NaOH buffer (pH 10.9) and 25% (v/v) ethylene glycol. Samples with brominating activity were pooled.

Preparative PAGE. The concentrated extract after immunoaffinity chromatography was fractionated by native PAGE at pH 7.5. The gel segment containing the enzyme was cut-out and the protein was eluted for 15 h with 10 mM-ammonium acetate buffer (pH 6.8).

M_r determination. The M_r values of purified BPO 1 and BPO 2 were estimated by molecular-sieve chromatography with a 90 × 2 cm column of Sephacryl S 300 HR (Pharmacia-LKB) equilibrated with 50 mM-ammonium acetate buffer (pH 6.8). Ferritin (M_r 440 000), gamma globulin (168 000), bovine serum albumin (68 000) and cytochrome *c* (12 000) were used as standards.

Fig. 1. Chromatography on DEAE-Sephacel showing the separation of the two bromoperoxidase activities, BPO 1 and BPO 2, present in extracts from *S. aureofaciens* ATCC 10762. Fractions of vol. 4 ml were collected. ●, Absorbance at 280 nm; □, bromoperoxidase activity; —, NaCl concentration.

Results

Purification of BPO 1 and BPO 2 from *S. aureofaciens* ATCC 10762

No brominating activity could be detected in the crude extract or in fractions obtained by ammonium sulphate precipitation. After the first chromatographic step, brominating activity could be measured. Fractions assayed for brominating activity after chromatography on DEAE-Sephacel exhibited two peaks (Fig. 1). The peak fractions were pooled separately, and the enzymes were purified (Table 1).

After chromatography on Sephacryl S 300 HR, BPO 1 activity could be detected by activity staining with phenol red on native PAGE. The protein-staining band corresponding to this activity was hardly visible; however, a strong protein band which did not show any brominating activity was present (Fig. 2). From 1700 g wet wt of bacteria about 90 μg of BPO 1 were isolated.

Purification of BPO 2 by procedures similar to those used for BPO 1 gave low enrichments of enzyme activity. Therefore, an immunoaffinity purification step was included. Specific interaction between the enzyme and antibodies raised against the bromoperoxidase from *S. aureofaciens* Tü24 led to strong binding of cross-reactive protein on the immunoaffinity column. Because desorption of the enzyme required 0.05 M-glycine/NaOH buffer (pH 10.9), 25% ethylene glycol was added to increase enzyme stability. With immunoaffinity chromatography a 500-fold overall purification was obtained, but PAGE detected an additional protein, co-purifi-

Fig. 2. Native PAGE of bromoperoxidase from *S. aureofaciens* Tü24, and BPO 1 and BPO 2 from *S. aureofaciens* ATCC 10762. (a) Activity staining with phenol red for brominating activity; (b) protein staining with Serva Blue R. Lanes: 1, 3.2 µg bromoperoxidase from *S. aureofaciens* Tü24; 2, 3.2 µg BPO 1; 3, 32 µg partially purified BPO 1 after molecular-sieve chromatography; 4, 3.2 µg BPO 2; 5, mixture of 1.6 µg bromoperoxidase from *S. aureofaciens* Tü24 and 1.6 µg BPO 2.

Table 1. Purification of BPO 1 and BPO 2 from *S. aureofaciens* ATCC 10762

The enzymes were purified from 1700 g (wet wt) bacteria. –, Could not be determined.

Purification step	Total protein (mg)	Total activity (U)	Specific activity [U (mg protein) ⁻¹]	Purification (-fold)	Recovery (%)
Crude extract	37800	–	–	–	–
(NH ₄) ₂ SO ₄ ppt.	13912	–	–	–	–
BPO 1					
DEAE-Sephacel	479.7	7.06	0.015	1	100
Heat treatment	321.8	14.7	0.046	3.06	208.2
Sephacryl S 300	37.38	14.07	0.367	25.06	199.3
1st Phenyl-Superose	0.53	10.05	19.0	1266.6	142.4
2nd Phenyl-Superose	0.19	6.68	35.18	2345.3	94.6
Chelating Sepharose	0.087	4.0	45.23	3015.3	56.7
BPO 2					
DEAE-Sephacel	425.5	9.7	0.022	1	100
Heat treatment	256.6	8.88	0.034	1.55	91.3
Phenyl-Superose	19.54	9.9	0.885	4.02	101.7
Immunoaffinity chrom.	0.22	3.1	10.53	478.6	31.9
Preparative PAGE	0.107	1.65	15.4	700	16.95

cation of which with BPO 2 was probably due to hydrophobic interactions with the gel matrix. For final purification of BPO 2, immunoaffinity chromatography was followed by preparative PAGE. From 1700 g (wet wt) of bacteria about 100 µg of BPO 2 was isolated. Each of the purified enzymes gave a single band on SDS-PAGE (Fig. 3).

Enzymic activities of BPO 1 and BPO 2

The two purified enzymes showed brominating activity in the presence of sodium azide (Fig. 2a); therefore, they

belong to the group of non-haem bromoperoxidases. That they showed peroxidase activity only in the presence of bromide was demonstrated by activity staining after PAGE. The specific brominating activity of BPO 1 was 45 U (mg protein)⁻¹, that of BPO 2 was 15 U (mg protein)⁻¹.

The pH optimum for the bromoperoxidase activity of BPO 1 in 1 M-sodium acetate buffer was pH 5.5; that for BPO 2 was pH 4.5.

The stability of the enzymes at higher temperatures was examined by incubation in 0.03 M-Tris/H₂SO₄ buffer (pH 8.3) at 70 and 80 °C followed by assay at

Fig. 3. SDS-PAGE of bromoperoxidase from *S. aureofaciens* Tü24 and of BPO 1 and BPO 2 from *S. aureofaciens* ATCC 10762. Lanes: 1, 6.4 µg bromoperoxidase from *S. aureofaciens* Tü24; 2, 6.4 µg BPO 1; 3, partially purified BPO 1 after molecular-sieve chromatography; 4, 6.4 µg BPO 2; 5, mixture of 3.2 µg bromoperoxidase from *S. aureofaciens* Tü24 and 3.2 µg BPO 2; 6, mixture of 3.2 µg bromoperoxidase from *S. aureofaciens* Tü24 and 3.2 µg BPO 1; 7, SDS-PAGE marker proteins. The gel was stained with Serva Blue R.

25 °C. After 4 h at 70 °C, 64% of the original brominating activity of BPO 2 was still present. BPO 1 showed no inactivation after 4 h at 70 °C. Also, after 5 min at 80 °C, BPO 1 showed no loss of activity, whereas BPO 2 retained only 50% of its activity.

The K_m values of BPO 1 and BPO 2 for bromide and H_2O_2 are listed in the general comparison of properties of all three bromoperoxidases (see Table 3).

Molecular properties of the three bromoperoxidases

The M_r values estimated from gel chromatography on Sephacryl S 300 HR were 65 000 for BPO 1 and 90 000 for BPO 2. When a mixture of BPO 2 and bromoperoxidase from *S. aureofaciens* Tü24 was applied to a Superdex 200 column (Pharmacia-LKB) the two enzymes could not be separated. The M_r of the subunits of BPO 1 and BPO 2 determined by SDS-PAGE were identical to those of the bromoperoxidase from *S. aureofaciens* Tü24 (31 000, Fig. 3). Presumably, therefore, BPO 1 consists of two identical subunits and BPO 2 has three identical subunits. During PAGE native BPO 1 migrated more slowly than BPO 2, which was electrophoretically similar to the bromoperoxidase from *S. aureofaciens* Tü24. Mixtures of native BPO 2 and bromoperoxidase from *S. aureofaciens* Tü24 showed two closely spaced bands on PAGE gels (Fig. 2), but only one band on SDS-PAGE gels (Fig. 3). In isoelectric focusing experiments, BPO 1 gave a single band with a pI of 4.5, and BPO 2 gave a band of pI 3.5.

Amino acid composition of Tü24 bromoperoxidase and partial sequences of BPO 1 and BPO 2 from ATCC 10762

The result of the amino acid analysis of BPO from *S. aureofaciens* Tü24 is presented in Table 2. The enzyme

Table 2. Amino acid composition of bromoperoxidase from *S. aureofaciens* Tü24

Amino acid	No. of residues per subunit
Aspartic acid	32
Threonine*	20
Serine*	15
Glutamic acid	33
Proline	12
Glycine	25
Alanine	31
Valine	17
Methionine	1
Isoleucine	9
Leucine	23
Tyrosine	13
Phenylalanine	16
Lysine	7
Histidine	6
Arginine	12
Tryptophan†	2
Cysteine‡	0

* Values obtained by extrapolating to time zero, assuming first-order decay.

† Tryptophan was determined by the method described by Liu (1972).

‡ Cysteine was determined as cysteic acid according to the method described by Hirs (1967).

contained no cysteine and only one methionine; acidic amino acids predominated over basic residues.

The NH_2 -terminal amino acid sequence of BPO 1 was NH_2 -Pro-Ile-Ser-Thr-Thr-Arg-Asp-Gly-Val-Glu-Ile-Phe-Tyr-Lys-Asp-Asp-Gly-Gln-Gly-Arg-; that of BPO 2 was NH_2 -Pro-Phe-Ile-Thr-Val-Gly-Gln-Glu-Asn-Ser-Thr-Ser-Ile-Asp-Leu-Tyr-Tyr-Glu-Asp-His-, which is identical to the sequence of the first twenty NH_2 -terminal amino acids of the bromoperoxidase from *S. aureofaciens* Tü24 (van Pée, 1988).

Fig. 4. Ouchterlony gel diffusion assay. The central well contained antiserum raised against bromoperoxidase from *S. aureofaciens* Tü24. Well 1 contained bromoperoxidase from *S. aureofaciens* Tü24, well 2 BPO 2 from *S. aureofaciens* ATCC 10762 and well 3 BPO 1 from *S. aureofaciens* ATCC 10762.

Immunological investigations of the three bromoperoxidases

The titre of the serum prepared against bromoperoxidase from *S. aureofaciens* Tü24, as determined by the Ouchterlony assay, was 1:32. When the two bromoperoxidases from *S. aureofaciens* ATCC 10762 were examined with the same serum in the Ouchterlony assay, BPO 1 showed no cross-reaction whereas BPO 2 exhibited an identical cross-reaction (Fig. 4). Similar results were obtained in immunoblotting experiments (data not shown).

Partial purification of bromoperoxidase activity from *S. aureofaciens* Tü24

Extracts of cells of *S. aureofaciens* Tü24 grown on the medium described for *S. aureofaciens* ATCC 10762 did not contain a bromoperoxidase corresponding to BPO 1 from the latter strain. After partial purification only an enzyme immunologically identical with BPO 2 could be detected spectrophotometrically or by activity-staining native PAGE gels with phenol red.

Discussion

Since the two bromoperoxidases isolated and purified from *S. aureofaciens* ATCC 10762 were not inhibited by azide, they belong to the class of non-haem haloperoxidases. That they show peroxidase activity in the presence of bromide was demonstrated by activity staining. Stimulation of peroxidase activity by bromide

has been reported previously for bromoperoxidase from the alga *Corallina pilulifera* (Itoh *et al.*, 1985). However, the mechanism of this activation is not known.

Even after ammonium sulphate fractionation no brominating activity could be detected in crude extracts. This phenomenon has been observed with other bacterial bromoperoxidases (van Pée & Lingens, 1985*b*) and may be due to inhibitory substances of unknown structure. Inactivation of such substances would account for the increased recovery after heat treatment.

As the enzyme was assayed at pH 5.5, proteins eluted from the DEAE-Sephacel column started to precipitate in the assay mixture and made it impossible to measure brominating activity. This could be avoided by dialysing against sodium acetate buffer (pH 5.5) before applying the protein solution to the DEAE-Sephacel column.

BPO 1 and BPO 2 could be separated by ion-exchange chromatography on DEAE-Sephacel; however, when the proteins were eluted too fast, the two brominating activities were not separated. This could explain why Krenn *et al.* (1988) did not detect two brominating enzymes at this purification step.

Krenn *et al.* (1988) reported that the bromoperoxidase they isolated from *S. aureofaciens* ATCC 10762 was homogeneous after ammonium sulphate precipitation, ion-exchange chromatography on DEAE-Sephacel and molecular-sieve chromatography. However, at this stage of purification BPO 1, which accounts for the main brominating activity in extracts from *S. aureofaciens* ATCC 10762, is not the major protein. On polyacrylamide gels BPO 1 was visible only when large amounts of protein were loaded. Krenn *et al.* (1988) obtained 1 mg of homogeneous bromoperoxidase from 100 g of cells, whereas we could isolate only 87 µg of homogeneous BPO 1 from 1700 g of cells. However, after molecular-sieve chromatography we still had 37 mg of protein. From these findings we conclude that the protein Krenn *et al.* (1988) thought was homogeneous bromoperoxidase was actually a different protein and the two bromoperoxidases were present only in very small quantities which could not be detected by staining for protein. As the activity stain used by Krenn *et al.* (1988) is not very sensitive, only one bromoperoxidase could be detected. The improved phenol red assay used by us is even more sensitive than staining of proteins with Serva Blue R.

The properties of the three bromoperoxidases are summarized in Table 3. BPO 1, which eluted first during chromatography on DEAE-Sephacel, showed no immunological cross-reaction with serum raised against bromoperoxidase from *S. aureofaciens* Tü24. On the other hand, the bromoperoxidase (BPO 2) that was eluted at a higher salt concentration cross-reacted with the serum. Therefore, BPO 2 from *S. aureofaciens* ATCC 10762 and the bromoperoxidase from *S. aureofaciens*

Table 3. Comparison of BPO 1 and BPO 2 from *S. aureofaciens* ATCC 10762, bromoperoxidase isolated from *S. aureofaciens* ATCC 10762 by Krenn *et al.* (1988) and bromoperoxidase from *S. aureofaciens* Tü24

ND, Not determined.

Property	<i>S. aureofaciens</i> ATCC 10762		<i>S. aureofaciens</i> Tü24 bromoperoxidase	<i>S. aureofaciens</i> ATCC 10762 bromoperoxidase (Krenn <i>et al.</i> , 1988)
	BPO 1	BPO 2		
M_r	65000	90000–95000	90000–95000	65000
Subunit M_r	31000	31000	31000	32000
No. of subunits	2	3	3	2
pI	4.5	3.5	3.5	3.5
Specific activity [U (mg protein) ⁻¹]	45.2	15.4	8.9	65
Immunological cross-reactivity	–	+	+	ND
K_m (Br ⁻), M	3.9×10^{-2}	1.9×10^{-2}	5.9×10^{-2}	2.0×10^{-2}
K_m (H ₂ O ₂), M	5.5×10^{-3}	1.6×10^{-3}	3.1×10^{-3}	8.0×10^{-3}
Heat-stability (70 °C, 4 h)	100%	64%	100%	20%
(80 °C, 5 min)	100%	50%	100%	ND

Tü24 must possess many identical epitopes. The similarity of these two enzymes was confirmed when the sequence of the first twenty amino acids of the NH₂-terminus of BPO 2 proved identical with that of the bromoperoxidase from *S. aureofaciens* Tü24 (van Pée, 1988), whereas the sequence of BPO 1 was dissimilar.

Although BPO 2 and the bromoperoxidase from *S. aureofaciens* Tü24 exhibited similar immunological properties, isoelectric points, and NH₂-terminal amino acid sequences the two enzymes were not identical and could be separated by native PAGE. However, these two bromoperoxidases are so closely related that BPO 2 cannot be the protein of which Krenn *et al.* (1988) reported the amino acid composition, as the latter was very different from the composition we obtained (see Table 2) for the bromoperoxidase from *S. aureofaciens* Tü24. Therefore, we assume that the bromoperoxidase described by Krenn *et al.* (1988) corresponds to BPO 1.

No enzyme corresponding to BPO 1 was found in *S. aureofaciens* Tü24 (van Pée *et al.*, 1987). As this strain produces 7-chlorotetracycline, it is very likely that BPO 2 and the immunologically identical bromoperoxidase from *S. aureofaciens* Tü24 are the chlorinating enzymes involved in 7-chlorotetracycline biosynthesis. Although these enzymes were isolated as bromoperoxidases and do not chlorinate monochlorodimedone, they may be able to chlorinate a more appropriate substrate, as shown for the chloroperoxidase from *Pseudomonas pyrracinia* (Wiesner *et al.*, 1988). Thus the possibility that they are chlorinating enzymes remains.

Dr Kieß, Institut für Lebensmitteltechnologie, Universität Hohenheim and Dr Hauer, BASF, Ludwigshafen, for determining the partial amino acid sequences, and Mrs Sury for skilled technical assistance. This work was supported by the Deutsche Forschungsgemeinschaft (O.P.), by a Heisenberg grant from the Deutsche Forschungsgemeinschaft (K.-H.v.P.), by the Bundesministerium für Forschung und Technologie (FRG) under the contract 0319416A, and by the Fonds der Chemischen Industrie.

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We are grateful to Mrs Nitsche, Institut für Biochemie, Universität Stuttgart, for help with the amino acid analysis. We wish to thank

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