Detection of a bromoperoxidase in *Streptomyces phaeochromogenes*

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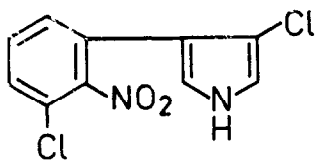
A bromoperoxidase could be detected after fractionation in the chloramphenicol producing actinomycete, *Streptomyces phaeochromogenes*. This enzyme is capable of catalyzing the bromination of the antifungal antibiotic pyrrolnitrin [3-chloro-4-(2-nitro-3-chlorophenyl)pyrrole] in the 2-position of the pyrrole ring. The enzyme had a pH optimum of 5.0. This procaryotic bromoperoxidase requires the presence of H_2O_2 and can also brominate monochlorodimedone, but cannot catalyze chlorination. This enzyme is the first haloperoxidase described from procaryotic sources.

Bromination *Bacterial haloperoxidase* Streptomyces phaeochromogenes *Chloramphenicol* *Pyrrolnitrin*

1. INTRODUCTION

Among many microbial natural halometabolites a few bromo derivatives such as bromonitrins [1,2], pyrrolomycins [3] and bromo analogues of chloramphenicol [4] are known. The enzymatic incorporation of chlorine into organic metabolites is known to be catalyzed by myeloperoxidase [5] and by chloroperoxidase from *Caldariomyces fumago* [6]. Bromoperoxidases, enzymes that in the presence of hydrogen peroxidase oxidize Br^- but not Cl^-, were isolated from several marine algae [7–9], but to date no enzyme capable of chlorination or bromination has been discovered in bacteria.

Here we describe the bromination of pyrrolnitrin (form I) by an enzyme from *Streptomyces*



phaeochromogenes, a chloramphenicol producing actinomycete. The bromination of pyrrolnitrin was used to confirm the nature of the reaction.

2. MATERIALS AND METHODS

2.1. *Chemicals*

Pyrrolnitrin was a gift of Ciba-Geigy (Basel, Switzerland). H_2O_2 (30%) was purchased from Merck (Darmstadt, FRG). Monochlorodimedone was prepared from dimedone by chlorination with sodium hypochloride [10]. Protamine sulfate (salmine-sulfate) was purchased from Fluka (Buchs, Switzerland).

2.2. *Organism and culture conditions*

S. phaeochromogenes NRRL B-3559 was received from Northern Regional Research Laboratories (Peoria, IL). This chloramphenicol producing strain was grown in 2-l Erlenmeyer flasks, containing 1 l medium at 30°C for 96 h on a rotary shaker. The mineral salt medium described [11], with glucose as the carbon source and L-isoleucine as the nitrogen source, was used. Cells were harvested by centrifugation, yielding about 12 g/l (wet wt).

2.3. *Enzyme assays*

Brominating activity was measured as in [12] with monochlorodimedone (44 μM) as substrate in

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the presence of H_2O_2 (7.2 mM) and bromide (82 mM) and a suitable amount of enzyme in 0.1 M sodium acetate buffer (pH 5.5). The reaction was started by the addition of H_2O_2. The decrease in monochlorodimedone absorbance at 290 nm ($\epsilon = 1.99 \times 10^4\ M^{-1} \cdot cm^{-1}$) with time was recorded on a Uvikon 810 spectrophotometer (Kontron). One unit of bromoperoxidase activity was defined as the formation of 1 μmol monobromomonochlorodimedone/min. Pyrrolnitrin was brominated in a 110 ml assay containing pyrrolnitrin (22.9 μM), sodium bromide (82 mM), H_2O_2 (72 mM), and 35.8 munits crude bromoperoxidase in 0.1 M sodium acetate buffer (pH 5.5). The reaction was started by the addition of H_2O_2. After incubation for 30 min at 25°C, another 35.8 munits of crude bromoperoxidase were added and the incubation was continued for a further 30 min.

2.4. *Isolation and purification of brominated pyrrolnitrin*

The reaction mixture was extracted twice with 2 vols ethyl acetate. The combined extracts were dried over anhydrous $MgSO_4$ and evaporated to dryness. The residue was dissolved in as little methanol–water (70:30, v/v) as possible, and purified by high-performance liquid chromatography (HPLC). The HPLC separations were performed on an HPLC apparatus (Knauer, Berlin, FRG) equipped with a dual detector and stainless steel columns (8 × 250 mm) filled with LiChrosorb RP8 (5–20 μm). The solvent system was methanol–water (70:30, v/v). A flow rate of 2 ml/min was maintained and the effluent was monitored at 254 nm.

2.5. *Spectral characterisation*

UV, spectrophotometer Uvikon 810 (Kontron, FRG); GC–MS, mass-spectrometer Varian 3700 (Varian, Bremen, FRG); glass capillary column, 25 m, SE 30; ^{1}H-NMR, Bruker WM 250 (Bruker, Karlsruhe-Forchheim, FRG).

2.6. *Partial purification of bromoperoxidase*

One part of cells (wet wt) was suspended in two parts of 50 mM sodium acetate buffer (pH 5.5) and disrupted with a Branson sonifier J-17 A for six 30-s periods. The cell debris was removed by centrifugation for 30 min at 22100 × g and 4°C. To the crude extract, protamine sulfate was added to 0.2% and the mixture was stirred for 20 min. The precipitate was removed by centrifugation for 30 min at 22100 × g. The supernatant was brought to 35% saturation with solid $(NH_4)_2SO_4$. After stirring for 30 min the precipitate was removed by centrifugation and the supernatant was brought to 70% saturation with solid $(NH_4)_2SO_4$. After stirring for 30 min the precipitate was collected by centrifugation and was dissolved in 50 mM sodium acetate buffer (pH 5.5) and dialyzed for 15 h against 5 l of the same buffer. The solution was passed onto a column (10.0 × 2.7 cm) of DE 52 and equilibrated with 50 mM sodium acetate buffer (pH 5.5). The sample was washed onto this column with 450 ml of this buffer and a 1.2-l gradient (0.2–0.6 M sodium acetate buffer, pH 5.5) was applied. Fractions (6.4 ml) were assayed for protein (A_{280}) and haloperoxidase activity. Those fractions (67–115) having an activity of more than 15% of the maximal activity were pooled and concentrated using an Amicon concentrator with a PM-30 membrane. The concentrated, pooled fractions were dialyzed for 15 h against 5 l of 10 mM potassium phosphate buffer (pH 7.0) and stored at −20°C.

3. RESULTS

When the crude extract was used, neither monochlorodimedone nor pyrrolnitrin was halogenated. With the supernatant of the protamine sulfate precipitation a negative result was obtained, too. Using the redissolved pellet of the 35–70% $(NH_4)_2SO_4$ precipitation, no halogenating activity was detectable either. However, when the eluate of the DE 52 column was tested, about two-thirds of the way through the salt gradient, protein, exhibiting brominating activity, was eluted. The pooled fractions had a specific activity of 0.12 units/mg for the bromination of monochlorodimedone. When Cl^- was used instead of Br^- none of the two organic substrates was halogenated. The addition of bromide and fluoride to the monochlorodimedone assay at pH 5.5 resulted in partial inhibition, as is shown in fig.1. The pH optimum is 5.0. At pH 4.5 the enzyme is already totally inactive, whereas at pH 7.0 there is still 30% of the maximal activity (fig.2).

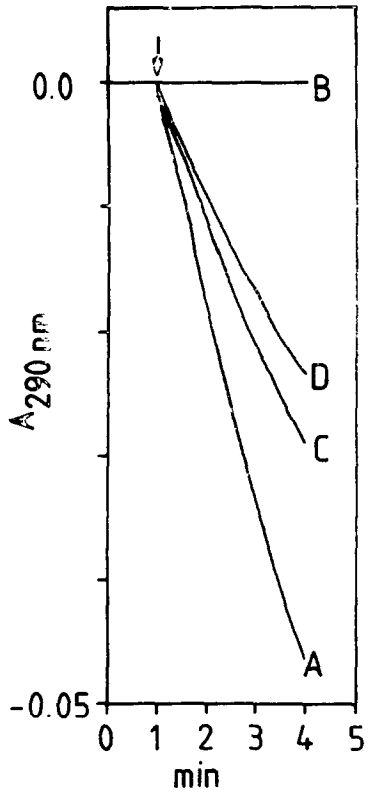


Fig.1. Monochlorodimedone assay with bromoperoxidase from *S. phaeochromogenes*. The assays were carried out as described in section 2. The curves compare the rate of bromination of monochlorodimedone (44 μM), when the assays are initiated with H_2O_2 (arrows). Curve A shows the bromination in the presence of 82 mM bromide and H_2O_2 (7.2 mM). The reactions with 82 mM chloride (B), 82 mM bromide and 82 mM chloride (C) and 82 mM bromide in the presence of 82 mM fluoride (D) at pH 5.5 are shown.

The product of the enzymatic bromination of pyrrolnitrin was purified by HPLC. The elemental composition of the obtained compound was established by GC–MS. The molecular ion appeared as a quartet at *m/e* 334/336/338/340 (intensity ratio, 10/16/7/1). This is characteristic of a monobromodichloro-substituted compound. The UV absorption spectrum in MeOH (neutral and acidic 0.1 N HCl) exhibited a shoulder at 274 and 240 nm. In alkaline solution (0.1 N NaOH) the spectrum showed a maximum at λ_{max} = 274 nm and a shoulder at 310 nm.

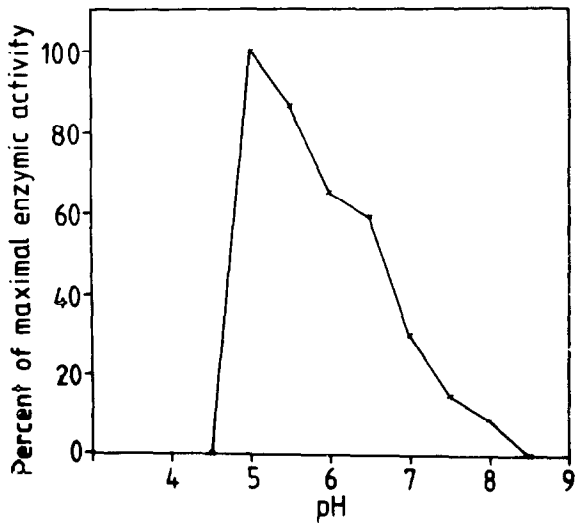
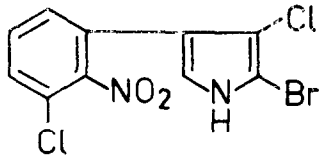


Fig.2. The effect of pH on the brominative activity of *S. phaeochromogenes* bromoperoxidase using the monochlorodimedone assay in 0.1 M potassium phosphate buffer.

The detailed structure was analyzed by ^{1}H-NMR. The signal for pyrrole C-H was equivalent to one proton at 7.03 ppm in $(CD_3)_2CO$, which can be assigned to the 2-position; a substituent in 5-position (*ortho* to the biaryl linkage) should strongly influence the 6′-H signal at 7.602 ppm (pyrrolnitrin at 7.605 ppm) [2]. From these spectral findings we deduce the structure 2-bromo-3-chloro-4-(2-nitro-3-chlorophenyl)pyrrole (form II).



4. DISCUSSION

Although several bromoperoxidases from marine algae [6–8] and two chlorinating enzymes [4,5] are already known, to date no haloperoxidase has been discovered in bacteria. In crude extracts from *S. phaeochromogenes* no halogenating activity can be detected either. However, when the extract is fractionated, using a DE 52 column with sodium acetate buffer (pH 5.5) the monochlorodimedone test can be employed successfully. This is

probably due to the fact that crude extracts of *S. phaeochromogenes* contain catalase which competes very efficiently with bromoperoxidáse for H_2O_2. Only after sufficient removal of this catalase can halogenating activity be measured.

Surprisingly, only brominating activity could be detected with monochlorodimedone as substrate. To check this result, we used pyrrolnitrin (form I) as substrate, because from our previous studies on pyrrolnitrin biosynthesis [2] we had some experience on the purification and structural elucidation of pyrrolnitrin derivatives. The obtained product [2-bromo-3-chloro-4-(2-nitro-3-chlorophenyl)pyrrole (form II)] confirmed the result, obtained with the monochlorodimedone test. The enriched enzyme was only capable of bromination although *S. phaeochromogenes* produces chloramphenicol, a chloride-containing metabolite.

Whether chlorination in *S. phaeochromogenes* is bromonium ion-induced as postulated for the formation of some chlorometabolites in marine algae [13], or whether chlorinating activity is not detectable because of a yet unknown inhibitor or because of an interfering enzyme is not yet known.

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