Detection of a new chloroperoxidase in *Pseudomonas pyrrocinia*

Wolfgang Wiesner, Karl-Heinz van Pee and Franz Lingens

Institut für Mikrobiologie der Universität Hohemheim, Garbenstraße 30, D-7000 Stuttgart 70, FRG

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A new chloroperoxidase could be detected in *Pseudomonas pyrrocinia* ATCC 15 958, a bacterium that produces the antifungal antibiotic pyrrolnitrin. This enzyme was separated from a ferriprotoporphyrin IX containing bromoperoxidase which was also produced by this bacterium. The enzyme is capable of catalyzing the chorination of indole to 7-chloroindole. This procaryotic chloroperoxidase requires the presence of H_2O_2 and can also brominate monochlorodimedone, but cannot catalyze its chlorination. This enzyme is the first chloroperoxidase described from procaryotic sources.

Chloroperoxidase (Pseudomonas pyrrocinia) *Bacterial haloperoxidase* *Pyrrolnitrin*

1. INTRODUCTION

The enzymatic incorporation of chlorine into organic metabolites is known to be catalyzed by myeloperoxidase [1] and by chloroperoxidase from *Caldariomyces fumago* [2]. Bromoperoxidases, enzymes that in the presence of hydrogen peroxide oxidize Br^- but not Cl^-, were isolated from several marine algae [3–7] and from bacteria [8–10]. These enzymes are all heme-proteins, with the exception of the bromoperoxidases from *Corallina pilulifera* [6] and *Ascophyllum nodosum* [7] which represent a novel class of haloperoxidases. The latter enzyme contains vanadium as a prosthetic group instead of protoporphyrin IX.

Here we describe the detection of the first bacterial chloroperoxidase from *Pseudomonas pyrrocinia*, a bacterium that produces the chlorine-containing antibiotic pyrrolnitrin and several chlorinated indole derivatives such as 7-chloroindole [11].

Dedicated to Professor Erich Hecker on the occasion of his 60th birthday

2. MATERIALS AND METHODS

2.1. *Chemicals*

Monochlorodimedone was prepared from dimedone by chlorination with sodium hypochloride [12]. Indole and H_2O_2 (30%) were purchased from Merck (Darmstadt, FRG). 7-Chloroindole was prepared from 7-chlorotryptophan according to Lübbe et al. [11]. 6-Chloroindole was purchased from Sigma (St. Louis, USA) and 4- and 5-chloroindole were from Jansen Chimica (Beerse, Belgium).

2.2. *Microorganism and culture conditions*

P. pyrrocinia ATCC 15 958 from which chloroperoxidase was isolated was grown for 3 days at 30°C and aeration (0.3 v/v per min) and stirring (150 rpm) in a 100-l fermentor which was inoculated with 5 × 1 l cultures from the late exponential growth phase. The mineral salt medium described by Lübbe et al. [11] was used. Cells were harvested by centrifugation.

2.3. *Enzyme assays*

Brominating activity was measured as described

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by Hewson and Hager [13] with monochlorodimedone (44 μM) as substrate in 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). 1 unit of bromoperoxidase activity was defined as the formation of 1 μmol monobromomonochlorodimedone/min. Indole was chlorinated in a 100 ml assay containing indole (50 μM), potassium chloride (8.2 mM), sodium azide (5 mM), H_2O_2 (7.2 mM), and 10 mU of partially purified chloroperoxidase in 0.1 M sodium acetate buffer (pH 4.0). The reaction was started by the addition of H_2O_2. After incubation for 16 h at 25°C the reaction mixture was extracted twice with 40 ml ethyl acetate.

2.4. *Spectral characterization*

UV, spectrophotometer Uvikon 810 (Kontron, FRG); GC-MS, mass-spectrometer Varian 3700 (Varian, Bremen, FRG); glass capillary column, 25 m, SE 30.

2.5. *Partial purification of* P. pyrrocinia *chloroperoxidase*

All steps were performed at 4°C. One part of cells (wet wt) was suspended in two parts of 0.1 M potassium phosphate buffer (pH 7.0) 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 18000 × *g* and 4°C. The crude extract was dialysed against 10 mM potassium phosphate buffer (pH 7.0). The solution was passed onto DE-52 equilibrated with 10 mM potassium phosphate buffer (pH 7.0). The sample was washed onto the column with 1500 ml of buffer and a 400-ml gradient (10–500 mM potassium phosphate buffer, pH 7.0) was applied. Fractions (3.2 ml) were assayed for protein (A_{280}) and haloperoxidase activity. Those fractions (21–35) having an activity of more than 25% of the maximal activity were pooled and dialysed against 10 mM potassium phosphate buffer (pH 8.5). This sample was applied to a DE-Sephadex A-25 column, equilibrated with 10 mM potassium phosphate buffer (pH 8.5). The unadsorbed fractions were collected and concentrated by ultrafiltration using a PM-30 membrane (Amicon).

3. RESULTS

No halogenating activity was detected in crude extracts. However, when the eluate of the DE-52 column was tested, brominating activity was eluted. The pooled fractions had also peroxidase and catalase activity because the chloroperoxidase could not be separated from the bromoperoxidase [10] at this stage. This, however, was achieved by employing a DEAE-Sephadex A-25 ion-exchange column with potassium phosphate buffer, pH 8.5. The unadsorbed, pooled and concentrated fractions had a specific activity of 0.26 units/mg for the bromination of monochlorodimedone. When Cl^- was used instead of Br^- this organic substrate was not halogenated. The addition of F^- to the monochlorodimedone assay at pH 5.5 resulted in partial inhibition but Cl^- had no effect on the bromination. The brominating activity was not inhibited by the addition of sodium azide, whereas the previously isolated bromoperoxidase [10] was totally inhibited by sodium azide. This indicates that chloroperoxidase from *P. pyrrocinia* has no heme prosthetic group. The chlorination of indole by chloroperoxidase is illustrated in figs 1 and 2. The elemental composition of the obtained compound was established by GC-MS. The molecular ion of the major product (B) appeared as a doublet at *m/e* 151/153 (intensity ratio, 3/1). This is characteristic of a monochloro-substituted compound. These spectral data and the retention time

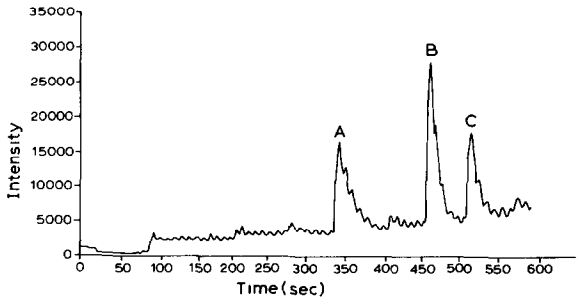

Fig.1. Gas chromatogram of the reaction mixture containing indole, H_2O_2, potassium chloride, sodium azide and chloroperoxidase. The peaks were: A, indole; B, 7-chloroindole; C, monobromoindole.

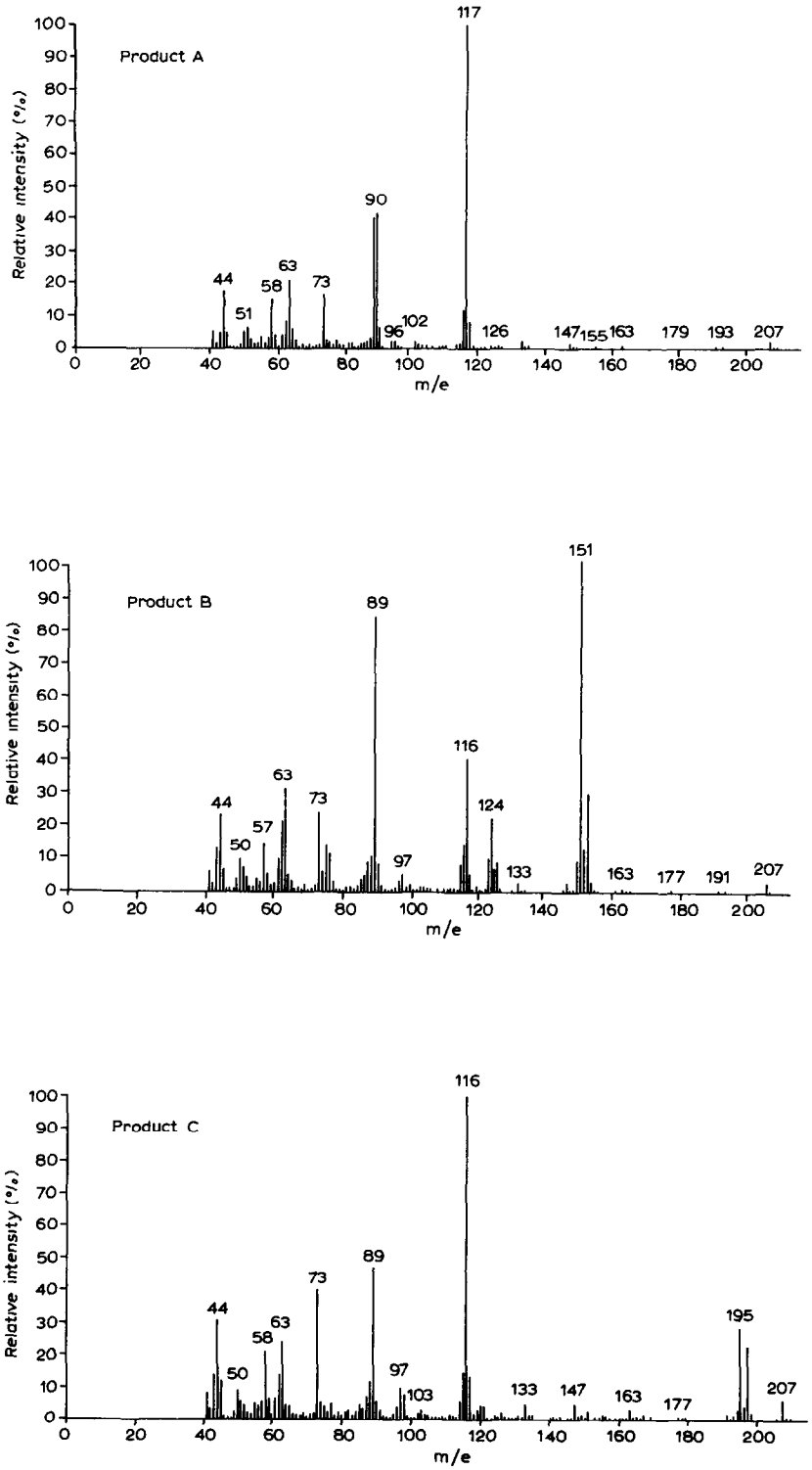

Fig.2. Mass spectra of the reaction products of chloroperoxidase. Products: A, indole; B, 7-chloroindole; C, monobromoindole.

Table 1

Retention times of chloroindoles

Compound	Retention time (min)
4-Chloroindole	4.6
5-Chloroindole	4.9
6-Chloroindole	4.7
7-Chloroindole	3.4
Enzymatically formed product	3,4

(table 1) by gas chromatography were identical with those from an authentic sample of 7-chloroindole, prepared by degradation of 7-chlorotryptophan by *P. pyrrocinia* [11]. The molecular ion of the second product (C) appeared as a doublet at *m/e* 195/197 (intensity ratio, 1/1). This is characteristic of a monobromo-substituted compound.

4. DISCUSSION

Several bromoperoxidases have been isolated from marine organisms [3–7]. These enzymes are thought to be responsible for the production of brominated natural products that commonly occur in marine organisms. Halometabolites from terrestrial organisms normally contain chlorine. Therefore it was very surprising that until now only brominating enzymes could be detected in bacteria such as *Streptomyces phaeochromogenes* [8], *P. aureofaciens* [9] and *P. pyrrocinia* [10]. However, when the crude extract from *P. pyrrocinia* is fractionated, using a DE-52 column with potassium phosphate buffer (pH 7.0) and a DEAE-Sephadex-A25 column with potassium phosphate buffer (pH 8.5), the chloroperoxidase can be separated from the bromoperoxidase [10].

Chloroperoxidase from *P. pyrrocinia* has no peroxidase and catalase activity but only brominating activity with monochlorodimedone as substrate. Another striking difference between bromoperoxidase and chloroperoxidase from *P. pyrrocinia* is the fact that chloroperoxidase was not inhibited by the addition of sodium azide. This suggests that chloroperoxidase is not a heme-protein. Chloroperoxidase did not catalyze the chlorination of monochlorodimedone but was able to chlorinate indole to 7-chloroindole. Chloroperoxidase from *C. fumago*, however, is reported to chlorinate monochlorodimedone and to form oxindole but no chloroindole with indole as substrate [14]. The detected bromoindole is probably due to very small contaminations of bromide, present in the potassium chloride and a very high affinity of chloroperoxidase to bromide ions.

The isolation of enzymatically formed 7-chloroindole suggests that chloroperoxidase could play a role in the formation of the 7-chloroindole derivatives and in chlorinated phenylpyrrole compounds isolated from *P. pyrrocinia*.

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