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Cloning and high-level expression of a chloroperoxidase gene from *Pseudomonas pyrrocinia* in *Escherichia coli*

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A chloroperoxidase gene from *Pseudomonas pyrrocinia* was cloned into *Escherichia coli* using the cosmid vector pJB8. The gene coding for the chloroperoxidase could be localized to a 1.5 kb fragment of DNA which was subcloned into the high-copy-number plasmid pUC18. In one subclone increased halogenating activity could be found which was 570-fold greater than in *P. pyrrocinia*. The halogenating enzyme was identified as the chloroperoxidase by SDS-polyacrylamide gel electrophoresis.

Chloroperoxidase; DNA cloning; Enzyme overproduction; (*Pseudomonas pyrrocinia*)

1. INTRODUCTION

Pseudomonas pyrrocinia produces the chlorinated antibiotic pyrrolnitrin [1]. From this strain a chlorinating enzyme was isolated [2]. This chloroperoxidase catalyzes the chlorination of monodechloroaminopyrrolnitrin to aminopyrrolnitrin [2] which is a direct precursor of pyrrolnitrin. Furthermore, chloroperoxidase chlorinates indole to yield 7-chloroindole [3]. Chloroperoxidase is also able to brominate and iodinate suitable organic substrates.

The chloroperoxidase from *P. pyrrocinia* belongs to the class of non-heme haloperoxidases, a novel class of halogenating enzymes. In contrast to the well-characterized heme-containing haloperoxidases, little is known about the properties of the non-heme enzymes. The chloroperoxidase consists of two identical subunits of 32 kDa. It contains no chromophore or metals in equimolar amounts [2], and it is unclear whether any metal is indeed necessary for the catalytic activity.

These characteristics and the possibility of using the chloroperoxidase for selective halogenation of organic compounds make it of interest and indicate it to be a powerful tool to aid more detailed studies of this enzyme.

Such investigations require large amounts of protein. Therefore, we decided to clone the chloroperoxidase gene, in order to obtain large amounts of the enzyme, through the phenomenon of enzyme overproduction.

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2. MATERIALS AND METHODS

2.1. *Bacterial strains, plasmids and culture conditions*

The bacterial strains used were *P. pyrrocinia* ATCC15958, a bacterium that produces the antifungal antibiotic pyrrolnitrin [1], *E. coli* VCS257, a derivative of DP50 supF [4], included in the DNA packaging kit from Gigapack, and *E. coli* TG2 whose derivation is unclear.

Cosmid pJB8 [5] served as vector for the construction of the gene library; the high-copy-number plasmid pUC18 [6] was used as cloning vehicle in the transformation experiments.

The *E. coli* strains were grown at 37°C in LB broth [4] or on LB agar (1%) supplemented with ampicillin at concentrations of 50–100 μg/ml. Transformants with pUC18 derivative plasmids were selected on ampicillin plates containing 40 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) and 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG). *P. pyrrocinia* was cultured at 30°C in liquid LB and on plates containing mineral salt medium as described in [7].

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2.2. *Preparation of DNA*

Total DNA from *P. pyrrocinia* was isolated according to Marmur [8] with some modifications. Preparative amounts of plasmid DNA were obtained using the method of Birnboim and Doly as described by Maniatis et al. [4] and further purified by CsCl-EtBr centrifugation. For analytical purposes, plasmids were isolated either by an alkaline lysis procedure followed by spermine precipitation [9] or by the boiling method of Holmes and Quigley [10].

2.3. *General DNA manipulations*

All enzymes used in cloning experiments were from Boehringer Mannheim. Agarose gel electrophoresis, DNA digestion with restriction enzymes, dephosphorylation with calf intestinal alkaline phosphatase (CIAP) and ligation were performed as described by Maniatis et al. [4]. Southern and colony blotting were performed using Hybond-N membranes (Amersham) according to the manufacturer's directions with slight modifications. Hybridization experiments were carried out overnight at 20°C using an oligonucleotide as probe or at 68°C for 48 h with nick-translated DNA as probe [4]. The oligonucleotide was labelled with [γ-^{32}P]ATP and polynucleotide kinase [4]. Nick translation was performed with Bio-11-dUTP from Gibco/BRL as suggested by the supplier. DNA fragments were isolated from agarose gels by the freeze-squeeze method [11]. Transformation of *E. coli* with plasmid DNA was according to the $CaCl_2$ procedure [4].

2.4. *Cosmid cloning*

Total DNA of *P. pyrrocinia* was partially digested with *Sau*3A and treated with CIAP. pJB8 DNA was digested with either *Hin*dIII or *Sal*I. The linear plasmids were dephosphorylated and cut with *Bam*HI. Ligation was carried out with 6 μg total DNA fragments of 32–47 kb in size and 0.6 μg of left and right cosmid 'arms'. The recombinant DNA was packaged in vitro using a DNA packaging kit from Boehringer Mannheim. Transduction of *E. coli* VCS257 was carried out as described by Maniatis et al. [4]. Plasmid-containing cells were selected on LB plates supplemented with 50 μg/ml ampicillin. Screening of the gene library was performed by colony hybridization using a mixed 17-base oligonucleotide as probe. The oligonucleotide corresponded to amino acid residues 12–17 of the NH_2-terminal end of chloroperoxidase [2].

2.5. *Preparation of crude extracts*

1 g cells (wet wt) was suspended in 3 ml of 0.1 M potassium phosphate buffer (pH 7.0). The cell suspension was sonicated for six 30-s periods. Cell debris was removed by centrifugation at 37000 × *g* and the supernatant assayed for halogenating activity.

2.6. *Partial purification of the chloroperoxidase*

Crude extracts were either treated at 60°C for 30 min or after dilution (1:10) passed onto a column (V = 10 ml) of DEAE-cellulose DE 52 (Whatman), equilibrated with 10 mM potassium phosphate buffer (pH 7.0). The sample was washed onto the column with this buffer, the chloroperoxidase being eluted with 0.2 M potassium phosphate buffer (pH 7.0). Fractions with halogenating activity were combined.

2.7. *Enzyme assay*

Brominating activity was assayed by the method of Hager et al. [12] with monochlorodimedone (44 μM) as substrate in the presence of H_2O_2 (7.2 mM), bromide (82 mM), NaN_3 (8.9 mM), and a suitable amount of enzyme in 0.1 M sodium acetate buffer (pH 5.5). The reaction was initiated by the addition of enzyme and the decrease in monochlorodimedone absorbance at 290 nm ($\epsilon = 1.99 \times 10^4\ M^{-1} \cdot cm^{-1}$) was monitored.

2.8. *Protein determination*

Protein concentration was determined by the method of Lowry et al. [13] using bovine serum albumin as a standard.

2.9. *SDS-polyacrylamide gel electrophoresis (SDS-PAGE)*

SDS-PAGE (10% gel) was performed as described by Laemmli [14]. Samples contained approx. 100 μg protein. The subunits of chloroperoxidase were identified using standard proteins as molecular size markers.

3. RESULTS

3.1. *Cloning and identification of the chloroperoxidase gene*

Cloning of the chloroperoxidase gene was carried out by constructing a gene library of *P. pyrrocinia* in *E. coli* using the cosmid pJB8 as vector. 2448 ampicillin-resistant colonies were obtained. To check the clones for the presence of recombinant DNA, plasmid DNA of 12 colonies was isolated. In all cases, the recombinant plasmids were 40–50 kb in size and contained different DNA inserts, as judged by restriction analysis. The colonies of the gene library were screened with the radiolabelled chloroperoxidase gene-specific oligonucleotide. Thus, 12 positive clones were detected. However, in Southern blot analysis, plasmid DNA of only three clones hybridized with the oligonucleotide.

Crude extracts of the three clones were assayed for halogenating activity, but none was detectable. Nevertheless after ion-exchange chromatography on DEAE-cellulose, the eluate of one clone (clone 32) did show halogenating activity. The pooled fractions had a total activity of 0.12 units for the bromination of monochlorodimedone.

To identify the halogenating enzyme, the eluate was analyzed by SDS-PAGE. A protein band of 32 kDa was considered as the presumptive chloroperoxidase subunits.

3.2. *Subcloning experiments*

Plasmid DNA of clone 32 was digested with *Eco*RI. The fragment containing the chloroperox-

Table 1

Halogenating activity in crude extracts (a) prepared from 1 g cells, in heat-treated extracts (b), and after ion-exchange chromatography (c)

	Spec. act. (U/mg)	Total activity (U)
P. pyrrocinia (c)	0.09	0.38
Clone 32 (c)	0.012	0.12
Subclone (a)	0.0075	0.21
Subclone (b)	11.25	315
Subclone (c)	12.35	218

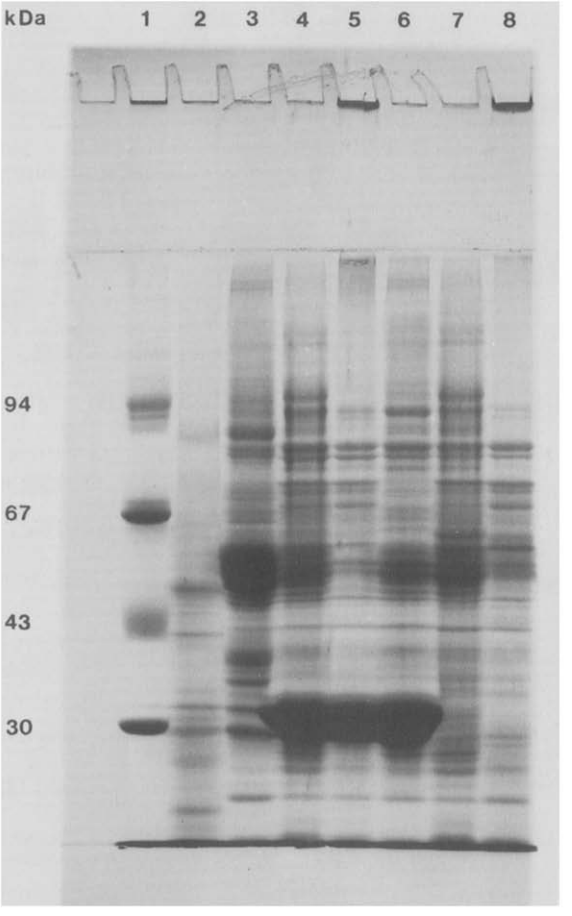

Fig.1. SDS-PAGE of crude extracts (a), heat-treated extracts (b), and extracts after chromatography (c). Lanes: 1, molecular mass size standards; 2, *P. pyrrocinia* (c); 3, clone 32 (c); 4, subclone (a); 5, subclone (b); 6, subclone (c); 7, *E. coli* TG2 (a); 8, *E. coli* TG2 (b).

idase gene was identified by Southern blot hybridization using the specific oligonucleotide as probe. This DNA fragment was subcloned into the *Eco*RI site of pUC18. The ligation mixture was introduced into *E. coli* TG2 by transformation. Several transformants containing recombinant plasmids were examined for the expression of the chloroperoxidase gene by measuring halogenating activity in crude extracts, after heat treatment, and after ion-exchange chromatography.

In one subclone increased halogenating activity could be detected. The total activity was 570-fold higher than in *P. pyrrocinia* and even 1816-fold higher than in clone 32 (table 1).

The extracts were again analyzed by SDS-PAGE (fig.1) and in all samples except the extract of *E. coli* TG2 without plasmid, a protein of molecular mass 32 kDa, which corresponded to the chloroperoxidase subunits, was present. The intensity of the chloroperoxidase band in extracts of the sub-

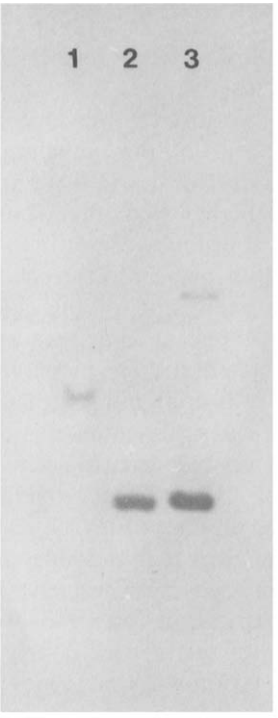

Fig.2. Southern blot hybridization, using the *Eco*RI insert from the plasmid of the subclone as probe. Lanes: 1, total DNA from *P. pyrrocinia* digested with *Eco*RI; 2, plasmid DNA from clone 32 digested with *Eco*RI; 3, plasmid DNA from the subclone digested with *Eco*RI.

clone correlated with the increased halogenating activity.

3.3. *Hybridization of the cloned chloroperoxidase gene with total DNA of* P. pyrrocinia

In order to confirm the origin of the chloroperoxidase gene, localized on the cloned 1.5 kb DNA fragment, Southern hybridization was carried out with an *Eco*RI digest of *P. pyrrocinia* total DNA and *Eco*RI-cleaved plasmid DNA of clone 32, using the 1.5 kb insert as probe (fig.2). The nick-translated biotinylated insert DNA hybridized to a 2.1 kb fragment of total *P. pyrrocinia* DNA. Furthermore, it could be shown that the insert was part of the plasmid of clone 32. The probe hybridized with two bands of *Eco*RI-digested plasmid DNA of the subclone. The upper band was uncleaved plasmid DNA.

4. DISCUSSION

The chloroperoxidase gene of *P. pyrrocinia* was cloned into *E. coli* using the cosmid pJB8. Subcloning allowed us to localize the gene on a 1.5 kb DNA fragment.

A striking feature is the overproduction of chloroperoxidase in the subclone. The halogenating activity in partially purified extracts was 570-fold higher than that found in *P. pyrrocinia*. This is probably due to a gene dosage effect, as pUC18 is present in the cell at a high copy number. The expression of the chloroperoxidase gene in *E. coli* appears to depend on readthrough transcription from the *lac* promoter on pUC18, since *E. coli* cells containing a plasmid, in which the fragment was inserted in the opposite direction, showed no halogenating activity. The high expression of the chloroperoxidase gene was achieved in the absence of IPTG, an inducer of the *lacZ* gene and thus it is possible that the cloned gene was expressed constitutively. In contrast to the high expression in the subclone, the chloroperoxidase gene was poorly expressed in clone 32. It is known that genes derived from *Pseudomonas* species are usually expressed at low levels in *E. coli* [15] owing to differences in promoter structure. Therefore, it is likely that the chloroperoxidase gene in clone 32 was transcribed from its own promoter resulting in the poor gene expression.

In these studies we have observed the same phenomenon as that found in previous investigations on halogenating enzymes from Streptomycetes and Pseudomonads. Halogenating activity usually cannot be measured in crude cell extracts. This is due to one or several inhibitors which can sometimes be removed by heat treatment or ion-exchange chromatography.

The large amounts of chloroperoxidase produced by the subclone enable us to carry out detailed studies on this interesting enzyme. The isolation of the chloroperoxidase gene offers the possibility of investigating not only the protein but also the gene and its regulatory sequences.

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