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Expression of the vanadium-dependent bromoperoxidase gene from a marine macro-alga *Corallina pilulifera* in *Saccharomyces cerevisiae* and characterization of the recombinant enzyme

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Abstract

The vanadium-dependent bromoperoxidase from the marine macro-alga *Corallina pilulifera* was heterologously expressed in *Saccharomyces cerevisiae*. The enzyme was purified and crystals in "tear drop" form were obtained. The catalytic properties of the recombinant enzyme were studied and compared with those of the native enzyme purified from *C. pilulifera*. Differences in thermal stability and chloroperoxidase activity were observed. The recombinant enzyme retained full activity after preincubation at 65 °C for 20 min, but the native enzyme was completely inactivated under the same conditions. The chlorinating activity of the native enzyme was more than ten times higher than that of the recombinant enzyme. Other properties, such as K_m values for KBr and H_2O_2, and optimal temperature and pH, were similar for each source of *C. pilulifera* bromoperoxidase.

Keywords: Macro-alga; *Corallina*; Rhodophyta; Haloperoxidase; Bromoperoxidase

1. Introduction

Haloperoxidases are enzymes catalyzing the halogenation of organic substrates. They are named after the most electronegative halide they use: e.g. bromoperoxidase (BPO) acts on bromide and iodide, whereas chloroperoxidase (CPO) oxidizes chloride, bromide and iodide. Previous studies indicated that many kinds of marine macro-algae including Rhodophyta, Phaeophyceae, and Chlorophyceae, have haloperoxidase activities (Yamada et al., 1985; Wever et al., 1997; Ohshiro et al., 1999). In the Rhodophyta species *Corallina pilulifera*, a high level of BPO was found and the enzyme has been well characterized (Itoh et al., 1985, 1986, 1987a,b). This enzyme was shown to be vanadium-dependent (Krenn et al., 1989), and cDNAs for two distinct BPO isomers were cloned and expressed in *Escherichia coli* (Shimonishi et al., 1998). One of the genes (*bpo1*) codes for a protein of 598 amino acids with a calculated molecular mass of 65,312 Da, and the other gene (*bpo2*) codes for a protein of 597 amino acids with an identity of about 90% to BPO1. The three-dimensional structure of BPO from Rhodophyta *C. officinalis,* which is very similar to the BPO from *C. pilulifera*, has been determined (Isupov et al., 2000).

Vanadium-dependent haloperoxidases have been purified from other algae (Wever et al., 1985; de Boer et al., 1986), lichen (Plat et al., 1987), and fungi (van Schijndel et al., 1993; Barnett et al., 1998). The corresponding genes from the fungal species *Curvularia inaequalis* (Simons et al., 1995) and *Embellisia didymospora* (Barnett et al., 1998) were cloned, and their deduced amino acid sequences were shown to be similar to each other. Molecular biological studies were also performed on the genes encoding vanadium-dependent haloperoxidases from the Phaeophyta, *Ascophyllum nodosum* (Weyand et al., 1999) and *Fucus distichus* (Vreeland et al., 1998), and the deduced amino acid sequences had high similarity. Although the sequence identity of these enzymes to those of *C. pilulifera* is very low, it has been shown that the amino acid residues of the active sites were conserved among the vanadium dependent haloperoxidases. Moreover, the active sites of vanadium-dependent haloperoxidases were shown to be similar to those of acid phosphatases (Hemrika et al., 1997).

Among the vanadium-dependent haloperoxidases, BPO from *C. officinalis* (Sheffield et al., 1993) and CPO from

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C. inaequalis (van Schijndel et al., 1994) were shown to be stable in organic solvents. BPOs from *C. officinalis* (Andersson et al., 1997) and *A. nodosum* (ten Brink et al., 1998) were shown to catalyze the stereoselective oxidation of organic sulfides to the corresponding sulfoxides. Moreover, CPO from *C. inaequalis* was demonstrated to oxidize and bleach a commercial dye, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid), in the presence of hydrogen peroxide (ten Brink et al., 2000). These properties make the vanadium-dependent haloperoxidases potentially useful as industrial catalysts for biotransformations and as bleaching enzymes for laundering.

Molecular biological studies of macro-alga are considered to be difficult because sugars, polyphenols, alginates, fucan sulfates, and tannins in the macro-alga disturb the isolation of nucleic acids (Weyand et al., 1999). Previously we succeeded in cloning and expressing the BPO gene of *C. pilulifera* (Shimonishi et al., 1998). Although it was reported that the algal BPO gene of *F. distichus* was also expressed in *E. coli* (Vreeland et al., 1998), little information is available. The fungal CPO gene from *C. inaequalis* was expressed in the yeast *Saccharomyces cerevisiae* (Hemrika et al., 1999). In this study, we describe the recombinant expression of *C. pilulifera* BPO using the yeast system (Hemrika et al., 1999), and compare the recombinant enzyme with the native enzyme produced by *C. pilulifera*.

2. Results

2.1. Expression of C. pilulifera BPO gene in S. cerevisiae

We constructed two plasmids, pTNT28 and pTNT30, for expressing *C. plilulifera* BPO genes. Both recombinant yeast strains harboring each plasmid did not show BPO activity in the cell-free extract without vanadate. That is, BPO was produced as the apo form in *S. cerevisiae*, and preincubation of the apo enzyme with Na_3VO_4 was necessary for activating the enzyme, similar to the activity of CPO from the fungus *C. inaequalis* produced by recombinant *S. cerevisiae* (Hemrika et al., 1999). It took about 2 h at 30 °C to activate the apo-enzyme to full activity (data not shown). Since the specific activity in the cell-free extract of *S. cerevisiae* with pTNT30 was 1.2 times as high as that with pTNT28, we used the strain *S. cerevisiae* BJ1991/pTNT30 in the subsequent experiments. The specific activity in the cell-free extract of this strain was about 0.05 μkat/mg.

2.2. Purification and crystallization of BPO from S. cerevisiae BJ1991/pTNT30

BPO was purified 122-fold from *S. cerevisiae* BJ1991/pTNT30 and the specific activity of the final purified preparation was 6.28 μkat/mg (Table 1). The N-terminal amino acid sequence (9 residues) of the purified enzyme was identical to that deduced from the *bpo1* nucleotide sequence except for the N-terminal methionine residue. Although SDS–PAGE showed some minor bands (Fig. 1), we tried to crystallize BPO according to

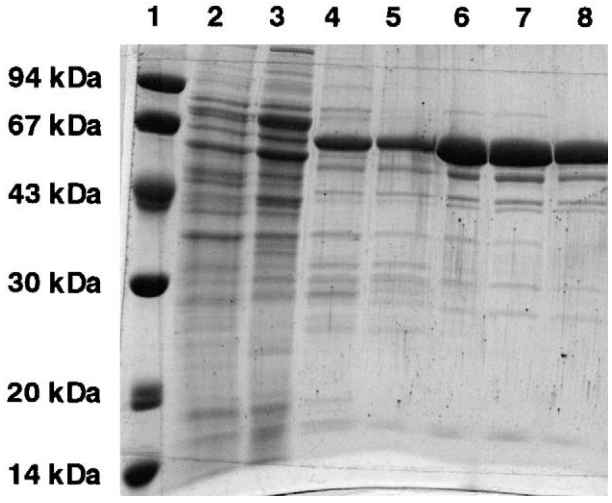

Fig. 1. SDS–PAGE of BPO from the recombinant *S. cerevisiae* BJ1991/pTNT30. Lane 1, marker proteins; lane 2, cell-free extracts (10 μg) of *S. cerevisiae* BJ1991/pTNT30; lane 3, pooled fractions after $(NH_4)_2$ SO_4 fractionation (10 μg); lane 4, pooled fractions after DEAE-Sepharose (5 μg); lane 5, pooled fractions after Sepharose CL-4B (5 μg); lane 6, pooled fractions after 1st Q-Sepharose (5 μg); lane 7, pooled fractions after 2nd Q-Sepharose (5 μg); lane 8, pooled fractions after Cellulofine GC700 (5 μg).

Table 1
Purification of BPO from the recombinant *S. cerevisiae* BJ1991/pTNT30

Purification steps	Protein (mg)	Total activity (μkat)	Specific activity (μkat /mg)	Purification (fold)	Yield (%)
Cell-free extract	5440	282	0.052	1	100
$(NH_4)_2SO_4$ fractionation	1570	154	0.098	1.9	54.5
DEAE-Sepharose	56.8	86.3	1.52	29.4	30.7
Sepharose CL-4B	16.7	63.7	3.81	73.9	22.6
1st Q-Sepharose	6.5	36.2	5.57	108	12.8
2nd Q-Sepharose	6.4	36.2	5.66	109	12.8
Cellulofine GC700	5.3	33.3	6.28	122	11.8

the method previously reported for BPO from *C. officinalis* (Brindley et al., 1998). As a result, the crystals were formed within 12 h and large crystals (0.5×0.6 mm) grew in 8 days from 0.9 M ammonium dihydrogen phosphate in 0.05 M Tris–HCl (pH 5.0) (Fig. 2). When the crystals were re-dissolved again in the buffer, the enzyme solution had the same specific activity as the final purified preparation in Table 1, indicating that the crystals observed in Fig. 2 were BPO. Fig. 3 shows the patterns of the native PAGE of BPO purified from *C. pilulifera*, the recombinant BPO and the re-dissolved BPO after crystallization. As a result, the mobility of the enzyme from *C. pilulifera* was not the same as those from *S. cerevisiae*. The electrophoresis pattern of the recombinant BPO was similar to that of re-dissolved BPO.

2.3. Comparison of enzyme properties between the native and recombinant BPOs

The optimal temperature and thermal stability were investigated for the native enzyme produced by *C. pilulifera* and the recombinant enzyme. Both enzymes showed the highest activity at 60 °C [Fig. 4(A)]. Concerning the thermal stability, the recombinant enzyme retained full activity after heat treatment at 65 °C for 20 min as shown in Fig. 4(B). However, under the same experimental conditions, the native enzyme was completely inactivated.

Kinetic parameters and chlorinating activities were determined for the native and the recombinant enzymes. As shown in Table 2, the K_m values of both enzymes for H_2O_2 and KBr were almost the same. In contrast, chlorinating activity of the native enzyme was over ten times higher than that of the recombinant enzyme. However, the chlorinating activities of the native and recombinant enzymes were very low compared to the BPO activities. The K_m value for KCl of the native enzyme could not be determined because the activity increased with the concentration of KCl in the reaction mixture up to 1.5 M.

Other enzyme properties such as, the optimal pH, pH stability, and the effect of inhibitors on the activities were almost similar to each other. In addition, analysis of the sugar chains indicated that neither of the enzymes had carbohydrate residues.

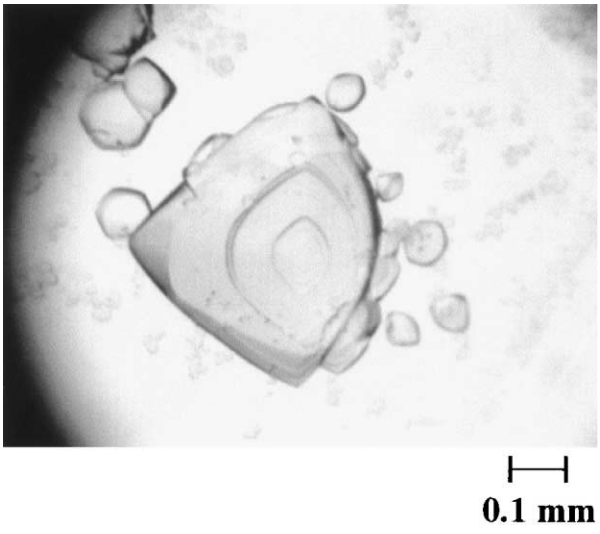

Fig. 2. Crystals of BPO from the recombinant *S. cerevisiae* BJ1991/pTNT30.

3. Discussion

Previously, the BPO gene of *C. pilulifera* was expressed in *E. coli*, and the enzyme was purified from the recombinant *E. coli* strain in our laboratory (Shimonishi et al., 1998). However, no activity was detected in the cell-free extract of the recombinant *E. coli* even in the presence of vanadate. Activity could be detected after the initial purification step, DEAE-Sepharose chromatography. The reason is probably that catalase in cell-free extracts of the *E. coli* degrades hydrogen peroxide added as the substrate for the enzyme assay, and that ion exchange chromatography separates BPO and catalase. In contrast to the

Fig. 3. Native PAGE of BPO purified from *C. pilulifera*, the recombinant BPO, and the re-dissolved BPO after crystallization. The enzyme samples were dissolved in 10% glycerol, 6% β-mercaptoethanol, 0.005% bromophenol blue, and 0.05 M Tris–HCl buffer (pH 6.8). Electrophoresis was carried out with 5% polyacrylamide gels. Lane 1, recombinant BPO purified from the yeast (5 μg), lane 2, re-dissolved BPO after crystallization (5 μg), lane 3, BPO purified from *C. pilulifera* (5 μg).

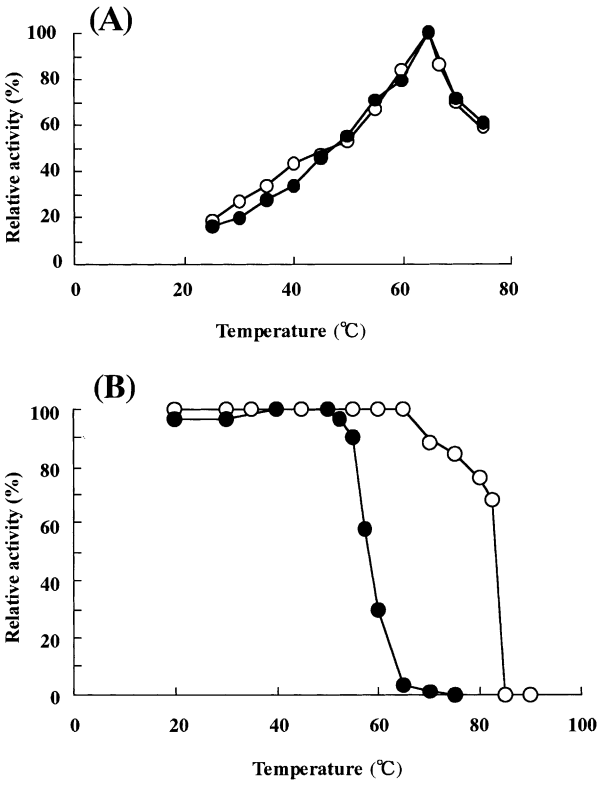

Fig. 4. Effects of temperature on activities (A) and stabilities (B) of the native and the recombinant BPOs. (A) The reaction mixture contained 0.1 M MES buffer (pH 6.5), 0.1 M KBr, 0.06 mM MCD, 1 mM Na_3VO_4, 2 mM H_2O_2, and 20 ng of the holo-enzyme in a total volume of 1 ml. The enzyme reaction was carried out at the indicated temperature. (B) Before the determination of enzyme activity, the holo-enzyme was preincubated at the indicated temperatures for 20 min. The enzyme reaction was carried out at 30 °C as described above. The activity was assayed in term of A_{290} reduction. Symbols: The enzyme activities of the native BPO from *C. pilulifera* (●) and the recombinant enzyme from *S. cerevisiae* BJ1991/pTNT30 (○).

expression system by *E. coli*, BPO activity was detected in the cell-free extract of the recombinant *S. cerevisiae* in this study. Moreover, the specific activity in the cell-free extract 0.052 μkat/mg of the recombinant yeast was higher than that after DEAE-Sepharose chromatography 0.038 μkat/mg in the expression system by *E. coli*. These data indicate that the *S. cerevisiae* expression system is more suitable for the BPO gene of *C. pilulifera* than that of *E. coli*. We are now planning to make the mutant enzymes, and believe that the yeast-expression system will be useful to evaluate their BPO activities.

During the estimation of CPO activities in this study, we found that BPO purified from *C. pilulifera* showed low CPO activity when 1.5 M KCl was present in the reaction mixture. Previously it was demonstrated that BPO from *C. pilulifera* did not act on chloride (Itoh et al., 1986); however, the studies were carried out using a concentration of only 20 mM chloride. It was not possible to calculate the K_m value for KCl because the activity did not reach limiting values at 1.5 M chloride. CPO activity of the native enzyme was higher than that of the recombinant enzyme. In addition, the thermal stability of the recombinant enzyme was higher than that of the native enzyme. Although neither of the enzymes had sugar chains, there was a difference in the N-terminal amino acid residue between each enzyme: the N-terminal amino acid residue of the native enzyme was blocked, and that of the recombinant enzyme was not blocked or identified. Native PAGE showed the difference in apparent molecular weight between the native enzyme and the recombinant one (Fig. 3). We think that one possible reason is that BPO from *C. pilulifera* has the unknown moiety attached to the N-terminal residue of the enzyme. However, the reasons for the differences in the thermal stability and CPO activity are unclear. Recently, a crystallographic study of BPO from a different *Corallina* species, *C. officinalis*, showed that BPO consists of homododecamer (Isupov et al., 2000), which is consistent with the previous result with BPO from *C. pilulifera* (Itoh et al., 1986). Since these two Coralline algae are closely related to each other, we think it is possible that X-ray structure analyses of the crystalline enzyme obtained in this study will be performed based upon the data of the enzyme from *C. officinalis*. It is expected that three-dimensional structure will elucidate the reasons for the unsolved results described above regarding the differences in CPO activity and thermal stability between the native and mutant enzymes.

4. Experimental

4.1. Plasmid harboring algal BPO gene and micro-organisms

The plasmid pKK223–3/*bpo1* contained the BPO gene of *C. pilulifera* (Shimonishi et al., 1998), and it was transferred into a yeast-*E. coli* shuttle vector with the inducible *Gal1* promoter (Hemrika et al., 1999). *E. coli* JM109 was used for DNA recombinant manipulation. *S. cerevisiae* strain BJ1991 (*Matα, leu2, trp1, ura3–251, prb1–1122, pep4–3*) was used as the host of the expression plasmid.

4.2. Construction of the BPO gene expression vector

Plasmid DNA isolation, transformation, restriction endonuclease digestion and other recombinant DNA techniques were performed as described by Sambrook et al., 1989). DNA fragments were purified from agarose gels using a Sephaglas BandPrep kit (Amersham Pharmacia Biotech., Little Chalfont, UK). A yeast-*E. coli* shuttle vector with the inducible *Gal1* promoter, pTNT14 (Hemrika et al., 1999) was used for the construction of the *bpo* expression vector. The 1.9-kb fragment was amplified from the *E. coli* BPO expression vector

pKK223–3/*bpo1* using primers the 5′BPOBam (5′-GAGAGAGGATCCAATTATGGGTATTCCAGCTC-3′; the *Bam*HI restriction site is underlined and the ATG initiation codon is in bold) and the 3′BPOXba (5′-GAGAGATCTAGAAATTTAGAAACCGTGTCCA -3′; the *Xba*I restriction site is underlined). PCR was performed with the Expand High Fidelity PCR system (Roche Diagnostics, Mannheim, Germany) as recommended by the manufacturer at 96 °C for 45 s, 60 °C for 45 s, and 72 °C for 2 min in a total of 30 cycles. Plasmid pTNT14 and the PCR product were digested with *Bam*HI and *Xba*I and the digested products were separately isolated and purified from an agarose gel. Both the 1.1-kb *Bam*HI-*Bam*HI and the 0.7-kb *Bam*HI-*Xba*I fragments were used in a ligation reaction together with the *Bam*HI-*Xba*I digested pTNT14 in an attempt to construct the *bpo* expression plasmid. Isolation of plasmid DNA from transformed *E. coli* JM109 colonies, however, revealed that only the 0.7-kb *Bam*HI-*Xba*I fragment was successfully ligated into the expression vector, giving pTNT20.

pTNT20 was subsequently digested with *Bam*HI and the 1.1-kb *Bam*HI-*Bam*HI fragment was ligated into this digested plasmid and transformed to *E. coli* JM109. Plasmid DNA was isolated from *E. coli* and checked by restriction enzyme analysis to confirm the presence and correct orientation of the 1.1-kb *Bam*HI-*Bam*HI fragment, giving pTNT28. Plasmid pTNT30 was constructed in which the *S. cerevisiae* CYC1 transcription termination region was cloned into the polylinker of pTNT28 downstream of the BPO gene. The 0.3-kb CYC1 terminator was amplified by PCR from the plasmid pPICZa (Invitrogen, Groningen, The Netherlands) using the primers 5′PICXba (5′-GAGAGATCTAGAGTCCCC CTTTTCCTTTGTCG-3′; the *Xba*I restriction site is underlined) and 3′PICPst (5′-GAGAGACTGCAGTC CAGCTTGCAAATTAAAGC-3′; the *Pst*I restriction site is underlined). PCR was performed with the same reagents described above at 96 °C for 45 s, 60 °C for 45 s, and 72 °C for 1 min in a total of 30 cycles. pTNT28 and the PCR amplified fragment were digested with *Xba*I and *Pst*I and the 0.3-kb CYC1 terminator fragment was ligated into the linearised vector giving pTNT30.

Transformation of *S. cerevisiae* was performed according to the method previously reported (Hemrika et al., 1999). Transformants were selected on the minimal medium (YGLT medium) containing 0.67% Yeast nitrogen base w/o amino acids (Difco, Sparks, MD), 2% glucose, 2% agar supplemented with 40 μg/ml of L-tryptophan and 50 μg/ml of L-leucine.

4.3. Cultivation of the recombinant yeast

The yeast cells were precultured in a 300 ml-Erlenmeyer flask containing 100 ml of YGLT medium at 30 °C for 2 days with rotary shaking (120 rpm). Precultured broth (100 ml) was inoculated into a 2-l shaking flask containing the production medium, 2% yeast extract, 2% peptone, and 4% galactose in a total volume of 1000 ml. Cultivation was done at 30 °C for 2 days with reciprocal shaking at 100 strokes per min. The cells were harvested by centrifugation at 10,000×*g* for 20 min and stored at −20 °C until use.

4.4. Purification of BPO from the recombinant yeast

The enzyme was purified according to a modification of the previous method (Itoh et al., 1985). All purification procedures were carried out at 4 °C or on ice. As the basal buffer, 50 mM Tris-SO_4 buffer (pH 7.4) containing 1 mM phenylmethanesulfonyl fluoride and 0.5 mM disodium ethylenediaminetetraacetate was used.

4.4.1. Preparation of cell-free extract and $(NH_4)_2SO_4$ fractionation

The cells were suspended in approximately five volumes of basal buffer and five times disrupted with 0.5-mm glass beads through a Dyno-Mill homogeneizer (Willy A. Bachofen, Basel, Switzerland). The cell-free extracts were obtained by centrifugation at 10,000×*g* for 40 min, and were brought to 30% saturation with $(NH_4)_2SO_4$. After 30 min, the precipitant was removed by centrifugation (10,000×*g*, 40 min) and $(NH_4)_2SO_4$ was added to the resulting supernatant to 60% saturation. After 30 min, the precipitant was recovered by centrifugation (10,000×*g*, 40 min), dissolved in a minimum volume of basal buffer and dialyzed against the same buffer.

4.4.2. DEAE-Sepharose column chromatography

The dialyzed enzyme solution was applied onto a DEAE-Sepharose column (5×15 cm) which had been equilibrated with basal buffer. The column was washed well with 0.2 M KCl in basal buffer, and the bound proteins were eluted with 0.3 M KCl in basal buffer at a flow rate of 100 ml/h. The active fractions were combined, concentrated by ultrafiltration, and dialyzed against basal buffer.

4.4.3. Sepharose CL-6B column chromatography

The dialyzed enzyme solution was applied onto a Sepharose CL-6B column (1.8×94 cm) which had been equilibrated with 0.15 M NaCl in basal buffer. The chromatography was performed at a flow rate of 2 ml/h, and the active fractions were combined and concentrated by ultrafiltration.

4.4.4. Q-Sepharose column chromatography

The concentrated enzyme solution was applied onto a Q-Sepharose column (1.7×12.5 cm) which had been equilibrated with 0.15 M NaCl in basal buffer. After the column was washed with 0.15, 0.2, and 0.3 M NaCl in basal buffers, respectively, the enzyme was eluted with 0.4 M NaCl in basal buffer. Chromatography was

performed at a flow rate of 2 ml/h, and the active fractions were combined and desalted by ultrafiltration. The concentrated enzyme solution was once again applied onto a Q-Sepharose column (1.5×6.5 cm) which had been equilibrated with 0.3 M NaCl in basal buffer. After the column was washed with 0.3 and 0.35 M NaCl in basal buffers, the enzyme was eluted with 0.4 M NaCl in basal buffer. Chromatography was performed at a flow rate of 2 ml/h, and the active fractions were combined, concentrated, and desalted by ultrafiltration.

4.4.5. Cellulofine GC700 column chromatography

The concentrated enzyme solution was applied onto a Cellulofine GC700 column (1.5×90 cm) which had been equilibrated with 0.15 M NaCl in basal buffer. Chromatography was performed at a flow rate of 2 ml/h, and the active fractions were combined and concentrated by ultrafiltration.

4.5. Crystallization

Crystals of BPO from the recombinant *S. cerevisiae* were obtained by using the hanging-drop vapor diffusion method with Linbro plates at 4 °C. The well solution contained 1.8 M ammonium dihydrogen phosphate in 0.1 M Tris–HCl buffer (pH 5.0). The protein drop consisted of an equal volume of the well solution and 23 mg/ml protein in 1 mM Tris–SO_4 buffer (pH 7.4).

The crystals were dissolved again in 0.1 M Tris–SO_4 buffer (pH 7.0) after washing them with 0.1 M Tris–HCl buffer (pH 5.0) containing 1.8 M ammonium dihydrogenphosphate. The specific activity of the dissolved enzyme solution was determined to confirm that the crystals observed in the wells were BPO.

4.6. Enzyme assays

Prior to determining its activity, the enzyme solution was preincubated with 1 mM Na_3VO_4 at 30 °C for 2 h. BPO activity was measured spectrophotometrically by the bromination of monochlorodimedone (MCD, $\varepsilon = 19.9\ mM^{-1}\ cm^{-1}$ at 290 nm) as described before (Yamada et al., 1985). The reaction mixture contained 100 mM MES buffer (pH 6.5), 100 mM KBr, 60 μM MCD, 2 mM H_2O_2, 1 mM Na_3VO_4, and enzyme. The enzyme activity was determined at 30 °C by following the decrease in absorbance at 290 nm. To assay chlorinating activity, 1.5 M KCl was added to the reaction mixture instead of KBr. BPO from *C. pilulifera* (the native enzyme) was purified according to the method described previously (Itoh et al., 1985).

4.7. Other analytical methods

Protein concentration was determined by the method of Bradford (1976) using a Bio-Rad protein assay reagent with bovine serum albumin as a standard. SDS–PAGE was carried out by the methods of Laemmli (1970) employing a 12.5% gel for separation. Protein bands were visualized by staining with Coomassie Brilliant Blue G-250 dissolved in 50% methanol–10% acetic acid and destained in 30% methanol–10% acetic acid. Staining of the gel for glycoprotein was performed by using the G. P. SENSOR kit (HONEN, Yokohama, Japan). In this kit, an aldehyde group generated by oxidizing glycoprotein sugar chains with periodic acid was reacted with hydrazine group of biotin hydrazine, and horseradish peroxidase-labeled avidin was added to form avidin-biotin complex and to generate color.

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