Cloning and expression of the gene for a vanadium-dependent bromoperoxidase from a marine macro-alga, *Corallina pilulifera*

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Abstract The cDNAs for a vanadium-dependent bromoperoxidase were cloned from a marine macro-alga, *Corallina pilulifera*. The open reading frame of one clone (*bpo1*) encoded a protein of 598 amino acids with a calculated molecular mass of 65 312 Da in good agreement with that of 64 kDa determined for the native enzyme. The deduced amino acid sequence coincided well with partial sequences of peptide fragments of the enzyme. From the same cDNA library we also isolated another cDNA clone (*bpo2*) encoding a protein of 597 amino acids with an identity of about 90% to BPO1, suggesting a genetic diversity of the bromoperoxidase gene of *C. pilulifera* growing in a relatively narrow area. The carboxy-terminal 123 residues of the enzyme (BPO1) showed an identity of 45% to that of the marine macro-alga *Ascophillum nodosum*. The homology search of the sequences of bromoperoxidases from *C. pilulifera* (this study) and *A. nodosum*, and chloroperoxidase from the fungus *Curvularia inaequalis* indicated highly conserved sequences PxYxSGHA and LxxxxAxxRxxxGxHxxxD. Furthermore, it was found that the histidine residue directly bound to vanadium, other residues building up the metal center and catalytic histidine residue forming the active site of the chloroperoxidase from *C. inaequalis* are conserved in the primary structure of the bromoperoxidase from *C. pilulifera*. The cloned *bpo1* was introduced into *Escherichia coli*, and the expressed BPO1 was purified from the recombinant strain. The N-terminal amino acid sequence of the purified BPO1 was identical to the deduced sequence from the cDNA except the N-terminal methionine.

Key words: Bromoperoxidase; Vanadium; Cloning; Primary structure; Marine alga; *Corallina pilulifera*

1. Introduction

The haloperoxidase is one of the best characterized enzymes in marine algae and catalyzes the halogenation of organic substrates. A variety of halometabolized compounds are found in marine algae. Our previous study indicated that many marine algae around Japan have a high level of haloperoxidase activity [1]. Especially, a high level of bromoperoxidase (BPO) with an ability to oxidize iodine and bromide was found in *Corallina pilulifera* [2]. The enzyme has been enzymologically well characterized [2–6] and shown to be a vanadium-dependent bromoperoxidase [7]. Haloperoxidases are classified into three groups by means of the type of the prosthetic group. The first group contains heme such as protoporphyrin IV, the second one contains vanadium, and the third one does not require any metal ions or cofactors. The first type, the heme-type chloroperoxidase cDNA, was cloned and sequenced from the fungus *Caldaliomyces fumago* [8]. The genes for bacterial haloperoxidases belonging to the third group have been cloned [9–12]. Recently, cDNA for the fungal vanadium-dependent haloperoxidase has also been cloned and sequenced from *Curvularia inaequalis* [13], and partial amino acid sequences of a vanadium-dependent haloperoxidase were determined from the marine brown macro-alga, *Ascophillum nodosum* [14].

Here, we determined the sequences of peptides from a vanadium-dependent BPO from the marine alga *C. pilulifera*. Based on the partial amino acid sequences, we cloned a full-length cDNA for the enzyme, deduced its entire primary structure, and expressed BPO with the cloned gene in *Escherichia coli*.

2. Materials and methods

2.1. Internal amino acid sequence

BPO was purified from *C. pilulifera* extract as described previously [2]. Purified enzyme was cleaved with *Staphylococcus aureus* V8 protease, and *Achromobacter* protease I (lysyl endopeptidase) and cyanogen bromide as reported previously [15–17]. Resulting peptide fragments were purified by reverse-phase HPLC and subjected to amino acid sequence analysis with a protein sequencer (PPSQ-10, Shimadzu) [18].

2.2. Amplification of the genomic DNA encoding BPO by polymerase chain reaction

The genomic DNA of *C. pilulifera* was isolated as described previously [19] and used as a template for the polymerase chain reaction (PCR) amplification of the BPO gene. We chose the two partial amino acid sequences of *C. pilulifera* BPO, AFAEGSPFHPSYG and LADNVAIGRNMAGVHYFFDQFQELLLGGQ, for preparation of the primers. Two upstream mixed primers, primers 1 and 2, corresponding to AEGSPFHP were synthesized with a DNA synthesizer (model 391A, Perkin-Elmer): primer 1, 5′-GC(AGCT)GA(AG)G-G(AGCT)TC(AGCT)CC(AGCT)TT(TC) CA(TC)CC-3′; primer 2, 5′-GC(AGCT)GA(AG)GG(AGCT)AG(TC)CC(AGCT)TT(TC)CA-(TC)CC-3′. Similarly, a downstream mixed primer, 5′-AA(AG)-TA(AG)TG(AGCT)AC(AGCT)CC(AGCT)GCCAT(AG)TT-3′ (primer 3), was synthesized according to the sequence NMAGVHYF.

The reaction mixture contained 0.5 μg of the genomic DNA as a template, 100 pmol each of primers 1, 2 and 3, and 1 unit of *Taq* DNA polymerase (Toyobo). The PCR cycle was repeated 30 times

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Abbreviations: BPO, bromoperoxidase; PCR, polymerase chain reaction; nt, nucleotide(s)

The nucleotide sequences reported in this paper have been submitted to the DDBJ/EMBL/GenBank Data Bank under the accession numbers D87657 and D87658.

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under the following conditions: 1 min, 94°C; 1 min, 51°C; 1 min, 72°C. The PCR product was purified by agarose gel electrophoresis and subcloned into the TA cloning vector (pCR II, Invitrogen) to yield plasmid pcB2, which encodes the expected nucleotide sequence (Fig. 1, nt 1420–1664).

2.3. Isolation of poly(A)$^+$ RNA

Poly(A)$^+$ RNA of *C. pilulifera* was isolated according to the method of Xing et al. [20]. Briefly, fresh *C. pilulifera* was collected from shallow waters at the shore of Hakuto Kaigan (Tottori Prefecture, Japan) and frozen at −80°C until use. The lyophilized alga (8.9 g) was crushed with a porcelain mortar under cooling with liquid nitrogen and suspended in 40 ml of a lysis buffer (2% SDS, 1% 2-mercaptoethanol, 50 mM EDTA and 150 mM Tris-HCl, pH 7.5) for 2 min. After addition of guanidinium chloride to a final concentration of 0.8 M, the suspension was mixed vigorously and extracted with phenol/chloroform and then with chloroform. To the aqueous layer was added LiCl to a final concentration of 3 M. The precipitate collected by centrifugation was resuspended into 0.8 ml of 1/2 concentration of lysis buffer and potassium acetate was added to a final concentration of 0.5 M. The resulting solution was extracted again with phenol/chloroform and then with chloroform. To the aqueous layer was added LiCl to a final concentration of 3 M. The precipitate collected by centrifugation was dissolved into 1.2 ml of distilled H_2O and precipitated with ethanol. Total RNAs (1.3 mg) collected by centrifugation were further purified using Oligotex-dT30 (Japan Roche) to give poly(A)$^+$ RNA [21].

2.4. Isolation and sequence analysis of cDNA clones for BPO

Oligo(dT)-primed cDNAs for *C. pilulifera* poly(A)$^+$ RNAs synthesized as described previously [22] were ligated with the *Eco*RI adaptor using the cDNA rapid adaptor ligation module (Amersham). The ligated cDNA was inserted into *Eco*RI-digested λgt10 with the cDNA rapid cloning module-λgt10 (Amersham). The recombinant phage vectors were packaged in vitro with Gigapack III Gold packaging extract (Stratagene). Screening of the cDNA library (5×10^4 pfu) was carried out by plaque hybridization with the *Eco*RI fragment of pcB2 as a probe. Plaques were transferred to nitrocellulose membrane, and the membrane was treated for 4 h at 60°C with 6×NET (0.9 M NaCl, 90 mM Tris-HCl, pH 8.3, 6 mM EDTA), 0.1% SDS, 50 μg/ml salmon sperm DNA, and then hybridized overnight at 60°C with the 0.25-kb *Eco*RI fragment of pcB2 labeled with [α-^{32}P]dCTP by the random-priming method [8] in 6×NET containing 0.1% SDS, 100 μg/ml salmon sperm DNA and 5×Denhardt's solution. After washing at 27°C in 1×SSC containing 0.1% SDS, the membrane was washed well with the same solution at 60°C and exposed to Fuji X-ray film RX with intensifying screens for 3 days. Positive clones were isolated and subcloned into plasmid vector pBluescript (Stratagene) for nucleotide sequence analysis.

The DNA sequence was determined at least twice from opposite directions by an automated DNA sequencer (model 373A, Perkin-Elmer) with a PRISM Dye Terminator Cycle Sequencing Kit (Perkin-Elmer).

2.5. Northern blot analysis

Poly(A)$^+$ RNAs (2 μg) purified from *C. pilulifera* were denatured with formaldehyde and electrophoresed in 1% agarose gel containing 0.7 M formaldehyde [23]. RNAs were transferred to nylon membrane and the BPO mRNA was hybridized with the 0.5-kb *Eco*RI fragment of BPO2 labeled with [α-^{32}P]dCTP for 15 h at 42°C in 50% formamide, 5×Denhardt's solution, 5×SSC, 50 mM sodium phosphate, pH 6.5, 0.1% SDS and 250 μg/ml salmon sperm DNA. This filter was washed three times with 0.2×SSC containing 0.1% SDS for 20 min at 60°C and then subjected to autoradiography.

2.6. Expression of BPO gene in E. coli

The expression vector pKK223-3 was digested with *Eco*RI and blunt-ended using Klenow fragment, and after phenol extraction, the resulting fragment was treated with *Pst*I (4.6 kb). Using PCR primers 4 and 5 (ATGGGTATTCCAGCTGACAAC and TCCAGGTCGAAGGTGAGAC), the 5′ region of BPO1 was amplified. The generated 0.4-kb fragment, which includes the translation initiation codon (ATG), was digested with *Xho*I (0.35 kb). The 3′ region of BPO1 was prepared using *Xho*I and *Pst*I (1.6 kb). The two obtained BPO1 fragments were inserted into the prepared expression vector pKK223-3 containing blunt-ended and *Pst*I sites. The transformed *E. coli* JM109 with the resultant plasmid was precultured at 37°C for 16 h in a test tube containing 5 ml of LB medium with 50 mg/l ampicillin. The preculture was inoculated into the main culture, and 1 mM IPTG was added to the main culture after 5 h. The cultivation was done at 37°C for 16 h in a 2-l flask containing 800 ml of the same medium.

2.7. Purification of BPO from the transformed E. coli

The grown cells were harvested by centrifugation (10 000×*g*, 20 min) and the harvested cells were suspended in 50 mM Tris-SO_4 buffer (pH 7.5) with 1 mM $NaVO_3$. This buffer containing 1 mM $NaVO_3$ was used as the basal buffer. The cells (94 g, wet weight) were disrupted with a sonic oscillator (20 kHz) and the cell debris was removed by centrifugation (12 000×*g*, 30 min). The supernatant was dialyzed against the buffer and put onto a DEAE-Sepharose Fast Flow column (diameter 4.8×29.5 cm) equilibrated with the buffer. The flow rate was 150 ml/h. After washing the column with the buffer and subsequently with the buffer containing 0.2 M NaCl, the desired proteins were eluted with the buffer containing 0.3 M NaCl. The active fractions were concentrated by the ultrafiltration and dialyzed against the buffer containing 0.8 M $(NH_4)_2SO_4$. The enzyme solution was applied onto a phenyl-Sepharose CL-4B column (diameter 1.5×16 cm) equilibrated with the buffer containing 0.8 M $(NH_4)_2SO_4$. The flow rate was 40 ml/h. After washing the column with the equilibrated buffer, the enzyme was eluted with the buffer containing 0.2 M $(NH_4)_2SO_4$. The active fractions were concentrated by ultrafiltration and dialyzed against the buffer containing 0.1 M NaCl. The enzyme solution was put into a Source 30Q column (diameter 1.4×1 cm) equilibrated with the dialyzed buffer. The flow rate was 10 ml/h. After washing the column with the buffers containing 0.1 M and 0.2 M NaCl, the desired protein was eluted with the buffer containing 0.3 M NaCl and concentrated by the ultrafiltration. Finally, the concentrated enzyme was put into a Superdex 200 HR10/30 column at a flow rate of 0.25 ml/min. The mobile phase was the buffer with 0.15 M NaCl.

BPO activity was measured spectrophotometrically by the bromination of monochlorodimedone as described before [1]. The protein concentration was determined with a Bio-Rad Protein Assay kit (Bio-Rad, Richmond, CA, USA).

2.8. N-Terminal amino acid sequence analysis

The purified enzyme was subjected to SDS-PAGE according to Laemmli [24] and subsequently blot-transferred to poly(vinylidene difluoride) membrane (clear blot membrane-P, ATTO, Tokyo) using CAPS transfer buffer as described by Matsudaira [25]. For the N-terminal amino acid sequence analysis, the membrane was stained with 0.1% Coomassie brilliant blue G-250 in 50% methanol for 10 min and destained in methanol/acetic acid/water (50:10:40, v/v). After the rinsed membrane by deionized water was air-dried, the protein band was subjected to protein sequencing (PPSQ-10, Shimadzu).

3. Results and discussion

Previously, we purified a novel vanadium-dependent bromoperoxidase from a marine red macro-alga, *C. pilulifera* [2]. For elucidation of the protein structure and reaction mechanism of the novel enzyme, we attempted to determine the protein structure using molecular cloning and protein chemical techniques in the first step. For amplification of the BPO gene by PCR we tried to obtain the amino-terminal sequence of the native enzyme but could not determine it, suggesting the possibility that the amino-terminal residue of BPO is masked. Thus, we sequenced two peptide fragments obtained by digestion with V8 protease as LVSPNAADEFDGEIAYG and LYLMALGRDIEFSEFS, two peptides with lysyl endopeptidase as LADNVAIGRNMAGVHYFSDQFQELLLGGQ and EIADLPLG, and four fragments cleaved by cyanogen bromide as AFAEGSPFHPSYG, PPAEIRRFXGEVTVGNLF, PPAPVLMSPELIAE and PPAPVLTSPELIAE. Among the determined partial amino acid se-

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                                                                CGCTTTTGTTGGCGGCACTCCAGTGCCATCCAAGCGG  -91
CAGAATGCGACACTTACTTCCTCATTCTGTCCTCGGATTCCTGTGACTTCCACTAAGCCAGCTGCTCGTCATGCTCATGTGGCCCCTACA   -1

ATGGGTATTCCAGCTGACAACCTCCAAAGTCGCGCCAAGGCTTCATTCGATACGCGTGTAGCTGCGGCCGAGCTTGCACTCAACCGCGGA   90
 M  G  I  P  A  D  N  L  Q  S  R  A  K  A  S  F  D  T  R  V  A  A  A  E  L  A  L  N  R  G    30

GTTGTACCATCGTTTGCAAATGGGGAAGAGCTTCTCTACCGCAACCCGGACCCTGACAACACTGATCCGAGCTTCATCGCTAGCTTCACA  180
 V  V  P  S  F  A  N  G  E  E  L  L  Y  R  N  P  D  P  D  N  T  D  P  S  F  I  A  S  F  T    60

AAGGGTCTTCCGCATGACGACAATGGCGCTATTATCGACCCCGACGACTTCTTGGCCTTCGTTCGTGCAATCAATAGTGGCGATGAAAAG  270
 K  G  L  P  H  D  D  N  G  A  I  I  D  P  D  D  F  L  A  F  V  R  A  I  N  S  G  D  E  K    90

GAGATCGCCGACCTCACATTGGGGCCAGCTCGCGACCCGGAGACTGGCTTACCAATCTGGCGCTCGGATCTGGCGAATTCTCTCGAGCTC  360
 E  I  A  D  L  T  L  G  P  A  R  D  P  E  T  G  L  P  I  W  R  S  D  L  A  N  S  L  E  L   120

GAAGTGCGAGGATGGGAGAACAGCTCTGCCGGTCTCACCTTCGACCTGGAGGGCCCGGACGCGCAGTCGATTGCCATGCCACCGGCGCCT  450
 E  V  R  G  W  E  N  S  S  A  G  L  T  F  D  L  E  G  P  D  A  Q  S  I  A  M  P  P  A  P   150

GTGCTCACGAGCCCTGAGCTCGTCGCCGAGATAGCAGAGCTGTACCTGATGGCGCTTGGACGCGAAATCGAGTTCAGCGAGTTTGATTCC  540
 V  L  T  S  P  E  L  V  A  E  I  A  E  L  Y  L  M  A  L  G  R  F  I  E  F  S  E  F  D  S   180

CCAAAGAACGCAGAGTATATTCAGTTTGCTATTGATCAGCTTAACGGGCTGGAGTGGTTCAACACACCGGCAAAGCTCGGAGATCCGCCT  630
 P  K  N  A  E  Y  I  Q  F  A  I  D  Q  L  N  G  L  E  W  F  N  T  P  A  K  L  G  D  P  P   210

GCGGAAATCCGTCGCCGTCGCGGTGAGGTGACTGTTGGAAACTTGTTCCGCGGTATTCTTCCAGGCTCTGAGGTCGGCCCGTACCTCAGC  720
 A  E  I  R  R  R  R  G  E  V  T  V  G  N  L  F  R  G  I  L  P  G  S  E  V  G  P  Y  L  S   240

CAGTACATCATCGTTGGTAGCAAGCAGATTGGCTCAGCGACAGTTGGTAACAAAACTCTCGTGAGCCCCAATGCTGCTGATGAGTTTGAT  810
 Q  Y  I  I  V  G  S  K  Q  I  G  S  A  T  V  G  N  K  T  L  V  S  P  N  A  A  D  E  F  D   270

GGTGAAATCGCCTACGGAAGCATCACCATTAGCCAGCGTGTGCGTATCGCCACGCCTGGACGCGACTTCATGACCGACTTGAAGGTATTC  900
 G  E  I  A  Y  G  S  I  T  I  S  Q  R  V  R  I  A  T  P  G  R  D  F  M  T  D  L  K  V  F   300

CTTGACGTCCAGGACGCTGCGGACTTCCGAGGCTTTGAGTCGTATGAGCCGGGAGCACGCCTCATCCGGACGATCCGCGATCTTGCGACG  990
 L  D  V  Q  D  A  A  D  F  R  G  F  E  S  Y  E  P  G  A  R  L  I  R  T  I  R  D  L  A  T   330

TGGGTGCACTTTGACGCACTGTACGAGGCCTACCTCAATGCGTGCCTAATTCTGTTGGCGAACGGCGTGCCGTTCGATCCCAACCTTCCG 1080
 W  V  H  F  D  A  L  Y  E  A  Y  L  N  A  C  L  I  L  L  A  N  G  V  P  F  D  P  N  L  P   360

TTCCAGCAGGAGGACAAGCTCGATAACCAGGACGTGTTTGTGAACTTCGGATCCGCACACGTGCTGAGTCTGGTGACTGAAGTGGCTACG 1170
 F  Q  Q  E  D  K  L  D  N  Q  D  V  F  V  N  F  G  S  A  H  V  L  S  L  V  T  E  V  A  T   390

CGCGCGTTGAAGGCGGTACGGTACCAGAAGTTTAACATTCATCGTCGCCTGCGCCCTGAGGCTACCGGTGGTCTGATTAGCGTTAACAAA 1260
 R  A  L  K  A  V  R  Y  Q  K  F  N  I  H  R  R  L  R  P  E  A  T  G  G  L  I  S  V  N  K   420

ATCGCACCGCAGAAGGGCGAGAGCATTTTCCCTGAGGTTGATCTTGCTGTTGAAGAGCTTGGAGATATCTTGGAGAAAGCTGAAATTAGC 1350
 I  A  P  Q  K  G  E  S  I  F  P  E  V  D  L  A  V  E  E  L  G  D  I  L  E  K  A  E  I  S   450

AATAGGAAGCAGAACATAGCTGACGGAGATCCTGACCCTGATCCTTCATTCCTGTTGCCGATGGCATTCGCCGAGGGCAGCCCATTCCAT 1440
 N  R  K  Q  N  I  A  D  G  D  P  D  P  D  P  S  F  L  L  P  M  A  F  A  E  G  S  P  F  H   480

CCGTCCTACGGAAGCGGCCACGCTGTGGTTGCTGGCGCATGTGTGACGATCCTGAAGGCGTTCTTCGACTCCGGCATCGAGATCGATCAG 1530
 P  S  Y  G  S  G  H  A  V  V  A  G  A  C  V  T  I  L  K  A  F  F  D  S  G  I  E  I  D  Q   510

GTGTTCGAGGTCGACAAAGATGAGGACAAGCTTGTGAAGTCGTCTTTCAAGGGAACTCTCACTGTTGCCGGTGAATTGAACAAGCTCGCC 1620
 V  F  E  V  D  K  D  E  D  K  L  V  K  S  S  F  K  G  T  L  T  V  A  G  E  L  N  K  L  A   540

GACAATATTGCGATCGGGCGTAACATGGCAGGTGTTCACTACTTCTCTGACCAGTTCGAGTCACTTCTGCTCGGTGAGCAGGTTGCGATT 1710
 D  N  I  A  I  G  R  N  M  A  G  V  H  Y  F  S  D  Q  F  E  S  L  L  L  G  E  Q  V  A  I   570

GGAATCTTGGAAGAGCAAAGTCTGACGTATGGCGAGAACTTCTTCTTCAACTTGCCGAAGTTTGATGGAACTACAATCCAGATCTAATTT 1800
 G  I  L  E  E  Q  S  L  T  Y  G  E  N  F  F  F  N  L  P  K  F  D  G  T  T  I  Q  I  *      598

TAACTGGACACGGTTTCTAAATTTCTTAACAATTTAGATATATCTAAACACTTAGTTATCTCTGTGCATGAGACAACTTCAAGCGGCAGC 1890
TTAGGCTAGCGTTTATTGAAAAAAAAAAAAAAAAAAAAAAAAAA                                               1934
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Fig. 1. Nucleotide sequence of bromoperoxidase cDNA (BPO1) from *C. pilulifera* and deduced amino acid sequence of the enzyme. Nucleotides are numbered starting from A of the tentative translation initiation codon ATG and amino acid residues are numbered from the initiating methionine. The partial amino acid sequences determined with the enzyme from *C. pilulifera* BPO are underlined, and the sequences used for the preparation of PCR primers are indicated by double underlining. Broken lines indicate deduced amino acid residues from the nucleotide sequence different from those determined by amino acid sequence analysis with the enzyme.

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CP.BPO1   MGIPADNLQSRAKASFDTRVAAAELALNRGVVPSFANGEELLYRNPDPDNTDPSFIASFT    60
CP.BPO2   ........................A.................CETG--.........    58

CP.BPO1   KGLPHDDNGAIIDPDDFLAFVRAINSGDEKEIADLTLGPARDPETGLPIWRSDLANSLEL   120
CP.BPO2   ..........................................................    118
CI.CPO                         MGSVTPIPLPKI.EPEEYNTNYILFWNHVGLE.NRVTHT    39

CP.BPO1   EVRGWENSSAGLTFDLEGPDAQSIAMPPAPVLTSPELVAEIAELYLMALGREIEFSEFDS   180
CP.BPO2   ......................V........M....I.............D........   178
CI.CPO    VGGPLTGPPLSARALGMLHL.IHD.YFSICPP.DFTTFLSPDTENAAYRLPSPNGANDAR    99

CP.BPO1   PKNAEYIQFAIDQLNGLEWFNTPAKLGDPPAEIRRRRGEVTVGNLFRGILPGSEVGPYLS   240
CP.BPO2   ....AF.RS..ER...................................................   238
CI.CPO    EAV.GAALKMLSS.YMKPVEQPNPNP.ANISDNAYAQLGLVLDRSVLEAPG.VDRESASF   159

CP.BPO1   QYIIVGSKQIGSATVGNKTLVSPNAADEFDGEIAYGSITISQRVRIATPGRDFMTDLKVF   300
CP.BPO2   ...................F.....................................   298
CI.CPO    MFGEDVADVFFALLNDPRGASQEGYHPTPGRYKFDDEP.HPVVLIPVD.NNPNGPKMPFR   219

CP.BPO1   LDVQDAADFRGFESYEPGARLIRTIRDLATWVHFDALY-EAYLNACLILLANGVPFDPNL   359
CP.BPO2   .....G.............................-.....................   357
CI.CPO    QYHAPFYGKTTKRFATQSEHFLADPPG.RSNADET.E.DD.VRV.IAMGG.QALNSTKRS   279

                    *
CP.BPO1   PFQQEDKL-DNQDVFVNFG------------------------------S-AHVLSLVT   386
CP.BPO2   ........-..H.......--------------------------------.-........   384
CI.CPO    .W.TAQG.YWAY.GSNLI.TPPRFYNQIVRRIAVTYKKEEDLANSEVNNADF.RLFA..D   339

                      *       *
CP.BPO1   EVATRALKAVRYQKFNIHRRLRPEATGGLISVNKIAPQKG-ESIFPEVDLAVEELGDILE   445
CP.BPO2   ................................KSFLA.SDI.....SEL....SS..D   444
AN.BPO    GX.EL.QRSSWT.NWQV..FA....L.------------..TLHLTIKGE.NADFDLSL..    59
CI.CPO    VAC.D.GIFSWKE.WEF-EFW..LSGVRD-------------------------------   368

                                                          **
CP.BPO1   K-AEISNRKQNI-ADGDPDPDPSFLLPM----AFAEGSPFH---PSYGSGHAVVAGACVT   496
CP.BPO2   DV..-..E...R-...IVS..K.....----.....---.....................   495
AN.BPO    N-..LLK.VAA.N.AQN.NNEVTY...Q----VYQ..T.T.---.........TQN..FA.   111
CI.CPO    ---------------..R..HGDP.W.TLGAPATNTNDI..KPPF.A.P....TFG..VFQ   413

CP.BPO1   ILKAFFDSGIEIDQVFEVDKDEDKLVKSSFKGT-LTVAGELNK-----------------   538
CP.BPO2   .......ANFQ..K.....T.............-.........-----------------   537
AN.BPO    V...LIQLQRGG.AIRP.YP.D.G.KLID.RQSC..FG..IK.-----------------   155
CI.CPO    MVRRYYNGRVGTWKDD.P.NIAIDMMI.EELNGVNRDLRQPYDPTAPIEDQPGIVRTRIV   473

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CP.BPO1   --------LADNIAIGRNMAGVHYFSDQFESLLLGEQVAIGILEEQSLTYGENFFFNLPK   590
CP.BPO2   --------....V.......................I.....................   589
AN.BPO    --------..V.V.F..Q.L.I..RF.GIQG.....TITVRT.HQELM.FA.EST.EFRL   206
CI.CPO    RHFDSAWE.MFEN..S.IFL...WRF.AAAARDILIPTTTKDVYAVDNNGATV.QNVEDI   532

CP.BPO1   FDGTTIQI                                                      598
CP.BPO2   ........                                                      597
AN.BPO    .T.EV.KLFQDGTFTIDGFKCPGLVYTGVENCV                             239
CI.CPO    RYT.RGTREDPEGLFPIGGVPLGIEIADEIFNNGLKPTPPEIQPMPQETPVQKPVGQQPG   592
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Fig. 2. Alignment of the amino acid sequences of bromoperoxidases BPO1 (CP.BPO1) and BPO2 (CP.BPO2) from *C. pilulifera* and BPO from *A. nodosum* (AN.BPO) and chloroperoxidase from *C. inaequalis* (CI.CPO). Dots indicate amino acid residues identical to those of bromoperoxidase BPO1. Gaps (-) have been inserted to achieve maximum homology. Asterisks indicate the highly conserved amino acid residues constituting the vanadium-containing active center determined with the enzyme from *C. inaequalis* [29].

quences of the enzyme, we selected two sequences, AEGSPFHP from AFAEGSPFHPSYG and NMAGVHYF from LADNVAIGRNMAGVHYFFDQFQELLLGGQ, for preparation of PCR primers. Using the amino acid sequences we successfully isolated a genomic clone pcB2 encoding a part of the BPO protein (Fig. 1, nt 1420–1664). To obtain a full-length cDNA we screened an oligo(dT)-primed cDNA library with [α-^{32}P]dCTP-labeled 0.25-kb *Eco*RI fragment of pcB2 as a probe. Three positive clones were obtained and the size of each insert was estimated by agarose gel electrophoresis. As the molecular mass of the subunit of BPO was 64 kDa [2], we selected two clones BPO1 and 2 containing inserts of 2062 and 2053 bp, respectively, sufficient to harbor the full-length BPO cDNA. The nucleotide sequence of *bpo1* is shown in Fig. 1 with the deduced amino acid sequence. The first ATG codon (1–3) was tentatively designated the translation initiation co-

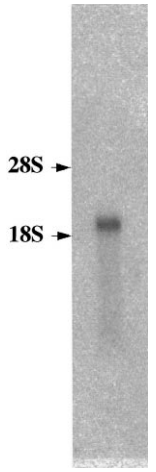

Fig. 3. Northern blot analysis of poly(A)$^+$ RNA from *C. pilulifera*. 2 μg of poly(A)$^+$ RNA from *C. pilulifera* was electrophoresed in 1% agarose/formaldehyde gel and transferred to nylon membrane. The BPO mRNA was hybridized with the 0.5-kb *Eco*RI fragment of BPO2 labeled with [α-^{32}P]dCTP. Positions of 18S and 28S rRNAs are shown on the left.

don, since the nucleotide sequence surrounding the initiation codon almost agrees with the consensus sequence for eukaryotic initiation codons described by Kozak [26]. The deduced primary structure contained most amino acid sequences determined with peptide fragments of the BPO protein. The open reading frame is 1794 bp and encodes a protein of 598 amino acids. The calculated molecular mass of this protein, 65 312 Da, is in good agreement with the molecular mass of 64 kDa estimated by SDS-polyacrylamide gel electrophoresis. We have also isolated an *Eco*RI fragment of *C. pilulifera* gene for BPO, the sequence of which completely agreed with that of the cDNA without any intervening sequence (data not shown). The other clone *bpo2* was also sequenced (data not shown) and its amino acid sequence was deduced from the nucleotide sequence as shown in Fig. 2. The open reading frame of this clone consisted of 1791 bp of nucleotides and encodes a protein of 597 amino acids. The calculated molecular mass of this protein is 65 199 Da. The homology between BPO1 and 2 was about 90% for both nucleotide and amino acid sequences (Fig. 2); the main difference between the amino acid sequences of BPO1 and 2 was present between residues 421 and 465 of BPO1 and the counterparts of BPO2. From the present result, there are two possibilities: (1) at least two genetically different strains of *C. pilulifera* exist in a relatively narrow growing area, although we carefully collected the morphologically single strain of *C. pilulifera* at the same sea shore; (2) an individual of *C. pilulifera* has multiple genes for BPO. Such multiple genes for BPO might cause the difference between amino acid residues deduced from the nucleotide sequence and those determined by amino acid sequence analysis with the enzyme indicated by the broken lines in Fig. 1. Since we previously purified BPO to homogeneity judged by SDS-PAGE [2], two different molecular species of BPO might exist in the purified enzyme preparation. As shown in Fig. 3, however, a single band with a size of 2 kb was observed using poly(A)$^+$ RNA from *C. pilulifera*, suggesting little heterogeneity in size of the BPO gene.

The cell-free extract of the transformed *E. coli* strain had no BPO activity (Table 1). This result was observed in the case of haloperoxidases produced by other bacterial strains, such as *Pseudomonas aureofaciens* [27] or *Streptomyces aureofaciens* [28], because catalase in cell-free extracts rapidly degraded the substrate for BPO, hydrogen peroxide. In our case, the activity was also detected after DEAE-Sepharose chromatography. Finally, the enzyme was purified (approximately 80% purity) as shown in Fig. 4. The amino acid sequence at the N-terminus of the enzyme was found to be Gly-Ile-Pro-Ala-Asp-Asn-Leu-Gln-Ser-Arg-Ala-Lys-Ala-Ser-Phe-Asp, which entirely agrees with the sequence predicted from the *bpo1* nucleotide sequence except for the N-terminal methionine residue. The N-terminal amino acid of BPO expressed in *E. coli* was not masked as found in BPO purified from *C. pilulifera*. The molecular weight of the purified enzyme judged from SDS-PAGE was approximately 64 kDa which coincided with that of *C. pilulifera* and that deduced from the nucleotide sequence of *bpo1*. Table 1 shows the result of enzyme purification. When the purified enzyme was dialyzed against 0.1 M citrate-phosphate buffer (pH 3.8) containing 1 mM EDTA, the enzyme activity decreased to 13%. And it was restored after the dialyzed enzyme was incubated with 1 mM $NaVO_3$ overnight at room temperature. This result confirmed that the BPO produced by *E. coli* was absolutely dependent on vanadate for its activity, like the *C. pilulifera* BPO. As shown in Fig. 4 and Table 1, BPO was not overexpressed in this transformant. To enhance the amount of BPO produced in the transformed strain, further efforts are necessary.

The entire primary structure of chloroperoxidase from *Curvularia inaequalis* [13] and the partial 238-residue sequence of BPO from *A. nodosum* [14] have been described as presented in Fig. 2. The partial sequence (residues 404–598) of BPO1 or the corresponding residues of BPO2 showed a high similarity to the sequence of BPO from *A. nodosum*, and the shorter sequence (BPO1, residues 458–493; BPO2, residues 457–492) of *C. pilulifera* BPO exhibited a similarity to that of chloroperoxidase from *C. inaequalis*. The alignment of these sequences revealed the presence of highly conserved consensus sequences, PxYxSGHA and LxxxxAxxRxxxGxHxxxD. Recently, X-ray structure of a vanadium-containing chloroperoxidase has been demonstrated from *C. inaequalis* [29] and the catalytic mechanism including the vanadium binding site proposed [30]. This study clearly identified that a histidine

Table 1
Purification of BPO from *E. coli*

	Protein (mg)	Total activity (U)	Specific activity (U/mg)
Cell-free extracts	17 600	–	–
DEAE-Sepharose	264	613	2.32
Phenyl-Sepharose	9.92	363	36.6
Source 30Q	1.66	159	95.9
Superdex	0.341	97.5	286

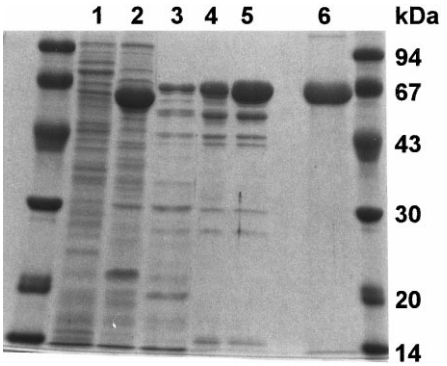

Fig. 4. SDS-PAGE of BPO from *E. coli*. Lane 1, cell-free extract; lane 2, DEAE-Sepharose FF eluate; lane 3, phenyl-Sepharose CL-4B eluate; lane 4, Source 30Q eluate; lane 5, Superdex eluate; lane 6, purified BPO from *C. pilulifera*.

residue directly bound to vanadium, residues of arginine, serine, glycine, aspartic acid and lysine building up the metal center and a catalytic histidine residue constitute the active site of the chloroperoxidase. Importantly, these residues are well conserved in the sequences of BPO from *C. pilulifera* and BPO from *A. nodosum* as shown by the asterisks in Fig. 2, suggesting that the active sites of vanadium-dependent haloperoxidases are structurally very similar. The preliminary X-ray analysis of BPO from *C. officinalis* has also been reported though its nucleotide sequence was not determined [31]. Since this species is similar to *C. pilulifera*, it is expected that our results must bring about the elucidation of the tertiary structure of algal BPO in the near future.

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