In Vitro Characterization of a Recombinant Blh Protein from an Uncultured Marine Bacterium as a β-Carotene 15,15′-Di-oxygenase

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Codon optimization was used to synthesize the blh gene from the uncultured marine bacterium 66A03 for expression in Escherichia coli. The expressed enzyme cleaved β-carotene at its central double bond (15,15′) to yield two molecules of all-trans-retinal. The molecular mass of the native purified enzyme was ~64 kDa as a dimer of 32-kDa subunits. The Km, kcat, and kcat/Km values for β-carotene as substrate were 37 μM, 3.6 min⁻¹, and 97 mM⁻¹ min⁻¹, respectively. The enzyme exhibited the highest activity for β-carotene, followed by β-cryptoxanthin, β-apo-4′-carotenal, α-carotene, and γ-carotene in decreasing order, but not for β-apo-8′-carotene, β-apo-12′-carotene, lutein, zeaxanthin, or lycopene, suggesting that the presence of one unsubstituted β-ionone ring in a substrate with a molecular weight greater than C35 seems to be essential for enzyme activity. The oxygen atom of retinal originated not from water but from molecular oxygen, suggesting that the enzyme was a β-carotene 15,15′-dioxygenase. Although the Blh protein and β-carotene 15,15′-monooxygenases catalyzed the same biochemical reaction, the Blh protein was unrelated to the mammalian β-carotene 15,15′-monooxygenases as assessed by their different properties, including DNA and amino acid sequences, molecular weight, form of association, reaction mechanism, kinetic properties, and substrate specificity. This is the first report of in vitro characterization of a bacterial β-carotene-cleaving enzyme.

Vitamin A (retinol) is a fat-soluble vitamin and important for human health. In vivo, the cleavage of β-carotene to retinal is an important step in vitamin A synthesis. The cleavage can proceed via two different biochemical pathways (1, 2). The major pathway is a central cleavage catalyzed by mammalian β-carotene 15,15′-monooxygenases (EC 1.14.99.36). β-Carotene is cleaved by the enzyme symmetrically into two molecules of all-trans-retinal, and retinal is then converted to vitamin A in vivo (3–5). The second pathway is an eccentric cleavage that occurs at double bonds other than the central 15,15′-double bond of β-carotene to produce β-apo-carotenals with different chain lengths, which are catalyzed by carotenoid oxygenases from mammals, plants, and cyanobacteria (6). These β-apo-carotenals are degraded to one molecule of retinal, which is subsequently converted to vitamin A in vivo (2).

β-Carotene 15,15′-monooxygenase was first isolated as a cytosolic enzyme by identifying the product of β-carotene cleavage as retinal (7). The characterization of the enzyme and the reaction pathway from β-carotene to retinal were also investigated (4, 8). The enzyme activity has been found in mammalian intestinal mucosa, jejenum enterocytes, liver, lung, kidney, and brain (5, 9, 10). Molecular cloning, expression, and characterization of β-carotene 15,15′-monooxygenase have been reported from various species, including chickens (11), fruit flies (12), humans (13), mice (14), and zebra fishes (15).

Other proteins thought to convert β-carotene to retinal include bacterioopsin-related protein (Brp) and bacteriorhodopsin-related protein-like homolog protein (Blh) (16). Brp protein is expressed from the bop gene cluster, which encodes the structural protein bacterioopsin, consisting of at least three genes as follows: bop (bacterioopsin), brp (bacteriorhodopsin-related protein), and bat (bacterioopsin activator) (17). brp genes were reported in Haloarcula marismortui (18), Halobacterium sp. SRC-1 (19), Halobacterium halobium (17), Haloquadratum walsbyi, and Salinibacter ruber (20). Blh protein is expressed from the proteorhodopsin gene cluster, which contains proteorhodopsin, crtE (geranylgeranyl-diphosphate synthase), crtl (phytoene dehydrogenase), cttB (phytoene synthase), cttY (lycopene cyclase), idi (isopentenyl diphosphate isomerase), and blh gene (21). Sources of blh genes were previously reported in Halobacterium sp. SRC-1 (19), Haloarcula marismortui (18), Halobacterium salinarum (22), uncultured marine bacterium 66A03 (16), and uncultured marine bacterium HF10 49E08 (21). β-Carotene biosynthetic genes cttE, cttB, cttY, idiA, and idi encode the enzymes necessary for the synthesis of β-carotene from isopentenyl diphosphate, and the Idi, IdpA, CrtE, CrtB, CrtI, and CrtY proteins have been characterized in vitro (23–28). Blh protein has been proposed to catalyze or regulate the conversion of β-carotene to retinal (29, 30), but there is no direct proof of the enzymatic activity.

In this study, we used codon optimization to synthesize the blh gene from the uncultured marine bacterium 66A03 for expression in Escherichia coli, and we performed a detailed biochemical and enzymological characterization of the expressed Blh protein. In addition, the properties of the enzyme were compared with those of mammalian β-carotene 15,15′-monooxygenases.

[1] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S4.
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Characterization of Bacterial β-Carotene 15,15'-Dioxygenase

EXPERIMENTAL PROCEDURES

**Reagents**—The expression vector pET-24a(+) was purchased from Novagen (San Diego). The expression host, *E. coli* ER 2566, and all restriction enzymes were purchased from New England Biolabs (Hertfordshire, UK). Luria-Bertani (LB) medium was purchased from BD Biosciences. β-Carotene and the other carotenoid substrates were purchased from Sigma and Carotenature (Lupsingen, Switzerland), respectively. The pre-stained ladder for SDS-PAGE and the gel filtration calibration kit were purchased from MBI Fermentas (Hanover, MD) and Amersham Biosciences, respectively. Western blot detection system and His-tagged monoclonal antibody were purchased from Intron Biotechnology (Seongnam, Gyeonggi, Korea) and Bio-Rad, respectively. Isotopically labeled water (H$_2^{18}$O) and molecular oxygen (18O$_2$) were purchased from Sigma and Cambridge Isotope Laboratories (Andover, MA), respectively. All other reagents were purchased from Sigma.

**DNA Cloning and Site-directed Mutagenesis**—The amino acid sequence used for codon optimization was obtained from the *blh* gene of the uncultured marine bacterium 66A03 (GenBank® accession number AAY68319). Codons of the *blh* gene sequence were optimized by selection based on probabilities obtained from the codon usage table without consideration of internal mRNA secondary structures or DNA repeats (31). The entire gene was synthesized (Genofocus, Daejeon, Korea), cloned into the pET-24a(+) expression vector using the restriction enzymes EcoRI and XhoI, and transformed into *E. coli* ER2566 as an expression host. Mutations of the conserved four histidine residues in the Blh protein were generated by site-directed mutagenesis using the QuickChange kit and protocol (Stratagene, Beverly, MA). DNA sequencing was performed at the Macrogen facility (Seoul, Korea).

**Expression and Purification**—The recombinant *E. coli* for expression of the wild-type and mutant enzymes was cultivated in 500 ml of LB medium (1.0% tryptone, 0.5% yeast extract, and 1.0% sodium chloride) in a 2,000-ml flask containing 20 μg/ml kanamycin at 37 °C with shaking at 200 rpm. When the absorbance of the culture reached 0.5 at 600 nm, isopropyl β-D-thiogalactopyranoside was added to a final concentration of 0.1 mM to induce expression of the recombinant enzyme, and the culture was incubated at 16 °C for 16 h. The cells were harvested from the culture broth by centrifugation at 6,000 × g for 30 min at 4 °C, washed twice with 0.85% NaCl, and then resuspended in a lysis buffer (50 mM NaH$_2$PO$_4$, 300 mM NaCl, pH 8.0) containing 1 mg/ml lysozyme. The resuspended cells were disrupted using a sonicator. The cell debris was removed by centrifugation at 13,000 × g for 20 min at 4 °C, and the supernatant was filtered through a 0.45-μm filter. The filtrate applied to His-Trap HP affinity chromatography (Amersham Biosciences) equilibrated with 50 mM sodium phosphate buffer containing 300 mM NaCl. The column was washed extensively with the same buffer, and the bound protein was eluted with a linear gradient between 10 and 200 mM imidazole at a flow rate of 1 ml/min. The purification step using chromatography was carried out in a cold room at 4 °C with a fast protein liquid chromatography system (Bio-Rad). The active fractions were collected and dialyzed against 100 mM Tricine–KOH buffer (pH 8.0). After dialysis, the resulting solution was used as the purified enzyme.

**Amino Acid Sequencing**—Partial amino acid sequences of the Blh protein were investigated. After separation via SDS-PAGE and staining of the sample, each chosen band was isolated, destained, and washed. In-gel digestion was then performed with 500 ng of sequencing-grade chymotrypsin in 100 μl Tris-HCl buffer (pH 8.0) with 10 μl CaCl$_2$ at 37 °C for 16 h. The digested peptides were extracted with 5% formic acid in acetonitrile and cleared by centrifugation at high speed for 5 min. The supernatant was dried in a SpeedVac for mass analysis. After desalting with Zip-Tip filter (Millipore, Billerica, MA), the digested peptides were loaded onto a fused silica microcapillary C18 column (75 μm × 150 mm).

Liquid chromatography separation was conducted using linear gradient elution with 0.1% formic acid in H$_2$O (solvent A) and formic acid in acetonitrile (solvent B). The elution program was as follows: a solvent composition of 97:3 (A:B) from 0 to 5 min; 60:40 from 5 to 72 min; 10:90 from 72 to 87 min; and 97:3 from 87 to 120 min. The flow rate was a constant 200 nl/min. The separated peptides were subsequently analyzed on a model LTQ linear ion-trap mass spectrometer (ThermoFinnigan, San Jose, CA). The electrospray voltage was set to 2.0 kV, and the threshold for switching from MS to MS/MS was 250. The normalized collision energy for MS/MS was 35% of the main $R_F$ amplitude, and the duration of activation was 30 ms. All spectra were acquired in data-dependent mode. Each MS scan was followed by MS/MS scans of the three most intense peaks from the full MS scan. The repeat count of peak for dynamic exclusion was 1, and its repeat duration was 30 s. The dynamic exclusion duration was set to 180 s, and the exclusion mass width was ±1.5 Da. The list size of dynamic exclusion was 50.

**Data Base Analysis**—Data base searches were performed for all MS/MS spectra using an *E. coli* protein data base with the addition of the amino acid sequence of the Blh protein. SEQUEST was used as the peptide-searching program, which included the dynamic modifications of oxidized methionine (+16 Da) and carboxyamidomethylated cysteine (+57 Da). SEQUEST criteria for peptide selection were based on the values of XCorr, which requires values greater than 1.8, 2.3, and 3.5 for +1, +2, and +3 charge state peptides, respectively, with a ΔCn above 0.1. The criterion for protein selection was a consensus score above 10.1.

**Western Blot Analysis**—The soluble fraction of recombinant *E. coli* lysate was subjected to Western blot analysis. The fraction was separated by SDS-PAGE on 12% gels and then transferred to a polyvinylidene difluoride membrane using a Mini-Protein II transfer apparatus (Bio-Rad) according to the manufacturer’s instructions. For immunodetection, the membranes were first blocked with 5% (w/v) skim milk in phosphate-buffered saline containing Tween 20 (PBST) and then incubated with His-tagged monoclonal antibody for 1 h. The

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2 The abbreviations used are: Tricine, N-[2-hydroxy-1,1-bis(hydroxy-methyl)ethyl]glycine; HPLC, high pressure liquid chromatography; MS, mass spectrometry; MS/MS, tandem MS; HR-MS, high resolution liquid chromatography-mass spectrometry.
Characterization of Bacterial β-Carotene 15,15′-Dioxygenase

The influence of temperature on enzyme stability for retinal formation was monitored in 100 mM Tricine-KOH buffer (pH 8.0) for 18 h at temperatures from 35 to 55 °C. A sample was withdrawn at each time interval and was assayed for the residual relative activity in 100 mM Tricine-KOH buffer (pH 8.0) containing 50 μM β-carotene and 0.04 unit/ml enzyme at 40 °C for 60 min. The experimental data for thermal deactivation of enzyme were fitted to a first-order curve, and the half-lives of the enzyme were calculated using Sigma-plot software (version 9.0, 2004).

Determination of Kinetic Parameters for Various Carotenoid Substrates—α-Carotene, β-carotene, γ-carotene, β-cryptoxanthin, zeaxanthin, lutein, β-apo-4′-carotenal, β-apo-8′-carotene, β-apo-12′-carotenal, and lycopene were used to determine the kinetic parameters of the enzyme. The reaction was performed in 100 mM Tricine-KOH buffer (pH 8.0) at 40 °C for 30 min using various concentrations of substrates (5–800 μM). The kinetic parameters were determined by fitting the data of the Michaelis-Menten equation.

Isotope Labeling for Determining the Mechanism of Oxygenation—To investigate the reaction mechanism of the enzyme, isotopically labeled water (H218O) or molecular oxygen (18O2) was used. For labeling experiments with H218O, freeze-dried purified enzyme and β-carotene were suspended in the H218O solution. For labeling experiments with 18O2, the enzyme and substrate were suspended in the H2O solution and then saturated by gasping the solution with 18O2 on ice for 5 min. The enzyme reaction was performed in 100 mM Tricine-KOH buffer (pH 8.0) containing 50 μM β-carotene and 0.04 unit/ml enzyme at 40 °C for 60 min. At the end of the incubation, an equal volume of acetonitrile was added to the reaction solution. The resulting solution was centrifuged at 10,000 × g for 10 min at 4 °C, and the supernatant was evaporated. The samples were resuspended in acetonitrile and analyzed by high resolution liquid chromatography-mass spectrometry (HR-MS).

Computational Analysis—Theoretical weights and pI values for proteins were calculated using the Compute pI/Mw tool at the ExPASy web site. Prediction of transmembrane helices in the protein was performed using the TMHMM program (33).

Molecular Modeling of the Blh Protein—The Blh protein had no homologous sequences. Thus, we predicted the secondary structure of the Blh protein using the protein structure prediction server (PSIPRED) (34) and compared it with other secondary structures in the Protein Data Bank. As a result, we selected 10 secondary structure candidates from among those analyzed and averaged the candidates. Based on the selected secondary structure, a simulated backbone was constructed, and side chains were added using the side chain prediction program SCWRL (35). The three-dimensional structure of the Blh protein from the uncultured marine bacterium 66A03 was generated using the Accelrys Discovery Studio Modeler (Accelrys, San Diego) (36). The generated structure was checked by PROCHECK (37), and then structure minimization was conducted using Chemistry at Harvard Molecular Mechanics (38). After energy minimization, molecular dynamics modeling was performed at 300 K, 1 atm for 500 ps with 1 fs each step. All simulation experiments were carried out on an HP XW6200 Workstation with dual Intel Xeon 3.2-GHz processors.
Characterization of Bacterial β-Carotene 15,15'-Dioxygenase

Uncultured marine bacterium 66A03 Blh protein
Uncultured marine bacterium HF10 49008 Blh protein
Halobacterium sp. NRC-1 Blh protein
Halococcus marismortui ATCC 43049 Brp-like protein
Haloragdus walsbyi DSM 16790 Brp-like protein

Results

DNA Sequence of the Codon-optimized blh Gene—Codons of the blh gene encoding the β-carotene-cleaving enzyme from the uncultured marine bacterium 66A03 were optimized, and the entire gene was synthesized. Among the 828 bp of the blh gene, including a stop codon, 213 bp (25.8%) were changed by codon optimization (supplemental Fig. 1). The synthesized gene was cloned into the pET-24a(+) vector and expressed in E. coli. The expressed Blh protein consisted of 275 amino acids, including membrane spanning domains, is shown in Fig. 1. The

β-Carotene, β-cryptoxanthin, and lutein were docked in the Blh models using the Surflex X docking program (Tripos, St. Louis, MO). Each docking run consisted of 100 independent docks with 1,000 iteration cycles. A random start was used to generate the substrate position within the docking box. The substrate orientation giving the lowest interaction energy was chosen for additional rounds of docking.

Analytical Methods—High pressure liquid chromatography (HPLC) analyses of carotenoids and retinoids were performed based on a previously reported method (10). The same volume of acetonitrile was added to the reaction solution, and the resultant solution was mixed and kept on ice for 5 min. After centrifugation at 10,000 × g for 10 min at 4 °C, substrates and products of the supernatant were analyzed by an HPLC system (Agilent 1200 series, Santa Clara, CA) equipped with a UV detector. The substrates β-carotene and lycopene were detected at 460 and 445 nm, respectively, and lutein and zeaxanthin were determined at 450 nm using a Zorbaxsil column (250 × 4.6 mm, Agilent). The column was eluted with the mixture of hexane and tert-butyl methyl ether with 97:3 (v/v) as the mobile phase with a flow rate of 1 ml/min. The products (3R)-3-hydroxy-retinal, retinal, and α-retinal were detected at 370 nm with retention times of 1.3, 1.8, and 2.2 min, respectively, and the product acycloretinal was detected at 400 nm using an YMC-ODS A column (250 × 2.0 mm, YMC, Kyoto, Japan). The column was eluted with a 90:10 (v/v) mixture of acetonitrile and water as the mobile phase at a flow rate of 0.4 ml/min. The retinal isomers 13-cis-retinal, 9-cis-retinal, and all-trans-retinal were detected at 370 nm using an YMC-ODS A column (250 × 4.6 mm, YMC) with retention times of 26.1, 26.3, and 27.3 min, respectively. The column was eluted with 80% acetonitrile as the mobile phase at a flow rate of 1.0 ml/min.

The HR-MS data were obtained using a JMS-SX102A spectrometer (Jeol, Tokyo, Japan) operated at an accelerating voltage of 10 kV. The electron impact ionization mass spectra were collected in the positive ion mode. The HR-MS was performed under the acquisition conditions as follows: ion source temperature 230 °C, ionization energy 70 eV, and ionization current 300 μA.
A band obtained from the soluble fraction of the cell extract confirmed the isolation of a highly purified recombinant protein. The Blh protein was expressed as a soluble form in E. coli, and the protein was purified without detergent even though it is a member of the bacteriorhodopsin family. Its soluble form may result from the codon optimization, which is known to increase the expression and solubility of enzymes (39).

The molecular weight of native enzyme, based on the weights of reference proteins, was estimated using gel filtration chromatography. The native Blh protein had a mass of 64 kDa as a dimer of 32-kDa subunits (Fig. 2B). A chromatogram for the gel filtration of the Blh protein confirms its purity and molecular weight (Fig. 2C).

The Blh protein on a SDS-PAGE was cut out and subjected to trypsinization. The digested peptides were recovered and characterized on a nano liquid chromatography-MS/MS to obtain amino acid sequence data. The following high confidence peptide sequences were obtained: YNIAFELIG, SRRHFS-FVWKQL, and FIGLPHGALD (supplemental Fig. 2). A comparison between these data and the Blh sequence showed that all three peptides were present in the sequence (Fig. 1).

Determination of the Retinal Product of the Blh Protein—The enzymatic conversion of β-carotene into retinal was examined by varying the reaction time and concentrations of enzyme and substrate, based on a standard reaction performed in 100 mM Tricine-KOH buffer (pH 8.0) containing 50 μM β-carotene and 0.04 unit/ml enzyme at 40 °C for 60 min (Fig. 3, A–C). The products formed at different reaction times and concentrations of enzyme and substrate were analyzed by an HPLC system using an YMC-ODS A column and showed the same retention time as an all-trans-retinal standard. However, other retinal isomers including 13-cis-retinal and 9-cis-retinal showed different retention times, indicating the enzymatic product of β-carotene is all-trans-retinal. With increasing reaction time from 10 to 60 min, the substrate β-carotene decreased whereas the product all-trans-retinal increased (Fig. 3D).

The enzyme cleaved β-carotene at its central double bond (15,15′) to yield two molecules of retinal. The formation of ret-
Characterization of Bacterial β-Carotene 15,15'-Dioxygenase

The enzymatic conversion of β-carotene into all-trans-retinal was examined at pH values ranging from 6.0 to 9.0. The maximum enzyme activity was observed at pH 8.0 (Fig. 5A). The effect of temperature on the enzyme activity is shown in Fig. 5B. The maximum activity was recorded at 40 °C. Above this temperature, the enzyme activity decreased significantly, exhibiting 66% of the maximum activity at 50 °C. Below 40 °C,
the enzyme activity decreased with decreasing temperature, exhibiting 15% of the maximum activity at 25 °C.

The thermostability of the Blh protein was measured at five incubation temperatures. The activity of the enzyme was very stable at below 45 °C but significantly decreased at above 50 °C with increasing reaction time (Fig. 5C). The enzyme followed the first-order kinetics of thermal inactivation, and the half-lives of the enzyme at 35, 40, 45, 50, and 55 °C were 17.6, 15.0, 12.5, 6.1, and 1.5 h, respectively.

Kinetic Analyses of the Blh Protein for Various Carotenoid Substrates—The kinetic parameters of the purified recombinant Blh protein on \( \beta \)-carotene, \( \gamma \)-carotene, \( \beta \)-cryptoxanthin, zeaxanthin, lutein, \( \beta \)-apo-4'-carotenal, \( \beta \)-apo-8'-carotenal, \( \beta \)-apo-12'-carotenal, and lycopene were determined and are shown in Table 1. The corresponding Michaelis-Menten curves are shown in supplemental Fig. 3. The Blh protein exhibited the highest catalytic efficiency \( (k_{cat}/K_m) \) with \( \beta \)-carotene among the substrates tested. This implies that \( \beta \)-carotene is an authentic substrate for the enzyme. The second-most kinetically favored substrate \( (k_{cat}/K_m) \) was \( \beta \)-cryptoxanthin, followed by \( \beta \)-apo-4'-carotenal, \( \alpha \)-carotene, and \( \gamma \)-carotene. \( \beta \)-Cryptoxanthin was cleaved into retinal and (3R)-3-hydroxy-retinal. If the 15,15'-double bond of zeaxanthin or lutein were cleaved, the products would have been (3R)-3-hydroxy-retinal and (3R)-3-hydroxy-retinal, or two molecules of (3R)-3-hydroxy-retinal, respectively. Their common product, (3R)-3-hydroxy-retinal, was formed by the enzymatic conversion of \( \beta \)-cryptoxanthin but was not formed by
cleavage of zeaxanthin or lutein (supplemental Fig. 4). Neither retinal, as the enzymatic product of apo-8′-carotenal, nor apo-12′-carotenal and acycloretinal, as the products of lycopene, were detected. These results demonstrate that the Blh protein has no activity on zeaxanthin, lutein, apo-8′-carotenal, apo-12′-carotenal, or lycopene.

**Origin of the Oxygen Atom of Retinal in β-Carotene Cleavage by the Blh Protein** — The molecular weight of retinal as a product was determined after the enzyme was incubated with β-carotene in the presence of H₂¹⁸O or H₂O₂. For labeling experiments with H₂¹⁸O, freeze-dried enzyme and β-carotene were suspended in the H₂¹⁸O solution. The mass spectrum of retinal formed under these conditions was the same as that using H₂O, and the molecular weight of retinal was determined as 284 m/z (Fig. 6A), indicating that the oxygen atom of retinal did not originate from water. However, after the enzyme reaction was performed in an ¹⁸O₂ atmosphere, the formed retinal was labeled with the ¹⁸O atom, and the molecular mass was 286 m/z (Fig. 6B). The fragment molecular masses of labeled retinal containing isotope ¹⁸O such as 213 and 241 m/z were 2 m/z greater than those of unlabeled retinal containing normal 18O such as 211 and 239 m/z. Thus, the oxygen atom of retinal as a reaction product of the Blh protein originated from molecular oxygen rather than from water.

**DISCUSSION**

Although most recent critical debates about β-carotene-cleaving enzymes have tended to center around mammalian β-carotene 15,15′-dioxygenases, we became interested in β-carotene-cleaving enzymes from other sources, especially bacteria. Hence, we used codon optimization to synthesize the blh gene from the uncultured marine bacterium 66A03 for...
The expressed enzyme converts \( \beta \)-carotene into all-trans-retinal. Characterization of the bacterial Blh protein as a new member of the \( \beta \)-carotene cleavage enzymes is a worthwhile undertaking.

The Blh protein is a putative membrane protein closely related to bacteriorhodopsin (40). Bacteriorhodopsin is a membrane protein containing seven transmembrane \( \alpha \)-helices and a covalently bound molecule of retinal. The Blh protein is predicted by the TMHMM program to have seven transmembrane segments, whereas human \( \beta \)-carotene 15,15'-dioxygenase has no transmembrane segments. These results suggest that the Blh protein is a membrane protein. The theoretical pI values of the Blh proteins from marine bacteria (8.89–9.56) were much higher than those of the mammalian \( \beta \)-carotene 15,15'-dioxygenases (5.49–6.30), indicating that the two proteins are different (Table 2). There were 48–56% hydrophobic amino acids in the bacterial Blh proteins and 33–34% in mammalian \( \beta \)-carotene 15,15'-dioxygenases.
Characterization of Bacterial β-Carotene 15,15′-Dioxygenase

TABLE 2
Theoretical pH values and hydrophobic amino acid contents of Blh and Brp-like proteins and β-carotene 15,15′-monooxygenases

| Protein                   | Source                              | pH   | Hydrophobic amino acids | %   |
|---------------------------|-------------------------------------|------|-------------------------|-----|
| Blh protein               | Uncultured marine bacterium 66A03   | 9.56 | 52.4                    |
|                           | Uncultured marine bacterium HF10 49E08 | 9.30 | 48.4                    |
|                           | Halobacterium sp. NRC-1             | 8.89 | 55.2                    |
| Brp-like protein          | Haloarcula marismortui ATCC 43049   | 9.12 | 56.3                    |
|                           | Halococcus walsbyi DSM 16790        | 9.50 | 52.2                    |
| β-Carotene 15,15′-monooxygenase | Chicken                          | 6.01 | 32.8                    |
|                           | Human                               | 6.30 | 33.7                    |
|                           | Mouse                               | 5.49 | 33.3                    |
|                           | Rat                                 | 5.90 | 32.9                    |

* The hydrophobic amino acids are alanine, leucine, valine, isoleucine, phenylalanine, and methionine.

TABLE 3
Biochemical properties of the Blh protein and β-carotene 15,15′-monooxygenases

| Organism                      | Optimum temperature | Optimum pH | Km       | Vmax    | kcat    | kcat/Km | Molecular mass  | Native enzyme   | No. of amino acids | Half-life at 45°C | Ref. |
|-------------------------------|---------------------|------------|----------|---------|---------|---------|---------------|----------------|--------------------|-------------------|-----|
| Uncultured marine bacterium   | 37 °C               | 8.0        | 37.0     | 45.0    | 3.60    | 97      | 32            | Dimer (64 kDa)  | 275                | 12.5              | This study       |
| Human                         | 37 °C               | 8.0        | 7.1      | 10.4    | 0.66    | 93      | 64            | Tetramer (250 kDa)| 547                | 2.4              | 41              |
| Mouse                         | 37 °C               | 9.0        | 6.0      | 0.04    |         |         | 64            |                 | 566                | 45, 48            |
| Chicken                       | 37 °C               | 8.0        | 8.0      | 10.0    | 0.03    |         | 60            |                 | 526                | 32               |
| Rat                           | 37 °C               | 7.5        | 3.3      | 0.03    |         |         | 64            |                 | 566                | 4                |

Thus, the Blh proteins are more hydrophobic than mammalian β-carotene 15,15′-monooxygenases.

The subunit molecular weight of the Blh protein was calculated as 32 kDa based on the 281-residue amino acid sequence plus a hexahistidine tag at the carboxyl terminus. The native protein has a total molecular mass of about 64 kDa as a dimer (Fig. 2). β-Carotene 15,15′-monooxygenases from humans, mice, chickens, and rats consisted of 547, 566, 526, and 566 amino acid residues, respectively, and their molecular masses were 60–64 kDa (Table 3). The molecular mass of the native human β-carotene 15,15′-monooxygenase is 250 kDa (41).

Mammalian β-carotene 15,15′-monooxygenases are reported to be Fe2+ dependent protoporphyrin (non-heme iron) enzymes and are inhibited by iron-chelating agents (4, 42, 43). Phenanthroline behaves as an iron-chelating agent and strongly inhibited the conversion of β-carotenoids into retinal by the Blh protein. Furthermore, among the metal ions tested, Fe2+ had the strongest effect on the activity of the Blh protein. Fe3+ is bound by four conserved histidine residues in the active sites of mammalian β-carotene monoxygenase and apocarotenoid oxygenase (43, 44) and in the molecular model of the Blh protein (Fig. 7A). In the holo Blh protein, Fe2+ is coordinated with His-21, His-78, His-188, and His-192, which are absolutely conserved across all Blh and Brp-like proteins (Fig. 1). The four conserved histidine residues were replaced with alanine to produce alanine-substituted mutants. Enzyme activities were determined for the mutants and were compared with that of wild-type enzyme. The relative activities of the H21A, H78A, H188A, and H192A mutants were 3, 0, 0, and 5%, respectively. Thus, we can propose that the four histidine residues in the Blh protein are metal-binding residues. The enzyme activity in the presence of 10 μM Fe3+ before dialysis was almost the same as after dialysis. These results imply that Fe2+ is tightly bound to the Blh protein.

The maximum activity of the Blh protein was observed at pH 8.0 and 40 °C (Fig. 5). The maximum activities of β-carotene 15,15′-monooxygenases from mammalian sources occurred at pH 7.5–8.5, except for the mouse enzyme that had a maximum activity at pH 9.0 (45). The optimum reaction temperature of mammalian β-carotene 15,15′-monooxygenases from rabbits (42), hogs (46), pigs (47), chickens (11), mice (48, 49), and humans (13, 41) was 37 °C. The thermostability of the human β-carotene 15,15′-monooxygenase was investigated, and the half-lives of the human enzyme at 35, 40, 45, and 50 °C were 4.5, 3.4, 2.4, and 1.4 h, respectively. The half-lives of the Blh protein were approximately 4–5-fold higher at these temperatures than those of the human β-carotene 15,15′-monooxygenase.

The Blh protein from the uncultured marine bacterium converted the substrate β-carotene into two molecules of retinal, and it converted β-apo-4′-carotene into one molecule of retinal. The enzyme cleaved the central double bonds (15,15′) of α-carotene, γ-carotene, and β-cryptoxanthin to yield not only one molecule of retinal but also one molecule of each α-retinal, acycloretinal, and (3R)-3-hydroxy-retinal. β-Carotene has two β-ionone rings; α-carotene has β-ionone and ε-ionone rings, and γ-carotene and β-apo-carotenals have one β-ionone ring. β-Cryptoxanthin has β-ionone and hydroxyl β-ionone rings; zeaxanthin has hydroxy ε-ionone and hydroxyl β-ionone rings, and lutein has two hydroxyl β-ionone rings. However, lycopene does not have a β-ionone ring. The Blh protein exhibited activity for β-carotene, β-cryptoxanthin, β-apo-4′-carotenal (C30), α-carotene, and γ-carotene in decreasing order, but not for β-apo-8′-carotenal (C30), β-apo-12′-carotenal (C30), lutein, zeaxanthin, or lycopene. Based on the chemical structures of the carotenoid substrates, enzyme activity was found only for substrates containing one unsubstituted β-ionone ring with a molecular weight greater than C35. The mammalian β-carotene 15,15′-monooxygenases from humans and rats had activity for β-carotene, β-cryptoxanthin, and β-apo-8′-carotenal (C30) but not lycopene (41, 50). We found that human β-carotene 15,15′-monooxygenase had activity for β-apo-4′-carotenal (C35). Although the kcat/Km values of the
Blh protein and the human \( \beta \)-carotene \( 15,15' \)-monooxygenase for \( \beta \)-carotene were similar, the \( k_{cat} / K_m \) value of the Blh protein for \( \beta \)-cryptoxanthin (28 mm\(^{-1}\) min\(^{-1}\)) as a substrate was 14-fold higher than that of the human enzyme (1.9 mm\(^{-1}\) min\(^{-1}\)) (41).

To interpret the substrate specificity of the Blh protein on carotenoid substrates, a simulated backbone of the enzyme structure was constructed, and side chains were added based on averaged similar secondary structures in the protein data base. Simulated molecular docking was performed with \( \beta \)-carotene, \( \beta \)-cryptoxanthin, and lutein substrates using the Surflex X docking program. When \( \beta \)-carotene was bound in the active site, the \textbf{para} position of one \( \beta \)-ionone ring of \( \beta \)-carotene, located in the interior of the active site, and the \textbf{para} position of the other unhydroxylated \( \beta \)-ionone ring interacted with the oxygen atom of the carbonyl group of Thr-179, at a distance of 3.01 Å (Fig. 7B). When \( \beta \)-cryptoxanthin was bound in the active site, the distance between the hydroxyl group of the (3R)-3-hydroxy-\( \beta \)-ionone ring of \( \beta \)-cryptoxanthin and the oxygen atom of the carbonyl group of Thr-179 decreased to 2.31 Å (Fig. 7C). The shorter distance may explain the observed higher affinity of the Blh protein for \( \beta \)-cryptoxanthin than for \( \beta \)-carotene. When lutein (or zeaxanthin) was bound in the active site, two hydroxyl groups of two (3R)-3-hydroxy \( \beta \)-ionone rings of lutein (or zeaxanthin) were noneffective in anchoring to the active site because the distance to the oxygen atom of Thr-179

**FIGURE 7.** Molecular model of the Blh protein. The oxygen atom at carbonyl group of Thr-179 interacted with the \textbf{para} position in the \( \beta \)-ionone ring of \( \beta \)-carotene or with the hydroxyl group in the (3R)-3-hydroxy-\( \beta \)-ionone ring of \( \beta \)-cryptoxanthin. A, active site of the Blh protein. Docking of \( \beta \)-carotene (B), \( \beta \)-cryptoxanthin (C), and lutein (D) into the active site of the Blh protein is shown.
Characterization of Bacterial β-Carotene 15,15′-Dioxygenase

A, dioxygenase mechanism. The oxygen atom of the product is provided by molecular oxygen rather than water via dioxetane intermediate (7). B, monooxygenase mechanism. The oxygen atom of the product is provided by molecular oxygen and water via an epoxide intermediate (51).

was too long (Fig. 7D). As a result, the Blh protein exhibited no activity for lutein or zeaxanthin. The Blh protein showed activity for β-apo-4′-carotene but not for β-apo-8′-carotene. In the molecular modeling studies, β-apo-4′-carotene was large enough to interact with Thr-179, but β-apo-8′-carotene was too small to interact (data not shown). However, it is necessary to obtain the actual structure of the enzyme complexed with these substrates to provide further evidence for this conclusion. To confirm our molecular modeling and structural analyses, determination of the crystal structure of the Blh protein and mutational analysis of the active site residues must be performed.

Carotenoid oxygenases can be divided into mono- and dioxygenases based on two different reaction mechanisms (Fig. 8). The oxygen atom of the product from the monooxygenase is provided by molecular oxygen and water via an epoxide intermediate, whereas that from the dioxygenase is provided by molecular oxygen rather than from water via a dioxetane intermediate (51, 52). The oxygenation mechanism of chicken β-carotene 15,15′-dioxygenase was investigated with isotopic molecular oxygen and water. The oxygen atom in the terminal aldehyde group of retinal as a product was provided by molecular oxygen and water (51), indicating that the enzyme catalyzes the oxidative cleavage using a monooxygenase rather than a dioxygenase mechanism. The other mammalian β-carotene 15,15′-dioxygenases were postulated to use the same mechanism. However, some controversies on the reaction mechanism was suggested because oxygen between retinal and water was exchanged within 5% (51). In contrast, the oxygen atom of the retinal formed by the reaction of the Blh protein originated from molecular oxygen rather than from water without oxygen exchange between retinal and water, indicating that the Blh protein used a dioxygenase mechanism unlike the mammalian β-carotene 15,15′-monooxygenases. The Blh protein cleaved β-carotenoid substrates at its central double bond (15,15′) and showed the highest activity for β-carotene among the substrates tested. Thus, the Blh protein can be identified as a β-carotene 15,15′-dioxygenase.

In the molecular model, the active site of the Blh protein exhibited coordination of Fe^{2+} with His-21, His-78, His-188, and His-192 (Fig. 7A). According to the cleaving mechanism of apocarotenoid oxygenase (52), the O_{2} molecule in the active site of the Blh protein binds to the coordination shell of Fe^{2+} in a side-on fashion. The side-on complex of Fe-O_{2} then attacks and cleaves between the C-15 and C-15′ atoms when bound to the Blh protein.

Most enzymes that catalyze the same reaction sequence are structurally similar. However, structurally and mechanistically unrelated enzymes that catalyze the same biochemical reactions do occur. In many cases, one of these analogous enzymes is found in the bacteria and the other in eukaryotes (53). Although the bacterial Blh protein and the mammalian β-carotene 15,15′-monooxygenases could both convert β-carotene into retinal, the properties of the bacterial Blh protein such as DNA and amino acid sequences, molecular weight, form of association, reaction mechanism, kinetic properties, and substrate specificity were different from the mammalian β-carotene 15,15′-monooxygenases. Thus, the Blh protein is a bacterial analog to the mammalian β-carotene 15,15′-monooxygenases.

This is the first report of in vitro characterization of a bacterial β-carotene-cleaving enzyme. This study contributes to the understanding of bacterial β-carotene-cleaving enzymes and provides a stepping stone for further studies. Moreover, this study will be useful in evolutionary studies investigating the relationships between bacterial and mammalian enzymes and industrial applications of retinal biosynthesis.

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Characterization of Bacterial β-Carotene 15,15'-dioxygenase

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