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ORIGINAL RESEARCH PAPER

Utilization of the recombinant human β-carotene-15,15′-monooxygenase gene in *Escherichia coli* and mammalian cells

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Abstract In animals, β-carotene 15,15′-monooxygenase (BCMO) is the key enzyme involved in the metabolism of plant β-carotene to retinal. In the present study, we utilized β-carotene-producing *Escherichia coli* to screen for mutants with higher BCMO activity which was monitored by color changes derived from β-carotene cleavage. Recombinant wild-type and T381L mutant BCMO proteins were purified to near homogeneity in *E. coli*, and their enzymatic activities were determined by HPLC analysis. The catalytic efficiency for β-carotene and retinal production of the mutant were 1.5-fold and 1.7-fold higher than those of wild-type, respectively. Further BCMO function in mammalian cells was analyzed by a retinoic acid receptor reporter assay, which responds to the metabolic conversion of β-carotene to retinoic acid in vivo. Overall, these tools can be used to screen more active BCMO for the industrial and pharmacological purpose of retinal production from β-carotene.

Keywords β-Carotene 15,15′-monooxygenase · β-Carotene · Retinal · Retinoic acid · Retinoic acid receptor

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Introduction

In vertebrates, vitamin A (retinol) is important for vision, embryonic development, cell differentiation, and for membrane and skin protection (de Luca 1991; Fisher and Voorhees 1996). For these processes, vitamin A must be converted into retinoic acid, which binds to the retinoic acid receptor that modulates the expression of the RA target genes (Chambon 1996). Fat-soluble vitamin A is present in two forms in nature: animal foods, such as fish oils and liver, and vegetables, in the form of β-carotene. In animals, vitamin A is formed by the oxidative cleavage of β-carotene into two molecules of all-*trans*-retinal, a reaction catalyzed by β-carotene 15,15′-monooxygenase (BCMO) (Wyss 2004). Although the enzyme has been known for nearly 50 years, its mechanism of action has been controversial as to whether the cleavage is symmetric or asymmetric (Leuenberger et al. 2001). In animals, the highest enzyme activity is in the intestinal mucosa (Lakshman et al. 1989) but

it is also detected in liver, kidney, lung, and fat tissues (During et al. 1996; van Vliet et al. 1996). Recently, BCMO cDNA was cloned from *Drosophila* (von Lintig and Vogt 2000) and vertebrates (Wyss et al. 2000; Paik et al. 2001; Redmond et al. 2001; Yan et al. 2001; Lindqvist and Andersson 2002). Although native and recombinant BCMO have been extensively characterized, its intrinsic activity does not meet optimal conditions for industrialization. Thus, efforts are required to improve its enzymatic activity. In this study, we cloned the human BCMO gene and screened mutant BCMO with higher enzymatic activity using β-carotene-producing *Escherichia coli*. Kinetic parameters of one mutant (T381L) were compared to those of wild-type. Finally we determined whether recombinant BCMO is active in mammalian cells by the RAR reporter gene assay. Overall, our in vivo systems could be used to screen more active BCMO for the industrial and pharmacological purpose of retinal production from β-carotene.

Materials and methods

Cloning the human BCMO gene

Human kidney cell line A704 (Korean Cell Line Bank) cells were grown in 100 mm dishes in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum. Total cellular RNA was isolated using an RNeasy Total RNA Purification kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. A one-step RT-PCR was performed using the Titan One Tube RT-PCR system (Roche Molecular Biochemicals, Basel, Switzerland). Purified RNA (10 ng) was mixed with 200 μM of each dNTP, 400 nM 5′- and 3′-primers, and avian myeloblastosis virus reverse transcriptase high-fidelity DNA polymerase mixture. The primers used to amplify the open reading frame of the BCMO gene were the following: forward primer 5′-GGGCTCGAGATGGATATAATATTTG GCAGGA-3′, and reverse primer 5′-GGGGG ATCCTCATCAGGTCAGAGGAGCCCC-3′. Mutant BCMO (Thr381→Leu: T381L) was created by recombinant PCR. The PCR product was digested with *Xho*I and *Bam*HI enzymes, and inserted into the mammalian expression vector Flag fusion-modified pcDNA3 (Invitrogen, Carlsbad, CA), the bacterial expression vectors pET-15b (Novagen, Madison, WI), and pGEX-4T2 (Amersham Pharmacia, Piscataway, NJ).

Expression and purification of human BCMO

The human BCMO gene (wild-type or mutant T381L) inserted into the vector pET-15b was introduced into *E. coli* BL21(DE3). After BL21(DE3) was cultured in Luria–Bertani (LB) broth with ampicillin (100 mg/l), expression of the recombinant proteins was induced by 1 mM IPTG. Purification was carried out using HiTrap Chelating HP column (Amersham Pharmacia) according to the manufacturer's instructions.

Determination of kinetic parameters

β-Carotene (3–50 μM) was added to 0.8% (w/v) Tween 40 in chloroform and homogenized at 10,000 rpm for 10 s using a homogenizer. Chloroform was evaporated by N_2 and then water was added. The resulting solution was used as the substrate solution. The enzyme solution was prepared in 100 mM Tricine/KOH buffer (pH 8.0) containing 167 mM NaCl, 13.3 μM Fe_2SO_4, 6.7 mM Tris(2-carboxylethyl)phosphine hydrochloride (TCEP), and 1.3% (w/v) 1-*S*-octyl-β-D-thioglucopyranoside (OTG). The enzyme solution (150 μl) containing 250 ng purified enzyme and substrate solution (50 μl) were mixed at 3:1 (v/v) and used as the reaction solution. The reaction was performed with the reaction solution (200 μl) at 37°C for 30 min. After incubation, the reaction was stopped by 3.7% (v/v) formaldehyde and additional incubation was done at 37°C for 10 min (Wyss et al. 2001). The apparent K_m (μM) and V_{max} (μmol min^{-1} mg^{-1}) were calculated by standard methods.

HPLC analysis

HPLC analysis of retinal was performed as the previously described (During et al. 1996). Two hundred micro liter of acetonitrile was added to the reaction solution. The resulting solution was mixed and kept on ice for 5 min. After centrifugation at 10,000*g* for 10 min at 4°C, the supernatant was

analyzed by HPLC using a YMC-PAC ODS-A column (250 × 4.6 mm, YMC, Kyoto, Japan). The column was eluted with acetonitrile/water (90:10 v/v) at 1 ml min^{-1}. The eluant was monitored at 370 nm.

Western blot analysis

After induction of glutathione-*S*-transferase (GST)-BCMO with 1 mM IPTG, *E. coli* was harvested, resuspended in 1 × SDS sample buffer (Laemmli 1970), boiled for 5 min, and subjected to SDS-PAGE. HeLa cells, transfected with Flag-tagged BCMO expression vector, were lysed in 100 µl lysis buffer consisting of 10 mM Tris/HCl (pH 7.4), 1 mM EDTA, 1 mM PMSF, 1 mM dithiothreitol, and a protease inhibitor mixture (Roche Molecular Biochemicals). Proteins were electrophoresed on 10% (v/v) SDS-PAGE gels. Proteins on gels were transferred to nitrocellulose, and blotted with anti-GST antibody for *E. coli* extracts (rabbit polyclonal; Santa Cruz Biotechnology) at 1:100, anti-Flag antibody for HeLa extracts (mouse monoclonal; Sigma) at 1:1,000, or anti-β-actin antibody (mouse monoclonal; Sigma) at 1:5,000. Nitrocellulose blots were further incubated with peroxidase-conjugated mouse or rabbit IgG secondary antibodies (Amersham Pharmacia Biotech). All antibodies were diluted in PBS containing 5% (v/v) skim milk and 0.1% Tween 20. Protein bands were detected with the ECL system (Amersham Pharmacia Biotech).

Color shift assay in β-carotene-accumulating *E. coli*

The pGEX-4T2 vector was used to express human BCMO as a fusion protein with bacterial GST. Recombinant BCMO in pGEX-4T2 was introduced into β-carotene-accumulating *E. coli* DH5α transformed with pAC-BETA04 containing *crtE*, *B*, *I*, *Y*, and *ipi* genes, under selection with 50 µg/ml ampicillin (for pGEX-4T2) and chloramphenicol (for pAC-BETA04). After plating the bacteria on LB medium containing 1 mM IPTG to induce the expression of BCMO, several almost white or colorless colonies were found and subjected to further analysis. The colonies were grown under a red light in LB medium until the cultures reached $OD_{600} = 1$. Expression of BCMO was induced by the addition of 1 mM IPTG for 10 h. The bacterial pellets, harvested by centrifugation, showed a color shift from yellow to almost white.

Transient transfection and luciferase assay

HeLa cells were seeded in a 12-well culture plate at 1.5×10^5 cells/well. Transfection was performed using Lipofectamine Plus reagent (Invitrogen), with RARE-tk-luciferase and increasing amounts of Flag-BCMO in pcDNA3 vector, together with the β-galactosidase expression vector (internal control). After 4 h transfection, cells were washed, fed with 5% (v/v) charcoal-stripped medium, and incubated for an additional 20 h in the presence of all-*trans* retinoic acid (1 µM, positive control) or increasing concentrations of β-carotene. Cells were washed with ice-cold PBS, collected, resuspended in 100 µl luciferase lysis buffer (Promega, Madison, WI), and subjected to three freeze-thaw cycles. Luciferase activity was measured by adding 20 µl luciferin into 30 µl lysates, using an analytical luminescence luminometer according to the manufacturer's instructions (Promega). β-Galactosidase activity was determined in 96-well plates that were read at 405 nm using an enzyme-linked immunosorbent assay reader. Luciferase activity was normalized to that of β-galactosidase. All the data presented here represent the mean of at least three independent transfections. Fold stimulation was calculated by dividing luciferase activity of treated cells by that of untreated cells.

Results and discussion

Cloning of human BCMO cDNA

For cloning the cDNA encoding human BCMO, we designed PCR primers deduced from the human BCMO sequence in the GenBank/EBI Data Bank (NM_017429). We performed RT-PCR using a total RNA preparation from human kidney cell line A704. The PCR product was separated by electrophoresis through 1% agarose and visualized with ethidium bromide (Fig. 1). Sequence analysis of the PCR product indicated that the cDNA encoded a protein of

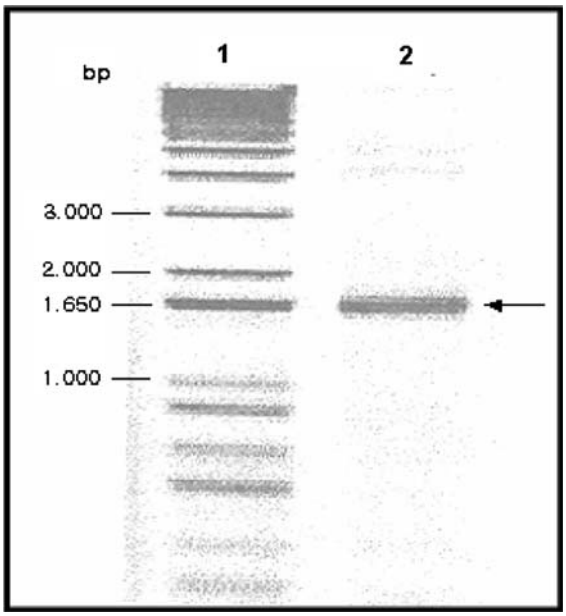

Fig. 1 Isolation of BCMO cDNA. (**a**) RT-PCR product of the BCMO gene. Total cellular RNA isolated from A704 cells was used to amplify human BCMO cDNA using one-step RT-PCR. The PCR product was separated by electrophoresis through a 1% agarose gel. Lane 1, 1 kb DNA marker (Gibco-BRL); lane 2, PCR product of human BCMO cDNA

547 amino acids, with a calculated molecular mass of about 62 kDa (data not shown).

Human BCMO activity in *E. coli*

The β-carotene-accumulating *E. coli* strain was transformed with recombinant human BCMO in pGEX-4T2, and positive clones were selected on LB agar supplemented with ampicillin and chloramphenicol. One of the selected clones was grown under a red light and treated with 1 mM IPTG to induce GST-BCMO. When treated with IPTG, the induction of GST-BCMO was clearly observed by Western blotting using an anti-GST antibody (Fig. 2a) and Coomassie Blue staining (Fig. 2b). Consistent with protein induction, bacterial pellets treated with IPTG showed a color shift from yellow to almost white or light yellow (Fig. 2c). This color shift assay was used to screen for more active BCMO enzymes, followed by PCR-based random mutagenesis. From the initial screening, we isolated a mutant which showed reduced yellow color and found that threonine at

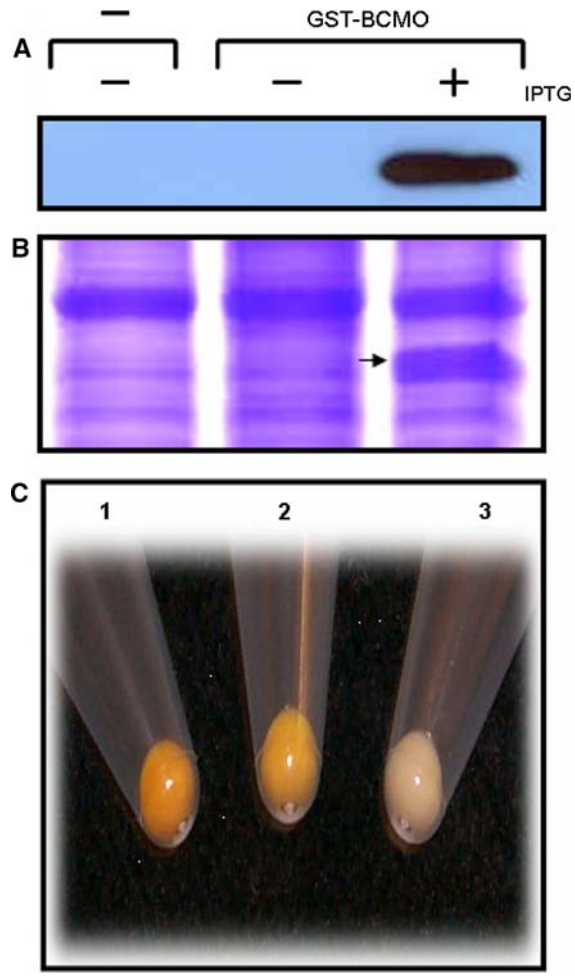

Fig. 2 Color shift assay in β-carotene-accumulating *E. coli*. (**a**, **b**) Induction of GST-BCMO by IPTG. Induction was monitored by Western blotting using an anti-GST antibody (**a**) and by Coomassie Blue staining (**b**). Arrow indicates the induced GST-BCMO band. (**c**) Color shift assay. *E. coli* cells, either treated or untreated with IPTG, were harvested. The cell pellets showed a color shift from deep yellow (β-carotene) to faint yellow (retinoids) when GST-BCMO was induced with IPTG. Lane 1, original *E. coli* producing β-carotene; lane 2, GST-BCMO-transformed *E. coli*; lane 3, GST-BCMO-transformed *E. coli* treated with 1 mM IPTG for 10 h

381 was changed to leucine by DNA sequencing (data not shown).

Purification of human BCMO protein

To purify the recombinant human BCMO protein (wild-type or T381L mutant), we subcloned the wild-type or mutant cDNA into pET-15b expression

vectors of *E. coli*. For induction in *E. coli*, transformed cells with the pET-15b were treated with IPTG and subjected to lysis by sonication. The induced His-tag BCMO proteins, determined by 10% (v/v) SDS-PAGE, was purified to near homogeneity on a HiTrap Chelating HP column (Fig. 3). The purified proteins were observed at about 65 kDa.

Determination of kinetic parameters

Although the BCMO protein was purified to near homogeneity, it had a strong tendency to precipitate and form inclusion bodies during purification and dialysis. Thus, we recovered a very small amount of soluble BCMO for the enzyme assay. The reaction was performed by mixing β-carotene (3–50 μM) with 250 ng purified BCMO (wild-type or T381L mutant). Then, the apparent K_m and V_{max} of the wild-type for β-carotene were 31 μM and 1.1 μmol min^{-1} mg^{-1}, whereas those of mutant were 27.5 μM and 1.5 μmol min^{-1} mg^{-1}, respectively (Table 1), indicating that the catalytic efficiency of the mutant for β-carotene was 1.5-fold higher than that of wild-type. Although the K_m values showed no significant differences between the wild-type and mutant, the V_{max} values

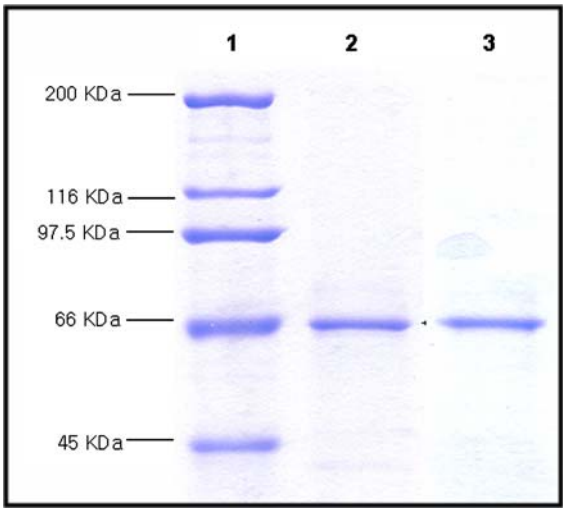

Fig. 3 Charactization of BCMO protein. Coomassie Blue staining of purified BCMO from *E. coli*. The 6 × His-tagged fusion protein of BCMO was purified using a HiTrap™ Chelating HP column. The purified protein was subjected to 10% SDS-PAGE and observed at about 65 kDa. Lane 1, protein marker (Bio-Rad); lane 2, wild-type; lane 3, T381L mutant

Table 1 Kinetic parameters of wild-type and T381L mutant BCMO

Enzyme	K_m (μM)	V_{max} (μmol min^{-1} mg^{-1})
Wild-type	31 ± 2.5	1.1 ± 0.05
T381L mutant	28 ± 1.0	1.5 ± 0.07

Data represent the means of three experiments

exhibited meaningful differences within 5% error range. Moreover, when 1 mg enzyme l^{-1} and 200 mg β-carotene l^{-1} were reacted, the wild-type and T381L mutant produced 20 and 35 mg retinal l^{-1} after 180 min, respectively. These results suggest that the mutant has a higher BCMO activity than the wild-type.

Although V_{max} value was lower than the reported value (Lindqvist and Andersson 2002) under our assay conditions, an increase in V_{max}/K_m of the T381L mutant compared to that of wild-type suggests a possibility that our initial screening in *E. coli* can be used to improve enzyme activity.

Human BCMO activity in mammalian cells

To validate whether the recombinant human BCMO is active in mammalian cells, we employed the reporter gene assay in the HeLa cells which express endogenous RAR (Cho et al. 2006). HeLa cells were transiently transfected with the RAR-responsive luciferase reporter plasmid (RARE-tk-luciferase) and BCMO expression vector, and then treated with either all-*trans* retinoic acid (RA) or β-carotene. The subsequent luciferase assay indicated that the reporter gene activity increased significantly in response to increasing concentrations of β-carotene in the presence of BCMO expression (Fig. 4a). In contrast, no obvious effect of β-carotene was observed in the absence of BCMO expression. These findings were further supported by dose–response studies in which luciferase reporter gene activity increased markedly in a BCMO dose-dependent manner (Fig. 4b, top). The gradual expression of BCMO was monitored by Western blotting using an anti-Flag antibody (Fig. 4b, bottom). Overall, these data suggest that recombinant human BCMO is functional in mammalian cells. Furthermore, this assay system could be used to screen for more active BCMO enzymes as described in *E. coli* systems.

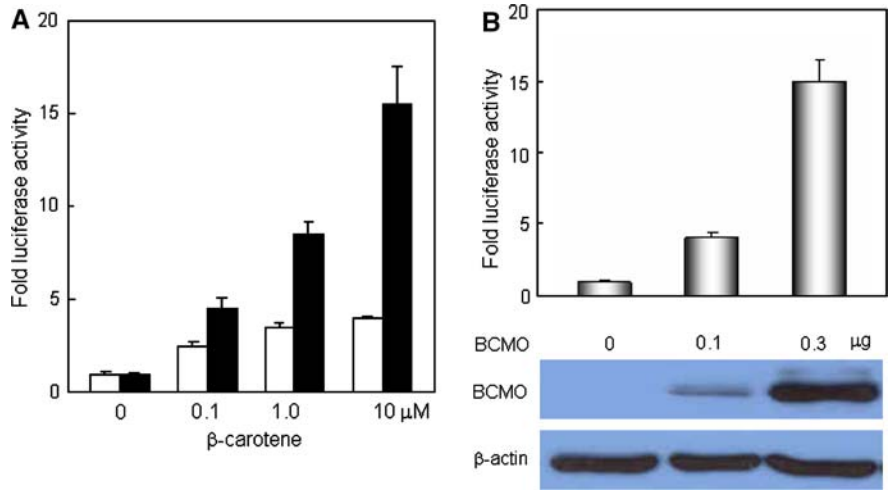

Fig. 4 Luciferase reporter assay in(mammalian cells. (**a**) Effect of β-carotene concentration on reporter gene activity. HeLa cells were transfected with 0.1 μg RARE-tk-luciferase and 0.3 μg BCMO expression vector, and treated with increasing concentrations of β-carotene. Cell extracts were prepared and subjected to a luciferase assay. Fold luciferase activity is shown: open bar, mock pcDNA3 vector; closed bar, BCMO cDNA inserted into pcDNA3. Data are presented as means ± standard deviation of triplicate experiments. (**b**) Effect of BCMO((expression on reporter gene activity. HeLa cells were transfected with 0.1 μg RARE-tk-luciferase and increasing amounts of BCMO expression vector, and treated with 10 μM β-carotene. Fold luciferase activity is shown in the upper panel. The expression of Flag-tagged BCMO was determined by Western blotting using an anti-Flag antibody (lower panel). β-Actin was used as an internal control for Western blotting

In summary, we have cloned the human BCMO gene by RT-PCR and subcloned it into several expression vectors. Although a recombinant human BCMO was purified to near homogeneity in *E. coli*, it had a strong tendency to precipitate during purification and dialysis. Since the recovery of soluble BCMO for in vitro enzyme assays is time-consuming and costly, we developed simple in vivo assay systems to solve these problems: a color shift assay in *E. coli*, and a reporter gene assay in mammalian cells. From the initial screening in the β-carotene-accumulating *E. coli*, we isolated a mutant which showed reduced yellow color. Enzyme assays using purified proteins showed an increase in V_{max} of the T381L mutant compared to that of wild-type, suggesting a possibility that the color screening in *E. coli* can be used to improve enzyme activity. Overall, our in vivo systems can be used to screen for more active BCMO enzymes, followed by further genetic modifications such as error-prone PCR and DNA shuffling.

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