Biotechnol Lett (2011) 33:1195–1200
DOI 10.1007/s10529-011-0556-1

ORIGINAL RESEARCH PAPER

Production of β-apo-10′-carotenal from β-carotene by human β-carotene-9′,10′-oxygenase expressed in *E. coli*

Yeong-Su Kim · Soo-Jin Yeom · Deok-Kun Oh

Received: 10 January 2011 / Accepted: 28 January 2011 / Published online: 8 February 2011

Abstract The gene encoding human β-carotene-9′,10′-oxygenase, which cleaves the 9′,10′ double bond in β-carotene into β-apo-10′-carotenal, was cloned and expressed in *Escherichia coli*. Under aqueous conditions, the optimum organic solvent for the formation of detergent micelles was toluene. The optimum pH, temperature, detergent type, and the optimum concentrations of detergent, substrate, and enzyme for β-apo-10′-carotenal production were 8.0, 37°C, Tween 40, 2.4%, 300 mg β-carotene/l, and 0.25 U/ml, respectively. Under the optimum conditions, 43 mg β-apo-10′-carotenal/l was produced after 21 h with a conversion of 14%. This is the first report to describe the enzymatic production of β-apo-10′carotenal.

Keywords β-Apo-10′-carotenal · β-Carotene · β-Carotene-9′ · 10′-Oxygenase

Introduction

The cleavage of β-carotene occurs through two different biochemical pathways (During and Harrison 2004). The first is a central cleavage catalyzed by β-carotene 15,15′ oxygenases (BCO1s) from mammals (Nagao et al. 1996; Redmond et al. 2001; Lindqvist and Andersson 2002) and marine bacteria (Kim et al. 2009), which cleave β-carotene at its central double bond (15,15′) into two molecules of retinal. The second is an eccentric cleavage that occurs at double bonds other than the central 15,15′ double bond of β-carotene. This cleavage, catalyzed by carotenoid oxygenases from mammals, plants, and cyanobacteria (Scherzinger and Al-Babili 2008), yields β-apocarotenal with different chain lengths that are converted to retinal or oxidized β-apocarotenoic acids.

Apocarotenal is found in spinach and citrus fruits. It is orange to orange-red and is used in foods, pharmaceuticals and cosmetics. Apocarotenal has been converted from β-carotene by carotenoid oxygenases. Among these enzymes, β-carotene-9′10′-oxygenase (BCO2) cleaves the 9′,10′ double bond in β-carotene to form β-apo-10′carotenal and β-ionone. Although BCO2 activities have been found in mammals such as humans (Lindqvist et al. 2005), mice (Kiefer et al. 2001), ferrets (Hu et al. 2006), and cattle (Tian et al. 2009), the enzymatic production of β-apo-10′-carotenal has not yet been attempted.

In the present study, the organic solvent, pH, temperature, detergent type, and concentrations of detergent, substrate, and enzyme for the formation of detergent micelles were optimized. Under the optimum conditions, β-apo-10′carotenal was produced by a recombinant human BCO2.

Electronic supplementary material The online version of this article (doi:10.1007/s10529-011-0556-1) contains supplementary material, which is available to authorized users.

Y.-S. Kim · S.-J. Yeom · D.-K. Oh (✉)
Department of Bioscience and Biotechnology, Konkuk University, 1 Hwayang-dong, Gwangjin-gu, Seoul 143-701, Republic of Korea
e-mail: deokkun@konkuk.ac.kr

Materials and methods

Reverse transcription-PCR (RT-PCR)

Human brain mRNA was obtained from Clontech Laboratories (Mountain View, CA). Reverse transcription (RT) was performed with an oligo (dT) primer using a RT-PCR kit (Intron Biotechnology, Seongnam, Korea).

Cloning, expression, and purification

The cDNA sequence of human BCO2 was obtained from a cDNA pool in the *Homo sapiens* (GenBank accession number BC041656). The PCR product was subcloned into the pET-28a(+) plasmid vector (Novagen, Darmstadt, Germany) using restriction enzyme *Nde*I and *Hin*dIII and then transformed into *Escherichia coli* ER2566 (New England Biolabs, Hertfordshire, UK). DNA sequencing analysis was performed by Macrogen (Seoul, Korea). The recombinant *E. coli* for protein expression were cultivated in a LB medium at 37°C with 20 μg kanamycin/ml until the OD_{600} reached 0.6. IPTG was then added at 0.1 mM to induce enzyme expression and then the culture was grown at 16°C for 16 h.

The cells were harvested from the culture broth by centrifugation at 6,000×*g* for 20 min at 4°C, washed twice with 0.85% (w/v) NaCl and then resuspended in a lysis buffer (pH 8.0) containing 50 mM NaH_2PO_4 and 300 mM NaCl with 1 mg lysozyme/ml. The resuspended cells were disrupted by a sonicator. The cell debris were removed by centrifugation at 13,000×*g* for 20 min at 4°C and the supernatant was filtered through 0.45 μm filter. All purification steps were carried out in a cold room at 4°C. The filtrate containing applied to a His-Trap HP affinity chromatography column (Amersham Biosciences, Uppsala, Sweden) equilibrated with the lysis buffer containing 10 mM imidazole (pH 8.0). The column was washed extensively with the same buffer, and the bounded protein was eluted with the lysis buffer containing 250 mM imidazole (pH 8.0). The active fraction was collected and dialyzed against 100 mM Tricine/KOH buffer (pH 8.0). After dialysis, the resulting solution was used as the purified enzyme.

Enzyme assay

The reaction solution contained 150 mM NaCl, 10 μM Fe_2SO_4, 5 mM Tris(2-carboxylethyl)phosphine hydrochloride (TCEP), and 1% (w/v) 1-*S*-octyl-*β*-D-thioglucopyranoside (OTG) as described previously (Kim et al. 2008). Unless otherwise stated, retinal production was performed at 37°C for 2 h in 100 mM Tricine/KOH buffer (pH 8.0) containing 300 mg *β*-carotene/l, 2.4% (w/v) Tween 40, and 0.3 U enzyme/ml. One unit (U) of enzymatic activity was defined as the amount of enzyme required to liberate 1 μmol *β*-apo-10′-carotenal per min at 37°C and pH 8.0.

Analytical methods

HPLC analyses of *β*-carotene and *β*-apo-10′-carotenal were performed based on the method of During et al. (1996). 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, substrate and product of the supernatant were analyzed by HPLC using a Zorbaxsil column (Agilent). The column was eluted with hexane/tert-butyl methyl ether (97:3 v/v) at 1 ml/min. Detection was at 460 nm. High resolution liquid chromatography-mass spectrometry (HR-MS) data were obtained using a JMS-SX102A spectrometer (Jeol, Tokyo, Japan) operated at accelerating voltage of 10 kV, ion source temperature of 230°C, ionization energy of 70 eV, and ionization current of 300 μA. The electron impact ionization mass spectra were collected in the positive ion mode.

Results and discussion

Gene cloning and enzyme purification

The *BCO2* gene (1,518 bp) from *H. sapiens,* with the same sequence as that reported in GenBank (accession number BC041656), was cloned and expressed in *E. coli*. The amino acid sequence of BCO2 from humans showed 77, 73, and 72% identity with those of BCO2s from cattle (Berry et al. 2009), mice (Kiefer et al. 2001), and rats (GenBank accession number BC133725), respectively. The crude extract

obtained from harvested cells was purified as a soluble protein by His-Trap HP affinity chromatography. The enzyme was purified 12-fold, a yield of 7.2%, and a specific activity of 0.08 U/mg. The molecular mass of the purified enzyme, determined as a single band by SDS–PAGE, was approx. 58 kDa (data not shown), consistent with the calculated value of 58,159 Da based on the 512 amino acids, including hexa-histidine residues. The native enzyme was estimated to be a tetramer with a molecular mass of approx. 240 kDa determined by gel filtration chromatography based on the masses of reference proteins.

The enzyme reaction product was identified as β-apo-10′-carotenal by HR-MS (data not shown). These results indicated that the purified enzyme had eccentric cleavage activity for the 9′,10′ double bond in β-carotene.

Effects of pH and temperature on enzyme activity

The maximum activity of human BCO2 was observed at pH 8.0 and 37°C (data not shown). The activity of BCO2 from ferrets was also maximal at pH 8.0 and 37°C (Hu et al. 2006). The optimum reaction pH and temperature of BCO1s were 8.0 and 37°C, respectively and were similar to other carotenoid oxygenase family members, from rabbits (Lakshmanan et al. 1972), hogs (Fidge et al. 1969), pigs (Nagao et al. 1996), mice (Redmond et al. 2001), and humans (Lindqvist and Andersson 2002). Thermal inactivation at pH 8.0 followed first-order kinetics with half-lives ($t_{1/2}$) of 326, 247, 191, 84, and 22 min at 35, 40, 45, 50, and 55°C, respectively.

The K_m, k_{cat}, and k_{cat}/K_m values for β-carotene as a substrate at pH 8.0 and 37°C were 67 μM, 0.32 min^{-1}, and 4.7 mM^{-1} min^{-1}, respectively. The previously reported K_m value for β-carotene was 3.5 μM for ferret BCO2 (Hu et al. 2006).

Optimization of organic solvent and detergent, enzyme, and substrate concentrations for β-apo-10′-carotenal production

Various organic solvents and detergents were investigated for the formation of detergent micelles for β-apo-10′-carotenal production (Table 1). Benzene gave the highest level of β-apo-10′-carotenal production. However, because of the toxicity of benzene, toluene, which yielded nearly the same results as benzene, was selected as the optimal solvent. Of the various detergents, Tween 40 gave the highest concentration of β-apo-10′-carotenal and was selected for use in forming detergent micelles. When the concentration of Tween 40 was varied from 0.6 to 6.0% (w/v), the highest concentration of β-apo-10′-carotenal occurred at 2.4% (w/v) (data not shown). These results are similar to those obtained in retinal production by BCO1s (Kim et al. 2007, 2008, 2010).

β-Apo-10′-carotenal production from 300 mg β-carotene/l reached a plateau at about 0.3 U enzyme/ml (Fig. 1a). Thus, 0.3 U/ml was considered the optimum enzyme concentration for β-apo-10-carotenal production. The maximum production of β-apo-10′-carotenal was observed at 300 mg β-carotene/l (Fig. 1b). At higher concentrations, the increased hydrophobicity within the detergent

Table 1 Effect of organic solvent and detergent in the reaction solution containing detergent micelles on β-apo-10-carotenal production

Organic solvent	Relative production (%)	Detergent	Relative production (%)
Toluene	97 ± 1.3	Brij58	34 ± 0.4
Benzene	100 ± 0.8	Tween 20	85 ± 2.2
Chloroform	79 ± 2.9	Tween 40	100 ± 1.7
Dichloromethane	16 ± 0.2	Tween 80	92 ± 1.1
2-Butanone	38 ± 0.9	Span 20	21 ± 0.5
Acetone	19 ± 0.5	Span 80	16 ± 0.8
		Triton-X100	8.7 ± 3.3

The reaction were performed in 100 mM Tricine/KOH buffer (pH 8.0) containing 2.4% (w/v) detergent, 0.3 U enzyme/ml, and 300 mg β-carotene/l at 37°C for 2 h. The relative production of 100% was 8.7 mg β-apo-10-carotenal/l. The data represent the means and standard deviations from three separate experiments

micelles might have prevented contact between the enzyme at the outside surface of the micelle and the β-carotene located in the hydrophobic core (Shikama 2006), thereby decreasing β-apo-10′-carotenal production at β-carotene concentrations above 300 mg/l. The optimal concentrations of β-carotene for retinal production using BCO1s from mice (Kim et al. 2007), humans (Kim et al. 2008), and marine bacteria (Kim et al. 2010) were reported to be 200, 200, and 350 mg/l, respectively.c

β-Apo-10′-carotenal production from β-carotene by human BCO2 under the optimum conditions

Under the optimum conditions, the enzyme produced 43 mg β-apo-10′-carotenal/l in 21 h, with a conversion of 14% (w/w) (Fig. 2). β-Carotene and β-apo-10′-carotenal decomposed over time in the reaction solution because they are unstable. As a control, the β-carotene concentration was measured in the absence of enzyme. The amount of β-carotene lost during the reaction after 21 h was 7 mg/l. The amounts of β-carotene used in Fig. 2 compensated for the loss in the control. The decreased amount in β-carotene was 82 mg/l (152 μM) from the initial 300 mg β-carotene/l by the enzyme after 21 h. Based on the conversion stoichiometry, the estimated amount of β-apo-10′-carotenal was 57 mg/l (152 μM), corresponding to a conversion yield of 19%. Thus, the difference in the amount of β-apo-10′-carotenal as the estimated amount minus the produced amount was 14 mg/l, which was apparently lost during the reaction.

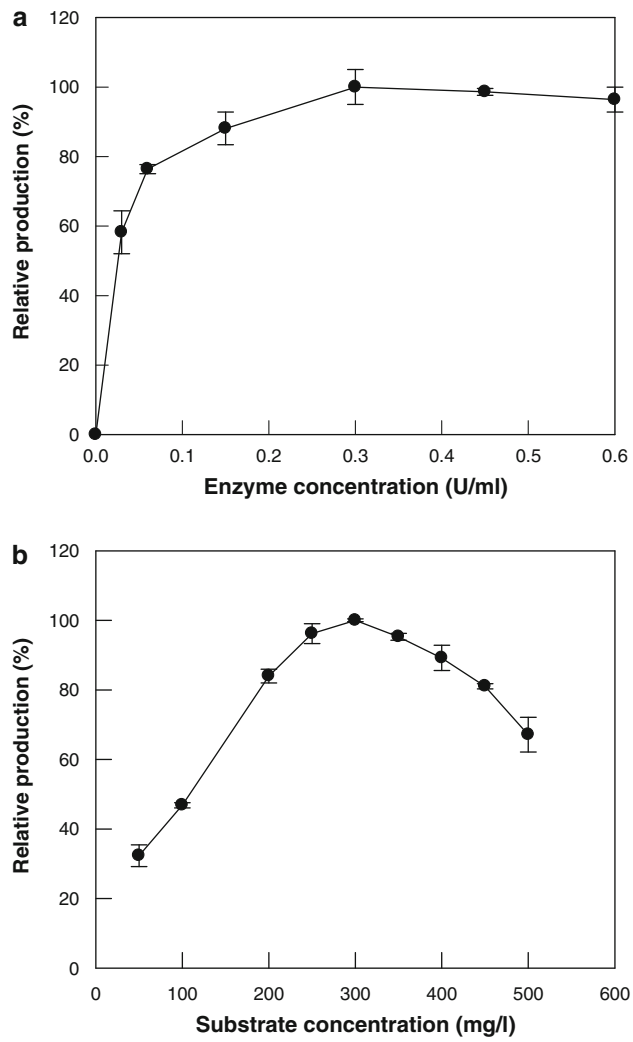

Fig. 1 **a** Effect of enzyme activity on β-apo-10-carotenal production. The reaction were performed in 100 mM Tricine/KOH buffer (pH 8.0) containing 2.4% (w/v) Tween 40, enzyme, and 300 mg β-carotene/l at 37°C for 2 h. The relative production of 100% was 8.5 mg β-apo-10-carotenal/l. **b** Effect of substrate concentration on β-apo-10-carotenal production. The reaction were performed in 100 mM Tricine/KOH buffer (pH 8.0) containing 2.4% (w/v) Tween 40, 0.3 U enzyme/ml, and β-carotene at 37°C for 2 h. The relative production of 100% was 8.5 mg β-apo-10-carotenal/l. The data represent the means of three experiments and the *error bars* represent standard deviation

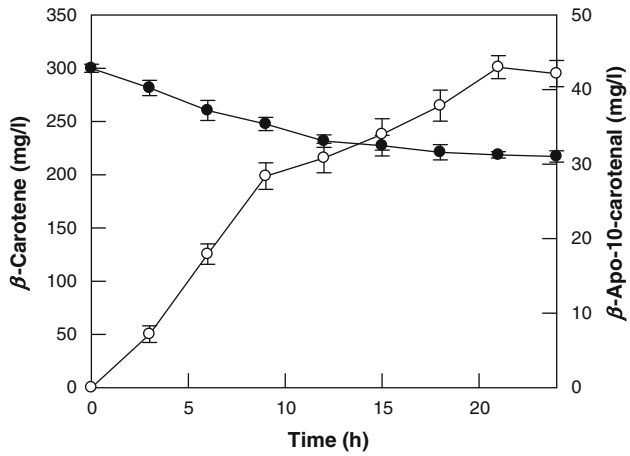

Fig. 2 Time course of β-apo-10-carotenal (*open circle*) production from β-carotene (*filled circle*) under the optimum conditions. The reaction was performed in 100 mM Tricine/KOH buffer (pH 8.0) containing 300 mg β-carotene/l, 2.4% (w/v) Tween 40, and 0.3 U enzyme/ml at 37°C for 24 h. The data represent the means of three experiments and the *error bars* represent standard deviation

In summary, the type of organic solvent and detergent and the concentrations of detergent, substrate, and enzyme in an aqueous solution containing detergent micelles were optimized for β-apo-10′-carotenal production from β-carotene by human BCO2. Under optimum conditions, the enzyme produced 43 mg β-apo-10′-carotenal/l from 300 mg β-carotene/l after 21 h. This is the first report to describe the enzymatic production of β-apo-10′-carotenal.

Acknowledgments This study was supported by a grant (2009-0084490) from the Basic Research Program, Ministry of Education, Science and Technology.

References

Berry SD, Davis SR, Beattie EM, Thomas NL, Burrett AK, Ward HE, Stanfield AM, Biswas M, Ankersmit-Udy AE, Oxley PE, Barnett JL, Pearson JF, van der Does Y, Macgibbon AH, Spelman RJ, Lehnert K, Snell RG (2009) Mutation in bovine beta-carotene oxygenase 2 affects milk color. Genetics 182:923–926

During A, Harrison EH (2004) Intestinal absorption and metabolism of carotenoids: insights from cell culture. Arch Biochem Biophys 430:77–88

During A, Nagao A, Hoshino C, Terao J (1996) Assay of beta-carotene 15,15′-dioxygenase activity by reverse-phase high-pressure liquid chromatography. Anal Biochem 241:199–205

Fidge N, Smith F, Goodman D (1969) Vitamin A and carotenoids. The enzymic conversion of beta-carotene into retinal in hog intestinal mucosa. Biochem J 114:689–694

Hu KQ, Liu C, Ernst H, Krinsky NI, Russell RM, Wang XD (2006) The biochemical characterization of ferret carotene-9′,10′-monooxygenase catalyzing cleavage of carotenoids in vitro and in vivo. J Biol Chem 281:19327–19338

Kiefer C, Hessel S, Lampert JM, Vogt K, Lederer MO, Breithaupt DE, von Lintig J (2001) Identification and characterization of a mammalian enzyme catalyzing the asymmetric oxidative cleavage of provitamin A. J Biol Chem 276:14110–14116

Kim YS, Kim NH, Kim HJ, Lee JK, Kim SW, Oh DK (2007) Effective production of retinal from beta-carotene using recombinant mouse beta-carotene 15,15′-monooxygenase. Appl Microbiol Biotechnol 76:1339–1345

Kim NH, Kim YS, Kim HJ, Oh DK (2008) Optimized formation of detergent micelles of beta-carotene and retinal production using recombinant human beta, beta-carotene 15,15′-monooxygenase. Biotechnol Prog 24:227–231

Kim YS, Kim NH, Yeom SJ, Kim SW, Oh DK (2009) In vitro characterization of a recombinant Blh protein from an uncultured marine bacterium as a beta-carotene 15,15′-dioxygenase. J Biol Chem 284:15781–15793

Kim YS, Park CS, Oh DK (2010) Retinal production from beta-carotene by beta-carotene 15,15′-dioxygenase from an unculturable marine bacterium. Biotechnol Lett

Lakshmanan M, Chansang H, Olson J (1972) Purification and properties of carotene 15,15′-dioxygenase of rabbit intestine. J Lipid Res 13:477–482

Lindqvist A, Andersson S (2002) Biochemical properties of purified recombinant human beta-carotene 15,15′-monooxygenase. J Biol Chem 277:23942–23948

Lindqvist A, He YG, Andersson S (2005) Cell type-specific expression of beta-carotene 9′,10′-monooxygenase in human tissues. J Histochem Cytochem 53:1403–1412

Nagao A, During A, Hoshino C, Terao J, Olson JA (1996) Stoichiometric conversion of all trans-beta-carotene to retinal by pig intestinal extract. Arch Biochem Biophys 328:57–63

Redmond T, Gentleman S, Duncan T, Yu S, Wiggert B, Gantt E, Cunningham FJ (2001) Identification, expression, and substrate specificity of a mammalian beta-carotene 15,15′-dioxygenase. J Biol Chem 276:6560–6565

Scherzinger D, Al-Babili S (2008) In vitro characterization of a carotenoid cleavage dioxygenase from *Nostoc* sp. PCC 7120 reveals a novel cleavage pattern, cytosolic localization and induction by highlight. Mol Microbiol 69: 231–244

Shikama K (2006) Nature of the FeO2 bonding in myoglobin and hemoglobin: a new molecular paradigm. Prog Biophys Mol Biol 91:83–162

Tian R, Pitchford WS, Morris CA, Cullen NG, Bottema CD (2009) Genetic variation in the beta, beta-carotene-9′,10′-dioxygenase gene and association with fat colour in bovine adipose tissue and milk. Anim Genet 41: 253–259