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BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

Effective production of retinal from β-carotene using recombinant mouse β-carotene 15,15′-monooxygenase

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Abstract The gene encoding β-carotene 15,15′-monooxygenase from *Mus musculus* (house mouse), which cleaves β-carotene into two molecules of retinal, was cloned and expressed in *Escherichia coli.* The expressed enzyme was purified by His-tag affinity and resource Q ion exchange chromatography columns to a final specific activity of 0.51 U mg^{-1}. The optimum pH, temperature, substrate and detergent concentrations, and enzyme amount for effective retinal production were determined to be 9.0, 37°C, 200 mg l^{-1} β-carotene, 5% (w/v) Tween 40, and 0.2 U ml^{-1} enzyme, respectively. Under optimum conditions, the recombinant enzyme produced 72 mg l^{-1} retinal in a 15-h reaction time, with a conversion yield of 36% (w/w). The specific activity of the purified enzyme and retinal production obtained in the present study were the highest results ever reported.

Keywords β-Carotene · β-Carotene 15,15′-monooxygenase · Optimization of reaction conditions · Retinal production

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Introduction

β-Carotene 15,15′-monooxygenase (EC 1.14.99.36), formerly known as β-carotene 15,15′-dioxygenase, cleaves β-carotene symmetrically into two molecules of all-*trans*-retinal and is the key enzyme in β-carotene metabolism for vitamin A formation (Nagao and Olson 1994; von Lintig and Wyss 2001). The enzyme was first isolated as β-carotene oxygenase by identifying a product of β-carotene cleavage as retinal (Olson and Hayaishi 1965). The characterization of the enzyme and the reaction mechanism from β-carotene to retinal were also investigated (Goodman et al. 1966, 1967).

β-Carotene 15,15′-monooxygenase activity has been found in mammalian intestinal mucosa and jejunum enterocytes and in the liver, lung, kidney, and brain (Glover 1960; Wolf 1995; During et al. 1996). Molecular cloning, expression, and characterization of the enzyme have been reported from various species, including *Drosophila melanogaster* (von Lintig and Vogt 2000), chickens (Wyss et al. 2000), mice (Paik et al. 2001; Redmond et al. 2001; Boulanger et al. 2003; Poliakov et al. 2005), humans (Yan et al. 2001), and zebrafish (Lampert et al. 2003). For mouse β-carotene 15,15′-monooxygenase, the K_m and V_{max} values were reported to be 1.2 μM and 104.8 pmol μg^{-1} h^{-1}, respectively, by Poliakov et al. 2005 and 6 μM and 36 pmol μg^{-1} h^{-1}, respectively, by Redmond et al. 2001. The molecular weight of the mouse enzyme is 63 kDa (Redmond et al. 2001). The association state of subunits in the native enzyme, a tetramer, has only been reported for the human enzyme (Lindqvist and Andersson 2002).

The activity of β-carotene 15,15′-monooxygenase has been assayed using β-carotene detergent micelles under the aqueous condition. To make β-carotene detergent micelles, the β-carotene was dispersed in a solvent such as acetone

and hexane and then formed micelles with a detergent such as Tween 20, Tween 80, and 1-*S*-octyl-β-D-thioglucopyranoside (OTG; Devery and Milborrow 1994; During et al. 1996; Paik et al. 2001; Lindqvist and Andersson 2002). The biotransformation of β-carotene to retinal has been performed using small amount of β-carotene because the biotransformation was focused on enzyme characterization rather than retinal production (Devery and Milborrow 1994; During et al. 1996; Paik et al. 2001; Lindqvist and Andersson 2002). As a result, the highest previously reported concentration of retinal was not high as 0.12 mg l^{-1} (Paik et al. 2001).

In this study, the gene encoding a mouse β-carotene 15,15′-monooxygenase was cloned and expressed in *Escherichia coli*. We investigated optimum conditions, such as pH, temperature, detergent and substrate concentrations, and enzyme amount for effective retinal production. High production of retinal was achieved under the established conditions.

Materials and methods

Reverse transcription PCR

The mouse liver messenger ribonucleic acid (mRNA) was obtained from Clontech Laboratories (Mountain View, CA). Reverse transcription (RT) of mouse liver mRNA was performed using an oligo(dT) primer using RT polymerase chain reaction (PCR) kit (Intron Biotechnology, Seongnam, Korea) according to the manufacturer's protocol, and then complementary deoxyribonucleic acid (cDNA) was made.

Cloning and DNA sequence analysis

Based on the cDNA sequence of the β-carotene 15,15′-monooxygenase from *Mus musculus* reported in GenBank (accession number NM 021486), the forward primer 5′-TT*GAATTC*ATGGAGATAATATTTGGCCAGAATAAG-3′ (base pair 179–205) and reverse primer 5′-TT*CTCGAG*AAGACTTGAGCCACCATGAC-3′ (base pair 1876–1857) were designed as primers to introduce the underlined *Eco*RI and *Xho*I restriction sites, respectively, and were synthesized by Bioneer (Daejon, Korea). The β-carotene 15,15′-monooxygenase-coding cDNA (1,698 bp) was amplified and obtained from a liver cDNA pool by PCR using the primers. The PCR product was subcloned into the pET-24a (+) plasmid vector (Novagen, Darmstadt, Germany) using the restriction enzymes of *Eco*RI and *Xho*I and then transformed into *E. coli* ER2566 (New England Biolabs, Hertfordshire, UK). The pET-24a(+) plasmid vector having T7 promotor is the powerful system in *E. coli*, and it contained six His-tag coding sequence at the C terminus. DNA sequencing was performed using the facility of Macrogen (Seoul, Korea).

Expression and purification

The recombinant *E. coli* for protein expression was cultivated with shaking of 200 rpm in a 2,000-ml flask containing 500 ml of Luria–Bertani (LB) medium with 20 μg ml^{-1} kanamycin at 37°C. When the absorbance of bacteria reached 0.6 at 600 nm, isopropyl-D-thiogalactopyranoside was added at a final concentration of 0.1 mM, and the recombinant protein was induced and expressed at 16°C and 150 rpm for 16 h. The cells were harvested from culture broth by centrifugation at 6,000×*g* for 20 min at 4°C, washed twice with 0.85% NaCl, and then resuspended in lysis buffer (pH 8.0) containing 50 mM NaH_2PO_4, 300 mM NaCl, and 1 mg ml^{-1} lysozyme. The resuspended cells were disrupted by a sonication. The cell debris was removed by centrifugation at 13,000×*g* for 20 min at 4°C, the supernatant was filtered through a 0.45-μm filter, and the filtrate was used as a crude extract. All purification steps were carried out in a cold room at 4°C with a fast protein liquid chromatography system (Bio-Rad Laboratories, Hercules, CA). The crude extract applied to a His-Trap HP affinity chromatography column (Amersham Biosciences, Uppsala, Sweden) equilibrated with 50 mM sodium phosphate buffer containing 300 mM NaCl and 10 mM imidazole (pH 8.0). 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 with a flow rate of 1 ml min^{-1}. The active fractions were collected, and dialysis was performed with 50 mM Tris–HCl buffer (pH 8.0). After dialysis, the resulting sample was loaded onto a Resource Q ion exchange column (Amersham Biosciences), previously equilibrated with 50 mM Tris–HCl buffer (pH 8.0). The column washed extensively with the same buffer, and the bound protein was eluted with linear gradient between 0 to 500 mM NaCl with a flow rate of 1 ml min^{-1}. After dialysis against 50 mM boric acid buffer (pH 9.0), the obtained solution was used as a purified enzyme.

Enzyme assay

β-Carotene (800 mg l^{-1}) was added to 20% (w/v) Tween 40, dissolved in chloroform. The mixture solution was homogenized with 10,000 rpm at 10 s using homogenizer (IKA, Kuala Lumpur, Malaysia). The chloroform was evaporated by N_2 gas, and then water was added. The resulting solution was used as the substrate solution. The enzyme solution was prepared in 66.7 mM boric acid buffer (pH 9.0) containing 166.7 mM sodium chloride, 13.3 μM Fe_2SO_4, 6.7 mM Tris(2-carboxylethyl)phosphine hydrochloride, and 1.3% (w/v) OTG. The enzyme solution and substrate solution were mixed with the ratio of 3: 1 (v/v), and then the mixture was used as the reaction solution. The reaction was performed with the reaction solution at 37°C

for 3 or 15 h. 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). One unit of the enzyme activity was defined as the amount of enzyme required to liberate 1 μmol retinal per min at 37°C and pH 9.0.

Optimization of reaction conditions for retinal production by β-carotene 15,15′-monooxygenase

To find the optimum reaction pH and temperature for the activity of β-carotene 15,15′-monooxygenase, pH was varied from 7.5 to 10.0 using 50 mM Tricine–HCl buffer (pH 7.5−8.5) and 50 mM boric acid buffer (pH 8.0−10.0) at 37°C, and temperature was varied from 25 to 45°C at pH 9.0. The reactions were performed with 200 mg l^{-1} β-carotene, 5% (w/v) Tween 40, and 0.01 U ml^{-1} enzyme for 3 h.

The effect of detergent concentration in the reaction solution on retinal production was investigated by varying Tween 40 concentration from 3 to 7% (w/v). The reactions were performed with 200 mg l^{-1} β-carotene and 0.01 U ml^{-1} enzyme at 37°C for 15 h. To determine optimum substrate and detergent concentrations in the reaction solution, the concentration of β-carotene was varied from 100 to 300 mg l^{-1} with the constant concentration of 5% (w/v) Tween 40. Another set experiment was used with the constant ratio of β-carotene to Tween 40 such as 100 mg l^{-1} and 2.5% (w/v), 150 mg l^{-1} and 3.75%, 200 mg l^{-1} and 5%, 250 mg l^{-1} and 6.25%, and 300 mg l^{-1} and 7.5%, respectively. The reactions were performed in 50 mM boric acid buffer (pH 9.0) containing 0.01 U ml^{-1} enzyme at 37°C for 15 h.

The effect of enzyme amount on retinal production was tested at concentrations of 0.005, 0.01, 0.03, 0.05, 0.10, 0.15, 0.20, and 0.25 U ml^{-1} enzyme. The reactions were performed in 50 mM boric acid buffer (pH 9.0) containing 200 mg l^{-1} β-carotene and 5% (w/v) Tween 40 at 37°C for 15 h.

The time course of retinal production was performed in 50 mM boric acid buffer (pH 9.0) containing 200 mg l^{-1} β-carotene, 5% (w/v) Tween 40, and 0.20 U ml^{-1} enzyme at 37°C for 30 h. The reaction mixtures were sampled, and the concentrations of β-carotene and retinal were determined.

HPLC analyses of β-carotene and retinal

High-performance liquid chromatography (HPLC) analyses of β-carotene and retinal were performed based on the previously reported method (During et al. 1996). The same volume of acetonitrile was added to the reaction solution. The 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 system (Agilent 1200, Santa Clara, CA) equipped with a UV detector. Retinal was determined at 370 nm using a YMC-PAC ODS-A column (250×4.6 mm, YMC, Kyoto, Japan). The column was eluted with the mixture of acetonitrile and water with 90:10 (v/v) as the mobile phase with a flow rate of 1 ml min^{-1}. β-Carotene was determined at 460 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^{-1}.

Results

Cloning, expression, and purification of β-carotene 15, 15'-monooxygenase from *M. musculus*

The β-carotene 15,15′-monooxygenase gene (1,698 bp) from *M. musculus* (house mouse), with the same sequence as that reported in GenBank (accession number NM 021486), was cloned and expressed in *E. coli*.

The crude extract obtained from harvested cells as a soluble protein was purified by His-Trap HP affinity and Resource Q ion exchange columns (Table 1). The β-carotene 15,15′-monooxygenase was purified with a final purification of 30-fold, a yield of 25%, and a specific activity of 0.51 U mg^{-1} (μmol mg^{-1} min^{-1}). The molecular mass of the expressed protein analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis was about 65 kDa (Fig. 1), consistent with the calculated value of 64,686 Da based on the 572-residue amino acid structure plus a hexa-histidine tag at the carboxyl terminus.

Effects of pH and temperature on the activity of β-carotene 15,15′-monooxygenase

The enzyme activity of β-carotene 15,15′-monooxygenase, converting β-carotene to retinal, was examined at pH ranges of 7.5 to 10.0. Enzyme activity was maximal at pH 9.0. At pH 8.5 and 10.0, the activity was approximately 70% of the maximum. The assay for mouse β-carotene 15,15′-monooxygenase was performed at pH 8.0 using Tricine–HCl buffer (Paik et al. 2001; Redmond et al. 2001;

Table 1 Purification of β-carotene 15,15′-monooxygenase

Step	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Yield (%)	Purification (fold)
Crude extract	254.4	4.22	0.02	100	1
His-Trap HP	12.2	1.89	0.16	45	9
Resource Q	2.1	1.06	0.51	25	30

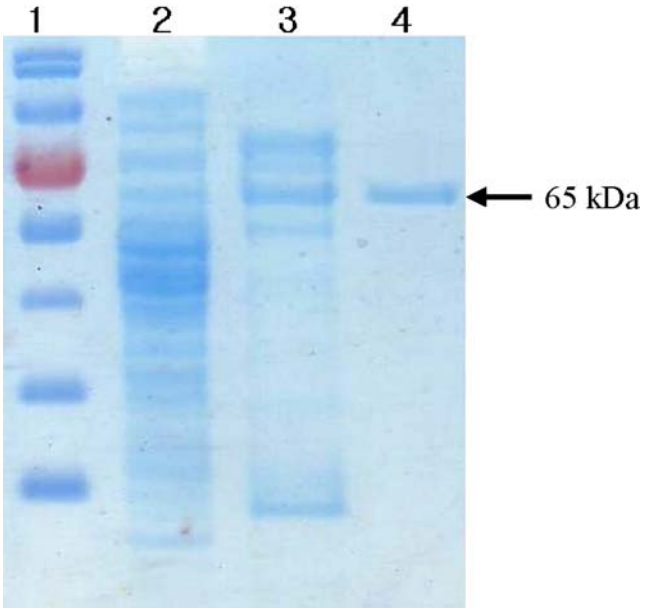

Fig. 1 SDS-PAGE analysis of β-carotene 15,15′-monooxygenase from each purification step. *Lane 1*, prestained marker proteins (170, 130, 100, 72, 55, 40, 33, and 24 kDa); *lane 2*, crude extract; *lane 3*, His-Trap HP column product; *lane 4*, Resource Q column product (purified enzyme)

Poliakov et al. 2005). However, the activity of the mouse enzyme at varying pH has not been described.

The effect of temperature on enzyme activity was investigated, and maximum activity was recorded at 37°C. Above this temperature, activity decreased significantly, being only 14% of the maximum at 45°C. Below 37°C, enzyme activity decreased with decreasing temperature and was only 22% of the maximum at 25°C. The optimum pH and temperature of most β-carotene 15,15′-monooxygenases are 37°C and around 8.0, respectively (Goodman et al. 1967; Fidge et al. 1969; Lakshmanan et al. 1972; Singh and Cama 1974; Nagao et al. 1996; Paik et al. 2001; Lindqvist and Andersson 2002), except the rabbit enzyme of 45°C (Ershov et al. 1993).

Effect of detergent and substrate concentrations on retinal production by β-carotene 15,15'-monooxygenase

As β-carotene is hydrophobic (During and Harrison 2004), detergents like Tween 40 are used to help dissolve it (Wyss et al. 2001). The Tween 40 concentration in the reaction mixture was varied from 3 to 7% (w/v) at 200 mg l^{-1} β-carotene (Fig. 2a), and the optimum concentration for retinal production was found to be 5% (w/v) at the end of the reaction of 15 h. The relative retinal production was defined as the value relative to the maximum retinal production.

The concentration of the β-carotene substrate was varied over the range of 100 to 300 mg l^{-1}, with a constant detergent concentration of 5% Tween 40 and a constant ratio of substrate to detergent of 40 mg l^{-1} β-carotene per 5% Tween 40 for 15 h as the end of the reaction (Fig. 2b). The relative conversion yield in 100 mg l^{-1} β-carotene is approximately 20% higher than in 200 mg l^{-1}. The optimum substrate and detergent concentrations for retinal production were 200 mg l^{-1} β-carotene and 5% Tween 40, respectively.

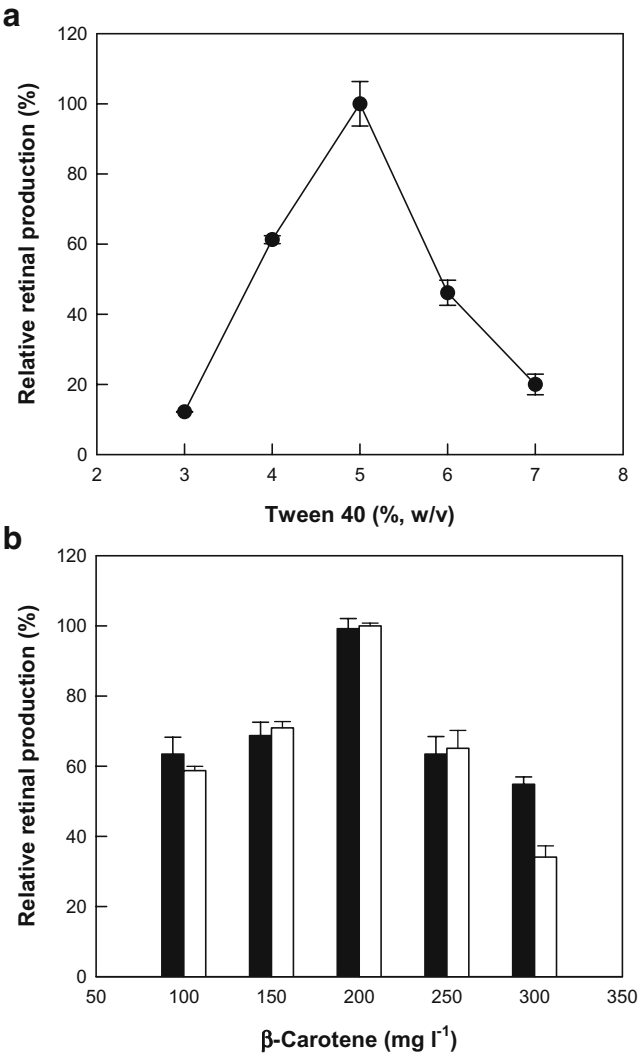

Fig. 2 Effects of substrate and detergent concentrations the reaction solution on retinal production. Data represent the means of three experiments, and *error bars* represent standard deviation. **a** Effect of detergent concentration. The reactions were performed in 50 mM boric acid buffer (pH 9.0) containing 200 mg l^{-1} β-carotene, Tween 40, and 0.01 U ml^{-1} enzyme at 37°C for 15 h. **b** Effects of substrate concentration with constant concentration of 5% (w/v) Tween 40 (*shaded bars*) and with the constant ratio of β-carotene to Tween 40 with 40 mg l^{-1} of β-carotene per 1% Tween 40 (*unshaded bars*). The reactions were performed in 50 mM boric acid buffer (pH 9.0) containing 0.01 U ml^{-1} enzyme, β-carotene, and Tween 40 at 37°C for 15 h. The used β-carotene and Tween 40 concentrations with the constant ratio were 100 mg l^{-1} and 2.5% (w/v), 150 mg l^{-1} and 3.75%, 200 mg l^{-1} and 5%, 250 mg l^{-1} and 6.25%, and 300 mg l^{-1} and 7.5%, respectively. Data represent the means of three experiments, and *error bars* represent standard deviation

Effect of enzyme amount on retinal production by β-carotene 15,15'-monooxygenase

Enzyme activity for retinal production was tested at various enzyme concentrations with 200 mg l^{-1} β-carotene and 5% Tween 40 at 37°C and pH 9.0 for 15 h (Fig. 3). Below 0.20 U ml^{-1} enzyme, retinal production increased with increasing enzyme amount, while retinal production reached a plateau above 0.20 U ml^{-1} enzyme. Thus, 0.20 U ml^{-1} enzyme showed maximum retinal production. This pattern may be due to the interfacial saturation at a high enzyme concentration in the detergent-micelles system (Al-Zuhair et al. 2004).

Retinal production by β-carotene 15,15'-monooxygenase from β-carotene under optimal conditions

Optimum conditions for retinal production were determined to be pH 9.0, 37°C, 200 mg l^{-1} β-carotene, 5% Tween 40, and 0.20 U ml^{-1} enzyme. A time course was determined for the conversion of β-carotene to retinal using the enzyme under these conditions (Fig. 4). With increasing time, the concentration of retinal increased because of the cleavage of β-carotene by the enzyme. After 15 h, retinal production reached a maximum (72 mg l^{-1}), with a conversion yield of 36%.

Discussion

Virus- (Yan et al. 2001; Lindqvist and Andersson 2002) and cell-based systems (Wyss et al. 2000; Paik et al. 2001; During and Harrison 2004) have been used mainly as expression hosts of β-carotene 15,15′-monooxygenase. In the present study, we successfully expressed the mouse β-carotene 15,15′-monooxygenase in *E. coli* ER2566 as a soluble protein using the pET-24a(+) plasmid vector (Fig. 1). The use of *E. coli* as a host for the expression of β-carotene 15,15′-monooxygenase increases the utility of retinal production because a recombinant protein production system in *E. coli* has commercial advantages, such as the use of inexpensive culture media and ready achievement of high cell concentrations, vs virus- and cell-based systems.

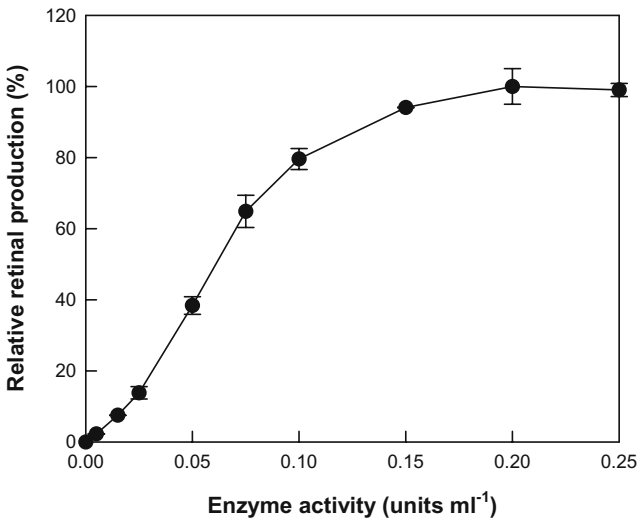

Fig. 3 Effect of enzyme activity on retinal production. The reactions were performed in 50 mM boric acid buffer (pH 9.0) containing 200 mg l^{-1} β-carotene, 5% Tween 40, and enzyme at 37°C for 15 h. Data represent the means of three experiments, and *error bars* represent standard deviation

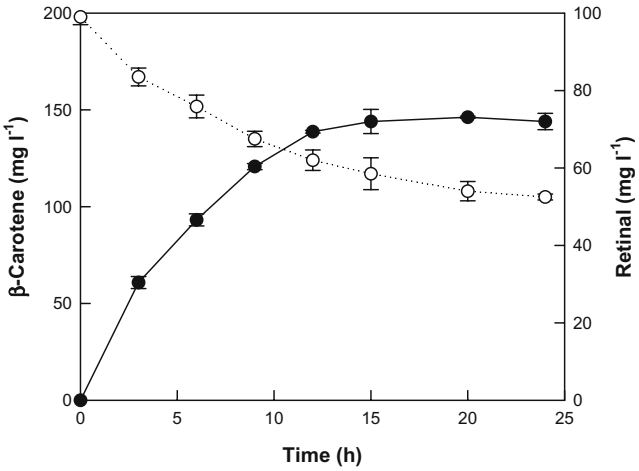

Fig. 4 Time course of retinal production (*filled circles*) from β-carotene (*open circles*) under optimal conditions. The reaction was performed in 50 mM boric acid buffer (pH 9.0) containing 200 mg l^{-1} β-carotene, 5% (w/v) Tween 40, and 0.20 U ml^{-1} enzyme at 37°C for 30 h. Data represent the means of three experiments, and *error bars* represent standard deviation

The recombinant β-carotene 15,15′-monooxygenase was purified to apparent homogeneity. The purified enzyme exhibited a specific activity of 510 nmol mg^{-1} min^{-1} (Table 1). The highest previously reported specific activity was 10 nmol mg^{-1} min^{-1} for the human enzyme expressed in insect cells (Lindqvist and Andersson 2002). The specific activity in the present study was 51-fold higher than that of the human enzyme and 15,300-fold higher than that of the partially purified native enzyme from chicken (Wyss et al. 2001). When OTG and hexane, which were applied in a study of the human enzyme (Lindqvist and Andersson 2002), were used as the detergent and solvent to dissolve the substrate, the mouse enzyme-specific activity decreased from 510 to 25 nmol mg^{-1} min^{-1}. Thus, the higher specific activity of the mouse enzyme was primarily due to the superior detergent and solvent system in the present study and partly due to the higher purity of the enzyme. As β-carotene micelles dissolved in solvent are evaporated to perform the enzyme reaction in an aqueous system, the solubility of hydrophobic substrates like β-carotene is important in the formation of stable micelles. After evaporation, the substrate solution with Tween 40 and

chloroform was maintained in a liquid state, whereas that using OTG and hexane changed to the solid state because of differences in solubility. According to the Merck Index, β-carotene's solubility is 10 mg ml^{-1} in chloroform and 1.1 mg ml^{-1} in hexane. The increased retinal productivity in the present detergent system may have been due to the higher solubility of β-carotene in chloroform than in hexane.

β-Carotene 15,15′-monooxygenase worked normally in emulsions but did not work in the water solution because of the hydrophobicity of β-carotene. The enzyme at the outside surface on Tween 40 micelles worked with the nonpolar substrate β-carotene located in the hydrophobic core of the micelles, resulting to the production of a polar retinal. Retinal was released from the micelles into the water solution (El-Gorab 1973).

Mouse β-carotene 15,15′-monooxygenase expressed in Chinese hamster ovary cells produced 0.12 mg l^{-1} of retinal over a period of 1 h (Paik et al. 2001), the highest concentration of retinal previously reported. The concentration of retinal in the present study under optimum conditions was approximately 600-fold higher than that in the study of Paik et al. (2001). β-Carotene and retinal of the reaction solution were decomposed with progressing time. As a control, the concentration of β-carotene was determined in the same reaction solution without the enzyme. The data of β-carotene used in Fig. 4 were compensated for the control. When the retinal solution without enzyme was incubated under the same reaction conditions, its concentration was reduced to 75% after 10 h and 50% after 24 h regardless of retinal concentration. Based on stoichiometry for the conversion of β-carotene into retinal, the final retinal concentration, calculated from the consumed β-carotene of 95 mg l^{-1} (177 mM), was estimated to be 100 mg l^{-1}, corresponding to 50% conversion yield. Retinal of 28 mg l^{-1} was seemed to be losing during the reaction time.

Compared to previous reports, our study demonstrates several novel aspects in retinal production. While expression of active β-carotene 15,15′-monooxygenase in bacteria has been reported to be problematic (Wyss et al. 2001; Lindqvist and Andersson 2002), we successfully used an *E. coli* system to express the active enzyme. Moreover, the purified enzyme showed the highest reported specific activity. This is the first report focusing on the industrial enzymatic production of retinal by optimizing reaction conditions.

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