Biochemical Properties of Purified Recombinant Human β-Carotene 15,15′-Monooxygenase*

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β-Carotene 15,15′-monooxygenase (BCO), formerly known as β-carotene 15,15′-dioxygenase, catalyzes the first step in the synthesis of vitamin A from dietary carotenoids. We have biochemically and enzymologically characterized the purified recombinant human BCO enzyme. A highly active BCO enzyme was expressed and purified to homogeneity from baculovirus- infected Spodoptera frugiperda 9 insect cells. The $K_m$ and $V_{max}$ of the enzyme for β-carotene were 7 μM and 10 nmol retinal/mg × min, respectively, values that corresponded to a turnover number ($k_{cat}$) of 0.66 min$^{-1}$ and a catalytic efficiency ($k_{cat}/K_m$) of $\sim 10^9$ M$^{-1}$ min$^{-1}$. The enzyme existed as a tetramer in solution, and substrate specificity analyses suggested that at least one unsubstituted β-ionone ring half-site was imperative for efficient cleavage of the carbon 15,15′-double bond in carotenoid substrates. High levels of BCO mRNA were observed along the whole intestinal tract, in the liver, and in the kidney, whereas lower levels were present in the prostate, testis, ovary, and skeletal muscle. The current data suggest that the human BCO enzyme may, in addition to its well-established role in the digestive system, also play a role in peripheral vitamin A synthesis from plasma-borne provitamin A carotenoids.

Retinol, also referred to as vitamin A, is a fat-soluble polysaturated essential for tissue development, growth, and vision. Retinol can be either ingested or synthesized within the body from dietary carotenoids. Preformed retinol is found almost exclusively in animals in the form of esters of fatty acids. It is hydrolyzed during the process of digestion, absorbed in the free form, re-esterified with fatty acids within the intestinal mucosa, and transported to the liver via the lymphatic route associated with chylomicrons (1). The major substrate for the in vivo synthesis of retinol is the plant carotenoid β-carotene. Of the more than 600 different carotenoids isolated from nature, ~50 possess biological activity; hence, these compounds are termed provitamin A carotenoids (2–4).

In vivo studies in humans show that the majority of the ingested β-carotene is cleaved at the central carbon 15,15′-double bond to form two molecules of retinal (retinaldehyde) (5, 6), and consequently, the enzyme that catalyzes this first step in vitamin A synthesis in the intestinal mucosa was named β-carotene 15,15′-dioxygenase (BCO) (7). Although the enzyme activity was first described in the mid-1950s, it was not until 1965 that the research groups of Goodman (8) and Olson (7) independently demonstrated that the cleavage of β-carotene to retinal could be studied in vitro by using soluble enzyme preparations from the intestine and liver. Characterization of the native enzyme was performed with a 100,000 × g supernatant fraction or an ammonium sulfate precipitate thereof. It was shown that the reaction is dependent on molecular oxygen and that nicotinamide dinucleotide cofactors are not required for catalysis. Also, the facts that the reaction was inhibited by the addition of iron chelating agents and that cyanide, which inhibits ferric protoporphyrin enzymes, did not attenuate the conversion of β-carotene to retinaldehyde suggested that BCO was a nonheme iron-containing enzyme. Furthermore, the native enzyme had the ability to cleave the 15,15′-double bond of a variety of carotenoids other than β-carotene, including α-carotene, β-apocarotenol, and β-apocarotenoids (9–11). The Michaelis-Menten constant ($K_m$) for the native enzyme of a variety of animal species relative to β-carotene has been determined to be in the 1–10 μM range. The pH optimum for the reaction was in the slightly alkaline range, and the enzyme was inhibited by sulfhydryl alkylating reagents such as p-chloromercuribenzoate and N-ethylmaleimide. BCO was either protected or activated by sulfhydryl reducing agents such as cysteine and glutathione (7, 10–13).

The human BCO cDNA encodes a hydrophilic protein of 547 amino acids with a predicted molecular weight of 62,637 (14). Amino acid comparison of the human BCO with the mouse (15), rat (GenBank™ accession number NM_053648), chicken (16), zebrafish (GenBank™ accession number AJ290390), and Dro sophila (17) enzymes show sequence identities of 85, 84, 67, 56, and 22%, respectively. Based on biochemical and amino acid sequence data, it has been proposed that BCO belongs to the nonheme iron-containing dioxygenase family. However, recently it was demonstrated that the reaction mechanism of enzymatic cleavage of the central carbon 15,15′-double bond in β-carotene involves a monooxygenase-type mechanism (18). By using a partially purified chicken intestine BCO preparation and isomerically pure α-carotene as substrate, it was shown that both $^{17}$O$_2$ and H$_2^{18}$O were incorporated into the two retinal products. Thus, the BCO enzyme was renamed β-carotene 15,15′-monooxygenase (19). The human BCO gene structure and chromosomal localization were also recently reported (14). However, no biochemical characterization of a purified human enzyme has been described.

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In the current study, we describe a detailed biochemical and enzymological characterization of the purified recombinant human BCO enzyme. Furthermore, a comprehensive analysis of tissue-specific expression is described.

**EXPERIMENTAL PROCEDURES**

**Materials—**All-trans-β-carotene, lycopene, all-trans-retinol, all-trans-retinoic acid, o-phenanthroline, N-ethylmaleimide, and p-chloromercuribenzoate were purchased from Sigma. Zeaxanthin and β-cryptoxanthin were obtained from Indofine (Somerville, NJ). a,β-Bipyridyl was from Aldrich. Aconitase was HPLC grade (Merck).

**Insect Cell Culture, Expression, and Purification of Recombinant Human BCO Protein—**Spodoptera frugiperda cells were maintained in monolayer cultures with Sf-900 II SFM (catalog number 10902-096; Invitrogen), or Grace's insect medium (catalog number 11605-094; Invitrogen) supplemented with 10% fetal calf serum. Using the Bac-To-Bac® baculovirus expression system (Invitrogen), and the pFastBacI-hBCO-His plasmid (above), recombinant baculovirus was constructed by transfection of the pBluescript-hBCO vector, resulting in plasmid (20). The complete coding sequence of human BCO cDNA was obtained from a liver DNA pool (CLONTECH Laboratories, Inc., Palo Alto, CA) by PCR using the following primers: hBCO2 (sense) 5'-AAAATGATCATCCTCCGACCGAGCC-3' and BacoCOr (antisense) 5'-CG-GAATTTCTCAGTGCAAGGACCGCTGG-3', corresponding to nucleotides 191–211 and 1863–1842, respectively, in the human cDNA sequence (GenBank™ accession number AK001592). The PCR product was then subcloned into the pCR2.1-TOPO vector (Invitrogen) using the extra deoxyadenosine added by the Taq polymerase and then subcloned in the pCMV-Sport2.0 vector (Invitrogen) using EcoRI.

To obtain pure recombinant human BCO enzyme, the hBCO enzyme amount varied between 60 and 1000 µg from 100 ml of insect cell culture media, which were established and screened by an enzyme-linked immunosorbent assay using the synthetic peptide as antigen as described previously (23). Hybridomas were established and screened by an enzyme-linked immunosorbent assay using the synthetic peptide as antigen as described previously (23). The anti-BCO monoclonal antibody, designated mAb-1-11, was determined to belong to the IgG1/κ subclass as revealed by an enzyme-linked immunosorbent assay-based mouse typer substrisotyping kit (Bio-Rad).

**Gel Electrophoresis, Immunoblotting, and Protein Quantitation—**SDS-polyacrylamide electrophoresis was performed according to the method of Laemmli (24), and the proteins were either detected in the gel with Coomassie Brilliant Blue R or with silver staining using a Silver Stain Plus kit (Bio-Rad). Alternatively, the proteins were transferred to a nitrocellulose membrane and detected with a mouse monoclonal antibody (mAb-1-11) using an ECL kit (Amersham Biosciences). The protein concentrations were determined using a Coomassie Plus protein assay reagent (Pierce).

**Gel Filtration Chromatography—**The purified enzyme (50 µg) was applied on a Sphacry S-300 (Amersham Biosciences) column (7 x 300 mm) equilibrated with 100 mM Tricine-KOH buffer (pH 8.0), containing 125 mM NaCl, 10 µM FeSO₄, 5 mM TCEP, and 1% (w/v) OTG with a flow rate of 17.5 ml/h, and 0.3-ml fractions were collected. 75 µl of each fraction was used directly in BCO enzyme assays, and 7.5 µl of each fraction was analyzed by immunoblotting. The columns were calibrated with proteins of known molecular mass: albumin (67 kDa), aldolase (158 kDa), catalase (232 kDa), and blue dextran (2 x 10⁶ Da).

**Immunocytochemistry—**The human hepatocellular carcinoma cell line, HepG2 (ATCC HB-8065), maintained in Dulbecco's modified Eagle's medium (catalog number 11995-065; Invitrogen) supplemented with 10% fetal calf serum, 10 mM HEPES, and 1% penicillin/streptomycin was cultured on 12-mm diameter microscope glass covers. The cells were transiently transfected with pCMV-hBCO using Fugene 6 (Roche Diagnostics Corporation, Indianapolis, IN) according to the manufacturer's instructions. 4 h after transfection, the cells were washed with cold PBS (10 ml) and fixed with cold 4% (w/v) paraformaldehyde in 2-ml Eppendorf tubes, and the solvent was evaporated. The assay buffer and enzyme, in some cases preincubated with various potentially inhibitory compounds for 30 min on ice, were then added to the carotenoid/detergent mix, and the tubes were incubated for 5 min at 37 °C with gentle agitation (70 rpm). HPLC analyses of reaction products were performed essentially as described by Durig et al. (21). Briefly, 25 µl of 37% (v/v) formaldehyde was added, and the incubation was continued for 10 min at 37 °C. For extraction of the products, 250 µl of acetonitrile was added, and the tube was vortexed and kept on ice for 5 min. After centrifugation for 10 min at 10,000 × g at 4 °C, the supernatant was separated on a 4.6 x 150-mm Phenomenex LUNA 3 µm C18 reversed-phase column (Waters, Milford, MA) in a mobile phase consisting either of 90% acetonitrile, 10% water, 0.1% (w/v) ammonium acetate or of 85% acetonitrile, 15% water, 0.1% (w/v) ammonium acetate with a flow rate of 1 ml/min (Waters 501 HPLC pump) and UV detection (Waters 484 tunable absorbance detector) at 380 nm. The enzyme kinetics were calculated using GraFit Version 5.0.1 (Erithacus Software Limited, Horley, UK). This program fits the data to the Michaelis-Menten equation using nonlinear regression analysis. For spectral scans of the products, the 4.6 x 150-mm XTerra MS C18 3.5 µm column was used under the same conditions as above, but with a LC-10AT liquid chromatograph, SPD-M10A VP detector array, and Class-VP chromatograph data system, version 4.2 (Shimadzu, Columbia, MD).

Retinal formed during the reactions was quantified from its peak height by using a standard curve obtained by incubating 1-50 pmol of all-trans-retinal in a 100 mM Tricine-KOH (pH 8.0) buffer, containing 125 mM NaCl, 10 µM FeSO₄, 5 mM TCEP, 1% (w/v) OTG, and 250 ng of heat-inactivated BCO for 15 min at 37 °C with gentle agitation (70 rpm). The products were then treated with the same wash/elution sequence as above to BCO assay and were separated on a 4.6 x 150-mm Phenomenex LUNA 3 µm C18 column in a mobile phase consisting of 90% acetonitrile, 10% water, and 0.1% (w/v) ammonium acetate.

**Monoclonal Antibody Production—**The synthetic peptide [CRN-RKQELPVRAVTKG, corresponding to amino acid residues 7–23 in human BCO, was coupled to keyhole limpet hemocyanin using m-maleimidebenzoyl-N-hydroxysuccinimide ester (Pierce) (22), and used for immunization of mice as described previously (23). Hybridomas were established and screened by an enzyme-linked immunosorbent assay using the synthetic peptide as antigen as described previously (23). The anti-BCO monoclonal antibody, designated mAb-1-11, was determined to belong to the IgG1/κ subclass as revealed by an enzyme-linked immunosorbent assay-based mouse typer substrisotyping kit (Bio-Rad).

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The purified BCO protein was expressed in a FastBac vector with a hexahistidine tag. To further examine the purified protein, a human digestive system 12-lane MTN was exposed at room temperature for 1 h. RNA Blotting—A human digestive system 12-lane MTN blot (catalog number 7782-1) was obtained by PCR and radiolabeled with [α-32P]dCTP (Amersham Biosciences) using a Rediprime II kit (Amersham Biosciences). ExpressHyb hybridization solution (CLONTECH) was used for 2 h before hybridization at 68 °C and for overnight hybridization at 68 °C with 2× 10^6 cpm probe/ml. Washing was performed according to the blot manufacturer. Autoradiography was carried out at −80 °C with Hyperfilm MP (Amersham Biosciences) and Quantas Rapid intensifying screens (DuPont) for 3 days. The same filters were subsequently hybridized with a human β-actin probe and exposed at room temperature for 1 h.

RESULTS
Identification and Analysis of Human BCO cDNA and Protein—Using the Drosophila BCO as query sequence (17), we performed BLAST searches of the GenBank data base and identified a human protein (GenBank accession number AK001592) with 22% sequence identity to the Drosophila protein. To investigate whether this was the human BCO homologue, we isolated the corresponding cDNA from human liver cDNA by the PCR. DNA sequencing of multiple independently amplified cDNAs revealed an aspartate instead of a glycine in position 302 of the BCO protein. These results were in concordance with the human genomic BCO sequences present in the Celera data base, and with the recently published gene sequence (GenBank accession number NP_059125) (14). The cDNA was subcloned into a FastBac vector with a hexahistidine tag added to the C terminus of the protein. The recombinant protein was expressed in S. frugiperda 9 insect cells and purified to homogeneity by Co2⁺ column chromatography. Fig. 1A shows a Coomassie-stained polyacrylamide gel of uninfected (lane 1) and infected (lane 2) insect cell homogenates, and 100 ng of the purified histidine-tagged protein of ~64 kDa (lane 3), consistent with the predicted molecular weight of 63,460 (including the six histidines). To further examine the purified protein, 650 ng of the protein was analyzed by SDS-polyacrylamide gel electrophoresis followed by silver staining. The purified protein showed a major band of ~64 kDa (lane 4). Fig 1B shows an immunoblot with a monoclonal antibody (mAb-1-11) against a synthetic peptide derived from the putative human BCO protein. Taken together, these data show that isolation of a highly purified recombinant protein was achieved.

Fig. 2 shows the results of an in vitro time course experiment in which 2.5 μl β-carotene was used as substrate with 60 ng of purified BCO protein. The products at the different time points were analyzed by reverse-phase HPLC and found to migrate with the same retention time as an all-trans-retinal standard, and the formation of product was linear up to 20 min. Preincubation of the purified protein at 95 °C for 5 min eliminated enzyme activity (data not shown). Fig. 3 shows the spectral properties of the enzyme reaction products by photodiode array detector on-line analysis after separation by HPLC. The enzyme-dependent product after incubation with β-carotene (Fig. 3, solid line) has an absorbance spectrum virtually identical to that of authentic all-trans-retinal (Fig. 3, dashed line C). Identical absorbance spectrum and retention time were seen for one of the enzyme-dependent reaction products when β-cryptoxanthin was used as substrate, indicating that all-trans-retinal is one of the two reaction products produced from this substrate (data not shown). Taken together, these data showed that the purified recombinant protein catalyzed the central cleavage of β-ionone ring containing carotenoids, suggesting that the PCR-amplified cDNA encoded a human homologue of the Drosophila BCO.

Characterization of Purified Recombinant BCO Enzyme—The biochemical properties of the purified protein were investigated by performing enzyme assays, gel filtration chromatography, and immunocytochemistry. Table I summarizes data on the effect of seven different detergents on BCO enzyme activity. The data show that maximal enzyme activity was achieved when OTG was used at a final concentration of 1% (w/v). Table II shows the effect of four different sulphydryl reducing agents on enzyme activity. The data are consistent with the observations that sulphydryl reducing agents are imperative for maximal enzyme activity in vitro (11–13) and that TCEP was the most effective agent when used at concentrations above 0.5 mM. In contrast to the native enzyme (11), inclusion of ferrous iron in the enzyme assay buffer was not essential for maximal activity of the recombinant human enzyme (data not shown).

The data shown in Fig. 4 indicate that the enzyme demonstrates a slightly alkaline pH optimum, similar to the activity present in intestinal homogenates of rat (12), rabbit (10), hog
FIG. 3. Absorbance spectrum of BCO reaction product. Photodiode array on-line analysis of BCO reaction products after separation on HPLC was performed as described under “Experimental Procedures.” A, the absorbance spectrum of authentic all-trans-retinol. B, the absorbance spectrum of all-trans-retinoic acid. C, the enzymedependent product after incubation with β-carotene (solid line) has a virtually identical absorbance spectrum to that of authentic all-trans-retinal (dashed line). The scale of the left y axis represents absorbance units of retinoid standards; the scale of the right y axis represents absorbance units of enzyme dependent product. mAU, milliabsorbance units.

TABLE I

| Detergent                        | Concentration | Relative activity |
|----------------------------------|---------------|------------------|
| 1-S-Octyl-β-D-thioglucopyranoside| 0.5           | 100%             |
| Triton X-100                     | 0.5           | 16               |
| Sodium dodecyl sulfate           | 0.5           | 4                |
| Tween 20                         | 0.5           | 4                |
| CHAPS                            | 0.5           | 3                |
| Octyl-β-glucoside                | 0.5           | 29               |
| Sodium cholate                   | 0.5           | 12               |
| 1-S-Octyl-β-D-thioglucopyranoside| 0.05          | 12               |
|                                  | 0.1           | 16               |
|                                  | 0.25          | 29               |
|                                  | 0.75          | 149              |
|                                  | 1             | 151              |

* 100% activity defined as the activity seen with 65 ng of BCO and 25 μM β-carotene in a 1-h reaction, 5.6 nmol/mg × min.

TABLE II

| Reducing agent                  | Concentration | Relative activity |
|----------------------------------|---------------|------------------|
| None                             | 0             | 19               |
| Tris(2-carboxyethyl)phosphine    | 5             | 100%             |
| Reduced glutathione              | 5             | 23               |
| Dithiothreitol                   | 5             | 32               |
| β-Mercaptoethanol                | 5             | 26               |
| Tris(2-carboxyethyl)phosphate    | 0.05          | 22               |
| hydrochloride                    | 0.25          | 108              |
|                                  | 0.5           | 139              |
|                                  | 2.5           | 105              |

* 100% activity defined as the activity seen with 1 μg of BCO and 25 μM β-carotene in a 15-min reaction, 1.35 nmol/mg × min.

FIG. 4. pH optimum of purified recombinant human BCO. The assays were performed in duplicate at the indicated pH in 100 mM Tricine-KOH, 150 mM NaCl buffers in the presence of 100 ng enzyme and 8 μM β-carotene for 30 min at 37 °C as described under “Experimental Procedures.” 100% equals 3.85 nmol/mg × min. Each value represents the average of three independent experiments.

FIG. 5. Inhibition of BCO enzyme activity by metal chelating and sulphydryl alkylating agents. A, o-phenanthroline; B, α,α-bipyridyl; C, N-ethylmaleimide; D, p-chloromercuribenzoate. The assays were performed in duplicate in the presence of inhibitors at the indicated concentrations with 500 ng of enzyme and 8 μM β-carotene for 30 min at 37 °C as described under “Experimental Procedures.” 100% equals 0.9 nmol/mg × min. Each value represents the average of two independent experiments.

The experiments of Fig. 5 show that the enzyme is sensitive to the metal chelating agents α,α-bipyridyl and o-phenanthroline, as well as the sulphydryl alkylating agents N-ethylmaleimide and p-chloromercuribenzoate. These findings are consistent with data regarding the biochemical properties of the intestinal BCO activity from different species. These results suggested that the purified recombinant human BCO enzyme possessed similar biochemical properties as compared with the partially purified native enzyme from a variety of animal species.

To ascertain whether BCO is a monomer or oligomer in solution, gel filtration chromatography experiments were performed with the purified enzyme. Fig. 6 shows a typical elution profile as assessed by enzymatic activity and immunoblotting of different Sephadex S-300 column chromatography fractions. When chromatography was performed under buffer conditions identical to those used in the enzyme assays, a majority of the enzymatically active and immunodetectable protein migrated at ~230 kDa. These data suggested that the purified BCO enzyme was a tetramer in its enzymatically active form. It should be pointed out that because the gel filtration experiments were performed with 1% OTG in the elution buffer, it is conceivable that the enzyme migrated on the column in experimentally created detergent-protein micelles; hence, the oligo-
In Vitro Kinetic Analysis of Purified BCO with Carotenoids as Substrates—To analyze the substrate specificity and kinetic constants for purified recombinant BCO, we performed in vitro assays with the carotenoid substrates \( \beta \)-carotene, \( \beta \)-cryptoxanthin, zeaxanthin, and lycopene. Incubation conditions were designed to ensure less than 10% substrate conversion with concentrations in the 0.25–32 \( \mu \)M range for \( \beta \)-carotene and in the 2.5–256 \( \mu \)M range for \( \beta \)-cryptoxanthin. The formation of product was linear with respect to protein over a 60–1000 ng range and time over a 20-min period (Fig. 2 and data not shown). Fig. 8 shows the reaction velocity (nmol product formed/mg protein \( \times \) min) as a function of substrate concentration (\( \mu \)M) plotted for a 15-min reaction with 250 ng of BCO (in duplicate). Product quantitation was performed as described under “Experimental Procedures”; however, when \( \beta \)-cryptoxanthin, zeaxanthin, and lycopene were used as substrates, the mobile phase was 15% water and 0.1% ammonium acetate in acetonitrile (for retinal, \( R_t = 12.2 \) min; for 3-hydroxyretinal, \( R_t = 3 \) min). The \( K_m \) and \( V_{\text{max}} \) of the enzyme with \( \beta \)-carotene as substrate were 7.1 ± 1.8 \( \mu \)M and 10.4 ± 3.3 nmol retinal/mg \( \times \) min, respectively (Fig. 8C), values that correspond to a turnover number (\( k_{\text{cat}} \)) of 0.660 min \(^{-1}\), assuming that each subunit is catalytically active. This output corresponds to a catalytic efficiency (\( k_{\text{cat}}/K_m \)) of 93,000 M \(^{-1}\) min \(^{-1}\). \( \beta \)-Cryptoxanthin was also accepted as substrate with an apparent \( K_m \) of 30.0 ± 5.8 \( \mu \)M and a \( V_{\text{max}} \) of 0.9 ± 0.2 nmol/mg \( \times \) min, values that correspond to a turnover number of 0.057 min \(^{-1}\) and a catalytic efficiency of 1,900 M \(^{-1}\) min \(^{-1}\). Zeaxanthin and lycopene were not substrates for the BCO enzyme under the assay conditions used.

Tissue Distribution of BCO mRNA—RNA blotting of human poly(A)+ RNA show that the BCO mRNA is expressed throughout the intestinal tract, with the highest level in jejunum, the segment of the small intestine that has been shown to possess the highest BCO enzyme activity (Fig. 9A) (26). The predominant mRNA is ~2.6 kb, and a less abundant mRNA of ~5.8 kb is also detected. Fig. 9B shows that in addition to the small intestine, BCO mRNA is also present at high levels in the liver and kidney and that lower levels are in the prostate, testis, ovary, colon, and skeletal muscle.

**DISCUSSION**

This paper describes the cloning, expression, purification, and characterization of human BCO, a cytosolic enzyme that catalyzes the first step in the in vivo synthesis of vitamin A by cleaving the central carbon 15,15′-double bond in provitamin A carotenoids. The identification of the present BCO enzyme as the human homologue of BCO characterized from a variety of species was based on multiple criteria, including: 1) the amino acid sequence has a high sequence identity with previously cloned BCO proteins belonging to the nonheme iron containing monooxygenases; 2) the absorbance spectra of the enzyme dependent reaction product with \( \beta \)-carotene as substrate is identical to that of all-trans-retinal; 3) the recombinant enzyme shares biochemical properties with the native BCO enzyme reported from a variety of animal species; and 4) the BCO mRNA is present in three independent experiments.
present at high levels along the whole intestinal tract, with the highest levels in jejunum, in concordance with reports on BCO enzyme activities in different segments of the intestine.

Since the initial in vitro characterizations of the intestinal and liver BCO enzymes by the research groups of Goodman and Olson almost 40 years ago, a considerable body of work has focused on the biochemical properties of the native BCO enzyme of partially purified preparations from different animal species such as rat (7, 12), rabbit (11), hog (13), guinea pig (11), and chicken (18, 19). However, no data on the biochemical properties of the native human isoenzyme have been reported. Furthermore, earlier efforts to purify the native mammalian enzyme to homogeneity have proven unsuccessful. A breakthrough in the area of BCO research came in 2000, when von Lintig and Vogt (17) isolated a cDNA encoding the fruit fly Drosophila melanogaster BCO by employing an expression cloning strategy using Escherichia coli cells genetically engineered to synthesize β-carotene de novo. This work was followed shortly after by other studies by Wyss et al. (15, 16), who partially purified the BCO enzyme from chicken intestine and cloned the chicken and mouse BCO cDNAs. At the same time, we performed GenBank data base searches and identified a human BCO cDNA that was found to encode a hydrophilic protein of 547 amino acids with a predicted molecular weight of 62,637 and that has a significant sequence homology with the fruit fly, chicken, and mouse isozymes.

Because expression of enzymatically active BCO in bacteria proved difficult, we chose the baculovirus system as a source of recombinant protein. The human BCO enzyme used in the present study was expressed in insect cells and purified to apparent homogeneity. The purified enzyme possesses a specific activity of 10 nmol retinal formed/mg protein/min when β-carotene is used as substrate under optimal reconstitution conditions. This specific activity is 300-fold higher than that of

![Image](https://example.com/image.png)
the partially purified native enzyme that was enriched 226-fold from chicken intestinal mucosa (15), and 300–1600-fold higher than that of the bacterially expressed and purified mouse BCO histidine (27) or glutathione S-transferase fusion proteins (28). The recombinant human enzyme catalyzes the cleavage of \( \beta \)-carotene and \( \beta \)-cryptoxanthin, but not of zeaxanthin or lycopene, suggesting that at least one half-site of an unsubstituted \( \beta \)-ionone ring substrate is imperative for cleavage of the central carbon 15,15'-double bond (Fig. 8C). This requirement coincides with the fact that \( \beta \)-carotene and \( \beta \)-cryptoxanthin are considered to have nutritional value as provitamin A carotenoids, in contrast to zeaxanthin and lycopene. Interestingly, the BCO enzyme shows a 4-fold higher \( K_m \) when \( \beta \)-cryptoxanthin is used as substrate as compared with \( \beta \)-carotene. Because \( \beta \)-carotene has two unsubstituted \( \beta \)-ionone ring half-sites and \( \beta \)-cryptoxanthin consists of one unsubstituted \( \beta \)-ionone ring half-site and one 3-hydroxylated \( \beta \)-ionone ring, it is conceivable that each half-site may bind to separate BCO subunits. This hypothesis is plausible because our data show that the enzymatically active BCO enzyme is an oligomer in solution. Another explanation could simply be that one molecule of enzyme binds one molecule of substrate in an extended hydrophobic pocket with the active site positioned in the middle. It should be mentioned that our substrate specificity data on the recombinant human isozyme corroborates data obtained with the partially purified native enzyme that was enriched 226-fold among these family members.

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