

**Reconstitution of β -Carotene Hydroxylase activity of thermostable CYP175A1
monooxygenase**

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Abstract

CYP175A1 is a thermostable P450 Monooxygenase from *Thermus thermophilus* HB27, demonstrating *in vivo* activity towards β -carotene. Activity of CYP175A1 was reconstituted *in vitro* using artificial electron transport proteins. First results were obtained in the mixture with a crude *E. coli* cell extract at 37°C. In this system β -carotene was hydroxylated to β -cryptoxanthin. The result indicated the presence of electron transport enzymes among the *E. coli* proteins, which are suitable for CYP175A1. However, upon *in vitro* reconstitution of CYP175A1 activity with purified recombinant flavodoxin and flavodoxin reductase from *E. coli*, only very low β -cryptoxanthin production was observed. Remarkably, with another artificial electron transport system, putidaredoxin and putidaredoxin reductase from *Pseudomonas putida*, purified CYP175A1 enzyme hydroxylated β -carotene at 3- and also 3'-positions, resulting in β -cryptoxanthin and zeaxanthin. Under the optimal reaction conditions, the turnover rate of the enzyme reached 0.23 nmol β -cryptoxanthin produced per nmol P450 per min.

Key words; Cytochrome P450; CYP175A1; β -carotene hydroxylase; thermostable

Abbreviations: CYP175A1, cytochrome P450 from *Thermus thermophilus* strain HB27; P450BM-3, cytochrome P450 from *Bacillus megaterium*; *T. thermophilus*, *Thermus thermophilus* strain HB27; *E.coli*, *Escherichia coli*; HPLC, High performance liquid chromatography; 11-Desoxycortisol, 4-pregnene-17 α , 21-diol-3, 20-dione; IPTG, isopropyl β -D-1-thiogalactopyranoside.

The cytochrome P450 constitutes a large superfamily of soluble and membrane-bound heme-thiolate proteins currently comprising well over 4500 distinct sequences from all phyla including animals, plants, fungi, and bacteria [1]. Cytochromes P450 catalyzes the monooxygenase reaction, which introduces oxygen into generally non-polar, aromatic or aliphatic molecules, thereby leading to hydroxylation, aromatization, epoxidation or cleavage of carbon-carbon bonds. Because of its unique and versatile catalytic properties, P450 enzymes are of great interest as potential biological catalysts for industrial usage. However, thermal and chemical instability of P450 enzymes often interferes with practical applications.

A promising new strategy to obtain industrially useful P450 catalysts is to search for thermostable P450 enzymes in thermophilic bacteria. The first reported thermostable P450 enzyme, CYP119 was isolated from acidothermophilic archaeon *Sulfolobus solfataricus* [2]. The protein has been expressed in *E. coli* and shown to oxidize fatty acids when catalysis is supported by hydrogen peroxide or a surrogate (mesophilic) electron donor system consisting either of putidaredoxin and putidaredoxin reductase [3], or of thermophilic ferredoxin and pyruvate ferredoxin reductase [4], although the biological substrate is still unknown. More recently, a second thermostable P450, CYP175A1, was isolated from *Thermus thermophilus* HB27 and the crystal structure of the heterologously overexpressed protein in *E. coli* was analyzed [5]. The P450 protein showed indeed high thermostability and structure determination revealed considerable similarity to the heme domain of cytochrome P450 BM-3 from *Bacillus megaterium* [5]. Previous *in vivo* experiments revealed that the enzyme catalyzes the hydroxylation of β -carotene at 3- and 3'-positions, thereby producing β -cryptoxanthin and zeaxanthin [6]. Xanthophylls, the oxygenated products of β -carotene, are important food and feed additives or precursors for pharmaceuticals. For examples, zeaxanthin and its stereoisomer lutein are reported to prevent age-related macular degeneration (AMD) [7]. Some studies suggest that astaxanthin can be more than 1000 times more effective as antioxidant than vitamin E [8]. In species such as salmon or shrimp,

astaxanthin is even considered as essential to normal growth and survival, and has been attributed vitamin-like properties [9].

Hitherto, two different types of carotene hydroxylase genes were identified as xanthophyll formation enzymes. One type of the enzymes belongs to hydroxylase/desaturase gene family [10], and the other belongs to the cytochrome P450 super family. In addition to CYP175A1, an α -carotene hydroxylase (producing lutein) P450 gene was recently identified [11]. Nevertheless, neither of the cytochrome P450 enzymes has been reconstituted *in vitro* yet and the catalytic properties of P450 type carotene hydroxylase have not been investigated in detail.

To analyze the unique functional properties of CYP175A1 as a P450-type β -carotene hydroxylase, and to explore its potential for industrial or biotechnological use, we have searched for functional reconstitution conditions using purified proteins and analyzed its enzymatic properties.

Materials and methods

Reagents. β -Carotene, soybean lecithin, retinoic acid, retinal, retinol, and geranylgeranylpyrophosphate were purchased from Sigma (Steinheim, Germany). β -Ionone was purchased from Merck (Darmstadt, Germany). Beta-cryptoxanthin was provided by Roche Vitamins (Basel, Switzerland). Zeaxanthin and 3-Hydroxy- β -ionone were from BASF AG (Ludwigshafen, Germany). 4-Hydroxy- β -ionone was synthesized from α -ionone as previously described [12].

Flavodoxin, flavodoxin reductase, putidaredoxin, and putidaredoxin reductase. The expression vectors for flavodoxin (pET11a-fld) and flavodoxin reductase (pET11a-fpr) were kindly provided by Prof. Michael R. Waterman (Vanderbilt University, Tennessee). The proteins were expressed in *E. coli* strain BL21 (DE3) and then purified as elsewhere [13,14]. The concentrations of the enzymes were determined photometrically, using extinction

coefficients of $7.1 \text{ mM}^{-1} \text{ cm}^{-1}$ at 456 nm [15] for flavodoxin reductase, and $8.42 \text{ mM}^{-1} \text{ cm}^{-1}$ at 465 nm for flavodoxin [16]. Putidaredoxin and putidaredoxin reductase were kindly provided by Dr. Luet-L. Wong (University of Oxford). Their concentrations were determined photometrically using extinction coefficients of $11.1 \text{ mM}^{-1} \text{ cm}^{-1}$ at 415 nm for putidaredoxin and $10.0 \text{ mM}^{-1} \text{ cm}^{-1}$ at 454 nm for putidaredoxin reductase [17].

CYP175A1. The construction of the expression plasmid used in this study was described previously [5]. *E. coli* strain BL21 (DE3) codon plus was transformed with the expression plasmid, carrying *CYP175A1* gene and grown overnight in 5 ml LB medium containing 50 $\mu\text{g/ml}$ ampicillin and 25 $\mu\text{g/ml}$ chloramphenicol. The overnight culture was used to inoculate 500 ml of TB medium [18] with antibiotics and was followed by further incubation at 37°C and 220 rpm. After the cell culture reached late log-phase (absorbance at 600 nm 0.6-0.8, around 4 hours), 1 mM of IPTG was added and incubation conditions were changed to 30°C at 120 rpm. The expression of CYP175A1 was monitored by CO-difference spectra and by Coomassie Brilliant Blue staining after SDS-PAGE. Cells were harvested by centrifugation and used for direct analysis or as material for purification. For the measurement of enzyme activity, the cells were suspended in a four-fold volume (v/w) of 40 mM potassium phosphate buffer (pH 7.4), and extracted after sonication. Cytochrome P450 contents was estimated from CO-difference spectra using an extinction coefficient of $0.091 \text{ mM}^{-1} \text{ cm}^{-1}$ [19].

Purification of CYP175A1 was performed as follows. Cells were suspended in 10 volumes of buffer containing 50 mM Tris-HCl (pH 7.5), 200 mM sodium chloride, 1 mM DTT, 1mM EDTA, 0.1 mM PMSF and treated with lysozyme (1 mg/ml final concentration) for 30 min at 4°C. After lysozyme treatment, the cell suspension was further sonicated, and then centrifuged. The supernatant was fractionated by ammonium sulfate between 35% and 50% saturation. After ammonium sulfate fractionation, the pellet was solubilized in minimum volume of 50 mM Tris-HCl buffer (pH7.5) and was applied onto Phenyl Sepharose column

(Pharmacia LKB, Uppsala, Sweden). The column was washed first with 35% saturated ammonium sulfate in 50 mM Tris-HCl buffer (pH 7.4), and then with the same buffer lacking ammonium sulfate, followed by elution with 1% sodium cholate. The fractions with red color were combined and loaded onto hydroxyapatite column. P450 enzyme was eluted from the column by a linear gradient of 50-300 mM potassium phosphate (pH 7.4) containing 5% glycerol. The eluted enzyme was concentrated by Amicon Ultrafiltration (Millipore GmbH, Schwalbach, Germany) and stored at -80°C until use. The purity of enzyme was estimated by SDS-PAGE and spectrophotometrically. The molar concentration of purified enzyme was determined using an absorption coefficient of $104 \text{ mM}^{-1} \text{ cm}^{-1}$ at 418 nm [5].

Hydroxylation of β -carotene and β -ionone. Suspensions of β -carotene in soybean lecithin (Sigma) were prepared as described elsewhere [20] and used on the same day as activity measurement. Unless stated otherwise, 50 μl of β -carotene suspension containing 20 mg/ml lecithin and 0.1 mM β -carotene was mixed with 50 μl of enzyme solution, containing CYP175A1 and the respective electron transfer proteins. The sets of P450 electron transfer system were as follows. To measure the activity of CYP175A1 without purification, the reaction mixture contained 35 μl of *E. coli* cell extract, and 1 mM NAD(P)H. To measure the activity of purified CYP175A1, 13 μM flavodoxin/14 μM flavodoxin reductase/1 mM NADPH, or 20 μM putidaredoxin/4 μM putidaredoxin reductase/1 mM NADH, or 0.1 mM hydrogen peroxide in 40 mM potassium phosphate, pH 7,4 were used. After incubation for 30 min, the reaction was terminated by addition of 100 μl of 0.1% 2, 6-di-tert-butyl-4-methylphenol (BHT, Sigma) in tetrahydrofuran/methanol (1/1) and the incubation mixtures were extracted three times by 400 μl of ethyl acetate. The organic phase was combined and dried in vacuum and analyzed by HPLC using Nucleosil C18 column (260 mm, 4 mm diameter, particle size 5 μm , Macherey-Nagel, Düren, Germany) with acetonitril/methanol/isopropanol (85/10/5) as mobile phase and flow rate 1 ml/min. LC-MS

analysis was performed by a Series 1100 LC-MSD system (Agilent, Waldbronn, Germany) with binary pump, degasser, autosampler and mass selective detector equipped with an electrospray ion source. Chromatographic separation was achieved on a Nucleosil C18 column (3.0 mm I.D. x 100 mm) using the same mobile phase as described for HPLC analysis at a flow rate of 0.5 ml/min. The mass spectrometer was tuned with the autotune procedure provided by the HP Chemstation software. Electrospray parameters were as follows: polarity positive, capillary voltage 3500 V, drying gas flow 10 l/min nitrogen, drying gas temperature 350EC, nebulizer pressure 25 psig, fragmentor 140 V. The mass spectrometer was operated in selected ion monitoring mode (SIM resolution high) using the respective molecular ions $M^{•+}$ and MH^+ and the fragment $[M-92]^{•+}$ corresponding to loss of toluene from the molecular ion, m/z 476.2, 568.2 and 569.2 for zeaxanthine and m/z 460.2, 552.3 and 553.3 for cryptoxanthine. Peak width was set at 0.30 min.

To measure the hydroxylation of β -ionone, 10 μ l of 10 mM β -ionone solution in DMF/acetonitril (1/1) was mixed with 500 μ l of enzyme preparation containing 0-1 μ M P450 and the same set of cytochrome P450 electron transfer system as described above, and incubated at 37°C for 30 min. The reaction mixture was extracted twice with diethylether and analyzed as described in [21] using GC-MS.

Binding assay. The spectral changes associated with the interaction of several substances with CYP175A1 were measured in 35 mM potassium phosphate buffer, pH 7.4, containing 0.1% Emulgen 913, 0.1 mM EDTA, 0.1 mM dithiothritol and 20% glycerol. Spectral changes were recorded using a UNICAM UV4-500 spectrophotometer (AnalyticalTechnology Inc., Cambridge, UK).

Results and discussion

Expression and purification of CYP175A1

Heterologous expression of CYP175A1 in *E. coli* was followed by the SDS-PAGE analysis. A protein with apparent molecular weight of 47 kDa became visible on SDS-PAGE about 20 h after IPTG induction, and the corresponding band increased thereafter (data not shown). In cells harvested 44 h after IPTG induction, CYP175A1 amounted to about 100-300 nmol P450 per liter culture. Crude *E. coli* cell extracts used for activity measurements contained 40-50 mg total protein and 4-6 nmol P450 per ml (0.4-0.6% of total protein). After purification, CYP175A1 fractions showed a single band by SDS-PAGE and an absorption maximum at 418 nm and an absorbance ratio at 417 nm/280 nm of 1.12 during spectroscopic analysis.

*β -Carotene hydroxylation by CYP175A: Activity in the *E. coli* crude cell extract*

The natural redox partner(s) of CYP175A1 in *T. thermophilus* are currently unknown. Biological function of CYP175A1 was previously investigated by functional complementation of a plasmid, carrying the *CYP175A1* gene, with a plasmid, harbouring three β -carotene producing genes in *E. coli* [6]. β -Carotene was hydroxylated to β -cryptoxanthin and zeaxanthin in *E. coli*, indicating the presence of artificial electron transport proteins in *E. coli*, which are able to transfer electrons to the heme iron of the monooxygenase. Therefore, a cell extract from *E. coli* was used first for *in vitro* analysis of the enzyme activity (Fig. 1). As a negative control, the *E. coli* BL21 (DE3) cell extract without P450 enzyme was used under the same conditions. HPLC analysis of the products resulted in a small peak with the same retention time as β -cryptoxanthin (Crypto). This product was consistently associated with the expression of CYP175A1 (+P450, -P450 in Fig. 1). Quantitative estimation of the product revealed 0.013 nmol of β -cryptoxanthin produced in 30 min at 37°C with 0.19 nmol P450. The activity disappeared completely at 65°C (65°C),

although CYP175A1 was expected to be thermostable from its melting point at 88°C [5]. Obviously, the artificial electron transport system(s) from mesophilic *E. coli* were not optimal and temperature sensitive.

Activity in the reconstituted system of purified proteins

Depending on their redox partners, P450 enzyme systems can be divided into two classes. Class I system comprises a ferredoxin and a NADH-dependent, FAD-containing ferredoxin reductase. Class II system comprises a NADPH-dependent diflavin reductase (FAD and FMN).

Flavodoxin and flavodoxin reductase from *E. coli* belonging to class II, have been proven as artificial electron transport partner for several P450s [13,14]. Thus these two proteins were tested first with CYP175A1 to analyze its activity *in vitro*. However, from the 100 µl of reaction mixture containing 0-1.28 nmol purified cytochrome P450, 13 µM flavodoxin and 14 µM flavodoxin reductase, we detected only small amounts of zeaxanthin and β-cryptoxanthin, and their production did not appear to depend on further increase of the P450 concentration or NADPH. Therefore, we have concluded that the production of the hydroxylated products in this case is non-enzymatic (data not shown). That suggested another proteins in *E. coli* extract, providing electrons to the heme iron of CYP75A1.

Next, we have tested the class I redox system, represented by putidaredoxin and putidaredoxin reductase from *Pseudomonas* sp. using NADH as electron donor. Figure 2 shows typical HPLC chromatograms of reaction products obtained with the fully reconstituted system at 37°C. Substances with the same retention times as zeaxanthin (8.9 min) and β-cryptoxanthin (13.6 min) increased according to the concentration of CYP175A1 in the reaction mixture (dotted line, without P450; dashed line, 6 µM; linear line 12 µM). The identity of the two xanthin-products was confirmed by LC-MS analysis.

Production of β -cryptoxanthin increased linearly up to 5 minutes and slowed down thereafter (Fig. 3). As shown in figure 4, the production of β -cryptoxanthin as a function of β -carotene concentration did not follow the Michaelis-Menten type kinetics. Production of β -cryptoxanthin increased according to the β -carotene concentration up to 50 μM , and decreased at higher substrate concentration. In these experiments, the same amount of soybean lecithin (10 $\mu\text{g/ml}$) was used in each incubation mixture to rule out indirect effects of reconstitution conditions. With 50 μM β -carotene, we have measured the β -cryptoxanthin formation with different amount of putidaredoxin (data not shown). Addition of putidaredoxin is saturable, and the apparent affinity constant was calculated to 22 μM .

Interaction of CYP175A1 enzyme with substances, containing a β -ionone ring.

In effort to find other suitable substrates for CYP175A1, we tested several chemical compounds by a spectrophotometrical method. Included were substances, containing β -ionone ring (β -carotene, retinoic acid, retinal, retinol, and β -ionone), as well as other related substances like geranylgeranylpyrophosphate and α -ionone. As a structure of CYP175A1 shows a very high similarity to that of the heme domain of P450 BM-3 from *Bacillus megaterium*, its substrates, fatty acids were also tested. However, among all substances tested, only β -ionone induced changes in the absorption spectrum in the Soret region with an isosbestic point at 405 nm. This result can suggest a very narrow substrate specificity of CYP175A1 and its certain role in carotene metabolism.

The β -ionone binding constant was calculated to 72 μM and the maximal change of absorption at 422 nm was to 20 $\text{cm}^{-1} \text{mM}^{-1}$ (Fig. 5). Type I spectrum change is typically induced by substrates. We have measured the hydroxylation of β -ionone by CYP175A1 under several different conditions. As a result, no indication for β -ionone as a substrate of CYP175A1 was observed after incubation with putidaredoxin/putidaredoxin reductase,

flavodoxin/flavodoxin reductase or *E. coli* bacterial cell extract at 37°C. No oxidized products were identified by GC-MS analysis.

β -carotene, vitamin A and related substances contain the unsaturated side chain of different lengths additionally to β -ionone ring, which lead to further decrease of their solubility in water. Therefore, the measurements were performed in the detergent containing buffer in order to increase the substance solubility and to decrease turbidity.

Unexpectedly, neither β -carotene nor vitamin A and related substances induced spectral rearrangements of CYP175A1.

Discussion

The gene coding for a thermostable P450 monooxygenase CYP175A1 is located in the same gene cluster as several carotenogenic genes in *Thermus thermophilus*. This suggests involvement of this monooxygenase into metabolism of carotenoids. Earlier *in vivo* experiments revealed a 3-hydroxylase activity of this monooxygenase towards β -carotene. *In vitro* reconstitution of this activity using an artificial electron transport system, consisting of putidaredoxin and putidaredoxin reductase, confirmed previous results. The turnover rate after optimization of reaction conditions was 0.23 min^{-1} . Activity of the desaturase type β -carotene hydroxylase from pepper fruits was reconstituted *in vitro*, however there is no information about turnover rates or specific activity of this enzyme [24].

Reconstitution of CYP175A1 activity *in vitro* was rather complicated because β -carotene is insoluble in water and can be hardly dispersed with co-solvents. Additionally, as CYP175A1 originated from a thermophilic microorganism may require a higher temperature for its function.

We have studied the interaction of the substrate and the enzyme by spectrophotometric analysis. The absence of a typical spectral change induced by β -carotene binding can be explained by a high hydrophobicity of this substrate. Substances with a large hydrophobic

moiety may require the interaction with phospholipid vesicles to access the active center of CYP175A1 [23].

In contrast, β -ionone, having a much higher solubility, induced a spin-state shift. However, β -ionone structure represents only one part of the β -carotene molecule and probably not enough for hydroxylation.

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Figure legends

Fig. 1. HPLC analysis of reaction products of β -carotene hydroxylation in *E. coli* cell extract. Peaks correspond to β -cryptoxanthin (Crypto). The reaction mixture contained 50 μ l *E. coli* extract, 50 μ M β -carotene, 10 mg/ml soybean lecithin in 40 mM potassium phosphate buffer (pH 7.4), and 1 mM NADH. (+**P450**) *E. coli* cell extract was prepared from the bacteria transformed by CYP175A1 expression vector. The mixture contained 0.19 nmol P450. (-**P450**) *E. coli* cell extract was prepared from control *E. coli* cells without P450 monooxygenase. (**65°C**) Incubation at 65°C for 30 min.

Fig.2. HPLC analysis of reaction products in the reconstitution system containing CYP175A1 and putidaredoxin/putidaredoxin reductase. β -Carotene was incubated with 12 μ M (linear line), 6 μ M (dashed line) or without (dotted line) of the P450 enzyme. Peaks correspond to zeaxanthin (Zea), β -cryptoxanthin (Crypto).

Fig. 3. Time course of β -cryproxanthin production in the reconstitution system with putidaredoxin and putidaredoxin reductase. Reaction mixture containing 40 mM potassium phosphate buffer (pH 7.4), 20 μ M putidaredoxin, 4 μ M putidaredoxin reductase, 50 μ M β -carotene, 10 mg/ml soybean lecithin and 1mM NADH was pre-incubated at 37°C for 3 min and the reaction was started by addition of P450 enzyme (12 μ M).

Fig. 4. Optimization of β -carotene concentration. Different concentration of β -carotene was suspended with soybean lecithin and incubated with 6 μ M CYP175A1, 20 μ M putidaredoxin, 4 μ M putidaredoxin reductase and 1 mM NADH at 37°C for 10 min. The concentrations of β -carotene indicate the final concentrations in the reaction mixture.

Fig. 5. Spectral changes of CYP175A1 induced by β -ionone. Four micromolar P450 solution was divided into two cuvettes and varying quantities of β -ionone solution in DMSO were added to the sample cuvette. To the reference cuvette, equal volume of DMSO was added. The final concentrations of β -ionone were (a); 0, (b); 0.5, (c); 10.5, (d); 21.1, (e); 40.7, (f); 89.4, (g); 185.2 μ M. Detailed experimental condition was described in Materials and Methods.

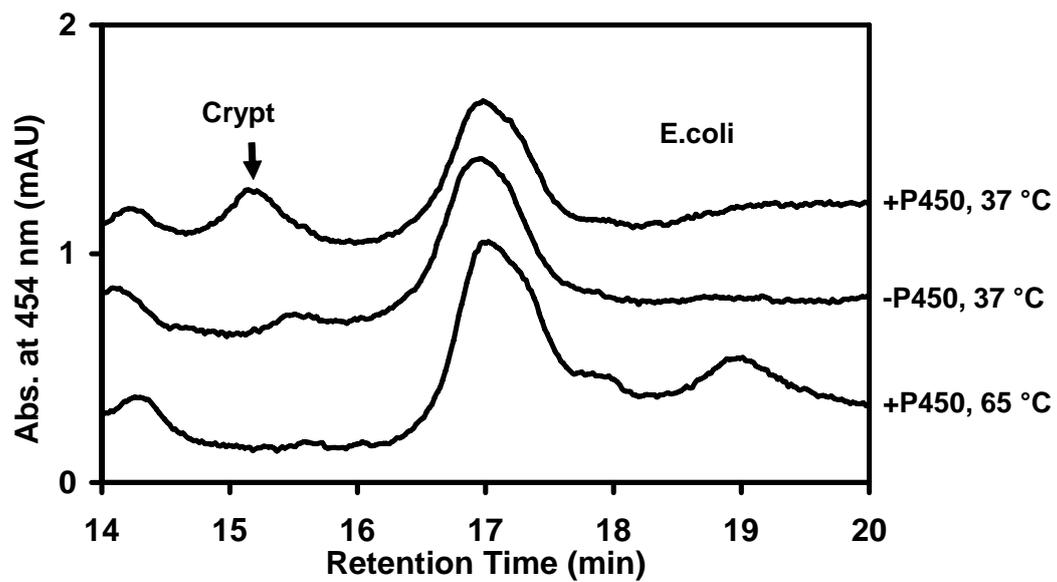


Figure 1.

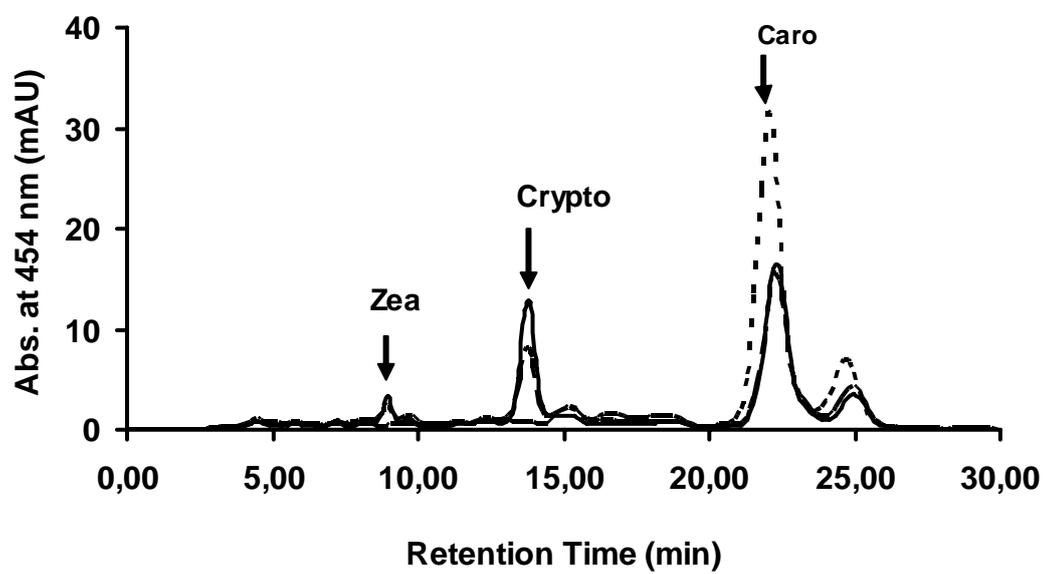


Figure 2.

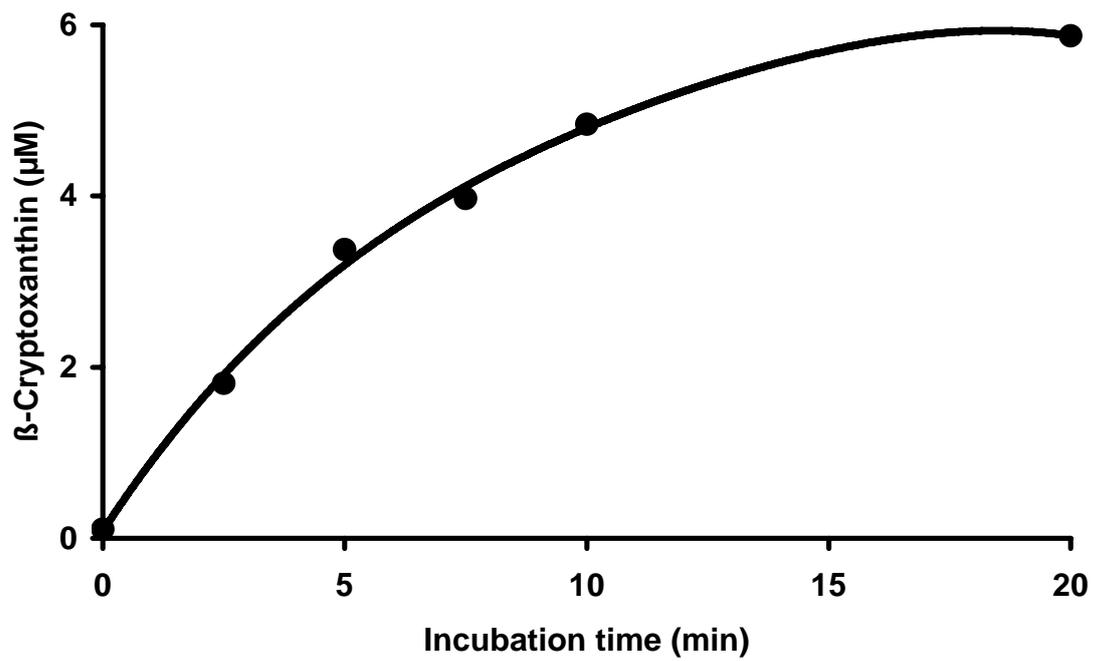


Figure 3.

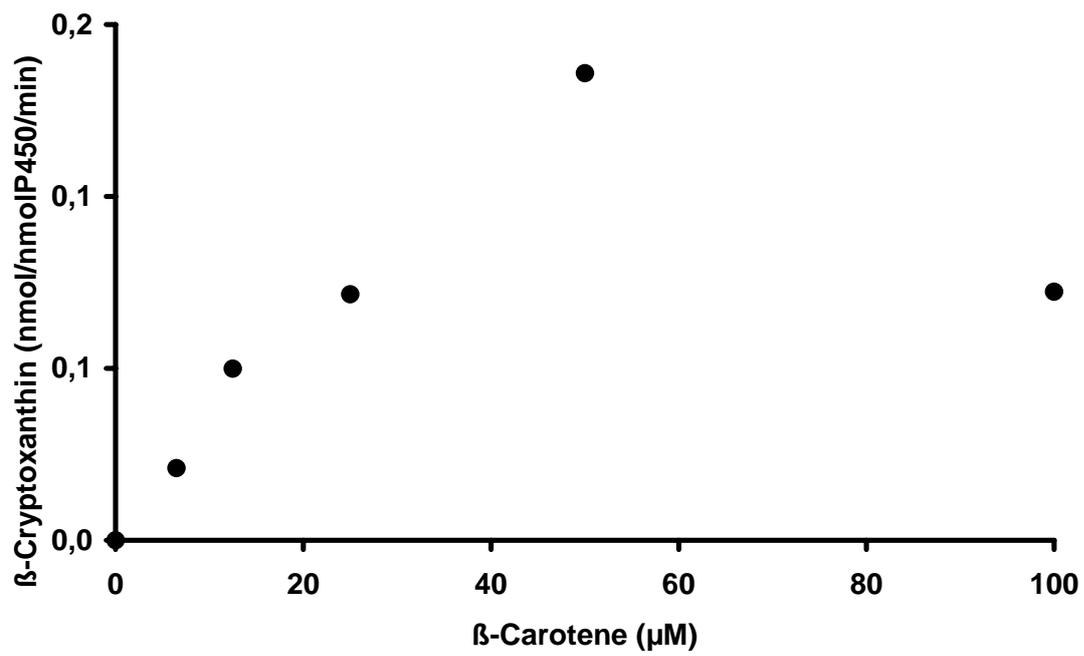


Figure 4.

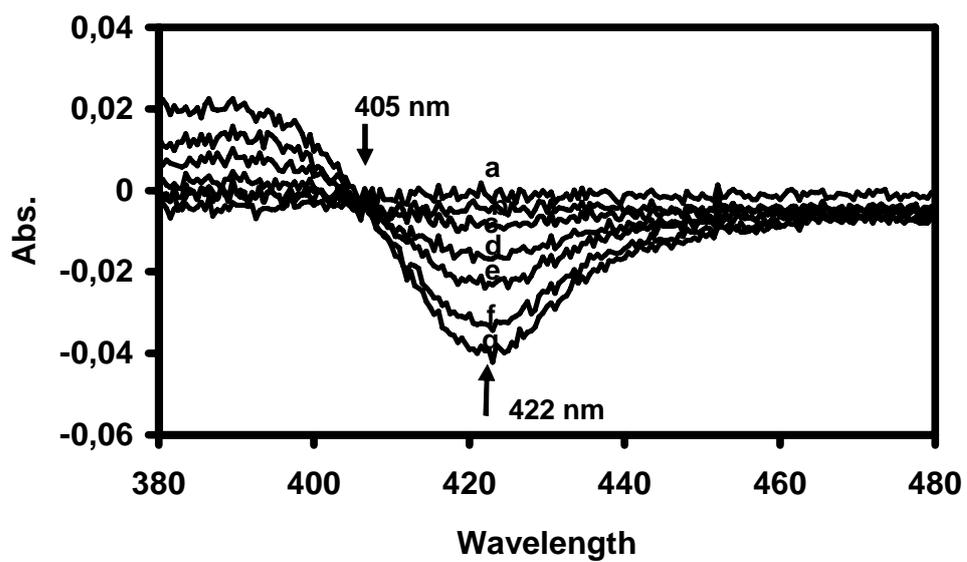


Figure 5.