Carbohydrate Polymers 101 (2014) 1147–1153

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Characterizing the natural canthaxanthin/2-hydroxypropyl-β-cyclodextrin inclusion complex

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ARTICLE INFO

Article history:
Received 26 September 2013
Received in revised form 16 October 2013
Accepted 21 October 2013
Available online 27 October 2013

Keywords:
Dietzia natronolimnaea HS-1
Carotenoid pigment
Hydroxypropyl-β-cyclodextrin
Inclusion complex characterization
Solubility enhancement
Chemical stability

ABSTRACT

A fundamental study on the phase-solubility, storage stability and antioxidant activity of the inclusion complex of 2-hydroxypropyl-β-cyclodextrin (HP-β-CD) with canthaxanthin (CTX) biosynthesized by *Dietzia natronolimnaea* HS-1 in a continuous bioreactor was designed. A variety of in vitro chemical methods including reducing power (RP), nitrite-scavenging activity (NSA), and 1,1-diphenyl-2-picryl-hydrazyl (DPPH), 2,2′-azinobis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS) and hydroxyl radicals scavenging capacities was used to determine antioxidant activities of the CTX and CTX/HP-β-CD complex. Results revealed that the apparent stability constant of the inclusion complex was 669 M^{-1} in a 1:1 stoichiometric ratio. The complexation of CTX with HP-β-CD significantly improved its water solubility and storage stability. An increased dose-dependent manner was found for the NSA and RP values of the CTX and CTX/HP-β-CD complex. The CTX/HP-β-CD complex showed higher scavenging capacity than native CTX against DPPH, ABTS and hydroxyl radicals. It can be used as a promising strategy to improve the food application of CTX.

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1. Introduction

Carotenoids are colored lipid-soluble compounds. Large natural abundance of these pigments is probably because of their relatively simple biosynthetic pathways in microorganisms (bacteria, algae and fungi), plants and animals (Gharibzahedi, Razavi, & Mousavi, 2013b). They are classified to two major groups: the carotenes which are composed of only carbon and hydrogen; and the xanthophylls, which are oxygenated derivatives. In the xanthophylls, oxygen can be present as OH groups (as in zeaxanthin), or as oxy-groups (as in canthaxanthin (CTX)); or in a combination of both (as in astaxanthin (ASX)) (Eonseon, Polle, Lee, Hyun, & Chang, 2003). Due to the recent discovery of their anticancer and antioxidant characteristics, wider use of these pigments as pharmaceuticals and nutraceuticals is expected. They can be also applied as natural colorants and are preferred to synthetic food colorants (Chandi & Gill, 2011; Gharibzahedi, Razavi, & Mousavi, 2013a). In comparison with carotenoids extraction from plant tissues or chemical synthesis, the industrial production of these pigments through microbial fermentations seems to be of paramount interest mostly due to the problems of seasonal and geographic variability in the production and marketing of several of the colorants of plant origin, and also the economic advantages of microbial processes using natural low-cost substrates as carbon source (Gharibzahedi, Razavi, & Mousavi, 2013d).

CTX (β-β′-carotene-4,4′-dione) is an abundant pigment in marine sources (crustaceans, fishes and microalgae), plants, fungi and bacteria. It is a high-value carotenoid pigment with many functions in food, feed nutraceuticals and cosmetics industries (Khodaiyan, Razavi, Emam-Djomeh, Mousavi, & Hejazi, 2007). *Dietzia natronolimnaea* HS-1 bacterium among the all introduced sources is recognized as a promising producer of natural CTX (Khodaiyan, Razavi, & Mousavi, 2008). It is Gram positive, catalase positive and oxidase negative with orange colonies that isolated during a routine screening of pigmented microorganisms (Khodaiyan et al., 2007). CTX is a highly unsaturated molecule and can easily be degraded by oxidants such as oxygen, light, and heat during the processing steps and storage conditions (Gharibzahedi, Razavi, & Mousavi, 2012a). On the other hand, the lipophilic nature of CTX led to its poor solubility and bioavailability in aqueous systems (Qian, Zhang, Pi, & Chen, 2009). One of the most surprising methods to improve chemical stability, solubility and bioavailability of many functional hydrophobic compounds such as CTX is the preparation of cyclodextrin (CD) inclusion complexes (Carlotti et al., 2008). CDs are cyclic oligosaccharides composed of (α-1,4)-linked-glucopyranose units and can be represented as a truncated cone structure with a hydrophilic outer surface and a hydrophobic cavity at their center. α-, β- and γ-CDs are the most common CDs which respectively consist of six, seven and eight glucopyranose units (Jullian et al., 2008). Wang, Zhang, Deng, and Wang (2007)

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http://dx.doi.org/10.1016/j.carbpol.2013.10.074

showed that the presence of primary and secondary hydroxyl groups to the exterior increase the water solubility of these compounds and the corresponding host-guest complexes. They reported a 50-fold increase in the water solubility of hydrophobic drugs with 2-hydroxypropyl-β-CD (HP-β-CD) in compared to β-CD (Wang et al., 2007). Although the water solubility, storage stability and antioxidant activity of inclusion complex of AST with HP-β-CD have been studied (Yuan, Du, Jin, Z& Xu, 2013; Yuan, Jin, Xu, Zhuang, & Shen, 2008), there is no specific study on the development of CTX/HP-β-CD complex and evaluation of its physicochemical and antioxidant characteristics. Thus, the objective of this study was to characterize the solubility, stability and, antioxidant and free-radical scavenging activities of HP-β-CD inclusion complexes with CTX produced by *D. natronolimnaea* HS-1 in a continuous fermentor.

2. Materials and methods

2.1. Materials, chemicals and reagents

D-glucose, yeast extract, peptone, malt extract, agar and antifoam 289 for *D. natronolimnaea* HS-1 cell growth were purchased from Sigma–Aldrich (Sigma–Aldrich Co., United States). The CTX standard and pure ethanol (99.9%, v/v) were provided by the *Bioprocess Engineering Laboratory* (BPEL, University of Tehran, Iran) and Bidestan Co. (Qazvin, Iran), respectively. Beet molasses was purchased from the Marvdasht Sugar Industry (Fars Province, Iran).

Sodium nitrite ($NaNO_2$), ferric chloride ($FeCl_3$), potassium ferricyanide ($K_3Fe(CN)_6$), trichloroacetic acid (TCA), ferrous sulfate, sodium phosphate, invertase enzyme, NaOH, HCl, H_2O_2, NaCl, Griess reagent, methanol (MeOH), MeOH (HPLC grade), dichloromethane (HPLC grade) and acetonitrile (HPLC grade) were supplied from Merck Chemical Co. (Darmstadt, Germany). HP-β-CD, butylated hydroxyanisole (BHA), 1,1-Diphenyl-2-picrylhydrazyl (DPPH$^{\bullet}$), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2′-azinobis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS$^{\bullet+}$), phenanthroline and potassium persulphate ($K_2S_2O_8$) were provided from Sigma–Aldrich (Oakville, ON, Canada). Citric acid was purchased from Gharbgostar Chemical Industries (Kermanshah, Iran). Vitamin C (VC) was also obtained by Fisher Scientific (Ottawa, Ontario, Canada).

2.2. Microorganism and feed medium preparation

In this work, the bacterium strain of *D. natronolimnaea* HS-1 was obtained from the BPEL. This strain was kept on yeast/malt (YM) agar plates containing 10 (g/l) D-glucose, 5 (g/l) peptone, 5 (g/l) yeast extract, 3 (g/l) malt extract, and 15 (g/l) agar. Single colonies every month were transferred to a fresh plate for 4 days incubation period, and then maintained under cold-storage (4 °C) conditions (Gharibzahedi, Razavi, & Mousavi, 2012a). After the preparing preculture in liquid YM medium (i.e., the above formulation without agar added), the inoculum was transferred into Erlenmeyer flasks containing 26.16 g/l beet molasses hydrolyzed by invertase (0.62%, w/v) and 10 g/l yeast extract. The flasks to produce CTX were finally incubated at 180 rpm and 28 ± 1 °C for 7 days in an orbital shaking incubator (Model JEIO TEC SI-4000R, Korea) to inoculate a continuous fermenter.

2.3. Continuous culture setup

The culture experiments for CTX production were conducted in a 2.5 l bioreactor with a 1.5 l of fermentation medium. The pH-value during the culture fermentation was controlled at 7.0 using 2 M HCl and 2 M NaOH solutions. The bioreactor was operated isothermally at 29 ± 1 °C, with a stirring rate of 130 ± 15 rpm and an aeration rate of 3 vvm. Foam was controlled by the addition of 25% antifoam 289 and cultivation was started using a 15% (v/v) inoculum. (Gharibzahedi, Razavi, & Mousavi, 2013c). The continuous fermentation strategy was as follows: operation was initially conducted in batch mode until a significant amount of biomass was produced, after which the feed medium was inserted into the bioreactor in continuous mode with a dilution rate of 0.14 h^{-1}. The concentration of enzymatic hydrolyzed molasses (EHM) in both initial and feed media was 45.90 g/l. The culture volume during the continuous fermentation process was kept constant by shifting out fermentation broth and adding fresh medium at the same flow rate. A constant volume of sample was withdrawn from the culture for analysis.

2.4. Carotenoid pigment extraction

Ten milliliters aliquots of culture were taken and centrifuged at 7500 × *g* for 7.5 min at 4 °C and thus collected the obtained supernatant. In the next step, the cell pellets were washed twice with physiological water (NaCl; 9 g/l in deionized water) and centrifuged. These cells were re-suspended three times in ethanol (3 ml) by vortexing for 5 min and centrifuged again to extract the synthesized pigment. A water bath (45 ± 2 °C) was used to completely extract the pigments (Gharibzahedi, Razavi, Mousavi, & Moayedi, 2012b). Subsequently, the carotenoid extracts containing CTX in order to get culture filtrate passed through a 0.2 μm hydrophobic fluorophore membrane (Sigma–Aldrich Co., USA).

2.5. Preparation of CTX/HP-β-CD complexes

The inclusion complexes were prepared by dissolving CTX (0.5 mM) and HP-β-CD (0.5 mM) in ethanol (20 ml). The mixture was sealed under a nitrogen atmosphere and stirred for 24 h. It was then sonicated in an ultrasonic bath (30 kHz, 100 W, SAIRAN Micro10sonic, Iran) for 5 min to thoroughly blend the mixture. After the removing ethanol using a vacuum-rotary evaporator at 30 °C (model VV2000, Heidolph, Schwabach, Germany), the residue was dissolved in water (50 ml) and filtered. The orange filtrate was firstly frozen at −40 °C for 24 h and then lyophilized and the resultant powers collected as the complex of CTX-HP-β-CD.

2.6. Analytical methods

2.6.1. Canthaxanthin analysis

A Knauer (Berlin, Germany) HPLC system including a k-1001 HPLC pump, a k-1001 solvent organizer, an on-line degasser, a dynamic mixing chamber and a UV–vis detector (K-2600, Knauer, Germany) was applied for the evaluation of individual carotenoids and CTX amount based on the modified method of Razavi, Blanchard, and Marc (2006). The separation was performed on a Lichrospher 100 RP-18 silica column (5.0 mm, 250 mm × 4 mm) at 35 °C. The acetonitrile/MeOH/dichloromethane solvent mixture (71:22:7, v/v/v) was used as isocratic mobile phase at a flow rate of 2 ml/min. The volume of solutions injected was 10 μl. A pre-column of the same material to protect the column was also used.

2.6.2. Phase solubility measurement

The phase solubility was measured according method explained by Higuchi and Connors (1965) with minor modifications. In brief, aqueous solutions of HP-β-CD at different concentrations from 0 to 12 mM in 100 mM phosphate buffer (pH 7.4) were prepared and then excess amount of CTX added to these solutions. The flasks were covered with aluminum foil to minimize the photochemical degradation. The solutions were sonicated in an ultrasonic bath (30 kHz, 100 W, SAIRAN Micro10sonic, Iran) at 25 °C for 1 h and

then equilibrated for 48 h. In the next step, the aqueous solutions were centrifuged at 13,000 rpm for 10 min. The obtained supernatants were filtered through 0.2 μm membrane filters to remove un-dissolved solids and analyzed using an UV/vis spectrophotometer (Jasco, V-630, Tokyo, Japan). The stability constant (K_c) of the CTX/HP-β-CD complexed was determined from the slope and intercept of the straight line (so, the CTX solubility of at 25 °C in the absence of HP-β-CD) of the phase solubility diagram based on the equation (Eq. (1)):

$$K_c = \left(\frac{\text{slope}}{S_0(1-\text{slope})}\right) \quad (1)$$

2.6.3. Stability determination

The inclusion complex of CTX/HP-β-CD and CTX were dissolved in water and ethanol, respectively. The samples were separately transferred into opaque glass bottles, gently flushed with nitrogen gas, capped and stored in the dark at 25 and 4 °C for 28 days. Absorbance amount of the samples was daily determined at 474 nm during one month of storage.

2.6.4. Antioxidant and free-radical scavenging activities

2.6.4.1. Reducing power assay. The modified method of Oyaizu (1986) was applied to investigate the reducing power (RP) of the CTX/HP-β-CD complex and native CTX. The different concentrations of the samples (20–220 μg/ml) with 2.0 ml sodium phosphate buffer (0.2 M, pH 6.6) and 2.0 ml of $K_3Fe(CN)_6$ (1%, w/v) were mixed. In the next step, the mixtures were incubated at 50 °C for 20 min and rapidly cooled. 2.0 ml of 10% TCA solution was added to the mixture. 2.0 ml of upper layer of the solution was taken out and mixed with 2.0 ml of deionized water and 0.4 ml of $FeCl_3$ (0.1% w/v). The absorbance after 10 min reaction was measured using an UV–vis spectrophotometer (Jasco, V-630, Tokyo, Japan) at 700 nm. VC and BHA were used as positive controls.

2.6.4.2. Nitrite scavenging activity assay. Nitrite scavenging activities (NSAs) of the CTX/HP-β-CD complex and native CTX were measured based on the applied method by Saha, Lajis, and Israf (2004) with small modifications. In brief, the samples were diluted with the distilled water for reaching to a appropriate concentration. 2.0 ml of citric acid buffer (pH 3.0) and 0.1 ml of 200 μg/ml $NaNO_2$ were added to a 10 ml-tube containing 3.0 ml of each sample. In the next step, water was transferred to the tube up to 10 ml and was immediately incubated for 60 min in the water bath at 37 °C. Equal volume of Griess reagent was then added to the obtained mixture and the absorbance (OD_p) after 10 min was measured using an UV–vis spectrophotometer (Jasco, V-630, Tokyo, Japan) at 538 nm. Also, the absorbance of the standard (without $NaNO_2$ and without sample (OD_s)) and control (without $NaNO_2$ (OD_c)) were also determined. VC and BHA were applied as the positive control compounds. The NSAs of two samples were calcultaed as follows (Eq. (2)):

$$\text{NSA} = \left(\frac{OD_s - (OD_p - OD_c)}{OD_s}\right) \times 100\% \quad (2)$$

2.6.4.3. DPPH radical-scavenging capacity. DPPH free-radical scavenging by the CTX/HP-β-CD complex and CTX was evaluated according to the procedure described by Wang, Li, Zeng, and Liu (2008). Briefly, DPPH solutions (2.0 ml) in ethanol (2×10^{-4} mol/l) and 2.0 ml of tested samples with various concentrations were mixed in the tubes. Then, the mixture was incubated for 60 min in the dark at 24 ± 1 °C. The absorbance decrease was recorded against ethanol using an UV/vis spectrophotometer (Jasco, V-630, Tokyo, Japan) at 517 nm. VC and BHA were applied as the positive control compounds. The scavenging activities of two samples on DPPH• were calculated using the following equation (Eq. (3)):

$$\text{SA}(\text{DPPH}_r^{\bullet}) = \left(\frac{A_0 - (A_i - A_j)}{A_0}\right) \times 100\% \quad (3)$$

where $\text{SA}(\text{DPPH}_r^{\bullet})$ is scavenging activity of the studied samples (%), A_0 is the absorbance of 2.0 ml DPPH solution and 2.0 ml sample solvent, A_i is the absorbance of 2.0 ml DPPH solution and 2.0 ml sample solution and A_j is the absorbance of 2.0 ml sample solution and 2.0 ml ethanol.

2.6.4.4. Hydroxyl radical scavenging activity. The hydroxyl radicals were generated by the Fenton reaction to analysis the hydroxyl radical scavenging activity (HRSA). 1.5 ml phenanthroline (5 mM) was added to the mixture of 2.5 ml sample solution, 4.0 ml phosphate buffer (0.75 M, pH 7.4) and 1.0 ml ferrous sulfate (7.5 mM). After the vortexing, 1.0 ml H_2O_2 (1.0%, w/v) was added and then incubated at 37 °C for 1 h in a water bath. The absorbance of the resulting solution (A_x) was reordered using an UV/vis spectrophotometer (Jasco, V-630, Tokyo, Japan) at 536 nm. The absorbance (A_s) after replacing the sample solution with deionized water was also measured. Both the sample solution and H_2O_2 were finally replaced by the same volume of deionized water, and the absorbance (A_0) was obtained (Yuan et al., 2013). VC and BHA were used as positive controls. The HRSAs of CTX/HP-β-CD complex and CTX were calculated as follows (Eq. (4)):

$$\text{HRSA} = \left(\frac{(A_0 - A_x)}{A_0}\right) \times 100\% \quad (4)$$

2.6.4.5. $ABTS^{\bullet+}$ radical scavenging capacity. The Trolox equivalent antioxidant capacity (TEAC) was determined using $ABTS^{\bullet+}$ radical cation decolorization assay as described by Debnath et al. (2012) with minor modifications. In this experiment, $ABTS^{\bullet+}$ was dissolved in deionized water at a final concentration of 7 mM and mixed with a $K_2S_2O_8$ solution at a final concentration of 2.45 mM. The reaction mixture was left to stand in the dark at room temperature for 12–16 h. Before use, the prepared $ABTS^{\bullet+}$ solution was diluted with sodium phosphate buffer (pH 7.40) and equilibrated to 30 °C in order to obtain an absorbance of 0.7 ± 0.02 at 734 nm. For measuring antioxidant capacity, 0.1 ml of various concentrations of the HP-β-CD complex and CTX (0.1–2.2 mg/ml) or standard compounds (BHA and VC, 0.03–0.10 mg/ml) was mixed with 0.9 ml of $ABTS^{\bullet+}$ solution. After incubation at room temperature, the absorbance was estimated using an UV/vis spectrophotometer (Jasco, V-630, Tokyo, Japan) at 734 nm. The $ABTS^{\bullet+}$ scavenging activity was determined using the following equation (Eq. (5)):

$$ABTS^{\bullet+} \text{ scavenging activity } (\%) = \left(1 - \frac{(a-b)}{(c-d)}\right) \times 100 \quad (5)$$

where a is absorbance of ($ABTS^{\bullet+}$ solution + sample/standard), b is absorbance of ($K_2S_2O_8$ + sample/standard), c is absorbance of ($ABTS^{\bullet+}$ solution + deionized water/ethanol) and d is ($K_2S_2O_8$ + deionized water/ethanol).

2.7. Statistical analysis

All analytical experiments were carried out in three replicates and the results presented as a mean ± standard deviation (SD). Analysis of variance (ANOVA) procedure followed by Duncan's test using SPSS 13 (SPSS Inc., Chicago, Illinois, USA) software.

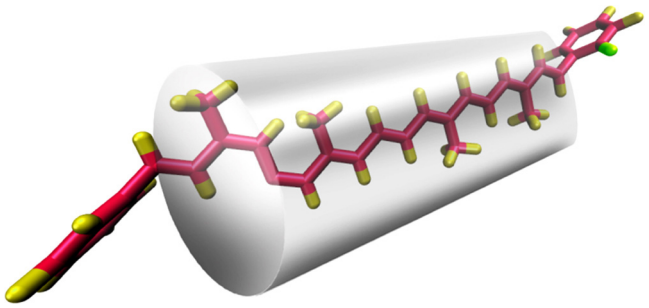

Fig. 1. Proposed model for CTX/HP-β-CD complex.

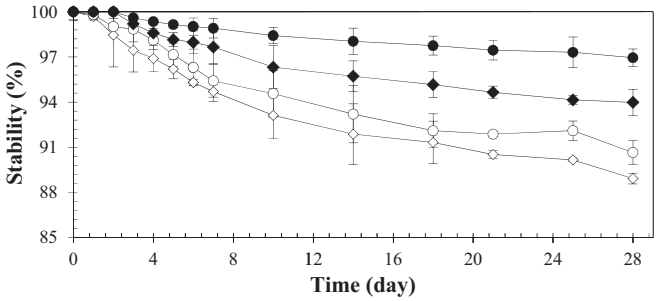

Fig. 2. Storage stability of native CTX (white symbol) and CTX/HP-β-CD complex (black symbol) at 4 (● and ○) and 25 °C (◊ and ♦).

3. Results and discussion

3.1. Phase solubility and stability constant

A quantitative stoichiometric analysis of the inclusion complexation of CTX in HP-β-CD was performed based on the described method by Higuchi and Connors (1965). The phase-solubility diagram is a widely useful technique for evaluation of the inclusion effect of CDs complexation with poorly water-soluble drugs and antioxidants and also determination of the stability constant (K_c) and thermodynamic parameters involved in the complexes formation (Davis & Brewster, 2004). The 1:1 CTX/HP-β-CD complex is an association-typical structure as a single CTX molecule place within the cavity of a HP-β-CD (Fig. 1), with a K_c for the equilibrium between the free and associated species. The phase-solubility diagram of natural CTX with HP-β-CD at 25 °C showed that the aqueous solubility of CTX increased linearly as a function of HP-β-CD concentration, feature of A_L-type complexes, showing formation of a water-soluble complex, although slopes lower than unity can be indicative of 1:1 stoichiometry. Correlation coefficient squared value (r^2) for the solubility curve was 0.987. Regression equation was as follows:

$$Y = 0.3268X + 0.725 \quad (6)$$

where Y is the CTX concentration (mM) and X is the HP-β-CD concentration (mM).

The calculated constant for apparent stability of the CTX/HP-β-CD complex was 669 M^{-1}, which indicated that the interactions between this natural pigment and HP-β-CD were very strong. The better protective microenvironment by the HP-β-CD cavity can be attributed to the opening enlargement of native β-CD and destruction of the strong intramolecular hydrogen bond network by the hydroxypropyl substitutions which lets guest molecules access the HP-CD cavity easily and give a higher stability constant (Brewster & Loftsson, 2007). Several researchers also reported the 1:1 stoichiometry of the complex of HP-β-CD with tanshinone IIA (Ling, Xuehua, Weijuan, & Chenrui, 2007), vanillin (Karathanos, Mourtzinos, Yannakopoulou, & Andrikopoulos, 2007), quercetin (Jullian, Moyano, Yanez, & Olea-Azar, 2007), caffeic acid (Zhang, Li, Zhang, & Chao, 2009), sulforaphane (Wu, Liang, Yuan, Wang, & Yan, 2010), and *trans*-ferulic acid (Wang, Cao, Sun, & Wang, 2011). A 9-fold increase in the CTX solubility in deionized water with the presence of 12.0 mM HP-β-CD was observed. Pfitzner, Francz, and Biesalski (2000) and Yuan et al. (2008) previously showed that the aqueous solubility of carotenoid pigments was highly enhanced with the formation of carotenoids/CD complexes.

3.2. Storage stability

In the previous work, we found that CTX biosynthesized by *D. natronolimnaea* HS-1 at a continuous bioreactor possessed strong antioxidant and free radical-scavenging activities (Gharibzahedi et al., 2013a). Yuan et al. (2013) showed that this potential is depended on the storage stability. The decomposition rates of free CTX and CTX/HP-β-CD complex at two temperatures of 4 and 25 °C are shown in Fig. 2. As considered in this figure, the complexation of CTX with HP-β-CD can effectively protect it against the pro-oxidants. Temperature also had a pronounced effect on the decomposition rate of CTX of the complex. The CTX stability for CTX/HP-β-CD complex at 4 and 25 °C was 96.95 and 93.98%, respectively. However, the decomposition rate for the native CTX at 4 and 25 °C respectively was 9.35 and 11.08% after 28 days of storage. In general, the complexation CTX with HP-β-CD can be helpful to improve its stability under storage conditions. A physiological, water-soluble complex of carotenoids (lycopene, β-carotene, lutein and zeaxanthin) with methyl-β-CD for the purpose of cell supplementation was developed by Pfitzner et al. (2000). These researchers found that the different carotenoid/methyl-β-CD complex solutions under cell culture conditions had higher stability than the uncomplex carotenoids. For low decomposition rate of the guest molecule in HP-β-CD can assume that when CTX was enclosed in the hydrophobic cavity, it experienced an apolar environment that should reduce the photolytic reaction. On the other hand, the small protective effect could be due to some screening effects of CD particles for the passage of light and free radicals to reach the guest molecule (Bayomi, Abanumay, & Al-Angary, 2002). The increase of CTX degradation by the increasing storage can be attributed to the high presence of free radicals and singlet-oxygen in the environment (Gharibzahedi et al., 2013a). The stability reduction of CTX in the CTX/HP-β-CD inclusion complex by promoting the storage temperature might be also due to the increase of mass transfer coefficient of CTX in aqueous solution and the diffusion enhancement of this pigment from the surface and cavities of HP-β-CD. Similar temperature effect on the stability of bioactive compounds complexed with β-CD was previously reported (Karathanos et al., 2007; Tommasini et al., 2004; Yuan et al., 2013). Moreover, it was demonstrated that the encapsulation of nutraceutical antioxidants in *Hypericum perforatum* extract such as epicatechin, catechin and euercetin in β-CD can improve their thermal stability (Kalogeropoulos, Yannakopoulou, Gioxari, Chiou, & Makris, 2010).

3.3. Antioxidant capacity

The RP is a sensible assay for the reflection of antioxidant activity of a natural chemical compound. The reduction of the Fe^{3+}/ferricyanide complex to the ferrous (Fe^{2+}) form by the presence of antioxidants can spectrophotometrically monitor by recording the absorbance at 700 nm as higher value of absorbance for the reaction compound represents stronger RP (Yuan et al., 2013). Fig. 3a compares the RP values of CTX and CTX/HP-β-CD complex with the positive control compounds (VC and BHA) by the increasing concentration in the studied range. BHA due to its

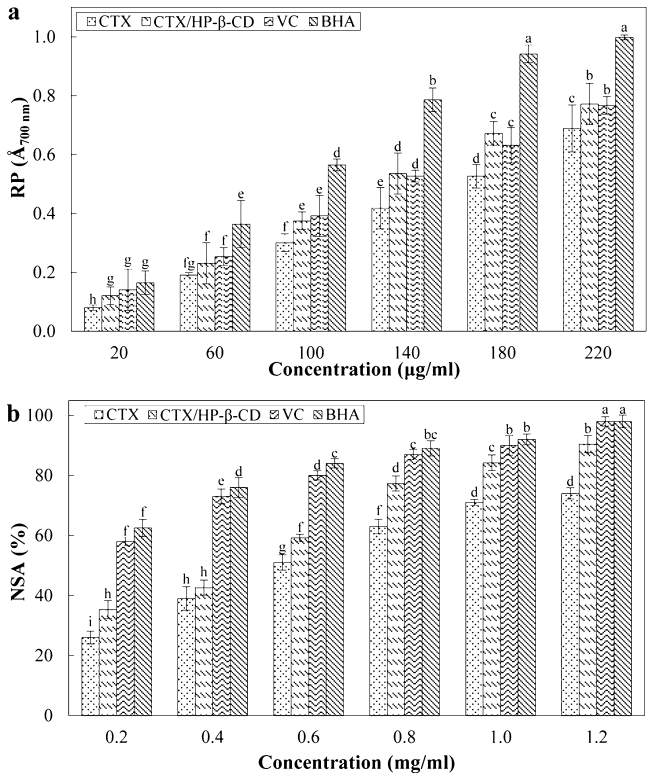

Fig. 3. RP (a) and NSA (b) activities of the CTX, CTX/HP-β-CD complex and positive control compounds (VC and BHA).

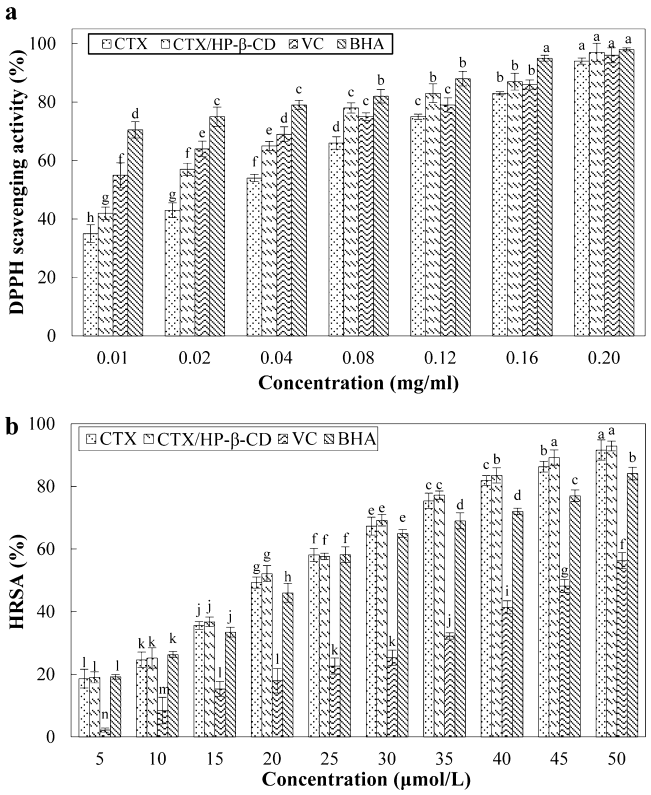

Fig. 4. DPPH (a) and hydroxyl (b) radical scavenging activities of the CTX, CTX/HP-β-CD complex and positive control compounds (VC and BHA).

more hydrogen-donating ability had the highest RP value among the other compounds ($p < 0.05$). Although the RP value of CTX was lower than CTX-HP-β-CD complex ($p < 0.05$), but the RP activities of both CTX and CTX-HP-β-CD complex increased with concentration up to 8.6- and 6.3-fold, respectively. Since CTX is weakly soluble and assembled in water, the assembled CTX molecules could not fully close and react with the Fe^{3+}/ferricyanide ions in the test (Gharibzahedi et al., 2013a). However, the biosynthesized pigment by *D. natronolimnaea* HS-1 at high concentrations had a favorable potential to reduce Fe^{3+}/ferricyanide ions. It is very interest that no significant differences were found in the RP values between the CTX/HP-β-CD complex and VC at the same concentration (Fig. 3a). This fact can be probably attributed to the higher solubility of lipophilic molecules of CTX in the complex structure due to the formation of strong intermolecular hydrogen bonding (Ling et al., 2007).

Nitrosamine as a pro-carcinogenic substance is produced via the nitrite adding to meat products and its reaction with amine compounds in the processing of these products (Gharibzahedi et al., 2013a; Lee, Kim, Jeong, & Park, 2006). In this study, we found that the CTX/HP-β-CD complex because of the effective scavenging of nitrosamine or nitrite can introduce as a functional ingredient for adding to the different drugs and foods. An increase dose-dependent manner was observed for the NSAs of the CTX, CTX/HP-β-CD complex, VC and BHA (Fig. 3b). Inhibition of the nitrosamines formation by CTX can be possibly because of the hydrogens donating ability from its hydroxyl groups to nitrite molecule ($NO_2^- + 16H^+ + 12e^- \leftrightarrow 2NH_4^+ + 4H_2O$). Comparison of the antioxidant activities of CTX and CTX/HP-β-CD complex at a concentration from 0.20 to 1.20 mg/ml demonstrated that the CTX/HP-β-CD complex was better able than CTX to scavenge the nitrosamine/nitrite ($p < 0.05$; Fig. 3b). Alvarez-Parrilla, de La Rosa, Torres-Rivas, Rodrigo-Gracia, and González-Aguiar (2005) investigated the complexation of β-CD with polyphenols present in apple such as chlorogenic acid, quercetin and rutin with evidence of increase in their antioxidant capacity.

Evaluation of scavenging activity of DPPH free radical is one of the most common procedures in order to determine the antioxidant capacities of various compounds. It is based on the loss of color when the unpaired electron of the nitrogen atom in the DPPH radical is reduced by receiving a hydrogen atom from an antioxidant compound (Krings & Berger, 2001). In this condition, the color change of the solution from purple to yellow can be spectrophotometrically quantified from the changes in absorbance at 517 nm (Gharibzahedi et al., 2013a). As shown in Fig. 4a, the effect of CTX and CTX/HP-β-CD complex on DPPH discoloration was dose-dependent from 0.01 to 0.20 mg/ml. The percentage of DPPH radical scavenging was increased by increasing the concentration of native CTX and its complex with HP-β-CD (Fig. 4a; $p < 0.05$). Yuan et al. (2013) found the similar results for ASX and ASX/HP-β-CD complex. DPPH free-radical quenching property by the CTX is due to the chemical nature of this xanthophyll pigment and its hydrogen donating ability (Krings & Berger, 2001). Overall, the inclusion complex of CTX/HP-β-CD had the higher antioxidant ability than free CTX to scavenge DPPH free radical (Fig. 4a). Two reasons for this fact can be mentioned: (I) the effective stabilization of radical species in the HP-β-CD cavity (Jullian et al., 2008), and (II) the formation of a hydrogen bond between secondary hydroxyl groups of HP-β-CD and —OH on the aromatic ring of the quest molecule (Strazisar, Andrensek, & Smidovnik, 2008).

The hydroxyl radical can be generated from hydrogen peroxide and superoxide anion in the presence of metal ions such as copper or iron. It is the most reactive free radical which can react with aromatic compounds in biological systems and form a double bond in their structure and a hydroxycyclo-hexadienyl radical. The resultant radical produces a peroxyl radical by reacting with oxygen or disintegrates to phenoxyl-type radicals by water elimination (Gharibzahedi et al., 2013a). A concentration-dependent

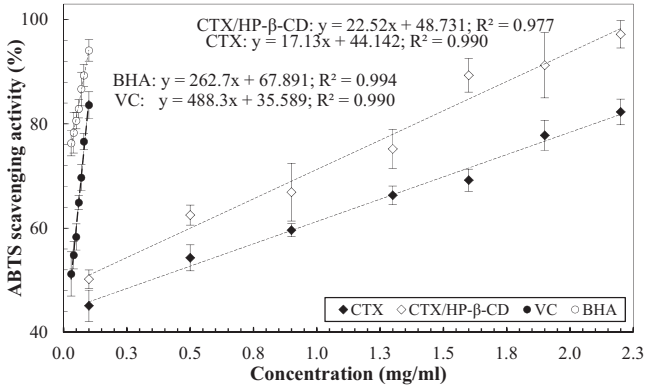

Fig. 5. ABTS radical scavenging activity of the various concentrations of CTX, CTX/HP-β-CD complex and positive control compounds (VC and BHA).

pattern was found for the HRSA percentage by CTX and CTX/HP-β-CD inclusion complex (Fig. 4b). The HRSAs of all samples were surprisingly more than VC and BHA ($p < 0.05$) due to the combined effects of reducing power, donation of hydrogen atoms and scavenging of active oxygen (Wang et al., 2008). The HRSA investigation of AST also showed that this pigment had a higher HRSA than VC at a same concentration (Yuan et al., 2013). Higher HRSA values of CTX/HP-β-CD inclusion complex in compared to the CTX (Fig. 4b) may be an outcome of the protection from the rapid oxidation by hydroxyl free radicals conferred by the HP-β-CD.

Fig. 5 compares ABTS-radical scavenging activity of the various concentrations of CTX and CTX/HP-β-CD complex with positive controls of VC and BHA. As considered in this figure, the used control compounds had the higher antiradical activity than the carotenoid pigment and its HP-β-CD complex. The levels of $ABTS^{\bullet+}$ scavenging for VC and BHA at a low concentration about 0.10 mg/ml respectively were 83.6 and 94.1%. However, a $ABTS^{\bullet+}$ scavenging activity of 82.3 and 97.2% was respectively obtained for the CTX and CTX/HP-β-CD complex at a concentration of 2.2 mg/ml. Mortesen et al. (1997) pointed out that the presence of functional groups on the terminal rings in xanthophylls structure such as CTX could be a reasonable explanation for modulating the $ABTS^{\bullet+}$ scavenging activity. An electron-withdrawing effect by the carbonyl oxygen atom can reduce the unpaired electron density in the conjugated systems, which led to a higher energy of the intermediate and the transition states for the reactions with free radicals. It was demonstrated that HP-β-CD as a secondary antioxidant can enhance the $ABTS^{\bullet+}$ antiradical capacity of the biological system (Nunez-Delicado, Sanchez-Ferrer, & Garcia-Carmona, 1997). This increases the half-life of natural antioxidants in food and extends the keeping time of food.

4. Conclusion

In this work, HP-β-CD as a successful carrier for the incorporation of CTX extracted from *D. natronolimnaea* HS-1 was applied. The complexed form of CTX in a molar ratio of 1:1 with HP-β-CD is more resistant than free CTX to the damage caused by oxidative stresses and reveals improved water-solubility for stable innovative formulations of food or pharmaceutical materials. Further studies in order to confirm the stable inclusion complexes between the HP-β-CD and CTX using proton nuclear magneticresonance (H-NMR) spectroscopy, differential scanning calorimetry (DSC) and X-ray diffraction (XRD) are needed.

Acknowledgments

The authors gratefully acknowledge the financial support from the University of Tehran and Iranian Center of Excellence for Application of Modern Technologies for producing functional foods and drinks. Thanks are also due to Dr. Mohammad Reza Gharib-Zahedi (Department of Chemistry, Sharif University of Technology) for providing his valuable guidance and technical assistance.

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