J. Korean Soc. Appl. Biol. Chem. **53**(5), 559-565 (2010)

Article

β-CD-mediated Encapsulation Enhanced Stability and Solubility of Astaxanthin

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Received April 13, 2010; Accepted June 16, 2010

To expand the industrial applications of astaxanthin in the food and cosmetic sectors, inclusion complexes (ICs) of astaxanthin were prepared with various types of cyclodextrins (CDs) and characterized by HPLC with respect to water-solubility and stability. Also, their characteristics were determined through SEM scanning electron microscopy and Furier transform infrared spectrometry. Among the various CDs, β-CD was selected as the host material for the inclusion of astaxanthin. The optimum ratio of astaxanthin and β-CD for IC formation was determined as 1:200. The water-solubility of the IC of astaxanthin and β-CD (As-β-CD) was enhanced up to 110-fold compared to free astaxanthin at pH 6.5 and 25°C. The stability of the β-CD-encapsulated astaxanthin against heat, light, and oxidation was greatly enhanced by over 7-9 folds compared to free astaxanthin. Furthermore, the β-CD-encapsulated astaxanthin had high stability even at 100°C. Compared to free astaxanthin, the IC formation of free astaxanthin greatly enhanced its stability under various storage conditions such as pH, temperature, ultraviolet irradiation, and the presence of oxygen.

Key words: astaxanthin, β-cyclodextrin, inclusion complex, solubility, stability

Reactive oxygen species and free radicals are produced in living bodies during normal metabolic processes, and their production can be accelerated by air pollution, smoke, physiological stress, exposure to ultraviolet (UV) light among others. Reactive oxygen species and free radicals are associated with the aging process and the initiation of many diseases [Guerin *et al*., 2003]. Natural antioxidants such as carotenoids may be used to help the human body reduce oxidative damage. Astaxanthin (3,3'-dihydroxy-β,β'-carotene-4,4'-dione) has a similar structure to β-carotene as a keto-carotenoid found in marine organisms such as crustaceans and algae [Shimidzu *et al*., 1996]. Astaxanthin is related to other well-known carotenoids such as β-carotene, zeaxanthin, and lutein. Its antioxidant activity is equivalent to about 550 times that of á-tocopherol [Shimidzu *et al*., 1996]. Its powerful antioxidant activity is related to its structure with 11 conjugated carbon-carbon double bonds and hydroxyl and keto endings. In addition, various biological activities such as immuno-modulatory activity [Bendich, 1991], antitumor effects [Jyonouchi *et al*., 2000], and eyesight protection [Snodderly, 1995] have been reported for astaxanthin. Despite its biological activities, astaxanthin has not been widely applied in practical use. Because astaxanthin is an unsaturated compound with double bonds, it is easily damaged by heat, light, and oxidation during production and storage, causing the loss of its biological activity [Chen *et al*., 2007]. Furthermore, the poor aqueous solubility of astaxanthin considerably limits its use in nutraceutical and cosmetic applications.

There have been few studies on improving the stability and solubility of astaxanthin. Chitosan was used as an additive to increase the stability of astaxanthin by the formation of microcapsules [Yun *et al*., 2003]. Higuera-Ciapara *et al.* [2004] and Kittikaiwan *et al.* [2007] developed

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doi:10.3839/jksabc.2010.086

physiological and stable capsules of astaxanthin with chitosan. It was also reported that astaxanthin was stabilized by microencapsulation using latex [Lee, 2003]. Up until now, the use of encapsulation technology for astaxanthin has been in its early stages. It is desirable to simultaneously increase the stability and solubility of astaxanthin for nutraceutical and cosmetic uses.

Inclusion complex (IC) formation has been recognized as a method to increase the stability and the solubility of ingredients in food and medicine [Lee *et al*., 1988; Kim *et al*., 1992; Lee *et al*., 1994; Lee *et al*., 2002; Mele *et al*., 2002; Chun *et al*., 2003; Kim *et al*., 2003; Polyakov *et al*., 2004; Seo and Kim, 2004; Lyng *et al*., 2005; Liu and Zhu, 2006; Yang *et al*., 2006; Chen *et al*., 2007; Jullian *et al*., 2007; Karathanos *et al*., 2007]. Cyclodextrin (CD) has been widely used to prepare inclusion complexes as a host molecule [Lee *et al*., 1988; Kim *et al*., 1992; Lee *et al*., 1994; Lee *et al*., 2002; Mele *et al*., 2002; Chun *et al*., 2003; Kim *et al*., 2003; Polyakov *et al*., 2004; Seo and Kim, 2004; Lyng *et al*., 2005; Liu and Zhu, 2006; Yang *et al*., 2006; Jullian *et al*., 2007; Karathanos *et al*., 2007]. Since CD has a lipophilic central cavity and a hydrophilic surface, it can encapsulate lipophilic materials. Several studies on the inclusion of carotenoids using various types of CDs have been performed. Previous studies indicate that the physical and chemical properties of lipophilic pigments can be improved by β-CD-mediated IC formation [Mele *et al*., 2002; Polyakov *et al*., 2004; Chen *et al*., 2007]. Chen *et al.* [2007] prepared water-soluble and stable ICs of astaxanthin using β-CD. However, quantitative analysis on the solubility of astaxanthin has not been carried out. In earlier literature [Chen *et al*., 2007], the solubility of astaxanthin was only evaluated with the naked eye to determine whether it would or would not dissolve under specific conditions. However, this approach was not suitable for the quantitative analysis of astaxanthin, through an absorbance measurement over a short time. The IC formation of astaxanthin using hydroxypropyl-β-CD (HP-β-CD) was previously investigated by Yuan *et al.* [2008]. While astaxanthin degraded completely within 32 h at 50°C under oxygen and light, the astaxanthin in the IC of HP-β-CD was protected from light and oxygen. HP-β-CD is known as a highly water soluble derivative. However, its inclusion efficiency is lower than β-CD and is very expensive [Cho *et al*., 2006; Yuan *et al*., 2008]. Thus its industrial applications are limited. In this work, to expand industrial applications of astaxanthin in the food and cosmetic sectors, ICs of astaxanthin were prepared with various types of CDs and characterized by HPLC with respect to water-solubility and stability. The characteristics of the ICs were also determined through SEM and FT-IR spectrometry. In addition, the water-solubility and stability of the β-CD encapsulated astaxanthin were quantitatively examined at various conditions such as temperatures, pH, light, and oxidation. We report that both the aqueous solubility and stability of astaxanthin were significantly improved by β-CD-mediated IC formation.

Materials and Methods

Materials and reagents. Astaxanthin, 2-hydroxypropyl-β-cyclodextrin (HP-β-CD), and 2-hydroxypropyl-γ-cyclodextrin (HP-γ-CD) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Alpha-cyclodextrin (α-CD), β-cyclodextrin (β-CD), and γ-cyclodextrin (γ-CD) were obtained from Wako (Osaka, Japan). Industrial grade β-cyclodextrin (Cyclo B) was purchased from Corn Products (Westchester, IL, USA). HPLC-grade methanol and acetonitrile were obtained from Burdick & Jackson (Muskegon, MI, USA). All other chemicals and regents were of analytical grade.

Preparation of the inclusion complexes of astaxanthin with cyclodextrins. The astaxanthin (98%, 10 mg) was dissolved in dichloromethane : acetone (3:1, v/v, 20 mL), and each cyclodextrin (2 g) was dissolved in distilled water (20 mL). The astaxanthin solution was then added to the cyclodextrin solution. The mixture was magnetically stirred for 7 h at 50°C. After stirring and cooling, it was stored for 24 h at 4°C. The liquid phase in the mixture was removed through paper filtration using a Whatman (Maidstone, UK) No. 41 filter. Next, the ICs were washed three times with 5 mL of deionized water and then 5 mL of ethanol. Finally, they were freeze-dried at −80°C under vacuum. All experiments were repeated three times and the data are expressed as means±standard errors (SE).

Structural analysis of the inclusion complexes. The particle sizes of the ICs were measured using a Quanta 200 scanning electron microscope (FEI, Einhoven, Netherlands). Structural changes were observed with a FT-IR spectrometer (Spectrum GX, Perkin Elmer, CT, USA). The spectra were measured in a frequency range between 4000~400 cm^{-1}.

Chromaticity analysis of the inclusion complexes. The lightness (L), redness (a), and yellowness (b) of the ICs were measured using a colorimeter (Tri-Stimulus Colorimeter, JC-801S, Color Techno System Co., Tokyo, Japan).

Water-solubility analysis of the inclusion complexes. The ICs were dispersed in deionized water at a concentration of 4% at various pHs (2, 6.5, 8) and temperatures (4, 25, 45°C). The mixture was shaken for 1 h, and water was removed through filtration. Afterwards,

it was dissolved in methanol and analyzed by an HPLC system (Agilent 1100, Agilent Technologies Inc., CA, USA) using a reverse phase C18 column with methanol-water (95:5, v/v) solvent as the mobile phase [Kim *et al*., 2008a]. The solubility was expressed as the amount of dissolved astaxanthin per 1 mL.

Long-term stability of the inclusion complexes. The effects of temperature, pH, light, and oxidation on the stability of the ICs in aqueous solution (1%, w/v) were studied for 28 days at various conditions. The effects of pH on stability were examined in a pH range of 3-8. To evaluate the effects of light, a UV lamp (253.7 nm, 19.8 W, Sankyo Denki Co., Japan) was used as an illumination source, which irradiated at a distance of 62 cm. To evaluate the effects of oxidation, some samples were exposed to air for 28 days. Samples were taken at intervals of 6 days and analyzed using a HPLC system [Jeon, 1999]. In addition, in order to evaluate the effects of heating, inclusion complex solutions (1%, w/v) were kept at various conditions (65~100°C for 30~60 min). All test samples were dissolved in methanol and treated under an ultrasonic environment as provided by a sonicater (40 kHz; Power sonic 510, Hwashin, Korea) for 5 min. They were then analyzed using the HPLC system.

Results and Discussion

The preparation of the CD-mediated inclusion complexes. The surface morphologies and structural changes of the various ICs, which were prepared at different reaction ratios (1:1, 1:5, 1:10) of free astaxanthin to various CDs such as α-CD, β-CD, γ-CD, HP-β-CD, and HP-γ-CD, were observed using SEM and an FT-IR spectrometer, respectively. At ratios of 1:1 to 1:10, the particle sizes of the ICs were not uniform and their color was very dark red brown. Moreover, the structures of the ICs could be not determined in the FT-IR spectrum (data not shown). In this study, an inclusion was not identified up to the ratio of 1:10, as similarly described in a report by Chen *et al.* [2007]. This was probably attributable to the addition of too much astaxanthin for inclusion to occur at the ratios of 1:1-1:10. Therefore, the reaction ratios were readjusted to higher values of 1:125, 1:150, and 1:200 of guest to host molecule. As a result, ICs were identified at the ratio of 1:200. With this ratio, the particles were uniform and several specific peaks appeared in the FT-IR spectrum (data not shown).

The sizes of all materials were observed by SEM as follows: astaxanthin (20-50 μm), α-CD (70 μm), β-CD (500 μm), γ-CD (50 μm), HP-β-CD (50 μm), and HP-γ-CD (50 μm). The particle sizes of the ICs decreased compared to the original size of the CDs that were used.

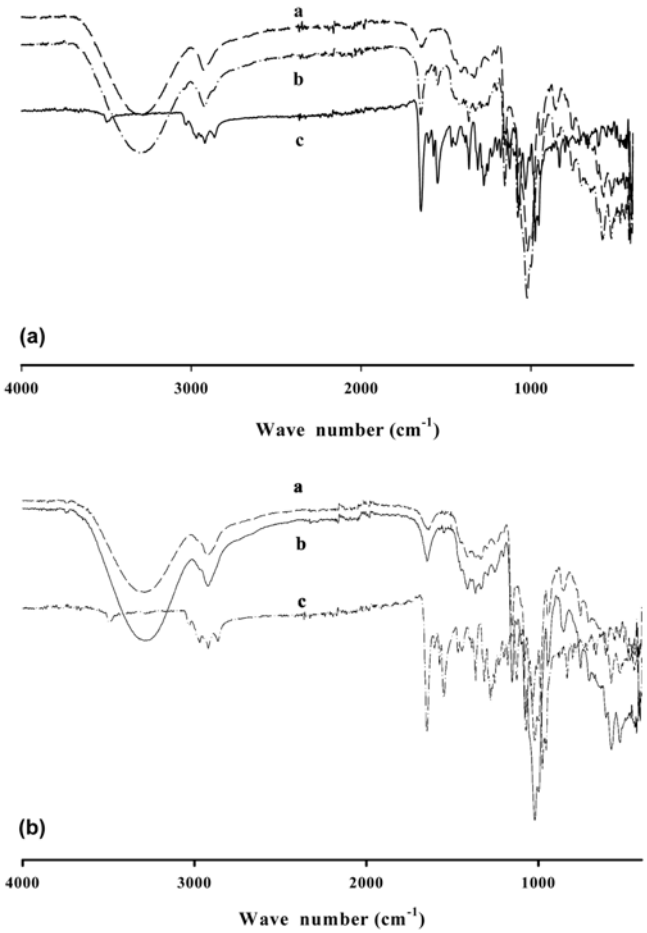

Fig. 1. FT-IR spectra for inclusion complexes of astaxanthin and β-CD (a) and industrial grade β-CD (CycloB) (b). (a) a: β-CD, b: As-β-CD, c: free astaxanthin. (b) a: industrial grade β-CD (CycloB), b: As-CycloB, c: free astaxanthin.

The results of the present study were similar to those reported by Lee *et al.* [2002], in which the size of β-CDs decreased with the addition of water and ethanol. Furthermore, compared to the host molecules, the shapes of the ICs were changed as observed by SEM, implicating that astaxanthin was included into the host molecules. The ICs formed at the ratio of 1:200 of astaxanthin and host molecule showed a uniform shape and particle size. Structural changes of IC formation were identified by FT-IR spectra to examine whether or not inclusion occurred. When α-CD and γ-CD were used as the host molecules, no structural changes were observed (data not shown). Only the ICs prepared with astaxanthin and β-CD (As-β-CD) showed characteristic peaks in the FT-IR spectra, as shown in Fig. 1(a). The peak of 1000 cm^{-1} was assigned to a C-O bond in the molecule of β-CD. The characteristic peaks of C=O and C=C bonds were observed at 1745 cm^{-1} and 1500 cm^{-1}, respectively, in the astaxanthin molecule. This indicated that the IC of

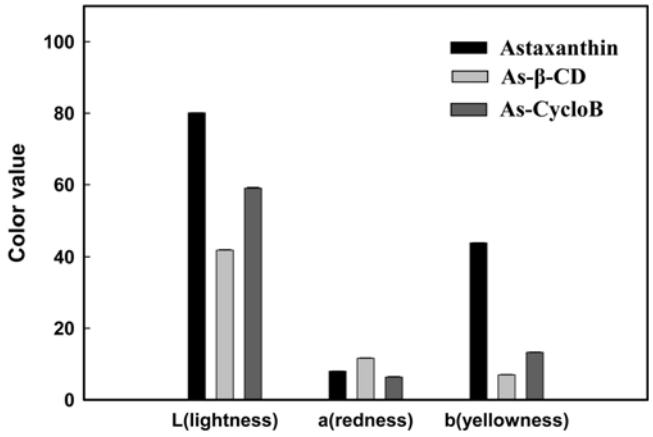

Fig. 2. Color values of free astaxanthin and inclusion complexes (As-β-CD, As-CycloB).

As-β-CD was obtained, which agrees with results reported by Chen *et al.* [2007] and Yuan *et al.* [2008]. IC formation was also identified using HP-β-CD and HP-γ-CD, but the particle color was very dark (data not shown). This indicated that astaxanthin was not included into HP-β-CD and HP-γ-CD. Through analyses by SEM and FT-IR, β-CD was found to be an optimum host material for the IC formation of astaxanthin. Moreover, the inclusion rate was over 90%, which was calculated by measuring the astaxanthin that remained after the inclusion reaction.

For the wide use of encapsulation technology, the cost of the key raw material, CD, must be considered. ICs (As-CycloB) of astaxanthin were prepared using a food grade β-CD, namely CycloB (minimum purity of 97%), which is widely used in the food and cosmetic sectors. The inclusion complexes made with CycloB showed characteristic peaks in the FT-IR spectra (Fig 1(b)). Compared to previously prepared β-CD-mediated ICs, the color of the CycloB-mediated ICs was lighter.

The chromaticity tests of free astaxanthin showed L (lightness) and b (yellowness) values of 80 and 45, respectively. After inclusion, the L values and b values of As-β-CD and As-CycloB decreased, as shown in Fig. 2, indicating as the formation of inclusion complex with β-CD and CycloB.

Water-solubility of the inclusion complex. Since astaxanthin is a hydrophobic material that is structurally associated with protein by an ester bond, it has very low solubility in aqueous solution. It was previously reported that astaxanthin is very unstable against heating, light, acidic pH, and oxidation, and has very poor solubility in water [Kim *et al*., 2008b]. Therefore, astaxanthin is mostly contained in softgel capsules. Due to its poor aqueous solubility and instability, its application as an ingredient in food and cosmetic emulsions is limited. This has been a significant technical problem for expanding the use of astaxanthin.

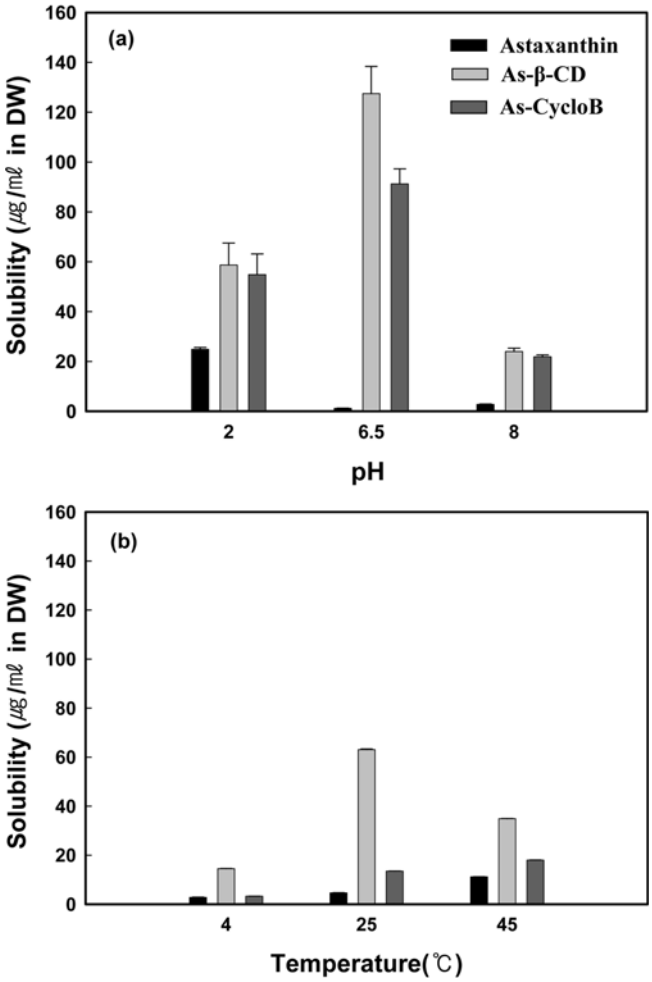

Fig. 3. Water-solubility of astaxanthin and inclusion complexes (As-β-CD, As-CycloB) at various pHs and room temperature (a) and at various temperatures at pH 6.0 (b).

In the present study, the water-solubilities of ICs (As-β-CD and As-CycloB) were investigated at various pHs and temperatures. The tests were carried out at the pH levels of most commercial beverages, such as pH 2, 6.5, and 8, as shown in Fig. 3(a). According to the results, the solubility of As-β-CD was improved up to about 100-fold that of free astaxanthin at pH 6.5, and up to 2~7 folds that of free astaxanthin at pH 2 and 8. The enhanced solubility of As-CycloB was similar to that of As-β-CD. The remarkably enhanced solubility of the ICs at pH 6.5 implicates the potential applicability of astaxanthin in milk-like beverages. More interestingly, free astaxanthin showed a higher solubility at acidic conditions (pH 2.0), while the ICs tended to have higher solubility at a neutral condition. It was previously reported that CD was hydrolyzed in acidic aqueous solution [Chung, 2000]. Therefore, it appears that partial hydrolysis of the ICs occurred under the acidic condition, and astaxanthin was

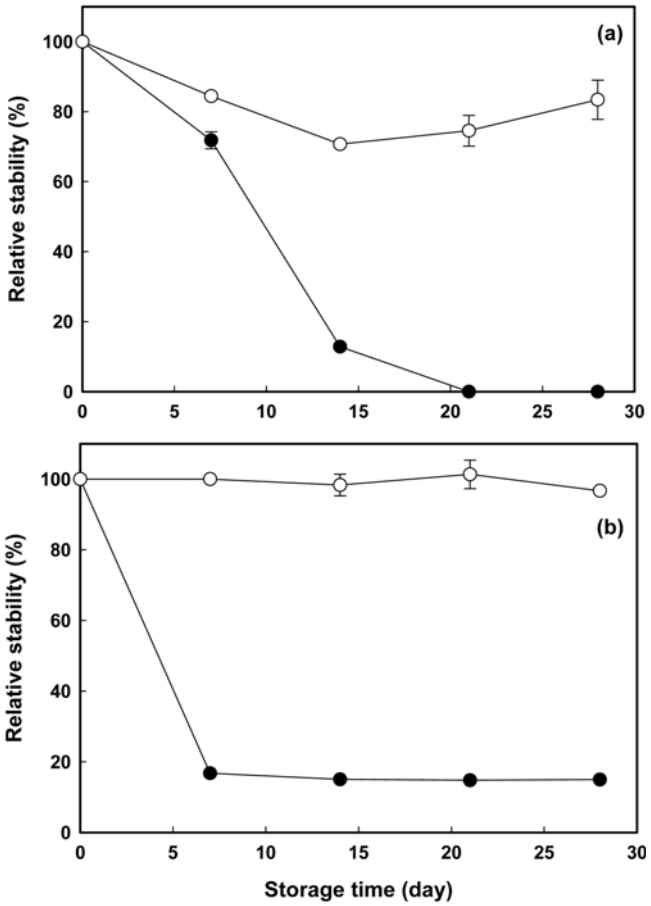

Fig. 4. Effects of UV irradiation (a) and air exposure (b) on the long-term stability of free astaxanthin (-●-) and inclusion complex As-β-CD (-○-) at room temperature.

degraded in the more acidic solution at pH 2, which was confirmed by the HPLC chromatograms (data not shown).

As shown in Fig. 3(b), the water-solubilities of the ICs were measured at various temperatures. The water-solubility of As-β-CD was 13 times higher that of free astaxanthin at 25°C. The water-solubility of As-β-CD increased up to 3-fold, compared 5-fold, compared to the case at 45 and 4°C, respectively. In the case of As-CycloB, the results were similar to those of As-β-CD. The solubility of free astaxanthin increased as a function of temperature in a range of 25 to 45°C, but the solubility of As-β-CD tended to decrease at 45°C. It seems that the when the ICs were dispersed in water at 45°C, astaxanthin was separated out of the ICs and then degraded in the aqueous condition of 45°C. Chen *et al.* [2007] reported that the aqueous solubility of ICs was slightly enhanced at room temperature (<0.5 mg/mL).

The stability of the inclusion complex. The effects of light, pH, and heat on the stability of As-β-CD were investigated to determine whether stability was improved

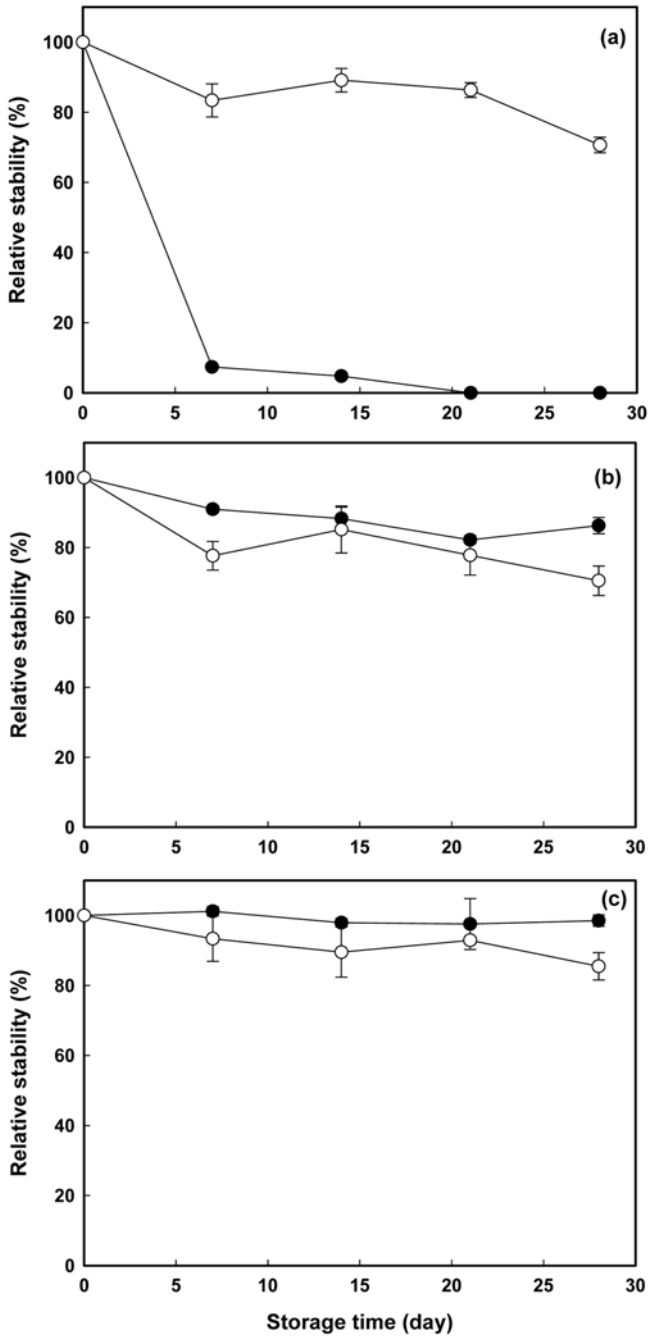

Fig. 5. Effects of pH on the long-term stability of free astaxanthin (-●-) and inclusion complex As-β-CD (-○-) at 4°C. (a) pH 3, (b) pH 5, (C) pH 8.

by CD-mediated IC formation. ICs in aqueous solution were studied for 28 days at various conditions. Samples were taken at intervals of 6 days and quantitatively analyzed using a HPLC system. UV irradiation degraded 90% and 100% of the free astaxanthin after 14 and 21 days, respectively. This is in accordance with results reported by Lee [2003] and Kim [2000] in which astaxanthin was degraded over 80% within 4 weeks after UV irradiation. The β-CD-encapsulated astaxanthin (As-

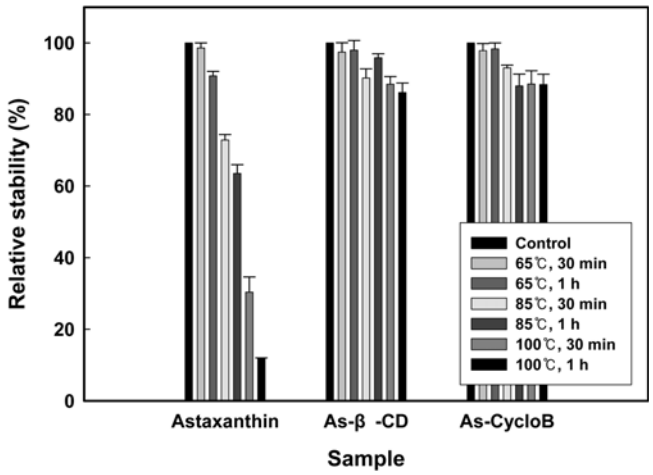

Fig. 6. Effects of heating on the stability of free astaxanthin and inclusion complexes.

β-CD) remained at over 80% after 21 days of UV irradiation, as shown in Fig. 4(a). In addition, the peak pattern in the FT-IR spectrum was not changed even after UV irradiation (data not shown). These data suggest that astaxanthin was effectively protected by β-CD encapsulation against UV irradiation.

It was reported that astaxanthin is easily degraded by exposure to air, and its stability against temperature and pH is highly dependent upon oxidation [Kim, 2000]. In the present study, the effects of air exposure were investigated during the storage of the ICs. While oxygen exposure degraded 83% of the initial amount of free astaxanthin, the β-CD-encapsulated astaxanthin (As-β-CD) did not degrade even after 21 days of oxygen exposure (Fig. 4(b)). This indicated that As-β-CD remained stable against oxidation.

Figure 5 shows the stability of the ICs in acidic pH (pH 2). Both free astaxanthin and the IC-type As-β-CD did not degrade at pH 5 and 8. Under the acidic condition (pH 2), while the free astaxanthin was degraded to over 90% of its initial amount after 7 days, the β-CD-encapsulated astaxanthin remained at over 90% after 14 days. These results indicate that the stability of astaxanthin was significantly improved under acidic conditions by β-CD encapsulation.

To investigate heat stability during industrial processing, free astaxanthin and IC-type astaxanthin were treated with heat under sterilization conditions. The free astaxanthin degraded more rapidly at a higher temperature. However, As-β-CD and As-CycloB, remained heat stable at 65°C for 1 h, respectively (Fig. 6). In addition, peak patterns did not change in the FT-IR spectrum (data not shown). As-β-CD and As-CycloB remained at over 80% at 85 and 100°C for 1 h, while free astaxanthin was degraded to the level of 10% at 100°C for 1 h. These results are similar to those of Chen *et al.* [2007] and Yuan *et al.* [2008]. Chen *et al*. [2007] reported that astaxanthin included with β-CD did not degrade for 6 days at 30 and 57°C. However, this approach was not suitable for quantitative analysis of astaxanthin, through an absorbance measurement over a short time. The stability of β-CD-encapsulated astaxanthin against heating, light, and oxidation was greatly enhanced over 7-9 folds compared to free astaxanthin. Also, the β-CD-encapsulated astaxanthin had high stability even at a high temperature of 100°C.

The inclusion complex (As-β-CD) prepared with astaxanthin and β-CD showed characteristic peaks in the FT-IR spectrum, and the color of the particles became lighter. Its solubility was enhanced up to 100 fold that of free astaxanthin at pH 6.5, and up to 2~7 folds that of free astaxanthin at pH 2 and 8, respectively. The stabilities of As-β-CD and As-CycloB against heating, light, and oxidation were greatly enhanced up to 7-9 folds compared to free astaxanthin. In conclusion, β-CD-mediated encapsulation significantly enhanced the water-solubility of astaxanthin as well as its stability against heating, light, oxidation, and acidic pH. These results are a foundation for industrial applications of astaxanthin in the food and cosmetic sectors.

Acknowledgements. This research was financially supported by the Ministry of Education and Science Technology (MEST) and the Korea Institute for Advancement of Technology (KIAT) through the Human Resource Training Project for Regional Innovation.

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