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ScienceDirect
Biochemical and Biophysical Research Communications 357 (2007) 187–193
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# *Cis* astaxanthin and especially 9-*cis* astaxanthin exhibits a higher antioxidant activity *in vitro* compared to the all-*trans* isomer

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Received 15 March 2007
Available online 28 March 2007

**Abstract**

In recent years, a number of studies have implicated the potent antioxidant property of astaxanthin in various experimental systems; however, these studies employed only the all-*trans* isomer. On the other hand, it has been reported that all-*trans* natural astaxanthin is readily isomerized to *cis–trans*, especially 9-*cis* and 13-*cis* isomers, under certain conditions by chemical analysis; however, the biological activities of the *cis* isomers of astaxanthin are little known. In the present study, we investigated the antioxidant activity of 9-*cis* and 13-*cis* astaxanthin compared to the all-*trans* isomer *in vitro*. In a stable radical DPPH scavenging activity test and in rat microsome and rabbit erythrocyte ghost membrane lipid peroxidation systems induced by AAPH and *t*-BuOOH, respectively, the results apparently showed that *cis*-astaxanthin, especially 9-*cis* astaxanthin, exhibited a higher antioxidant effect than the all-*trans* isomer. In addition, during polyunsaturated fatty acid (PUFA) oxidation, both DHA and linoleic acid hydroperoxides formation were markedly inhibited by astaxanthin isomers addition in the order 9-*cis* > 13-*cis* > all-*trans*. Furthermore, 9-*cis* also exhibited the most effective inhibition of the generation of ROS induced by 6-hydroxydopamine (6-OHDA) in human neuroblastoma SH-SY5Y cells among the astaxanthin isomers, as well as on the degradation of collagen type II induced by DHA and linoleic acid hydroperoxides. The above-mentioned results suggest, for the first time, that *cis* isomer astaxanthin, especially 9-*cis* astaxanthin, has a much higher antioxidant potency than that of the all-*trans* isomer.


*Keywords:* Free radical; Astaxanthin isomers; Antioxidant activity

Astaxanthin, generally known as all-*trans* astaxanthin, is a red-orange carotenoid pigment, naturally found in many well-known aquatic animals, such as shrimp, crab, and salmon. Due to belonging to the xanthopyll class of carotenoids, astaxanthin is closely related to β-carotene, lutein, and zeaxanthin, sharing with them many of the general metabolic and physiological activities attributed to carotenoids. On the other hand, astaxanthin has unique chemical properties based on its molecular structure. The presence of the hydroxyl (OH) and keto (C=O) moieties on each ionone ring explains some of its unique features, namely, a higher antioxidant activity. In recent years, a number of studies on all-*trans* astaxanthin have *in vitro* and *in vivo* demonstrated its antioxidant effect, for example, the quenching effect on singlet oxygen, a strong scavenging effect on superoxide, hydrogen peroxide, and hydroxyl radical [1,2]. In addition, all-*trans* astaxanthin also showed strong activity as an inhibitor of lipid peroxidation [3,4].

To date most of the researchers studying the properties of astaxanthin employed only the all-*trans* isomer; however, all-*trans* natural astaxanthin is readily isomerized to *cis–trans*, especially 9-*cis* and 13-*cis* for steric reasons (Fig. 1). Yuan et al. have indicated that increased temperature or in organic solvents [5], in the presence of acid [6],

*Abbreviations:* HPLC, high-performance liquid chromatography; LC–MS, liquid chromatography–mass spectrometry; ROS, reactive oxygen species; DHA, docosahexaenoic acid; TBARS, thiobarbituric acid-reactive substances.

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doi:10.1016/j.bbrc.2007.03.120

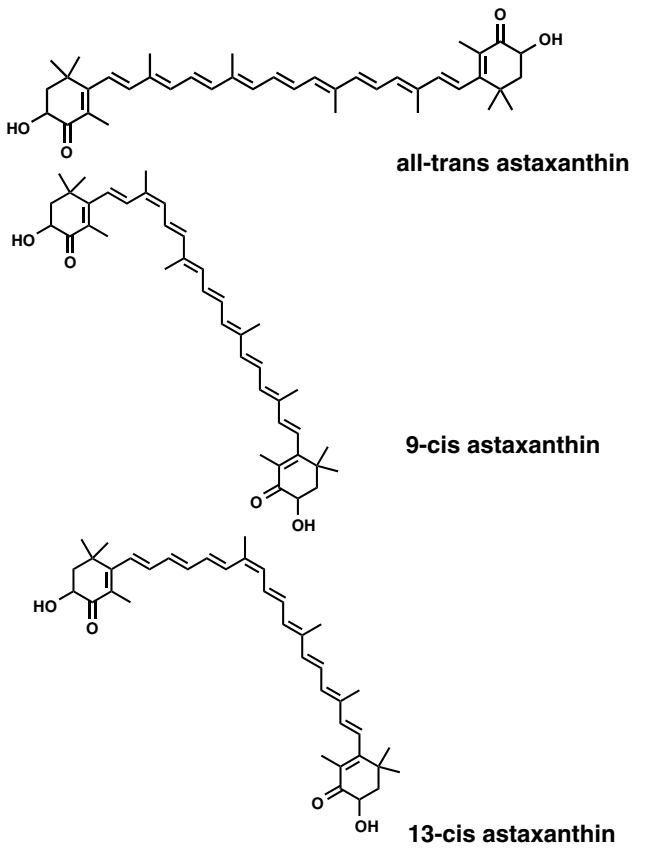


Fig. 1. Structure of all-*trans* astaxanthin, 9-*cis* astaxanthin, and 13-*cis* astaxanthin.

and exposure to light or ions, such as copper(II) [7], are several important factors which could easily induce all-*trans* astaxanthin isomerization, forming *cis*-isomers. As mentioned above, a few of the all-*trans* astaxanthin functional properties have been revealed, while, to the best of our knowledge, in reference to the biological activity of *cis*-astaxanthin, little has been investigated.

Based on these backgrounds, with great interest we *in vitro* investigated here the antioxidant activity of 9-*cis* and 13-*cis* astaxanthin isomers compared to all-*trans* astaxanthin. In the present study, we examined the DPPH scavenging activity of astaxanthin isomers and their inhibition effect on lipid membrane peroxidation, as well as the effect on DHA (ω-3) and linoleic acid (ω-6) initial stage peroxidation by hydroperoxide quantitative detection. In addition, we also investigated the ability of astaxanthin isomers to suppress the generation of ROS in human neuroblastoma SH-SY5Y cells and to degrade collagen type II induced by DHA and linoleic acid hydroperoxides.

## Materials and methods

*Materials*. The synthetic all-*trans*-astaxanthin and DHA were kindly provided by Fuji Chemical Industries (Toyama, Japan) and NOF Co. (Kawasaki, Japan). Methyl linoleate 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN), 2,2′-azobis(2,-amidinopropane) (AAPH), butylated hydroxyltoluene (BHT), and 6-hydroxydopamine hydroperoxide (6-OHDA) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Thiobarbituric acid (TBA) was from MERCK (Damastadt. Germany), and collagen type II was purchased from MCK (Tokyo, Japan).

*Isolation of 9-cis and 13-cis astaxanthin*. All-*trans* astaxanthin diluted in tetrahydrofuran (THF) solvent was incubated at 50 °C for 2 h. The reaction solutions were injected into HPLC-UV using a column of Develosil ODS HG-5 (8.0 × 250 mm, Nomura Kagaku, Japan). Semi-preparative HPLC was performed at room temperature using a mobile phase consisting of methanol (95%) and water (5%) with a linear program. The flow rate was set at 2.0 ml/ml, and both 9-*cis* astaxanthin and 13-*cis* astaxanthin peaks were collected by monitoring at 471 nm. The concentrations of 9-*cis* astaxanthin and 13-*cis* astaxanthin were calculated by liquid chromatography–mass spectrometry (LC–MS) compared to a standard curve of all-*trans* astaxanthin.

*DPPH radical scavenging activity*. The experiment was performed following the previously described method [8]. Briefly, an aliquot of astaxanthin isomers (all-*trans*, 9-*cis* and 13-*cis*) (final concentration of 10 μM) in THF was added to DPPH (final concentration of 1 mM) in ethanol/100 mM Tri–HCl buffer (pH 7.5) (3:1, v/v). The mixture was allowed to stand for 40 min at room temperature in the dark. The absorbance at 517 nm by DPPH was measured by HPLC using a TSKgel-Octyl-80Ts column (4.6 × 150 mm, Tosoh, Tokyo, Japan) with a mobile phase of methanol/water (70:30, v/v) at a flow rate of 1 ml/min. The same amount of THF, without astaxanthin isomers, was added as a blank.

*Microsomal lipid peroxidation*. Fresh microsomes were prepared by the previously described method by Palozza [9]. Briefly, fresh microsomes were obtained from Wister rats (6 weeks, 180–200 g) by tissue homogenization with 5 volumes of ice-cold phosphate buffer (pH 7.4) containing 5% EDTA and 5% BHT. Microsomal vesicles were isolated by removal of the nuclear fraction at 19,000 rpm for 10 min, and the mitochondrial fraction was removed at 28,000 rpm for 10 min. The microsomal fraction was sedimented at 61,000 rpm for 60 min, washed once in 0.15 M KCl, and collected again at 61,000 rpm for 30 min. The membranes were homogenized again into 0.1 M Tri–HCl buffer (pH 7.4), and stored at −80 °C. Microsomal proteins were determined by a BCA assay kit (Pierce).

Astaxanthin isomers (final concentration of 10 nmol/mg protein) in THF were added to the microsomes, and lipid peroxidation was initiated by the addition of 50 mM AAPH. The same amount of THF, without astaxanthin isomers, was added as the control. Reaction mixtures were shaken in air at 37 °C. Lipid peroxidation products were determined as malondialdehyde (MDA) formation by measurement at 532 nm.

*Rabbit erythrocyte membrane ghost system*. Rabbit erythrocyte membrane ghosts were prepared by following the previously reported method [10]. Commercially available rabbit blood (100 ml) was obtained from Japan Biotest Institute Co., Ltd., and diluted with 150 ml of isotonic buffer solution (10 mM phosphate buffer/152 mM NaCl). After centrifugation (3500 rpm, 10 min), the blood was washed three times with 10 ml of isotonic buffer solution and lysed in 10 mM phosphate buffer, pH 7.4. Erythrocyte membrane ghosts were pelleted by centrifugation (11,000 rpm, 40 min), and the precipitate was diluted to give a suspension. Astaxanthin isomers (final concentration of 10 nmol/mg protein) in THF were added to the erythrocyte membrane ghosts, and peroxidation was induced by *tert*-butyl hydroperoxide. The same amount of THF, without astaxanthin isomers, was added to the control. After incubation at 37 °C for 20 min, 1 ml of 2.0 M TCA/1.7 M HCl and 2 ml of 0.67% TBA solution were added to stop the reaction. The quantity of TBA-reacting substance (TBARS) was determined at 532 nm.

*Fatty acid peroxidation reaction*. An appropriate amount of astaxanthin isomers (10 μM) was added to 1 mM DHA or methyl linoleate solution. Peroxidation was initiated by adding AMVN (1 mM). Reaction solutions in THF were incubated under air in the dark at 37 °C with continuous shaking. At regular intervals, aliquots of the sample (50 μl) were withdrawn and stored at −80 °C immediately. Lipid hydroperoxide levels were determined by HPLC monitored at 234 nm. As a preliminary experiment, a major DHA peroxidation product fraction was isolated by

HPLC monitored at 234 nm and identified by measuring the hydroperoxide activity of each isolated fraction using a Lipid Hydroperoxide assay kit (Cayman).

*DHA hydroperoxides preparation*. DHA hydroperoxides (DHA-OOH) were prepared by the reaction of soybean lipoxygenase with DHA. A 83.6-mg sample of DHA (purity 70%, NOF Co., Japan) and 8 mg lipoxygenase (100 mg, Sigma type I-B) were added to 220 ml of 200 mM borate buffer (pH 9.0). The reaction was carried out for 15 min by stirring in a dish filled with $O_2$ at room temperature. The reaction was terminated by HCl addition to pH < 4.0, and the formed peroxides were extracted twice with an equal amount of chloroform/methanol (1:1). The collected chloroform layer was evaporated. The obtained peroxides were quantified by lipid hydroperoxide kit (Cayman) compared to a standard curve prepared by authentic 13-HPODE.

*Linoleic acid hydroperoxides preparation*. Methyl linoleate hydroperoxide (MLOOH) was prepared by the reaction of soybean lipoxygenase with methyl linoleate (ML). A 200-mg sample of ML and sodium deoxycholate (1.62 g) was dissolved in 240 ml of 200 mM borate buffer (pH 9.0). Lipoxygenase (100 mg, Sigma type I-B) was added to the solution and incubated for 3 h at room temperature. The formed peroxide was extracted twice with an equal amount of chloroform/methanol (1:1). The collected chloroform layer was evaporated. The obtained peroxide was purified by thin layer chromatography (TLC) and developed with *n*-hexane/ether (6:4). The peroxide was extracted with $CHCl_3$ and then the solvents were evaporated. The amount of MLOOH was calculated from the molar coefficient, $\varepsilon_{234nm} = 25000\ M^{-1}\ cm^{-1}$, using the value of linoleic acid hydroperoxide.

*Cell culture and fluorescence assay of the ROS generated in SH-SY5Y cells*. The SH-SY5Y cells, a human dopaminergic neuroblastoma cell line, were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal bovine serum (FBS) in a $CO_2$ incubator at 37 °C.

The generation of the ROS in the SH-SY5Y cells was measured as accumulated oxidized carboxy-$H_2$DCFDA within a certain period of time as described by Ouyang and Shen [11]. Briefly, the cells (which had reached approximately 80% confluence) seeded on six-well plates were washed twice with serum-free DMEM and thereafter incubated for 4 h in DMEM without serum in the presence of 100 nM astaxanthin isomers. After washing with DMEM without serum, the cells were loaded with carboxy-$H_2$DCFDA for 30 min, prior to exposure to 50 μM 6-OHDA for 45 min. Followed by treatment with 6-OHDA, the cells were washed once with PBS (+) and PBS (−), respectively, and then collected into vials. The fluorescence of dichlorofluorescein (DCF) in the supernatant was measured by an EPICS Elite Flow Cytometer (Beckman Coulter, Inc., USA). The data were analyzed using the Bonferroni/Dunn multiple comparison procedure.

## Results

### *Antioxidative activity of astaxanthin isomers to DPPH radicals scavenging and lipid peroxidation*

The scavenging ability against stable DPPH free radicals can be used to evaluate the antioxidant activities due to their hydrogen-donating ability. In this study, 1 mM DPPH was incubated with 10 μM astaxanthin isomers, respectively, in ethanol/100 mM Tri–HCl buffer (pH 7.5) (3:1, v/v) in the dark at room temperature. As shown in Fig. 2A, after 40 min incubation, compared to a blank in which astaxanthin was absent, although all of the astaxanthin isomers exhibited a scavenging effect on DPPH radicals, 9-*cis*-astaxanthin and 13-*cis* astaxanthin showed higher activity than all-*trans* astaxanthin, especially 9-*cis* astaxanthin, the activity of which was about 4-fold higher than that of all-*trans* astaxanthin.

As a hydrosoluble hydroxyl radical initiator, AAPH was employed to induce membrance lipid peroxidation in the present study. Fig. 2B (upper panel) shows the effect of adding astaxanthin isomers to rat fresh microsomal membranes treated with 50 mM AAPH with respect to lipid peroxidation. The microsomes were incubated under air at 37 °C in the absence or presence of 10 nmol astaxanthin/mg protein, and the thiobarbituric acid-reactive substance (TBARS) level was measured at 535 nm. Within 2-h incubation, all of the astaxanhin isomers exhibited their inhibition effect on microsomal peroxidation compared to the astaxanthin absence control. However, 2 h later, all-*trans* astaxanthin almost lost its effect, being shown as a similar curve to the +APPH control, while the addition of *cis* astaxanthin, especially 9-*cis* astaxanthin, markedly inhibited TBARS generation up to 8 h.

Like many other biological membranes, red blood cell membranes are prone to lipid peroxidation due to the high content of polyunsaturated fatty acids (PUFA) such as linolenic acid (18:2) and docosahexaenoic acid (22:6), which may increase a biological system's susceptibility to lipid peroxidation. As shown in Fig. 2B (bottom panel), 9-*cis*-astaxanthin addition showed the strongest protection effect against lipid peroxidation induced by *tert*-butyl hydroperoxide among the astaxanthin isomers.

An appropriate amount of astaxanthin isomers (10 μM) was added to DHA or methyl linoleate solution (1 mM). Peroxidation was initiated by adding 1 mM AMVN known as a lipophilic radical initiator . Reaction solutions in THF were incubated under air in the dark at 37 °C with continuous shaking. After incubation, DHA-OOH and MLOOH were detected by HPLC analysis at 234 nm. Fig. 2C show that, in both unsaturated fatty acid peroxidation systems, all of these astaxanthin isomers acted as inhibitors; of them, 9-*cis* isomer showed about a 2-fold higher activity compared to all-*trans* isomer.

### *Effects of astaxanthin isomers on 6-hydroxydopamine-induced ROS generation in SH-SY5Y cells*

6-Hydroxydopamine (6-OHDA)-induced apoptosis in dopaminergic neuronal cells is a common cell model of Parkison's disease (PD) [12]. It also has been known that 6-OHDA-induced neuron degeneration involves oxidative stress, and that ROS-mediated signaling pathways play an essential role in 6-OHDA-induced cell death [11]. In the present study, the SH-SY5Y cells pre-incubated with or without 100 nM astaxanthin isomers were loaded with carboxy-$H_2$DCFDA for 30 min, which is a fluorescent indicator used to display the accumulated ROS generated in the cells. Then the cells were immediately exposed to 50 μM 6-OHDA for 45 min. The results are shown in Fig. 3. Higher DCF fluorescence in the control cells induced by 6-OHDA and its significant reduction in the pre-incubated cells with 100 nM astaxanthin isomers for 4 h demonstrated the ability of astaxanthin, especially the 9-*cis* isomer, to suppress ROS generation in the cells.

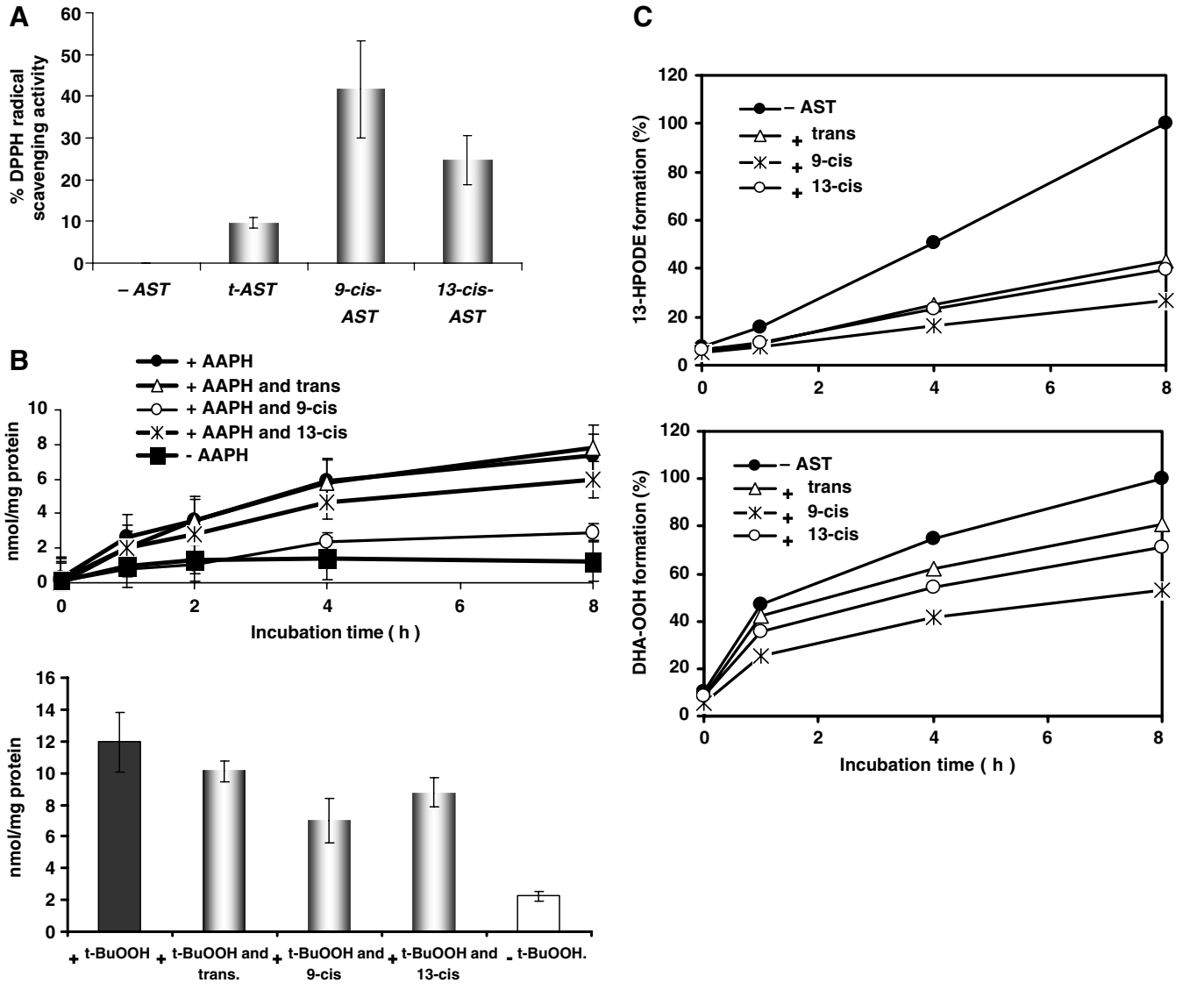


Fig. 2. Antioxidative activity of astaxanthin isomers to DPPH radicals scavenging and lipid peroxidation. (A) DPPH radical scavenging assay. (B) Lipid peroxidation in membrane model systems using microsomes (upper panel) and rabbit erythrocyte membrane ghosts (bottom panel). (C) Formation of DHA and methyl linoleate hydroperoxides in solution. The details are described in Materials and methods. The results are the means of three experiments.

*Effects of astaxanthin isomers on degradation of collagen II induced by DHA and linoleic acid hydroperoxides*

Collagen type II (CII) is a primary protein component of cartilage and its degradation is a major, early and irreversible event in rheumatoid arthritis (RA) and osteoarthritis (OA). Recently, it is suggested that chondrocyte-derived lipid peroxidation might mediate collagen II degradation by an *in vitro* model [13]. In the present study, we performed a reaction of collagen II degradation induced by lipid hydroperoxides and, using this experiment model, we investigated the protective effect of astaxanthin isomers on the collagen II degradation. After incubation at 37 °C for 24 h, in both 13-HPODE (Fig. 4A) and DHA-OOH (Fig. 4B, left panel) reaction systems the addition of 9-*cis* astaxanthin exhibited the strongest inhibitive effect on collagen II degradation. In addition, the protective effect was shown to be a dose-dependent with the addition of 9-*cis* astaxanthin (Fig. 4B, right panel).

## Discussion

Numerous studies have demonstrated the antioxidant potential of carotenoids that can significantly reduce free radicals and the oxidative load to help the body maintain a healthy state. Of the carotenoids, astaxanthin, especially the natural form all-*trans* isomer has been shown to possess super antioxidative property even surpassing the antioxidant benefits of beta-carotene, zeaxanthin, canthaxanthin, vitamin C, and vitamin E. The protection against oxidation of all-*trans* astaxanthin is suggested by the quenching of singlet oxygen and scavenging of free radicals to prevent and terminate chain reactions. As demonstrated by Shimidzu [14], all-*trans* astaxanthin has a singlet oxygen

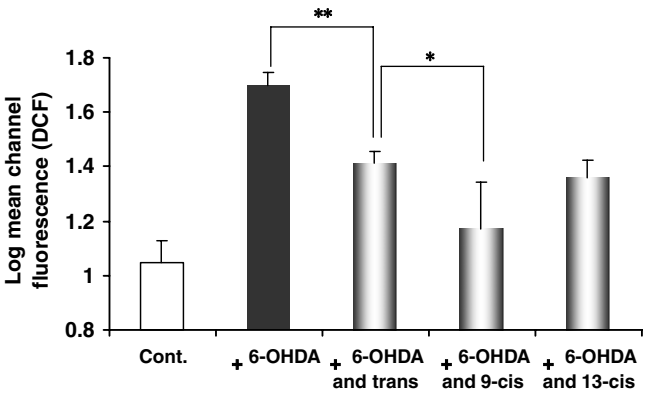


Fig. 3. Effect of astaxanthin isomers on 6-OHDA-induced ROS generation in SH-SY5Y cells. The cells were pre-incubated with or without 100 nM astaxanthin isomers for 4 h, then loaded with carboxy-$H_2$DCFDA for 30 min, followed by exposure to 50 μM 6-OHDA for 45 min. The carboxy-$H_2$DCFDA-loaded cells without 6-OHDA-treatment were used as control. Each value represents the mean SD of triplicate determinations. $^{*}P < 0.05$; $^{**}P < 0.005$.

quenching activity over 500 times greater than that of alpha-tocopherol. Using a homogenate of rat mitochondria, astaxanthin also exerted a 100-fold greater activity than vitamin E in inhibiting lipid peroxidation. In recent years, increasing studies on all-*trans* astaxanthin have revealed its other biological functions such as anti-inflammatory activity [15], antihypertensive and neuroprotective potentials; in addition, all-*trans* astaxanthin is more potent in the prevention of carcinogen-induced neoplastic transformation in 10T1/2 cells than any of the previously studied carotenoids [16].

All-*trans* astaxanthin is unstable and is readily isomerized to its analogues, especially the 9-*cis* and 13-*cis* isomers, by environmental factors, such as heat, acid, and metal ions, suggesting that *cis*-isomers of carotenoids may be naturally formed in certain organisms. In the alga *Haematococcus pluvialis*, although astaxanthin exists mainly as all-*trans* astaxanthin esters of various fatty acids, *cis*-astaxanthin esters were also detected in the algal pigment extracts

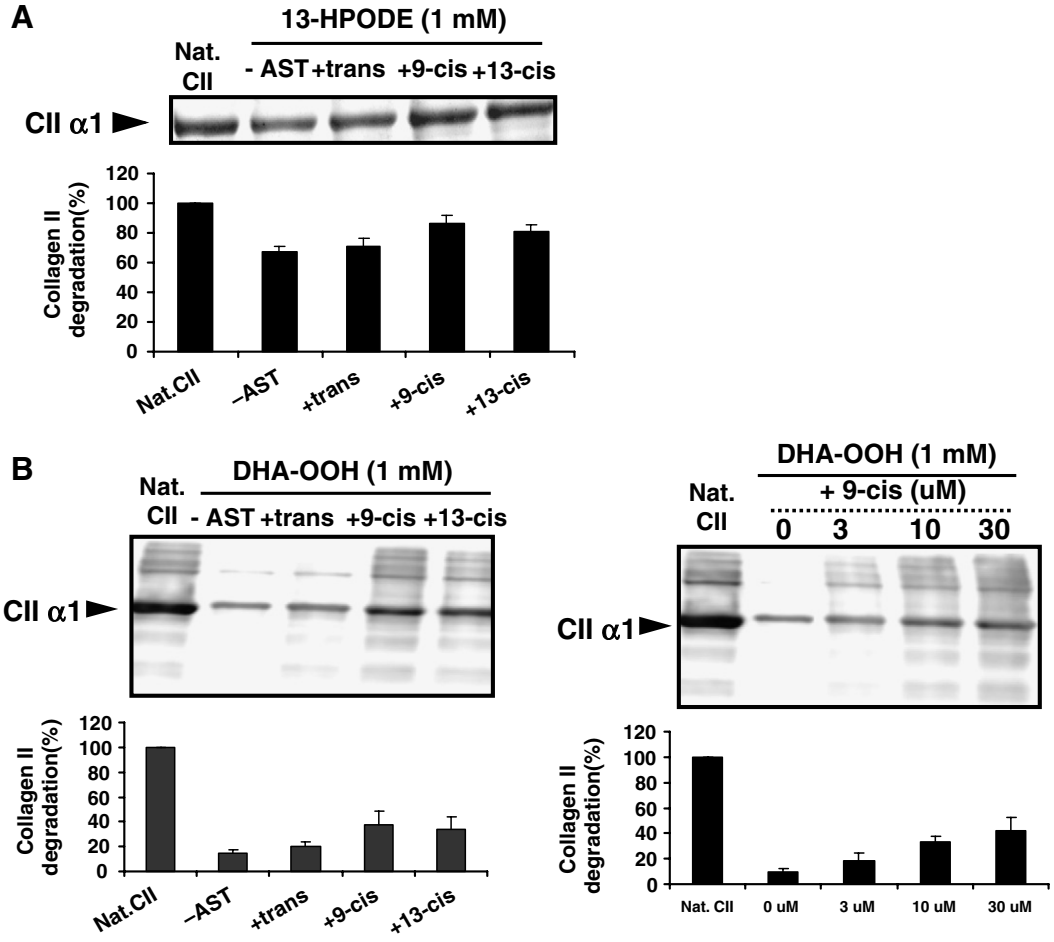


Fig. 4. Effect of astaxanthin isomers on lipid hydroperoxide-induced collagen II degradation. Ten micromolar astaxanthin isomers were added to the reaction solution in PB (pH 7.4), including 0.2 mg/ml native collagen II and 1.0 mM 13-HPODE (A) or 1 mM DHA-OOH (B, left panel). After the incubation at 37 °C for 24 h, collagen II detection was carried out by CBB staining or Western blot using 4–8% SDS–PAGE and human anti collagen II antibody (mono, CMN). Based on the results (B, left panel), the effect of 9-*cis* astaxanthin at a dose-dependent addition was examined (B, right panel).

[17,18]. The saponified extract solution from *H.pluvialis* has been reported to contain *cis*-isomers rather than the all-*trans* isomer [19]. The free *cis*-astaxanthin might come from the hydrolysis of *cis*-astaxanthin esters or the isomerization of all-*trans* astaxanthin.

In reference to the biological activity of *cis*-astaxanthin, the only one reported is that they might have lower provitamin A activity, which is not strong compared to the all-*trans* isomer astaxanthin [20]. Thus it is postulated to minimize the isomerization of all-*trans* astaxanthin to form *cis*-isomers during the extraction of pigments. However, none have investigated whether *cis*-astaxnathin possesses other biological activities. Because most of the beneficial properties of all-*trans* astaxanthin are attributed to its ability to prevent oxidation processes by quenching singlet oxygen or to terminate free radical chain reactions, it is of interest to investigate the structure–activity relationship of astaxanthin isomers.

In this present study, we examined *in vitro* the antioxidant property of 9-*cis* and 13-*cis* astaxanthin, compared with its all-*trans* isomer. In both DPPH-HPLC assay and membrane lipid peroxidation systems using microsomes and erythrocyte ghosts, *cis* astaxanthin, especially 9-*cis*, exhibited higher free radical-scavenging activity and markedly suppressed the membrane lipid peroxidation rather than the all-*trans* isomer (Figs. 2A and B). In membrane lipid peroxidation experiments, TBA-reacting substance (TBARS) assay was employed to estimate the lipid peroxidation degree. The assay exhibits mostly the end-products of lipid peroxidation, such as aldehydes and ketones, whereas as is well-known at the early stage of the lipid peroxidation process, hydroperoxides are initially formed [21]. Thus it is important to examine whether astaxanthin isomers also have some effect on the initial stage in lipid peroxidation, namely lipid hydroperoxide formation. In agreement with the report by Terao [3], all-*trans* astaxanthin exerted a significant effect on methyl linoleate hydroperoxide (MLOOH) formation, whereas, in the present experiment, the inhibitive extent was clearly lower than that of 9-*cis* astaxanthin. In contrast to an ω-6 unsaturated fatty acid methyl linoleate, ω-3 PUFA is thought to be much more prone to peroxidation [21]. During ω-3 PUFA DHA peroxidation, DHA hydroperoxide (DHA-OOH) formation is also markedly suppressed by astaxanthin isomers in the order 9-*cis* > 13-*cis* > all-trans (Fig. 2C). Reactive oxygen species (ROS) regenerated a large amount via multiple oxidative chain reactions by mitochondria. These ROS need to be neutralized in order to maintain the proper functions of this cellular component and to protect the cells from oxidative modification. To the best of our knowledge, few have reported the ROS generation-suppressing effect of astaxanthin. In our study, astaxanthin, and especially the 9-*cis* isomer, were shown to have a significant effect on ROS generation induced by 6-hydroxydopamine (6-OHDA) in human neuroblastoma SH-SY5Y cells (Fig. 3). As is well-known, the Parkinson's disease (PD) is one of the most common neurodegenerative disorders, and 6-hydroxydopamine (6-OHDA)-induced oxidative stress and apoptosis in dopaminergic neuronal cells is a common cell model of PD. Therefore, our results might indicate further evidence of the neuroprotective effect of astaxanthin. Decreased collagen II and increased lipid hydroperoxide have been detected in patients with osteoarthritis, which indicate the relationship of oxidative stress to the progression of the disease [22]. We have demonstrated here the strongest protective effect of 9-*cis* astaxanthin on collagen II degradation induced by lipid hydroperoxides (Fig. 4), suggesting the possible prophylactic effect of astaxanthin for arthritis.

In this present study, the above-mentioned results have suggested that 9-*cis* and 13-*cis* asxatanthin might have a higher antioxidant activity than the all-*trans* isomer *in vitro*; however, we cannot now explain the corrective mechanism. Galit Levin et al. have suggested that 9-*cis* β-carotene exhibited a higher antioxidant activity than the all-*trans* isomer [23]. In that report on the higher reactivity of 9-*cis* β-carotene against free radicals, they stated it could be explained that, compared to the all-*trans* form, the *cis* form was shown to possess a higher potential energy, and therefore, was more susceptible to various reactions. Nevertheless, whether this postulation for astaxanthin isomers is ture, there is no doubt that structural parameters besides the length of the polyene chain may determine the antioxidant properties of carotenoids, although the detailed mechanism is not clear.

In oral astaxanthin administration to humans, it is reported [24] that after 6–7 h, a maximum level of astaxanthin in the plasma is detected, in which the increased concentration of *cis*-astaxanthin, especially the 13-*cis* isomer, and a decrease in the all-*trans* isomer are indicated. Also, in our *in vitro* experiment, after several hours incubation at 37 °C, the all-*trans* astaxanthin was found to be about 10% isomerized to *cis*-isomers, mostly the 13-*cis* isomer. Inversely, when the 9-*cis* and 13-*cis* astaxanthin are incubated under the same condition, a similar level of conversion to the all-*trans* isomer was detected from 13-*cis* incubation, whereas no isomerization was shown during 9-*cis* astaxanthin incubation, indicating that 9-*cis* isomer is the most stable form in a similar organism environment. Taking these reports together, as well as *in vivo* results, it is necessary to compare the antioxidant activity of astaxanthin isomers in further studies.

In conclusion, we suggested here that *cis* astaxanthin and especially, the 9-*cis* isomer might have a higher antioxidant activity than the all-*trans* isomer *in vitro* through a radical DPPH scavenging activity test, lipid peroxidation, ROS generation in human neuroblastoma SH-SY5Y cells and collagen II degradation induced by lipid hydroperoxides.

## Acknowledgments

The authors thank Fuji Chemical Industries (Toyama, Japan) and NOF Co. (Kawasaki, Japan) for all-*trans* astaxanthin and DHA provision, respectively. We also thank

Dr. Yoji Kato (University of Hyogo) and Naruomi Yamada (University of Nagoya) for their valuable advice.

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