Research Report

Astaxanthin inhibits reactive oxygen species-mediated cellular toxicity in dopaminergic SH-SY5Y cells via mitochondria-targeted protective mechanism

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ABSTRACT

Astaxanthin is a powerful antioxidant that occurs naturally in a wide variety of living organisms. The aim of this study is to investigate the effect and the mechanism of astaxanthin on reactive oxygen species (ROS)-mediated apoptosis in dopaminergic SH-SY5Y cells. The treatment with DHA hydroperoxide (DHA-OOH) or 6-hydroxydopamine (6-OHDA), either of which is ROS-inducing neurotoxin, led to a significant decrease in viable dopaminergic SH-SY5Y cells by MTT assay, whereas a significant protection was shown while the cells were pretreated with astaxanthin. Moreover, 100 nM astaxanthin pretreatment significantly inhibited apoptosis, mitochondrial abnormalities and intracellular ROS generation occurred in either DHA-OOH- or 6-OHDA-treated cells. The neuroprotective effect of astaxanthin is suggested to be dependent upon its antioxidant potential and mitochondria protection; therefore, it is suggested that astaxanthin may be an effective treatment for oxidative stress-associated neurodegeneration.

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1. Introduction

Astaxanthin (AST) (Fig. 1), a red-orange carotenoid pigment, naturally occurs in many aquatic animals such as shrimp, crab and salmon. As attributed to carotenoid and closely associated to β-carotene, lutein, and zeaxanthin, astaxanthin shares with them many of the general metabolic and physiological activities. 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 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 radicals and an inhibitory effect on lipid peroxidation (Miki, 1991; Palozza and Krinsky, 1992). In addition to these, several other biologic activities of astaxanthin, including anti-cancer, anti-inflammatory, anti-diabetic, immunomodulatory activities and a neuroprotective effect, also have been reported (Hussein et al., 2006).

Parkinson's disease (PD) is a neurodegenerative disorder characterized by a preferential loss of the dopaminergic neurons. The mechanism responsible for degeneration of dopaminergic neurons is incompletely understood; however, an increasing body of evidence suggests that oxidative stress,
mitochondrial inhibition and impairment of the ubiquitin-proteasome system may be largely involved as major biochemical processes in the degenerative cascade (Fahn and Cohen, 1992; Schapira, 2001; Balaban et al., 2005; Leroy et al., 1998). In our previous study, we demonstrated that reactive oxygen species (ROS)- and mitochondrial dysfunction-mediated apoptotic signaling increased within a few hour after treatment with DHA hydroperoxides (DHA-OOH) and resulted in dopaminergic SH-SY5Y cell death (Liu et al., 2008). In addition, the in vitro experimental model using neurotoxic compounds, such as 6-Hydroxydopamine (6-OHDA), has also revealed that the neuronal cell death was regulated by ROS generation, mitochondrial inhibition and other oxidative stress-related signaling molecules (Hanrott et al., 2006; Chalovich et al., 2006; Jia et al., 2008).

Currently, most efforts to prevent and treat neurodegenerative disorders focus on diet, lifestyle modification and drugs that target the disease processes. Astaxanthin is a powerful antioxidant, and Hussein, G. et al. recently reported that astaxanthin prevented the ischemia-induced impairment of spatial memory in mice (Hussein et al., 2005). Although these facts suggested that astaxanthin might be a potent candidate for a natural neuroprotective agent, further basic evidence to demonstrate the neuroprotective effect of astaxanthin is needed.

The human dopaminergic neuronal cell line SH-SY5Y processes many qualities of substantia nigra neurons (Takahashi et al., 1994). Therefore, in this study we investigated whether astaxanthin would prevent DHA-OOH- or 6-OHDA-induced cytotoxicity in SH-SY5Y cells. We examined the effects of astaxanthin on cell viability, apoptosis in DHA-OOH- or 6-OHDA-treated cells. The possible mechanisms of astaxanthin protection were investigated by measuring mitochondrial change and intracellular ROS generation in the cells.

2. Results

2.1. Effect of astaxanthin on DHA-OOH- or 6-OHDA-induced cell death

SH-SY5Y cells are widely used to study pathogenesis because this cell line expresses such representative dopaminergic markers as tyrosine hydroxylase and dopamine transporter (Wang et al., 2008 and Hasegawa et al., 2003). Therefore, SH-SY5Y cells can be a suitable model system to study the role of astaxanthin against ROS-mediated dopaminergic cell death.

In this study, SH-SYSY cells were pretreated with astaxanthin for 4 h and 24 h at different concentration, washed, and then treated with DHA-OOH or 6-OHDA for an additional 24 h. Astaxanthin itself had no apparent effect on cell viability at the concentration of 25–1000 nM even for 24 h (Fig. 2A). DHA-OOH (10 μM, 24 h) and 6-OHDA (100 μM, 24 h) induced a significant decrease in cell viability by 80% and 70%. The pretreatment of SH-SYSY cells with astaxanthin for 4 h resulted in a dose-dependent protection against DHA-OOH- or 6-OHDA-induced toxicity at the concentration of 25–100 nM range, and the most significant protection was found at the concentration of 100 nM, with respectively 65% and 84% of the control. The pretreatment with astaxanthin at the concentration of 500 nM and 1000 nM caused a similar or reduced effect compared to that of 100 nM. In addition, the protective effect of astaxanthin was slightly enhanced by a longer pretreatment for 24 h in both the case of DHA-OOH- and 6-OHDA-treated cells (Figs. 2B and C).

2.2. Effect of astaxanthin on DHA-OOH- or 6-OHDA-induced apoptosis

DNA fragmentation, nuclei condensation and PARP cleavage are well-used hallmarks of cell apoptosis. In this study, we investigated the effect of astaxanthin on the DHA-OOH- or 6-OHDA-induced apoptosis by the hallmark measurements. As shown in Fig. 3A, the results of the agarose gel electrophoresis revealed that the DNA fragmentation in the 6-OHDA-treated SH-SYSY cells was clearly inhibited while the cells were pretreated with 100 nM astaxanthin for 4 h, whereas astaxanthin pretreatment had a slight protective effect on the DHA-OOH-treated cells. Exposure of SH-SYSY cells to 10 μM DHA-OOH or 100 μM 6-OHDA for 1 h led to an obvious apoptotic nuclei formation. Apoptotic nuclear characteristics were decreased significantly when cells were pre-incubated with 100 nM astaxanthin for 4 h. Astaxanthin alone had no apparent effect (Fig. 3B). Furthermore, following 4 h treatment with 100 nM astaxanthin, both DHA-OOH- and 6-OHDA-induced PARP cleavage, which was shown as a band production at 89 kDa, appeared reduced also (Fig. 3C).

2.3. Effect of astaxanthin on mitochondrial abnormalities induced by DHA-OOH or 6-OHDA

We previously demonstrated that DHA-OOH-induced apoptosis involved mitochondrial dysfunction (Liu et al., 2008). It is also generally accepted that the 6-OHDA-induced neuronal apoptosis is mediated by the mitochondrial dysfunction.
pathway characterized by several events including cytochrome c release, attenuation of mitochondrial membrane potential and mitochondrial protein carbonyls (Jia et al., 2008; Smith and Gass, 2007). Hence, we investigated whether astaxanthin could inhibit the DHA-OOH or 6-OHDA-induced change in the mitochondria of SH-SY5Y cells.

Cytochrome c release was assessed through determining the expression of cytochrome c in the cytosolic fraction of the cells. The results in Fig. 4 showed that DHA-OOH and 6-OHDA increased cytochrome c release to 9 fold and 7 fold, respectively, relative to control. The releases were decreased to 2 fold and 3 fold of control, respectively, by astaxanthin treatment at 100 nM.

Fig. 5 showed representative images of SH-SY5Y cells with DiOC6 fluorescence staining. Both DHA-OOH and 6-OHDA led to a decrease in green DiOC6 fluorescence, reflecting the toxins-induced decrease in mitochondrial membrane potential. Pretreatment with 100 nM astaxanthin significantly

Fig. 2 – Protective effect of astaxanthin (AST) on DHA-OOH- or 6-OHDA-induced decrease in cell viability measured by MTT assay. (A) SH-SY5Y cells were incubated with different concentrations of astaxanthin for 4 or 24 h, and cell viability was assessed by MTT assay. (B and C) Cells were incubated with or without 25–1000 nm astaxanthin for 4 or 24 h. Then the medium were removed and the cells were washed three times with FBS-free DMEM prior to the addition of 10 μM DHA-OOH or 100 μM 6-OHDA for additional 24 h. Values are percentage to the control (no drugs) and are mean±SE (n=9) of three independent experiments in triplicates. *p<0.01 and **p<0.001 versus control, †p<0.05 and ††p<0.01 versus DHA-OOH or 6-OHDA.
Prevented the decrease in mitochondrial membrane potential as demonstrated by increased green DiOC6 staining of astaxanthin-pretreated cells.

Treatment with 10 μM DHA-OOH or 100 μM 6-OHDA for 4 h caused a significant increase in protein carbonyls, an index of protein oxidation, in mitochondrial fraction but not cytosolic fraction.

Fig. 3 – Protective effect of astaxanthin (AST) on DHA-OOH- or 6-OHDA-induced apoptosis. (A) DNA fragmentation assessment. SH-SY5Y cells were incubated with 10 nM and 100 nM astaxanthin for 4 h, and washed, then exposed to 10 μM DHA-OOH or 100 μM 6-OHDA for additional 4 h. The DNA fragments were purified and then separated on an agarose gel. (B) Images and statistical analysis of nucleic morphology change. SH-SY5Y cells pretreated with 100 nM astaxanthin for 4 h and subsequently treated with 10 μM DHA-OOH or 100 μM 6-OHDA for 1 h were stained with Hoechst 33258, and observed using a fluorescence microscope. Statistical analysis of representative images was performed with three independent experiments. Values are mean ± SE. ***p<0.001 versus control, *p<0.05 and **p<0.01 versus DHA-OOH or 6-OHDA. (C) PARP cleavage was assessed by western blot analysis.
Fig. 4 – Protective effect of astaxanthin on cytochrome c release. The cytosolic fraction of DHA-OOH or 6-OHDA-treated cells with or without 100 nM astaxanthin pre-incubation was used to examine the cytochrome c release by western blot (upper). Statistical analysis of cytochrome c expression was performed with three independent experiments (lower). Values are mean±SE. ***p<0.001 versus control, ##p<0.01 versus DHA-OOH or 6-OHDA.

Fig. 5 – Protective effect of astaxanthin on decrease in mitochondria membrane potential. Representative images of DiOC6 staining of cells pretreated with 100 nM astaxanthin for 4 h and followed by 10 μM DHA-OOH or 100 μM 6-OHDA exposure for 4 h. The images were observed under fluorescent microscopy. Each panel is representative of two separate experiments.
fraction, providing the further evidences that both DHA-OOH and 6-OHDA should be mitochondria-targeting neurotoxins. Pretreatment with 100 nM astaxanthin for 4 h appeared to inhibit the toxins-induced mitochondrial protein carbonyl increase (Fig. 6). Astaxanthin alone had no effect on protein oxidation in either mitochondrial fraction or cytosolic fraction in the cells.

2.4. Effect of astaxanthin on DHA-OOH- or 6-OHDA-induced ROS generation

ROS generation has been demonstrated to be a common feature occurring in DHA-OOH- or 6-OHDA-treated cells and is also proposed as one of the initial trigger leading to activation of apoptotic signaling. In this study, we examined the effect of astaxanthin on ROS generation in SH-SY5Y cells exposed to DHA-OOH or 6-OHDA. Intracellular ROS levels were determined with DCF fluorescence by flow cytometry. As shown in Fig. 7, exposure of SH-SY5Y cells to DHA-OOH and 6-OHDA led to a 3.5 fold and 1.8 fold increases, respectively, in DCF signal compared with the control group, whereas, astaxanthin pretreatment significantly inhibited the increase in DCF fluorescence in both toxins-treated cells.

2.5. Uptake of astaxanthin into SH-SY5Y cells

To identify in which fraction of the SH-SY5Y cells astaxanthin concentrates and exhibits the protective effect on oxidative stress-mediated cell damage, we investigated relative proportion of astaxanthin uptake into the cells. By HPLC analysis, astaxanthin was detected at 0%, 9.42%, 7.9%, and 72.56% of the total administration levels in the cytosolic, mitochondrial, membrane fraction of the cells and the culture medium, respectively (Fig. 8), suggesting that astaxanthin concentrating in the mitochondrial fraction may contribute directly to the protection against the mitochondrial abnormality and cell death by its potent antioxidant property.

3. Discussion

Advances in understanding the neurodegenerative pathogenesis are creating new opportunities for the development of neuroprotective therapies. In this work, we have demonstrated that astaxanthin, a natural carotenoid and a rich component in aquatic animals, significantly protected DHA-OOH- or 6-OHDA-induced cellular toxicity in human neuroblastoma dopaminergic SH-SY5Y cells.

PD is characterized by a profound loss of dopaminergic neurons in the substantia nigra. Even though the cause of PD remains largely unknown, several lines of evidence strongly suggest the involvement of oxidative stress (Mariani et al., 2005). In deed, increased levels of 8-OHdG (Yasuhara et al., 2007), MDA (Dexter et al., 1989), lipid hydroperoxides (Dexter et al., 1994) and protein carbonyls (Alam et al., 1997) have been demonstrated in the PD substantia nigra. DHA hydroperoxide
(DHA-OOH), the primary product derived from DHA peroxidation, has been previously demonstrated to be a potent apoptosis inducer in SH-SY5Y cells. The high levels of oxidative stress and DHA content in the brain, in addition to the high susceptibility of DHA to oxidative stress, suggest that DHA-OOH neurocytotoxicity may be an important causative event leading to the neurodegeneration. On the other hand, 6-OHDA is a potent neurotoxin that leads to degeneration of dopaminergic neurons and has been used in several studies as an in vitro and in vivo model neurotoxin to elucidate the pathological mechanism of PD (Blum et al., 2001). Both DHA-OOH- and 6-OHDA-induced neuronal apoptosis has been suggested to be mediated by ROS generation and mitochondrial abnormalities. In view of the increasing evidence that ROS generation and mitochondrial dysfunction are accepted as common events leading to neuronal apoptosis in PD (Foster et al., 2006), our in vitro results raise the possibility that astaxanthin could modify PD progression via an anti-apoptotic mechanism.

Currently, most efforts to prevent and treat neurodegenerative disorders focus on diet, lifestyle modification and drugs that target the disease processes (Logroscino 1996; Luchsinger and Mayeux, 2004; Mattson 2007). Astaxanthin is a natural carotenoid with super antioxidant activity. Of the carotenoids, astaxanthin has been shown to possess antioxidant property even surpassing the antioxidant benefits of β-carotene, zeaxanthin, canthaxanthin, vitamin C and vitamin E (Pashkow et al., 2008). For example, astaxanthin has a singlet oxygen quenching activity over 500 times greater than that of α-tocopherol (Shimidzu et al., 1996), as well as a 100-fold greater activity than vitamin E in inhibiting lipid peroxidation (Kurashige et al., 1990). In recent years, increasing studies on astaxanthin have also revealed such other pharmacological activities as on inflammation, cancer and diabetes (Pashkow et al., 2008).

There is evidence that some antioxidants have neuroprotective effect in the in vitro models of Parkinson’s disease. However, the lack of efficacy to penetrate blood–brain barrier has led the antioxidants to fail to exhibit the in vivo effect. Tso and Lam detected astaxanthin in the brain of rats fed with natural astaxanthin (Tso and Lam, 1996), suggesting that astaxanthin could cross the blood–brain barrier in mammals. Together with the neuroprotective effect in this study, it is suggested that astaxanthin may be used as a brain nutrient to protect the brain content from oxidative stress, neuronal apoptosis, and even brain aging.

In the present study, we have demonstrated the protective effects of astaxanthin on DHA-OOH- or 6-OHDA-induced cellular toxicity in SH-SY5Y cells. The protective ability is quite powerful at the nmol/L levels, which seems to be more potent than that of other natural compounds at the μmol/L levels in the same 6-OHDA-treated SH-SY5Y cell line, such as isoborneol (Tian et al., 2007), pyrroloquinoline quinone (PQQ) (Hara et al., 2007), and baicalein (Lee et al., 2005). In addition,
the major mechanism responsible for the protection of astaxanthin may be the suppression of ROS generation. Mitochondria are a major source of intracellular ROS and are particularly vulnerable to oxidative stress in the neuronal cells (Szeto, 2006). Our data demonstrated the protective role of astaxanthin in mitochondria dysfunction and ROS generation, but astaxanthin pretreatment did not affect the change of Bcl-2 and p-p38 expression induced by either DHA-OOH or 6-OHDA (data not shown), which are the upstream feature of mitochondrial ROS generation. We detected the largest concentration of astaxanthin in the mitochondria compared to other fractions in SH-SY5Y cells, which agrees with the report that detected astaxanthin only in mitochondrial fraction using a human mesangial cell line (Manabe et al., 2008). Mitochondria nutrients have been defined as those which protect mitochondria from oxidative damage and improve mitochondrial function (Liu, 2008). The data shown in this study have demonstrated the effect of astaxanthin on protecting the mitochondria in SH-SY5Y cells from oxidative damage; however, whether astaxanthin could have the ability to improve mitochondria function has not been investigated but is expected to be examined in future studies.

In conclusion, our studies demonstrate that astaxanthin prevented DHA-OOH- or 6-OHDA-induced neuronal apoptosis, mitochondrial abnormalities and intracellular ROS generation in dopaminergic SH-SY5Y cells. The effects on reducing oxidative damage in the mitochondria of the cells suggest that astaxanthin may be a potent candidate for an anti-apoptotic compound that modifies PD progression.

4. Experimental procedures

4.1. Materials

Astaxanthin was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). DHA (purity=98%) was purchased from the Cayman Chemical Co. (Ann Arbor, MI). 6-Hydroxydopamine hydrochloride was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The antibody against mouse cytochrome c was obtained from BD Pharmingen (USA).

4.2. DHA hydroperoxide preparation

The DHA hydroperoxides (DHA-OOH) were prepared by the reaction of lipoxidase (from soybeans, Wako Pure Chemical Industries, Ltd., Japan) with docosahexaenoic acid as previously described (Liu et al., 2008). The reaction mixture containing 83.6 mg docosahexaenoic acid, 16 mg lipoxidase and 220 ml of borate buffer (200 mM, pH 9.0) was used, and the reaction was carried out in a flask filled with oxygen at room temperature. After incubation for 10 min, to terminate the reaction, HCl was added to the mixture until the solution pH was below 4.0. The formed hydroperoxides were extracted twice with an equal amount of chloroform/methanol (1:1), and the collected chloroform layer was then evaporated. The obtained DHA-OOH was dissolved in ethanol. The identification was performed by HPLC analysis monitored at A334, and the concentration was quantified using a lipid hydroperoxide kit (Cayman) and compared to a standard curve prepared using authentic 13-HPODE.

4.3. Cell culture and cell viability

Human dopaminergic neuroblastoma SH-SY5Y cells were grown in Cosmedium-001 (Cosmo-Bio, Tokyo, Japan) containing 5% fetal bovine serum. The cells were seeded on plates coated with polylysine and cultured at 37 °C. The cell viability was quantified by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, different concentrations of astaxanthin were added to the cells for 4 h. Following the removal of excess astaxanthin agent, the cells were washed three times with FBS-free DMEM prior to the addition of 10 μM DHA-OOH or 100 μM 6-OHDA for 24 h, followed by the further incubation with 0.5% MTT solution (5 mg/ml) for 4 h. The cells were then lysed with 0.04 N HCl in isopropyl alcohol, and the absorbance was read at wavelengths of 550 nm (peak) and 630 nm (bottom).

4.4. Analysis of DNA fragmentation

The SH-SY5Y cells were incubated with 100 nM astaxanthin for 4 h, followed by the treatment with 10 μM DHA-OOH or 100 μM 6-OHDA for 4 h. The SH-SY5Y cells treated were collected and suspended in 0.2 ml of lysis buffer (20 mM Tris–HCl, pH 7.5, 10 mM EDTA, and 0.5% Triton X-100) and incubated at room temperature for 10 min. The samples were then centrifuged at 12,000 × g for 10 min, and the supernatant containing the DNA cleavage products was incubated with 0.2 mg/ml proteinase K at 37 °C for 1 h followed by 0.1 mg/ml RNase A for 30 min at 50 °C. The DNA fragments were purified by phenol/chloroform extraction and ethanol precipitation, then separated on an ethidium bromide (0.5 mg/ml)-containing 2% agarose gel.

4.5. Nuclear staining assay

To determine the apoptotic nuclei, SH-SY5Y cells were incubated with 100 nM astaxanthin for 4 h, washed, and the subsequently treated with 10 μM DHA-OOH or 100 μM 6-OHDA for 1 h. The cells were stained with a fluorescent DNA-binding dye, Hoechst 33258, and observed using a fluorescence microscope (Olympus Optical Co., Ltd., Tokyo, Japan).

4.6. Subcellular fraction of SH-SY5Y cell

Subcellular isolation from the cells was carried out as described by Pallotti et al. (2004). Briefly, cells are harvested by centrifugation at 600 × g for 10 min, washed with PBS, and resuspended with 5 volumes of Solution A (0.25 M sucrose, 20 mM HEPES-KOH, pH 7.5, 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.1 mM PMSF). The cellular suspension was homogenized with a glass-glass homogenizer with 20 up and down passes of the pestle. The homogenate was then centrifuged at 750 × g for 10 min. The pellet was used as the cell membrane fraction. The resulting supernatant was collected and then centrifuged at 10,000 × g for 15 min. The pellet was used as the mitochondrial fraction.
The supernatant was centrifuged at 105,000 ×g for 60 min, and the resulting supernatant was used as cytosolic fraction.

4.7. Measurement of the mitochondrial membrane potential

The mitochondrial membrane potential was measured by DiOC6 staining. Briefly, the toxins-treated SH-SYSY cells with or without astaxanthin pretreatment were incubated with 30 nM DiOC6 for 30 min at 37 °C. The DiOC6 staining was observed using a fluorescence microscope (Olympus Optical Co., Ltd., Tokyo, Japan).

4.8. Protein carbonyls

The mitochondrial and cytosolic fraction in the SH-SYSY cells were separated as described in Section 4.6. The proteins in each fraction were isolated by RIPA lysis buffer. Protein carbonyls were assayed using the dinitrophenylhydrazine (DNPH) reaction followed by Western blotting.

4.9. Analysis of ROS production

The intracellular ROS level was detected by flow cytometry using DCHF-DA that is oxidized by hydrogen peroxide or low-molecular-weight peroxides to produce the fluorescent compound 2,7-dichlorofluorescein (DCF). In this study, the SH-SYSY cells (which had reached approximately 80% confluence) were seeded on six-well plates and thereafter incubated for 4 h in serum-free DMEM in the presence of 100 nM astaxanthin. After washing with serum-free DMEM, the cells were loaded 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).

4.10. Analysis of astaxanthin concentration in different fractions of the cell

Astaxanthin contents in cell membrane, mitochondrial and cytosolic fractionation and media were quantified by high performance liquid chromatography (HPLC). Five dishes (9-cm cell culture dishes) of confluent SH-SYSY cells were incubated with 100 nM astaxanthin for 4 h. The media were collected. The cells were washed with PBS for three times, and then were fractionated into cell membrane, mitochondria and cytosol as described in 2.6. The media and fractionations were dissolved in 200 ml of acetone and filtered through a 0.45-mm polytetrafluoroethylene membrane filter; then 20 ml of solution was subjected to HPLC-UV using a column of Develosil ODS HG-5 (4.6×250 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 0.8 ml/ml, and astaxanthin peak was collected by monitoring at 471 nm. Astaxanthin was quantified relative to calibration with a standard sample. Obtained quantity of astaxanthin in media and each cellular fraction was adjusted by added total astaxanthin quantity and expressed as % of added total astaxanthin.

4.11. Western blot analysis

The homogenates prepared from the cells were treated with the SDS sample buffer and then immediately boiled for 5 min. The protein concentrations were determined using the BCA protein assay kit (Pierce). The proteins were separated by SDS-PAGE in the presence of 2-mercaptoethanol and electro-transferred onto a nitrocellulose membrane (Hybond ECL) (Amersham Biosciences). To detect the immunoreactive proteins, we used horseradish peroxidase-conjugated anti-rabbit or mouse or goat IgG and ECL blotting reagents (Amersham Biosciences).

4.12. Statistical analysis

All data were analyzed using Bonferroni/Dunn’s multiple comparison procedure.

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