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Selenium-Containing Allophycocyanin Purified from Selenium-Enriched *Spirulina platensis* Attenuates AAPH-Induced Oxidative Stress in Human Erythrocytes through Inhibition of ROS Generation

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ABSTRACT: Both selenium and allophycocyanin (APC) have been reported to show novel antioxidant activities. In this study, a fast protein liquid chromatographic method for purification of selenium-containing allophycocyanin (Se-APC) from selenium-enriched *Spirulina platensis* and the protective effect of Se-APC on 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH)-induced oxidative stress have been described. After fractionation by ammonium sulfate precipitation, and separation by DEAE-Sepharose ion-exchange and Sephacryl S-300 size exclusion chromatography, Se-APC with purity ratio (A652/A280) of 5.30 and Se concentration of 343.02 $\mu g\, g^{-1}$ protein was obtained. Se-APC exhibited stronger antioxidant activity than APC by scavenging ABTS (2,2′-azinobis-3-ethylbenzothiazolin-6-sulfonic acid) and AAPH free radicals. The oxidative hemolysis and morphological changes induced by AAPH in human erythrocytes were effectively reversed by coincubation with Se-APC. Lipid oxidation induced by the pro-oxidant agent cupric chloride in human plasma, as evaluated by formation of conjugated diene, was blocked by Se-APC. The accumulation of malondialdehyde, loss of reduced glutathione, and increase in enzyme activities of glutathione peroxidase and reductase induced by AAPH in human erythrocytes were effectively suppressed by Se-APC. Furthermore, Se-APC significantly prevented AAPH-induced intracellular reactive oxygen species (ROS) generation. Taken together, our results suggest that Se-APC demonstrates application potential in treatment of diseases in which excess production of ROS acts as a casual or contributory factor.

KEYWORDS: selenium, selenium-containing allophycocyanin, antioxidant, purification

INTRODUCTION

Trace element selenium (Se) is an essential nutrient. It has received considerable attention in the past decades. A number of investigations and clinical studies have demonstrated that Se supplementation could reduce the incidence of human cancers, including prostate, lung, colon and liver cancers.[1] Se is taken up from the diet in inorganic forms, such as selenite and selenate, or organic forms like selenocysteine and selenomethionine. Se has been shown to affect the functions of several specific intracellular selenoproteins by being a component of their essential constituent selenocysteine.[2] Selenocompounds are enzymatically and/or nonenzymatically metabolized in the biological environment, and finally incorporated into Se-containing proteins. Se functions in the active sites of a large number of Se-containing enzymes, such as glutathione peroxidase (GPx) and thioredoxin reductase (TrxR), and acts as an essential component of several major metabolic pathways, including thyroid hormone metabolism, antioxidant defense systems, and immune function.[3,4] Oral supplementation of Se in the form of sodium selenite had a marked beneficial effect on the overall antioxidant potential of the animals, levels of blood glutathione and activities of various antioxidant enzymes.[5] An important role of these antioxidant enzymes is to protect cells against the effects of free radicals that are produced during the normal oxygen metabolism. The human body has developed an antioxidant defense system to control the levels of free radicals that can damage cells and contribute to the development of some chronic diseases.[6]

Spirulina platensis (*S. platensis*) is a blue-green microalga belonging to the Cyanobacteria family in the shape of a spiral coil, living in both sea and fresh water. *S. platensis* has been widely used as a functional food product because of its high contents of protein and other nutritional elements. Our previous works have shown that *S. platensis* was a good carrier for Se accumulation.[7,8] Se was mainly incorporated into the proteins of *S. platensis* cells. It has been found that over 85% of Se in *S. platensis* existed in organic forms, and most of them were incorporated into the protein components.[7] Moreover, selenomethionine was identified as one of the major organic Se compounds in Se-enriched *S. platensis*.[9] Allophycocyanin (APC) is a light-harvesting pigment—protein complex found in the core of the phycobilisomes of *S. platensis*. This water-soluble protein is broadly used in biochemical techniques as a fluorescent probe, particularly for flow cytometry.[10] Studies have also reported that APC showed hepatoprotective, antioxidant, radical scavenging, antiarthritic and anti-inflammatory properties, as evidenced in both *in vitro* and *in vivo* experimental models.[11,12] Although demonstrating widespread application, APC still remains the least-studied phycobiliprotein due to its extremely low content, which accounts for less than 10% of the total cellular phycobiliproteins. Various methods have been reported for the purification of

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APC by employing chromatographic steps or an aqueous two phase extraction method.[13−15] However, limited information on Se-containing APC (Se-APC) is available. Considering these, we report an effective method for purification of Se-APC from Se-enriched *S. platensis* in this study. The procedures involve ammonium sulfate precipitation, ion exchange chromatography and gel filtration chromatography. The obtained Se-APC with high purity was further characterized by UV−vis, fluorescence spectra, and SDS−PAGE and identified by MALDI-TOF-TOF mass spectrometry.

Our previous works have demonstrated that phycocyanin significantly prevented the hIAPP-induced overproduction of intracellular ROS.[16] Moreover, Se-containing phycocyanin exhibited higher antioxidant and antiproliferative activities than phycocyanin.[17] Similar to our findings, Se-containing proteins extracted from Se-enriched *Ganoderma lucidum* were also found to exhibit much higher antioxidant activities than those without Se.[18] In this study we tried to get the evidence of the protective effect of Se-APC on 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH)-induced erythrocyte hemolysis. It was shown that Se-APC could attenuate AAPH-induced oxidative damage in human erythrocytes through inhibition of ROS generation. These results suggest that Se-APC demonstrates application potential in treatment of diseases in which excess production of ROS acts as a casual or contributory factor.

MATERIALS AND METHODS

Materials. The microalga *S. platensis* was kindly provided by the Research Center of Hydrobiology of Jinan University, China. AAPH, cupric chloride ($CuCl_2$), ABTS (2,2′-azinobis-3-ethylbenzothiazolin-6-sulfonic acid), bicinchoninic acid (BCA) kit, dichlorofluorescein diacetate (DCFH-DA) and DEAE-Sepharose were purchased from Sigma-Aldrich. Assay kits for determination of malondialdehyde (MDA), glutathione (GSH), glutathione peroxidase (GPx) and glutathione reductase (GR) activities were purchased from Beyotime Institute of Biotechnology. The ultrapure water used in all experiments was supplied by a Milli-Q water purification system from Millipore.

Cultivation of High Se-Enriched *S. platensis* Cells. The cultivation of *S. platensis* cells was carried out in 250 mL Erlenmeyer flasks containing 100 mL of Zarrouk medium (pH 9.0) at 30 ± 2 °C with a light illumination of 4000 lx and a 14:10 h light:dark cycle. A stepwise Se addition method was used in the culture of Se-enriched *S. platensis*, where Se was added to the medium on day 7 (100 mg L^{-1}), day 8 (150 mg L^{-1}), and day 9 (200 mg L^{-1}), respectively, with an accumulative concentration of 450 mg L^{-1}.[7,8]

Purification of Se-APC by FPLC. The freeze-dried Se-enriched *S. platensis* cells were suspended in 50 mM Na−phosphate buffer (pH 7.0). The cell suspension was frozen (at −20 °C) and thawed repeatedly 10 times and then sonicated for 3 min (Sonics VCX 600 system, 200 W). Then the sonicated cell preparation was centrifuged at 10000*g* for 30 min, and the supernatant containing Se-APC was collected for further purification. The Se-APC containing supernatant was fractionated by precipitation with three saturations of ammonium sulfate at 30%, 50% and 65%. The precipitates obtained from 30% and 50% saturations of ammonium sulfate were discarded, while the precipitates obtained from 65% saturation of ammonium sulfate were allowed to stand overnight at 4 °C . The precipitated proteins containing mainly Se-APC were collected by centrifugation at 10000*g* (Millipore microcentrifuge) for 30 min at 4 °C. The pellet was then redissolved in 5 mL of Na−phosphate buffer (5 mM, pH 7.0) and dialyzed overnight at 4 °C against the same buffer. The crude Se-APC concentration was determined by using a spectroscopic method.[19] The dialyzed Se-APC solution was centrifuged at 4500*g* for 1 h and applied into a Biologic Duo-Flow chromatography system equipped with a Bio-Rad Econo-column (1.5 × 50 cm) filled with DEAE-Sepharose Fast Flow resin. The column was developed with two linear concentration gradients of NaCl (0−0.15 M, 0.15−0.22 M). The flow rate was 1.5 mL min^{-1}. Se-APC was eluted in the second NaCl gradient, and the effluent was collected in 2 mL fractions. These fractions were monitored by recording the absorption spectrum from 250 to 700 nm. The purity ratio (A_{652}/A_{280}) could then be determined. The pooled fractions were further purified and desalted on a Sephacryl S-300 column (1.5 × 40 cm) after centrifugation at 4500*g* for 1 h. Se-APC was eluted with 2 mM Na−phosphate buffer (pH 7.0) at 1 mL min^{-1}. The effluent was collected in 1 mL fractions. Those fractions with purity ratio (A_{652}/A_{280}) > 5.0 were pooled. The purification procedures for APC from *S. platensis* were the same as that for Se-APC from high Se-enriched *S. platensis* as described above.

Spectroscopic Measurements. All UV−vis absorption spectra were recorded by a UV−vis−NIR spectrophotometer (CARY 500) with a 1 cm path length. Purity ratio (A_{652}/A_{280}) was calculated based on the UV−vis spectra. Fluorescence spectra were recorded on a Cary Eclipse fluorescence spectrometer (VARIAN).

SDS−PAGE analysis. SDS−PAGE on 15% polyacrylamide gel was carried out using the Laemmli buffer system. Protein sample was mixed with equal volume of sample buffer containing 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol, 2% (w/v) SDS, 0.002% (w/v) bromophenol blue and 60 mM Tris (pH 6.8), and boiled for 10 min. Electrophoresis was carried out at room temperature, and the gel was stained with Colloidal Coomassie blue.

Peptide Mass Fingerprint (PMF) Analysis by MALDI-TOF-TOF Mass Spectrometry and Database Searching. Protein bands were excised from Colloidal Coomassie blue stained gels and cut into small pieces with a surgical needle carefully. Destaining of gel pieces was performed using 100 mM NH_4HCO_3 with 50% methanol (v/v). The destaining step was repeated 3 times. The gel pieces were dehydrated with ACN and dried by SpeedVac (SAVANT refrigerated condensation trap system). The dried gel samples were incubated in a trypsin solution containing 50 mM NH_4HCO_3 and 40 $\mu g\ \mu L^{-1}$ trypsin for 30 min on ice. The gel samples were incubated at 32 °C overnight after addition of buffer solution (25 mM NH_4HCO_3) to cover all gel pieces. The digested peptides were then extracted from the gel pieces with buffer solution and washing solution (ACN containing 5% TFA) by sonication for 10 min (Branson 5210 system). The extraction was repeated twice. The supernatants containing the digested peptides were pooled and dried by SpeedVac.

The digested peptide samples were dissolved in 0.1% TFA then applied to a Zip Tip u-C_{18} column (Millipore) for desalting. The desalted peptide samples were redissolved in 50% ACN containing 0.1% TFA and were added into a 192-well MALDI plate and air-dried prior to analysis in the MALDI-TOF-TOF system. Dried α and β subunit fractions of Se-APC were collected from HPLC by SpeedVac prior to trypsin digestion. The digested subunit samples were directly added into the 192-well MALDI plates for analysis. MALDI-TOF-TOF analysis was using an ABI Applied Biosystems 4700 Proteomics Analyzer (Amersham Biosciences). Mass spectra were obtained using a laser (337 nm, 200 Hz) as desorption ionization source. Data were obtained in the reflection positive mode using delayed extraction. Spectra were calibrated using trypsin auto lysis products (m/z 842.51 and 2211.10) as internal standards. After MS acquisition, 10 strongest peptides per spot were selected automatically for MS−MS analysis. Identification of proteins was accomplished by searching against NCBI nonredundant protein sequence database.

The peptide mass tolerance was set as 0.5 Da (50 ppm), and variable modifications of oxidation and carbamidomethylation were considered. Automatic data analysis and database searching were achieved by the GPS Explore software (Applied Biosystem Inc.). The probability score generated was used as criterion for identification. Proteins with total score >59 or best ion score >29 were considered to be credible.[20]

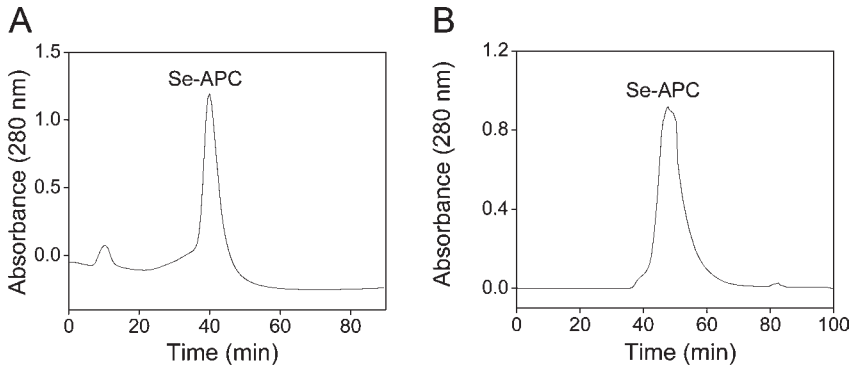

Figure 1. DEAE-Sepharose chromatography of Se-APC from *Spirulina platensis*. The sample was eluted with increasing NaCl concentration gradient, first at 0−0.15 M, followed by 0.15−0.22 M. The flow rate was 1.5 mL min^{-1} (A). Sephacryl S-300 chromatography of *Spirulina platensis* Se-APC. The sample was eluted with 2 mM Na−phosphate buffer (pH 7.0) at 1 mL min^{-1} (B).

Determination of Total Se. ICP-AES method was used to determine Se concentration.[20] The sample was digested with 1 mL of H_2O_2 and 3 mL of concentrated nitric acid in a digestive stove (Qian Jian Measuring Instrument Co., Ltd., China) at 180 °C for 3 h. The digested product was reconstituted to 10 mL with Milli-Q water and used for total Se determination.

$ABTS^+$ Free Radical Scavenging Activities. $ABTS^+$ free radical scavenging activities of antioxidants were measured according to the method previously described.[17] Briefly, 50 μL of the tested samples was mixed with 1 mL of ABTS reagent with absorbance of 0.70 ± 0.02 at 734 nm, and then the absorbance at 734 nm was measured after the initiation of mixing for 6 min.

Preparation of Erythrocyte Suspension. The AAPH free radical scavenging activities of antioxidants were measured as the inhibition of erythrocyte hemolysis according to the procedures described by Cheung et al.[21] with some modifications. Briefly, the blood was obtained from healthy volunteers. Erythrocytes were separated from the plasma by centrifugation at 1500*g* for 10 min, then washed three times with PBS buffer (pH 7.4), and finally resuspended using the same buffer to a hematocrit level of 20%. An aliquot of 0.1 mL of 20% erythrocyte suspension was mixed with 0.1 mL of 100 mM AAPH and 0.2 mL of PBS buffer (absorbance *A*) or 0.2 mL of test compounds (absorbance *B*). The mixture was shaken gently and incubated at 37 °C for 2 h. After incubation, the mixture was diluted with 8 mL of PBS buffer, while complete hemolysis was achieved by dilution with 8 mL of distilled water. Then the mixture was centrifuged at 1500*g* for 10 min, and the absorbance of the supernatant was measured at 540 nm. The % hemolysis inhibition was calculated as follows: % inhibition = $(1 - A/B) \times 100\%$.

Plasma Oxidation Assay. Plasma samples were obtained after centrifugation of the heparinized blood from healthy volunteers at 1500*g* for 10 min. Aliquots were stored at 4 °C until used. Lipid oxidation was carried out at 37 °C by treating plasma samples with 200 μM $CuCl_2$ as oxidant for the indicated time. To evaluate the protective role of the Se-APC, samples were preincubated with the Se-APC at different concentrations (range 0.06−0.3 μM) for 15 min. The copper-induced oxidation in 40-fold diluted plasma samples was monitored by recording the formation of diene at 245 nm for 2 h at 37 °C.[22]

Atomic Force Microscopy (AFM) Imaging. A 0.5 mL aliquot of the treated blood sample was manually spread onto a polished microscope glass slide to create the blood film. The film was then air-fixed rapidly by waving the slide vigorously. The film was examined under the optical microscope and regions with a single layer of red blood cells but without undue overlap of neighboring cells were marked using a lens mounted inking device.[23] An atomic force microscope (Thermomicroscopes Autoprobe CP Research, Veeco Instruments, Sunnyvale, CA, USA) was used to detect the morphology of erythrocytes.

Determination of ROS Generation. A fluorometric assay (DCFH-DA assay) was used to determine the relative levels of ROS, such as superoxide radical, hydroxyl radical, and hydrogen peroxide, as described previously. 2′,7′-Dichlorofluorescein diacetate is a nonpolar compound that diffuses rapidly into the cells, where it hydrolyzes to the fluorescent polar derivative DCFH, which is a reduced form. The reduced form of DCFH is oxidized to the highly fluorescent DCF in the presence of esterases and ROS. Treated cells were harvested by centrifugation at 1500*g* for 15 min, washed twice with PBS buffer, and then suspended in PBS buffer. The cell suspension was incubated with DCFH-DA to a final concentration of 10 μM at 37 °C for 30 min. Intracellular ROS generation was monitored by measuring the fluorescence intensity of cells with a BioTek microplate reader, with excitation and emission wavelengths set at 488 and 525 nm, respectively.

Determination of Lipid Peroxidation, GSH Content, Enzyme Activities of GPx and GR. At the indicated time, the reaction mixture was centrifuged and 0.6 mL of distilled water was added to the erythrocyte pellet in order to lyse the erythrocytes. Aliquots of the samples were transferred into polyethylene tubes and stored at −80 °C until analysis. The protein concentrations were determined using a BCA protein assay kit.

Lipid peroxidation was estimated based on thiobarbituric acid (TBA) reactivity. Samples were evaluated for MDA production using a spectrophotometric assay for TBA. The extinction coefficient of 153 $mM^{-1} \cdot cm^{-1}$ at 532 nm for the chromophore was used to calculate the MDA-like TBA produced. The content of GSH in the lysate was determined at 412 nm by the reaction with DTNB.

For the assay of the GPx and GR activities, the methods have been previously described.[24] Briefly, GPx activity was measured by quantifying the rate of oxidaion of reduced glutathione to oxidized glutathione by the hydrogen peroxide. One unit of GPx activity was defined as the decrease in 1 mM GSH (except the effect of nonenzymatic reaction) in system of enzymatic reaction of 1 mg of protein/min. Determination of GPx activity was based on the oxidation of GSH by GPx, coupled to the disappearance of NADPH by GR. GR activity was determined by following the decrease in absorbance at 340 nm due to the oxidation of NADPH utilized in the reduction of oxidized glutathione.

Statistical Analysis. Experiments were carried out at least in triplicate, and results were expressed as means ± standard deviations (SDs). Statistical analysis was performed using SPSS statistical package (SPSS 13.0 for Windows; SPSS, Inc., Chicago, IL). The difference between two groups was analyzed by two-tailed Student's *t* test, and that between three or more groups was analyzed by one-way analysis of variance multiple comparisons. Differences with $P < 0.05$ (*) were considered statistically significant.

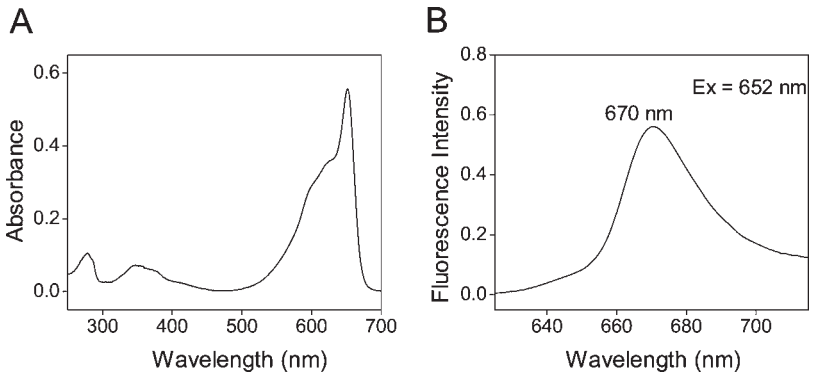

Figure 2. Spectroscopic characterization of purified Se-APC: UV−VIS spectra of Se-APC obtained from Sephacryl S-300 chromatography (A); emission spectrum of purified Se-APC obtained from Sephacryl S-300 chromatography (B). The spectra were recorded in PBS buffer (2 mM, pH 7.0) and normalized.

RESULTS AND DISSCUSION

Purification of Se-APC by FPLC. The results for purification of Se-APC from Se-enriched *S. platensis* are shown in Figures 1 and 2. First, a three-step precipitation procedure using ammonium sulfate at 30%, 50% and 65% saturation was used. Contaminating proteins were removed in the initial 30% saturation of ammonium sulfate, and Se-PC fraction was precipitated in the 50% saturation of ammonium sulfate. Most of the Se-APC remaining in the supernatant was further precipitated by 65% saturation of ammonium sulfate.[25] The crude Se-APC obtained was determined at about 24.8 mg/g dry weight of *S. platensis* by using a spectroscopic method. This fraction was then subjected to DEAE-Sepharose chromatography. The elution involved two steps. The first step with a linear NaCl concentration gradient of (0−0.15 M) removed most contaminating proteins in the sample. Se-APC was eluted in the second step in which a NaCl concentration gradient of (0.19−0.21 M) was employed. A good separation of Se-APC from other algal proteins was achieved (Figure 1A). Comparing with previous works also employing DEAE-Sepharose chromatography, a more effective removal of contaminating proteins in the first step may be the critical reason of higher purity ratio obtained in this study. Sephacryl S-300 size exclusion chromatography was used to further purification of Se-APC. As shown in Figure 1B, a sharp Se-APC peak with purity ratio of 5.30 was obtained. After purification by ion exchange and size exclusion chromatography, Se-APC with a Se concentration of 343.02 μg g^{-1} was finally obtained.

Characterization of Se-APC by UV−Vis and Fluorescence Spectroscopy and SDS−PAGE. The characteristic absorption peak at 652 nm and strong absorbance below 300 nm in the UV−VIS spectra of Se-APC fractions shown in Figure 2A corroborate the presence of the Se-APC protein. The decrease in relative intensity of peaks at 280 and 652 nm indicates the improvement in the purity of Se-APC in terms of purity ratio (date not shown). The fluorescence spectrum of purified Se-APC showed maximum emission at 670 nm (Figure 2B). Since both APC and Se-APC have the same fluorescence spectra, it suggests that the incorporation of Se into APC does not affect the fluorescence property of this protein.[26] Results of SDS−PAGE analysis displayed two major bands corresponding to α and β subunits of Se-APC with molecular masses of 17.1 kDa and 18.4 kDa, respectively (Figure 3).

Identification of α and β Subunits of Se-APC and APC by Peptide Mass Fingerprint (PMF). Peptide mass fingerprint

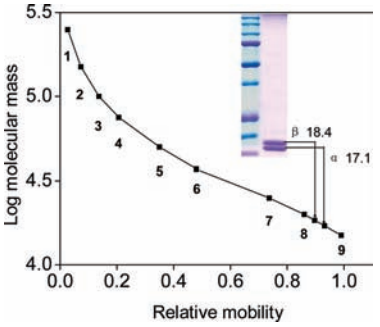

Figure 3. SDS−PAGE analysis of purified Se-APC. The molecular masses of recombinant protein markers used were (1) 250 kDa; (2) 150 kDa; (3) 100 kDa; (4) 75 kDa; (5) 50 kDa; (6) 37 kDa; (7) 25 kDa; (8) 20 kDa; (9) 15 kDa (Precision Plus protein standards, Bio-Rad).

(PMF) using MALDI-TOF-TOF mass spectrometry was used to identify α and β subunits separated by SDS−PAGE. In this study, the introduction of the Zip Tip column for desalting the digested protein samples and the use of sonication to facilitate extraction of the peptides resulted in superior performance of the subsequent PMF analysis. Table 1 reports the accession numbers and protein molecular masses of the identified subunits. The number of peptides identified in each subunit and the protein score of each PMF analysis are also presented. The protein bands excised from the SDS−PAGE gel of purified Se-APC and APC could be well identified by MALDI-TOF-TOF, which confirmed the purity of Se-APC and APC purified using this FPLC system. Most importantly, it could be observed that the incorporation of Se into APC did not affect the identification of the subunits by MALDI-TOF-TOF.

Antioxidant Activity of Se-APC. Antioxidant activity of Se-APC was evaluated by ABTS$^{\bullet}$ free radical scavenging assay. In this assay, the relatively long-lived ABTS$^{\bullet}$ free radicals generated by the direct oxidation of ABTS with manganese dioxide are decolorized when they react with hydrogen-donating antioxidants. This assay provides a direct comparison of the antioxidant activities of tested samples as presented in terms of percentage inhibition. As shown in Figure 4A, concentration response curves of Se-APC and APC were obtained at final concentrations ranging from 0 to 5.6 μM. Se-APC inhibited ABTS oxidation higher than those of APC, suggesting a stronger antioxidant activity of Se-APC under the hydrophilic conditions. Furthermore, Se-APC and APC showed

Table 1. Identification of α and β Subunits of Se-APC and APC by MALDI-TOF-TOF[a]

protein	subunit	accession no.	protein MW (Da)	no. of peptides identified	protein score
APC	α	gi|1633287	17078.8	9	133
	β	gi|291568829	18429.9	12	156
Se-APC[b]	α	gi|1633287	7078.8	7	129
	β	gi|291568829	18429.9	10	147

[a] Results searched from NCBI nonredundant protein sequence database. [b] Se-APC was identified as APC since the NCBI database contains no sequence information on Se-containing proteins from selenized *Spirulina*.

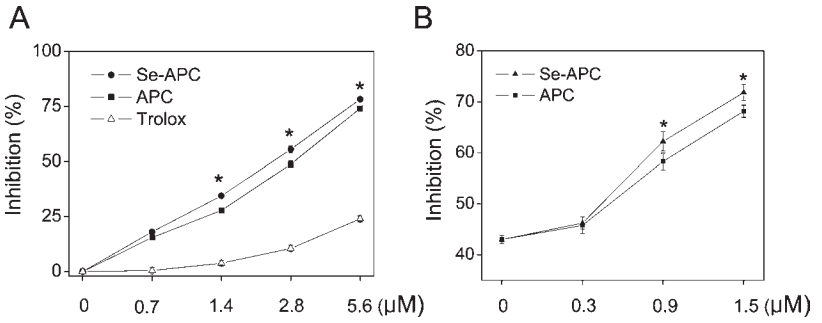

Figure 4. Antioxidant activities of Se-APC as determined by ABTS assay (A) and erythrocyte hemolysis assay (B). Values expressed are means ± SD of triplicates. A significant difference between Se-APC and APC treatments at the same concentration is indicated at the $P < 0.05$ (*) level.

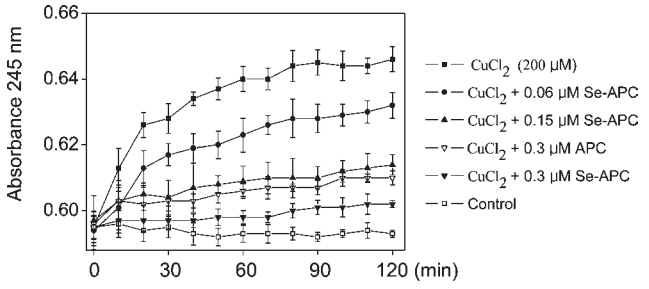

Figure 5. Time-dependent diene accumulations during copper-induced oxidation of plasma for 2 h at 37 °C. Plasma samples were preincubated with Se-APC (0.006−0.3 μM) and APC (0.3 μM) for 15 min, and then 200 μM $CuCl_2$ was added. As positive control, cells were treated with $CuCl_2$ only.

significantly ($P < 0.05$) higher inhibition on ABTS oxidation than well-known antioxidants, namely, Trolox. For instance, Se-APC and APC inhibited ABTS oxidation by 55.5% and 44.8% at 2.8 μM respectively, which were significantly higher than those of Trolox (10.5%).

Attenuation of Erythrocyte Hemolysis and Plasma Oxidation by Se-APC. Antioxidant activity of Se-APC was further conformed by erythrocyte hemolysis assay and plasma oxidazability assay. AAPH is a well-known water-soluble radical initiator, which forms alkylperoxyl radicals and alkylperoxides that are capable of modifying both lipids and DNA.[27] In hemolysis assay, AAPH radicals attack erythrocyte membrane components and cause changes in the structure and function of cell membranes.[28] As shown in Figure 4B, Se-APC and APC showed dose-dependent protection on erythrocytes against AAPH-induced hemolysis. Moreover, Se-APC inhibited AAPH-induced hemolysis more significantly than APC. For instance, Se-APC inhibited hemolysis by 62.3% at 0.9 μM and up to 71.9% at 1.5 μM, which were significantly higher than those of APC (58.3% and 68.2%). The difference in antioxidant activities between Se-APC and APC obtained by this assay was quite consistent with those obtained by ABTS assay. In the plasma oxidizability assay, the kinetics of conjugated diene formation at 245 nm upon exposure of plasma to $CuCl_2$ is shown in Figure 5. Treatments of plasma with 200 mM $CuCl_2$ led to lipoprotein oxidation and diene accumulation. The presence of increasing concentrations of Se-APC (0.06−0.3 μM) resulted in a progressive inhibition of copper-induced diene formation with a concomitant increase of plasma resistance to oxidation. At the same concentrations, such as 0.3 μM, Se-APC demonstrated stronger inhibition on copper-induced diene formation than APC. Significant difference ($P < 0.05$) between Se-APC and APC treatments was observed during the time of 50−120 min. These results clearly demonstrate the higher antioxidant activity of Se-APC than APC.

To characterize the erythrocyte damage induced by AAPH and the protection of Se-APC, morphological changes of erythrocytes were examined by AFM. It is clear that the typical biconcave shape and smooth surfaces of the normal erythrocytes can be readily observed from Figure 6. When erythrocytes were treated with 100 mM AAPH, the shape of the whole erythrocyte changed dramatically and cell surface became rougher than normal erythrocytes. Irreversible hemolysis and the erythrocyte collapse were also observed. However, pretreatment of erythrocytes with 1.2 μM Se-APC before the presence of AAPH alleviated the damage of erythrocyte and the morphology of erythrocytes reversed to somewhat concave, suggesting that Se-APC effectively block the oxidative stress induced by AAPH in erythrocytes.

Se-APC Inhibits AAPH-Induced ROS Generation. ROS generation is a biomarker of oxidative stress. ROS consists of highly reactive molecules, including oxygen ions, free radicals, and peroxides. ROS is generated as a natural byproduct of normal cellular metabolism and has important roles in cell signaling. It was constantly generated and eliminated in all biological systems, and they play important roles in a variety of normal biochemical functions and abnormal pathological processes. Excess intracellular ROS may

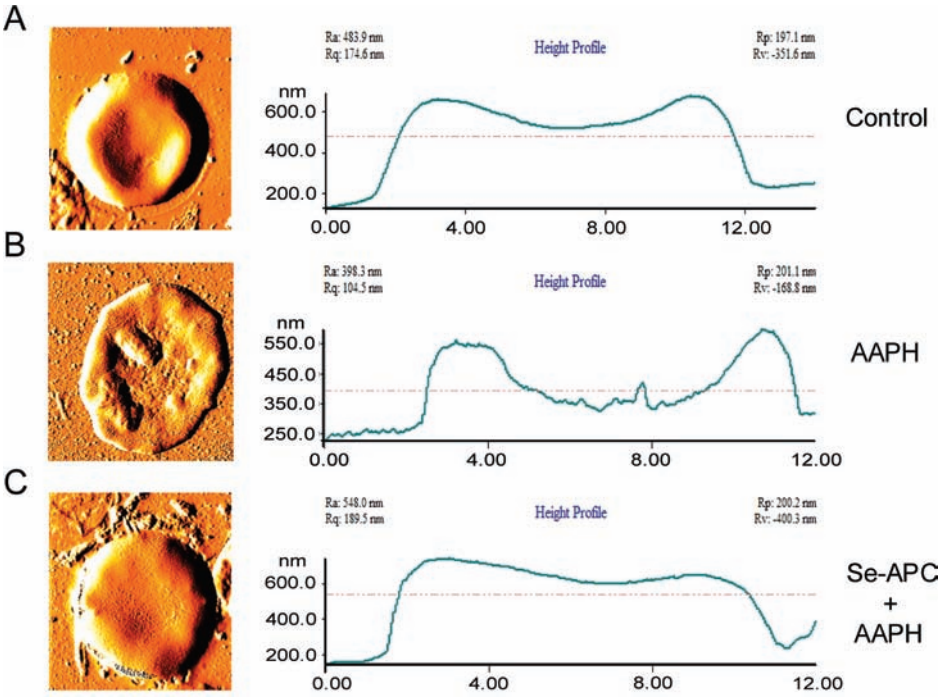

Figure 6. AFM images of erythrocyte sample. AFM images of normal erythrocyte (A). AFM images of erythrocytes were treated with 100 mM AAPH for 2-h only (B). Erythrocytes were pretreated with 1.2 μM Se-APC for 30 min and further treated with 100 mM AAPH for 2 h (C).

attack cellular membrane lipids, proteins, and DNA, inhibit their normal functions, and finally cause oxidative damage. To further explore the biomarkers' basic contribution to the protective effects of Se-APC on erythrocytes, AAPH-induced ROS generation and the effects of Se-APC were examined in erythrocytes. Intracellular ROS was detected by using a fluorescein-labeled dye, DCFH-DA. As show in Figure 7, erythrocytes treated with AAPH showed a significant increase in ROS generation by comparing with the control. However, pretreatment of erythrocytes with Se-APC for 0.5 h significantly reduced ROS generation in erythrocytes exposed to AAPH. For instance, 0.3 μM Se-APC reduced the DCF fluorescence from 292 $\pm$ 15% of control to 195 $\pm$ 18% (Figure 7). Furthermore, no significant differences were found in erythrocytes treated with 1.5 μM Se-APC compared with control erythrocytes. These results indicate that Se-APC protects human erythrocytes from AAPH-induced hemolysis through inhibition of ROS generation.

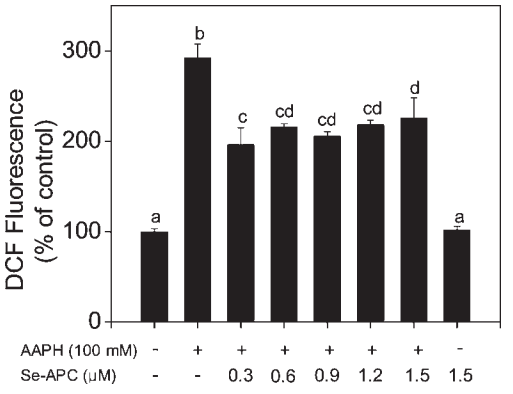

Figure 7. Protective effects of Se-APC on AAPH-induced ROS generation in erythrocytes. Erythrocytes were pretreated with different concentrations (0.3−1.5 μM) of Se-APC for 30 min and further treated with 100 mM AAPH for 2 h. As positive control, erythrocytes were treated with AAPH for 2 h only. Values expressed are means $\pm$ SD of triplicates. A significant difference between treatments is indicated at $P < 0.05$.

Se-APC Prevents AAPH-Induced Changes in Level of MDA and GSH, and Activities of Antioxidant Enzymes. Excess ROS can damage cell structure, leading to DNA damage, lipid peroxidation and protein degradation. As one of the end products of lipid peroxidation, MDA could alter the structure and function of cell membrane, block cellular metabolism and lead to cytotoxicity.[29] As shown in Figure 8A, treatment of AAPH significantly caused lipid peroxidation in erythrocytes. MDA content increased from 0.62 $\pm$ 0.04 to 1.06 $\pm$ 0.09 nmol mg^{-1} proteins after 2 h incubation with 100 mM AAPH. However, the addition of 0.9 μM Se-APC significantly inhibited the MDA formation, and no significant differences were found in erythrocytes treated with 1.5 μM Se-APC compared with control erythrocytes. These results imply that Se-APC could alleviate the damage of AAPH on erythrocyte membranes.

Oxidative stress occurs when free radical production exceeds antioxidant defense systems. In order to prevent or repair oxidative damage, the cells develop both enzymatic and nonenzymatic defense systems. GSH is the main nonenzymatic antioxidant within the cell and plays an important role in protection against oxidative stress. It is usually assumed that GSH depletion reflects intracellular oxidation. In contrast, an increase in GSH level could be expected to protect the cells against a potential oxidative insult.[30] In this study, GSH content in erythrocytes incubated in PBS buffer (control) remained almost unchanged during the 2 h of incubation at 37 °C. The presence of 100 mM AAPH caused a significant consumption of the cytosolic GSH with a decrease from 63.15 $\pm$ 9.80 to 19.33 $\pm$ 3.50 nmol mg^{-1} proteins after 2 h incubation. However, the addition of 0.3 μM Se-APC significantly suppressed the AAPH-induced depletion of the cytosolic GSH. Furthermore, no significant

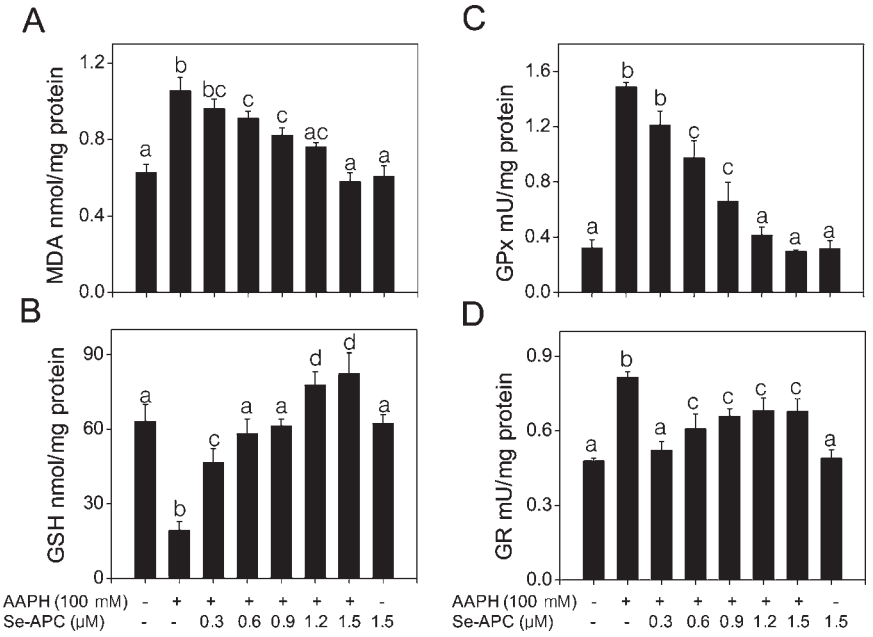

Figure 8. Changes in MDA content (A), GSH concentration (B), antioxidant enzyme activities of GPX (C) and GR (D) in erythrocyte. Erythrocytes were pretreated with different concentrations (0.3−1.5 μM) of Se-APC for 30 min and further treated with 100 mM AAPH for 2 h. As positive control, cells were treated with AAPH for 2 h only. Values expressed are means ± SD of triplicates. A significant difference between treatments is indicated at $P < 0.05$.

differences were found in erythrocytes treated with 1.5 μM Se-APC compared with control erythrocytes (Figure 8B).

Cellular antioxidant enzymes play a crucial role in the defense system against oxidative stress. Changes in activities of antioxidant enzymes can be considered as biomarkers of cell antioxidant response.[31−33] In the enzymatic defense systems, GPx is a primary antioxidant enzyme responsible for detoxification of intracellular free radicals. Depletion of GPx increased the susceptibility of cells to stress-induced cell death.[34] GPx catalyzes GSH oxidation to oxidized glutathione at the expense of peroxides,[35] and GR recycles oxidized glutathione back to GSH.[36] Significant increase in the activities of GPx and GR was observed after a 2 h treatment with 100 mM AAPH (Figure 8C,D), which indicates a positive response of the cell defense system to face an oxidative insult.[32,37] Similar effects of selenite on intracellular GPx activity in human endothelial cells in response to oxidative damage have been reported.[38] Moreover, Se-MeSeCys,[39] flavonoid quercetin,[33] olive oil phenol hydroxytyrosol[32] and a coffee melanoidin[37] were also found to be able to protect cells against oxidative damage by preventing severe changes in the enzymatic antioxidant system. In line with those results, we showed that pretreatment of erythrocytes with Se-APC prevents an increase in the activities of GPx and GR induced by AAPH, whereas erythrocytes treated with 1.5 μM Se-APC did not change the activities of GPx and GR. Taken together, these results indicate that Se-APC could prevent or repair oxidative damage in erythrocytes by protecting both enzymatic and nonenzymatic defense systems.

In conclusion, we demonstrated a fast protein liquid chromatographic method for purification of Se-APC from Se-enriched *Spirulina platensis* and its protective effect on AAPH-induced oxidative stress. It was shown show that Se-APC could attenuate AAPH-induced oxidative damage in human erythrocytes through inhibition of ROS generation. These results suggest that Se-APC could be used as a functional food that demonstrates application potential in treatment of diseases in which excess production of ROS acts as a casual or contributory factor.

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