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Purification and characterization of selenium-containing phycocyanin from selenium-enriched *Spirulina platensis*

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Abstract

A fast protein liquid chromatographic method for purification of selenium-containing phycocyanin (Se-PC) from selenium-enriched *Spirulina platensis* was described in this study. The purification procedures involved fractionation by ammonium sulfate precipitation, DEAE-Sepharose ion-exchange chromatography and Sephacry S-300 size exclusion chromatography. The purity ratio (A_{620}/A_{280}) and the separation factor (A_{620}/A_{655}) of the purified Se-PC were 5.12 and 7.92, respectively. The Se concentration of purified Se-PC was 496.5 μg g^{-1} protein, as determined by ICP-AES analysis. The purity of the Se-PC was further characterized by UV–VIS and fluorescence spectrometry, SDS–PAGE, RP-HPLC and gel filtration HPLC. The apparent molecular mass of the native Se-PC determined by gel filtration HPLC was 109 kDa, indicating that the protein existed as a trimer. SDS–PAGE of the purified Se-PC yielded two major bands corresponding to the α and β subunits. A better separation of these two subunits was obtained by RP-HPLC. Identification of the α and β subunits separated by SDS–PAGE and RP-HPLC was achieved by peptide mass fingerprinting (PMF) using MALDI-TOF-TOF mass spectrometry.

Keywords: Selenium; Phycocyanin; *Spirulina platensis*; Purification; Chromatography

1. Introduction

Selenium (Se) has received considerable attention as an essential micronutrient for human. This trace element is required for the activity of a number of selenium-dependent enzymes such as glutathione peroxidase, which catalyzes the reduction of hydrogen peroxide and phospholipid hydroperoxides. It is suggested that Se is associated with anticancer and other physiological functions (Ip et al., 2000; Miller et al., 2001). Se is taken up from the diet in inorganic form such as selenite (SeO_3^{2-}) or organic forms such as selenocysteine and selenomethionine. These Se compounds are enzymatically and/or non-enzymatically metabolized in the biological environment, and Se is finally incorporated into Se-containing proteins (Rayman, 2000).

Because of the high content of proteins and other nutritional elements in *Spirulina platensis*, this microalga is widely cultivated for the production of health food products. *S. platensis* is an important source of phycocyanin (PC), a blue photosynthetic pigment with strong antioxidative and anti-inflammatory activities as shown in *in vitro* and *in vivo* studies (Benedetti et al., 2006; Bhat and Madyastha, 2000, 2001; Farooq et al., 2004; Gonzalez et al., 1999). PC has been used for the treatment of Alzheimer's disease and Parkinson's disease (Rimbau et al., 2001) and the prevention of experimental oral and skin cancers (Morcos et al., 1988). Recently, PC was reported to induce apoptosis in lipopolysaccharide-stimulated RAW 264.7 macrophages (Reddy et al., 2003) and human chronic myeloid leukemia cell line-K562 (Subhashini et al., 2004).

Our previous works have shown that *S. platensis* was a good carrier for Se accumulation (Chen et al., 2005, 2006; Huang et al., 2002; Zheng et al., 2003). It was found that

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the accumulated Se was mainly incorporated into proteins of *S. platensis* cells. PC is the major protein component of *S. platensis*. Several methods for purification of PC from microalgae have been described (Benedetti et al., 2006; Bermejo et al., 2006; Boussiba and Richmond, 1979; Hilditch et al., 1991; Minkova et al., 2003; Patel et al., 2005; Tchernov et al., 1999; Zhang and Chen, 1999). However, limited information on Se-containing PC is available. In this study, we report an effective method for purification of Se-PC from Se-enriched *S. platensis*. The procedures involved ammonium sulfate precipitation, ion exchange chromatography and gel filtration chromatography. The purified Se-PC was further characterized and identified by UV–VIS and fluorescence spectra, SDS–PAGE, reversed phase and gel filtration HPLC and MALDI-TOF-TOF mass spectrometry.

2. Results and discussion

2.1. Purification of Se-PC by FPLC and determination of Se concentration

The results of purification of Se-PC from Se-enriched *S. platensis* were shown in Figs. 1–3 and Table 1. Since PC is the major soluble protein component of *S. platensis* cell extract, conventional protein purification techniques were employed. A two-step precipitation procedure using ammonium sulfate at 30% and 50% saturation was used (Zhang and Chen, 1999). The initial 30% saturation of ammonium sulfate removed contaminating proteins, whereas 50% saturation of ammonium sulfate precipitated the major Se-PC fraction. Most of the allophycocyanin (APC) remained in the supernatant, which could be further precipitated by 65% saturation of ammonium sulfate (Zhang and Chen, 1999). As shown in Table 1, these steps improved the purity ratio and the separation factor of Se-PC to a considerable level of 2.74 and 3.03, respectively.

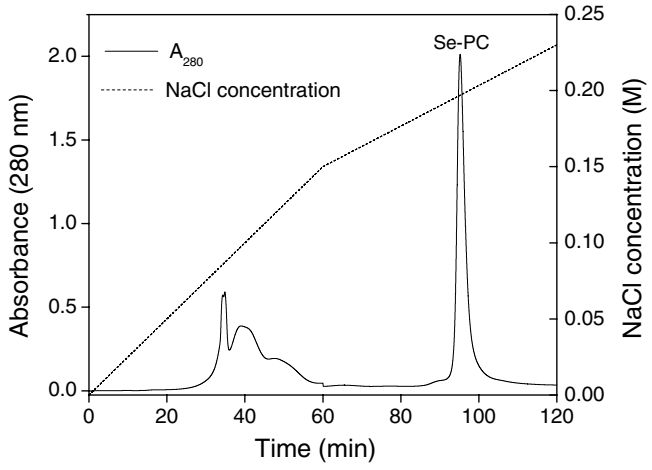

Fig. 1. DEAE-sepharose chromatography of *Spirulina platensis* Se-PC. The sample was eluted with increasing NaCl concentration gradient, first at 0–0.15 M, followed by 0.15–0.22 M. The flow rate was 1.5 ml min^{-1}.

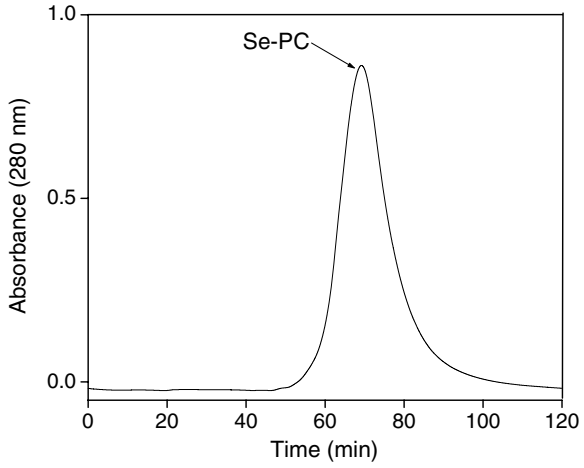

Fig. 2. Sephacryl S-300 chromatography of *Spirulina platensis* Se-PC. The sample was eluted with 2 mM Na–phosphate buffer (pH 7.0) at 1 ml min^{-1}.

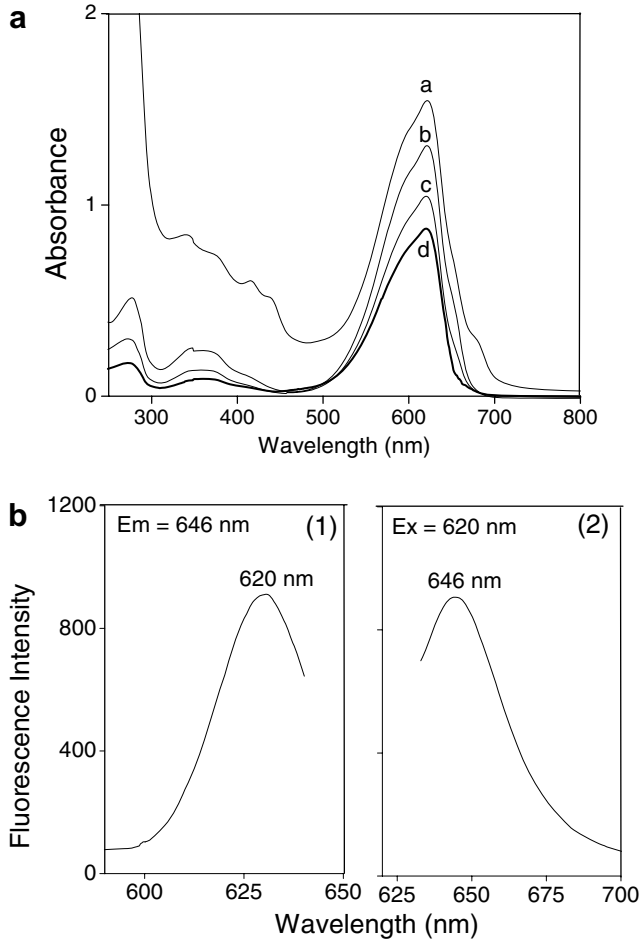

Fig. 3. Spectroscopic characterization of purified *Spirulina platensis* Se-PC: (a) UV–VIS spectra of Se-PC obtained in different steps of purification. a, crude extract; b, fraction obtained from 50% ammonium sulfate precipitation; c, pooled fractions obtained from DEAE-Sepharose chromatography; d, pooled fractions obtained from Sephacryl S-300 chromatography. (b) Excitation spectrum (1) and emission spectrum (2) of purified Se-PC obtained from Sephacryl S-300 chromatography. The spectra were recorded in PBS buffer (2 mM, pH 7.0) and normalized.

Table 1
Purity ratio, separation factor and Se concentration of Se-PC obtained in different purification steps

Purification step	Purity ratio A_{620}/A_{280}	Separation factor A_{620}/A_{655}	Se concentration μg g^{-1} protein
Crude extract	0.62	2.41	233.8
Precipitation with 50% saturation of $(NH_4)_2SO_4$	2.74	3.03	368.5
DEAE-sepharose fast flow chromatography	4.69	5.33	448.2
Sephacryl S-300 chromatography	5.12	7.95	496.5

The crude Se-PC fraction obtained was 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-PC 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-PC from other algal proteins was achieved (Fig. 1). The purity ratio and separation factor of Se-PC were enhanced to 4.69 and 5.33, respectively. Comparing with previous works also employing DEAE-Sepharose chromatography (Patel et al., 2005; Zhang and Chen, 1999), the higher purity ratio obtained in this study was probably due to a more effective removal of contaminating proteins in the first step. Further purification of Se-PC was achieved by Sephacry S-300 size exclusion chromatography. As shown in Fig. 2, a sharp Se-PC peak was obtained, with purity ratio and separation factor of 5.12 and 7.95, respectively. As compared with other published methods (Benedetti et al., 2006; Bermejo et al., 2006; Boussiba and Richmond, 1979; Hilditch et al., 1991; Minkova et al., 2003; Patel et al., 2005; Tchernov et al., 1999; Zhang and Chen, 1999), our purification method gave a higher purity of Se-PC.

Under photoautotrophic culture conditions, the organic Se in *S. platensis* accounted for about 80% of the total Se accumulated in the algal cells and was comprised of about 60% of the water-soluble protein-bound form (Chen et al., 2005). As shown in Table 1, after purification by ion exchange and size exclusion chromatography, a Se concentration of 496.5 μg g^{-1} in the final Se-PC fraction was obtained. This allowed the subsequent structure and seleno-amino acid sequence analysis to be carried out.

2.2. Characterization of Se-PC by UV–VIS and fluorescence spectroscopy, SDS–PAGE and HPLC

The characteristic absorption peak at 620 nm and strong absorbance below 300 nm in the UV–VIS spectra of various Se-PC fractions shown in Fig. 3(a) corroborate the presence of the Se-PC protein. The decrease in relative intensity of peaks at 280 nm and 655 nm indicates the successive improvement in the purity of Se-PC in terms of purity ratio (A_{620}/A_{280}) and separation factor (A_{620}/A_{655}). The fluorescence spectrum of purified Se-PC showed maximum emission at 646 nm (Fig. 3(b)). This is in agreement with the fluorescence property of PC reported in the literatures (Benedetti et al., 2006; Bermejo et al., 1997, 2006; Glazer, 1989; Minkova et al., 2003). Since both PC and Se-PC have the same fluorescence spectra, it suggests that the incorporation of Se into PC does not affect the fluorescence property of this protein. Moreover, Se-PC showed no fluorescence emission in the range of 660–700 nm, indicating a successful separation of APC from Se-PC. SDS–PAGE analysis revealed two major bands corresponding to α and β subunits of Se-PC. Their molecular masses were estimated to be 17.5 kDa and 18.5 kDa, respectively (Fig. 4). The molecular mass of Se-PC monomer (αβ) was thus calculated to be 36 kDa. However, the molecular mass of the native Se-PC was 109 kDa, as determined by gel filtration HPLC chromatography (Fig. 5). These data indicated that the aggregation state of the purified Se-PC was trimer $(\alpha\beta)_3$. The results are consistent with those reported by Patel et al. (2005), Benedetti et al. (2006) and Bermejo et al. (2006).

Analytical methods frequently used for characterization of purity of PC are UV–VIS spectroscopy (in terms of purity ratio and separation factor), fluorescence spectroscopy and SDS–PAGE of the denatured proteins (Benedetti et al., 2006; Bermejo et al., 2006; Boussiba and Richmond, 1979; Minkova et al., 2003; Patel et al., 2005; Tchernov et al., 1999; Zhang and Chen, 1999). However, spectroscopic techniques just give a rough estimation of the purity and nature of proteins, while SDS–PAGE provides only approximated value of the molecular mass of the protein under study (Simó et al., 2005). Due to the similar amino acid sequences and similar molecular masses, both PC and APC and their α and β subunits (i.e., PC-α; PC-β; APC-α and APC-β) present in *S. platensis* could not be separated by SDS–PAGE (Zolla and Bianchetti, 2001).

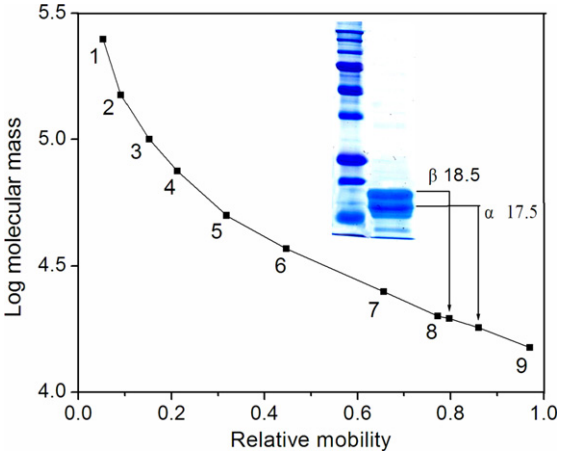

Fig. 4. SDS–PAGE analysis of purified *Spirulina platensis* Se-PC. The molecular masses of recombinant protein markers used were (1) 250 kDa; (2) 150 kDa; (3) 100 kDa; (4) 75 kDa; (5) 50 kDa; (6) 37 kDa; (7) 25 kDa; (8) 20 kDa; (9) 15 kDa (Precision Plus protein standards, Bio-Rad).

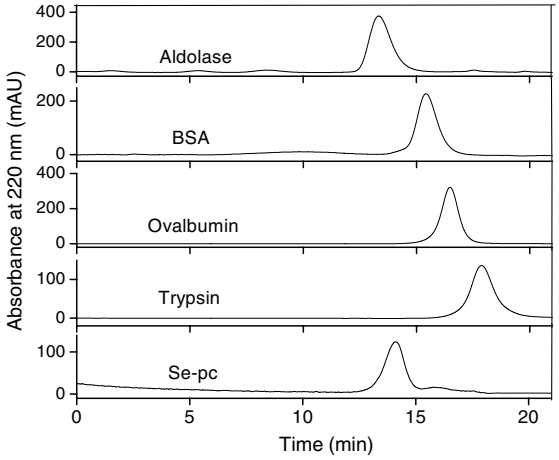

Fig. 5. Gel filtration HPLC chromatographic profiles of purified Se-PC and protein markers. Protein markers included aldolase (158 kDa), BSA (67 kDa), ovalbumin (43 kDa) and trypsin (23.8 kDa). The relationship between the retention time (T) and log molecular mass (M) was $T = -5.4661\ M + 41.769$, $R^2 = 0.9998$.

Recently, new capillary electrophoresis-mass spectrometry (CE-MS) procedures were developed to analyze proteins in *S. platensis* microalgae (Simó et al., 2005). Another method based on HPLC–MS with the complete resolution of the protein components of phycobilisome from cyanobacterium *Synechocystis* 6803 was described (Zolla and Bianchetti, 2001).

In order to better characterize Se-PC isolated from *S. platensis* by ion exchange and size exclusion chromatography, the purified protein was subjected to RP-HPLC analysis employing a H_2O-ACN-TFA elution gradient system modified from that of Zolla and Bianchetti (2001). With respect to the Waters Symmetric C_{18} and Vydac C_4 columns, a better separation of Se-PC was obtained using a Zobax C_8 column. As shown in Fig. 6(a), when detector was set at 214 nm, 280 nm and 600 nm, two major peaks corresponding to the α (retention time: 30.7 min) and β (retention time: 33.7 min) subunits of Se-PC were revealed in the chromatograms. Both subunits were eluted from the C_8 column in a narrow range of ACN concentration of 48.9–53.9% (v/v), as expected by their close similarity in molecular mass, hydrophobicity and chemical structure. No other obvious peaks could be detected in the three chromatograms, confirming the purity of Se-PC purified using this FPLC system.

Fig. 6(b) shows the on-line spectra of α and β subunits recorded by diode-array detector. The spectra of the two subunits showed obvious absorbance bands at 630–660 nm, typically due to the presence of tetrapyrrole chromophoric groups (phycocyanobilin) covalently bound to the polypeptide backbone. The absorbance at 280 nm corresponded to the total proteins. The intensity ratio of band 630–660 nm to 280 nm of β subunit was twice of that of α subunit, indicating that the ratio of phycocyanobilin content of the two subunits of Se-PC (β:α) was about 2:1, a value in agreement with previous findings (Bermejo et al., 1997).

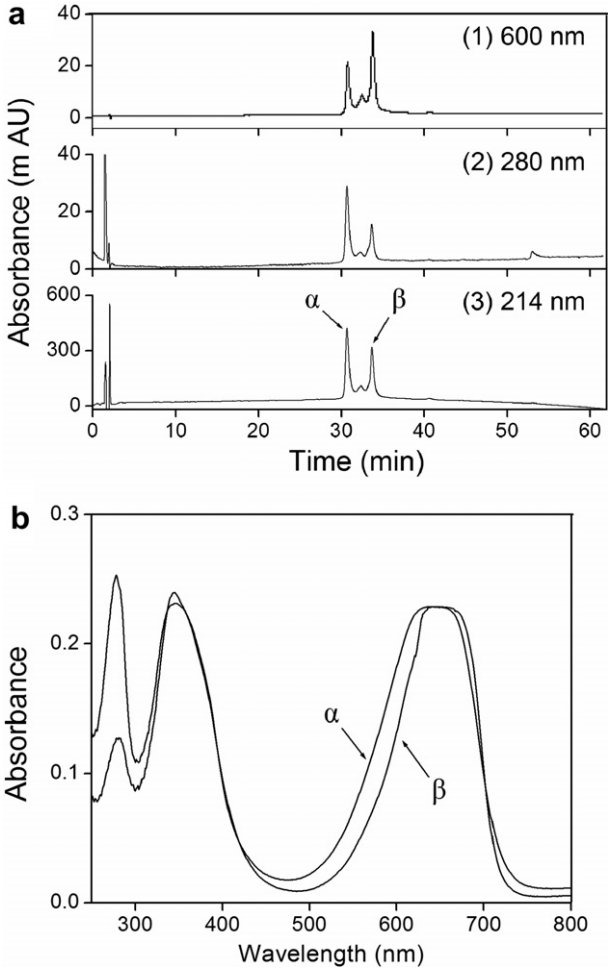

Fig. 6. RP-HPLC chromatographic profiles of purified *Spirulina platensis* Se-PC recorded at 600 nm, 280 nm and 214 nm, respectively (a), and on-line absorption spectra of α and β subunit peaks (b).

2.3. Identification of α and β subunits of Se-PC and PC by peptide mass fingerprint (PMF)

The identification of α and β subunits separated by SDS–PAGE and RP-HPLC was achieved by peptide mass fingerprint (PMF) using MALDI-TOF-TOF mass spectrometry. 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 2 reported the accession numbers, protein molecular masses and isoelectric points (pIs) of the identified subunits. The number of peptides identified in each subunit and the protein score of each PMF analysis were presented. The protein bands excised from the SDS–PAGE gel of purified Se-PC and PC and the fractions collected from RP-HPLC could be well identified by MALDI-TOF-TOF, which confirmed the purity of Se-PC and PC purified using this FPLC system. Furthermore, more peptides were identified and higher protein scores were obtained in HPLC fractions, indicating that the optimized HPLC method was superior than SDS–PAGE for resolution of

Table 2
Identification of α and β subunits of Se-PC and PC by MALDI-TOF-TOF[a]

Separation method	Protein	Subunit	Accession No.	Protein mol mass (Da)	Protein pI	No. of peptide identified	Protein score
SDS–PAGE	Se-PC[b]	α	gi|1654094	17589.8	6.58	5	144
		β	gi|18252399	18082	4.96	8	157
	PC	α	gi|1654094	17589.8	6.58	8	97
		β	gi|18252399	18082	4.96	6	108
HPLC	Se-PC[b]	α	gi|1654094	17589.8	6.58	7	323
		β	gi|18252399	18082	4.96	10	178
	PC	α	gi|1654094	17589.8	6.58	5	272
		β	gi|18252399	18082	4.96	6	157

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

α and β subunits in Se-PC and PC. Most importantly, it could be observed that the incorporation of Se into PC did not affect the identification of the subunits by MALDI-TOF-TOF.

3. Experimental

3.1. 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 Zarrouk medium (pH 9.0) at 30 ± 2 °C with a light illumination of 4000 lx and a 14:10 h light:dark cycle. For Se-enriched culture, a stepwise Se addition method was employed (Chen et al., 2005, 2006). Se was added to the culture medium on day-7 (100 mg L^{-1}), day-8 (150 mg L^{-1}), and day-9 (200 mg L^{-1}) with a final accumulative concentration of 450 mg L^{-1}. *S. platensis* cells were harvested and freeze-dried on day-11.

3.2. Purification of Se-PC 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 for 10 times and then sonicated for 3 min (Sonics VCX 600 system, 200 W). The sonicated cell preparation was centrifuged at 10,000*g* for 30 min, and the Se-PC containing supernatant was collected for further purification.

The Se-PC containing supernatant was fractionated by precipitation with solid ammonium sulfate at 30% saturation followed by 50% saturation. The precipitate obtained from 30% saturation of ammonium sulfate was discarded. The supernatant was further brought to 50% saturation of ammonium sulfate and allowed to stand overnight at 4 °C. The precipitated proteins containing mainly Se-PC were collected by centrifugation at 10,000*g* (Millipore microcentrifuge) for 30 min at 4 °C. The pellet was re-dissolved in 5 ml Na–phosphate buffer (5 mM, pH 7.0) and dialyzed overnight at 4 °C against the same buffer. The dialyzed Se-PC solution was centrifuged at 4500*g* for 1 h and applied to a Bio-Logic DuoFlow chromatography system equipped with a Bio-Rad Econo-column (1.5 × 50 cm) packed with DEAE-Sepharose Fast Flow resin. The column was developed with two linear concentration gradients of NaCl. The first gradient employed was 0–0.15 M, followed by the second gradient of 0.15–0.22 M. The flow rate was 1.5 ml/min. Se-PC was eluted in the second NaCl gradient and the effluent was collected in 2-ml fractions. The purity of fractions was monitored by recording the absorption spectrum from 250 to 800 nm. The purity ratio (A_{620}/A_{280}) and separation factor (A_{620}/A_{655}) could then be determined. All fractions having purity ratio (A_{620}/A_{280}) > 4.0 and separation factor (A_{620}/A_{655}) > 5.0 were pooled. After centrifugation at 4500*g* for 1 h, the pooled fractions were further purified and desalted on a Sephacryl S-300 column (1.5 × 40 cm). Se-PC 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_{620}/A_{280}) > 5.0 and separation factor (A_{620}/A_{655}) > 7.0 were pooled.

The purification procedures for PC from *S. platensis* were the same as that for Se-PC from high Se-enriched *S. platensis* as described above.

3.3. Spectroscopic measurements

All UV–VIS absorption spectra were recorded on a UV–VIS–NIR spectrophotometer (CARY 500) with a 1 cm path length. Purity ratio (A_{620}/A_{280}) and separation factor (A_{620}/A_{655}) were calculated based on the UV–VIS spectra. Fluorescence spectra were recorded on a F4500 Fluorescence spectrometer (Hitachi).

3.4. SDS–PAGE analysis

SDS–PAGE on 15% polyacrylamide gel was carried out using the Laemmli buffer system (Laemmli, 1970). Protein sample was mixed with equal volume of sample buffer containing 2% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol, 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.

3.5. HPLC analysis

HPLC was performed using a Waters 6690 chromatography system. The molecular mass of the purified Se-PC was estimated by gel filtration HPLC on a Progel™-TSK column G5000PW (30 cm × 7.5 mm ID) combined with a TSK-GFL guard column PWH (7.5 cm × 7.5 mm ID). Samples were eluted with 0.1 M Na–phosphate buffer (pH 6.8) at a flow rate of 0.6 ml min^{-1} and monitored at 220 nm. Molecular mass was determined from a calibration curve using aldolase (158 kDa), BSA (67 kDa), ovalbumin (43 kDa) (Gel Filtration Calibration Kit, Amersham Pharmacia Biotech) and trypsin (23.8 kDa) (Sigma T4665) as protein markers.

For characterization of purity, Se-PC samples in Na–phosphate buffer (5 mM, pH 7.0) were injected onto a Zobax C_8 column (150 × 4.6 mm), previously equilibrated with a solution composed of 80% buffer A (0.1% TFA in water) and 20% buffer B (0.1% TFA in ACN). PC subunits were eluted from the column according to the following program: 0–60 min, linear gradient to 80% buffer B; 60–65 min, linear gradient to 95% buffer B; 65–70 min, linear gradient to 20% buffer A; 70–75 min, 20% buffer B. The flow-rate was 1.0 ml min^{-1} and the effluent was monitored at three different wavelengths, i.e., 214 nm, 280 nm and 600 nm. The eluted subunit fractions were collected manually and used for MALDI-TOF-TOF identification.

3.6. In-gel digestion

Protein bands were carefully excised from colloidal Coomassie blue stained gels and cut into small pieces with a surgical needle. Destaining of gel pieces was performed using 100 mM NH_4HCO_3 in 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 μg/μL trypsin for 30 min on ice. After addition of buffer solution (25 mM NH_4HCO_3) to cover all gel pieces, the gel samples were incubated at 32 °C overnight. 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 and the supernatants containing the digested peptides were pooled and dried by SpeedVac.

3.7. Peptide mass fingerprint (PMF) analysis by MALDI-TOF-TOF mass spectrometry and database searching

The digested peptide samples were dissolved in 0.1% TFA and applied to a Zip Tip u-C_{18} column (Millipore) for desalting. The desalted peptide samples were re-dissolved in 50% ACN containing 0.1% TFA and were added onto a 192-well MALDI plate and air-dried prior to analysis in the MALDI-TOF-TOF system. The α and β subunit fractions of Se-PC collected from HPLC were dried by SpeedVac prior to trypsin digestion. The digested subunit samples were directly added onto the 192-well MALDI plates for analysis.

MALDI-TOF-TOF analysis was performed 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 acquired in the reflection positive mode using delayed extraction. Spectra were calibrated using trypsin autolysis 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 carried out by searching against NCBI non-redundant 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 performed 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.

3.8. Determination of total Se, inorganic Se and organic Se concentrations

Se concentration was determined by ICP-AES method (Chen et al., 2005). The sample was digested with 3 ml concentrated nitric acid and 1 ml H_2O_2 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 H_2O and used for total Se determination.

3.9. Determination of algal growth and protein concentrations

The dry weight of algal biomass was determined by drying the cells at 70 °C in a vacuum oven until constant weight was obtained. The protein concentrations were determined by the Bradford method (Bradford, 1976).

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