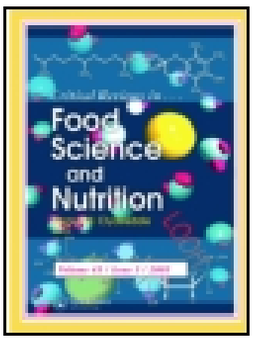

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Purification and bioactivities of phycocyanin

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Abstract

Phycocyanin is an important light-harvesting pigment antenna protein in *Cyanobacteria*, *Rhodophyta*, *Cryptophyta* and *Glaucophyta*, and has a variety of bioactivities. The introduction of the purification and bioactivities of phycocyanin contributes to a significant improvement in developing it in final processed products. In fact, the knowledge of phycocyanin has experienced a rapid increase in the past twenty years, and has promoted the relevant technological revolution with a decisive contribution to the final application. The purpose of this review is to critically introduce the present knowledge of the purification and bioactivities of phycocyanin, and is to illustrate the main problems and prospects.

Keywords

Phycocyanin; Purification; Bioactivities; Main problems and prospects

INTRODUCTION

Phycobiliprotein is a main light-harvesting pigment antenna protein that mainly exists in *Cyanobacteria*, *Rhodophyta*, *Cryptophyta* and *Glaucophyta*. 60% of soluble proteins in alga is phycobiliprotein, which includes phycocyanin, phycoerythrin and allophycocyanin (Binder et al., 1972; Sidler, 2004; Yu et al., 2010). Phycobiliprotein can efficiently transmit the light energy to chlorophyll a, and thus alga are capable of undergoing photosynthesis (Apt et al., 1995; French and Young, 1952; Glazer, 1985; Holzwarth, 1991).

Phycocyanin is one of phycobiliproteins, which is beautiful sky blue and can be used as an effective ingredient of food additives, the healthcare food, cosmetics and pharmaceuticals (Eriksen, 2008; Wu and Yang, 2006). Phycocyanin not only can improve the immunity of the human body and promote the regeneration of animal blood cells, but also has a strong fluorescence because the chromophore group exists in its molecular structure (Glazer et al., 1973; Ilani and Berns, 1971; Seppälä et al., 2007; Sode et al., 1991; Zhang et al., 1999). Thus, it is widely used in the fields of molecular biology, immunology, cytology and molecular diagnose (Eriksen, 2008; Xia et al., 2001). However, the purification of phycocyanin is complex and unstable, and this limits its industrial production and application (Minkova et al., 2003; Patel et al., 2005; Yu et al., 1997; Zhang and Chen, 1999). Thus, studies on the large-scale purification technology of phycocyanin have become a focus in recent twenty years, significant improvements have been achieved and corresponding technologies have also been continuously

innovated. Moreover, a big progress has been made in the studies on the bioactivities of phycocyanin, including *in vitro* anticancer activity, the chemotherapy sensitivity, the photosensitized tumor suppressor activity, the anti-inflammatory effect, the anti-oxidative and anti-irradiative effect, the immunomodulatory function, the promotion of the cell growth, and the neuroprotective effect, etc. (Abd El-Baky and El-Baroty, 2012; Jaiswal et al., 2008; Pardhasaradhi et al., 2003; Wang et al., 2001). Thus, this paper is the first time to critically review the progress of the purification and bioactivities of phycocyanin.

STRUCTURE OF PHYCOCYANIN

Phycocyanin comprises of the polypeptide chain and the chromophore phycobilin (Fig. 1). The polypeptide chain is composed of α- and β-subunit whose molecular weights are between 18 and 20 kDa (Holzwarth, 1991; Sidler, 2004; Xu, 2003; Yu et al., 2002; Yu et al., 2010). The phycocyanin monomer (αβ) contains three chromophores, positioned in Cys-84 of α-subunit and Cys-84 and Cys-155 of β-subunit. α- and β-subunit forms monomer (αβ) by intermolecular interaction, which is further aggregated to be $(\alpha\beta)_3$ and $(\alpha\beta)_6$ by a connecting polypeptide. Phycocyanin mainly exists in the forms of trimer and hexamer. Moreover, monomers can form dimer or higher aggregates than hexamer. The chromophore phycobilin is synthesized from protoheme by a soluble heme oxygenase that can convert protoheme to biliverdin IXα. By the other soluble enzymes, phycobilin 15,16-dihydrobiliverdin IXα, (3*Z*)-phycoerythrobilin and (3*Z*)-phycocyanobilin can be sequentially produced from biliverdin IXα (Fig. 2) (Beale and

Cornejo, 1984a; Beale and Cornejo, 1984b; Beale and Cornejo, 1991a, b; Beale and Cornejo, 1991c; Cornejo and Beale, 1988; Rhie and Beale, 1992; Rhie and Beale, 1994; Rhie and Beale, 1995).

CRUDE EXTRACTION OF PHYCOCYANIN

The crude extraction of phycocyanin includes the cell disruption and the salting-out precipitation, etc., in which the former step is crucial and important. The cell disruption of alga is divided into the mechanical and non-mechanical methods. Mechanical methods include ultrasonication, bead mill and high pressure homogenization. Non-mechanical methods include the chemical osmosis and the repeated freeze-thaw. Ultrasonication is often used in the laboratory study, whereas the bead mill and high-pressure homogenization are suitable for the large-scale disruption of algal cells. The enzymolysis and chemical osmosis are widely used in the laboratory study. Santiago-Santos et al. (2004) carried out the crude extraction of *Spirulina* phycocyanin by enzymolysis, and they found that the repeated freeze-thaw was more suitable for the large-scale disruption of *Spirulina* cells than the ultrasonification. Lin et al. (1998) carried out the crude extraction of *Spirulina* phycocyanin with sodium dodecyl benzene sulfonate. Peng et al. (1999) obtained a large amount of the crude phycocyanin product by the repeated freeze-thaw with the sequential centrifugation collection. Because *Spirulina* is a single-cell microorganism and has a thick cell wall, a better effect of the cell disruption can be achieved by combining lysozyme with the repeated freeze-thaw method(Li et al., 2007). Zhang et al. (1999)

used the potassium chloride-lysozyme and the repeated freeze-thaw method to disrupt the cell wall of *Spirulina*, and then used the salting-out precipitation to separate and purify phycocyanin. The specific extraction process is as follows: (1) the enzymatic method: use the distilled water to dilute the algal powder or algal mud, and then treat them by both KCl and lysozyme at 30°C to disrupt the cell wall. The resultant extract was subject to filter under vacuum conditions. After its pH was adjusted to 3.5-4.0 with an acetate buffer, it was placed in the refrigerator to precipitate overnight. Centrifuge at 8000 rpm for 10 min to obtain the supernatant. The phycocyanin powder was prepared by further evaporation drying. (2) the freeze-thaw and salting-out method: the algal powder or mud was washed three times by the distilled water and frozen at -30°C, and then take them out to thaw urgently at 37°C. Repeat above steps 3-5 times, and then centrifuge at 8000 rpm for 10 min to obtain the supernatant. Add 50% of ammonium sulfate to precipitate phycocyanin. Moreover, the direct extraction of phycocyanin by phosphate buffer saline (PBS) is also used. Zhang et al. (1999) extracted phycocyanin from *S. platensis* using the PBS buffer, and the specific procedure is as follows: (1) the preparation of pH 6.0 buffer, (2) weigh 20 g of *S. platensis* powder and add them to 200 ml of the PBS buffer, and then put samples to a magnetic stirrer to stir for 4 h and centrifuge at 10000 g for 10 min to obtain the supernatant, (3) the resultant supernatant was filtered and evaporated with a rotary vacuum evaporator until the blue solid appeared. The blue solid was placed in a desiccators for 1 h to dry out completely, and was ground into the blue powder. Siegelman et al. (1978) reported that phycoerythrin was firstly

precipitated using $(NH_4)_2SO_4$ with a saturated degree of 25%, and then phycocyanin using $(NH_4)_2SO_4$ with a saturated degree of 30%, and finally allophycocyanin with $(NH_4)_2SO_4$ with a saturated degree of 50%.

CHROMATOGRAPHIC PURIFICATION

Compared to the crude extraction, the purification of phycocyanin is more immature, but studies pertinent to it are active. The chromatographic technology is widely used to purify phycocyanin because it is the most effective protein purification technology, and is easily scaled up to the industrial production. Chromatographic technologies used widely in the purification of phycocyanin are introduced below:

Ion-exchange chromatography and size exclusion chromatography

The ion-exchange chromatography for the solute separation is based on the difference in the electrostatic interaction force between the charged solute and the ion-exchange solvent. Owning to the negative charge of phycocyanin in a weakly acidic medium, it is widely purified by the anion-exchange chromatography. The size exclusion chromatography is based on the difference in the molecular weight of protein. Generally, the ion-exchange chromatography is often used in conjugation with the size exclusion chromatography. Chen and Reis purified phycocyanin from *S. maxima* by combining the size exclusion chromatography with the ion-exchange chromatography (Chen et al., 2006; Reis et al., 1998). Chen et al. (2006) purified phycocyanin

with a weakly acidic DEAE-Sepharose Fast Flow anion-exchange chromatography column. The purification fold of phycocyanin was 5.12, and could be further improved to 7.92 when the size exclusion chromatography was combined. The content of selenium phycocyanin obtained finally was 496.5 μg/g. DEAE-Sephadex is often used to purify phycocyanin. Patil et al. (2006) purified phycocyanin by combining the ion-exchange chromatography with an aqueous two-phase extraction technology. The purification fold of phycocyanin was improved from 1.18 to 3.92 by a DEAE-sephadex-filled anion-exchange chromatographic column (φ 2.5 cm × 15 cm), and the recovery rate of phycocyanin was up to 73%. This method removes the need for precipitation, centrifugation and dialysis, and so it opens up the possibility for the large-scale isolation and purification of phycocyanin. The anion-exchange chromatographic column (1.5 cm × 10 cm) filled with the Q-Sepharose Fast Flow is also used to purify phycocyanin, and the purification fold and purity of phycocyanin obtained from the crude extract were 2.2 and 93%, respectively. Thus, the anion-exchange chromatography is an effective mean for the large-scale purification of phycocyanin. Patel et al. (2005) directly purified phycocyanin from three cyanobacterial species, ie, *Spirulina* sp. (freshwater), *Phormidium* sp. (marine water) and *Lyngbya* sp. (marine water), by an efficient single step chromatographic method. Phycocyanin from the above cyanobacterial species was purified to homogeneity by the ion-exchange chromatography on a DEAE-Sepharose CL-6B column. The pure phycocyanin was obtained from *Spirulina* sp., *Phormidium* sp., and *Lyngbya* sp. with purity ratios (A_{620}/A_{280}) of 4.42, 4.43, and 4.59, respectively.

Phycocyanin and allophycocyanin were separated and purified from *S. platensis* by the precipitation with ammonium sulfate, ion-exchange chromatography and size exclusion chromatography (Zhang and Chen, 1999). The pure phycocyanin and allophycocyanin were finally obtained with an A_{620}/A_{280} value of 5.06 and an A_{655}/A_{280} value of 5.34, respectively. By repeated freeze-thaw cycles with sequential purification by a three-step process: ammonium sulfate fractionation, Sephadex G-150 size exclusion chromatography and DEAE cellulose ion-exchange chromatography, phycocyanin can be successfully extracted from a novel isolate *Oscillatoria quadripunctulata* (Soni et al., 2006). The purified phycocyanin shows a maximal absorbance at 620 nm, and a purity ratio (*R*) of 3.31 is achieved. The ratios of phycocyanin to phycoerythrin and of phycocyanin to allophycocyanin are 4.90 and 3.92, respectively. The recovery efficiency of phycocyanin from the crude extract is above 68%. Moraes et al. (2009) made a study on the use of ammonium sulfate precipitation, ion-exchange chromatography and gel filtration chromatography to purify phycocyanin by their different combinations. The finally designed combination includes the crude extraction, the precipitation with ammonium sulfate and the ion-exchange chromatography. When the elution step was studied, the pH of elution was further considered in order to obtain the phycocyanin with a final purity of 4.0 and a purification factor of 6.35.

Hydroxyapatite column chromatography

Hydroxyapatite (HAP) is a natural or synthetic calcium phosphate crystal, and has a good biological compatibility (Markov and Ivanov, 1974; Sun, 2005; Zardi et al., 1985). Hydroxyapatite column chromatography for the separation and purification of biomacromolecules, such as proteins, enzymes and nucleic acids, has a good selectivity (Fountoulakis et al., 1999; Tsuru et al., 1991). When the other separation technologies are invalid, the application of this technology are often able to display a good separation performance. Hydroxyapatite is cheap and can be used as the filler of the preparative chromatography. Yin et al. (2000) obtained 80 mg of highly pure phycocyanin by a preparative hydroxyapatite chromatographic column (12 mm × 10 cm) to absorb the crude extract of phycocyanin. This method can also make phycocyanin and allophycocyanin separate better. The purification fold of phycocyanin was improved from 1.2 to 4.8 by a preparative hydroxyapatite chromatographic column (3 cm × 35 cm). Hu et al. (2002) used fresh *Spirulina* filaments as the raw material, and used the segmented gradient salting-out to purify phycocyanin, enabling the purity of the extracted phycocyanin higher than generally recognized standard. (1) Put fresh filaments and an appropriate amount of glass beads into the flask, and stir them fully to disrupt cells. Centrifuge the broken cell suspension in a high-speed centrifuge, and discard the pellet. The supernatant was further centrifuged at 25000 rpm for 60 min to discard the pellet again. (2) Add the solid ammonium sulfate to the supernatant until a saturated degree of 25%, and stir fully. The precipitate was discarded after the supernatant was centrifuged at 10000 rpm for 10

min. Add ammonium sulfate to the supernatant until a saturated degree of 55%, and stir fully. The supernatant was discarded after the sample was centrifuged at 10000 rpm for 10 min. The precipitate was dissolved into a PBS solution, and then add an appropriate amount of solid ammonium sulfate until a saturated degree of 27%, and stir fully. The precipitate was discarded after the above sample was centrifuged at 10000 rpm for 10 min. Add ammonium sulfate to the supernatant until a saturated degree of 33%, and stir fully. The precipitate was obtained after the above sample was centrifuged at 10000 rpm for 10 min. The obtained precipitate was dissolved with a PBS solution. Add ammonium sulfate of 28-32% saturated degree to precipitate phycocyanin. The resultant phycocyanin was dissolved with a PBS solution, and was subject to further dialysis. (3)The dialysate of phycocyanin was loaded on a hydroxyapatite chromatography column, and was then eluted using different concentrations of PBS buffers. Fractions with a purification factor of more than 4.5 were collected and desalted by dialysis. (4) The obtained phycocyanin was filtered by a Sephadex G-100 chromatographic column, and fractions with a purification factor of more than 5.0 were concentrated and freeze-dried. Wang et al. (1999) obtained phycocyanin with a purification fold of up to 14 by Sephadex G-200 column chromatography with sequential DEAE-Sephadex A25, hydroxyapatite and Sephadex G-200 column chromatography. Siegelman and Kycia (1978) obtained phycocyanin (A_{565nm}/A_{280nm} = 5.0), phycoerythin (A_{565nm}/A_{280nm} = 4.5) and allophycocyanin (A_{565nm}/A_{280nm} = 6.0) from phycobiliprotein by the repeated purification using the hydroxyapatite column chromatography.

Benedetti et al. (2006) set up an efficient procedure for purifying phycocyanin from a blue-green algae *Aphanizomenon flos-aquae*. Using this procedure, phycocyanin could be purified by a simple single-step chromatographic run using a hydroxyapatite column (A_{620}/A_{280} of 4.78), considering its use for the health-promoting property while removing the other specific algal components.

Hydrophobic interaction chromatography and reverse micelle extraction

Hydrophobic interaction chromatography (HIC) is an elution chromatography using surface coupling weak hydrophobic groups of the adsorbent as a stationary phase. The principle of this chromatography is based on the difference in the interaction between separated solutes and hydrophobic adsorbents to purify biological macromolecules (Jennissen, 2001; Soni et al., 2008). Santiago-Santo et al. (2004) obtained 0.19×10^{-9} mol/ml of phycocyanin with a purification factor of 3.5 and a yield of 17% with a chromatographic column (1.5 cm×20 cm) filled with a methylated macroporous hydrophobic medium. Soni et al. (2008) obtained 0.332 mg/ml of phycocyanin with a purification factor of 3.5 by the above method. Although the hydrophobic interaction chromatography improves the purity of protein, the product obtained finally is diluted, and its concentration was therefore decreased. The reverse micelle is a collection colloid that hydrophilic groups of amphoteric surfactants spontaneously gathered inward in non-polar organic solvents. Liu et al. (2008) purified phycocyanin from *Spirulina* by the reverse micelle extraction. They used a CTAB/n-pentanol-octane reverse micelle solution to separate and purify

phycocyanin from *Spirulina*. Owning to the high extraction efficiency and distribution coefficient of phycocyanin in this system, the extracted phycocyanin has a high purity. (1) Accurately weigh 0.5 g of *Spirulina* powder, and add 100 ml of 0.01 mol/l phosphate buffer to prepare the separated sample. The sample was frozen and thawed repeatedly. The broken *Spirulina* cell suspension was centrifuged at 4000 rpm for 20 min to obtain the crude extract containing phycobiliprotein. (2) Dissolve hexadecyltrimethylammonium bromide (CTAB) into the mixture of n-octane and n-pentanol in an appropriate proportion, and wash the mixture 2-3 times with a PBS buffer to obtain a clear CTAB reverse micelle solution. (3) Aliquots of the crude phycobiliprotein extract and the solution of the reverse micelle were added to a stoppered centrifuge tube. The centrifuge tube was upside down at 25°C for 10 min at a frequency of 60 min^{-1} to stratify still. The water phase was used as the extraction liquid of the reverse micelle. (4) Centrifuged tubes filled with oil phase (the remaining portion of the reverse micelle) were added with equal volumes of inorganic salt solutions with different pHs. Centrifuged tubes were upside down at 25°C for 120 min at a frequency of 60 min^{-1} to separate the water phase from the oil phase. The water phase was used as the extraction liquid of the reverse micelle. Yin et al. (2011) established an effective procedure of combining the high-speed counter current chromatography with reverse micelle solvent system to separate phycocyanin from *S. platensis*. By optimizing the stationary phase of the reverse micelle solvent system (0.10 g/ml cetyltrimethylammonium bromide [CTAB]/ isooctane-hexylalcohol), the mobile phase A (0.05 mol/l sodium phosphate

buffer, pH 4.0, containing 0.2 mol/l KCl) and the mobile phase B (0.05 mol/l sodium phosphate buffer, pH 8.0, containing 0.4 mol/l KCl), 78.7 mg of phycocyanin was purified from 200 mg of the crude extract of *S. platensis*, and the purity of the product was 4.25 based on the absorptive ratio of A_{620}/A_{280}, which was increased by 6.85-fold compared to the crude extract.

Expanded bed absorption chromatography

Expanded bed absorption chromatography has been developed as a new type of the chromatographic technology in recent 10 years. It is one of the most important large-scale bio-integrated separation technologies that can simultaneously clarify the liquid, and concentrate and purify the desirable product (Hjorth et al., 2008; Kennedy, 2001; Sun, 2005). Niu et al. (2007) collected the effluent from the crude extract of algal proteins obtained by ammonium sulfate precipitation and the expanded bed absorption chromatography column filled with 50 ml of phenyl-sepharose as the absorbent. The purification fold of phycocyanin was improved from 0.69 to 3.0, and the amount of phycocyanin obtained was 29.9-51.0 g by one-step expanded bed absorption chromatography. Ramos et al. (2011) achieved the large-scale purification of phycocyanin from *Synechocystis aquatilis* using the expanded bed adsorption chromatography. Phycobiliprotein was firstly extracted from *S. aquatilis* cells by the osmotic shock, and was separated by passing the centrifuged cell suspension through expanded bed absorption chromatography column using Streamline-DEAE as the absorbent. The eluted phycocyanin-rich solution is finally purified by the packed-bed chromatography using DEAE-cellulose. The

optimal extraction was achieved using a phosphate buffer (pH 7.0). The operation conditions of the expanded bed absorption chromatography were optimized at a small scale using a column of 15 mm internal diameter, and the process of the expanded bed adsorption chromatography by increasing the internal diameter of columns (15, 25, 40, 60 and 90 mm) was scaled up. By combining with the conventional ion-exchange chromatography with DEAE-cellulose as the absorbent, the pure phycocyanin with a yield of 74% was finally obtained, maintaining a high protein recovery rate while reducing both processing costs and times. They also developed a method to purify phycocyanin from *Anabaena marina* (Ramos et al., 2010). In their design, the separation was performed by the expanded bed absorption (EBA) chromatography using Streamline-DEAE. Optimal conditions for EBA were obtained at a small scale using a 15 mm internal diameter column, yielding 0.9 mg phycocyanin/ml absorbent. This process was then scaled up by 36-fold, the success of the scale-up process was demonstrated. Their experiments demonstrate that small diameter column is useful to simulate the behavior of larger diameter column for use in scaled-up systems. Expanded bed absorption chromatography is demonstrated to be a scalable technology, allowing large quantities of phycocyanin to be obtained.

BOUSSIBA IMPROVEMENT METHOD BASED ON BELLMAN DYNAMIC PROGRAMMING PRINCIPLE

Boussiba improvement method is a new trial for the separation and purification of phycocyanin from alga. Chen et al. (2002) optimized process parameters and obtained optimal conditions of

the phycocyanin extraction from *Spirulina* based on the protein separated technology from *Spirulina* suggested by Boussida et al. (1979). (1) Weigh 10 g of *S. platensis* powder, dissolve them into 1 L water, and stir adequately. Cell walls were disrupted by the colloidal mill and high pressure homogenization, and then the extraction was carried out in a thermostat water bath. (2) The extraction broth was centrifuged at 12000 rpm for 40 min to obtain the supernatant which was subsequently subject to the isoelectric point separation. (3) The final product phycocyanin was obtained by the spray-dried technology.

PULSED ULTRASOUND ASSISTED EXTRACTION TECHNOLOGY

Phycocyanin can be extracted by the pulsed ultrasound assisted extraction technology using *S. platensis* as the raw material according to the following procedure (Qu et al., 2007): weigh the degreased *S. platensis* powder → dissolve them into water → pulsed ultrasound assisted extraction→ filter quickly by the filter paper → take out the filtered supernatant → analysis and determination of phycocyanin. Optimized parameters of the pulsed ultrasound assisted extraction of phycocyanin were as follows: ultrasonic duration 90 min, ultrasonic power 1400 W, ultrasonic time 6 s, interval time 9 s, circulation pump speed 12 r/s, and extract temperature 20°C. The yield of phycocyanin is 13.45% when the feeding amount is 20 g.

BIOACTIVITIES OF PHYCOCYANIN

In vitro anticancer activity of phycocyanin

Schwartz and Sklar (1986) demonstrated that *Spirulina* phycocyanin could obviously inhibit the growth of cancer cells. Wang et al. (2001) found that phycocyanin had a strong inhibition to the growth of Hela cells *in vitro*. With the increase in its concentration from 10 to 80 mg/ml, the inhibitory rate increased from 3.5% to 31% gradually. Moreover, they detected the cell cycle of living cells by flow cytometry (FCM) to determine *in vitro* anti-cancer activity of phycocyanin. They found that the transformation and accumulation of Hela cells were from S, G2, M to G1 (the quiescent phase of the DNA synthesis) by the agency of phycocyanin. This indicates that the synthesis of DNA degrades gradually, and that phycocyanin inhibits the growth of cancer cells. Zhang et al. (2000) determined the effect of *Spirulina* phycocyanin on the growth of cultured human leukemia cell lines HL-60, K-562 and U-937 *in vitro* by the semi-solid agar culture and MTT assay, and found that *Spirulina* phycocyanin had different degrees of inhibition to these three leukemia cell lines, which had a significant concentration-dose effect. Reddy et al.(2003) found that phycocyanin could selectively inhibit the activity of peroxidase-2 (COX-2) that was known to be high expression in inflammatory and tumor cells. They used the lipopolysaccharide (LPS) to stimulate RAW264 phagocytic cell lines, and observed the role of *Spirulina* phycocyanin. Phycocyanin is demonstrated to have a dose-dependent inhibition to the proliferation of RAW2647 cell lines. Iijima et al. (1983) firstly found that the survival rate of mice significantly increased, and that the lymphocytic activity was significantly higher than the control group after the mice with liver tumor cells were subject to the oral administration of

phycocyanin. Before this, the report was only presented by Tang et al. (1999) that the recombinant allophycocyanin (rAPC) had an obvious inhibition to S180 sarcoma cells of mice, and that the inhibitory rate was 45-64% when the mice that were subcutaneously inoculated with S180 sarcoma cells were injected by different concentrations of allophycocyanin. The gavage and abdominal injection had no effect on the number of white blood cells of tumor-bearing mice, and on the thymus index.

Although phycocyanin has a significant physiological activity, it is generally accepted that another important physiological active substance, *Spirulina* polysaccharide, has a much better effect than it. Guan and Guo (2001) prepared a novel biomaterial by immobilizing phycocyanin to the support medium polystyrene, and studied the inhibition of this novel biomaterial to liver cancer 7402 cell lines and human pancreatic cancer cell lines SW1990 *in vitro*. When the initial mass concentration of the fixed phycocyanin was 20 μg/hole, the inhibitory rate of liver cancer 7402 cell lines reached 55%. With the increase in the mass concentration of phycocyanin, the inhibitory rate of cancer cells decreased. But when the mass concentration of phycocyanin increased to 0.5 mg/hole and 1 mg/hole, the inhibitory rate rose again to 55% and 66%, respectively. In the serum-free media containing the natural *Spirulina* phycocyanin (0.5 mg/hole), only a small amount of cells presented vacuoles and disintegration, and the inhibitory rate of cancer cells was about 6%. They observed by the electronic microscopy that on the condition of high concentration of protein, phycocyanin immobilized on the surface of

polystyrene had a certain resistance to the surface adherent growth of liver cancer cells (Guan et al., 2000). The fixed phycocyanin can avoid the endocytosis and consumption of cells, and thus it ensures a prolonged exposure to cell surface receptors and improves the inhibition of cancer cells. The inhibitory role of the light immobilized selenium phycocyanin was compared with a natural light fixed phycocyanin. The fixed phycocyanin played an important role in inhibiting the growth of hepatocellular carcinoma cells at a low concentration (< 0.05 mg/hole). The quickest and strongest inhibition was presented by the immobilized selenium phycocyanin at a concentration of more than 0.05 mg/hole. The inhibitory rate of hepatocellular carcinoma cells by selenium phycocyanin was up to 56%, whereas it was only 26% by the light fixed phycocyanin. This indicates that selenium and phycocyanin function together within a range of a high concentration. Roy and his coworkers (2007) detected the effect of phycocyanin on the proliferation of doxorubicin-sensitive (S-HepG2) and -resistant (R-HepG2) HCC (hepatocellular carcinoma) cell lines. They found a 50% decrease in the proliferation of S- and R-HepG2 cells treated with 40 and 50 μM of phycocyanin for 24 h, respectively. Phycocyanin enhanced the sensitivity of R-HepG2 cells to doxorubicin. R-HepG2 cells treated with phycocyanin showed typical apoptotic features, such as membrane blebbing and DNA fragmentation. Moreover, they observed that R-HepG2 cells treated with 10, 25 and 50 μM of phycocyanin for 24 h showed 18.8, 39.72 and 65.64% cells in sub-G_0/G_1-phase, respectively, the cytochrome *c* release, the decrease in membrane potential, the caspase 3 activation, and PARP [poly (ADP-ribose)

polymerase]. These studies indicate the down-regulation of the anti-apoptotic protein Bcl-2 and the up-regulation of the pro-apoptotic Bax (Bcl2-associated X-protein) protein in R-HepG2 cells treated with phycocyanin, and demonstrate that phycocyanin can induce the apoptosis in R-HepG2 cells, and its potential as an anti- HCC agent. Effect of phycocyanin on rat histiocytic tumor cell lines was investigated in detail by Pardhasaradhi et al. (2003). In their experiments, AK-5 cell lines were induced into the apoptotic death program when they were treated with phycocyanin, which was involved in the activation of caspase-3. The phycocyanin-mediated apoptotic death is induced through the generation of reactive oxygen radicals. Free radical scavengers inhibited the phycocyanin-induced apoptotic death in AK-5 cell lines and Bcl-2 could regulate the ROS generation. These experiments demonstrate that phycocyanin can induce the apoptotic death in AK-5 cell lines, which is inhibited by the Bcl-2 expression through the regulation of the free radical generation. Phycocyanin was possible chemotherapeutic agent through its apoptotic activity against tumor cells. Moreover, it was observed to ameliorate doxorubicin-induced oxidative stress and apoptosis in adult rat cardiomyocytes (Khan et al., 2006). Phycocyanin from *S. platensis* can result in a significant down-regulation of MDR1 (multidrug resistance-1) expression in phycocyanin-treated HepG2 cells through reactive oxygen species and cyclooxygenase-2 (COX-2) mediated pathways (Nishanth et al., 2010). Further studies revealed the involvement of NF-κB and AP-1 in phycocyanin-induced down-regulation of MDR1. Also, the inactivation of signal transduction pathways involving in Akt, ERK, JNK

and p38 by phycocyanin was observed. Thus, the efficacy of phycocyanin in overcoming the MDR1 mediated drug resistance in HepG2 cells by down-regulation of reactive oxygen species and COX-2 pathways via the involvement of NF-κB and AP-1 was demonstrated. Li et al. (2006) investigated the effect of highly purified phycocyanin on the growth and proliferation of HeLa cells *in vitro*. They found that there was a significant decrease in the number of cells that survived for HeLa cells treated with phycocyanin compared to control cells. phycocyanin can induce characteristic apoptotic features, including cell shrinkage, membrane blebbing, microvilli loss, chromatin margination and condensation into dense granules or blocks, and the degradation of the genomic DNA of HeLa cells. Moreover, they found that phycocyanin could promote the expression of Fas and ICAM-1 (intercellular cell- adhesion molecule 1) protein, while it held back the Bcl-2 (B-cell lymphocytic-leukaemia proto- oncogene 2) protein expression.

Chemotherapy sensitivity of phycocyanin

The drug tolerance presented in tumor cells is one of the main causes of failure of the clinical cancer chemotherapy(Yang, 1996). Xin et al. (1998) studied *in vitro* sensitizing effect of phycocyanin on methotrexate and cisplatin, and found that there was a good linear relationship between the concentration of phycocyanin and the viability of Hep-2 cells ($r = 0.935$). Phycocyanin at 100 mg/L has no obvious toxicity to Hep-2 cells. The combination of phycocyanin with methotrexate significantly enhanced the cytotoxicity of methotrexate. The synergistic effect is also improved with the increase in the methotrexate concentration. There is a

significant difference in the cell survival rate. Phycocyanin at 100 mg/L or verapamil at 10 mg/L has no significant cytotoxicity. After the individual interaction of phycocyanin, or verapamil, with methotrexate occurred, both of which improved the cytotoxicity of methotrexate and had a very significant difference. However, the sensitizing effect was not significant. Moreover, the toxicity of verapamil is much higher than that of phycocyanin.

Photosensitized tumor suppressor activity of phycocyanin

Morcos et al. (1988) found that the cell survival rate of myeloma cells in mice was only 15% when the mice were treated by 0.25 g/L of phycocyanin with sequential 514 nm laser irradiation (300 J/m^2), whereas it was 69% or 71% when myeloma cells were only treated by laser radiation or phycocyanin. Cai et al. (1995) irradiated using 630 nm copper laser (12 J/cm^2) the cultured human colon carcinoma cell lines HR8348 treated by phycocyanin at the concentration of 100 μg, 50 μg and 25 μg, the survival rates of cancer cells determined by MTT method were 22.2%, 37.6% and 89.7%, respectively, showing a concentration-dose effect. After S180 xenografted mice were injected with 2 mg of phycocyanin and orally administrated with 20 mg of phycocyanin, the survival rates were 58% and 53%, respectively, compared to the control group. Thus, *in vivo* and *in vitro* experiments show that phycocyanin has a photosensitization and a non-toxic side effect and can be used as a high-quality photosensitizer. This may be caused by its molecular structure. The carrier protein of phycocyanin covalently binds with open-chain

tetrapyrrole chromophores, thus it has a similar structure with hematoporphyrin derivatives widely used in China, such as hematoprophyrin.

Anti-inflammatory effect

Experimental studies on the anti-inflammatory effect of phycocyanin have been widely carried out worldwide. Huang et al. (2002) observed the antagonistic effect of selenium phycocyanin on the carbon tetrachloride (CCl_4)-induced acute liver injury. The acute liver injury model of mice was induced by 2% of CCl_4 oil gavage. Each group of mice was intraperitoneally injected with selenium phycocyanin, *Spirulina* phycocyanin and inorganic selenium, the levels of glutathione peroxidase (GPx), superoxide dismutase (SOD), malondialdehyde (MDA), alanine aminotransferase (ALT) and nitric oxide (NO) were determined in blood and liver tissues after mice were injected for 7 d. Results showed that the contents of inorganic selenium, GPx and SOD significantly increased in blood and liver of the selenium-phycocyanin group of mice compared to those of the CCl_4 group of mice. Additionally, the contents of ALT and MDA in blood and liver tissues and the level of NO_2^-/NO_3^- significantly decreased. Further analysis indicated that the level of inorganic selenium in the blood of mice was positively related to the activity of GPx. The activity of GPx in the blood of mice was negatively related to the levels of MDA, ALT and NO_2^- /NO_3^-. Thus, selenium phycocyanin has a significant antigonistic function to the oxidative damage of the mouse liver caused by CCl_4 due to the dual efficacy of the anti-inflammation and the enhancement of the activity of the selenium enzyme combined with

selenium phycocyanin. Romay et al. (2000) was the first time to report that phycocyanin had the anti-inflammatory and oxygen free radical-scavenging functions, and to demonstrate that the anti-inflammatory effect of phycocyanin is dose-dependent in 12 inflammatory experimental models. Phycocyanin can reduce the inflammatory tissue edema. The anti-inflammatory effect may be related to the reduction in the release of histamine, the activity of myeloperoxidase (MPO), the level of the prostaglandin E2 (PGE2), the level of the phospholipase A2 (PLA2), the scavenging of the oxygen free radical and the inhibition of COX-2. Gonzalea et al. (1999)

observed the histopathology and ultrastructure of colon by constructing the colitis model by enema with 1 ml of 4% acetic acid and determining the MPO activity after phycocyanin of 150, 200 and 300 g/kg was orally administrated for 24 h. They found that phycocyanin could reduce the activity of MPO and inhibit the invasion of inflammatory cells to decrease the injury of the colonic tissue. Vadira et al. (1998) reported that phycocyanin had a protective effect on the CCl_4-induced liver injury of mice, and could make the level of the serum liver enzyme almost identical to that of the control group. Phycocyanin has also a protective effect on the hepatic enzyme, and hence makes the loss of glucose-6-phosphatase and microsomal cytochrome P140 decrease. The anti-inflammatory effect of the phycocyanin extract was investigated in the acetic acid-induced colitis in rats (Gonzalez et al., 1999). Phycocyanin could substantially reduce the activity of MPO which was increased in the control group of colitis, could inhibit the infiltration of inflammatory cells, and could to some extent reduce the colonic damage. Cherng et al. (2007)

determined the effect of phycocyanin on the production of nitrite, the index of NO, and the iNOS expression in lipopolysaccharide-treated RAW 264.7 macrophages. The significant inhibition of phycocyanin to the LPS-induced nitrite production, and to the iNOS protein expression accompanied by an attenuation of the tumor necrosis factor-α (TNF-α) formation, was observed. However, phycocyanin has no effect on the interleukin-10 production in macrophages. Moreover, phycocyanin suppressed the activation of the nuclear factor-κB (NF-κB) through preventing the degradation of the cytosolic IκB-α in LPS-stimulated RAW 264.7 macrophages. These studies provide an additional explanation for the anti-inflammatory activity and therapeutic effect of phycocyanin. The inhibition of the allergic inflammatory response by phycocyanin may be perhaps mediated by the inhibition of histamine release from mast cells (Remirez et al., 2002).

Anti-oxidative and anti-irradiative effect

Phycocyanin has a significant antioxidative activity of scavenging hydroxyl and hydrogen peroxide free radicals (Bhat and Madyastha, 2000; Lu et al., 2006; Romay et al., 2003; Zhou et al., 2003). It can also inhibit the generation of the liver microsomal lipid peroxide by Fe^{2+}-Vc or 2,2-amidinopropane hydrochloride (Bhat and Madyastha, 2000). Meanwhile, the antioxidative activity is a basis of anti-inflammatory and anti-tumor effect (Halliwell, 1990; Romay et al., 1998). Phycocyanin can remove free radicals generated in the damaged cells, and this makes them avoid the oxidative DNA damage caused by free radicals. If damaged DNAs cannot be

repaired timely and accumulate to a certain extent, the cell apoptosis program will start and induce the cell apoptosis (Rimbau et al., 2001; Rimbau et al., 1999). Phycocyanin can remove the tumor necrosis factor (TNF)-α reaction caused by the oxidative stress (Romay et al., 2001). Zhang et al. (1996a) found that the survival rate of mice could be enhanced by about 28% compared to the control group when the mice were injected with phycocyanin of 50 mg/kg, and were then irradiated by the lethal dose of ^{60}Co ray. Moreover, they found that phycocyanin might stimulate the formation of granulopoiesis progenitor cells and hematopoietic stem cells of irradiated mice, might increase the number of nucleated bone marrow cells and the total number of peripheral blood cells of mice, but might have no significant effect on the number of peripheral red blood cells and hemoglobin. Romay et al. (2003) showed that phycocyanin might scavenge alkyl, hydroxyl and peroxide, might inhibit the microsomal lipid peroxidation caused by Fe^{2+} and Vc. This oxidative function is relevant to the formation of the tumor necrosis factor (TNF-α) and NO. Bhat and Madyastha (2001) considered that phycocyanin might scavenge ONOO-, and it is relevant to color-generating groups.

Immunomodulatory function, promotion of the growth of cells and neuroprotective effect

Tang et al. (1998) confirmed that phycocyanin could promote the transformation of the phytohemagglutinin (PHA)-stimulated lymphocyte and recover the formation ability of E rosette of T cells injured by cyclophosphamide. It may significantly increase the number of antibody-forming cells and the antibody-producing ability in spleen cells of normal mice and

immunocompromised mice treated by hydrocortisone. Zhang et al. (1996b) showed that *Spirulina* phycocyanin could enhance the proliferation and generation of the level of colony forming units (CFU-GM) of granulocyte progenitor cells. *In vitro* experimental results show that *Spirulina* phycocyanin can not directly stimulate the formation of granulocyte progenitor cells, and so increases the sensitivity of granulocyte progenitor cells to the colony-stimulating factor (GM-CSF) to make them enter the stage of the cell proliferation. Rimbau et al. (1999) found that after mice with the nerve injury caused by kainic acid were administrated by phycocyanin, their neural dysfunction was decreased. Therefore, phycocyanin can be used as a repair agent of the injury of nerve cells caused by the oxidative stress, and can be used to treat the neurodegenerative disease, such as the Parkinson's disease.

MAIN PROBLEMS AND PROSPECTS

It can be concluded that using the combination of repeated freeze-thaw with the ultrasonification to disrupt algal cells, and then using ammonium sulfate salting-out to precipitate the crude protein, are a good pretreatment technology widely accepted for separating phycocyanin. However, due to a large energy consumption and a small treatment amount of the ultrasonication, it cannot be applied to the large-scale industrial production. The combination of the high pressure homogenization with the physical crushing may be a better choice. Overall, the large-scale purification technology of phycocyanin is immature. The present chromatographic purification focuses on the joint use of two or more chromatographic techniques. The

combination of the anion-exchange chromatography with the size exclusion chromatography is often used to purify phycocyanin. This method can obtain a high purity of phycocyanin, but many purification steps will greatly reduce its yield. Especially, some chromatographic techniques, such as the hydrophobic interaction chromatography or the size exclusion chromatography, are not beneficial to the enrichment and purification of the product due to the dilution of the product during these processes. A critical problem of the large-scale production of *Spirulina* phycocyanin is how to reduce the purification step to improve its yield and purity. The use of the membrane separation technology to purify phycocyanin not only is simple, but also can improve the purity, yield and safety of the product. However, it has been reported less so far.

The large-scale purification of phycocyanin is still difficult and remains to be further explored. Simultaneously, the application of phycocyanin to functional foods and pharmaceuticals is still blank. Thus, the future research and development of phycocyanin may focus on the following aspects: (1) To explore a new large-scale preparative technology of phycocyanin suitable for the industrial production to reduce the cost of phycocyanin. (2) To expand the application of phycocyanin from functional foods to drugs and medical diagnostic reagents for its deeper development based on the research results of phycocyanin bioactivities.

CONCLUSIONS

The purpose of this review is to critically introduce the purification and bioactivities of phycocyanin. It will provide the detailed information for researchers and other persons pertinent to final products containing phycocyanin. With the improvement of research approaches, studies on the purification and bioactivities of phycocyanin will become deeper and deeper. We believe that this will become a trend in future.

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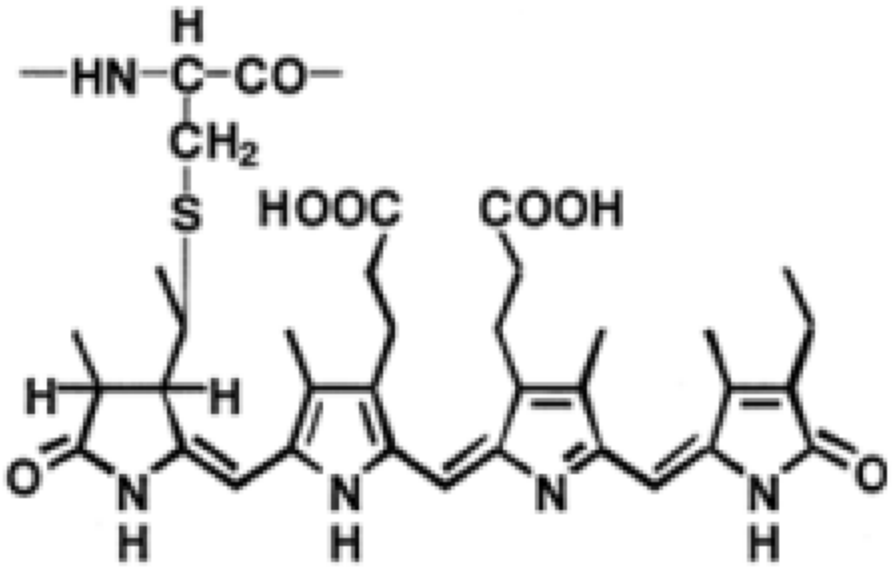

Figure 1 Structure of phycocyanin. Each of phycocyanin subunits contains a polypeptide chain and one (α-subunit) or two (β-subunit) chromophores-phycobilin. The connection between the polypeptide chain and the chromophore is a thioester bond.

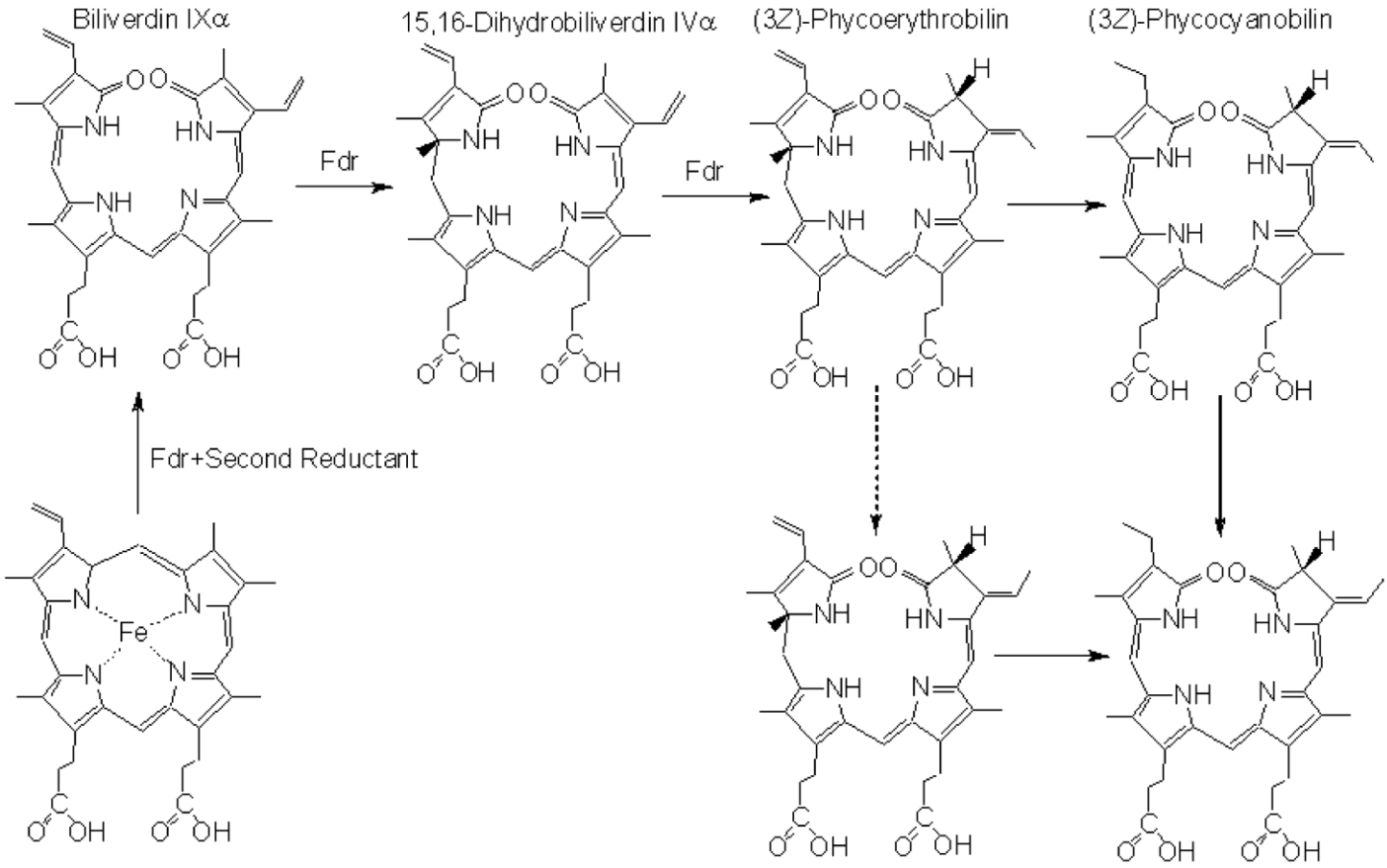

Figure 2 Biosynthetic pathway of phycobilin. Enzyme-catalyzed reactions that have been characterized in *Spirulina* sp. extracts are heme oxygenase, biliverdin IXα reductase, 15,16-dihydrobiliverdin IXα reductase, (3*Z*)-phycoerythrobilin-to-(3*Z*)-phycocyanobilin isomerase, and phycobilin 3-ethylidine *cis-trans* isomerase. The isomerization of (3*E*)-phycoerythrobilin to (3*E*)-phycocyanobilin has also been detected *in vitro*. It is not known whether the ethylidine *cis-trans* isomerase can accept (3*Z*)-phycoerythrobilin as the substrate. Abbreviation: Fdr - the reduced ferredoxin.