

Full Length Research Paper

# Isolation and antibacterial activity of anabaena phycocyanin

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**The isolation and antibacterial activity of anabaena phycocyanin were investigated. The result indicates that three kinds of protein ingredients: PC-A, PC-B and PC-C were obtained using high performance liquid chromatography. The estimated molecular masses of PC-A and PC-B were 14 to 18 kD. PC-B and PC-C had certain antibacterial activity on *Bibrio parahemolyticus*, *Bacillus mucilaginosus* and *Sarcina lutea*. In addition, PC-C had certain antibacterial activity on *Vibrio harveyi*. PC-A did not possess antibacterial activity in the study.**

**Key words:** Anabaena, phycocyanin, liquid chromatogram, antibacterial.

## INTRODUCTION

Phycocyanins are photosynthetic pigments of cyanobacteria. Pure phycocyanin are widely used as fluorescent labelling reagents (Glazer, 1994; Telford et al.2001), and as natural colorants for food and cosmetics (Yoshida et al., 1996) due to their antioxidant and anti-inflammatory properties are also potential therapeutic agents (Zhang et al., 2000; Romay et al., 2003). Recent studies indicated that phycocyanin from spirulina have obvious curative effect on stimulating red blood cells generation, acts as anti-cancer and anti-tumour and improves the body immunity (Schwartz and Sklar, 2002; Li et al., 2007; Yang et al., 2006). As the key substance, phycocyanin extraction quantity and quality were crucial to phycocyanin effective application. Many researchers explored all kinds of methods to extract and purify phycocyanin, such as hot water method and ultrasonic extraction, ion exchange chromatography and expanded bed adsorption chromatography which achieved certain results (Yang et al., 2005; Niu et al., 2007; Patil and Raghavarao, 2007). Nevertheless, hot water method was time-consuming and had a low extraction rate (Yao et al., 2009). Meanwhile, ultrasonic extraction was difficult to control and steps trivia, and can change the structure of

phycocyanin, thereby degrading the quality of the extracted compounds (Yang et al., 2008). Anabaena found in freshwater algae, are cultivated to make the process operation simpler, of higher output, low cost, and to make phycocyanin content higher than spirulina. However, to the best of our knowledge, very few studies had been done to systematically explore Anabaena phycocyanin extraction and purification and antibacterial function. Previously studies reported the optimization extraction conditions with repeated freezing and thawing method (Su et al., 2012).

In this study, we investigated the anabaena culture and phycocyanin isolation using repeated freezing and thawing method and high performance liquid chromatography. In addition, we also determined the antibacterial function using 96 orifice plate method.

## MATERIALS AND METHODS

Anabaena (*Anabaena cylindrica*) was provided by the biological algae species pool of Wuhan Institute of Hydrobiology. Acetonitrile were purchased from the imported reagents corporation. All other analytical grade chemicals were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai China). Apparatus used for the analysis were ultraviolet (UV) spectrophotometer (Shimadzu UV-2401), high performance liquid chromatography (waters 600E) and enzyme linked immunosorbent assay (ELISA) from the U.S.

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thermoelectric MK-3.

### The expansion cultivating of anabaena

The experiment was conducted at the biological experiment teaching center at Zhejiang Ocean University, Zhoushan, China, from August, 2011 to October, 2012. Anabaena (10 mL) were inoculated into BG11 medium (40 mL) and cultured in artificial climate incubator, grown at 25°C under a 12-h photoperiod and photosynthetic photon flux density (PPFD) of 180  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ . Relative humidity was maintained at ~80%, with shaking one to two times daily. By gradually expanding the training, logarithm of the growing period of anabaena was taken for phycocyanin extraction.

### Extraction of phycocyanin crude products

Anabaena culture solution was centrifuged (1,0000 g, 20 min) at 4°C, the supernatant was removed and the precipitation was collected (Ishikawa, 1979). Then to get the precipitation of fresh anabaena algae, anabaena (fresh weight 1 g) were immersed in pH7.0 phosphoric acid buffer with solid/liquid ratios 1:1 (g/mL) in 50 mL beaker. The different beakers were frozen 30 min at -20°C. Moreover, anabaena was frozen and taken out from the refrigerator and dissolved in the water at 38°C (frozen and dissolved process was repeated five times) (Gao et al., 2012); then anabaena obtained were homogenized with pH7.0 phosphoric acid buffer for 5 min in a mortar at 0°C. The homogenate was centrifuged (10,000 g, 30 min) at 4°C and the supernatant was collected of which phycocyanin crude extracts were obtained. Phycocyanin crude extracts was saturated to 60% (v/v) with ammonium sulfate. The precipitate was collected by centrifuging at 5000 g for 20 min to obtain crude phycocyanin. Crude phycocyanin was stored at -20°C until further study.

### Determination of phycocyanin

Phycocyanin crude extract was diluted to appropriate concentration and distilled water was added to reach a final volume of 4 mL. The UV spectrophotometer was used to determinate the absorption value of phycocyanin crude extracts at 280 and 620 nm, and the specific activity was calculated according to the formula  $\text{OD}_{620}/\text{OD}_{280}$ .

### High performance liquid chromatography purification of phycocyanin

1 g of phycocyanin crude extracts was diluted to 2 mL ultrapure water, and filtered with 50 mm membrane filtration. High performance liquid chromatography (Waters600e) for the purification of proteins, 300SB-C8 semi-preparative reversed-phase column (4.6 x 250 mm, American waters company), waters2478 detector were used. Mobile phase respectively containing 0.1% trifluoroacetic acid (TFA) (v/v) water (A solution) and 0.1% TFA (v/v) acetonitrile (B solution); a linear gradient elution, column temperature 40°, B liquid ratio from 5 to 95% linear elution in 30 mins were used. The flow rate was 1 mL/min collection for each peak elution by freeze drying using freeze drying machine.

### Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of phycocyanin ingredient

SDS-PAGE was carried out according to Okadjima et al. (1993). 10% (w/v) separation gel and 4.8% (w/v) concentration of glue was

used and the proteins in the gel were silver-stained (Blum et al., 1987) at mark 10 to 120 KDa.

### Antibacterial analysis of phycocyanin composition

During the various steps of protein purification, antibacterial activity was monitored by a liquid growth inhibition assay on the Gram-positive strain and on the Gram-negative strain (Bulet et al., 1991) by the 96 orifice plate method. Bacteria and fungus used in present study were obtained from China General Microbiological Culture Collection Center (Beijing, P. R. China). Nine species (Gram positive and Gram negative) of bacteria were used. The Gram positive bacteria were *Bacillus mucilaginosus*, *Sarcina lutea*, *Staphylococcus aureus*, *Micrococcus luteus*. The Gram negative bacteria were *Vibrio harveyi*, *Proteus P. vulgaris*, *Escherchia coli*, *Bibrio parahemolyticus* and *Bacillus subtilis Cohn*.

Configure LB culture medium, 121°C sterilized for 20 min, One single colony of each type of bacteria (from the nutrient agar culture) was inoculated with a sterile loop, and was transferred into 10 mL sterile nutrient broth. The broth cultures were incubated in a shaking incubator at 37°C for 16 to 20 h. The control group was 10 ul axenic culture medium, 10 ul medium dissolved sample group and its corresponding sample. Sample after enzyme mark instrument detection, initial data were recorded. The absorbance value were detected at 12 and 24 h at 37°C and 100 rpm using ELISA (Yang et al., 2011; Velidandi et al., 2011).

## RESULTS AND DISCUSSION

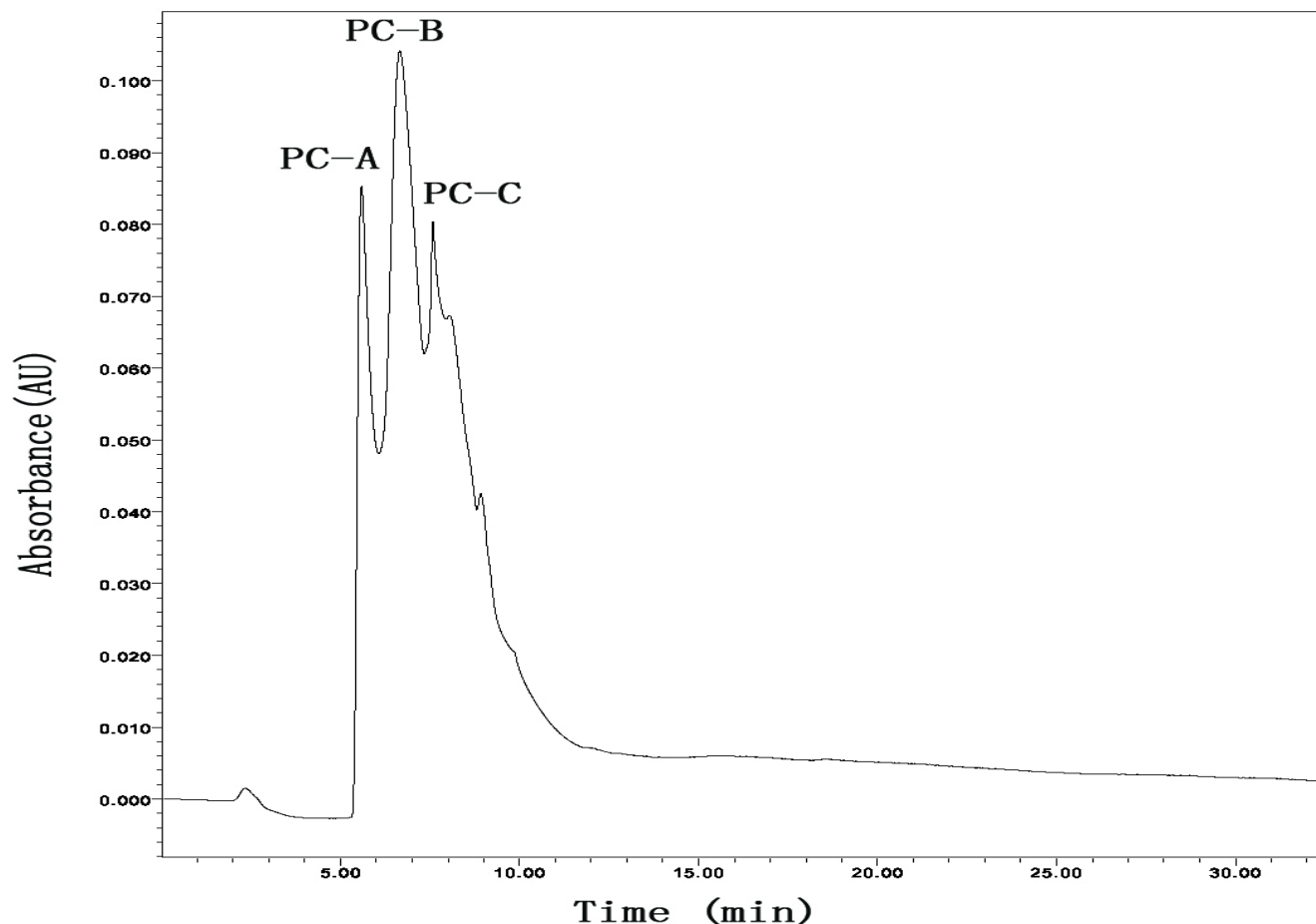
According to previous research that phycocyanin has maximum absorption value at 620 nm (Yang et al., 2005; Su et al., 2012; Kaledona et al., 2007), phycocyanin crude products as samples and pH 7.0 phosphate buffer solution as control were scanned at the wavelengths of 200 to 800 nm using a Shimadzu UV-2401 spectrophotometer. In further research, the concentrations of phycocyanin were measured by measuring the absorbance at 280 and 620 nm; the specific activity was 1.52 which is higher than 0.93 obtained by Liao and Zhang, (2011) and Yang et al. (2005).

### High performance liquid chromatography further purification of phycocyanin

Crude phycocyanin were separated and purified by liquid chromatography for molecular sieve experiment; molecular sieve column was 300SB-C8 (4.6 x 250 mm, American waters company). From Figure 1, it can be seen that using high-performance liquid chromatographic separation of 3 maximum absorption peak, respectively in each peak collecting proteins, three ingredients were obtained; labeled PC-A, PC-B and PC-C for the next step of SDS - PAGE electrophoresis and bacteriostatic test .

### Phycocyanin ingredients SDS - PAGE electrophoresis

SDS-PAGE electrophoresis results show that, PC-A and



**Figure 1.** The elution curve of phycocyanin crude products by semi-preparative reversed-phase column after the separation of 60% ammonium sulfate precipitation.

PC-B molecular masses occurred between 14 to 18 kDa (Figure 2). It is consistent with that of spirulina (16 to 18 kDa) and arthonema africanum (17 to 19 kDa) (Liao and Zhang, 2011; Minkova et al., 2007). However, the PC-C was not certain in the electrophoresis graph. Table 1 shows the PC-A, PC-B and PC-C bacteriostasis experiment for observation of the antibacterial activity.

#### Phycocyanin crude extract ingredients bacteriostasis experiment

By high performance liquid chromatography, purification was obtained in the three ingredients PC-A, PC-B and PC-C. Antibacterial activity of PC-A, PC-B and PC-C were investigated. It was found that PC-B and PC-C had certain antibacterial activity on *Bibrio parahemolyticus*, *Bacillus mucilaginosus* and *Sarcina lutea*. In addition, PC-C had certain antibacterial activity on *Vibrio harveyi*. Antibacterial activity was not found in PC-A in the experiment.

#### Conclusion

This paper presents a system procedure that ensures a simple, fast and efficient separation and isolation of large quantities phycocyanin and partially purified from anabaena. The extraction of phycocyanin, by repeated freeze thaw method, solid-liquid ratio of 1:1, freezing time of 30 to 60 min, and ammonium sulfate saturation of 60% obtained higher purity. For the first time, the bacteriostatic of the purification component was analysed. In further investigation, PC-B and PB-C will be a new molecular sieve for mass spectrometry and protein sequencing analysis, for the following protein gene cloning and expression.

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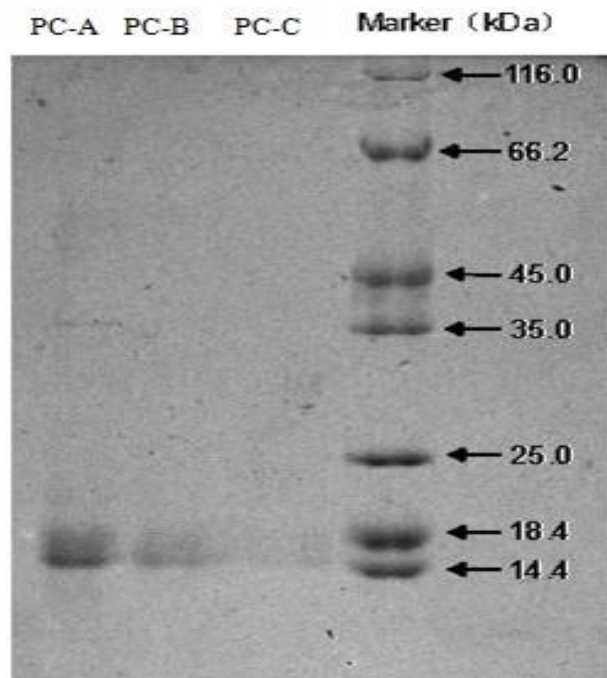


Figure 2. SDS-PAGE analysis of phycocyanin ingredients.

Table 1. Antibacterial activity of phycocyanin ingredients.

Bacteria	PC-A	PC-B	PC-C
<i>Vibrio harveyi</i>	—	—	+
<i>Proteus P. vulgaris</i>	—	—	—
<i>Escherchia coli</i>	—	—	—
<i>Bibrio Parahemolyticus</i>	—	+	+
<i>Bacillus subtilis (Ehrenberg) Cohn</i>	—	—	—
<i>Bacillus mucilaginosus</i>	—	+	+
<i>Sarcina lutea</i>	—	+	+
<i>Staphylococcus aureus</i>	—	—	—
<i>Micrococcus luteus</i>	—	—	—

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