Contents lists available at ScienceDirect

Food and Bioproducts Processing

journal homepage: www.elsevier.com/locate/fbp

Short communication

Aqueous two-phase countercurrent distribution for the separation of c-phycocyanin and allophycocyanin from *Spirulina platensis*

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ABSTRACT

C-phycocyanin (C-PC) and allophycocyanin (APC) with similar molecular structures were separated, respectively from *Spirulina platensis* cell homogenate by single extraction and multi-stage countercurrent distribution (CCD) using an aqueous two-phase system (ATPS) composed of polyethylene glycol (PEG) and potassium phosphate (KPi). The partition coefficients of C-PC and APC were 10.64 and 0.57, respectively, and the extraction selectivity of C-PC was 18.67 from 0.5% (w/w) *S. platensis* crude extract by single extraction using PEG6000/KPi ATPS (pH 7.0) with 34% (w/w) tie line length (*TLL*). In ten-stage CCD under the same ATPS extraction condition with 2% (w/w) *S. platensis* crude extract, the purity of C-PC increased nearly twice and the recovery of APC increased more than nine-fold compared with single extraction. The results displayed that most C-PC (82.1%) followed the mobile phase was enriched in the top phases of the last three tubes, while more APC (41%) remained in the stationary phase was enriched in the bottom phases of the first three tubes in the ten-stage CCD. Hence, aqueous two-phase CCD technology provided an effective and low cost method for C-PC and APC separation from *S. platensis* cell homogenate directly.

Keywords: Aqueous two-phase system; Countercurrent distribution; C-phycocyanin; Allophycocyanin; *Spirulina platensis*

1. Introduction

Spirulina platensis has drawn attention as a nutritious food for human due to its rich proteins content (Colla et al., 2007; Oliverira et al., 2009; Radmann et al., 2007), in which phycobiliproteins (a family of hydrophilic, brilliantly colored, and stable fluorescent pigment proteins) mainly including C-PC and APC have promising applications in biomedical research, diagnostics and therapeutics (Farooq et al., 2004; Subhashini et al., 2004; Li et al., 2007). It has been proved that C-PC and APC can be applied in immunomodulating agents and anticarcinogenic activities (Ou et al., 2010; Ge et al., 2006). However, the phycobiliproteins cannot be used widely because the separation of C-PC and APC with large amounts was complex and tedious due to their amino acid sequence similarity (Apt et al., 1995; Brejc et al., 1995). C-PC and APC have similar three dimensional structures, whose basic building blocks are composed of α and β subunits of molecular weights in the range of 18,000 and 20,000. Three or six (αβ)-monomers are arranged around a symmetry axis and form an $(\alpha\beta)_3$-trimer or $(\alpha\beta)_6$-hexamer (Padyana et al., 2001; Padyana and Ramakumar, 2006; McGregor et al., 2008; Adir et al., 2001). Moreover, the difference in the isoelectric points between C-PC and APC is not significant due to the similar ratio of acidic amino acids to basic amino acids in the two proteins (Abalde et al., 1998; Gómez-Lojero et al., 1997). Therefore, the slight differences between the two proteins in molecular weight and isoelectric point caused C-PC and APC separation and purification in trouble. Conventional methods for phycobiliproteins separation usually involved several kinds of chromatography such as ion-exchange, gel-filtration and hydroxylapatite chromatography (Chen et al., 2006; Minkova et al., 2003; Reis et al.,

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Received 21 January 2011; Received in revised form 23 May 2011; Accepted 2 August 2011
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doi:10.1016/j.fbp.2011.08.002

1998; Samtiago-Santos et al., 2004), which were generally time-consuming, low loading quantities, complex and difficult to be scaled up (Niu et al., 2007). In order to make phycobiliproteins to be successful biopharmaceutical products, it is worth to find an effective and low cost separation method of phycobiliproteins.

ATPS extraction is a better alternative for many biomolecules in bioseparation process, especially in the early downstream stages. For the target proteins separation and purification from a complex mixture, there were some advantages of ATPS extraction such as scaling up feasibility, process integration capability and biocompatibility (Rito-Palomares et al., 2001; Benavides and Rito-Palomares, 2004; Hernandez-Mireles and Rito-Palomares, 2006). However, the resolution of a single ATPS extraction for the purification of closely similar products such as C-PC and APC was an issue that needed to be lucubrated (Patil et al., 2008). As a classic partition technique, CCD was applied in aqueous two-phase systems (CCD-ATPS) for the similar proteins separation in initial establishment of the process conditions with high resolution (Truust and Johansson, 1996; Hernandez-Mireles et al., 2008).

In this paper, CCD-ATPS was applied for respective separation of C-PC and APC from *S. platensis* cell homogenate. The initial conditions of ATPS extraction for C-PC and APC selective separation including polymer molecular weight and *TLL* were established. Then, the practical strategy of CCD-ATPS with various extraction stages was applied in an effective separation of C-PC and APC from *S. platensis* cell homogenate. The purity and content of C-PC and APC were monitored, respectively at each stage of CCD-ATPS by absorption spectroscopy. This CCD-ATPS method significantly increased product loading quantities and got a good resolution of C-PC and APC. So both the high purify of C-PC and the high recovery of APC were achieved by a simple and low cost operation, respectively.

2. Experimental

2.1. Materials

PEG2000, 4000 and 6000 were purchased from Merck Chemical Company (Shanghai, China). KPi including potassium dihydrogen phosphate and dipotassium hydrogen phosphate, and other chemicals were obtained from local sources as analytical grade. *S. platensis* was provided by Yunnan Here Biotechnology Company (Yunnan, China) as dried powder. Aqueous solutions were prepared with deionized and doubly distilled water.

2.2. Aqueous two phase diagram determination

Phase boundaries (binodal curves) of PEG/KPi ATPS were obtained by cloud-point measurements in a water bath temperature of $25 \pm 0.5\,^{\circ}C$. Samples of PEG 2000, 4000, 6000 aqueous solution with known concentration were prepared, respectively and added drop-wise to potassium phosphate buffer also with known concentration at pH 7.0 until the two-phase region was reached (turbid samples). Then water was added drop-wise until the one phase region was reached (transparent samples). The compositions lie on the binodal curve, in which the change from two-phase to one phase system occurs. A sample was regarded as one phase when it was completely clear and no phase separation could be detected. The system was repeatedly weighed and the compositions at the phase boundary were calculated. The compositions of the tie lines were determined by the volume ratio of the top phase to the bottom phase followed the lever rule.

2.3. Preparation of S. platensis crude extract

A given quantity of dry *S. platensis* cells were suspended in a given quantity of 0.01 M phosphate buffer (pH 7.0), which was calculated as the concentration of the initial *S. platensis* crude extract with its unit of mass % (w/w). The cell suspension was frozen (at $-20\,^{\circ}C$) and thawed repeatedly for five times. Cell fragments were removed by centrifugation at 5000 rpm for 30 min at $4\,^{\circ}C$, and the supernatant was collected as the crude phycobiliproteins by the centrifugation. The blue and transparent supernatant was used as *S. platensis* crude extract in following experiments, which was rich in C-PC and APC.

2.4. Aqueous two phase extraction and countercurrent distribution

ATPS extraction was carried out by adding compositions predetermined (based on the phase diagram). Weighed quantities of PEG and salt were added to a given quantity of *S. platensis* crude extract making the total weight of the system 100% on w/w basis. The mixture was stirred thoroughly for about half an hour at $25 \pm 0.5\,^{\circ}C$ to equilibrate and allowed for phase separation.

Partitioning of C-PC and APC under optimized ATPS conditions was investigated by manual CCD (Fernandes et al., 2001) as shown in Fig. 1. A set of n tubes each were labeled with numbers from 0 to $n-1$. The tubes contained a 10 g two-phase system (the volume ratio between top and bottom phases was kept 1.0 ± 0.05), and tube 0 contained *S. platensis* crude extract. All systems were equilibrated at $25 \pm 0.5\,^{\circ}C$, and the top phase as the mobile phase contained phycobiliproteins in tube 0 was mixed successively with the fresh bottom phases of tubes 1 to $n-1$ after phase separation, while the bottom phase as the stationary phase contained phycobiliproteins in tube 0 was mixed successively with the fresh top phases of tubes 1 to $n-1$. The crude phycobiliproteins system in tube 0 initially was equilibrated by inverting the test tube $2n-1$ times and then centrifuged. After phase equilibration and separation, C-PC and APC from tube 0 were transferred successively to the subsequent tube. This procedure was continued until all $2n-1$ transfers (including tube 0) had been carried out, and n complete systems were obtained.

The top and bottom phases both containing phycobiliproteins were separated by centrifugation at 5000 rpm for 20 min. The concentrations of C-PC and APC in the two phases were analyzed for estimating the purity and the content, respectively. All the experiments were repeated three times and were carried out at a scale of 10 g.

2.5. Spectroscopic measurements

Absorbance spectra of C-PC and APC were measured using UV–VIS Spectrophotometer of Model UVPC2501 (Shimadzu, Japan). The ratio of A_{620}/A_{280} gives the purity of C-PC ($P_{C\text{-}PC}$) and A_{652}/A_{280} gives the purity of APC (P_{APC}), wherein A_{620} is the maximum absorbance of C-PC, A_{652} is the maximum absorbance of APC and A_{280} is the absorbance of total proteins. All these absorbance values were measured against a blank phase system. The concentrations of C-PC and APC in the