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Recovery of B-phycoerythrin using expanded bed adsorption chromatography: Scale-up of the process

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Abstract

B-phycoerythrin is a major light-harvesting phycobiliprotein in some marine algae. It is widely used as a fluorescent probe and analytical reagent, and could also be used as a natural dye in foods and cosmetics. In the present work, an expanded-bed methodology for large-scale recovery of B-phycoerythrin from the microalga *Porphyridium cruentum* is developed using Streamline-DEAE as adsorbent. The operation of expanded bed technology was first optimized on a small scale using a column of 15 mm internal diameter. The chromatographic parameters studied were the sample load, viscosity and expansion degree. The optimal conditions proved to be a sample load of 0.88 mg B-PE/mL Streamline DEAE, an expanded bed volume twice the settled bed volume and a sample viscosity of 1.068 mP. The expanded bed adsorption process was then scaled up 16 times by increasing the column diameter, while maintaining the sedimented bed height, linear flow rate and protein load at constant values. The success of the scale-up process was verified by determining the protein breakthrough capacity and product recovery. A comparative study was made of the performance of four columns of 15, 25, 40 and 60 mm internal diameters. The standard deviation of the breakthrough curves ranged from 0.81 to 0.76; the higher the internal diameter the lower the standard deviation. The yield of the EBA chromatography was in the range of 71–78%. These results show that small diameter columns can be effectively used for mimicking the behaviour in scaled up systems providing a useful tool for method scouting studies. The results also show that the large-scale assay (60 mm diameter column) was as efficient as the lab scale one (15 mm diameter column) in recovering B-phycoerythrin from the unclarified crude extract. Results show that expanded bed chromatography is a scaleable technology that allows large quantities of B-PE to be obtained without product loss. It maintains a high protein recovery while reducing both processing costs and times, when compared with previous methodologies.

Keywords: Phycoerythrins; *Porphyridium cruentum*; Phycobiliproteins; Expanded-bed adsorption chromatography; Scale-up

1. Introduction

Phycobiliproteins are water-soluble proteins that form light-harvesting antenna complexes (phycobilisomes) and act as photosynthetic accessory pigments in red algae, this being the usual source of these compounds [1,2]. In terms of their absorption properties, the phycobiliproteins are divided into four main classes: phycoerythrins (PEs, $\lambda_{max} \sim 540$–570 nm), phycoerythrocyanin (PEC, $\lambda_{max} \sim 570$ nm), phycocyanins (PCs, $\lambda_{max} \sim 610$–620 nm) and allophycocyanins (APC, $\lambda_{max} \sim 650$–655 nm) [3]. The main application of phycobiliproteins is as fluorescent markers of cells and macromolecules in biomedical research and highly sensitive fluorescence techniques [4–6]. Phycobiliproteins have also been shown to have therapeutic value due to their protective effect and anticarcinogenic activity, thus being reported that phycoerythrin has photosensitive activity and can be used to kill cancer cell [7–9]. In addition, they have potential as natural colorants for use in food, cosmetics and pharmaceuticals, particularly as substitutes for synthetic dyes, which are generally toxic or otherwise unsafe [10–14]. B-PE has been shown to be particularly useful due to its high fluorescence efficiency and its intense and unique pink colour [15,16]. However, the widespread use of B-PE and other phycobiliproteins has been somewhat limited by the high cost of these purified macromolecules. For this reason, it is necessary to adopt new methodologies to obtain effectively phycobiliproteins in general and B-PE in particular for the application in life and research.

Abbreviations: PE, phycoerythrin; C-PC, C-phycocyanin; APC, allophycocyanin; i.d., internal diameter; DEAE, diethylamino ethyl cellulose; EBAchromatography, expanded-bed adsorption chromatography

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The conventional schemes for phycobiliprotein purification use multiple unit operations, each operation reducing the yield. These schemes recover phycobiliproteins extracts by a combination of difference and non-scaleable methods. Conventional methodologies involve two steps: the first consists of sample pretreatment in order to liberate the intracellular material, obtaining a crude extract ready for the second step, in which the phycobiliproteins are separated using mainly conventional chromatography. Sample pretreatment consists of disintegration of the cell-wall, for which different methods have been used: ultrasound [1,17–20], lysozyme [21–23], mechanical breakage [24], treatment with rivanol [25], treatment with Triton X-100 and mechanical homogenization [26], ultrasound and extraction with acetone [27]. After cell-wall disintegration, all methods continue with the precipitation of the phycobiliproteins using ammonium sulfate. Centrifugation and dialysis of the samples is usually then necessary to obtain a crude extract ready for the next step. This second step involves one or more chromatographic processes. For phycoerythrin purification, several chromatographic methods are described in the literature. These methods involve adsorption, hydrophobic interaction, gel filtration or ion exchange chromatography [28,17,29,19,20,30]. The high number of unit operations increases the production cost and time necessary to perform purification.

Expanded bed adsorption (EBA) chromatography is an alternative bioseparation technique that greatly reduces the number of purification operations for protein adsorption to gel media, capturing proteins from particle-containing feedstock [31]. In a fluidised bed a stationary phase (adsorbent particles) is fluidised in a liquid stream upwards. This stream increases the distance between the adsorbent particles, thus allowing particles contained in the feed to pass freely in the interstitial volume of the matrix. The position of each particle in the column depends on the particle size and density as well as on the fluid viscosity, density and velocity [31,32]. Provided the absorbent is chosen in a suitable range of particle size and/or density a so-called classified bed is obtained. A classified bed has some very important advantages such as reduced mobility of the solid phase, reduced axial mixing in the liquid phase and plug flow performance comparable to a packed bed [31,33]. Perfectly classified fluidised beds are termed expanded bed. The use of this technique not only simplifies the clarification step, but also produces concentrated and partially pure product ready for the next purification step, usually chromatography on a packed bed in a single operation [34–38]. The use of expanded beds simplifies the downstream-processing flow sheets for the recovery of a wide variety of proteins, with concomitant savings in equipment and operating costs [39,38].

Commercially available expanded beds range in size from 25 mm to over 1 m in diameter, facilitating scale-up across a wide range of operating volumes. Works performed elsewhere has demonstrated the successful scale-down of an EBA system to 10 mm [40,41] and 1.9 mm diameter [42]. Moreover, a set of analytical methods has been developed and validated to simulate performance of EBA operations [43–47]. In the present work, an expanded-bed methodology for large-scale recovery of B-PE from *Porphyridium cruentum* is developed. The objective is to maximize the phycoerythrin recovery more than the purity of solution, since the procedure is intended to replace low-resolution steps that result in phycobiliprotein loss. For this, first the operation of expanded bed technology was optimized on a small scale, and the chromatographic parameters of sample load, viscosity and expansion degree were studied. Next, the process was scaled up 16 times by increasing the column diameter, while parameters such as the sedimented bed height, linear flow-rate and protein load remained constant. The success of the scale-up process was verified by determining the protein breakthrough capacity and product recovery. Results show that expanded bed chromatography is a scaleable technology that allows large quantities of B-PE to be obtained without product loss. It maintains a high protein recovery while reducing both processing costs and times, when compared with previous methodologies.

2. Materials and methods

2.1. Microalgal biomass and chemicals

The red microalga *P. cruentum* UTEX 161 was used. The biomass was obtained from chemostat cultures as described elsewhere [48,20]. For phycobiliprotein purification, microalgal cells were removed from the growth medium by centrifugation at 2000 × *g* for 5 min, and stored at −20 °C. Streamline-DEAE anion-exchanger was from GE Healthcare (Uppsala, Sweden). Sodium azide and all other chemicals were from Sigma Diagnostics (St. Louis, MO, USA) and used without further purification.

2.2. Spectroscopic measurements

Absorbance measurements and absorption spectra were recorded on a Perkin–Elmer (Beaconsfield, UK) Lambda-20 UV–vis spectrophotometer with a 1 cm light path. All spectra were recorded at room temperature. The amounts of B-PE and R-PC in the different extracts and biliprotein-containing solutions were calculated from measurements of the absorbance at 565, 620 and 650 nm using the following equations to correct the pigment's spectral overlap [49,50].

$$\text{R-PC}\,(\text{mg mL}^{-1}) = \frac{(\text{OD}_{620\,\text{nm}} - 0.7\text{OD}_{650\,\text{nm}})}{7.38} \quad (1)$$

$$\text{B-PE}\,(\text{mg mL}^{-1}) = \frac{(\text{OD}_{565\,\text{nm}} - 2.8\,[\text{R-C}])}{12.7} \quad (2)$$

2.3. Equilibrium adsorption

Adsorption isotherms were determined by shake flask experiments obtaining the static binding capacity of Streamline DEAE as described elsewhere [39]. The dynamic binding capacity was determined from the breakthrough profile. For this purpose a crude extract solution of B-PE (0.033 mg mL^{-1} and 1.068 mP viscosity) in 50 mM acetic acid–sodium acetate buffer, pH 5.5 (starting buffer) was used. Initially, starting buffer was pumped upwards through the bed at increasing flow rate till 198 cm h^{-1} until the bed stopped expanding. After this, the B-PE crude extract solution was applied to the expanded bed and the breakthrough curve was calculated as the amount of applied B-PE per ml adsorbent at different C/C_0, where C_0 corresponds to the B-PE concentration in the crude extract and C the B-PE concentration in the flowthrough. Frontal loading was terminated when the outlet concentration of protein rose to equal that in the inlet.

2.4. Expanded bed operation

The column was filled with the necessary volume of adsorbent (Streamline DEAE) to a constant settled height of 15 cm (H_0). Distilled water was then pumped upwards through the bed until it stopped expanding. A Heidolph PD-5001 peristaltic pump (Schwabach, Germany) regulated the flow-rate. When the bed was stable (after approximately 20–30 min) the column was equilibrated