Bioresource Technology 100 (2009) 5312–5317

Contents lists available at ScienceDirect

Bioresource Technology

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

Strategy for a protein purification design using C-phycocyanin extract

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ARTICLE INFO

Article history:
Received 16 January 2009
Received in revised form 13 May 2009
Accepted 17 May 2009
Available online 11 June 2009

Keywords:
C-phycocyanin
Process design
Purification
Ion exchange chromatography
Gel filtration

ABSTRACT

A variety of techniques have been developed for the separation and recovery of proteins. The cost of purifying the product is frequently determined by the desired quality of the final product, which is evaluated by measuring the purity. In this work the design of a protein purification process for C-phycocyanin, a phycobiliprotein that can be used in the food and medical industries, was established. The study evaluated the use of ammonium sulfate precipitation, ion exchange chromatography and gel filtration to purify C-phycocyanin in a variety of sequences. The final design included the C-phycocyanin extraction step, precipitation with ammonium sulfate and ion exchange chromatography. When the elution step was studied, the kind of elution and pH were considered in order to obtain a product with a final purity of 4.0 with a purification factor of 6.35, so that, at the end of the strategy, C-phycocyanin of analytical grade would be obtained.

1. Introduction

One critical element in the successful commercial exploitation of innovations in the biotechnology and related industries is the downstream processing of industrially important proteins and other biopolymers (Schügerl and Hubbuch, 2005; Desai, 2000). Therefore, rapid and effective optimizing methodologies for the purification process are of great importance in process development (Ishihara and Yamamoto, 2005).

When the purity requirement is specified, the goal of the process design is to obtain maximum recovery at the lowest cost, or in other words, an optimum downstream process is a purification strategy that produces the required quality (Wheelwright, 1987).

In an efficient design for a protein-drug purification process by chromatography, many parameters, such as the mobile phase, column configurations, the resin and operational variables must be considered (Ishihara et al., 2007). Ion exchange chromatography (IEC) is an important technique in the separation and purification of proteins. The major advantage of this technique is that it provides mild separation conditions that enable the proteins to maintain their conformation. The interaction between the protein and the ion exchange resin has a strong salt dependence (Hallgren, 1999).

It is as important to study the adsorption conditions as it is to study the elution conditions, considering that this will determine the separation of the main protein from the other contaminants. For example, in linear gradient elution IEC, the slope of the gradient and the flow rate as well as the column length all affect the separation behavior in a complicated way. Thus, if the process is not well understood, it will not be easy to choose the right conditions for providing the required resolution, an allowable process time and desired the buffer consumption. Another typical elution method, stepwise (or step gradient) elution IEC, is commonly employed for process IEC. In this elution method, concentration of the elution buffer which is usually determined by a trial-and-error approach is the key variable (Ishihara et al., 2007).

Recent research has used the ion exchange resin Sepharose Fast Flow to purify proteins like C-phycocyanin. It is based on 90 μm highly cross-linked 6% agarose beads with high chemical and physical stability. The exceptional flow characteristics make this ion exchanger the first choice for separating crude mixtures in the early stages of a purification scheme (Silveira et al., 2008).

C-phycocyanin (C-PC) is a natural blue dye extracted from cyanobacteria such as *Spirulina platensis* and has received attention because it has many commercial applications in foods and cosmetics (Vonshak, 1997). Recent studies have demonstrated the hepatoprotective (Romay et al., 2003), anti-inflammatory (Romay et al., 2003; Reddy et al., 2003; Bhat and Madyastha, 2001) and antioxidant (Estrada et al., 2001; Bhat and Madyastha, 2000) properties of C-PC. Due to their limited distribution in nature, these pigments are rather expensive, and obtaining them as pure compounds is a potentially attractive endeavor (Reis et al., 1998).

Purity is defined as the ratio of active substance to the total quantity of material (Wheelwright, 1987). For C-phycocyanin, the A_{620}/A_{280} ratio is considered a good indicator of the purity of the preparation especially when other forms of protein contaminants are taken into account (Liu et al., 2005). A purity of 0.7 is consid-

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0960-8524/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biortech.2009.05.026

ered food grade, 3.9, as reactive grade and greater than 4.0, analytical grade (Patil et al., 2006; Palomares et al., 2001). Pure C-phycocyanin has been obtained using the precipitation technique combined with a chromatographic method (Reis et al., 1998; Liu et al., 2005; Patel et al., 2005). In most of the research, C-phycocyanin of analytical grade is obtained (Reis et al., 1998; Boussiba and Richmond, 1979; Abalde et al., 1998), but three or four purification steps are used, including at least two chromatography techniques, which include mainly ammonium sulfate precipitation, a hydroxyapatite column and ion exchange. Some papers report the use of hydrophobic interaction or gel filtration (Bhaskar et al., 2005; Eriksen, 2008). The development of a fast and efficient process to purify C-phycocyanin from *S. platensis* is of great interest, as are the evaluation of the steps of this process and the optimization of the parameters.

Although some papers report on the extraction and purification of C-phycocyanin, none report on the design of the process or the maximization of a purification step, but only on protocols to purify this bioproduct. This paper reports on a strategy for purifying proteins from real systems with C-phycocyanin as the target and indicates the importance of the elution step in the ion exchange process.

2. Methods

2.1. Chromatography media and column

A strong anion-exchange column was used: Q-Sepharose Fast Flow™ (6% cross-linked agarose, column size 20 cm × 1 cm I.D., total bed volume V_t = 8 ml) and Sephacryl S-100HR™ (cross-linked allyl dextran with *N*,*N*'-methylene bisacrylamide, column size 40 cm × 1 cm I.D., total bed volume V_t = 12 mL) for the columns and media, both purchased from GE Healthcare (Uppsala, Sweden).

2.2. C-phycocyanin extraction

The cyanobacterium *S. platensis* LEB 52 (Costa et al., 2000) culture was grown in outdoor photo-bioreactors with a capacity of 450 L, using Zarrouk's synthetic medium (Zarrouk, 1966) diluted to 20%. The biomass was dried at 40 °C to a moisture content of 12% and then frozen. Mechanical rupture was carried out by milling in a ball mill. The particles in the outflow of the ball mill were separated with a set of sieves. The biomass collected was between 0.106 and 0.125 mm (Moares et al., 2009).

The extraction of C-phycocyanin was carried out according to Silveira et al. Silveira et al. (2007), who used water as solvent, added to biomass at a biomass-to-solvent ratio of 0.08:1 in erlenmeyer flasks at an impeller rotational speed of 100 rpm for 4 h at 25 °C.

2.3. Protein purification

2.3.1. Study of the elution step in IEC

In this paper, all the techniques used to establish the purification strategy (precipitation, ion exchange chromatography and gel filtration) were studied in preliminary experiments (data not shown). Whenever the IEC elution conditions were not yet known, the elution was performed with a linear gradient, from 0 to 1 M of NaCl in the initial buffer and a down flow of 45 cm/h. Two different volumes were tested for the gradient elution, 36 mL and 50 mL. Another kind of elution, in which the column was first eluted with 0.1 M NaCl in 0.025 M Tris–HCl pH7.5 was also evaluated, and the sample was then eluted with 0.3 M NaCl in the same buffer. In all the assays, samples were collected periodically and their concentration was measured (Eq. (1)), considering the wavelength of C-phycocyanin absorption (620 nm) and the wavelength of allophycocyanin absorption (652 nm) and a correction factor for these two values for C-phycocyanin concentration. The extract purity (Eq. (2)) of each sample was also evaluated, and the wavelength of the C-phycocyanin maximum absorption was compared to that of the whole proteins (A_{280}). The recovery (Eq. (3)) was calculated using the ratio of amount of C-phycocyanin collected to the amount of C-phycocyanin loaded. The purification factor (PF) was given by the ratio of the extract purity before and after the process.

$$\mathrm{C-PC(mg/mL)} = \frac{(A_{620} - 0.474(A_{652}))}{5.34} \quad (1)$$

$$\mathrm{EP} = A_{620}/A_{280} \quad (2)$$

$$\mathrm{REC}(\%) = \frac{\text{C-PC(mg/mL)} \times \text{collected volume(mL)}}{\text{C-PC initial extract(mg/mL)} \times \text{initial volume(mL)}} \times 100 \quad (3)$$

2.3.2. Study of the purification process design

The best sequence for purification of C-phycocyanin from *S. platensis* was studied. The protein purification design consisted of three main techniques in sequence, including precipitation with ammonium sulfate, ion exchange chromatography and gel filtration, as presented in Fig. 1.

2.3.2.1. Precipitation. C-PC was fractionated with solid $(NH_4)_2SO_4$ at room temperature with 0–20%/20–50% of saturation. Ammonium sulfate was added to the solution containing C-phycocyanin until 20% saturation was attained. The solution was maintained overnight and then centrifuged. The precipitate was discarded and ammonium sulfate was added to supernatant until 50% saturation was attained. The solution was maintained overnight and then centrifuged. The precipitate, rich in C-phycocyanin, was dissolved in 0.05 M sodium phosphate buffer pH 7.0 at an initial volume/final volume ratio of 0.52, and dialyzed against the same buffer, as optimized earlier by the author's research group (Silva et al., 2009).

2.3.2.2. Ion exchange chromatography. The extract containing the C-phycocyanin obtained in an earlier step was loaded onto a Q-Sepharose Fast Flow column previously equilibrated in 0.025 M Tris–HCl buffer pH7.5 at 40 cm/h (Silveira et al., 2008). The nonadsorbed proteins were removed by washing with the same equilibrium buffer. Elution was carried out using a linear gradient of 0–1 M NaCl with a gradient volume of 50 mL in 0.025 M Tris–HCl buffer pH 7.5. Samples were collected every 5 min and the C-phycocyanin concentration and total protein content determined. The experiments were performed at 25 °C.

2.3.2.3. Gel filtration. The assays involving gel filtration were carried out using bed height of 15 cm (12 mL) of Sephacryl S-100 HR equilibrated in 0.05 M sodium phosphate buffer pH 7.0. The fraction from an earlier step was loaded at 8 cm/h. The volume injected was not more than 1–4% of the bed height (Amersham Biosciences, 2002). The fractions were collected and the concentration, recovery and purity measured.

The best sequence was chosen based on the purity at the end of the process.

3. Results and discussion

3.1. A study of elution in ion exchange chromatography

The influence of the type and volume of the salt gradient used to elute the C-phycocyanin from the Q-Sepharose Fast Flow ion exchange resin was evaluated in terms of the increase in purity (EP), purification factor (PF) and recovery, as presented in Table 1.