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Purification of phycocyanin from *Arthrospira platensis* by hydrophobic interaction membrane chromatography

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ABSTRACT

The cyanobacterium *Arthrospira platensis* is an important source of phycocyanin, which has many commercial applications in foods, cosmetics and pharmaceutics. In this study, hydrophobic interaction membrane chromatography (HIMC) was used for the first time to separate phycocyanin from allophycocyanin and obtain analytical grade phycocyanin, avoiding packed bed (i.e., packed column) chromatography steps. The extraction of phycobiliproteins was carried out on freeze-dried biomass samples suspended in NaCl 0.1 M, and the phycobiliprotein crude extract was obtained by centrifuging the suspension to eliminate cell debris. The crude extract, after the addition of an appropriate amount of ammonium sulphate, was loaded on an ammonium sulphate responsive commercial hydrophilic polyvinylidene fluoride (PVDF) membrane for the HIMC purification process. Applying a two-step HIMC purification procedure, a phycocyanin purity ratio of 4.20 and a yield of 67.0% was attained.

1. Introduction

Photosynthetic cyanobacteria generally contain chlorophyll *a* only and, to enhance light capture, they mainly use the accessory phycobiliprotein pigments (chromophore-protein covalently linked compounds). The most common phycobiliproteins are phycocyanins, allophycocyanins and phycoerythrins. The main components of these pigments are protein subunits α and β, which form the monomeric unit (αβ). Various units self-assemble into the phycobiliproteins, which, in vivo, are organized to form larger protein complexes, the phycobilisomes [1]. These water soluble bright blue (phycocyanins and allophycocyanins) and red (phycoerythrins) pigments, intensely fluorescent, are high value products, having several commercial applications.

Phycocyanin obtained from *Arthrospira platensis* (Spirulina) is used as natural food additive, natural cosmetic dye and fluorescent marker in biomedical research. The use of phycocyanin as nontoxic photosensitizer in photodynamic therapy (PDT) of tumours has been reported, as well as phycocyanin's therapeutic activities as an antioxidant, anti-inflammatory, neuroprotective, anti-cancer and immunomodulatory agent [2–4].

The commercial value of phycocyanin is dependent on its purity, which is usually evaluated by the ratio between the value of absorption maximum of phycocyanin (PC) (around 615–620 nm) and the absorbance value at 280 nm, which is related to the total amount of proteins in the product (A_{PC}/A_{280}). Phycocyanin with purity > 0.7 is considered as food grade, > 3.9 as reactive grade and > 4.0 as analytical grade [5,6]. In addition to this purity parameter, the separation factor between phycocyanin and allophycocyanin (APC) is sometimes reported [7–11], and it is evaluated by the ratio between the value of absorption maximum of phycocyanin and allophycocyanin (A_{PC}/A_{APC}).

The purification of phycocyanin is a complex and time consuming process and a number of purification methods have been proposed [2,3,12–28]. Usually, high purity is attained through a number of purification steps, often involving different packed bed chromatographic methods, which reduce product yield and increase the costs, hindering the exploitation at a large scale [2]. Several simplified chromatographic purification procedures, more suitable for a large scale phycocyanin production, have been proposed [10,17,19,21–23]. Conversely, only few protocols completely avoiding the expensive and time consuming packed bed chromatography purification steps have been suggested [5–7,28]. However, among these, only the procedures exploiting aqueous two phase extraction steps coupled to ultrafiltration (which also can be a time consuming step) allow to obtain analytical

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grade phycocyanin [6,7].

Membrane chromatography (MC) has emerged as a cost-effective alternative to column chromatography [29] and has gained increasing importance, particularly for processing large volumes of diluted streams, for polishing purposes in antibody and therapeutic protein production, and for purifying large biomolecules (e.g. nucleic acids, proteins, antibodies). Applications, advantages and drawbacks of MC versus packed-bed chromatography have been discussed in references [30–33].

This paper reports the application of hydrophobic interaction membrane chromatography for phycocyanin purification [34], using a salt responsive commercial hydrophilic PVDF membrane. This membrane was not originally designed for MC, and it is commercialized simply as a microfiltration device, enabling high flow rates and throughput, low extractables, broad chemical compatibility and very low protein binding. In particular, a very low protein binding represents a great advantage, because: (*i*) it minimizes unspecific protein binding on the membrane, and (*ii*) it allows to both induce and modulate protein/membrane interactions, thus promoting reversible and selective retention of the target protein on the membrane by changing the environmental conditions (i.e., ammonium sulphate concentration).

Ghosh described [35,36] the separation of human plasma proteins by HIMC, exploiting hydrophilic PVDF membranes and inducing selective and reversible protein binding to the membrane using ammonium sulphate, a strong anti-chaotropic salt which increases the hydrophobic effects, promotes binding, but avoids protein denaturation and loss of biological activity occurring in strongly hydrophobic environments [30].

Here, we report evidences that HIMC can be exploited to separate phycocyanin from allophycocyanin and to obtain, within few minutes, analytical grade phycocyanin avoiding both packed bed chromatography and ultrafiltration steps.

2. Materials and methods

2.1. Organism and culture conditions

The cyanobacterium *Arthrospira platensis*, strain M2 from the culture collection of the Institute of Ecosystem Study (CNR) at Sesto Fiorentino (Italy), was grown on Zarrouk's medium [37] using glass columns (i.d. = 50 mm, 600 mL working volume) immersed in a thermostated water bath (30 °C) and operated in batch mode. Cultures were continuously illuminated from one side at a light intensity of 150 μmol photons $m^{-2} s^{-1}$.

2.2. Chemicals and laboratory equipment for extraction and purification

Sodium chloride, ACS reagent, was purchased from VWR Chemicals; ammonium sulphate (AS), RPE analytical grade, was purchased from Carlo Erba Reagents.

NaCl and AS solutions were prepared with 18 MΩ Milli-Q water.

In order to reduce the risk of PVDF membrane clogging during the HIMC process, disposable Corning bottle-top vacuum filter systems (cellulose acetate membrane, average pore size 0.22 μm, catalogue code CLS430767) were used to filter 0.1 M NaCl solutions and 3 M AS stock solutions as well as the deionized water used to clean PVDF membranes.

HIMC procedure was performed with hydrophilic PVDF microfiltration membranes (Durapore, average pore size 0.45 μm, diam. 47 mm, thickness 125 μm, filter code HVLP 04700, purchased from Sigma-Aldrich) using a glass vacuum microfiltration assembly which was composed by: 250 mL glass funnel, 47 mm sintered glass support, anodized aluminium clamp, 100 mL glass vacuum flasks for phycobiliproteins recovering, or 1 L glass vacuum flask for washing.

2.3. Phycobiliprotein extraction

Phycobiliprotein crude extract was obtained suspending freeze-dried *A. platensis* powder in NaCl 0.1 M (typically 100–150 mg Spirulina in 10–11 mL of extraction solution). The suspension was maintained at 4 °C for 24 h, then centrifuged for 45 min (12,000 rcf, T = 10 °C). The supernatant containing phycocyanin and allophycocyanin (crude extract stock solution) was collected, stored at 4 °C, and usually used within three days.

2.4. Spectrophotometric determination of phycobiliproteins

Phycocyanin and allophycocyanin were determined using Eqs. (1) and (2) [38]; phycocyanin purity was evaluated using Eq. (3) and phycocyanin/allophycocyanin separation factor using Eq. (4). Absorbance values were corrected by subtracting the absorbance at 750 nm [39]. Finally, phycocyanin recovery yield (%) was determined using the ratio between the amount of recovered phycocyanin and the amount of loaded phycocyanin applying Eq. (5) [19].

$$[PC] = \frac{(A_{615} - 0.474 \times A_{652})}{5.34} \quad (1)$$

$$[APC] = \frac{(A_{652} - 0.208 \times A_{615})}{5.09} \quad (2)$$

$$P = \frac{A_{615}}{A_{280}} \quad (3)$$

$$SF = \frac{A_{615}}{A_{652}} \quad (4)$$

$$Y\,(\%) = \frac{PC\left(\frac{mg}{mL}\right) \times \text{collected volume (mL)}}{PC \text{ initial extract}\left(\frac{mg}{mL}\right) \times \text{initial volume (mL)}} \times 100 \quad (5)$$

A_{615}, A_{652} and A_{280} are the absorbance at 615 nm (phycocyanin absorption maximum), 652 nm (allophycocyanin absorption maximum) and 280 nm (absorbance related to the whole protein content), respectively. The absorbance values at these wavelengths were used throughout this study, even if untreated extracts and poorly purified/separated phycobiliprotein solutions showed the positions of the absorption maximum of phycocyanin and allophycocyanin at wavelengths different from 615 and 652 nm. The latter condition can occur as a result of the overlap of the absorption bands of phycocyanin and allophycocyanin both present in solution. Indeed, the absorption band of allophycocyanin appears as a shoulder of the phycocyanin band around 650 nm, whenever both proteins are present in solution and allophycocyanin is the minor component.

Absorbance measurements were carried out with an UVmc2 spectrophotometer (SAFAS). Suprasil quartz 1 cm light path cuvettes were used.

2.5. Evaluation of PVDF membrane ability to retain phycobiliproteins and of the proper ammonium sulphate concentration to separate phycocyanin from allophycocyanin

Crude extract solutions at various AS concentrations were prepared keeping phycobiliprotein (phycocyanin + allophycocyanin) content constant (about 1 mg). Each solution was prepared by mixing an aliquot of the crude extract stock solution with an appropriate volume of AS 3 M stock solution and 0.1 M NaCl solution, to reach the desired AS concentration in a final volume of 10 mL. Each phycobiliprotein solution was loaded on a PVDF membrane and filtered using a glass vacuum microfiltration assembly, to evaluate the retention ability of the membrane, according to the procedure reported below.

The glass vacuum microfiltration device was assembled with a PVDF membrane. The membrane was washed with 100 mL of deionized water

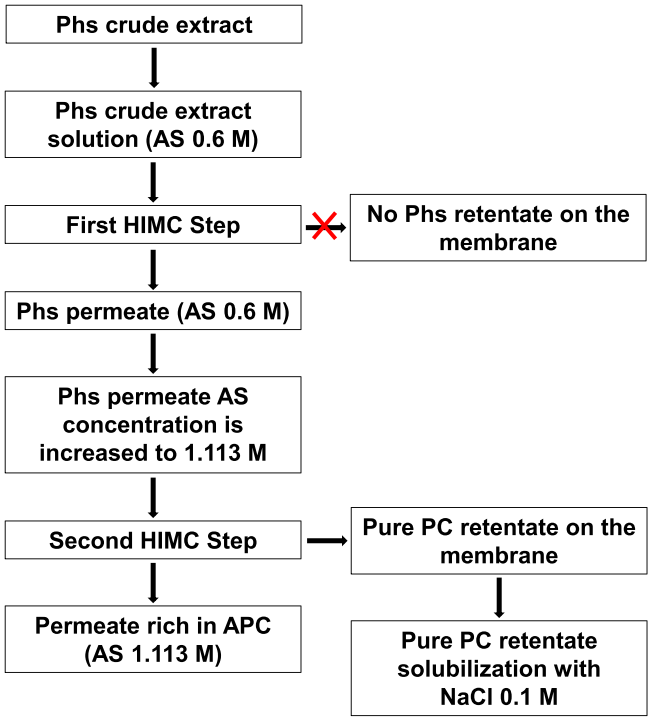

Fig. 1. Two-step hydrophobic interaction membrane chromatography (HIMC) procedure used to separate phycocyanin (PC) from allophycocyanin (APC) and obtain analytical grade phycocyanin (AS = ammonium sulphate; Phs = phycobiliproteins).

and then conditioned with 5 mL of an AS solution having the same AS concentration as in the phycobiliprotein solution to be processed. Both deionized water and AS solution were collected in the washing flask and eliminated. The phycobiliprotein solution (10 mL) was loaded on the membrane, and the phycobiliprotein filtered solution (from here on called permeate) recovered in a clean 100 mL flask. The washing flask was connected again to the vacuum microfiltration assembly and the eventual blue pigment retained by the membrane (from here on denominated retentate) washed with 10 mL of AS solution having the same AS concentration as the phycobiliprotein solution processed. The retentate was desorbed and recovered as filtrate with 10 mL of NaCl 0.1 M in a clean 100 mL flask. Both the retentate and the permeate were characterized by absorbance measurements.

2.6. Phycocyanin purification procedure

After having evaluated the proper AS concentration allowing separation of phycocyanin from allophycocyanin, we attempted to optimize the HIMC conditions to obtain phycocyanin of analytical grade purity. To achieve this goal two HIMC steps were required. A schematic diagram of the purification process is depicted in Fig. 1. Purification was performed at room temperature.

2.6.1. First membrane chromatography step

Proteins that require less strong anti-chaotropic conditions (i.e., lower AS concentration) than phycocyanin and allophycocyanin to interact with the PVDF membrane were removed from phycobiliprotein solutions according to the procedure reported below.

The glass vacuum microfiltration device was assembled with a PVDF membrane. The membrane was washed with 100 mL of deionized water and then conditioned with 5 mL of AS 0.6 M (this AS concentration was optimized in preliminary experiments not reported here). An aliquot of 5 mL of crude extract in AS 0.6 M, with a total phycobiliproteins content of about 2 mg was loaded on the membrane and filtered; the permeate was recovered in a clean 100 mL flask. Then a volume of 5 mL of AS 0.6 M was loaded on the membrane, filtered and the solution recovered in the same permeate flask in order to maximize phycobiliprotein recovery. No blue adsorbed pigment was observed on the membrane at AS 0.6 M. The solution volume was carefully measured (it was about 9.7 mL) and AS 0.6 M was added to have a final volume of 20 mL.

2.6.2. Second membrane chromatography step

Ammonium sulphate 3 M and NaCl 0.1 M were added to 10 mL of the permeate in AS 0.6 M, obtained in the first step of the HIMC procedure (Section 2.6.1), in order to have a phycobiliprotein solution with the concentration of AS suitable for purifying phycocyanin and separating it from allophycocyanin. For this purpose the concentration of AS must be maintained 1.11–1.12 M (see also Section 3.1). In this work AS concentration was kept equal to 1.113 M. The phycocyanin and allophycocyanin total content was about 0.94 mg.

In order to obtain analytical grade phycocyanin, this solution was loaded on a new PVDF membrane, applying the usual procedure: the PVDF membrane was washed with 100 mL of deionized water and then conditioned with 5 mL of 1.113 M AS solution. After collecting in the washing flask and eliminating both deionized water and AS solution, the phycobiliprotein solution in AS 1.113 M was loaded on the membrane, filtered and the phycobiliprotein permeate recovered in a clean 100 mL flask. The washing flask was connected again to the vacuum microfiltration assembly and the retentate on the membrane washed with 10 mL of AS 1.113 M. The retentate was then desorbed and recovered with 10 mL of NaCl.0.1 M in a clean 100 mL flask. Both retentate and permeate were characterized by absorbance measurements.

2.7. Evaluation of maximum binding capacity of PVDF membrane

In order to evaluate the maximum binding capacity of PVDF membrane in the conditions applied to obtain phycocyanin of analytical grade, the procedure described in Section 2.6.1 (*First membrane chromatography step*) was repeated as many times needed until a volume of phycobiliprotein solution sufficient to carry out the experiment was obtained. A single membrane was used in this step, washing and reconditioning it after each extract loading cycle.

AS 3 M was added to the permeate phycobiliprotein solution in order to have a final AS concentration of 1.113 M, before applying the second HIMC step. Increasing volumes of the phycobiliprotein solution in AS 1.113 M were consecutively loaded on a new membrane, which was cleaned with deionized water and conditioned with AS 1.113 M after each loading cycle, following the procedure reported in Section 2.6.2.

2.8. Electrophoresis analyses

The total concentration of proteins in the crude extract and in the purified phycocyanin solution was determined according to Bradford method, using bovine serum albumin as standard.

2.8.1. Non-denaturing gel electrophoresis (Native-PAGE)

Native-PAGE was carried out loading 25 μg of protein on 8% (v/v) running and 4% stacking home-made polyacrylamide slab gels (30% Acrylamide/Bis Solution, 37.5:1, Biorad), 1.5 mm thick, using Tris–glycine buffer (pH 8.3). Gels were run at 4 °C and the protein bands were visualized by staining with Bio-Safe™ Coomassie Stain (Biorad).

2.8.2. Denaturing gel electrophoresis (SDS-PAGE)

Sodium dodecyl sulfate-PAGE (SDS–PAGE) was carried out loading 25 μg of protein on a 15% (v/v) running and 4% stacking, home–made polyacrylamide slab gel (30% Acrylamide/Bis Solution, 37.5:1, Biorad), 1.5 mm thick, containing 0.1% (w/v) SDS using Laemmli buffer [40]. Samples were pre-incubated with loading buffer (2% (w/v) SDS, 10%

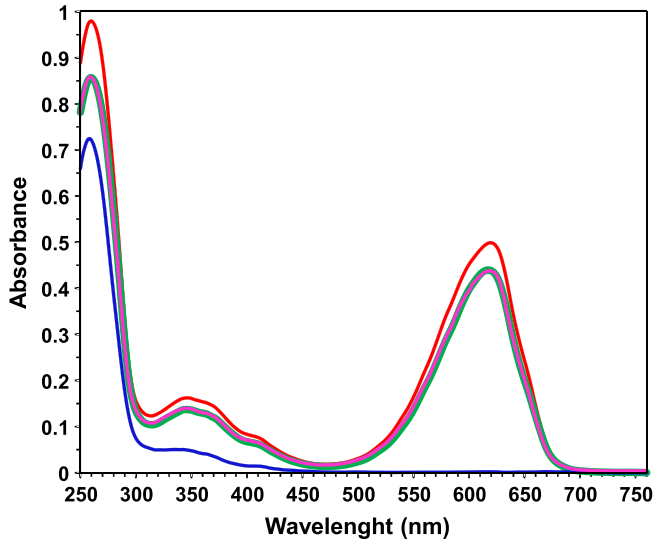

Fig. 2. Absorbance spectra of: the permeate of a phycobiliprotein solution in NaCl 0.1 M (pink curve), the permeate of a phycobiliprotein solution in ammonium sulphate (AS) 0.5 M (green curve, almost coincident with the pink curve), the permeate of a phycobiliprotein solution in AS 1.7 M (blue curve). The absorbance spectrum of the untreated (i.e. not filtered) phycobiliprotein solution in NaCl 0.1 M is also reported (red curve) as control. All the solutions have the same phycobiliprotein concentration. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(v/v) glycerol, 4.5% (v/v) b-mercaptoethanol, 0.025% (w/v) bromophenol blue and 0.06 M Tris (pH 6.8)), for 5 min at 95 °C. Gels were run at room temperature and the protein bands were visualized by staining with Bio-Safe™ Coomassie Stain (Biorad). The molecular weight of subunits was determined by calibrating the gel with molecular weight markers (All blue prestained protein standards, Biorad).

3. Results and discussion

3.1. Evaluation of PVDF membrane ability to retain phycobiliproteins and separate phycocyanin from allophycocyanin

The ability of PVDF membrane to selectively and reversibly retain phycobiliproteins was evaluated as well as the possibility to separate phycocyanin from allophycocyanin using a proper AS concentration. For this purpose, phycobiliprotein solutions at various AS concentrations were loaded on the membrane and its ability to retain the blue proteins monitored through absorbance measurements. Some absorbance spectra are shown in Fig. 2.

PVDF membrane was not able to retain phycobiliproteins in the absence of AS (Fig. 2, pink curve) or at low AS concentration (Fig. 2, green curve). However, reduced absorbance values (compared to the absorbance of a control solution, Fig. 2, red curve) were achieved for these permeates, which evidenced a partial loss of the phycobiliprotein content. The loss of phycobiliproteins appeared mostly due to their adsorption on the sintered glass support of the vacuum microfiltration assembly, which appeared slightly blue to a visual inspection, than due to interactions with the PVDF membrane (which remained white). Increasing AS concentration, phycobiliprotein content in the permeate progressively decreased, while the membrane turned blue. At sufficiently high AS concentration, the membrane completely retained phycobiliproteins and no phycocyanin or allophycocyanin were detectable in the permeate (Fig. 2, blue curve). To recover phycobiliproteins on the membrane the extraction solution (NaCl 0.1 M) was used.

Moreover, by an accurate tuning of AS concentration, the conditions to separate phycocyanin from allophycocyanin were established. A

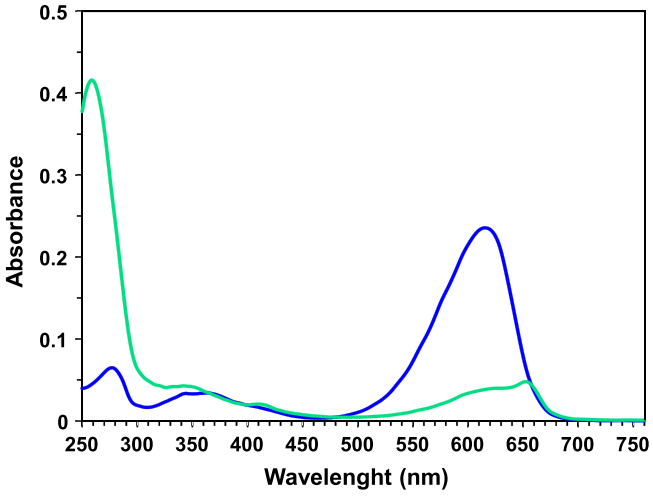

Fig. 3. One-step hydrophobic interaction membrane chromatography of a solution of phycobiliproteins in ammonium sulphate (AS) 1.113 M: absorbance spectrum of the retentate recovered with NaCl 0.1 M (blue curve) and of the permeate still in AS 1.113 M (blue-green curve). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

good separation was obtained at AS 1.11–1.12 M. Outside this optimal range, a higher concentration of AS reduces both the degree of separation of phycocyanin and allophycocyanin and the purity of phycocyanin; a lower concentration of AS causes a decrease of the interaction of phycocyanin with the membrane, which is crucial for its purification. At AS 1.113 M (the concentration used in this work, Section 2.6.2) phycocyanin was retained by the membrane, while allophycocyanin was not. Allophycocyanin was present in the permeate (Fig. 3, blue-green curve) as well as most biomolecules contained in the loaded extract; for example, the presence of nucleic acids is clearly evidenced by the absorption band at about 260 nm. Phycocyanin was recovered by loading 10 mL of NaCl 0.1 M on the membrane (Fig. 3, blue curve). This one-step HIMC procedure permitted to separate phycocyanin from allophycocyanin. The separation factor between phycocyanin and allophycocyanin (A_{615}/A_{652}) changed from 2.14 of the untreated extract to 3.57 (± 0.28, SD, n = 3) of the retentate, while phycocyanin purity (A_{615}/A_{280}) increased from 0.93 to 3.70 (± 0.14, SD, n = 3), not enough to reach the analytical grade.

3.2. Optimization of membrane chromatography procedure to obtain phycocyanin of analytical grade

To obtain phycocyanin of analytical grade (i.e. $A_{615}/A_{280} > 4$), a different, optimized, procedure including two hydrophobic interaction membrane chromatography steps was applied.

In a very preliminary design of the optimized purification procedure (see Supplementary data, Fig. S1), an attempt to enhance the purity of the product using a membrane stack was performed. The procedure follows that reported by Ghosh [35,36] to separate human plasma proteins. This single step hydrophobic interaction membrane chromatography procedure gave poor results, very likely because the simplicity of the filtering equipment was not suitable to ensure proper membrane stack operation. A visual inspection was already sufficient to conclude that the purification process was not good enough. In fact, the uniform blue colour visible on a single membrane (in the presence of a sufficient AS concentration) was replaced by an irregular blue patchy appearance when a two membrane stack was used. The retentate phycocyanin purity was only about 3.2.

Better results were achieved using one membrane at a time and by applying a second purification step. In this procedure (see Supplementary data, Fig. S2), a retentate solution, obtained loading on

the membrane an extract solution in AS 1.113 M and recovering it with NaCl 0.1 M, was further purified by a second membrane chromatography step after restoring 1.113 M AS concentration.

Although a phycocyanin purity around 4.0 (analytical grade) was achieved following this procedure, a further attempt to improve the purification protocol was carried out. The improved procedure is sketched in Fig. 1 and described in detail in Section 2.6. It still consists of two distinct membrane chromatography steps.

The design of these two purification steps was inspired by some phycobiliprotein salting-out procedures (for example [8,21]), in which (*i*) AS saturation degree sufficiently low to avoid phycobiliprotein precipitation is used to precipitate undesired proteins; after that, (*ii*) phycobiliprotein precipitation is attained increasing AS saturation degree. The fractionation of proteins using this multi-step salting-out procedure permits to obtain more pure phycobiliproteins than direct phycobiliprotein salting-out at high AS saturation degree.

Similarly, through the first membrane chromatographic step at AS concentration 0.6 M (Section 2.6.1), those proteins requiring more mild anti-chaotropic conditions than phycocyanin and allophycocyanin to be retained by the PVDF membrane were removed from the phycobiliprotein solutions. The second membrane chromatographic step, at AS 1.113 M (Section 2.6.2), allowed to separate phycocyanin from allophycocyanin, thus increasing phycocyanin purity. Following this simple and rapid (a few minutes) procedure a purity of 4.20 (± 0.07, SD, n = 16) was achieved. The yield was 67.0% (± 3.0, SD, n = 16) and the separation factor between phycocyanin and allophycocyanin of 3.76 (± 0.08, SD, n = 16) (untreated extract had P = 0.93, SF = 2.14).

Table 1 reports various examples of procedures found in the literature for phycocyanin purification, included the protocol presented in this paper, specifying the crucial purification steps adopted and the final phycocyanin yield and purity achieved.

Phycocyanin purity was further demonstrated by native-Page (Fig. 4A) and SDS–PAGE (Fig. 4B). Only one intense band was present in purified phycocyanin when examined by native-PAGE (Fig. 4A, lane 2) while crude extract (Fig. 4A, lane 1) showed less defined bands, evidencing the presence of significant amounts of other proteins. Moreover, the correspondence of phycocyanin band position, in the native–PAGE of the crude extract and purified phycocyanin sample, evidenced the integrity of the protein complex following the purification procedure. SDS-PAGE analysis of the crude extract (Fig. 4B, lane 1) evidenced the presence of many protein bands which were greatly reduced in their number (Fig. 4B, lane 2) in the purified phycocyanin. Two intense bands were visible both for the crude extract and purified phycocyanin sample, corresponding to α- and β-subunits, with the expected molecular weights around 15–20 kDa. SDS-PAGE of the crude extract (Fig. 4B, lane 1) showed less defined, broadened bands in the same molecular weight range, due to the concomitant presence of phycocyanin and allophycocyanin α- and β-subunits, which have different, even if similar, molecular weights.

3.3. Evaluation of maximum binding capacity of PVDF membrane

The binding capacity of a membrane is an important parameter in membrane chromatography, especially in view of large scale production or for industrial applications. The evaluation of the maximum binding capacity of the PVDF membrane used in this study permitted also to investigate how phycocyanin purity and yield, and the phycocyanin/allophycocyanin separation factor were affected by the amount of phycobiliproteins loaded on the membrane.

Phycocyanin content in the retentate increased by increasing the volume of a phycobiliprotein solution (in AS 1.113 M) loaded on the membrane (Fig. 5A), reaching a plateau at about 1.26 mg phycocyanin, corresponding to a membrane capacity of about 10.50 mg/mL (membrane volume was 0.120 mL, calculated by using the “effective” membrane diameter value of 35 mm and its thickness of 125 μm). Allophycocyanin content in the retentate was low and increased only slightly (Fig. 5A). Conversely, increasing the volume loaded on the membrane, phycocyanin content in the permeate exhibited roughly a quadratic growth (Fig. 5B). The non-linear increase of permeate phycocyanin content was due to a more extensive phycocyanin retention by the membrane at lower loaded volumes; in this conditions allophycocyanin permeate content is higher than phycocyanin one, while at larger loaded volumes, approaching the maximum binding capacity of the PVDF membrane, larger amounts of phycocyanin filtered through it, in the permeate. Permeate allophycocyanin content increased linearly instead (Fig. 5B), as a consequence of poor interactions with the membrane.

Retentate phycocyanin purity (Fig. 5C, full squares) and phycocyanin/allophycocyanin separation factor (Fig. 5C, empty squares) were preserved to the largest extract volumes loaded on the membrane. The greater variation of these parameters observed at the smaller volumes was probably due to an uncomplete membrane conditioning and/or unspecific binding processes; some variability was observed during the first cycles of use of a new membrane, the variability decreased after some subsequent cycles of use of the membrane. Finally, the yield of phycocyanin recovered in the retentate decreased roughly linearly loading larger extract volumes (Fig. 5D). Yield decreased from 67.0% for 15–25 mL to about 30% for 97 mL of extract loaded on the membrane.

These results evidenced that increasing the extract volumes (i.e. the phycobiliproteins amount) loaded on the membrane did not greatly affect retentate phycocyanin purity and the separation factor. Moreover, the re-use of the same membrane resulted not only as a possible chance, but, even more, as an advantageous procedure. On the contrary, as the membrane has a limited binding capacity, the retained phycocyanin amount increased along with the loaded extract volume until a plateau was reached. The corresponding yield progressively decreased, because the phycocyanin amount of the loaded volumes increased proportionally (i.e. linearly) with the volume itself, but it was no more retained by the membrane; it filtered through the membrane, in the permeate. However, loading the permeate on the clean membrane enhances the amount of phycocyanin that can be recovered. For example, loading again on the membrane the permeate obtained from the largest extract volume (97 mL) used in the second purification step further phycocyanin was retained and could be recovered (about 22%). Phycocyanin purity reached 4.24 (Fig. 5C, full red square) and phycocyanin/allophycocyanin separation factor 4.12 (Fig. 5C, empty red square). No further attempts were made to recover phycocyanin still remaining in the permeate.

4. Conclusions

Analytical grade phycocyanin (purity 4.20, yield 67.0%) was attained applying HIMC using an ammonium sulphate responsive commercial hydrophilic PVDF membrane, without applying column chromatography or ultrafiltration steps. This is the first time that hydrophobic interaction membrane chromatography is exploited for phycocyanin purification and separation from allophycocyanin. The results indicate that this approach can represent a useful method for phycocyanin purification, facilitating its scaling up due to the simplicity of procedure. We are confident that this method can be further improved, for example, studying the effects of temperature, pH, type of salt/buffer and salt/buffer concentration or exploiting other anti-chaotropic salts or membranes; and that HIMC can be also applied to achieve an efficient purification procedure for other phycobiliproteins of economic interest (such as phycoerythrin and allophycocyanin).

Table 1
Procedures applied to purify phycocyanin from cyanobacterial extracts.

Crucial purification treatments[a]	Species	Purity (A_{PC}/A_{280})	Y (%)	Ref.
Column Chromatographic methods				
1. $(NH_4)_2SO_4$ precipitation 2. Hydroxyapatite chromatography 3. Ion exchange chromatography	*A. platensis*	4.15	–	[14] Boussiba et al. (1979)
1. Hydrophobic interaction chromatography 2. Ion exchange chromatography	*Synechochoccus* sp.	4.85	76.6	[15] Abalde et al. (1998)
1. Five rivanol sulphate treatments 2. $(NH_4)_2SO_4$ precipitation 3. Gel filtration chromatography 4. $(NH_4)_2SO_4$ precipitation	*A. fusiformis*	4.30	45.7	[16] Minkova et al. (2003)
1. Two rivanol sulphate treatments 2. $(NH_4)_2SO_4$ precipitation 3. Gel filtration chromatography 4. $(NH_4)_2SO_4$ precipitation	*A. africanum*	4.52	55.0	[17] Minkova et al. (2007)
1. Three $(NH_4)_2SO_4$ precipitation steps 2. Ion exchange chromatography	*Spirulina* sp.	4.42	45.6	[8] Patel et al. (2005)
1. Three $(NH_4)_2SO_4$ precipitation steps 2. Ion exchange chromatography	*Phormidium* sp.	4.43	35.2	[8] Patel et al. (2005)
1. Three $(NH_4)_2SO_4$ precipitation steps 2. Ion exchange chromatography	*Lyngbya* sp.	4.59	36.8	[8] Patel et al. (2005)
1. $(NH_4)_2SO_4$ precipitation 2. Hydroxyapatite chromatography	*A. flos-aquae*	4.78	–	[18] Benedetti et al. (2006)
1. Two $(NH_4)_2SO_4$ precipitation steps 2. Gel filtration chromatography 3. Ion exchange chromatography	*O. quadripunctulata*	3.31	44.2	[9] Soni et al. (2006)
1. Two $(NH_4)_2SO_4$ precipitation steps 2. Hydrophobic interaction chromatography	*P. fragile*	4.52	62.0	[10] Soni et al. (2008)
1. Expanded bed adsorption chromatography 2. Ion exchange chromatography	*A. platensis*	3.64	8.7	[11] Niu et al. (2007)
1. Two $(NH_4)_2SO_4$ precipitation steps 2. Ion exchange chromatography	*A. platensis*	4.00	–	[19] Moraes et al. (2009)
1. Ion exchange chromatography in Expanded bed mode	*Spirulina* sp.	1.60	79.0	[20] Moraes et al. (2015)
1. Ion exchange chromatography in Fixed bed mode	*Spirulina* sp.	1.70	62.0	[20] Moraes et al. (2015)
1. Two $(NH_4)_2SO_4$ precipitation steps 2. Ion exchange chromatography	*A. platensis*	5.59	67.0	[21] Yan et al. (2011)
1. Chitosan affinity precipitation 2. Activated charcoal adsorption 3. Ion exchange chromatography	*A. platensis*	4.30	42.3	[22] Liao et al. (2011)
1. Expanded bed adsorption chromatography 2. Ion exchange chromatography	*A. platensis*	> 4.00	59.0	[23] Bermejo et al. (2012)
1. Two $(NH_4)_2SO_4$ precipitation steps 2. Triton X-100 precipitation 3. Gel filtration chromatography 4. Ion exchange chromatography	*Lyngbya* sp.	5.53	60.2	[24] Sonani et al. (2014)
1. Two $(NH_4)_2SO_4$ precipitation steps 2. Ion exchange chromatography	*Synechococcus* sp.	4.03	–	[25] Sonani et al. (2017)
Aqueous two phase extraction				
1. Three aqueous two phase extraction steps	*A. platensis*	4.02	78.6	[27] Patil et al. (2008)[b]
Aqueous two phase extraction + Column Chromatographic methods				
1. Chitosan affinity precipitation 2. Activated charcoal adsorption 3. Aqueous two phase extraction	*A. platensis*	5.10	66.0	[26] Patil et al. (2006)[b]
1. Chitosan affinity precipitation 2. Activated charcoal adsorption 3. Aqueous two phase extraction 4. Ion exchange chromatography	*A. platensis*	6.69	–	[26] Patil et al. (2006)
1. Ion exchange chromatography 2. Aqueous two phase extraction	*Nostoc* sp.	3.55	–	[27] Johnson et al. (2014)[b]
Aqueous two phase extraction + membrane process				
1. Two aqueous two phase extraction steps 2. Ultrafiltration 3. $(NH_4)_2SO_4$ precipitation	*A. maxima*	3.80	29.5	[28] Rito-Palomares et al. (2001)
1. Three aqueous two phase extraction steps 2. Ultrafiltration	*A. platensis*	4.05	85.0	[6] Patil et al. (2007)
1. Three aqueous two phase extraction steps 2. Ultrafiltration	*A. platensis*	4.02	–	[7] Patil et al. (2008)
Membrane process: micro- and ultrafiltration				
1. Two microfiltration steps 2. Ultrafiltration	*Spirulina* sp.	1.07	82.0	[5] Chaiklahan et al. (2011)
Membrane process: membrane chromatography				
1. Two hydrophobic interaction membrane chromatography steps	*A. platensis*	4.20	67.0	This paper

[a] "Subsidiary" treatments, such as centrifugation or dialysis, are not reported.
[b] If aqueous two-phase extraction is the unique or the last purification step, polyethylene glycol (PEG) has still to be removed before product utilization.

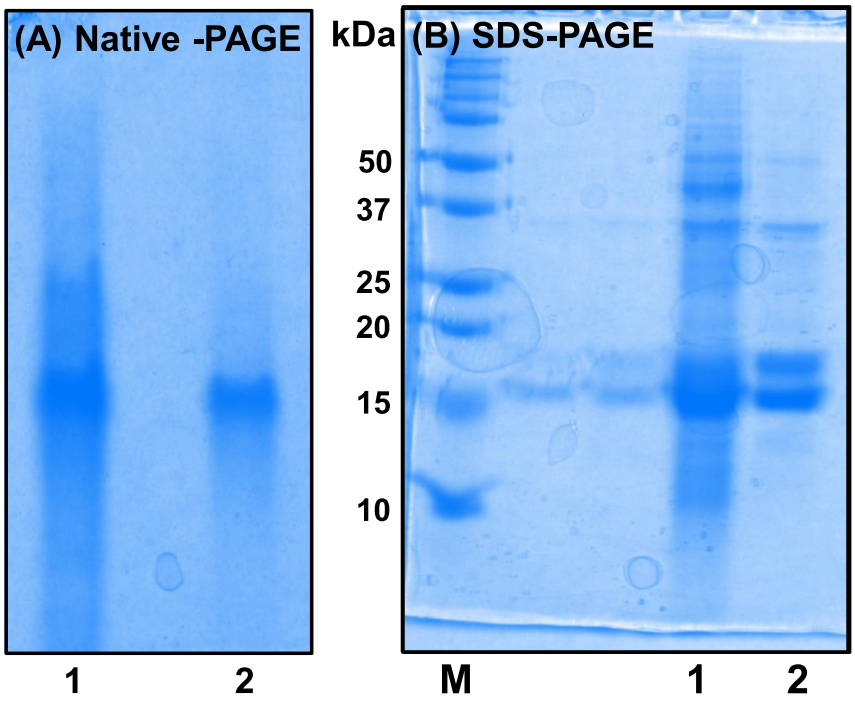

Fig. 4. Non-denaturing gel electrophoresis (Native-PAGE) (A) and denaturing gel electrophoresis (SDS-PAGE) (B) of phycobiliprotein crude extract (lane 1, 25 μg) and purified phycocyanin (lane 2, 25 μg). In SDS-PAGE gel (B), lane M is relative to molecular markers.

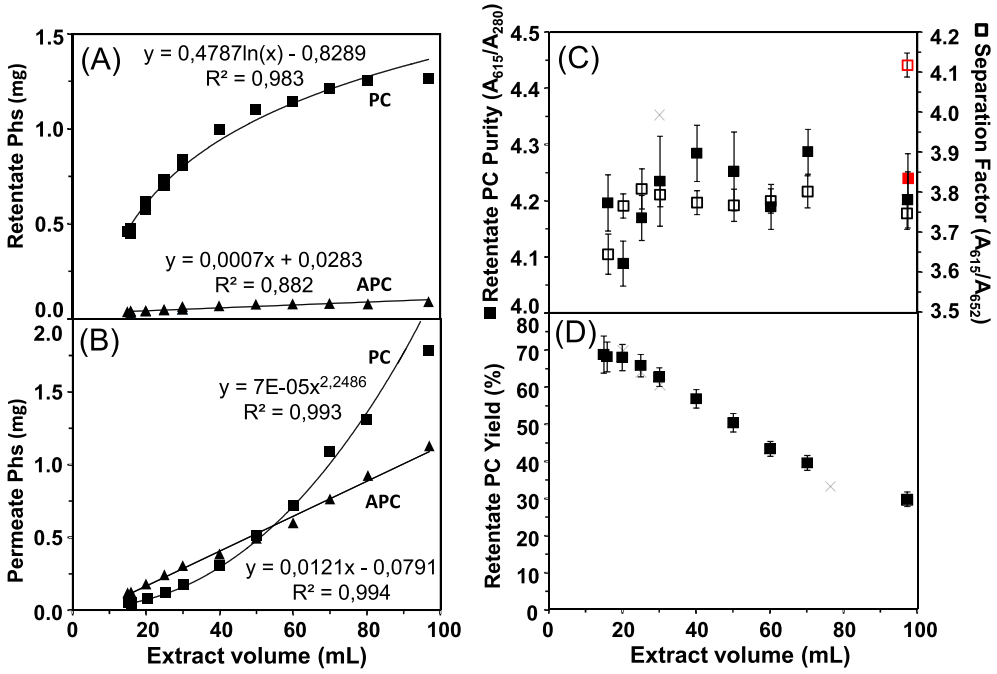

Fig. 5. Polyvinylidene fluoride (PVDF) membrane binding capacity: (A) retentate phycocyanin (PC, squares) and allophycocyanin (APC, triangles) content, (B) permeate PC (squares) and APC (triangles) content, (C) purity (full squares) and separation factor (empty squares) of PC in the retentate and (D) the corresponding yield (%) as a function of the extract volume loaded on the membrane. The full and empty red squares in (C) represent purity and separation factor of PC recovered after having loaded the largest volume permeate in ammonium sulphate 1.113 M (97 mL) a second time on the membrane. Data are the average of 3 experiments. Phs in panels (A) and (B) is used as acronym for phycobiliproteins (i.e. phycocyanin or allophycocyanin). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Conflict of interest

The authors RL, GCZ and GT declare that they have submitted a patent application.

Author contributions

All Authors declare that they have contributed to the manuscript in the following three sections: (1) the conception and design of the study, or acquisition of data, or analysis and interpretation of data; (2) drafting the article or revising it critically for important intellectual content; and (3) final approval of the version to be submitted.

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Appendix A. Supplementary data

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