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Fast preparative isoelectric focusing of phycocyanin subunits in layers of granulated gels

A new method is presented for the fast preparative separation of the light-harvesting photosynthetic pigment C-phycocanin into its α and β subunits, which is based on isoelectric focusing in layers of granutaled gels containing 7 M urea. The method has been successful in cases where other separation procedures failed. The recovery of the separated chains of the light -sensitive biliprotein amounts to 70 ± 10 % when the separation is carried out under light exclusion and in an argon atmosphere. A simple and inexpensive setup for work under an atmosphere of protective gas is described.

Rhodophyta, *Cryptophyta* and cyanobacteria contain phycobilins, a special class of light-harvesting pigments [1–3]. Phycobilins are biliproteins, composed of subunits with covalently linked bile pigment chromophores (*e. g.* [2–5]). Especially interesting is the blue phycocyanin (PC) which is composed of two subunits, the α-chain and the β-chain. For many research applications a separation of these subunits is required. The methods applied so far for isolation are: (i) Ion exchange chromatography on Biorex-70 with an 8 to 9 M urea gradient according to Glazer and Fang [6]. (ii) Gel chromatography on Bio-Gel P-60 with 63 mM formic acid as solvent system [7]. (iii) Preparative gel electrophoresis in 7 M urea [8]. Problems arise due to the lability of the phycocyanobilin chromophores. Bleaching is observed especially when biliprotein solutions of low concentrations are used and long separation times are required due to a high dilution factor. We present a new method which combines some advantages of the above separation procedures, especially speed and resolution, while avoiding the disadvantage of excessive dilution inherent to most chromatographic procedures [9].

Basically, the method described is preparative isoelectric focusing in a granulated gel [10] containing 7 M urea. For gel preparation, 6 g Sephadex G-75 (Pharmacia, Uppsala, Sweden) are allowed to swell for 3–4 h at 80 °C in 200 mL of double distilled water. Then 100 g of analytical grade urea (Serva, Heidelberg, FRG) are slowly dissolved while stirring carefully with a glass rod. The gel is allowed to settle and the supernatant is carefully decanted, leaving a residue of 113 mL which is carefully degassed in the aspirator vacuum for 5 min, followed by addition of 2 mL Servalyt (analytical grade, pH 3–7, Serva). A gel layer is prepared by pouring the gel suspension into the trough followed by water evaporation to about 20 % weight loss using an infrared lamp (Philips Infrared Lamp R 95 E, 220 V, 150 W, distance 35 cm) and a fan.

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The electrophoresis apparatus consisted of an LKB basis unit fitted with gel trough, electrode strips and lid (LKB 2117; LKB productors, Bromma, Sweden). For light exclusion, the electrofocusing bed has to be kept in a dark room or covered by a hood. To avoid chromophore losses through oxidation, all work is carried out under an argon atmosphere; the gas is introduced through a pin situated between the lid (with venting holes) covering the gel bed and a glass plate which had been rubber-sealed on top of this lid at a distance of about 3 mm. In some experiments 1 M phosphoric acid or 1 % Servalyt pH 3–10 were used as analyte. The catholyte in these experiments was 1 M sodium hydroxide solution or 1 % Servalyt pH 3–10. Better results were obtained using anolyte and catholyte solutions, originally described for ultrathin electrofocusing gels [11]. The pH gradient was established by prefocusing at 1500 V at maximally 15 W using an electronically regulated E 532 power supply (Bachofer, Reutlingen, Federal Republic of Germany) for about 4 h (in analogy to [10]). This measure considerably shortens the residence time of the sample in the gel during separation.

Typically, 15–40 mg (maximally 500 mg) of pure PC are suspended in 2.5 ml (5 ml) of double distilled water and urea is added to a concentration of 7.5 M. Complete separation of the subunits is in most cases already obtained within 3 h. The blue colored zones visible in the gel (Fig. 1) are scratched out and eluted with 8 M urea. The denaturating agent is subsequently removed quantitatively using a Bio-Gel P-2 (or P-6) column (Bio-Rad, Munich, FRG), pre-equilibrated with 80 mM potassium phosphate buffer, pH 6. The method of choice, however, is dialysis against 100 mM potassium phosphate buffer,

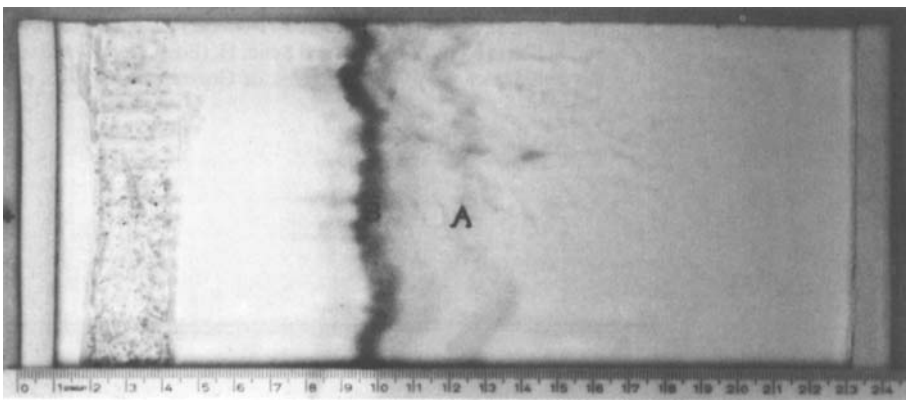

Figure 1. Separation of *Mastigocladus laminosus* C-phycocyanin into its α- and β-subunits by preparative isoelectric focusing in granulated gels containing 7 M urea. Servalyt carrier ampholytes, pH 3–7 (1 %). Separation time 4 h, following prefocusing for the same time or overnight; initial settings: 1500 V, power limited to 15 W. A, α-subunit; B, β-subunit near anode (+).

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Table 1. Isoelectric points (p*I*) and recovery of subunits of C-phycocyanin

Biliprotein	p*I*	Yield (%)
Mastigocladus laminosus		
α-subunit	6.10 ± 0.05	70 ± 10
β-subunit	5.00 ± 0.05	70 ± 10
Spirulina geitleri		
α-subunit	5.00 ± 0.05	70 ± 10
β-subunit	5.60 ± 0.05	70 ± 10

pH 7, for 16 h first at 20 °C, then at 4 °C. The removal of urea is monitored *via* the index of refraction.

The isoelectric points determined in urea are summarized in Table 1. A purity check by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 2) showed that the separated subunits contained less than 5 % impurities such as other subunits and / or colorless peptides. The intactness of the separated subunits was monitored by spectroscopy employing the ratio: absorption at maximum 616 nm (α-sub) or 604 nm (β-sub)/A 350 of about 6:1, proving the nativeness of the chromophores. Since the phycocyanobilin chromophores of the separated subunits remain intact the samples can be used for spectroscopic measurements (*e. g.* [12]), which are sensitive to structural defects (*i. e.* chromophore bleaching). Subunit separation of phycocyanin by isoelectric focusing is superior to other methods. Subunits of C-phycocyanin from *Spirulina geitleri* could be separated on Fractogel PSK 55 (E. Merck, Darmstadt, FRG), in 1/16 M formic acid (R. Fischer, unpublished results). A separation of *Mastigocladus laminosus* C-phycocyanin subunits was easily achieved by isoelectric focusing but neither fractionation on Fractogel nor Biorex 70 (an ion exchange resin) proved successful [6]. Gel chromatography on Biogel P-60 [7] required low flow rates due to the high compressibility of the gel resulting in excessively long separation times up to 3 to 4 days. By visual inspection the separation was satisfactory; however, according to optical measurements, only the α-fraction was pure whereas the β-fraction was insufficiently enriched, probably due to β-chromophore bleaching (R. Fischer, unpublished).

Generally, subunits are much more labile than trimers or whole phycobilisomes. Since bleaching is minimal upon short separation times, preparative isoelectric focusing appears to be an optimal method. We found approximately 70 % ± 10 % recovery of biliproteins based on absorption measurements (see Table 1).

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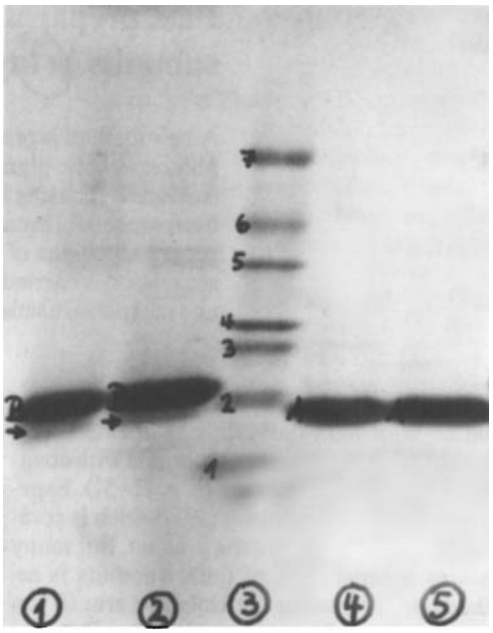

Figure 2. Sodium dodecyl sulfate (SDS) – polyacrylamide gel electrophoresis of *Mastigocladus laminosus* C-phycocyanin subunits obtained by preparative isoelectric focusing in layers of granutaled gel. The α-subunit is pure, and the β-subunit contains less than 5 % impurities (α-subunit and colorless peptides, respectively). Lanes (1) and (2): β-subunit containing less than 5 % impurities (α-sub., see arrow + colorless peptides); lane (3): SDS marker proteins (Sigma, St. Louis, MO, USA): (1) lactalbumin, M_r 14 200; (2) trypsin inhibitor, M_r 20 100; (3) trypsinogen, M_r 24 000; (4) carbonic anhydrase, M_r 29 000; (5) glycerinaldehyde-3-phosphate dehydrogenase, M_r 36 000; (6) ovalbumin, M_r 45 000; (7) bovine serum, M_r 66 000. Lanes (4) and (5): α-subunit.

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