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Separation of closely related intrinsic membrane polypeptides of the photosystem II light-harvesting complex (LHC II) by reversed-phase high-performance liquid chromatography on a poly(styrene–divinylbenzene) column

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Abstract

The three closely related intrinsic membrane polypeptides of the photosystem II light-harvesting complex (LHC II) were successfully resolved on a PRP-1 poly(styrene–divinylbenzene) column using a three-stage linear water–acetonitrile gradient containing 0.1% trifluoroacetic acid. The hydrophobic proteins of photosystem I (PS I-200) and photosystem II core particles were also separated by this method, showing that membrane proteins of different sizes and hydrophobicities can be resolved in this system.

1. Introduction

Mixtures of hydrophobic membrane proteins are usually analyzed by polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulphate (SDS), which separates denatured, detergent-bound proteins on the basis of their molecular mass in the absence of complicating factors such asglycosylation [1]. Although this method is reasonably fast (several hours), it would be very useful to have a rapid alternative method which would separate membrane proteins on some basis other than molecular mass. Reversed-phase (RP) HPLC is extensively used for separation of soluble proteins, but has had only limited application to the separation of intrinsic membrane proteins [2–7] because of the many technical difficulties due to their highly hydrophobic nature [2–4].

The photosystem (PS) II light-harvesting complex (LHC II) is the major chlorophyll–protein complex of green plant photosynthetic membranes [8,9]. It has three hydrophobic polypeptides of M_r 25 000–28 000, all of which have three membrane-spanning helices [10] and are very similar in amino acid sequence [11,12]. Since they are intrinsic membrane polypeptides, they can only be released from the membrane with high concentrations of detergent, and are insoluble in aqueous solutions in the absence of detergent. In tomato, the type I LHC II polypeptide is four amino acids longer than the type II polypeptide, and has one less positive charge; the type III polypeptide is 11 amino acids shorter and has two fewer positive charges [12]. The

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three types have 74% residue identity. Optimum separation of the denatured polypeptides on SDS-PAGE requires overnight electrophoresis in the presence of 4 *M* urea [12]. In this paper we report that a PRP-1 poly(styrene–divinylbenzene) reversed-phase column can be used to separate the three spinach LHC II polypeptides rapidly and in a different order from SDS-PAGE. To our knowledge, this is the first report of the use of this type of column for separation of intrinsic membrane proteins.

2. Methods

LHC II was isolated from washed thylakoids (photosynthetic membranes) solubilized with 1.2% Triton X-100 according to Ryrie *et al.* [13]. PS II core preparations were prepared according to ref. 14. In some cases, LHC II was prepared as a by-product of this procedure. PS I preparations with different amounts of associated antenna chlorophyll (PS I-200, PS I-100) were isolated according to Mullet *et al.* [15] and Haworth *et al.* [16].

Aliquots of LHC II corresponding to 100 μg chlorophyll (approximately 200 μg protein) were precipitated in 80% aqueous acetone at room temperature and collected by centrifugation. The pellet was dried under a stream of nitrogen, dissolved in 50–100 μl of acetonitrile–formic acid (2:1, v/v) and filtered through a 0.45-μm nylon filter (Cole-Parmer). The filtrate was injected immediately onto the HPLC column to avoid acid-induced degradation of the polypeptides. PS I and II preparations were treated the same way.

The HPLC system consisted of a Waters 600E gradient system (Waters, Milford, MA, USA) with an U6K injector and a Waters 994 photodiode array detector. The detector was routinely set to 215 nm. HPLC-grade acetonitrile and trifluoroacetic acid (TFA) were purchased from BDH (Canada). Distilled water was filtered through a 0.22-μm GSWP filter (Millipore). Reversed-phase chromatography was carried out on a 10-μm poly(styrene–divinylbenzene) PRP-1 column, (150 × 4.1 mm, 7.5 nm pore size, Hamilton, NY, USA) at a flow-rate of 0.5 ml/min and a temperature of 25°C. A guard column of the same material was used in all experiments. The three-stage linear gradient started with 90% A (0.1% TFA in water) and 10% B (0.1% TFA in acetonitrile) reaching 63% B after 52.5 min, 70% B after 80 min and 100% B after 90 min. Other columns investigated were a PRP-3 poly(styrene–divinylbenzene) column (50 × 4.1 mm; 30 nm pore size, Hamilton) and a silica-based RP-8 column (250 × 4 mm; 30 nm pore size, 10 μm particle size) from Merck (Darmstadt, Germany).

SDS-PAGE separations were according to ref. 12 on a 14% polyacrylamide gel containing 4 *M* urea, 0.8 *M* Tris, pH 8.8. HPLC fractions were dried under nitrogen before being solubilized in 2% SDS, 10 m*M* dithiothreitol, 65 m*M* Tris, pH 6.8.

3. Results

The LHC II polypeptides were completely solubilized in acetonitrile–formic acid (2:1, v/v) after removal of pigments by acetone extraction. Separation of the three polypeptides was achieved with a PRP-1 column with a pore size of about 7.5 nm, using a three-stage linear acetonitrile–water gradient in the presence of 0.1% TFA (Fig. 1). Fig. 1 shows the separation of two major peaks (1 and 2) and a shoulder (3), which is clearly visualized on an expanded scale (Fig. 1, inset).

Fig. 2a shows a similar sample of purified spinach LHC II separated by high-resolution SDS-PAGE containing 4 *M* urea. Type I, II and III polypeptides are numbered according to their relative molecular masses which are approximately 27 000, 26 000 and 25 000 in spinach [14]. Samples were collected from HPLC separations such as the one in Fig. 1, evaporated, dissolved in electrophoresis buffer and separated by urea-SDS-PAGE. Fig. 2b shows that peak 1 contains the type II polypeptide. The central fraction of peak 2 contained only the type I polypeptide (Fig. 2e), while the shoulder (peak 3) was enriched for the type III polypeptide (Fig. 2c, d). Taking into account the difficulty in collecting fractions that correspond exactly to the peaks

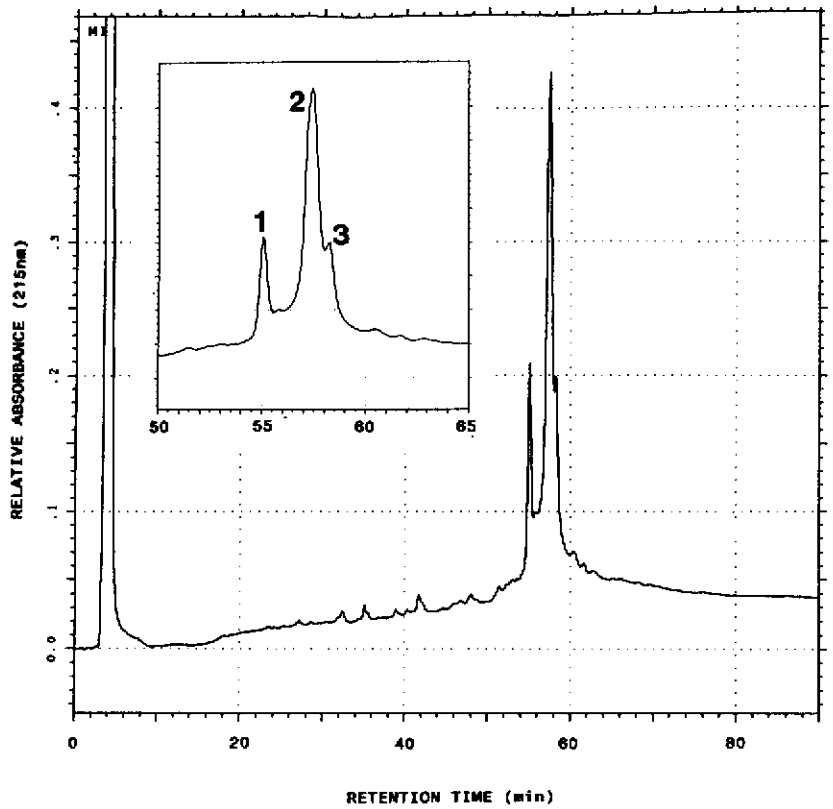

Fig. 1. Separation of the three types of spinach LHC II polypeptides on a PRP-1 column with a three-stage 10 to 90% gradient of acetonitrile in water, 0.1% TFA (see Methods). Inset: expanded scale showing relevant segment of the profile. See text.

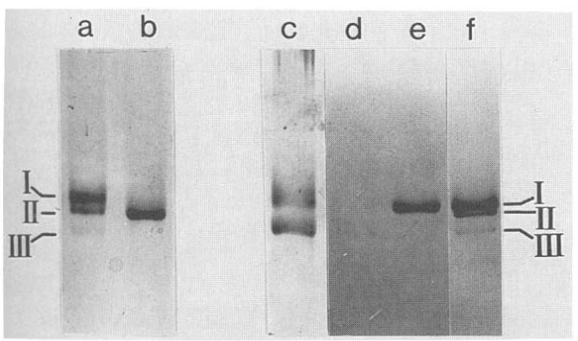

Fig. 2. SDS-PAGE separation of LHC II fractions from the PRP-1 HPLC column. (a–b) and (c–f) are from two different runs. All samples Coomassie-stained except for (c) which was silver stained. (a) Purified LHC II, (b) peak 1, (c and d) peak 3, (e) peak 2, (f) purified LHC II.

in question, we can conclude that peaks 1, 2 and 3 correspond to types II, I and III, respectively. This shows that the PRP-1 column is resolving the three polypeptide types in a different order from SDS-PAGE, and therefore is separating them on some basis other than molecular mass.

LHC II was isolated from several different plants and separated by HPLC (Fig. 3) and by SDS-PAGE (Fig. 4). Each plant has a somewhat different profile on HPLC, as would be expected from its SDS-PAGE separation. Pea has one major peak on HPLC (Fig. 3a) and apparently no type II band on SDS-PAGE (Fig. 4c). The barley type III polypeptide separates well from types I and II on SDS-PAGE (Fig. 4a), although the latter are not as well resolved from each other as they are in spinach. On HPLC (Fig. 3b), there is one major peak and a well-separated shoulder which is probably the type III poly-

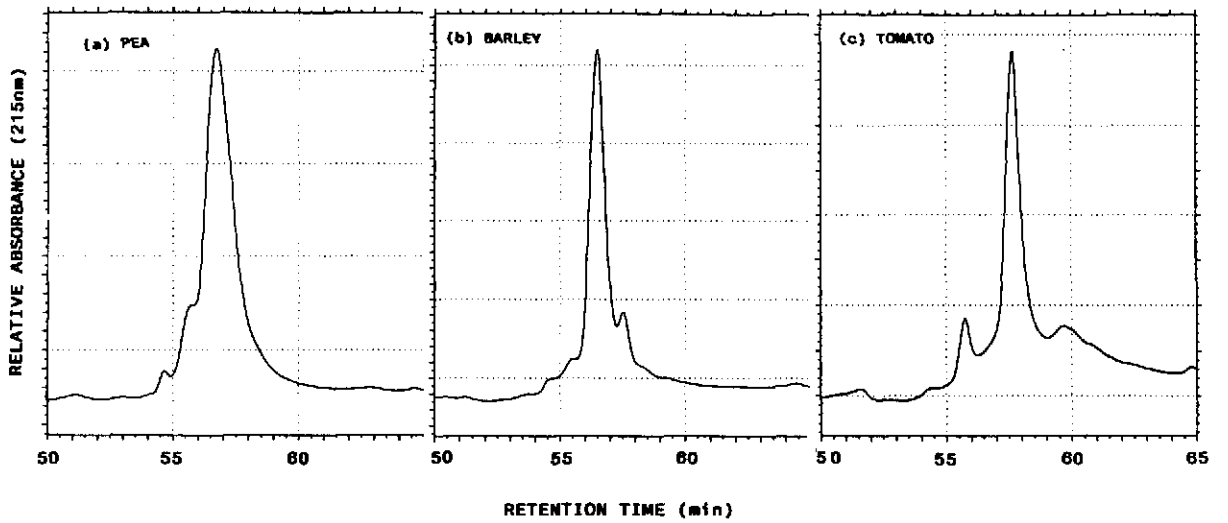

Fig. 3. HPLC separation of LHC II preparations from several plants. (a) Pea, (b) barley, (c) tomato.

peptide by analogy with spinach. The HPLC profile of tomato LHC II (Fig. 3c) is similar to that of spinach, as is its SDS-PAGE profile (Fig. 4d), although the two tomato type III bands are not resolved on HPLC the way they are on SDS-PAGE.

In order to determine if the HPLC separation method might be useful for resolving more complex mixtures of photosynthetic membrane proteins, it was challenged with a sample of a crude PS I preparation (PS I-200) (Fig. 5a) containing 17–20 polypeptides [15], and a PS II core preparation (Fig. 5b) containing 6–8 major polypeptides by SDS-PAGE [14]. There was no apparent problem with solubilization of either sample, in spite of the fact that the core proteins of both photosystems are even more hydrophobic than the LHC II proteins [8]. These results show that a large number of membrane proteins of different sizes and hydrophobicities can be separated by this method.

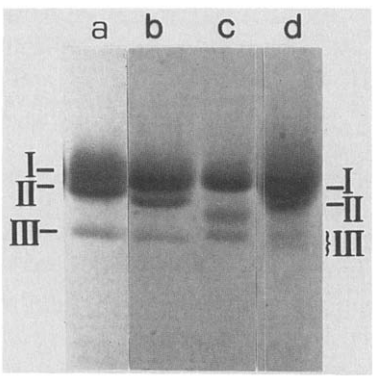

Fig. 4. SDS-PAGE separation of LHC II preparations from several plants. (a) Barley, (b) spinach, (c) pea, (d) tomato.

Over 200 injections of 50–100 μg protein on the same column over a period of months gave reproducible peak patterns. This included several large-scale preparations of LHC II, all of which gave the same retention times for the three polypeptides. There was rarely an indication of protein adsorbed to the separating column and no evidence for specific loss of any LHC II polypeptide. Occasionally some protein would be removed from the guard column after cleaning with acetonitrile or methanol. Although most trial separations involved variations of the water–acetonitrile gradient program, it was found that the addition of as little as 5% propanol to the acetonitrile caused a large shift in peak positions (data not shown). Although the presence of propanol resulted in higher back pressure in the column, it might be useful for resolving a minor protein peak migrating close to or underneath a major peak in the acetonitrile–water system.

Two other columns were tested for membrane

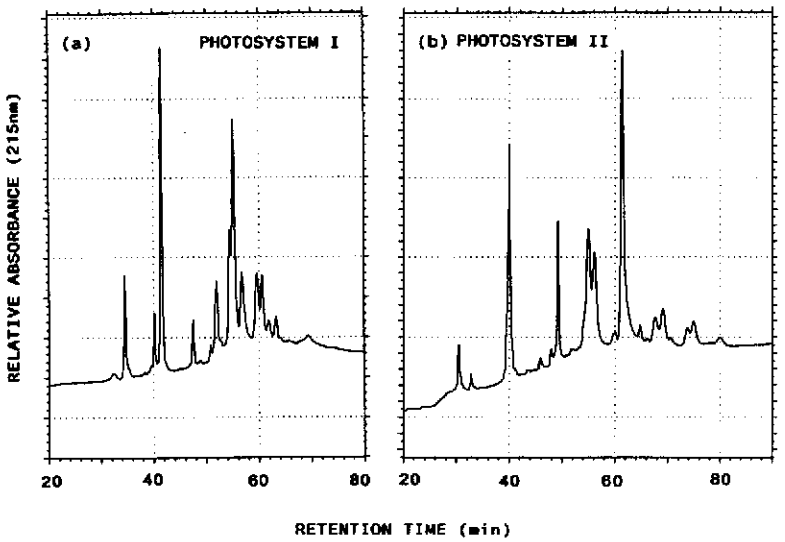

Fig. 5. HPLC separation of polypeptides from photosynthetic particles. (a) Photosystem I-200, (b) Photosystem II core particles.

protein separation. A short PRP-3 column (50 mm × 4.1 mm) with a pore size of 30 nm separated types I and II polypeptides, but no resolution of type III was obtained with any gradient conditions tried. A silica-based wide-pore RP-8 column (30 nm pore size, 10 μm particle size, 250 × 4 mm) did not give reproducible results.

4. Discussion

The HPLC separation method we report here is applicable to a wide range of photosynthetic membrane proteins. It is capable of separating LHC II proteins which have a high degree of sequence identity and differ in length by as little as four amino acids. With a preparation time of about 10 min and a running time of less than 90 min, this HPLC separation is faster than SDS-PAGE methods of comparable resolving power. The volatile buffer system means that it can be used preparatively for sequence analysis. Most importantly, however, it separates polypeptides on a different basis from SDS-PAGE. The polymeric reversed-phase column is probably more successful for separating membrane proteins because there are no silanol groups which could interact with polar groups on the protein [17,18]. Our results suggest such columns may be very useful for a wide range of membrane proteins with different molecular weights and hydrophobicities.

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