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SEPARATION OF MEMBRANE COMPONENTS BY PARTITION IN DETERGENT-CONTAINING POLYMER PHASE SYSTEMS

ISOLATION OF THE LIGHT HARVESTING CHLOROPHYLL *a/b* PROTEIN

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SUMMARY

The application of phase partition, using Triton X-100 in combination with the aqueous two-phase system dextran–polyethylene glycol, is described for the separation and isolation of the chlorophyll–protein complexes which are the main hydrophobic proteins of the chloroplast thylakoid membrane. The chlorophyll *a* protein of photosystem I is easily extracted into the upper phase while the chlorophyll *a/b* proteins of the light-harvesting complex are more resistant to extraction, thus remaining at the interface. The separation method can easily be scaled up into a preparative method. Essentially pure light-harvesting complex from spinach chloroplast could be obtained within 3 h.

INTRODUCTION

The study of the structure and function of biological membranes has been hampered by the lack of efficient methods to separate functional components of the membrane, most of which are amphipatic in nature. Integral membrane proteins are characterized by a hydrophobic domain, which interacts with the hydrophobic core of the membrane lipids, and a hydrophilic domain which is suspended in the surrounding aqueous medium. Detergents are frequently used to solubilize membrane proteins by replacing the membrane lipids and incorporating the protein molecules into detergent micelles[1]. This process is quite effective and if non-ionic detergents are used the biological activity of the proteins can often be preserved. The subsequent separation steps present many problems, however. The detergents in the mixed micelles mask the surfaces of the membrane components and thereby swamp out differences in behaviour between different solubilized components, resulting in low resolution. Often membrane components are unstable and occur at a very low concentration in tissues. In order to prepare large quantities one must be able to use relatively large scale separation methods. Standard methods such as chromatography, electrophoresis and centrifugation are, however, both time consuming and difficult to apply on a large scale.

The present article describes the application of phase partition to the isolation

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of membrane proteins, using detergents in combination with aqueous polymer two-phase systems[2–4]. The two-phase systems comprise two water-soluble polymers and water; they can be supplemented with salts and other low-molecular-weight compounds in order to optimize conditions for preservation of biological activity. These phase systems have been used for fractionation of a large number of bioparticles and biomolecules such as cells, cell organelles, membrane vesicles, enzymes and nucleic acids[3–6], both on a small scale for analytical purposes and on a large scale for preparation purposes handling several litres of phases[2,7]. The resolution obtained by one single partition step can be improved considerably by a multi-stage process such as counter-current distribution[2] or by partition chromatography[2,8]. The introduction of biospecific ligands covalently bound to one of the polymers can also be used to increase selectivity[9–11]. Partition can also be used to study interactions between biomolecules and is particularly useful for detecting weak interactions between proteins[12,13] and for binding assays[14,15].

When detergents are included in the phase system, for example, dextran–polyethylene glycol–water, they will undergo partition, depending on their chemical natures and concentrations. Below the critical micelle concentration a detergent will partition as a monomer or as small aggregates, while above this concentration the partition of the micelles is dominant. In fact, the critical micelle concentration can be determined by measuring the partition of a detergent as a function of its concentration as demonstrated for Triton X-100 in the dextran–polyethylene glycol–water system, Fig. 1[16].

The partition coefficients of detergents above the critical micelle concentration are dependent to a great extent on their hydrophilic moieties, as expected since this constitutes the exposed surface of the micelles. Thus, for example, several non-ionic detergents contain a polyethylene glycol chain as the hydrophile. Micelles containing these detergents have exposed polyethylene glycol chains on their surfaces and therefore prefer the upper polyethylene glycol rich phase of the dextran–polyethylene

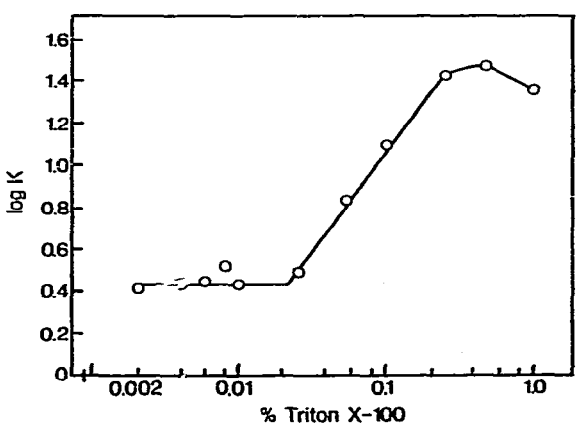

Fig. 1. Partition coefficient of Triton X-100 in a dextran–polyethylene glycol–water two-phase system as a function of Triton concentration. K = Concentration in upper phase/concentration in lower phase. The critical micelle concentration is 0.023% (w/w). Phase system: 8% (w/w) Dextran 70, 8% (w/w) PEG 6000 and 2 mM potassium phosphate buffer, pH 6.0.

glycol system. In contrast, a detergent like digitonin, which has a carbohydrate as the hydrophile, prefers the lower dextran-rich phase of the same system. Proteins which are solubilized by detergents and incorporated into micelles would partition partly as the micelle surface, and by selection of detergents one should be able to manipulate the partition of the membrane proteins.

The basis of separation by detergent-containing phase systems is the interaction between the membrane proteins and the detergents. Also, there will be a competition between the tendency of the protein to be confined in one of the phases due to interaction with the polymers and the tendency for it to be incorporated into a micelle and partition as the micelles (Fig. 2).

This approach has been used to purify a membrane-bound enzyme, phospholipase A, from *Escherichia coli*[17] and monoamine oxidase from mitochondria[18]. In the present article we describe how successive extractions with detergent phase systems can be used to separate the chlorophyll proteins of the chloroplast thylakoid membranes, and discuss further developments of this technique.

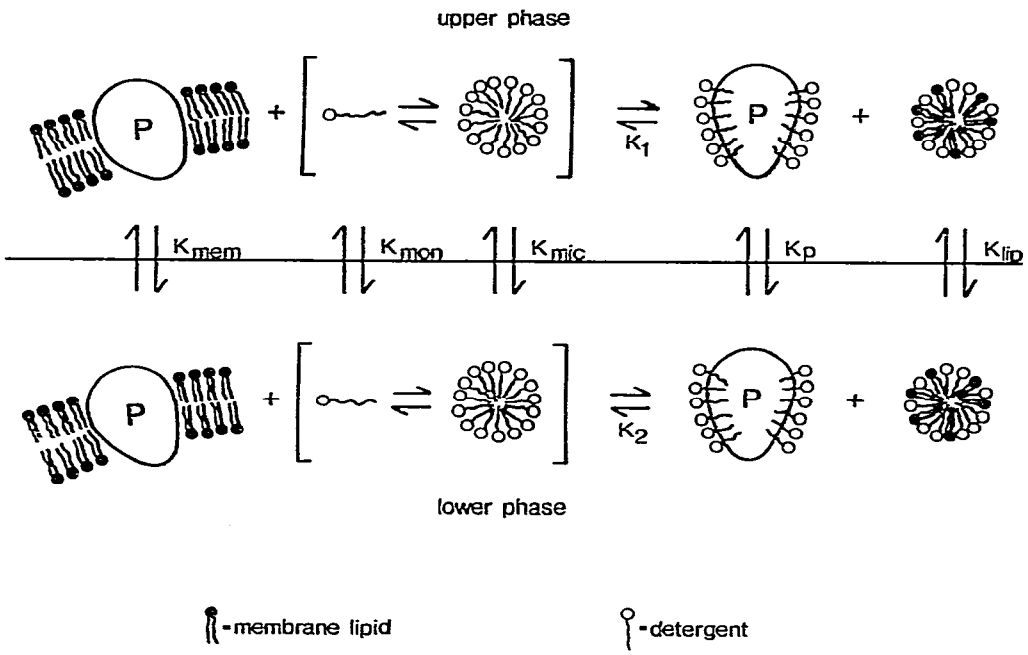

Fig. 2. Highly simplified model of different equilibria when membrane and detergent are partitioned in a two-phase system. P = Intrinsic membrane protein. K_{mem}, K_{mic}, K_{mon} and K_{lip} are partition coefficients for the membranes, detergent monomer, detergent micelle, solubilized protein and detergent–lipid comicelles respectively; K_1 and K_2 are apparent equilibrium constants for the solubilization processes in the two phases.

MATERIALS AND METHODS

Preparation of chloroplast thylakoid membranes

Spinach leaves (25 g) were blended in a knife homogenizer in a medium comprising 50 m*M* sodium phosphate buffer, pH 7.4, 5 m*M* $MgCl_2$ and 500 m*M* sucrose.

The brie was filtered through a nylon net and the fluid was centrifuged at 1000 *g* for 1 min. The pellet was resuspended in the same medium and centrifuged at the same speed for 10 min. It was then suspended in 50 m*M* sodium phosphate buffer, pH 7.4, 5 m*M* $MgCl_2$ and 50 m*M* sucrose and washed three times at 2000 *g* for 5 min. This treatment removes the envelope and most stromal protein from the chloroplast thylakoid membrane. The final pellet was suspended in distilled water immediately before addition to the polymer mixture.

Phase systems and polymer solutions

A 4-g phase system containing 7% (w/w) dextran 500, 4.4% (w/w) polyethylene glycol 6000 (PEG 6000), 10 m*M* sodium phosphate buffer, pH 6.8, and 100 m*M* NaCl was prepared by mixing 1.40 g 20% (w/w) dextran 500, 0.44 g 40% (w/w) PEG 6000, 0.4 ml 0.1 *M* sodium phosphate buffer, pH 6.8 (equimolar concentrations of mono- and disodium phosphate), and 0.4 ml 1.0 *M* NaCl and made up to 3.00 g with double distilled water. Finally, 1 g of sample (2.5 mg chlorophyll) suspended in water was added to yield the 4-g system. The polymer composition of the upper phase is essentially 7% polyethylene glycol as seen from the phase diagram[2]. An upper phase solution can therefore be obtained by mixing 17.5 g 40% (w/w) PEG 6000, 10 g 1 *M* NaCl and 5 g 0.2 *M* sodium phosphate buffer, pH 6.8, and made up to 100 g by water, yielding a composition of 7% (w/w) PEG 6000, 100 m*M* NaCl and 10 m*M* sodium phosphate buffer, pH 6.8 (solution 1). Another upper phase solution with 2% Triton X-100 (solution 2) was obtained by replacing 20 g of the water with the same amount of 10% (w/w) Triton X-100. By mixing solutions 1 and 2 in different proportions, upper phases of various Triton X-100 concentrations can be obtained.

Analytical extraction

The original 4-g system containing the chloroplast membranes was thoroughly mixed and allowed to settle, usually facilitated by a low-speed centrifugation. In this phase system without detergent the membranes partition to the interface or the lower phase. The upper phase was then removed and replaced by an equal volume of another upper phase containing a certain concentration of Triton X-100 (obtained by mixing suitable proportions of solutions 1 and 2). The two phases were again mixed and allowed to settle as described above. The upper phase now containing some green material was collected and used for analyses. The procedure was then repeated several times using an upper phase with a constant or increasing detergent concentration. The extraction procedure was continued until all material had been extracted into the upper phase.

Preparative extraction

The extraction procedure described above can easily be scaled up into a preparative extraction. Chloroplasts were prepared from 100 g of spinach leaves. A pellet of washed chloroplasts (2.5 mg chlorophyll) was suspended in 6 ml water and added to a mixture 8.4 g 20% (w/w) dextran 500, 2.64 g 40% PEG 6000, 1.20 g 0.2 *M* sodium phosphate buffer, pH 6.8, 2.40 g 1 *M* NaCl and 3.36 g water. This yields a 24-g phase system with the same composition as for the analytical extraction. After mixing, the solution was centrifuged at 3000 *g* for 5 min to facilitate phase settling. The clear upper phase was removed and replaced by 12 ml of a solution of 7% (w/w) PEG 6000 and 1% (w/w)

Triton X-100 in 0.1 *M* NaCl and 0.01 *M* sodium phosphate buffer, pH 6.8 (1:1 mixture of solutions 1 and 2). After mixing and phase separation, the upper phase was removed and collected. The same procedure was repeated six times. After each extraction step the chlorophyll *a*/*b* ratio was estimated in order to check that the extraction follows the profile from the analytical procedure (Fig. 4b).

After the seventh extraction 12 g of solution 1 were added. After mixing and separation, the upper phase was removed and the same extraction repeated once. These final steps were performed in order to remove excess of unbound Triton X-100. After the last partition cycle the remaining thylakoid material collects as a thick layer at the interface. The upper and lower phases were removed by pipette and discarded. The remaining interfacial material was diluted with 6 ml water and centrifuged at 10,000 *g* for 10 min. The supernatant was discarded and the pellet washed with another 6 ml water. The supernatant, which may be pale green, was removed and discarded. The pellet was suspended in 0.03–0.1 % Triton X-100 and centrifuged at 10,000 *g* for 10 min. (Various amounts of Triton X-100 are needed to solubilize the pellet, probably because different amounts of Triton are bound to the interfaced material after the extractions.) A white-grey pellet was obtained. The supernatant was recovered and made 0.1 *M* with respect to $MgCl_2$. This precipitated most of the green material which was collected by centrifugation.

Chlorophyll determination

Chlorophyll was usually determined in acetone according to the method of Arnon[19]. For quick estimation of chlorophyll *a*/*b* ratios of the upper phases obtained in the preparative procedure the absorbances at 675 and 650 nm in 1 % Triton were used. The ratio is then obtained from a standard curve as shown in Fig. 3.

Gel electrophoresis

Two types of sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis (PAGE) were used for analyses of the material after the extractions. The relative content of chlorophyll-protein complexes was determined by the method of Ander-

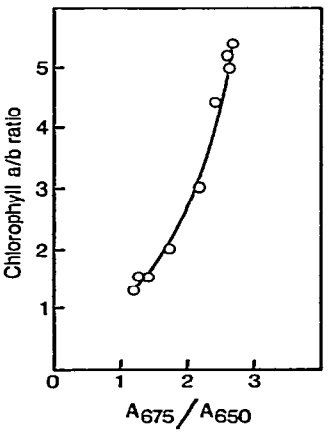

Fig. 3. Plot of absorbance ratio, A_{675}/A_{650}, measured for chloroplast membranes in 1 % Triton against chlorophyll *a*/*b* ratio as determined by Arnon[19]. The different fractions were obtained from a procedure like that described in Fig. 4b.

son[20]. The chloroplast material was solubilized at 4°C in 0.15 *M* Tris–HCl (pH 8.8) containing 13% glycerol and 0.5–1% SDS to give a SDS/chl weight ratio of 10:1–20:1. The lower SDS concentration was used for the starting chloroplast material. Tube gels were employed with a stacking gel containing 4% acrylamide, 56 m*M* Tris–SO_4 (pH 6.14) and 0.1% SDS and a resolving gel containing 8% acrylamide and 0.43 *M* Tris–HCl (pH 9.35). The upper reservoir buffer (pH 8.64) was 41 *M* Tris adjusted with boric acid and 0.1% SDS. The lower reservoir buffer (pH 9.35) was 0.43 *M* Tris adjusted with HCl. Electrophoresis at 4°C was carried out for 2 h at 2 mA per gel. The relative distribution of chlorophyll on the gels was estimated by scanning unstained gels at 675 and 650 nm.

For polypeptide analyses the polyacrylamide electrophoresis system of Laemmli[21] was used. A polyacrylamide gradient slab (10–16%) was used. The chloroplast material and standards were solubilized in Laemmli's solubilizing buffer containing 0.25% (v/v) mercaptoethanol and heated at 75°C for 3 min. A current of 10 mA was applied to each gel.

The gels were stained in Coomasie brilliant blue (0.1%, w/v) in methanol–acetic acid–water (50:7:43, v/v/v). Apparent molecular weights were obtained by comparison with known standards: bovine serum albumin (68,000): ovalbumin (45,000); chymotrypsinogen (25,000); trypsin inhibitor (21,000) and myoglobulin (14,300).

RESULTS

When thylakoid membranes are partitioned in dextran–polyethylene glycol containing 0.1 *M* NaCl they are found at the interface and the lower phase. The polyethylene glycol rich upper phase is non-turbid and colourless. If this is removed and successively replaced by new upper phases containing increasing amounts of Triton X-100, thylakoid material will be extracted into the upper phase. In the first extractions (1–5) only very little chlorophyll-containing material is extracted (Fig. 4a). Increasing amounts are found in the upper phase after extractions 6–11, followed by a decline to extraction 15. The amount of material then increases again and reaches a peak after extraction 21. The material has thus been divided into two main fractions. That between extractions 1 and 12 has a high chlorophyll *a*/*b* ratio, while the other between extractions 16 and 22 has a chlorophyll *a*/*b* ratio of 1–1.2. This suggests a separation of chlorophyll proteins of the chloroplast thylakoids.

Chloroplasts of higher plants exhibit three main chlorophyll–protein complexes which are the main intrinsic proteins of the thylakoid membrane[20]. Two of these, the reaction centre complexes of photosystems I and II, are chlorophyll *a* proteins, and one, the light-harvesting complex, is a chlorophyll *a*/*b* protein. The different chlorophyll *a*/*b* ratios of the various fractions after the extractions suggest a selective release of the chlorophyll–protein complexes from the membrane. The analyses with the SDS–PAGE technique, which allows the chlorophyll to remain protein bound, confirmed this assumption. Fig. 5a shows the chlorophyll–protein complexes of chloroplast before fractionation. Two major chlorophyll *a* bands can be seen, one associated with photosystem I (CPI) and the other assumed to be associated with photosystem II (CPa). Three major chl *a*/*b* bands which are all due to multiple forms of the light-harvesting complex (LHCP)[20]. Some minor bands of low electrophoretic

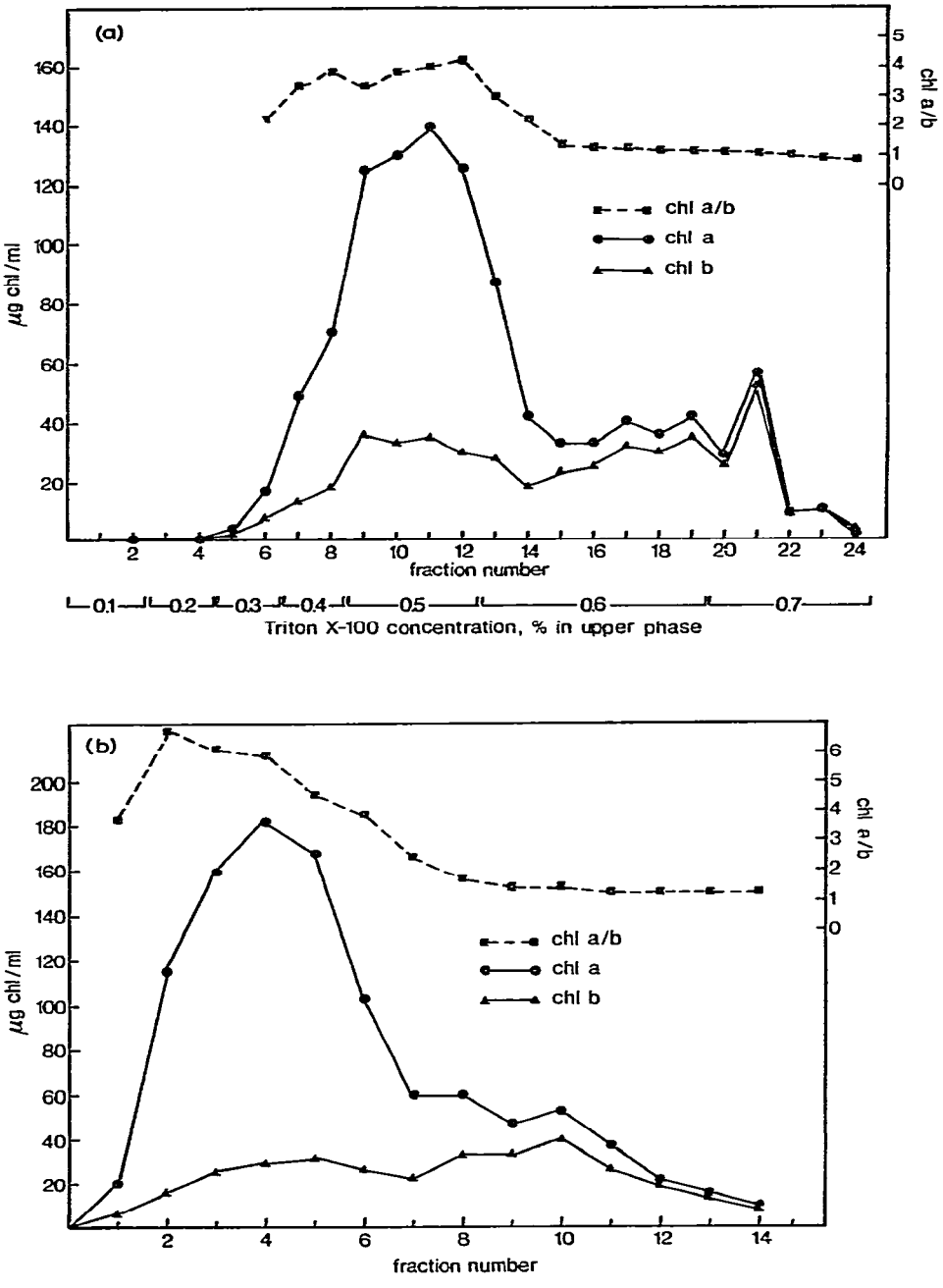

Fig. 4. Analytical extraction of chloroplast thylakoids in an aqueous polymer phase system. Extractions were performed repeatedly with top phases of (a) increasing concentrations of Triton X-100 (0.1–2%) or (b) a constant concentration of Triton X-100 (1%).

mobility can also be detected and are probably multiple forms of the light-harvesting complex[22] and of the CPI complex[20]. SDS–PAGE analyses revealed a high enrichment of the CPI complex in the left peak while the peak to the right only showed the chlorophyll *a*/*b* protein of the light-harvesting complex.

The extraction procedure can be changed in an arbitrary manner and different profiles are obtained if the detergent concentration of the upper phase is changed. Fig. 4b shows a profile when the Triton concentration was maintained constant at 1 %. Here, also, the material is separated into two main parts, one enriched in CPI and the other in LHCP. By analysing several extraction profiles where different concentrations of Triton have been used it can be concluded that it is not the absolute concentration of the detergent which is the deciding factor, but rather the ratio between the sum of the amounts of detergent used in previous extractions and the original amount of membrane material. This means that a more efficient extraction can be obtained by increasing the upper phase volume without changing the detergent concentration.

Preparative isolation of light-harvesting chlorophyll protein

From the extraction profile in Fig. 4b it can be seen that after seven extractions with an upper phase containing 1 % Triton most of the chlorophyll *a* protein has been removed and the remaining material has a chlorophyll *a*/*b* ratio of 1–1.2. Gel elec-

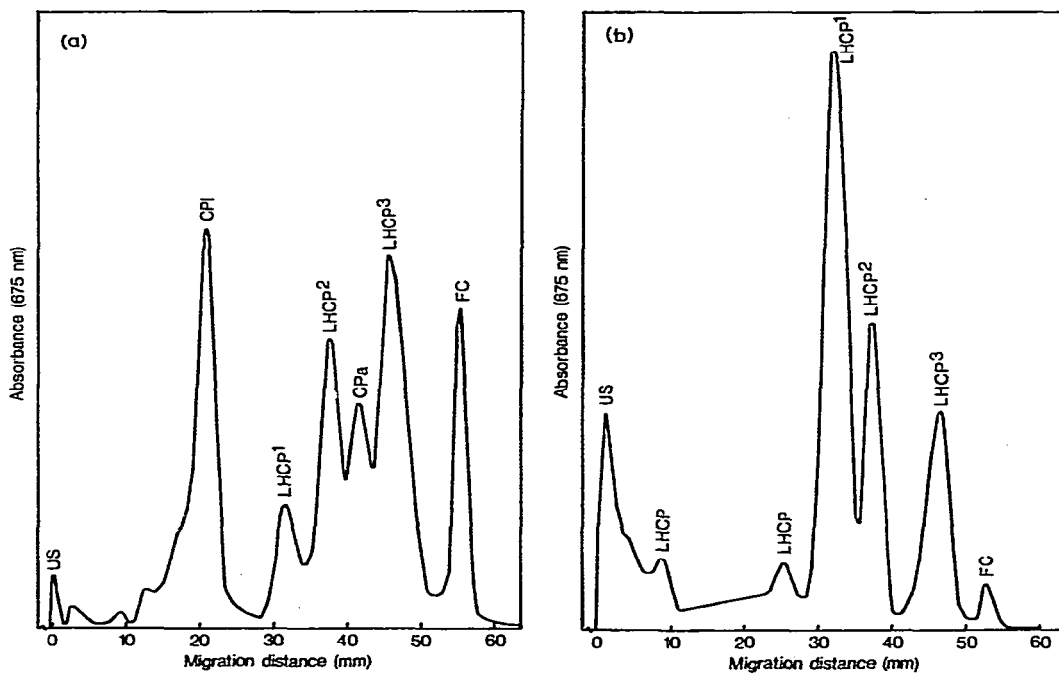

Fig. 5. The relative distribution of chlorophyll–protein complexes resolved by SDS–PAGE at 4°C. Unstained gels were scanned at 675 nm. a, Spinach chloroplasts; b, material isolated by the preparative extraction procedure. Note the absence of the two chlorophyll *a* bands of CPI and CPa. CPI = Photosystem I chlorophyll *a*–protein complex. CPa = photosystem II chlorophyll *a*–protein complex; LHCP = light-harvesting complex; FC = free chlorophyll; US = unsolibilized material.

trophoresis showed that this remaining fraction consisted mainly of light-harvesting chlorophyll–protein complex. In order to purify this protein we therefore stopped the extraction with Triton in the upper phase and after the seventh extraction an upper phase without Triton was used. Thereby any Triton which was not firmly bound to the remaining protein was removed by the subsequent two extractions by upper phase without Triton. After this procedure the light-harvesting chlorophyll *a/b* protein present at the interface can easily be recovered after dilution and centrifugation. The polymers are removed by repeated washings.

The chlorophyll–protein complexes of this material are shown in Fig. 5b. The dominant bands are the three chlorophyll *a/b* bands of the light-harvesting complex. The absence of the two main chlorophyll *a* bands (CPa and CPI) demonstrates their selective release from the membrane by the extraction procedure. There are also two minor chlorophyll *a/b* bands which probably belong to multiple forms of the light-harvesting complex with a higher aggregation number compared to LHCP[1].

Triton X-100 enhances the formation of LHCP[1] (ref. 23) and this explains why LHCP[1] is the main chlorophyll *a/b* band in contrast to the starting chloroplasts where LHCP[3] is the dominant chlorophyll *a/b* band. The extracted material is more difficult to solubilize and even a SDS/chlorophyll ratio of 20 gives some unsolubilized material remaining at the start of the gel. This material shows a similar chlorophyll *a/b* ratio compared to the other bands, which in combination with the low amount of free chlorophyll excludes the possibility of hidden chlorophyll *a* bands.

The results of these electrophoretic analyses lead to the isolation of the light-harvesting complex without contamination by other chlorophyll–protein complexes. However, a contamination by non-pigmented proteins cannot be excluded. Therefore, electrophoresis under more denaturating conditions was done followed by straining with Coomasie brilliant blue. Fig. 6 shows that the material from the pre-

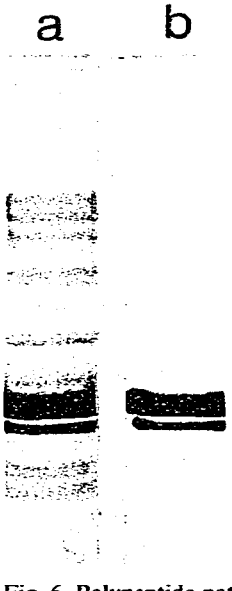

Fig. 6. Polypeptide pattern of chloroplast thylakoids (a) and the material isolated by the preparative extraction procedure with Triton X-100 in an aqueous polymer phase system (b).

parative extraction procedure exhibits only the 25 and 23 kD polypeptides typical of the light-harvesting complex[20]. Even after overloading, only traces of other thylakoid proteins were detected. Thus, the preparative extraction results in a pure light-harvesting chlorophyll *a/b* protein.

DISCUSSION

The selective extraction of membrane components in detergent-containing phase systems is probably influenced by several factors. Fig. 2 shows a simplified model of possible equilibria in a mixture of an intrinsic membrane protein and a detergent in a two-phase system. The detergent monomers are in equilibrium with its micelles; the protein and membrane lipids in the membrane react with the detergent to form complexes between the protein and the detergent and mixed micelles of lipids and detergent. Each species of molecules is involved in a partition equilibrium. For simplicity, membranes adsorbed at the interface are not illustrasted but they should also be considered. The final result will depend on all the equilibria involved.

By choosing suitable salts in the phase system we can manipulate the partition of the membrane vesicles. Thus, by using 0.1 *M* NaCl as the dominating salt, as in the experiments above, the membrane partitions into the lower phase or at the interface, *i.e.*, $K_{mem} = 0$. With a detergent like Triton X-100, which has polyethylene glycol as the hydrophile, the micelles of the detergent prefer the upper phase, *i.e.*, K_{mic} is large, and consequently the partition coefficient of the solubilized protein, K_p, will also be large. For different membrane proteins the apparent partition will therefore depend on both the equilibrium constant, K_2, and the partition coefficient, K_p. In the present experiments the chlorophyll protein CPI is extracted before the chlorophyll protein LHCP. Whether this is due to differences mainly in K_2 or K_p is difficult to judge from the experiments made so far. It is likely, however, that K_2 is of major importance since LHCP, which is localized mainly in the partition region[24], may partly be protected against detergent action.

A particular advantage of partition methods is that they can easily be scaled up. This is also demonstrated by the procedure used here. Only low-speed centrifugation is used to facilitate separation of the phases; we could therefore easily separate chlorophyll–protein complexes from several hundred grams of leaf material.

The method is also rapid, the preparation of light-harvesting chlorophyll protein from chloroplasts can be completed within 3 h. This compares favourably with other methods which require longer times and also involve extensive ultracentrifugations[25]. This gain in time is achieved because the solubilization and separation steps occur simultaneously. The separation of the solubilized proteins from the residual material is caused by the phase partition which occurs in a matter of seconds, and the bulk settling of the phases is completed after a few minutes of low speed centrifugation.

A general problem with membrane protein isolation is the removal of the detergent after separation. Centrifugation or adsorption on detergent adsorbing beads has frequently been used. In the present procedure for isolation of LHCP, however, this problem does not arise because the detergent is removed in the final extractions with upper phase without detergent leaving the LHCP at the interface or in the bottom phase.

Future developments should involve the application of this principle to other detergents. By employing two different detergents, one preferring the upper phase and the other the lower phase, an increased selectivity may be obtained, depending on the nature of interaction between the detergents and the membrane components. Also, it would be of interest to use detergents with a K value of 1. The complexes with proteins may partition more according to the nature of the exposed protein surfaces than to the detergent. Thus, by employing an excess of detergent the partition coefficient, K_p, in Fig. 2 would govern the partition behaviour of proteins. In both approaches it would be of interest to apply multistage procedures such as countercurrent distribution and partition chromatography to improve resolution.

The present paper describes a complete purification of the chlorophyll *a/b* protein of the light-harvesting complex. It would be of interest to try to separate the two chlorophyll *a* proteins CPI and CPa in order to develop a preparative procedure for the presumptive photosystem II chlorophyll *a* protein, CPa. The method could also be applied for the isolation of hydrophobic proteins of other biological membranes.

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