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FRACTIONATION OF THYLAKOID MEMBRANE COMPONENTS BY EXTRACTION IN AQUEOUS POLYMER TWO-PHASE SYSTEMS CONTAINING DETERGENT

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SUMMARY

A combination of detergent extraction and aqueous polymer two-phase partition has been used to fractionate thylakoid membrane components. The membranes were initially forced to partition to the lower phase, and subsequently, on solubilization, the components were selectively extracted to the upper phase by a stepwise increase in the detergent concentration. The detergents partitioned either strongly to the upper phase (Triton X-100) or almost equally between the phases (octyl glucoside). By this simple method polypeptide complexes could be purified several-fold in just a few rapid steps.

INTRODUCTION

Isolation of membrane components is essential for the investigation of their structure and function. The shortage of efficient methods for the separation of intrinsic components is a great obstacle in bioscience. Integral proteins spanning the membrane consist of a hydrophobic part, which interacts with the inner hydrophobic part of the membrane, and hydrophilic domains, which are in contact with the surrounding aqueous medium. The use of detergents in membrane solubilization is often quite effective and, if non-denaturing detergents are used, the biological activity of the components may often be preserved. However, the subsequent separation steps present many problems. The detergent in the micelles formed on solubilization, which also contain lipids and proteins from the membrane (mixed micelles), conceals the membrane components and makes their isolation difficult.

Recently, however, a new method was presented in which the membrane to be fractionated was added to a polymer two-phase system containing the detergent Triton X-100 at low concentrations[1]. The phase system was adjusted so that the membranes partitioned to the interface and the lower phase while the components solubilized by the detergent partitioned to the upper phase. In this way the solubilization and separation could be achieved simultaneously. The method was used to isolate and purify the light harvesting chlorophyll *a*/*b* protein (LHC-II) of photosystem II (PS II) from spinach chloroplasts.

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The aim of this work was to investigate further the potential of this technique. Chloroplast thylakoid membrane were selected for study, and Triton X-100 and octyl glucoside (octyl-β-D-glucopyranoside) were used for the solubilization. The results show that a selective fractionation of membrane components, which differs for the two detergents, can be achieved.

MATERIALS AND METHODS

Polyethylene glycol 6000 (PEG 6000), now renamed PEG 8000, was obtained from Union Carbide, New York, NY, U.S.A. Dextran T-500, batch No. GI 21917, was supplied by Pharmacia, Uppsala, Sweden. ^{14}C-labelled octyl glucoside was a generous gift from Lennart Krabisch Department of Medical and Physiological Chemistry.

Thylakoid membranes were isolated as described previously[3]. Photosystem II enriched inside-out thylakoid membranes originating from the thylakoid grana region, and photosystem I enriched right side-out thylakoids originating from the stroma region, were isolated by a combination of mechanical disintegration and phase partition as described previously[4]. In order to collect the material after the phase partition, the phases were diluted two-fold with 10 m*M* magnesium chloride. Under these conditions the material sediments after centrifugation for 15 min at 10 000 *g*.

Single-tube gradient extraction

An 8-g phase system containing 7% (w/w) Dextran T-500, 4.4% (w/w) PEG 6000, 100 m*M* sucrose, 10 m*M* sodium phosphate buffer (pH 6.8), 100 m*M* sodium chloride and 2 m*M* magnesium chloride was prepared by mixing 2.80 g of 20% (w/w) Dextran T-500, 0.88 g of 40% (w/w) PEG 6000, 0.6 g of 1 *M* sucrose, 0.3 g of 0.2 *M* sodium phosphate buffer (pH 6.8), 0.8 g of 1.0 *M* sodium chloride, 60 μl of 0.1 *M* magnesium chloride and made up to 6.00 g with double-distilled water. Finally, 2 g of sample (5.0 mg chlorophyll) suspended in buffer (100 m*M* sucrose, 10 m*M* sodium phosphate buffer, (pH 6.8) and 5 m*M* magnesium chloride) was added to yield the 8-g system. The polymer composition of the upper phase was essentially 7% PEG 6000. An upper phase solution could therefore be obtained by mixing 17.5 g of 40% (w/w) PEG 6000, 10 g of 1.0 *M* sucrose, 5 g of 0.2 *M* sodium phosphate buffer (pH 6.8), 10 g of 1.0 *M* sodium chloride, 137 μl of 1.46 *M* magnesium chloride and made up to 100 g with double-distilled water, yielding a composition of 7% (w/w) PEG 6000, 100 m*M* sucrose, 10 m*M* sodium phosphate buffer (pH 6.8), 100 m*M* sodium chloride and 2 m*M* magnesium chloride (solution 1). Another upper phase solution with 2% (w/w) of either Triton X-100 or octyl glucoside (solution 2) was obtained by replacing 20 g of the water with the same amount of 10% (w/w) Triton X-100 or octyl glucoside. By mixing solutions 1 and 2 in different proportions, systems of various detergent concentrations could be obtained.

The original 8-g system containing the chloroplast membranes was thoroughly mixed, and the phases were settled by low speed centrifugation (1500 *g*). In this phase system, without detergent, the membranes partition to the interface or the lower phase. The upper phase was removed and a new upper phase with detergent, as described above, was added. The two phases were mixed and settled again by centrifugation. The upper phase, now containing some green material, was collected and

analysed. The procedure was then repeated several times using an upper phase with constant or increasing detergent concentration.

Counter-current distribution

For multiple extractions at a fixed detergent concentration an apparatus for counter-current distribution in a centrifugal acceleration field[5] was used. The purpose of the centrifugation is only to facilitate the settling of the phases. The centrifugation time was 70 sec at 80 *g*, the mixing time 30 sec, and 55 transfers were made. The lower phase, the interface and 10% of the upper phase were kept stationary. The polymer composition was the same as for the extraction described above.

Chlorophyll determination

Chlorophyll was either determined according to Arnon[6] or estimated from the absorbances at 670 nm and 650 nm[1].

CM-Sepharose chromatography

In order to eliminate Dextran and PEG from the samples and to concentrate the material, the pH was adjusted to 4 and the material adsorbed on a CM-Sepharose column (1 cm × 1 cm), equilibrated with 5 m*M* sodium acetate buffer (pH 4). Unbound material was washed off with 3–5 column volumes of 5 m*M* sodium acetate buffer (pH 4). The proteins were eluted with a buffer comprising 0.125 *M* Tris–HCl (pH 6.8), 4% SDS and 20% glycerol.

Gel electrophoresis

Polypeptide analysis was performed by polyacrylamide gel electrophoresis according to the method of Laemmli[7], using a 12–22% polyacrylamide gradient slab. The eluted samples were made 5% with respect to mercaptoethanol, and kept at 75°C for 3 min. The gels were stained with Coomassie brilliant blue and scanned with an Ultroscan laser densitometer (LKB); the areas under the peaks were measured.

Partitioning of octyl glucoside

The partitioning of octyl glucoside was determined by using ^{14}C-labelled octyl glucoside. Phase systems with the same composition as the one used for single-tube extractions were prepared with octyl glucoside in the concentration range 0.07–1.5%. Samples from the upper and lower phases were analysed by liquid scintillation counting.

RESULTS

Partition of thylakoid membranes in the PEG–Dextran system depends, among other factors, on the ionic composition. Excess sodium chloride was included to force the thylakoids into the lower phase. If the upper phase is removed and successively replaced by new upper phases with the same salt concentration containing constant or increasing amounts of Triton X-100, thylakoid material is solubilized. The solubilized material will have partition properties that are dependent on the nature of the membrane component and the detergent used[1].

Octyl glucoside was found to partition almost equally between the phases in

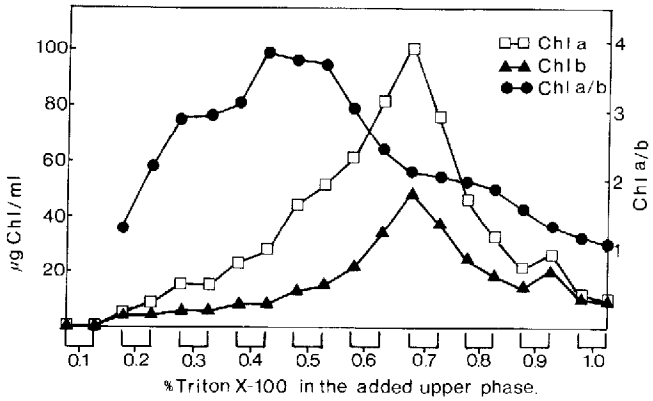


Fig. 1. Gradient extraction of chloroplast thylakoids in an aqueous polymer two-phase system with Triton X-100. Extractions were performed repeatedly with two extractions at each concentration of Triton X-100. Pooled fractions at 0.1, 0.3, 0.5, 0.8, and 1.0% Triton X-100 were analysed by gel electrophoresis (see Fig. 2).

the concentration range 0.07–1.5%. Triton X-100 has previously[1] been showed to partition strongly to the upper phase above CMC (0.025%) with a partition coefficient of 8 at 0.05% Triton X-100 and 25 at 0.4% Triton X-100.

Analysis of the chlorophyll distribution after an extraction using Triton X-100 is shown in Fig. 1. At low concentrations of Triton X-100 the chlorophyll *a*/*b* ratio rises to *ca.* 4 and then at higher concentrations it drops to *ca.* 1. These results are similar to those previously obtained by Andersson and Albertsson[1].

Subthylakoid material originating either from the grana stacks or from the stroma lamellae region were also tested. The extraction profiles (not shown) of these two fractions did not differ greatly. However, both fractions were more easily extracted than thylakoids. Mechanically disrupted thylakoids were also more easily extracted. This indicates that the vesicular size is an important factor in the solubilization process.

In an attempt to improve the fractionation, multistep extractions at constant detergent concentration were performed. Counter-current distribution was used for this experiment. After optimization of the system, it was found that a Triton X-100 concentration of 0.7% in the upper phase gave the best fractionation with respect to the chlorophyll distribution when 4 mg of chlorophyll (thylakoid membranes) were applied to the counter-current distribution (Fig. 3). Subthylakoid vesicles were also examined by the counter-current procedure, and the results confirmed those obtained from the single-tube extractions (not shown).

The fractions were analysed by electrophoresis. Prior to electrophoresis the samples were concentrated and separated from the polymers because these changed the mobility of the polypeptides. This could be achieved by adsorption of the proteins on an ion-exchange column, followed by elution in a small volume. There was no noticeable difference in polypeptide pattern or migration between a control sample (without polymer) and a sample in which the polymers had been removed by passing it through a column (Fig. 4, slots a and b).

There were distinct differences between the polypeptide patterns of the frac-

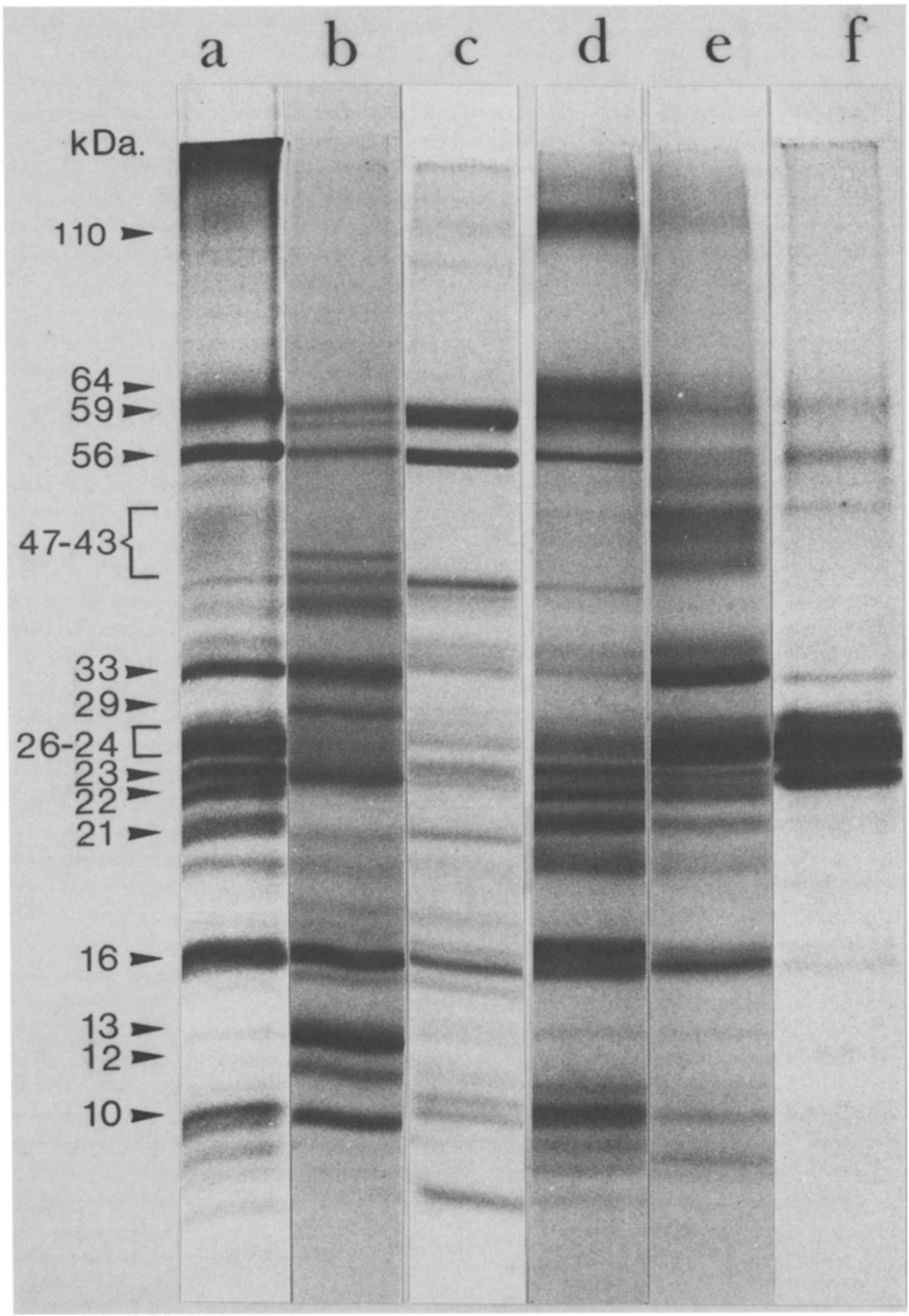
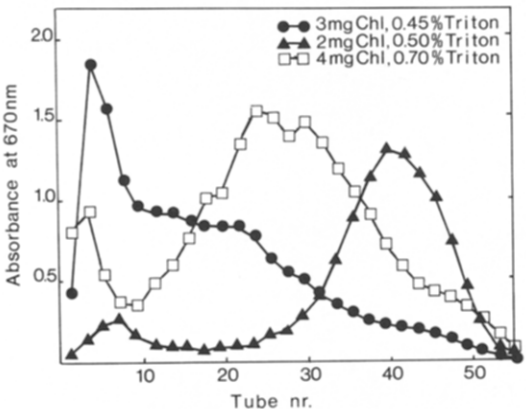


Fig. 2. Polypeptide pattern of thylakoids and of different fractions obtained by the gradient extraction of thylakoids with increasing amounts of Triton X-100 in the added upper phase as shown in Fig. 1. Slots: a = Thylakoid membranes; b = 0.1%; c = 0.3%, d = 0.5%, e = 0.8%, and f = 1.0%, Triton X-100 fractions in Fig. 1.



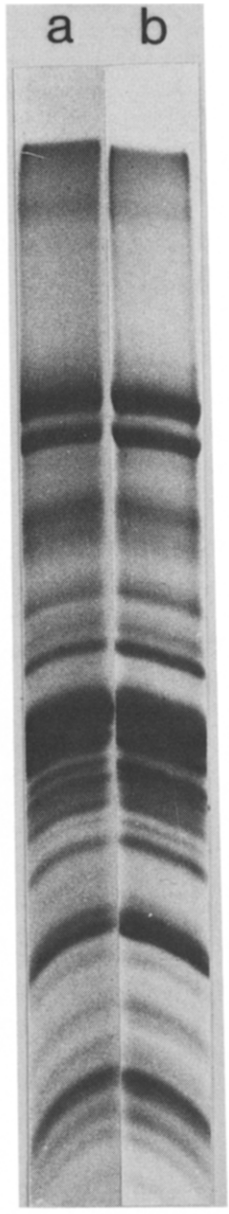


Fig. 3. Counter-current distribution of chloroplast thylakoids with three different Chl to Triton X-100 ratios (55 transfers).

Fig. 4. Polypeptide pattern of chloroplast thylakoids prior to (a) and after (b) CM-sepharose column.

tions from thylakoids extracted with increasing levals of Triton X-100 (Fig. 2, slots b–f). The fraction obtained at 0.1% Triton X-100 (the percentage values refer to the amount of detergent in the added upper phase; (Fig. 2, slot b), contained three prominent bands (12, 13 and 29 kDa) which were enriched some hundred times compared

with the starting material (Fig. 2, slot a). Two of these polypeptides with apparent molecular weights of 12 kDa and 13 kDa may correspond to ATP-ase subunits chloroplast factor 1 (CF_1) and chloroplast factor 0 (CF_0), but the identity of the third polypeptide of 29 kDa is not known. A number of other polypeptides were also enriched in this fraction, *e.g.* the 33, 23 and 16 kDa polypeptides that are presumed to play a role in photosynthetic oxygen evolution[10]. The identity of these polypeptides was inferred from co-electrophoresis with purified proteins (not shown). Virtually no chlorophyll-containing polypeptides were present in this fraction.

The first fraction seen containing chloropohyll was obtained at 0.3% Triton X-100. The two predominant polypeptides in this fraction, at 59 and 56 kDa, correspond to $CF_1\alpha$ and $CF_1\beta$ (Fig. 2, slot c). The 33, 23 and 16 kDa polypeptides enriched in the 0.1% Triton fraction were now depleted.

The fraction obtained at 0.5% Triton X-100 (Fig. 2, slot d) showed two polypeptides, one at 110 kDa corresponding to chlorophyll protein 1 (CP1) and one at 64 kDa corresponding to apo-CP1, both associated with photosystem 1. This is consistent with the high chlorophyll *a*/*b* ratio found in this fraction (see Fig. 1). Two polypeptides in the 21 and 22 kDa region were also strongly enriched. Polypeptides were once again seen in the 23 and 16 kDa region.

The 0.8% Triton X-100 fraction showed a clear enrichment of photosystem II components, such as the reaction centre (47–43 kDa) and LHC-II (26–24 kDa). This is consistent with the low chlorophyll *a*/*b* ratio found in this fraction (Fig. 1). There is also a polypeptide enriched in the 33 kDa region.

In the last fraction (1.0% Triton, Fig. 2, slot f) the most pronounced polypeptides were LHC-II, but there were also two other polypeptides of 27 and 23 kDa.

Interestingly, polypeptides in the region of 33, 23, 16 and 10 kDa, found enriched in the chlorophyll-free fraction, and depleted in the first chlorophyll-containing fraction, reappeared at higher Triton X-100 concentrations. This may indicate that there are two or more polypeptides with these molecular weights.

The polypeptide bands were quantified by gel-scanning in each fraction. Polypeptides of $CF_1\beta$, LHC-II and an unknown 20 kDa polypeptide demonstrate the separation that can be obtained. These polypeptides show similar, well separated maxima along the extraction profile (Fig. 5).

A gradient extraction of thylakoids with octyl glucoside is shown in Fig. 6. In contrast to the Triton X-100 extraction the material rich in chlorophyll *b* is extracted first. In addition, the polypeptide patterns show distinct differences. In the second fraction, at 1.0% octyl glucoside in the added upper phase (Fig. 7, slot b), the LHC-II bands (24–26 kDa) were the most prominent. A 16 kDa polypeptide also shows a pronounced enrichment in this fraction. LHC-II reaches its maximum at 1.3% (Fig. 7, slot d), although these polypeptides were rather dominating throughout the extraction.

Another difference, compared with the Triton X-100 extraction, was that only *ca.* 50% of the material could be extracted to the upper phase even when high concentrations were used (2.0%). The residue in the bottom phase (Fig. 7, slot e) showed pronounced enrichment of CP1 (64 kDa). Note the similarity in polypeptide pattern between this fraction and the one obtained with 0.5% Triton X-100 (Fig. 2, slot d). Although the general extraction pattern differed between the two detergents used, the same polypeptides dominated in the non-green fractions, which were obtained at

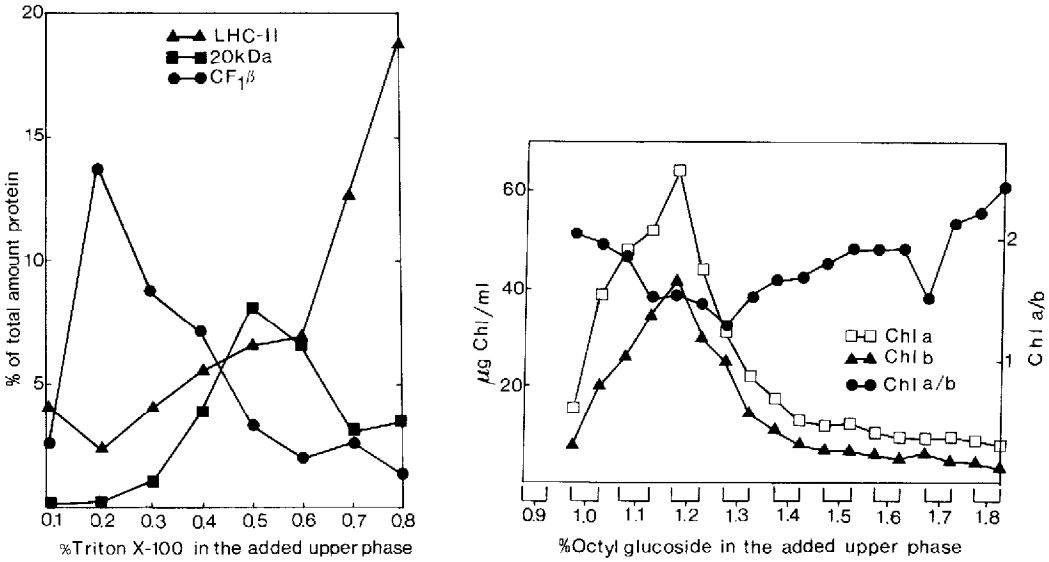


Fig. 5. Proportions of three polypeptides, CF1β, LHC-II and an unknown 20 kDa polypeptide, in each fraction of a gradient extraction experiment like that of Fig. 1. Values are related to the total amount of protein in each fraction.

Fig. 6. Gradient extraction of chloroplast thylakoids in an aqueous polymer two-phase system with octyl glucoside. Extractions were performed repeatedly with two extractions at each concentration of octyl glucoside. Fractions obtained at 0.9, 1.0, 1.2 and 1.3% octyl glucoside, and the residue in the lower phase, were pooled for gel electrophoresis (see Fig. 7).

the lowest concentration of both detergents. Some of these polypeptides originate from the inner side of the thylakoid membrane[8]. This suggests that the detergent destabilizes the membrane prior to its solubilization, so that internally located proteins can leak out. The ready extraction of these polypeptides in a medium containing 100 m*M* sodium chloride is consistent with their electrostatic binding to the membrane[10].

DISCUSSION

The results presented show that a stepwise and selective fractionation of the proteins from the thylakoid membrane can be obtained by combining detergent solubilization of the membrane with partition in an aqueous two-phase system. When Triton X-100 was used, apparently previously undetected polypeptides came to the fore. Our findings demonstrate that groups of polypeptides seem to be extracted together independent of the detergent used (compare Fig. 2, slot d with Fig. 7, slot e).

These results suggest that the polypeptides in each group constitute complexes or are in close priximity in the native membrane. This is in agreement with the results reported by several investigators who have shown that the thylakoid membrane consists of a few supramolecular complexes (for a review, see ref. 2).

Interestingly, the extraction pattern with octyl glucoside as detergent is dif-

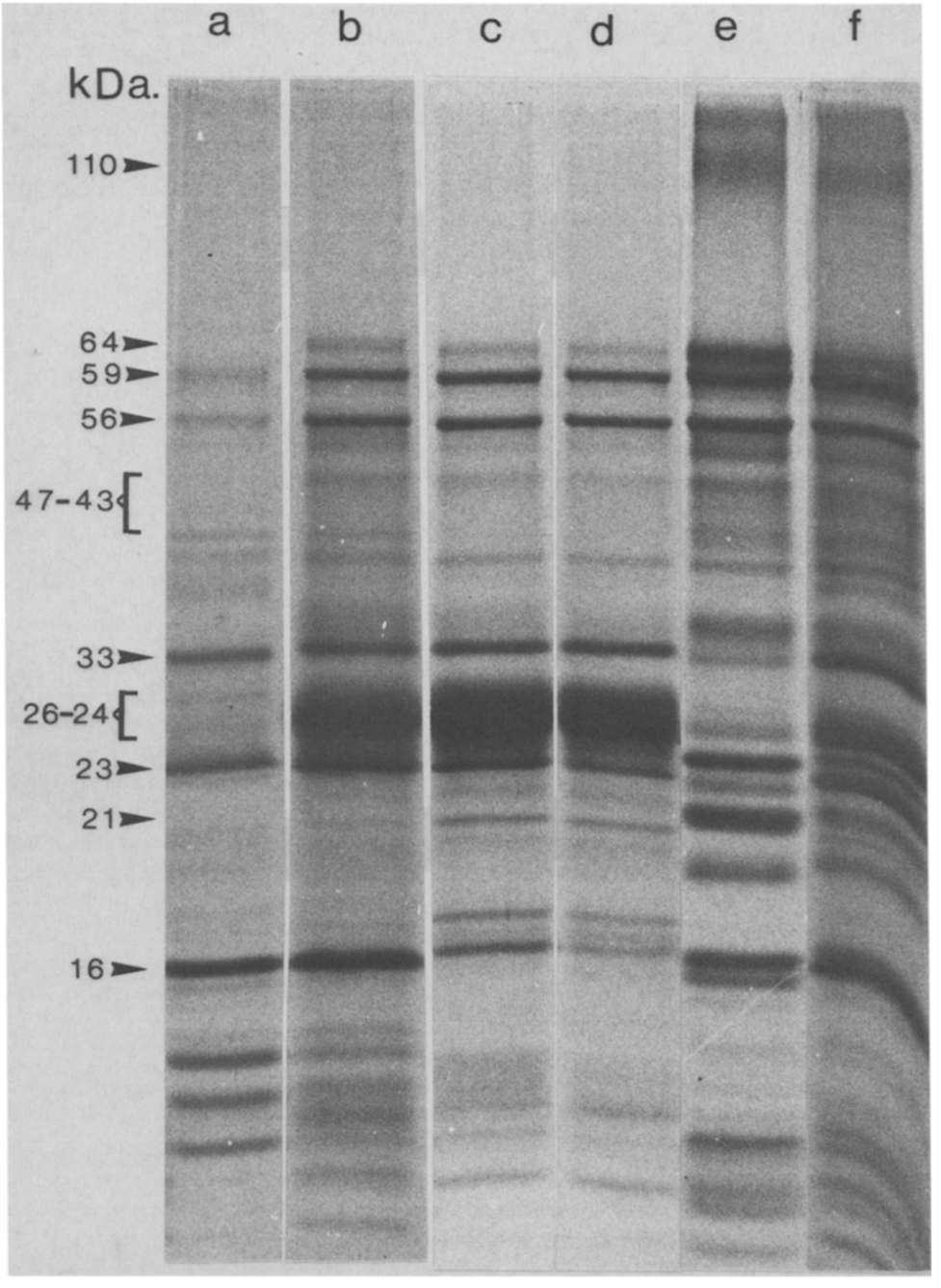


Fig. 7. Polypeptide pattern of thylakoids and of different fractions obtained by the extraction of thylakoids with an increasing amount of octyl glucoside in the added upper phase, described in Fig. 6. Slots: a = 0.9%, b = 1.0%, c = 1.2%, d = 1.3%, e = residue in the lower phase; f = thylakoids.

ferent from that with Triton X-100. One explanation for this result is that octyl glucoside acts in the same way as Triton X-100 in the solubilization, but that the mixed micelles formed partition differently in the two detergents used.

Mixed micelles with Triton X-100 expose the PEG chain of the detergent and therefore favour the upper phase, whereas the micelles with octyl glucoside will expose a completely different surface. This is supported by the difference in partition between Triton X-100 micelles, which prefer the upper phase, and octyl glucoside, which is more equally distributed between the phases. In addition the PEG chain of Triton X-100 might cover the protein surface of the mixed micelle and thereby prevent contact between the proteins and the phase components. In the case of octyl glucoside, the protein surface may be more accessible for interaction with the surrounding phase.

An alternative explanation is that the two detergents show different selectivity in solubilizing the thylakoid membrane. This view is supported by the results obtained by Camm and Green[9]. When thylakoid membranes were solubilized with octyl glucoside the extract was very rich in photosystem II chlorophyll proteins, leaving a residue highly enriched in CP1, the main chlorophyll protein of photosystem I. The opposite solubilization pattern is obtained when Triton X-100 is used (see *e.g.* ref. 11). It is also possible that the different extraction patterns for octyl glucoside and Triton X-100 are caused by a combination of differences in solubilization properties and in partition behaviour of the mixed micelles.

Since the method is simple, rapid and can easily be scaled up, it should be ideally suited to use as a first step in the purification of membrane proteins.

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