Separation of Chloroplast Thylakoid Membrane Polypeptides by Electrophoresis on Polyacrylamide Gradient Gels with a Length of 60 cm

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^{14}C labeled thylakoid membranes of *Vicia faba* chloroplasts were separated by one-dimensional electrophoresis in gel cuvettes of different length using a 12–18% polyacrylamide gradient gel system containing 0.1% sodiumodecylsulfate and 6 M urea. The use of gels with a length of 22 cm and 60 cm led to the detection of about 40 and 80 polypeptide bands, respectively, after dye staining and 50 and more than 100 polypeptide bands, respectively, after autoradiography. The conditions used strongly denaturate chlorophyll proteins and avoid the formation of different polypeptide conformations. Polypeptides differing in molecular mass from about 3000 to 70000 were separated in sharp bands.

Introduction

SDS[2] polyacrylamide gel electrophoresis has been intensively used to analyse the polypeptide components of intact as well as of functionally deficient chloroplast thylakoid membranes of higher plants and green algae. Using one-dimensional gradient gels with a length up to 30 cm and dye staining of the polypeptides, SDS-polypeptide extracts were separated in maximal 50 polypeptide bands [1, 2]. The number of components separated has been increased to about 80 bands by two-dimensional electrophoresis including isoelectric focusing [3, 4]. However, the latter technique often led to the separation of multiple molecular forms of pure proteins due to chemical and conformational microheterogeneities [5]. This increases the number of polypeptide spots, which must be separated and is undesirable in many cases.

Besides this problem one-dimensional electrophoresis has some further advantages over two-dimensional separations concerning reproducibility and expense. In the present experiment an attempt is made to improve its resolving power. This has been achieved using gels with a length up to 60 cm and a polyacrylamide gel system containing 6 M urea, known to denaturate polypeptide oligomers completely including the chlorophyll proteins [6].

The results obtained were compared with results of usual 22 cm-gradient gels.

Material and Methods

Preparation of metabolically [^{14}C] radiolabeled chloroplast thylakoid membranes:

Seedlings of *Vicia faba* were grown for 2 weeks at 20 °C in an air atmosphere (4000 cm^3) enriched with 0.096 MBq [^{14}C] $CO_2 \cdot cm^{-3}$. The light to dark ratio was 16 to 8 h. Leaves were immediately harvested and homogenized at 4 °C with a buffer of 0.075 M Tris-HCl, 0.5 M Sucrose, 1 mM Na-diethyldithiocarbamate and 2% humanserum albumin. The suspensions were filtered through two layers of cheesecloth, chloroplasts were sedimated by centrifugation at 4000 × *g* for 15 min and then osmotically shocked by resuspending them in an excess of bidistilled water. Nuclei and cell debris were sedimented at 800 × *g* for 5 min and discarded before the swollen thylakoid membranes were sedimented at 20000 × *g* for 15 min. The membrane polypeptides were solubilized with triton X-100 at a ratio of detergent/chlorophyll (w/v) of 10 and precipitated with pure aceton. The pellet was solubilized with a buffer containing 50 nM Na-borate, pH 9.0, 8 M urea, 1 mM diisopropylfluorophosphate and 5 mM dithiothreit.

Abbreviations: Chl_a-AP 1, chlorophyll *a* – apoprotein 1 (nomeclature of chlorophyll-proteins according to Machold *et al.* [8]; CF_1, coupling factor; Cyt *f*, cytochrome *f*; LHC, light-harvesting complex; Pl, proteolipid; RuBPC, ribulose-1,5-bisphosphate-carboxylase; SDS, sodium dodecyl sulfate.

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Following centrifugation for 15 min at 20000 × *g* the dissolved polypeptides were loaded on polyacrylamide gels (80 µg protein/slot).

In order to estimate the apparent molecular mass of membrane polypeptides the following standard proteins were co-electrophoresed: bovine serum albumine (68000), catalase (60000), ovalbumine (45000), aldolase (40000), chymotrypsinogen A (25000), myoglobin (17000), and cytochrome *c* (12400).

The gels were fixed in a solution of methanol/acetic acid/water = 5/1/5 (v/v/v), stained in Coomassie brilliant blue G 250 (0.1% in fixative) and destained in 7% acetic acid. Following incubation in a mixture of glycerol, methanol acetic acid and water (1,5:2:1:15) the gels were dessiccated and the labeled proteins visualized by autoradiography on HS 11 X-ray sensitive film (VEB Fotochemische Werke, Berlin). The absorbance of films was scanned with a Zeiss-Schnellphotometer III (VEB Carl Zeiss, Jena).

Polyacrylamide gel electrophoresis

Two different slab gel apparatus modified according to Studier [7] were used. The dimension of the 24 cm gel cuvette was 240 × 200 × 0.8 mm and of the 62 cm cuvette was 620 × 250 × 0.8 mm. Cooling of the gels was achieved by constant stirring of the lower reservoir buffer.

Polyacrylamide gel electrophoresis was performed using a buffer system, previously termed system III by Machold *et al.* [8]:

Upper reservoir buffer: 0.056 M borate-HCl, pH 8.0, 0.1 % SDS.

Stacking gel: 2.99 M Tris-HCl, pH 8.9, 6% acrylamide, acrylamide/bisacrylamide = 30/0.8, 0.1% SDS, 6 M urea, length 12 mm.

Separation gel: 2.99 M Tris-HCl, pH 8.9, gradient 12–18%, acrylamide/bisacrylamide = 30/0.8, 0.1% SDS, 6 M urea.

Lower reservoir buffer: 0.112 M borate-HCl, pH 0.8.
Current (initial) 1 mA/mm^2.
Current (after penetration) 3,5 mA/mm^2.
Temperature: 15 °C.
Time: 24 h (22 cm-gel), 72 h (60 cm-gel).

Chemicals: SDS (Ferak Berlin) was recrystallized from ethanol. Acrylamide, N,N′-methylene-bis-acrylamide, Tris-(hydroxymethyl)-aminomethan and Coomassie brilliant blue G 250 were obtained from Serva, Heidelberg.

Results

Stroma free chloroplast thylakoid membranes, isolated from plants grown in an atmosphere enriched with [^{14}C] CO_2 were disintegrated in the presence of 2% SDS, 2% DTT and 8 M urea and separated on an

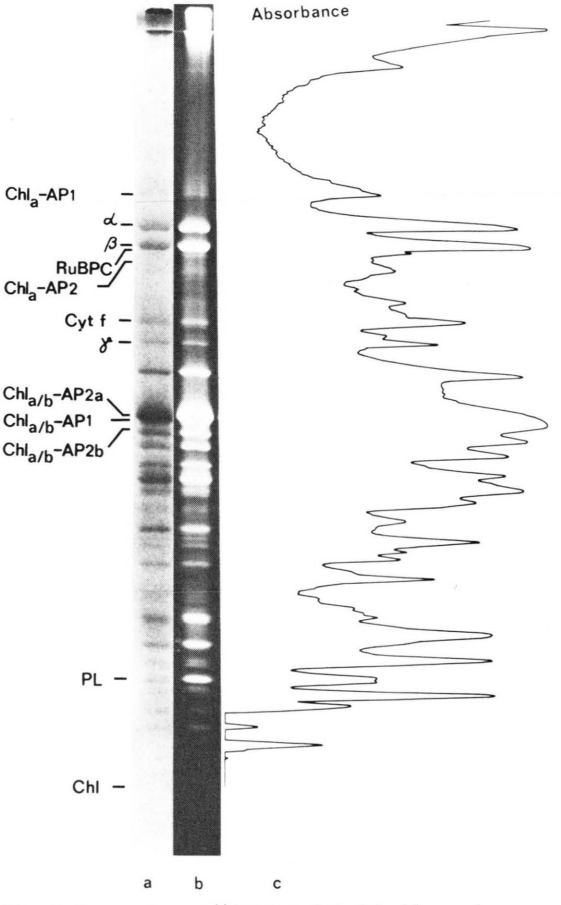

Fig. 1. Separation of ^{14}C-labeled thylakoid membrane proteins in a 22 cm-polyacrylamide gradient gel (12–18%).
a) Coomassie blue-stained gel;
b) autoradiogram;
c) densitometric tracing of the autoradiogram.

usual 22 cm-slab gel as described under Material and Methods. Following dye staining about 40 polypeptide bands became visible (Fig. 1a). The autoradiogram of the same gel (Fig. 1b) shows about 50 bands indicating a higher sensitivity of autoradiography compared with dye staining. Some of the polypeptides separated in the gel system used have been previously identified functionally as shown in Fig. 1, *i.e.* chlorophyll proteins [8], ATP synthase subunits, cytochrome *f* and RubP carboxylase subunits [9].

Since it could not be excluded that polypeptides were superimposed by other ones after separation in 22 cm-gels, thylakoid membrane polypeptides were separated in 60 cm-gels (Fig. 2). Compared with the results obtained with the 22 cm-gels the resolution of polypeptides became highly improved in all regions of the 60 cm-gel. Following dye staining about 60 polypeptide bands were visible.

Autoradiography of this gel following desiccation led to the detection of additional bands. 104 ones, unambiguously perceptible on the reduced scale of length of photography and densitogram shown in Fig. 2, were marked with running numbers. All of the bands containing functionally identified polypeptides are listed in Table I. However, a higher number of polypeptide bands then designated in Fig. 2 is visible in the original autoradiogram and the corresponding densitogram. As an example, Fig. 3 shows the densitograms of two gel regions containing polypeptides of the light-harvesting complex (Fig. 3a) and of the coupling factor CF_1 (Fig. 3b). Compared with the results of 22 cm-gels between the α-subunit (number 4) and the β-subunit (number 7) of CF_1 at least three additional polypeptides (number 4a, 5, 6) and between $Chl_{a/b}$-AP 1 (number 26) and $Chl_{a/b}$-AP 2b (number 29) two additional bands (number 27 and 28) are visible. Comparable additional polypeptide bands can be found in all gel regions of the 60 cm-gel.

Table I. Apparent molecular weight of functionally identified polypeptides in 60 cm-gels.

Polypeptide Number		Apparent molecular weight ($\times 10^{-3}$)
3	Chl_a-AP 1	69
4	α-subunit, ATP-synthase	59
7	β-subunit, ATP-synthase	54
11	Chl_a-AP 2	49
19	γ-subunit, ATP-synthase	35
25	$Chl_{a/b}$-AP 2a	27
26	$Chl_{a/b}$-AP 1	26.5
29	$Chl_{a/b}$-AP 2b	25.5
40	δ-subunit, ATP-synthase	23.0
85	proteolipid, ATP-synthase	7.5

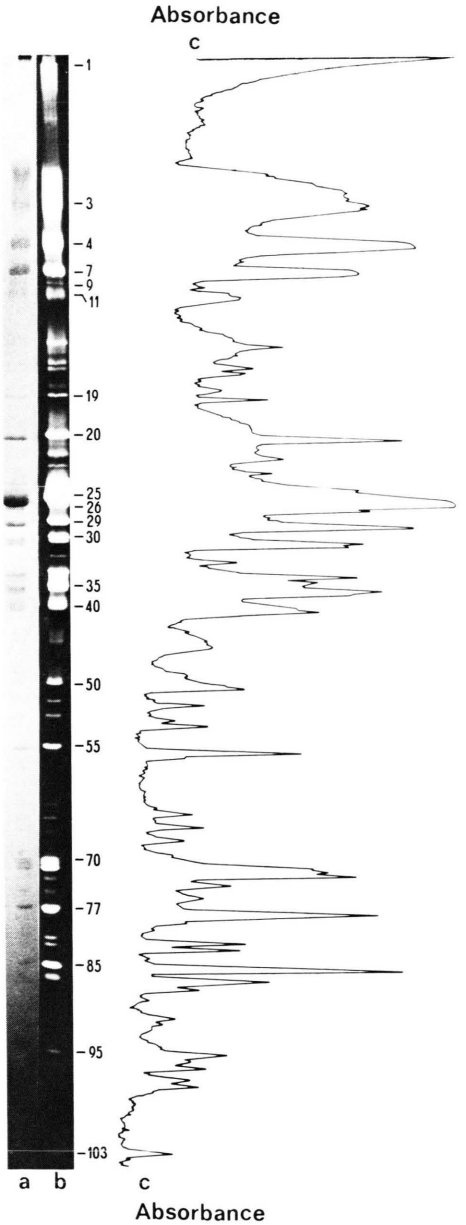

Fig. 2. Separation of ^{14}C-labeled thylakoid membrane proteins in a 60 cm-polyacrylamide gradient gel (12–18%).
a) Coomassie blue-stained gel;
b) autoradiogram;
c) densitometric tracing of the autoradiogram.

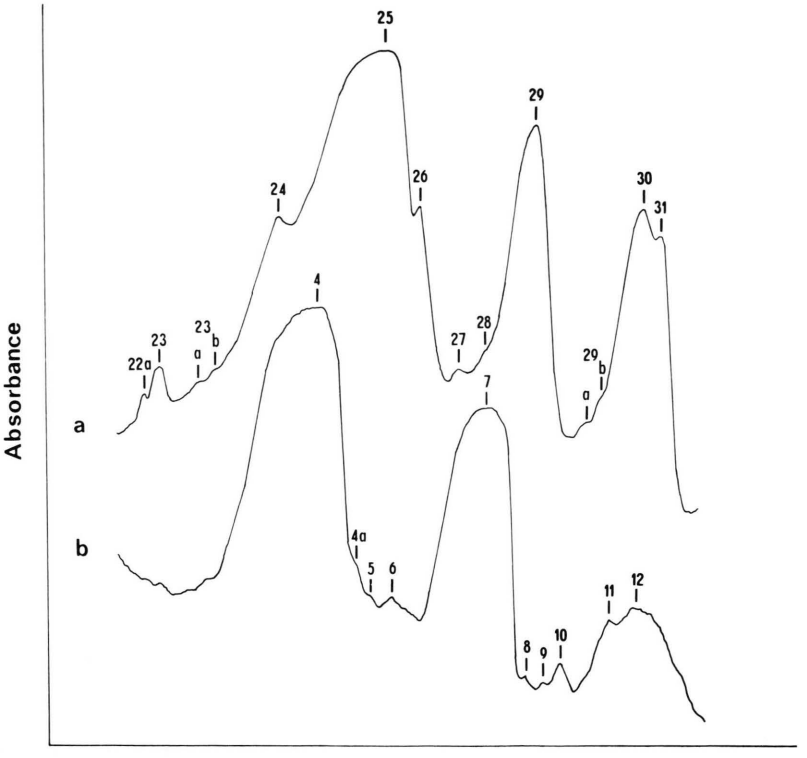

Fig. 3. Sections of the densitometric tracing of Fig. 2c showing minor components in the gel regions containing
a) polypeptides of the LHC;
b) α- and β-subunit of the ATP synthase CF_1 (number 4: α-subunit, 7: β-subunit of CF_1, 25: $Chl_{a/b}$-AP 2a, 26: $Chl_{a/b}$-AP 1, 29: $Chl_{a/b}$-AP 2b).

These results demonstrate the high resolving power of the gel system used. Furthermore it is obviously that many polypeptides are superimposed by other ones, if SDS-solubilized thylakoid membrane proteins are separated in 22 cm-gels.

Discussion

Polyacrylamide gel electrophoresis is a wide used method to separate thylakoid membrane proteins. Originally, using 10 cm homogeneous rod gels Thornber *et al.* [10] separated SDS-solubilized thylakoid membranes into about 15 polypeptide bands. Later on the resolution of one-dimensional electrophoresis was considerably improved by applying of polyacrylamide gradient gels with length up to 30 cm [2, 11]. After dye staining maximal 50 highly focused polypeptide bands were visible [1]. Since in these gels the distance between most of the polypeptide bands is very small the use of radioactive labeled polypeptides combined with sensitive autoradiography led to some improvement only [12], as shown again in the present paper. However, a really improved separation of complex polypeptide mixtures can be achieved by further increase of the gel length. According to Rodbard *et al.* [13] the resolution of electrophoretical gel systems increases proportionally to $\sqrt{l}$ (l = path length) and $\sqrt{t}$ (t = duration of electrophoresis), respectively, which means that the resolution should be doubled when the path length increases fourfold. This is in good agreement with the findings presented here. Thylakoid polypeptides were separated on 22 cm- and 60 cm-gels into about 50 and 120 components, respectively, as detectable after autoradiography.

The polypeptide bands found in the 22 cm- and 60 cm-gels represent different polypeptide species and, as far as known, no aggregates. Urea promotes the denaturation of polypeptides during electrophoresis [15, 14]. Therefore, the use of 6 M urea leads to the denaturation of all chlorophyll-protein-complexes and avoids the formation of aggregates and multiple bands of homogeneous proteins, which appear in other gel systems [6]. Therefore, the 60 cm-gel system, presented here, is well suitable to analyse complex polypeptide mixtures. Certainly, it

can not be excluded that inspite of the high efficiency some polypeptides separated by this method still may be superimposed. Recently the improved method was used to estimate the subunit stoichiometry of the chloroplast ATP synthase complex (CF_1-CF_o) in thylakoid membranes of *Vicia faba* L. [12].

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