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PROTEINS AND POLYPEPTIDES OF ENVELOPE MEMBRANES FROM SPINACH CHLOROPLASTS

I. ISOELECTRIC FOCUSING AND SODIUM DODECYL SULFATE POLYACRYLAMIDE GEL ELECTROPHORESIS SEPARATIONS

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Key words: Chloroplast envelope; Ribulosebisphosphate carboxylase; Isoelectric focusing; Polypeptide; Protein; (Spinach chloroplast)

Polypeptides of spinach chloroplast envelopes were separated by electrophoresis in an SDS-polyacrylamide gradient gel. At least 37 polypeptides were resolved; nine were prominent. Two (M_r 54000 and 16000) were also found in the stroma fraction and identified by peptide mapping and isoelectric focusing in the second dimension as the large and small subunits of ribulose-1,5-bisphosphate carboxylase. Proteins of the chloroplast envelope were also separated by isoelectric focusing. An adaptation of a previous method (Ames, G.F.L. and Nikaido, K. (1976) Biochemistry 15, 616–623), using solubilization in SDS and isoelectric focusing in the presence of a high concentration of Nonidet P-40, gave the best separation and resolved the envelope membranes into at least 21 proteins. The major band (p*I* 6.85) contained both subunits of the carboxylase and at least two additional polypeptides which corresponded to the prominent bands found in SDS gel electrophoresis of chloroplast envelopes.

Introduction

The two membranes that comprise the chloroplast envelope support a great variety of physiological functions that are sustained by the nature, amount and organization of the biochemical constituents [1–15]. Although the lipid and pigment composition of the envelope has been studied in detail [12], the function and organization of these components within the two membranes are not known. Surprisingly, the envelope contains little protein per unit mass compared to thylakoid membranes [12]. Electrophoretic patterns on SDS-polyacrylamide gels [4,5,9,10,12,13,16–23] show a series of high molecular weight bands ($M_r > 60000$) and four prominent polypeptides. The 29000 Da polypeptide has been demonstrated to be the phosphate translocator [20,21]. The 12000–14000 Da polypeptide was identified as the small subunit of ribulose-1,5-bisphosphate carboxylase [16] with anti-ribulose-1,5-bisphosphate carboxylase serum [22,23]. The identity of the 52000 Da polypeptide as the large subunit of ribulose-1,5-bisphosphate carboxylase, however, is controversial [16,22,23]. The isoelectric points of the proteins of chloroplast envelopes have not yet been reported, probably due to the difficulty of solubilizing the hydrophobic envelope proteins in nonionic detergents.

The aim of this investigation is first to obtain reproducible polypeptide separations of spinach chloroplast envelopes by SDS-polyacrylamide gel

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electrophoresis and to characterize the M_r of all polypeptides; secondly, to compare some of the polypeptides common to the envelope, stroma and thylakoid fractions with a similar M_r; and thirdly, to characterize the proteins of the chloroplast envelope by their isoelectric points. A preliminary report of these findings has been published [24].

Materials and Methods

Pure grade chemicals were supplied by Fluka with the exception of agarose, ammonium persulfate and Coomassie R-250 (Merck), urea (BDH), SDS (Serva), *Staphylococcus aureus* V_8 protease (Miles Laboratories), Ampholine (LKB), LMW electrophoresis calibration kit (Pharmacia) and the color reagent for the protein assay [25] which was purchased from Bio-Rad.

Chloroplast envelope preparation

Spinach (*Spinacia oleracea*) leaves were obtained from the local market. Envelopes were isolated from intact chloroplasts according to the method of Joyard and Douce [6,12]. The envelope, thylakoid and stromal proteins were estimated by the dye-binding method of Bradford [25] using bovine serum albumin as standard.

SDS electrophoresis

Electrophoresis was carried out in an Ortec model 4200 vertical slab gel ($100 \times 70 \times 3$ mm) system. Polyacrylamide gels were prepared essentially according to the method of Laemmli [26] with the following modifications: the separation gel consisted of a linear 8–14% (w/v) acrylamide gradient, accompanied by a 5–15% (w/v) sucrose gradient. The ratio of acrylamide to *N,N'*-methylenebisacrylamide was 30 : 0.8. The catalyst concentrations were 0.02% (w/v) ammonium persulfate and a 0.04–0.02% (v/v) *N,N,N',N'*-tetramethylenediamine gradient in the gel. The gel was overlayered with water and electrode buffer, and kept at room temperature to complete the polymerization. On the following day, the 3% stacking gel was poured and the slot former inserted.

The samples for electrophoresis were solubilized in 50 mM Tris-HCl (pH 6.8), 2% (w/v) 2-mercaptoethanol, 0.1% (w/v) bromophenol blue, 10% (v/v) glycerol and incubated at 40°C for 30 min. An LKB 2103 power supply provided a constant current of 20 mA until the dye front reached the lower gel. The current was then increased to 30 mA until the tracking dye was about 1 cm from the bottom of the separating gel. Total electrophoresis time was 4–5 h.

The gel was fixed in 7% (v/v) acetic acid for 30 min (twice) and then stained in 0.25% Coomassie blue R-250, 25% (v/v) ethanol, 7% (v/v) acetic acid for 45 min. The gel was destained in 10% (v/v) ethanol and 7% (v/v) acetic acid until the background was clear, and finally stored in 7% acetic acid. The staining and destaining procedures were adapted from those of Weber and Osborn [27]. The destaining gel was scanned at 600 nm with a Zeiss-Disc ZK4 gel scanner. Calibration proteins were: phosphorylase *b* (94000); bovine serum albumin (67000); ovalbumin (43000); carbonic anhydrase (30000); soybean trypsin inhibitor (20100); α-lactalbumin (14400).

Proteolysis in the presence of SDS

SDS-polyacrylamide gel electrophoresis in the first dimension was performed as described above except that staining time was reduced to 15 min. The gel was destained by diffusion overnight. Bands of interest were excised from the gel with a razor blade, washed in 10 ml of distilled water for 30 min and incubated in 10 ml of 125 mM Tris-HCl (pH 6.8), 0.1% SDS for 30 min at room temperature with rotary shaking. After incubation, gel slices were placed on a stacking gel without slots and covered with a molten 1% agarose solution in 125 mM Tris-HCl (pH 6.8), 0.1% SDS. The separating gel for the second-dimension electrophoresis contained 15% acrylamide. After the gel was placed in the electrophoresis apparatus and overlayered with electrode buffer, a solution containing 100 μg protease in 125 mM Tris-HCl (pH 6.8), 0.1% SDS (w/v), 0.01% bromophenol blue (v/v), 20% glycerol (v/v) was layered on the hardened agarose according to Ref. 28. Electrophoresis was performed for 5 h at 10 mA. Digestion occurred directly in the stacking gel during the co-electrophoresis of the protease and the polypeptides. The electrophoresis was completed in the separating gel at 30 mA for 2 h and 40 mA for 1 h. Gel staining and destaining were carried out as described above.

Isoelectric focusing

Glass tubes (130 × 5 mm) were filled, leaving an adequate space for the sample, with gel solution containing 8 M urea, 5% (w/v) sucrose, 4.5% (w/v) acrylamide, 0.2% (w/v) *N,N'*-methylenebisacrylamide, 2% (w/v) Nonidet P-40, 2% (v/v) Ampholine (3.5–10), 0.06% (v/v) *N,N,N',N'*-tetramethylenediamine and 0.25% (w/v) ammonium persulfate.

Envelope membranes were solubilized in several concentrations of Nonidet P-40 (0.05–5%, v/v), corresponding to protein/Nonidet P-40 weight ratios of 1.3–0.03, for 30 min at 4, 20 and 40°C. Before loading onto the gel, sucrose was added to about 10% (w/v). Envelope membranes were also solubilized according to the method of Ames and Nikaido [29] in solutions of different SDS percentages (from 0.05 to 7.5%) and protein/SDS weight ratios (from 2.5 to 0.13) for 30 min at 4, 20 and 40°C. The solubilized proteins were diluted with 2 vol. of the following mixture (sample dilution buffer): 9.5 M urea, 2% (v/v) Ampholine (3.5–10) and 8% (w/v) Nonidet P-40. The Nonidet P-40/SDS ratio had to be greater than or equal to 8 [29].

The prepared chloroplast envelope proteins (200 μg) were loaded on each rod gel. SDS/Nonidet P-40-treated samples were overlayered with 50 μl of a solution containing 1 M urea, 2% (v/v) Ampholine (3.5–10), 2% (w/v) Nonidet P-40; Nonidet P-40-treated samples were covered with 50 μl containing 1% (w/v) Nonidet P-40, 2% (v/v) Ampholine (3.5–10), 5% (v/v) glycerol. Electrode solutions were 0.15 M ethanolamine for the upper chamber (cathode) and 0.1 M HCl for the lower chamber (anode) [30]. Electrofocusing was performed at room temperature with a Bio-Rad Model 155 electrophoresis system, at 250 V for 15 h and 500 V for 1 h. At the end of the run, gels were fixed for 30 min in 7% (v/v) acetic acid (twice) and then equilibrated for 30 min in 25% (v/v) ethanol, 7% (v/v) acetic acid and stained for at least 45 min in the staining solution. The gel was destained in the equilibrating solution and scanned at 600 nm. A blank, unstained gel was sliced into pieces of 5 mm which were eluted in 1 ml of distilled water for 4 h and the pH of the eluted gel sections was determined [30].

Results and Discussion

SDS-polyacrylamide gel electrophoresis

The separation of envelope, thylakoid and stromal polypeptides, obtained in a linear acrylamide gel gradient, is shown in Fig. 1. The purified envelope fraction contains at least 37 polypeptide bands (Fig. 1E). A densitometric tracing of the gel (Fig. 2) showed nine predominant polypeptides (99 000, 88 000, 75 000, 65 000, 54 000, 34 000, 29 000, 27 000 and 16 000 Da) and a large number of minor polypeptides which were present either in small amounts (170 000, 148 000, 137 000, 120 000, 70 000, 45 000, 42 000–38 000, 36 000) or were close to another band. The latter polypeptides appeared as shoulders or as small peaks depending on the envelope preparations (arrows in Fig. 2). Under the selected conditions, the apparent M_r of each polypeptide was reproducible: the standard deviation ranged from 300 to 1800

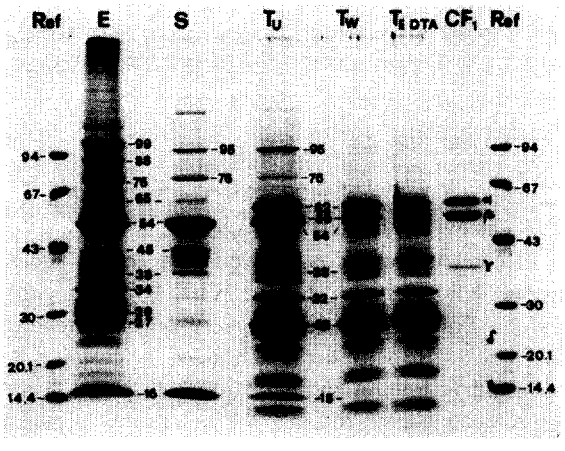

Fig. 1. Electrophoretic separation of polypeptides from different chloroplast fractions: E, envelopes (50 μg proteins); S, stroma (40 μg); Tu, unwashed thylakoids (40 μg); T_w, once-washed thylakoids (40 μg); T_{EDTA}, once-washed thylakoids treated with 0.75 mM EDTA (40 μg); CF_1, supernatant of EDTA-treated thylakoids (10 μg); Ref, standard proteins (12 μg). Apparent M_r values are indicated in kDa. α (62 kDa), β (56), γ (38), δ (23), ε (15) correspond to the subunits of CF_1. The different chloroplast fractions were obtained in the same step sucrose gradient according to Refs. 6 and 12. Unwashed thylakoids were collected, diluted with a suspension medium containing 0.3 M sucrose, 10 mM Tricine-NaOH (pH 7.6) and centrifuged at 17000 × *g* for 5 min. The pellet (washed thylakoids) was then resuspended in the same medium to about 2 mg chlorophyll/ml.

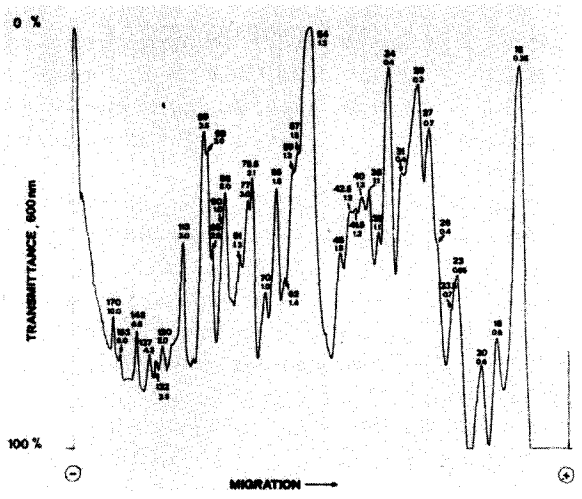

Fig. 2. Densitometric tracing of envelope polypeptides (E in Fig. 1) separated by SDS-polyacrylamide gradient gel electrophoresis. Peaks and shoulders are characterized by their apparent M_r and standard deviation ($n = 7-10$).

Da for polypeptides of 16000–70000 Da. Above this value the standard deviation increased (Fig. 2). Unlike previous reports [5,9,10,16,18,19,21–23] it was now possible to assign an M_r to all the polypeptides, including the minor ones.

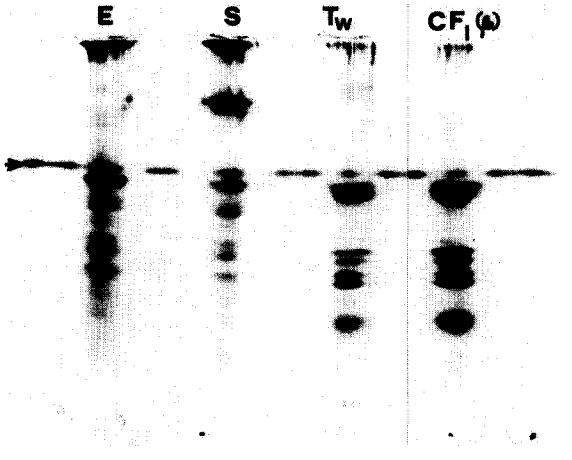

Fig. 3. Peptide mapping of the 54000–56000 Da polypeptides from the envelope (E), stromal (S), washed thylakoid (T_w) fractions and from the supernatant of the EDTA-treated thylakoids (CF_1). Depending on the fraction used, one to five bands (corresponding to 54000–56000 Da polypeptide) obtained in the first dimension were pooled on top of the second-dimension gel and the *S. aureus* V_8 protease (100 μg) was overlayered as described in Materials and Methods. The position of the protease in the gel is indicated by the arrow.

When the polypeptides of the stromal and thylakoid fractions were separated electrophoretically in a linear acrylamide gel gradient (Fig. 1, S and T_w), the densitometric tracing indicated that these two fractions contained at least 37 and 24 polypeptides, respectively (not shown). The electrophoretic pattern of envelope and stromal fractions (Fig. 1E and S) consisted of at least six major band groups corresponding to similar apparent M_r polypeptides (75 000–76 000, 65 000, 54 000, 38 000–45 000, 27 000 and 16 000). The electrophoretic pattern of envelope and thylakoid fractions (Fig. 1E and T_w) also contained polypeptides having similar apparent M_r (54 000–56 000, 38 000, 32 000–34 000 and 26 000–27 000).

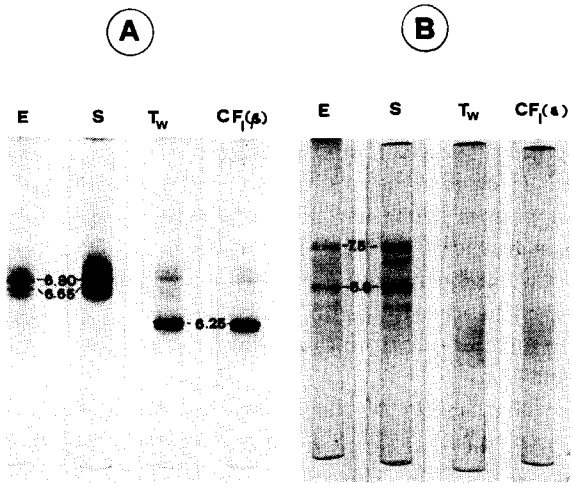

Fig. 4. Isoelectric focusing separation of the 54000–56000 (A) and 15000–16000 Da (B) polypeptides from different fractions obtained in a first-dimensional SDS-polyacrylamide gel electrophoresis. In this particular experiment, solubilization of the different fractions (E, envelope; S, stroma; T_w, washed thylakoids; CF_1, coupling factor: supernatant of the EDTA-treated thylakoids) was carried out as described in Materials and Methods. After the electrophoretic separation in the first dimension, the polypeptides concerned were visualized by a solution of 0.25 M KCl [32] for 10–15 min. The whitish and opaque bands were cut out, and soaked twice in 1 ml H_2O for 10 min. After the removal of water, the gel slices were crushed, macerated and eluted in 400 μl of 50 mM Tris-HCl (pH 6.8) for 1 h under gyratory motion. Then solid urea, Nonidet P-40 and Ampholine (3.5–10) were added to about 6 M, 4% (w/v) and 2% (v/v), respectively. The entire suspensions were loaded on top of the rod gels (80×5 mm) and overlayered with 1% (v/v) Ampholine (3.5–10), 1% (w/v) Nonidet P-40 and 10% (v/v) glycerol. Other conditions as in Materials and Methods. Figures correspond to p*I* values.

To test if the 54000–56000 Da polypeptides, common to all three fractions, had exactly the same electrophoretic mobility, an appropriate amount of each of the three initial samples was loaded on a gel to obtain very thin bands (for the 54000–56000 Da polypeptides). The results of six experiments confirmed that the electrophoretic mobility of the 54000 Da polypeptides from the envelope and stromal fractions was identical and that the apparent M_r of the corresponding polypeptide from the thylakoid fraction was always slightly higher (56000 Da). In addition, a partial digestion of the 54000 Da polypeptide with protease from *S. aureus* V_8 showed that the peptide fragments from envelope and stromal fractions were identical while the pattern from the thylakoid polypeptide was different (Fig. 3E, S and T_w). This was further demonstrated by the behavior of these polypeptides in a second dimension, i.e., in a pH gradient polyacrylamide gel. Isoelectric focusing separations of the envelope and stromal polypeptides showed a similar pattern consisting of at least 3 bands (Fig. 4A, E and S). The corresponding pattern of the thylakoid polypeptides (Fig. 4A, T_w) was different (at least two major and approximately five minor bands of different p*I* values) but was similar to the pattern of the 56000 Da polypeptide from the β-subunit of CF_1, (CF_1 in Fig. 4A).

The identity of the 54000 Da polypeptide is controversial [16,22]. We have demonstrated that this polypeptide corresponds to the large subunit of ribulose-1,5-bisphosphate carboxylase in three different ways. First, the electrophoretic mobility of this envelope polypeptide corresponds exactly to that of the main band of the stroma (Fig. 1E and S). Secondly, the peptide maps of both envelope and stromal bands are identical (Fig. 3E and S). Thirdly, isoelectric focusing in the second dimension reveals an identical pattern (Fig. 4A, E and S). These results are consistent with those of Pineau et al. [22] and Joyard et al. [23] who have excluded the possibility of contamination of the envelope vesicles by the stroma. Since the large subunit of the carboxylase is synthesized inside the chloroplast and consequently does not cross the envelope like the small subunit, it is difficult to explain its presence in the envelope fraction. The simplest explanation is that the holoenzyme of the carboxylase has an affinity for envelope membranes and is not removed by the procedures used to isolate envelopes. Association of the carboxylase with the envelope might also be of physiological significance: the assembly of both subunits may be an envelope event.

Since the enzymatic digestion was limited with polypeptides having an M_r less than 20000, the M_r 15000–16000 polypeptides from the envelope and stromal fractions (Fig. 1) were subjected to isoelectric focusing in a second dimension. Fig. 4B shows that the 15000 Da polypeptides were resolved into identical bands. This polypeptide can be assigned to the small subunit of the carboxylase [16,22,23]. Moreover, the presence of several bands in Fig. 4B may be due to isofocusing variants as was observed for the small subunit of the carboxylase in *Pisum sativum* [31]. If the M_r of the spinach small subunit precursor is similar to that of *P. sativum* [33] and *Chlamydomonas* [34], the 20000 Da polypeptide found in our gel (Figs. 1 and 2) might be the precursor itself. Similarly, the 34000 Da polypeptide (Fig. 2) which probably corresponds to the 33000 and 32000 Da polypeptides described by Pineau and Douce [16] and Ellis [35], respectively, might be the precursor of the major polypeptide of the light-harvesting chlorophyll *a*/*b*-protein complex (see Discussion in Ref. 36). The phosphate translocator (M_r 29000) which is as yet the best characterized polypeptide in chloroplast envelopes [20,21] was well defined in our gel (Figs. 1 and 2).

The nature of the 54000–56000 Da band encountered in thylakoid membranes was also investigated by comparing the electrophoretic separation of unwashed, washed and EDTA-extracted thylakoids as shown in Fig. 1. A single washing of thylakoids (Fig. 1, cf. T_u and T_w) resulted in the disappearance of the lighter portion of the double band (54000 Da) and a significant loss of the 15000 Da polypeptide band. Thus, thylakoid washing(s) eliminated the two subunits of the ribulose-1,5-bisphosphate carboxylase and revealed the β- and ε-subunits of the coupling factor (Fig. 1, T_u, T_w, T_{EDTA} and CF_1; see also Ref. 36) which were identified by peptide mapping (Fig. 3, T_w and CF_1) and isoelectric focusing in the second dimension (Fig. 4B, T_w and CF_1). The isoelectric focusing pattern of the 16000 Da polypeptides from washed thylakoids and CF_1 were completely

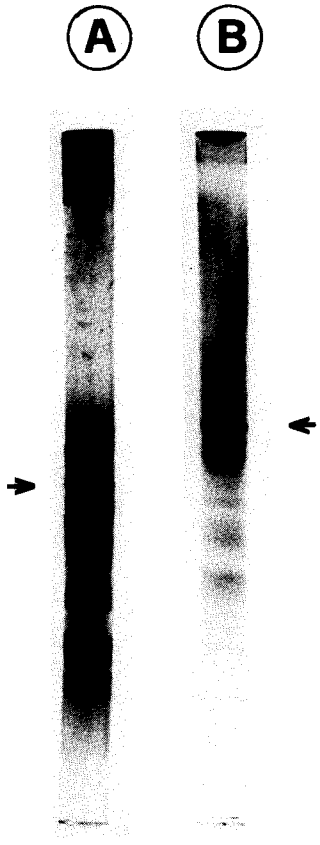

Fig. 5. Separation of chloroplast envelope proteins by polyacrylamide gel isoelectric focusing. (A) Membranes (200 μg proteins) were solubilized at 40°C for 30 min in 2% Nonidet P-40 (weight ratio of protein to detergent, 1:15). (B) Membranes (200 μg proteins) were solubilized at 40°C for 30 min in 0.1% SDS (weight ratio of protein to detergent, 1:0.75). For other conditions see Materials and Methods.

different from that of the envelope and stromal fractions (Fig. 4B). Due to the small amount of 16 000 Da polypeptide available in the first-dimension separation of washed thylakoids and CF_1 (T_w and CF_1 in Fig. 1) it was not possible to demonstrate unequivocally their identity.

Polyacrylamide gel isoelectric focusing

In the presence of nonionic detergents such as Nonidet P-40 or Triton X-100, the best separations (thin bands, minimum of streakings) were obtained by incubating chloroplast envelopes in 2% Nonidet P-40, at a protein/Nonidet P-40 weight ratio of 0.07 and at 40°C for 30 min (Fig. 5A). Nonionic detergents, however, do not permit complete solubilization of membrane proteins [30,37]. Thus, we have adopted the technique proposed by Ames and Nikaido [29] involving SDS solubilization followed by isoelectric focusing in the presence of a high concentration of Nonidet P-40. The resolution of the envelope proteins was good (not shown) for all concentrations of SDS between 0.05 and 0.2% in the solubilization medium and for a protein/SDS weight ratio varying between 2.5 and 0.7. At higher SDS concentrations it was found that the staining intensity of the acidic proteins decreased and the alkaline region of the gel became darker. Solubilization of chloroplast envelopes in 0.1% SDS and a protein/SDS weight ratio of 1.33 for 30 min at 40°C resulted in

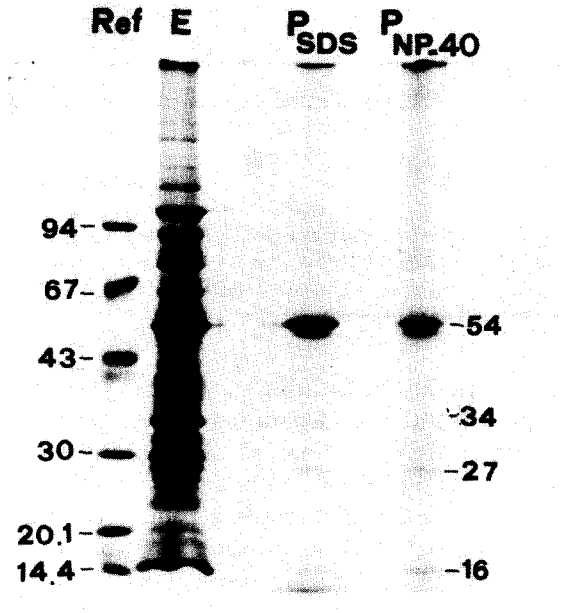

Fig. 6. Electrophoretic separations in SDS-polyacrylamide gel (second dimension) of proteins obtained by isoelectric focusing (first dimension). The proteins obtained by isoelectric focusing in the first dimension (arrows in Fig. 5) were revealed by staining, cut off with a razor blade, washed for 30 min in water, then equilibrated for 30 min in 0.125 M Tris-HCl (pH 6.8) and 0.1% SDS. The slices were placed on the stacking gel of the second dimension and sealed with 1% agarose in the same Tris buffer. Other conditions as in Materials and Methods, Ref, standard proteins (kDa) as in Fig. 1; E, control envelope polypeptides; P_{SDS} and P_{NP-40}, SDS-electrophoretic separations of the main proteins focused after solubilization in SDS/Nonidet P-40 and Nonidet P-40, respectively.

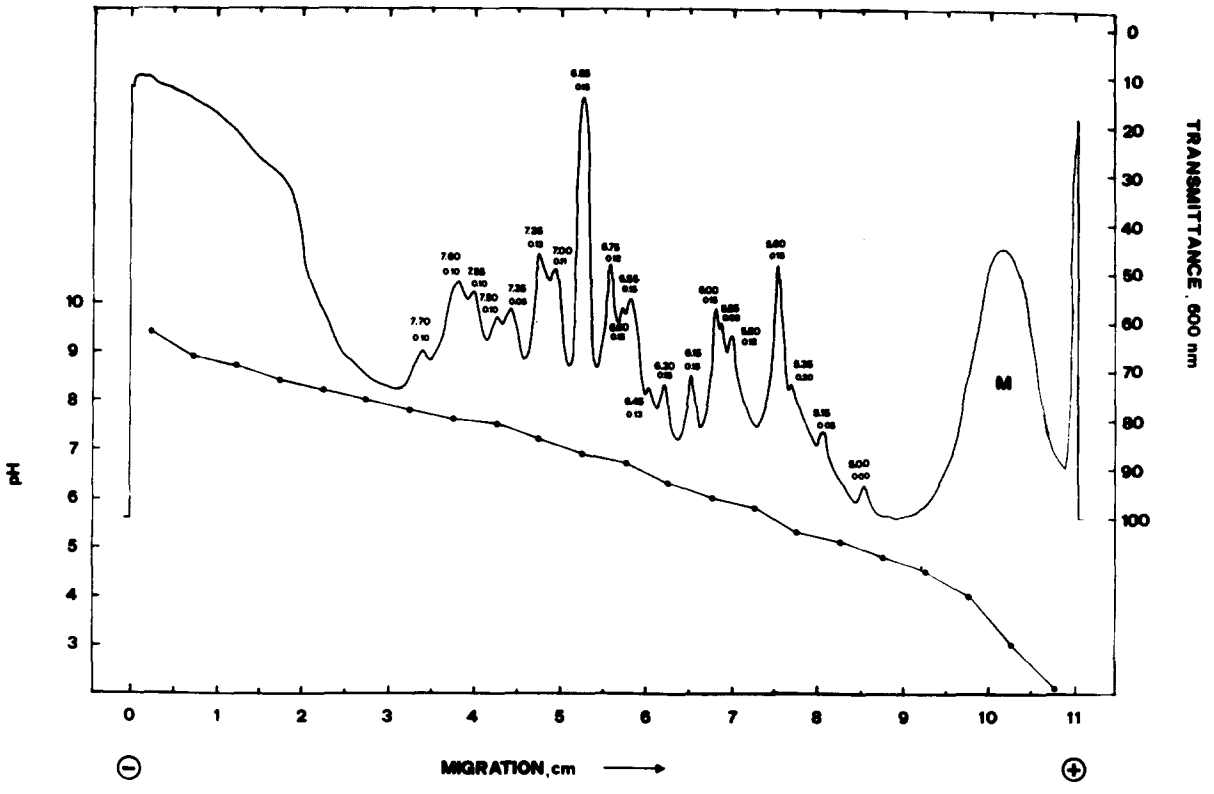

Fig. 7. Densitometric tracing of envelope proteins separated by polyacrylamide gel isoelectric focusing. The chloroplast envelopes were solubilized in SDS (0.1%)/Nonidet P-40 (5.3%) at a protein/SDS weight ratio of 1.33. Peaks and shoulders are characterized by their p*I* (see pH gradient) and standard deviation ($n = 5$–20). The M band contains detergent/carotenoid micelles.

the best isoelectric focusing separation (Fig. 5B).

Since SDS is a denaturing detergent it was important to verify that envelope proteins had electrofocusing behavior after addition of Nonidet P-40 to the SDS-solubilization medium, identical to those solubilized in Nonidet P-40 alone. As shown in Fig. 6, the main band obtained by isoelectric focusing after solubilization with Nonidet P-40 alone or SDS/Nonidet P-40 (arrows in Fig. 5) was further separated by SDS-polyacrylamide gel electrophoresis into at least five identical polypeptides. It is noteworthy that the two proteins focusing at the same pH gave rise to polypeptides having M_r values of 54000 (accompanied by a satellite band having a smaller M_r), 34000, 27000 and 16000. The 54000 and 16000 Da polypeptides belong to ribulose-1,5-bisphosphate carboxylase as attested by the SDS gel electrophoretic separation of control envelope proteins (Fig. 6E). These results show quite clearly that the native charges of the chloroplast envelope proteins were preserved and identical after both solubilizing treatments.

As shown in Fig. 7, the isoelectric focusing separation of spinach chloroplast envelopes displayed at least 21 proteins, ranging from p*I* 5 to 8. The number of native proteins was of course less than that of polypeptides. The scanning profile revealed only one prominent protein having a p*I* of 6.85. As shown previously (Fig. 6), it corresponded mainly to ribulose-1,5-bisphosphate carboxylase (M_r 54000 and 16000). It is significant that the p*I* of one of the variants of the small carboxylase subunit from *Chlamydomonas reinhardii* was found to be 6.85 [38]. Six proteins with the following p*I* values (7.25, 7.00, 6.75, 6.55, 6.00 and 5.60) were less intensely stained. The other bands, although minor, were always encountered in the scanning

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The aim of this investigation is first to obtain reproducible polypeptide separations of spinach chloroplast envelopes by SDS-polyacrylamide gel

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