ISOELECTRIC FOCUSING OF MEMBRANE PROTEINS FROM SPINACH CHLOROPLASTS

Ilse NOVAK-HOFER and Paul-André SIEGENTHALER
Laboratoire de physiologie végétale et biochimie Institut de Botanique, Université de Neuchâtel rue de Chantemerle 20, 2000 Neuchâtel, Switzerland

Received 29 September 1975

1. Introduction

The protein components of thylakoids from higher plants [1–6] as well as from some algae [7–9] have been studied by various methods of sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Up to about 20 polypeptides can be separated by this method and it has been shown by several authors that photosystem I and photosystem II particles are composed of distinct sets of polypeptides [1,3–6,9]. In some cases, a functional identification or a chemical characterization of some of these components has been attempted, complete identification however is far from being achieved yet [3,8,10].

Disintegration of the thylakoid by the anionic detergent SDS and separation by SDS-PAGE result in a denaturation of the proteins and their resolution into subunits. A less destructive extraction and separation procedure may provide better information on the properties of membrane proteins in their native state. No method has been found yet, to extract quantitatively all of the chloroplast membrane proteins without denaturing them and to keep them in the shape of molecules rather than aggregates. It is known however, that the nonionic detergent Triton X-100 extracts a large part of these proteins without disruption of their native conformation and without inactivation [11,12]. Furthermore, it has been shown that isoelectric focusing of thylakoid suspensions in presence of Triton X-100 releases several pigment-protein complexes from the membranes [13].

In this paper, we attempted to disclose the protein composition of various membrane fractions (intact chloroplasts, washed thylakoids, photosystem I and -II particles) obtained by solubilization with Triton X-100. We achieved this by subjecting the fractions to isoelectric focusing in the presence of 1% Triton X-100. After 12 h of isoelectric focusing, up to 25 protein bands can be detected by Coomassie brilliant blue staining. The different fractions show characteristic protein patterns.

2. Materials and methods

2.1. *Plant material*

Spinacia oleracea var. Nobel was grown on a modified Hoagland solution in a phytotron [14]. Leaves were harvested after 8 weeks of growth.

2.2. *Preparation of chloroplasts and chloroplast fractions*

All procedures were carried out at + 4°C. Chloroplasts were isolated according to Kalberer et al. [15] by grinding the leaves for a short time in 330 M sucrose, 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES) pH 7.6, filtering through 8 layers of cheesecloth and centrifuging the filtrate for 30 sec at 2000 *g*. The resulting pellet of intact chloroplasts was resuspended in the homogenization medium and washed once by centrifugation for 5 min at 2000 *g*. Washed thylakoids were prepared by suspending intact chloroplasts in ice cold distilled water, centrifuging for 10 min at 10 000 *g* and washing the pellets three times in 50 mM Tris-HCl pH 7.5. The final pellet was suspended in 250 mM sucrose and 50 mM *N*-tris(hydroxcymethyl)methylglycine(Tricine) pH 7.5.

Photosystem I and -II particles (TSF-I and TSF-II

according to Vernon et al.) were isolated by the action of Triton X-100 and subsequent differential centrifugations [16]. The pellets (TSF-II: 100 000 *g* for 1 h; TSF-I: 100 000 *g* for a total of 16 h) were suspended in 250 mM sucrose and 50 M Tricine, pH 7.5. Preparations were checked for their photochemical activities which were in agreement with the results of Vernon and Shaw [16].

The different fractions were adjusted to the same protein concentrations using a modified Lowry procedure [17] for protein determination. The suspensions were passed through a glass homogenizer in the presence of 1% Triton X-100 and stirred for one hour at + 4°C. Without further centrifugation, electrofocusing was performed with samples containing 50–100 μg of protein.

2.3. *Isoelectric focusing on polyacrylamide gels*

We used vertical gel slabs (3 mm thickness) with a length of 7.5 cm and a width of 10 cm. Acrylamide and *N,N'*-methylenebisacrylamide were purchased from Eastman Kodak and used without further purification. We used the electrofocusing system devised by Allen [18] with the exception that 1% Triton X-100 was incorporated in the gel. The final mixture consisted of 6.85 ml 4.4% Triton X-100, 10 ml of acrylamide solution (24 g acrylamide and 0.84 g *N,N'*-methylenebisacrylamide per 100 ml aqueous solution), 2.4 ml of *N,N,N',N'*-tetramethylethylenediamine (1 ml TEMED per 100 ml aqueous solution), 0.75 ml LKB Ampholine carrier ampholyte (pH 3–10) and 10 ml of ammoniumperoxodisulphate (70 mg per 100 ml aqueous solution), thus yielding final concentrations in the gel of 6% acrylamide, 2.5% ampholine and 1% Triton X-100. Electrode solutions were 0.1 M HCl for the upper bath (anode) and 0.15 M ethanolamine for the lower bath (cathode). Electrofocusing was performed for 12 h at 4°C with an Ortec Model 4200 electrophoresis system (kindly supplied by Professor E. Stutz) at 20 V with a current decreasing from 18 to 2 mA.

At the end of electrofocusing, one part of the gel was fixed for 30 min in 12.5% trichloroacetic acid and then stained for at least 3 h in a solution containing acetic acid, ethanol and 0.2% Coomassie brilliant blue solution (1:2.5:2.5 vol., respectively). Destaining was performed by washing the gels in a mixture of acetic acid, ethanol and water (1:2.5:6.5 vol., respectively). Gels were scanned with a Zeiss-Disc-Zk 4 gel scanner, recording the transmittance at 600 nm. The unstained part of the gel was sliced into pieces of 0.5 cm which were eluted in 1 ml of distilled water for one hour and the pH of the eluted gel sections was then determined. The method allows to determine an approximative isoelectric point for a single protein peak within 0.7 pH-units.

3. Results

Fig.1 shows typical scanning profiles of gels along

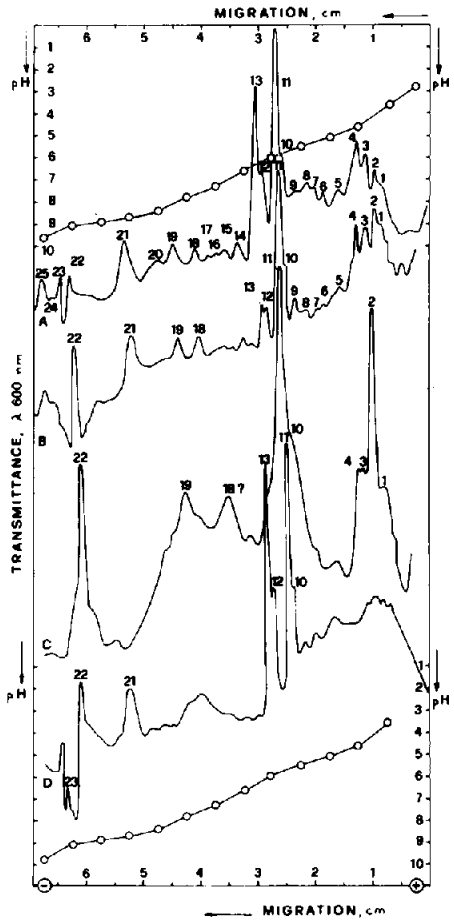

Fig.1. Densitograms and pH-gradient of various membrane fractions of spinach chloroplasts after polyacrylamide gel isoelectric focusing. (A) intact chloroplasts, (B) washed thylakoids, (C) photosystem II enriched fraction (TSF-II), (D) photosystem I enriched fraction (TSF-I).

with the respective pH-gradient measured after 12 hours of electrofocusing. Separation of intact chloroplasts (fig.1A) shows the presence of 25 protein bands. They are numbered from the anode (acidic part of the gel) to the cathode (basic part of the gel). There is a first group of 4 narrowly spaced bands in the range of pH 3.8–4.8 (No. 1–4). Between pH 5.0 and 5.7, five weakly stained proteins are visible (No. 5–9). Two strongly colored double peaks follow at about pH 5.9 and 6.3, respectively (No. 10–13). Between pH 6.7 and 8.8, eight proteins are spaced (No. 14–21) and a last group of 4 proteins is located from pH 9 to 10 (no. 22–25).

Washed thylakoid preparations (fig.1B) differ from intact chloroplasts by a strong diminution of proteins No. 12–13 and to a lesser extent of proteins No. 14–17.

In an attempt to identify the function of some of these membrane proteins, we have examined the composition of particles which are enriched either in photosystem I or in photosystem II activities. Photosystem I and -II preparations can be distinguished by different groups of proteins. Photosystem II-particles (fig.1C) have a clear dominance of protein No. 2 focusing at about pH 4.0. Proteins No. 3–5 as well as No. 10–11, 18–19 and 22 are also present. Photosystem I particles (fig.1D) also contain proteins No. 10, 11 and 22. In addition, however, proteins No. 12–13, 21 and 23 appear more distinctly in this fraction than in the photosystem II particles. Both of these fractions contain some minor bands in the pH 5.0–5.7 region which cannot be assigned unequivocally to one of the two photosystem preparations.

4. Discussion

Although the protein patterns obtained by isoelectric focusing in the presence of Triton X-100 should still be improved, mainly in order to produce gels totally devoid of background staining, the reproducible resolution of up to 25 protein bands from the thylakoid membranes show that this method is a powerful tool for studying thylakoid membrane proteins.

Contrary to SDS-PAGE which gives information on the polypeptide composition of the membrane on a molecular weight basis isoelectric focusing characterizes these molecules by their isoelectric points. The bands separated by SDS-PAGE represent mainly subunits of membrane proteins [10] and this method resolves a maximal number of polypeptides. Extraction and separation by isoelectric focusing in the presence of Triton X-100 is much less destructive than the SDS method and should extract a number of membrane proteins without denaturation.

Both photosystem I and -II fractions show a characteristic protein composition, i.e. proteins No. 2, 18, 19 for photosystem II and proteins 12, 13, 21, 23 for photosystem I. The peaks No. 12 and 13 in the photosystem I preparations may represent contaminations from the stroma, since they are found only in rather small amounts in the total lamellar preparations. Protein No. 2 (focusing at about pH 4) is dominant in the photosystem II fraction. The position of this protein can be correlated with a recent observation by Ninnemann and Strasser [13] who found a zone of protein bands in the pH 4–5 region of the isoelectric focusing patterns only in flashed induced leaves (active photosystem II) compared to flashed leaves which have no water splitting capacity.

Photosystem I and -II fractions have also some proteins in common, such as No. 10, 11 and 22, especially No. 10 and 11 which are strongly present in both fractions. Concerning the presence of protein peaks common to both photosystem I and -II fractions, the results reported in the literature on SDS-PAGE are often contradictory. For instance, Nolan and Park [6] show quantitative rather than qualitative differences between these two fractions. On the other hand, Klein and Vernon [3] report protein patterns with distinct peaks characterizing photosystem I and -II particles without obvious common bands. At present, we feel that it is not possible to solve this problem, because we do not know how the subunits obtained by the SDS-method are effectively arranged in situ. We used the TSF-I and -II fractions of Vernon and Shaw [16], but we separated the membrane proteins of these particles by isoelectric focusing in the presence of Triton X-100 instead of SDS-PAGE. With our method, we find a distinctly common double peak focusing at about pH 6. These proteins common to both photosystem fractions might reflect an organization related to the native state of the photosystems.

Most work so far reported in this field was aimed towards resolving a maximal number of polypeptides of the thylakoid membrane (SDS-method). Our

approach is geared towards releasing by a milder treatment, a reduced number of membrane proteins presumably still in their native state. The subunit structure of these proteins could then be analyzed by SDS–PAGE. Thus taking advantage of both methods, we intend to use a combination of isoelectric focusing in the presence of Triton X-100 and of SDS-PAGE in order to provide further insight into the nature of thylakoid membrane proteins.

Acknowledgements

This work was supported in part by the Fonds National Suisse de la Recherche Scientifique (grant No. 3.2470.74 to P.A.S.).

References

[1] Remy, R. (1971) FEBS Lett. 13, 313–317
[2] Machold, O. (1971) Biochim. Biophys. Acta 238, 324–331.
[3] Klein, S. M. and Vernon, L. P. (1974) Photochem. Photobiol. 19, 43–49.
[4] Anderson, J. M. and Levine, R. P. (1974) Biochim. Biophys. Acta 333, 378–387.
[5] Hofer, I., Strasser, R. J. and Sironval, C. (1974) in: Proc. 3rd Intern. Congress on Photosynthesis, Rehovot, Israel, 1974, (Avron, M., ed.), Vol. III, pp. 1685–1690, Elsevier Sci. Publ. Co., Amsterdam.
[6] Nolan, W. G. and Park, R. B. (1975) Biochim. Biophys. Acta 375, 406–421.
[7] Hoober, J. K. (1970) J. Biol. Chem. 245, 4327–4334.
[8] Chua, W. and Bennoun, P. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 2175–2179.
[9] Apel, K., Bogorad, L. and Woodcock, C. F. L. (1975) Biochim. Biophys. Acta 387, 568.
[10] Machold, O. (1975) Biochim. Biophys. Acta 382, 494–505.
[11] Wasserman, A. R. (1974) in: Methods in Enzymology (Colowick, S. P. and Kaplan, N. O., eds.), Vol. XXXII (B), pp. 406–422, Academic Press, New York, San Francisco and London.
[12] Makino, S., Reynolds, S. A. and Tanford, C. (1973) J. Biol. Chem. 248, 4926–4932.
[13] Ninnemann, H. and Strasser, R. J. (1974) in: Proc. 3rd Intern. Congress on Photosynthesis, Rehovot, Israel, (1974), (Avron, M., ed.), Vol. III, pp. 1639–1646, Elsevier Sci. Publ. Co., Amsterdam.
[14] Siegenthaler, P. A. and Depéry, F. (1976) Eur. J. Biochem. in the press.
[15] Kalberer, P. P., Buchanan, B. B. and Arnon, D. I. (1967) Proc. Natl. Acad. Sci. U.S.A. 57, 1542–1549.
[16] Vernon, L. P. and Shaw, E. R. (1971) in: Methods in Enzymology (Colowick, S. P. and Kaplan, N. O., eds), vol. XXIII(A), pp. 277–289, Academic Press, New York and London.
[17] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[18] Maurer, R. (1971) Disc-electrophoresis, 2nd Edn., p. 134, Walter de Gruyter, Berlin.