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Chloroplast envelope proteins from *Chlamydomonas*: separation into outer and inner envelopes and analysis by two-dimensional gel electrophoresis

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Fractions enriched in outer and inner chloroplast envelope membranes were isolated from the alga *Chlamydomonas reinhardtii*. Functionally active chloroplasts were lysed after equilibration in a hypertonic solution and outer and inner envelopes were separated. The protein content of each fraction was analysed by two-dimensional gel electrophoresis using isoelectric focusing/SDS polyacrylamide gel electrophoresis. The method is capable of separating components over a wide range of molecular mass and p*I* without interference by lipid binding. Each fraction contained a characteristic protein pattern. A 32 kDa protein was found exclusively associated with the inner envelope indicating that algal chloroplast envelopes share a common feature with those of higher plants.

The two membranes which form the chloroplast envelope play a vital role in several aspects of chloroplast metabolism. They are the site of synthesis of various chloroplast lipids [1], they regulate the exchange of metabolites between the cytosol and chloroplast [2] and they are involved in the transfer of proteins of cytosolic origin into the chloroplast [3].

The complexity of the operations taking place on the envelope and the large number of proteins involved makes a better knowledge of the envelope constituents more and more necessary. Pea [4] and spinach [5,6] envelopes have been separated into fractions enriched in outer and inner membranes and their respective constituents have been analysed by various gel electrophoresis techniques. None of these has proved entirely satisfactory. Often solubilisation problems resulted in gel patterns which gave incomplete information on the constituents of the samples.

We describe a separation of *Chlamydomonas reinhardtii* envelopes into fractions enriched in outer and inner membranes and the analysis of the protein constituents by two-dimensional (2D) gel electrophoresis using isoelectric focusing (IEF) as the first dimension. The procedure applied here is adapted to membrane proteins and results in an excellent solubilisation of the envelopes and negligible interference by the high lipid content of the envelope.

Chloroplasts were isolated from a cell-wall-less strain of *Chlamydomonas reinhardtii* (CW 15-277, from the Chlamydomonas Genetics Center, Department of Botany, Duke University, Durham, NC, U.S.A.), using the method of Mendiola-Morgenthaler et al. [7]. Cells were harvested in the second hour of illumination when the chloroplasts are still relatively small and compact as bigger

Abbreviation: Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

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chloroplasts incubated in a hypertonic solution broke before their membranes had time to separate. Envelopes membranes were obtained by lysis at −20°C of intact chloroplasts in a hypertonic solution containing 10 mM Tricine, pH 7.8, 2 mM EDTA and 0.6 M sorbitol. The envelopes were recovered by flotation on a step gradient of sucrose as described by Keegstra et al. [4]. Total envelope membranes were recovered in the lighter sucrose layer, as a yellow clump devoid of eyespot contaminants, and further separated into two distinct fractions by isopycnic centrifugation on a linear sucrose density gradient (0.3 M–1.1 M sucrose). They were recovered as two yellow bands at buoyant densities of 1.05 g/ml for the lighter fraction and 1.1 g/ml for the heavy fraction.

The yield of the lighter fraction is small as only 10% of the total envelope proteins are associated with it. As the chloroplasts are not broken during their incubation in 0.6 M sorbitol but undergo a change of their elongated crescent shape towards a more compact form, it is possible that the separation of the two envelope membranes is not uniform. Furthermore, freeze-fracture electron microscopy of ultrarapidly frozen *Chlamydomonas* cells has revealed the existence of large particles linking together the two envelope membranes [8]. This particularity of green algae which is not so marked in higher plant envelopes, could explain partly the poor separation of the two envelopes and the recovery of most of the material in the heavy fraction. When envelope membranes fractionated after lysis of chloroplasts in an hypertonic solution were analysed by one-dimensional (1D) gel electrophoresis, the bands were rather diffuse in part of the gels. Lipid extraction from the samples frequently changed the relative intensity of several bands. The disappearance of two bands at high molecular mass associated with a strong enhancement of a band at 57 kDa was observed. However, on 1D gels, the most striking difference between light and heavy fractions was the absence from the light one of a major 32 kDa protein which was found exclusively in the heavy fraction (data not shown).

The complexity of the gel patterns of envelope membranes and the poor resolution of the bands in a 1D gel system led us to apply 2D gel electrophoresis using the method of O'Farrell [9] adapted to membrane proteins by Ames and Nikaido [10], with slight modification as a more sophisticated method of analysis. In the samples, the concentration of SDS was raised to 2.2% SDS, at which the envelope components were completely solubilised, and that of the non-ionic detergent was adapted to 8.8% Nonidet P-40; the ampholytes were Servalyt 2-11 (Serva, Heidelberg). The second dimension was carried out as Laemmli gels with gradients from 7.5% to 20% acrylamide. The silver staining method of Morrissey [11] was used to detect the proteins.

Careful examination of the 2D gels after staining does not reveal any insoluble material carried into the second dimension from the origin of the first dimension. The bands of 1D gels are present in the 2D gels, most of them separated into several components with the same molecular mass but different p*I*. A large number of distinct spots corresponding to different proteins present in small amounts are distributed throughout the gel but mostly in p*I* ranges 5–6 and 7.5–8.5. The separation of the individual constituents is excellent, even those with molecular mass inferior to 15 kDa, as practically no interference with proteins by lipids occurs.

Comparison of 2D gels of fractionated envelopes (Figs. 1 and 2) shows clearly that the two fractions contain distinct sets of proteins. The lighter fraction has fewer components than the heavier one and its gel patterns are different. Its main constituents are proteins with molecular mass of 130, 114, 102, 96, 72, 64, 57, 44, 38, 30, 28 and 13 kDa. The latter has a p*I* of 5.2 and is a different protein from that of 13 kDa, p*I* 5.0, associated with the heavy fraction. All these components are found in traces in the heavy fraction where the pattern resembles more the gel of total envelopes.

The major band at 57 kDa in 1D gels is resolved in 2D gels into seven components distributed between light and heavy fractions (Figs. 1 and 2): 5 weak spots with p*I* 9 (light fraction), 8.5, 8 (heavy fraction), 6.5 (both fractions) and 5.4 (heavy fraction) and two major ones at p*I* 6.1 (light fraction) and 5.3 (both fractions). The component with p*I* 6.1 and molecular mass of 57 kDa was identified as the large subunit of the ribulose-1,5-bisphosphate carboxylase/oxygenase by im-

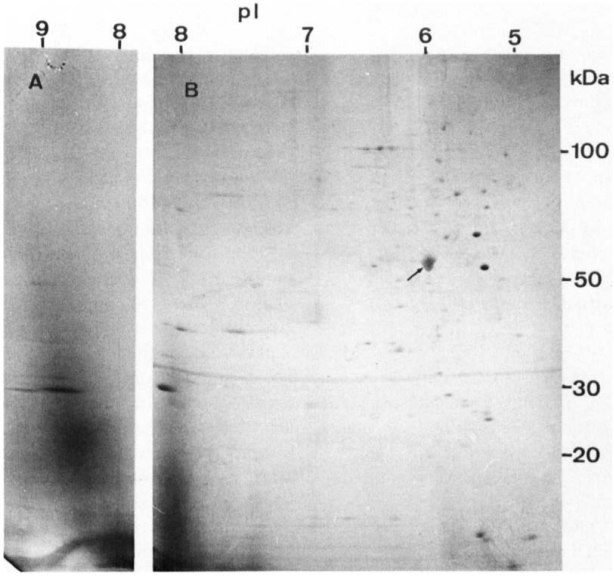

Fig. 1. Analysis of outer envelope membranes by two-dimensional isoelectric focusing/SDS polyacrylamide (7.5–20% gradient) gel electrophoresis. (A) p*I* range 8–9.5; (B) p*I* range 5–8. Arrow indicates the large subunit of the ribulose-1,5-bisphosphate carboxylase/oxygenase of *Chlamydomonas*.

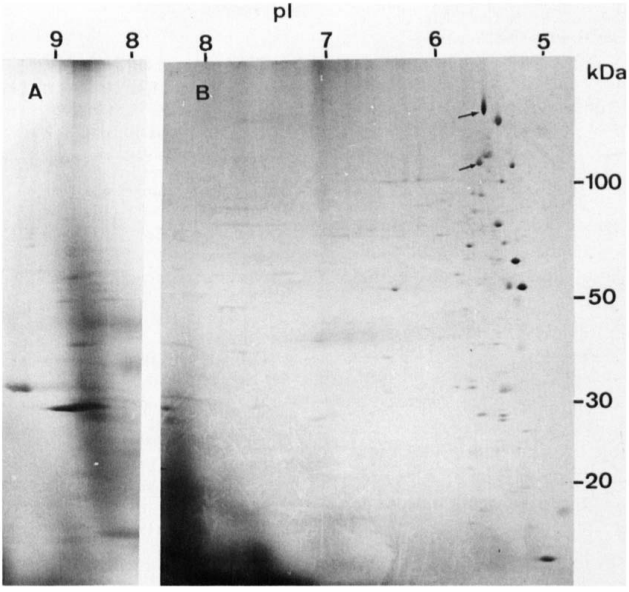

Fig. 2. Analysis of inner envelope membranes by two-dimensional isoelectric focusing/SDS polyacrylamide (7.5–20% gradient) gel electrophoresis. (A) p*I* range 8–9.5; (B) p*I* range 5–8. Arrows indicate the spots reacting with antibodies raised against the large subunit of the ribulose-1,5-bisphosphate carboxylase/oxygenase of *Chlamydomonas*.

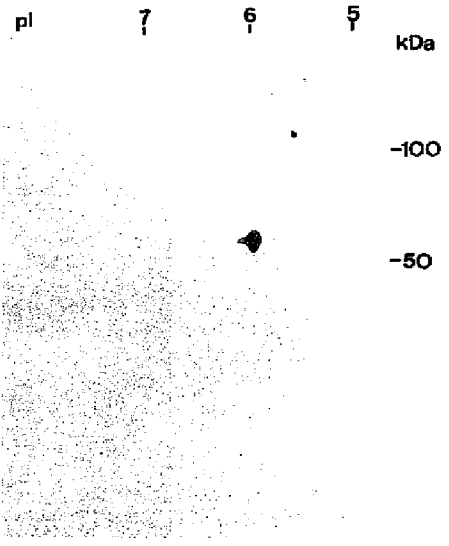

Fig. 3. Electrophoretic blot on nitrocellulose of a two-dimensional isoelectric focusing/SDS polyacrylamide gel of whole envelope membranes prior to separation into outer and inner fractions. Rabbit antibodies raised against the large subunit of the ribulose-1,5-bisphosphate carboxylase/oxygenase of *Chlamydomonas* were used as first antibodies.

munoblotting of 2D IEF/SDS-PAGE gels with rabbit-antibodies raised against the large subunit of the *Chlamydomonas* enzyme (Fig. 3). Its concentration varies between the different preparations and is related to the concentration of two spots with high molecular mass and slightly more acidic p*I*. Both spots also react with the antibodies raised against the large subunit, suggesting that part of it is still involved in complexes.

Three major proteins with molecular mass of 64 kDa and p*I* 5.5, 57 kDa and p*I* 5.3 and 30 kDa and p*I* 8.5–9 are found in equal amounts in both fractions. The 30 kDa protein stains intensively with silver and is split into a doublet (Fig. 1A). At present it is impossible to apportion any of these three proteins to either fraction. The 32 kDa protein of the heavy fraction is usually not detectable in 2D gels run under the conditions used here for isoelectric focusing where the pH gradient range lies between 4 and 8. However, when the basic region of the first dimension is extended toward pH 10 by adding the appropriate ampholytes (Servalyt 9–11), the 32 kDa protein becomes clearly visible (Fig. 2A).

Our results show that the methods of isolation and fractionation of chloroplast envelopes developed for higher plants are applicable with some modifications to algae. As in pea and spinach, the lysis of *Chlamydomonas* chloroplasts previously equilibrated in a hypertonic solution allows the separation of the envelopes into a light and a heavy fraction on a linear sucrose gradient. This property and the results of the analysis by gel electrophoresis are in close analogy with data obtained for higher plants' envelopes. The light and heavy fractions of *Chlamydomonas* chloroplast envelopes described here possess the major features of preparations enriched in outer and inner envelope membranes.

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