RESOLUTION OF CHLOROPLAST LAMELLAR PROTEINS BY ELECTROPHORESIS IN POLYACRYLAMIDE GELS. DIFFERENT PATTERNS OBTAINED WITH FRACTIONS ENRICHED IN EITHER CHLOROPHYLL *a* OR CHLOROPHYLL *b*

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1. Introduction

Many experiments have been performed to analyse the lamellar proteins of chloroplasts; however these investigations have been hampered by the lack of suitable means for disrupting the lipophilic structures of the lamellae.

Several procedures for the solubilization of lamellar proteins have been reported utilizing solvents such as formic acid [1], or a mixture of phenol/formic acid/water [2] or various detergents [3–8, 13].

A solubilization of membranes is best obtained with an anionic detergent sodium dodecyl sulfate (SDS); consequently we have chosen this solvent for disrupting the lamellae of chloroplasts isolated from spinach, wheat and barley. The qualitative analysis of solubilized lamellar proteins was then performed by disc electrophoresis in polyacrylamide gels. From the three materials investigated, similar electrophoretic patterns of lamellae were obtained resolving about ten protein components.

To characterize these solubilized proteins in relationship with the two photosystems, fractions enriched in chlorophyll *a* or in chlorophyll *b* were prepared using Triton X 100, and were analysed after subsequent solubilization with SDS by disc electrophoresis in polyacrylamide gels. Proteins bands corresponding to the two kinds of fractions were obtained.

2. Materials and methods

2.1. *Plant material*

Wheat (Triticum sativum L., var. Florence aurore) and barley (Hordeum vulgare L. var. Ceres) seeds were washed in 10% sodium hypochlorite, rinsed with distilled water and germinated for 7 days in aquiculture on standard Arnon and Hoagland nutritive solution [9, 10]. Plants were cultivated under a 12 hr photoperiod at 4000 lux; the temperature was maintained at 20° and relative humidity at 70%. Spinach plants (Spinacia oleracea L. var. america) were grown under the same conditions with the exception of an illumination of 20,000 lux.

2.2. *Subcellular fractionation*

a) *Chloroplast isolation*: Chloroplasts were isolated from leaves after homogenization in a Waring blendor at full speed, three times 5 sec. The buffer system was: tris 5×10^{-2} M, $MgCl_2$ 5×10^{-2} M, Sorbitol 0.4 M, pH 7.5.

The slurry was filtered through six layers of gauze and one layer of blutex 50. The filtrate was centrifuged for 5 min at 200 *g* to remove nuclei and cell debris. Chloroplasts were isolated from the supernatant by sedimentation at 1,000 *g* for 10 min.

b) *Chloroplast lamellae preparation*: Chloroplast pellets were washed once by resuspension in the homogenization buffer and sedimented as described above. The isolated chloroplasts were then osmotically broken by two washings in the buffer system minus sorbitol and one washing in tris 0.1 M supplemented with mer-

captoethanol 5×10^{-2} M. After each washing, the membranes were sedimented at 30,000 *g* for 15 min. All the green material was recovered in the pellet after this centrifugation.

2.3. *Chloroplast membrane solubilization*

The degree of membrane solubilization by SDS was measured by a decrease in the absorbance at 530 nm as aliquots of 0.3 M SDS were added to 3 ml samples containing about 200 μg of chlorophyll (in tris–mercaptoethanol buffer). A membrane preparation is usually characterized by its protein concentration, but with chloroplast membranes it appeared to be easier to refer to the chlorophyll concentration.

2.4. *Preparation of membrane fractions enriched in chlorophyll a or chlorophyll b*

A procedure similar to the one described by Briantais [11] was employed. Wheat and spinach lamellae were treated with detergent Triton X 100 in the ratio Triton X 100/chlorophyll = 3 (w/w).

The detergent treated lamellae were then layered on a sucrose gradient 20–50% in the buffer system previously described. This gradient was centrifuged at 60,000 *g* for 1 hr.

After centrifugation, two well separated layers of chlorophyll lamellar fractions were obtained. The chlorophyll concentration of these fractions was determined spectrophotometrically. Absorption spectra of both fractions were recorded in the buffer solution at 20° on a Cary model 14 spectrophotometer fitted with a scattered light transmission attachment.

2.5. *Gel electrophoresis*

Gels were prepared and run according to the general method described by Davis [9] except spacer and sample gels were not used. Sucrose (0.25 M) was added to the samples to facilitate layering.

The gels contained 10% acrylamide, 0.1% SDS and were run at room temperature with a current of 8 mA per gel for 4 to 5 hr. They were stained and fixed by an immersion in a 0.25% solution of Coomassie brilliant blue in methanol–acetic acid–water (5:1:5, v/v/v) for at least 1 hr. The gels were then soaked in 7.5% acetic acid and 5% methanol for 15 min and finally destained electrophoretically in the same solvent. The electrophoresis buffer was sodium phosphate 0.1 M pH 7.2 plus 0.1% SDS (w/v). After destaining, the gels were scanned at 600 nm on a Joyce-Loeble densitometer.

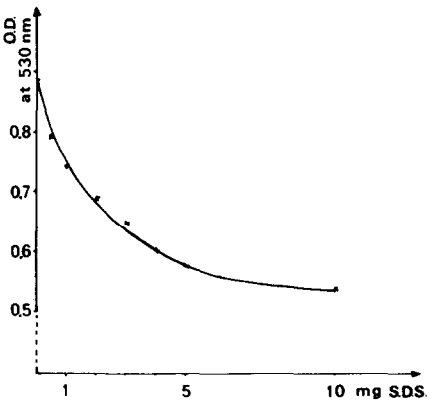

Fig. 1. Solubilization of chloroplast lamellae by SDS.

3. Results

3.1. *Solubilization of chloroplast lamellar proteins by SDS*

The solubilization of membrane proteins is followed by a drop in turbidity when incremental amounts of SDS are added to a lamellar suspension (fig. 1). A rapid decrease in turbidity is observed up to 1 mg of SDS per 200 μg chlorophyll, then a slow decline appears to reach a plateau at about 5 mg of SDS. At this SDS/chlorophyll ratio of 25, the solubilization is complete and when electrophoresis is performed little material remains at the origin of the gels. Moreover, chlorophyll is released from its protein complex and migrates separately.

3.2. *Electrophoretic patterns of chloroplast lamellar proteins from different sources*

Fig. 2 shows the densitometric determination obtained by SDS acrylamide gel electrophoresis of solubilized spinach, wheat and barley chloroplast membrane proteins. A number of features of these patterns are worth noting:

1) Patterns obtained from spinach, wheat and barley are similar, indicating the reproducibility of SDS action on the chloroplast membranes.

2) The heterogeneity of membrane proteins is revealed by the large number of separate components. Ten components can be observed. They are labelled from one (cathode side) to ten (anode side).

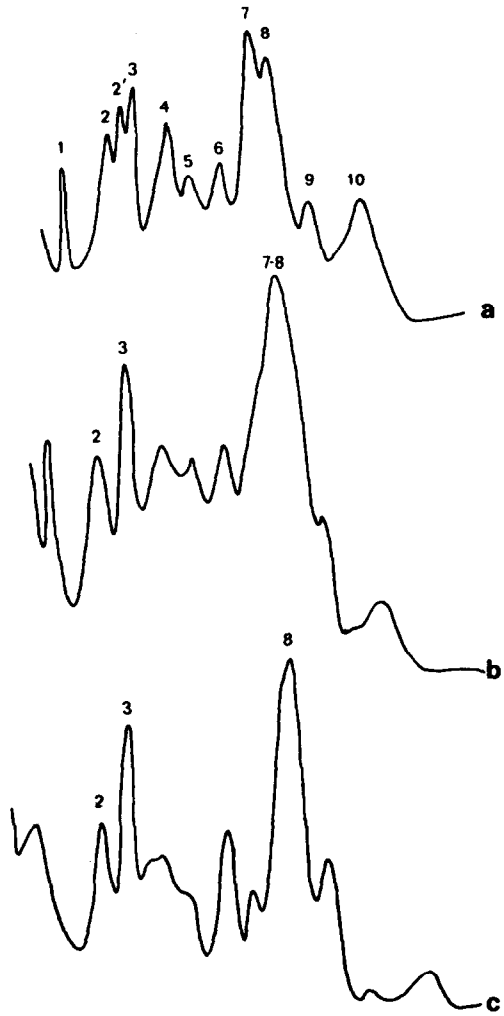

Fig. 2. Polyacrylamide gel electrophoresis of lamellar proteins. Densitometric tracings at 600 nm. Migration was from the origin (left) to the anode (right). (a) Spinach chloroplasts, (b) barley chloroplasts, (c) wheat chloroplasts.

3) Quantitatively two groups of major components corresponding to peaks 2, 2', 3 and 7, 8, 9, are represented in large amounts.

4) The major difference noted among the three sources of chloroplasts is the presence of an additional component (2') in spinach. Furthermore peaks 7 and 8 in wheat and barley appear to be superimposed.

These electrophoreses are performed with solubilized lamellae i.e. a mixture of proteins, lipids, polysaccharides and pigments. The effect of the removal of pigments by two successive extractions in acetone at $-20°$ is investigated for spinach lamellar suspensions. It is noted that this treatment by acetone weakens protein peaks 1, 2 and 7 (fig. 3), consequently the technique was no longer employed.

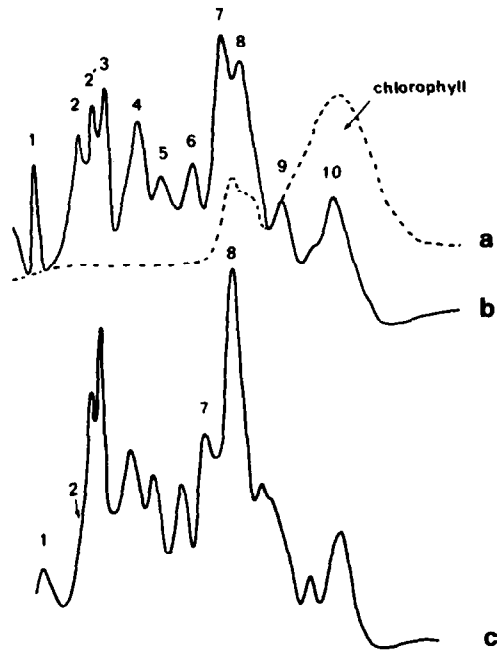

Fig. 3. Densitometric tracings of gel electrophoresis of SDS solubilized spinach lamellae. a) Before Coomassie blue staining, b) after Coomassie blue staining, c) after removal of pigments by acetone.

3.3. *Proteins associated with Triton fractions enriched in chlorophyll a or in chlorophyll b*

The identification of these proteins, their association with photosystem I and photosystem II poses certain problems. In order to elucidate this question lamellar fraction enriched in chlorophyll *a* or in chlorophyll *b* are prepared and analysed.

These fractions are obtained by action of Triton X 100 on wheat chloroplast membranes and isolated after sucrose density gradient centrifugation. Two fractions are obtained. The heavy fraction enriched in chlorophyll *b* presents chlorophyll *a*/chlorophyll *b* ratios varying between 1.9 and 2.3. The chlorophyll *a*/chlorophyll *b* ratios for the light fraction enriched

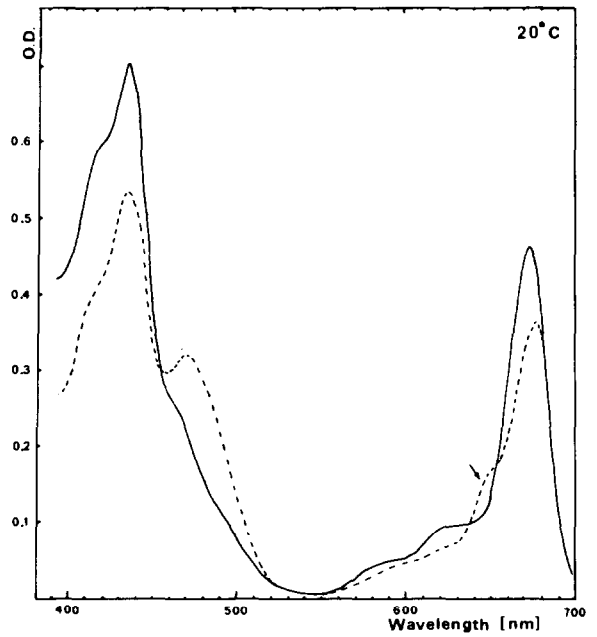

Fig. 4. Absorption spectra at 20° in phosphate buffer of Triton X 100 fractionated lamellae. —— light fraction; - - - heavy fraction.

in chlorophyll *a* are between 4.7 and 7.5. For untreated lamellae this ratio is about 3. Absorption spectra of each fraction are represented in fig. 4. The absorption bands of chlorophyll *b* located at 650 nm and 470 nm, are visible in the heavy fraction, whereas they do not appear in the light fraction (a slight shoulder in the 470 nm region is noted). In the blue region the two fractions have their maximum at 438 nm. In the red region the absorption maximum of the light fraction is at 675 nm; the heavy fraction is slightly shifted (3 nm) to the longer wavelengths.

The SDS solubilization of these fractions, followed by polyacrylamide gel electrophoresis gives quite different densitometric tracings (fig. 5). A comparison between the light fraction (fig. 5a) and the non-Triton treated lamellae (fig. 2c) reveals identical bands 3, 5, 6; however band 8 is decreased in the former. The heavy fraction behaves quite differently (fig. 5b), peaks 7, 8 are markedly represented as well as peak 2. The differences for the other components are less important.

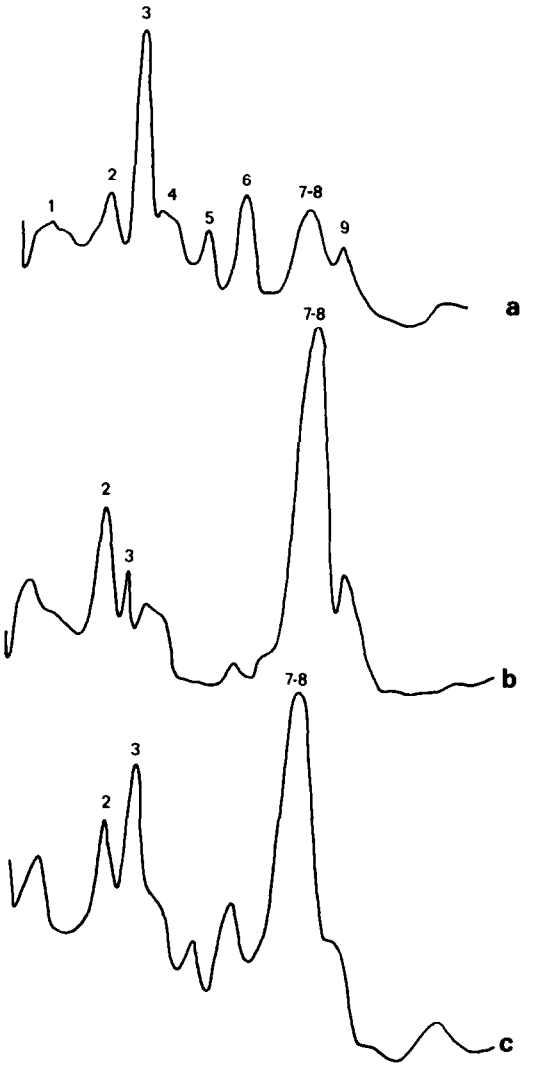

Fig. 5. Densitometric tracings of gel electrophoresis of: a) light fraction (enriched in chlorophyll *a*), b) heavy fraction (enriched in chlorophyll *b*), c) mixture of light and heavy fraction.

When the mixture of the two fractions is layered on the same gel (fig. 5c), a pattern similar to the one obtained with non-treated lamellae is recovered (fig. 2). Similar results have been obtained with spinach lamellae.

4. Discussion

The present study reveals an heterogeneity among lamellar proteins of chloroplasts. At least ten protein subunits are found. The heterogenous nature of a structural lamellar protein isolated from wheat and bean has already been reported by Mani and Zalik [13]. However, contrary to our findings, they did not obtain similar electrophoretic patterns with the two lamellar materials investigated.

Bailey and coworkers [3, 5], using an electrophoretic technique, have also reported the presence of chlorophyll–protein complexes related to photosystem I and photosystem II. In addition, they have noted several supplementary protein components which were attributed to stromatic contamination. However, in our case, it seems improbable that these protein components are due to stromatic contaminants in the lamellar preparations for the two following reasons. First, the lamellae fractions were purified by sucrose density gradient. Second, electrophoresis of SDS-treated stromatic proteins showed a very different pattern compared to the electrophoresis of SDS-treated lamellar proteins.

Little is known on the identity of electrophoresis separated lamellar proteins. It is likely that components 3 and 7, 8 are the chlorophyll associated proteins. In some experiments indeed some chlorophyll remained bound to these components (fig. 3-1(a) and 1(b)).

The purpose of this investigation is principally to report the qualitative differences between the proteins of fractions derived from photosystem I and photosystem II. Components 3, 5, 6 are found to be the major components of photosystem I while components 2 and 8 appear to originate from photosystem II.

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