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PHYSICOCHEMICAL STUDY OF STRUCTURAL PROTEINS OF CHLOROPLAST FROM *ZEA MAYS* L.

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SUMMARY

1. Structural proteins from maize chloroplasts were obtained in aqueous solution by a simple method using the ionic detergent sodium dodecyl sulfate. They were shown to be completely free from lipid and pigments by gas chromatography and spectrophotometry.

2. Gel electrophoresis, in the presence of the detergent, showed about ten bands, one being the most important with respect both to absorbance and nitrogen content (about 40 % of the total protein); its molecular weight was 25 000. Sephadex filtration allowed a partial separation, thus providing a good purification of the main component.

3. Both the crude solution and the purified fraction seemed to be homogeneous by ultracentrifugation analysis, with sedimentation coefficients of 2.8 and 2.3 S, respectively. The crude solution exhibited a high diffusion coefficient, probably related to a polydisperse associated system.

INTRODUCTION

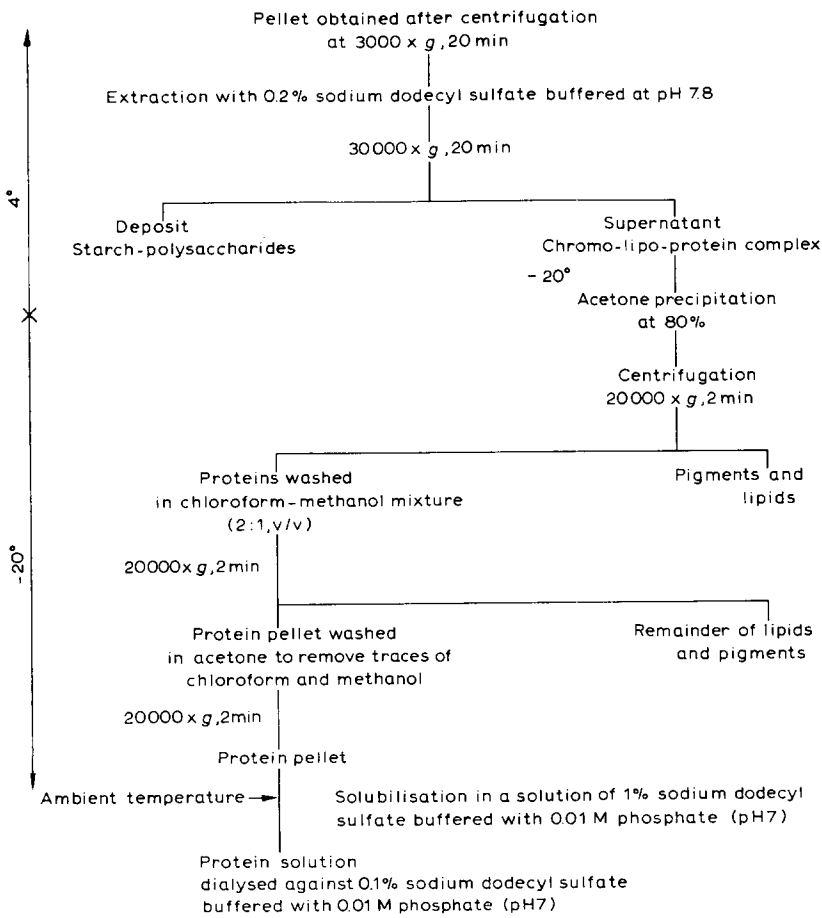
It is now well established that the photochemical reactions of photosynthesis occur in the lamellae and grana structures of the chloroplasts. The biological significance of these structures has given rise to much research in order to find out their biochemical composition[1,2]. Although the various pigments and lipids of these structures are already well known, there is little information on their constituent proteins. However, the protein fraction plays an essential part, as judged by its quantitative importance (50 % of the mass of lamellae) and by the fundamental role allotted to it. This lack of information is, on the whole, due to the insolubility of these proteins in an aqueous medium when they are entirely freed of their associated lipids and pigments. Although it was first considered that only one constituent protein existed in lamellae structures, it soon became evident that several proteins were present, at least ten according to the results of MENKE AND JORDAN[3] and BOQUET *et al.*[4]. Several methods have been described which enable a fraction freed of its lipids and pigments to be solubilized, but not all the lamellar proteins are obtained[5–7]. The purpose of this work was to obtain the complete solubilisation of such proteins, and to establish some

of their physicochemical characteristics in order to follow methodically the separation of the various components.

MATERIALS AND METHODS

Zea mays L grains (INRA 260 variety) were made to germinate in mould in a glass house at a temperature of 25° in day light. The young plantlets were lifted 12 days later at the three-leaf stage.

The plastid structures were separated at 4° by grinding the limbs in a mortar with sand in a solution of 0.35 M NaCl buffered with 0.01 M phosphate at pH 7.8. The ground material was then filtered through cloth and centrifuged for 20 min at 3000 × *g* in order to obtain a deposit containing mainly plastid structures. The deposit was washed 3 times with the original buffer solution to eliminate the soluble substances still present[8]. The protein components were then extracted from these structures by the method shown in Scheme 1.



Electrophoreses were carried out in polyacrylamide gel by the method described by SHAPIRO *et al.*[9] and systematically repeated by WEBER AND OSBORN[10] for determining molecular weights. The gels which gave the best separation contained 10 % acrylamide, 0.25 % bisacrylamide and 0.1 % sodium dodecyl sulfate (the sodium dodecyl sulfate was recrystallised in absolute ethanol, thus giving better results). The gels were 50 mm long and 5 mm in diameter. Migration occured under a current of 4 mA per tube for 4 h. The electrophoresis buffer was a 0.1 M phosphate buffer with 0.1 % sodium dodecyl sulfate at pH 7.2. The gels were stained with Coomassie blue, which was the most efficient stain, and destained with a mixture of 30 % methanol, 7 % acetic acid and water in the presence of a Dowex-type ion exchange resin. The same gels were used for analytical purposes as well as for the molecular weight determinations. In the latter case, the mobility of a component was calculated in relation to a migration indicator which in this case was bromophenol blue[10].

$$\text{Mobility} = \frac{\text{distance of protein migration}}{\text{lenght after destaining}} \times \frac{\text{length before staining}}{\text{distance of dye migration}}$$

The sedimentation analyses were performed in a Spinco E analytical ultracentrifuge, fitted with an AN-D rotor. The determinations were made in a synthetic boundary cell at 59780 rev./min and 10°. Two types of film were taken simultaneously with a Schlieren optical system and by ultraviolet absorption.

Diffusion coefficients were calculated according to the method of EHRENBERG[11].

Specific volumes were determined with an Anton PAAR microdensitometer. Gas chromatography of the lipids was performed on a 1520 B aerograph. The protein solution was refluxed for 24 in chloroform–methanol (2:1, v/v). After evaporation of the solvent, the extract thus obtained was taken up in hexane, saponified and the fatty acids collected were then analysed.

Detection of the reducing sugars was performed according to the method of NELSON[12], after solutions had been hydrolyzed with 1 M HCl for periods of 1–18 h at 100°.

Protein nitrogen was determined after wet ashing by the Kjeldhal method.

RESULTS

The first objective was to solubilise in an aqueous medium all the proteins of the plastid structures. The nitrogen determinations of the protein solution obtained by the method previously described showed that almost all the proteins were thus solubilised, since over 95 % of the initial protein nitrogen was found in the final solution.

The absorption spectrum of the protein solution showed a complete absence of pigments. Fig. 1 shows the presence at pH 7 of an absorption maximum at 278 nm, followed by a marked shoulder at 291 nm. At pH 12, two maxima appear at 275 and 283 nm and the shoulder at 291 nm is raised (tyrosine ionisation); a slight absorption is observed at 340 nm, and a hump at 410 nm. These latter results are in agreement with the observations of BIGGINS AND PARK[13] on protein extracts from spinach chloroplasts.

Gas chromatographic analysis confirmed the almost complete absence of lipids in the preparations. In a solution containing 70 mg of proteins, a maximum 300 μg

of fatty acids was detected, thus representing less than 0.5 % of the initial lipid content (based on LICHTENTHALER AND PARK'S[1,2] calculations). A maximum content of 3 % of reducing sugars was found with regard to a glucose scale.

The protein solution, brought to 2.5 mg of proteins per ml, was analysed directly by electrophoresis on a polyacrylamide gel. The pattern obtained is shown in Fig. 2.

It is important to stress that no stain remains at the start of the gel column.

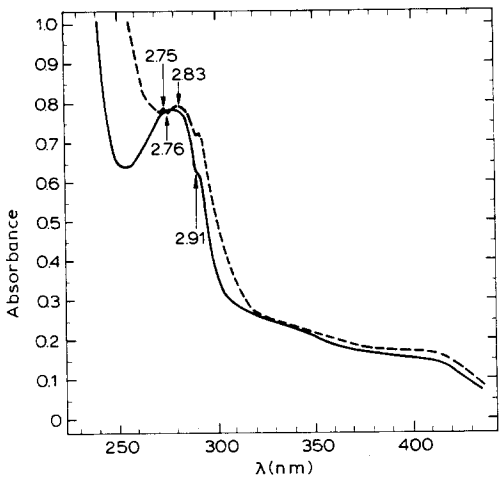


Fig. 1. Absorption spectrum of proteins in a 0.1 % sodium dodecyl sulfate solution, 0.01 M phosphate buffer pH 7 (——) and pH 12 (---). The protein content is 0.55 mg/ml.

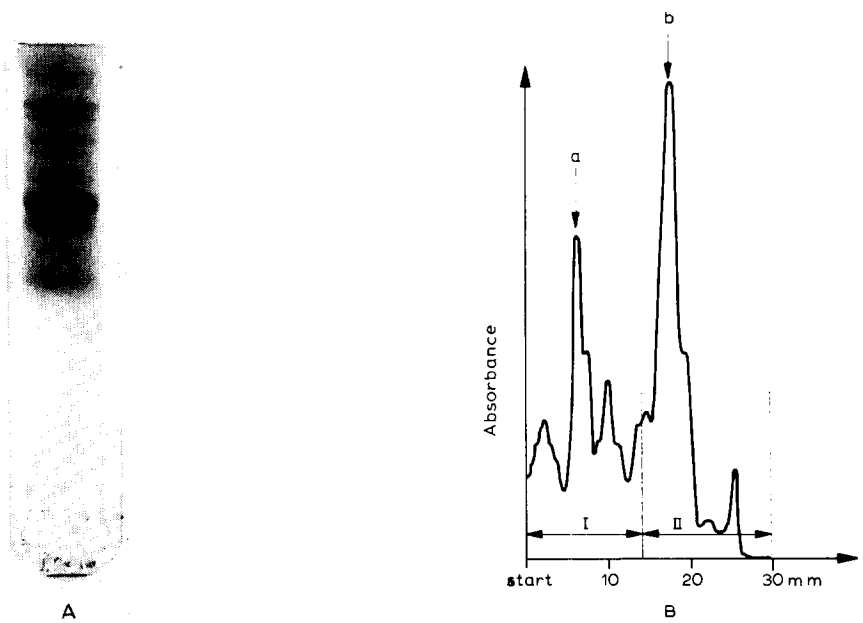


Fig. 2. Gel electrophoresis of chloroplast lamellae proteins. A. Pattern after staining with Coomassie blue. B. Densitogram of the protein pattern recorded on a Chromoscan 5-11 apparatus.

The analysis of ^{14}C labeled proteins also showed that 90 % of the radioactivity of the sample was found in the gel, confirming that the proteins had effectively entered.

Of the ten bands observed, two, the *a* and *b* bands, were more densely stained. According to the absorbance readings, they represented 17 and 40 %, respectively, of the total proteins (staining with Coomassie blue being proportional to the amount of proteins).

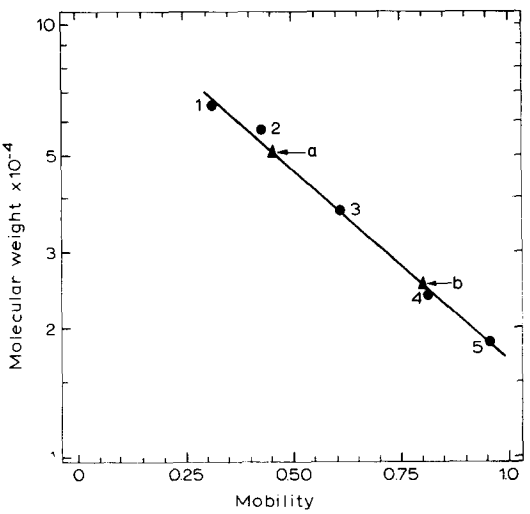
Band *a* is narrow, very clear and highly coloured; Band *b* the most important one, is large, its limits less clear and is equally intense. Appropriate tracers were used to determine the molecular weights of these various bands (Fig. 3).

The main band, *b*, had a molecular weight in the order of 25 000, whereas that of Band *a* was estimated to be about 50.000. The very fast front band, which migrates more rapidly than cytochrome *c*, has a molecular weight of about 10 000.

The protein solution was studied by analytical ultracentrifugation in order to confirm its heterogeneity, and the molecular weight of the various components.

As will be seen from Fig. 4, the proteins migrated as a single peak. Its sedimentation coefficient varied with the protein concentration, and was estimated to be 2.8 S by extrapolation to zero concentration (Fig. 5). This sedimentation peak exhibits a high rate of diffusion. The diffusion coefficient, calculated from the Schlieren peaks by extrapolation at zero concentration was $18 \cdot 10^{-7}$ cm²/sec. It was noted that, during the experiment, the area under the Schlieren peaks remained constant, indicating that no large aggregates sedimented from the boundary.

By filtering through Sephadex G-200, the protein solution was separated into



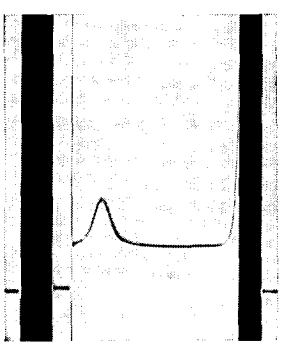


Fig. 3 Curve of electrophoretic mobility as a function of molecular weight. The controls used at a concentration of 2 mg/ml are, 1, bovine serum albumin, 2, pyruvate kinase, 3, yeast alcohol dehydrogenase, 4, trypsin, 5, lactoglobulin.
a and b represent the main bands of the plastid structures (Fig. 2).

Fig. 4. Sedimentation velocity pattern of maize chloroplast structural proteins in 0.1 % sodium dodecyl sulfate, 0.1 % NaCl, pH 7 at 20°. Picture taken in a synthetic boundary cell after 24 min at 59780 rev./min.

two peaks, I and II (Fig. 6). An examination of the elution profile at 280 nm showed these two peaks to be asymmetrical, which probably reflects their heterogeneity.

Electrophoresis on polyacrylamide gel confirmed the presence of the heaviest molecules in the first elution peak, whereas the lighter molecules, Band *b* and following, were in the second peak (Fig. 2B)

By taking the top part only of the second peak, it was possible to obtain a very slightly contaminated band *b*.

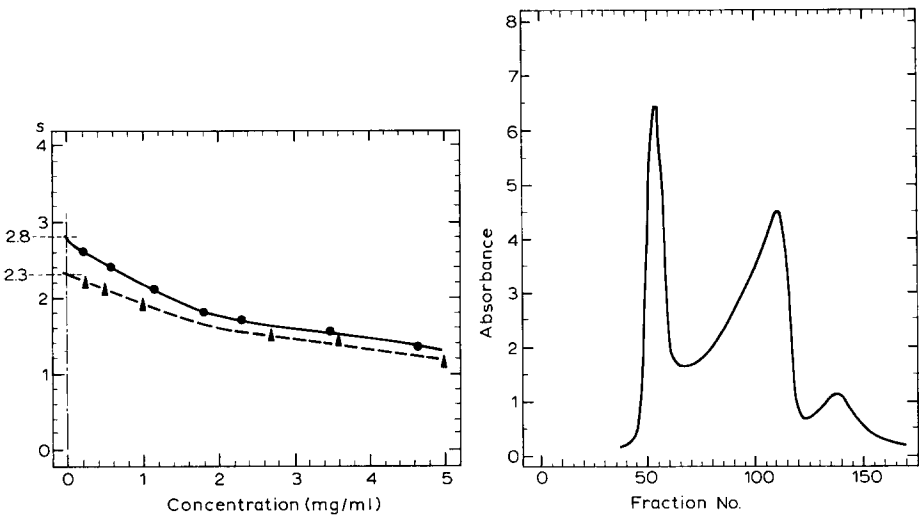


Fig. 5. Sedimentation coefficient curves as a function of the protein content, for the crude solution (●) and for Fraction II obtained after filtering with Sephadex G-200 (▲).

Fig. 6. Elution profile obtained by filtering the crude solution of the structural proteins with Sephadex G-200. The elution buffer was 0.1 % sodium dodecyl sulfate, 0.1 % NaCl and 0.01 M phosphate buffer at pH 7.

Ultracentrifugation analyses were made on Fraction II of the crude solution (second peak obtained with G-200). They gave an $s^{\circ}_{20,w} = 2.3$, less therefore than that of the crude solution. The diffusion coefficient, which was also less, was $10 \cdot 10^{-7}$ cm^2/sec (Fig. 5).

DISCUSSION

Since the work of SMITH[14], the use of detergents for studying structural proteins, either free or associated with lipids and pigments, has grown considerably. The method developed here makes use of sodium dodecyl sulfate, and is akin to that described by BIGGINS AND PARK[13], however, we obtained complete solubilisation of the precipitated proteins, whereas BIGGINS AND PARK only attained 75 %. Our result may be explained by the very good lipid extraction by the chloroform–methanol step, since, if this stage is omitted, solubilisation drops to around 80 %.

With all the plastid structural proteins in aqueous solution at our disposal, we were able to make a valid analytical study of all of them. On the assumption that

each band was pure, since the entire sample enters the gel, there should be about ten different proteins. This number is higher than those found in the literature.

The electrophoresis made by MANI AND ZALIK[7] on wheat and bean chloroplasts exhibited two and five components, respectively. Many investigations have also been made on lipid-containing protein structures; THORNBERG *et al.*[15] in particular obtained a picture close to ours for spinach chloroplasts, with two predominating complexes. During a previous investigation, THORNBERG *et al.*[16] found six bands after electrophoresis of a fraction of lamellae proteins of the same origin. A part of these proteins was ascribed to fraction I of the chloroplasts. BRAUNITZER AND BAUER[17] essentially found three protein bands in spinach and observed a great similarity between bands from higher plants. WEBER[5], working on *Antirrhinum majus*, found only one migration band. It should be emphasised that, in most of these experiments, solubilisation is only partial and part of the sample is retained at the start of the gel, thus a complete picture of all the structural proteins is not given.

In our case, two of the components were more important, one of which, clearly predominating, represented 40 % of total proteins. The studies previously undertaken on maize structural proteins by BOQUET *et al.*[4] and COLLOT *et al.*[18] showed that there was a particular frequency in the distribution of the N- and C-terminal amino acids, alanine and glycine, respectively, being the most abundant with frequencies in the order of 40 %. It therefore seems likely that these two amino acids in fact represent the ends of this predominant protein.

The more or less purified "structural protein" from both mitochondria and chloroplasts, frequently represent, quantitatively, 50 % of the total structural proteins (CRIDDLE[19]). This result agrees well with our result.

The data obtained with ultracentrifugation showed a fall in the sedimentation coefficient from 2.8 to 2.3 S ,from the crude solution to fraction II. This tallies with the electrophoretic data, since it is logical to assume that mean molecular weight of Fraction II, which contains the lightest components, is less than that of the total solution. The small discrepancy between the two sedimentation coefficients may be explained (a) by the absence of high molecular weight substances and (b) by the predominant protein in both solutions.

Although the crude solution is heterogeneous under electrophoresis, it sediments as a single peak. This could be due to the strong association of the various components, as well as the presence of a dominant protein which, because of its importance, masks the weaker protein peaks. It should be noted that the very high diffusion coefficient of this solution ($18 \cdot 10^{-7}$ cm^2/sec) probably reflects this associated heterogeneity.

The diffusion coefficient of Fraction II is lower ($10 \cdot 10^{-7}$ cm^2/sec) showing that a purer product is present, which is confirmed by electrophoresis. The specific volume is 0.722 cm^3/g. which gives a molecular weight of 22 000 $\pm$ 2000g/M by Svedberg's equation. This value agrees perfectly with the results of BIGGINS AND PARK[15] who found a molecular weight of 22 000 g/M for the proteins of spinach chloroplasts, and also with those of CRIDDLE AND PARK[6] who used a different method of preparation of the same material, and obtained a molecular weight of 23 000 g/M. The sedimentation coefficients found by MANI AND ZALIK[7] for wheat and bean of 1.3 and 1.2 S, respectively, are very different. These authors used a medium containing 1 % sodium dodecyl sulfate and the significant effect of the detergent concentration on sedi-

mentation must be stressed here. SMITH AND PICKELS[20], using sodium dodecyl sulfate concentrations of 0.25 to 2.5 %, found a variation of 2.32 to 1.69 S in the sedimentation coefficient. Likewise ITOH *et al.*[21] noticed, in the case of a spinach protein–pigment complex, a change of 3.5 to 1.9 S in the sedimentation coefficient with dodecyl benzene sulfonate concentrations of $2.9 \cdot 10^{-3}$ M to $2 \cdot 10^{-2}$ M. Furthermore, a lighter component appeared (1.2 S) at concentrations below $2.5 \cdot 10^{-2}$ M. It is therefore necessary to interpret the sedimentation coefficient values obtained in the presence of detergents with extreme caution.

The results from this study show that the plastid structures of maize contain a large number of distinct proteins which have a strong tendency to associate, as shown by ultracentrifugation. This property must play a part in the building of the plast structures.

There is, however, a predominant amount of a partially purified component with a relatively low weight in the region of 25 000 g/M.

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