BBA 4156

ISOLATION OF WATER-SOLUBLE CHLOROPHYLL PROTEIN FROM THE LEAVES OF *CHENOPODIUM ALBUM*

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(Received March 26th, 1963)

SUMMARY

1. A water-soluble, photosensitive chlorophyll protein was obtained from the leaves of *Chenopodium album*.

2. The substance, purified by ammonium sulfate fractionation and column chromatography, showed a single-peaked sedimentation pattern on ultracentrifugal analysis.

3. The native chlorophyll protein (prepared in the dark) showed absorption bands at 277, 429 and 667 mμ. Irradiation caused a marked change of the spectrum. New peaks appeared at 362, 399, 567 and 743 mμ.

4. The chlorophyll protein contained chlorophylls *a* and *b*. The irradiated protein had three additional pigments which were not found in the native one.

INTRODUCTION

Attempts have been made to isolate chlorophyll in the state in which it exists in living green plants. Among various achievements that have been made along this line of approach is the isolation of chloroplastin and protochlorophyll holochrome. The former is a real chlorophyll protein obtained by treating green leaves with digitonin[1] or other detergents. The substance has been purified to give a single peak on ultracentrifugal analysis. Since the molecular weight of chloroplastin is reported to be as high as 240000 (see refs. 2 and 3) and the dispersion of the substance has only been obtained in the presence of detergent, it would be reasonable to consider that the substance obtained is still far from being the ultimate unit of the chlorophyll-protein combination in the chloroplasts. Protochlorophyll holochrome isolated and purified from etiolated leaves belongs to the same category[4,5].

Recently, we have isolated from fresh leaves of *Chenopodium album*, a water-soluble protein containing chlorophyll as the pigment component. This substance is very sensitive to light and changes its absorption spectrum even in diffuse light. The method of purification and the properties of the new substance will be briefly described in the following.

METHODS

Preparation of chlorophyll protein

To obtain the chlorophyll protein in its native state, care must be taken to work under weak green light during the course of the following procedures.

5 kg of fresh Chenopodium leaves were homogenized in 3 l of 0.01 M disodium phosphate solution. Rough fragments were filtered off through gauze and the green juice thus obtained was centrifuged at 10000 rev./min in a continuous flow centrifuge. Acidification, at this step, to pH 5.0 by addition of about 15 ml acetic acid facilitates sedimentation, without any deteriorative effect on the final product. The somewhat turbid liquid thus obtained was made 0.3 saturated with ammonium sulfate and filtered on a Buchner funnel with a thin layer of kieselguhr as filter aid. The filtrate was made 0.6 saturated with ammonium sulfate and filtered again in the same way to collect the precipitate. The filtrate was discarded and the precipitate was dissolved in 250 ml phosphate buffer (pH 7.8). This solution showed, on 50-fold dilution, an absorbancy of 0.8–1.0 at 667 mμ in a 1-cm cell. This brown solution was dialyzed against 0.01 M phosphate buffer (pH 7.8) for 24 h in a cold room, for chromatography. A column (diameter 2 cm, length 10 cm) of Amberlite CG 50, equilibrated with 0.01 M phosphate buffer (pH 7.8) was found to be most suitable for this purpose. Beneath the uppermost brown zone, a green zone was formed which was washed in turn with 0.01 M and 0.02 M buffer solutions (pH 7.8) and then eluted with 200 ml 0.5 M solution of the same buffer. This green solution showed, on 50-fold dilution, an absorbancy of 0.7–1.0 at 667 mμ in a 1-cm cell. After this, the ammonium sulfate fractionation was repeated and the fraction precipitating between 0.3- and 0.6-saturation was dissolved in 0.05 M phosphate buffer (pH 7.8) and stored in the dark in a frozen state.

Chromatography of pigment components

The pigment components were extracted with 80 % acetone from the chlorophyll protein in its native and irradiated states, and analyzed by paper chromatography according to JEFFREY's method[6], slightly modified, with petroleum benzin–isopropyl alcohol (98 : 2, v/v) as developing solvent.

RESULTS

Ultracentrifugal analysis

The analysis was carried out with a Spinco Ultracentrifuge Model E at 14°, using a solution of the purified sample dissolved in and dialyzed against 0.2 M NaCl solution.

A single-peaked sedimentation pattern was obtained indicating the homogeneous dispersion of the substance in solution. From the observed rate of sedimentation, the sedimentation constant was calculated to be $s'_{20} = 2.70$. On visual inspection, the upper limit of green color was found to migrate with the sedimentation peak of the substance in the Schlieren figure (Fig. 1).

The relatively small size of the molecule is inferred from the above data, and also from the rather high dispersibility of the substance as shown by the rapid flattening of the Schlieren peak observed during the ultracentrifugation. The solubility and stability in plain aqueous media, *i.e.* in the absence of any detergent, are striking

characteristics of this chlorophyll protein. Neither repeated precipitation with ammonium sulfate nor various chromatographic treatments affect the solubility of the substance.

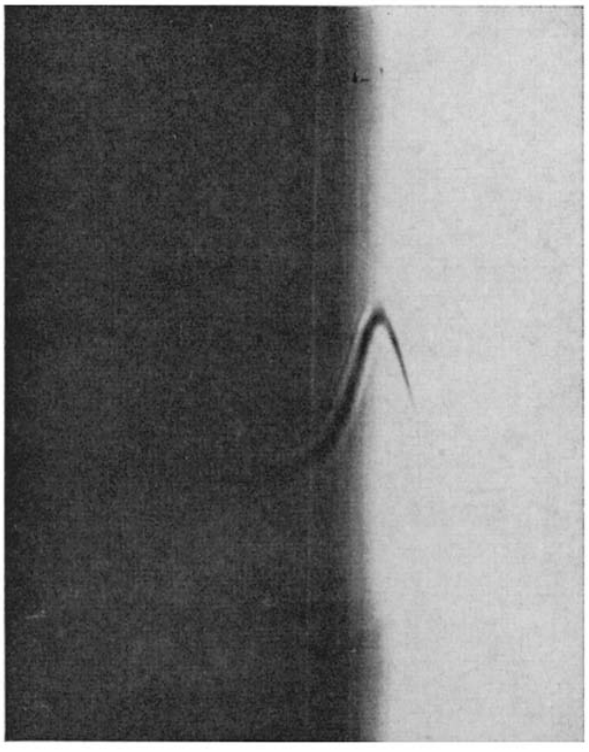

Fig. 1. Sedimentation figure of chlorophyll protein 667. Chlorophyll protein prepared in the dark. Photograph taken 32 min after attaining the maximum speed of 59000 rev./min.

Absorption spectra

The absorption spectrum of the native chlorophyll protein, measured with a Cary Spectrophotometer Model 14, showed absorption maxima at 277, 429 and 667 mμ (Fig. 2). The absorption at 277 mμ was probably due to the protein content; the maxima at 429 and 667 mμ represented respectively, the Soret- and α-bands of the

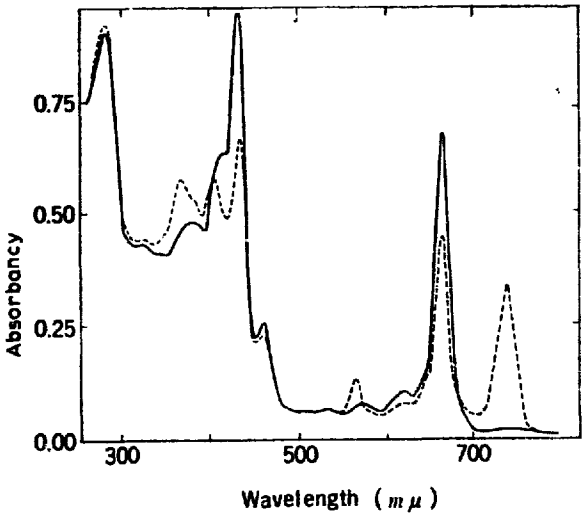

Fig. 2. Absorption spectra of chlorophyll proteins 667 and 743. ———, prepared in the dark; ------, irradiated for 30 sec with 10000-lux light.

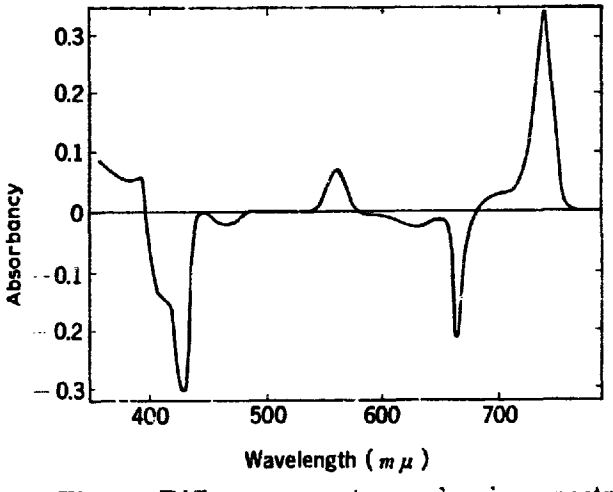

Fig. 3. Difference spectrum showing spectral changes on irradiation for 30 sec with 10000-lux light.

chlorophyll complex. Of special interest in connection with this chlorophyll protein was its photo-sensitivity. Irradiation with 10000-lux light for 30 sec caused a marked change of the spectrum. New peaks appeared at 362, 399, 567 and 743 mμ, while the α- and the Soret-bands diminished considerably in intensity (Figs. 2 and 3). Further illumination (*e.g.* 2 min) makes the 743-mμ absorption even greater than that at 667 mμ.

Chromatophore groups of chlorophyll protein

Addition of alcohol (or acetone) to the solution of the chlorophyll protein changed its absorption spectrum at alcohol concentrations above 50%. The maximum at 667 mμ shifted to 663 mμ. In the case of the irradiated preparation, the peaks at 567 and 743 mμ disappeared. The absorption spectrum of the alcoholic extract is shown in Fig. 4. It differed from the absorption curves of both the native and irradiated forms of the protein and those of the pure chlorophylls *a* and *b* dissolved in the same solvent (80% methanol). It must be noted that the pigments in this complex seemed to be rather firmly attached to the protein moiety, their complete removal being attained only after repeated extraction with (cold) methanol. The results of paper chromatographic analysis of the acetone extracts of the native and irradiated chlorophyll proteins are shown in Fig. 5a, b. The presence of several pigment components besides chlorophylls *a* and *b* was thus discovered. No distinct spot identifiable as carotenoid was recognized in the chromatograms.

Saponification of the extracted pigments in etheric solution, followed by washing with water, also failed to demonstrate the presence of carotenoids in the resulting etheric phase. Neither was there found any absorption corresponding to that of phytofluene.

Each spot was eluted with acetone and examined spectrophotometrically. Spots 1

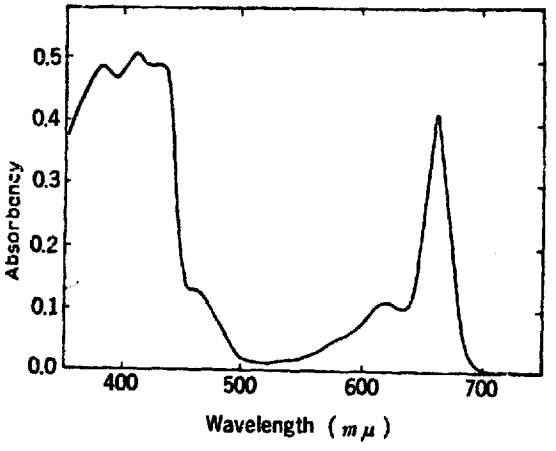

Fig. 4. The absorption spectrum of 80% methyl alcoholic extract from chlorophyll protein prepared in the dark.

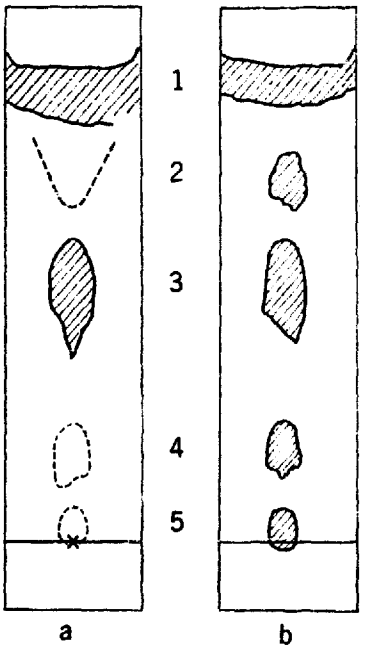

Fig. 5. Paper chromatograms of the pigments obtained from chlorophyll proteins, (a) prepared in the dark and (b) irradiated.

and 3 in both chromatograms were shown to represent chlorophylls *a* and *b* (Fig. 6). The irradiated sample showed three additional spots (Spots 2, 4 and 5) which did not appear in the native material. Among these, Spot 5 was the most distinct; it was bluish in color, showing absorption maxima at 353, 568 and 735 mμ. These may have been responsible for the absorption peaks at 567 and 743 mμ of the irradiated chlorophyll protein. The color of Spots 2 and 4 was fainter, with absorption maxima at 690 and 650 mμ, respectively. A pale green spot was also visible in the chromatogram of the native sample at the position corresponding to Spot 2 of the irradiated one. The absorption of this pigment was found to be similar to that of chlorophyll *a*.

The detailed characterization of the spectral properties of these pigments and elucidation of their chemical nature will be continued in the coming season when abundant material will become available.

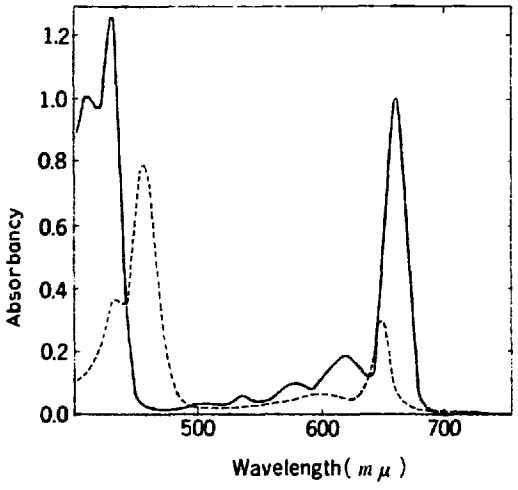

Fig. 6. Absorption spectra of chlorophylls *a* and *b* in acetone extracts obtained from Spot 1 and Spot 3 of the paper chromatogram (Fig. 5, a). ———, Spot 1 (chlorophyll *a*); -------, Spot 3 (chlorophyll *b*).

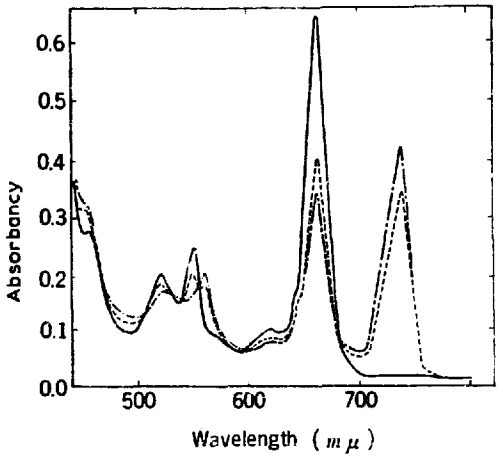

Fig. 7. Photoxidation of cytochrome 553 with chlorophyll protein. ———, dark (with cytochrome absorption at 553 mμ), -------, irradiated (1 min); –·—·–, irradiated (2 min).

Other constituents

The protein nature of the substance was indicated by positive results in ninhydrin and Biuret tests, as well as by a prominent absorption peak at 277 mμ. The protein residue obtained by repeated extraction of the chlorophyll protein with 80 % alcohol or acetone was pale brown. A part of this residue was soluble in 0.05 M disodium phosphate solution. The solution showed, when reduced with dithionate, absorption maxima at 563, 533 and 428 mμ. The absorption spectrum resembled that of cytochrome b_6 reported to exist in the chloroplasts[7,8]. We could not separate this substance from the chlorophyll protein either by column chromatography or ammonium sulfate fractionation. The final decision as to whether this heme protein represented an actual component of the chlorophyll protein or merely a contamination must await further investigation.

Photoxidation of algal cytochrome 553

The chlorophyll protein catalyzed the photoxidation of the algal cytochrome 553. (KATOH[9] has observed a similar photoxidation of his Porphyra cytochrome 553 in the

presence of chromatophore fragments of the same alga.) The solution of the reduced cytochrome (crystalline sample obtained from *Porphyra tenera*[10]) was exposed to light in the presence of the chlorophyll protein. The absorption of the cytochrome at 553 mμ disappeared; at the same time the peak of the irradiated form of the chlorophyll protein appeared at 567 mμ (Fig. 7). The irradiated chlorophyll protein was found to be equally effective in photoxidizing the cytochrome.

DISCUSSION

All the above-described data indicate that the green substance under investigation was a chlorophyll protein of relatively low molecular weight in a state of homogeneous molecular dispersion.

The name "Chlorophyll protein 667" is proposed for the new substance according to the usual way of denoting various chlorophyll components of the chloroplasts. The illuminated product will be denoted as "Illuminated chlorophyll protein 743". There is a possibility that this substance represents one of the various states in which chlorophyll is known to exist in living chloroplasts (FRENCH[11-14], ALLEN[15], KOK[16,17], THOMAS[18]). Its isolation marks the first case in which a genuine chlorophyll protein is obtained in a water-soluble form without the use of any detergent or organic solvent. Investigation of the photochemical changes of the chlorophyll protein, as well as of the possibility that it participates in various light-induced reactions of chloroplasts, are in progress.

ACKNOWLEDGEMENTS

We want to express our hearty thanks to Professor A. TAKAMIYA and Dr. S. KATOH of the University of Tokyo and Professor K. OKUNUKI of the University of Osaka for their kind help and advice. We are also indebted to Professor H. TAMIYA for his encouragement during this work.

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