Biochimica et Biophysica Acta, 635 (1981) 341—347

BBA 48058

CHEMICAL, PHYSICOCHEMICAL AND SPECTROPHOTOMETRIC PROPERTIES OF CRYSTALLINE CHLOROPHYLL-PROTEIN COMPLEXES FROM *LEPIDIUM VIRGINICUM* L.

TERUYO MURATA [a,*] and CHIKAKO ISHIKAWA [b]

[a] *Department of Chemistry, Faculty of Science and* [b] *Department of Hygienic Chemistry, School of Pharmaceutical Science, Toho University, Miyama 2-2-1, Funabashi, Chiba 274 (Japan)*

(Received July 15th, 1980)
(Revised manuscript received November 3rd, 1980)

Key words: Chlorophyll-protein purification; Crystallization; Molecular weight determination; Absorption spectrum; (Lepidium virginicum)

Summary

Two kinds of water-soluble chlorophyll-protein complexes were prepared from leaves of *Lepidium virginicum* L., one (CP661) from the plant cultivated in a green house from seeds collected near Mono Lake, CA, and the other (CP-663) from a plant collected at Narashino, Chiba, Japan, by ammonium sulfate fractionation followed by column chromatography on DEAE-cellulose and Sephacryl S-200. The chlorophyll · proteins were further purified by crystallization. CP661 has absorption peaks at 661, 468, 439, 419, 380, 339 and 272 nm. CP663 had absorption peaks at 663, 469, 438, 419, 379, 338 and 272 nm. Estimated molecular weights were 78 000 for CP661 and 80 000 for CP663 by gel filtration chromatography and 83 000 for CP661 and 107 000 for CP663 by an equilibrium sedimentation method. 1 mol chlorophyll · protein contained 4 mol chlorophyll *a* and *b* with ratios of 1.0 in CP661 and 1.6 to 1.9 in CP663, but no carotenoids. These characters are different from those of chlorophyll-protein complexes which are prepared from the thylakoid membranes of chloroplasts with detergents.

Introduction

A water-soluble chlorophyll · protein was first prepared from *Chenopodium album* by Yakushiji et al. [1]. The chlorophyll · protein is called Chenopodium

* To whom correspondence should be addressed.

CP668 according to a red absorption peak at 668 nm. It contains chlorophyll *a* and *b* but no carotenoids. Light illumination converts its absorption spectrum, depressing bands at 429 and 668 nm and producing new bands at 362, 399, 567 and 743 nm.

A different type of chlorophyll · protein was prepared from inflorescence of cauliflower [2]. The protein has an absorption peak at 674 nm. The absorption spectrum is insensitive to light illumination. Chlorophyll *a* to *b* ratio is 6. The same type of chlorophyll · protein was prepared from leaves of *Brassica nigra* (wild mustard) [3].

We prepared another type of chlorophyll · protein with a red absorption peak at 661 or 663 nm from *Lepidium virginicum* L. [3]. These proteins are crystallizable [4], and insensitive to light illumination [3]. An analysis of the absorption spectrum in the red region in the curve-fitting method [5] reveals that the chlorophyll · protein contains only three chlorophyll forms, Cb650, Ca662 and Ca670, according to the terminology of French et al. [6].

In this paper, chemical, physicochemical and spectrophotometric properties of the chlorophyll · proteins of *Lepidium virginicum* L. are studied.

Materials and Methods

Leaves were collected from *Lepidium virginicum* L. which was cultivated in a green house from seeds collected near Mono Lake, CA, U.S.A. Leaves (1 kg) were homogenized with a Waring blender in 1 l of 0.1 M phosphate buffer *. The homogenate was filtered through three layers of cheese-cloth. Proteins in the green filtrate were fractionated with $(NH_4)_2SO_4$. A fraction precipitating between 40 and 90% saturation of $(NH_4)_2SO_4$ was collected by centrifugation at 13 000 × *g* for 10 min. The pellet obtained was dissolved in 200 ml of 0.13 M phosphate buffer and dialyzed against 4 l of the same buffer solution. The dialyzed solution was passed through a DEAE-cellulose column (3 cm diameter × 5 cm high) which had been equilibrated with 0.13 M phosphate buffer. The effluent was diluted with 2 vol. distilled water, and was applied to another DEAE-cellulose column (3 cm diameter × 11 cm high) which had been equilibrated with 0.04 M phosphate buffer. The chlorophyll · protein was adsorbed on the column underneath a brown layer at the top. After the column was washed with 400 ml of 0.05 M and then 200 ml of 0.1 M phosphate buffer, the chlorophyll · protein was eluted with 0.2 M phosphate buffer. The solution obtained (200 ml) was made 60% saturation of $(NH_4)_2SO_4$ and centrifuged at 13 000 × *g* for 10 min. The pellet was discarded, and the supernatant was made 90% saturation of $(NH_4)_2SO_4$ and centrifuged at 13 000 × *g* for 10 min. The pellet obtained was dissolved in 5 ml of 0.1 M phosphate buffer and centrifuged at 13 000 × *g* for 15 min to remove insoluble materials. The supernatant was applied to a column of Sephacryl S-200 (2.5 cm diameter × 85 cm high) which had been equilibrated with 0.1 M phosphate buffer. The chlorophyll · protein was eluted with the same buffer solution, and fractions having an A_{662nm}/A_{272nm} ratio higher than 1.0 were collected. The protein solution thus

* Except otherwise stated, the phosphate buffer used throughout the present study was a sodium-potassium phosphate buffer, pH 7.2.

obtained was made 90% saturation of $(NH_4)_2SO_4$. The precipitate was collected by centrifugation at 13000 × *g* for 15 min and dissolved in 0.1 M phosphate buffer (3 ml). After dialysis of the green solution against 90% saturation of $(NH_4)_2SO_4$ (300 ml) at 5°C for 20 days, crystals of the chlorophyll · protein formed. The crystals, dark green in colour, were contaminated by white amorphous precipitate. By repeated centrifugation at 1000 × *g* for 3 min and resuspension in 90% saturation of $(NH_4)_2SO_4$, the amorphous precipitate was removed. Then, the crystallized chlorophyll · protein was dissolved in 0.1 M phosphate buffer (1 ml), and recrystallized by means of dialysis against 90% saturation of $(NH_4)_2SO_4$ (300 ml). The crystals were separated from amorphous precipitate by recentrifugation and resuspension as in the case of crystallization. The chlorophyll · protein thus obtained as crystals was used for chemical, physicochemical and spectrophotometric characterization. The yield of the chlorophyll · protein was 60 mg.

Discontinuous polyacrylamide gel electrophoresis for the chlorophyll · protein was performed by the method of Davis [7], with a slab gel apparatus (Toyo SPG-1400). Concentrations of polyacrylamide for the separation and the concentration gel were 7.5 and 3.1%, respectively. The electrophoresis was performed at a constant voltage of 60 V for 4 h in a cold room. After green-colored bands were marked, the gel plate was stained for 1 h in methanol/acetic acid/water (50 : 7 : 43, v/v) containing 0.02% Coomassie Brilliant Blue R-250, and destained in methanol/acetic acid/water (20 : 7 : 73, v/v).

An isoelectric point was determined by means of electrophoresis on an ampholine column having a pH range of 3—10 [8]. A direct current, 4 mA, was maintained for 48 h while the applied voltage increased from 700 to 1500 V. Temperature was kept at 5°C.

To extract chlorophyll from the chlorophyll · proteins, 2 ml of chlorophyll · protein solution in 0.05 M phosphate buffer and 6 ml of 2-butanone (methyl ethyl ketone) were mixed and stirred. A green-colored 2-butanone layer containing the extracted chlorophyll were separated from a colorless aqueous layer containing the apoprotein by centrifugation at 1000 × *g* for 5 min. The butanone solution was dehydrated by passage through a column of anhydrous Na_2SO_4 and then evaporated. The residue was dissolved in 80% aqueous acetone, and the amounts of chlorophyll *a* and *b* were determined according to the method of Arnon [9].

The amount of protein was determined according to the dry weight. The chlorophyll · protein in 0.05 M CH_3COONH_4 was evaporated first at 80°C and then at 110°C in vacuo over P_2O_5 until the constant weight was obtained.

Amino acids were analyzed with an amino acid analyzer (Hitachi KLA-5), after the protein was hydrolyzed in 6 M HCl at 110°C for 24 h. The content of half-cystine was determined by the carboxymethylation method [10]: The protein in 6 M guanidine-HCl was reduced by dithiothreitol (50 mol/mol protein) at 50°C for 6 h, and treated with iodoacetic acid (100 mol/mol protein) in the dark at room temperature for 20 min. The content of tryptophan was spectrophotometrically determined [11].

An N-terminal amino acid was determined by the dansylation method [12]. Dansylamino acids were identified by two-dimensional thin-layer chromatography on polyamide [13].

Molecular weights of the proteins were estimated by gel filtration column chromatography on Sephadex G-200 (1.5 cm diameter × 90 cm high). The eluent contained 0.2 M NaCl and 0.05 M sodium-potassium phosphate buffer, pH 7.0. The molecular weights were also estimated by the method of equilibrium sedimentation in an analytical ultracentrifuge (Beckman, model E). The protein in 0.2 M NaCl and 0.05 M sodium-potassium phosphate buffer, pH 7.0 was centrifuged at 9341 rev./min for 10 h.

Absorption spectra were measured with a recording spectrophotometer (Hitachi 200-10).

DEAE-cellulose was purchased from Brown. Cellulose tubes for dialysis were from Visking. Sephacryl S-200 and Sephadex G-200 were from Pharmacia. Proteins of molecular weight standard were from Boehringer Mannheim. Ampholine was from LKB. Polyamide layer sheets were from Cheng Chin Trading Co., Ltd. Other chemicals were from Wako Pure Chemical Industries, Ltd.

Results

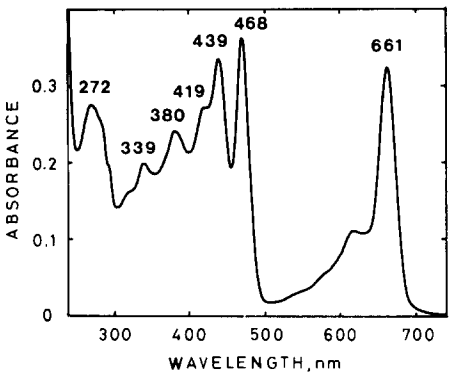
Absorption spectra

Fig. 1 shows an absorption spectrum of the chlorophyll · protein prepared from *L. virginicum* cultivated from seeds collected in California and dissolved in 0.05 M phosphate buffer. Absorption maxima were at 661, 468, 439, 419, 380, 339 and 272 nm. The complex band around 272 nm is ascribed to the aromatic amino acids. The bands at 661, 439, 419, 380 and 339 nm are due to chlorophyll *a*, and the band at 468 nm to chlorophyll *b*. The red absorption band of chlorophyll *b*, though unclear in Fig. 1, appeared as a shoulder around 650 nm, when the absorption spectrum was measured at liquid nitrogen temperature [5]. The absorption spectrum in Fig. 1 was unique in that the Soret band of chlorophyll *b* at 468 nm was the highest in the visible region. According to the wavelength of red absorption peak, this protein is named CP661.

Fig. 2 shows an absorption spectrum of the chlorophyll-protein prepared from *L. virginicum* collected at Narashino, Chiba. Absorption maxima were at 663, 469, 438, 419, 379, 338 and 272 nm. In this protein, the chlorophyll *b* band at 469 nm was lower than the chlorophyll *a* bands at 663 and 438 nm. According to the red absorption peak, this protein is named CP663. The difference in absorption spectrum between the two kinds of proteins may be produced by different ratios of chlorophyll *a* to *b*.

Discontinuous polyacrylamide gel electrophoresis

Only one green band appeared in the discontinuous polyacrylamide gel electrophoresis of each of the crystalline chlorophyll · proteins. Relative mobility to bromophenol blue was 0.77 for CP661 and 0.80 for CP663. No bands other than the green-colored one were stained by Coomassie Brilliant Blue R-250. This indicated that there were no detectable impurities in the chlorophyll · protein preparations. When the crystalline proteins were stored in a refrigerator for more than 2 months, another green-colored band appeared at a relative mobility of 0.42 for CP661 and 0.45 for CP663. This band might be due to a polymer of the chlorophyll · protein.



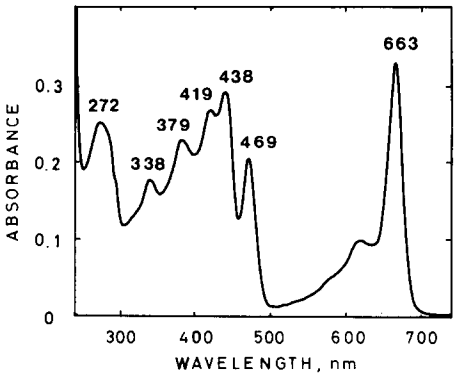


Fig. 1. Absorption spectrum of crystalline chlorophyll · protein CP661 dissolved in 0.05 M sodium-potassium phosphate buffer, pH 7.2.

Fig. 2. Absorption spectrum of crystalline chlorophyll · protein CP663 dissolved in 0.05 M sodium-potassium phosphate buffer, pH 7.2.

Physicochemical properties

Molecular weights were estimated to be 78 000 for CP661 and 80 000 for CP663 by gel filtration chromatography. They were 83 000 for CP661 and 107 000 for CP663 by the method of sedimentation equilibrium. A value used for the partial specific volume of the protein was 0.75.

The isoelectric points of the chlorophyll · proteins were examined by means of the isoelectric focusing in the ampholine column. A single band with respect to absorbance at 660 nm, appeared at pH 4.5 in CP661 and at pH 4.2 in CP663. Therefore, the isoelectric point was determined to be 4.5 for CP661 and 4.2 for CP663.

Compositions

The chlorophyll content in the protein was calculated on the basis of the dry weight of protein and spectrophotometrically determined amounts of chlorophyll *a* and *b*. The molecular weight estimated by the gel filtration chromatography was used in the calculation. This indicated that 1 mol of CP661 contained 4 mol chlorophyll with a chlorophyll *a* to *b* ratio of 1.0. 1 mol CP663 contains 4 mol chlorophyll with a chlorophyll *a* to *b* ratio ranging from 1.6 to 1.9 according to the preparation.

No carotenoids were detected by the paper chromatography of the 2-butanone extracts of the chlorophyll · proteins. The absorption spectra of the protein solutions (Figs. 1 and 2) and the 2-butanone extracts did not show any sign of the presence of carotenoids. With respect to the lack of carotenoids, the chlorophyll · proteins are similar to the water-soluble and crystallizable bacteriochlorophyll · protein [14].

The amino acid compositions of the chlorophyll · proteins are presented in Table I. CP661 and CP663 had very similar amino acid compositions. High contents of aspartic acid in both of the proteins were noted. The polarity index [15] was 50.3 in CP661 and 49.1 in CP663. This indicates that the chlorophyll · proteins are very hydrophilic ones.

TABLE I

AMINO ACID COMPOSITIONS OF CRYSTALLINE CHLOROPHYLL · PROTEINS

Amino acid	CP661 (mole%)	CP663 (mole%)
Lysine	6.16	5.68
Histidine	0.11	0.10
Arginine	2.16	2.45
Aspartic acid	16.33	16.31
Threonine	7.51	7.30
Serine	9.84	9.72
Glutamic acid	8.22	7.57
Proline	7.17	8.35
Glycine	8.93	9.40
Alanine	3.30	3.98
Half-cystine *	0.21	0.29
Valine	5.60	5.69
Methionine	0.06	0.10
Isoleucine	5.66	5.37
Leucine	7.06	6.73
Tyrosine	2.62	2.42
Phenylalanine	3.26	3.15
Tryptophan **	5.82	5.38
Polarity index ***	50.3	49.1

* Determined according to Waxdal et al. [10].
** Determined according to Goodwin and Morton [11].
*** Calculated according to Capaldi and Vanderkooi [15].

The N-terminal residue was glycine in CP661 and isoleucine in CP663. This is in contrast to the case of crystallizable bacteriochlorophyll protein of green bacteria in which the N-terminal residue is alanine [14].

Discussion

Two kinds of crystallizable chlorophyll · proteins were prepared from the leaves of *L. virginicum* having different origins; CP661 from the plants originating from seeds collected in California, and CP663 from the plants growing in the campus of Toho University in Japan. These proteins were more or less different in their absorption spectrum, chlorophyll *a* to *b* ratio, estimated molecular weight, isoelectric point and crystalline shape [4]. Although the plants of different origins belong to the same species, *L. virginicum* L., they look somewhat different. For example, hairs on the leaf surface are thicker and longer in the plant of California origin. The plants which belong to the same species but have different characteristics form different varieties, although this has not been established in this species. These findings suggest that the two kinds of chlorophyll · proteins are not identical.

The chlorophyll *a* to *b* ratio was 1.0 in CP661, but varied from 1.6 to 1.9 in CP663. Since the molar ratio of chlorophyll to protein is 4, two chlorophyll *a* and two chlorophyll *b* molecules are bound in CP661. CP663, however, must be a mixture of protein molecules having different compositions of chlorophyll *a* and *b*. This fact may suggest that a part or all of the binding sites are not strictly specific to the chlorophyll species in CP663.

The absorption spectra of the water-soluble chlorophyll · proteins are unique. Irrespective of the high contents of chlorophyll *b*, the red absorption band of this chlorophyll is not clearly separated from that of chlorophyll *a*. Sugiyama and Murata [5] analyzed the red absorption bands of the proteins by the curve-fitting method and inferred that there were three chlorophyll forms, Cb650, Ca662 and Ca670, but no Ca677 and Ca684. Cb650, Ca662 and Ca670 form a compound absorption band, in which the chlorophyll *b* band is not easily discriminated.

The water-soluble chlorophyll · proteins are distinctly different from the chlorophyll-protein complexes which are prepared from the thylakoid membrane of chloroplasts by detergent treatments; i.e., the light-harvesting chlorophyll *a/b* protein [16], the P-700-chlorophyll *a* protein [16] and the reaction center II chlorophyll · protein [17]. Carotenoids are absent in the water-soluble proteins, but present in the detergent-solubilized proteins [16]. The red absorption peak appears at much shorter wavelengths in the water-soluble (661 or 663 nm) than in the detergent-solubilized proteins (around 675 nm). The N-terminal amino acid is glycine in CP661 and isoleucine in CP663, while it is serine in the light-harvesting chlorophyll *a/b* protein [16] and alanine and aspartic acid in the P-700-chlorophyll *a* protein [16]. The polarity index calculated from the amino acid composition was 50 in CP661 and 49 in CP663, while it is 38 in the light-harvesting chlorophyll *a/b* protein [16] and 39 in the P-700-chlorophyll *a* protein [16]. These facts indicate that CP661 and CP663 are much more hydrophilic than the detergent-solubilized chlorophyll · proteins.

The functions of the water-soluble chlorophyll · proteins are not known. The very hydrophilic nature of the proteins may suggest that the proteins are solubilized in the stroma but are not bound to the thylakoid membrane of the chloroplasts. Although speculative, a possible function of the proteins is to transfer chlorophyll molecules from membrane to membrane, as the lipid exchange proteins transfer phospholipids [18].

References

1 Yakushiji, E., Uchino, K., Sugimura, Y., Shiratori, I. and Takamiya, F. (1963) Biochim. Biophys. Acta 75, 293—298
2 Murata, T., Toda, F., Uchino, K. and Yakushiji, E. (1971) Biochim. Biophys. Acta 245, 208—215
3 Murata, T. and Murata, N. (1971) Carnegie Inst. Wash. Year Book 70, 504—507
4 Murata, T., Itoh, R. and Yakushiji, E. (1980) Biochim. Biophys. Acta 593, 167—170
5 Sugiyama, K. and Murata, N. (1978) Biochim. Biophys. Acta 503, 107—119
6 French, C.S., Brown, J.S. and Lawrence, M.C. (1972) Plant Physiol. 49, 421—429
7 Davis, B.J. (1964) Ann. N.Y. Acad. Sci. 121, 404—427
8 Vesterberg, O. and Svensson, H. (1966) Acta Chem. Scand. 20, 820—834
9 Arnon, D.I. (1949) Plant Physiol. 24, 1—15
10 Waxdal, M.J., Konigsberg, W.H., Henley, W.L. and Edelman, G.M. (1968) Biochemistry 7, 1959—1966
11 Goodwin, T.W. and Morton, R.A. (1946) Biochem. J. 40, 628—632
12 Gray, W.R. and Hartley, B.S. (1963) Biochem. J. 89, 379—380
13 Kimura, S. (1974) Japan Anal. (in Japanese) 23, 563—575
14 Thornber, J.P. and Olson, J.M. (1968) Biochemistry 7, 2242—2249
15 Capaldi, R.A. and Vanderkooi, G. (1972) Proc. Natl. Acad. Sci. U.S.A. 69, 930—932
16 Thornber, J.P., Alberte, R.S., Hunter, F.A., Shiozawa, J.A. and Kan. K.-S. (1976) Brookhaven Symp. Biol. 28, 132—148
17 Satoh, K. (1979) Biochim. Biophys. Acta 546, 84—92
18 Kader, J.C. (1977) in Cell Surface Reviews (Poste, G. and Nicolson, G.I., eds.), Vol. 3, pp. 127—204, North-Holland, Amsterdam.