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WATER-SOLUBLE CHLOROPHYLL PROTEIN OF *BRASSICA OLERACEA* VAR. BOTRYS (CAULIFLOWER)

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SUMMARY

A water-soluble chlorophyll protein was prepared from *Brassica oleracea* var. Botrys (cauliflower) and purified by $(NH_4)_2SO_4$ fractionation and by chromatography on a DEAE-cellulose column. The chlorophyll protein contained chlorophylls *a* and *b* in the ratio 6:1, and no carotenoids. The molecular weight, determined by means of gel filtration on Sephadex G-100, was 78000. The chlorophyll protein showed absorption peaks at 273, 340, 384, 420, 438, 465, 628, 674 and 700 nm. Since the three bands at 384, 420 and 438 nm all have approximately the same height, the spectrum is different from that of chlorophyll *a* in organic solvents. The fluorescence of the chlorophyll protein showed a peak at 683 nm, with shoulders at 706 and 745 nm at room temperature, and peaks at 685, 706 and 744 nm at the temperature of liquid N_2. An apo-protein was prepared by removing the chlorophylls with 2-butanone and purified by precipitation with $(NH_4)_2SO_4$. The apo-protein thus prepared had an absorption band at 273 nm but none at longer wavelengths. The apo-protein could be combined with chlorophylls, forming a chlorophyll protein which had spectral characteristics similar to those of the original.

INTRODUCTION

A water-soluble chlorophyll protein was first extracted by YAKUSHIJI *et al.*[1] from the leaves of *Chenopodium album*. The protein, called Chenopodium CP668, contained chlorophylls *a* and *b*, but no carotenoids, and had main absorption bands at 277, 429 and 668 nm. It was sensitive to the light; upon illumination of the protein the absorption bands at 429 and 668 nm decreased and new peaks appeared at 362, 399, 567 and 743 nm. TAKAMIYA *et al.*[2] showed that the photoconversion of chlorophyll protein from the 668-nm form to the 743-nm form proceeded in the presence of oxidants such as O_2, ferricyanide, ferricytochrome *c* and 2,6-dichlorophenolindophenol. TAKAMIYA *et al.*[3] also showed, by means of flash experiments, that the triplet state of chlorophyll *a* was involved as an intermediate during the photoconversion.

MURATA *et al.*[4] succeeded in separating the apo-protein and chlorophyll as well as reconstituting them to form the original chlorophyll protein complex. They found, during the course of the investigation, that photoconversion of the chlorophyll protein

from the 668-nm form to the 743-nm form was due to an oxidation of chlorophyll *a* and some modification of the apo-protein moiety.

Since the discovery of the chlorophyll protein in Chenopodium (Chenopodium CP668), we have been searching for water-soluble chlorophyll proteins in other plants. Recently, we have succeeded in preparing it from cauliflower. The spectral features of the chlorophyll protein are different from those of Chenopodium CP668 and the absorption spectrum is not changed by light illumination. In this paper, the methods of preparation and some characteristics of the chlorophyll protein of cauliflower are described.

METHODS

Preparation of chlorophyll protein

1.2 kg of cauliflower were blended in 1.5 l of 0.1 M phosphate buffer solution, pH 7.2. Fragments were filtered out with cheese cloth. To the pale green juice (2.0 l) thus obtained, 300 g $(NH_4)_2SO_4$ were added followed by 125 ml of 2 % 2-ethoxy-6,9-diaminoacridine lactate solution. After being set aside for 30 min, the solution was filtered on a Buchner funnel through a thin layer of talcum. The filtrate was made 70 % saturated with respect to $(NH_4)_2SO_4$ and filtered again in the same way. The precipitate was dissolved in 400 ml of 0.05 M phosphate buffer solution and the resultant green solution showed an absorbance of 0.3–0.4 at 674 nm in a 1-cm cuvette. This solution was dialyzed against 0.01 M phosphate buffer solution for 24 h at 5°. A column of DEAE-cellulose was prepared in the chloride form by passing 0.5 M NaCl solution through it and then equilibrating it with 0.01 M phosphate buffer, pH 7.2. The dialyzed solution of chlorophyll protein was applied to the column and the chlorophyll protein was adsorbed on the DEAE-cellulose under a top brown layer. The column was washed with 0.01 M and then 0.02 M phosphate buffer solutions. The chlorophyll protein was eluted with 0.1 M phosphate buffer, pH 7.2. This solution (100 ml) showed an absorbance of 0.4 at 674 nm in a 1-cm cuvette. The resulting green solution was then fractionated with $(NH_4)_2SO_4$, the fraction between 0.3 and 0.6 saturation being retained. The DEAE-cellulose column chromatography and the $(NH_4)_2SO_4$ fractionation were repeated several times. Finally, the protein was purified by means of gel filtration on a Sephadex G-100 column.

Extraction of chlorophyll and preparation of the apo-protein

The apo-protein was prepared according to the method described previously[4]. One part of chlorophyll protein solution, previously dialyzed against 0.01 M phosphate buffer, pH 7.2, was mixed with two parts of 2-butanone (methyl ethyl ketone) and shaken. The green 2-butanone layer containing chlorophylls and the colorless aqueous layer containing the apo-protein were separated by centrifugation. The butanone solution containing chlorophyll was dried with anhydrous Na_2SO_4 and then the butanone was evaporated. The residue was dissolved in an acetone–water mixture (4 : 1, v/v). The absorption of this solution was measured and the chlorophyll *a* to *b* ratio was determined according to the method of VERNON[5]. The apo-protein was precipitated from the aqueous solution with $(NH_4)_2SO_4$ at 70 % saturation. In order to remove 2-butanone, the $(NH_4)_2SO_4$ precipitation was repeated several times, and the product was then purified by gel filtration on a column of Sephadex RH-100. The temperature during the preparation of the apo-protein was maintained below 5°.

Reconstitution of chlorophyll proteins

Reconstitution of chlorophyll proteins was carried out according to the method described previously[4]; a small amount of an ethanolic solution of chlorophyll was mixed with the aqueous solution of apo-protein. The volume of chlorophyll–ethanol solution did not exceed one-fifth of that of the apo-protein solution. After being set aside for 20 min, the mixture was saturated with respect to $(NH_4)_2SO_4$ and filtered through a thin layer of talcum. The precipitate was dissolved in 0.05 M phosphate buffer (pH 7.2), whereby free chlorophyll remained adsorbed onto the talcum. The $(NH_4)_2SO_4$ precipitation was repeated several times in order to remove free chlorophyll from the protein preparation. The mixture of chlorophylls *a* and *b* used for the reconstitution was prepared from spinach according to the method devised by TAKAMIYA AND OGURA (unpublished results; see ref. 4 for details). Chlorophylls *a* and *b* were separated by means of cellulose powder column chromatography by elution with a mixture of light petroleum and 2-propanol (98:2, v/v).

Measurement of absorption and fluorescence spectra

Absorption spectra were measured by means of a Hitachi recording spectrophotometer EPS-2U and a Hitachi Perkin–Elmer spectrophotometer 139. Fluorescence spectra were measured according to the method of MURATA *et al.*[6].

RESULTS

Physical characteristics

The molecular weight of the cauliflower chlorophyll protein was determined by means of gel filtration on a column of Sephadex G-100 (2.5 cm diameter, 95 cm length, pH 7.0 and $I = 0.5$). As indicators of molecular weight γ-globulin, albumin, chymotrypsinogen, cytochrome *c* and Blue dextran 2000 were used. The molecular weight of the chlorophyll protein was estimated to be 78000.

The sedimentation constant of the chlorophyll protein was measured by means of a Hitachi Analytical Ultracentrifuge Model UCA-1A at 20°, using a solution of the purified sample dissolved in and dialyzed against 0.2 M NaCl solution. The peak of the Schlieren pattern and the boundary of the green color moved together, suggesting that the chlorophyll protein was a single component. From the observed rate of sedimentation, the sedimentation constant was calculated to be $s_{20,w} = 4.7 \cdot 10^{-13}$.

The isoelectric point of the chlorophyll protein was determined by means of electrophoresis on an ampholine column (for pH range 3–10). A sucrose density gradient was formed in the column of 1% ampholine solution and the chlorophyll protein was placed near the mid-point of the column. A direct current, 700 V, 4 mA, was applied for 48 h. The temperature was maintained at 2°. A single band with respect to the absorption at 674 nm was observed. The peak position was at pH 4.6. Thus, the isoelectric point was $pI = 4.6$.

Absorption spectra of cauliflower chlorophyll protein

Fig. 1 (a) shows an absorption spectrum of purified cauliflower chlorophyll protein. Absorption maxima occur at 273, 340, 384, 420, 438, 465, 595, 628, 674, and 700 nm. The band at 273 nm is produced by absorption due to protein. The bands at 384, 420, 438, 595, 628 and 674 nm are due to chlorophyll *a* and the relatively small

band at 465 nm to chlorophyll *b*. The red band of chlorophyll *b* around 650 nm was not observed, probably because of a low content of chlorophyll *b* compared with chlorophyll *a*.

Another characteristic feature of the absorption spectrum was the relatively weak absorption of the Soret band of chlorophyll *a* at 438 nm compared with the other chlorophyll *a* bands at 384, 420 and 674 nm. The spectrum was distinctly different from the absorption spectrum of chlorophyll *a* in organic solvents where the Soret band is always higher than the other bands.

As will be shown later, the band at 700 nm is not attributed to an absorption of chlorophyll, but may be due to a decomposed chlorophyll. The 700-nm band was produced in the light at an early stage of purification. Fig. 1 (b) shows an absorption spectrum of the chlorophyll protein partially purified (once through the DEAE-cellulose column) in the dark. There was practically no absorption band at 700 nm, whereas an absorption spectrum similar to that shown in Fig. 1 (a) was observed at the same stage of purification if the procedures were carried out in the light. After the partial purification in the dark, no further production of the 700 nm band was observed in the light. However, a very slow formation of the 700 nm band in the dark was found when the fully-purified chlorophyll protein was stored in a refrigerator for more than 2 weeks. This conversion did not seem to be stimulated by light.

The apo-protein and pigment components were separated by means of 2-butanone, whilst with acetone the chlorophylls were not completely extracted from the protein. When 1 part of buffer solution of the protein was shaken with 2 parts of 2-butanone, two layers were formed. The pigment components were found in the upper, 2-butanone, layer and the apo-protein remained in the lower, water, layer. 2-Butanone was evaporated after drying with anhydrous Na_2SO_4 and the substances remaining were dissolved in 80 % acetone or diethyl ether. Fig. 2 shows the absorption spectra of the 2-butanone extract. In the 80 % acetone solution of chlorophylls prepared from the 2-butanone extract of the chlorophyll protein lacking the 700-nm band there were absorption bands of chlorophyll *a* at 663, 433, 413, and 380 and absorption bands of chlorophyll *b* at 460 nm (Fig. 2a). The pigment components were separated by means of paper chromatography with petroleum ether, according to the method of JEFFREY[7]. Chlorophylls *a* and *b* were found, but no carotenoids. The ratio of chlorophylls *a* to *b* in the chlorophyll protein was 6.3.

In the extract of the protein having the 700 nm band, on the other hand, there

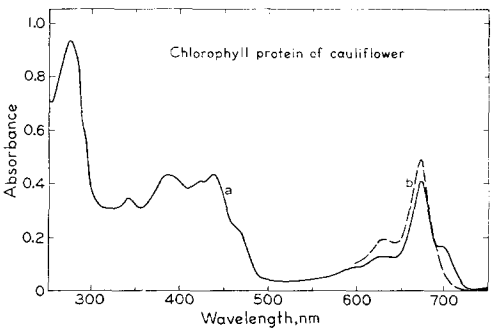


Fig. 1. Absorption spectra of cauliflower chlorophyll protein a, purified in the light; b, partially purified in the dark.

was another absorption band at 685 nm (Fig. 2b) in addition to the bands of chlorophylls *a* and *b* observed in the former sample. Apparently, it is not an absorption band of chlorophyll, but it may be due to a decomposition product of chlorophyll *a*. On the paper chromatography of the extract, another spot was found between the spots of chlorophylls *a* and *b*. The absorption spectrum of this component, dissolved in ether, showed main peaks at 428 and 685 nm. This pigment must be responsible for the absorption band at 700 nm in the chlorophyll protein.

The apo-protein remaining in the aqueous layer was purified by means of $(NH_4)_2SO_4$ fractionation and then dissolved in 0.05 M phosphate buffer, pH 7.2. Its absorption spectrum (Fig. 3) showed a peak at 273 nm and shoulders at 284 and 291 nm (the absorption due to aromatic amino acids) and no other absorption peaks in the visible region of the spectrum.

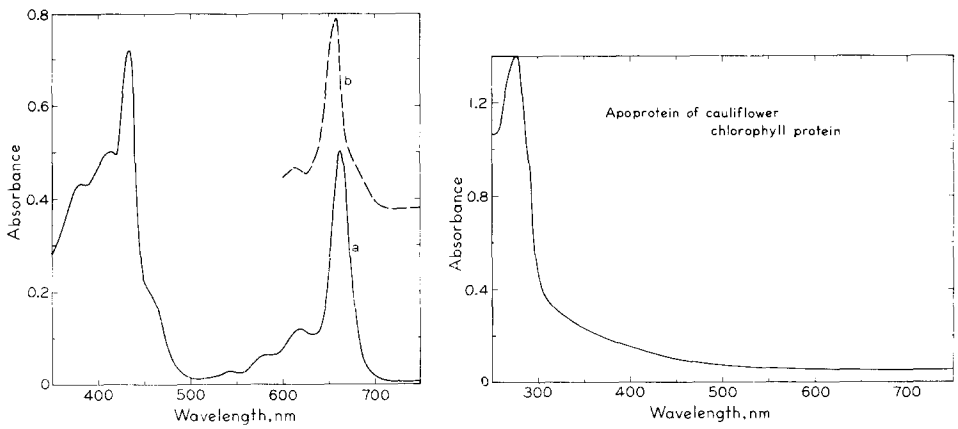


Fig. 2. Absorption spectra of a 2-butanone extract of the chlorophyll protein. a, Extract of the partially purified protein, lacking the 700-nm band, dissolved in 80% acetone; b, extract of the protein having the 700-nm band, dissolved in diethyl ether.

Fig. 3. Absorption spectrum of the apo-protein prepared from cauliflower chlorophyll protein.

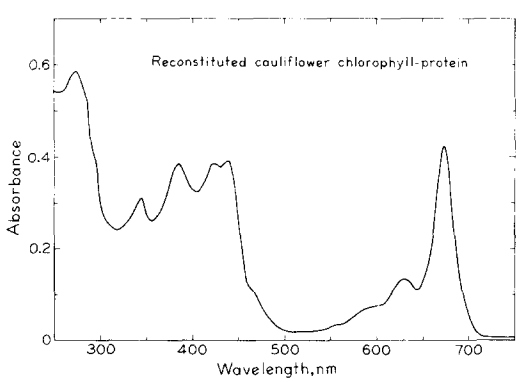


Fig. 4. Absorption spectrum of reconstituted chlorophyll protein prepared from the apo-protein and a mixture of chlorophylls *a* and *b* ($a/b = 3$).

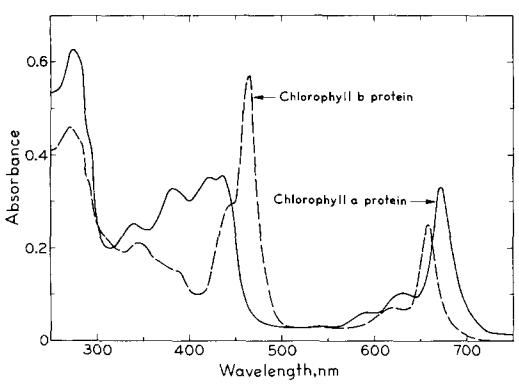
Properties of reconstituted chlorophyll protein

The apo-protein prepared from cauliflower chlorophyll protein could be combined with chlorophylls *a* and *b*, to form water-soluble chlorophyll proteins. When a mixture of chlorophylls *a* and *b* (3 : 1) was used, the chlorophyll protein formed had spectral characteristics similar to those of the original protein (Fig. 4). However, the absorption band at 700 nm was lacking even if the original protein had the 700-nm band. A red peak appeared at 672 nm, whereas it was found at 674 nm in the original protein.

Chlorophyll *a* and chlorophyll *b* proteins were prepared by mixing the apo-protein and chlorophyll *a* or *b*, and then fractionating the protein repeatedly with $(NH_4)_2SO_4$. The absorption spectrum of reconstituted chlorophyll *a* protein was similar to that of the original chlorophyll protein except for the absence of the 465 nm band of chlorophyll *b* and the 700 nm band (Fig. 5a). The main absorption maxima were at 383, 420, 435 and 672 nm. These bands had approximately the same heights, thus exhibiting a spectrum different from that of chlorophyll *a* in organic solvents, where the Soret band at 430 nm is distinctly higher than the other bands. In the spectrum of chlorophyll *b* protein the absorption maxima are located at 438, 463 and 657 nm (Fig. 5,b). In this case, however, the Soret band at 463 nm was much higher than the other bands.

Fluorescence spectra of cauliflower chlorophyll protein

Fig. 6 shows the fluorescence spectra excited by blue light. At room temperature, there was a peak at 683 nm and shoulders at 706 and 745 nm. At the temperature of liquid N_2 there were three preaks, at 685, 706 and 744 nm. The shift of the 683 nm band as a result of cooling may be due to increased self-absorption. The emission band at 683 nm appears to be emitted by the main absorption band at 674 nm. The band at 706 nm may originate from the absorption band at 700 nm. The fluorescence peak at 744 nm may be a sattelite band of the 683 nm and/or the 706 nm fluorescence.



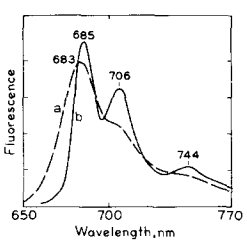


Fig. 5. Absorption spectra of chlorophyll *a* protein (a) and chlorophyll *b* protein (b).

Fig. 6. Fluorescence spectra of cauliflower chlorophyll protein at room (a) and liquid N_2 temperature (b). The excitation light was a broad band of blue light with the maximum at 445 nm, obtained from a 150-W incandescent lamp filtered through V-V44 (Toshiba) B-460 (Hoya Glass) and HA-50 (Hoya Glass). A red cut-off filter, V-R65 (Toshiba), was placed between the sample and an analyzing monochromator. The band-width of the analyzing monochromator was 5 nm at liquid N_2 temperature and 10 nm at room temperature.

Chlorophyll protein from leaves

The same type of water-soluble chlorophyll protein was also prepared by the same method from the leaves of cauliflower. Its absorption spectrum was similar to that of the protein prepared from the inflorescence. A further study on the chlorophyll protein from leaves was not carried out.

DISCUSSION

We have been searching for water-soluble chlorophyll proteins prepared without the use of detergents since the first discovery of this type of chlorophyll protein from *Chenopodium album*[1]. The chlorophyll protein of cauliflower investigated in the present study falls in this category of chlorophyll proteins. It has a molecular weight of 78000, an isoelectric point at pI = 4.6 and a red absorption peak at 674 nm, while the protein from Chenopodium has a molecular weight of 78000 (unpublished), an isoelectric point at pI = 7.2 (unpublished) and an absorption peak at 668 nm[2].

A chlorophyll protein similar to that prepared from the inflorescence of cauliflower is also present in the leaves of the same plant. Another water-soluble chlorophyll protein with an absorption peak at 674 nm was prepared from the leaves of wild mustard (unpublished). It is inferred, therefore, that this type of chlorophyll protein was present not only in the inflorescence of cauliflower, but also in the leaves of some kinds of higher plants.

The Chenopodium CP668 changes its absorption spectrum upon illumination. On the other hand, the cauliflower chlorophyll protein is insensitive to light. Only during an early stage of purification (in the room light) was an absorption band observed at 700 nm.

The pigment corresponding to the 700 nm band is not caused by any of the known chlorophylls, but may be a decomposition product of chlorophyll *a*. In fact, the band at 700 nm was absent in the reconstituted chlorophyll proteins prepared from the apo-protein and purified chlorophyll *a*, chlorophyll *b* or a mixture of them.

The absorption spectrum of the original chlorophyll protein is different from that of chlorophyll *a* dissolved in organic solvents. The red band at 674 nm, the Soret band at 438 nm and two other bands at 420 and 383 nm had approximately the same heights. In solutions of chlorophyll in organic solvents, the Soret band is always higher than any of the other bands. In fact, the absorption spectrum of a 2-butanone extract of the chlorophyll protein showed the Soret band to be higher than any other bands. The absorption peak of the red band (674 nm) appears at a wavelength longer by 10–15 nm than those of chlorophyll *a* in organic solvents (663 nm in acetone, 660 nm in ether). The large shift of the red absorption band and the change in relative heights of the absorption peaks may be caused by strong interaction between the chlorophyll molecules in the chlorophyll protein.

A similar absorption spectrum was obtained for the chlorophyll *a* protein. This suggests that the interaction between chlorophylls *a* and *b*, even if present, is not necessary to induce such spectral changes of chlorophyll *a*, but that the interaction between chlorophyll *a* molecules is essential.

In the chlorophyll *b* protein, there were no such dramatic spectral changes of chlorophyll *b* with respect to the ratio of the heights of the Soret to the other bands. However, the red band shifted by about 10 nm to longer wavelengths when compared

with the same band of chlorophyll *b* in organic solvents. This fact may suggest a strong interaction between the chlorophyll *b* molecules in the chlorophyll *b* protein.

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