Purification of Protochlorophyllide Holochrome

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Abstract. Phototransformable protochlorophyllide holochrome was prepared from etiolated bean leaves. The detergent Triton X-100 in the presence of glycerol and tricine-KOH buffer (pH 8) enhanced the extractability, specific activity, and phototransformability of the holochrome. Purification was achieved by polyethylene glycol-6000 precipitation and hydroxylapatite, DEAE-cellulose, and agarose chromatography. The presence of Triton X-100 permitted removal of the carotenoid contamination from the holochrome. The 678-nm absorption maximum of newly formed chlorophyllide a holochrome shifts to 672 nm in a temperature-dependent manner. The purified holochrome contains 0.24 g of protein per 

μ mole of protochlorophyllide. Estimation of the molecular weight of the holochrome by gel filtration on agarose revealed the presence of aggregates of approximately 550,000 and 300,000. There are at least 2 chromophores per 550,000 molecular weight.

The last step in chlorophyllide a biosynthesis is the reduction of protochlorophyllide. The reaction requires light in angiosperm leaves, and the photosensitizer is protochlorophyllide (3). The photoactive system was extracted from etiolated bean leaves into aqueous solution (12) and was called protochlorophyll holochrome (22). Later it was found that the purified holochrome contains only the nonesterified form of protochlorophyll, i.e., protochlorophyllide or magnesium vinylpyropheophytin a, (19). The first in vivo product of the phototransformation of protochlorophyllide is chlorophyllide, which is later esterified with phytol to form chlorophyll a (25). Accordingly we have used the term protochlorophyllide holochrome (PCH) for the extractable photoactive complex. The biochemistry and physiology of PCH has been reviewed recently (3, 10).

Smith and coworkers (20) partially purified PCH. They showed that the holochrome is a protein-protochlorophyllide complex which needs no external cofactor for the light-mediated reduction to chlorophyllide a holochrome. They reported a sedimentation coefficient of 16 to 17 and a molecular weight in the range of 700,000. Their most highly purified preparations contained 0.96 g protein per μ mole protochlorophyllide. About 50% of the protochlorophyllide was phototransformable. The preparations contained a mixture of several carotenoids which could not be removed from the holochrome (21). Boardman (2), using similar preparative methods, obtained essentially the same results (s20,w = 18; molecular weight 600,000 ± 50,000; 1.1 to 1.7 g protein per μ mole protochlorophyllide; 50-60% transformation). Purification by electrophoretic methods suggested that the protein/protochlorophyllide ratio could be reduced further. We detail here a method for the isolation and purification of PCH, utilizing newer methods for protein separations, which provides a preparation of considerably higher purity and better transformability than previously reported.

Materials and Methods

Bean seedlings (Phaseolus vulgaris L. cv. light red kidney; Michigan Bean Corporation, Saginaw, Michigan) were grown in the dark at 23° to 25° on vermiculite previously soaked with Kratz-Myers D-medium (13) diluted 1:4 with tap water. The primary leaves were harvested after 9 to 11 days of growth. All subsequent manipulations were carried out in a cold room (3°) equipped with a green safelight. Tricine was prepared by the method of Good (8). The buffer (pH 8.0), subsequently referred to as “tricine buffer,” contained 0.01 M tricine, 0.008 M KOH, 0.002 M MgSO4, and 0.001 M Na2 EDTA. Calcium phosphate (brushte) was prepared according to Siegelman et al. (17) and transformed to hydroxylapatite by titration with 2 M KOH at 25° until a constant pH of about 9 was reached. Shortly before use the hydroxylapatite was washed free of fines by repeated decantation to permit high flow rates. It was regenerated by washing with 1 M K2HPO4 and ethanol. DEAE-cellulose (DE-11, Whatman) was prewashed according to the manufacturer’s direction and titrated with 1 M KOH to pH 8.0 in the presence of 0.25 M KCl. The effluent of the chromatographic columns (except for the agarose column) was monitored with

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both ultraviolet and visible flow monitors (1isco Analyzers, Model UA-2, with a dual beam and a visible optical unit, respectively) and a conductivity meter (Conductivity Meter type CDMd, Radiometer). A constant flow rate was maintained by a peristaltic pump between the column and the first monitor. At the relatively high flow rates used, the monitor light did not phototransform the PCH. Other chemicals were obtained from the following sources: Triton X-100 from Ruger Chemical Company, polyethylene glycol-6000 from Baker Chemical Company, Polyclar AT (polyvinylpyrrolidone) from General Aniline and Film Corporation, Bio-Gel A-1.5 m, 200 to 400 mesh (8 % agarose) from Bio-Rad Laboratory, and sodium dodecylsulfate from Matheson Company.

Measurement of Protochlorophyllide and Chlorophyllide a Holochrome. The absorption spectra of the various preparations were measured in a 1-cm cell at 10° to 12° with a Cary Model 14 spectrophotometer before and after a saturating red irradiation. The spectrophotometric cell was irradiated in the center of a petri dish (height 6 cm, diam 12 cm) filled with a water-ice mixture and covered externally with aluminum foil. There was a 2-cm aperture on 1 side for the beam of a 150-W internal reflector projection lamp (Sylvania DFA). A 3-second irradiation saturated the phototransformation. The time between irradiation and measurement of the sample was about 30 seconds. A 10-second irradiation had no significant effect on the absorption spectrum of the newly formed chlorophyllide a holochrome.

The relative amount of PCH in a particular preparation, subsequently called “activity,” was calculated from the absorption spectrum obtained before irradiation. The base line used for measurement was a straight line connecting the absorbance values at 595 and 670 nm. The activity of a preparation is defined as the product of the volume in ml and the corrected absorbance at 639 nm (A639). A sample of 1 ml with \( A_{639} = 1.0 \) in a 1-cm cell has 1.0 unit of activity. The Beer-Lambert law was obeyed.

The relative amount of chlorophyllide a holochrome was calculated from the increase of the absorbance at 678 nm measured after irradiation (\( A_{678} \)). The degree of phototransformation is expressed as the ratio \( A_{678}/A_{639} \). The molar concentrations of protochlorophyllide and chlorophyllide a were calculated from absorption spectra of 80% acetone extracts of the holochrome obtained before and after phototransformation. The millimolar absorbivities \( \varepsilon_{678} = 31.1 \) and \( \varepsilon_{665} = 73.3 \) for protochlorophyllide and chlorophyllide a, respectively, were used. They were calculated from the specific absorbivities of the components in 80% acetone used by Boardman (2).

**Extraction and Purification.** 1) Samples of 40 g of leaves were homogenized in an Omni-Mixer (type OM, Ivan Sorval) at full speed for 4 minutes with 20 g of Polyclar AT and 160 ml of extraction medium (0.05 M tricine, 0.05 M KOH, 0.002 M MgSO4, 0.001 M Na2EDTA, 0.06% (v/v) Triton X-100, 25% (v/v) glycerol; pH 8.6). The temperature of the homogenate was maintained below 5° by immersing the homogenizing vessel in an ice-solid CO2 bath. The homogenate was clarified by filtration through a 15-cm milk filter and centrifuged at 78,500 \( \times \) g in a Spinco (Model L-2) centrifuge (No. 30 rotor) at 0° for 1 hour. A lipid layer was removed from the top of the centrifuge tubes, and the supernatant was retained.

2) A 50% polyethylene glycol-6000 solution (14) was added to the crude extract to give a final concentration of 15%; then the mixture was stirred for 5 minutes, allowed to stand for 30 minutes, and centrifuged at 48,000 \( \times \) g \(-2^\circ\) in a Sorvall RC-2B centrifuge for 1 hour. The precipitate was redissolved in tricine buffer and again centrifuged at 48,000 \( \times \) g for 30 minutes.

3) A hydroxylapatite column (7.5 \( \times \) 15 cm) was prepared the day before use and equilibrated with tricine buffer containing 0.2 M KCl. The PCH solution from step 2 was made 0.2 M with respect to KCl and applied to the column. After the column was washed with 2.5 liters of tricine buffer containing 0.2 M KCl, the adsorbed PCH was eluted with tricine buffer containing 0.25 M potassium phosphate buffer (pH 8.0). The flow rate was maintained at 20 ml/min. All fractions containing PCH were combined.

4) The PCH solution from the previous step was desalted on a Sephadex G-25 column (7.5 \( \times \) 25 cm) equilibrated with the tricine buffer, and then applied to a column of DEAE-cellulose (5 \( \times \) 55 cm) equilibrated with the tricine buffer. The adsorbed PCH occupied about 30% of the bed volume. Immediately after desorption the PCH was eluted by a convex exponential KCl gradient in the tricine buffer [initial concentration 0.05 M (0.3 1), final concentration 0.25 M (2.0 1)]. The flow rate was maintained at 20 ml/min, and 20-ml fractions were collected. Fractions with a \( A_{270}/A_{639} \) ratio between 25 and 28 were pooled and concentrated by ultrafiltration.

5) A portion of the concentrated solution (2 ml) containing about 2.0 units of PCH was applied to an 8% agarose column (1.75 \( \times \) 72 cm) equilibrated with tricine buffer containing 0.1 M KCl. The same buffer solution was used for elution. The flow rate was maintained at 10.0 ml/hr by constant hydrostatic pressure with a Mariotte flask, and 20-ml fractions were collected.

**Measurement of Protein.** An aliquot of the PCH solution was diluted to a volume of 2 ml, and the protein was precipitated by addition of 2 ml 10% trichloracetic acid. The solution was centrifuged and decanted. The precipitated protein was resuspended in 10 ml of ethanol and centrifuged again. The colorless precipitate was dissolved in 4 ml of biuret reagent (9) and made to 5.0 ml with
water. The solution was centrifuged, and the absorbance at 560 nm was measured versus a biuret blank. The protein concentration was calculated by using a value of E - 0.049 obtained with crystalline bovine serum albumin. Semi-micro Kjeldahl nitrogen analyses performed on the highest purity fractions, gave essentially similar protein values.

Electrophoresis. Polyacrylamide gel columns (0.6 × 7.5 cm) were prepared by the method of Davis (6); the spacer gel was omitted. The gels were equilibrated with tris-glycine buffer (pH 8.3) by electrophoresis for 2 hours (500 V, 5 mA/tube) without sample. Samples for analysis (10–100 μl, containing 20–100 mg protein) were made 0.2 M in sucrose before application to the column. Electrophoresis was performed at 500 V and 3 mA/tube (about 50 min) at 3°C. The protein on the column was fixed with 15% trichloroacetic acid (12 hr) and then stained with 0.1% Coomassie Brilliant Blue in 15% trichloroacetic acid for 3 hours at room temperature. The excess stain was removed by washing the gel columns several times with 10% trichloroacetic acid. Protein samples were treated with sodium dodecyl sulfate (1%) and were examined by disc electrophoresis in the presence of sodium dodecyl sulfate (0.1%) according to the method of Shapiro et al. (15).

**Results and Discussion**

Extraction and Stability of the Protophlorophylide Holochrome. After considerable preliminary testing of buffer type, ionic strength, and pH, we finally adopted an extraction solution containing tricine-KOH buffer, MgSO₄, EDTA, glycerol, and Triton X-100 (pH 8.6). The pH of the homogenate decreased to 8.0 following the extraction of the tissue. About 0.8 to 1.0 units of PCH could be obtained from 1 g of leaves. The optimal pH for maximum yield and stability was in the range 7.5 to 8.5. The tricine-KOH buffer used by Smith et al. (20) and Boardman (2) gave similar results; however, it was not used because of low buffering capacity at pH 8. The nonionic detergent Triton X-100, which is now widely used to solubilize chloroplast components (e.g., 4, 23), was found to increase yield, specific activity, and transformability of the PCH considerably (table 1). PCH preparations extracted with the combination of glyceral and Triton X-100 had absorption maxima at slightly longer wavelengths (639 nm for PCH, 678 nm for chlorophyllide a holochrome, respectively) than those extracted without the detergent. There was no change in the position of the peaks after removal of Triton X-100. Extraction of PCH without Triton X-100 and glyceral apparently leads to a partially denatured holochrome, which is responsible for the lower degree of transformability and the change in the position of the absorption maxima. The detergent digitonin was reported (18) to give higher yields of PCH; however, the strong inhibition of the phototransformation was only partly overcome by glyceral. A further advantage of Triton X-100 is its reversible binding to organic structures (7), which permits its removal during the purification of PCH. The favorable effect of Triton X-100 was dependent on the presence of glyceral, which apparently protected the PCH against inactivation (table 1). The optimal concentration of Triton X-100 was between 0.05 and 0.1% ; higher concentrations did not increase the yield of PCH in the extract but instead hastened the loss of transformability. Storage of the crude PCH extract at 3°C resulted in a 15% loss of activity and transformability in 24 hours (table 1) even in the presence of Triton X-100 and/or glyceral. The loss of transformability was more than 50% without glyceral. The crude extract could be stored frozen (−15°C) for at least 1 month without loss of activity and transformability. Preparations of PCH containing 2-mercaptoethanol (0.05 M) completely lost their transformability after a few hours at 3°C, which indicated a destructive effect. They were observed to have a strong light-red fluorescence under ultraviolet light and a shift of the 639-nm absorption maxima of PCH to 630 to 635 nm indicating a conversion to

<table>
<thead>
<tr>
<th>Table 1. Effect of Triton X-100 and Glycerol on the Extraction and Stability of Protophlorophylide Holochrome</th>
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<tbody>
<tr>
<td><strong>Extraction</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Triton, + Glycerol</td>
</tr>
<tr>
<td>Triton</td>
</tr>
<tr>
<td>Glycerol</td>
</tr>
<tr>
<td>Triton, + Gly</td>
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</tbody>
</table>

1 One unit/ml = A₆₃⁹/cm before irradiation. Dark-grown bean leaves (20 g) were extracted as described in the text in the presence or absence of Triton X-100 and/or glyceral.
2 A₆₃⁹ before, A₆₇₈ after irradiation.
a nontransformable PCH. Chlorophyllide a holochrome and PCH are only weakly-red fluorescent. The purification did not stabilize the holochrome.

**Purification.** A rapid purification procedure was essential since the phototransformable PCH was unstable irrespective of the degree of purity. The sequence of steps outlined above was found to be satisfactory. Starting with a frozen crude extract, the whole purification procedure required about 24 to 30 hours. A typical purification is summarized in table II. The absorption spectra of the crude extract before and after phototransformation had a 480-nm shoulder due to carotenoids that was almost completely removed by either polyethylene glycol precipitation or ultrafiltration-dialysis (15–20 hr). The hydroxylapatite chromatography only slightly increases the specific activity; however, it allows the removal of Triton X-100 by washing the absorbed PCH with a large volume of tricine buffer containing 0.2 M KCl. A typical elution profile from the DEAE-cellulose column is shown in figure 1. The arrows indicate the small portion used for the further purification to obtain highest specific activity values. The eluate from the DEAE-cellulose column showed a distinct protein absorption band at 278 nm.

![Figure 1: Chromatography of protochlorophyllide holochrome on DEAE-cellulose.](image1)

**Table II. Purification of Protochlorophyllide Holochrome**

<table>
<thead>
<tr>
<th>Stage of purification</th>
<th>Conc units/ml</th>
<th>A&lt;sub&gt;478&lt;/sub&gt;/A&lt;sub&gt;639&lt;/sub&gt;</th>
<th>Protein mg/ml</th>
<th>Specific activity units/μg protein</th>
<th>A&lt;sub&gt;280&lt;/sub&gt;/A&lt;sub&gt;439&lt;/sub&gt;</th>
<th>Yield % units</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Crude extract</td>
<td>0.272</td>
<td>1.7</td>
<td>15.3</td>
<td>17.8</td>
<td>…</td>
<td>100</td>
</tr>
<tr>
<td>2. Polyethylene glycol precipitation</td>
<td>0.285</td>
<td>1.8</td>
<td>10.5</td>
<td>27.2</td>
<td>…</td>
<td>70</td>
</tr>
<tr>
<td>3. Hydroxylapatite chromatography</td>
<td>0.110</td>
<td>1.8</td>
<td>3.5</td>
<td>31.5</td>
<td>…</td>
<td>50</td>
</tr>
<tr>
<td>4. DEAE-cellulose chromatography</td>
<td>0.090</td>
<td>2.0</td>
<td>1.6-1.9</td>
<td>47-56</td>
<td>…</td>
<td>5</td>
</tr>
<tr>
<td>5. Agarose chromatography</td>
<td>0.050</td>
<td>1.8</td>
<td>0.43</td>
<td>116</td>
<td>12</td>
<td>4</td>
</tr>
</tbody>
</table>

1 One unit/ml = A<sub>639</sub>/cm before irradiation.
2 A<sub>439</sub> before, A<sub>478</sub> after irradiation.

The A<sub>280</sub>/A<sub>290</sub> ratio of the fractions with high specific activity from the DEAE-cellulose chromatography was 1.4 to 1.6. The elution pattern from the agarose column is shown in figure 2. The PCH was eluted in 2 distinct components as indicated by protein absorbance at 280 nm and protochlorophyllide absorbance at 440 and 639 nm. The finding of 2 separate fractions of PCH by agarose chromatography indicated 2 different molecular weight fractions of PCH. The agarose column was calibrated with the molecular weight markers thyroglobulin (670,000) and catalase (240,000) which eluted with peak volumes at 62 and 100 ml respectively. The PCH elution maxima were at 70 and 90 ml, corresponding to molecular weights of the order of 550,000 and 300,000 respectively. The absorbance ratio (A<sub>290</sub>/A<sub>439</sub>) was constant from the beginning of PCH elution to a volume of 94 ml. The constancy of the ratio provides evidence for the homogeneity of the protein in this portion of the chromatogram. The transformability was about 1.8 for all fractions from the agarose column, which is lower than the value of 2.0 found in the previous purification step. The decrease is probable related to the instability of PCH during the time required for the agarose column chromatography.

![Figure 2: Chromatography of protochlorophyllide holochrome on agarose.](image2)
absorbance on standing in darkness. The wavelength shift required 30 minutes at 12° for completion and reached a limit at about 672 nm. At higher temperature, 25°, the shift is completed in 10 to 15 minutes. There is no concomitant shift of the Soret band. The wavelength shift of the newly formed chlorophyllide a holochrome was found at all stages of purification. It is probably indicative of a local environmental change between the chromophore and the protein. The observed in vivo shift of the long wavelength band of newly formed chlorophyllide (1,5,16) is probably due to the same reaction, rather than to vesicle formation. The absorption spectrum of PCH following the agarose purification is shown in figure 4.

**Fig. 3.** Absorption spectra of the protochlorophyllide holochrome from the DEAE-cellulose chromatography. (a) Before (——) and after (----) phototransformation; (b) difference spectrum of phototransformed vs nontransformed.

The homogeneity of the agarose-purified PCH was examined by disc electrophoresis. Samples applied to either 3, 5, or 7% polyacrylamide gels gave only a single diffuse band which fluoresced weakly in the red under ultraviolet light. The diffuseness of the PCH band on gel columns was an indication of an aggregating-disaggregating system. Disc electrophoresis of PCH with a detergent (13) revealed 1 strong sharp band and occasionally 2 weakly detectable slower moving bands. Apparently in the presence of sodium dodecylsulfate PCH has only 1 type of subunit.

**Spectral Properties of Purified PCH and Chlorophyllide a Holochrome.** The absorption spectrum of PCH following the DEAE-cellulose purification is shown in figure 3. The principal absorption maxima are at 440 and 639 nm with absorbance shoulders at about 615, 418, 382, and 332 nm. The absorbance maximum at 278 nm is 4.6 times greater than the 440 nm maximum. Immediately after the phototransformation a principal absorbance maximum was at 678 nm due to the newly-formed chlorophyllide a. The broad absorbance band at 625 to 630 nm and the Soret band at 438 nm are due primarily to the chlorophyllide superimposed on the absorbance of nontransformable PCH. The 678-nm peak of the newly formed chlorophyllide a holochrome shifts slowly towards shorter wavelengths in a temperature-dependent manner with a slight decrease in absorbance.

**Fig. 4.** Absorption spectra of the protochlorophyllide holochrome from the agarose chromatography prior to the 94-ml elution volume. (—) before and after (-----) the phototransformation.

**Chromophore Absorbance.** A comparison was made between the absorbance of PCH and chlorophyllide a holochrome and that of the extracted chromophores. Aliquots of PCH, of the same sample used to obtain the spectra of figure 3, were made to 80% acetone before and after the phototransformation. The samples were centrifuged to remove denatured protein, and absorption spectra were measured in 5-cm cells (fig 5) to compensate for the dilution due to acetone. The maxima of the 2 spectra are at the expected wavelengths (3). There was a shift of absorption maxima to shorter wavelengths, but there appeared to be only a small change in the heights of the absorption peaks. The absorption spectra of PCH and the acetone extracts (fig 3 and 5) permit the calculation of a transformation ratio (A_{478}/A_{499}). A value of 2.0 for the ratio is equivalent to about a 75% transformation of protochlorophyllide a to chlorophyllide a based on
known absorptivities (3). The specific activity of the highest purity PCH (fig 4, table II) is equivalent to 0.24 g of protein per μmole protochlorophyllide, thus the aggregate of molecular weight about 550,000 contains at least 2 protochlorophyllide chromophores. The smaller aggregate apparently contains only 1 chromophore. The difference spectra permit closer examination of the spectral changes. The presence of 2 chromophores in the larger aggregate is in agreement with the spectral measurement. The absorbance of the newly formed chlorophyllide a appears to arise concomitantly with a strong decrease in Soret absorbance.

Extraction with Triton X-100 facilitated the removal of carotenoids from PCH. No spectroscopically evident carotenoids were observed following partial purification. They apparently are neither an essential constituent of PCH nor required for the phototransformation. There is no spectroscopic evidence for any other visible pigment in purified PCH.

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**Literature Cited**


