Purification of the enzyme NADPH:protochlorophyllide oxidoreductase

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A procedure for the purification of the enzyme NADPH:protochlorophyllide oxidoreductase is described. This involves fractionation of sonicated oat etioplast membranes by discontinuous-sucrose-density-gradient centrifugation, which gives membranes in which the enzyme is present at a high specific activity. The enzyme is solubilized from the membranes with Triton X-100, followed by gel filtration of the extract; enzyme activity is eluted in fractions corresponding to a mol.wt. of approx. 35 000. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of the enzyme-containing fractions from gel filtration shows two peptides, of mol.wts. approx. 35 000 and 37 000.

The formation of chlorophyll by higher plants is well known to be a light-requiring process, this being apparently necessary to effect the formation of 5-aminolaevulinate and to photoconvert protochlorophyllide *a* into chlorophyllide *a*. Owing to the marked difference in spectroscopic properties between protochlorophyllide and chlorophyllide, this photoreduction can be readily detected in etiolated leaf tissues, and extensive studies *in vivo* have been carried out on this reaction (see Kirk & Tilney-Bassett, 1978).

The first studies *in vitro* on protochlorophyllide phototransformation were carried out by Smith and Krasnovsky and co-workers (Smith & Benitez, 1953; Krasnovsky & Kosobutskaya, 1952), with their isolation of a photoactive soluble protein–protochlorophyllide complex from etiolated bean leaves. This was designated ‘protochlorophyllide holochrome’. Subsequent physical measurements on this and related preparations (see Boardman, 1966) led to characterization of the holochrome as a species of mol.wt. approx. 0.6×10^6 – 1.0×10^6 . By using the detergent Triton X-100, a highly purified preparation of mol.wt. approx. 0.6×10^6 was later isolated from etiolated bean tissues (Schopfer & Siegelman, 1968). Subsequent use of saponin to facilitate solubilization led to the isolation of a soluble photoactive protochlorophyllide–protein complex from etiolated barley having a mol.wt. of only 63 000. Application of the same procedure to etiolated bean tissue gave a preparation with mol.wt. 100 000 or greater (Henningsen & Kahn, 1971).

Further characterization of the protochlorophyllide holochrome subunit has been claimed from

studies using SDS/polyacrylamide-gel electrophoresis. Canaani & Sauer (1977) reported identification of the subunit of bean protochlorophyllide holochrome by this technique as a pigment-binding polypeptide of mol.wt. 45 000. More recently Redlinger & Apel (1980), using related techniques, reported four different pigment-binding polypeptides of mol.wts. ranging from 14 000 to 70 000, all of which are concerned with the photoconversion reaction in barley extracts. A similar multiplicity of pigment-binding polypeptides was also reported earlier for maize by Guignery *et al.* (1974).

It is apparent from this listing that considerable disagreement exists about the apoprotein of photoconvertible protochlorophyllide or protochlorophyllide holochrome. Work from our laboratory (Griffiths, 1978) has ascribed to this protein an enzymic role as NADPH:protochlorophyllide oxidoreductase, with the photoactive protochlorophyllide holochrome of earlier literature equated with the protochlorophyllide reductase enzyme–substrates complex. It was therefore considered that identification of the enzyme should result in the simultaneous unequivocal identification of the apoprotein of the holochrome. In the present paper, procedures for the purification of protochlorophyllide reductase from etioplasts are described. It is shown, by a variety of techniques, that the enzyme from oats is co-purified with two closely related peptides, of mol.wts. 35 000 and 37 000.

Experimental

Materials

Sephadex 6B, Sephadex G-150 and Blue Dextran 2000 were obtained from Pharmacia Fine

Abbreviation used: SDS, sodium dodecyl sulphate.

Chemicals, Uppsala, Sweden. Thyroglobulin, bovine serum albumin and ovalbumin were purchased from Sigma (London) Chemical Co., Poole, Dorset, U.K., and cytochrome *c* was from Boehringer Mannheim, Germany. Other chemicals employed were of A.R. grade where possible.

Biological material

Seeds of oats (*Avena sativa* var. Pennal) or barley (*Hordeum vulgare* var. Proctor), obtained from local seed merchants, were imbibed and germinated between layers of absorbent paper, sown in trays of damp Levington's potting compost (Fisons, Harston, Cambridge, U.K.) and grown in the dark at 20°C for 5 or 6 days. The etiolated shoots were harvested under a dim green safe-light and all further operations were performed under a green safe-light in a 4°C cold-room.

Isolation of etioplast membranes

Approx. 200 g of etiolated material was homogenized for 20 s in an Ato-Mix blender with 700 ml of buffer 1, containing 0.5 M-sucrose, 20 mM-Tes (2-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]-amino)ethanesulphonic acid), 20 mM-Hepes, [4-(2-hydroxymethyl)-1-piperazine-ethanesulphonic acid], 2 mM-MgCl₂, 1 mM-EDTA and 5 mM-cysteine, at pH 7.5. The homogenate was filtered through four layers of muslin and four layers of Miracloth (Calbiochem, San Diego, CA, U.S.A.) and centrifuged at 500 g for 90 s to sediment nuclei, unbroken cells and debris. Etioplasts were sedimented from the supernatant by acceleration to 8000 g, followed by switching off the motor. The etioplast pellet was resuspended in buffer 2, containing 20 mM-sucrose, 20 mM-Tes, 20 mM-Hepes and 2 mM-MgCl₂, at pH 7.5, causing lysis of the plastids, and the etioplast membranes were sedimented by centrifugation at 9000 g for 15 min, then resuspended in 5–10 ml of buffer 1 or 2 as appropriate.

Sonication of etioplast membranes and sucrose-density-gradient centrifugation

Etioplast membranes, prepared as described above and suspended in buffer 2, were sonicated three times for 4 s periods in an ultrasonic disintegrator (MSE), with a 2 cm-diameter probe with settings of low power and amplitude no. 5. Sonicated membranes, in 2 ml portions, were applied to continuous (15–45%, w/w) or discontinuous (20/40%, w/w) gradients of sucrose dissolved in buffer 2. Continuous linear gradients were prepared in 25 ml tubes and centrifuged for 120 min at 2°C and 100 000 g in a 3 × 25 ml swinging-bucket rotor in an MSE Superspeed 50 centrifuge. Discontinuous gradients were prepared in 50 ml tubes and centrifuged for 120 min at 2°C and 75 000 g in an 8 × 50 ml angle rotor in an MSE Superspeed 65 centrifuge.

Either gradients were fractionated after centrifugation or, in the case of preparative discontinuous gradients, material that was collected at the 20/40%-sucrose interface was removed with a Pasteur pipette, centrifuged at 38 000 g for 30 min and the resulting pellet resuspended in 1 ml of buffer 1 to give a purified sonicated-membrane preparation.

Gel filtration of solubilized protochlorophyllide reductase

Earlier attempts at purification of the soluble enzyme by gel filtration were carried out on Sepharose 6B packed in a 2.5 cm × 35 cm column, this choice of material having been influenced by the results of earlier workers in the field of protochlorophyllide holochrome isolation. However, with the realization (see below) that the reductase could, under the appropriate conditions, be consistently eluted as a protein of mol.wt. approx. 40 000. Sephadex G-150, with a much greater resolution in this molecular-weight range, was substituted for the Sepharose 6B.

A column was equilibrated with running buffer, containing 100 mM-KCl, 50 mM-Tricine {*N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine}, 20 mM-Hepes, 2 mM-MgSO₄, 1 mM-EDTA, 0.4 mM-Triton X-100, 10% (v/v) glycerol and 5 mM-cysteine, at pH 8.0, by allowing 2 bed vol. to pass through before sample application; buffer was delivered at a rate of 24 ml/h by a peristaltic pump and eluate was collected in 3 ml fractions. Columns were calibrated by following the elution of Blue Dextran 2000 and proteins of known molecular weight monitored by their u.v. absorbance at 280 nm; the standard proteins chosen showed identical elution profiles irrespective of the presence of Triton X-100 in the running buffer. The elution volume of a species was taken as the volume of eluate from the start of the run to that eluting the highest concentration of that species.

Protochlorophyllide reductase was solubilized by mixing a suspension of etioplast membranes or sonicated membranes with an equal volume of the above running buffer containing 4 mM-Triton X-100. The mixture was kept on ice for 30 min, then centrifuged at 38 000 g for 30 min to sediment insoluble material; 2 ml of the supernatant was applied to a column.

SDS/polyacrylamide-gel electrophoresis

SDS/polyacrylamide-gel electrophoresis was performed as described by Laemmli (1970). A 12% (w/v) acrylamide/0.32% (w/v) bisacrylamide separating gel and 6% (w/v) acrylamide/0.16% (w/v) bisacrylamide stacking gel were used. Gels were run for 15 h at 60 V in a 4°C cold-room. Fractions obtained by gel filtration were concentrated before electrophoresis, either by dialysis

against Aquacide in Visking tubing or by ultra-filtration through an Amicon Minicon B15 protein separator.

Assay of protochlorophyllide reductase activity

Protochlorophyllide reductase was assayed in the presence of exogenous protochlorophyllide and NADPH by measuring the $\Delta(A_{672}-A_{710})$ in a dual-beam spectrophotometer after repeated xenon flashing as described by Griffiths (1978). Enzyme rates are expressed in units, defined as the amount of enzyme that catalyses formation of 1 nmol of chlorophyllide/min under standard assay conditions.

Spectroscopy

Visible-light-absorption spectra of fractions were recorded on a sensitive split-beam spectrophotometer as previously described (Griffiths, 1978).

Protein determination

Protein was measured as described by Bramhall *et al.* (1969).

Results

It has previously been shown (Griffiths, 1978) that the activity of protochlorophyllide reductase in etioplasts from barley is associated exclusively with the membrane fraction. On fractionation of oat etioplasts by osmotic shock in dilute buffer, followed by centrifugation, a membrane fraction is obtained containing more than 90% of the reductase activity of the original etioplast, whereas less than 5% is recovered in the supernatant fraction (Table 1). The specific activity of the enzyme in the membranes is 2.4 units/mg of protein, compared with 0.5 units/mg in the original homogenate.

Fig. 1 shows the distribution of peptides in the various fractions obtained during the isolation of oat etioplast membranes. Although a large number of the leaf proteins present in the total homogenate (track 1) are missing from the membrane fraction

(track 5), some peptides, notably those of mol.wts. 67 000, 37 000 and 12 000–20 000, appear to be selectively concentrated by the membrane isolation procedure. Far too many peptides, however, are

Fig. 1. Peptide content of fractions obtained during the preparation of purified sonicated etioplast membranes from oats

Fractions from each stage of the purification were analysed by SDS/polyacrylamide-gel electrophoresis as described in the Experimental section. Tracks 1–5 contain fractions produced during the preparation of etioplast membranes and show the following: (1) crude homogenate; (2) supernatant produced by acceleration of crude homogenate to 8000 g; (3) etioplasts; (4) soluble fraction from lysed etioplasts; (5) etioplast membranes. Tracks 6–8 contain fractions produced by centrifugation of sonicated etioplast membranes on discontinuous (20/40%, w/w) sucrose gradients as follows: (6) protein-rich fraction from top of gradient; (7) purified sonicated membranes from 20/40%-sucrose boundary; (8) pelleted material at bottom of gradient; (9) positions of standard proteins [bovine serum albumin (mol.wt. 68 000), carbonic anhydrase (29 000), cytochrome c (11 700)].

Table 1. Purification of protochlorophyllide reductase-enriched fractions

Etiolated oat shoots were homogenized and the etioplast fraction was isolated as described in the text. Etioplast membranes were prepared by osmotic breakage of the etioplasts and these were purified by centrifugation on discontinuous layers of sucrose as described. Samples of the various fractions were assayed for the total and specific activity of protochlorophyllide reductase.

Fraction	Protein (mg)	Protochlorophyllide reductase	
		Total activity (units)	Specific activity (units/mg of protein)
Crude homogenate	2898	1564	0.5
Etioplasts	218	432	2.0
Etioplast-free supernatant	1840	784	0.4
Etioplast membranes	172.8	422	2.4
Etioplast soluble fraction	60	23	0.4
Sucrose-gradient-purified membranes	2.16	30	13.9

present in the latter fraction to enable an assignment of protochlorophyllide reductase to be made at this stage.

After sonication of the membranes followed by centrifugation for 120 min on linear sucrose density gradients, the enzyme on analysis of the fractions was found to be concentrated in two bands corresponding to 34 and 24% sucrose respectively. The bulk of the protein, however, under these conditions, barely entered the gradient and appeared at the end of the centrifugation in the top 15%-sucrose fraction. Knowing the sedimentation characteristics of the enzyme, it was now possible to devise a more convenient procedure for isolation of the enzyme-enriched fractions. After some preliminary runs, a procedure involving centrifuging sonicated etioplast membrane extracts for 2 h at 75 000 *g* on discontinuous gradients of 40 and 20% sucrose was finally adopted. The distribution of the enzyme after such a procedure is shown in Fig. 2. As expected, under these conditions a large amount of the protein at the end of the run is in the low-sucrose layer at the top of the tube (fractions 10 and 11). The reductase activity, however, is now concentrated as a single band at the 40/20%-sucrose boundary (fraction 5). This band, which has been found to be made up of small membrane fragments, is readily visible under dim green light, and so in subsequent runs could be easily removed with very little contamination from the rest of the tube contents, providing a convenient method for preparing an enzyme-enriched fraction.

Included in Fig. 1 are details of the electrophoretic analysis of the peptides found in selected

fractions taken from a sample centrifuged by the adopted procedure. Comparing the distribution of peptides throughout the gradient with those present in the original membranes (Fig. 1), it is seen that the centrifugation has served to co-purify the reductase and a number of peptides, principally two of mol.wts. approx. 35 000 and 37 000, and to a lesser extent two further peptides of mol.wts. 66 000 and 70 000 (Fig. 1, track 7). The fraction taken from the top of the tube is enriched in protein (Fig. 2), particularly in low-molecular-weight peptides (12 000–20 000) from the original extract (Fig. 1, track 6), whereas the pellet at the bottom of the tube contains the same peptides (Fig. 1, track 8) as the original membranes (Fig. 1, track 5), indicating that this pellet represents non-sonicated membranes.

In order to purify the reductase from the sucrose-gradient membrane fraction, it was thought necessary to solubilize the enzyme in a native condition and to subject this to conventional protein-purification procedures. After much preliminary work, involving a whole range of solubilizing agents, the non-ionic detergent Triton X-100 was chosen as being the most suitable detergent for our purpose. Gel filtration was adopted as a protein-fractionation procedure, since ion-exchange chromatography could not be used, owing to the extreme sensitivity of the reductase to increasing ionic strength (Mapleston, 1978). Sepharose 6B was selected for the gel filtration, in view of the relatively high molecular weight value of protochlorophyllide holochrome previously published by various workers.

A Triton X-100 extract of the purified membrane

Fig. 2. Distribution of protochlorophyllide reductase activity and protein after centrifugation of oat etioplast membranes on discontinuous sucrose-density gradients

A suspension of sonicated etioplast membranes was applied to discontinuous (20/40%, w/w) gradients of sucrose as described in the text and centrifuged for 120 min at 75 000 *g*. The samples were then fractionated and each fraction (3 ml) was assayed for reductase activity (●), protein (■) and sucrose (○) concentrations.

preparation was chromatographed and eluted with detergent-supplemented buffer as described in the Experimental section. Fig. 3 records the elution characteristics of the reductase activity from the Sepharose 6B column, and includes details of the molecular-weight calibration of the column obtained in a separate experiment by the manufacturer's recommended procedure, with the standard proteins cytochrome *c* (11 700), ovalbumin (43 000) human serum transferrin (80 000) and thyroglobulin (600 000). Protochlorophyllide reductase was eluted from this column by a volume corresponding to a mol.wt. of approx. 38 000. This value is considerably lower than had been expected from most of the previously published data for protochlorophyllide holochrome. The specific activity of protochlorophyllide reductase at the peak fraction of activity elution is approx. 14 units/mg of protein.

The various fractions from the Sepharose chromatography were, after assay for enzymic activity, concentrated by dialysis against Aquacide and electrophoresed in SDS on polyacrylamide gels.

Fig. 3. Elution characteristics from Sepharose 6B of protochlorophyllide reductase solubilized from purified sonicated oat etioplast membranes

A suspension of purified sonicated etioplast membranes from oats (protein concn. approx. 2 mg/ml) was solubilized by incubation with an equal volume of 4 mM-Triton X-100 in buffer, and insoluble material removed by centrifugation as described in the Experimental section. A 2 ml sample of the supernatant was applied to the column, which was run in the dark at 4°C. Selected fractions (1 ml) of eluate were assayed for protochlorophyllide reductase activity. Calibration of the column in terms of molecular weights was carried out separately by using standard proteins as described in the text.

The resulting stained peptide patterns of the various column fractions and the original pre-chromatographed membrane sample are included in Fig. 4. Whereas at least four major peptides are present in the original membranes (track 2), successive elution of these in different fractions, on the basis of molecular size, occurs during the chromatography. The fraction representing the peak of protochlorophyllide reductase activity eluted between 65 and 71 ml contains predominantly the two peptides of apparent mol.wts. 35 000 and 37 000 (Fig. 4, track 5).

Fig. 5 records the absorption spectrum of the protochlorophyllide reductase-enriched fraction before and after illumination. The peak at approx. 630 nm for the original unilluminated material is characteristic of aqueous protochlorophyllide solutions (Griffiths, 1978). The lack of any light-induced absorbance change, despite the high reduc-

Fig. 4. Peptide composition of selected fractions from gel filtration, on Sepharose 6B, of Triton-solubilized, sonicated and purified oat etioplast membranes

The peptide composition of selected fractions as described in Fig. 3 was determined by SDS/polyacrylamide-gel electrophoresis. At four stages of the elution, adjacent pairs of fractions were combined and the resulting 6 ml samples of eluate concentrated and electrophoresed. Tracks: (1) position of protein standards [bovine serum albumin (mol.wt. 68 000), carbonic anhydrase (29 000), cytochrome *c* (11 700)]; (2) Triton-solubilized membranes; (3) fraction eluted between 0 and 6 ml; (4) fraction eluted between 39 and 45 ml; (5) fraction eluted between 65 and 71 ml; (6) fraction eluted between 99 and 105 ml.

Fig. 5. Absorption spectrum of protochlorophyllide reductase-enriched fraction derived by gel filtration of Triton-solubilized purified sonicated etioplast membranes

The absorption spectrum of the fraction containing most protochlorophyllide reductase activity obtained in Fig. 3 is shown. Illumination for 30 s resulted in no change of the spectrum.

tase activity in this fraction, indicates the absence of a photoactive complex and probably implies that the enzyme is eluted free of one or both of its substrates. The presence of protochlorophyllide absorption in the spectrum suggests that the eluted enzyme lacks NADPH. In fact, addition of NADPH to this corresponding fraction on other occasions has resulted in the pigment becoming photoconvertible, supporting this suggestion.

One important consequence of demonstrating that enzymically active, and therefore native, protochlorophyllide reductase behaves as a low-molecular-weight protein concerns our suggestion of the identity of the reductase enzyme-substrates complex with 'protochlorophyllide holochrome'. As mentioned above, holochrome preparations to date are reported as having molecular weights considerably greater than the 38000 that we find for the enzyme here. Comparing the current procedure with earlier techniques, which had led to identification of high-molecular-weight forms of both protochlorophyllide holochrome (Schopfer & Siegelman, 1968; Canaani & Sauer, 1977) and protochlorophyllide reductase (Griffiths & Mapleston, 1978) indicates that much higher Triton X-100/protein ratios were employed for enzyme solubilization in the present work. To check if this could account for the discrepancy between the present data and earlier reports, two preparations of oat etioplast membranes of different protein concentrations were extracted with Triton X-100, followed by gel filtration. Sonication of the membranes was not employed, as earlier work, resulting in the high molecular-weight estimates, was carried out without any sonication step. It was feared that the sonication may have perhaps produced artificially small particles, owing to the generation of lysophosphatides, which have a detergent action (Hauser, 1971).

Fig. 6. Elution characteristics of protochlorophyllide reductase solubilized from oat etioplast membranes on Sephadex G-150

Soluble preparations of protochlorophyllide reductase from oat etioplast membranes were prepared and chromatographed on Sephadex G-150, and fractions were collected and assayed as described in the Experimental section. The protein concentration of the suspension of membranes taken for solubilization in Figs. 6(a) and 6(b) was 6.5 and 2 mg/ml respectively. The column was calibrated for molecular weights as described in Fig. 3.

In order to improve the resolution of proteins in the lower-molecular-weight range, the Sepharose 6B column was replaced by a cross-linked dextran (Sephadex G-150) column of similar dimensions. The protein/Triton X-100 ratios (w/w) of the two different chromatographed samples were 5:1 and 1.5:1 respectively. Figs. 6(a) and 6(b) record the

elution profile of protochlorophyllide reductase activity for each sample, together with the molecular-weight calibration of the column determined as described above. It is apparent from the elution profiles that the Triton/protein ratio has a marked influence on the elution characteristics of protochlorophyllide reductase activity. Two activity peaks are apparent in the samples, one corresponding to a high molecular weight ($> 100 \times 10^3$) eluted close to the void volume and a second peak eluted later. The relative proportion of the total activity in each of the two peaks varies considerably depending on the sample. From the sample solubilized at high Triton concentration (protein/Triton 1.5:1), most of the reductase activity was retarded by the column, to be eluted later at a position corresponding to a mol.wt. of approx. 35 000 (Fig. 6*b*). Only a trace of activity was eluted close to the void volume. In contrast, the sample solubilized with the protein/Triton ratio of 5:1 gave an increase in amount of enzyme activity eluted early as a high-molecular-weight form, with a corresponding decrease in the yield of the low-molecular-weight form eluted later (Fig. 6*a*). It is therefore apparent that the ratio of Triton to protein used in the initial extraction of the enzyme from the membrane markedly influences the elution characteristics of gel filtration. Increasing the Triton/protein ratio leads to a greater proportion of the activity penetrating the gel to be eluted by a volume approaching a limit corresponding to a mol.wt. of approx. 35 000.

On analysis of the various column fractions by SDS/polyacrylamide-gel electrophoresis it became apparent that separation of the material on the column strictly according to molecular weight was only achieved for the sample solubilized at the high Triton X-100 concentration. In samples solubilized at low concentrations of Triton X-100, peptides appeared to be eluted largely unresolved by the column with peptides of low molecular weights (by SDS/polyacrylamide-gel electrophoresis) appearing in early-eluted fractions which should, from the column calibration, have contained material of much higher molecular weights. It is obvious therefore that at the lower Triton concentrations the membrane proteins are present as mostly unresolved large mixed micelles, whereas in the high-Triton samples individual protein molecules are present which are separated on the column strictly according to molecular weight.

This is borne out by peptide analysis of the two activity peaks from the chromatography of the sample solubilized at high protein/Triton ratio (Fig. 6*a*). This shows (Fig. 7) that the enzyme-containing fraction eluted late from the column (Fig. 6*a*, 302 ml after void volume) contains, as expected, peptides in the mol.wt. range approx. 30 000–50 000, with the

Fig. 7. Peptide composition of selected fractions from gel filtration on Sephadex G-150 of etioplast membranes solubilized at a high protein/Triton ratio

The peptide composition of selected fractions obtained as described in Fig. 6(*a*) was determined by SDS/polyacrylamide-gel electrophoresis. At two stages in the elution, adjacent pairs of fractions were combined and the resulting 6 ml samples of eluate concentrated and electrophoresed. Tracks: (1) fraction eluted between 0 and 6 ml; (2) fractions eluted between 27 and 33 ml; (3) position of protein standards [ovalbumin (mol.wt. 43 000) carbonic anhydrase (29 000)].

doublet at approx. 35 000 (Fig. 7, track 2) corresponding to the non-micellar form of the enzyme. In contrast, the high-molecular-weight fractions containing the reductase (Fig. 6*a*, 3 ml after void volume) have peptides (Fig. 7, track 1) covering a wide range of molecular weights, but again including the doublet at approx. 35 000. This fraction therefore corresponds to the incompletely solubilized micellar form of the enzyme.

The process of membrane isolation and solubilization by different amounts of Triton, followed by gel filtration, was repeated with barley etioplasts to check whether the findings on oat etioplasts are of

Fig. 8. Elution characteristics of protochlorophyllide reductase solubilized from barley etioplast membranes on Sepharose 6B

Soluble preparations of protochlorophyllide reductase from barley etioplast membranes were prepared and run on Sepharose 6B. The protein concentration of the suspension of membranes taken for solubilization in Figs. 8(a) (●) and 8(b) (○) was 8 and 2 mg/ml respectively.

general significance. Again, barley has been used extensively for protochlorophyllide holochrome isolation in the past, so its analysis here should provide a direct comparison of results from our technique for the isolation of the reductase with earlier data from holochrome isolations.

The barley etioplast membranes of protochlorophyllide reductase (specific activity 0.5 units/mg of protein) were solubilized in Triton X-100 at detergent/protein ratios of 6.1:1 and 1.5:1 respectively. The elution of the reductase from the Sepharose 6B column, for the two samples, together with the column calibration, are shown in Fig. 8 (traces *a* and *b*). As in the experiment with oats, an increase in the amount of the enzyme entering the column during chromatography and becoming eluted in a volume corresponding to a mol.wt. of approx. 35 000, showing that the same general phenomena described above for oats are also observed in barley. Peptide analysis of the column fractions by SDS/polyacrylamide-gel electrophoresis bore out the similarities in behaviour even further.

Discussion

Several reports on the characterization of protochlorophyllide holochrome have appeared since it was first isolated in 1952. All these studies have been

directed towards purification of a photoactive protochlorophyllide-protein complex. However, owing to the inherent instability of this association a reliable procedure for the preparation of a homogeneous photoactive protochlorophyllide complex has yet to be established. This situation is in marked contrast with the proven and tested methods available for purification of chlorophyll-protein complexes from practically all photosynthetic tissue (see Thornber, 1979).

What has emerged from holochrome isolation studies is that earlier preparations of high molecular weight, such as the 600 000-mol.wt. macromolecule found by Schopfer & Siegelman (1968), are really aggregated forms, since considerably smaller photoactive preparations (subunits) have more recently been identified by the application of modern protein-analysis techniques. Thus, using SDS/polyacrylamide-gel electrophoresis Canaani & Sauer (1977) reported the detection of a 45 000-mol.wt. polypeptide as the holochrome subunit in bean extracts, whereas more recently Redlinger & Apel (1980), using related techniques, reported another four protochlorophyllide-binding proteins in barley extracts, all apparently concerned with photo-conversion. These data, coupled with the earlier reports by Duranton's group (Guignery *et al.*, 1974) of yet a further four protochlorophyllide-binding proteins from maize extracts must, however, in view of their inconsistency, be regarded as highly tentative. It is quite likely that some of these claims reflect artefacts of the isolation techniques, since all rely on detection of protochlorophyllide associated with protein during electrophoresis of membrane extracts in SDS. It is well documented that protochlorophyllide is only loosely bound to protein in the holochrome (Manetas & Akoyunoglou, 1976; Redlinger & Apel, 1980) or reductase, probably via a salt linkage between the C-7 propionate carboxy group of the pigment and some cationic site on the protein (Griffiths, 1980). It would therefore be expected to be stripped off the protein in even the slightest trace of detergent. Non-specific adsorption of the pigment to sites on other proteins by hydrophobic interaction might then occur, producing complexes capable of surviving the electrophoresis in SDS. That such artificial complexes between metalloporphyrins and proteins can be created in SDS is already well established (see Thomas *et al.*, 1976).

In the current work these problems have been overcome by employing a less ambiguous criterion for identification of the protochlorophyllide reductase protein, namely by assaying its enzymic activity by the procedure developed in our laboratory (Griffiths, 1978). The procedure for purification of the enzyme outlined here was developed only after considerable preliminary work involving selection of

the most suitable starting material which, on processing, produced the most pure enzyme sample, choice and concentration of detergent to use, together with selection of the protein-fractionation procedure most suited to our application (Maplestone, 1978). Oat plants were finally adopted as starting material, as they gave etioplast membrane preparations which could readily be fractionated on sucrose gradients after sonication. The success of our technique in purifying the reductase is, we consider, largely due to the use of highly purified membrane preparations as the source of extractable enzyme. The introduction of sonication and the density-gradient membrane purification step served to separate the enzyme from a group of low-molecular-weight (12 000–20 000) peptides, which would not otherwise have been so readily achieved by gel filtration. The sacrifice in yield of enzyme as a result of this step (Fig. 2) is thought justified in view of this high degree of purification.

A wide range of detergents were screened before this work (Maplestone, 1978) and our experiences fully confirm the findings of Stumman (1978) regarding saponin as a good detergent for extraction of protochlorophyllide holochrome, i.e. protochlorophyllide–protein complex still retaining photo-conversion activity. However, although low concentrations of saponin gave protochlorophyllide reductase of high molecular weight on gel filtration, raising the saponin concentration to try to separate protochlorophyllide reductase of low molecular weight invariably led to inactivation of the enzyme. We found Triton X-100 to be a superior detergent for completely solubilizing the enzyme with minimum loss of enzymic activity. A point worth emphasizing, however, is that our Triton-solubilized and purified enzyme fraction did not show any photoactivity on spectroscopic analysis (Fig. 5), probably owing to loss of NADPH from the enzyme complex, and would thus not have been detected in the traditional holochrome assay. Techniques for the isolation of protochlorophyllide reductase therefore do not necessarily lead to the isolation of protochlorophyllide holochrome as traditionally defined. The term protochlorophyllide holochrome has been useful over the past few decades to describe photoactive protochlorophyllide without any knowledge of the molecular structure involved. In fact, many preparations have been described that are obviously very different, but by possessing photoconvertible protochlorophyllide qualify as protochlorophyllide holochrome. In view of the recent advances made in identifying protochlorophyllide reductase, perhaps a plea should be made in favour of replacing the old protochlorophyllide holochrome terminology with the term 'protochlorophyllide reductase enzyme–substrates complex', which takes on more significance.

The co-purification of protochlorophyllide reductase in the present work with two peptides of mol.wts. approx. 35 000 and 37 000 implies that the enzyme is purified in a state free of any detergent molecules, since Triton solubilizes proteins usually by forming micelles of approx. 140 Triton molecules (Reynolds & Stoekenius, 1977), which should have resulted in any complex being of mol.wt. at least 80 000. The presence of detergent micelles and varying extents of aggregation could well have accounted for the high molecular-weight values for protochlorophyllide holochrome reported by earlier groups (Schopfer & Siegelman, 1968) and for the enzyme solubilized here at the lower detergent/protein ratio (Figs. 6a and 7a). The fact that the enzyme from barley (Fig. 7) and also from bean extracts (R. P. Oliver & W. T. Griffiths, unpublished work) behaved in a similar manner to the enzyme from oat gives support to our conclusion on its characterization, and introduces a certain degree of uniformity to the subject for the first time.

Confirmation of this identification of the polypeptide(s) of protochlorophyllide reductase has been briefly reported by using different experimental techniques (Oliver & Griffiths, 1980).

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