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COMBINED PREPARATION OF FERREDOXIN, FERREDOXIN–NADP+ REDUCTASE AND PLASTOCYANIN FROM SPINACH LEAVES

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SUMMARY

A large-scale procedure has been developed for the combined preparation of ferredoxin, ferredoxin–NADP+ reductase (NADPH:ferredoxin oxidoreductase, EC 1.6.99.4) and plastocyanin from spinach leaves. By modification, simplification and adjustment of common isolation procedures for the individual enzymes, it has proved possible to obtain these proteins in a highly purified state and in good yield.

INTRODUCTION

We have previously reported that a purified photosynthetic pigment system I preparation, obtained from digitonin-treated chloroplasts, is able to reduce NADP+ in light with ascorbate–dichlorophenolindophenol as the electron donor, provided that ferredoxin, ferredoxin–NADP+ reductase (NADPH:ferredoxin oxidoreductase, EC 1.6.99.4) and plastocyanin are added[1–3].

A detailed study of this pigment system therefore called for large amounts of these photosynthetic electron-transferring proteins. Although isolation procedures for the individual enzymes have been described in the literature[4–18], it was felt that by modification of these methods a procedure could be developed which (a) would permit simultaneous isolation of the proteins from one batch of spinach leaves, (b) was applicable to large amounts of starting material and (c) would give a higher yield, particularly of the relatively "expensive" enzymes ferredoxin and plastocyanin.

With this approach, we have developed a large-scale preparative procedure in which ferredoxin, ferredoxin–NADP+ reductase and plastocyanin are purified together in the initial stages, and separation and purification of the individual enzymes are effected in as few steps as possible. The procedure considerably reduced the time needed for the enzyme isolation and, in addition, involved a higher yield of ferredoxin and plastocyanin than previous methods. The latter was accomplished by, among other things, using a new homogenization method, keeping plastocyanin in the reduced form throughout the isolation procedure and introducing recycling chromatography.

EXPERIMENTAL PROCEDURE

(A) *Enzyme assays*

Plastocyanin was determined spectrophotometrically by measuring the difference in absorbance at 597 nm between the oxidized (ferricyanide) and reduced (ascorbate) sample ($\Delta A_{597\,nm}$). The amount of plastocyanin was calculated by use of the molar absorbance coefficient of oxidized plastocyanin: $\varepsilon_M = 9.8 \cdot 10^3$ (ref. 16). The purity of the sample was indicated by the absorbance ratio $A_{278\,nm}/\Delta A_{597\,nm}$ ("absorption index"). The purity of ferredoxin was represented by the absorbance ratio $A_{422\,nm}/A_{274\,nm}$ or $A_{422\,nm}/A_{276.5\,nm}$ (see below). The amount of ferredoxin was calculated from the absorbance at 422 nm using the molar absorbance coefficient $\varepsilon_M = 10.32 \cdot 10^3$ (ref. 19). Pyridine nucleotide transhydrogenase activity of ferredoxin–NADP+ reductase was assayed by the method of KEISTER *et al.*[13]. One enzyme unit was defined as the amount of enzyme which produces an increase in absorbance of 0.01 at 340 nm in 3 min.

NADPH-diaphorase activity of ferredoxin–NADP+ reductase was measured by the procedure of KEISTER *et al.*[13], using dichlorophenolindophenol as the electron acceptor. One enzyme unit was defined as the amount of enzyme causing a decrease in absorbance of 1.0 per min at 620 nm. The enzyme assays were carried out at 25° in a model CF4R Optica spectrophotometer. Spectra were recorded in a model 14R Carry spectrophotometer.

Specific activity was expressed in units per mg of protein. The protein content of the enzyme preparations was determined by the ultraviolet absorbance procedure of Warburg and Christian, with the Folin–Ciocalteu reagent, or by the biuret method, using crystalline bovine serum albumin as a standard[20]. Nitrogen was determined by a micro Kjeldahl method[21]. As the ultraviolet absorbance procedure gives a somewhat higher protein value for ferredoxin–NADP+ reductase than the other methods, this procedure was not used for calculation of the specific activity of the pure enzyme.

(B) *Preparation of ferredoxin, ferredoxin–NADP+ reductase and plastocyanin*

All stages in the purification process were performed at 0–4°; most of them were carried out in a cold room. Freshly picked spinach leaves were washed in tap water and distilled water, drained by centrifuging in nylon cloth in a domestic spin-drier, packed in plastic bags in batches of 1 kg and stored in a deep-freeze until required.

20 kg of frozen leaves, corresponding to about 13 kg of dry deveined leaves, were pounded and thawed by immersing the plastic bags in running tap water. The thawed leaves were then ground for about 5 min in 0.05 M phosphate buffer (pH 8.0) containing 0.1 % Triton X-100 (1 l of buffer per kg of spinach leaves), in a "PUC" colloid mill type JV 10 (Probst and Class GMBH, Rastatt). The effect of various homogenization media on the enzyme yield was tested. The above medium was found to be the most suitable as it permitted complete extraction of the proteins. After grinding, the dark-green homogenate was drawn off and centrifuged at 2000 × *g* for 30 min in M.S.E. centrifuges, type Mistral 6L. The residue was washed once by homogenizing for 5 min with 5 l of 0.05 M phosphate buffer (pH 8.0) containing 0.1 % Triton X-100, in the colloid mill and then discarded. The supernatant and wash fluid were combined and transferred in batches of 3 l to plastic buckets, cooled in an ice-salt

mixture. The extract was chilled to 0° (10 to 15 min) before acetone (Merck, p.a.), cooled overnight in the deep-freeze (−25°), was added slowly and with mechanical stirring up to a final concentration of 35 %. The addition of acetone was performed at such a rate that the temperature did not rise above 0° (20 to 30 min). The suspension was centrifuged at 2000 × *g* and at 0° for 15 min, and the dark-green residue was discarded. The enzymes were then precipitated from the supernatant solution by the slow addition of acetone, cooled in the deep-freeze, to a final concentration of 80 %. The precipitate settled rapidly when stirring was discontinued. The greater part of the supernatant fluid could be decanted before collection of the precipitate by centrifuging for 10 min at 2000 × *g*. The supernatant fluid was discarded and the residue suspended in 600 ml of 0.01 M Tris buffer (pH 7.3). Extraction of the enzymes was facilitated by using a Potter–Elvehjem homogenizer. The suspension was centrifuged at 23000 × *g* for 20 min, and the residue was once again extracted with 200 ml of 0.01 M Tris buffer (pH 7.3), by stirring for several hours. The supernatant and wash solution were dialyzed overnight against 5 mM Tris buffer (pH 7.3). The precipitate that appeared during dialysis was removed by centrifugation.

To the acetone-free, dialyzed extract 5 M NaCl solution was added to bring the Cl^- concentration up to 0.2 M. The extract was then applied to a column (42 cm × 5 cm) of DEAE-cellulose (DE22, Whatman), equilibrated with 0.15 M Tris–HCl buffer (pH 7.3), containing 0.07 M NaCl (final concentration of Cl^- 0.2 M). The column was washed with the same buffer (about 1 l) until the absorbance at 280 nm, as measured by a Uvicord II absorptiometer (LKB, Stockholm), had decreased again to a constant low level.

The passed solution ("0.2 M Cl^- eluate of first DEAE-cellulose column"), yellowish-brown in color, was used for the preparation of ferredoxin–$NADP^+$ reductase and plastocyanin (see below).

The DEAE-cellulose column was successively washed with 500 ml of 0.15 M Tris buffer (pH 7.3), containing 0.27 M Cl^- and about 500 ml of 0.15 M Tris buffer (pH 7.3), containing 0.30 M Cl^-. Washing with the latter solution was continued until the reddish band of ferredoxin had descended nearly to the bottom of the column. The ferredoxin was then eluted with 0.15 M Tris buffer (pH 7.3), containing 0.35 M Cl^-. Ferredoxin-containing fractions having an absorbance ratio $A_{422\,nm}/A_{274\,nm}$ of 0.1 or higher were combined and concentrated with an Amicon ultrafiltration cell, model 401, using a Diaflo ultrafiltration membrane, type UM-1 (Amicon Corporation, Cambridge, Mass.). The concentrated solution (6 ml) was passed through a Sephadex G-75 (superfine, bead form, Pharmacia, Uppsala) column (90 cm × 3 cm), equilibrated against 0.01 M Tris buffer (pH 7.3), using the LKB recycling chromatography system (ReCyChrom, LKB, Stockholm). The flow rate was 10–15 ml/h. Usually two cycles were sufficient to separate the ferredoxin from a faster-moving colorless and a slower-moving yellow protein. The faster-moving component was removed from the system after the first cycle. The ferredoxin effluent was collected in 3-ml fractions, which were analyzed for ferredoxin content and absorbance at 274 nm. The fractions having an absorbance ratio $A_{422\,nm}/A_{274\,nm}$ of 0.42 or higher were combined, concentrated by means of ultrafiltration (Amicon ultrafiltration cell, model 50) to a volume of 25 ml, deaerated under reduced pressure and stored at −20° in a nitrogen atmosphere. The yield of ferredoxin was between 350 and 400 mg, and the ratio of the absorbance at 422 and 274 nm was 0.45. The yield is even higher (375 to 425 mg) if we accept the

value $\varepsilon_M = 9.68 \cdot 10^3$, which is based on the new molecular weight of spinach ferredoxin (11650) (ref. 22). The ultraviolet absorption peak of the purest fractions from the Sephadex column was at 276.5 nm, and the absorbance ratio $A_{422\,nm}/A_{276.5\,nm}$ was 0.49 (*cf.* ref. 10).

The "0.2 M Cl^- eluate of the first DEAE-cellulose column" (see above), containing the two other enzymes, was concentrated to about 300 ml by ultrafiltration and fractionated with ammonium sulfate at 40–75 % saturation. The pH was kept within the range 7.3–7.6 by adding 0.1 M NaOH. The precipitate from the ammonium sulfate fractionation was used for the preparation of ferredoxin–NADP$^+$ reductase, the supernatant for the isolation of plastocyanin.

The supernatant containing plastocyanin was dialyzed for about 16 h against 1 mM phosphate buffer (pH 7.0) to remove excess salt. The plastocyanin was kept in the reduced state (1 ml of 0.1 M sodium ascorbate was added when the protein was found to be partially oxidized), and the dialyzed solution was applied to a column (18 cm × 3 cm) of DEAE-cellulose equilibrated with 0.01 M phosphate buffer (pH 7.0). The column was washed with 250 ml of 0.01 M phosphate buffer (pH 7.0) before elution was started by gradually increasing the molarity of the phosphate buffer to 0.2 M. After the removal of protein impurities with 250 ml of 0.1 M and 250 ml of 0.15 M phosphate buffer (pH 7.0), as indicated by the absorbance at 280 nm, plastocyanin was recovered from the column with 0.2 M phosphate buffer. The fractions having absorption indices lower than 5.5 were combined and concentrated by ultrafiltration. The concentrated solution was passed through a Sephadex G-75 column (90 cm × 3 cm), equilibrated against 0.01 M phosphate buffer (pH 7.0), using the LKB recycling chromatography system. Two cycles were sufficient to separate the plastocyanin from a faster-moving yellow fraction. The fractions having absorption indices lower than 2.2 were combined and saved. The yield of plastocyanin with an absorbance index of 2 was 130 mg. Though an absorption index of 0.85 was attainable in the purest fractions by continued gel filtration or by chromatography on hydroxylapatite, samples of plastocyanin with such a high degree of purity were found to be unstable. Similar observations have been made by KATOH *et al.*[16] and by GORMAN AND LEVINE[17].

After addition of some potassium ferricyanide to convert the protein into the oxidized form, the plastocyanin solution was dialyzed against 1 mM phosphate buffer (pH 7.0) and then stored as a frozen solution at −20°. Under these conditions the protein was found to remain stable for several months.

The precipitate from the ammonium sulfate fractionation, which contained ferredoxin–NADP$^+$ reductase, was collected by centrifugation and dissolved in 100 ml of 0.01 M Tris buffer (pH 7.3). The solution was passed through a Sephadex G-25 column, equilibrated with 0.01 M Tris buffer (pH 7.3) to remove excess salt. The enzyme, dissolved in 0.01 M Tris buffer (pH 7.3) containing 5 mM mercaptoethanol, was then adsorbed on a DEAE-cellulose column (42 cm × 5 cm), equilibrated with the same buffer. After washing with 1.5 l of 0.01 M and 2 l of 0.05 M Tris buffer (pH 7.3) containing 5 mM mercaptoethanol, ferredoxin–NADP$^+$ reductase was eluted from the column with a linear gradient of 0.1 to 0.2 M Tris buffer (pH 7.3) containing 5 mM mercaptoethanol (3 l). In this way it proved possible to separate ferredoxin–NADP$^+$ reductase from several other enzymes, *e.g.* nitrite reductase, which was eluted at a higher molarity of Tris buffer. The yellow fractions containing ferre-

doxin–$NADP^+$ reductase, as indicated by their pyridine nucleotide transhydrogenase activity and NADPH-specific diaphorase activity, were combined, concentrated by ultrafiltration to about 200 ml and fractionated with ammonium sulfate at pH 7.5. The purest fractions, usually precipitating between 60–65 % and 65–70 %saturation, were dissolved in a small volume of 0.01 M Tris buffer (pH 7.3) and then subjected to gel filtration on a Sephadex G-100 column (90 cm × 3 cm), equilibrated with the same buffer. The best fractions from the column (specific transhydrogenase activity higher than 600) were combined and applied to a column of hydroxylapatite (Bio-Gel HT, Bio-Rad Laboratories, Richmond, Calif.) of 6 cm × 3.5 cm, equilibrated with 1 mM phosphate buffer (pH 6.5). The column was washed with 100 ml of 0.01 M potassium phosphate buffer (pH 7.0) and 100 ml of 0.1 M potassium phosphate buffer (pH 7.0). Subsequently the phosphate concentration was raised to 0.2 M and washing continued until the enzyme had descended nearly to the bottom of the column (about 100 ml). Elution was then achieved by increasing the phosphate concentration to 0.25 M. The purified enzyme was stored in a deep-freeze at −20°, if desired after gel filtration (Sephadex G-25) or dialysis against 0.01 M Tris buffer (pH 7.3), to remove excess phosphate. The specific activity of pure ferredoxin–$NADP^+$ reductase was 830 in the transhydrogenase assay, and 260 in the diaphorase assay. The yield was about 180 mg.

TABLE I

SUMMARY OF THE PURIFICATION PROCEDURE FOR SPINACH FERREDOXIN, PLASTOCYANIN AND FERREDOXIN–$NADP^+$ REDUCTASE

Starting material corresponding to 13 kg of deveined spinach leaves.

Stage of purification	*Absorbance ratio* $A_{422\,nm}/A_{274\,nm}$	*Ferredoxin (mg)*
Eluate DEAE-cellulose column	0.13	*
Effluent recycling chromatography on Sephadex G-75	0.45	390
	Absorption index $A_{278\,nm}/\Delta A_{597\,nm}$	*Plastocyanin (mg)*
Eluate DEAE-cellulose column	200–300	*
Dialyzed supernatant of ammonium sulfate fractionation	33	215
Eluate 2nd DEAE-cellulose column	4.9	180
Effluent recycling chromatography on Sephadex G-75	2.0	130
	Specific transhydrogenase activity of ferredoxin–NADP+ reductase	*Units*
Eluate DEAE-cellulose column	24	800 000
Ammonium sulfate precipitate (40–75 % saturation)	55	590 000
Eluate 2nd DEAE-cellulose column	170	450 000
Ammonium sulfate precipitate (60–70 % saturation)	400	300 000
Effluent Sephadex G-100 column	660	210 000
Eluate hydroxylapatite column	830	150 000

* These values could not be determined spectrophotometrically because of the presence of contaminating pigments.

All three enzymes were shown to be homogeneous; no traces of contaminants could be found by disc-gel or starch-gel electrophoresis. The procedure described above has been repeated several times in our laboratory with consistent and reproducible results. In case of insufficient centrifuge capacity it may be advantageous to use a continuous-action rotor for the large-scale centrifugation at the initial purification step.

Representative data concerning yields and purities obtained in the preparation of ferredoxin, plastocyanin and ferredoxin–NADP$^+$ reductase are summarized in Table I.

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