A novel procedure for the rapid purification of plastocyanin from pea (*Pisum sativum*) leaves

Anthony R. ASHTON* and Louise E. ANDERSON

Department of Biological Sciences, University of Illinois at Chicago Circle, Chicago, IL 60680, U.S.A.

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Plastocyanin is soluble at high concentrations ($>3\text{ M}$) of $(\text{NH}_4)_2\text{SO}_4$ but under these conditions will adsorb tightly to unsubstituted Sepharose beads. This observation was utilized to purify plastocyanin from pea (*Pisum sativum*) in two chromatographic steps. Sepharose-bound plastocyanin was eluted with low-ionic-strength buffer and subsequently purified to homogeneity by DEAE-cellulose chromatography.

Plastocyanin, a copper-containing protein of the photosynthetic electron-transport chain that donates electrons to the Photosystem I complex (Lien & San Pietro, 1979), has been purified from a number of plants, including spinach (*Spinacia oleracea*) and French bean (*Phaseolus vulgaris*) (Kato, 1971; Milne & Wells, 1970). Purification schemes used have included fractionation with acetone and $(\text{NH}_4)_2\text{SO}_4$ (Kato, 1971), followed by repeated chromatography on ion-exchange resins and gel filtration. These procedures usually require at least three and up to five chromatographic steps (Gorman & Levine, 1966). In the present paper we describe a method for the purification of plastocyanin that employs two chromatographic steps. This procedure was used to purify pea (*Pisum sativum*) plastocyanin, a previously neglected component of one of the most studied photosynthetic organisms.

Experimental

Materials

Sepharose 4B, Sephacryl S-200, Sephadex G-150 and G-25 were obtained from Pharmacia, Piscataway, NJ, U.S.A., Whatman cellulose powder for column chromatography (CF 1) from Reeve Angel, Clifton, NJ, U.S.A., and Bio-Gel P-200 and P-4, from Bio-Rad Laboratories, Richmond, CA, U.S.A. DEAE-cellulose (Serva) was obtained from Gallard Schlesinger Corp., Carle Place, NY, U.S.A. $(\text{NH}_4)_2\text{SO}_4$ (special enzyme grade) was obtained from Schwarz-Mann, New York, NY, U.S.A., Triton X-100, Tris and Hepes from Sigma Chemical

Co., St. Louis, MO, U.S.A. All other chemicals were Analytical-Reagent grade. Pea seeds (*Pisum sativum* L., var. Little Marvel) were obtained from Northrup and King, Chicago, IL, U.S.A.

Methods

Pea plants were grown in vermiculite in a greenhouse. Chloroplasts were prepared as described by Cockburn *et al.* (1968), but in the absence of sodium isoascorbate. The chloroplasts were lysed by resuspension in water and the suspension was then adjusted to 50 mM-Hepes (potassium salt)/10 mM-KCl/2 mM MgCl_2 /1 mM-EDTA (potassium salt), pH 7.4 (Hepes/KCl buffer) with concentrated buffer. Thylakoid membranes were sedimented by centrifugation (20 000 g for 15 min).

Plastocyanin was detected by its absorbance at 597 nm. During purification, plastocyanin was quantified by measuring the absorbance difference between oxidized [$1\text{ mM-K}_3\text{Fe}(\text{CN})_6$] and reduced (10 mM-ascorbate) samples. The A_{278} was used as a measure of protein concentrations. Alternatively the dye-binding method of Bradford (1976) was used.

Gel electrophoresis was conducted as described by Laemmli (1970). The conductivity of solutions was measured at 20°C with a Radiometer conductivity meter type CDM 2e and a CDC 114 cell (0.64 cm).

Batch adsorption to various chromatographic matrices. Various matrices (5 ml portions) were added to 40 ml portions of a 3.5 M- $(\text{NH}_4)_2\text{SO}_4$ solution in 50 mM-Tris/HCl/10 mM-KCl/2 mM- MgCl_2 /1 mM-EDTA, pH 7.4 (Tris/KCl buffer) containing plastocyanin. The suspension was incubated for 1 h and the resin collected by filtration on to Miracloth and washed with 20 ml of 3.5 M- $(\text{NH}_4)_2\text{SO}_4$ in Tris/KCl buffer. These washings were pooled with the sample filtrate. The various

Abbreviations used: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

* Present address: C.S.I.R.O. Division of Plant Industry, P.O. Box 1600, Canberra City, A.C.T. 2601, Australia.

chromatographic matrices were then washed sequentially with 20 ml each of 2.9M- and 1.9M- $(\text{NH}_4)_2\text{SO}_4$ and finally Tris/KCl buffer.

All procedures in the purification of plastocyanin were conducted at $1^\circ\text{--}4^\circ\text{C}$.

Results and discussion

Plastocyanin was extracted from thylakoid membranes with 50mM-Tris/HCl/200mM-KCl, pH 9.0, containing 1% Triton X-100 or the HEPES/KCl buffer containing 1% Triton X-100. $(\text{NH}_4)_2\text{SO}_4$ (200g/litre) was dissolved in the detergent extract and insoluble material collected by filtration through Whatman no. 1 filter paper. $(\text{NH}_4)_2\text{SO}_4$ (300g/litre) was dissolved in the filtrate, and the precipitate, which contains membrane-associated thioredoxin (Ashton *et al.*, 1980), collected by centrifugation (20000g for 10 min). Plastocyanin did not precipitate and could be detected by the blue colour of the supernatant solution. Passage of the $(\text{NH}_4)_2\text{SO}_4$ solution through a column of Sepharose 4B pre-equilibrated with 2.9M- $(\text{NH}_4)_2\text{SO}_4$ in Tris/KCl buffer caused plastocyanin to be adsorbed to the top of the column. Continued washing of the column with 2.9M- $(\text{NH}_4)_2\text{SO}_4$ in Tris/KCl buffer (1 column volume) did not elute the plastocyanin, which remained near the top of the column. The plastocyanin could be eluted with buffer containing 1.9M- $(\text{NH}_4)_2\text{SO}_4$, but was routinely eluted with Tris/KCl buffer containing no $(\text{NH}_4)_2\text{SO}_4$ (Fig. 1). The plastocyanin elutes with the Tris/KCl buffer.

Fig. 1. Elution of plastocyanin from a Sepharose 4B column

A 430 ml sample (containing 18 A_{597} units of plastocyanin) was applied to a Sepharose 4B column ($4.9\text{cm}^2 \times 7.5\text{cm}$) equilibrated with 2.9M- $(\text{NH}_4)_2\text{SO}_4$ in Tris/KCl buffer. The column was washed with 80 ml of 2.9M- $(\text{NH}_4)_2\text{SO}_4$ in Tris/KCl buffer, then plastocyanin eluted with Tris/KCl buffer, ●, A_{278} ; ○, A_{597} (oxidized minus reduced); △, conductivity, [note 1 mS (SI unit) $\equiv$ 1 mmho)].

The recovery of plastocyanin in the Tris/KCl eluate was greater than 95%. The A_{278}/A_{597} ratio of the eluate is approx. 43, suggesting that the plastocyanin is <5% pure. However, gel electrophoresis reveals that plastocyanin is the major protein in the eluate and similarly comprises 40% of the protein as determined by the Coomassie Blue-binding procedure of Bradford (1976) can be attributed to plastocyanin [based on plastocyanin content estimated by A_{597} (oxidized minus reduced)]. Clearly most of the material absorbing at 278 nm is not protein. The bulk of this material does not elute from the subsequent DEAE-cellulose column. The plastocyanin-containing fractions were pooled, oxidized with $\text{K}_3\text{Fe}(\text{CN})_6$ and dialysed against Tris/KCl buffer. The non-diffusible solution could then be adsorbed to a DEAE-cellulose column equilibrated with Tris/KCl buffer. A linear gradient (0–200mM-KCl) in the Tris/KCl buffer was used to elute the plastocyanin (Fig. 2). Some material absorbing at 278 nm is not retained by the column. The gradient elutes more material absorbing at 278 nm, followed by the plastocyanin peak, which shows a constant A_{278}/A_{597} ratio. In some preparations plastocyanin is the only protein that is eluted by the KCl gradient. The plastocyanin eluted from the DEAE-cellulose column shows only one band (mol.wt. 11000) after electrophoresis in 15% (w/v) acrylamide gels containing SDS. Gel filtration of the plastocyanin through a Sephacryl S-200 column shows a single protein peak. Pea plastocyanin preparations have A_{278}/A_{597} ratios ranging from 1.1 to 1.5. This is comparable with absorbance ratios of other plastocyanins (Ramshaw *et al.*, 1973). Oxidized pea plastocyanin has absorbance

Fig. 2. Elution of plastocyanin from a DEAE-cellulose column

Plastocyanin, eluted from a Sepharose 4B column, as described in Fig. 1 and dialysed against Tris/KCl buffer was applied to a DEAE-cellulose column ($4.9\text{cm}^2 \times 5\text{cm}$) and eluted with a KCl gradient from 0 to 200mM-KCl. ○, A_{278} ; ●, A_{597} ; △, conductivity (note 1 mS $\equiv$ 1 mmho).

Table 1. *Adsorption of plastocyanin on various chromatographic matrices*

Adsorption and elution of plastocyanin was as described in Experimental section. Approx. 3 A_{597} units were present in each experiment. The 3.5 M-(NH₄)₂SO₄ fraction contains material that did not bind to the column. Batch adsorption was less effective than column adsorption. Thus batch treatment with Sepharose adsorbed approx. 15% of the plastocyanin adsorbed by an equal volume of Sepharose in a column.

(NH ₄) ₂ SO ₄ fraction (M)	Chromatographic matrix ...	Percentage of total plastocyanin in (NH ₄) ₂ SO ₄ eluate						
		Sephacryl S-200	Sepharose 4B	Cellulose	Sephadex G-25	Sephadex G-150	Bio-Gel P-4	Bio-Gel P-200
1.5		7.2	35.5	72.9	84.4	82.4	89.9	96.6
2.92		4.8	12.5	14.9	3.0	10.7	4.3	2.0
1.95		45.6	38.8	8.3	8.0	5.3	4.8	1.0
0.0		42.4	13.2	3.9	4.5	1.5	1.0	0.4

peaks at 460nm, 597nm and 770nm, which disappear on reduction.

A number of chromatographic supports were examined for the ability to adsorb plastocyanin from solutions containing (NH₄)₂SO₄. Sepharose 4B and Sephacryl S-200 were the most effective column adsorbents, binding plastocyanin at the top of the column, whereas cellulose and Sephadex G-150 or G-25 were much less effective and easily became saturated with plastocyanin. Polyacrylamide-based resins P-4 and P-200 showed little affinity for plastocyanin. Table 1 lists the results of batch adsorption of plastocyanin from 3.5 M-(NH₄)₂SO₄ (90% satd.). Sephacryl S-200 was the most effective adsorbent, whereas Sepharose 4B also bound substantial amounts of plastocyanin. The other adsorbents did not bind appreciable amounts of plastocyanin, although cellulose and the Sephadex resins were again relatively better adsorbents than the polyacrylamide resins.

Why does plastocyanin adsorb to Sepharose as we have described? It is probably not zone precipitation as described by Porath (1979), since adsorption occurs at constant ionic strength, and furthermore, adsorption of plastocyanin is strictly dependent on the nature of the chromatographic support, whereas zone precipitation should be independent of the nature of the support.

Fujita *et al.* (1980) have reported that yeast enzymes bind to cellulose at high ionic strength and can be eluted by hydroxy-group-rich ligands such as glycerol or sucrose in a procedure they describe as 'hydrogen-bond chromatography'. Inclusion of 1 M-sorbitol in the presence of (NH₄)₂SO₄, however, retards rather than enhances the elution of plastocyanin from Sepharose 4B, indicating that these two phenomena are different.

The plastocyanin-Sepharose interaction is reminiscent of hydrophobic chromatography in that adsorption occurs at high ionic strength and elution is accomplished at low ionic strength (Hofstee & Otilio, 1978). Hydrophobic interactions may not

be the sole driving force, however, since the adsorption of plastocyanin can occur in the presence of 1% (v/v) Triton X-100. Furthermore, chromatography at 24°C causes a more rapid elution of plastocyanin rather than the retardation expected for a purely hydrophobic interaction.

The data in Table 1 indicate that carbohydrate-based resins are necessary for significant adsorption, suggesting that the pyranose rings are important in the adsorption process. It is noteworthy that agarose (Sepharose 4B), which is the best unmodified carbohydrate support, consists of alternating D-galactose (or 6-O-methyl-D-galactose) and 3,6-anhydro-L-galactose residues (Percival, 1970), which could provide the site for hydrophobic interactions with plastocyanin.

We have found that pea ferredoxins can also be adsorbed to Sepharose 4B at high ionic strength in a process that is very similar to that which we have described for plastocyanin (Ashton & Anderson, 1980). In this procedure, however, the two electron carriers are separated before Sepharose 4B chromatography, since all the ferredoxin of the chloroplast is found in the stromal fraction after lysis of the chloroplasts, whereas the plastocyanin remains attached to the thylakoid membranes. Preliminary experiments show that ferredoxin and plastocyanin can be separated on the Sepharose 4B column (ferredoxin being more tightly bound than plastocyanin), suggesting that a combined purification of plastocyanin and ferredoxin is feasible.

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