Isolation and Identification of Poplar Isoplastocyanins

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An improved four-stage isolation and purification procedure for preparing poplar isoplastocyanins is described in detail. Absorbance (UV-VIS) spectroscopy and isoelectric focusing (IEF) are used to determine the protein purity and identity. The present procedure increases twice the total plastocyanin (PC) yield. Four PC isoform fractions are consecutively isolated at the third chromatographic step: oxidized PC*a*(II) and PC*b*(II) and reduced PC*b*(I) and PC*a*(I). PC*a*(II) and PC*b*(II) obtained at the fourth chromatographic step are highly purified PC isoforms which show the purity index (*p.i.*) $A_{278}/A_{597} \leq 0.85$. Isoelectric points (p*I* values) of the PC isoforms are found to be at pH 3.92 ± 0.04 for PC*a* and at pH 3.85 ± 0.02 for PC*b*. The results of appropriate biological experiments that include the highly purified poplar PC isoforms could give answers to the questions about the physiological significance of PC dimorphism for photosynthesis.

Key words: Photosynthesis, Plastocyanin, Dimorphism

Introduction

A variety of methods have been developed for isolation and purification of plastocyanin (PC) from both intact leaves and isolated chloroplasts (Freeman and Guss, 2001; Yokum, 1982). Briefly, most of these methods utilize solvent extraction and precipitation with acetone or ammonium sulfate, followed by ion-exchange chromatography on DEAE cellulose, and DEAE Sephadex and/or gel filtration. Total PC yields of 6–10 mg kg^{-1} have been achieved, depending on the starting material (Freeman and Guss, 2001).

Isolation of PC has been successfully realized by a method elsewhere described (Plesničar and Bendall, 1970). The modification of this method is proved to be important for revealing PC dimorphism in poplar (Dimitrov, 2007). The respective procedure is briefly described in Dimitrov *et al.* (1987) and Donchev and Dimitrov (1988).

In the present paper we describe an improved variant of the method mentioned above (Dimitrov *et al.*, 1987; Donchev and Dimitrov, 1988). The total PC yield is two times higher than that cited before (Freeman and Guss, 2001). Besides, together with the PC*a*(II) and PC*b*(II) fractions two additional PC*a*(I) and PC*b*(I) fractions are isolated. PC*a*(II) and PC*b*(II) show a higher degree of purity compared to our previous results (Dimitrov *et al.*, 1987; Donchev and Dimitrov, 1988). The purity and the redox states of the fractions were determined by their specific UV-VIS absorption spectra. The identity of the protein fractions was found out by isoelectric focusing (IEF).

Materials and Methods

General

PC*a* and PC*b* were prepared from fresh, depetiolated leaves of poplar (*Populus nigra* var. *italica*) following the method of Dimitrov *et al.* (1987) and Donchev and Dimitrov (1988), with some modifications. UV-VIS spectra were recorded at room temperature on a Specord UV-VIS spectrophotometer (Carl Zeiss, Jena, Germany) as described by Donchev *et al.* (2004). IEF was performed as by Donchev (1989) and Dimitrov *et al.* (2008). All stages of the procedure were carried out at 2–4 °C.

Experimental

An excess amount of liquid nitrogen was poured out to 5 kg leaf material. The mixture received was mechanically ground into a powder using a laboratory grinder "Mechanica-309" (Poland). The powder material was again cooled by liquid nitrogen. To the cooled material 10 l of 0.1 M tris(hydroxymethyl)aminomethane (Tris)-

HCl buffer, pH 7.6, containing 2 μM phenylmethylsulfonyl fluoride (PMSF), 0.0025 M ε-amino-*n*-caproic acid and 1 ml β-mercaptoethanol, were added. The extract was vigorously stirred for 30 min. Then the obtained extract (≈ 10 l) was filtered through 8 gauze layers and was additionally separated by centrifugation on a K-23 centrifuge (VEB MLW Zentrifugenbau Engelsdorf, Leipzig, Germany) for 10 min at 3600 × *g*. 2.2 Volumes of cooled acetone were added to the supernatant with stirring and the mixture was kept in an ice-box for protein sedimentation for one night. On the other day the supernatant was decanted and the sediment was separated from the residual supernatant by centrifugation for 10 min at 3600 × *g*. The sediment contained all watersoluble proteins including PC and could be kept at –25 ºC for a long time without loosing its nativity.

To further execute the procedure, 200 g of the sediment were homogenized in 300 ml 0.1 M Tris-HCl buffer, pH 7.6, by stirring for 1 h on a magnetic stirrer in an ice-box. The homogenate was centrifuged for 10 min at 3600 × *g*. The supernatant contained the extracted proteins. The sediment was re-suspended in 300 ml of the same buffer for extraction of the residual protein amounts.

The brown protein solution was dialyzed for 36 h against 15 l of 0.05 M Tris-HCl buffer, pH 7.6, conductivity σ = 3.03 mS cm^{-2} (20 ºC). After dialysis pH and σ values were corrected up to the initial values of the dialyzing buffer.

The protein solution (1 l) was loaded on a first chromatographic column (DE-52 Whatman cellulose, 5.0 × 25 cm) equilibrated with 0.05 M Tris-HCl buffer, pH 7.6. The elution of the protein fractions started with a linear gradient of NaCl, from 0.0 to 0.19 M in two volumes of 500 ml each of the equilibrating buffer, followed by isocratic elution with 1 l of 0.05 M Tris-HCl buffer, containing 0.19 M NaCl, pH 7.6, σ = 18.8 mS cm^{-2} (20 ºC) (Fig. 1). The elution curve was registered on a double beam flow photometer "ISCO" (Instrumentation Specialties Co., Lincoln, NB, USA) supplied by an optic cell Type 6 and a monitor "UA-5". Fractions of 15 ml each were collected by an "LKB"-2112 collector.

PC was eluted from the column as a colourless reduced fraction [PC(I)] soon after beginning of the isocratic elution. PC was identified by the specific absorption band of its oxidized state [PC(II)] at 597 nm in the differential spectrum [PC(II) minus PC(I)]. At high protein concentrations the identification could be done visually after addition of a few drops of concentrated $K_3[Fe(CN)_6]$ which made the PC solution dark blue coloured.

The integrated PC fraction (≈ 500 ml) was dialyzed for 36 h against 0.02 M Na-phosphate buffer, pH 6.9, σ = 2.4 mS cm^{-2} (20 ºC). After oxidation with some excess of $K_3[Fe(CN)_6]$ the PC fraction was loaded on a second chromatographic column (DE-52 Whatman cellulose, 3.5 × 25 cm) equilibrated with 0.02 M Na-phosphate buffer, pH 6.9, σ = 2.4 mS cm^{-2} (20 ºC) (Fig. 2). The elution started with a linear gradient of Na-phosphate buffer concentrations, pH 6.9, from 0.02 to 0.2 M in two volumes of 300 ml each. The linear gradient shifted the blue PC fraction up to two thirds of the column length. $K_3[Fe(CN)_6]$ was held on the top of the column. Next, an isocratic elution with 0.2 M Na-phosphate buffer, pH 6.9, σ = 16.4 mS cm^{-2} (20 ºC) was applied. About one third of the obtained PC fraction was in the oxidized state [PC(II)], the remaining was fully reduced [PC(I)]. PC(I) was identified spectrally and visually by oxidation with $K_3[Fe(CN)_6]$.

The combined PC fractions (≈ 350 ml) were dialyzed 24 h against 0.03 M Na-phosphate buffer, pH 6.9, σ = 3.25 mS cm^{-2} (20 ºC). After oxidation with some excess of $K_3[Fe(CN)_6]$ the PC fraction was loaded on a third chromatographic column (DE-52 Whatman cellulose, 2.0/3.5 × 50 cm for lower or higher protein amounts, respectively) equilibrated with 0.03 M Na-phosphate buffer, pH 6.9, σ = 3.25 mS cm^{-2} (20 ºC) (Fig. 3). The elution started with 0.1 M Na-phosphate buffer, pH 6.9, σ = 8.8 mS cm^{-2} (20 ºC). During the chromatography the blue protein fraction was separated into two oxidized PC*a*(II) and PC*b*(II) sub-fractions which were eluted well separated from the column. Further elution with 0.15 M buffer resulted in obtaining two colourless reduced PC*b*(I) and PC*a*(I) fractions. Small amounts of all PC fractions were desalted and concentrated by ultrafiltration through an "Amicon YM-3" membrane (Beverly, MA, USA) and were checked for purity by their UV-VIS spectra (Fig. 4) and IEF (Fig. 5).

The remaining amounts of PC*a*(II) and PC*b*(II) were dialyzed against 12 l 0.03 M Na-phosphate buffer, pH 6.9, σ = 3.25 mS cm^{-2} (20 ºC) and were subjected one by one to chromatography on a fourth column (DE-52 Whatman cellulose, 2.0 × 50 cm) equilibrated with 0.03 M Na-phosphate buffer,

pH 6.9, σ = 3.25 mS cm^{-2} (20 °C). An isocratic elution with 0.1 M of the same buffer was carried out. Both PC*a*(II) (not shown) and PC*b*(II) showed similar chromatographic and spectral feathers (Figs. 6 and 7, respectively). The highly purified PC*a*(II) and PC*b*(II) fractions were desalted and concentrated by ultrafiltration through an "Amicon YM-3" membrane. After UV-VIS analysis (Fig. 7) they were divided in small (1 ml) volumes at the concentration of 1 mg ml^{-1} and were kept under argon at –25 °C.

Results and Discussion

First of all, the present procedure allows an isolation and superfine purification of PC*a*(II) and PC*b*(II). The procedure is sufficiently effective and reproducible under the conditions of the routine laboratory practice.

The isolation of the total PC fraction was carried out on the first DE-52 Whatman cellulose column (Fig. 1). The PCs were eluted in reduced state after the NaCl concentration was gradually increased to 0.19 M in the isocratic phase. The elution mobility of the protein fractions during the ion-exchange chromatography was determined from the p*I* values of the proteins and the parameters of the elution buffer (pH, ionic strength) (Scopes, 1985). Under the conditions of the first chromatographic column (Fig. 1) the PCs were eluted as the last fraction because of their low p*I* values (< 4.0, see below) which were preceded by two sizable non-PC fractions (Fig. 1). They were eluted slightly ahead the PCs and exhibited a UV-VIS Soret (405 nm) peak. The elimination of many other contaminants like polyphenols and protein fractions with close p*I* values took place on the second DE-52 Whatman cellulose column (Fig. 2). Again a large contaminant fraction that exhibiting a UV-VIS Soret (405 nm) peak was eluted slightly ahead of the total PC fraction. The real separation of the two isoplastocyanins, however, occurred under the chromatographic conditions of the third DE-52 Whatman cellulose column (Fig. 3). Moreover, after PC*a*(II) and PC*b*(II) were eluted in their reduced forms from the column, they underwent partial oxidation during ultrafiltration.

In Fig. 4 the spectra of PC*a*(II), PC*b*(II), PC*b*(I) and PC*a*(I) from the third column corresponding to the fraction numbers 1–4 are shown. They were registered in the range of 240–700 nm in a

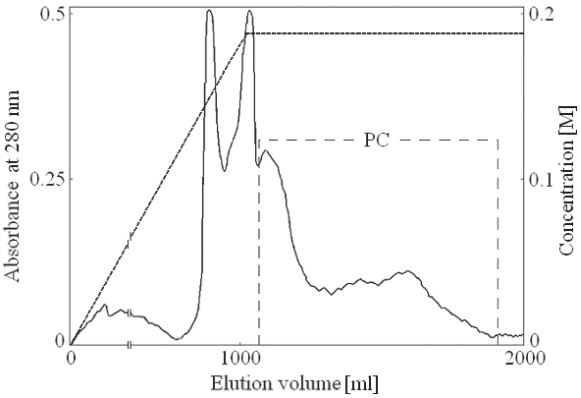

Fig. 1. Isolation of the total PC fraction from a water/acetone extract of poplar leaves on the first chromatographic column (DE-52 Whatman cellulose, 5.0 × 25 cm): (———), elution curve; (------), consecutive linear gradient (0.0–0.19 M of NaCl) and isocratic (0.19 M of NaCl) elution in 0.05 M Tris-HCl buffer, pH 7.6.

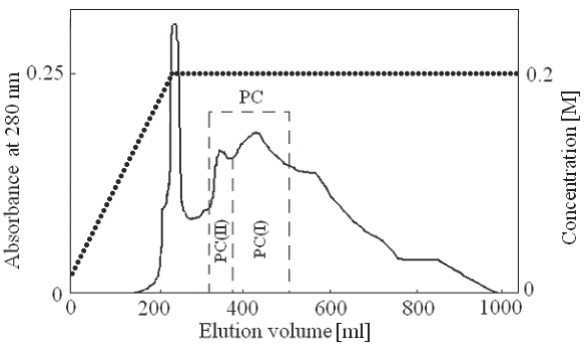

Fig. 2. Purification of the total PC fraction on the second chromatographic column (DE-52 Whatman cellulose, 3.5 × 25 cm): (———), elution curve; (• • • • •), consecutive linear gradient (0.02–0.2 M) and isocratic (0.2 M) elution with Na-phosphate buffer, pH 6.9.

regime of transmittance (0–100%) and were used for qualitative comparison between the fractions. The spectra of PC*a*(II) and PC*b*(II) (Fig. 4, fraction numbers 1 and 2, respectively) were typical for an oxidized PC (Donchev and Dimitrov, 1988). At this stage of purification the oxidized isoforms showed purity indexes as follows: $p.i._{PCa(II)} \approx 1.1–1.4$ and $p.i._{PCb(II)} \approx 1.0–1.2$. The spectra of PC*b*(I) and PC*a*(I) (Fig. 4, fraction numbers 3 and 4, respectively) pointed out to partial oxidation of the PCs and contamination with another type of protein. Evidence is provided by the spectral shoulder at 405 nm (24000 cm^{-1}) and disappearing

of the characteristic sub-structure of the spectral profile in the range of 250–270 nm (40000–37000 cm^{-1}).

p*I* values of PC*a* and PC*b* were determined by IEF on standard 1-mm Servalit polyacrylamide gel (PAAG) plates (Serva, Heidelberg, Germany) with a pH gradient of 3–6. The pH gradient profile was determined by excision of series of 1 × 0.5 mm gel slices followed by extraction of ampholytes with 0.1 M KCl attended by pH measurements (Dimitrov *et al.*, 1993). The p*I* values of the PC fractions were determined as averages of the measurements on five individual plates at equal conditions. The measured values were $pI_{(PCa)}$ = (3.92 ± 0.04) pH and $pI_{(PCb)}$ = (3.85 ± 0.02) pH, *i.e.* the difference between both p*I* values is 0.07 pH units. In Fig. 5 the isophoregrams of one plate are shown. On the positions 1–4 the PC fractions of the third chromatographic column were loaded in order of their elution. It is evident that PC*a*(II) and PC*b*(II) (positions 1 and 2, respectively) were homogeneous and fully purified fractions. PC*b*(II) exhibited a little lower p*I* value, *i.e.* it is "more acidic". The p*I* values of fractions 3 and 4 were equal of those in positions 2 and 1, respectively. That means that fractions 3 and 4 could be identified as PC*b*(I) and PC*a*(I), respectively. At higher protein concentrations at least two weaker "satellites" with p*I* ≤ 3.6 pH in position 3 could be observed. Obviously, PC*b*(I) and PC*a*(I) were incompletely purified at this chromatographic step. That is reflected in their absorption spectra (Fig. 4).

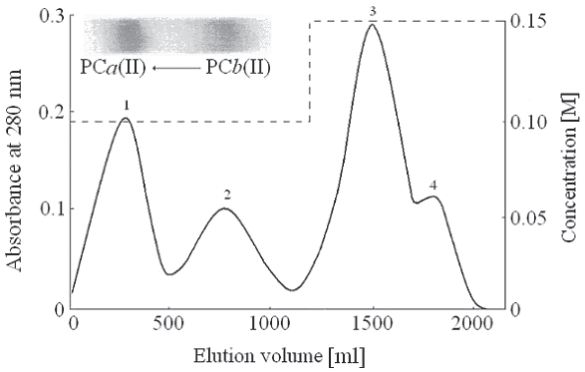

Fig. 3. Separation of the oxidized and reduced forms of PC*a* and PC*b* on the third chromatographic column (DE-52 Whatman cellulose, 2.0 × 50 cm, the inset picture above on the left): (———), elution curve: 1, PC*a*(II); 2, PC*b*(II); 3, PC*b*(I); 4, PC*a*(I); (- - - - - - -), the terrace-like isocratic (0.1–0.15 M) elution with Na-phosphate buffer, pH 6.9.

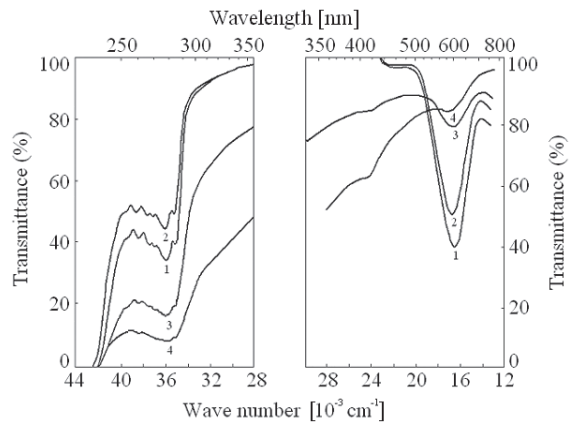

Fig. 4. UV-VIS spectra of the oxidized and reduced forms of PC*a* and PC*b* (fractions 1, 2, 3, and 4) of the third chromatographic column. 1, PC*a*(II); 2, PC*b*(II); 3, PC*b*(I); 4, PC*a*(I).

The additional purification of PC*a*(II) and PC*b*(II) on the fourth column (Fig. 6) increased the purity of the two isoproteins. The achieved values of *p.i.* ≤ 0.85 claimed etalon for PC purity (Fig. 7).

The presented procedure follows the principle approach for isolation of a total PC fraction (Plesničar and Bendall, 1970), modified later to isolate poplar PC*a* and PC*b* (Dimitrov *et al.*, 1987; Donchev and Dimitrov, 1988). The presented development of our method improves significantly

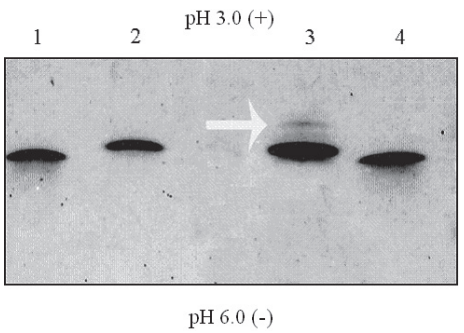

Fig. 5. Isophoregrams of the oxidized and reduced forms of PC*a* and PC*b* (fractions 1, 2, 3, and 4) of the third chromatographic column. Positions 1, 2, 3, and 4 correspond to PC*a*(II), PC*b*(II), PC*b*(I), and PC*a*(I), respectively. The white arrow indicates two weaker "satellites" on 3rd position. Standard polyacrylamide gel (PAAG) plate (Serva), pH 3.0–6.0 (24 °C).

the isolation procedure for poplar PC isoforms, as follows:

(i) The two-fold "temperature shock" treatment of the biomass by liquid nitrogen doubles the yield of the total PC fraction (≈ 20 mg kg^{-1}).

(ii) Some specific inhibitors like β-mercaptoethanol – a phenol-oxidases inhibitor (Scopes, 1985), PMSF – a specific inhibitor of trypsin and chymotrypsin (Fahrney and Gold, 1963), and ε-amino-*n*-caproic acid – a specific inhibitor of carboxy-peptidase B (Dessaint *et al.*, 1979), added to the starting 0.1 M Tris-HCl buffer decrease the protein loss during the extraction procedure. They prevent PC proteins from enzymatic hydrolysis and oxidation of the phenol groups by formation of dark pigments from endogenous phenol oxidases.

(iii) Consecutive application of a linear gradient (0.0–0.19 M) and isocratic (0.19 M) elution with NaCl shifts the total PC fraction twice faster compared to the isocratic 0.5 M Tris-HCl buffer elution (Dimitrov *et al.*, 1987; Donchev and Dimitrov, 1988). Two well defined non-PC fractions that show a UV-VIS Soret (405 nm) peak are eluted ahead of the total PC fraction (Fig. 1).

(iv) The linear gradient (0.02–0.2 M) and the isocratic (0.2 M) elution with Na-phosphate buffer, pH 6.9, in the second chromatographic column (Fig. 2), separate more effectively a large contaminant fraction that exhibits a UV-VIS Soret (405 nm) peak compared to the isocratic conditions (Dimitrov *et al.*, 1987; Donchev and Dimitrov, 1988).

(v) Terrace-like isocratic (0.1–0.15 M) elution with Na-phosphate buffer, pH 6.9, in the third chromatographic column (Fig. 3) separates additionally two reduced PC sub-fractions [PC*b*(I) and PC*a*(I)] while the single isocratic elution with 0.1 M phosphate (Dimitrov *et al.*, 1987; Donchev and Dimitrov, 1988) results in obtaining PC*a*(II) and PC*b*(II) only.

(vi) The additional fourth column chromatography (Fig. 6) results in isolation of PC*a*(II) and PC*b*(II) with a high degree of purity (*p.i.* ≤ 0.85) (Fig. 7) – a result unattainable elsewhere up to now.

At the molecular level, poplar PC is the most studied plastocyanin type (Freeman and Guss, 2001). Among all known isoplastocyanins, only poplar PC*a* and PC*b* differ in their electrostatics (Dimitrov *et al.*, 1987). This difference causes the successful separation of the two isoforms by the method presented here. The PC isoform separation from other plants was carried out by other methods like chromatofocusing (Dimitrov *et al.*, 1990), "finger print" (Dimitrov *et al.*, 1993), preparative electrophoresis (Burkey *et al.*, 1996) or proteomics (Kieselbach *et al.*, 2000). The presented method is applicable for poplar PC and enables the laboratory personnel to produce large

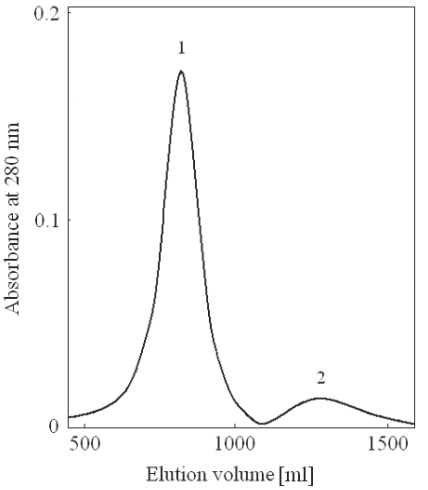

Fig. 6. Last purification of PC*b*(II) (fraction 2 of Fig. 3) on the fourth chromatographic column (DE-52 Whatman cellulose, 2.0 × 50 cm). Isocratic elution with 0.1 M Na-phosphate buffer, pH 6.9: 1, highly purified PC*b*(II); 2, PC*b*(I) with traces of contaminants.

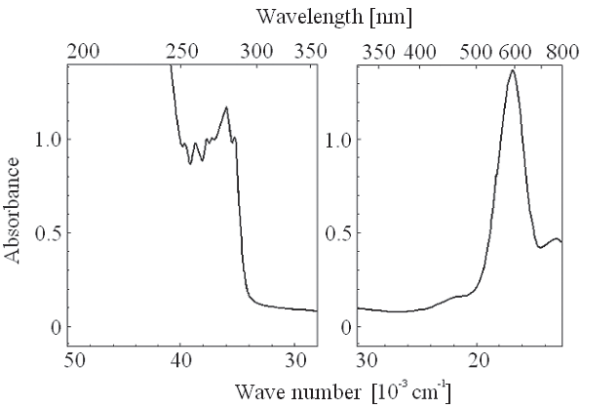

Fig. 7. UV-VIS absorption spectrum of highly purified PC*b*(II) from the fourth chromatographic column (fraction 1 of Fig. 6) in 10 mM Na-phosphate buffer, pH 6.9, *p.i.* ≈ 0.85.

and qualitative PC*a* and PC*b* amounts for further investigation of their physiological significance.

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Burkey K. O., Gizlice Z., and Carter T. E. (1996), Genetic variation in soybean photosynthetic electron transport capacity is related to plastocyanin concentration in the chloroplast. Photosynth. Res. **49**, 141–149.

Dessaint J. P., Katz S. P., and Waksman B. H. (1979), Cathepic carboxypeptidase B as a major component in "T-cell activating factor" of macrophages. J. Immunopharmacol. **1**, 399–414.

Dimitrov M. I. (2007), Plastocyanin dimorphism. DSc thesis. Bulgarian Academy of Sciences, Institute of Biophysics, Sofia.

Dimitrov M. I., Egorov C. A., Donchev A. A., and Atanasov B. P. (1987), Complete amino acid sequence of poplar plastocyanin *b*. FEBS Lett. **226**, 17–22.

Dimitrov M. I., Donchev A. A., and Egorov T. A. (1990), Microheterogeneity of parsley plastocyanin. FEBS Lett. **265**, 141–145.

Dimitrov M. I., Donchev A. A., and Egorov T. A. (1993), Twin plastocyanin dimorphism in tobacco. Biochim. Biophys. Acta **1203**, 184–190.

Dimitrov M. I., Donchev A. A., Toromanov G. R., Getov V. I., and Toncheva-Panova T. G. (2008), Plastocyanin microheterogeneity in *Scenedesmus acutus* MT8. Ind. J. Biochem. Biophys. **45**, 310–316.

Donchev A. A. (1989), Structural and physicochemical characteristics of isoplastocyanins from *Populus nigra* var. *italica*. PhD thesis. Bulgarian Academy of Sciences, Institute of Biophysics, Sofia.

Donchev A. A. and Dimitrov M. I. (1988), Molecular heterogeneity of poplar plastocyanin. C. R. Acad. Bulg. Sci. **41**, 69–72.

Donchev A., Shosheva A., Dimitrov M., Toromanov G., Getov V., Kachalova G., and Bartunik H. (2004), Isolation and analysis of poplar cytochrome b_6f subcomplexes. C. R. Acad. Bulg. Sci. **57**, 67–74.

Fahrney D. E. and Gold A. M. (1963), Sulfonyl fluorides as inhibitors of esterases. I. Rates of reaction with acetylcholinesterase, α-chymotrypsin and trypsin. J. Am. Chem. Soc. **85**, 997–1000.

Freeman H. C. and Guss J. M. (2001), Plastocyanin. In: Handbook of Metalloproteins, Vol. 2 (Messerschmidt A., Huber R., Poulos T., and Wieghardt K., eds.). John Wiley and Sons, New York, pp. 1153–1169.

Kieselbach T., Bystedt M., Hynds P., Robinson C., and Schröder W. P. (2000), A peroxidase homologue and novel plastocyanin located by proteomics to the *Arabidopsis* chloroplast thylakoid lumen. FEBS Lett. **480**, 271–276.

Plesničar M. and Bendall D. S. (1970), The plastocyanin content of chloroplasts from some higher plants estimated by a sensitive enzymatic assay. Biochim. Biophys. Acta **216**, 192–199.

Scopes R. (1985), Extract preparation. In: Protein Purification (Scopes R. K., ed.). Mir, Moscow, pp. 34–196.

Yokum C. F. (1982), Purification of ferredoxin and plastocyanin. In: Methods in Chloroplast Molecular Biology, Section M: Membrane Proteins and Protein Complexes, Chapter 79 (Edelman M., Hallick R. B., and Chua N. H., eds.). Elsevier Biomedical Press, Amsterdam, New York, Oxford, pp. 973–981.