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Note

High-performance liquid chromatography of chloroplast pigments

One-step separation of carotene and xanthophyll isomers, chlorophylls and pheophytins

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Owing to their light-harvesting and protective functions[1], the chlorophylls and carotenoids of the photosynthetic membrane are of key interest in studies on thylakoid architecture[2], chloroplast development[3] and senescence[4], adaptation to environmental conditions[5] and effects of pollution-induced stress[6]. In higher plants, the pattern of plastid pigments is highly conserved. In addition to the porphyrin-derived pigments chlorophyll *a*, chlorophyll *b* and pheophytin *a*[7], the chloroplasts generally contain β-carotene, lutein, violaxanthin and neoxanthin as major and lutein epoxide and antheraxanthin as minor carotenoids[8]. Zeaxanthin, formed from violaxanthin via antheraxanthin under light[9,10] may be present at different levels depending on the light exposure of the plant. Only a few deviations from this general pattern have been observed; spruce needles, for example, further contain α-carotene[11] and *Lactuca* species and some closely related genera synthesize lactucaxanthin[12]. Thus, the plastid pigments include groups of isomeric carotenoids with either β–β-, or β–ε- or ε–ε-end-groups (*i.e.*, β- or α-carotene, zeaxanthin or lutein or lactucaxanthin, antheraxanthin or lutein epoxide) and cover a wide range of polarity.

In recent years, high-performance liquid chromatographic (HPLC) techniques have facilitated the separation and quantification of plastid pigments, but fast and efficient one-step procedures for higher plants have not yet been fully developed. Clearly, separation procedures developed for carotenoids of similar polarity[13,14] are of little use for mixtures of plastid pigments. The HPLC systems in refs. 15–21 allowed the major groups of plastid pigments to be separated but were either not successful in or not tested for the separation of carotenoid isomers. With other procedures[22,23] carotenoid isomers could be separated, but the pigment extracts examined contained no chlorophylls. In one study[24] more than 40 chlorophyll and carotenoid pigments from phytoplankton species were separated. This procedure covered an extended polarity range and even resolved the highly polar algal pigments chlorophyll *c*, peridinin and fucoxanthin. Lutein and zeaxanthin were partially resolved, but the resolution of α- and β-carotene was not satisfactory.

In this paper a fast one-step separation of chlorophylls and carotenoids from higher plant chloroplast using HPLC is described. By modifying the HPLC system of

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Krinsky and Welankiwar[22] we improved the separation efficiency for chlorophylls *a* and *b* and isomeric carotenoids. Also, we improved the stability of H^+-sensitive pigments during separation. Pigment extracts from plants differing in their carotenoid isomer content were used to demonstrate the separation power of the system.

EXPERIMENTAL

Plant material

Endive (*Cichorium endivia*) and lettuce (*Lactuca sativa*) were obtained from local markets and spruce needles (*Picea abies*) from the shaded lower branches of a 15-year-old tree growing near the Institute. The needle generation of 1987, harvested in October 1987, was examined.

Chloroplasts of *Lactuca sativa* with low or high zeaxanthin content were prepared as follows. *Lactuca sativa* plants were kept in the dark at 4°C overnight to allow for conversion of zeaxanthin into violaxanthin[10], and chloroplasts were prepared from this material as in ref. 25. The chloroplasts were resuspended in 50 m*M* citrate–sodium hydroxide buffer (pH 5.2) and aliquots were incubated for 15 min at room temperature in the dark with or without 15 m*M* ascorbic acid. The former but not the latter condition induced the enzymatic de-epoxidation of violaxanthin via antheraxanthin to zeaxanthin[26,27]. The incubated chloroplasts were sedimented by centrifugation and washed twice with 50 m*M* HEPES–sodium hydroxide buffer (pH 7.5).

Pigment standards for HPLC

Lutein, violaxanthin, neoxanthin and β-carotene, extracted from *Cichorium endivia* leaves, were separated on Kieselgel G plates with hexane–isopropanol–water (100:10:0.25). Commercially available α- and β-carotene were also used.

Chemicals

Acetonitrile, hexane and methanol (Promochem, Wesel, F.R.G.) were of Chrom AR grade. For HPLC, acetonitrile and methanol were filtered through HULP-type and hexane through HAWP-type Millipore filters (pore size 0.45 μm), degassed at reduced pressure and stored under helium. Kieselgel G was purchased from Merck (Darmstadt, F.R.G.) and α- and β-carotene from Sigma (St. Louis, MO, U.S.A.).

Pigment extraction

Pellets of pre-treated and washed lettuce chloroplasts were quantitatively extracted with acetone and the extract was clarified by centrifugation and used immediately for chlorophyll determination[28] and HPLC analysis. Green leaves of *Cichorium endiva* (1 g) were homogenized together with 1 g of calcium carbonate in an ice-cold mortar, suspended in acetone at room temperature and the mixture was quantitatively extracted in an Allihn's glass filter tube (pore size 10–16 μm) (Schott, Mainz, F.R.G.) under gentle suction. Spruce needles (100 mg) were frozen in liquid nitrogen, mixed with 100 mg of calcium carbonate and homogenized for 1 min in the 5-ml cuvette of a dismembrator (Type II) (Bachofer, Reutlingen, F.R.G.). The still frozen powder was suspended in acetone and the mixture was quantitatively extracted and filtered as described above. The pigment extracts (adjusted to 10–20 μg/ml of

chlorophyll *a*) were either used immediately for HPLC analysis or were stored under nitrogen at −20°C in the dark for a few days without pigment alteration.

Liquid chromatographic system

The chromatograph (all components from Waters Millipore, Eschborn, F.R.G.) consisted of two Model 510 pumps, a U6K universal liquid chromatograph injector, a Model 680 automated gradient controller, a Model 490 programmable multi-wavelength detector and Model 740 data module.

Liquid chromatographic conditions

Pigment separations were performed at room temperature on a Waters Nova-Pak C_{18} Radial-Pak cartridge (dimensions 8 × 100 mm, filled with 4-μm spherical particles, end-capped) combined with a Waters RCM-100 radial compression separation system. The cartridge was protected with a Guard-Pak precolumn insert of μBondapak C_{18} (end-capped). The following solvent mixtures were used: (A) acetonitrile–methanol–0.2 *M* Tris–HCl buffer (pH 8.0) (74:6:1); (B_1) methanol–hexane (5:1) or (B_2) methanol–hexane (7:1). For pigment separation, isocratic chromatography with 100% A (from 0 to 4 min) was followed by a linear gradient from 100% A to 100% B (from 4 to 9 min), isocratic chromatography with 100% B (from 9 to 18 min) and a linear gradient from 100% B to 100% A from 18 to 20 min). The flow-rate was 2 ml/min. Pigment samples in acetone (10–20 μl containing chlorophyll *a*, chlorophyll *b* and total carotenoids in approximate amounts of 200, 60 and 60 pmol, respectively) were injected. The back-pressure increased during the runs from initial values of 150–200 p.s.i. ($1 \cdot 10^6$–$1.4 \cdot 10^6$ N m^{-2}) to final values of 300–400 p.s.i. The principal absorbance detector wavelength was 440 nm, which suits both chlorophylls and carotenoids, but 400 nm (to detect pheophytin *a* and acid-treated epoxy-carotenoids) and 280 nm were also routinely checked.

Absorbance spectroscopy

Absorbance spectra were monitored with a DW-2000 spectrophotometer (SLM Instruments, Urbana, IL, U.S.A.).

RESULTS AND DISCUSSION

Fig. 1 shows the HPLC separation of pigments from three plants selected for differences in their pigment patterns, *i.e.*, *Cichorium endivia*, *Picea abies* and *Lactuca sativa*. The major peaks, *i.e.*, peaks 1, 2, 7, 10, 11 and 13, observed for all three plants, are assigned to neoxanthin, violaxanthin, lutein, chlorophyll *b*, chlorophyll *a* and β-carotene, respectively. Their retention times agreed with those of the purified standards, and their elution sequence corresponded to that observed with various reversed-phase HPLC systems[16,17,18–21]. Our neoxanthin standard (λ_{max} in ethanol 466, 438, 414 and 330 nm) exhibited a well defined fine structure in its visible spectrum but no strong *cis* peak in its UV absorbance spectrum, as reported for 9′-*cis*-neoxanthin[21,29]. The neoxanthin isolated from our pigment extracts by HPLC appears to have the same configuration. This assumption, based on identical retention times, is supported by the well established occurrence of 9′-*cis*-neoxanthin in chloroplasts[21]. A small peak occurring just before neoxanthin for both the neoxanthin

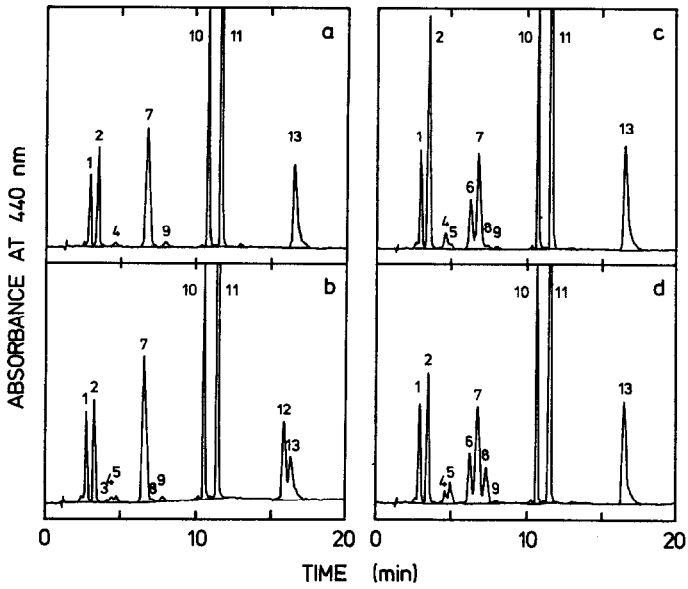

Fig. 1. HPLC separation of pigments extracted from leaves of *Cichorium endivia* (a), needles of *Picea abies* (b) and chloroplasts of *Lactuca sativa* before (c) and after (d) partial de-epoxidation of violaxanthin via antheraxanthin to zeaxanthin. Solvent systems A and B_1 (see Experimental) were used. Peaks: 1 = neoxanthin, 2 = violaxanthin, 3 and 4 = lutein epoxides (?), 5 = antheraxanthin, 6 = lactucaxanthin, 7 = lutein, 8 = zeaxanthin, 9 = ?, 10 = chlorophyll b, 11 = chlorophyll a, 12 = α-carotene, 13 = β-carotene.

standard and plant pigment extracts (Fig. 1) could be strongly enhanced during standard preparation by prolonged drying of the developed TLC plate. Therefore, this compound appears to be, at least in part, an isomerization artifact.

In addition to the generally occurring pigments, α-carotene was resolved from the needles of *Picea abies* (Fig. 1b, peak 12). The ratio of α- to β-carotene is known to vary and to increase in shade-adapted needles[30], as in the material we used. Lactucaxanthin, observed in significant amounts in *Lactuca sativa* chloroplasts[31] was clearly separated from lutein, as shown in Fig. 1c and d (peak 6). Subjection of *Lactuca sativa* chloroplasts to violaxanthin de-epoxidation (Fig. 1d) allowed peak 5 to be assigned to antheraxanthin, the intermediate, and peak 8 to zeaxanthin, the final product of the de-epoxidation sequence[32]. Owing to their retention times, the minor peaks 3 and 4 might tentatively be assigned to lutein epoxides[21], but their identification has not been attempted.

Short-term treatment of pigment extract with 2 m*M* hydrochloric acid caused 99% of the chlorophyll *a*, 13% of the chlorophyll *b*, 81% of the violaxanthin, 40% of the pigments of peaks 3–5 and 9% of the neoxanthin to be converted into pheophytins or 5,8-epoxy-carotenoids[33], respectively, whereas lutein, zeaxanthin and α- and β-carotene were not affected. Fig. 2 shows the acid-induced pigment change and the occurrence of pheophytin b (peak 10) and pheophytin a (peak 11). Two further peaks also detected after acid treatment (Fig. 2b, peaks x_1 and x_2) are assumed to represent 5,8-epoxy-carotenoids.

To obtain the described pigment separations, the original procedure of Krinsky and Welankiwar[22] was subjected to several modifications. We added 1.25% of 0.2 *M* aqueous Tris buffer (pH 8) to solvent system A to remove traces of acid (brought in by acetonitrile) and to protect the highly acid-labile pigments chlorophyll *a* and violaxanthin from conversion. This modification was a prerequisite for reproducible quantification of the pigments (S.D. < 2%). Also, we changed the step gradient in ref. 22 to a 5-min linear gradient, which allowed the time interval between the elution of chlorophyll *b* and chlorophyll *a* to be trebled. Finally, we lowered the hexane content in solvent system B, thus improving the lifetime of the cartridge and also the separation efficiency of the system for chlorophylls and carotenes. Comparison of Figs. 1b and 2a demonstrates the latter effect for identical pigment extracts. On lowering the hexane content in solvent system B further (methanol–hexane: 5:1 for Fig. 1 and 7:1 for Fig. 2) each of the chlorophyll peaks was resolved into a major and a minor peak, comparable to those observed in ref. 21. While the retention times of α- and β-carotene increased, their separation was also improved.

While searching for separation procedures for higher plant pigments, we chose to optimize the HPLC system in ref. 22 rather than that in ref. 24 because it worked with one rather than two columns, with solvent systems of relatively low viscosity and, as a consequence, with back-pressures far below the rated limit of the RCM columns.

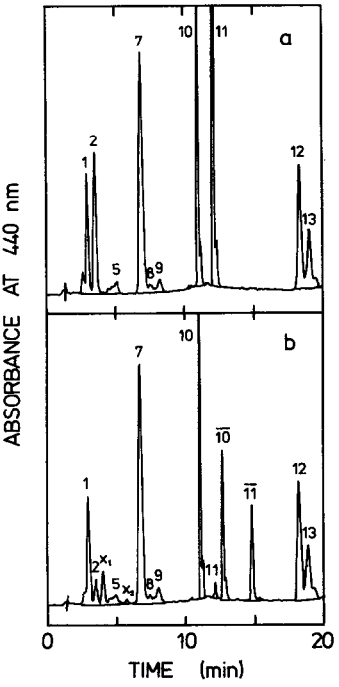

Fig. 2. HPLC separation of a pigment extract from *Picea abies* before (a) and after (b) acid treatment. Solvent systems A and B_2 (see Experimental) were used. For acid treatment, the pigment solution in acetone (0.96 ml) was mixed with 0.1 *M* hydrochloric acid (0.02 ml) and immediately neutralized with 0.2 *M* Tris–HCl buffer (pH 8.0) (0.02 ml). Peaks 1–13 as in Fig. 1; 10 = pheophytin *b*, 11 = pheophytin *a*, x_1 = ?, x_2 = ?

In spite of the simpler experimental setting up, an improved resolution of the critical isomer pairs lutein–zeaxanthin and α–β-carotene was obtained here.

The described HPLC procedure for the one-step separation of chloroplast pigments, requires less than 20 min and should help in the elucidation of several questions of physiological significance, such as pheophytin formation, the activity of the violaxanthin de-epoxidation and zeaxanthin epoxidation reactions and the regulation of α- and β-carotene levels in α-carotene-containing plants.

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