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DETERMINATION OF PLASTOQUINONE-9 IN CHLOROPLASTS BY HIGH PRESSURE LIQUID CHROMATOGRAPHY

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Abstract—A method for the specific determination of nmol quantities of plastoquinone-9 in chloroplasts is described. HPLC of a heptane extract of chloroplasts separated the plastoquinone-9 from other material and permitted a final spectrophotometric assay. Levels of plastoquinone-9 and plastoquinol-9 were determined in dark adapted and illuminated chloroplasts.

INTRODUCTION

Plastoquinone-9 (2,3-dimethyl-5-solanesyl-1,4-benzoquinone) is present in leaves of higher plants in both the oxidised (quinone) (PQ-9) and the reduced (dihydroquinol) form (PQH_2-9). Methods for quantitative determination of PQ-9 and PQH_2-9 are of two kinds. There are those in which the plastoquinones (PQs) and similar compounds are extracted from leaf material, purified by TLC and the separated materials determined spectrophotometrically [1]. The other approach [2] involves the extraction of PQs, chlorophylls, carotenoids etc. into petrol and partitioning of this with aqueous methanol so as to partially remove chlorophyll and carotenoids. The amount of oxidized PQ is then estimated spectrophotometrically.

This paper describes a rapid and highly specific method for assaying PQ-9 in chloroplast preparations. It relies on a separation by HPLC and a final spectrophotometric assay. As described the range of the method is 10–40 nmol PQ-9 and requires chloroplasts containing *ca* 0.5 mg chlorophyll.

RESULTS

Figure 1 (a) and (b) are typical HPLC elution profiles for chloroplast extracts. Peak III from the silica column and peak E from the reverse phase column had retention times identical to purified reference PQ-9. Peak E was shown, by subsequent silica column chromatography, to contain α-tocopherol in addition to PQ-9. Peaks I and D contained all the carotenoid remaining in the extracts

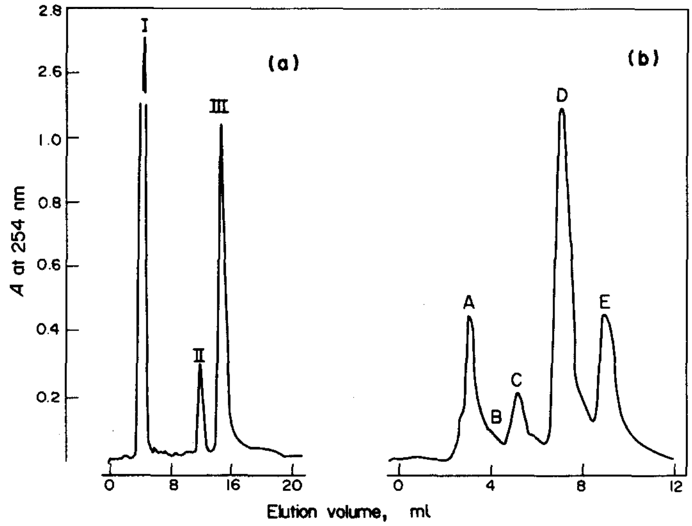

Fig. 1. HPLC elution profiles of *n*-heptane extracts of spinach chloroplasts. Dark adapted chloroplasts containing *ca* 1.2 mg chlorophyll extracted and the extract passed through μ Porasil Sep-pak (see Experimental) (a) HPLC on μ Porasil (silica) column. (b) HPLC on C_{18} (reverse phase) column.

Table 1. Levels of PQ-9 in chloroplasts

Species	PQ-9 total (nmol/mg chl)	mol PQ-9/100 mol chlorophyll	PQ-9 in dark adapted chloroplasts nmol (mg Chl)$^{-1}$	PQ-9 in dark adapted chloroplasts % of PQ-9 total
Spinach	37.0 ± 5.0 (13)	4.1	29.2 ± 3.1 (26)	79
				81.3 ± 8.1 (9)
Pea	43.6 ± 5.1 (7)	4.9	36.4 ± 4.0 (7)	83
				85.7 ± 4.9 (6)
Silver beet	58.1 ± 1.7 (4)	6.5	47.4 ± 4.5 (4)	82
Cucumber	44.1 ± 2.4 (7)	4.9	39.7 ± 4.9 (5)	90
				90.0 ± 4.8 (4)
Cos lettuce	35.3 ± 3.5 (5)	3.9	27.3 ± 3.2 (6)	77

PQ-9 total was obtained by prior incubation of chloroplasts in the dark for 10 min at 25° suspended in 0.4 M sucrose containing DCPIP (1 μmol/mg chlorophyll). Dark adapted chloroplasts were obtained by omitting DCPIP. Values are means ± s.d. (number of samples). PQ-9 in dark-adapted chloroplasts, expressed as % of PQ-9 total was obtained from the mean values. In addition for spinach, peas and cucumber sufficient assays were made of both PQ-9 total and PQ-9 in dark adapted chloroplasts from the same sample to calculate means and standard deviations.

after Sep-Pak treatment. Peak II contained phylloquinone. Peak III from the silica column appeared, from the following to contain PQ-9 with only a small amount of impurity. (a) The UV spectrum and the reduced minus oxidised spectrum of this material were the same as for the purified reference PQ-9 and resembled closely the data in [2]. (b) The material when chromatographed on the reverse phase system and monitored at 254 nm showed one major peak and two subsidiary peaks amounting to *ca* 5% of the major peak. (c) ^{1}H NMR spectra (100 MHz, CDCl$_3$) of the purified reference PQ-9 and the material in peak III were identical and appeared to be the same as that of Sommer and Koffler [9]. The spectra were consistent with little or no contamination with PQs containing shorter isoprenoid side chains or PQ-Bs (which contain an aliphatic ester). (d) CI mass spectra of the purified reference PQ-9 and the material in peak III were identical, m/e (rel. int.) 749 [MH$^+$] (4), 748 [M$^+$] (3), 747 [M$^+$] (2), 151 [base peak] and major peaks (greater than 30% base peak) at 109, 123, 137, 164, 179. There was evidence that peak III contained small amounts of material other than PQ-9, peak heights less than 10% of base peak.

For the complete analytical method, working in the range 30–40 nmol PQ-9, duplicate determination of PQ-9 levels in chloroplast suspensions agreed within 5% and the recovery of purified reference PQ-9 (10–25 nmol) added to chloroplast suspensions was quantitative. For the extraction of PQ-9 from aqueous methanol, heptane was found to be a more efficient solvent than petrol (40–60°) as used by Redfearn and Friend [2]. Three extractions with heptane removed all the PQ-9 from the aqueous methanol whereas after three extractions with petrol variable amounts of PQ-9 (15–20% total) remained and could be extracted with heptane.

Table 1 shows the total PQ-9 i.e. PQ-9 plus PQH$_2$-9 concentrations in chloroplast preparations from a variety of species together with the level of oxidised form in dark adapted chloroplasts. Since PQH$_2$-9 in the chloroplast samples is not directly estimated by our procedure it is necessary to make two determinations in order to calculate the PQH$_2$-9 present. The first measures the PQ-9

Table 2. Change of redox state of PQ-9 following illumination

Source of chloroplasts	PQ-9 (nmol/mg chlorophyll) Total (i)	PQ-9 (nmol/mg chlorophyll) PQ-9 in dark adapted (ii)	PQ-9 (nmol/mg chlorophyll) PQ-9 after 10 min illumination (iii)	% Oxidised in light	mol PQH$_2$/500 mol chlorophyll after 10 min illumination (iv)
Spinach	35.3	32.8	23.2	66	6.8
Pea	51.7	45.8	35.6	69	9.0
Silver beet	59.3	44.7	36.2	61	12.9

PQ-9 total and PQ-9 in dark adapted chloroplasts were determined as in Table 1. Illumination was with red light (140 nEs^{-1}cm^{-2}). The value in column (iv) was obtained by subtracting the value in column (iii) from that in (i).

present in the sample, the second determines the PQ-9 present in the sample following oxidation of PQH_2-9 to PQ-9 by treatment with dichlorophenolindophenol (1 μmol/mg chlorophyll). The PQH_2-9 originally present is then calculated by difference. The procedure is in principle the same as that used by Redfearn and Friend [2]. Attempts were made to determine PQH_2-9 directly but were discontinued when it was found that PQH_2-9 was not quantitatively extracted from the aqueous methanol into heptane (*ca* 25% loss) and PQH_2-9 in ethanol was unstable as judged by spectral changes occurring over 24 hr at 0°. However, it was ascertained that under the conditions used for routine assay of PQ-9 PQH_2-9 did not undergo significant oxidation during the work up if pyrogallol was present in the methanol used to denature the chloroplasts. Thus it was possible to measure the oxidation reduction state of PQ-9 in the chloroplasts. Indeed the data in Table 2 indicate that significant proportion of the PQ-9 can be reduced by illumination of chloroplasts in the absence of Photosystem I acceptor.

DISCUSSION

The method described is specific for PQ-9. The values in Table 1 for total PQ-9 are expectedly somewhat lower than the values of PQ (said to be mainly PQ-9) recorded for sugar beet chloroplasts (72–132 nmol PQ/mg chlorophyll) by Redfearn and Friend [2]. More recently, Barr, Henninger and Crane [1] obtained values of 30–40 nmol PQ-9/mg chlorophyll for spinach chloroplasts and Lichtenthaler [4] reported data allowing the calculation of values of 58 nmol PQ-9/mg chlorophyll for intact spinach chloroplasts and 38 for sonicated chloroplasts. The values reported in Table 1 were obtained with broken washed chloroplasts, i.e. those without outer envelope or stroma, and do not indicate the locations in the chloroplast preparations of the PQ-9 as no attempt was made to remove any plastoglobuli e.g. by sonication [4]. There is considerable variation between the PQ-9 content of the different species and the highest values came from commercially grown silver beet which was older than the other materials used. This may reflect different quantities of PQ-9 in the plastoglobuli [6] but from Table 2 it can be seen that a higher PQ-9 content gives rise to a higher content of PQ-9 reducible in the light.

Table 1 indicates that in the dark PQ-9 is largely in the oxidised form. Redfearn and Friend [2] have provided data previously on this matter, but their values although limited to one species (sugar beet) showed much more variation. Part of this variation could have been due to the presence of materials other than PQ-9 in the final extract. HPLC of an extract prepared by the method of Redfearn and Friend [2] gave an elution profile similar to that seen in Fig. 1 (a). One of the non-PQ-9 components i.e. peak II showed a small change in absorbance at 255 nm on addition of borohydride and would have contributed, therefore, to the total absorbance change at 255 nm attributed to plastoquinone by Redfearn and Friend [2]. This material could account for the higher values for PQ reported by these workers as well as possibly the greater variation in its redox state.

Since PQ-Bs, -Cs and -Ds have been reported to be present in spinach chloroplasts [1] although in smaller amounts than PQ-9, it was interesting to ascertain whether the PQ-9 fraction, i.e. peak III of Fig. 1a contained any of these PQs. The data presented in the Results section indicate that peak III was PQ-9 with very little contamination. It is especially interesting that when peak III was subject to reverse phase HPLC only traces of material other than PQ-9 and absorbing at 254 nm were detected.

It is doubtful whether PQ-Bs, -Cs and -Ds were present in the chloroplast preparations used here. The materials which separated on reverse phase HPLC (Fig. 1b) did not have UV spectra resembling those reported for these PQs. Extracts prepared and then subjected to TLC by the procedure of Barr *et al.* [1] gave PQ-9, which was shown by HPLC (silica column) to be impure, and only one other band with a spectrum resembling a PQ. It was present in very much smaller amount than PQ-9 and on HPLC (silica column) was resolved into 4 components, the main one having a retention time corresponding to peak II (Fig. 1a). Lichtenthaler [4] comments that one can occasionally detect small quantities of the PQ-Cs and -Bs in older tissues and draws attention to the possibility of their formation in some chromatographic procedures.

Table 2 indicates that the procedure may be used to determine the redox state of PQ-9 of chloroplasts under different experimental conditions as well as giving a reliable measure of the total PQ-9 present. In the absence of an electron acceptor for Photosystem I, only a proportion of the total PQ-9 is reduced after 10 min of intense actinic light. Under the conditions used 6.8–12.9 molecules of PQH_2-9 were present after illumination for every 500 molecules of chlorophyll. It is not known if this PQH_2-9 corresponds to the reactive PQ pool as determined by the kinetic methods used by Stiehl and Witt [7] and Amez *et al.* [8] but the value of 6.8 molecules PQH_2-9/500 molecules chlorophyll spinach is quite close to 7 and 7.5 molecules of readily reducible PQ per reaction centre of Photosystem II or P700 in spinach [7] and several algae [8]. The values for silver beet and peas (Table 2) are much higher.

The quantitative data in this paper have been obtained for chloroplast preparations containing *ca* 0.5 mg chlorophyll and 10–30 nmol PQ-9. The sensitivity of the UV assay would permit a reduction in the amount of chloroplast preparation required for the determination but this has not been attempted.

EXPERIMENTAL

Spinach (*Spinacia oleracea*, hybrid 102), peas (*Pisum sativum*, var. Massey gem), cucumber (*Cucumus sativus*, var. Supermarket), Cos lettuce (*Sativa roman*) seed was obtained from Arthur Yates & Co., Sydney, Australia. Spinach was grown hydroponically on Hoagland's soln under a 12 hr light, 12 hr dark regime in the glasshouse and leaves harvested at 18–21 days. Peas and cucumber were grown in the glasshouse on vermiculite moistened with Hoagland's solution. Cucumber leaves and the top 1–2 cm of pea plants were harvested at 14–18 days. Cos lettuce was grown in soil for 4 weeks. Silver beet (*Beta vulgaris*) and occasionally spinach were purchased at the local market.

Chloroplast preparations. All operations were at 0–4°. *Ca* 80 g leaf material (the centre mi ribs of spinach and silver beet removed) were homogenised for 10–15 sec in a Waring blender at 3/4 speed with 150 ml of 0.4 M sucrose, 10 mM NaCl and 50 mM Tris–HCl (pH 8.0). The slurry was strained through eight layers of cheese-cloth containing a thin lining of cotton wool. Cell debris was removed by centrifuging for 1 min at 200 *g*. Chloroplasts were then sedimented at 2000 *g* for 10 min, washed by resuspension in 80 ml NaCl (10 mM) and resedimented at 7000 *g* for 10 min. They were finally resuspended in 0.4 M sucrose yll/ml) and stored on ice. Approx yields of rophyll/g starting material) were: spinach 00; cucumber, 350; peas, 400 and Cos lettuce

outine determination of PQ-9 in chloroplast preparations. Chloroplast suspensions (0.75 ml, 500–600 μg chlorophyll) were pipetted rapidly into MeOH (3 ml) at −20° containing pyrogallol (1 mg/ml). The aq. MeOH mixture was extracted (× 3) with *n*-heptane (5 ml) by shaking by hand for 1 min and separating the layers by centrifugation. The heptane fractions were pooled and taken to dryness under red. pres. at 30°. The residue was dissolved in *ca* 1 ml *n*-heptane and stored at −20° until processed by HPLC.

Immediately prior to HPLC the *n*-heptane solns were passed through a μ Porasil Sep-pak (Waters Associates) using *ca* 10 ml CH_2Cl_2–*n*-heptane (30:70, v/v) as eluant. This removed chlorophylls and a large proportion of carotenoids and improved the resolution on HPLC. The eluates were evapd under N_2, the residue dissolved in 100 μl heptane and applied to the appropriate column. HPLC (Si column) was performed on either a Waters Associates μ Porasil column (6 × 30 mm) with CH_2Cl_2–*n*-heptane (6:94) at 2 ml/min or a Brownlee LiChrosorb Si 10 column (4.5 mm × 25 cm) with CH_2Cl_2–*n*-heptane (3:97, v/v) at 1 ml/min. PQ-9 was detected by its absorption at 254 nm using either a Phillips SP6-500 UV–Vis Spectrophotometer with an 8 μl flow cell or an Altex Model 153 Detector.

The appropriate eluate fractions from HPLC were taken down in a rotary evaporator, the residue dissolved in EtOH and the PQ-9 determined by the spectrophotometric procedure described by Redfearn and Friend [2] which involves measuring the ΔA_{255nm} on adding $NaBH_4$. The $\varepsilon_{oxid} - \varepsilon_{red}$ value used was 14 800 [2].

Reverse phase HPLC was performed on a Waters μ Bonda Pak C_{18} column [1.5 ml/min, MeOH–*iso*-propanol (3:1) as eluant]. Chlorophyll was determined according to Arnon [3].

PQ-9 was obtained from Roche Products Pty. Ltd. (Dee Why, N.S.W.) in 1974 (stored at −20°). On HPLC (Si column) it gave rise to three peaks absorbing at 254 nm. The major one (*ca* 60% of total peak areas) had the spectral properties of PQ-9 [2] and is referred to as 'purified reference PQ-9'.

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