Isolation of Plastoquinone from Spinach by HPLC

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Abstract

We report a method for the purification of plastoquinone-9 (PQ), a prenylquinone cofactor involved in the photosynthetic electron transport chain. The described procedures relies on spinach-chloroplast isolation followed by PQ extraction and chromatographic fractionation. Extraction of PQ was achieved using partition of chloroplast suspension with methanol:petroleum ether. This procedure removed large amounts of green pigments from the extract and thus facilitates the subsequent chromatographic isolation of PQ. To obtain pure PQ, the developed extraction was combined with a two-step chromatographic approach using orthogonal stationary phases, i.e. alumina and octadecylsilane (C18). A small scale protocol for analytical reverse-phase high performance liquid chromatography (RP-HPLC), which may be implemented in most laboratories equipped with conventional systems, is described. The reported methodology represents a valuable tool for the fast production of small amounts of PQ, for which there are no commercial standards available.

Keywords: Spinacia oleracea, plastoquinone-9, HPLC.

Introduction

In plants, algae and cyanobacteria the light-driven redox reactions responsible for the primary energy transduction by photosynthesis involve three major membrane protein complexes named photosystem II (PSII), cytochrome $b_{6}$, and photosystem I (PSI). Active centers of these complexes communicate via two redox carriers: membrane-soluble plastoquinone-9 (PQ) connects the reducing activity of PSII to the oxidizing activity of the $b_{6}$ complex, while the soluble protein plastocyanin, is reduced by the $b_{6}$ activity and oxidized by PSI [1,2].

Besides its main role as proton and electron carrier, PQ is an active player in the short and long term light adaptation responses of photosynthetic cells [3-6]. Recently the involvement of PQ as singlet oxygen scavenger has been reported in high light stressed Chlamydomonas reinhardtii cultures [7].

In spite of the reborn interest for the different PQ functions, which are at present intensively investigated, PQ reference standards are no longer commercially available and the purification methods reported in the literature are often fragmentary and dated, being based on protocols published in the 60-80’s [8-11]. In the present paper we describe a procedure in which PQ is directly extracted from water suspensions of chloroplasts by partition with methanol:petroleum ether. This procedure removed large amounts of green pigments from the extract and thus facilitates the subsequent chromatographic isolation of PQ. To obtain pure PQ, the developed extraction was combined with a two-step chromatographic approach using orthogonal stationary phases, i.e. alumina and octadecylsilane (C18). A small scale protocol for analytical reverse-phase high performance liquid chromatography (RP-HPLC), which may be implemented in most laboratories equipped with conventional systems, is described. The reported methodology represents a valuable tool for the fast production of small amounts of PQ, for which there are no commercial standards available.

Materials and Methods

Chloroplast preparation and solvents

Chloroplasts were prepared from spinach leaves (Spinacia oleracea L.) following standard protocols described in Barr and Crane [8]. The total chlorophyll (Chl) content was determined spectrophotometrically upon extraction of a few μL of chloroplast suspension with acetone, accordingly to Arnon [12]. Acetone, methanol, petroleum ether (bp 40-60°C), diethyl-ether, ethanol and acetonitrile of analytical or HPLC grade, used as solvent for PQ extraction and isolation, were from Sigma-Aldrich.

Extract and fractionation of plastoquinone

As an alternative to the Barr and Crane method [8], we tested for PQ extraction from chloroplasts a protocol set up by Venturoli et al. [13] for the extraction of ubiquinone-10 from membranes of photosynthetic bacteria. To this aim, 100 mL of sugar free chloroplasts were diluted in water to a final Chl concentration (a+b) of 1 mg/mL. A 20 time volume excess of a methanol:petroleum ether 3:2 mixture was directly added to the liquid chloroplast sample and vigorously shaken for 1 min. After phase separation, the petroleum ether was collected, and the extraction repeated twice by re-adding fresh petroleum ether to the methanol-water lower phase. The petroleum ether fractions were combined, dried by rotary evaporation and resuspended in 10 mL of petroleum ether.

The extract obtained was fractionated by open column chromatography on acid washed alumina (SIGMA-Aldrich) following the scheme of Barr and Crane [8]. 100 g of alumina were deactivated by adding 6 ml of double distilled (d.d.) water, resuspended in petroleum ether and placed into a glass column with diameter and length of 2.5 cm and 40 cm, respectively. The petroleum ether extract, containing PQ, was loaded into the column and 7 fractions (1-7) were obtained by eluting in sequence with 100 mL of 0, 0.2, 2, 4, 8, 16 and 20% of diethyl-ether in petroleum ether (v/v).

HPLC plastoquinone isolation

Analytical HPLC analysis was performed at 40°C by using a Jasco Pu-1580 pump, a C-18 reverse phase column (Waters Spherisorb 5 μm ODS2, 4.6 x 250 mm), and a Jasco UV 970 detector operating at 255 nm. The mobile phase (flow rate 1.5 mL/min) was a mixture of acetonitrile:ethanol 3:1, as described in Yoshida et al. [14].

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Results and Discussion

The method of PQ purification described in the present work is discussed in the following by considering separately: (i) the extraction with organic solvents; (ii) the fractionation by aluminium oxide; (iii) the isolation by HPLC and recovery of pure components.

Extraction with organic solvent

From 500 g of spinach leaves, 95 mL of chloroplasts containing chlorophyll (a + b) at a concentration of 1.34 mg/mL were obtained. After washing chloroplasts in water to get a sugar free chloroplast suspension, at variance to established protocols [8], extraction of PQ was achieved by partition between a methanol/water phase and a petroleum ether phase (see Materials and Methods for details). This procedure, extensively used for ubiquinone extraction form chromatophores of photosynthetic bacteria [13], was found to be effective also in the case of PQ. Although a small portion of PQ remained in the methanol/water phase, resulting in a slightly lower extraction yield as compared to the established method [8], this extraction procedure provided significant advantages. First, time-consuming chloroplast lyophilization could be avoided, which speeded up the procedure and prevented possible unwanted reactions during lyophilization. Second, since large amounts of green pigments remained trapped in the methanol-water phase, the subsequent purification steps were considerably facilitated. In order to further separate PQ from chlorophyll and other pigments, the obtained extracts were then treated on an aluminium oxide column as described below.

Fractionation by aluminium oxide

The method of PQ purification described in the present work is discussed in the following by considering separately: (i) the extraction with organic solvents; (ii) the fractionation by aluminium oxide; (iii) the isolation by HPLC and recovery of pure components.

Fractionation by aluminium oxide

The use of an acidic alumina column, besides removing to a large extent green pigments and carotenoids, has the advantage that plastoquinone oxidation takes place during this procedure. Indeed it has been shown [15,16] that a substantial fraction (30-40%) of the extracted plastoquinone is in the doubly reduced and protonated form, known to present an absorption maximum at 255 nm (ε255 = 17.94 M-1 cm-1), which facilitates the separation and the collection of the purified PQ.

The residue from the methanol/petroleum ether extraction was loaded on the top of a 100 g alumina column, and eluted with fractions of 100 mL of petroleum ether with increasing v/v percentages of diethyl ether (see Extraction and fractionation of plastoquinone). At variance to what reported in established protocols [8], PQ eluted in fraction 5. This difference in the elution profile was likely due to the much lower concentration of green pigments in the methanol-water phase, the subsequent purification steps were considerably facilitated. In order to further separate PQ from chlorophyll and other pigments, the obtained extracts were then treated on an aluminium oxide column as described below.

Isolation and recovery of plastoquinone

The dried residue of the eluted fraction 5 from the aluminium oxide column was dissolved in ethanol and subjected to HPLC analysis; the obtained chromatograms are shown in Figure 1. Oxidized PQ is known to present an absorption maximum at 255 nm (ε255 = 17.94 M-1 cm-1) that shifts to 290 nm upon reduction (ε290 = 3.39 M-1 cm-1) [17]; this implies that the detection of the oxidized or the reduced PQ peaks are maximized at 255 nm and 290 nm, respectively. As shown in the upper panel of Figure 1, a large peak with a retention time of 19.2 minutes is present in the chromatogram at 255 nm (black trace), characteristic of the oxidized PQ [14]. Essentially no peak appeared in the chromatogram at 290 nm (red trace) at the retention time expected for reduced plastoquinone (8.1 min, see below and [14]). This confirms that during the fractionation through the aluminium oxide column, reduced PQH2 gets oxidized [19]. After addition of NaBH4 to reduce PQ [10,14], the elution peak at 19.2 minutes in the 255 nm chromatogram (lower panel of Figure 1, black trace) decreased, and the reduced PQ eluted earlier with a retention time of 8.1 min, better detected at 290 nm (lower, left panel of Figure 1, red trace). Although reduction of the oxidized PQ form upon NaBH4 addition was incomplete, the elution profiles showed peaks with the same retention times and the same qualitative response to reducing conditions as those reported in [14].

**Figure 1**: HPLC elution profiles after fractionation with aluminium oxide. Upper panel: chromatogram of the alumina column fraction 5 from the methanol : petroleum ether extract of water suspended chloroplasts. Red and black chromatograms refer to elution profiles detected at 290 or 255 nm, respectively. In the lower panel, black downward arrow indicates the elution of the PQ oxidized form (retention time of 19.2 min); the peak of the oxidized form at 255 nm is decreased upon reduction. Red upward arrow indicates the elution of the PQ reduced form (retention time of 8.1 min) whose peak at 290 nm becomes visible upon reduction.

**Figure 2**: UV spectra of HPLC purified PQ. Upper panel: spectrum of PQ collected from the HPLC. Lower panel: oxidized-reduced difference spectrum. The oxidized PQ eluting at 19.2 min (see Figure 1) has been collected from analytical HPLC runs, dried under nitrogen flow, and resuspended in ethanol.
UV absorption spectrum of purified PQ was estimated from the oxidized-minus-reduced difference spectrum at 255 nm (ox-red ΔA(255nm) = 15 mM-1 cm-1) for the difference band. Injection of a known amount of PQ in methanol to petroleum ether followed by orthogonal chromatographic steps. Since PQ is not commercially available, our procedure is expected to facilitate the production and usage of this molecule for bioassay or quantification purposes in a normally equipped laboratory.

The concentration of the HPLC purified PQ was estimated from the oxidized-minus-reduced difference spectrum at 255 nm (ox-red ΔA(255nm) = 15 mM-1 cm-1) for the difference band. Injection of a known amount of PQ in methanol:petroleum ether followed by orthogonal chromatographic steps. Since PQ is not commercially available, our procedure is expected to facilitate the production and usage of this molecule for bioassay or quantification purposes in a normally equipped laboratory.

Conclusions

The involvement of PQ in photosynthetic electron transport, redox signaling pathways and the recent evidence that PQ can act as singlet oxygen scavenger under high light stress are renewing the interest of researchers for this cofactor.

In the present work we have shown that PQ isolation can be easily done using partition of chloroplast suspension with methanol:petroleum ether followed by orthogonal chromatographic steps. Since PQ is not commercially available, our procedure is expected to facilitate the production and usage of this molecule for bioassay or quantification purposes in a normally equipped laboratory.

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