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Note

High-performance liquid chromatographic determination of pyrroloquinoline quinone with electrochemical detection and its application to bacterial samples

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Pyrroloquinoline quinone (PQQ) was originally found in microorganisms as a cofactor of some dehydrogenases[1,2]. It was soon shown that PQQ-dependent enzymes are more widespread among other organisms, *i.e.*, plants[3,4], mammals[5] and human[6]. Moreover, recent studies have suggested that PQQ also functions as a component of electron-transport systems[7], as a growth-stimulating factor in microorganisms[8] and more interestingly as a modulator of collagen cross-linking in higher animals[9]. More versatile functions of PQQ will probably be revealed in future studies.

To facilitate studies on PQQ, a simple and reliable column high-performance liquid chromatographic (HPLC) analysis of PQQ would be extremely useful. HPLC analysis with UV detection[10] and fluorimetric detection after PQQ derivatization[10] have already been reported, but these methods have low selectivity and are tedious for routine assays. Thus, a biological assay with apoenzyme of bacterial glucose dehydrogenase (GDH, E.C. 1.1.99.17) is widely used for the determination of PQQ in biological samples[10–12]. In this instance, conditions for preincubation of the apoenzyme with PQQ should be carefully controlled for a reliable assay.

As PQQ functions as a redox cofactor, electrochemical detection (ED) would seem to be extremely useful for the determination of PQQ, but no convenient method has yet been reported. This might be mainly because the selective and quantitative conversion of PQQ to the corresponding reduced form (pyrroloquinoline quinol, $PQQH_2$), is not readily achieved using most commercially available electrodes.

In this paper, we describe a reliable method for the determination of PQQ using a coulometric, porous graphite electrode, which makes it possible to reduce PQQ stoichiometrically. Its application to some biological samples is also described.

EXPERIMENTAL

Chemicals

All reagents were of the highest purity available. PQQ (in potassium chloride solution) was obtained from Mitsubishi Gas Chemical (Niigata, Japan). $PQQH_2$ was prepared from PQQ using phenylhydrazine as a reducing agent, as described by Duine *et al.*[13].

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High-performance liquid chromatography

A Model 880-PU solvent delivery system (Japan Spectroscopic, Tokyo, Japan) and a Model 7125 sample injector (Rheodyne, Cotati, CA, U.S.A.) were used with the HPLC system. The analytical column (150 × 4.6 mm I.D.) was packed with ODS-A-302 (5-μm particle size) (YMC, Kyoto, Japan) and kept at 70°C with a Model CH 110 temperature-control module (Kyoto Kuromato, Kyoto, Japan). Chromatography was performed at a flow-rate of 0.55 ml/min with a mobile phase of methanol–ethanol–concentrated phosphoric acid–water (26:0.6:0.4:73, v/v, pH 2.0) under a flow of nitrogen.

A Model 5100 A Coulochem electrochemical detector was used, equipped with Model 5020 guard cell, Model 5021 conditioning cell and Model 5011 high-sensitivity analytical cell (ESA, Bedford, MA, U.S.A.). In-line stainless-steel filters were used in the conditioning cell and the analytical cell. PQQ was detected with the following redox parameters: guard cell potential at −0.75 V, conditioning cell potential at −0.7 V, detector 1 (coulometric electrode) at −0.7 V and detector 2 (amperometric electrode) at +0.3 V. The output gain of detector 2 was set at 8000. The output response time to the recorder was set at 10 s.

Biological assay of PQQ

The assay procedures are essentially the same as described by Geiger and Gorisch[12]. Briefly, the apoenzyme of glucose dehydrogenase was prepared from membranes of *Escherichia coli* K-12, to which a bacterial sample was added, and preincubated at 25°C for 60 min. The reaction was started by adding glucose, and the rate of 2,6-dichlorophenol–indophenol reduction was recorded.

Samples

Gluconobacter suboxydans ATCC 621 (obtained from the Institute for Fermentation, Osaka, Japan), was grown aerobically at 30°C in a medium containing 0.6% glutamate, 0.5% glycerol, 35 m*M* potassium phosphate (pH 6.8), minerals and vitamin mixtures as described by Ameyama *et al.*[14]. After brief centrifugation of the culture, a 200-μl portion of the clear supernatant was treated with a Bond-Elut SCX cartridge (100 mg) (Uniflex, Tokyo, Japan), and PQQ was eluted with 600 μl of water. The eluate was pooled and 10 μl were injected into the HPLC system. The recovery of authentic PQQ was 94 ± 2.0% (mean ± S.D.) for three determinations. Prior to use, the SCX cartridge was washed with 5 ml of 1*M* hydrochloric acid and then with 10 ml of water.

RESULTS

Electrochemical detection of PQQ

The voltammetric characteristics of PQQ were generated by making repetitive injections of a constant amount of PQQ solution but varying the electrochemical potential of detector 1. As shown in Fig. 1A, the potential at −0.2 V seems to be sufficient for the quantitative reduction of PQQ, but some interfering materials probably derived from the sample solvent made it impossible to detect PQQ by HPLC with the high sensitivity range as described below. These interfering materials were substantially eliminated at −0.7 V, and it is therefore recommended that PQQ be reduced at the applied potential of −0.7 V for detector 1 (Fig. 1).

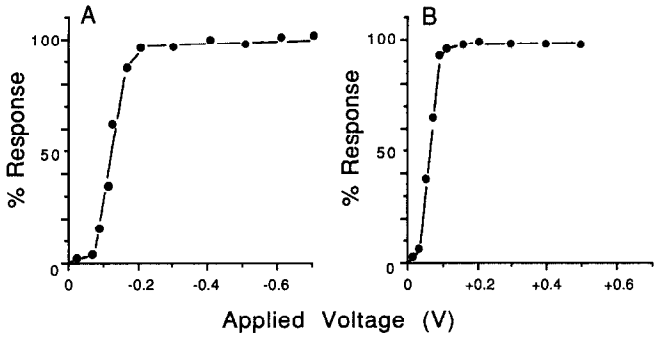

Fig. 1. Current–voltage curves for PQQ. (A) Samples of PQQ (2.5 nmol) were monitored at detector 1 with increasing negative potentials from −0.02 to −0.7 V. (B) Setting detector 1 at −0.7 V, detector 2 was varied from 0 to +0.4 V. Injection volume, 10 μl; gain, 10; response time, 10 s. For conditions, see Experimental.

To analyse the product spectrophotometrically, the analyte reduced at −0.7 V (detector 2 at 0 V) was directly collected under a nitrogen atmosphere. The spectrum of the reduced analyte thus obtained was found to be identical with that of $PQQH_2$, which was chemically reduced using the reported procedures[13] (data not shown). The resulting product fully retained its biological activity, as judged by the assay using apo-enzyme of bacterial glucose dehydrogenase[12] (data not shown).

Application of a more negative potential for detector 1 resulted in extensive degradation of PQQ; the resulting product(s) was biologically inactive and could not be converted back to the original oxidized form (PQQ).

In order to achieve selective detection in the redox mode, detector 2 (amperometric electrode) was operated at an applied potential of +0.3 V.

Column HPLC

When the chromatography was effected with the eluent composition methanol-concentrated phosphoric acid-water (26:0.4:73.6, v/v/v, pH 2), as described by Duine *et al.*[10], unknown peaks interfered with the PQQ determination. Flow-rates between 0.45 and 0.65 ml/min did not improve the separation. Addition of 0.6% (v/v) of

TABLE I

EFFECT OF ETHANOL AND COLUMN TEMPERATURE ON THE HPLC SEPARATION OF PQQ

Ethanol concentration (%)	*Column temperature (°C)*	k'_1[a]	k'_2[a]
0	45	1.94	1.50
	70	1.00	1.56
0.6	45	1.07	1.52
	70	0.74	1.46

[a] $k'_1 = (t_{RPQQ} - t_{R0})/t_{R0}$, $k'_2 = (t_{Ri} - t_{R0})/t_{R0}$ where t_{R0} = retention time of solvent front, t_{RPQQ} = retention time of PQQ and t_{Ri} = retention time of interfering compounds.

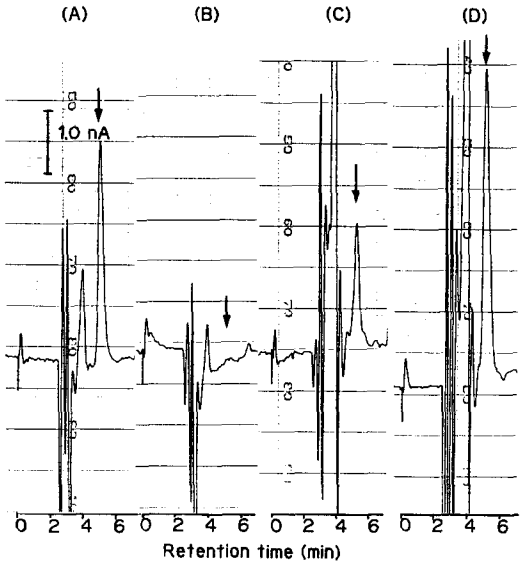

Fig. 2. Chromatograms of authentic PQQ and culture fluids obtained from *Gluconobacter suboxydans*. (A) Authentic PQQ, 1 pmol; (B) culture fluid grown for 0 h; (C) fluid obtained after 23 h of culture; (D) mixture of authentic PQQ and culture fluid (23 h). Culture fluids were treated with a Bond-Elut SCX cartridge, then subjected to HPLC. Injection volume, 10 μl; gain, 8000; response time, 10 s. For conditions, see Experimental. The arrows correspond to PQQ.

ethanol resulted in a shorter retention of PQQ without affecting the retention of the interfering peaks (Table I). A column temperature of 70°C also greatly improved the separation of PQQ.

A typical chromatogram of PQQ is shown in Fig. 2A. Under the conditions used, the retention time of PQQ was 5.3 min and good linearity was obtained between the peak height and the amount of sample injected over the range 0.25–2.0 pmol ($r = 0.997$). A nitrogen atmosphere over the mobile phase in the reservoir is also important for avoiding auto-oxidation of $PQQH_2$ by air.

The method was applied to the analysis of biological samples. We attempted to determine PQQ in the culture fluids produced by *G. suboxydans* (Fig. 2B and C). The samples were simply pretreated using SCX cation-exchange cartridges (Bond-Elut) before being subjected to HPLC. As shown in Fig. 2B, the culture fluid just after inoculating the cells (0 h) showed no peak corresponding to PQQ, indicating that possible contamination of PQQ from the glassware or medium, as reported by Van Kleef, *et al.*[15], was almost negligible under the experimental conditions. It is therefore concluded that the corresponding PQQ peak shown in Fig. 2C was formed by *G. suboxydans* cells during the cell growth. It is also shown in Fig. 2D that the mixture of authentic PQQ and the sample obtained from a 23-h culture gave a single peak.

Table II compares our HPLC method with a published enzymatic method[12]; the values for the two methods well agreed with each other. These results also reinforced the reliability of our method. Using this method, a concentration of 100 n*M* PQQ in culture fluid can be detected. The coefficient of variation of the peak height and the retention time of PQQ were 10% and 0.4%, respectively, based on the average of three independent analyses on the same culture sample.

TABLE II

CONTENTS OF PQQ IN CULTURE FLUIDS DETERMINED BY THE HPLC METHOD AND BIOLOGICAL ASSAY.

Gluconobacter suboxydans was grown aerobically at 30°C for the indicated periods in mineral medium containing 0.5% glycerol. Culture fluids were treated with a Bond-Elut cartridge before being subjected to HPLC or biological assay using the apo-enzyme of glucose dehydrogenase[12]. HPLC conditions as described under Experimental. Injection volume, 10 μl.

Cell growth		*PQQ in culture medium (nM)*	
Time (h)	*Absorbance (610 nm)*	*HPLC method*	*Biological assay*
0	–	0	0
18	0.72	37.1	35.1
23	1.22	141	134
39	1.13	105	101

DISCUSSION

Judging from the function of PQQ as a redox cofactor, an electrochemical detector seems to be the first choice for the sensitive and selective detection of PQQ with the HPLC system. In our experience, however, most such detectors currently available from commercial sources did not work well, mainly because stoichiometric reduction to $PQQH_2$ was not readily achieved under mild conditions. This may explain why no successful results have been reported previously. As shown in this study, the microporous electrode with a large surface area worked excellently at a mild electrochemical potential, where further degradation of the reduced analyte was avoidable. Quantitative PQQ reduction was actually achieved at applied potentials between -0.2 and -0.7 V, but a potential of -0.7 V was the most suitable for the sensitive and selective detection of PQQ in the HPLC system; presumably some contaminants in biological samples were irreversibly destroyed at this potential, but the exact reason is not known.

At present, the sensitive determination of PQQ in biological samples is exclusively achieved by biological assay methods. Our method is simpler and more convenient than the methods described previously. Further analyses using our HPLC method may reinforce the occurence of PQQ in biological samples.

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