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Immunochemical Studies on Expression of Quinoproteins in *Escherichia coli*

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Abstract An immunochemical method has been developed as the most sensitive tool for studying the expression of quinoproteins containing pyrroloquinoline quinone (PQQ) in *E. coli*. The PQQ was conjugated to bovine serum albumin (BSA), and the conjugant was purified by using a KwikSep™ dextran desalting column chromatography. The PQQ-BSA conjugant was immunized to rabbits, and the IgG fractions of the antisera were purified. The most sensitive antibody againt PQQ-BSA conjugant recognized some nanogram quantity of the antigen on the blot, but had little cross reactivity with BSA. Using this batch of the antibody, all the immunochemical assays of quinoproteins in *E. coli* were performed. Some six different PQQ-specific spots were detected by Western blot analysis of the soluble proteins in *E. coli* after two-dimensional gel electrophoresis. Their molecular weights on the blot were estimated to be about 100-, 90-, 72-, 58-, 52-, and 50-kDa. Their pI values fell in the range from 4.8 to 5.5. These results strongly suggest that quinoproteins are present in *E. coli*, and that the protein moieties were covalently bound to PQQ.

Key words: Pyrroloquinoline quinone (PQQ), quinoprotein, *Escherichia coli*, immunochemical assay, anti-PQQ antibody

Since pyrroloquinoline quinone (PQQ) was first discovered from a facultative methylotrophic bacterium, *Pseudomonas* TP1, as an organic cofactor of methanol dehydrogenase in 1979 [11], several research groups extensively studied on its biological distribution, physico-chemical integrities, biosynthetic pathways, physiological functions, gene cloning, and gene manipulation [2, 11]. PQQ is widely distributed in all the kingdoms in nature, i. e. fungi, plants, and animals, besides microorganisms. It is well known that quinoproteins containing PQQ are physiologically and pathologically important in many cellular activities including growth stimulation for microorganisms and mammals, oxidation of sulfhydryl-residues, gene regulation in mice, and for vitamin-like activity [2, 11]. It has also been reported that PQQ is linked covalently or noncovalently to a protein moiety. Methylotrophic bacteria are able to synthesize free PQQ from L-glutamic acid and L-tyrosine as precursors [2]. Other quinoproteins like aromatic amino acid decarboxylase are shown to have a covalently bound form of PQQ [1]. Different view points on the availability of covalently-bound PQQ in some quinoproteins have been raised. It has been suggested that the redox cofactors from amine oxidase (bovine serum) and from methylamine dehydrogenase (some methylotrophic bacteria) could be 3,4,6-trihydroxyphenylalanine (TOPA), and tryptophan tryptophylquinone (TTQ), respectively [2, 4]. This controversy seems to be derived by the application of various assays for PQQ (or quinoprotein) among research groups.

A number of assays or identification methods for PQQ in biological samples have been developed including bioassay with bacterial apoenzyme, high performance liquid chromatography (HPLC), the redox cycling method, and gas chromatography/mass spectrometry (GC-MS) [2, 4, 11]. Recently, immunochemical approaches using a poly- and/or monoclonal antibody have been reported [3, 13]. It has been generally accepted for a long time that *E. coli* is apparently unable to synthesize free PQQ [7], however, Duine and his colleagues revealed the presence of PQQ in glutamic decarboxylase in *Escherichia coli* ATCC 11246 [17]. Thus, it is worthy to study the characteristics of quinoprotein(s) in *E. coli*. In this study, the presence of quinoprotein(s) in *E. coli* was immunochemically detected by two-dimensional gel electrophoresis.

Preparation of Total Proteins from *E. coli*

The bacterial strain used in this study was *E. coli* DH5α. The strain was cultured in LB medium (w/v 1% Bacto-

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tryptone, 0.5% yeast extract, 1% NaCl, pH 7.2) on a shaking incubator at 37°C for 12 h. Cells were collected by centrifugation (4,000 ×g, 4°C, 10 min), and pellets were resuspended in cell lysis buffer [50 mM Tris-Cl (pH 8.0), 10 mM EDTA]. The suspension was sonicated, and centrifuged at 12,000 ×g for 15 min. Soluble proteins were obtained by sodium acetate precipitation and concentration as previously described [9, 18]. Two-dimensional gel electrophoresis was performed as follows: The first dimension was run in a pH 5–7 gradient tube gel, and then grafted horizontally onto the top of a polymerized SDS slab gel. For determining the pH gradient, one of the forced tube gels was cut into 1-cm slices, with each slice suspended in 1-ml deionized water for 30 min. The pH was determined with pH paper. The second dimension was run on a vertical slab at constant voltage (5 volts/cm). After the run, the proteins were stained with silver salts.

Preparation of PQQ-BSA and PQQ-Lipase Conjugants

Citro *et al.* (1989) first reported that PQQ could not elicit a mammalian immune system due to the limitation of its molecular size [3]. They conjugated the PQQ to gelatin through the 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide (EDC)-mediated reaction to raise anti-PQQ antibody in a rabbit, and successfully elicited a fairly good antibody against the PQQ-gelatin conjugant, which reacted with as low as eight nanograms of the PQQ-gelatin conjugant. In this study, PQQ's carboxylic residue was conjugated to amino residue of a carrier polypeptide, BSA (or lipase), by an EDC-mediated reaction. Thus, PQQ and BSA (or lipase) were suspended in 3 ml of conjugation buffer (10 mM phosphate buffer, pH 6.5), the solution was mixed with EDC, and the mixture was reacted at room temperature for 2 h. To differentiate conjugants from nonconjugants, the absorbance of the agents was measured at 250 nm and 280 nm, and the ratio of A_{250}/A_{280} was calculated (Table 1). Based upon the ratio, the conjugants of PQQ-BSA (0.93) or PQQ-lipase (0.91) were fractionated from nonconjugants by employing a KwikSep™ dextran desalting column chromatography (Pharmacia Biotech, Uppsala, Sweden). The PQQ-BSA conjugant was used as an immunogen for the production of anti-PQQ antibody, while the PQQ-lipase conjugant was used as an antigen for the titration of the antibody raised against the PQQ-BSA conjugant in a rabbit. This method was found be a fairly good way to conjugate PQQ to a carrier molecule (BSA), since highly sensitive anti-PQQ antibody was raised against the conjugant in a rabbit.

Table 1. The absorbancy ratio of immunogens.

Immunogens	Ratio (A_{250}/A_{280})
BSA	0.82
Lipase	0.80
PQQ	1.23
PQQ-BSA conjugant	0.93
PQQ-lipase conjugant	0.90

The absorbance of three chemicals (PQQ, BSA, PQQ-BSA) was measured at 250 and 280 nm, respectively. The ratio of A_{250}/A_{280} was indicated in an appropriate blank.

Production of Antibody against PQQ-BSA Conjugant

The PQQ-BSA conjugant (200 μg) was mixed with Freund's complete adjuvant and the mixtures were hypodermically injected into the hindback (30 sites) and paws (2 sites) of a rabbit. The immunization was performed four times with two weeks interval. The dosage of the immunogen was decreased by half, and incomplete adjuvant was substituted for the complete one from the second immunization process. Ten ml of blood was collected from the ear vein of the rabbit after the fourth immunization. Antiserum was isolated from the whole blood by centrifugation and the IgG fraction of the serum was isolated using protein-A Sepharose column chromatography [6]. Anti-BSA antibody was also raised in a rabbit by the same procedures described above and used as a control. The titer of antibody against PQQ-BSA (or anti-BSA antibody) was determined by dot blotting. Different amounts of PQQ-BSA conjugant were dissolved in TBST [20 mM Tris-Cl (pH 7.5), 500 mM NaCl, 0.05% Tween 20], they were spotted on nitrocellulose membrane, and completely dried. Following the manufacturer's directions in the Bio-Rad Immunoblot™ assay kit [15], the dried membrane was equilibrated in TBST and incubated in blocking solution (20 mM Tris-Cl, pH 7.5, 150 mM NaCl, 5% non-fat dry milk). Antibody against PQQ-BSA conjugant (or anti-BSA antibody) was diluted to 1:100 by blocking solution and used as the first antibody. Goat anti-rabbit IgG horseradish peroxidase conjugant diluted to 1:5,000 by blocking solution and peroxidase substrates (1-chloro-4-naphthol and hydrogen peroxide) were used to measure the titration of antisera. All the pre-immune sera did not react with any one of the four immunogens (BSA, lipase, PQQ-BSA, PQQ-lipase) used in this experiment. Results showed that anti-PQQ antibody detected PQQ-BSA conjugant up

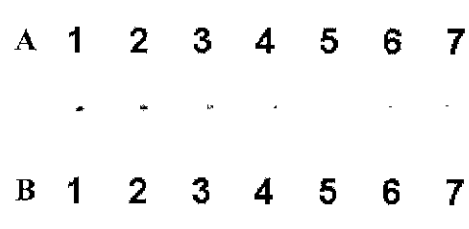

Fig. 1. Titer comparison of antiserum against PQQ-BSA.

Various amounts of PQQ-BSA conjugant (125 ng to 2 ng) were dropped on Nitrocellulose membrane, and then the membrane was treated with the antibody against PQQ-BSA conjugant (A) or anti-BSA (B) antibody. 1: 125 ng; 2: 62.5 ng, 3: 31.3 ng; 4: 15.6 ng; 5: 7.8 ng, 6: 3.9 ng; 7: 2 ng

to 2 ng (Fig. 1). When anti-BSA antibody was used, it detected only PQQ-BSA conjugant of 125 ng quantity (Fig. 1). However, the pre-immune serum (control) did not detect the activity of PQQ-BSA conjugant at any dilution used in both cases. This suggested that our antibodies were specifically raised against immunogens and sensitive enough to detect PQQ of ng ranges.

Immunochemical Assay of *E. coli* Total Protein with Anti-PQQ Antibody

Total proteins of *E. coli* were isolated by sonication and analyzed by SDS-PAGE. Many protein bands in a broad range of molecular weights were detected. The quinoproteins among total proteins were determined by the western blot immunochemical assay method using anti-PQQ antiserum. For more accurate analysis, two-dimensional gel electrophoresis was performed (Fig. 2A), which can reveal pI values of proteins as well as their molecular weights. After two-dimensional separation of the total extract of *E. coli*, detection with anti-PQQ antibody was conducted. Western blot immunoassay was done by following the manufacturer's directions in the Bio-Rad Immunoblot™ assay kit [15]. After the one- or two-dimensional electrophoresis, the proteins were transferred to a nitrocellulose membrane at constant voltage (5 volts/cm) for 12 h. Then, the membrane was soaked in protein blocking solution, and immunoassay was processed as described above. The results with antibody against PQQ-BSA conjugant demonstrated that at least six different polypeptides interacted with anti-PQQ antibody (Fig. 2B). Among the six spots, a 50-kDa spot (Fig. 3, spot 6) was identified as glutamate carboxylase by polyclonal and monoclonal antibodies [16]. Although the molecular weight of this enzyme has been determined, the pI of the enzyme has not yet been reported until now. Our results showed that the pI of the 50-kDa glutamate decarboxylase was 4.8. Analysis of the molecular weights of the rest of the spots (spots 1-5) suggested that these spots might be isoforms or subunits of several different polypeptides previously reported, i.e. probably a monomer of aromatic amino acid decarboxylase (52 kDa) from *Micrococcus* [12], a large subunit of amine dehydrogenase (58 kDa) from *Pseudomonas putida* [5], n-methylputrescine oxidase (72 kDa) from tobacco root [8], an alpha-subunit of bacterial methylamine oxidase (90 kDa) [10], and aromatic amino acid decarboxylase (100 kDa) from pig kidney [14]. A spot in a circle appeared on the blot when anti-BSA antibody was used for detection (Fig. 2C), which overlapped with the spot in a circle in Fig. 2B. It seems that a protein has a cross reactivity with antibody against the PQQ-BSA conjugant and anti-BSA antibodies.

In this study, we present the results of sensitive and convenient immunoassays for identifying quinoproteins containing PQQ in *E. coli* and report the identification of

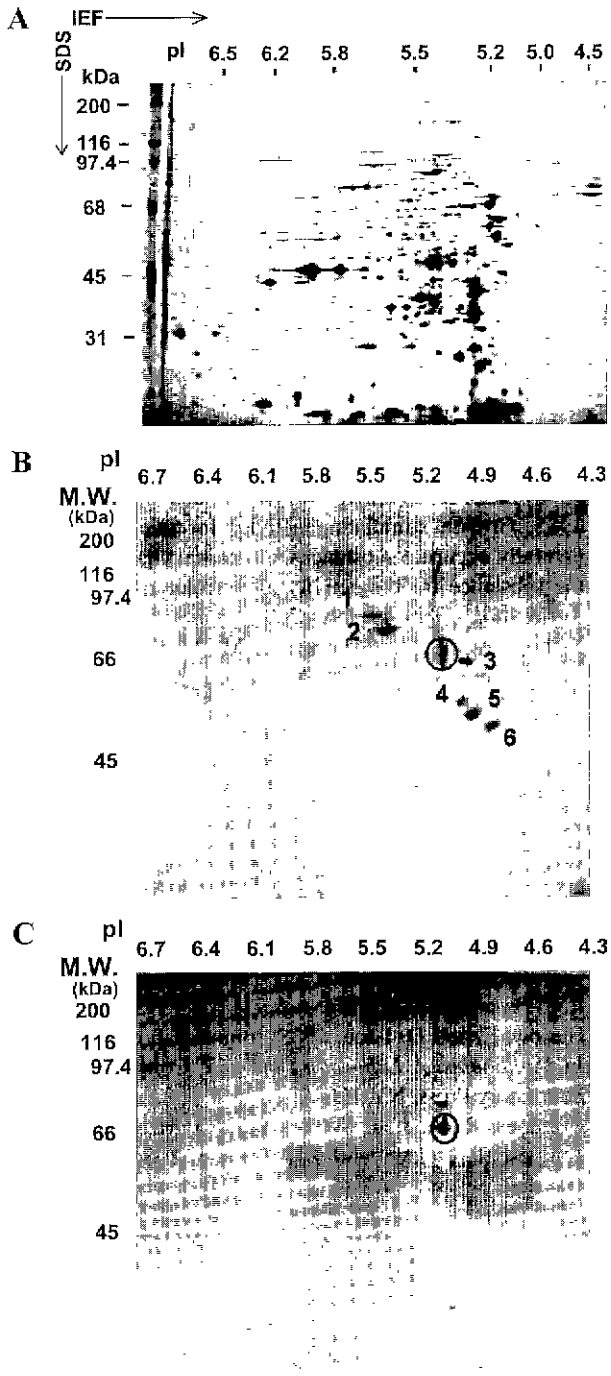

Fig. 2. Two-dimensional gel electrophoresis and western blot analysis with PQQ-specific antibody in *E. coli* total proteins

Panel A: Total proteins were isolated from *E. coli*. They were separated on first-dimensional isoelectric focusing, and second-dimensional SDS-PAGE. The spots were visualized by silver salt. The pI range was separated from pH 5 to pH 7. *Panel B*: Proteins were separated, then transfered onto NC membrane. The proteins were detected by antibody against PQQ-BSA conjugant. The pI range was separated from pH 5 to pH 7. Spots 1-6: Detected proteins: Circle: A protein that was cross reacted with both antibody against PQQ-BSA conjugant and anti-BSA antibody. *Panel C*: The proteins were detected by anti-BSA antiserum. The pI range was separated from pH 5 to pH 7. Circle: A protein that was cross reacted with both antibody against PQQ-BSA conjugant and anti-BSA antibodies.

quinoproteins from total proteins of *E. coli*, using anti-PQQ antibody. These results will facilitate the development and application of immunochemical assays to the studies in gene expression of quinoproteins in organisms.

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