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Short communication

Isolation and purification of PQQ-dependent lactate dehydrogenase from *Gluconobacter* and use for direct electron transfer at carbon and gold electrodes

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ABSTRACT

This research details the isolation and purification of a new type of lactate dehydrogenase that is dependent upon the coenzyme pyrroloquinoline quinone (PQQ). PQQ-dependent enzymes have been of interest in the literature over the last decade due to the fact that many of them can undergo direct electron transfer (DET) at electrode surfaces which is of interest for biosensor and biofuel cell applications. In the paper, we detail the isolation of PQQ-dependent lactate dehydrogenase (PQQ-LDH) from two sources of *Gluconobacter* (*Gluconobacter* sp. 33 and *Gluconobacter suboxydans*). This paper also shows the first evidence that PQQ-LDH can undergo direct electron transfer at gold and carbon electrode surfaces for future use in biosensors and biofuel cells.

1. Introduction

Over the last two decades, the ability for an enzyme to undergo DET has been of increasing interest for both enzymatic biosensors and enzymatic biofuel cells. It was first shown in 1997 by Ikeda et al. that a PQQ-dependent dehydrogenase could undergo direct electron transfer [1]. However, it hasn't been until recently that DET of PQQ-dependent dehydrogenases has been of interest for biofuel cells and biosensors.

All PQQ-containing enzymes belonging to the group of quinohemoenzymes contain the bound cofactor PQQ along with one or more heme-c moieties. The cofactor PQQ is coordinated with the apoenzyme via Ca^{2+} ions and electrons are transferred from the substrate via PQQ to the heme groups and then to the current collector [2]. PQQ-dependent enzymes containing heme-c can undergo rapid electron transfer reactions under appropriate conditions [3,4]. Heme-c serves as a universal mediator of internal electron transfer from the specific catalytic site to the electrode and creates ideal opportunities to incorporate quinohemoenzymes for use as DET enzymatic schemes.

The acetic acid bacteria *Gluconobacter*, contains multiple PQQ-dependent dehydrogenases capable of undergoing DET [5]. These enzymes include PQQ-dependent alcohol dehydrogenase (PQQ-ADH), PQQ-dependent aldehyde dehydrogenase (PQQ-AldDH), PQQ-dependent glucose dehydrogenase (PQQ-GDH), and PQQ-dependent glycerol dehydrogenase (PQQ-GlyDH). However, there are a number of unclassified PQQ-dependent enzymes in *Gluconobacter* [5]. This paper details the isolation and purification of one of those unclassified PQQ-dependent enzymes with substrate specificity to lactate.

The majority of PQQ-dependent enzymes in *Gluconobacter* are membrane bound enzymes [5]. Membrane bound enzymes require cell lysis and subsequent sample preparation techniques before the enzyme of interest can be further purified [6]. The aim of the purification procedure was to isolate the enzyme with the maximum possible yield based on the percentage recovered activity compared with the total activity in the original extract. In addition, the preparation should possess the maximum catalytic activity, i.e. there should be no degraded or inactivated enzyme present and it should be of the maximum possible purity (it should contain no other enzymes or large molecules). This paper details the isolation of a membrane bound PQQ-dependent dehydrogenase with lactate specificity followed by ion exchange chromatography purification of the protein. Once sufficient purity was obtained, direct electron transfer of the PQQ-dependent lactate dehydrogenase at gold and carbon electrodes was investigated followed by the use of PQQ-LDH in a lactate/air biofuel cell.

2. Experimental

2.1. Reagents

All reagents purchased were stored, and used as received from Sigma-Aldrich unless otherwise noted. All solutions were prepared with 18 MΩ water. *Gluconobacter* sp. 33 (DSM 3504), *Gluconobacter* sp. 33 (ATCC 15163), and *Gluconobacter suboxydans* (ATCC 621) were purchased from DSMZ or ATCC as noted. Cell culture reagents: D-glucose, $Ca(CO_3)_2$, D-mannitol, yeast extract, $(NH_4)_3HPO_4$, $MgSO_4{\cdot}7H_2O$, H_3PO_4, and $(NH_4)OH$ cell culture grade reagents were used for all media solutions. Cell culture grade reagents were also used for all buffer preparations: NaCl, Tris–HCl, trizma base, sucrose, $CaCl_2$, Triton X-100,

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KH_2PO_4, and K_2HPO_4. Chromatography column packings: DEAE-Toyo-Pearl 650 M® (anion exchange resin), CM-Toyo-Pearl® (cation exchange resin), and hydroxyapatite (dual mode ion exchange resin —GE Healthcare). Activity assay reagents: 2,6-dichlorophenol indophenol (DCIP), phenazine methosulfate (PMS), and cell culture grade lactate. SDS-PAGE reagents: HEPES running buffer, GelCode Blue® staining solution, loading buffer, LoneRanger® molecular weight ladder, and pre-cast gels (5% stacking gel/12% resolving gel) were purchased from Pierce.

2.2. Enzyme activity assay procedure

For each enzyme of interest the reaction mixture consists of 1.5 mL of 50 mM potassium phosphate buffer pH 7.3, 0.2 mL of 60 μM PMS, 0.1 mL of 70 μM DCIP, 0.01 mL of enzyme, and 0.2 mL of a 0.2 M substrate solution [7]. For PQQ-LDH, lactate is the substrate. The change in absorbance for each sample is measured at 37 °C at time=0 min and time=2 min at 600 nm on a Thermo Brand® spectrophotometer.

2.3. SDS-PAGE procedure

SDS-PAGE was performed using a pre-cast gel (5% stacking gel/12% resolving gel) purchased from Pierce and run in an Emperor Penguin™ water cooled, dual gel vertical system from Owl Separations® electrophoresis apparatus for 33 min starting at 100 mA/gel ending at 40 mA/gel at 150 V using HEPES running buffer and LoneRanger® molecular weight ladder also purchased from Pierce. The samples were prepared in a 4:1 ratio of sample to loading buffer (proprietary reagent purchased from Pierce) followed by boiling for 3 min and allowing to cool before loading onto the gel. GelCode Blue® (proprietary staining solution purchased from Pierce) was used to stain the gels following electrophoresis followed by a 18 MΩ water rinse.

2.4. Gluconobacter sp. 33 growth optimization

In order to obtain PQQ-LDH, commercially purchased *Gluconobacter* sp. 33 (DSM 3504) and *Gluconobacter* sp. 33 (ATCC 15163), had to be cultivated aerobically. The cultivation media consist of the following (g/L): yeast extract—5, D-mannitol—10, $(NH_4)_2HPO_4$—1.0, and $MgSO_4{\cdot}7H_2O$—2.0 with the pH adjusted to 5.5. The aeration (150 rpm) and temperature (30 °C) of the cultivation period were controlled using a shaking incubator with enhanced capability to control the temperature to 0.01 °C. In order to compare different lines of *Gluconobacte*r sp. 33 (DSMZ 3504 and ATCC 15163) and different strains (ATCC 621) two lines of *Gluconobacter* sp. 33 and *G. suboxydans* were grown up in 250 mL wide mouth volumetric flasks with 150 mL of media in each flask. Each flask was inoculated with 100 μl aliquots of the respective bacteria. Following the 20 hour growth period, cells were collected via centrifugation (5000 g at 4 °C) and rinsed twice with 0.9% NaCl solution before being placed in a −20 °C freezer [7]. Viable cells are rose colored and are stable in the freezer for up to 6 months. For each extraction 4 L of cells are required.

2.5. Cell lysis: PQQ-LDH

While there is no literature source for the isolation and purification of PQQ-LDH, an ion exchange chromatography method had to be developed using *Gluconobacter* cell lysate as the starting material. Literature sources based on the isolation and purification of PQQ-dependent glycerol dehydrogenase, also a membrane bound PQQ-dependent dehydrogenase were used as a guideline in developing the method for PQQ-LDH [8]. *Gluconobacter* cells (four L) were suspended in 50 mM Tris–HCl buffer pH 9.0 buffer. The cell suspension was then sonicated using a sonic dismembrator for 1 min at 4 °C (using a pulsed frequency at the maximum power output). The sonicated cell solution was then centrifuged for 10 min at 7500 g at 4 °C in order to remove cell debris and collect the cell membranes. Next, the resultant supernatant containing the cell membranes was centrifuged for 8 h at 12,000 g at 4 °C in order to collect the cell membranes of interest (pellet). The PQQ-LDH containing pellet was resuspended in 50 mL of 5 mM Tris–HCl buffer pH 9.0 containing 0.2% Triton X-100 in order to solubilize the enzyme of interest (PQQ-LDH). It was then incubated at 4 °C with gentle stirring for 1 h, followed by centrifugation for 1 h at 7500 g at 4 °C and the resultant supernatant was collected.

2.6. Ion exchange chromatography: PQQ-LDH

The lysed *Gluconobacter* sample containing solubilized PQQ-LDH was first applied to a DEAE-Toyo-Pearl column (2.5×25 cm) equilibrated with 5 mM Tris–HCl buffer pH 9.0. The enzyme was eluted by a gradient of NaCl (50–200 mM) in 75 mM Tris–HCl buffer pH 9.0 containing 0.2% Triton X-100. Fractions containing active PQQ-LDH were collected, concentrated, and underwent dialysis against 5 mM Tris–HCl buffer pH 9.5 containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$. This sample was applied to a DEAE-Toyo-Pearl column (1.5×10 cm) equilibrated with 5 mM Tris–HCl buffer pH 9.5 containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$. The enzyme was eluted by a gradient of NaCl (50–200 mM) in 75 mM Tris–HCl buffer pH 9.5 containing 0.2% Triton X-100, 1 mM $CaCl_2$ and 1 mM $MgCl_2$. Fractions containing active PQQ-LDH were collected, concentrated, and underwent dialysis against 5 mM phosphate buffer pH 9.3. This sample was applied to a hydroxyapatite column equilibrated with 5 mM phosphate buffer pH 9.3 and PQQ-LDH was eluted by a gradient of NaCl (5–100 mM) potassium phosphate buffer pH 9.5 containing 0.2% Triton X-100, 5 mM $CaCl_2$ and 5 mM $MgCl_2$. The purified enzyme has a gold/red color (due to the heme-c/PQQ content) and stable for approximately one week at 4 °C in solution. When lyophilized, enzyme stability is increased to approximately one month. Specific activity assays along with SDS-PAGE is used to determine the activity and purity of PQQ-LDH containing samples.

2.7. DET at gold electrodes modified with adsorbed enzyme

Gold electrodes (0.5 cm i.d.) were prepared prior to modification by polishing them on a Buehler polishing cloth followed by a methanol rinse and rinsing with 18 MΩ water to insure the electrode surface is uniformly clean and there is no prior fouling. In order to prepare the electrode for enzyme adsorption the gold electrodes were cleaned by electrochemically cycling in 0.2 M NaOH between −1.8 V and 2.3 V (5 cycles) and between −1.8 V and 0.8 V (3 cycles) at a scan rate of 0.1 V/s. The electrodes were then thoroughly washed with 18 MΩ water prior to use.

For each experiment, 0.10 mg/mL of PQQ-LDH enzyme was dissolved in 10 mL potassium phosphate buffer pH 6.0 with 1 M NaCl as supporting electrolyte. A control was run in the presence of buffer only. Substrate specific to the enzyme (lactate) was added in increasing increments ranging from 2.0 mM to 6.0 mM for PQQ-LDH, and cyclic voltammetry was performed. A Ag/AgCl electrode was used as a reference and platinum mesh was used as the counter electrode. Cyclic voltammetry was used to investigate the electrochemistry of the working electrode using a CH Instruments potentiostat interfaced to a PC and was performed by sweeping from 0.3 V to 0.7 V at a scan rate of 0.01 V/s.

2.8. DET at carbon electrodes

Screen printed carbon electrodes were purchased from DropSens® and used as received. The working (4 mm i.d.) and counter electrodes are made of carbon, whereas reference electrode and electric contacts are made of silver. For each experiment, 0.10 mg/mL of PQQ-LDH was dissolved in 10 mL potassium phosphate buffer pH 6.0 with 1 M NaCl

as supporting electrolyte. A control was run in the presence of buffer only. Lactate substrate was added in increasing increments ranging from 10 mM to 30 mM and cyclic voltammetry was performed. Cyclic voltammetry was used to investigate the electrochemistry of the working electrode using a CH Instruments potentiostat interfaced to a PC and was performed by sweeping from −0.4 V to 0.4 V at a scan rate of 0.1 V/s.

2.9. Modified Nafion procedure

Casting solutions for making the mixture-cast membranes of Nafion® and quaternary ammonium bromides were prepared as discussed in Minteer et al. [9–11]. The modified Nafion® membranes were formed in a two step process. The first step was to cast a suspension of Nafion® with tetrabutylammonium bromide salt dissolved in suspension at a concentration of tetrabutylammonium bromide salt in a three-fold excess of the concentration of sulfonic acid sites in the Nafion® suspension. 18 MΩ water (approximately 7 mL) was added to the weighing boats and allowed to soak overnight. The water was removed and the films were rinsed thoroughly with 18 MΩ water and dried overnight. Following the HBr salt extraction, the films were resuspended in 1.0 mL of lower aliphatic alcohols. The suspended film was then employed in forming the enzyme casting solutions of membrane and enzyme.

2.10. Bioanode fabrication

PQQ-dependent enzymatic bioanodes were fabricated using 1.0 cm^2 Toray® carbon fiber paper. The PQQ-dependent enzyme was immobilized at the electrode in a TBAB modified Nafion® membrane in a 1:2 ratio of enzyme to polymer. The casting solution consisted of 50 µL (0.10 mg PQQ-LDH suspended in 1 mL of 5 mM phosphate buffer pH 7.2 with 1 mM $CaCl_2$) enzyme to 100 µL polymer mixed thoroughly then coated onto the carbon electrode and allowed to dry. Once the electrode was dry, it was ready to be used in a complete cell apparatus.

2.11. Physical cell apparatus

The physical test cell consisted of custom fabricated “U” shaped cylindrical glass tubing with 2.6 cm diameter, 14.8 cm height, and 7.6 cm length. Approximately 50 mL of solution was contained on either side of a Nafion® 212 membrane acting as a salt bridge. The cathode side of the test cell contained various pHs of phosphate buffer

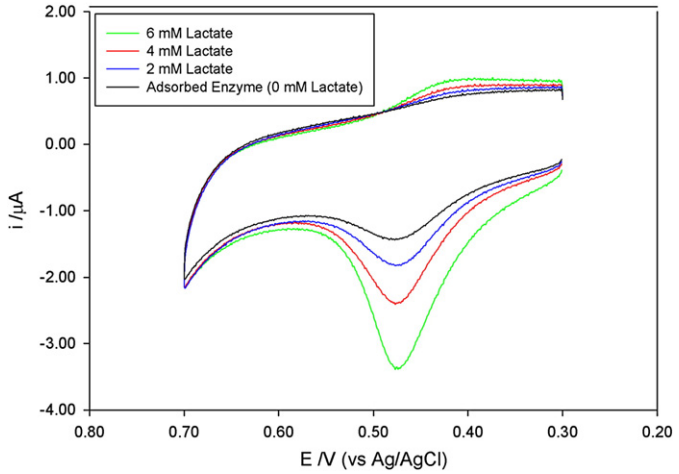

Fig. 1. Representative cyclic voltammograms of PQQ-LDH adsorbed at a gold electrode (scan rate 0.1 V/s) in buffer and 2 mM, 4 mM, and 6 mM lactate in buffer. A plot of lactate oxidation peak current (A) vs. concentration of lactate in millimolar concentrations results in a linear calibration plot with an equation of $y=(1.81\pm0.17\times10^{-7})x+4.47\pm0.62\times10^{-7}$ and a R^2 of 0.984.

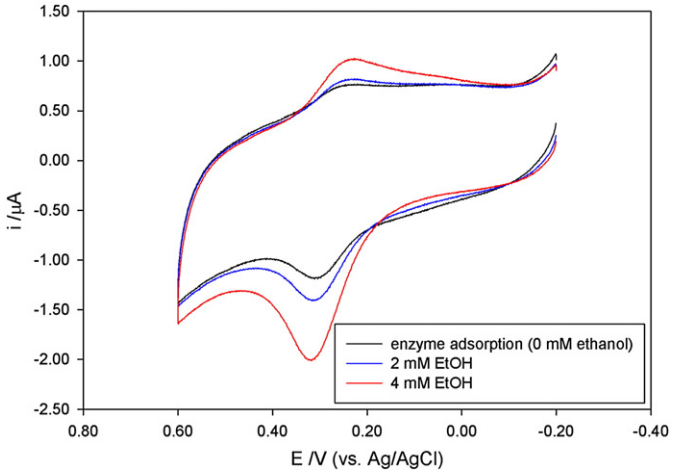

Fig. 2. Representative cyclic voltammograms of PQQ-ADH adsorbed at a gold electrode (scan rate 0.1 V/s) in buffer and 2 mM and 4 mM ethanol in buffer.

ranging from 5.5 to 8.5 with 1 M NaCl as supporting electrolyte with dissolved oxygen present. The cathode material is an ELAT electrode with 20% Pt on Vulcan XC-72 (E-Tek). The anode side of the test cell is filled with various pHs of phosphate buffer ranging from 5.5 to 8.5 with 1 M NaCl as supporting electrolyte and various concentrations of fuel ranging from 10.0 mM to 50.0 mM lactate at 20 °C. The modified electrode with enzyme acts as the anode. The complete cell was allowed to equilibrate for 2–6 h before data collection. All data were collected and analyzed for the test cell with a CH Instruments potentiostat interfaced to a PC computer.

3. Results and discussion

The purification scheme for PQQ-LDH, similar to PQQ-ADH and PQQ-AldDH, is based on previous literature for another membrane bound PQQ-dependent dehydrogenase (PQQ-GlyDH) [8]. Unlike the initial cell lysis of PQQ-ADH and PQQ-AldDH, for PQQ-LDH, cell membranes of interest were first collected then lysed as opposed to lysing the *Gluconobacter* bacteria cells as a whole. This adds to the specificity of the starting material as opposed to total cell lysis. PQQ-LDH was purified using anion exchange chromatography in addition to using hydroxyapatite which is a dual mode ion exchange resin. The presence of Triton X-100 is necessary in several of the chromatographic steps in order to solubilize the enzyme of interest while not affecting the overall ionizable groups of the enzyme.

The buffers employed in the ion exchange chromatography (IEC) purification scheme of PQQ-LDH range between pH 9.0 and 9.5. Based on this, the p*I* of PQQ-LDH can be assumed to be less than 9.0 due to the fact there is enzyme adsorption onto the anion exchange column resin. Specific activity in micromoles per minute per mg was determined for PQQ-LDH from all three different bacteria sources. The specific activity of PQQ-LDH from *Gluconobacter* sp. 33 (DSMZ 3504) was 3.7 µmol per minute per mg. This was the highest PQQ-LDH activity observed. The observed activities of PQQ-LDH from *Gluconobacter* sp. 33 (ATCC 15163) and *Gluconobacter oxydans* sp. *suboxydans* (ATCC 621) were 0.90 and 0.52 µmol per minute per mg, respectively. The overall yield of PQQ-LDH was approximately 0.5 mg for each preparation from 4 L of starting cell culture material. SDS-PAGE experiments resulted in a gel containing purified PQQ-LDH containing four bands with two bands with molecular weights/subunits correlating to known literature about PQQ-dependent dehydrogenases (80 kDa and 60 kDa). The exact structure of PQQ-LDH is unknown, except for the fact that there are large subunits at approximately 80 kDa and 60 kDa, most likely heme-c containing moieties similar to those found in PQQ-ADH (which is known). The

largest subunit at 80 kDa is most likely the one responsible for oxidation of the substrate while the subunit at 60 kDa most likely carries out ubiquinone reduction in this case allowing the enzyme to undergo DET similar to PQQ-ADH [12].

The ability of PQQ-LDH to undergo DET was investigated by voltammetry at both gold and carbon electrodes. Similarly to literature sources for DET of PQQ-ADH [12–14], DET of PQQ-LDH is also carried out via the heme-c moieties contained within the enzyme as shown in the representative cyclic voltammograms shown in Fig. 1. Fig. 1 shows the ability of PQQ-LDH to undergo direct electron transfer at gold electrodes. There are clear voltammetric peaks at 0.477 V vs. Ag/AgCl for lactate oxidation. This potential is significantly higher than alcohol oxidation at PQQ-dependent alcohol dehydrogenase at a gold electrode (0.317 V vs. Ag/AgCl) as shown in Fig. 2. It can be assumed that the location of the heme-c moieties responsible for DET in PQQ-LDH when orientated towards a gold electrode surface is concentrated in one subunit similar to PQQ-ADH, because there is only one redox peak present. It is important to note that although the oxidation potential is lower for alcohol than lactate, the slope of the calibration curve for lactate is 88% larger than ethanol. The larger size of each enzyme could inhibit rapid conductance of electrons from the oxidation of substrate to the heme-c containing subunit responsible for DET.

For self-powered sensor and biofuel cell applications, it is desirable to have the redox couple for the DET of the heme-c moieties to be as low as possible. Therefore, adsorption configurations on carbon substrates were investigated next to allow the redox couple of heme-c to occur at even lower potentials and lend to simpler and inexpensive fabrication methods compared to configuration on gold substrates. Previous research has shown the ability of PQQ-ADH to undergo DET at screen printed carbon electrodes [15]. Fig. 3 shows the ability of PQQ-LDH to also undergo DET at carbon electrodes. There are clear voltammetric peaks at 0.164 V vs. silver/silver chloride for adsorbed enzyme (compared to 0.137 V for adsorbed PQQ-dependent alcohol dehydrogenase). This voltammetric peak shifts with increasing concentration of lactate. Therefore, since DET can occur between PQQ-LDH and a carbon electrode, PQQ-LDH was used as the catalyst at the anode of lactate/air biofuel cell.

Lactate/air biofuel cells were tested under variable environmental conditions by looking at the effects of fuel concentration and pH. When the lactate/air biofuel cell was tested under a range of fuel concentrations, V_{max} (the maximum rate when all of the enzyme molecules have substrate bound) for the enzymatic system immobilized at the

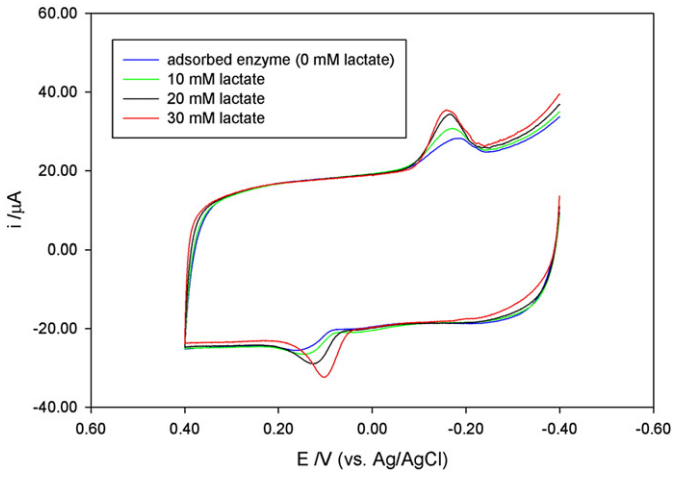

Fig. 3. Representative cyclic voltammograms of PQQ-LDH adsorbed on a carbon screen printed electrode (scan rate 0.1 V/s). A plot of lactate oxidation peak current (A) vs. concentration of lactate in millimolar results in a linear calibration plot with an equation of $y=(2.59\pm0.45\times10^{-7})x+4.81\pm0.85\times10^{-6}$ and a R^2 of 0.942.

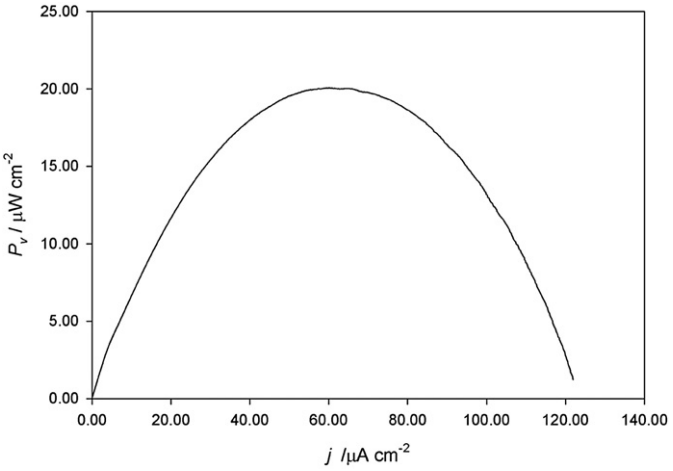

Fig. 4. Representative power curve for lactate/air biofuel cell in 10 mM lactate in pH 7.15 phosphate buffer.

electrode surface can be approximated in relation to the power density produced. Fig. 4 is a representative power curve of a lactate/air biofuel cell employing a 10 mM lactate solution as fuel. The average power density for this lactate/air biofuel cell is 18.0±0.16 μW/cm^2. The average power density increases to 22.0±0.18 μW/cm^2 for 25 mM fuel, but then levels off and is not statistically different from 50 mM lactate fuel (20.3±0.24 μW/cm^2). Once an enzymatic system reaches its V_{max} value, regardless of how much fuel is added to the system, the enzyme is unable to convert any more substrate to product at a higher rate than already operating at thus the power produced will level off [16]. The open circuit potential of the lactate/air biofuel cell was 0.85±0.02 V and testing was done over a 45 day period without degradation in performance. This stability is similar to the stability observed for ethanol/oxygen biofuel cells employing PQQ-alcohol dehydrogenase immobilized in tetrabutylammonium bromide modified Nafion on carbon electrodes [17], but is considerably longer than other biofuel cells that have shown operational half life periods of 2.5 days for an ethanol biofuel cell with PQQ-dependent alcohol dehydrogenase immobilized on carbon electrodes with glutaraldehyde [18]. It is also important to note that the open circuit potential of the lactate/air biofuel cell is also higher than observed for ethanol biofuel cells employing PQQ-dependent alcohol dehydrogenase. Researchers have observed open circuit potentials up to 0.76±0.01 V for an ethanol/air biofuel cell with a platinum cathode [15] and up to 0.27 V for an ethanol biofuel cell employing co-immobilized glucose oxidase and micro-peroxidase at the cathode [18].

In addition to being tested under a range of fuel concentrations appropriate to each respective enzymatic system, the effects of operating pH for the lactate/air biofuel cell were also examined. There is no correlation between pH and power output for the lactate/air biofuel

Table 1
Bioanode Performance as a function of fuel concentration and pH

	Maximum current density (A/cm^2)	Maximum power density (W/cm^2)
Fuel concentration		
10 mM lactate	$1.02\times10^{-4}\pm1.77\times10^{-5}$	$1.80\times10^{-5}\pm1.55\times10^{-6}$
25 mM lactate	$1.38\times10^{-4}\pm1.12\times10^{-5}$	$2.20\times10^{-5}\pm1.78\times10^{-6}$
50 mM lactate	$1.20\times10^{-4}\pm1.76\times10^{-5}$	$2.03\times10^{-5}\pm2.40\times10^{-6}$
pH effects in 10 mM lactate		
5.5	$1.86\times10^{-4}\pm4.22\times10^{-5}$	$8.21\times10^{-6}\pm2.93\times10^{-6}$
6.5	$8.70\times10^{-5}\pm5.98\times10^{-5}$	$1.18\times10^{-5}\pm3.48\times10^{-6}$
7.15	$1.02\times10^{-4}\pm1.77\times10^{-5}$	$1.80\times10^{-5}\pm1.55\times10^{-6}$
8.5	$1.55\times10^{-4}\pm9.90\times10^{-6}$	$7.92\times10^{-6}\pm3.11\times10^{-6}$

cell with 10 mM lactate as shown in Table 1. The pH independence of the PQQ-LDH bioanode is expected due to the fact that the modified Nafion® immobilization membrane for each enzyme creates a buffered environment and is resistant to changes. This pH stability is of special interest considering that this type of pH stability has not been observed for PQQ-dependent alcohol dehydrogenase either for use in a sensor [19] or in a biofuel cell [18].

4. Conclusions

PQQ-LDH is a membrane bound PQQ-dependent dehydrogenase which can be derived from various strains of *Gluconobacter*. This is the first time that PQQ-LDH has been isolated and purified from any bacteria. PQQ-LDH contains the typical subunits containing a PQQ moiety along with subunit containing multiple heme-c indicative of all quinohemoproteins including PQQ-ADH. The presence of the multiple heme-c moieties PQQ-LDH allows for DET to occur at both gold and carbon electrode surfaces enabling the fabrication of lactate bioanodes. PQQ-LDH shows a linear correlation between substrate concentration and peak current. Initial data have shown that the PQQ-LDH bioanode will operate under a range of fuel concentrations and pHs (5.0–8.5) and without the performance being greatly adversely affected. The modified Nafion® immobilization membrane plays an important role in maintaining the activity of each respective enzyme in a range of pHs as compared to enzyme free in solution in addition to creating a more rugged electrode configuration compared to enzymes free in solution. With the discovery of this new PQQ-dependent/heme-c containing enzyme, new DET-based bioelectrodes can be fabricated using lactate as substrate.

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