Brazilian Journal of Microbiology (2010) 41: 796-804
ISSN 1517-8382

ISOLATION AND PURIFICATION OF COMPLEX II FROM *PROTEUS MIRABILIS* STRAIN ATCC 29245

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Submitted: May 21, 2009; Approved: March 16, 2010.

ABSTRACT

A respiratory complex was isolated from plasma membrane of pathogenic *Proteus mirabilis* strain ATCC 29245. It was identified as complex II consisting of succinate:quinone oxidoreductase (EC 1.3.5.1) containing single heme b. The complex II was purified by ion-exchange chromatography and gel filtration. The molecular weight of purified complex was 116.5 kDa and it was composed of three subunits with molecular weights of 19 kDa, 29 kDa and 68.5 kDa. The complex II contained 9.5 nmoles of cytochrome b per mg protein. Heme staining indicated that the 19 kDa subunit was cytochrome b. Its reduced form showed absorptions peaks at 557.0, 524.8 and 424.4 nm. The α-band was shifted from 557.0 nm to 556.8 nm in pyridine ferrohemochrome spectrum. The succinate: quinone oxidoreductase activity was found to be high in this microorganism.

Key words: *Proteus mirabilis*, complex II, cytochrome b

INTRODUCTION

Proteus mirabilis belongs to family *Enterobacteriaceae* and is facultative anaerobic, rod shaped, and gram negative bacterium (14). It is mainly found in GI tract, soil, infections of bladder, lung, urinary tract and wounds. It infects and persists for a long period of time (26). It also causes pneumonia, chills, fever, cough and chest pain (4, 12, 27).

In bacterial cells, the generation of energy in the form of adenosine triphosphate (ATP) is mainly motivated by the activity of respiratory chain enzymes of inner plasma membrane (5). The respiratory chain of bacteria usually composed of enzyme complexes I to IV, ubiquinone, cytochromes, and ATP synthase (complex V), transfers electrons from NADH and succinate at one end to molecular oxygen at the other (2). Van Wielink *et al.* (22) observed the presence of cytochrome b, a1 and d in spectral investigation of the plasmic membrane of *Proteus mirabilis*. Van Wielink *et al.* also (20) characterized the presence of cytochrome b and c in plasmic membranes of *Proteus mirabilis* by means of 77K spectra in both aerobic and anaerobic conditions. The function of cytochrome b was investigated in *Proteus mirabilis* (21) revealed the presence of cytochrome b in Q or b-cycle between the two HQNO inhibition sites. Thus a few types of respiratory components of *Proteus mirabilis* have been studied with their enzymatic and structural features. However the presence of the succinate: quinine oxidoreductase (complex II) of *Proteus mirabilis* has not so far been completely studied.

Complex II of the bacterial electron transport chain is of special interest. It functions as a dehydrogenase in the respiratory system and plays an important role in the tricarboxylic acid cycle (TAC) (17). This membrane associated complex catalyses the oxidation of succinate to fumarate and reduce ubiquinone to ubiquinol, and has been characterized in bacteria and heterophilic eukaryotes (19). Complex II (succinate:quinone oxidoreductase) of aerobic respiratory chain

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oxidizes succinate to fumarate and passes electrons directly into the quinone pool. It serves as the only direct link between activity in the citric acid cycle and electron transport in the membrane (1). Membrane-bound succinate dehydrogenases (succinate:quinone reductases, SQR) couples the oxidation of succinate to fumarate to the reduction of quinone to quinol and also catalyzes the reverse reaction. SQR (respiratory complex II) is involved in aerobic metabolism as part of the citric acid cycle and of the aerobic respiratory chain (11). Iron sulfur clusters and flavin adenine dinucleotide (FAD) are located in the water soluble moiety, while cytochrome b is present in membrane-bound moiety. The purified succinate quinine oxidoreductase from *Bacillus subtilis* contains two hemes b and the other gram negative bacteria such as *E. coli* (6, 9) and *P. denitrificans* (15) contains one molecule of heme b per FAD which showed that cytochrome b was generally present with the association of succinate: quinine oxidoreductase.

In present study we report the isolation and purification of complex II found in the plasmic membrane of *Proteus mirabilis* strain ATCC 29245 and its activity *in vitro*.

MATERIALS AND METHODS

Materials

DEAE-Bio-Gel and Bio-Gel-P100 were purchased from Bio-Rad Pakistan. DEAE-Sephadex and Sodium succinate from Sigma, Triton-X100 from Acros (USA), Commassie Brilliant Blue dye from Fischer, DCPIP and Tris salt from Fluka and Pyridine and Sodium dithionite from BDH (U.K) and Nutrient Broth, Nutrient agar, Potassium dichromate, SDS and EDTA were purchased from Merck. All other chemicals used in this study were of the extra pure grade.

Organism and culture conditions

The gram –ve facultative bacterium *Proteus mirabilis* strain (ATCC 29245) was used in the present study. The strain was provided by PCSIR Laboratories Lahore, Pakistan. The bacterium was aerobically grown on nutrient medium (Merck) in conical flasks at 37^{o}C on shaker (C_{24} KC Refrigerated Incubator shaker USA) at 250 rpm. Growth of Proteus was studied after the regular interval of 2 hours by taking its optical density by spectrophotometer (CICIL/UV-Visible Spectrophotometer HitachiU-2001) at 595 nm. The cultivation of *Proteus mirabilis* was performed in 200 liters of the above nutrient medium with a stainless steel fermenter of 500 L volume. The culture was harvested at the early exponential phase by centrifugation at 4000 rpm for 25 min and suspended in 50mM Tris HCl buffer (pH: 8.4) containing 50mM EDTA.

Purification of membrane bound complex- II

Frozen cells (about 25gm in a centrifugally packed state) were suspended in 100ml of 50mM Tris HCl buffer (pH: 8.4) and 20ml of 50mM EDTA (buffer A). The suspension was sonicated with a sonic oscillator (Soniprep 150 SANYO UK) at 12-14 KHz for total period of 15 min with intervals of 1 min at 4^{o}C. After sonication the suspension was subjected to centrifuge by using centrifuge machine (HITACHI-CP 80 MX) at 15000 rpm at 4^{o}C for 15 min. Supernatant contained membrane fraction and cytoplasmic fraction where as pellets were of unbroken cells . The supernatant then ultra centrifuged at 35000 rpm and 4^{o}C for 60 min. The reddish pellet obtained was of cell membrane whereas the supernatant was of cytoplasm. Then cell membrane pellet was suspended in 25ml of 50mM Tris-HCl buffer (pH 8.4) with 2.5ml of 50mM EDTA and 4.2 ml of 20% (wt/vol) Triton-X 100 (with final concentration of detergent is 3%). The resulting mixture was stirred using a magnetic stirrer for 1 hour to solubilize the total cell membrane proteins. This suspension was then ultra-centrifuged at 45000 rpm and 4^{o}C for 45 min. Reddish supernatant thus obtained was total membrane proteins and subjected to a first ion-exchange chromatography (Biologic LP System, BIO-RAD) on a DEAE-Sephadex column(4.0 by 16.0 cm) equilibrated with buffer A. The column was washed with 400ml of buffer A containing 1% of Triton X-100, then enzyme was eluted by using linear gradient solution of 600ml each of buffer A containing 1% of Triton X-100 and buffer B containing 1% Triton X-100 and 1.0 M NaCl. The eluates having enzymatic activity were collected (BIO-RAD, Model

2110 Fraction collector) and dialyzed against 1 liter of buffer A and then subjected to a second ion exchange chromatography on DEAE-Bio-Gel (1.5 by 8.0 cm) equilibrated with buffer A. The adsorbed enzyme was eluted with a linear gradient of NaCl (0-1.0 M NaCl) produced in 600ml of buffer A containing 1% Triton X-100. Thus the fractions 30-34 (5ml each) with the enzymatic activity as shown in (Figure 2) were collected and concentrated by lyophilization to an appropriate size and subjected to gel filtration with a Bio-Gel P-100 column equilibrated with 50mM Tris-HCl buffer (pH:8.4) containing 1% Triton X-100 and 0.5 M NaCl. The yellowish-red complex II fraction was collected and used as purified preparation.

Spectroscopy

Absorption spectra of respiratory proteins were studied by using a quartz cuvette with the CICIL/UV-Visible Spectrophotometer Hitachi U-2001 at room temperature in visible range. Cytochrome b from *Proteus mirabilis* strain ATCC 29245 was suspended in 50mM Tris-HCl (pH: 8.4) containing 1% Triton X-100. Cytochrome b was oxidized with 1M Potassium dichromate solution and reduced by adding few pellets of sodium dithionite. For obtaining ferrohemochrome spectra, the 0.7mL enzyme was suspended in 0.5mL of 0.2 N NaOH, 1.8mL of distilled water and 0.5ml of pyridine and then reduced with a small amount (500mg)of sodium dithionite.

Polyacrylamide Gel Electrophoresis

Native polyacrylamide gel electrophoresis was performed in the presence of 0.4% Triton X-100 and Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed under denaturing conditions at room temperature by the method of Schagger and von Jagow (18). In the estimation of the apparent molecular weight by SDS-polyacrylamide gel electrophoresis, a set of protein markers (PAGE ruler pre-stained protein ladder, SM0671 Fermentas) with known molecular weights were used. The presence of cytochrome b in the native gel was detected by heme staining reagent (3).

Assay of Redox Activity

Cytochrome b oxidoreductase activity was measured at room temperature spectrophotometrically (CICIL/UV-Visible Spectrophotometer. Hitachi U-2001) in time scanned mode. The oxidation of succinate to fumarate with concomitant transport of electron to DCPIP dye (which acts as electron accepter) was determined by monitoring the decrease in absorbance at 600nm for 5 min. The reaction mixture contained 9µL of 0.1M EDTA, 3mL of 50mM Tris-HCl buffer (pH 8.4), 159µL of 1mM (dichlorophenolindophenol) DCPIP dye, and 60µL of 0.1M sodium succinate. Addition of the enzyme (40µL of Complex II) initiated the reaction.

RESULTS

Growth profile of *Proteus mirabilis* strain ATCC 29245

Growth profile of *Proteus mirabilis* showed that maximum growth could be achieved after 12-14 hours of incubation at 37°C (Fig.1). Biomass was collected after 12 hours of incubation to ensure maximum activity of complex II.

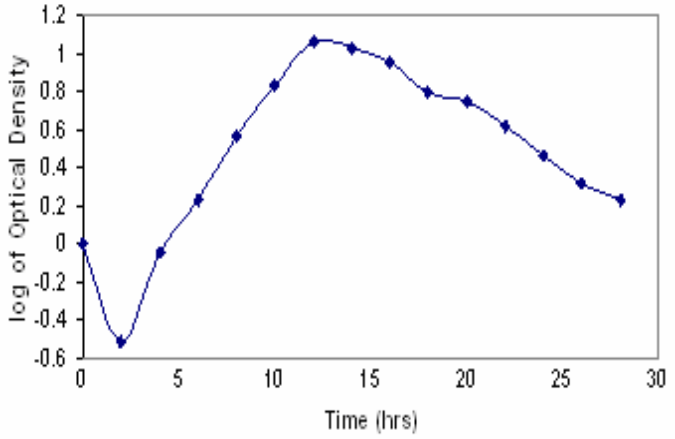

Figure 1. Growth profile of *Proteus mirabilis* strain ATCC 29245. It shows that maximum active biomass can be achieved in 12-14 hrs after incubation.

Purification of Complex II from *Proteus mirabilis* strain ATCC 29245

The membrane bound respiratory complex II was solublized with Triton X-100 and purified by ion-exchange chromatography and gel filtration. Purification factors and yields were shown in the Table 1. Purification was started with

solublized membranes containing a total amount of 235mg of protein and 310 nmol of total heme b. The final preparation appeared to be homogenous as revealed by nondenaturing polyacrylamide gel electrophoresis (Native PAGE) since a single band was observed after heme staining.

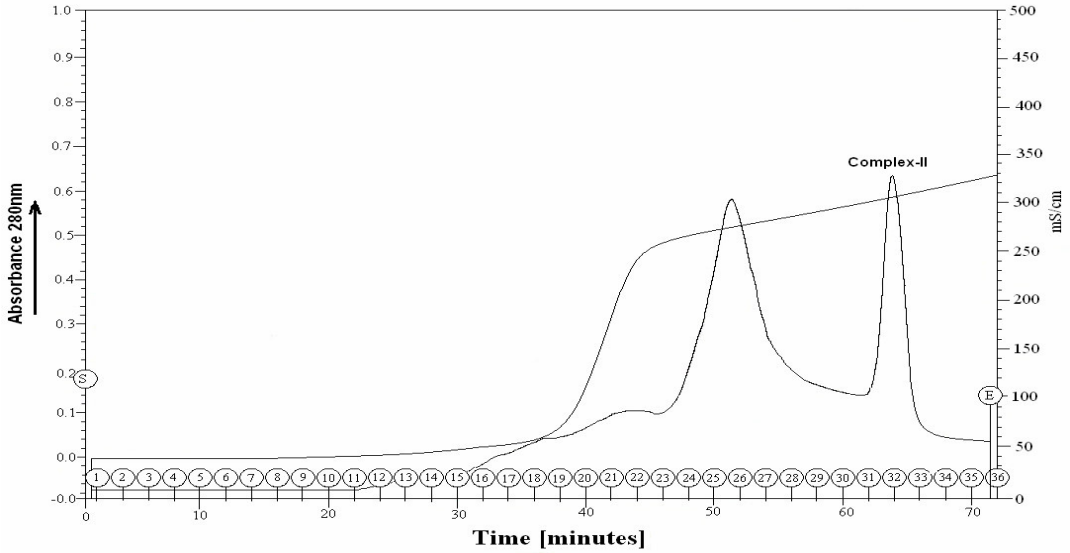

Figure 2. Anion exchange chromatography of complex II. The enzyme was eluted by using linear gradient solution of 600ml each of buffer A containing 1% of Triton X-100 and buffer B containing 1% Triton X-100 and 1.0 M NaCl.

Table 1. Purification of complex II from *Proteus mirabilis* strain ATCC 29245

Step	Total Vol. (ml)	Total Protein (mg)	Total heme b (nmol)	Heme b protein nmol/mg	Yield %
Solublized membranes	80.0	235	310	1.3	100
DEAE- Sephadex	25.0	39	119	3.05	38.34
DEAE- Bio Gel	10.0	8.7	41.3	4.08	13.3
Gel Filtration	8.0	4.2	35.6	8.5	11.48
Purified Enzyme	1.0	2.9	27.4	9.5	8.7

Spectral properties of Complex II from *Proteus mirabilis* ATCC 29245

Absorption spectrum of complex II is shown in the Figure 3. Figure 3A showed the oxidized spectrum of the membrane proteins and showed a single absorption peak at 411.0 nm. When the enzyme was reduced with the addition of sodium dithionite it showed peaks at 557, 524.8 & 424 nm as shown in figure 3B. The characteristic α-peak at 557 nm showed the presence of heme b. The figure 3C showed peaks at 556.8, 524.8 & 420 nm of pyridine ferrohemochrome spectrum, which further confirmed the presence of cytochrome b in complex II.

Polyacrylamide Gel Electrophoresis

The purified enzyme was subjected to Native PAGE (7) followed by Coomassie brilliant blue and the heme staining. A single band showed that the enzyme was purified to homogeneity (Fig. 4).

Apparent molecular weights of proteins were estimated by polyacrylamide gel electrophoresis in the presence of SDS by the method of Laemmli (10). Purified sample of solubilized membrane proteins was loaded upon 12.5% SDS-PAGE along with a standard marker proteins ladder (Fig. 5). The sample showed prominent protein band in the region of 68.5, 29 and

19 kDa.

Assay of Redox activity

The enzymatic properties of the *Proteus mirabilis* succinate: quinone oxidoreductase was analyzed by using the artificial electron acceptor DCPIP. When succinate was added to the oxidized enzyme under aerobic conditions, the heme b moiety of enzyme was fully reduced in 5 min (Fig.6) at 660nm.

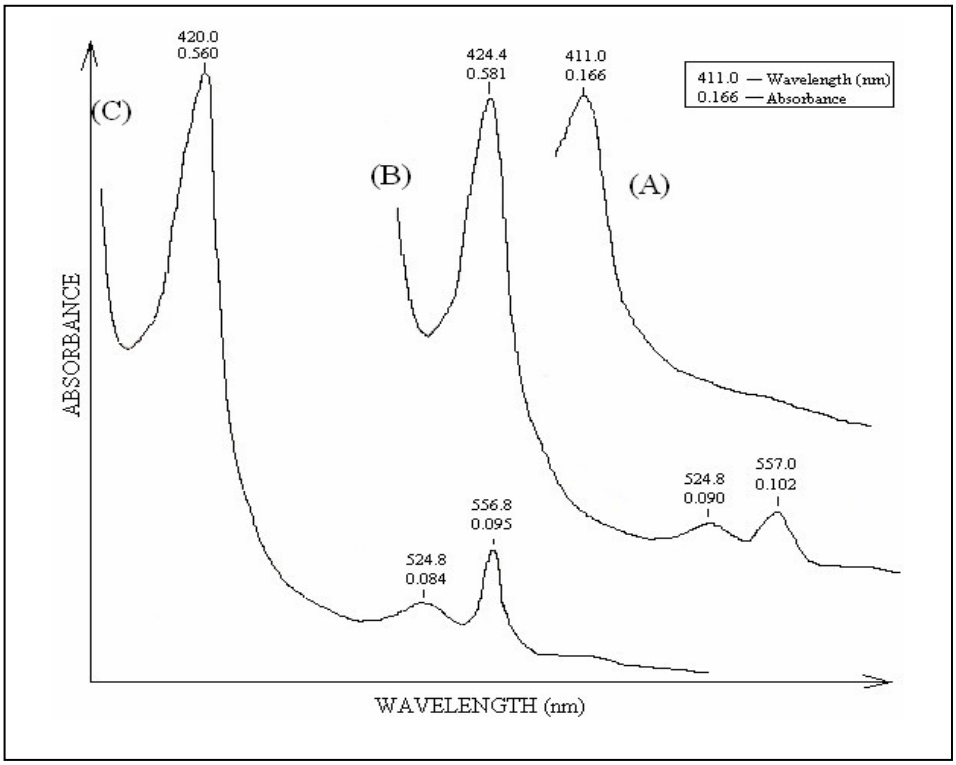

Figure 3. Spectral properties of the *Proteus mirabilis* strain ATCC 29245. Absorption spectra of cytochrome b from *Proteus mirabilis* strain ATCC 29245 at room temperature. The cytochrome b was suspended in 50 mM tris-HCl (pH 8.4) containing 1% Triton X-100. A: Oxidized absorption spectrum. The cytochrome b was oxidized by adding 1M potassium dichromate. B: Reduced spectrum of membrane-bound cytochrome b. The cytochrome b was reduced with sodium dithionite. C: Pyridine ferrohemochrome spectrum. The cytochrome b was suspended in 0.2 N NaOH and pyridine and reduced with a small amount of sodium dithionite.

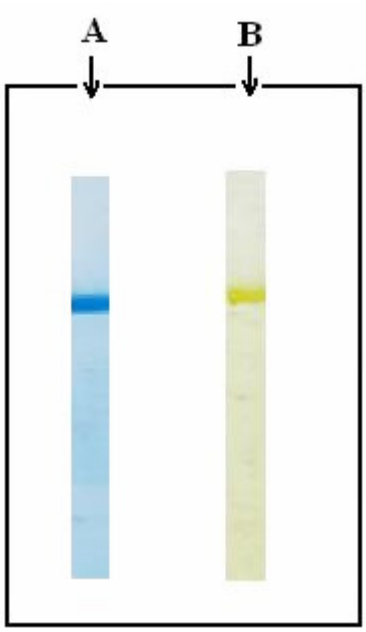

Figure 4. NATIVE PAGE of the *Proteus mirabilis* strain ATCC 29245. The purified cytochrome b was run on NATIVE PAGE and stained. Column A: Commassie brilliant blue staining of heme b, Column B: Heme Staining of heme b.

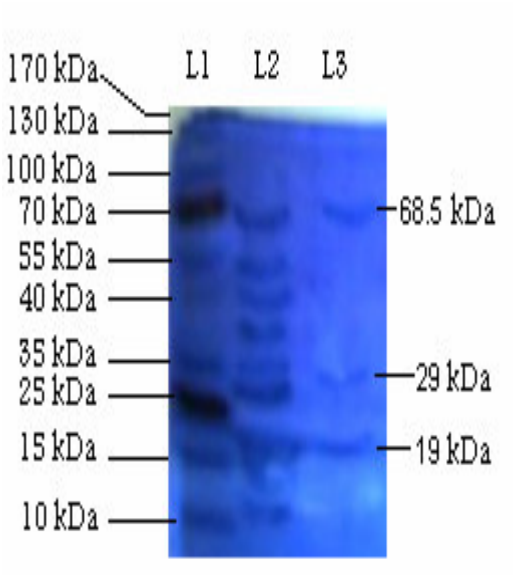

Figure 5. SDS-PAGE of the *Proteus mirabilis* strain ATCC 29245. The purified cytochrome b was loaded on 12.5% gel. The gel was stained with commassie brilliant blue after electrophoresis. Lane 1 (molecular mass marker proteins), lane 2 (sample 1, partially purified complex II after ion exchange chromatography) and lane 3, (sample 2, purified complex II). The molecular masses of three subunits of the *Proteus mirabilis* strain ATCC 29245 complex II were estimated to be 68, 28.5, and 19.5 kDa.

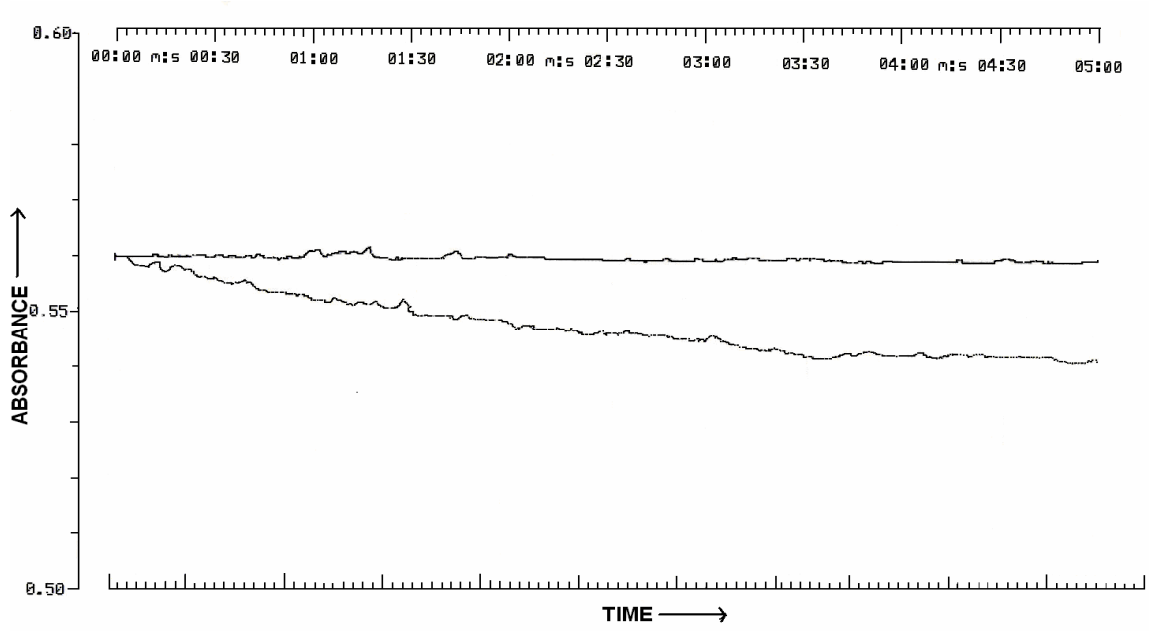

Figure 6. Enzyme assay of complex II from *Proteus mirabilis* strain ATCC 29245. The enzymatic reaction was time-scanned for 5 min at 660nm to observe the decrease in absorption of DCPIP dye which acts as artificial electron acceptor *in vitro*.

. DISCUSSION

Proteus mirabilis is a pathogenic microorganism so the study of its respiratory proteins is important. Growth profile of *Proteus mirabilis* was studied in shake flasks, incubated at 37^{o}C at 250rpm (diameter 2.5cm). The culture was found to grow exponentially between 8-14 hours. Maximum biomass was accumulated after 12 hours as shown in Figure 1. Decline phase started after 14 hours was due to the exhaustion of nutrients and accumulation of toxic by-products particularly release of H_2S gas (23).

Cytochrome b is a component of Complex II and is required for electron transfer from succinate to ubiquinone (8). The *b*-type cytochromes in bacteria function as essential

components of the respiratory complexes. The cytochrome b functions between two HQNO sites or more probably in a Q- or b-cycle (22). Presence of succinate: quinone oxidoreductase and many types of cytochrome b of complex II present in bacterial electron transport chain have been reported and confirmed in *Proteus mirabilis* but its isolation from plasma membrane could not be achieved. In the present study, we have reported for the first time the isolation of cytochrome b and succinate: quinone oxidoreductase in complex II from plasma membrane of *Proteus mirabilis* ATCC 29245. The nonionic detergent Triton X-100 was found to be the best suitable detergent for solubilization and stabilization of cytochrome b and succinate: quinone oxidoreductase of this bacterium. The enzyme is stable in the presence of Triton X-100 and has showed high succinate: quinone oxidoreductase activity. When the enzyme was oxidized with $K_2Cr_2O_7$, the spectra showed that the Soret absorption peak at 411nm; upon reduction with sodium dithionite, the Soret absorption maxima shifted at 424nm and the α-absorption peak appeared at 557nm while β-absorption peak appeared at 525nm as shown in figure 3. These absorption peaks were attributed to the reduced form of cytochrome b and are very close to the values observed in isolated cytochrome b from other bacteria as given in Table 2. The presence of single form of cytochrome b is indicated by the symmetrical α-absorption peak at 557nm in reduced form. This result suggests that in the isolated complex II from *Proteus mirabilis*, only one type of cytochrome b is involved in electron transfer.

Table 2. Comparison of soluble cytochrome b-557 from different bacteria

Property	***P. mirabilis*** **Present Work**	***B. subtilis*** **(Qureshi *et al.* 1996) (16)**	***E. coli*** **(Yariv *et al.* 1981) (25)**	***P. aerogenosa*** **(Moore *et al.* 1986) (13)**
λ -max at 25°C (Oxidized form)	411 nm	412nm	417nm	418
λ -max at 25°C (Reduced form)	424.4, 524.8, 557	425, 526, 556.5	425, 527, 557	424, 526, 557
λ -max at 25°C (Pyridine spectra)	420, 524.8, 556.8	420, 526, 556.5	N.A	N.A

A prominent protein stained bands in the region of about 68.5, 29, and 19 kDa of covalently bound succinate: quinone oxidoreductase in the membrane fraction of *Proteus mirabilis* appeared when the membrane proteins from *Proteus mirabilis* run on 12.5% SDS-PAGE along with standard marker proteins (Fig.5). The 19 kDa subunit of complex II from the *Proteus mirabilis* strain ATCC 29245 stained with heme-staining reagent, considering that the succinate: quinone oxidoreductase seems to combine heme *b* component in molecule which strongly suggests that the enzyme isolated with Triton X-100 maintains its intact structure.

Bacterial succinate: quinone oxidoreductase may have one or two hemes *b* in the molecule. Spectral properties of the succinate: quinone oxidoreductase enzyme from *Proteus mirabilis* strain ATCC 29245 also suggests the presence of only one type of heme b in the molecule. On the basis of total heme b content, the recovery in the purification was calculated to be about 9.5 nmol /mg of heme b, which yields 8.7% of the total solublized proteins. For further investigation and conformation of the presence of cytochrome b in *Proteus mirabilis*, energy-linked reduction of cytochrome *b* has been studied when complex II is fully reduced with a substrate such as succinate. It is generally accepted that succinate: quinone oxidoreductase (Complex II) is a membrane bound respiratory complex and is associated with cytochrome b (24). The enzymatic properties of the *Proteus mirablis* succinate: quinone oxidoreductase was analyzed by using the artificial electron acceptor DCPIP. When sodium succinate (an electron donor) was added to the oxidized enzyme, the heme b moiety of the enzyme was almost fully reduced in 5 min with the

decrease in absorbance of DCPIP dye .The overall activity was quite prominent and notable. It looks like; the succinate: quinone oxidoreductase enzyme in this bacterium is highly active.

In the present study we have found that the complex II of *Proteus mirabilis* strain ATCC 29245 has a single heme b in the molecule which was confirmed by spectroscopic studies.

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