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HQNO-sensitive NADH:DCIP Oxidoreductase of a Pathogenic *Bacillus cereus* Causing β-Hemolysis

Young Jae Kim* and Ki Tae Park[1]

Department of Microbiology, Changwon National University, Sarim-dong, Changwon, Kyungnam 641-773, Korea
[1]Ki Tae Park Korean Medicine Clinic, Sangnam-dong, Changwon, Kyungnam 641-773, Korea

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Membranes prepared from *Bacillus cereus* KCTC 3674, grown aerobically on a complex medium, oxidized NADH exclusively, whereas deamino-NADH was little oxidized. The respiratory chain-linked NADH oxidase system exhibited an apparent K_m value of about 65 μM for NADH. Interestingly, the activity of NADH:DCIP oxidoreductase on NADH oxidase system was decreased remarkably by Na^+ or K^+, and its optimal pH was 5.5. The activity of NADH:DCIP oxidoreductase was very resistant to the respiratory chain inhibitors such as rotenone, capsaicin, and $AgNO_3$, whereas it was inhibited by about 40% with 40 μM 2-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO). From the results, we suggest the possibility that the aerobic respiratory chain-linked NADH oxidase system of *B. cereus* KCTC 3674 may possess the HQNO-sensitive NADH:DCIP oxidoreductase lacking an energy coupling site.

Key words – *Bacillus cereus* KCTC 3674, aerobic respiratory chain, NADH:DCIP oxidoreductase

Introduction

Although the NADH dehydrogenases (NDH) in the bacterial respiratory chains are referred to as NADH:ubiquinone oxidoreductase, many bacteria have been known to possess quinones other than ubiquinone[2]. Thus, the name NADH:quinone oxidoreductase in bacteria seems more appropriate than NADH:ubiquinone oxidoreductase.

Three types of NADH:quinone oxidoreductases in the respiratory chain of bacteria have been reported[14]. They are the H^+-translocating NADH:quinone oxidoreductase (designated NDH-1), the Na^+-translocating NADH:quinone oxidoreductase (designated Na^+-NDH), and the NADH: quinone oxidoreductase lacking an energy coupling site (designated NDH-2). In general, NDH-1 or Na^+-NDH reacts with deamino-NADH as well as with NADH, shows high affinities for NADH, and possesses an energy coupling site[4,8]. In contrast, NDH-2 reacts very little with deamino-NADH, but with NADH without any energy coupling site, and shows low affinities for NADH[4,7,8]. *Escherichia coli*[8], *Vibrio alginolyticus*[9,10], and *Thermus thermophilus* HB-8[13] are known to possess two different types of NADH:quinone oxidoreductases. *Zymomonas mobilis* is known to possess only NADH:quinone oxidoreductase lacking the energy coupling site[7].

Respiratory chain inhibitors have proved to be a useful tool for probing the mechanism of electron transfer and proton or sodium translocation in the respiratory chain. Generally, NDH-1 is inhibited by the respiratory inhibitors rotenone and capsaicin, whereas NDH-2 is only slightly inhibited by these inhibitors[13,14]. Interestingly, Na^+-NDH is known to be very resistant to rotenone and capsaicin[14], but highly sensitive to 2-heptyl-4-hydroxyquinoline-*N*-oxide[10] and $AgNO_3$[1].

Takao Yagi showed that the respiratory inhibitor capsaicin inhibited H^+-translocating NADH:quinone oxidoreductases, and did not inhibit the NADH oxidase and NADH:quinone oxidoreductase of *Bacillus subtilis*[12]. To date, little detailed study has been done on the enzymatic and energetic properties of the aerobic respiratory chain-linked NADH oxidase system in the genus *Bacillus*. *B. cereus* produces several extracellular hemolysins, which are considered potential factors of virulence of the opportunistic pathogen[11]. In this paper, we report the results for enzymatic properties of the NADH:DCIP oxidoreductase on the aerobic respiratory chain-linked NADH oxidase system of *B. cereus* KCTC 3674.

Materials and Methods

Bacterial strain and conditions

The bacterial strain used in this study was *B. cereus* KCTC 3674[3,4,5]. The bacterium was grown aerobically at

*Corresponding author
Tel : +82-2-55-279-7464, Fax : +82-55-279-7460
E-mail : yjkim@changwon.ac.kr

37° C in liquid media containing 0.5% polypeptone and 0.5% yeast extract in 50 mM Tris-HCl buffer (pH 7.5). Preculture grown overnight was used to inoculate the main culture to give a turbidity of approximately 0.03.

Preparation of membranes from *B. cereus* KCTC 3674

For the preparation of membranes from *B. cereus* KCTC 3674, the protoplast formation was carried out at 37°C. Cells harvested in logarithmic growth phase were suspended in 50 mM potassium phosphate (pH 7.5) containing 5 mM EDTA at a concentration of 1 g (wet weight) per 80 ml. Lysozyme was added at a concentration of 300 μg/ml (freshly prepared). The mixture was incubated for 30 min, after which the protoplasts were harvested by centrifugation at 14,000×g for 30 min. Protoplasts were washed once in 50 mM potassium phosphate (pH 7.5) containing 5 mM EDTA, and centrifuged at 14,000×g for 30 min at 4°C. Washed protoplasts were resuspended in 50 mM potassium phosphate (pH 7.5) containing 5 mM EDTA to give a concentration of 20 ml/g, and protoplast suspensions were passed through a French pressure cell twice at 25,000 psi. Unbroken cells and cell debris were removed by centrifugation at 10,000×g for 10 min at 4°C, and the supernatant was centrifuged at 120,000×g for 2 h at 4°C to sediment the membrane fraction. A membrane pellet was washed in 50 mM potassium phosphate (pH 7.5) containing 5 mM EDTA. After sedimentation at 120,000×g for 2 h at 4°C, inverted membrane vesicles were rewashed in 50 mM potassium phosphate (pH 7.5) containing 10% glycerol. Membranes were resuspened in 50 mM potassium phosphate (pH 7.5) containing 10% glycerol at a protein concentration of about 25 mg/ml, and stored at -80°C.

Measurement of enzyme activities

The activity of the NADH oxidase was measured at 37°C from a decrease in A_{340} by using varian Cary 3E spectrophotometer. The assay mixture of NADH oxidase contained 125 μM NADH or deamino-NADH in 2 ml of 50 mM Tris-HCl (pH 8.5) containing 0.1 M KCl. The assay was started by addition of 200 μg of membrane protein. Activity was calculated by using a millimolar extinction coefficient of 6.22. The assay mixture for NADH:DCIP oxidoreductase contained 50 μg of membrane protein, 30 mM KCN, and 100 μM DCIP (2,6-dichlorophenol-indophenol) in 2 ml of 50 mM MES-KOH (pH 5.5). The reaction of the NADH:DCIP oxidoreductase was started by the addition of 125 μM NADH. The NADH:DCIP oxidoreductase activity was measured at 600 nm, and was calculated by using a millimolar extinction coefficient of 16.5.

Protein determination

Protein was measured by Bio-Rad protein assay, based on the method of Bradford. Bovine serum albumin was used as a standard.

Results

Enzymatic properties of the membrane-bound NADH oxidase

The effects of salts and pH on the NADH oxidase activity were examined with membranes prepared from *B. cereus* KCTC 3674. As shown in Fig. 1, the membranes oxidized NADH (●), but very little deamino-NADH (○) as a substrate. The rate of NADH oxidation was slightly activated by monovalent cations including Na^+ and K^+, but was little activated by Li^+ (data not shown). The maximum activity of NADH oxidase was obtained in the presence of 0.1 M NaCl or KCl. The optimal pH for NADH oxidation in the presence of 0.1 M NaCl was 8.5 (data not shown).

The respiratory chain-linked NADH oxidase system exhibited an apparent K_m value of about 65 μM for NADH (Fig. 2). In order to examine the enzymatic properties of NADH:quinone oxidoreductase of *B. cereus* KCTC 3674, an artificial electron acceptor 2,6-dichlorophenol-indophenol (DCIP) was introduced.

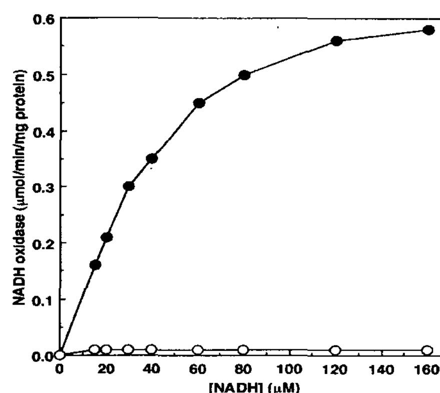
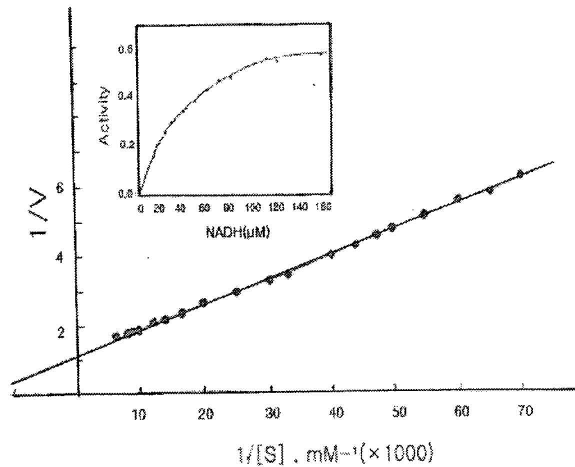


Fig. 1. Ability of NADH oxidase to oxidize NADH or deamino-NADH. The activities of NADH and deamino-NADH oxidases were measured in 2 ml of 50 mM Tris-HCl (pH 8.5) containing 0.1 M KCl and different concentrations of NADH (●) or deamino-NADH (○). The assay was started by addition of membrane suspensions containing about 200 μg of protein at 37°C.



Organisms	Km values (μM)
B. cereus KCTC 3674	65
E. coli	5.3~6.6, 48
V. alginolyticus	5~8, 25~30
Nap 1	27~35
Z. mobilis	66

Fig. 2. A Lineweaver-Berk plot of the NADH oxidase activity. The K_m values of *B. cereus* KCTC 3674, *V. alginolyticus*, and Nap 1 which is a Na^+ pump-defective mutant of *V. alginolyticus*[9] were measured in this study.

The effects of salts, pH, and respiratory inhibitors on the activity of the NADH:DCIP oxidoreductase

The activity of NADH:DCIP oxidoreductase was remarkably reduced by Na^+ and K^+ (Fig. 3A), and was, also, prominently reduced with an increase in the value of pH (Fig. 3B).

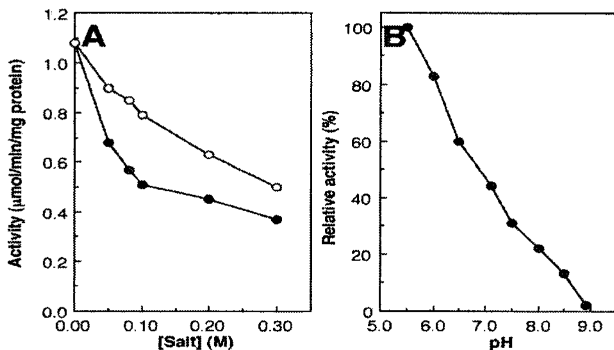


Fig. 3. Effects of salts and pH on the activity of NADH:DCIP oxidoreductase. (A) The assay mixture of NADH:DCIP oxidoreductase (2 ml) contained 50 mM MES-KOH (pH 5.5), 50 μg of membrane protein, 30 mM KCN, 100 μM DCIP, and various concentrations of NaCl (●), or KCl (○), (B) The activity of NADH:DCIP oxidoreductase was determined at various pHs. Buffers used at 50 mM was MES-KOH (pH 6 to 6.5) and Tris-HCl (pH 7.1 to 8.9). All assays were started by addition of 125 μM NADH at 37℃.

The respiratory inhibitor 2-heptyl-4-hydroxyquinoline-*N*-oxide(HQNO), which acts at the b cytochromes and also inhibits the Na^+-translocating NADH:quinone oxidoreductase, inhibited the activity of NADH:DCIP oxidoreductase by about 40% at a concentration of 100 μM (Fig. 4A). Rotenone and capsaicin, which inhibit the energy-transducing NADH: quinone oxidoreductase, inhibited the activity by about 10% at a concentration of 100 μM and 300 μM, respectively (Fig. 4B and 4C).

Effect of Ag^+ on the enzyme activities of NADH oxidase system

$AgNO_3$ is known to inhibit Na^+-translocating NADH: quinone oxidoreductase[1]. As shown in Fig. 5, the membrane-bound NADH oxidase activity was highly sensitive

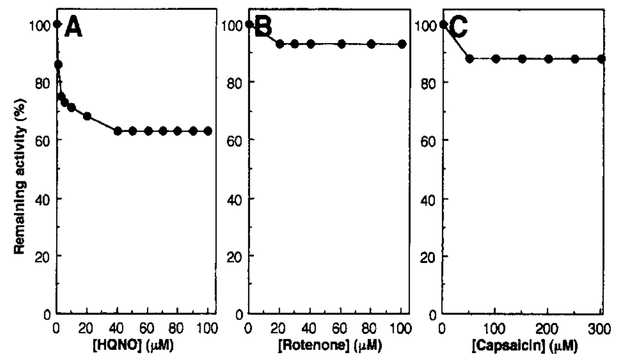


Fig. 4. Effects of respiratory chain inhibitors on the NADH: DCIP oxidoreductase. The activity of NADH:DCIP oxidoreductase was measured with various concentrations of HQNO (A), rotenone (B), and capsaicin (C). The assay mixture of NADH:DCIP oxidoreductase contained 50 μg of membrane protein, 30 mM KCN, 100 μM DCIP in 2 ml of 50 mM MES-KOH (pH 5.5). All assays were started by addition of 125 μM NADH.

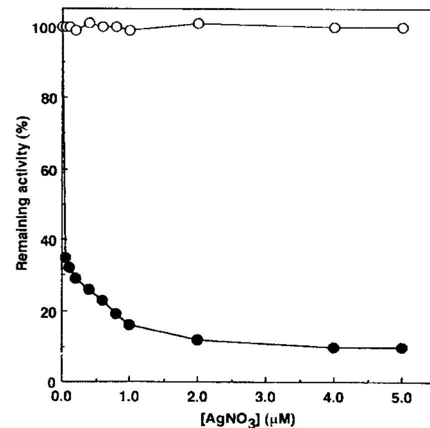


Fig. 5. Effect of $AgNO_3$ on the enzyme activities of the NADH oxidase and NADH:DCIP oxidoreductase. NADH oxidase (●) and NADH:DCIP oxidoreductase (○).

to Ag^+. In contrast, the activity of NADH:DCIP oxidoreductase was not affected by Ag^+ at all.

Discussion

The results of this study show the possibility that the aerobic respiratory chain-linked NADH oxidase system of *B. cereus* KCTC 3674 may possess the enzymatic system lacking an energy coupling site.

Membranes prepared from *B. cereus* KCTC 3674 oxidized NADH, but very little deamino-NADH as a substrate, and exhibited an apparent K_m value of approximately 65 μM for NADH. Generally, NADH:quinone oxidoreductase lacking an energy coupling site (NDH-2) oxidizes little deamino-NADH, and shows low affinities for NADH[7,8] (Fig. 2). The activities of NADH oxidase and NADH:ubiquinone-1 oxidoreductase of *B. subtilis* membranes are little affected by capsaicin[12]. In order to examine the enzymatic properties of NADH:quinone oxidoreductase of *B. cereus* KCTC 3674, an artificial electron acceptor 2,6-dichlorophenol-indophenol (DCIP) was introduced in this study. The activity of NADH:DCIP oxidoreductase of *B. cereus* KCTC 3674 was little affected by capsaicin and rotenone. $AgNO_3$ and HQNO are known to be the potent inhibitors of Na^+-translocating NADH:quinone oxidoreductase[1,10]. Interestingly, the NADH:DCIP oxidoreductase of *B. cereus* KCTC 3674 was sensitive to HQNO, whereas was not affected by Ag^+ at all. The membrane-bound NADH oxidase was highly sensitive to Ag^+. Thus, it is suggested that $AgNO_3$ does not inhibit the NADH:quinone oxidoreductase of *B. cereus* KCTC 3674, but inhibit the quinol oxidase.

B. cereus KCTC 3674 vigorously excretes proteases into the extracellular environment[3,6]. Thus, it is very difficult to prepare the functional inverted membrane vesicles for energy measurement experiment. Due to the lack of a functional *in vitro* system, little detailed study has been done on the energetic experiments in the genus *Bacillus*. To further examine whether the aerobic respiratory chain of *B. cereus* KCTC 3674 generates energy or not, we will try the preparation of inverted membrane vesicles for a functional *in vitro* energy measurement system.

Acknowledgments

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초록 : Beta hemolysis 유발 병원균 *Bacillus cereus*의 HQNO-sensitive NADH:DCIP oxidoreductase

김영재* · 박기태[1]
(창원대학교 자연과학대학 미생물학과, [1]박기태 한의원)

호기적으로 자란 *Bacillus cereus* KCTC 3674로 부터 조제된 막은 NADH만을 산화하고, deamino-NADH는 거의 산화하지 않았다. 호흡쇄와 연계된 NADH oxidase계는 K_m 값이 약 65 μM 이였다. NADH:DCIP oxidoreductase의 활성은 Na^+ 또는 K^+에 의해 감소되었다. 그 최적 pH는 5.5 였다. NADH:DCIP oxidoreductase의 활성은 rotenone, capsaicin, $AgNO_3$와 같은 호흡저해제에는 매우 저항적이였지만, 40 μM HQNO (2-heptyl-4-hydroxyquinoline-*N*-oxide) 존재하에서는 약 40% 저해되었다. 이들 결과로 부터, *Bacillus cereus* KCTC 3674의 호기적 호흡쇄와 연계된 NADH oxidase계는 energy coupling site가 결여된 HQNO-sensitive NADH:DCIP oxidoreductase를 소유하고 있는 것으로 추정된다.