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Partial Purification of the Outer Membrane-Associated 2-Furaldehyde Dehydrogenase from *Klebsiella pneumoniae*

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Klebsiella pneumoniae 균주의 세포외막으로부터 2-Furaldehyde Dehydrogenase의 부분정제에 관하여

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Abstract: From the outer membrane portion of Gram-negative *Klebsiella pneumoniae*, the activity of 2-furaldehyde dehydrogenase depending upon beta-nicotinamide adenine dinucleotide was detected. Cytoplasmic membrane was preferentially extracted from crude membrane with Mg^{2+} and Triton X-100, and then outer membrane was collected by ultracentrifugation. The crude enzyme was obtained by solubilization of outer membrane with lysozyme, ethylene diamine tetraacetate and Triton X-100. Thereafter 2-furaldehyde dehydrogenase was partially purified through column chromatography on QAE-Sephadex Q-50 and Sephadex G-150 and the enzyme activity was analyzed by means of high performance liquid chromatography. The optimal pH for the activity of the enzyme was about 9.5 and the optimal temperature was about 85℃. The partially purified enzyme retained its activity at 85℃ for 5 hours. The optimal concentration of Triton X-100 for the activity of the enzyme was about 1.5% in the reaction mixture.

Key words: *Klebsiella pneumoniae*, 2-furaldehyde dehydrogenase, beta-nicotinamide adenine dinucleotide, high performance liquid chromatography

It was reported that *Saccharomyces cerevisiae* could convert 2-furaldehyde mainly to 2-furfuryl alcohol and a little bit to 2-furoic acid (Morimoto *et al.*, 1986). And the metabolic pathway of 2-furoic acid to glutamic acid was proposed in *Pseudomonas* F2 which can not utilize 2-furaldehyde as a carbon source (Trudgill, 1969). It was reported recently that 2-furaldehyde is degraded by protocooperation of Gram-negative bacteria, *Pseudomonas testosteroni, Pseudomonas maltophilia, Klebsiella pneumoniae,* and *Pseudomonas fluoresens* (Han *et al.*, 1979) : the oxidation of 2-furaldehyde upto 2-furoic acid was detected in case of the first three strains of them.

Attempts were made to elucidate the mechanism of microbial conversion of 2-furalde-

hyde to 2-furoic acid in case of *Klebsiella pneumoniae*. At first we tried to detect and to characterize the activity of 2-furaldehyde dehydrogenase associated with outer membrane.

MATERIALS AND METHODS

Strain and Growth Condition

Klebsiella pneumoniae isolated by Han *et al.* (1979) was used. Cells were grown in minimal salts medium proposed by Hong *et al.* (1983) with mild aeration at 30℃, to which 0.1% of NH_4Cl, glucose and redistilled 2-furfuraldehyde were added, respectively.

Chemicals

2-Furaldehyde purchased from Fisher Chem. Co., U.S.A. was redistilled and stored at −20℃. Flavin adenine dinucleotide (FAD), beta-nicotinamide adenine dinucleotide phosphate (NADP), oxidized form and reduced form of nicotinamide adenine dinucleotide (NAD and NADH), ethylene diamine tetraacetate (EDTA), oxidized glutathione, 5, 5′-dithiobis-(2-benzoic acid)(DTNB), egg white lysozyme (EC 3.2.1.17) and bovine serum albumin were purchased from Sigma Chem. Co., U.S.A., Triton X-100 from BDH Chem., England and ercoll from Pharmacia Fine Chem., Sweden, respectively.

Fractionation of Subcellular Components from Crude Cell Extract

Cells grown for 12 hours were harvested at 4℃ with Pellicon Cassette System (Millipore, U.S.A.), and washed three times with 0.05 M Tris-maleate buffer (pH 7.0) containing 50 mM $MgSO_4 \cdot 7H_2O$ and 0.4 M NaCl, then kept in the same buffer overnight. The suspended cells were ruptured twice with French press (Aminco, U.S.A.) at 16,000 psi and the ruptured cell suspension was centrifuged at 2,300g, 4℃ to remove unbroken intact cells. Membrane portion was obtained by ultracentrifugation (Europa 65 Ultracentrifuge, MSE Scientific Instruments, England) at 100,000g for 60 minutes under 4℃.

Detection of the Activity of 2-Furaldehyde Dehydrogenase

Various electron acceptors were employed for the detection of activity: For the oxidase activity, ferricyanide assay was taken according to the method proposed by Rajagopalan *et al.* (1964 & 1970) and Wood *et al.* (1970) and for the glutathione-linked activity, reduced glutathione is reversely reacted with DTNB resulting in the increase of absorbance at 412 nm according to the method proposed by Owens *et al.* (1965). The beta-NAD and beta-NADP-linked activities were assayed by the increase in absorbance at 340 nm after the addition of beta-NAD and beta-NADP. And flavin-linked activity was assayed by the decrease in absorbance at 450 nm after the addition of FAD.

For the application to high performance liquid chromatographic analysis at 340 nm, reaction mixture was composed of 0.30 m*l* of 0.25 M buffer with 0.03 M 2-mercaptoethanol, 0.25 m*l* of crude extract, 0.05 m*l* 10 mM beta-NAD, 0.05 m*l* of 20 mM 2-furaldehyde and 0.1 m*l* of 10% Triton X-100. All reaction mixtures were preincubated at 35℃ for 90 min.

Separation of Outer and Cytoplasmic Membranes

Cytoplasmic membrane was obtained from crude membrane pellet employing Mg^{2+} and Triton X-100 according to the method proposed by Schnaitman (1971). The separation procedure of outer and cytoplasmic membranes was carried out by the method of Diedrich *et al.* (1977) with some modification: 0.1 M citrate-phosphate buffer (pH 7.0) containing 0.03 M 2-mercaptoethanol and 2% Triton X-100. After incubation for 30 minutes at 30℃, this suspension was stirred overnight magnetically, and then the outer membrane-peptidoglycan fraction was obtained as pellet by ultracentrifucation at 100,000g for 60 minutes under 4℃. This fraction was washed twice with the same buffer without Triton X-100. For the purifacation

of outer membrane, Percoll density gradient ultracentrification was employed: The membrane was suspended in small volume of the above buffer and 0.5 m*l* of the suspension was layered onto top of the gradient matrix and ultracentrifuged at 70,000g for 45 minutes under 4℃ using Europa 65 rotor, TFT 75.13.

Preparation of Crude Outer Membrane Protein

The washed outer membrane-peptidoglycan fraction was suspended in 0.1 M citrate-phosphate buffer (pH 7.0) containing 0.03 M 2-mercaptoethanol, 10 mM EDTA, 0.5% lysozyme and 0.5% Triton X-100 and crude outer membrane protein was obtained by the modified method of Mizuno and Kageyama (1978): The mixture warmed upto 30℃ within a few minutes and kept at room temperature for 60 minutes, and then incubated at 37℃ for 2 hours. Crude outer membrane protein was recovered from the supernatant, after the suspension was centrifuged at 8,000g for 60 minutes under 4℃.

Detection of 2-Furaldehyde Dehydrogenase Activity using High Performance Liquid Chromatography

5% (w/v) $ZnSO_4 \cdot 7H_2O$ and 0.15 M $Ba(OH)_2 \cdot 8H_2O$ were added to the reaction mixture, and then precipitate was removed by centrifugation at 8,000g, for 20 minutes under 4℃. The supernatant was analyzed by HPLC with flow rate of 1.0 m*l*/min using Li-Chrosorb RP-18 (Merck, W. Germany) column and 70% (v/v) methanol (MeOH:H_2O=70 : 30) and detected at 340 nm for NADH by Waters Model 440 Absorbance Detector.

Determination of Protein Concentration

The method of Lowry *et al.* (1951) was used with bovine serum albumin as a standard, and the method of Wang and Smith (1975) was also used for samples containing Triton X-100.

Table 1. *Effect of various electron acceptors on the oxidation of 2-furaldehyde by fractonated subcellular components.*

Added electron acceptors	Activity
Ferricyanide	−
Flavin adenine dinucleotide	−
beta-Nicotinamide adenine dinucleotide	+
beta-Nicotinamide adenine dinucleotide phosphate	−
Glutathione, oxidized form	−

RESULTS AND DISCUSSION

Detection of Enzyme Activity

Various assay methods were applied in various pH and temperature with crude cell extract, and then gradually checked out the obtained positive assay method with fractionated subcellular components. As shown in Table 1, the enzymatic oxidation mechanism is dependent specifically on beta-NAD: UV scanning spectrum (LKB UV-Visible Scanning Spectrophotometer, Sweden) of the filtrate of enzymatic reaction mixture shows broad band with a peak at 340 nm (Fig.1), due to NADH production and HPLC chromatogram detected at 340 nm shows gradual increase according to the reaction time (Fig.2).

Separation and Characterization of Outer and Cytoplasmic Membranes

Sucrose density gradient ultracentrifugation has been commonly used for the separation of

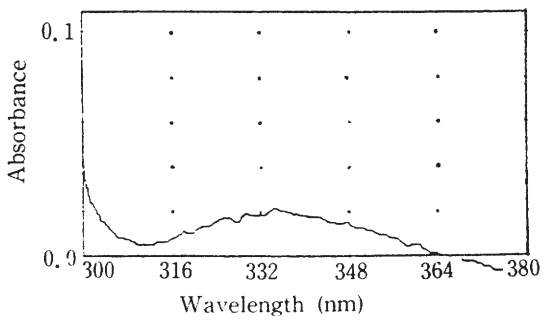

Fig.1. *UV scanning spectrum of the filtrate of enzymatic reaction mixture, incubated at 35℃ for 30 minutes in 0.25 M glycine-NaOH buffer (pH 9.0) containing 10 mM beta-NAD and 10 mM 2-furaldehyde.*

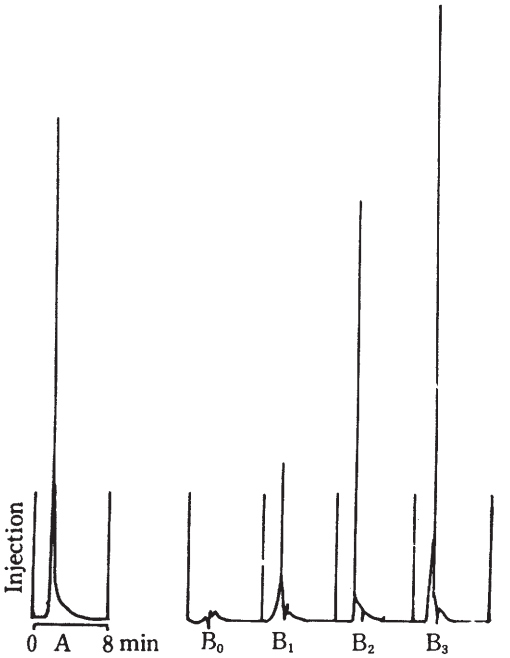

Fig.2. *Time-dependent changes of HPLC chromatogram of the filtrates of enzymatic reaction mixture, incubated in 0.25 M glycine-NaOH buffer (pH 9.5) containing 10 mM beta-NAD and 20 mM 2-furaldehyde; A, beta-NADH as a reference, B_0, B_1, B_2, and B_3, filtrates reacted at interval of 30 minutes, that is, 0, 30, 60, and 90 minutes, respectively.*

outer and cytoplasmic membranes. However, simple and clear methods were taken here, that is, differential extraction of membranes with Mg^{2+} and Trition X-100 and Percoll density gradient ultracentrifugation were beneficial in this case. Schnaitman (1971) proposed that direct addition of Mg^{2+} to the preferential solubilization process had no effect on the solubility of the cytoplasmic membrane proteins in Triton X-100. Therefore, the harvested cells were washed with buffer containing 50 mM Mg^{2+} as mentioned previously and the washed cells were kept in the same buffer overnight. As a result of Percoll density gradient ultracentrifugation, it was confirmed that the density of cytoplasmic membrane is about 1.06 g/m*l* and outer membrane about 1.10 g/m*l* in case of *Klebsiella pneumoniae*.

Partial Purification of 2-Furaldehyde Dehydrogenase

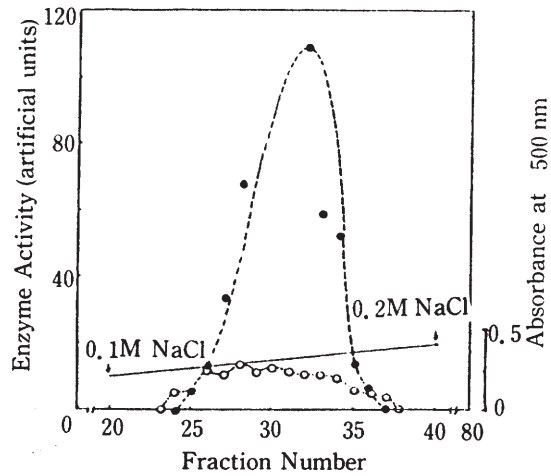

Fig.3. *Ion exchange chromatography of crude outer membrane protein on QAE-Sephadex Q-50; Column dimensions, 3.0×40 cm, Elution buffer, 0.1 M citrate-phosphate buffer (pH 7.0) containing 0.5% (v/v) Triton X-100, Fraction volume, 5 ml; ●, 2-furaldehyde dehydrogenase activity, ○, protein.*

From the crude outer membrane protein, 2-furaldehyde dehydrogenase was partially purified through QAE-Sephadex anion exchange column chromatography. As shown in Fig 3, the enzyme fraction was obtained at

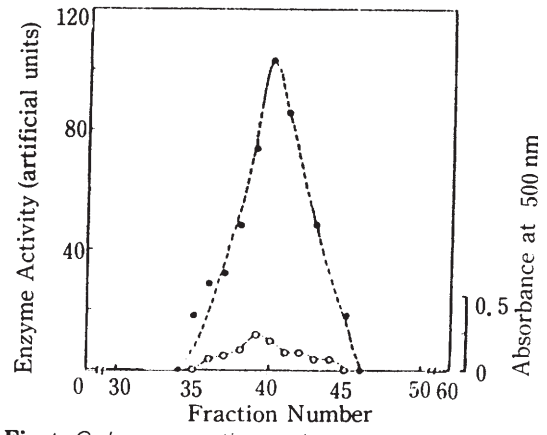

Fig.4. *Gel permeation chromatography of eluates from QAE-Sephadex on Sephadex G-150; Column dimensions, 2.0×80 cm, Elution buffer, 0.1 M citrate-phosphate buffer (pH 7.0) containing 0.5% (v/v) Triton X-100, Fraction volume, 5 ml; ●, 2-furaldehyde dehydrogenase activity, ○, protein.*

about 0.15 M NaCl concentration. Further purification was achieved through Sephadex G-150 gel permeation chromatography. The result was shown in Fig 4. A quantitative evaluation of the results obtained from the consecutive purification steps is given in Table 2. The specific activity of partially purified enzyme was estimated to be 16.98 units/mg protein, that is about five folds higher than that of the disintegrated cell extract. The final yield of enzyme activity was approximately 22% of the cell extract.

Effect of pH the Enzyme Activity

The enzyme acitivity was analyzed at various pH values to find out the effect of pH on the activity of the partially purified enzyme (Fig. 5). The enzyme showed its maximal activity at pH 9.5.

Effect of Temperature and Thermostability

In order to study the effect of temperature on the enzyme, the activity was assayed at various temperature between 25℃ and 100℃. As shown in Fig 6, the optimal temperature was about 85℃. The thermal stability of the partially purified enzyme was inspected by incubating the enzyme for 5 hours at 85℃ in 0.25 M glycine-NaOH buffer (pH 9.5). The enzyme was extraordinarily thermostable, and it had equal or higher activity in comparison with the non-incubated enzyme as shown in Fig

Table 2. *Partial purification of 2-furaldehyde dehydrogenase from Klebsiella pneumoniae.*

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Recovery (%)
Disintegrated cell extract	276	957	3.46	100
Membrane fraction	99.2	582	5.87	61
QAE-Sephadex Q-50 eluate	23.05	221	9.59	23
Sephadex G-150 eluate	12.85	217	16.89	22

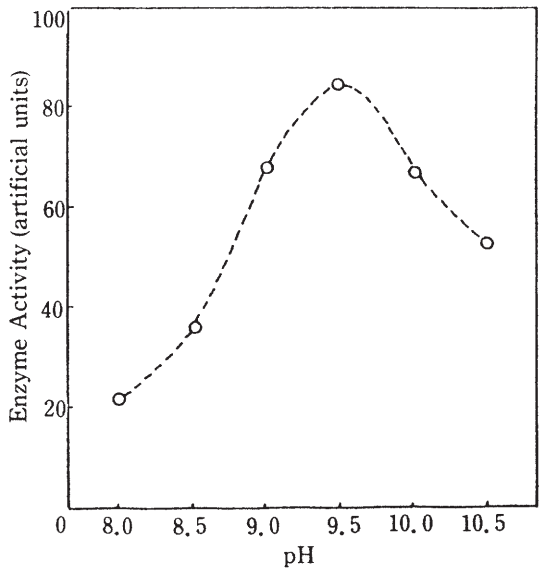

Fig.5. *Effect of pH on the activity of partially purified 2-furaldehyde dehydrogenase. 0.25 M citrate-phosphate buffer was used between pH 6.0 and pH 7.0, between pH 7.5 and pH 8.5, 0.25 M Tris buffer was used and 0.25 M glycine-NaOH buffer was also used between pH 9.0 and pH 10.5. All buffers contained 0.03 M 2-mercaptoethanol.*

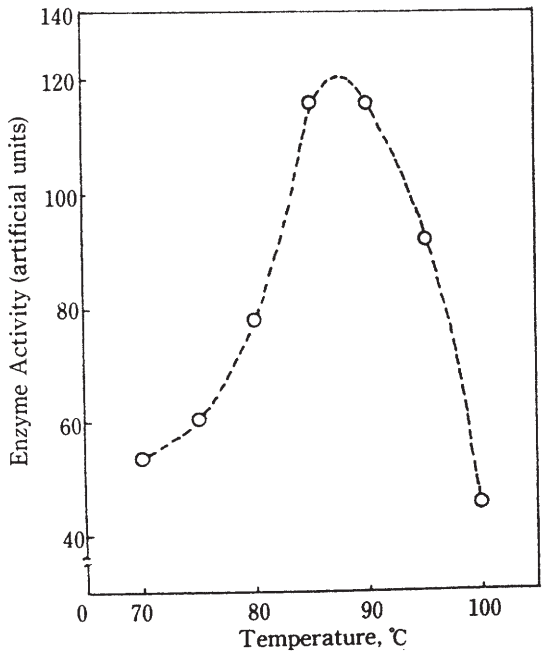

Fig.6. *Effect of temperature on the activity of partially purified 2-furaldehyde dehydrogenase.*

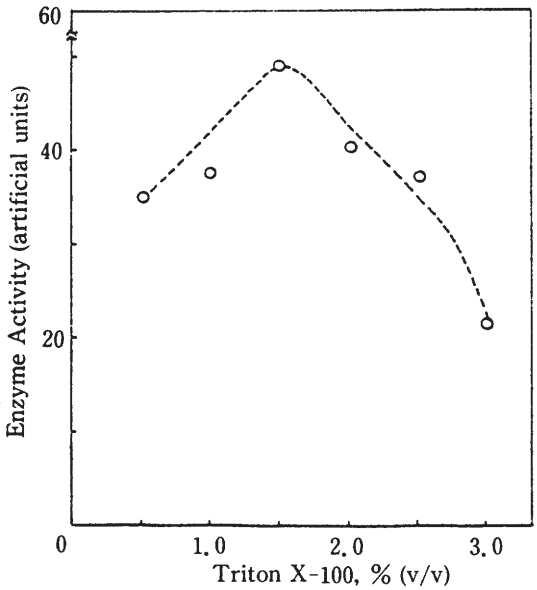

Fig.7. *Effect of Triton X-100 concentration on the activity of partially purified 2-furaldehyde dehydrogenase.*

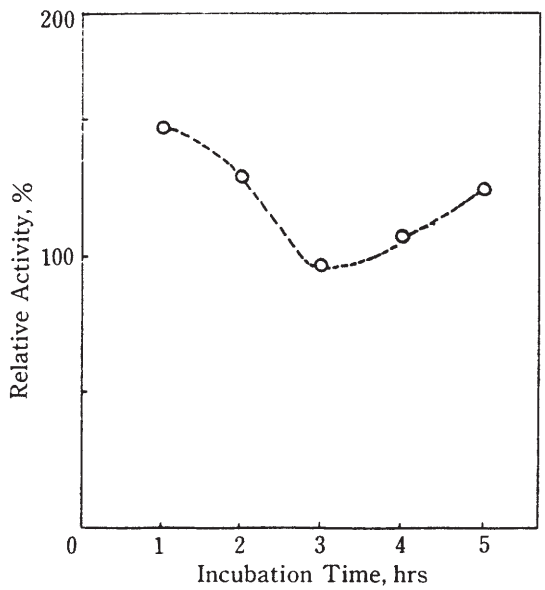

Fig.8. *Themal stability of partially purified 2-furaldehyde dehydrogenase. After enzyme solutions were preincubated under 85℃ at interval of one hour in 0.25 M glycine-NaOH buffer (pH 9.5), they were reacted with 10 mM beta-NAD and 20 mM 2-furaldehyde at 85℃ for 90 minutes in the same buffer.*

8. The high stability of enzyme is assumed to be due to the interactions among protein, lipid and detergent.

Effect of Nonionic Detergent Triton X-100

Integral membrane proteins are solvated by the lipids of the membrane, interact with the boundary lipids, and the conformational flexibility of many integral membrane proteins is usually constrained by lipid annulas. Thus many integral enzymes are in fact activated upon detergent solubilization, which immediately releases them from physical constraints imposed by the bilayer. Therefore, the effect of nonionic detergent Trition X-100 on the enzyme activity was investigated: The maximum activity was revealed 1.5% (v/v) Triton X-100, as shown in Fig.7.

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적 요

그람陰性인 *Klebsiella pneumoniae* 菌株의 細胞外膜으로부터 beta-nicotinamide adenine dinucleotide를 prosthetic 基로 하는 2-furaldehyde dehydrogenase의 活性이 檢出되었으며 이를 high performance liquid chromatography로 分析하였다.

Mg^{2+}와 Triton X-100을 利用하여 粗細胞膜分劃物로부터 細胞質膜을 選擇的으로 溶解시키고 난 後, 細胞外膜을 超遠心分離로써 얻었으며, 이 細胞外膜에 lysozyme, EDTA, Triton X-100을 使用하여 細胞外膜蛋白質을 얻었다. 그로부터 이 酵素

를 QAE-Sephadex Q-50 陰이온交換樹脂圓柱 크로마토그라피 및 Sephadex G-150 젤濾過圓柱 크로마토그라피를 通해 部分精製하였다.

部分精製된 酵素는 水素이온指數 9.5, 温度 85℃ 附近에서 最適活性을 보여주었으며 相當한 熱安定性을 가져 85℃에서 5 時間 동안 熱處理하였을 때에도 活性을 보여 주었다. 또한 反應混合物에 1.5% (v/v) 濃度의 Triton X-100이 存在할 때에 역시 最適活性을 보여 주었다.

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