KOR. JOUR. MICROBIOL, September, 1988 p. 197-206 Vol. 26, No. 3

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

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Klebsiella pneumoniae 균주의 세포외막에서 분리한 2-Furaldehyde Dehydrogenase의 특성에 관한 연구

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ABSTRACT: An outer membrane-associated 2-furaldehyde dehydrogenase, catalyzing the oxidation of 2-furaldehyde to 2-furoic acid from *Klebsiella pneumoniae* was purified to homogeneity and characterized. The enzyme showed its highly specific dependency on β-NAD$^+$. Enzyme activity was monitored during purification by using substrate 2-furaldehyde and coenzyme β-NAD$^+$ by means of high performance liquid chromatography. The outer membrane was successfully collected by the methods of Percoll density gradient ultracentrifugation and ultracentrifugation after preferential solubilization of the membrane with Mg^{2+} and Triton X-100. The enzyme was purified by the series of procedures including extraction of outer membrane protein with EDTA and lysozyme, and fractionation by column chromatography on QAE-Sephadex Q-50, and subsequently Sephadex G-100. The enzyme showed its optimal activity at 85°C, pH 9.5, and in the presence of 1.5% (vol/vol) Triton X-100. The enzyme exhibited a native molecular size of 88,000 by nondenaturing polyacrylamide gel electrophoresis and had an apparent Km of 4.72 mM for 2-furaldehyde.
KEY WORDS □ *Klebsiella pneumoniae*, 2-furaldehyde dehydrogenase, β-NAD$^+$, outer membrane

2-Furaldehyde is one of the abundantly disseminated recalcitrants in our environment and its toxicity has been well known. Recently, microorganisms capable of growing in the presence of this aldehyde, that is, four Gram-negative bacteria, *Pseudomonas testosteroni*, *P. maltophilia*, *P. fluorescens*, and *Klebsiella pneumoniae*, were isolated from soil by Han *et al.* (1979). Although many physiological characteristics were brought to light (Byun *et al.*, 1979; Han, 1982; Chae, 1982; Kim *et al.*, 1983, and Lee *et al.*, 1985), the enzymatic degradation mechanism of 2-furaldehyde was not understood yet even in the case of the four strains. They reported that 2-furaldehyde is degraded by protocooperation of the above-mentioned microorganisms and 2-furoic acid is an important intermediate during degradation process of 2-furaldehyde in the above strains (Han *et al.*, 1979).

On the other hand, Trudgill (1969) had proposed the metabolic pathway of 2-furoic acid to L-glutamic acid in *Pseudomonas* F2 which can not utilize 2-furaldehyde as a carbon source. Therefore, it could be deduced that the metabolic pathway of 2-furaldehyde by microorganisms might be generally similar to that proposed by Trudgill, after the oxidation to 2-furoic acid. Thus, first of all, the enzymatic conversion mechanism of

2-furaldehyde to 2-furoic acid, which is the first step in this process, must be elucidated to understand the whole degradative pathway of 2-furaldehyde. We have reported the existence of a 2-furaldehyde-oxidizing enzyme from one of the four isolated strains, *K. pneumoniae* (Lee *et al.*, 1986).

This paper describes the detection, purification, and characterization of β-NAD^+-dependent 2-furaldehyde dehydrogenase from *Klebsiella pneumoniae*.

MATERIALS AND METHODS

Organism and Culture Condition

Klebsiella pneumoniae, isolated from soil 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 °C for 12 hours, in which 0.1% (wt/vol) of KH_2PO_4, NH_4Cl, glucose and redistilled 2-furaldehyde (Fisher Chemical, USA), 0.2% (wt/vol) of KH_2PO_4, and 0.02% (wt/vol) of NaCl, $MgSO_4 \cdot 7H_2O$, and $CaCl_2 \cdot 2H_2O$ were contained.

Chemicals

2-Furaldehyde purchased from Fisher Chemical, USA was redistilled and stored at −20 °C. FAD, β-$NADP^+$, β-NADPH, β-NAD^+, β-NADH, EDTA, oxidized glutathione, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), egg white lysozyme (EC 3.2.1.17) and bovine serum albumin were purchased from Sigma Chemical, USA, Triton X-100 from BDH Chemical, England and Percoll from Pharmacia Fine Chemical, Sweden, respectively.

Crude Membrane Preparation and Measurement of Enzyme Activity

Crude membrane was prepared according to the method proposed by Lee *et al.* (1986). β-NAD-dependent enzyme activity was assayed by the increase in absorbance at 340 nm after the addition of β-NAD^+, according to the method proposed by Lee *et al.* (1986).

Separation of Cytoplasmic and Outer Membrane

According to the proposal of Schnaitman (1971), crude membrane was washed twice with 0.1 M citrate-phosphate buffer (*p*H 7.0). And then 10% (vol/vol) Triton X-100 (BDH Chemical, England) was treated to crude membrane with 2% (vol/vol) as the final concentration by the method of Diedrich *et al.* (1977) with some modification: The crude membrane was suspended in 0.1 M citrate-phosphate buffer (*p*H 7.0) containing 30 mM 2-mercaptoethanol and after incubation at 30 °C for 30 minutes, the suspension was stirred magnetically at 4 °C overnight. And then cytoplasmic membrane was obtained as supernatant and outer membrane-peptidoglycan complex as pellet by ultracentrifugation at 100,000 × g under 4 °C for 60 minutes. This fraction was washed twice with the same buffer without Triton X-100. For the purification of outer membrane, Percoll density gradient ultracentrifugation was employed: The membrane fraction was suspended in small volume of the above buffer and 0.5 m*l* of the suspension was layered on the top of the gradient matrix with initial density of 1.10 g/m*l*, that is, Percoll solution containing 2.5 M sucrose. After centrifugation at 70,000 × g for 45 minutes under 4 °C using fixed angle rotor (Europa 65 rotor, TFT 75.13, MSE Scientific Instruments, England), each membrane fraction was collected by puncturing the bottom of tubes with needle. Density marker beads (Pharmacia Fine Chemical, Sweden) was used in a counter tube to monitor the buoyant density.

Identification of Membrane Fractions

As a marker protein in the cytoplasmic membrane, NADH oxidase (EC 1.6.99.22) was assayed according to the method proposed by Booth and Curtis (1977), and as a marker compound in the outer membrane, 2-keto-3-deoxyoctonate (KDO) was estimated by the method proposed by Osborn (1963) and Osborn *et al.* (1972) using N-acetylmuramic acid as a reference substance (Matsushita *et al.*, 1978). For the cytochrome difference, each membrane fraction was scanned according to the method of Collins and Niederman (1976).

Enzyme Purification

Crude outer membrane protein was prepared according to the method proposed by Lee *et al.* (1986). The outer membrane protein was resus-

pended with the above-mentioned buffer and adjusted to the final volume of 20 m*l*, and this suspension was applied onto QAE-Sephadex Q-50 (Sigma Chemical, USA) column (3 × 40 cm) equilibrated with 0.1 M citrate-phosphate buffer (*p*H 7.0) containing 30 mM 2-mercaptoethanol and 0.1% (vol/vol) Triton X-100. And then, the column was washed out with one bed volume of the same buffer, followed by elution with a linear NaCl concentration gradient (0-0.3 M) under the flow rate of 20 m$l \cdot hr^{-1}$. 40 m*l* of the QAE-Sephadex Q-50 eluates, representing enzyme activity was concentrated ten-fold by ultrafiltration through a Diaflo membrane, type PM 10 (Amicon Co., USA). The concentrated enzyme solution was applied again onto Sephadex G-100 (Sigma Chemical, USA) column (2 × 80 cm) equilibrated with the same buffer used in anion exchange chromatography, and eluted with that buffer.

Determination of Molecular Weight

The molecular weight of the purified enzyme was determined by nondenaturing polyacrylamide gel electrophoresis (PAGE) on a set of 10%, 9%, and 8% polyacrylamide resolving gels, according to the procedure proposed by Hedrick and Smith (1968) and Chrambach and Rodbard (1971). Bovine milk α-lactalbumin (molecular weight, 14,200), bovine erythrocyte carbonic anhydrase (molecular weight, 29,000), chicken egg albumin (molecular weight, 45,000), and bovine serum albumin (molecular weight of monomer, 66,000; molecular weight of dimer, 132,000) were used as standard marker proteins.

Electrophoresis

The purification process, the purity of the enzyme was checked by PAGE. Nondenaturing gels were run as described by Davis (1964) and Bryan (1977) with a 10% polyacrylamide resolving gel. SDS-PAGE was performed as described by Laemmli (1970) with a 10% polyacrylamide resolving gel. Proteins were visualized by staining with Coomassie Blue R-250 and/or by silver staining (Merril *et al.*, 1984).

Enzyme Assays

During the purification, enzyme assay method by high performance liquid chromatography (HPLC) was employed. Reaction mixture, containing 0.30 m*l* of 0.25 M Tris buffer with 0.03 M 2-mercaptoethanol, 0.25 m*l* of enzyme solution, 0.05 m*l* of β-NAD$^+$, 0.05 m*l* of 20 mM 2-furaldehyde and 0.1 m*l* of 10% Triton X-100 was incubated at 35 °C for 90 minutes. After the addition of 5% (wt/vol) $ZnSO_4 \cdot 7H_2O$ and 0.15 M $Ba(OH)_2 \cdot 8H_2O$ to the reaction mixture, the precipitate was removed by centrifugation at 8,000 × g for 20 minutes. The supernatant was analyzed by HPLC with flow rate of 1.0 m$l \cdot min^{-1}$ using LiChrosorb RP-18 (Merck, W. Germany) column and 70% (vol/vol) aqueous methanol as a solvent and detected at 340 nm for β-NADH by Waters Model 440 Absorbance Detector.

Assays for the characterization of 2-furaldehyde dehydrogenase with various aldehyde substrates contained 20 mM substrate. Assays for effects of various *p*H, temperature, and Triton X-100 concentration were carried out with the above-mentioned assay method.

Protein Determination

Concentrations of protein were estimated by the method of Lowry *et al.* (1951), and for samples containing Triton X-100 and 2-mercaptoethanol the method proposed by Bradford (1976) was taken. In the case of Percoll density gradient ultracentrifugation, protein concentration in each effluent was estimated from the absorbance at 280 nm.

RESULTS AND DISCUSSION

Localization

Two layers were obtained from crude membrane preparation by Percoll density gradient ultracentrifugation, as shown in Fig. 1. Because of the polysaccharide moiety in lipopolysaccharide molecules, the density of outer membrane is greater than that of cytoplasmic membrane, which allows their separation from each other by various density gradient ultracentrifugation (Miura and Mizushima, 1969, Osborn *et al.*, 1972, Obrink *et al.*, 1977). The buoyant density at the upper layer was about 1.070-1.083 g/m*l*, and it was found to be enriched for NADH oxidase activity and cyto-

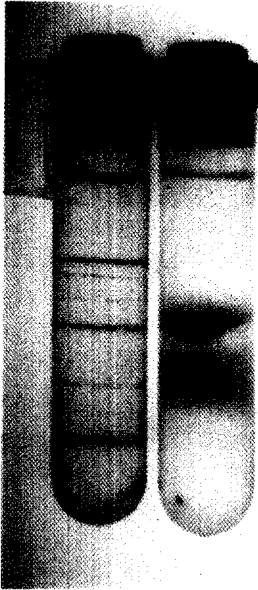

Fig. 1. *Separation of cytoplasmic and outer membranes by Percoll density gradient ultracentrifugation; left, density marker beads (buoyant density, g/ml, from the bottom of tube; violet, 1.133, green, 1.112, orange, 1.098, blue, 1.083, red, 1.070, yellow, 1.050, blue, 1.035); right, cytoplasmic at the upper layer and outer membrane at the lower layer*

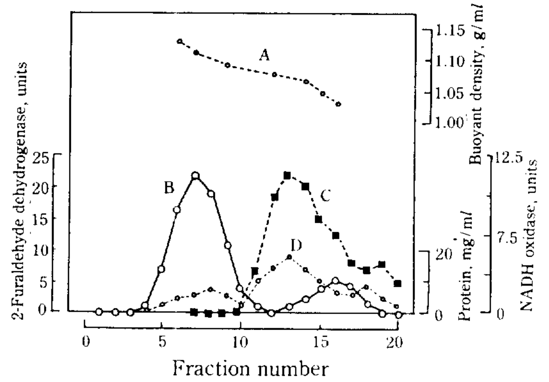

Fig. 2. *Fractionation of cytoplasmic and outer membranes by Percoll density gradient ultracentrifugation: Each fraction was prepared by collecting 0.45 ml of eluates by puncturing the bottom of tubes; A, buoyant density of marker beads (g/ml), B, 2-furaldehyde dehydrogenase activity (units), C, NADH oxidase activity (units), D, protein concentration (mg/ml) from* A_{280}

Table 1. *Comparison between cytoplasmic and outer membrane fraction of K. pneumoniae*

	Cytoplasmic membrane fraction	Outer membrane fraction
Buoyant density (g/ml)	1.070-1.083	1.090-1.105
Protein (mg/ml)	18.20	4.70
2-Keto-3-deoxyoctonate (μg/ml)	44.90	242.20
2-Furaldehyde dehydrogenase (units)	—	22.25
NADH oxidase (units)	9.60	—

Note: Each membrane fraction was prepared as described in Fig. 2. Cytoplasmic membrane was obtained from the fraction number 13, and outer membrane from 7 of Fig. 2.

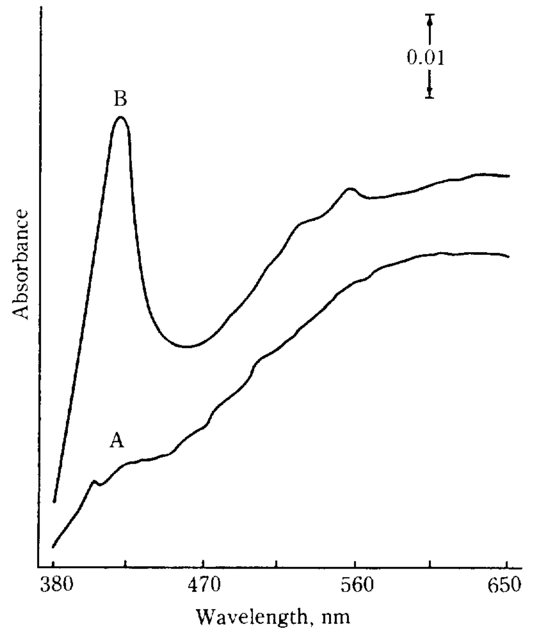

Fig. 3. *The reduced-minus-oxidized difference spectra of cytoplasmic and outer membranes detected by spectrophotometer; A, the outer membrane, B, the cytoplasmic membrane*

chrome content, and the buoyant density of lower layer was about 1.090-1.105 g/ml, and it was found to be enriched 2-keto-3-deoxyoctonate content and 2-furaldehyde dehydrogenase activity (Table 1, and Fig. 2 and 3). Therefore, the upper layer refers to cytoplasmic membrane and the lower layer to the outer membrane. And it was suggested that 2-furaldehyde dehydrogenase is as-

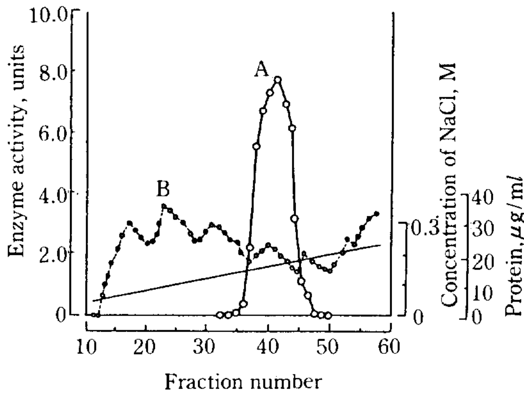

Fig. 4. *Ion exchange chromatography on QAE-Sephadex Q-50 of crude outer membrane protein; column dimension, 3.0 × 40 cm; elution buffer, 0.1 M citrate-phosphate buffer (pH 7.0) containing 0.1% (v/v) Triton X-100; each fraction volume, 4 ml; A, 2-furaldehyde dehydrogenase activity, B, protein (μg/ml)*

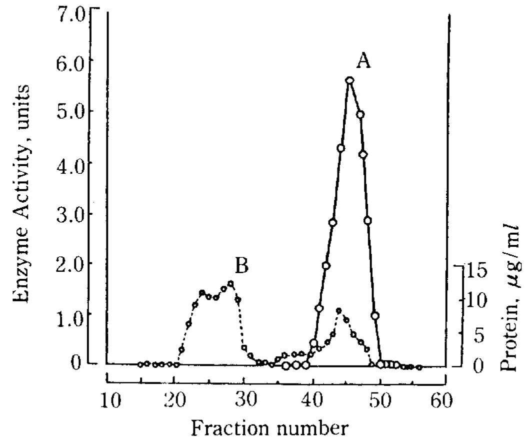

Fig. 5. *Gel permeation chromatography on Sephadex G-100 of the eluates from QAE-Sephadex anion exchange chromatography (fraction number 37-46); column dimension, 2.0 × 80 cm; elution buffer, 0.1 M citrate-phosphate buffer (pH 7.0) containing 0.1% (v/v) Triton X-100; each fraction volume, 5 ml, A 2-furaldehyde dehydrogenase activity, B, Protein (μg/ml)*

sociated with outer membrane in case of *K. pneumoniae*.

Purification

From the crude outer membrane protein, the enzyme was purified through QAE-Sephadex Q-50 anion exchange column chromatography. As shown in Fig. 4, the enzyme fraction was obtained at around 0.15 M NaCl concentration. Then, further purification was achieved through Sephadex G-100 gel permeation column chromatography (Fig. 5). The specific activity of the purified 2-furaldehyde dehydrogenase was estimated to be 806.29 units (mg protein)$^{-1}$, that is about 274.25 folds higher than that of the disintegrated cell extract (Table 2). The final yield of the enzyme activity was approximately 14.1% of the cell extract.

The molecular weight of 2-furaldehyde dehydrogenase was estimated to be 88,000 by non-denaturing PAGE with various concentrations of polyacrylamide resolving gels. 2-Furaldehyde dehydrogenase was tested for purity by non-denaturing PAGE (Fig. 6 and 7). Nondenaturing PAGE showed single band of protein when visualized by Coomassie Blue R-250 and silver staining.

Table 2. *Purification of 2-furaldehyde dehydrogenase from K. pneumoniae*

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Recovery (%)	Purification folds
Disintegrated cell extract	68.26	200.68	2.94	100.0	1.00
Solubilized outer membrane	25.97	96.09	3.70	47.9	1.26
Crude outer membrane protein	11.87	48.53	4.09	24.2	1.39
QAE-Sephadex Q-50 eluate	0.08	30.76	370.60	15.3	126.05
Sephadex G-100	0.03	28.22	806.29	14.1	274.25

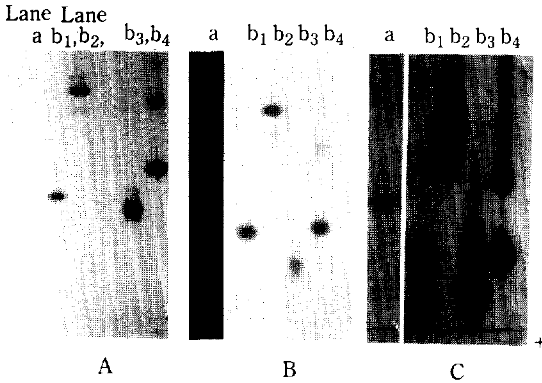

Fig. 6. *Nondenaturing polyacrylamide gel electrophoresis of 2-furaldehyde dehydrogenase, with various concentrations of polyacrylamide of running gel; A, 10%, B, 9%, C, 8%; lane a, 2-furaldehyde dehydrogenase, lanes b_1, b_2, b_3, b_4, and b_5, α-lactalbumin, carbonic anhydrase, chicken egg albumin, monomer and dimer of bovine serum albumin, and mixture of above marker proteins, respectively*

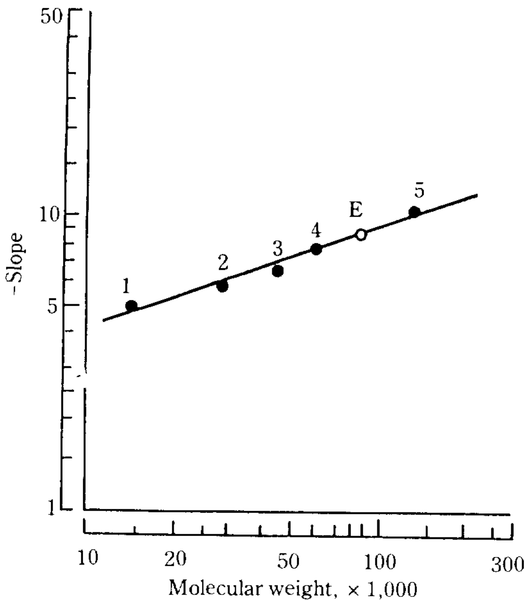

Fig. 7. *Logarithmic plotting for molecular weight estimation of 2-furaldehyde dehydrogenase by nondenaturing polyacrylamide gel electrophoresis. α-Lactalbumin (1), carbonic anhydrase (2), chicken egg albumin (3), monomer (4) and dimer (5) of bovine serum albumin were used as molecular weight marker proteins; E means 2-furaldehyde dehydrogenase.*

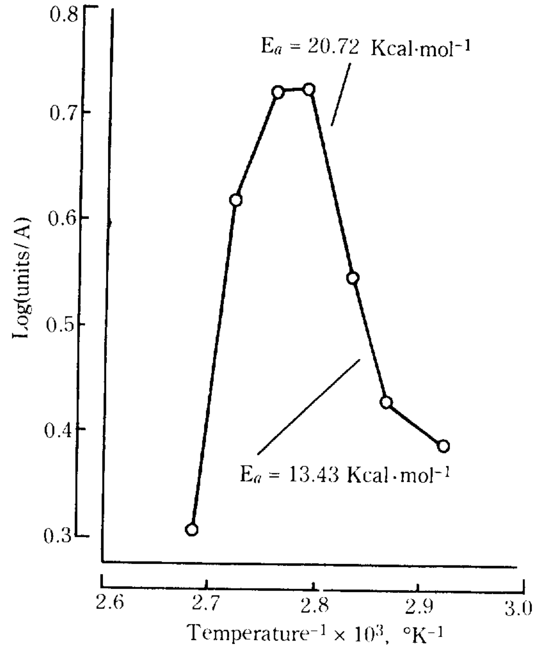

Fig. 8. *Effect of temperature on the activity of the purified 2-furaldehyde dehydrogenase*

Table 3. *Thermal stability of the purified 2-furaldehyde dehydrogenase*

Incubation time	Relative activity (%)
Control	100
1 hour	145
2 hour	130
3 hour	95
4 hour	105
5 hour	120

Note: The enzyme solutions were preincubated under 85 °C at the time interval of one hour in 0.25 M glycine-NaOH buffer (pH 9.5), and then they were reacted according to standard assay method.

Catalytic Properties of 2-Furaldehyde Dehydrogenase

The purified enzyme showed its maximal activity at about 85 °C (Fig. 8), and the enzyme is highly stable at that temperature when incubated at the time interval of one hour for five hours (Table 3). There had been a report about the high thermal stability of protein in the case of β-glucosidase upto 70 °C (Woodward and Wiseman, 1982)

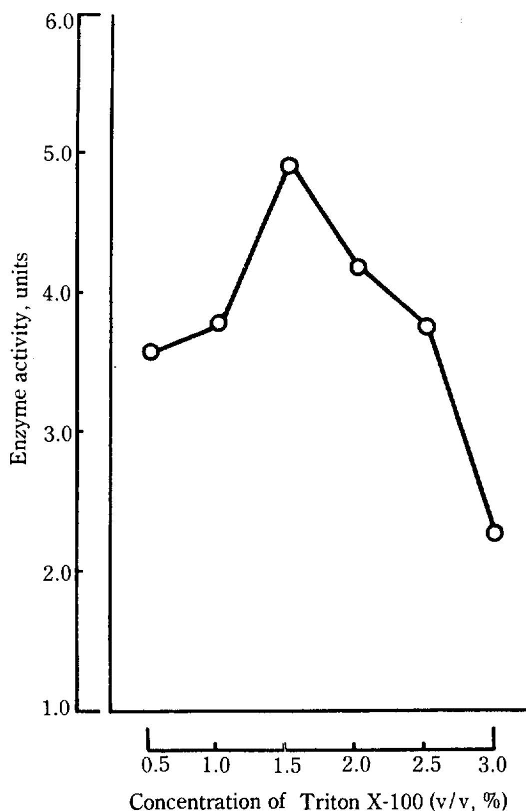

Fig. 9. *Effect of Triton X-100 (v/v, %) on the activity of purified 2-furaldehyde dehydrogenase*

due to its high content of carbohydrate moiety. Therefore, it seems that 2-furaldehyde dehydrogenase might have characters of glycoproteins. If not so, the high thermal stability of the enzyme may be ascribed to the interaction with the non-ionic detergent Triton X-100. Abvoe the cloud point of Triton X-100 at 64 °C, the solution of Triton X-100 turns suddenly turbid and there occurs a microscopic phase separation in the solution (Bordier, 1981). Thus the mixed micelles formed by the hydrophobic residues of protein and detergent were found to aggregate with the rest of the detergent while the hydrophilic residues remained in the aqueous phase. Thereby, when Triton X-100 was added to the reaction mixtures, the maximal activity was appeared at the final concentration of 1.5% (v/v) Triton X-100 as shown in Fig. 9, and the enzyme showed its optimal *p*H in the 0.25 M glycine-NaOH buffer, *p*H 9.5 (Fig. 10).

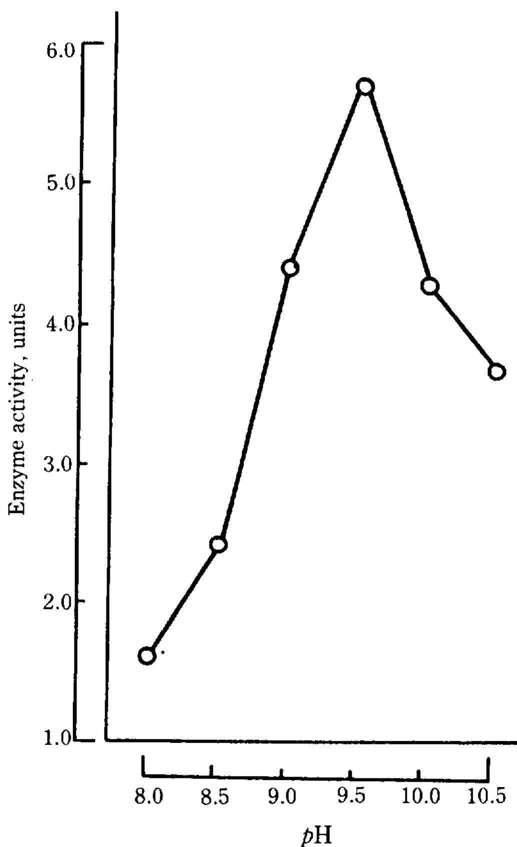

Fig. 10. *Effect of pH on the activity of purified 2-furaldehyde dehydrogenase*

Table 4. *Substrate specificity of 2-furaldehyde dehydrogenase*

Substrates (10 mM)	Units	Relative activity (%)
2-Furaldehyde	8.48	100
Formaldehyde	2.90	34
Benzaldehyde	1.22	14
Propionaldehyde	1.22	14
Acetaldehyde	1.03	12
Isovaleraldehyde	1.03	12
p-Dimethylaminobenzaldehyde	0.85	10

Note: Unit was defined as μmol NADH produced per minute.

Preparation of aldehyde dehydrogenases isolated from bovine liver (Westerhausen *et al.*, 1982), baker's yeast (Tamaki and Hama, 1982), and *Pseudomonas fluorescens* (Jakoby, 1958) had

been shown to oxidize a wide variety of aliphatic and some aromatic aldehydes. On the contrary, other aldehyde dehydrogenases had been reported to show absolute specificity for their substrates: Succinic semialdehyde-Co A (Nirenberg and Jakoby, 1960), γ-aminobutyraldehyde (Jakoby and Fredericks, 1959), malonic semialdehyde-Co A (Vagelos and Earl, 1959), formaldehyde (Schutte *et al.,* 1982), and β-aspartic semialdehyde (Black and Wright, 1955) dehydrogenases would be included. In the case of 2-furaldehyde dehydrogenase, when 10 mM of various aldehydes were added to the reaction mixtures containing 10 mM of β-NAD^+, and reacted under 85 °C for 90 minutes, the enzyme showed a distinct specificity on 2-furaldehyde only (Table 4).

With the various concentration of 2-furaldehyde from 1 mM to 10 mM, the Km value and Vmax value of the 2-furaldehyde dehydrogenase were determined from the Lineweaver-Burk Plot (Fig. 11). The Km value was estimated to be 4.72 mM. And k_{cat} value for the catalytic center activity to be 9.69×10^3 min^{-1} at pH ^.5 under 85 °C.

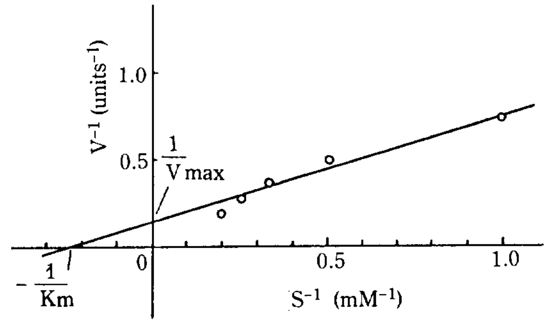

Fig. 11. *Lineweaver-Burk plot for 2-furaldehyde*

적 요

*Klebsiella pneumoniae*의 세포외막으로부터 2-furaldehyde를 2-furoic acid로 산화시키는 2-furaldehyde dehydrogenase를 분리하여 그 특성을 조사하였다. 이 효소는 β-NAD^+를 특이적으로 요구하였다. 분리과정중의 효소활성도는 2-furaldehyde를 기질로 사용하고 β-NAD^+를 조효소로 사용하면서 high performance liquid chromatography에 의해 측정되었다. 세포외막은 Percoll의 밀도흉배에 의한 초원심분리방법과 Mg^{2+}, Triton X-100으로 용해시킨 후, 초원심분리시키는 방법으로 수집되었다. 세포외막단백질은 EDTA와 lysozyme을 처리함으로서 얻어졌고, 효소는 QAE-Sephadex Q-50과 Sephadex G-100을 사용하면서 column chromatography 방법에 의해 분리되었다. 본 효소는 85℃, *p*H 9.5, 그리고 1.5% (vol/vol) Triton X-100의 존재하에서 최대활성을 보여주었다. 효소의 분자량은 nondenaturing polyacrylamide gel electrophoresis의 결과, 88,000으로 추정되었고, 2-furaldehyde에 대한 효소의 Km값은 4.72 mM 이었다.

ACKNOWLEDGEMENT

This work was supported by grants from the Ministry of Education (1987-1988).

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(Received July 25, 1988)