Purification and Characterization of Particulate Alcohol Dehydrogenase from *Gluconobacter suboxydans*[†]

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Particulate alcohol dehydrogenase of acetic acid bacteria that is mainly participated in vinegar fermentation was purified to homogeneous state from *Gluconobacter suboxydans* IFO 12528. Solubilization of enzyme from the bacterial membrane fraction by Triton X-100 and subsequent fractionation on DEAE-Sephadex A-50 and hydroxylapatite was successful in enzyme purification. A cytochrome c-like component was tightly bound to the dehydrogenase protein and existed as an enzyme-cytochrome complex. It was also confirmed that the alcohol dehydrogenase is not a cytochrome component itself. The molecular weight of the enzyme was determined to be 150,000, and gel electrophoresis showed the presence of three subunits having a molecular weight of 85,000, 49,000 and 14,400. The smallest subunit was corresponded to the cytochrome c-like component. Ethanol was oxidized in the presence of dyes *in vitro* but NAD or NADP were not required as hydrogen acceptor. Unlike NAD-linked alcohol dehydrogenase in yeast or liver and other primary alcohol dehydrogenases in methanol utilizing bacteria, the enzyme from the acetic acid bacteria showed its optimum pH at fairly acidic pH.

The world of acetic acid bacteria has been classified into two genera, *Acetobacter* and *Gluconobacter*.¹⁾ One of the aspects of this classification is based on their physiological characteristics in carbohydrate metabolism; for examples, production of acetic acid from ethanol is strong in *Acetobacter*, weak to negligible in *Gluconobacter*, production of gluconic acid from glucose is weak to negligible in *Acetobacter*, strong in *Gluconobacter*, and ketogenic activity is weak or none in *Acetobacter*, strong in *Gluconobacter*. Nonphosphorylative carbohydrate metabolism has been known as a predominant metabolic pathway in the acetic acid bacteria²⁾ as well as in other oxidative bacteria of such genera as *Pseudomonas*,^{3~5)} *Serratia*⁶⁾ and *Klebsiella*.²⁾ Some of the important problems, however, still remain to be elucidated, such as, why and how the organisms accumulate acetic acid in high concentration so rapidly in *Acetobacter*, and gluconic acid

and/or ketogluconic acids in *Gluconobacter*. Additionally, it has not been clarified why they are so strict aerobe and why they don't grow so fast and make a mass cell population even though a nutritionally rich medium is devised.

In the course of our investigation on the nonphosphorylative metabolic pathway of carbohydrates in acetic acid bacteria and other oxidative bacteria, many kinds of enzymes concerning ketogluconate metabolism have been prepared in pure form from these bacteria^{7~16)} and studied in some details.^{17~22)} Our understandings have proceeded that the enzymes involved in the nonphosphorylative ketogluconate metabolism of the oxidative bacteria is divided into two groups. One is pyridine nucleotide dependent enzymes such as 2-ketogluconate reductase^{7,9~11,15)} and 5-ketogluconate reductase^{8,11)} and the other is characterized as membrane bound particulate dehydrogenases.^{11~14,16)} The latter group involves glucose dehydrogenase, gluconate dehydrogenase, ketogluconate dehydrogenase, sorbitol dehydrogenase, alcohol dehydrogenase and so on.

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These particulate enzymes are independent on the pyridine nucleotide coenzymes in their enzyme activities and play an important role in the production of oxidative fermentation products such as gluconic acid, ketogluconic acids, sorbose, dihydroxyacetone, acetic acid and others.

A sole report concerning alcohol oxidation by acetic acid bacteria has been made by Sekuzu *et al.*²³⁾ and Nakayama²⁴⁾ that a highly purified cytochrome c component prepared from *A. peroxydans* is accompanied with alcohol dehydrogenase activity. In the course of our studies on ketogluconate metabolism of acetic acid bacteria, a series of particulate enzymes of glucose dehydrogenase, gluconate dehydrogenase, ketogluconate dehydrogenase, sorbitol dehydrogenase and alcohol dehydrogenase have been shown to be cytochrome-linked enzyme.^{11~14, 16, 21, 22)} In this paper, we wish to describe the purification and characterization of particulate alcohol dehydrogenase from *G. suboxydans*. An evidence will be presented that a protein dehydrogenating alcohol is not a cytochrome c component itself, but exists as an enzyme-cytochrome complex.

METHODS AND MATERIALS

Chemicals. All chemicals used in this study were commercial products. Yeast extract and NAD(P) and its reduced forms were generously supplied from the Oriental Yeast Ind. Co., Ltd. Sodium gluconate and glucose for culture medium were kind gift from the Fujisawa Pharmaceutical Ind. Co., Ltd. Hydroxylapatite was prepared according to Tiselius *et al.*²⁵⁾ Potato extract was prepared as mentioned by Ameyama and Kondo.²⁶⁾

Microorganisms. Bacterial strains of genus *Gluconobacter*, listed in Table I, were generous donation from the Institute for Fermentation, Osaka (IFO). As reported by Asai,²⁾ gluconobacters grow on glucose or gluconate and produce a marked amount of 5-ketogluconate as well as 2-ketogluconate. *G. suboxydans* IFO 12528 was selected for further extensive study of the particulate alcohol dehydrogenase and in other experiments on 5-ketogluconate reductase and 5-ketogluconate producing gluconate dehydrogenase.²⁷⁾ The growing cells and resting cells of gluconobacters utilized ethanol but the accumulation of acetic acid was not so high as acetobacters in cell level experiments.²⁸⁾

Medium and cultivation. The culture medium of gluconobacter was consisted of 20 g of sodium gluconate, 5 g of glucose, 3 g of glycerol, 3 g of yeast extract, 2 g of polypeptone and 200 ml of potato extract in 1 liter of tap water. The pH of the medium was spontaneously settled on 6.5 when all these ingredients were mixed. Gluconobacters were transferred from the potato extract-agar slant to 100 ml of the medium in 500 ml shaking flask and the cultivation was carried out at 30°C for 24 hr with reciprocal shaking. In case of large scale culture, 1.5 liters of inoculum (15 shaking flasks) were transferred into 30 liters of the medium in 50-liter jar fermentor. In this case, the potato extract was usually omitted from the medium and cultivation was performed at 30°C for 24 hr under vigorous aeration at 30 liters per min with 500 rpm agitation.

Enzyme assay. Following assay systems were available: ethanol was oxidized by particulate alcohol dehydrogenase (1) by spectrometry in the presence of 2,6-dichlorophenolindophenol and phenazine methosulfate, (2) by colorimetry in the presence of ferricyanide and (3) by polarometry with oxygen electrode or (4) by manometry in a conventional Warburg apparatus. In this study, assay method with ferricyanide was mainly employed in its simplicity for routine assay. This method was employed by Wood *et al.*²⁹⁾ in the assay of gluconate dehydrogenase of *Pseudomonas fluorescens* using ferricyanide as an electron carrier. Some modifications are made as also reported elsewhere.³⁰⁾ The rate of reduction of ferricyanide to ferrocyanide gives a quantitative amount of ethanol oxidized. The reaction mixture contained 10 μ moles of potassium ferricyanide, 0.5 ml of McIlvaine buffer, pH 5.5, enzyme solution and 100 μ moles of ethanol in a total volume of 1.0 ml. The reaction was carried out at 37°C by the addition of ethanol solution, and stopped by adding 0.5 ml of the ferric-Dupanol reagent.²⁹⁾ Then, 3.5 ml of water was further added to the reaction mixture and well mixed. The resulting stabilized Prussian blue color formed was measured spectrophotometrically at 660 nm after standing for 20 min at 37°C. One unit of enzyme activity was defined as the amount of enzyme catalyzing the oxidation of 1 μ mole of ethanol per min under these assay conditions and 4.0 absorbance unit equaled to 1 μ mole of ethanol oxidized.

Protein determination. Protein content was determined according to the method by Lowry *et al.*,³¹⁾ using a bovine serum albumin as the standard. A modified method described by Dulley and Grieve³²⁾ was employed instead for the samples which contained Triton X-100.

Electrophoresis. For estimation of purity of the enzyme preparations, disc gel electrophoresis was performed under essentially the same conditions as de-

scribed by Davis⁸³) using 7.5% of polyacrylamide gel and Tris-glycine buffer, pH 8.3. Sodium dodecyl sulfate (SDS)-acrylamide gel electrophoresis was performed to determine molecular weight of the purified enzyme under essentially the same conditions of Weber and Osborn.⁸⁴) Free boundary electrophoresis of the purified enzyme was performed to estimate the occurrence of dissociation of enzyme at pH 6.0 of phosphate buffer of 0.1 ionic strength. After passage of 10.0 mA current for 180 min, each 25 drops were collected and subjected to further analyses.

Analytical ultracentrifugation. Estimation of sedimentation velocity and examination of purity of the purified enzyme was carried out with a Hitachi model UCA-1A analytical ultracentrifuge at 59,780 rpm using a schlieren optics.⁸⁵) Temperature was maintained at 20°C throughout the measurement.

RESULTS AND DISCUSSION

Distribution of particulate alcohol dehydrogenase in gluconobacters

Distribution of particulate alcohol dehydro-

genase was surveyed with cell homogenate of the genus *Gluconobacter* as shown in Table I. High enzyme activity was found in cell homogenate from three strains of *G. dioxyaceticus*, both strains of *G. nonoxygluconicus*, two strains of *G. cerinus* IFO 3267 and IFO 3269 and *G. suboxydans* IFO 12528 showed the highest enzyme activity under these experimental conditions. It was also interesting that, despite of fairly well growth on the medium, a poor enzyme activity was observed with all strains of *G. suboxydans* var. α . As expected, all enzyme activity occurred in the particulate membrane fraction when the cell homogenate of several gluconobacters was centrifuged at $100,000 \times g$ for 60 min. These results also suggested the possibility to solubilize the enzyme by a surface active agent, and the solubilization could actually performed by Triton X-100. Extraction of the enzyme was also done with acetone dried cell as mentioned by Naka-

TABLE I. DISTRIBUTION OF PARTICULATE ALCOHOL DEHYDROGENASE IN GLUCONOBACTERS

Each strain of gluconobacters was inoculated on three shaking flasks (300 ml of the medium) and incubated at 30°C for 24 hr under reciprocal shaking. Enzyme activity was assayed with cell homogenate under the standard assay conditions. Results are expressed by total enzyme activity and the parenthesis means specific activity (units/mg protein).

Strain	A	D	H ^a	Strain	A	D	H ^a
<i>G. liquefaciens</i> IFO 12388	4		(0.1)	<i>G. gluconicus</i> IFO 3286	37		(0.2)
<i>G. melanogenus</i> IFO 12257	75		(0.6)	<i>G. roseus</i> IFO 3990	59		(0.8)
<i>G. melanogenus</i> IFO 12258	13		(0.1)	<i>G. albidus</i> IFO 3250	tr		
<i>G. melanogenus</i> IFO 3293	143		(0.6)	<i>G. albidus</i> IFO 3251	133		(0.6)
<i>G. melanogenus</i> IFO 3294	218		(1.3)	<i>G. albidus</i> IFO 3253	174		(1.2)
<i>G. sphaericus</i> IFO 12467	tr			<i>G. suboxydans</i> IFO 3130	24		(0.2)
<i>G. oxydans</i> IFO 3189	13		(0.1)	<i>G. suboxydans</i> IFO 3172	51		(0.3)
<i>G. oxydans</i> IFO 3287	261		(1.7)	<i>G. suboxydans</i> IFO 3289	120		(0.6)
<i>G. dioxyaceticus</i> IFO 3271	267		(1.5)	<i>G. suboxydans</i> IFO 3290	53		(0.3)
<i>G. dioxyaceticus</i> IFO 3272	428		(2.2)	<i>G. suboxydans</i> IFO 3291	118		(0.7)
<i>G. dioxyaceticus</i> IFO 3274	462		(2.0)	<i>G. suboxydans</i> IFO 12528	635		(4.2)
<i>G. cerinus</i> IFO 3262	64		(0.4)	<i>G. suboxydans</i> var. α IFO 3254	tr		
<i>G. cerinus</i> IFO 3263	4		(0.1)	<i>G. suboxydans</i> var. α IFO 3255	tr		
<i>G. cerinus</i> IFO 3264	57		(0.4)	<i>G. suboxydans</i> var. α IFO 3256	tr		
<i>G. cerinus</i> IFO 3265	16		(0.1)	<i>G. suboxydans</i> var. α IFO 3257	tr		
<i>G. cerinus</i> IFO 3266	tr			<i>G. suboxydans</i> var. α IFO 3258	tr		
<i>G. cerinus</i> IFO 3267	361		(2.2)	<i>G. capsulatus</i> IFO 3462	108		(1.0)
<i>G. cerinus</i> IFO 3268	71		(0.5)	<i>G. industrius</i> IFO 3260	tr		
<i>G. cerinus</i> IFO 3269	431		(2.3)	<i>G. nonoxygluconicus</i> IFO 3275	495		(2.6)
<i>G. cerinus</i> IFO 3270	129		(0.9)	<i>G. nonoxygluconicus</i> IFO 3276	450		(1.9)
<i>G. gluconicus</i> IFO 3171	tr			<i>G. rubiginosus</i> IFO 3244	432		(2.1)
<i>G. gluconicus</i> IFO 3285	57		(0.4)				

^a Particulate alcohol dehydrogenase activity is abbreviated as A D H.

yama.²⁴⁾ It was found that extraction and solubilization of the enzyme without the aid of detergent caused polymerization, most of which was dimer, and made the enzyme less stable as briefly mentioned later.

Enzyme formation during the cultivation

Time course of enzyme formation in cell of *G. suboxydans* IFO 12528 during the growth was estimated. As shown in Fig. 1, a maximal formation of the total enzyme activity of particulate alcohol dehydrogenase was observed at the end of exponential phase of bacterial growth. Similar results were obtained with other enzyme species of acetic acid bacteria such as *G. liquefaciens*,^{7,8)} *G. suboxydans* var. α ,²⁸⁾ *A. rancens*,¹⁰⁾ *A. ascendens*¹⁵⁾ and other oxidative bacteria of *P. aeruginosa*, *K. aerogenes* and *S. marcescens*.³⁶⁾ A typical sequential enzyme formation was also recorded in fermentation engineering experiments with *G. suboxydans* var. α performed in a well controlled jar fermentor.²⁸⁾ When glucose was added as the carbon source, of the particulate dehydrogenases, the first enzyme appeared was glucose dehydrogenase and a comparable amount of gluconate was accumulated in the

culture broth at the middle exponential phase. Gluconate in the culture broth spontaneously facilitated the formation of gluconate dehydrogenase, ketogluconate dehydrogenase and alcohol dehydrogenase. The pH of the medium was rapidly lowered to 4.3 within 24 hr and maintained at a constant level throughout the culture for about 120 hr so far tested. As mentioned briefly in the METHODS AND MATERIALS, the growing cells or resting cells of gluconobacters rapidly oxidize glucose, gluconate and other substrates including ethanol. There was a marked difference between gluconobacters and other oxidative bacteria that an appreciable oxidation of ethanol was seen in gluconobacters but not in other oxidative bacteria.²²⁾ The broken cell of gluconobacters (cell homogenate) revealed a marked activity of ethanol oxidation much more than intact cells. Two times or more enzyme activity of glucose oxidation or gluconate oxidation was also usually present in the cell homogenate comparing with intact cells.

Purification of particulate alcohol dehydrogenase

All operations were carried out at 0~5°C, unless otherwise stated. In the followings, centrifugation means a conventional run carried out at $12,000 \times g$ for 20 min. Potassium phosphate buffer, pH 6.0, containing 1 mM 2-mercaptoethanol was basically used in this work.

Preparation of particulate fraction

Cell paste of *G. suboxydans* IFO 12528 (350 g, wet wt.) which was harvested by continuous flow centrifugation from 90 liters of 24 hr culture was washed twice with cold distilled water and then suspended in 0.01 M buffer. The cell suspension was passed through a French pressure cell press at 20,000 psi. Cell debris was removed by centrifugation at $5000 \times g$ for 10 min. To the cell homogenate, ammonium sulfate was added to 0.75 saturation and pH was adjusted to 6.0. After standing for 30 min in an ice bath, the resulting precipitate was collected and stored in a deep freezer at -20°C. The precipitate could be

FIG. 1. Time Course of Particulate Alcohol Dehydrogenase Formation.

Three shaking cultured flasks (300 ml of the medium) were harvested after 12 hr interval. Bacterial growth was measured from the turbidity of culture broth at 660 nm and expressed by dotted line. Enzyme activity, (●), and protein content were measured with cell homogenate. Specific activity, (○), was expressed as units per mg protein.

stored without any loss of enzyme activity for at least 6 months so far examined. The precipitate was dissolved in a small volume of 0.01 M buffer and dialyzed against the same buffer to remove ammonium sulfate thoroughly. Almost of enzyme activity was recovered in this dialyzed solution.

Solubilization of enzyme from the particulate fraction

Solubilization of the enzyme was performed as essentially the same as mentioned before.¹¹⁾ The protein concentration of the dialyzed enzyme solution was adjusted to 30 mg per ml with the dialyzing buffer and the enzyme solution was incubated for 3 hr at 0°C with gentle stirring in the presence of 1% Triton X-100. The enzyme solution was then spun down to remove insoluble materials and a reddish supernatant was obtained as the solubilized enzyme.

DEAE-Sephadex column chromatography

The solubilized enzyme was dialyzed against 0.002 M buffer containing 0.1% Triton X-100 overnight. Dialyzed enzyme solution was applied on a column of DEAE-Sephadex A-50 (5×30 cm), which had been equilibrated with the same buffer and washed with additional 500 ml of the buffer. Enzyme activity was eluted from the column with a linear gradient of phosphate up to 0.1 M, and the enzyme activity was eluted at around 0.05 M of phosphate concentration. Pooled enzyme fraction was then dialyzed against 0.002 M buffer containing 0.05% Triton X-100 overnight. Chromatography on DEAE-Sephadex A-50 was repeated. After adsorption of the enzyme into a column of DEAE-Sephadex A-50 (1.5×20 cm), the column was first treated with 0.015 M buffer containing 0.05% Triton X-100 and elution of the enzyme was performed by a linear gradient elution made between 0.015 M and 0.1 M of phosphate buffer. Each buffer reservoir contained 500 ml and 0.05% Triton X-100 was present throughout this step. This second step of DEAE-Sephadex A-50 chromatography was found convenient to bring about in removing an impurity which was co-

chromatographed in the next step. Pooled enzyme fraction was dialyzed against 0.01 M buffer containing 0.05% Triton X-100 overnight.

Hydroxylapatite column chromatography

The dialyzed enzyme solution was divided into three portions and each portion was adsorbed separately into a hydroxylapatite column (1.5×5 cm), which had been equilibrated with the same buffer and the column was washed with 150 ml of the buffer. The column was then treated stepwise with 0.02 M, 0.05 M and 0.1 M buffer containing 0.1% Triton X-100. Almost of enzyme activity was eluted at 0.1 M concentration of phosphate. As can be seen in Fig. 2, elution pattern of this column chromatography gave a symmetric protein peak with accompanying corresponding enzyme activity and rose-red color intensity. Specific activity around the peak fractions showed a constant level and fractions of which specific activity over 175 were collected. Pooled enzyme solution was concentrated by a Toyo ultra filter UP-20. Summary of a typical purification of the enzyme is shown in Table II, and the enzyme was purified about

FIG. 2. Chromatography on Hydroxylapatite Column.

Protein, 180 mg, containing 23,600 units of enzyme activity was applied to a column (1.5×5 cm), which had been equilibrated with 0.01 M buffer containing 0.1% Triton X-100. Enzyme activity was eluted with 0.1 M buffer containing 0.1% Triton X-100. Fractions of 10 ml were collected at a flow rate of 3 ml per hr.

TABLE II. SUMMARY OF PURIFICATION OF PARTICULATE ALCOHOL DEHYDROGENASE FROM *Gluconobacter suboxydans*

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)
Homogenate	32,920	131,700	4	(100)
Solubilized fraction	5,665	108,200	19	82
DEAE-Sephadex A-50 (I)	2,080	104,000	50	79
DEAE-Sephadex A-50 (II)	550	71,400	130	54
Hydroxylapatite	290	56,550	195	43

50-fold with an yield of 43 per cent.

Properties of purified particulate alcohol dehydrogenase

Absorption spectra. Purified particulate alcohol dehydrogenase of *G. suboxydans* showed a cytochrome c-like absorption spectra (Fig. 3). A hemeprotein was present as almost reduced form at the final enzyme preparation, suggesting that the hemeprotein was quite stable and less autooxidizable. Absorption maxima at the wavelength of 553 nm, 522 nm and 417 nm were observed with the

FIG. 3. Absorption Spectra of Particulate Alcohol Dehydrogenase from *G. suboxydans*.

Finally purified enzyme with a specific activity of 195 was used at concentrations of 0.1 mg per ml for reduced form and 0.1 mg per ml for oxidized form, in 0.01 M buffer containing 0.1% Triton X-100.

reduced enzyme and a sole peak at 409 nm was observed with the oxidized enzyme. In the ultraviolet region, absorption spectra could not examine successfully due to the presence of an excess amount of Triton X-100 in the enzyme solution. These absorption spectra were fairly similar to those of cytochrome c judging from the literatures by Okunuki and his co-workers.³⁷⁻³⁹⁾ The absorption spectra were also quite similar to those from *A. peroxydans*,²⁴⁾ but an apparent molecular size of the enzyme was somewhat different as mentioned below. Difference spectrum was also taken with reduced form versus oxidized form as shown in Fig. 4. A marked reduction of absorption around 450~460 nm suggested the presence of flavocomponent in the purified enzyme. These results from spectral study indicate that the alcohol dehydrogenase is consisted of heterogeneous components of hemeprotein, flavoprotein and others, and has a function as an enzyme complex.

FIG. 4. Difference Spectrum of Particulate Alcohol Dehydrogenase from *G. suboxydans*.

Spectrum was taken at the enzyme concentration of 0.018 mg per ml of which specific activity was 195. Sodium dithionite-reduced enzyme was set at sample cuvette and oxidized enzyme at pH 9 was placed in the reference hole of a Hitachi recording spectrophotometer model 124.

Homogeneity. Homogeneity of the purified enzyme was estimated by an analytical ultracentrifuge and the sedimentation pattern of the enzyme showed a single symmetrical peak (Fig. 5). The faster moving peak which

FIG. 5. Sedimentation Pattern of Particulate Alcohol Dehydrogenase from *G. suboxydans*.

Finally purified enzyme with a specific activity of 195 was used at the enzyme concentration of 11.1 mg per ml in 0.01 M buffer containing about 0.1% Triton X-100. Photographs were taken at 44 min, 52 min and 60 min after reaching at 59,780 rpm. Upper frame, particulate alcohol dehydrogenase; lower frame, Triton X-100 at 5 mg per ml in 0.01 M buffer. Direction of sedimentation was right to left.

had a rose-red colored was corresponded to the particulate alcohol dehydrogenase, and the slower sedimenting component was confirmed to be Triton X-100 which was present in excess in the enzyme solution. As a reference, 5 mg per ml of Triton X-100 was separately run under the same analytical conditions as also shown in Fig. 5. An apparent sedimentation constant of the enzyme was calculated to be 5.9 S and that of Triton X-100 was found to be 1.9 S. Cytochrome c having an alcohol dehydrogenase activity from *A. peroxydans* showed 10.3 S of sedimentation constant²⁴⁾ and the value was almost twice as much as our preparation. Gel filtration of the enzyme on a Sephadex G-200 column was also done to estimate whether any impurity was present and to determine an apparent molecular weight. No appreciable minor component was detected from the gel filtration chromatogram and an apparent molecular weight was obtained to be approximately 150,000. Thus the purified particulate alcohol dehydrogenase was shown to be homogeneous and a cytochrome component was tightly bound to form an enzyme-

cytochrome complex.

Electrophoretic analyses. When the purified enzyme was subjected to a conventional polyacrylamide gel electrophoresis prepared at pH 8.3 and run at pH 9.5, three protein bands were stained as shown in Fig. 6, trace-1. Enzyme activity was detected on the slowest moving protein band when the gel was incubated in the presence of phenazine methosulfate, nitroblue tetrazolium and ethanol at pH 5.5,⁴⁰⁾ as shown in Fig. 5, trace-2. When an unstained gel was irradiated by a fluorescent

FIG. 6. Densitometric Traces of Polyacrylamide Gel Electrophoresis of Particulate Alcohol Dehydrogenase.

Finally purified enzyme with a specific activity of 195 was used and applied to a gel column (7.5% polyacrylamide) and run at pH 9.5 for 2 hr at 2 mA. Trace-1, protein staining. Enzyme protein (50 μ g) was applied and stained by coomassie brilliant blue. Trace-2, enzyme activity staining. Enzyme activity was detected by staining the gel in a medium containing 100 mM ethanol, 0.4 mg per ml of nitroblue tetrazolium, 0.14 mg per ml of phenazine methosulfate and 0.1 M buffer, pH 6.0. After the incubation for 5 to 10 min at 25°C in a dark, the reaction was stopped by dipping the gel into 7% acetic acid solution. Enzyme protein (50 μ g) was used and the unstained gel was bufferized at pH 6 before enzyme activity staining. Trace-3, localization of cytochrome component. Enzyme protein (1 mg) was applied and unstained gel was immediately traced with a filter of 550 nm. Trace-4, iron staining. Enzyme protein (50 μ g) was used and gel was stained by benzidine in a dark. After development of blue color, the gel was traced with a filter of 440 nm. A Toyo Densitorol DMU-33C was used in this densitometric scanning. Direction of electrophoresis was right of left.

light, strong intensity of fluorescence was observed only with the slowest moving protein band. This also indicated the presence of flavoprotein in the particulate alcohol dehydrogenase complex. When the unstained gel was traced with a filter of 550 nm, a middle protein band with the least stained by coomassie brilliant blue was found to be the cytochrome c-like component (Fig. 6, trace-3). This band was usually visible when a lot of protein was treated by gel electrophoresis. These results confirmed that the alcohol dehydrogenase is not a cytochrome component itself and the cytochrome component, however, has a function as an electron acceptor *in vivo*, since the cytochrome component of the enzyme was readily reduced by the addition of ethanol to the enzyme solution. When the gel column was stained with benzidine to detect iron by nonspecific chelation,⁴¹⁾ and followed by densitometry at 440 nm (Fig. 6, trace-4), all three protein bands contained an appreciable amount of iron. The results obtained here indicate that electrophoresis at alkaline pH makes dissociation of the enzyme complex into subunits. Similar observations were reported with a purified particulate gluconate dehydrogenase from *K. aerogenes*¹³⁾ and *P. aeruginosa*,¹⁴⁾ which contained tightly bound cytochrome component, when the enzymes were treated at alkaline pH. It was also supported from the fact that, when the particulate alcohol dehydrogenase was treated at pH 6.0 in a conventional apparatus of free boundary electrophoresis for 180 min at 0.1 ionic strength, no dissociation occurred and retained its catalytic and physicochemical properties identical with native enzyme.

Dissociation into subunits was also observed by SDS-gel electrophoresis in the determination of molecular weight of the enzyme. In the presence of SDS, the enzyme was dissociated into three subunits with a molecular weight of 85,000, 49,000 and 14,400 from the top to bottom of the gel column as shown in Fig. 7. The sum of molecular weight of each band gave 150,000 of total molecular weight, which was in good agreement with the result of molecular weight determination by gel filtration by

Sephadex G-200 column.

Dissociation into subunits was also observed in the presence of urea. Temporary denaturation of the enzyme was observed with increas-

FIG. 7. SDS-Gel Electrophoresis of Particulate Alcohol Dehydrogenase.

Finally purified enzyme with a specific activity of 195 was used and 10 μ of enzyme protein was applied (Upper column). Lower column, standard marker proteins. Standard marker proteins used were from right to left of the lower column were: bovine serum albumin, mol. wt. 68,000; ovalbumin, mol. wt. 43,000; trypsin, mol. wt. 23,300 and lysozyme, mol. wt. 14,300. Both columns were stained by coomassie brilliant blue. Direction of electrophoresis was right to left.

ing concentration of urea in the presence of 0.1 M 2-mercaptoethanol at pH 6.0, and immediate dilution gave a complete renaturation when traced by disappearance and reappearance of enzyme activity. Irreversible denaturation was observed by prolonged incubation of the enzyme with urea at 3 M for over 60 hr in the presence of 0.1 M 2-mercaptoethanol at pH 6.0. The urea-denatured enzyme solution was applied to a Sephadex G-150 column (1.5 $\times$ 30 cm), which had been equilibrated with 0.01 M buffer containing 3 M urea and 0.1 M 2-mercaptoethanol. Elution pattern of the urea-denatured enzyme was shown in Fig. 8-A. It was obvious that the enzyme was dissociated into three subunits as similarly observed by SDS-gel electrophoresis. Each three peaks and original denatured enzyme solution were run on an SDS-gel column separately as also shown in Fig. 8-B.

No attempt has been made to reconstitute the alcohol dehydrogenase complex from the dissociated and separately purified subunit. Two of three subunits of the enzyme has been

FIG. 8. Gel Filtration of Urea-denatured Enzyme on Sephadex G-150 Column (A) and SDS-Gel Electrophoresis of Denatured Components after Gel Filtration (B).

Finally purified enzyme with a specific activity of 195 (10 mg) was dialyzed for 4 days against 3 M urea in 0.01 M buffer containing 0.1 M 2-mercaptoethanol and 0.1% Triton X-100. The dialyzed solution was applied to a column of Sephadex G-150 (1.5 × 30 cm), which had been equilibrated with the dialyzing buffer. Fractions of each 0.5 ml were collected. SDS-Gel electrophoresis was performed after gel filtration and densitometric traces were shown with peak fractions and the original denatured enzyme solution. Trace-1, original denatured enzyme solution; Trace-2, fraction No. 27; Trace-3, fraction No. 42 and Trace-4, fraction No. 63. Direction of gel electrophoresis was right to left.

partially characterized, one is cytochrome c-like hemeprotein and the other is flavoprotein to which substrate, ethanol, first binds. The third subunit with the fastest mobility in polyacrylamide gel at pH 9.5 has not been characterized.

These results suggest that the purified enzyme preparation of the particulate alcohol dehydrogenase from *G. suboxydans* is composed of three different subunits and made up an enzyme complex of dehydrogenase and hemeprotein. It might be quite possible that the cytochrome c having alcohol dehydrogenase activity from *A. peroxydans*, of which molecular weight of 250,000,²⁴ is composed of heterogeneous subunits and the alcohol dehydrogenase activity is not due to cytochrome c component itself. It could be expected that if such enzyme is treated at alkaline pH, SDS, urea or other denaturants, dissociation into subcomponents probably

occur, and it will be clarified that a protein component free from cytochrome c has an enzyme activity as mentioned above. Indeed, similar results have been obtained with particulate alcohol dehydrogenase from other species of acetic acid bacteria such as *G. liquefaciens*, *A. aceti* and *Acetobacter* sp. in our laboratory as reported elsewhere.¹⁶

Properties of catalytic activity

The particulate alcohol dehydrogenase could be assayed *in vitro* in the presence of either one of the following dyes as a hydrogen acceptor; 2,6-dichlorophenolindophenol, phenazine methosulfate or potassium ferricyanide. NAD or NADP were not effective as hydrogen acceptor. Substrate specificity of this enzyme with potassium ferricyanide is summarized in Table III. Primary aliphatic alcohols having carbon chain length of 6 or less were rapidly oxidized but not methanol. Secondary and tertiary alcohols and aromatic alcohols could not oxidize to appreciable extent so far tested. Profile of substrate specificity of this enzyme was quite similar to that from cytochrome c of

TABLE III. SUBSTRATE SPECIFICITY OF PARTICULATE ALCOHOL DEHYDROGENASE FROM *Gluconobacter suboxydans*

The reaction rate with ethanol is expressed as 100.

Substrate	Relative rate
Methanol	2
Ethanol	100
<i>n</i> -Propanol	93
<i>n</i> -Butanol	81
<i>n</i> -Pentanol	64 ^a
<i>n</i> -Hexanol	41 ^a
Isopropanol	0
<i>tert</i> -Butanol	0
Benzylalcohol	0 ^a
Phenethylalcohol	0 ^a
Formaldehyde	0
Acetaldehyde	0
Propionaldehyde	0
Benzaldehyde	0 ^a
Glycerol	0
Glucose	0
Glycolate	0
Lactate	0

^a Enzyme reaction was carried out in the presence of 0.2% Triton X-100.

A. peroxydans.²⁴⁾ Substrate specificity of this enzyme as a typical primary alcohol dehydrogenase also likes to those from methanol utilizing bacteria,^{42~44)} except that the enzyme from acetic acid bacteria could not oxidize methanol at all and showed no ammonium ion requirement in the alcohol dehydrogenase activity. The profile of substrate specificity of this enzyme was also similar to those of NAD-linked alcohol dehydrogenase from yeast⁴⁵⁾ and horse liver.⁴⁶⁾ An apparent Michaelis constant for ethanol was examined to be 1.6×10^{-3} M (Fig. 9), which was almost same order obtained with the enzyme of *A. peroxydans*.²⁴⁾ This value is also similar to that of horse liver enzyme and about one tenth of that obtained with an yeast NAD-dependent alcohol dehydrogenase.⁴⁵⁾

FIG. 9. Effect of Substrate Concentration on Enzyme Activity.

Finally purified enzyme with a specific activity of 195 was used. Enzyme activity was measured at various concentrations of ethanol as indicated.

FIG. 10. Effect of pH on Enzyme Activity.

Finally purified enzyme with a specific activity of 195 was used. Enzyme activity was assayed under the standard assay conditions except that the pH of buffer (McIlvaine buffer) was varied as indicated.

Optimum pH of ethanol oxidation was shown in Fig. 10, and the enzyme showed its maximum activity at pH 5 to 6. Apart from this pH region, enzyme activity was gradually decreased. It was a marked difference in pH optima when the enzyme was compared with those of primary alcohol dehydrogenase of methanol utilizing bacteria^{42~44)} or NAD-linked alcohol dehydrogenase from yeast and mammals,^{45~46)} which unexceptionally show their maximal reaction rate at fairly alkaline pH. It is quite reasonable for acetic acid bacteria to have a pH optimum of alcohol oxidation at fairly acidic pH. Ethanol content could be well assayed at acidic pH by the particulate alcohol dehydrogenase of acetic acid bacteria and could be applied to determine the ethanol content of brewing samples without any preadjustment of samples' pH. An attempt for this purpose was reported elsewhere.³⁰⁾

Effect of Triton X-100 to the particulate alcohol dehydrogenase

Particulate alcohol dehydrogenase has thus purified to homogeneous state from the membrane fraction of acetic acid bacteria. Triton X-100 was used throughout in this study. It is thought that proteins localized in the particulate fraction or anchored to the cellular membrane have some hydrophobicity in its protein molecule and quite hard to make a solution without the aid of detergents.⁴⁷⁾ Nakayama²⁴⁾ prepared a cytochrome c having an alcohol dehydrogenase activity from cell of *A. peroxydans* to almost homogeneous state. He treated the cell as acetone dried cell and, thereafter, the purification of the enzyme was fairly well processed like a soluble enzyme without any aid of detergent. Judging from the data obtained in this study, our enzyme preparation had just a half of molecular size of Nakayama's preparation on the basis of sedimentation constant and molecular weight. Another preparation of particulate alcohol dehydrogenase from *A. aceti* also showed of its molecular weight to be approximately 150,000.¹⁶⁾ As shown in Fig. 11, dimeric

FIG. 11. Effect of Triton X-100 on Particulate Alcohol Dehydrogenase in Analytical Ultracentrifugation.

Finally purified enzyme with a specific activity of 195 was used. Upper frame, monomer of enzyme in the presence of excess amount of Triton X-100. Enzyme protein (11.0 mg per ml) in 0.01 M buffer containing about 0.1% Triton X-100. Lower frame, dimer of enzyme in the deficiency of Triton X-100. Monomer enzyme was once adsorbed into a small column of hydroxylapatite (1×1 cm), which had been equilibrated with 0.01 M buffer. After washing the column with the buffer to remove Triton X-100 thoroughly, the enzyme was eluted with 0.2 M buffer and enzyme solution was further concentrated, when necessary. Concentration of dimer was adjusted to 11.0 mg per ml. Both photographs were taken at 44 min after reaching at 59,780 rpm. Direction of sedimentation was right to left.

formation was observed when the enzyme was freed from excess Triton X-100 and run in analytical ultracentrifuge. An apparent sedimentation constant of the dimeric enzyme was found to be 9.8 *S* that was comparable to Nakayama's preparation (10.3 *S*) and molecular weight was also increased to 320,000 when analyzed by Sepharose 6B column. Dimeric enzyme was spontaneously dissociated

into monomer by the addition of excess amount of Triton X-100. A trace amount of monomeric enzyme can be seen in the sedimentation profile of dimeric enzyme (Fig. 11, lower frame).

In our preparation, Triton X-100 was found to be useful detergent to solubilize the enzyme from the particulate fraction and the detergent also showed some stabilizing effect when the enzyme was stored as a solution. The enzyme of particulate membrane fraction was quite stable, but once treated and solubilized by a detergent, the enzyme often became labile. It is quite probable that some molecules of Triton X-100 must be associated with the enzyme of hydrophobic region, since the enzyme preparation which was freed from the excess detergent still showed a deep absorption peak around 270 to 290 nm in the ultraviolet range. Partial decrease in specific activity was observed with the dimeric enzyme and resumed to its original activity by the addition of Triton X-100. Dimeric enzyme could be formed by interaction between hydrophobic regions of two enzyme molecules under the conditions that the detergent is deficient.

Further characterization on the particulate alcohol dehydrogenase in connection with vinegar fermentation and reconstitution study of the enzyme from its subunits are under progress. As described above, glucose dehydrogenase, gluconate dehydrogenase, ketogluconate dehydrogenase, glycerol dehydrogenase, sorbitol dehydrogenase^{11-14, 21, 22} have also been purified and characterized to compose an enzyme complex as flavo-cytochrome protein. A group of these enzymes are specifically coupled with electron transfer systems in acetic acid bacteria and other oxidative bacteria. In other words, fermentative production of gluconate, ketogluconate, acetate and so on by these bacteria are strongly associated with their respiratory systems. We believe that further characterization of these enzyme systems should lead us to complete understanding of the nonphosphorylative oxidative metabolism of carbohydrates in acetic acid bacteria and other oxidative bacteria.

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