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Purification and Characterization of Polyethylene Glycol Dehydrogenase Involved in the Bacterial Metabolism of Polyethylene Glycol

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Polyethylene glycol (PEG) dehydrogenase in crude extracts of a PEG 20,000-utilizing mixed culture was purified 24 times by precipitation with ammonium sulfate, solubilization with laurylbetaine, and chromatography with diethylaminoethyl-cellulose, hydroxylapatite, and Sephadex G-200. The purified enzyme was confirmed to be homogeneous by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The molecular weight of the enzyme, which appeared to consist of four identical subunits, was 2.4×10^5. The enzyme was stable below 35°C and in the pH range of 7.5 to 9.0. The optimum pH and temperature of the activity were around 8.0 and 60°C, respectively. The enzyme did not require any metal ions for activity and oxidized various kinds of PEGs, among which PEG 6,000 was the most active substrate. The apparent K_m values for tetraethylene glycol and PEG 6,000 were about 10.0 and 3.0 mM, respectively.

The bacterial metabolism of polyethylene glycol (PEG) was clarified by Kawai et al., who found that the first step of PEG metabolism was catalyzed by PEG dehydrogenase (6).

Several papers have reported on lower-molecular-weight PEG dehydrogenases which require flavin adenine dinucleotide, ferricyanide, or nicotinamide adenine dinucleotide for activity (3, 9-11). However, higher-molecular-weight PEG dehydrogenases have been unknown until recently. In a previous paper (6), we have partially purified the PEG dehydrogenase from a mixed culture of *Flavobacterium* and *Pseudomonas* species capable of growth on PEG 20,000 and have shown some of its properties.

In this paper we report the further purification and characterization of the 2,6-dichlorophenolindophenol (DCPIP)-dependent higher-molecular-weight PEG dehydrogenase involved in PEG metabolism by a mixed culture of *Flavobacterium* and *Pseudomonas* species.

MATERIALS AND METHODS

Materials. The PEGs used were the same as those listed previously (8). Laurylbetaine was a kind gift from Kao-Atlas Co. Ltd., Tokyo, Japan. Ferritin, catalase, aldolase, ribonucleic acid polymerases (α, β, and β'), phosphorylase a, fumarase, egg albumin (ovalbumin), and bovine serum albumin (for sodium dodecyl sulfate-polyacrylamide gel electrophoresis) were all purchased from Boehringer Mannheim GmbH, Mannheim, West Germany. Bovine serum albumin for gel filtration was a product of Sigma Chemical Co., St. Louis, Mo. The diethylaminoethyl-cellulose was a product of Brown Co. Hydroxylapatite was purchased from Seikagaku Kogyo Co. Ltd., Tokyo, Japan. Sephadex G-25 (coarse) and Sephadex G-200 (fine) were products of Pharmacia Fine Chemicals, Uppsala, Sweden, and collodion bags were obtained from Sartorius Membranfilter GmbH, Göttingen, West Germany. All other chemicals were commercial products and were used without further purification.

Microorganisms. A synergistic mixed culture (*Flavobacterium* and *Pseudomonas* species; 5, 6, 8) was used throughout this study. The mixed culture was carried on a PEG 6,000 slant and was always used as the mixed culture.

Cultivation. The mixed culture was grown on a PEG 6,000 medium (6) in a 2-liter flask containing 500 ml of medium. The flask was incubated with shaking at 28°C for 6 days, before cells were harvested by centrifugation and washed twice with physiological saline. The washed cells were stored at −20°C until use.

Assay of PEG-oxidizing activity. The enzyme activity was assayed by measuring the initial rate of DCPIP reduction. The standard reaction mixture contained 250 μmol of potassium phosphate buffer (pH 8.0), 2.5 μmol of potassium cyanide, 0.25 μmol of DCPIP, 100 mg of PEG 6,000, and an appropriate amount of the enzyme in a total volume of 2.50 ml and was incubated at 30°C. The reaction was started by the addition of the substrate, and the decrease in absorbance at 600 nm was followed with a Hitachi double-beam spectrophotometer 124 against a blank tube containing the reaction mixture without PEG 6,000.

Protein determination. The protein content was determined by the method of Lowry et al. (7), with bovine serum albumin as the standard.

Definition of unit and specific activity. A PEG

dehydrogenase unit was defined as the amount of enzyme that reduced 1 μmol of DCPIP per min under the standard conditions. The specific activity was expressed as enzyme units per milligram of protein.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Disc gel electrophoresis was carried out by the method of Weber and Osborn (12) as modified by Hayashi and Ohba (4). The native enzyme was dialyzed against 10 mM potassium phosphate buffer, pH 7.2, containing 1% sodium dodecyl sulfate, 25% glycerol, and 3% 2-mercaptoethanol at 30°C for 3 h. The dialyzed enzyme was then incubated in the same buffer at 50°C for 3 h before the run, and several micrograms of the dialyzed enzyme was applied to electrophoresis with 5% gel. The gel was stained with Coomassie brilliant blue. To estimate the molecular weight of the enzyme subunit, relative migration versus logarithm of the molecular weight was obtained with marker proteins, which were treated in a manner similar to that for the dialyzed enzyme described above.

Estimation of molecular weight by gel filtration. The molecular weight of PEG dehydrogenase was determined by gel filtration on Sephadex G-200 according to the method of Andrews (2). The column (1.5 by 56 cm) was equilibrated with 0.05 M potassium phosphate buffer, pH 8.0, containing 0.2% laurylbetaine and 0.05 M KCl. The purified enzyme, which was concentrated by ultrafiltration in a collodion bag, was eluted in 0.75-ml fractions at a flow rate of 3.8 ml/h. The elution volume for the purified enzyme was obtained by measuring the absorbance at 280 nm and also by measuring its catalytic activity. The marker proteins were applied to the same column and were eluted under the same conditions as the purified enzyme. Elution volumes for marker proteins were obtained by measurement of absorbance at 280 nm for egg albumin, bovine serum albumin, aldolase, and ferritin. That for catalase was determined by measuring its catalytic activity.

RESULTS AND DISCUSSION

Purification of the enzyme. All operations were performed at <4°C throughout the purification procedure. The basal composition of buffers used was as follows. Buffer A was composed of 0.05 M potassium phosphate buffer, pH 8.0, 0.5 mM ethylenediaminetetraacetate, and 10% glycerol. Buffer B contained the same components as buffer A plus 0.2% laurylbetaine.

(i) Preparation of crude extract. The washed cells (about 20 g, wet weight) were suspended in 200 ml of buffer A and disrupted in 100-ml portions by treatment on ice for 15 min with a Kaijo-Denki 19-kHz ultrasonic oscillator.

(ii) First ammonium sulfate fractionation. Ammonium sulfate was added to the turbid mixture containing the extract and cell debris (about 2.15 g of protein) to reach 30% saturation, which precipitated the particulate fraction of the sonic extract. After being kept on ice for 2.5 h, the mixture was centrifuged at 8,000 × *g* for 20 min. The precipitate was washed once with buffer A containing ammonium sulfate to 30% saturation.

(iii) Solubilization with laurylbetaine. The washed precipitate was collected by centrifugation at 8,000 × *g* for 20 min and was suspended with buffer A containing ammonium sulfate to 30% saturation and 0.4% laurylbetaine. After being kept on ice for 3 h, the suspension was centrifuged and the supernatant solution was collected. The precipitate was resuspended in buffer A containing ammonium sulfate to 30% saturation and 0.4% laurylbetaine and then was centrifuged.

(iv) Second ammonium sulfate fractionation. The combined supernatant solution (about 0.6 g of protein) was brought to 70% saturation with ammonium sulfate. The insoluble material was collected by centrifugation after standing at 4°C overnight and was dissolved in a small amount of buffer B. The solution was desalted on a Sephadex G-25 (coarse) column equilibrated with buffer B. Laurylbetaine (0.2%) was added to prevent aggregation.

(v) Diethylaminoethyl-cellulose column chromatography. The enzyme solution (about 0.5 g of protein) was applied to a diethylaminoethyl-cellulose column (2.8 by 25 cm) equilibrated with buffer B. After the column was washed thoroughly with the same buffer, the enzyme was eluted with buffer supplemented with 0.1 M potassium chloride at a flow rate of 95 ml/h. The active fractions were collected and subjected to the next procedure without dialysis.

(vi) Hydroxylapatite column chromatography. The enzyme solution (about 50 mg of protein) was placed onto a hydroxylapatite column (2.7 by 2 cm) equilibrated with buffer B. The column was washed with the same buffer; however, the concentration of potassium phosphate buffer was changed to 0.1 M. The enzyme was eluted by raising the concentration of potassium phosphate buffer to 0.2 M at a flow rate of 8 ml/h. The active fractions were combined and absorbed to dry powder of Sephadex G-200.

(vii) Sephadex G-200 column chromatography. The enzyme solution containing Sephadex G-200 gel was placed on a Sephadex G-200 column (2.5 by 55 cm) equilibrated with buffer B and was eluted with the same buffer at a flow rate of 7 ml/h. The active fractions were combined, and the homogeneity of the enzyme was confirmed as described below. The enzyme solution was faintly colored, but the ultraviolet absorption spectrum of the purified enzyme showed that it did not contain flavin as a cofactor. A summary of the purification is presented in Table 1.

Homogeneity of the purified enzyme and estimation of the molecular weight. Since laurylbetaine interfered with normal polyacrylamide disc gel electrophoresis, the purity of the enzyme was confirmed by the appearance of a single band of protein on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The purified enzyme migrated toward the anode as a single band of protein (Fig. 1). By comparing the relative mobility of this band with those of other marker proteins of known molecular weights, the molecular weight of the protein unit was estimated to be 6.0×10^4 to 6.2×10^4.

The molecular weight of this enzyme also was determined by gel filtration on Sephadex G-200 (Fig. 2). The single protein peak was obtained by measurement of absorbance at 280 nm, and it coincided with the peak of the enzyme activity. By comparing the elution volume of PEG dehydrogenase with those of marker proteins, the molecular weight of this enzyme was estimated to be about 2.4×10^5. From these results, the enzyme appeared to consist of four identical units.

Characteristics of the enzyme. The enzymatic properties of the purified enzyme were characterized.

(i) pH and heat stability. After being kept at various pH values at 5°C for 12 h, the enzyme was reacted with PEG 6,000 at pH 8.0. From the remaining activity at each pH, the enzyme was found to be stable between pH 7.5 and 9.0. However, about 40% of the initial activity was lost at pH 7.0 and 10.0. The thermal stability of the enzyme was measured in 0.05 M potassium phosphate buffer at pH 8.0 after preincubating the enzyme at various temperatures for 15 min. The activity was almost stable at <35°C, but 94% of the initial activity was lost at 60°C.

(ii) Effects of temperature and pH on enzyme activity. The initial velocity of DCPIP reduction by the enzyme increased at temperatures up to around 60°C and rapidly declined at those above 60°C (Fig. 3). The enzyme showed

TABLE 1. *Summary of purification of PEG dehydrogenase*

Fraction	Total protein (mg)	Total activity (U)	Sp act (mU/mg)	Purification (fold)	Yield (%)
Crude extract	2,150	108	50	1	100
Ammonium sulfate precipitate (0 to 30% saturation)	1,460	94.5	65	1.3	88
Solubilization with laurylbetaine	596	69.6	117	2.3	65
Ammonium sulfate precipitate (30 to 70% saturation)	486	47.6	98	2.0	44
Diethylaminoethyl-cellulose	51	45.9	905	18.1	43
Hydroxylapatite	21	24.3	1,140	22.8	23
Sephadex G-200	11	13.5	1,200	24.0	13

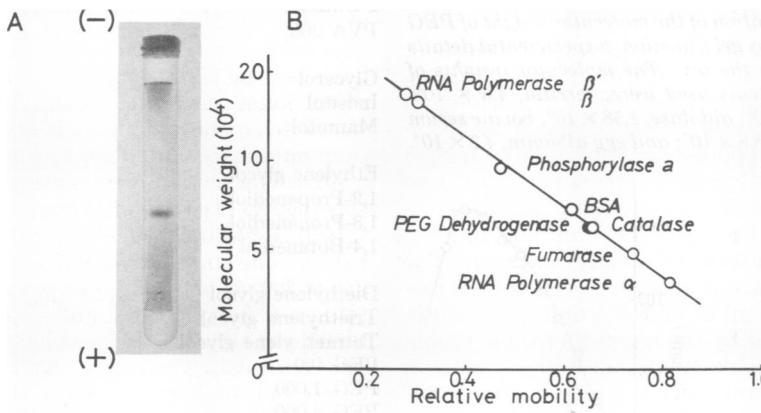

FIG. 1. *Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and molecular weight determination of the purified PEG dehydrogenase. The purified enzyme treated with sodium dodecyl sulfate was subjected to electrophoresis as described in the text. (A) Gel was stained with Coomassie brilliant blue solution. (B) Subunit molecular weights of marker proteins used are: ribonucleic acid (RNA) polymerase—α subunit, 3.9 × 10^4; β subunit, 1.55 × 10^5; and β′ subunit, 1.65 × 10^5; phosphorylase a, 9.4 × 10^4; bovine serum albumin (BSA), 6.8 × 10^4; catalase, 6.0 × 10^4; and fumarase, 4.9 × 10^4.*

an optimum reactivity at around pH 8.0 toward PEG 6,000 at a concentration of 5 mM (Fig. 3). Potassium phosphate buffer appeared to activate the enzyme markedly compared with other buffers.

(iii) Effects of various reagents and metal ions on activity. The various reagents and metal ions were previously added to the reaction mixture which did not contain PEG 6,000, and the mixture was preincubated at 30°C for 5 min. The reaction was started by the addition of PEG 6,000. Table 2 shows the relative activity. No reagent or metal activated the enzyme activity markedly. Although the sulfhydryl reagents *p*-chloromercuribenzoate and monoiodoacetate and heavy metals such as Hg^{2+} and Cu^{2+} inhibited the activity, the enzyme activity was not affected by 2-mercaptoethanol. The chelating agents ethylenediaminetetraacetate, *o*-phenanthroline, α,α'-dipyridyl, sodium fluoride, and sodium azide did not inhibit the enzyme activity at concentrations of 1 mM.

(iv) Substrate specificity. Table 3 presents the enzyme activity toward various hydroxyl compounds. Primary alcohols and PEGs which have a relatively longer carbon chain were dehydrogenated at higher rates among the substrates tested. PEG dehydrogenase oxidized *n*-propyl alcohol–*n*-heptanol, but methanol and ethanol were barely oxidized at a final concen-

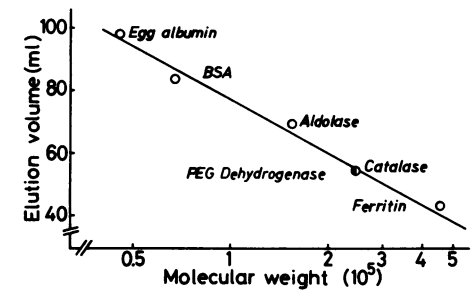

FIG. 2. *Estimation of the molecular weight of PEG dehydrogenase by gel filtration. Experimental details are described in the text. The molecular weights of the marker proteins used were: ferritin, 4.5 × 10^5; catalase, 2.4 × 10^5; aldolase, 1.58 × 10^5; bovine serum albumin (BSA), 6.8 × 10^4; and egg albumin, 4.5 × 10^4.*

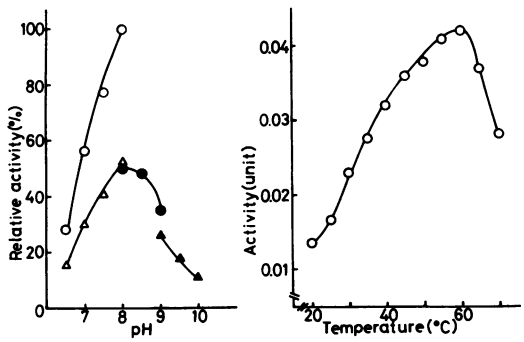

FIG. 3. *Effects of temperature and pH on PEG dehydrogenase activity.*

TABLE 2. *Effects of various chemicals and metal ions on PEG dehydrogenase*

Chemical	Concn (mM)	Relative activity (%)	Metal (1 mM each)	Relative activity (%)
None		100	Hg^{2+}	0
p-Chloromercuribenzoate	0.01	71	Cu^{2+}	0
	0.1	18	Pb^{2+}	64
Monoiodoacetate	0.1	100	Ba^{2+}	91
	1.0	38	Zn^{2+}	41
$NaAsO_2$	1.0	100	Cd^{2+}	36
NaN_3	1.0	100	Ni^{2+}	71
NaF	1.0	98	Mg^{2+}	84
o-Phenanthroline	1.0	95	Ca^{2+}	89
α,α'-Dipyridyl	1.0	114	Mn^{2+}	76
Ethylenediaminetetraacetate	1.0	100	Co^{2+}	72
KCN	1.0	91	Sr^{2+}	100
			Fe^{3+}	67

TABLE 3. *Relative activity of PEG dehydrogenase toward various hydroxyl compounds*

Substrate[a]	Relative activity[b] (%)
Methanol	0
Ethanol	1
1-Propanol	18
1-Butanol	87
1-Pentanol	111
1-Hexanol	115
1-Heptanol	114
2-Propanol	0
2-Butanol	0
PVA 200[c]	0
Glycerol	0
Inositol	0
Mannitol	0
Ethylene glycol	0
1,2-Propanediol	0
1,3-Propanediol	0
1,4-Butanediol	26
Diethylene glycol	4
Triethylene glycol	5
Tetraethylene glycol	25
PEG 400	49
PEG 1,000	26
PEG 4,000	20
PEG 6,000	100
PEG 20,000	10

[a] Final concentration, 5 mM.

[b] The oxidizing activity toward PEG 6,000 was defined as 100.

[c] PVA 200, polyvinyl alcohol 200.

tration of 5 mM. The mixed culture did not grow on methanol-pentanol at all. Among diol-type compounds, ethylene glycol and 1,2- and 1,3-propenediol were not oxidized, but 1,4-butanediol was oxidized to some extent. The enzyme did not act on secondary alcohols such as 2-propanol, 2-butanol, and polyvinyl alcohol or on glycerol or sugar alcohols. Hence the enzyme is assumed to be a kind of primary alcohol dehydrogenase linked with DCPIP. Since methanol, ethanol, ethylene glycol, propylene glycol, diethylene glycol, and triethylene glycol were not or only barely oxidized, the enzyme appeared to require the presence of a certain carbon chain length for its activity. The apparent Michaelis constants for tetraethylene glycol and PEG 6,000 were calculated to be 10.0 and 3.0 mM, respectively, from double-reciprocal plots of the relationship between the initial velocity of the reaction and the concentration of tetraethylene glycol or PEG 6,000. These values and the substrate specificity toward PEGs differ from those of the partially purified enzyme (6). Therefore, a few PEG dehydrogenases with different substrate specificities seem to be contained in PEG 6,000-grown cells. PEG 6,000 and its depolymerized compounds seem to induce different kinds of PEG dehydrogenases. In this paper, we have purified one of these PEG dehydrogenases, the activity of which is highest with PEG 6,000.

From the data on the substrate specificity and the requirement for an electron acceptor, this enzyme is quite different from the previously reported alcohol dehydrogenases (EC 1.1.1.1, 1.1.1.2, 1.1.1.71, 1.1.1.73, 1.1.1.164, and 1.1.99.8, a primary alcohol dehydrogenase of a methanol-utilizing bacterium [13], and a particulate alcohol dehydrogenase from acetic acid bacteria [1]).

LITERATURE CITED

1. **Ameyama, M., K. Tayama, E. Miyagawa, E. Shinagawa, K. Matsushita, and O. Adachi.** 1978. A new enzymatic microdetermination procedure for ethanol with particulate alcohol dehydrogenase from acetic acid bacteria. Agric. Biol. Chem. **42**:2063–2069.
2. **Andrews, P.** 1965. The gel filtration behaviour of proteins related to their molecular weights over a wide range. Biochem. J. **96**:595–606.
3. **Harada, T., and Y. Sawada.** 1977. Occurrence in *Alcaligenes* MC11 of a NAD-dependent dehydrogenase with high specificity towards ether-alcohol compounds. J. Ferment. Technol. **55**:553–559.
4. **Hayashi, K., and Y. Ohba.** 1972. SDS-polyacrylamide gel electrophoresis. (In Japanese) Protein Nucleic Acid Enzyme **17**:304–311.
5. **Kawai, F., M. Fukaya, Y. Tani, and K. Ogata.** 1977. Identification of polyethylene glycols (PEGs)-assimilable bacteria and culture characteristics of PEG 6,000 degradation by a mixed culture. J. Ferment. Technol. **55**:429–435.
6. **Kawai, F., T. Kimura, M. Fukaya, Y. Tani, K. Ogata, T. Ueno, and H. Fukami.** 1978. Bacterial oxidation of polyethylene glycol. Appl. Environ. Microbiol. **35**:679–684.
7. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
8. **Ogata, K., F. Kawai, M. Fukaya, and Y. Tani.** 1975. Isolation of polyethylene glycols-assimilable bacteria. J. Ferment. Technol. **53**:757–761.
9. **Ohmata, S., N. Kojima, and T. Sakai.** 1974. Degradation of polyethylene glycol by a bacterium isolated from soil. (In Japanese) J. Ferment. Assoc. Jpn. **32**:196–202.
10. **Payne, W. J.** 1963. Pure culture studies of the degradation of detergent compounds. Biotechnol. Bioeng. **5**: 355–365.
11. **Payne, W. J., and R. L. Todd.** 1966. Flavin-linked dehydrogenation of ether glycols by cell-free extracts of a soil bacterium. J. Bacteriol. **91**:1533–1536.
12. **Weber, K., and M. Osborn.** 1969. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. **244**: 4406–4412.
13. **Yamanaka, K., and K. Matsumoto.** 1977. Purification, crystallization and properties of primary alcohol dehydrogenase from a methanol-oxidizing *Pseudomonas* sp. No. 2941. Agric. Biol. Chem. **41**:467–475.