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Original Article

Partial purification and characterization of glyoxylate dehydrogenase from *Acetobacter aceti* JCM20276

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Summary

In this study, glyoxylate dehydrogenase was solubilized and partially purified from the membrane fraction of *Acetobacter aceti* JCM20276. The enzyme exhibited high substrate specificity towards glyoxylate. The optimal pH for the enzyme reaction was observed at pH 7 and it showed relatively high activity at 35–45℃. A combination of 2,6-dichlorophenolindophenol and phenazine methosulfate served as the most efficient electron accepter for the enzyme, and its activity was independent of cytochrome *c*, NAD, NADP, and ferricyanide. The addition of Fe^{3+} significantly inhibited the enzyme activity. The results suggest that the glyoxylate dehydrogenase from *A. aceti* JCM20276 is distinct from the glyoxylate dehydrogenases reported from fungi.

Abbreviations: PMS: phenazine methosulfate, DCIP: 2,6-dichlorophenolindophenol.

Introduction

Acetic acid bacteria are gram-negative bacteria belonging to the family *Acetobacteraceae* of the class *Alphaproteobacteria*. These bacteria are strictly aerobic and found in sugary, alcoholic, and acidic environments such as fruits, flowers, and particularly, fermented beverages[1]. Acetic acid bacteria can perform oxidative fermentation, which is a process of incomplete oxidation by membrane-bound dehydrogenases[2]. The membrane-bound dehydrogenase complexes are linked to the respiratory chain, which transfer electrons via ubiquinone and a terminal ubiquinol oxidase to oxygen as the final electron acceptor[1, 2].

Glyoxylate is an intermediate of several metabolic processes and plays an important role in the metabolism of organic acids[3]. In plants and other organisms, glyoxylate is formed by oxidation of glycolic acid, deamination of glycine, or metabolism of tricarboxylic acids in the TCA cycle[4]. The glyoxylate cycle intermediates allow bacteria, fungi, and plants to convert fatty acids into carbohydrates[5, 6]. Enzymatic oxidation of glyoxylate in humans produces calcium oxalate, which is correlated with kidney stone formation[7]. Glyoxylate levels are used as plasma markers for the clinical diagnosis of early diabetes[4]. Enzymatic transformation of glyoxylate to oxalate has been reported in various organisms such as NADP-dependent glyoxylate dehydrogenase (CoA-acylating) from *Pseudomonas oxalaticus*[8], NAD-dependent glyoxylate dehydrogenase from the plant pathogen *Sclerotium rolfsii*[9], cytochrome *c*-dependent flavohemoprotein glyoxylate dehydrogenase from a wood-destroying fungus *Tyromyces palustris*[10], and lactate dehydrogenase from rabbit muscles[11, 12].

The process of oxidation of glyoxylate to oxalate by a membrane-bound enzyme has been partially characterized in an acetic acid bacterium, *Acetobacter aceti* JCM20276 (formerly *Acetobacter dioxyacetonicus* A15)[13]. The solubilized crude enzyme, which was obtained from the particulate fraction of the bacterial cell lysate, shows high specificity to glyoxylate with either oxygen or 2,6-dichlorophenolindophenol (DCIP) as an electron acceptor. Although the exact enzymatic reaction is unclear, H_2O_2 was not produced under the conditions where oxygen is used as the electron acceptor, suggesting that the enzyme is indeed a glyoxylate dehydrogenase rather than glyoxylate oxidase[13]. This system was hypothesized to play an important role in the formation of oxalic acid from glucose and organic acids by *Acetobacter*. In this study, we obtained partially purified glyoxylate dehydrogenase from *A. aceti* JCM20276 with a purity higher than the previously reported one and investigated the properties of the enzyme.

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Materials and Methods

Materials

Glyoxylate, phenazine methosulfate (PMS), DCIP, and other chemicals were purchased from Wako Pure Chemical Co. (Osaka, Japan) and Nacalai Tesque (Kyoto, Japan).

Bacterial strain and culture

Acetobacter aceti JCM20276 was obtained from Japan Collection of Microorganism (Tsukuba, Japan). The bacterium was cultured in a lactate medium consisting of 0.6% sodium lactate, 0.3% yeast extract, and 0.2% polypepton.

Purification of glyoxylate dehydrogenase

After the culture was grown for 2 days at 30℃, cells were harvested by centrifugation. The cells (1 g of wet weight) were suspended in 5 ml of 10 mM potassium phosphate buffer (pH 7) and passed twice through a French press (Stansted fluid power Ltd, Essex, UK) at 16,000 psi. The enzyme was solubilized from the cell lysate by addition of 0.5% Triton X-100 with stirring for 60 min on an ice bath. After centrifugation at 10,000 g for 10 min, the supernatant containing the solubilized enzyme was dialyzed thoroughly against 20 mM Tris-HCl buffer (pH 8) containing 0.1% Triton X-100. The dialyzed enzyme solution was applied onto a Q-Sepharose column (100 ml) equilibrated with 20 mM Tris-HCl buffer (pH 8) containing 0.1% Triton X-100 and 10% glycerol. After that, the column was washed with the same buffer containing 0.1 mM NaCl. The enzyme was eluted using a linear gradient from 0.1 to 0.2 M NaCl in the same buffer.

Enzyme activity assay

Glyoxylate dehydrogenase was assayed in a reaction mixture containing 33 mM of glyoxylate, 0.2 mM of DCIP, 0.6 mM PMS, and 16 mM potassium phosphate buffer (pH 6) in a total volume of 1 ml. The reaction was carried out at 30℃ and absorbance at 600 nm was recorded during the reaction. One unit of activity was defined as the amount of enzyme that catalyzes the conversion of one μmole of the substrate per min.

Effects of pH and temperature

The pH profile was examined using three buffer systems (10 mM each): acetate buffer (pH 4–5), potassium phosphate buffer (pH 6–7), and Tris-HCl buffer (pH 8–9). The temperature profile was determined by testing the activity between 30℃ to 60℃ at pH 6. The thermostability was examined by determining the remaining activity after incubation at 25℃ to 60℃ for 1 h.

Effects of metal ions, inhibitors, and organic solvents

The effect of metal ions (1 mM each) and inhibitors (1 mM each) on the enzyme activity were tested. The enzyme was incubated with metal ions or inhibitors at 4℃ for 1 h before the assay. To test the effect of organic solvents, the enzyme was incubated with methanol, ethanol, acetone, and *n*-hexane (20% and 60%) at 4℃ for 1 h before the assay.

Results

Purification of glyoxylate dehydrogenase

In a previous study, Kasai et al. solubilized the enzyme with deoxycholate from an insoluble membrane fraction obtained by sonication of cells[13], suggesting that glyoxylate dehydrogenase is a membrane-bound protein. In this study, we employed a French press to obtain the intact membrane-bound enzymes and solubilized the enzyme by Triton X-100. The use of the non-ionic surfactant Triton X-100, instead of anionic deoxycholate, for enzyme solubilization permitted us to perform the subsequent ion-exchange chromatography with a Q-Sepharose column. The purification results are summarized in Table 1. The enzyme was partially purified to 11-fold with 12% recovery. The total amount of glyoxylate dehydrogenase obtained was 15.5 mg with a specific activity of 4,190 unit/mg. Unfortunately, further purification could not be carried out as a pilot gel-filtration trial using a portion of the purified enzyme resulted in significant loss of the enzyme activity.

Substrate specificity

The substrate specificity of the enzyme was examined with a wide range of metabolites including aldehydes, hy-

Table 1. Purification of glyoxylate dehydrogenase

Purification step	Total activity (unit)	Total protein (mg)	Specific activity (unit/mg)	Recovery (%)	Purification (-fold)
Crude lysate	555,000	1,420	391	100	1
Solubilized fraction	288,000	1,010	285	52	0.73
Q-Sepharose	65,000	15.5	4,190	12	11

droxy acids, and alcohols, as glyoxylate dehydrogenase-like activity may be exhibited by lactate dehydrogenases as well[11]. We found that the best substrate for our enzyme was glyoxylate. The enzyme also showed slight activity with formaldehyde and formate. The relative enzyme activity for glyoxylate, formaldehyde, and formate was 100%, 8.3%, and 1.3%, respectively. No enzyme activity was detected with glycolate, lactate, malate, oxalate, ethanol, methanol, and butanol. The enzyme preparation by Kasai et al. shows relatively high activity with formaldehyde (about 30% of the activity with glyoxylate)[13]. Therefore, our enzyme preparation exhibited much higher substrate specificity to glyoxylate compared to the previous one, presumably due to the higher purity of our preparation.

Effect of pH on activity and stability

The enzyme exhibited the maximal activity at pH 7 (Fig. 1A). The activity decreased greatly when the pH was decreased below 7 and decreased moderately when the pH was increased over 7. On the other hand, the optimal pH of the previously reported enzyme preparation was at pH 6[13]. The cytochrome *c*-dependent glyoxylate dehydrogenase from *T. palustris* shows the maximal activity at pH 8[10]. The NAD-dependent glyoxylate dehydrogenase from *S. rolfsii* exhibits the maximal activity in the alkaline condition at pH 9[9]. Therefore, the optimal pH of the enzyme from *A. aceti* is lower than the fungal enzymes. The effect of pH on the stability of the enzyme from *A. aceti* has not previously been reported. While this enzyme was most stable at pH 6 and relatively tolerant to alkaline conditions, it was unstable under acidic conditions (Fig. 1B).

Effect of temperature on activity and stability

The enzyme showed relatively high activity at 35–45℃ with the maximum at 35℃ (Fig. C). The enzyme activity dropped rapidly when temperature exceeded 45℃, and lost almost all the activity when temperature was over 55℃. As shown in Fig. 1D, the activity remained over 90% after incubation at 25–35℃ for 1 h and significantly dropped at over 45℃, suggesting its poor heat resistance.

Electron acceptors

We examined the effectiveness of electron acceptors for the enzymatic dehydrogenation of glyoxylate. The mixture of DCIP and PMS, which was not tested in the previous report[13], served as the best electron acceptor (Fig. 2A). The activity with only DCIP was about 3% of that with the DCIP-PMS mixture. Cytochrome *c*, NAD, NADP, and ferricyanide could not serve as electron acceptors for this enzyme. The previous enzyme preparation has been re-

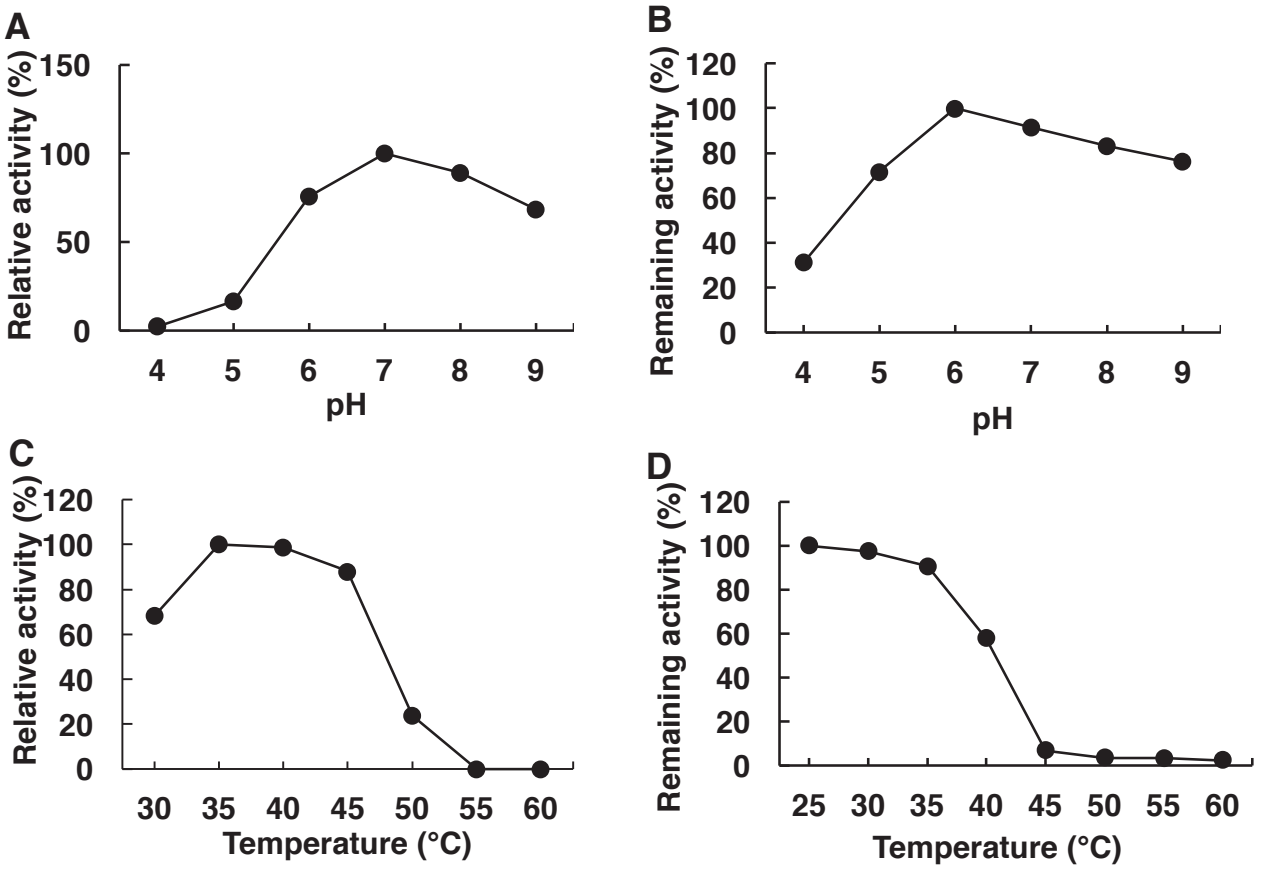

Fig. 1 Effect of pH on activity (A) and stability (B) of glyoxylate dehydrogenase. Effect of temperature on activity (C) and stability (D) of glyoxylate dehydrogenase.

ported to utilize ferricyanide as an electron acceptor with lower efficiency[13], which might be attributed to some contamination. In contrast, cytochrome *c* and ferricyanide are efficiently utilized by cytochrome *c*-dependent glyoxylate dehydrogenase from *T. palustris*[10], suggesting that the enzyme from *A. aceti* is distinct from the cytochrome *c*-dependent enzyme.

Effects of metal ions, inhibitors, and organic solvents

The effect of metal ions on the enzyme activity was investigated. The addition of Fe^{3+} markedly inhibited its activity, while other metal ions (Ca^{2+}, Mg^{2+}, Zn^{2+}, Mn^{2+}, Cu^{2+}) had no or minor, if any, effects on the enzyme activity (Fig. 2B). The effect of Fe^{3+} on the enzyme preparation is significantly different from that seen in case of the previously prepared enzyme, which could not be inhibited by Fe^{3+} [13]. The effect of potential inhibitors was tested as shown in Fig. 2C. The addition of 1 mM EDTA moderately suppressed the enzyme activity by about 30%. KCN and thiourea had slight effects on the enzyme activity, while sodium azide did not inhibit the enzyme (Fig. 2C). The effect of organic solvents at 20% and 60% (v/v) was also examined. The enzyme retained more than 94% of its original activity in ethanol, methanol, acetone, and *n*-hexane, each at 20% (Fig. 2D). Even at a high concentration (60%) of the solvents, the enzyme exhibited more than 50% of activity, suggesting that the enzyme was tolerant towards organic solvents to some extent.

Discussion

The data described above demonstrated that our en-

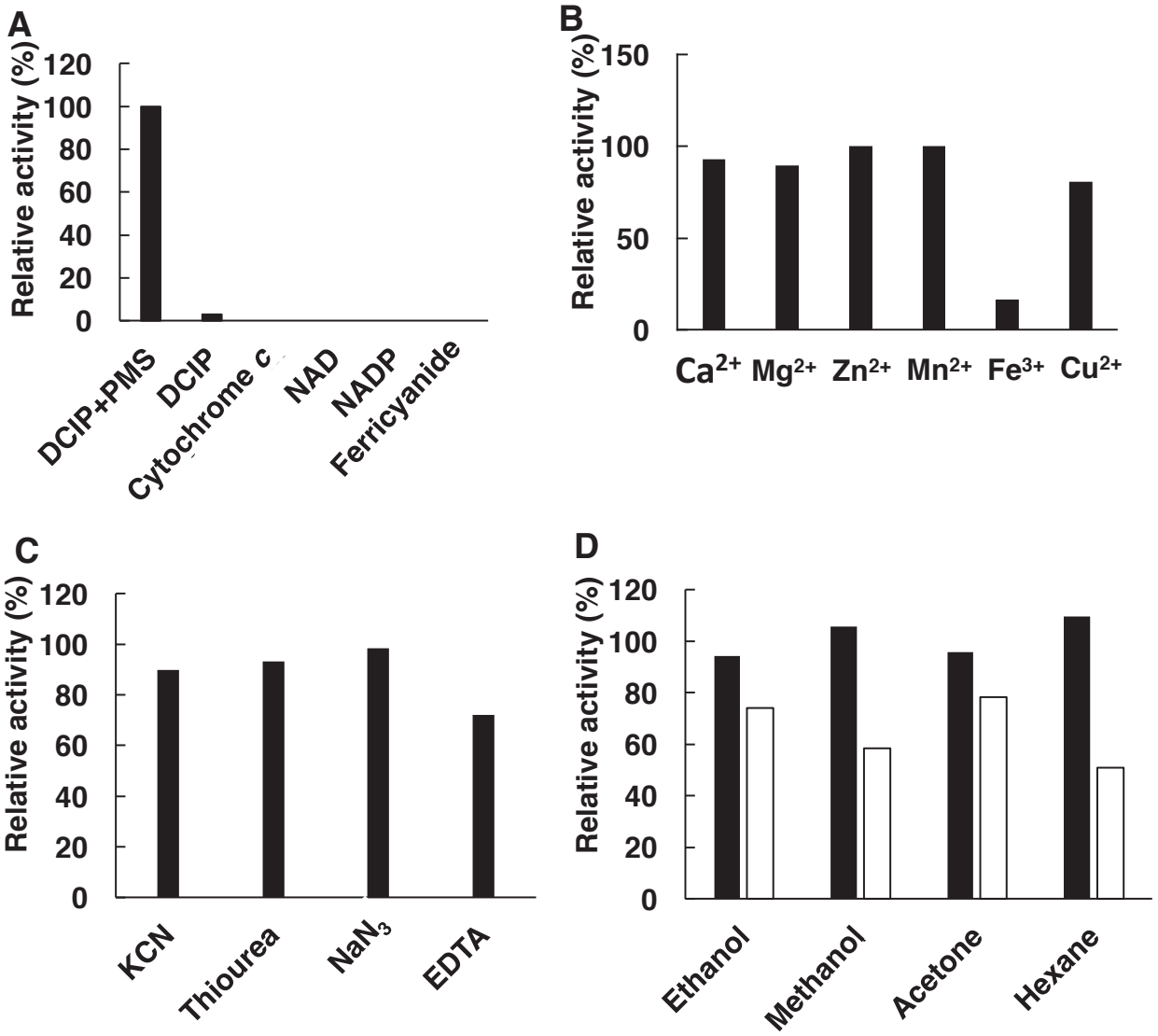

Fig. 2 Effectiveness of electron acceptors (A). The activity with DCIP and PMS was set to 100% in A. Effect of metal ions (B), inhibitors (C), and organic solvents (D) on glyoxylate dehydrogenase activity. The concentration (v/v) of organic solvent used were 20% (the filled bars) and 60% (the open bars) in D. Activity of the enzyme before incubation with metal ions (B), inhibitors (C), and organic solvents (D) was set to 100%.

zyme preparation from *A. aceti* JCM20276 acts as a glyoxylate dehydrogenase and it is essentially identical to the enzyme that was reported by Kasai et al[13]. However, we did observe some key differences between these two preparations such as the optimal pH, utilization of ferricyanide as an electron acceptor, and inhibition by Fe^{3+} [8]. This is not surprising because the two preparations were obtained by different purification procedures and there might be chances of contamination with different proteins. The enzyme preparation described in this study exhibited higher specificity to glyoxylate and did not show any activity towards ferricyanide, suggesting that we have succeeded in obtaining a more purified form of the enzyme as compared with the reported one. Importantly, our study revealed some new properties of the enzyme such as successful solubilization by Triton X-100, no activity towards alcohols and hydroxy acids, the effect of pH on the stability, optimal temperature for activity, thermostability, efficient utilization of DCIP-PMS as the electron acceptors, and relatively high tolerance towards organic solvents.

Acetic acid bacteria have many membrane-bound and cytosolic oxidoreductases[2]. For instance, there are 32 membrane-bound dehydrogenases, 11 with identified and 21 with unidentified substrate specificities in the genome of *Gluconobacter oxydans* ATCC621H[14, 15]. Many membrane-bound enzymes, such as alcohol dehydrogenases, reported from acetic acid bacteria are pyrroloquinoline quinone- or flavin-dependent enzymes containing heme *c* moieties as the electron transfer mediators[16, 17]. On the other hand, the nature of the prosthetic group of membrane-bound aldehyde dehydrogenases, which oxidizes short-chain aldehydes, is still unclear[2]. The glyoxylate dehydrogenase from *A. aceti* could potentially be similar to the short-chain aldehyde dehydrogenase reported from acetic acid bacteria[18–20] as the conversion of glyoxylate to oxalate catalyzed by glyoxylate dehydrogenase includes the oxidation of the aldehyde group, which is essentially identical to the reaction catalyzed by the short-chain aldehyde dehydrogenase. However, further studies are required to completely identify and characterize glyoxylate dehydrogenase from *A. aceti*, as genomic information indicates that there are still several membrane-bound oxidoreductases remaining to be identified and characterized in acetic acid bacteria.

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