Doctoral Dissertation

Studies on enzymes involved in glyoxylate metabolism in an acetic acid bacterium

September 2020

Doctoral Program in Advanced Life Sciences
Graduate School of Life Sciences
Ritsumeikan University

KUMSAB Jakkaphan

Doctoral Dissertation Reviewed
by Ritsumeikan University

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(酢酸菌におけるグリオキシル酸代謝に関与する酵素の研究)

September 2020

2020 年 9 月

Doctoral Program in Advanced Life Sciences

Graduate School of Life Sciences

Ritsumeikan University

立命館大学大学院生命科学研究科

生命科学専攻博士課程後期課程

KUMSAB Jakkaphan

クンサブ ジャッカファン

Supervisor: Professor MIHARA Hisaaki

研究指導教員：三原 久明 教授

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List of Abbreviations

CBB	Coomassie Brilliant Blue R-250
EDTA	Ethylene diaminetetraacetic acid
PCR	Polymerase chain reaction
Tris	Tris(hydroxymethyl)aminomethane
KPB	Potassium phosphate buffer
GR	Glyoxylate reductase
PMS	Phenazine methosulfate
DCIP	2,6-Dichlorophenolindophenol
DTT	Dithiothreitol
2-ME	2-Mercaptoethanol
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
SDS	Sodium dodecyl sulfate

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Synoptic Abstract

Glyoxylate is an important intermediate in the microbial glyoxylate bypass pathway. However, little is known about enzymes involved in glyoxylate metabolism other than the glyoxylate pathway in acetic acid bacteria. In this study, first, 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°C. 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. Next, an uncharacterized protein with moderate sequence similarities to *Gluconobacter oxydans* succinic semialdehyde reductase and plant glyoxylate reductases/succinic semialdehyde reductases was found in the genome of *A. aceti* JCM20276. The corresponding gene was cloned and expressed in *Escherichia coli*. The gene product was purified and identified as a glyoxylate reductase that exclusively catalyzed the NAD(P)H-dependent reduction of glyoxylate to glycolate. The strict substrate specificity of this enzyme to glyoxylate, the diverged sequence motifs for its binding sites with cofactors and substrates, and its phylogenetic relationship to homologous enzymes suggested that this enzyme represents a novel class of enzymes in the β-hydroxyacid dehydrogenase family. These studies may provide an important clue to clarify the metabolism of glyoxylate in acetic acid bacteria.

Chapter 1

Introduction

Acetic acid bacteria (AAB)

Acetic acid bacteria (AAB) are obligate aerobic bacteria with gram-negative or gram-variable, non-spore forming, ellipsoidal to rod-shaped cells. The bacterial sizes vary between 0.4 and 1 mm in width and between 0.8 and 4.5 mm in length. They are catalase positive and oxidase negative. The optimum temperature of growth is between 28-30°C. The optimum pH for the bacterial growth is 5.0-6.5 while AAB can grow at lower pH values between 3 and 4 (Holt et al., 1994). AAB are heterogeneous assemble, comprising both peritrichously and polarly flagellated organisms. They could be motile due to the presence of peritrichous or polar flagella (Sengun & Karabiyikli, 2011). Acetic acid bacteria are belonged to the family *Acetobacteraceae* of *Alphaproteobacteria*, which includes the genera *Acetobacter, Gluconobacter, Gluconacetobacter, Acidomonas, Asaia, Kozakia, Swaminathania, Saccharibacter, Neoasaia, Granulibacter, Tanticharoenia, Ameyamaea, Neokomagataea,* and *Komagataeibacter* (Yamada & Yukphan, 2008; Yukphan et al., 2009, 2008). AAB are widespread in nature on various plants such as fruits, cereals, herbs, and flowers. AAB are also present in sugary, acidic, and alcoholic fermented beverages and foods (Bartowsky & Henschke, 2008; Gupta et al., 2001; Mamlouk & Gullo, 2013; Moonmangmee et al., 2000; Raspor & Goranovič, 2008; Sengun & Karabiyikli, 2011). AAB have been widely studied because of their role in food production and biotechnological industry. AAB can perform oxidative fermentation, which is a process of incomplete oxidation where the substrates are oxidized by membrane-bound dehydrogenases. Carbohydrates were processed via the partial oxidation by AAB and the products (aldehydes, ketones, and organic acids) were released into culture media (Adachi et al., 2003). The electrons are abstracted from the substrate by a cofactor of specific enzymes, and then transferred to the terminal oxidase via respiratory ubiquinone. O_2 is constantly required for oxidative fermentation. O_2 availability is important for the fermentation rate and the rate of compound production

(Gullo et al., 2014; Qi et al., 2013; Schlepütz et al., 2013). The most typical example of oxidative fermentation is the acetic acid production from ethanol (Mamlouk & Gullo, 2013; Saichana et al., 2015). AAB are used as an alternative to the chemical synthesis for production of valuable biocatalysts useful compounds in the industry (Sengun & Karabiyikli, 2011). Several carbohydrates can be used by AAB as carbon sources. AAB showed catalytic activity on variety of organic acids, such as acetic, citric, fumaric, lactic, malic, pyruvic, and succinic acids. In addition, *Gluconobacter* preferred sugar as a carbon source, as compared with *Acetobacter*. *Gluconobacter* species can generate energy more efficiently by the metabolization of the sugars via pentose phosphate pathway (Saichana et al., 2015).

Acetobacter aceti

Acetobacter aceti is an important microorganism in food industry, which shows the ability to oxidize many types of substrates during fermentation process. *Acetobacter* strains have been known to produce acetic acid and exhibit resistance to acetic acid and ethanol. *Acetobacter* strains are known to be useful features for industrial production of acetic acid and vinegar (Saichana et al., 2015). The acetic acid is produced by incomplete oxidation of ethanol through processes catalyzed by membrane-bound enzymes. Ethanol is mainly utilized as an energy source and is rarely utilized as a carbon source during the incomplete oxidation (Kazunobu et al, 2016.; Sengun & Karabiyikli, 2011).

Glyoxylate and its importance in industry

Glyoxylate, which is also known as 2-oxoacetic acid, is the simplest aldehyde acid. glyoxylate has double features of both acid and aldehyde that can generate dozens of fine chemical products chemicals. glyoxylate is an important organic acid in the chemical, cosmetic, pharmaceutical, and food industries, widely used in medicine, spices, pesticides, paint, paper, food additives, and other fields. Monohydrate of glyoxylate is white crystals, of which melting point is 52°C, and it is easily soluble in water, while in water, it exists in the form of hydras dihydroxyacetic acid (S. Zhang, 2012). glyoxylate is produced in

several ways, for example, by nitric acid oxidation of glyoxal, by catalytic oxidation of ethylene or acetaldehyde, and by electrochemical reduction of oxalic acid. glyoxylate is difficult to determine quantitatively using traditional analytical methods such as acid-base or redox titrations, separation, or precipitation. The determination of glyoxylate in its electrochemical synthesis reaction mixture is a difficult process because this mixture contains carboxylic acids with convergent acidic dissociation constants and other compounds carrying organic groups that can undergo oxidation or reduction such as ethylene glycol, glyoxal, and glycolic acid (Pozdniakov et al., 2015, 2016). Spectrophotometric analysis of glyoxylate has been used to determine enzyme activity by a color reaction with 2-aminobenzaldehyde and glycine or by forming a color product with phenylhydrazine, but this method can be affected by the presence of other aldehydes in the mixture (Abdulwahed et al., 2020). Glyoxylate derivatives are high-value-added fine chemical products and widely used. At present, major glyoxylate derivatives are vanillin, ethyl vanillin, heliotropine, allantoin, hydroxyphenyl hydantoin, hydroxyphenylglycine, and hydroxy phosphonic acid (S. Zhang, 2012). Vanillin is used in perfume, food, and pharmaceutical industries. glyoxylate is also utilized in the production of pharmaceuticals such as β-lactam anti-biotics (methicillin, oxacillin, and nafcillin), anti-inflammatory agents (allantoin), and antihypertensive agents (atenolol). glyoxylate has been used in agrochemistry (glyphosate production). Allantoin, hydroxyphenyl hydantoin, and hydroxyphenylglycine are three kinds of glyoxylate derivatives with wide market demand and promising application prospects (Kumar et al., 2012). Allantoin widely exists in nature, but due to its low contents, complicated extraction processes are required, resulting in high cost. glyoxylate market is caused by increasing manufacture of allantoin, which is related to increasing demand for keratolytic (antimicrobial and antiparasitic) ingredients in skin care products, and vanillin, which is in demand in food industry. glyoxylate is of interest as an investigation object for both fundamental science and various industry branches (Pozdniakov et al., 2019; S. Zhang, 2012).

Glyoxylate in organisms

Glyoxylate is a compound of a central importance in metabolisms of various organisms. It is found in plants and involved in the metabolic cycle of animals. In plants and some organisms, glyoxylate is produced from the oxidation of glycolic acid, deamination of glycine, or metabolism of tricarboxylic acids in the Krebs cycle (Escher CL & Widmer F., 1997). It was also found in the transamination with amino acids in plants, animals, and microorganisms. Glyoxylate is converted to oxalate under biological systems (Christoph et al., 1998). In addition, glyoxylate cycle intermediate allows bacteria, fungi, and plants to convert fatty acids to carbohydrates. Glyoxylate have been reported conversion to formate and carbon dioxide in rat liver slices and conversion of glycolate to glycine in higher plants. Glyoxylate is an intermediate in the degradation of allantoin by fishes and uric acid by a strain of *Pseudomonas* (Pozdniakov et al., 2019).

Glyoxylate as a medical biomarker

Glyoxylate is served as an early marker in diabetes diagnosis with predictive qualities for associated complications and as potential to guide the development of new antidiabetic therapies, new treatment approaches and towards improved diabetes diagnosis (Nikiforova et al., 2014). Furthermore, glyoxylate is related to kidney stones formation. Oxalate is a metabolic waste product excreted by the kidney. It derives from two main sources, one from ascorbic acid and another from glyoxylate. Oxalate production from glyoxylate oxidation is higher than ascorbic acid. About 50–60% of urinary oxalate is derived from the enzymatic oxidation of glyoxylate. Hyperoxaluria is a common cause of kidney stones by the low solubility of calcium oxalate salts. Severe hyperoxaluria may lead to loss of renal function and systemic oxalosis. Therefore, the enzyme specific to glyoxylate will be useful for developing a new method to detect glyoxylate and kidney stone formation (Carpenter & Merkler, 2003; Asplin, 2002).

Glyoxylate dehydrogenase

Enzymatic transformation of glyoxylate to oxalate has been reported in various

organisms, such as NADP-dependent glyoxylate dehydrogenase (CoA-acylating) from *Pseudomonas oxalaticus* (Quayle, 1963), NAD-dependent glyoxylate dehydrogenase from the plant pathogen *Sclerotium rolfsii* (Balmforth & Thomson, 1984), cytochrome *c*-dependent flavohemoprotein glyoxylate dehydrogenase from a wood-destroying fungus *Tyromyces palustris* (Tokimatsu et al., 1998), and lactate dehydrogenase from rabbit muscles (Banner & Rosalki, 1967). 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) (Kasai et al., 1963). 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 (Kasai et al., 1963). 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, I 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.

Glyoxylate reductase (GR)

Glyoxylate reductase (GR) catalyzes the reduction of glyoxylate to glycolate using either NADPH or NADH as a coenzyme. GRs are highly conserved and present in most known organisms (Rumsby & Cregeen, 1999; Greenler et al., 1989; Ohshima et al., 2001; Booth et al., 2006). GRs usually catalyze the conversion of hydroxypyruvate into D-glycerate in addition to glyoxylate reduction. Due to their overlapped substrate specificity and metabolic role, those enzymes are called glyoxylate reductase/hydroxypyruvate reductase (GRHPR) (Kutner et al., 2018). Alternatively, they also convert succinic semialdehyde (SSA) into 4-hydroxybutyrate, albeit with lower catalytic efficiency than for the glyoxylate to glycolate conversion (Hawes et al., 1996). Thus, GLYR1 and GLYR2 are often termed plant GR/SSA reductases (GR/SSARs). The HIBADH-related family, a member of the β-hydroxyacid dehydrogenases (β-HAD)

superfamily, represents a large structurally and mechanistically related superfamily of enzymes found in all three domains of life (Njau et al., 2001; Tchigvintsev et al., 2012; Mitchell et al., 2019). The HIBADH-related family has evolved from a short-chain alcohol dehydrogenase and usually catalyzes the NAD(P)-dependent oxidation/reduction of various substrates containing β-hydroxy acid/β-aldehyde moieties. In addition to the plant GR/SSARs, the member of this family includes HIBADH (Y. Zhang et al., 2011), tartronate semialdehyde reductase (Tchigvintsev et al., 2012; Meyer et al., 2015), 2-(hydroxymethyl)glutarate dehydrogenase (Kasai et al., 1963), L-serine dehydrogenase (Ueshima et al., 2010), sulfolactaldehyde 3-reductase (Duan et al., 2014), and numerous uncharacterized homologs.

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Chapter 2

Characterization of partially purified glyoxylate dehydrogenase from *Acetobacter aceti*

In this Chapter 2, glyoxylate dehydrogenase from *Acetobacter aceti* JCM20276 was partially purified and characterized. The effect of pH and temperature on glyoxylate dehydrogenase activity were investigated. To understand the enzyme specificity to substrate, various substrates were examined. The effects of metal ions, inhibitors, organic solvents, and electron acceptors on the enzyme were also studied. This Chapter is adapted from Jakkaphan Kumsab, Ruta Tobe, and Hisaaki Mihara. (2019) Partial purification and characterization of glyoxylate dehydrogenase from *Acetobacter aceti* JCM20276. **Trace Nutrients Research**, 36: 81−86.

Materials and Methods

Materials

Glyoxylate, phenazine methosulfate (PMS), 2,6-Dichlorophenolindophenol (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 30ºC, 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°C 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 (10mM 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°C to 60°C at pH

6. The thermostability was examined by determining the remaining activity after incubation at 25°C to 60°C for 1 h.

Effects of metal ions, inhibitors, and organic solvents

The effect of metal ions (1mM each) and inhibitors (1 mM each) on the enzyme activity were tested. The enzyme was incubated with metal ions or inhibitors at 4°C 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°C 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 (Kasai et al., 1963), suggesting that glyoxylate dehydrogenase is a membrane-bound protein. In this study, I 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 (Figure 2-1). 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.

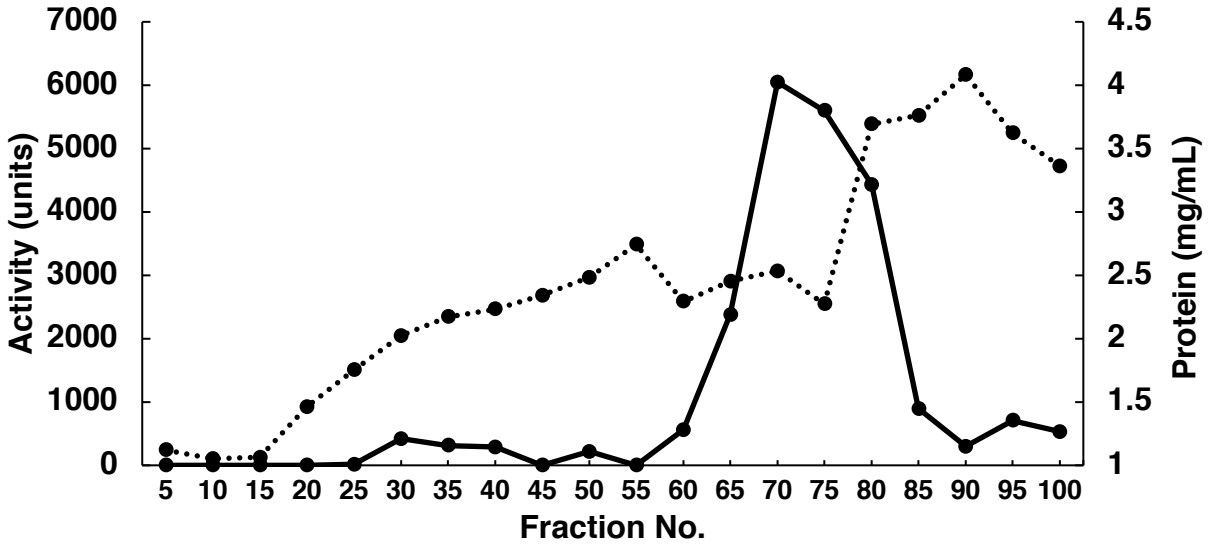

Figure 2-1. Purification of glyoxylate dehydrogenase with a Q-Sepharose column. The black line represents the enzyme activity and the dashed line represents protein concentration (mg/mL). The dialyzed enzyme solution was applied onto a Q-Sepharose column (100 mL) equilibrated with 20 mM Tris-HCl buffer (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. Each fraction (10 mL) was collected per tube.

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	14,20	391	100	1
Solubilized fraction	288,000	1,010	285	52	0.73
Q-Sepharose	65,000	15.5	4,190	12	11

Substrate specificity

The substrate specificity of the enzyme was examined with a wide range of metabolites including aldehydes, hydroxy acids, and alcohols, as glyoxylate dehydrogenase-like activity may be exhibited by lactate dehydrogenases as well (Banner & Rosalki, 1967). I found that the best substrate for our enzyme was glyoxylate (Table 2). 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) (Kasai et al., 1963). 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.

Table 2. Substrate specificity of glyoxylate dehydrogenase

Substrate	Relative activity* (%)
Glyoxylate	100
Glycolate	ND
Formate	1.3
Lactate	ND
L-Malate	ND
Oxalate	ND
Ethanol	ND
Methanol	ND
Butanol	ND
Formaldehyde	8.3

*ND, not detected.

Effect of pH on activity and stability

The enzyme exhibited the maximal activity at pH 7 (Figure 2-2). 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 (Kasai et al., 1963). The cytochrome *c*-dependent glyoxylate dehydrogenase from *T. palustris* shows the maximal activity at pH 8 (Tokimatsu et al., 1998). The NAD-dependent glyoxylate dehydrogenase from *S. rolfsii* exhibits the maximal activity in the alkaline condition at pH 9 (Balmforth & Thomson, 1984). 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 (Figure 2-3).

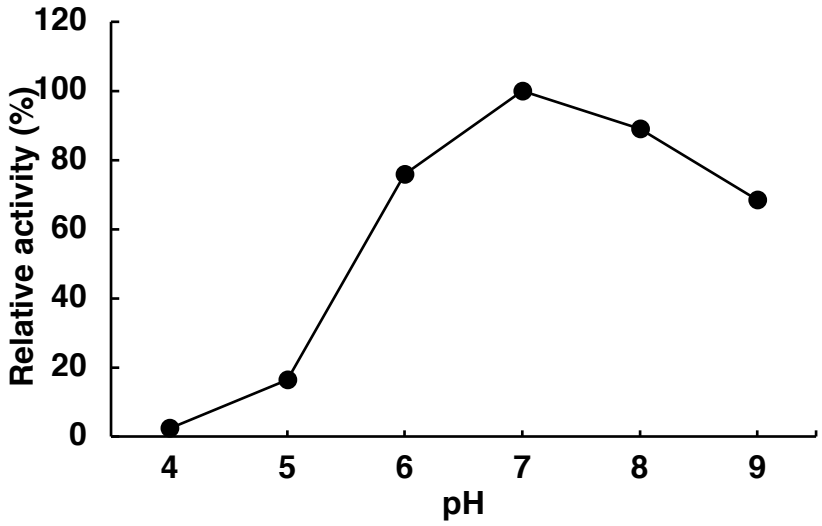

Figure 2-2. Effect of pH on glyoxylate dehydrogenase activity.

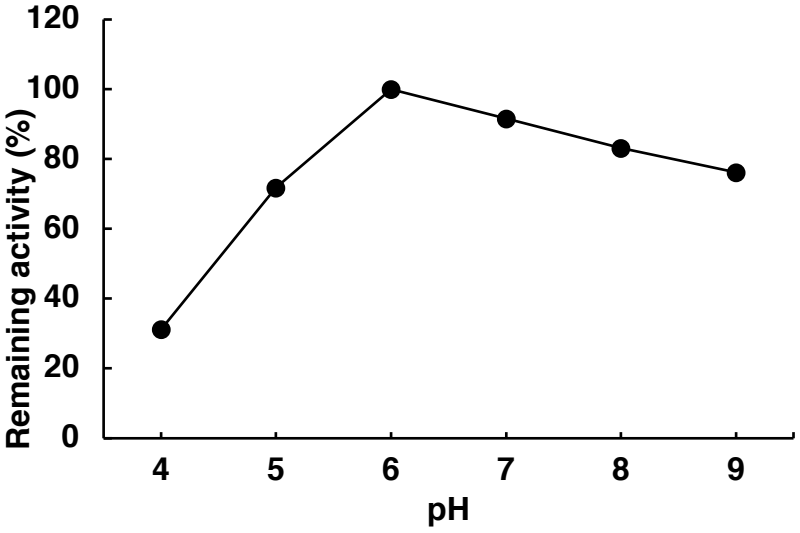

Figure 2-3. Effect of pH on glyoxylate dehydrogenase stability.

Effect of temperature on activity and stability

The enzyme showed relatively high activity at 35-45°C with the maximum at 35°C (Figure 2-4). The enzyme activity dropped rapidly when temperature exceeded 45°C, and lost almost all the activity when temperature was over 55°C. As shown in Figure 2-5, the activity remained over 90% after incubation at 25-35°C for 1 h and significantly dropped at over 45°C, suggesting its poor heat resistance.

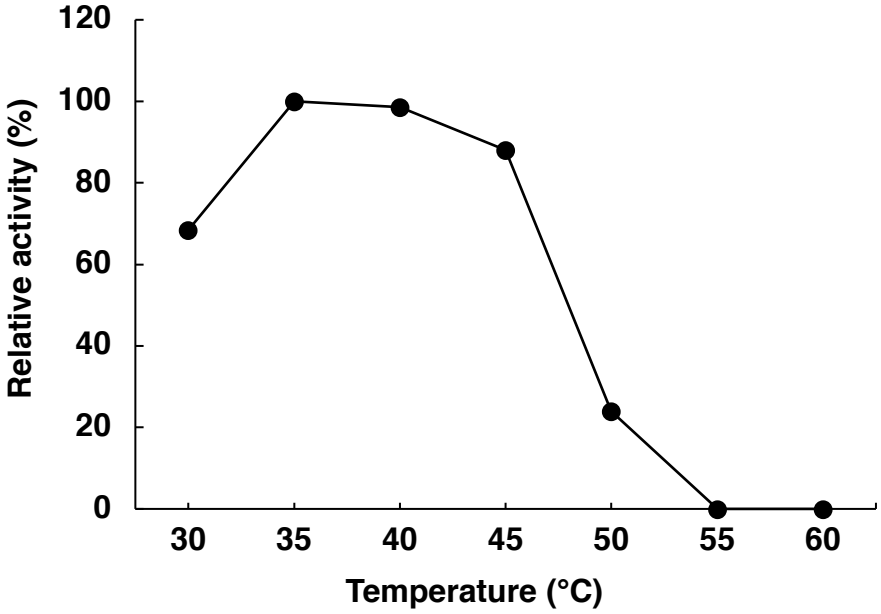

Figure 2-4. Effect of temperature on activity of glyoxylate dehydrogenase.

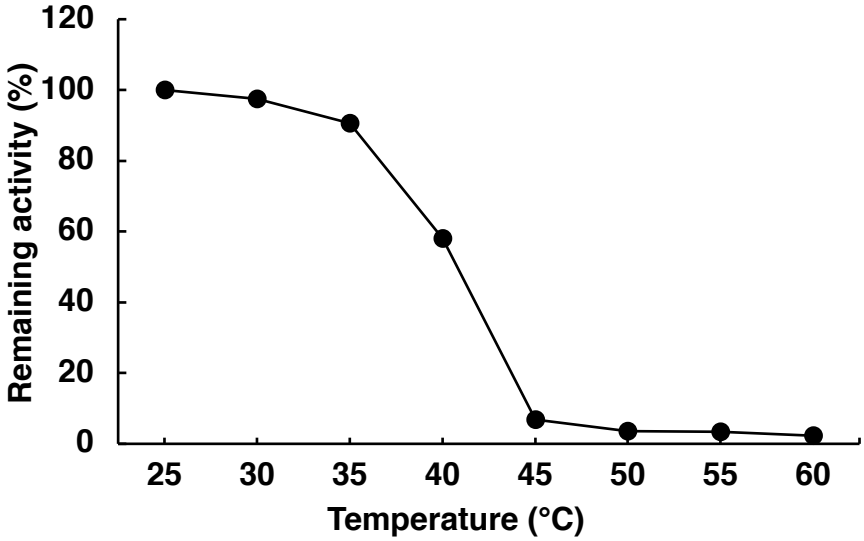

Figure 2-5. Effect of temperature on stability of glyoxylate dehydrogenase.

Electron acceptors

Enzymatic dehydrogenation of glyoxylate was examined the effectiveness of electron acceptors. The mixture of DCIP and PMS, which was not tested in the previous report (Kasai et al., 1963), served as the best electron acceptor (Figure 2-6). 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 reported to utilize ferricyanide as an electron acceptor with lower efficiency (Kasai et al., 1963), 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* (Tokimatsu et al., 1998), suggesting that the enzyme from *A. aceti* is distinct from the cytochrome *c*-dependent enzyme.

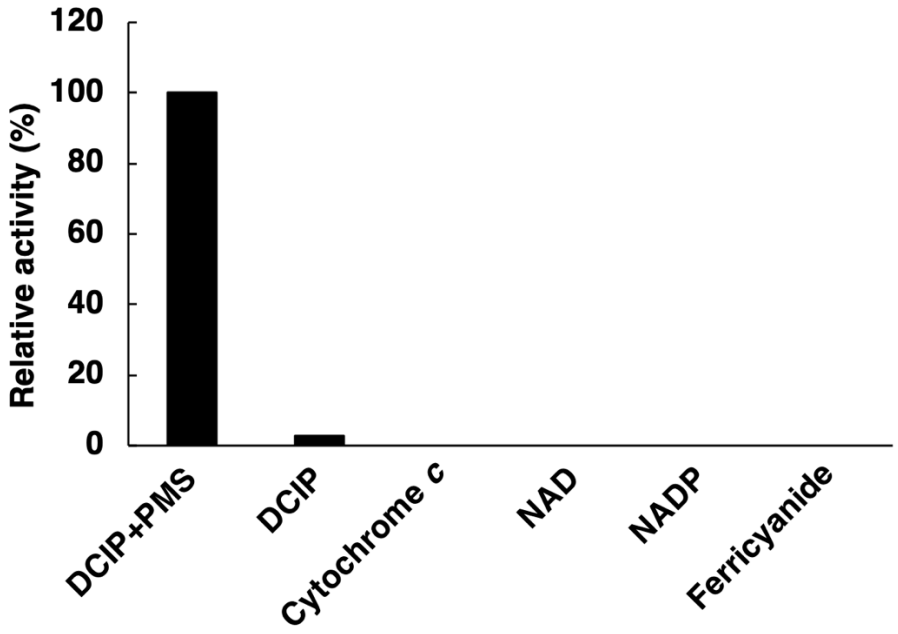

Figure 2-6. Effectiveness of electron acceptors on glyoxylate dehydrogenase activity. Enzyme activity were examined by adding phenazine methosulfate (PMS), 2,6-dichlorophenolindophenol (DCIP), cytochrome *c,* nicotinamide adenine dinucleotide (NAD), nicotinamide adenine dinucleotide phosphate (NADP), or ferricyanide. The activity with DCIP and PMS was set to 100%.

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+}, and Cu^{2+}) had no or minor, if any, effects on the enzyme activity (Figure 2-7). 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+} (Kasai et al., 1963). The effect of potential inhibitors was tested as shown in Figure 2-8. 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 (Figure 2-8). 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% (Figure 2-9). 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.

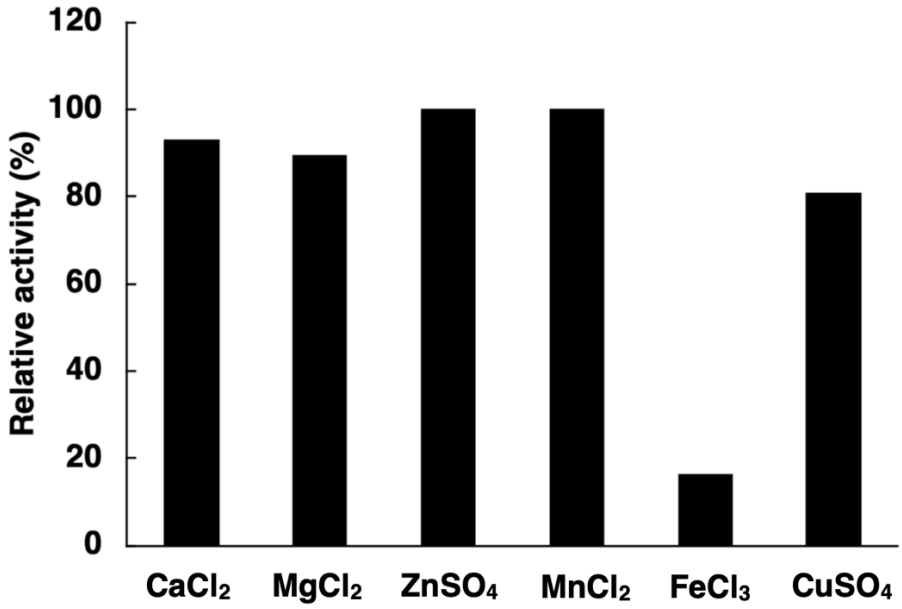

Figure 2-7. Effect of metal ions on glyoxylate dehydrogenase activity.

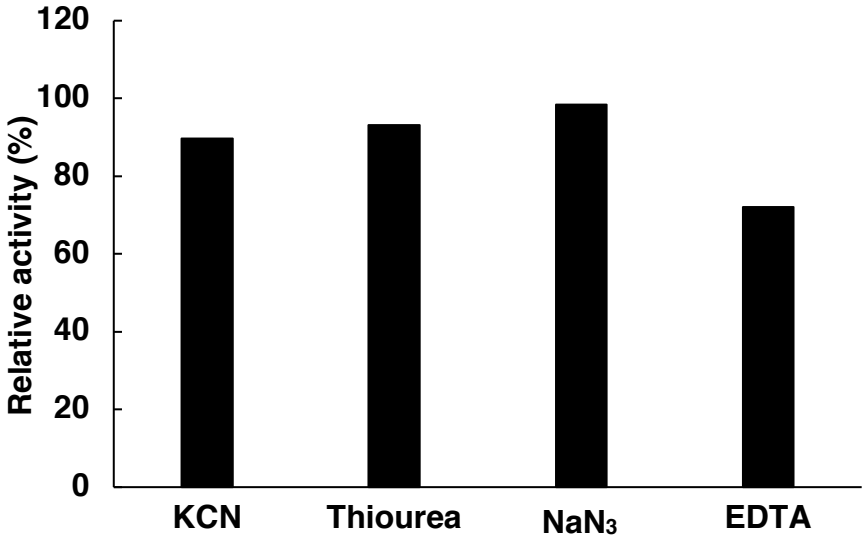

Figure 2-8. Effect of inhibitors on glyoxylate dehydrogenase activity. Enzyme activity was tested with KCN, thiourea, NaN_3, and ethylenediaminetetraacetic acid (EDTA).

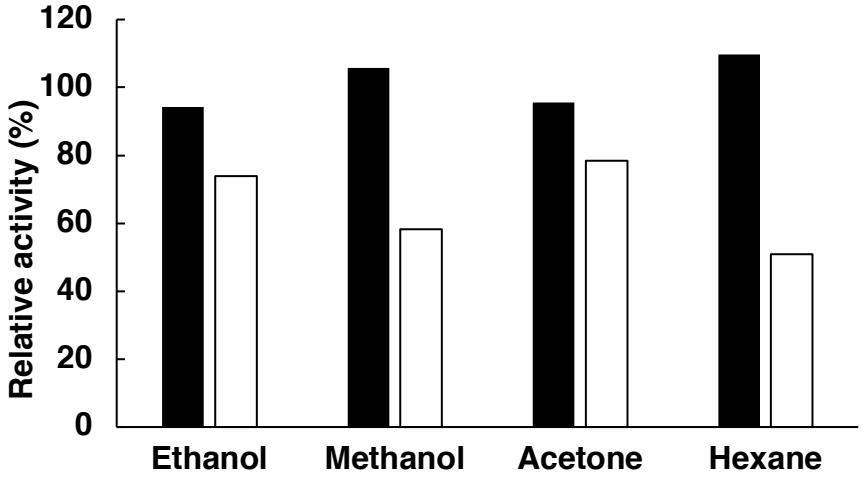

Figure 2-9. Effect of organic solvents on glyoxylate dehydrogenase activity. The concentration (v/v) of organic solvent used were 20% (black bars) and 60% (white bars).

Discussion

The data described above demonstrated that the enzyme 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. (Kasai et al., 1963). However, I 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+} (Quayle, 1963). 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 I 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 (Saichana et al., 2015). For instance, there are 32 membrane-bound dehydrogenases, 11 with identified and 21 with unidentified substrate specificities in the genome of *Gluconobacter oxydans* ATCC621H (Prust et al., 2005; Richhardt et al., 2013). 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 (Ameyama et al., 1981; Masud et al., 2010). On the other hand, the nature of the prosthetic group of membrane-bound aldehyde dehydrogenases, which oxidizes short-chain aldehydes, is still unclear (Saichana et al., 2015). The glyoxylate dehydrogenase from *A. aceti* could potentially be similar to the short-chain aldehyde dehydrogenase reported from acetic acid bacteria (Gómez-Manzo et al., 2010; Thurner et al., 1997) 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.

Conclusion

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°C. A combination of DCIP and PMS 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.

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Chapter 3

Characterization of a novel glyoxylate reductase from *Acetobacter aceti*

In this Chapter 3, glyoxylate reductase gene (*aac4036*) was cloned from *A. aceti.* The protein was expressed and purified by affinity column chromatography. The enzyme was biochemically characterized. In addition, the amino acid sequence of the enzyme was compared with its homologous proteins in other bacteria. This Chapter is adapted from Jakkaphan Kumsab, Ruta Tobe, Tatsuo Kurihara, Yuu Hirose, Taketo Omori, and Hisaaki Mihara (2020) Characterization of a novel class of glyoxylate reductase belonging to the β-hydroxyacid dehydrogenase family in *Acetobacter aceti,* **Bioscience, Biotechnology, and Biochemistry**, in press, doi:10.1080/09168451.2020.1797470.

Materials and Methods

Bacterial strains and culture conditions

A. aceti JCM20276 was provided by the RIKEN BRC through the National BioResource Project of the MEXT/AMED, Japan. *A. aceti* was grown in a lactate medium consisting of 0.6% sodium lactate, 0.3% yeast extract, and 0.2% polypepton. *E. coli* strains DH5α and BL21(DE3) were used for DNA manipulation and cloned gene overexpression, respectively. *E. coli* strains were grown aerobically at 37°C in lysogeny broth (LB) medium containing 100 μg/mL ampicillin.

Cloning of the AacGR gene, *aac4036*

A DNA fragment containing the *aac4036* gene was amplified by polymerase

chain reaction (PCR) with genomic DNA of *A. aceti* JCM20276 and the primers 5′-ATTACATATGGGACTTAGCAGCATGCAGATTC-3′ and 5′-TAATCTCGAGCTACGCCTTTAGCGATCGTTGG-3′. After digestion with NdeI and XhoI, the PCR product was ligated into pColdI (Takara Bio Inc., Shiga, Japan) to yield pAAC4036 encoding AAC4036 with a His-tag sequence at the N-terminal region. This construct was introduced into *E. coli* DH5α for plasmid manipulation.

Purification of AacGR

E. coli BL21(DE3) cells carrying pAAC4036 were cultured in LB medium supplemented with ampicillin (100 μg/mL) at 37°C. When the optical density at 600 nm (OD_{600}) reached 0.5, *aac4036* gene expression was induced with 1 mM isopropyl β-D-thiogalactopyranoside, and cells were cultured at 16°C overnight. The cells were harvested by centrifugation and resuspended in 50 mM Tris-HCl buffer (pH 8.0) containing 150 mM NaCl. The cells were disrupted by sonication for 10 min and centrifuged. The supernatant was then through a Ni-NTA column (GE Healthcare) equilibrated with the same buffer. The enzyme was eluted by a stepwise increase in the imidazole concentration (100 to 150 mM) in the same buffer. The enzyme fractions were then pooled and dialyzed with this buffer.

Native molecular mass determination

The molecular mass of **AacGR** was determined by size exclusion chromatography with a Superdex 200 HR column (GE Healthcare). A mobile phase of 50 mM Tris-HCl buffer (pH 8.0) with 150 mM NaCl was used at a flow rate of 0.5 mL/min. Proteins were monitored at 280 nm. The size marker proteins used were aprotinin (M_r 6,500), RNaseA (M_r 13,700), carbonic anhydrase (M_r 29,000), ovalbmin (M_r 44,000), conalbumin (M_r 75,000), and aldolase (M_r 158,000).

Enzyme assay

GR activity was assayed at 45°C in a reaction mixture containing 100 mM citrate buffer (pH 4.0), 1 mM glyoxylate, 0.15 mM NADH or NADPH, and 0.1-1 μg/mL of enzyme in a total volume of 1 mL. The absorbance at 340 nm was recorded during the reaction with a Shimadzu UV1800 spectrophotometer (Shimadzu, Kyoto, Japan). One unit of activity was defined as the amount of enzyme that catalyzed the reduction and the oxidation of one μmol of NAD(P)H and $NAD(P)^+$, respectively, per min. NAD(P)H-dependent reduction of a substrate by the enzyme was examined with various carboxylic acids, aldehydes, and dicarbonyls at pH of 4.0 and 6.0. $NAD(P)^+$-dependent oxidation of a substrate by the enzyme was examined with various hydroxy acids and amino acids at pH of 4.0, 6.0, and 9.0. Protein was determined using a BCA Protein Assay Kit (Merck, Tokyo, Japan), with bovine serum albumin as a standard.

Effects of pH and temperature on enzyme activity

The effect of pH on enzyme activity was tested by determining the enzyme activity in a reaction mixture containing 100 mM buffer with pH varying between 3.0 and 11.0. The effect of pH on enzyme stability was examined by determining the remaining activity of the enzyme after incubation for 1 h on ice in 100 mM buffers with pH varying from 3.0 to 13.0. The effect of temperature on enzyme activity was determined at various temperatures from 25 to 70°C. The effect of temperature on enzyme stability was examined by determining the remaining activity of the enzyme after incubation in 100 mM citrate buffer (pH 4.0) for 10 min at various temperatures from 45 to 75°C.

Effects of metal ions, inhibitors, and organic solvents on enzyme stability

The enzyme was incubated on ice for 1 h in 100 mM citrate buffer (pH 4.0) containing metal ions or inhibitors at a final concentration of 1 mM before the assay. A portion (3 μL) of the incubated solution was added to the assay mixture with a total volume of 800 μL, which accordingly contained 0.00375 mM metal ions or inhibitors. To test the effects of organic solvents on enzyme activity, the enzyme was incubated on

ice for 1 h in 100 mM citrate buffer (pH 4.0) containing 20-60% methanol, ethanol, or acetone, after which the remaining enzyme activity was determined in the same manner as described for the examination of the effects of metal ions or inhibitors on the enzyme.

Effects of preserving additives on AacGR stability

Measurements enzyme stability was performed with several additive (sucrose, glucose, sorbitol. and glycerol). The enzyme was incubated with 20% of preserving additives (sucrose, glucose, sorbitol, or glycerol). The mixture was kept at 4°C and -20°C for 0, 1, and 7 days before the remaining activity was measured.

Determination of product of AacGR

The purified AacGR was incubated with 1 mM glyoxylate and 1 mM NADPH in 100 mM of citrate buffer (pH 4.0) for 1, 5, 10, and 15 min. The reaction mixture was heated at 100°C in a water bath for 20 min. Glycolate was determined by mixing 50 μL of the reaction mixture with 1 mL of 0.01% 2,7-dihydroxynaphthalene in concentrated sulfuric acid. The color produced was measured at 540 nm with a spectrophotometer. The standard curve was made with various concentrations of glycolate.

Results and Discussion

Sequence analysis of AAC4036

Analysis of the genome sequence of *A. aceti* JCM20276 revealed that this bacterial strain had a gene, termed *aac4036* (GenBank/EMBL/DDBJ accession no. LC549188), that was predicted to encode a putative NAD(P)-dependent oxidoreductase. The putative gene product, AAC4036, showed the highest sequence identity (54%) with *G. oxydans* SSAR (GoxSSAR) (Meyer et al., 2015) among the biochemically characterized enzymes. GoxSSAR exhibits SSAR activity, with low activity toward

glyoxal, phenylglyoxal, and methylglyoxal (Meyer et al., 2015). AAC4036 also shared slight to moderate amino acid sequence identities with enzymes belonging to the HIBADH-related superfamily, such as *Geobacter sulfurreducens* SSARs (30%) (Zhang et al., 2011; Meyer et al., 2015), *A. thaliana* GLYRs (27%) (Zarei et al., 2017), *Eubacterium barkeri* 2-(hydroxymethyl)glutarate dehydrogenase (27%), and *Pyrobaculum calidifontis* 6-phosphogluconate dehydrogenase (26%) (Ueshima et al., 2010) (Figure. 3-1A). This implied that AAC4036 is a member of the HIBADH-related enzyme superfamily. InterProScan Search (Mitchell et al., 2019) (https://www.ebi.ac.uk/interpro/) results also suggested that AAC4036 belongs to the HIBADH-related family (IPR015815). I selected a few representative proteins from each class, including biochemically characterized enzymes, to analyze the phylogeny of AAC4036 (Figure 3-1B.). AAC4036 and GoxSSAR clearly belonged to a distinct clade and were the most divergent from the HIBADH clade. Since the AAC4036 and GoxSSAR clade was related to the clade containing plant GLYRs and *Geobacter* SSARs, I hypothesized that AAC4036 was likely to catalyze the reduction of an aldehyde substrate.

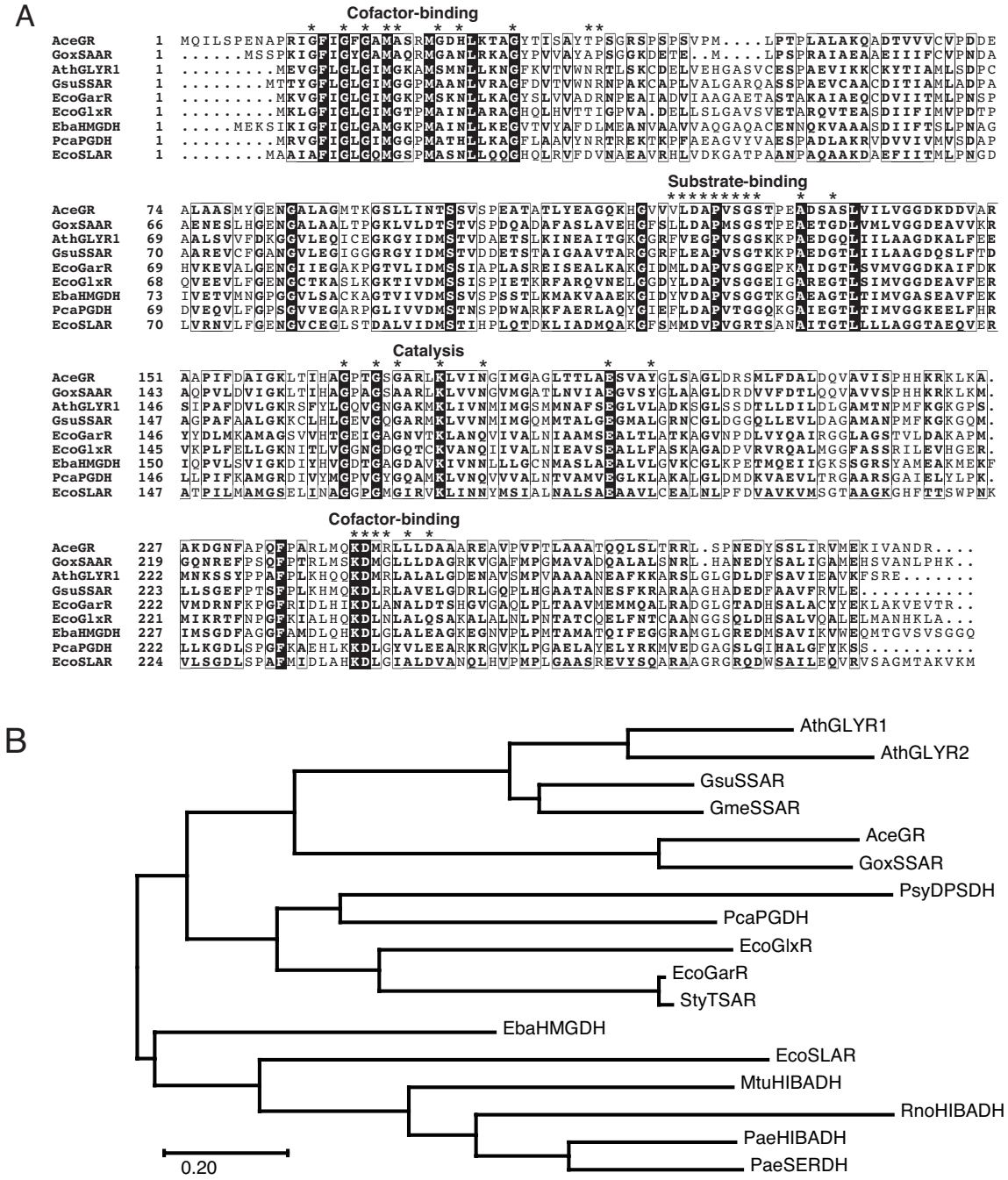

Figure 3-1. Sequence analysis of *Acetobacter aceti* glyoxylate reductase (AacGR) and β-hydroxyacid dehydrogenases (β-HADs). (A) Amino acid sequence alignment of AacGR. (B) Phylogenetic tree of AacGR (LC549188) and related enzymes. Multiple alignments were performed using Multalin (Corpet, 1988)and rendered using ESPript (Robert & Gouet, 2014). Residues conserved in all sequences are shown with reversed text.

Asterisks indicate functionally important residues (Njau et al., 2001). Phylogenetic analyses were conducted in MEGA X (Kumar et al., 2018). Proteins used for analyses were: GoxSSAR, *G. oxydans* SSAR (Q5FQ06); AthGLYR1, *A. thaliana* GR/SSAR1 (Q9LSV0); AthGLYR2, *A. thaliana* GR/SSAR2 (F4I907); GsuSSAR, *G. sulfurreducens* SSAR (Q74DE4); GmeSSAR, *G. metallireducens* SSAR (Q39R98); EcoGarR, *Escherichia coli* NADPH-dependent tartronate semialdehyde reductase (TSAR) (P0ABQ2); EcoGlxR, *E. coli* NADH-dependent TSAR (P77161); EbaHMGDH, *E. barkeri* 2-(hydroxymethyl)glutarate dehydrogenase (Q0QLF5); PcaPGDH, *Pyrobaculum calidifontis* 6-phosphogluconate dehydrogenase (A3MU08); EcoSLAR, *E. coli* 3-sulfolactaldehyde reductase (P0A9V8); PsyDPSDH, *Pseudomonas syringae* D-phenylserine dehydrogenase (E5RM11); StyTSAR, *Salmonella typhi* TSAR (Q8Z3K1); MtuHIBADH, 3-hydroxyisobutyrate dehydrogenase (HIBADH) (P9WNY5); RnoHIBADH, Rattus norvegicus HIBADH (P29266); PaeHIBADH, *Pseudomonas aeruginosa* HIBADH (P28811); and PaeSERDH, *P. aeruginosa* NAD-dependent L-serine dehydrogenase (Q9I5I6).

Heterologous expression and purification of AAC4036

To characterize the catalytic properties of AAC4036, *A. aceti* AAC4036 with an N-terminal His-tag was produced in *E. coli* BL21(DE3) cells harboring the pAAC4036 construct and purified by Ni-NTA column chromatography. Analysis of the soluble crude cell extract by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) showed that the overproduction of AAC4036 was achieved, with the protein produced having an approximate molecular mass of 33 kDa, which was consistent with the theoretical molecular mass (32.8 kDa) calculated from the amino acid sequence (Fig. 3-2). AAC4036 was purified to homogeneity, based on a single band occurring on the SDS-PAGE gel (Fig. 3-2). Oligomeric state of AAC0436 was determined by size exclusion chromatography using a Superdex 200 column. The result indicates the protein to be a tetramer with M_{r} of 126.4 kDa (Figure 3-3 and 3-4). During purification, I found that

AAC0436 catalyzed glyoxylate reduction while using NADPH as a cofactor. I obtained 6.5 mg of the enzyme with a specific activity of 1,300 units/mg (2.8-fold purity, with a recovery rate of 119%) (Table 3-1).

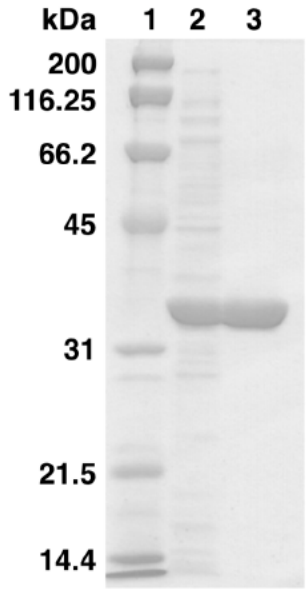

Figure 3-2. Analysis by SDS-PAGE of the AAC4036 (AacGR) protein purified from recombinant *E. coli* cells. Lanes 1, 2, and 3 indicate the size marker proteins, crude extract, and enzyme purified by Ni-affinity chromatography, respectively. The numbers on the left indicate the molecular masses of the marker proteins.

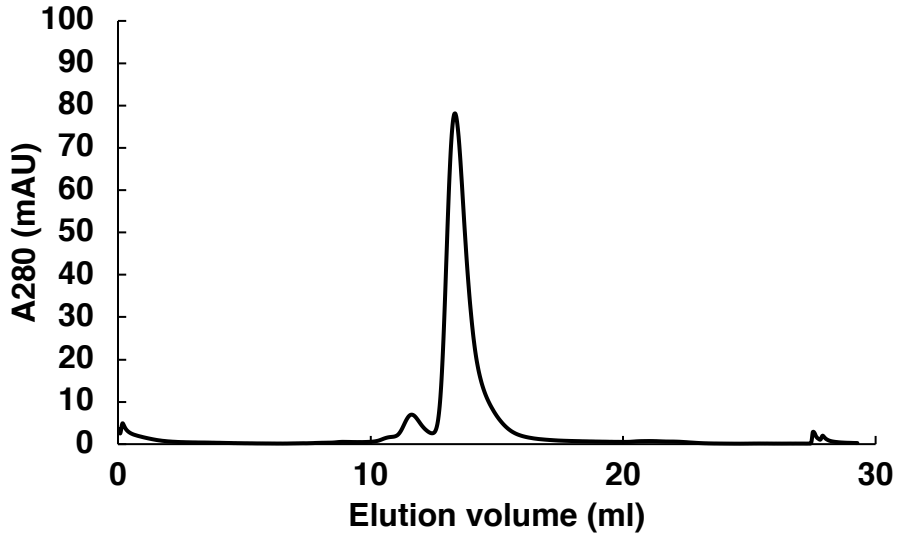

Figure 3-3. Size exclusion chromatography analysis of AacGR. The enzyme was eluted at the elution volume of 13.34 mL.

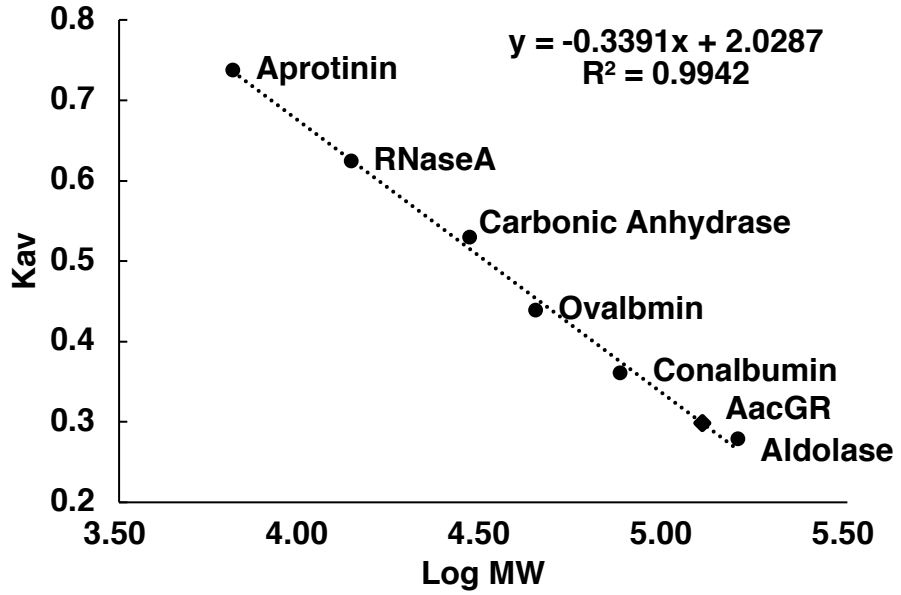

Figure 3-4. Molecular mass of AacGR. The size marker proteins used were aprotinin (M_r 6,500), RNaseA (M_r 13,700), carbonic anhydrase (M_r 29,000), ovalbumin (M_r 44,000), conalbumin (M_r 75,000), and aldolase (M_r 158,000).

Table 3-1. Purification of recombinant AacGR.

Purification step	Total activity* (units)	Total protein (mg)	Specific activity* (units/mg)	Recovery (%)	Purification (-fold)
Crude extract	7,120	15.4	463	100	1
Ni-NTA column	8,460	6.51	1,300	119	2.81

*The enzyme was assayed in a reaction mixture containing 100 mM citrate buffer (pH 4.0), 1 mM glyoxylate, and 0.15 mM NADPH at 45°C.

Substrate specificity

I initially hypothesized that AAC0436 might utilize SSA as a substrate because it shared 54% amino acid sequence identity with *G. oxydans* SSAR. However, I found that AAC0436 showed no activity towards SSA. When glyoxylate was tested as a substrate, the enzyme exhibited markedly high activity in the presence of NADPH or NADH as an electron donor. The specific activities of the enzyme with NADH (1,150 units/mg) and NADPH (1,300 units/mg) were similar, but slightly higher with NADPH. NAD(P)H-dependent substrate reduction by the enzyme was not observed with other small molecules, such as carboxylic acids (formate, acetate, oxalate, 3-hydroxypropionate, DL-glycerate, pyruvate, hydroxypyruvate, and phenylpyruvate), aldehydes (formaldehyde, acetaldehyde, glutaraldehyde, and succinic semialdehyde), and dicarbonyls (glyoxal, methylglyoxal, and phenylglyoxal). I also examined $NAD(P)^+$-dependent glycolate oxidation by the enzyme. The enzyme showed no detectable activity for the reverse reaction at acidic (pH 4.0) and neutral (pH 7.0) pH conditions, whereas it exhibited a specific activity of 1.1 units/mg (with NAD^+) and 2.4 units/mg (with $NADP^+$) when tested

at pH 9.0 with 50 mM glycolate. The specific activity of the enzyme for the forward reaction at pH 9.0 was determined to be 540 units/mg with 1 mM glyoxylate and NADPH. Therefore, the enzyme appeared to favor the forward reaction not only at pH 4.0 but also at pH 7.0 and 9.0. In contrast, all of the other hydroxy acids (DL-lactate, L-malate, (*S*)-hydroxyisobutyrate, and (*R*)-hydroxyisobutyrate) and amino acids (D-serine, L-serine, D-threonine, and L-threonine) were inert as substrates of the enzyme when examined at pH of 4.0, 6.0, and 9.0. These results suggest that AAC0436 is a distinctive GR (hereinafter called AacGR) that exclusively catalyzes the reversible NAD(P)H-dependent reduction of glyoxylate to glycolate.

Effect of pH on activity and stability of AacGR

The effect of pH on AacGR activity was examined over the pH range of 3.0–11.0 (Figure 3-5). The maximum activity was observed at a pH of 4.0, with approximately 50% of this activity retained at a pH of 3.5 and 8.0, respectively. The enzyme activity was significantly low in Tris–HCl buffer (pH 7 and 8), which might be due to an amino group of Tris reacting with the substrate aldehyde. The optimal pH of the reaction catalyzed by AacGR was lower than that of most known GRs/GRHPRs from plants (Hoover et al., 2007), fungi (Duan et al., 2014), bacteria (Ogino et al., 2008), and archaea (Ohshima et al., 2001), which have an optimum pH around 6-7, but it was comparable with that of the GR from *Chlamydomonas reinhardtii*, which exhibits relatively high activity at a pH of around 5.0 (Husic & Tolbert, 1987). AacGR exhibited a high pH stability, such that more than 80% of the activity was retained over the pH range of 4.0-11.0 for 1 h on ice (Figure 3-6). The fungal GR from *Paecilomyes thermophila* also shows a broad pH stability at pH of 4.5–10.0 (Duan et al., 2014).

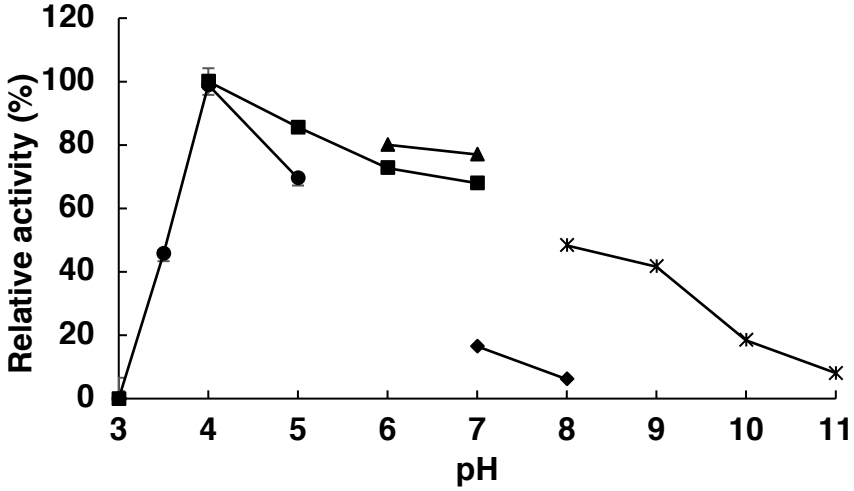

Figure 3-5. Effect of pH on the activity of AacGR. Buffers were: citrate, pH 3–5 (closed circles); citrate-phosphate, pH 4–7 (closed squares); phosphate, pH 6–7 (closed triangles); Tris–HCl, pH 7–8 (closed diamonds); and glycine–NaOH, pH 8–11 (asterisks).

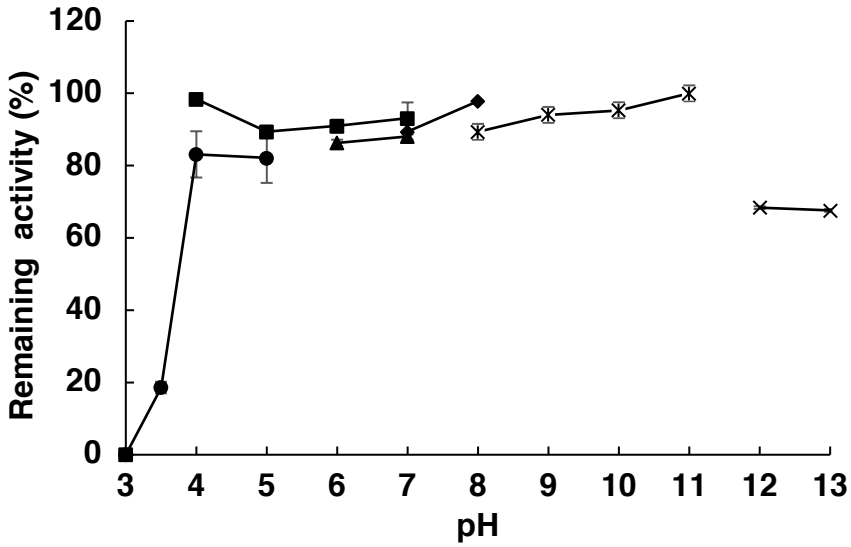

Figure 3-6. Effect of pH on stability of AacGR. Buffers were: citrate, pH 3–5 (closed circles); citrate-phosphate, pH 4–7 (closed squares); phosphate, pH 6–7 (closed triangles); Tris–HCl, pH 7–8 (closed diamonds); glycine–NaOH, pH 8–11 (asterisks); and KCl-NaOH, pH 12-13 (crosses).

Effect of temperature on activity and stability of AacGR

The effects of temperature on the activity of AacGR were examined from 25 to 70°C (Figure 3-7). AacGR showed relatively high activity over a broad temperature range (30-45°C), with maximum activity at 45°C. The enzyme was stable up to 65°C for 10 min and inactivated rapidly when the temperature exceeded 70°C (Figure 3-8). In contrast, *A. thaliana* GLYR1 is heat-labile and has an optimum reaction temperature of 22°C (Zarei et al., 2017). AacGR retained about 90% of its activity after 10-min incubation at 65°C, at which temperature it did not show activity in the reaction mixture. This observed discrepancy may be related to the reversible nature of the enzyme denaturation by heat, because the heat-treated enzyme was cooled on ice until the assay.

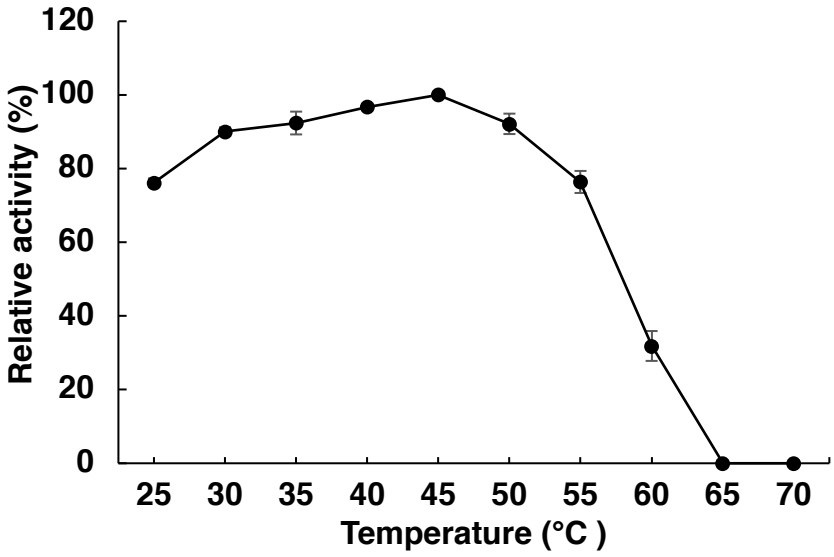

Figure 3-7. Effect of temperature on activity of AacGR.

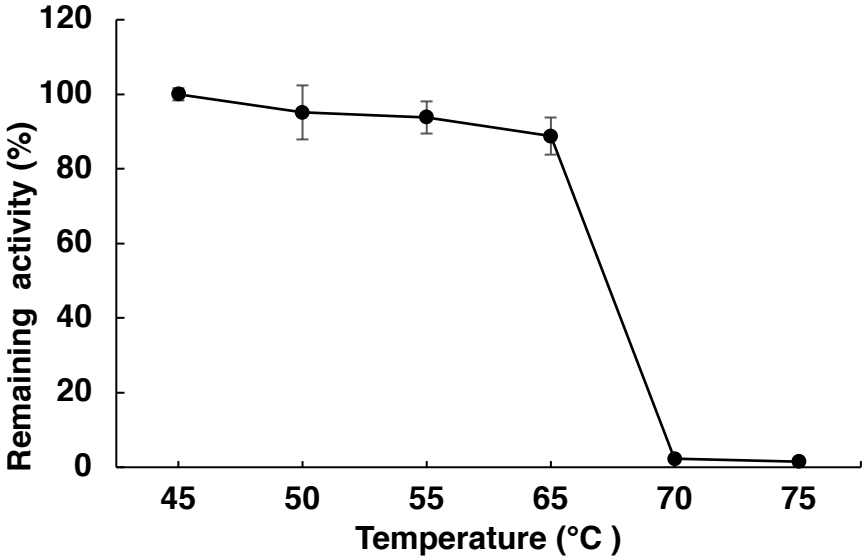

Figure 3-8. Effect of temperature on stability of AacGR.

Effects of metal ions, chemical compounds, and organic solvents on stability of AacGR

I examined the effects of various metal ions and chemical compounds on the stability of AacGR by incubating the enzyme for 1 h on ice in the presence of the additives. Most metal ions did not affect AacGR stability, except that $Fe^{3+}$and Hg^{2+} decreased enzyme activity by about 15 and 30%, respectively (Figure 3-9). On the other hand, enzyme activity was decreased to 20% by incubation with 60% ethanol (Figure 3-10).

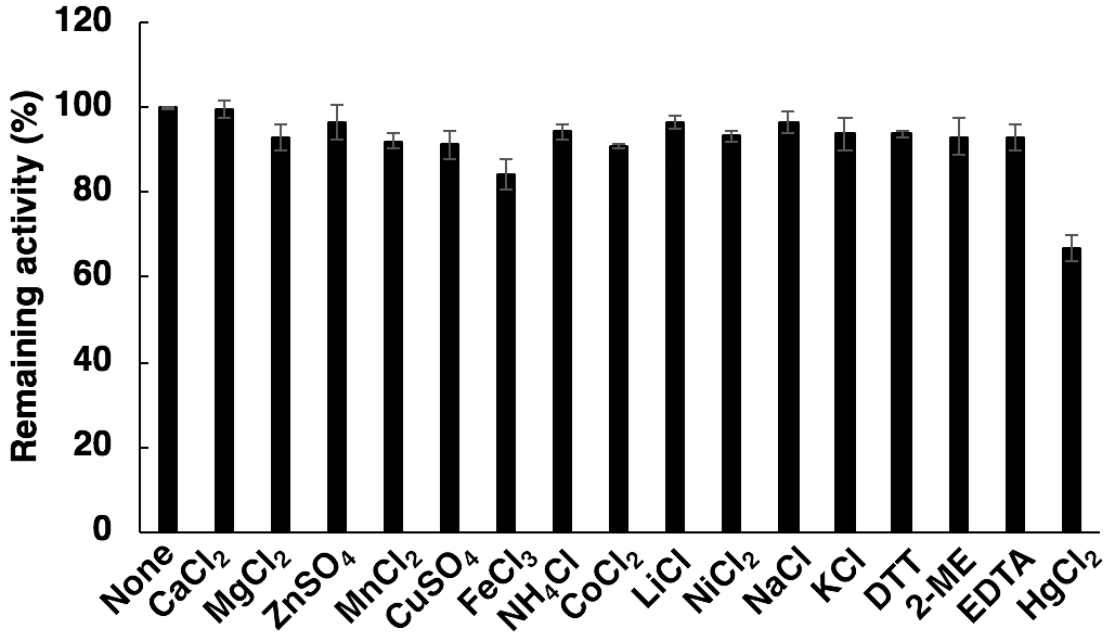

Figure 3-9. Effect of metal ions and inhibitors on AacGR activity. The activity of enzyme without a treatment (None) was set as 100%. Enzyme activity was examined with additives including dithiothreitol (DTT), 2-mercaptoethanol (2-ME), Ethylenediaminetetraacetic acid (EDTA).

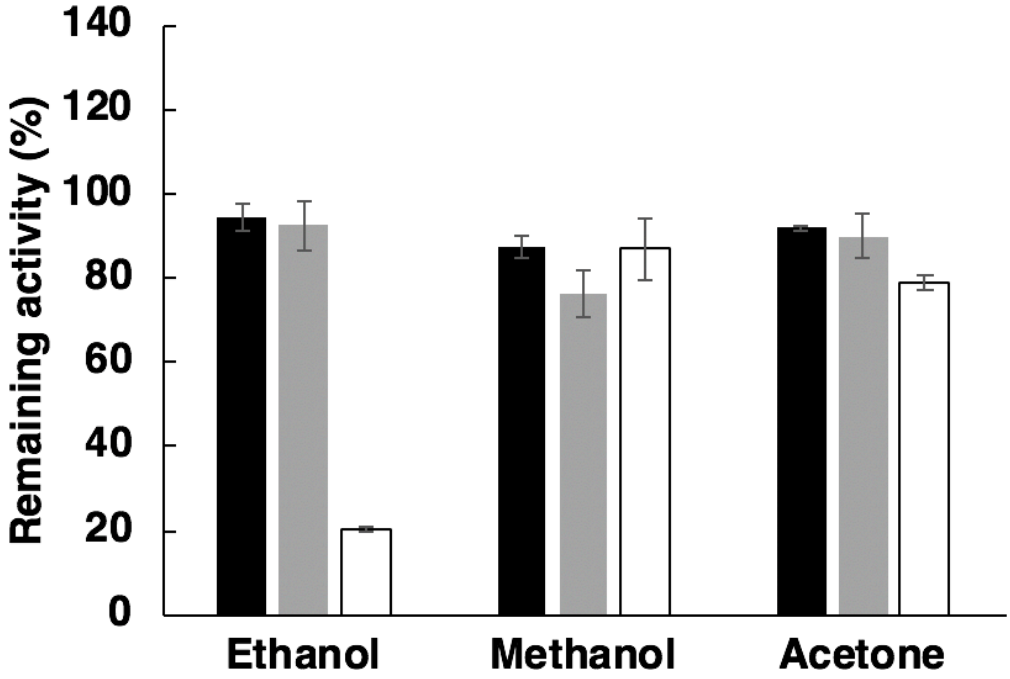

Figure 3-10. Effect of organic solvents on AacGR activity. The concentration (v/v) of organic solvents used were 20% (the black bars), 40% (the gray bars), and 60% (the open bars). The activity of enzyme without a treatment was set as 100%.

Kinetic analysis of AacGR

Kinetic constants of the AacGR-catalyzed reaction of glyoxylate were investigated using NADH or NADPH as a cofactor. The K_m values of AacGR for glyoxylate in the presence of NADH and NADPH were 0.58 and 0.38 mM, respectively (Table 3-2). The V_{max} value of AacGR for glyoxylate in the presence of NADPH (1,020 μmol·min^{-1}·mg^{-1}) was comparable to or slightly higher than that in the presence of NADH (951 μmol·min^{-1}·mg^{-1}). Kinetic constants of the enzyme for the reverse reaction (NAD(P)$^+$-dependent oxidation of glycolate) were also determined. In this case, the enzyme exhibited markedly high K_m values (> 300 mM) for glycolate using either cofactor (Table 2). The V_{max} values of AacGR for glycolate were markedly lower than those found for glyoxylate. The catalytic efficiency (k_{cat}/K_m) of AacGR for glyoxylate with NADPH was 5×10^4 times higher than that for glycolate with NADP$^+$. These results

suggest a clear preference of AacGR for NAD(P)H-dependent glyoxylate reduction. The high catalytic efficiency of AacGR is comparable to that of the GR from *Thermus thermophilus* HB27 (Ogino et al., 2008).

Table 3-2. Kinetics parameters of AacGR*

Substrate	Cofactor	K_m (mM)	V_{max} ($\mu mol \cdot min^{-1} \cdot mg^{-1}$)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1} \cdot M^{-1}$)
Glyoxylate	NADH	0.58	951	530	9.1×10^5
Glyoxylate	NADPH	0.38	1,020	570	1.5×10^6
Glycolate	NAD^+	309	8.7	4.8	1.6×10^1
Glycolate	$NADP^+$	334	18	10	3.0×10^1

*The forward reaction was examined in a reaction mixture containing 100 mM citrate buffer (pH 4.0), 0.15 mM NADH (or NADPH), and various concentrations of glyoxylate at 45 °C. The reverse reaction was tested in a mixture containing 100 mM glycine–NaOH buffer (pH 9.0), 0.2 mM NAD^+ (or $NADP^+$), and various concentrations of glycolate at 45 °C.

Effects of NaCl and sodium phosphate on AacGR

Effects of NaCl and sodium phosphate on AacGR were investigated. In the previous study of *T. thermophilus* GR, the effects of high concentrations of NaCl and sodium phosphate on the enzyme activity have been investigated (Ogino et al., 2008). AacGR retained more than 20% of its activity in the presence of 500 mM NaCl (Figure 3-11), while the activity of *T. thermophilus* GR is completely inhibited at >500 mM NaCl. The *T. thermophilus* GR was shown activation by the addition of 100-150 mM sodium phosphate (Ogino et al., 2008), whereas no activation was observed for AacGR by sodium phosphate (Figure 3-12).

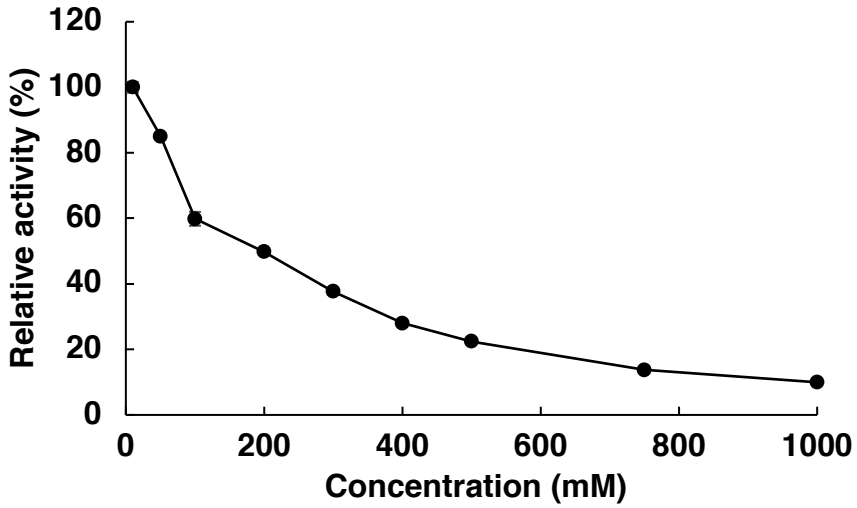

Figure 3-11. Effect of NaCl on AacGR activity.

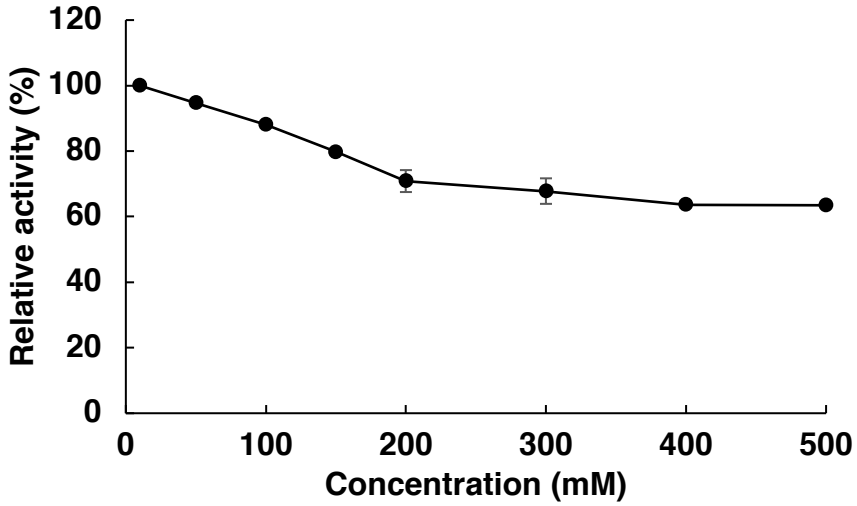

Figure 3-12. Effect of phosphate on AacGR activity.

Effects of preserving additives on AacGR stability

The remaining activity of AacGR was measured after storage of the enzyme with preserving additives for 0, 1, and 7 days. AacGR was only slightly inactivated when it was kept at low temperature. About 20% and 60% of the enzyme activity was lost by storage for 7 days at 4°C and -20°C, respectively. On the other hand, most of the additives showed protective effects with the remaining activity over 60% at 4°C and -20°C for 7 days of storage. These additives were useful for increasing the stability of AacGR in the enzyme solution (Figure 3-13, 3-14, 3-15, 3-16, and 3-17).

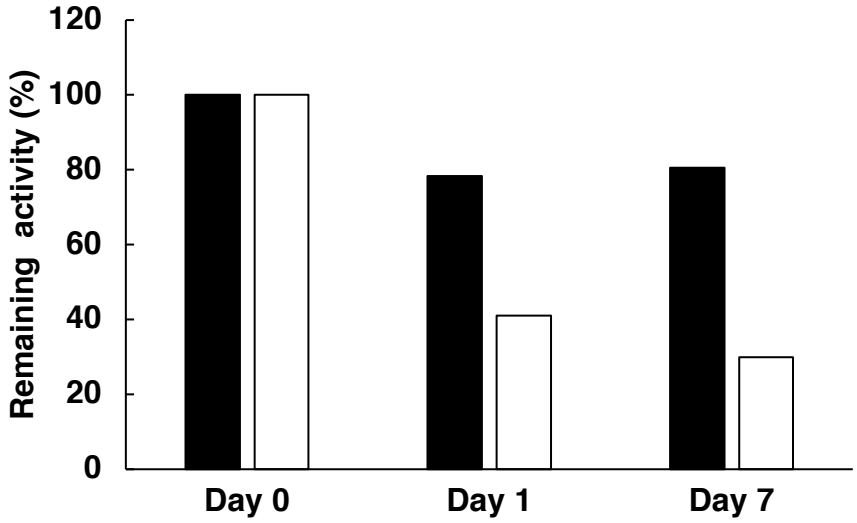

Figure 3-13. Stability of AacGR without additive. The enzyme was incubated at at 4°C (the black bars) or -20°C (the white bars). The activity of enzyme before incubation was set as 100%.

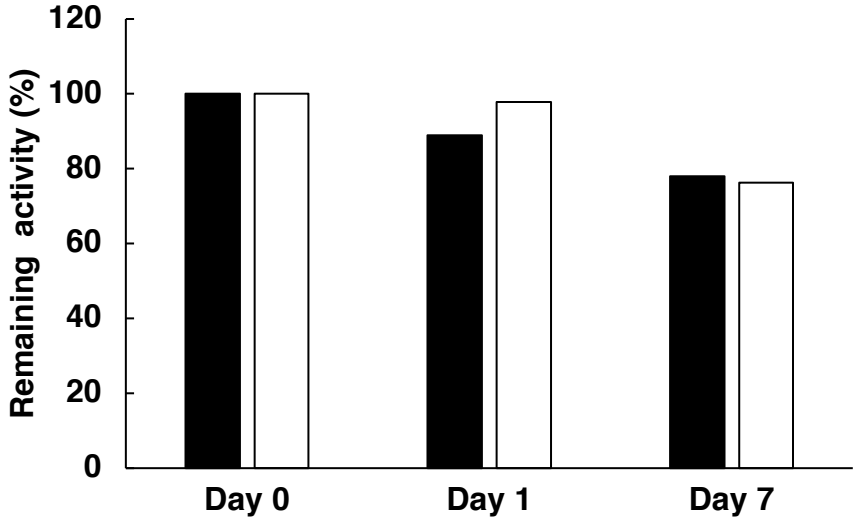

Figure 3-14. Effect of sucrose on AacGR stability. The enzyme was incubated at at 4°C (the black bars) or -20°C (the white bars). The activity of enzyme before incubation was set as 100%.

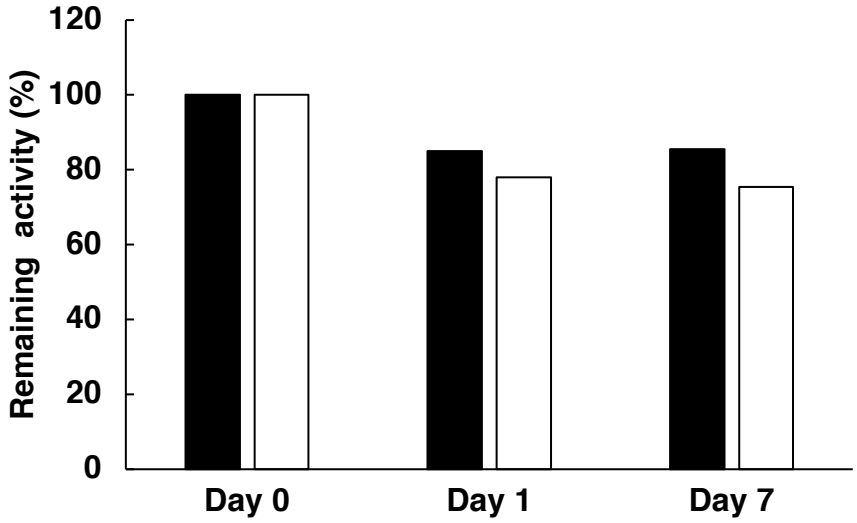

Figure 3-15. Effect of sorbitol on AacGR stability. The enzyme was incubated at at 4°C (the black bars) or -20°C (the white bars). The activity of enzyme before incubation was set as 100%.

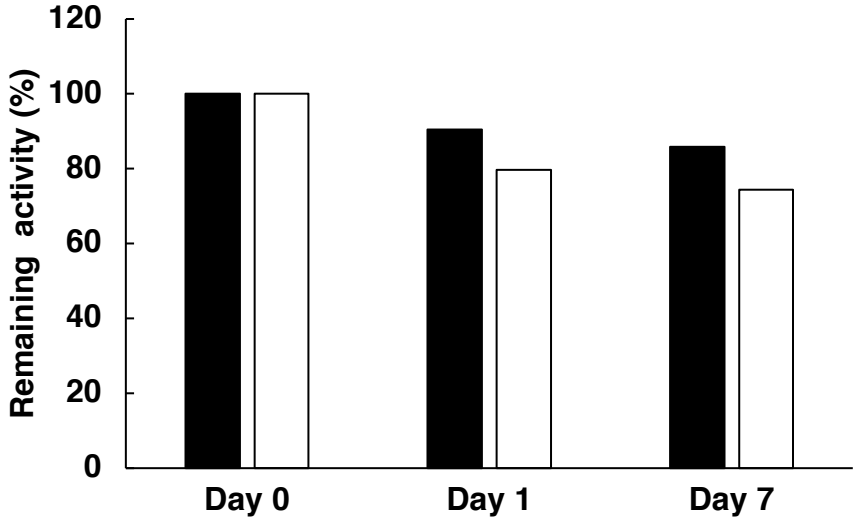

Figure 3-16. Effect of glycerol on AacGR stability. The enzyme was incubated at at 4°C (the black bars) or -20°C (the white bars). The activity of enzyme before incubation was set as 100%.

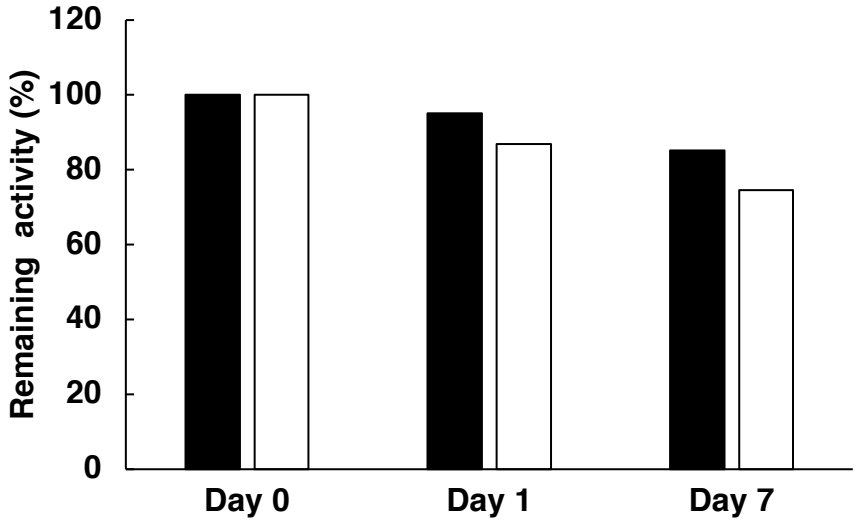

Figure 3-17. Effect of glucose on AacGR stability. The enzyme was incubated at at 4°C (the black bars) or -20°C (the white bars). The activity of enzyme before incubation was set as 100%.

Product identification of the enzymatic reaction by AacGR

The production of glycolate from glyoxylate in the reaction catalyzed by AacGR was examined using 2,7-dihydroxynaphthalene. The reaction mixture without the enzyme was used as a control, and the reaction mixture containing glyoxylate as a substrate was incubated with 0.01 mg of the enzyme. Figure 3-19 showed that the amount of glycolate increased in a time-dependent manner. This result demonstrated that AacGR catalyzed the reduction of glyoxylate to produce glycolate.

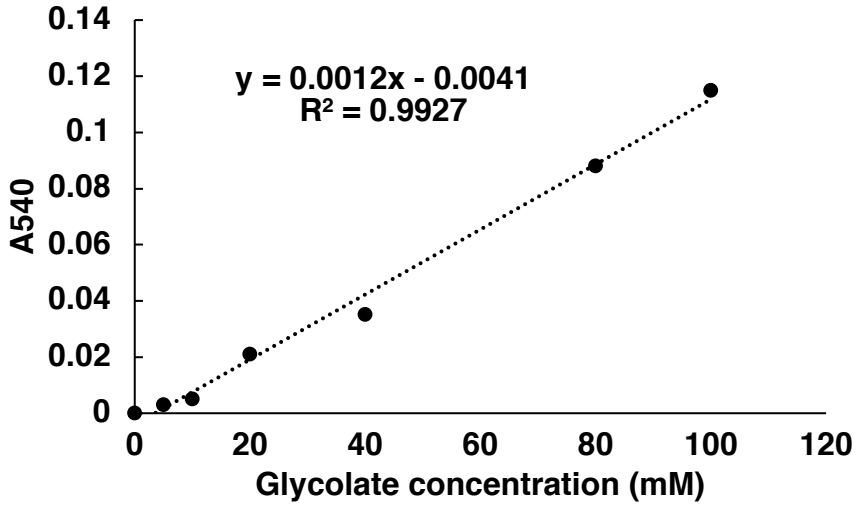

Figure 3-18. Standard curve for glycolate determination.

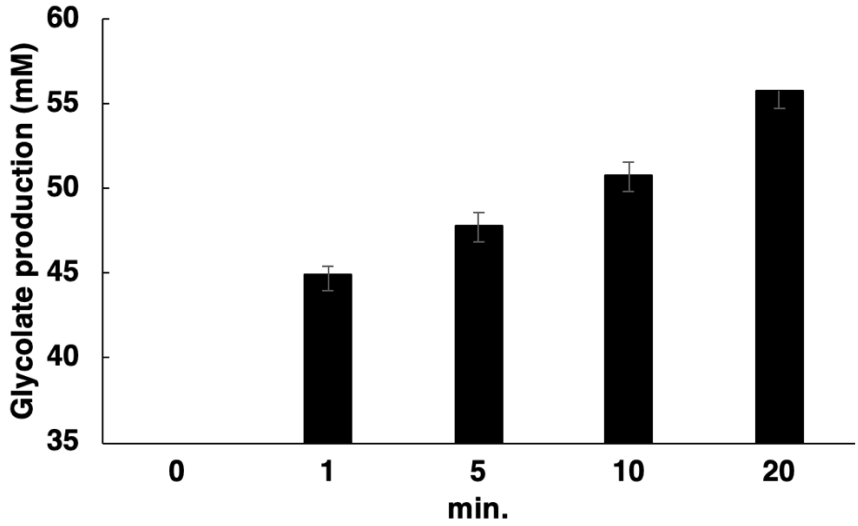

Figure 3-19. Determination of glycolate production by AacGR.

Comparison of AacGR with other related enzymes

In this study, I showed that AacGR exclusively catalyzes glyoxylate reduction using NADPH or NADH as a cofactor. To the best of our knowledge, there is no other example of a glyoxylate-specific NAD(P)-dependent reductase. Most GRs are GRHPR-type enzymes that have broad substrate specificity, most commonly to hydroxypyruvate, and belong to the D-2-hydroxyacid dehydrogenase family (Ohshima et al., 2001; Booth et al., 2006; Kutner et al., 2018), which is structurally and functionally distinct from the HIBADH family. Meanwhile, the HIBADH-related family of enzymes generally acts on the common 3-hydroxypropionate moiety of their substrates (in addition to HIBA), including D/L-glycerate (Hubbard et al., 1998), L-serine (Tchigvintsev et al., 2012), and D-phenylserine (Ueshima et al., 2010), to perform $NAD(P)^+$-dependent oxidation. Therefore, AacGR and the closely related SSARs from *G. oxydans* (Meyer et al., 2015) and *Geobacter* spp. AacGR exhibited closely related SSARs from *G. oxydans* (Meyer et al., 2015) and *Geobacter* spp. (Zhang et al., 2011) and the plant GLYRs (Zarei et al., 2017) differ from typical HIBADH-related enzymes in that they preferentially act on the aldehyde group at the C_2- and C_4-positions in their substrates (glyoxylate and SSA, respectively) to perform NAD(P)H-dependent reduction.

Enzymes in the β-HAD superfamily are typically around 300 amino acids in length, and contain four conserved regions: the N-terminal dinucleotide cofactor-binding, substrate-binding, catalysis, and second cofactor-binding regions (Njau et al., 2001) (Figure 3-1A). The multiple sequence alignment of AacGR with several representative enzymes from each class of the β-HAD superfamily revealed that AacGR and GoxSSAR have diverged cofactor- and substrate-binding sequences that differ from those of most members of the HIBADH-related family (Figure 3-1A). The amino acid sequence of residues 14-27 in AacGR is 'FIG**F**GAM**A**SRM**G**DH', while the sequence of the 'HIBADH signature' is '[LIVMFY](2)G**L**GX[MQ]**G**XX[MA][**SAV**]X[SNHR]' (accession no. PS00895 in the PROSITE database, https://prosite.expasy.org/prosite.html) (Njau et al., 2001; Sigrist et al., 2013). Specificity to NAD or NADP is known to depend on residues 18-20 on the C-terminal side of the

conserved GLGX[MQ]G motif (Hawes et al., 1996). In the majority of family members, the NAD-dependent enzyme contains the DXX motif, in contrast to the NRX motif in the NADP-dependent enzyme in the same region (Hawes et al., 1996) (Figure 3-1A). However, AacGR and GoxSSAR contain the 'TPS' and 'APS' motifs, respectively, in the corresponding position in their amino acid sequences. The absence of either Asp or Arg at this position in AacGR and GoxSSAR is consistent with their unusual dual cofactor specificity to NADH and NADPH, which is atypical for the β-HAD superfamily. In the substrate-binding consensus region, AacGR, GoxSSAR, GmeSSAR, GLYR1, and GLYR2 have the 'SG**S** triad' motif in place of the highly conserved 'SG**G** triad' found in typical β-HAD superfamily members, which provides a binding site for a carbonyl group in their substrates.

Among the biochemically characterized enzymes, AacGR shares the highest sequence identity (54%) with GoxSSAR. Unfortunately, the activity of GoxSSAR towards glyoxylate is unknown, but AacGR is at least distinct from GoxSSAR in that AacGR does not utilize SSA as a substrate, while GoxSSAR prefers to act on SSA (Meyer et al., 2015). Because of the typically wide substrate diversity of β-HADs and the observation that multiple homologs of them are present in the genomes of various species, some β-HAD superfamily members may have species-specific metabolic roles. There are marked differences in glyoxylate-related metabolism between *A. aceti* and *G. oxydans*: *A. aceti* JCM20276 and NBRC14818 (Sakurai et al., 2011) contains genes encoding isocitrate lyase (*aceA*) and malate synthase (*glcB*) in the glyoxylate pathway, whereas several TCA cycle and glyoxylate pathway genes are missing in *G. oxydans* (Prust et al., 2005).. Therefore, it is not surprising that AacGR exhibits distinct substrate specificity from that of GoxSSAR, even though both *A. aceti* and *G. oxydans* are 'acetic acid bacteria'. When the sequences of close, uncharacterized homologs of AacGR and GoxSSAR were compared, AacGR was assigned to a clade phylogenetically distinct from that of GoxSSAR (Figure 3-20). Based on these findings, I propose that AacGR constitute a new class of enzyme in the β-HAD superfamily. The metabolic function of AacGR is unknown in *A. aceti*. Our finding of the glyoxylate specificity of AacGR may provide an important clue to clarify bacterial glyoxylate metabolism. Future research on

the relationship between the structure and function of these enzyme will clarify the mechanisms of their substrate selectivity.

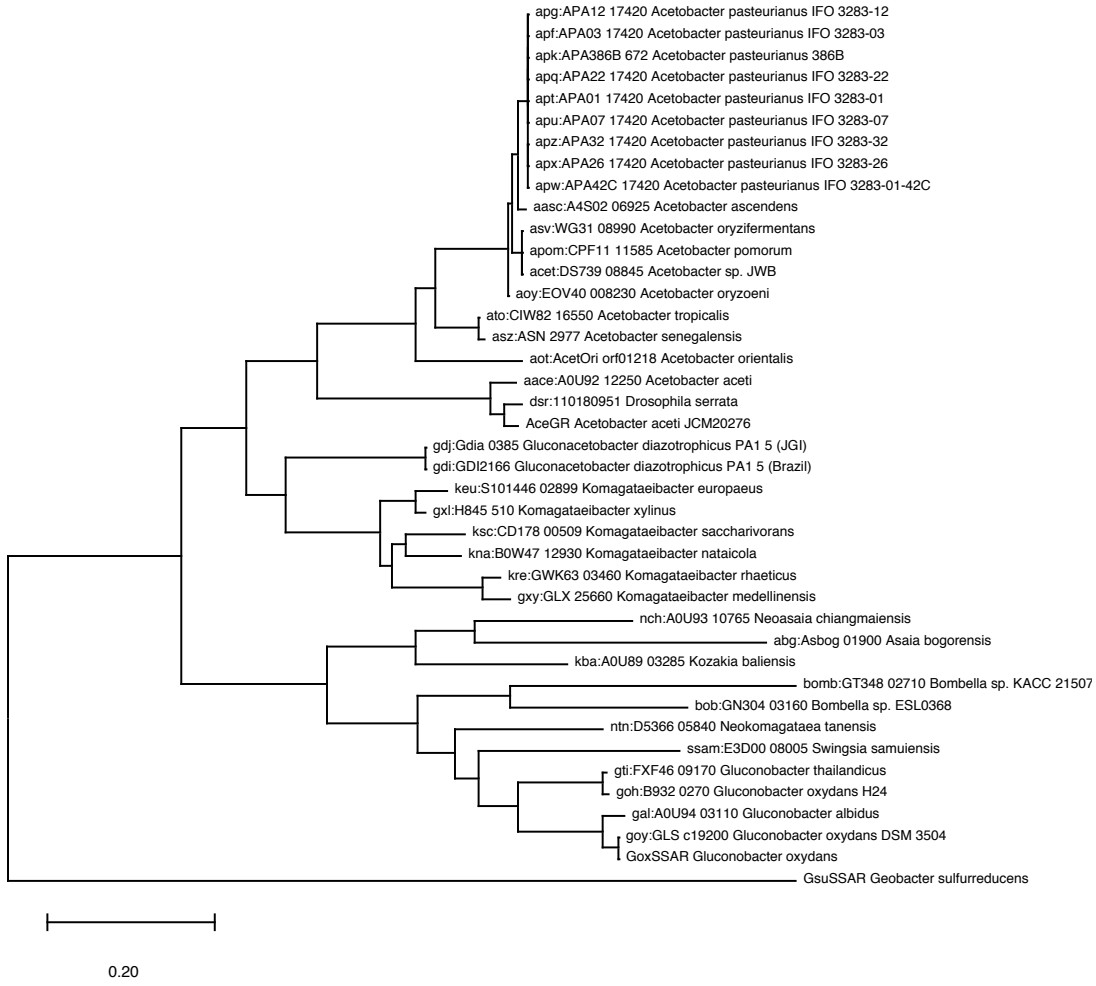

Figure 3-20. Phylogenetic analysis of AacGR with its uncharacterized close homologs. The evolutionary history was inferred by using the Maximum Likelihood method and JTT matrix-based model [1]. The tree with the highest log likelihood (-7227.75) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 41 amino acid sequences. There was a total of 319 positions in the final dataset. Evolutionary analyses were conducted in MEGA X [2][3]. Sequences with the indicated accessions were obtained from GenomeNet (https://www.genome.jp). AacGR, *Acetobacter aceti* glyoxylate reductase; GoxSSAR, *Gluconobacter oxydans* succinic

semialdehyde reductase; GsuSSAR, *Geobacter sulfurreducens* succinic semialdehyde reductase.

Conclusion

Enzymes related to β-hydroxyacid dehydrogenases/3-hydroxyisobutyrate dehydrogenases are ubiquitous, but most of them have not been characterized. An uncharacterized protein with moderate sequence similarities to *Gluconobacter oxydans* succinic semialdehyde reductase and plant glyoxylate reductases/succinic semialdehyde reductases was found in the genome of *Acetobacter aceti* JCM20276. The corresponding gene was cloned and expressed in *Escherichia coli*. The gene product was purified and identified as a glyoxylate reductase that exclusively catalyzed the NAD(P)H-dependent reduction of glyoxylate to glycolate. The strict substrate specificity of this enzyme to glyoxylate, the diverged sequence motifs for its binding sites with cofactors and substrates, and its phylogenetic relationship to homologous enzymes suggested that this enzyme represents a novel class of enzymes in the β-hydroxyacid dehydrogenase family. This study may provide an important clue to clarify the metabolism of glyoxylate in bacteria.

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Chapter 4

Conclusion

The objective of this study was to discover novel glyoxylate-dependent oxidoreductase enzymes and to find novel metabolisms on glyoxylate in *Acetobacter aceti*. *A. aceti* is widely use in the biotechnology industry. Understanding metabolisms in *A. aceti* will be useful for enhancing production of valuable products from this bacterium. Glyoxylate dehydrogenase and glyoxylate reductase will also be able to be developed as biosensing probes and used for medical purposes. Chapter 1 and Chapter 4 describes a general introduction and conclusion, respectively. The rest of the Chapters are adopted from the articles which have been published or accepted for publication in peer-reviewed journals during the PhD course of my study.

Chapter 2 is adapted from Jakkaphan Kumsab, Ruta Tobe, and Hisaaki Mihara. (2019) Partial purification and characterization of glyoxylate dehydrogenase from *Acetobacter aceti* JCM20276. **Trace Nutrients Research**, 36: 81−86.

Chapter 3 is adapted from Jakkaphan Kumsab, Ruta Tobe, Tatsuo Kurihara, Yuu Hirose, Taketo Omori, and Hisaaki Mihara (2020) Characterization of a novel class of glyoxylate reductase belonging to the β-hydroxyacid dehydrogenase family in *Acetobacter aceti,* **Bioscience, Biotechnology, and Biochemistry**, in press, doi:10.1080/09168451.2020.1797470.

In Chapter 2, the purification and characterization of glyoxylate dehydrogenase from *A. aceti* was described. Glyoxylate dehydrogenase was partially purified to 11-fold with 12% recovery by Q-Sepharose column chromatography. The specific activity of the enzyme was 4,190 unit/mg. I have characterized basic properties of the enzyme including the effects of pH and temperature on activity and stability of the enzyme. The enzyme showed the highest activity at pH 7. The enzyme was stable at pH 6 and relatively tolerant to alkaline conditions, but was unstable under acidic conditions. The enzyme showed relatively high activity at 35-45°C. The temperature profile showed an optimal activity at 35°C. The enzyme activity remained over 90% after incubation at 25-35°C for 1 h. The

effects of metal irons and organic solvents were also investigated. The addition of Fe^{3+} markedly inhibited its activity. The enzyme retained more than 94% of its original activity in ethanol, methanol, and acetone, each at 20%. 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. Several potential substrates and electron acceptors were examined. The best substrate for the 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 combination of DCIP and PMS served as the most effective electron acceptor.

In Chapter 3, the purification and characterization of glyoxylate reductase from *A. aceti* was described. The amino acid sequence analysis revealed AAC4036 was a putative NAD(P)-dependent oxidoreductase. The enzyme was related to the HIBADH superfamily. AAC4036 showed the highest sequence identity (about 54%) when compared with *G. oxydans* SSAR (GoxSSAR). The *A. aceti* glyoxylate reductase (AacGR) was cloned and expressed using the pColdI plasmid in *E. coli* BL21(DE3). The N-terminal His-tag enzyme was purified by 1 step of Ni-chelating column chromatography. The enzyme was revealed to be a tetramer as determined by Superdex 200 gel filtration. The enzyme demonstrated the highest activity when tested with glyoxylate as a substrate. The enzyme was very specific to glyoxylate. The maximum activity of AacGR was shown at pH 4.0 and 45°C. The enzyme was unstable under acidic conditions. The enzyme remained 90% of the activity after incubation at 65°C for 10 min. $Fe^{3+}$and Hg^{2+} inhibited the enzyme activity by 10% and 30%, respectively. The phylogenetic analysis suggested that AacGR and GoxSSAR belonged to a distinct clade and were most divergent from the HIBADH clade.

List of Publications

Jakkaphan Kumsab, Ruta Tobe, and Hisaaki Mihara. (2019) Partial purification and characterization of glyoxylate dehydrogenase from *Acetobacter aceti* JCM20276. **Trace Nutrients Research**, 36: 81−86.

Jakkaphan Kumsab, Ruta Tobe, Tatsuo Kurihara, Yuu Hirose, Taketo Omori, and Hisaaki Mihara (2020) Characterization of a novel class of glyoxylate reductase belonging to the β-hydroxyacid dehydrogenase family in *Acetobacter aceti,* **Bioscience, Biotechnology, and Biochemistry**, in press, doi:10.1080/09168451.2020.1797470.

Acknowledgements

I would like to express my sincere appreciation to Innovative Asia Scholarship provided by JICA for supporting living, academic and research allowances. I also would like to express my special thanks of gratitude to Professor Mihara Hisaaki, Lecturer Tobe Ryuta and all members in Applied Molecular Microbiology laboratory at Ritsumeikan University for the professional guidance and advices as well as provided equipment related to this work. Completion of my research could not be done without their support and encouragement.

KUMSAB Jakkaphan

Appendix

Culture media and chemical solutions

Appendix Table 1. Luria-Bertani broth

Compositions	%(W/V)
Yeast extract	0.5
Tryptone	1.0
NaCl	1.0

Appendix Table 2. Glucose agar

Compositions	Amount
Yeast extract	5.0 g
Bacto peptone	3.0 g
Glucose	30.0 g
$CaCO_3$	10.0 g
Agar	15.0 g
Water	1.0 L

Appendix Table 3. SDS sample buffer (1 L)

Compositions	Amount
0.5 M Tris-HCl pH 6.8	250 mL
2-Mercaptoethanol	100 mL
10% SDS	400 mL
Sucrose	100 g
Bromophenol blue	100 mg
Distilled water	Up to 1.0 L

Appendix Table 4. 5X CBB staining solution (50 mL)

Compositions	Amount
CBB G-250	25 g
99.5% Ethanol	12.5 mL
Phosphoric acid	25 mL
Distilled water	Up to 50.0 mL

Appendix Table 5. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) 10%

Compositions	Separating gel (mL)	Stacking gel (mL)
Water	4.0	1.35
30% Acrylamide	3.3	0.335
1.5 M Tris-HCl buffer (pH 8.8)	2.5	-
0.5 M Tris-HCl buffer (pH 6.8)	-	0.25
10% SDS	0.1	0.02
10% Ammonium persulfate	0.1	0.02
TEMED	0.004	0.002