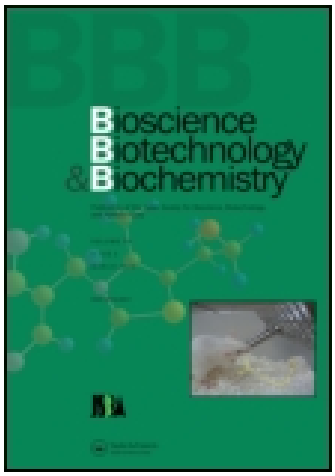

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Oxidation of Ethylene Glycol and Glycolic Acid by Glycerol Oxidase

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A glycerol oxidase from *Aspergillus japonicus* oxidized ethylene glycol to glyoxal by the same reaction pathway as alcohol oxidases from methanol yeast. The optimum pH and temperature for the oxidation of ethylene glycol were around 7.0 and 40°C, respectively. Those of glycolaldehyde were similar to those of ethylene glycol. The apparent K_ms for ethylene glycol and glycolaldehyde were 195 and 48.8 mM, respectively. The maximum velocities for ethylene glycol and glycolaldehyde were 89.1 and 62.2 μmol/min/mg of protein, respectively. Glycerol oxidase also oxidized glycolic acid, which is not oxidized by the alcohol oxidases, to glyoxylic acid like glycolate oxidases from green plants, and the apparent K_m and V_{max} for glycolic acid were 114 mM and 2.68 μmol/min/mg of proten, respectively. The glycerol oxidase was applicable to the production of glyoxal and glyoxylic acid.

Glyoxal is now chemically synthesized through either the oxidation of acetaldehyde by nitric acid or the dehydrogenation of ethylene glycol. Recently, Isobe and Nishise[1] have found that two alcohol oxidases (alcohol: oxygen oxidoreductase, EC 1.1.3.13) from methanol yeasts such as *Candida* sp. and *Pichia pastoris* oxidize ethylene glycol to glyoxal *via* glycolaldehyde and have demonstrated an attractive new enzymatic method for production of glyoxal from ethylene glycol using alcohol oxidase. Glyoxylic acid is currently manufactured either by the oxidation of glyoxal using nitric acid or *via* a three-step reaction which involves ozonolysis of dimethyl maleate, hydrogenation of the resulting hydroperoxide intermediate, and hydrolysis of the resulting methylglyoxylate hemiacetal. Seip *et al.*[2] have developed an enzymatic production of glyoxylic acid using glycolate oxidase from spinach. However, the yield by chemical synthesis is low due to the formation of by-products, and enzymatic methods have some drawbacks; specific activity of alcohol oxidases for glycolaldehyde is low[1] and glycolate oxidase slowly oxidizes glyoxylic acid.[2] During further screening of the enzymes that catalyze the oxidation of ethylene glycol to glyoxal, we found that the glycerol oxidase (glycerol: oxygen 1-oxidoreductase) from *Aspergillus japonicus*[3] oxidized ethylene glycol to glyoxal. This oxidase also oxidized glycolic acid to glyoxylic acid. The oxidative reaction of glycolic acid has been studied in detail using some glycolate oxidases (glycolate: oxygen oxidoreductase, EC 1.1.3.1) from green plants such as spinach,[4] pea, 5) pumpkin,[6] tabacco,[7] and cucumber,[8] and lactate oxidase[9] (L-lactate: oxygen 2-oxidoreductase (decarboxylating), EC 1.13.12.4) from *Mycobacterium smegmatis*, but not with fungal enzymes.

This paper describes some characterizations of the oxidative reaction of ethylene glycol and glycolic acid by glycerol oxidase and provides an evaluation of glycerol oxidase for the production of glyoxal and glyoxylic acid.

Materials and Methods

Chemicals. Ethylene glycol, glycolaldehyde dimer, glycolic acid sodium salt, glyoxylic acid monohydrate, 40% glyoxal solution, 3-methyl-2-benzothiazolinone hydrazone hydrochloride (MBTH), *N*-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES), and acetonitrile were purchased from Wako Pure Chemicals Industries, Ltd. (Osaka, Japan). Catalases from beef liver and glycerol oxidase from *Aspergillus japonicus* were from Boehringer Mannheim GmbH (Mannheim, Germany) and Kyowa Hakko Kogyo Co. Ltd., (Tokyo, Japan), respectively, and used without further purification. Mono Q HR 5/5 and Superose 6 HR 10/30 columns were from Pharmacia LKB Biotech (Uppsala, Sweden). All chemicals used were the highest grade products available from commercial sources.

Column chromatography of glycerol oxidase. Column chromatography of glycerol oxidase was done using a FPLC system (Pharmacia Fine Chemicals Co.) with a column of Mono Q HR 5/5 or Superose 6 HR 10/30 as follows. The glycerol oxidase (260 units) was chromatographed on a Mono Q column equilibrated with the same buffer as for DEAE-Sephadex A-25 column chromatography in Uwajima *et al.*,[10] except that in this case the column was washed with the same buffer for 10 min and the enzyme was eluted by a linear gradient with 50 mM borate buffer, pH 10, containing 0–0.45 M ammonium sulfate at a flow rate of 1.0 ml per min. Active fractions were combined and concentrated, and then filtrated on a Superose 6 column, also equilibrated with the same buffer as the Sephadex G-200 gel filtration of Uwajima *et al.*[10] at a flow rate of 0.4 ml per min.

Assay of enzyme activity and kinetic parameters. Glycerol oxidase activity was measured by following H_2O_2 formation at 37°C by the method of Uwajima and Terada,[3] except that the reaction volume was changed from 3 ml to 1 ml. The apparent kinetic parameters were calculated from initial velocity measurements by following H_2O_2 formation at 25°C.

Standard reaction conditions for formation of glyoxal and glyoxylic acid. Ethylene glycol, glycolaldehyde, or glycolic acid sodium salt dissolved in 0.2 M TES–NaOH, pH 7.0 (0.95 ml), containing 1300 or 2600 units of catalase was incubated at 20°C with shaking (140 rpm, 3 cm). The reaction was started by adding 50 μl of glycerol oxidase.

Identification of glycolaldehyde, glyoxal, and glyoxylic acid. Derivatives 1 and 2 of glycolaldehyde, glyoxal, or glyoxylic acid were prepared by the reaction of their carbonyl bonds with MBTH[11] as follows. Derivative 1; to glycolaldehyde, glyoxal, or glyoxylic acid in 0.2 M glycine–HCl buffer, pH 4.0 (0.75 ml), 0.3 ml of 1.0% MBTH solution was added and left at

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Abbreviations: MBTH, 3-methyl-2-benzothiazolinone hydrazone hydrochloride; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; HPLC, high pressure liquid chromatography.

room temperature for 10 min. Glyoxal developed a yellow color, but the other two compounds had no color. Derivative 2; 0.75 ml of 0.2% $FeCl_3$ solution was added to 0.20 ml of the derivative 1 and left at room temperature for 10 min. The color of the glycolaldehyde and glyoxylic acid became blue, but glyoxal did not. The derivatives were separated by a reversed phase HPLC using a Shimadzu Syn Pro Pep C_{18} column with an eluent of 20 mM sodium dihydrogen phosphate and acetonitrile under the same conditions as described previously.[1]

Measurement of glycolaldehyde, glyoxal, and glyoxylic acid. The concentrations of glycolaldehyde, glyoxal and glyoxylic acid were photometrically assayed using the MBTH derivatives 1 or 2 as described previously.[1]

Results

Column chromatography of glycerol oxidase sample

Many protein peaks were shown by a Mono Q column chromatography of glycerol oxidase-Kyowa, but the glycerol oxidase activity was eluted in one peak, which also showed oxidizing activity for both ethylene glycol and glycolic acid. The active fractions from the Mono Q column were put on a Superose 6 column. The oxidizing activities for the three substrates were filtrated at the same position on the Superose 6 column and their reaction ratios were also the same (Fig. 1). As these results indicate that glycerol oxidase might contain oxidizing activity for both ethylene glycol and glycolic acid, the following experiments were done without purification of the glycerol oxidase sample.

Oxidation of related compounds of ethylene glycol

One hundred millimolar ethylene glycol, glycolaldehyde, glyoxal, glycolic acid, glyoxylic acid, or oxalic acid was incubated with glycerol oxidase (0.65 unit) by the assay method of enzyme activity in Materials and Methods. Hydrogen peroxide was generated only from ethylene glycol, glycolaldehyde, and glycolic acid. These results indicate that they were oxidized by glycerol oxidase, but glyoxal, glyoxylic acid, and oxalic acid were not. The substrate specificity of glycerol oxidase for ethylene glycol-related compounds might be broader than that of alcohol oxidases from methanol yeast.[1]

For the identification of reaction products from ethylene glycol, glycolaldehyde and glycolic acid, 100 mM ethylene glycol, glycolaldehyde, or glycolic acid was incubated with 20 units of glycerol oxidase under the standard reaction conditions for formation of glyoxal and glyoxylic acid. The MBTH derivatives 1 and 2 of their reaction products were prepared as described in Materials and Methods, and then put on a reversed phase C_{18} column. As shown in Table I, the retention times for the reaction products of ethylene glycol were the same as those for the authentic glycolaldehyde and glyoxal. That of the reaction product of glycolaldehyde was also the same as that of authentic glyoxal. No peaks were found at the same retention time for authentic glyoxylic acid in ethylene glycol and glycolaldehyde. In addition, approximately 96.8 and 98.7 mM glyoxals were formed from 100 mM ethylene glycol and glycolaldehyde, respectively, when each substrate was incubated with 20 units of glycerol oxidase at 20°C for 5 h (Table II). Consequently, it was concluded that glycerol oxidase oxidized ethylene glycol to glyoxal *via* glycolaldehyde, but did not oxidize glyoxal to glyoxylic acid.

The retention times for MBTH derivatives 1 and 2 of the reaction product from glycolic acid were the same as those of the authentic glyoxylic acid (Table I), and approximately 24.6 mM glyoxylic acid was formed by incubating 25 mM glycolic acid with 200 units of glycerol oxidase at 20°C for 10 h (Table II). It was, therefore, concluded that the

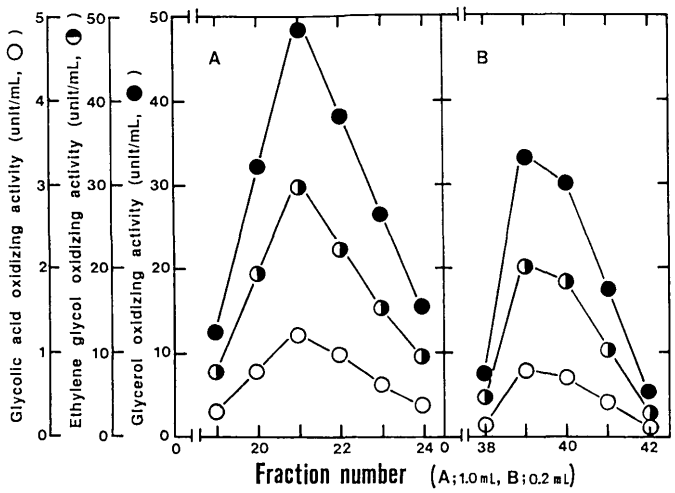

Fig. 1. Elution Pattern of Oxidizing Activity.

Column chromatographies of glycerol oxidase-Kyowa were done under the conditions described in Materials and Methods. The oxidizing activities were assayed under the same conditions as the enzyme activity, except that 20 mM glycerol, 0.5 M ethylene glycol, and 0.5 M glycolic acid were used. (A) Mono Q column chromatography. (B) Gel filtration on Superose 6. (●), glycerol oxidizing activity; (◑), ethylene glycol oxidizing activity; (○), glycolic acid oxidizing activity.

Table I. Identification of Reaction Product by Reversed Phase HPLC

Derivatives 1 and 2 were prepared as described in Materials and Methods, after incubation of ethylene glycol, glycolaldehyde, and glycolic acid under the standard reaction conditions for formation of glyoxal and glyoxylic acid, and separated by reversed phase HPLC as described in Materials and Methods.

	Retention time (min)	
	Derivative 1	Derivative 2
Authentic		
Glycolaldehyde	ND	16.4
Glyoxal	34.0, 34.6	ND
Glyoxylic acid	12.1	22.8
Substrate		
Ethylene glycol	34.0, 34.6	16.4
Glycolaldehyde	34.0, 34.6	16.4*
Glycolic acid	12.1	22.8

* Remaining substrate.

Table II. Conversion of Ethylene Glycol, Glycolaldehyde and Glycolic Acid

The reaction was done under the standard reaction conditions for formation of glyoxal and glyoxylic acid, except that ethylene glycol, glycolaldehyde, and glycolic acid were incubated with 20, 20, and 200 units of glycerol oxidases, respectively.

Substrate	Concentration (mM)	Reaction time (h)	Product	Concentration (mM)	Conversion (%)
Ethylene glycol	50	5	Glyoxal	49.8	99.6
	100	5	Glyoxal	96.8	96.8
Glycolaldehyde	50	5	Glyoxal	50.0	100.0
	100	5	Glyoxal	98.7	98.7
Glycolic acid	25	10	Glyoxylic acid	24.6	98.4
	50	10	Glyoxylic acid	40.8	81.6

oxidation reaction of glycolic acid by glycerol oxidase proceeded like that of glycolate oxidase[4-8] from green plants, but not like lactate oxidase,[9] which catalyzes the oxidative decarboxylation of glycolic acid and formed formate, CO_2, and H_2O as the major products.

Reaction of ethylene glycol and glycolaldehyde

Effects of pH on oxidation of ethylene glycol and glycolaldehyde were measured by following the formation of H_2O_2 as described in the assay of enzyme activity, except that 100 mM each of ethylene glycol and glycolaldehyde, and 0.2 M TES–NaOH buffer, pH 6.5 to 9.0, were used. The optimum pHs for oxidation of ethylene glycol and glycolaldehyde were found to be identical, around 7.0. The optimum temperatures for both oxidative reactions were approximately 40°C. The apparent K_ms for ethylene glycol and glycolaldehyde obtained were 195 and 48.8 mM at pH 7.0, respectively, by a plot of s/v against s. The K_m for glycolaldehyde is intermediate between both alcohol oxidases[1] from *Candida* sp. and *Pichia pastoris*, but that for ethylene glycol is much smaller than those of both alcohol oxidases.[1] The maximum velocities for ethylene glycol and glycolaldehyde, which were estimated on the basis of the data by Uwajima *et al.*[10] (142.2 units of glycerol oxidase activity is one mg of protein) were 89.1 and 62.2 μmol/min/mg of protein, respectively. These values are more than 15 and 60 times faster than those of alcohol oxidases from *Candida* sp.[1] These results indicate that the conversion of ethylene glycol to glyoxal by glycerol oxidase might proceed more rapidly than by alcohol oxidases[1], and that glycerol oxidase might be better than the alcohol oxidades for glyoxal production.

Reaction of glycolic acid

Effects of pH and temperature on oxidation of glycolic acid were measured under the same conditions as the ethylene glycol oxidation, except that 100 mM glycolic acid and 0.65 unit of glycerol oxidase were used. The optimum pH and temperature were found to be around 7.0 and 40°C, respectively, which were similar to the oxidation of ethylene glycol. The apparent K_m for glycolic acid obtained was 114 mM at pH 7.0, from a plot of s/v against s, and the maximum velocity was estimated to be 2.68 μmol/min/mg of protein. The oxidation of glycolic acid was competitively inhibited by glyoxylic acid, and the K_i was estimated to be 16.9 mM. These results indicate that glycerol oxidase might be available for glyoxylic acid production as well as glyoxal production, but conditions different from those for glyoxal production might be required for production of glyoxylic acid.

Formation of glyoxal from ethylene glycol

Effects of enzyme concentrations on the velocity of glyoxal formation were investigated under the standard reaction conditions for glyoxal formation using 5 to 50 units of glycerol oxidase (0.035 to 0.35 mg of protein). Addition of a large amount of glycerol oxidase did not effective increase the glyoxal formation rate. For example, 46.7, 56.4, and 68.7 mM glyoxal were formed from 100 mM ethylene glycol by 5, 20, and 50 units of glycerol oxidase, respectively, at 10°C for 3 h of incubation.

The optimum temperature for glyoxal formation was also investigated under the standard reaction conditions, except

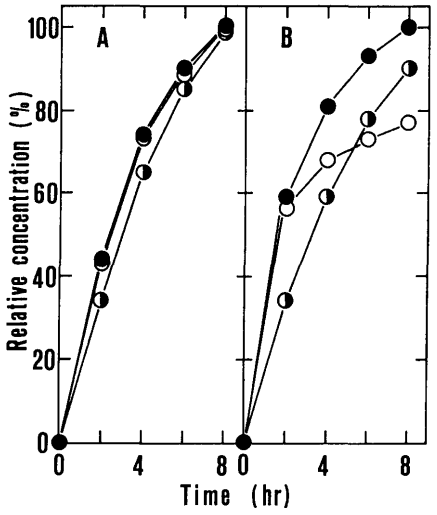

Fig. 2. Effects of Reaction Temperatures on Formation of Glyoxal and Glyoxylic acid.

The reaction was done under the standard reaction conditions for formation of glyoxal and glyoxylic acid, except that temperatures were changed from 10 to 30°C. A and B showed the formation of glyoxal from ethylene glycol and glyoxylic acid from glycolic acid, respectively. (◑), 10°C; (●), 20°C; (○), 30°C.

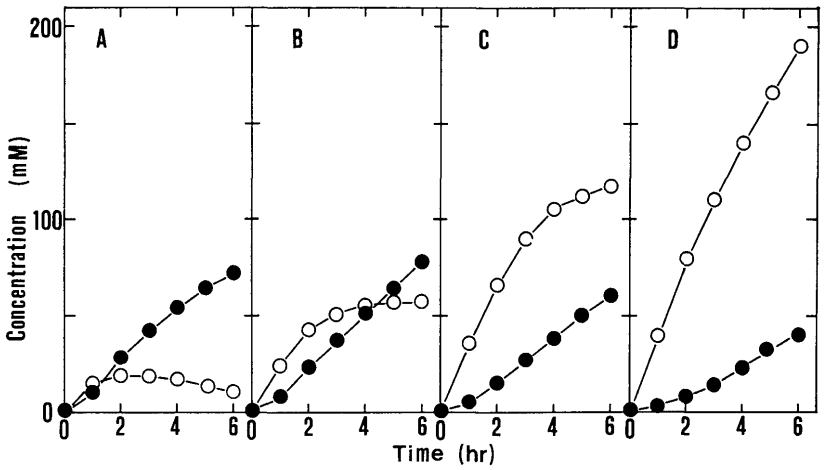

Fig. 3. Course of the Enzymatic Oxidation of Ethylene glycol to Glycolaldehyde and Glyoxal with Glycerol Oxidase.

The reaction was done under the standard reaction conditions for glyoxal formation, except that 0.1–1.0 M ethylene glycol was incubated with 6 units of glycerol oxidase at 20°C for 6 h. A, 0.1 M ethylene glycol; B, 0.25 M ethylene glycol; C, 0.5 M ethylene glycol; D, 1.0 M ethylene glycol. (○), glycolaldehyde; (●), glyoxal.

that 100 mM ethylene glycol was incubated with 5 units of glycerol oxidase at 10, 20, and 30°C. Glyoxal formed at 10°C was approximately 80% of that at 20°C for 2 h of incubation and became similar to that of 20°C at 6 h of incubation. The yield of glyoxal at 30°C was similar to that of 20°C at 2 or 6 h of incubation (Fig. 2A). As long incubation of ethylene glycol with glycerol oxidase is required for enzymatic preparation of glyoxal, 20°C was selected as the standard reaction condition described in Materials and Methods.

The effects of ethylene glycol concentration on the velocity of glycolaldehyde and glyoxal formations were shown in Fig. 3. The conversion rate of ethylene glycol to glycolaldehyde increased with the ethylene glycol concentration, but that of glycolaldehyde to glyoxal decreased against the ethylene glycol concentration. For example, 100 mM ethylene glycol was smoothly converted to glyoxal, but approximately 180 mM glycolaldehyde and 40 mM glyoxal were formed by incubation of 1 M ethylene glycol with 6 units of glycerol oxidase at 20°C for 6 h. These results indicate that glycolaldehyde oxidation might be a rate-limiting step on the glyoxal formation from ethylene glycol. When glycolaldehyde was used as substrate, the maximum amount of glyoxal was formed by approximately 250 mM glycolaldehyde, and the amount of glyoxal formed was decreased by increasing the glycolaldehyde concentration (Fig. 4). These results suggest that a high concentration of glycolaldehyde might competitively inhibit the glyoxal formation from ethylene glycol. The inhibition constant for the substrate action as a inhibitor of glycolaldehyde was estimated to be approximately 160 mM by a plot of $1/v$ against s. Accumulation of glycolaldehyde is undesirable in the production of glyoxal from ethylene glycol, so 100 mM ethylene glycol was used as the initial substrate concentration and 75 mM ethylene glycol was added every 3 h of incubation, while the pH was kept around 7.0. Approximately 170 mM glyoxal was formed after 15 h of incubation, although the velocity of glyoxal formation was gradually reduced by glycolaldehyde. It was, thus, conceivable that a consecutive reaction of glycerol oxidase with a low concentration of ethylene glycol might be effective for production of glyoxal.

Formation of glyoxylic acid from glycolic acid

The optimum reaction temperature for the glyoxylic acid formation was investigated under the same conditions as for the ethylene glycol oxidation, except that 500 mM glycolic acid was incubated with 75 units of glycerol oxidase. The glyoxylic acid formed at 10°C was approximately 60% of that at 20°C for 2 h of incubation. After 8 h of incubation, that at 10°C became approximately 85% of that at 20°C. The amount of glyoxylic acid formed at 30°C was similar to that at 20°C for 2 h of incubation, but then the glyoxylic acid formation rate at 30°C became slower than that at 20°C (Fig. 2B). Glycolic acid formation at 20°C was, therefore, better than at 10 or 30°C. The optimum temperature for glyoxylic acid formation was lower than for the initial oxidation reaction of glycolic acid, because a long-time incubation was required for the glyoxylic acid formation and glyoxylic acid inhibited the glycolic acid oxidation.

The optimum concentration of glycolic acid for glyoxylic acid formation was investigated by incubating 100 mM to 2 M glycolic acid with 105 units of glycerol oxidase at 20°C. As shown in Fig. 5, the maximum amount of glyoxylic acid was formed by approximately 0.4 and 1 M glycolic acid at 4 and 6 h of incubation, respectively, and the amount of glyoxylic acid formed was decreased against the higher concentration of glycolic acid. These results indicate that a higher concentration of glycolic acid might also act as an inhibitor. Since glycolic acid and glyoxylic acid act as an inhibitor of glycolic acid oxidation, control of the substrate or enzyme concentration might be required to significantly increase glyoxylic acid formation.

Effects of enzyme concentrations were investigated by incubating 500 mM glycolic acid with 10 and 130 units of glycerol oxidase at 20°C. When 30–130 units of glycerol oxidase was added, the amount of glyoxylic acid formed was not different for 1 h of initial incubation, but was

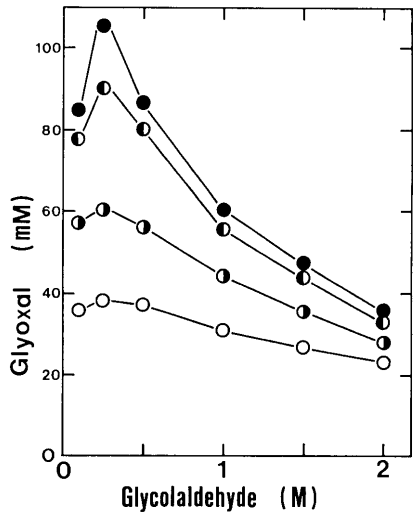

Fig. 4. Effects of Glycolaldehyde Concentration on Glyoxal Formation.

The reaction was done under the standard reaction conditions for glyoxal formation, except that 0.1 1.0 M glycolaldehyde was incubated with 6 units of glycerol oxidase at 20 C. (○), 2 h; (◑), 4 h; (◐), 6 h; (●), 8 h of incubation.

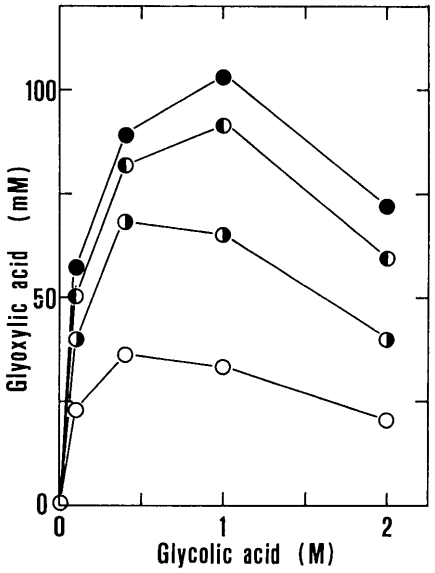

Fig. 5. Effects of Glycolic Acid Concentration on Glyoxylic Acid Formation.

The reaction was done under the standard reaction conditions for glyoxylic acid formation, except that 0.1 2.0 M glycolic acid was incubated with 105 units of glycerol oxidase at 10 C. (○), 2 h; (◑), 4 h; (◐), 6 h; (●), 8 h of incubation.

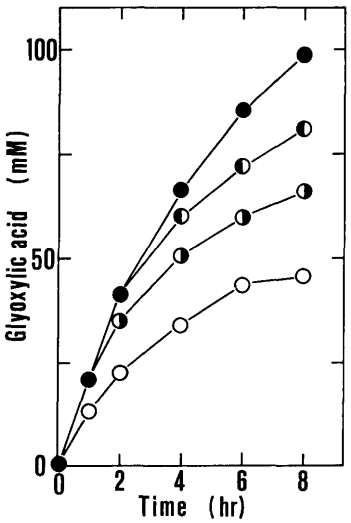

Fig. 6. Effects of Glycerol Oxidase Concentration on Conversion of Glycolic Acid to Glyoxylic Acid.

The reaction was done under the standard reaction conditions for glyoxylic acid formation, except that 0.5 M glycolic acid was incubated with 10–130 units of glycerol oxidase at 20°C for 18 h. (○), 10 units; (◑), 30 units; (◐), 65 units; (●), 130 units of glycerol oxidase.

increased by increasing the enzyme amount and using long-time incubation. Thus, for example, approximately 20% of glycolic acid was converted to glycoxylic acid by 130 units of glycerol oxidase in 8 h of incubation (Fig. 6). Addition of a large amount of glycerol oxidase was required to obtain a high concentration of glyoxylic acid, but the addition of a large amount of enzyme at the beginning might be ineffective. We compared two ways to add glycerol oxidase; one by adding 20 units each at every 3 h of incubation (*i.e.*, a total of 4 times); the other by adding 100 units only in the initial reaction. In the former case, approximately 120 mM glyoxylic acid was formed for 24 h of incubation and the glyoxylic acid formation continued, while in the latter case approximately 90 mM glyoxylic acid was formed for 15 h of incubation and then the formation did not continued. As consecutive reaction with glycolic acid by feeding glycerol oxidase was effective to increase the glyoxylic acid yield from glycolic acid, an immobilized glycerol oxidase might be useful for glyoxylic acid formation.

Discussion

This study demonstrated that glycerol oxidase from *Aspergillus japonicus* had oxidizing activity not only for glycerol but also for ethylene glycol, glycolaldehyde, and glycolic acid. Uwajima and Terada[3] have reported that glycerol oxidase did not oxidize ethylene glycol when 50 μmol of ethylene glycol (16.7 mM) was incubated with 2 μg of protein (0.28 unit) in a 3-ml reaction mixture. However, oxidation of ethylene glycol might be undetectable under their conditions, because the apparent K_m and V_{max} of glycerol oxidase for ethylene glycol were approximately 195 mM and 89.1 μmol/min/mg of protein, respectively. The oxidizing activities for glycerol, ethylene glycol, and glycolic acid were not separated on Mono Q and Superose 6 columns, and the ratios of their respective oxidizing activities were the same in all active fractions. These results indicate that glycerol oxidase from *Aspergillus japonicus* might be able to also oxidize ethylene glycol and glycolic acid, which were converted to glyoxal and glyoxylic acid, respectively. Thus, the glycerol oxidase might react with the hydroxyl group not only adjacent to the hydroxyl or aldehyde groups but also the carboxyl group, while alcohol oxidases[1] from methanol yeast connot oxidize the hydroxyl group adjacent to the carboxyl group. No oxidizing activities from glyoxal to glyoxylic acid or from glyoxylic acid to oxalic acid were found in the glycerol oxidase, indicating that glycerol oxidase might not react with the aldehyde group and might be available for the production of glyoxal or glyoxylic acid.

Some enzymatic properties of glycerol oxidase for oxidation of ethylene glycol, glycolaldehyde and glycolic acid, obtained in this study, can be compared with those of alcohol oxidase[1] and glycolate oxidases.[4-8] The maximum velocities of glycerol oxidase for ethylene glycol and glycolaldehyde were much faster than those of the alcohol oxidases.[1] No differences were found in optimum pHs of glycerol oxidase for oxidation of ethylene glycol and glycolaldehyde, while those of the alcohol oxidases[1] are much different for ethylene glycol and glycolaldehyde. In addition, no oxidizing activity of glyoxal to glyoxylic acid are found as alcohol oxidases.[1] Therefore, glycerol oxidase might be better than the alcohol oxidases[1] for enzymatic preparation of glyoxal from ethylene glycol. However, the glyoxal formation rate was inhibited by a high concentration of ethylene glycol or glycolaldehyde. As a high concentration of ethylene glycol is generally desirable for glyoxal production, the properties of glycerol oxidase might be disadvantageous for enzymatic preparation of glyoxal. Thus, an effective conversion method of ethylene glycol to glyoxal was investigated by taking advantage of glycerol oxidase. A consecutive reaction with a low concentration of ethylene glycol might be useful for enzymatic preparation of glyoxal using glycerol oxidase, because it might regulate the glycolaldehyde concentration by addition of ethylene glycol. It was, therefore, conceivable that immobilized glycerol oxidase would be promising for glyoxal production. The high specific activity of glycerol oxidase for ethylene glycol and glycolaldehyde might be very advantageous for use as an immobilized enzyme.

Seip *et al.*[2] and Hoefngel *et al.*[12] have reported that glycolate oxidase from spinach contains oxidizing activity for glyoxylic acid as well as glycolic acid, and some primary amines improve the glyoxylic acid yield. However, the amines were not required for enzymatic preparation of glyoxylic acid using glycerol oxidase, because in glycerol oxidase, the oxidative reaction of glyoxylic acid to oxalic acid was less than 0.1% of the glycolic acid oxidation (data not shown). This specificity of glycerol oxidase might be advantageous for glyoxylic acid production, but other properties such as the high K_m for glycolic acid and low K_i for glyoxylic acid might be undesirable for enzymatic preparation of glyoxylic acid. Since glyoxylic acid competitively inhibited the oxidation of glycolic acid, a high concentration of glycolic acid was used to improve the yield of glyoxylic acid. However, this led to the same kind of substrate inhibition as glycolaldehyde in glycolaldehyde oxidation. The yield of glyoxylic acid was improved by feeding of glycerol oxidase, while that of glyoxal was improved by feeding of ethylene glycol. These results are suggestive that regulation of the glycolaldehyde concentra-

tion might be essential for enzymatic preparation of glyoxal and that the ratios of concentrations of glycolic acid to glycerol oxidase might be important for glyoxylic acid formation.

Here, we described some properties of glycerol oxidase in the reactions with ethylene glycol and glycolic acid, and evaluated glycerol oxidase for enzymatic production of glyoxal and glyoxylic acid. Glycerol oxidase has several potential drawbacks for smooth conversion of ethylene glycol to glyoxal or glyclic acid to glyoxylic acid due to competitive inhibition by the substrate or product. However, the yield of glyoxal and glyoxylic acid were improved by repreated addition of substrate or enzyme. Therefore, a reactor using an immobilized glycerol oxidase might be more conducive to improve the yield of glyoxal or glyoxylic acid. An examination of the immobilization of glycerol oxidase and catalase, and their applications are under way.

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