Application of Glyceric Acid to Bio-related Functional Materials and Improvement of Microbial Production

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Abstract: Glyceric acid (GA) is an oxidative product of glycerol, and its d-isomer is obtained as a phytochemical from tobacco leaves and fruits of some plants. However, the production and applications of GA have not yet been fully investigated. In this review, recent developments in the microbial production of GA and its application to bio-related materials are summarized. The sodium salt of diacylated GA showed superior surface tension-lowering activity and antitrypsin activity. GA and its glucosyl derivative had positive effects on the viability and collagen production of skin cells in vitro, respectively. Glucosyl derivatives of GA showed protective effects against heat-induced protein aggregation. In addition, the microbial production of GA using raw glycerol as the starting material was investigated. The effect of methanol, a major impurity in raw glycerol, on GA production was investigated, and mutant strains to tolerate methanol in the culture were constructed. Enantioselective production of GA using newly isolated microbial strains has also been developed.

Key words: glyceric acid, glycerol use, surfactant, enzyme inhibitor, protective solutes, acetic acid bacteria, methanol, oxidative fermentation

1 Introduction

Sustainable development is an important global challenge for all industries. The use of renewable resources such as sugars and oils in the energy and chemical industries has therefore been expanding to establish a sustainable industry and address environmental issues, such as global warming. Triacylglycerols, the main component of plant oils, can be used to produce biodiesel, surfactants, and value-added chemicals, and the production and use of triacylglycerols have been increasing as they are produced from atmospheric carbon dioxide by plants and have long acyl chains in the fatty acid moiety of triacylglycerols. The use of fatty acids generates glycerol, another component of triacylglycerols, and therefore expanding use of glycerol is desired with an increase in the use of plant oils. Glycerol is widely used in various industries as a moisturizer, thickening agent, lubricant, building blocks of chemicals, etc. Many studies have also presented the alternative use of glycerol as a starting material for producing chemicals, such as alcohols, organic acids, and polymers. However, the rapid increase in the production of biodiesel fuels resulted in a surplus of impure glycerol, called raw glycerol. Raw glycerol contains alkali metals and methanol as impurities (Table 1)\(^1\)\(^-\)\(^5\), making it difficult to use as a starting material for chemical production.

Glyceric acid (GA), i.e., 2,3-dihydroxypropanoic acid, is a simple but attractive derivative of glycerol because of its structure with two kinds of functional groups and chiral isomers (Fig. 1). GA was originally found as a constituent of tobacco leaves in 1956, and its structure was reported as a d-\((R)\) isomer\(^6\). Subsequent studies using d-GA showed some biological activities, such as liver stimulation\(^7\). In addition, both enantiomers of GA are recognized as markers of symptoms of d-glyceric academia/acidurias or hyperoxaluria type 2\(^8\). Although some applications have been investigated, there have been few industrial and commercial applications of GA. Thus, to expand the use of GA, more attractive functions of GA and its derivatives are necessary.

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Thus, the use of raw glycerol without a refining process for GA production needs to be developed.

### 2.2 Synthesis and properties of acyl GAs

GA has two hydroxy groups; thus, their acylated compounds, namely diacyl GA or monoacyl GA, are expected to have amphiphilic properties. In addition, metabolites from *Penicillium funiculosum*, OR-1, contained mixtures of diacyl GAs with acyl chain lengths of C14 to C18 (Fig. 2A), which showed a strong inhibitory effect on trypsin digestion of casein. Habe *et al.* reported the synthesis and evaluation of dioleoyl GA, a main component of OR-1, from GA calcium salt. The dioleoyl GA showed a relatively lower inhibitory effect than OR-1, suggesting that another potent substance in OR-1 was responsible for trypsin inhibition. Other diacyl GAs with long acyl chains (C16 or C18:2) were synthesized via the benzyl ester derivative of GA (Fig. 2B). The long acyl chains in the compounds resulted in low water solubility. They showed no toxic effects on NHDFs and normal human dermal microvascular endothelial cells up to ~34 μM.
In contrast, diacyl GAs with medium acyl chains \( \text{C}_6 \) to \( \text{C}_{12} \) were synthesized by direct acylation of GA \( \text{Fig. 2B} \). Although the acid form of diacyl GAs with medium acyl chains showed little solubility in water, their sodium salts had good water solubility. Among them, dioctanoyl GA sodium salt showed great surface-tension lowering properties \( \text{Table 2} \). This also showed a greater inhibitory effect on trypsin activity than dioleoyl GA. This is probably due to the difference in water solubility. Another water-soluble sodium salt of diacyl GAs, namely didecanoyl GA sodium salt, also had inhibitory effects on trypsin activity comparable to that of dioctanoyl GA sodium salt. Additionally, sodium salts of diocanyl GA and didecanoyl GA form large associates with vesicle-like structures at high concentration \( \text{Table 2} \). The understanding of this unique property of diacyl GAs will lead to new applications of GA diacyl surfactants.

Monooacyl GAs were also synthesized from \( \text{n-GA} \). Conventional acylation with acyl chlorides provided a mixture of regioisomers, 2-O-acyl GA and 3-O-acyl GA. These could be separated via column chromatography, and their physicochemical properties were investigated. In contrast to diacyl GAs, monooacyl GAs showed solubility in water without saponification. The 2-O-acyl GAs showed great surface-tension lowering properties \( \text{Table 2} \); therefore, they are considered as a new class of green surfactants.

### Table 2

|                   | CMC\(^a\) (M) | \( \gamma_{\text{CMC}} \)\(^b\) (mN/m) |
|-------------------|---------------|-------------------------------------|
| Dihexanoyl GA sodium salt | \( 2.9 \times 10^{-3} \) | 33.9 |
| Dioctanoyl GA sodium salt | \( 8.2 \times 10^{-4} \) | 25.5 |
| Didecanoyl GA sodium salt | \( 2.3 \times 10^{-4} \) | 27.9 |
| 2-O-Lauroyl GA | \( 3.0 \times 10^{-4} \) | 25.6 |
| 2-O-Myristoyl GA | \( 9.3 \times 10^{-5} \) | 28.7 |
| 2-O-Palmitoyl GA | \( 2.9 \times 10^{-5} \) | 34.1 |

\(^a\) Critical micelle concentration.  
\(^b\) Surface tension at the CMC.

In contrast, diacyl GAs with medium acyl chains (C6 to C12) were synthesized by direct acylation of GA \( \text{Fig. 2B} \). Although the acid form of diacyl GAs with medium acyl chains showed little solubility in water, their sodium salts had good water solubility. Among them, dioctanoyl GA sodium salt showed great surface-tension lowering properties in water \( \text{Table 2} \). This also showed a greater inhibitory effect on trypsin activity than dioleoyl GA. This is probably due to the difference in water solubility. Another water-soluble sodium salt of diacyl GAs, namely didecanoyl GA sodium salt, also had inhibitory effects on trypsin activity comparable to that of dioctanoyl GA sodium salt. Additionally, sodium salts of dioctanoyl GA and didecanoyl GA form large associates with vesicle-like structures at high concentration \( \text{Table 2} \). The understanding of this unique property of diacyl GAs will lead to new applications of GA diacyl surfactants.
boxylic acid in GGA was amidated by \( N \)-dodecylamine in the presence of a condensing reagent of 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride, resulting in the synthesis of \( N \)-dodecyl glucosylglyceric acid amide (Fig. 3). This compound showed good solubility in some organic solvents such as ethanol, acetone, dimethylformamide as well as water. The water solution showed a good surface tension-lowering property of 27.8 mN/m at a critical micelle concentration of 0.157 mM. This compound had a great protective property against heat-induced aggregation of egg white at low concentrations. Commercialized alkanoyl-\( N \)-methylglucamides (for example, MEGA-8 and MEGA-10) are solubilizing reagents for membrane proteins. Considering the structural similarity, \( N \)-dodecyl glucosylglyceric acid amide could also have similar property.

3 Development of Microbial Production of GA

3.1 Oxidative fermentation of GA from glycerol

Acetic acid bacteria are widely used for vinegar production from ethanol. This process is called oxidative fermentation because it consumes oxygen not to completely oxidize ethanol to carbon dioxide, but to accumulate acetic acid in the culture media. This oxidative fermentation is also used to produce sorbose, dihydroxyacetone, 2-ketogluconate, etc. Habe et al. developed a microbial method to produce GA through oxidative fermentation (Fig. 4)\(^ {10, 11} \). By selecting microbial strains, the enantioselectivity of GA was varied from 70% to 99% enantiomeric excess (ee). Acetobacter tropicalis NBRC16470 produced \( D \)-GA with 99% ee, whereas Gluconobacter frateurii NBRC103465 produced \( L \)-GA with 72% ee (Fig. 5). The accumulation of GA in the culture broth of \( G. \) frateurii reached 136.5 g/L in 7 d upon fed-batch cultivation in a 5 L jar fermenter.
Habe et al. also developed a recovery process for GA from culture broth using the electrodialysis method\(^{26,27}\). Although the production process of GA from glycerol has been developed, the use of raw glycerol for GA production had to be developed. For example, it was demonstrated that GA production from raw glycerol by *Gluconobacter* spp. requires pretreatment of raw glycerol to remove impurities\(^{12}\).

### 3.2 Investigation and improvement of GA production from methanol-containing glycerol

When using raw glycerol in microbial production of GA, methanol, a major impurity of raw glycerol, could have a negative effect on microbial growth. Indeed, addition of 1 vol% methanol in the culture of *Gluconobacter* strains decreased GA production level by 30–51% compared to no addition of methanol\(^{28}\). Therefore, the effect of methanol on GA production by *G. frateurii* was investigated in detail. The draft genome sequence of *G. frateurii* strain NBRC103465 was first analyzed\(^{29}\). This strain possesses putative genes for methanol detoxification as well as those for GA production. The DNA microarray was designed based on the draft sequence, followed by transcriptome analysis to investigate the responses of *G. frateurii* cells to methanol in the culture for GA production\(^{30}\). In comparison to methanol-free conditions, the cells upregulated a homolog gene of class III alcohol dehydrogenase \(\text{adhC}_\text{Gf}\) in the presence of methanol. This enzyme is known to catalyze oxidation of formaldehyde to formic acid. The \(\text{adhC}_\text{Gf}\) was heterologously expressed in *Escherichia coli* and the gene product was confirmed to have dehydrogenase activity toward formaldehyde. Therefore, *G. frateurii* could detoxify formaldehyde arising from methanol oxidation by upregulating the class III alcohol dehydrogenase gene. Interestingly, no changes in the expression of genes responsible for GA production were observed. The effect of methanol on the enzymatic reaction of glycerol oxidation to glycoaldehyde was investigated\(^{31}\). This reaction is catalyzed by membrane-bound alcohol dehydrogenase \(\text{mADH}\)\(^{32}\), and therefore the activity of purified \(\text{mADH}\) from *G. oxydans* toward glycerol was evaluated in the presence of methanol. With 0.3 vol% methanol in the reaction, the dehydrogenase activity of \(\text{mADH}\) toward glycerol decreased by half; apparently, methanol caused a decrease in GA production. Therefore, it will be useful to develop mutant \(\text{mADHs}\) with decreased affinity toward methanol.

An approach for improving GA production was attempted by chemical mutagenesis of *G. frateurii* to produce methanol-resistant mutant strains\(^{33}\). Using N-methyl-N′-nitro-N-nitrosoguanidine, a methanol-resistant mutant library of *G. frateurii* NBRC103465 was constructed, followed by screening of these mutants by GA production level with a pH indicator. Upon evaluating >1000 colonies, Gf398 strain was found to exhibit superior methanol-resistant ability compared to that of parental strain. The Gf398 strain also produced more GA in the presence of 3–5 vol% methanol than the parental strain. The jar fermenter experiment of this mutant strain demonstrated GA production by fed-batch cultivation from methanol-containing glycerol, which was two-fold greater than the parental strain (unpublished data). Investigation of mutation points of the Gf398 strain will elucidate how the strain tolerates external methanol and detoxifies methanol and formaldehyde, and will lead to further development of useful microbial strains to use methanol-containing raw glycerol.

Another study of GA production by methylotrophic acetic acid bacteria, *Acidomonas methanolica*, provided a new approach for GA production from methanol-containing glycerol\(^{32}\). *A. methanolica* is a unique acetic acid bacterium that assimilates methanol; therefore, GA production from methanol-containing glycerol by this bacterium was expected. GA production was first confirmed, and subsequent experiments with methanol-containing glycerol revealed that *A. methanolica* produced more GA from glycerol with 1 vol% methanol than that without methanol. Interestingly, the enantioselectivity of the GA produced by *A. methanolica*, 44 % ee of \(\text{d-GA}\), was lower than those of other acetic acid bacteria such as *G. frateurii* and *Acetobacter tropicalis* (Fig. 5). This strain broadened the insight into GA production from methanol-containing glycerol as well as the stereoselectivity of GA by acetic acid bacteria.

### 3.3 Enantioselective production of GA by microbial resolution

Although \(\text{d-GA}\) can be utilized for various applications, \(\text{l-GA}\) is expected to be used as the starting material for generating \(\text{l-sugars}\) for biological and pharmacological applications\(^{34}\). Although conventional conversion of glycerol to GA by metal catalysts often yields a racemic mixture, oxidative fermentation of GA by acetic acid bacteria produces mainly \(\text{d-GA}\) (Fig. 5). Therefore, separation methods to obtain pure \(\text{l-GA}\) from racemic mixtures are necessary for \(\text{l-GA}\) production. Microbial resolution is an effective method to obtain chiral isomers from racemic mixture. Therefore, screening experiments for obtaining \(\text{d-GA}\)-asimulating bacteria were conducted to construct a microbial resolution method for \(\text{l-GA}\) production\(^{35}\). Accumulation of \(\text{l-GA}\) from a racemic mixture of GA was achieved by screening strains of *Serratia* sp. GA3R and *Pseudomonas* spp. GA72P, with > 89% ee of \(\text{l-GA}\) (Fig. 5). This demonstrated that enantioselective production of both \(\text{d-GA}\) and \(\text{l-GA}\) could be achieved by microbial fermentation by selecting GA-producing and GA-asimulating bacteria.
4 Summary

In this review, recent achievements in microbial production of GA and its application were summarized to promote the utilization of raw glycerol and to apply GAs to bio-related functional materials. GA itself as well as its acyl and glucosyl derivatives showed various bio-related functions, expanding the applications of GA to skincare and cosmetic products. Microbial production of both D- and L-GA was developed by GA-producing *Gluconobacter* strains, methylo-troph acetic acid bacteria, and newly isolated GA-assimilating bacterial strains. These developments will promote the use of GA in chemical and biological industries, leading to expanded use of raw glycerol in oleochemical and energy industries.

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