Synthesis and Characterization of a Novel Glycolipid with Glucosylglycerate as a Hydrophilic Showing Protective Effects on Heat-induced Protein Denaturation

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Abstract: A novel glycolipid featuring a glucosylglycerate moiety as a polar head group was synthesized in two steps from sucrose, glycerate, and N-dodecylamine. Glucosylglyceric acid was formed from sucrose and glyceric acid using sucrose synthase as a catalyst, followed by condensation with N-dodecylamine using 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM) as a condensing agent. A white solid compound was recovered with a yield of 21% after purification by hydrophobic column chromatography. The structure and purity of the isolated compound, identified as N-dodecyl glucosylglyceric acid amide (aGGA), were confirmed by 1H and 13C nuclear magnetic resonance and liquid chromatography-electrospray ionization-mass spectrometry. aGGA was soluble in several polar solvents, including acetone, dimethyl formamide, and short chain alcohols. The dissolution of aGGA in water reduced the surface tension to 27.8 mN m⁻¹ at a critical micellar concentration of 1.57 × 10⁻⁴ M. In addition, the presence of aGGA at concentrations as low at 0.68 mM protected egg white from heat-induced denaturation. These results suggest that aGGA could be useful as a protein-protecting surfactant.

Key words: glyceric acid, α-glucosylglycerate, N-dodecyl-(R)-2-O-α-D-glucopyranosylglyceric acid amide, surfactant, glycerol

1 Introduction

Glyceric acid (2,3-dihydroxypropanoic acid, GA), a minor constituent of certain plants, can be mass-produced from glycerol, a by-product of biodiesel and oleochemical manufacturing. Acetobacter tropicalis, a species of acetic acid bacteria, can produce enantiopure D-GA in an enantiomer excess of 99.3%. However, although some of the properties of GA and its derivatives have been investigated, no industrial applications have been made public.

Among the various GA derivatives that have been studied, α-glucosylglyceric acid (R)-2-O-α-D-glucopyranosyl glyceric acid; GGA), is particularly interesting. GGA consists of α-glucose and D-GA moieties and is accumulated in microorganisms such as cyanobacteria, archaea, and some γ-proteobacteria as a means of balancing osmotic pressure and protecting biological molecules, such as DNA and proteins, from the external environment. GGA is preferentially accumulated under high-salt and nitrogen-limited conditions.

GGA can be enzymatically synthesized from sucrose and D-GA via transglycosylation using sucrose phosphorylase as a catalyst. Sato et al. demonstrated that normal human dermal fibroblasts produced 19–41% more type I collagen
2 Experimental

2.1 Materials

d-Glyceric acid was prepared by the oxidative fermentation of glycerol by *Acetobacter tropicalis* NBRC14670, according to the method reported by Habe et al.4 Sucrose phosphorylase was obtained from Oriental Yeast (Osaka, Japan). N-Dodecyl-β-D-glucopyranosylglyceric acid amide (aGGA), was synthesized and its interfacial properties, effects on surface tension, and protective effects against protein denaturation were assessed.

2.2 Synthesis of aGGA

GGA-Na was synthesized from sucrose and d-GA by sucrose phosphorylase, as reported previously.10 Amidation of GGA was carried out in an H2O/methanol (1:1) solution in the presence of dodecylamine and DMT-MM as a condensation agent. Briefly, to a round-bottom flask containing sodium glucosylglycerate (0.195 g, 0.67 mmol) in pure water (11 mL), 0.67 mL of 1 M HCl, 11.67 mL of methanol, and 0.67 mmol (124 mg) of dodecylamine were added and stirred at room temperature. After dissolving dodecylamine in the mixture, 4 eq of DMT-MM (2.69 mmol) was added. The reaction proceeded at room temperature with stirring, and the progress was checked periodically by thin layer chromatography (TLC). After the spots corresponding to starting materials on the TLC plate disappeared, the methanol in the reaction mixture was evaporated under reduced pressure. To the resulting material, 1 M HCl and ethyl acetate were added, and the organic layer was collected and dried with anhydrous Na2SO4. The ethyl acetate was then concentrated to approximately 10 mL by evaporation. An excess amount of acetone was added to the concentrated ethyl acetate mixture, resulting in a white precipitate. After removal of the precipitate by filtration, the filtrate was concentrated in vacuo and dried. The crude product was further purified by hydrophobic chromatography using an AKTAprime plus (GE Healthcare UK Ltd., Little Chalfont, UK) equipped with a HiTrap Butyl FF column (GE Healthcare). The mobile phase was composed of solvent A (milli-Q water) and solvent B (ethanol), driven at a flow rate of 5 mL min⁻¹. Elution started with 0% B (100 mL) and was increased to 50% B (total 200 mL) with a final holding volume of 100 mL 50% B. The effluent was fractionated into 10-mL aliquots and evaluated for purity by TLC. Fractions containing aGGA were combined and dried under vacuum.

The obtained product was characterized by TLC, liquid chromatography–mass spectrometry (LC-MS) and 1H- and 13C-nuclear magnetic resonance (NMR). The yield of aGGA was 21%.

2.3 Solubility of aGGA in various solvents

Separately, 1 mL of six solvents (water, dimethyl formamide, 1,3-butandiol, ethanol, acetone, or diethyl ether) were added to 10 mg of synthesized aGGA. Each solution was mixed by vortex at room temperature. If the aGGA failed to dissolve at room temperature, the solution was incubated at 60°C for 30 min (except for diethyl ether). The solubility of aGGA was compared against that of GGA-Na.

2.4 Determination of surface tension

The surface tension of aqueous solutions containing aGGA was determined by the Wilhelmy plate method at

in the presence of 0.034–34 mM GGA sodium salt (GGA-Na). This GGA-induced stimulation of collagen synthesis is supported by the fact that α-glucosylglycerol, a structural analog of GGA, enhanced collagen synthesis in mice via an increase of IGF-I production.17 Sato et al.14 also evaluated GGA-Na as a protective solute *in vitro*. The protective effects of GGA against DNA scission by hydroxylradical was investigated in water using a hydroxyradical-generation system employing H2O2, FeCl3, and ascorbic acid. The protective effects of GGA-Na were dose-dependent and more effective than sugars alone, *e.g.*, glucose, sucrose, and trehalose, in protecting DNA from scission.14,18

The protective effects of GGA-Na against heat-induced protein aggregation were also evaluated. GGA-Na and GA-Na, at concentrations of 34 mM and 78 mM, respectively, suppressed the heat-induced aggregation of egg white to a greater degree than sugars such as glucose and trehalose.19 This suggests that the structure of GA may contribute to the protein-protective properties of GGA-Na as an enzyme stabilizer.19

The functional properties of GGA make it a potential candidate for use in biocompatible chemical products such as skin care products. However, since GGA is highly polar and hydrophilic, it is expected to have low compatibility with oil-based solvents or materials. To broaden its potential applicability in skin care products, GGA must be made amphiphilic.

Toward this end, a GGA derivative having an amphiphilic structure, N-dodecyl-(-)-2-O-α-D-glucopyranosylglyceric acid amide (aGGA), was synthesized and its interfacial properties, effects on surface tension, and protective effects against protein denaturation were assessed.

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25°C using a DY-500 surface tension meter (Kyowa Kaimen Kagaku Co., Niiza, Japan). The critical micelle concentration (CMC) of the compound was calculated from the crosspoint on the surface tension curve. The surface tension at CMC (γCMC) was also determined. The same experiment was performed with sucrose monolaurate and MEGA-10 for comparison purposes.

2.5 Estimation of emulsifying activity
The oil/water (O/W) emulsifying ability of aGGA was evaluated using a colorimetric method. In a test tube, 0.1 mL of oil (squalane, soybean oil, olive oil, decane, liquid paraffin, or silicone oil), constituting the hydrophobic phase, was added to 4 mL of distilled water containing 1 mg of aGGA. For comparison, the same experiments were performed with sodium dodecyl sulfate (SDS, a representative surfactant) and MEGA-10. The test tubes were vortexed thoroughly for 1 min and allowed to stand at room temperature. After 3 h, the lower 1 mL of the mixture was transferred to a cuvette, and its turbidity, measured at 620 nm, was expressed as an optical density (OD). Turbidity with no surfactant was measured as a control. Data are presented as mean values determined from at least three independent experiments.

2.6 Protein-protective properties of aGGA
To a reaction mixture composed of 50 mM sodium-phosphate buffer and 2% (w/v) egg white, aGGA was added to final concentrations of 0.1–0.001% (w/v). The mixture was heated at 70°C and the degree of protein aggregation was evaluated by measuring the OD of the mixture at 720 nm using an ultraviolet–visible spectrophotometer. This experiment was repeated with MEGA-10 for comparison.

2.7 Analytical procedures
TLC plates were developed with a solution of ethyl acetate/methanol/acetic acid/H2O (4/3/3/1) to monitor the reaction progress, and with a chloroform/methanol (1/1) solution for aGGA confirmation. Organic compounds on the TLC plates were visualized by heating the plates at 120°C for 5 min with a 5% (w/v) phosphate-molybdate solution in ethanol containing 5% (v/v) sulfuric acid and 0.6% phosphoric acid.

LC-MS was performed on a Shimadzu LC-MS 2020 system (Shimadzu, Otsu, Japan) equipped with a reverse-phase Synergi 4u column (150 mm × 2.0 mm; Phenomenex, Torrance, CA, USA). The mobile phase was composed of solvent A [0.1% (v/v) formic acid] and solvent B [acetonitrile] at a flow rate of 0.2 mL min⁻¹. Gradient elution was performed starting at 50% B for 2 min, increasing to 95% B over 10 min and holding for 3 min, then returning to 50% B over the following 3 min and holding for 2 min. The column was kept at 40°C during analysis. Effluents were ionized by electrospray ionization and detected in negative mode over a m/z range of 50–2,000. 1H and 13C NMR spectra in CD3OD were recorded with a Bruker Advance III (Bruker, Karlsruhe, Germany) at 400 MHz for 1H-NMR and 100 MHz for 13C-NMR.

3 Results and Discussion

3.1 Synthesis and solubility of aGGA
We examined the introduction of dodecylamine onto GGA (Fig. 1A) as a means of synthesizing aGGA (Fig. 1B) using DMT-MM as a condensation agent, one of the most general methods of amidation. After stirring this reaction overnight at room temperature, the TLC spot corresponding to GGA (Rf value: 0.41) disappeared, while spots arose with Rf values of 0.68–0.95. Following the removal of methanol by evaporation and subsequent extraction with ethyl acetate, the products in each layer were evaluated by TLC, revealing a single spot in the organic layer. The addition of acetone yielded a precipitate, which was removed by filtration. After drying and purifying via column chromatography, the filtrate was shown to contain a pure compound by NMR and LC-MS analyses. The 1H and 13C chemical shifts δ (ppm) of the compound were as follows: for 1H-NMR, 4.80 (d, 1H, J = 3.9 Hz, H-1), 4.16 (dd, 1H, J = 5.1, 3.0 Hz, H-7), 3.86 (dd, 1H, J = 11.8, 3.0 Hz, H-8a), 3.82–3.78 (m, 3H, H-3,6), 3.74 (t, 1H, J = 9.3 Hz, H-5), 3.68 (dd, 1H, J = 12.3, 5.9 Hz, H-8b), 3.47 (dd, 1H, J = 9.7, 3.9 Hz, H-2), 3.33 (d, 1H, J = 9.6 Hz, H-4), 3.23 (t, 2H, J = 7.0 Hz, H-9), 1.53 (q, 2H, J = 7.0 Hz, H-10), 1.29–1.32 (br, 18H, H-11) and 0.90 (t, 3H, J = 6.9 Hz, H-12) (Fig. 2A); for 13C-NMR, 172.5 (C-1'), 80.9 (C-2'), and 64.2 (C-3') in glyceraldehyde, 100.8 (C-1), 74.9 (C-3), 74.5 (C-5), 73.3 (C-2), 71.6 (C-4), and 62.5 (C-6) in glucose, 40.3 (C-1'), 33.1–23.7 (C-2'-C-11'), and 14.4 (C-12') in dodecyl moiety (Fig. 2B). Collectively, these data confirm aGGA synthesis. The total ion current chro-

(A)

(B)

Fig. 1 Chemical structures of (A) GGA-Na and (B) aGGA.
matogram showed that aGGA was eluted at approximately 5.2 min (Fig. 3A), as evidenced by the following ions: [M-H]⁻ (m/z = 434), [M + HCOO]⁻ (m/z = 480), and [2M-H]⁻ (m/z = 870) (Fig. 3B). These data indicate a molecular weight of 435. The final yield of aGGA was 21%.

The solubilities of aGGA and GGA-Na were evaluated in a variety of solvents (Table 1). The synthesized aGGA showed a lower solubility than GGA-Na in water owing to its more hydrophobic structure. In contrast, aGGA was soluble in both dimethyl formamide and ethanol at ambient temperature and in both 1,3-butandiol and acetone after mild heating. aGGA was not soluble in diethyl ether.

3.2 Surface properties of aGGA in water

The surface tension of aGGA in water was evaluated using the Wilhelmy plate method. Surface tension decreased with increasing concentrations of aGGA (Fig. 4). The crosspoint of the surface tension plot indicated that the CMC of aGGA was $1.57 \times 10^{-4}$ M, while the γCMC was 27.8 mN m⁻¹ (Table 2). For comparison, this experiment was repeated with two surfactants with structures similar to that of GGA, i.e., sucrose monolaurate having a C12-acyl chain as a hydrophobic group and MEGA-10 having an amide bond as a hydrophilic group (Fig. 4). The γCMC values were 37.0 mN m⁻¹ and 28.3 mN m⁻¹, respectively (Table 2). Although the CMC of aGGA was similar to those of sucrose monolaurate or polyoxyethylene monolaurate ($1.52 \times 10^{-4}$ M, Table 2), the γCMC of aGGA indicated a greater surface tension-lowering ability. The amide group in aGGA is able to make intermolecular hydrogen bonds, which enable aGGA to be packed tightly at air-water interface. This would result in showing lower surface tension

<table>
<thead>
<tr>
<th>Solvents</th>
<th>Dielectric constant</th>
<th>Test substances</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>GGA-Na</td>
</tr>
<tr>
<td>Water</td>
<td>80</td>
<td>+++</td>
</tr>
<tr>
<td>Dimethyl formamide</td>
<td>37</td>
<td>+</td>
</tr>
<tr>
<td>1,3-Butandiol</td>
<td>29</td>
<td>–</td>
</tr>
<tr>
<td>Ethanol</td>
<td>24</td>
<td>–</td>
</tr>
<tr>
<td>Acetone</td>
<td>21</td>
<td>–</td>
</tr>
<tr>
<td>Diethyl ether</td>
<td>4.3</td>
<td>–</td>
</tr>
</tbody>
</table>

+++, soluble at ambient temperature; ++, soluble after mild heating; +, partially soluble; -, not soluble.
than others having ester bonds. These results demonstrate the potential use of aGGA as surface tension-lowering agent.

3.3 Emulsifying properties of aGGA

The emulsifying properties of aGGA were evaluated by measuring the turbidity of O/W emulsions at 620 nm. The experiment was repeated with both SDS and MEGA-10 for comparison. Aqueous solutions of aGGA [0.025% (w/v), 0.57 mM] mixed with squalane, soy bean oil, or olive oil yielded stable dispersions incubating at room temperature for 3 h. This indicates better oil-emulsifying properties than SDS [0.025% (w/v), 0.87 mM]. The 0.025% (w/v) solution of aGGA exhibited similar or better oil-emulsifying properties than those of MEGA-10 [0.025% (w/v), 0.72 mM] (Fig. 5). In contrast, none of the surfactants were able to produce stable O/W emulsions with decane, liquid paraffin, or silicone oil.

3.4 Protein-protective effects of aGGA

We previously demonstrated the protective effects of GA-Na and GGA-Na against heat-induced protein aggregation. Here, the protective effect of aGGA on heat-induced protein denaturation, shown in Fig. 6A, was dose-dependent up to a concentration of 0.03% (w/v) (0.68 mM). At higher concentrations, e.g., 0.1% and 5% (w/v), any protective effect was no longer observable. MEGA-10 did not

Table 2: Physical properties of aGGA and synthetic surfactants.

<table>
<thead>
<tr>
<th>Surfactants</th>
<th>Carbon number of hydrophilic group</th>
<th>CMC (M)</th>
<th>γ-CMC (mN/m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>aGGA</td>
<td>12</td>
<td>1.57 x 10^-4</td>
<td>27.8</td>
</tr>
<tr>
<td>SDS</td>
<td>12</td>
<td>8.1 x 10^-3</td>
<td>ca. 38</td>
</tr>
<tr>
<td>Sucrose monolaurate</td>
<td>12</td>
<td>2.62 x 10^-4</td>
<td>37.0</td>
</tr>
<tr>
<td>Polyoxyethylene monolaurate (ethylene oxide adduct number, 12; o-methoxy)</td>
<td>12</td>
<td>1.52 x 10^-4</td>
<td>36.2</td>
</tr>
<tr>
<td>MEGA-10</td>
<td>10</td>
<td>4.39 x 10^-3</td>
<td>28.3</td>
</tr>
</tbody>
</table>

1 Data retrieved from reference 21. 2 Data retrieved from reference 22.
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exhibit a protective effect until dosed at levels of 0.1% and 5% (w/v) (Fig. 6B). Previously, we confirmed that 5% (w/v) GGA-Na (170 mM) showed a strong inhibitory effect on the heat-induced aggregation of egg white \(^4\), much like that observed with MEGA-10. These results show that aGGA will be useful as a functional, low-concentration surfactant for protecting some biological molecules.

4 Conclusions

\(N\)-Dodecyl glucosylglyceric acid amide (aGGA) was synthesized from sucrose, glycerate, and \(N\)-dodecylamine in two steps with an overall yield of 21%. The synthesized aGGA was water-soluble and exhibited surface tension-lowering properties similar to those of commercially available synthetic surfactants. aGGA was also soluble in select organic solvents and, much like SDS and MEGA-10, could be used to produce stable O/W emulsions with squalane, soy bean oil, and olive oil. These data support the use of aGGA as a surfactant and as a candidate ingredient in skin care and toiletry products.

Acknowledgments

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References

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