Synthesis and Characterization of Dioctanoyl Glycerate as Water-soluble Trypsin Inhibitor

Shun Sato1*, Shota Nagata2, Tomohiro Imura3, Tomuka Fukuoka1, Tomotake Morita1, Yutaka Takahashi2, Yukishige Kondo2, Dai Kitamoto1 and Hiroshi Habe1

1 Research Institute for Sustainable Chemistry, National Institute of Advanced Industrial Science and Technology (AIST) (Tsukuba Central 5-2, 1-1-1 Higashi, Tsukuba, Ibaraki 305-8565, JAPAN)
2 Department of Industrial Chemistry, Faculty of Engineering, Tokyo University of Science (1-3 Kagurazaka, Shinjuku, Tokyo 162-8601, JAPAN)
3 Research Institute for Chemical Process Technology, National Institute of Advanced Industrial Science and Technology (AIST) (Tsukuba Central 5-2, 1-1-1 Higashi, Tsukuba, Ibaraki 305-8565, JAPAN)

Abstract: Glyceric acids (GAs) esterified with long acyl chains (> C16) exhibit antitrypsin activity (Folia Microbiol. 46, 21-23 (2001)). However, their hydrophobic nature, derived from the long acyl chains, has limited the number of studies on their physical and biological properties. To improve the water solubility of diacyl GAs, GA was esterified with octanoyl groups (C8), and its physical properties were investigated. Synthesized diocanoyl GA was not water-soluble, whereas its sodium salt was. Surface tension measurements of diocanoyl GA sodium salt (diC8GA-Na) in water revealed that the critical micelle concentration (CMC) was 0.82 mM, and surface tension at the CMC was 25.5 mN/m. Additionally, diC8GA-Na inhibited casein digestion by trypsin to a greater extent than dioleoyl GA. These data suggest that water-soluble diacyl GAs may have utility as surfactants and bioactive compounds.

Key words: glyceric acid, diacyl glyceric acid, antitrypsin activity, surfactant, glycerol

1 Introduction

Glyceric acid (2,3-dihydroxypropanoic acid, GA) is a natural organic compound and minor constituent of specific plants3-6. Biological activities of GA and its derivatives, including diacyl GA and glucosyl GA, have been determined, and use of these compounds as building blocks for chemical and biological materials has been suggested.7-10 Additionally, GA can be mass-produced from glycerol, a by-product of biodiesel and oleochemical manufacturing.11 Microbial production of GA from glycerol by acetic acid bacteria provides a chiral skeleton in the C-2 of GA, as some strains produce enantiopure D-GA with 99% enantiomer excess (ee).7

To develop GA derivatives, we first studied the synthesis and functionality of acyl GAs. In our previous studies, diacyl GAs with acyl chain lengths of C16 and C18 were synthesized and their biological properties investigated.10-12 Of these, dioleoyl GA, a primary component of the antitrypsin compounds produced by fungi,13 exhibited antitrypsin activity. The trypsin inhibition efficiency of dioleoyl GA was low, but likely due to its low water solubility. The low water solubility of this compound has limited the evaluation of physical properties of diacyl GAs in aqueous environments.

Fukuoka et al. synthesized monoacyl GAs with acyl chain lengths ranging from C12 to C18 and investigated their interfacial properties.14 These compounds were water-soluble, and an evaluation of their interfacial characteristics was performed in water. Monolauroyl GA had a critical micelle concentration (CMC) of 0.3 mM, and the surface tension of water at the CMC was 25.6 mN/m. This compound also formed a stable oil-in-water (O/W) emulsion compared to monolaurin or sodium dodecyl sulfate (SDS). These data suggest that modification of the hydrophobicity of diacyl GAs may produce excellent surfactant molecules.

To investigate the interfacial properties and biological activities of diacyl GAs with shorter acyl chains, a diacyl GA molecule with octanoyl groups was synthesized. We found that dioctanoyl GA (diC8GA) was not soluble in water, whereas its sodium salt (diC8GA-Na) was water-soluble and had superior surface tension-lowering properties. We also evaluated its inhibitory effect on trypsin.
2 Experimental

2.1 Materials

\( \text{d-GA} \) with an ee of 99\% was prepared from glycerol by oxidative fermentation using \textit{Acetobacter tropicalis} NBRC16470\(^\text{[8]} \). The \( \text{d-GA} \) calcium salts were converted to free acid using a DOWEX (Dow Chemicals, Midland, MI) cation exchange resin, as described previously\(^\text{[10]} \). Octanoyl chloride was purchased from Tokyo Kasei (Tokyo, Japan). All other reagents and solvents were obtained from Wako Pure Chemicals (Osaka, Japan).

2.2 Synthesis of \( \text{diC}_8\text{GA-Na} \) and its sodium salt

Acylation of d-GA was performed in anhydrous acetone in the presence of dimethylaminopyridine and triethylamine, as described previously\(^\text{[10]} \). Briefly, 3.04 mL d-GA solution (10 mmol in acetone) was added to a 100 mL three-neck round-bottom flask and maintained at 0\(^\circ\)C on ice. Anhydrous acetone (35.7 mL), triethylamine (3.04 mL, 22 mmol), and dimethylaminopyridine (87.3 mg, 0.7 mmol) were added to the solution with stirring, and the flask was purged with dry air. Acylation was started by the drop-wise addition of octanoyl chloride (5.64 mL, 33 mmol) over 30 min at 0\(^\circ\)C. The solution continued to be incubated at 0\(^\circ\)C for an additional 30 min. Reaction progress was monitored by thin layer chromatography (TLC). After disappearance of the spot corresponding to GA, the reaction mixture was filtered using No. 40 filter paper (GE Healthcare UK Ltd., Little Chalfont, UK), and filtrates were evaporated in \( \text{vacuo} \). To the resulting material, 1 M HCl and ethyl acetate were added and the organic layer was collected. The fraction was dried using anhydrous Na\(_2\)SO\(_4\), and solvents were removed by evaporation. The crude oil produced was purified using silica gel chromatography with hexane, hexane/ethyl acetate (8:2, v/v) and ethyl acetate. Fractions containing \( \text{diC}_8\text{GA} \) were combined, dried using anhydrous Na\(_2\)SO\(_4\), and concentrated in \( \text{vacuo} \). The resulting yellow oil was characterized by nuclear magnetic resonance (NMR) and liquid chromatography-mass spectrometry (LC-MS), and 1.85 g of purified \( \text{diC}_8\text{GA} \) was obtained.

The synthesized \( \text{diC}_8\text{GA} \) was dissolved in a methanol/water (1:1) solution and converted to sodium salt by neutralization with an equivalent volume of 1 M NaOH. After neutralization, the pH of the solution was approximately 8. Methanol was evaporated, and the resulting \( \text{diC}_8\text{GA-Na} \) was recovered by lyophilization. The structure of the sodium salt was confirmed by \( ^1\text{H} \) NMR.

2.3 Determination of surface tension

The surface tension of the aqueous solution containing \( \text{diC}_8\text{GA-Na} \) was measured at 25\(^\circ\)C using the pendant drop method with an automatic interfacial tensiometer (DM500, Kyowa Interface Science, Niiza, Japan) and Drop Shape Analysis software (FAMAS v2.01, Kyowa Interface Science). The CMC of the compound was calculated from the crosspoint on the surface tension curve. The value of surface tension at CMC (\( \gamma_{\text{CMC}} \)) was also determined.

2.4 Estimation of emulsifying activity

The O/W emulsifying ability of \( \text{diC}_8\text{GA-Na} \) was evaluated by a colorimetric method, as described previously\(^\text{[15]} \). Briefly, in a test tube, 0.1 mL of soybean oil as the hydrophobic phase was added to 4 mL of distilled water containing 0.025\% (w/v) \( \text{diC}_8\text{GA-Na} \), which corresponded to 0.66 mM. Also, for comparison, 4 mL of distilled water containing 0.1 to 10 mM SDS were used, including 0.025\% (w/v) SDS (\( = 0.87 \) mM). 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 was measured at 620 nm and expressed as optical density (OD). Turbidity with no surfactant was measured as a control. Data were represented as mean values from at least three independent experiments.

2.5 Evaluation of antitrypsin activity

Antitrypsin activity was qualitatively evaluated by the disk-diffusion method of Habe et al.\(^\text{[19]} \). Each sample (200 \( \mu \)L), consisting of 10 \( \mu \)L of \( \text{diC}_8\text{GA-Na} \) solution, 4 \( \mu \)L of 1 M Tris (pH 7.5), 2 \( \mu \)L of trypsin (1 mg/mL), and 184 \( \mu \)L of H\(_2\)O were incubated at 37\(^\circ\)C for 15 min and tested for antitrypsin activity. Paper discs containing the above samples were placed on a 0.1\% (w/v) casein-containing agar plate (consisting of 1 g of casein, 15 g of agar, 5 mL of 1 M CaCl\(_2\), and 50 mL of 1 M Tris (pH 7.5) per L of water) and incubated for 12 h. Final concentrations of \( \text{diC}_8\text{GA-Na} \) were used in the range of 0.1 to 2 mM for comparisons with reference data using dioleoyl GA, and a positive control, leupeptin\(^\text{[18]} \). In place of \( \text{diC}_8\text{GA-Na} \), sodium octanoate and sodium glycercate were used as negative controls.

The diameters of the cleared zones with either no \( \text{diC}_8\text{GA-Na} \) or appropriate concentrations of the compound (\( D_{\text{cont}} \) and \( D_{\text{nat}} \), respectively) were measured. The diameter of the paper disk (\( D_{\text{disk}} \)) was 8 mm. The inhibition efficiency was calculated according the following equation:

\[
\text{Inhibition efficiency (\%)} = 100 - \left( \frac{D_{\text{nat}} - D_{\text{disk}}}{D_{\text{cont}} - D_{\text{disk}}} \times 100 \right)
\]

2.6 Analytical procedures

TLC was developed with chloroform/methanol (8:2), and the organic compounds were visualized by heating at 120\(^\circ\)C for 5 min with a 5\% (w/v) phosphomolybdate solution in ethanol containing 5\% (v/v) sulfuric acid and 0.6\% (v/v) phosphoric acid. The \( ^1\text{H} \) and \( ^{13}\text{C} \) NMR spectra in CDCl\(_3\) were recorded with a Bruker AV-400 at 27\(^\circ\)C (Bruker, Karlsruhe, Germany). LC-MS was performed on a Shimadzu LC-MS 2020 system (Shimadzu, Otsu, Japan) equipped with a reverse-phase Synergi 4u column (150 \( \times \) 2.0 mm, Phenomenex, Torrance, CA). Samples were eluted in 0.1\% (v/v)
3 Results and Discussion
3.1 Synthesis and water solubility of diC8GA and its sodium salt

In a previous report, monoacyl GAs with acyl chain lengths ranging from C12 to C18 were synthesized using the free acid form of GA in acetone. Thus, this approach was also used to synthesize dioctanoyl GA (Fig. 1). To obtain the diacyl compound, octanoylchloride was used in 1.7-fold excess to the hydroxyl groups in GA. The yellow oil obtained was diC8GA and purified with a yield of 51%. The structure of diC8GA was confirmed by NMR and LC-MS. The 1H and 13C chemical shifts (ppm) were as follows: 5.3 (t, 6H), 4.4 (m, 2H), 2.3 (m, 4H), 1.6 (quin, 4H), 1.2 (br, 16H), and 0.87 (t, 6H) for 1H-NMR, and 173.02, 172.72 and 172.28 (C=O), 69.73 (C2), 62.08 (C3), 33.7 (α-C), 24.53 (β-C), 22.3 (-CH2-), and 13.75 (-CH3) for 13C-NMR. The 1H NMR spectrum showed diacylation of the hydroxyl groups in GA (Fig. 2A), while the 13C NMR spectrum showed the presence of three types of carbonyl groups (Fig. 2B). Collectively, these data confirmed the synthesis of diC8GA. As shown in Fig. 3, the total ion current chromatogram (TIC) of LC-MS revealed that diC8GA was eluted at approximately 4.5 min, and [M-H]− (m/z = 357) and [2M-H]− (m/z = 715) were the main ion forms with additional adduct ions (formate and proton adducts). TIC traces showed a dominant peak at 4.5 min with no other peaks, indicating the high purity of diC8GA.

Since the yellow material was not water-soluble, it was converted to the corresponding sodium salt by neutralization with NaOH. The sodium salt was soluble in water, with concentrations higher than 1 mM (approximately CMC, see below) forming a stable colloidal dispersion.

The water solubility of monoacyl GAs depends on their acyl chain lengths. The free acid forms of monoacyl GAs with acyl chains ranging from C12 to C16 are soluble in water, whereas with a C18 acyl chain is insoluble in water. Due to the absence of hydroxyl groups, the free acid forms of diacyl GAs are understandably less water-soluble than monoacyl GAs. To investigate whether GAs with diacyl chains shorter than C8 are water-soluble in their free acid forms, studies on the synthesis and water solubility of dihexanoyl GA are ongoing.

3.2 Surface properties of diC8GA-Na in water

We next evaluated the surface tension of diC8GA-Na in water using the pendant drop method. As shown in Fig. 4, a decrease in the surface tension of diC8GA-Na was observed with increasing concentration. From the crosspoint of the surface tension plot, the CMC of diC8GA-Na was calculated as 0.82 mM, while the surface tension at the CMC was 25.5 mN/m. This suggests a high potential of diC8GA-Na with surface-tension lowering agent. To elucidate this in detail, analyses of the assembled structures in aqueous media are underway.

This is the first report on the surface properties of diacyl GA sodium salt. Our results suggest that diC8GA-Na can be...
used as a water-soluble surfactant. Because monoacyl GAs also have excellent surface properties, and the synthesis method for these compounds is the same as that for diacyl GAs, a wide variety of GA acyl derivatives with practical surface properties could be synthesized by modifying the number and length of acyl groups.

3.3 Emulsifying property of diC8GA-Na

The oil-emulsifying property of diC8GA-Na in water was evaluated by turbidity measurements of the solution at 620 nm after incubation of 3 h at room temperature. Each sample contained surfactant at 0.025% (w/v), which correspond to 0.66 mM for diC8GA-Na and 0.87 mM for SDS. Control, with no surfactant.

Fig. 5 Emulsifying activity of diC8GA-Na. Turbidity of oil-in-water emulsion was measured at 620 nm after incubation of 3 h at room temperature. Each sample contained surfactant at 0.025% (w/v), which correspond to 0.66 mM for diC8GA-Na and 0.87 mM for SDS. Control, with no surfactant.

Fig. 4 Surface tension-concentration plot of diC8GA-Na.

3.4 Antitrypsin activity of diC8GA-Na

Since diC8GA-Na was water-soluble, its inhibitory activity against trypsin was tested. Using the disk-diffusion method with diC8GA-Na, zones of clearance were observed surrounding the disks in a dose-dependent manner. With 2 mM diC8GA-Na, the zone of clearance on the agar plate was nearly undetectable. The inhibitory efficiency of diC8GA-Na was calculated based on the size of the zone of clearance as a function of concentration. As shown in Fig. 6, trypsin inhibition efficiency of 0.1 mM diC8GA-Na, which corresponded to one eighth of its CMC, was calculated to be 10%. The trypsin inhibition efficiency increased with diC8GA-Na concentration, and the half-maximal inhibitory concentration (IC50) was approximately 1 mM, which was slight above of its CMC (0.82 mM). At 2 mM diC8GA-Na, its inhibition efficiency increased sharply, reaching 90% of trypsin inhibition. This implies that the CMC might relate to expressing antitrypsin activity. Also, the trypsin inhibition efficiency of diC8GA-Na was 1.5-fold higher than dio-
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Presence of diC8GA-Na will allow us to investigate the insoluble diacyl GAs in the liquid system will lead to further understanding of antitrypsin mechanism by diacyl GAs.

4 Conclusion
In this study, we synthesized dioctanoyl GA and its sodium salt from GA and octanoyl chloride. Although diC8GA was not water-soluble, its sodium salt had superior surface properties in water compared to commercially available synthetic surfactants. This water-soluble sodium salt also exhibited superior antitrypsin activity compared to dioleoyl GA. These data support the use of GA derivatives as surfactants and bioactive compounds.

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