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Synthesis and Evaluation of Dioleoyl Glyceric Acids Showing Antitrypsin Activity

Hiroshi Habe* , Tokuma Fukuoka, Shun Sato, Dai Kitamoto and Keiji Sakaki

Research Institute for Innovations in Sustainable Chemistry, National Institute of Advanced Industrial Science and Technology (AIST) (Central 5-2, 1-1-1 Higashi, Tsukuba, Ibaraki 305-8565, JAPAN)

Abstract: Previously, Lešová *et al*. reported the isolation and identification of metabolite OR-1, showing antitrypsin activity, produced during fermentation by *Penicillium funiculosum*. The structure of OR-1 was a mixture of glyceric acid (GA), esterified with C_{14}-C_{18} fatty acids, and oleic acid (C18:1) as the most predominant fatty acid (*Folia Microbiol*. 46, 21–23, 2001). In this study, dioleoyl D-GA and dioleoyl L-GA were synthesized via diesterification with oleoyl chloride, and their antitrypsin activities were evaluated using both a disk diffusion method and spectral absorption measurements. The results show that both compounds and their equivalent mixtures possess antitrypsin activities; however, their IC_{50} values (approximately 2 mM) are much higher than that of OR-1 (4.25 μM), suggesting that dioleoyl GA does not play a major role in the OR-1 antitrypsin activity.

Key words: glyceric acid, oleoyl glyceric acid, antitrypsin activity, biodiesel fuel, glycerol use

1 INTRODUCTION

D-Glyceric acid (D-GA) is an organic acid originally found in a variety of plants[1-3]. Recently, we have been conducting research on the microbial production of D-GA[4-10], and we have demonstrated that D-GA can be mass-produced from glycerol using a biotechnological process at >100 g/L[7]. Because glycerol is a renewable resource that can be obtained at approximately 10% weight as a byproduct of biodiesel fuel production through the transesterification of vegetable oils and animal fats, the bioconversion of glycerol to valuable chemicals is potentially important. Because D-GA itself has several biological effects, including diuresis[2], liver-stimulating and cholesterolytic activities[11], and the acceleration of ethanol and acetaldehyde oxidation[12], GA appears to be a promising chemical. However, to promote the use of GA, further functional analysis of GA and GA derivatives is necessary.

There have been several studies on the synthesis of GA derivatives and their properties[13-15]. Previously, Lešová *et al*. (2001) reported the isolation and identification of metabolite OR-1, produced during fermentation by *Penicillium funiculosum*. The structure of OR-1 was identified by infrared spectrophotometry (IR), ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR), and gas chromatography (GC); it was revealed to be a mixture of GA esterified with C_{14}-C_{18} fatty acids. The main constituents of the fatty acids were oleic acid (C18:1), linoleic acid (C18:2), and palmitic acid (C16:0) as the most (37.0%), second-most (29.0%), and third-most (23.9%) predominant fatty acids, respectively. Functional and steady-state studies revealed that OR-1 behaved like an uncompetitive trypsin inhibitor[13]. Protein inhibitors are known to be effective in suppressing carcinogenesis in a plethora of *in vivo* and *in vitro* systems[16].

However, because only 100 mg of OR-1 was prepared from a 5-L culture of *P. funiculosum*[13] in one study, its productivity appears to be low. Additionally, OR-1 was revealed to be only a mixture of esters of GA, and, from the published data[13], it remains unknown whether it is diacyl-GA, a mixture of 2-*O*-monoacyl-GA and 3-*O*-monoacyl-GA, or their mixture. Hence, the contributions of the possible OR-1 constituents to antitrypsin activity also remain unknown.

In this study, we synthesized both D- and L-GA dioleate (Scheme 1a), because oleate was the most predominant fatty acid in OR-1. Additionally, we tested their trypsin inhibitory effects using a disk diffusion method and spectral absorption measurements. No information was found in the literature on the synthesis of dioleoyl GA.

*Correspondence to: Hiroshi Habe, Research Institute for Innovation in Sustainable Chemistry, National Institute of Advanced Industrial Science and Technology (AIST), Central 5-2, 1-1-1 Higashi, Tsukuba, Ibaraki 305-8565, JAPAN
E-mail: hiroshi.habe@aist.go.jp
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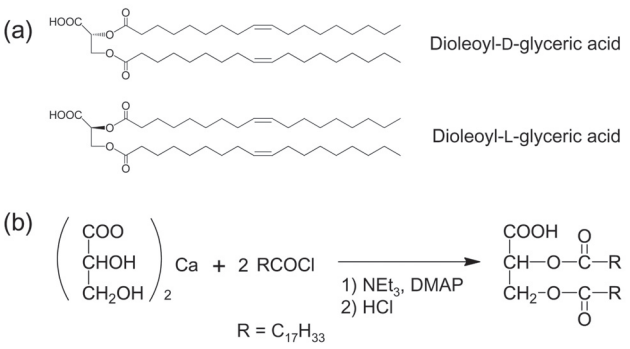

Scheme 1 (a) Chemical structures of dioleoyl glyceric acids and (b) synthesis of dioleoyl glyceric acids.

2 EXPERIMENTAL PROCEDURES

2.1 Materials

All reagents and solvents were commercially available and of the highest purity. D- and L-GA calcium salts were purchased from Sigma-Aldrich (St. Louis, MO). Casein (Hammerstein grade; Wako Pure Chemical Industries, Osaka, Japan), trypsin crystals from porcine pancreas (Wako), and leupeptin hemisulfate monohydrate (Wako) were used for the disk diffusion method. N^{α}-benzoyl-DL-arginine-4-nitroanilide (BAPNA) was obtained from Sigma-Aldrich.

2.2 Synthesis of dioleoyl D-glyceric acid and dioleoyl L-glyceric acid

For the synthesis of GA dioleate, we used commercially available D- and L-GA calcium salts as starting materials (Scheme 1b). GA calcium salt (0.43 g, 3 mmol) was dispersed in 20 mL of *N*,*N*-dimethylformamide (DMF), and triethylamine (TEA; 6.0 mL, 43 mmol), 4-dimethylamino pyridine (DMAP; 0.15 g, 1.2 mmol), and oleoyl chloride (3.0 g, 10 mmol) were subsequently added to the GA solution, in that order. The reaction mixture was stirred for 1.5 h at room temperature and for another 1.5 h at 60℃. After cooling to room temperature, 60 mL of ethyl acetate, 20 mL of 7% HCl aq., and 60 mL of water were added to the reaction mixture. The ethyl acetate phase was extracted, washed twice with brine, dried over magnesium sulfate, and evaporated. Approximately 4 g of the resulting yellowish oily material were purified by silica gel column chromatography using elution with *n*-hexane:ethyl acetate (10:1) and then *n*-hexane:ethyl acetate (3:1). The fractions containing the target compound were collected and evaporated. Yellowish oily compounds were obtained from D- (yield; 0.86 g, 45%) and L-GA (yield; 0.79 g, 41%).

The purity of the compounds was examined by thin-layer chromatography (TLC) using silica gel 60 F254 (Merck, Darmstadt, Germany) with a solvent system of chloroform: methanol (10:1) to develop the chromatogram; it was then analyzed with a GC-2010/AOC-20i (Shimadzu, Kyoto, Japan), fitted with a fused-silica chemically bonded capillary column (DB-1, 0.25 μm, 30 m; J&W Scientific, Folsom, CA, USA). Each sample (1 μL; 1 mg/mL) was injected into the column at 100℃. The column temperature was increased by 10℃ /min to 250℃. The flow rate of the helium carrier gas was 2.5 mL/min. The structural determination of the purified product dissolved in $CDCl_3$ was carried out using ^{1}H NMR analysis with an AV-400 NMR spectrometer (400 MHz, Bruker, Karlsruhe, Germany).

2.3 Disk diffusion method

Stock solutions of dioleoyl GA samples were prepared in dimethyl sulfoxide (DMSO) and appropriately diluted with DMSO before use. Each sample (200 μL), consisting of 10 μL of dioleoyl GA solution, 4 μL of 1 M Tris (pH 7.5), 2 μL of trypsin (1 mg/mL), and 184 μL of H_2O was incubated at 37℃ for 15 min and tested for antitrypsin activity. Paper discs containing the above samples were placed on a casein-containing agar plate (consisting of 1 g of casein, 15 g of agar, and 50 mL of 1 M Tris (pH 7.5) per liter of water) and incubated for 12 h. The final concentrations of dioleoyl GA were in the range of 0.1-3 mM. In place of dioleoyl GA, methyl oleate and GA calcium were used as negative controls and leupeptin as a positive control.

The diameters of the cleared zones with either no dioleoyl GA or appropriate concentrations of the compound (D_{cont} and D_{inhit}, respectively) were measured. The diameter of the paper disk (D_{disk}) was 6 mm. The inhibition efficiency was calculated according to the following equation:

$$\text{Inhibition efficiency}(\%) = 100 - [(D_{inhit} - D_{disk})/(D_{cont} - D_{disk}) \times 100]$$

2.4 Spectral absorption measurements for the tryptic hydrolysis of N^{α}-benzoyl-DL-arginine-4-nitroanilide (BAPNA)

The inhibitory effects of dioleoyl D-GA, dioleoyl L-GA, and a mixture of the two on trypsin activity were determined by measuring the absorption spectrum of p-nitroaniline (410 nm), a tryptic hydrolysate of BAPNA[17]. The molar extinction coefficient of *p*-nitroaniline at 410 nm is 8,800[17]. Stock solutions for the reactions were prepared as follows: trypsin solution, 25 mg of trypsin crystals were dissolved in 1 L of 50 mM 2-(*N*-morpholino) ethanesulfonic acid (MES)- NaOH buffer (pH 7); BAPNA solution, BAPNA was dissolved in DMSO to 100 mM; inhibitor solution, dioleoyl D-GA, dioleoyl L-GA, and methyl oleate were dissolved in DMSO to 10 mM each. The trypsin inhibition reaction mixture (1 mL total volume) consisted of 650 μL of 50 mM MES, 50 μL of BAPNA solution, 200 μL of inhibitor solution, and 100 μL of trypsin solution. The reactions were started by adding 100 μL of the trypsin solution to the reaction mixture and were followed for 15 min in a V-530 spectrophotometer (JASCO, Tokyo, Japan). The in-

hibition concentration $(IC)_{50}$ values represent the concentration of inhibitors (e.g., dioleoyl GA) at 50% inhibition.

3 RESULTS AND DISCUSSION

3.1 Synthesis of dioleoyl D-glyceric acid and dioleoyl L-glyceric acid

In the present study, we synthesized D- and L-GA dioleate. The successive esterification of the two hydroxyl groups of D- and L-GA with oleoyl chloride was achieved in the presence of DMF, TEA, and DMAP. Purified dioleoyl D-GA and dioleoyl L-GA were obtained with yields of 45 and 41%, respectively, according to the batch procedure described in 2.2. We executed the same procedure twice and carried out further purification. Finally, 0.9 and 0.95 g of colorless oily compounds were obtained as possible dioleoyl D-GA and dioleoyl L-GA, respectively. The purities of these compounds were 96.6 and 100%, respectively, according to the GC analysis (data not shown).

The compounds were identified as dioleoyl D-GA and dioleoyl L-GA by ^{1}H NMR analyses. Chemical shifts of these compounds are summarized in Table 1.

3.2 Disk diffusion method for the detection of antitrypsin activity

Because the disk diffusion test is an easy method for examining antitrypsin activity, we used it to evaluate the synthesized dioleoyl GA. Disk diffusion tests were performed using 0.1-3 mM dioleoyl GA. At a final concentration of 3 mM dioleoyl GA, a pale-white turbidity was observed in the tested samples. The clearing zone diameters in 0.1, 0.3, 1.0, and 3.0 mM inhibitors (D_{inhit}) were 20.0 ± 0.06, 18.7 ± 0.06, 16.7 ± 0.06, and 14.3 ± 0.06 mm for dioleoyl D-GA and those for dioleoyl L-GA were 19.3 ± 0.06, 18.7 ± 0.06, 17.3 ± 0.06, and 15.0 ± 0.10 mm, respectively. We measured that D_{cont} was 20.0 ± 0.10 mm. The inhibition efficiencies (%) are shown in Fig. 1. The results show that both dioleoyl D- and dioleoyl L-GA have an inhibitory effect on trypsin activity, because methyl oleate as well as GA calcium in the same concentration range either did not show or showed tiny cleared zones (data not shown). Additionally, there was little difference in the inhibitory effects of the D- and L-isomers.

We then compared their antitrypsin activities with that of leupeptin, a known protease inhibitor that can inhibit serine, cysteine, and threonine proteases[18]. Using the disk diffusion method, we found that the inhibition efficiencies

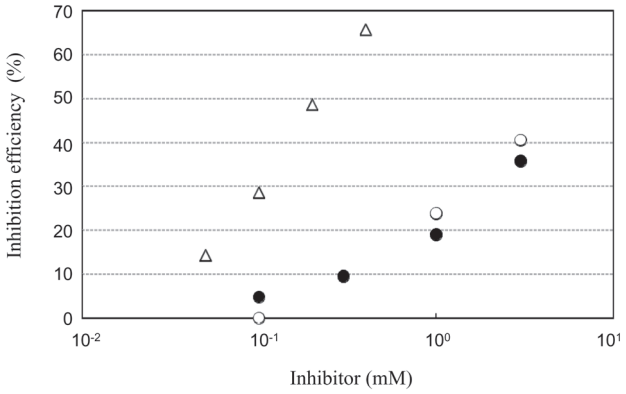

Fig. 1 Results of disk diffusion tests for detection of dioleoyl GA antitrypsin activity. Symbols: open triangles, leupeptin; closed circles, dioleoyl L-GA; open circles, dioleoyl D-GA.

Table 1 ^{1}H NMR data for dioleoyl glyceric acids ($CDCl_3$, 400 MHz)

	D-GA dioleate		L-GA dioleate	
	^{1}H-NMR δ (ppm)	J (Hz)	^{1}H-NMR δ (ppm)	*J* (Hz)
GA				
H-2	5.35 m		5.34 m	
H-3a	4.43 dd	5.6, 12.1	4.43 dd	5.6, 12.1
H-3b	4.55 dd	3.3, 12.1	4.55 dd	3.2, 12.0
Acyl group				
–CO-CH_2– (at C-2)	2.42 td	3.1, 7.6	2.42 td	3.0, 7.5
–CO-CH_2– (at C-3)	2.34 t	7.6	2.34 t	7.6
–CO-CH_2CH_2–	1.59-1.67 m		1.59-1.67 m	
–(CH_2)n–	1.23-1.33 br		1.27 m	
–CH=CH–	5.35 m		5.34 m	
–CH=CH-CH_2–	1.98-2.05 m		2.02 m	
–CH_3	0.88 t	6.8	0.88 t	6.8

in 0.05, 0.1, 0.2, and 0.4 mM leupeptin were 14.3, 28.6, 48.6, and 65.7%, respectively (Fig. 1), indicating that dioleoyl GA has a much weaker trypsin inhibition activity than leupeptin.

3.3 Inhibitory effect of dioleoyl GA on the tryptic hydrolysis of BAPNA

As the disk diffusion test described above is rather qualitative, we attempted to investigate the effect of the synthesized dioleoyl GA on the trypsin activity according to Erlanger *et al*.[17] As final concentrations of greater than 3 mM dioleoyl GA resulted in pale-white turbidity in the trypsin inhibition reaction mixtures, the spectral absorption measurements for the tryptic hydrolysis of BAPNA were carried out in the presence of 0, 0.5, and 2 mM dioleoyl GA (Fig. 2). Dioleoyl D-GA, dioleoyl L-GA, and a 1:1 mixture of both dioleoyl GAs had an inhibitory effect on the trypsin activity that is greater than the same concentrations of the methyl oleate samples. As shown in Fig. 2, the inhibition rate of dioleoyl GA for the hydrolysis of BAPNA at 5 mM decreased with increasing dioleoyl GA concentrations up to 2 mM. In this experiment, there was little difference in the inhibitory effects of the D isomer, the L isomer, or their mixture.

Even though we could not determine the exact IC_{50} values owing to the insolubility of dioleoyl GA, Fig. 2 suggests that the IC_{50} is around 2 mM. However, this value differs from the IC_{50} of OR-1, which is 4.25 μM[13]. The analytical data on the structure of OR-1[13] suggest several structural possibilities, including GA diesterified with homologous or heterologous fatty acids, and a mixture of 2-*O*-monoacyl-GA and 3-*O*-monoacyl-GA with homologous or heterologous fatty acids. However, the contributions of these possible OR-1 constituents to the antitrypsin activity are unknown. Our finding that the IC_{50} of dioleoyl GA is approximately 2 mM suggests that GA diesterified with fatty acids has little effect on the antitrypsin activity. The insolubility of dioleoyl GA in water is thought to make it ineffective. To investigate which constituent(s) of OR-1 actually have an effect on the antitrypsin activity, the synthesis of GA monoesterified with fatty acids and the evaluation of their antitrypsin activity will be necessary.

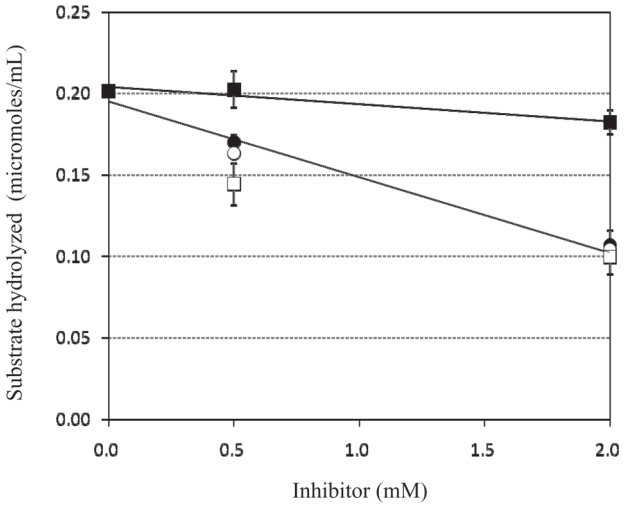

Fig. 2 Inhibitory rates of the hydrolysis of BAPNA as a function of the dioleoyl GA concentration. Symbols: closed squares, methyl oleate; open squares, a 1:1 mixture of dioleoyl D- and dioleoyl L-GA; closed circles, dioleoyl L-GA; open circles, dioleoyl D-GA. Incubation time, 15 min; BAPNA concentration at start, 5 mM; buffer used, 50 mM 2-(N-morpholino)ethan esulfonic acid (MES)-NaOH buffer (pH 7); temperature, 25°C.

4 CONCLUSIONS

We synthesized for the first time both dioleoyl D-GA and dioleoyl L-GA as possible constituents of the *P. funiculosum* metabolite OR-1[13]. Both dioleoyl GAs and their mixture exhibited antitrypsin activity, as shown by the disk diffusion method and spectral absorption measurements. However, their inhibitory effects on trypsin activity (IC_{50}〜2 mM) were much lower than that of OR-1 (IC_{50} 4.25 μM), suggesting that the OR-1 constituents that primarily contribute to the antitrypsin activity may be a mixture of 2-*O*-monoacyl-GA and 3-*O*-monoacyl-GA.

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