Enzymatic Synthesis of Surface Active and Biodegradable Glucosaminide Fatty Acid Esters

Tooru SAGOH¹, Kazunobu TOSHIMA¹, Kazuo KAWADA²
and Shuichi MATSUMURA^{1*}

¹ Department of Applied Chemistry, Faculty of Science and Technology, Keio University
(3-14-1, Hiyoshi, Kohoku-ku, Yokohama-shi 223-8522, JAPAN)

² School of Science, Kitasato University
(1-15-1, Kitasato, Sagami-hara-shi 228-8555, JAPAN)

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Abstract: Methyl, ethyl and butyl β -D-glucosaminide 6-O-fatty acid esters were prepared by the novel direct transglycosylation reaction of chitosan with methanol, ethanol and butanol using resting cells of the chitosan-assimilating strain, *Penicillium* sp. KS018 as the enzyme source of exo- β -D-GlcNase followed by the transesterification reaction with the fatty acid methyl ester using lipase. Transesterification by the lipase depended on enzyme origin, and reaction conditions, such as temperature and reaction pressure. Methyl, ethyl and butyl β -D-glucosaminide 6-O-fatty acid monoesters demonstrated excellent surface activity in aqueous solution. The antimicrobial properties of the 6-O-dodecanoyl-glucosaminides were stronger compared to dodecyl β -D-glucoside. The methyl, ethyl and butyl glucosaminide 6-O-fatty acid esters were all readily biodegradable by activated sludge.

Key words: enzymatic synthesis, transglycosylation, chitosan, sugar based surfactant, surface activity, glucosaminide fatty acid ester, biodegradation

1 Introduction

The greening of chemistry requires the discovery and development of new synthetic pathways using alternative feedstocks, reaction conditions and catalysts for improved selectivity and energy minimization and the designing bio/environmentally compatible chemicals. Some challenges for this include application of biocatalysts and use of naturally abundant and renewable resources, such as polysaccharides and plant oils, as alternative feedstocks.

Chitosan is produced by the deacetylation of chitin which is one of the most abundant biomass forms next to cellulose. D-Glucosamine is a monomeric component of chitosan and has wide application as a

hydrophilic moiety of amphiphilic molecules having biological properties and molecular recognition abilities. Such amphiphilic molecules that form supramolecular assemblies involve long chain alkyl glycosides and short or medium chain alkyl glycoside fatty acid esters (1,2). Long chain fatty acid esters with various sugars, such as glucose, sucrose and raffinose, show excellent surface activities and biodegradability (3). Some are used as edible surfactants, such as an emulsifier for food processing. The biodegradation mechanism of sugar esters, such as sucrose esters and the α -sulfatide sucrose ester, has also been extensively studied (4).

Though short chain alkyl D-glucosaminides are soluble in water and in organic solvents, the short chain alkyl D-glucosaminides may become versatile interme-

*Correspondence to: Shuichi MATSUMURA, Department of Applied Chemistry, Faculty of Science and Technology, Keio University, 3-14-1, Hiyoshi, Kohoku-ku, Yokohama-shi 223-8522, JAPAN
E-mail: matumura@aplc.keio.ac.jp

diates for the production of novel biologically active and environmentally benign amphiphiles. The direct preparation of such glycosides from chitosan and an alcohol using an enzyme may contribute to the establishment of a new field of green chemistry. Glycosides should be produced instead of free sugar units when the hydrolase enzyme has transglycosylation activity and an alcohol is present instead of water in the reaction mixture. The enzymatic preparation of the alkyl glycoside by transglycosylation of polysaccharides, such as xylan (5,6), chitosan (7-9) and cellulose (10), and an alcohol has been reported by us. The short chain alkyl β -D-glucosaminide 6-O-fatty acid ester (R'-RGlcN) was prepared by direct transglycosylation of chitosan and an alcohol using exo- β -D-glucosaminidase (exo- β -D-GlcNase) from the chitosan-assimilating strain *Penicillium* sp. KS 018 with subsequent esterification with fatty acid. Physico-chemical properties as surfactant, and biodegradability and antimicrobial activity were evaluated. The concept of this study and preparation of the surfactant, R'-RGlcN, are shown in **Scheme 1**.

2 Experimental

2.1 Materials and Measurements

Methyl octanoate and methyl dodecanoate were purchased from Tokyo Kasei Kogyo Co., Ltd. and distilled under reduced pressure before use. Chitosan was purchased from Katokichi Co., Ltd. (Japan). Porcine pancreatic lipase (41 U/mg protein, according to the supplier) was from Sigma Chemical Co. (St. Louis, MO, USA). *Candida antarctica* lipase immobilized on acrylic resin [Novozym 435 (triacylglycerol hydrolase + carboxylesterase) having 10,000 PLU/g (propyl laurate units: lipase activity based on ester synthesis)] and *Rhizomucor miehei* lipase immobilized on a macroporous anion exchange resin (Lipozyme RM IM) were kindly supplied by Novozymes Japan Ltd. (Chiba, Japan). Lipase AK (*Pseudomonas fluorescens*) was kindly supplied by Amano Pharmaceutical Co., Ltd. (Nagoya, Japan). The enzyme was dried in vacuum over P_2O_5 at 25°C for 1 d. 1H and ^{13}C NMR spectra were recorded with a JEOL spectrometer (Tokyo, Japan) at 300 and 75 MHz, respectively.

Short chain alkyl β -D-glucosamides (RGlcN) and their 6-O-fatty acid esters (R'-RGlcN) were analyzed by a high-performance liquid chromatography (HPLC) with a refractive index detector and a commercial

HPLC column (TSK-GEL NH₂-60, TOSOH, Co., Ltd., Tokyo, Japan, acetonitrile-water as the eluent) calibrated with the chemically synthesized authentic standards.

2.2 Isolation of Chitosan-Assimilating Microbe and Preparation of Resting Cells as Enzyme Source of Exo- β -D-GlcNase

Chitosan-assimilating microbes were isolated from soil using enrichment culture techniques with chitosan as the sole carbon source. The most active fungal strain, KS018, was selected as the chitosan-assimilating strain having transglycosylation activity. The KS018 strain, identified as *Penicillium* sp. by NCIMB Japan Co., Ltd. (Shimizu-shi, Japan), was grown in an inorganic medium (0.2% NH₄Cl, 0.02% K₂HPO₄, 0.02% MgSO₄·7H₂O, 0.0002% CaCl₂, 0.0001% FeSO₄·7H₂O, 0.0002% MnSO₄·4H₂O, 0.0007% ZnSO₄·7H₂O, and trace of CuSO₄ and Vitamin B₁·HCl) containing 0.5% chitosan as the growing substrate (200 mL) in a shake flask with reciprocal shaking at 30°C. Initial pH of the medium was adjusted to 6.0 with NaOH. At 4-days incubation, the cells were harvested by centrifugation (9,000 g, 15 min, 4°C), washed with saline to obtain 10 g of wet cells as the enzyme source of exo- β -D-glucosaminidase (exo- β -D-GlcNase) for the preparation of alkyl β -D-glucosaminide (RGlcN). The resting cells showed exo- β -D-GlcNase activity as measured using chitotritol (11).

2.3 Preparation of RGlcN by Transglycosylation of Chitosan and Alcohol Using Resting Cells of *Penicillium* sp. KS018

RGlcN was prepared by direct transglycosylation of chitosan and an alcohol using resting cells as biocatalyst as shown in **Scheme 1**. A typical preparation of methyl β -D-glucosaminide (MeGlcN) is as follows. A mixture of 50 mL 0.5% aqueous chitosan solution (pH 4.0), 30 mL methanol, 10 g wet cells and 10 mL acetate buffer (100 mM, pH 6.0) was incubated in a shake flask with stirring at 30°C for 48 h. Yield of MeGlcN in the incubation mixture was directly determined by HPLC using an authentic standard. The product was purified by column chromatography and its structure analyzed. After the reaction, the unreacted chitosan was filtered and the filtrate evaporated and purified by column chromatography [(SiO₂, CHCl₃/CH₃OH/28% NH₄OH = 10/4/1 (v/v), R_f=0.2] to obtain MeGlcN in a yield of

Scheme 1 Preparation of R'-RGlcN and Eco-Cycle System.

132 mg (527 mg/g chitosan). The isolated product was analyzed by HPLC, elemental analysis, IR, and ^1H NMR and ^{13}C NMR spectroscopies. Results for MeGlcN: ^1H NMR (270 MHz: D_2O): $\delta=2.50$ (1H, m), 3.20-3.35 (3H, m), 3.40 (3H, s), 3.59-3.79 (1H, dd, $J=5.6$), 3.78-4.02 (1H, dd, $J=2.2, 12.2$), 4.20 (1H, d, $J=8.28$).

Ethyl β -D-glucosaminide (EtGlcN) and butyl β -D-glucosaminide (BuGlcN) were prepared by the reaction of chitosan with ethanol and butanol, respectively, using resting cells. EtGlcN: ^1H NMR (270 MHz: D_2O): $\delta=1.15$ (3H, t, $J=7.28$), 2.52 (1H, m), 3.20-3.35 (3H, m), 3.40 (3H, s), 3.50-3.60 (1H, dd, $J=2.2, 5.6$), 3.78 (1H, dd, $J=2.2, 12.2$), 4.20 (1H, d, $J=8.28$). BuGlcN: ^1H NMR (270 MHz: D_2O): $\delta=0.93$ (3H, t, $J=8.0$), 1.41 (2H, m), 1.63 (2H, m), 2.64 (1H, dd, $J=9.6$), 3.34-3.50 (3H, m), 3.63-3.79 (2H, m), 3.90-4.02 (2H, m), 4.39 (1H, d, $J=8.2$).

2.4 Preparation of Methyl, Ethyl and Butyl 6-O-Acyl- β -D-Glucosaminides (R'-RGlcN) Using Lipase

RGlcN and the fatty acid methyl ester were stirred with lipase at constant temperature under a reduced pressure as shown in **Scheme 1**. Transesterification products were periodically analyzed by HPLC. After the reaction, an aliquot of ethanol was added to the reaction mixture and the insoluble enzyme was removed by suction filtration through a Celite pad. The filtrate was evaporated to obtain the crude product

which was purified by column chromatography (silica gel C-300, CHCl_3 - CH_3OH -28% $\text{NH}_4\text{OH}=20:5:1$, v/v). The chemical structure of the isolated product was found by HPLC, elemental analysis, and IR, ^1H NMR and ^{13}C NMR spectroscopy. MeGlcN (100 mg), three equivalents methyl dodecanoate (333 mg) and 100% Lipozyme RM IM (100 mg, relative to MeGlcN) were stirred at 70°C and 100 Torr for 7 h. After the reaction, 20 mL ethanol were added and the insoluble enzyme was removed by filtration through a Celite pad. The ethanol was evaporated to obtain the crude product (400 mg). This was further purified using silica gel column chromatography to obtain methyl 6-O-dodecanoyl- β -D-glucosaminide (C12-MeGlcN) in a yield of 63.4 % (122.3 mg) as a white powder. A series of R'-RGlcNs were prepared similarly. Results for R'-RGlcN are shown in **Table 1**.

2.5 Interfacial Properties

2.5.1 Static surface tension and occupation area of a molecule at surface ($A_{\min}$)

Static surface tension was measured with an automatic digital Kyowa Precise Surface Tensiometer by the CBVP Method (Kyowa Kagaku Co., Ltd., Tokyo, Japan) at 25°C . Measurement was carried out using the Wilhelmy vertical plate technique and sandblasted glass plate. The test solutions were aged at 25°C for at least 1 h before measurement. Each measurement was made three times and the mean value was taken.

The occupation area of a molecule at a surface ($A_{\min}$) was calculated according to the literature (12). The surface excess concentration ($\Gamma_{\max}$) in mole/ cm^2 , and the corresponding $A_{\min}$ in nm^2 , at the liquid/air interface were calculated using equations 1 and 2:

$$\Gamma_{\max} = (1/2.303 RT) (\partial\gamma/\partial \log C)_T \quad (1)$$

and

$$A_{\min} = 10^{14}/N\Gamma \quad (2)$$

where $(\partial\gamma/\partial \log C)_T$ is the slope of the surface tension vs. concentration curves below the cmc at a constant temperature and γ is the surface tension.

2.5.2 Foaming power and emulsification power

Foam properties were measured by the semi-micro TK method at 25°C according to Yano and Kimura (13). Initial foam volume in mL expressed the foam production and foam volume after 5 min expressed foam stability.

Emulsification power was determined by the height of the emulsion layer after shaking a 0.1% aqueous

Table 1 Analytical Data for 6-*O*-acylated β -D-glucosaminides.

Compound	¹ H NMR (δ)	Elemental analysis (%)					
		C		H		N	
		Found	Calcd.	Found	Calcd.	Found	Calcd.
C8MeGlcN	0.90 (3H, t, $J=8.0$), 1.20-1.36 (8H, m), 1.60 (2H, m), 2.40 (2H, t, $J=7.2$), 2.63 (1H, m), 3.25-3.40 (3H, m), 3.50 (3H, s), 4.15 (1H, d, $J=9.5$), 4.25 (1H, dd, $J=6.3$), 4.50 (1H, dd, $J=2.5$, 13.2)	56.40	56.00	9.15	9.03	4.39	4.32
C12MeGlcN	0.88 (3H, t, $J=8.0$), 2.50 (1H, m), 1.10-1.50 (16H, m), 1.60 (2H, m), 2.40 (2H, t, $J=7.7$), 2.60 (1H, m), 3.20-3.35 (3H, m), 3.40 (3H, s), 4.18 (1H, d, $J=8.5$), 4.25 (1H, dd, $J=5.6$), 4.63 (1H, dd, $J=2.5$, 13.2)	60.77	60.42	9.93	10.03	3.73	3.56
C8EtGlcN	0.85 (3H, t, $J=8.0$), 1.20-1.40 (11H, m), 1.65 (2H, m), 2.40 (2H, t, $J=7.8$), 2.70 (1H, m), 3.28-3.50 (3H, m), 3.55 (1H, s), 3.95 (1H, m), 4.20 (1H, d, $J=8.2$), 4.25 (1H, dd, $J=6.8$), 4.60 (1H, dd, $J=2.3$, 13.3)	57.63	57.50	9.37	9.19	4.20	4.12
C12EtGlcN	0.90 (3H, t, $J=8.2$), 1.20-1.45 (19H, m), 1.65 (2H, m), 2.40 (2H, t, $J=7.8$), 2.70 (1H, m), 3.28-3.50 (3H, m), 3.60 (3H, s), 3.95 (1H, m), 4.20 (1H, d, $J=8.2$), 4.25 (1H, dd, $J=6.6$), 4.60 (1H, dd, $J=2.5$, 13.1)	61.43	61.67	10.02	10.09	3.52	3.60
C12BuGlcN	0.90 (3H, t, $J=8.2$), 0.95 (3H, m), 1.30-1.50 (21H, m), 1.65 (2H, m), 2.40 (2H, t, $J=7.8$), 2.75 (1H, m), 3.25-3.50 (3H, m), 3.60 (1H, m), 3.90 (1H, m), 4.20 (1H, d, $J=8.5$), 4.30 (1H, dd, $J=6.7$), 4.55 (1H, dd, $J=2.5$, 13.4)	63.28	62.91	10.38	10.09	3.35	3.20

solution (5 mL) with hexane (5 mL) for 1 min at 25°C. Volumes of emulsion layer, toluene layer and aqueous layer were each measured after a 5-minute stand.

2·6 Antimicrobial Activity

The antimicrobial activity of the R'-RGlcN was evaluated by the agar dilution method (14). Gram-positive bacterial strains, *Staphylococcus aureus* KB210, *Bacillus subtilis* KB211 and *Micrococcus luteus* KB212, gram-negative bacterial strains, *Escherichia coli* KB213, *Salmonella typhimurium* KB20 and *Pseudomonas aeruginosa* KB115 and five fungal strains, *Candida albicans* KF1, *Saccharomyces cerevisiae* KF25, *Trichophyton mentagrophytes* KF213, *Penicillium chrysogenum* KF270 and *Aspergillus niger* KF103 were used. Nutrient agar and Sabouraud dextrose agar were used for bacterial and fungi, respectively. Antimicrobial activity was expressed as minimum inhibitory concentration (MIC).

2·7 Hydrolytic Stability

A hydrolytic stability test was carried out by dissolv-

ing R'-RGlcN in buffer solution at pH 4, 7, 8 and 10. The monoester at 1000 mg/L was incubated at 30°C in an incubator. Solutions of 0.1 M acetate buffer at pH 4.0, 0.07 M phosphate buffer at pH 7.0, and 0.05 M glycine-NaOH buffer at pH 8.0 and 10.0 were used for the nonenzymatic degradation test. Degradation of the monoester was analyzed by HPLC, and hydrolytic stability expressed as remaining % ester.

2·8 Biodegradation

R'-RGlcN biodegradability was evaluated by biochemical oxygen demand (BOD). BOD was determined with a BOD Tester (Model 200F; TAITEC Corp., Koshigaya-shi) using the oxygen consumption method, according to the Modified MITI Test (15). Activated sludge was obtained from a municipal sewage plant in Yokohama City.

3 Results and Discussion

3.1 Preparation of RGlcN by Transglycosylation of Chitosan and Alcohol Using Resting Cells

Resting cells of *Penicillium* sp. KS018 were capable of transglycosylation and MeGlcN was produced by the direct reaction of chitosan and methanol with a yield of 530 mg/g chitosan. No MeGlcN was produced using monomeric D-glucosamine or N-acetyl-D-glucosamine in place of chitosan. Transglycosylation between chitosan and methanol using resting cells of *Penicillium* sp. KS018 was analyzed with respect to the reaction conditions. Yield of RGlcN gradually increased with incubation time, and after a 2-day incubation, reached constant value. The yield increased with temperature up to 30°C and then decreased probably due to deactivation of the enzyme. Reaction temperature, giving the highest yield of RGlcN, was 30°C. The yield quickly increased to maximum value with alcohol concentration from 0.2 to 10% and then slightly decreased due to deactivation of the enzyme by high alcohol concentration. **Figure 1** shows yields of RGlcN using methanol, ethanol and butanol during a 2-day incubation at 30°C. Yield decreased with chain length of the alcohol.

3.2 Enzymatic Synthesis of Methyl, Ethyl and Butyl 6-O-Acyl- β -D-Glucosaminides

Methyl, ethyl and butyl β -D-glucosaminides (RGlcN) were reacted with methyl dodecanoate and octanoate by lipase to produce the corresponding 6-O-

Fig. 1 Preparation of RGlcN by Transglycosylation of Chitosan with Methanol, Ethanol and Butanol Using Resting Cells of *Penicillium* sp. KS 018 at 30°C for 2 Days.

acylated β -D-glucosaminides (R'-RGlcN). Esterification was significantly influenced by enzyme origin, reaction temperature, molar ratio of the two reactants, reaction time, enzyme concentration, reaction pressure and organic solvent.

3.2.1 Effects of enzyme origin

Lipases from various origins were screened for 6-O-monoesterification of RGlcN and methyl dodecanoate at 70°C. **Table 2** shows 6-O-esterification results for MeGlcN with methyl dodecanoate. Lipozyme RM IM showed the best results for the 6-O-monoester synthesis. Without lipase, no significant 6-O-esterification occurred suggesting the lipase (Lipozyme RM IM) to actually catalyzed esterification.

3.2.2 Effects of reaction temperature

The reaction temperature significantly influenced

Table 2 Effects of Enzyme Origin on Yield of Methyl 6-O-dodecanoyl- β -D-glucosaminide (C12-MeGlcN)^a.

Entry	Lipase	Molar ratio of MeDo/MeGlcN	Solvent	Pressure (Torr)	Time (h)	Yield (%)
1	Lipozyme RM IM	5/1	—	80	5	73
2	Novozym 435	5/1	—	80	5	63
3	lipase AK	5/1	—	80	5	15
4	PPL	5/1	—	80	5	10
5	Lipozyme RM IM	1/1	—	80	5	30
6	Lipozyme RM IM	1/1	THF	760	2	0
7	Novozym 435	1/1	THF	760	24	52
8	lipase AK	1/1	THF	760	24	0

a) Esterification carried out in bulk using methyl dodecanoate (MeDo) and MeGlcN with 100% lipase (relative to MeGlcN) at 70 °C

Fig. 2 Effects of Temperature on Yield of R'-RGlcN. MeGlcN and methyl dodecanoate reacted with 100% Lipozyme RM IM at 80 Torr for 7 h. ○: C12-MeGlcN, ●: C8-MeGlcN, ▲: C12EtGlcN.

yield of the 6-*O*-monoester (R'-RGlcN). **Figure 2** shows the relationship between yield and temperature for Me, Et and BuGlcN with methyl dodecanoate and octanoate. The highest yield was obtained by the esterification around 70°C. 6-*O*-Monoester yield decreased with temperature higher than 70°C probably due to deactivation of the enzyme.

3·2·3 Effects of substrate molar ratio

The molar ratio of fatty acid methyl ester and RGlcN was responsible for 6-*O*-monoester yield due to equilibrium shift of the reaction system. **Figure 3** shows the

Fig. 3 Effects of Molar Ratio of Methyl Dodecanoate (MeDo) and RGlcN on Yield of C12-RGlcN. RGlcN and methyl dodecanoate reacted with 100% Lipozyme RM IM at 70°C and 80 Torr for 7 h. ○: C12-MeGlcN, ●: C12-EtGlcN, ▲: C12-BuGlcN.

relationship between yield and molar ratio of the two reactants. The same was observed such that 6-*O*-monoester yield increased with methyl dodecanoate ratio. At least 3 equivalents methyl dodecanoate versus RGlcN were necessary to obtain a 60 % 6-*O*-monoester yield.

3·2·4 Time course of esterification

Figure 4 shows the time course of the transesterification of RGlcN and methyl dodecanoate by Lipozyme RM IM at a molar ratio of 5. A mixture of methyl dodecanoate (214 mg, 10 mmol), RGlcN (2 mmol) and Lipozyme RM IM (100% relative to RGlcN) was stirred at 70°C and 80 Torr. Aliquots of the reaction mixture were periodically withdrawn and directly analyzed by HPLC. Yield of the 6-*O*-monoester gradually increased to an equilibrium value after 6 h. The yield of monoester was then almost constant.

3·2·5 Effects of enzyme concentration

Enzyme concentration was responsible for the rate of the reaction as shown in **Fig. 5**. At least 100% immobilized lipase was necessary for efficient esterification. More enzyme caused no increase in ester yield. The present experiment requires a relatively large amount of enzyme. However, the enzyme could be repeatedly used without significant decrease in activity. In order to establish a practical method for esterification, a continuous bioreactor using the immobilized enzyme is now under study.

3·2·6 Effects of the solvent

Organic solvent may facilitate transesterification by

Fig. 4 Time Course of C12-RGlcN Yield. RGlcN and methyl dodecanoate at molar ratio of 1/5 reacted with 100% Lipozyme RM IM at 70°C and 80 Torr. ○: C12-MeGlcN, ●: C12EtGlcN.

Fig. 5 Effects of Enzyme Concentration on Yield of C12-MeGlcN.

MeGlcN and methyl dodecanoate (1/5, mol/mol) were reacted with 100% Lipozyme RM IM (relative to MeGlcN) at 70°C and 80 Torr for 5 h.

Fig. 6 Effects of Reaction Pressure on Yield of C12-MeGlcN.

MeGlcN and methyl dodecanoate (1/5, mol/mol) reacted with 100% Lipozyme RM IM (relative to MeGlcN) at 70°C for 5 h. ○: C12-MeGlcN, ●: C12-EtGlcN.

diffusion of the two substrates. Therefore, an equimolar mixture of the two substrates was used for the reaction instead of excess fatty acid esters when using an organic solvent. Lipases from various origins were compared with respect to monoester synthesis in tetrahydrofuran (THF) at 70°C. Only Novozym 435 exhibited activity for monoester synthesis in THF. However, no esterification occurred using Lipozyme RM IM which showed excellent activity under bulk conditions, possibly due to deactivation of the enzyme by organic solvent. The effects of the organic solvent on monoester yields were compared for various solvents, such as THF, acetonitrile, dimethyl formamide, toluene and tert-butanol, at 70°C for a 24-h reaction. The results are summarized in **Table 2**. The best results were obtained when THF was used as the solvent. It was confirmed that 6-*O*-dodecanoyl MeGlcN (C12-MeGlcN) was obtained in 54% yield by reaction of an equal molar methyl dodecanoate and MeGlcN in THF at 70°C for 24 h. Without solvent, excess methyl ester as solvent was essential for esterification.

3·2·7 Effects of reaction pressure

Reaction pressure influences esterification by equilibrium shift of the reaction system. **Figure 6** shows the effects of the reaction pressure on reaction of RGlcN and methyl dodecanoate at 70°C between 760 and 40 Torr. The highest yield was obtained when RGlcN and methyl dodecanoate reacted at 100 Torr.

3·3 Interfacial Properties of R'-RGlcN in Aqueous Solution

RGlcN octanoic and dodecanoic acid 6-*O*-monoesters (R'-RGlcN) exhibited surface activity, such as surface tension depression, micelle formation and foaming properties. From surface tension versus concentration plots for fatty acid 6-*O*-monoesters in distilled water, critical micelle concentration (cmc) determined from inflection of surface tension versus concentration curve, surface tension at cmc (γ_{cmc}), efficiency of adsorption at the surface (pC_{20}) [pC_{20} , negative log of C_{20} the surfactant molar concentration required to reduce surface tension by 20 mN/m] (16, 17), and A_{min} of fatty acid 6-*O*-monoesters are summarized in **Table 3** along with reference data for n-dodecyl poly(oxyethylene) ether (n=6) ($C_{12}EO_6$) measured under the same conditions. Cmc was dependent on length of the acyl chain length and the aglycones of RGlcN. The shorter the acyl chain, the higher was cmc. γ_{cmc} of RGlcN fatty acid 6-*O*-monoesters were lower compared to $C_{12}EO_{23}$.

Foaming properties were examined by the semi-micro TK method at cmc at 25°C and are summarized in **Fig. 7**. Excellent foam production and stability were observed for C12-EtGlcN. C12-BuGlcN showed high foam production but low stability similar to the polyethylene glycol monoalkyl ether, such as $C_{12}EO_6$ and n-dodecyl poly(oxyethylene) ether (n=25) ($C_{12}EO_{25}$).

Table 3 Surface Activity of 6-*O*-acylated Glucosaminides at 25°C.

Compound	cmc (mmol/L)	γ_{cmc} (mN/m)	pC ₂₀	$A_{min} \times 10^2$ (nm ²)
C8-MeGlcN	8.58	28.2	3.2	43.8
C8-EtGlcN	5.39	29.5	3.4	49.8
C8-BuGlcN	2.76	26.0	3.9	48.7
C12-MeGlcN	1.68	26.8	3.7	24.9
C12-EtGlcN	0.473	28.0	4.7	37.1
C12-BuGlcN	0.0719	27.6	5.7	55.9
C ₁₂ EO ₆	0.110	31.3	5.5	66.7

Emulsification power was determined by shaking 0.1% aqueous sample solution (5 mL) and hexane (5 mL) for 1 min at 25°C. Volumes of the emulsion, toluene and aqueous layers after a 5-minute stand are shown in **Fig. 8**. The dodecyl ester showed excellent emulsification power compared to conventional polyoxyethylene dodecyl ether type nonionics. C12-BuGlcN showed the strongest emulsification power for the hexane-water system.

3.4 Hydrolytic Degradation

The characteristic feature of the ester-type surfactant may be the hydrolytic degradability, possibly responsible for quick biodegradation. A hydrolytic degradation test was carried out by dissolving the esters in buffer solutions at pH 4, 7, 8 and 10. The results are shown in **Fig. 9**. R'-RGlcNs were quite stable at pHs 4 and 7 but

Fig. 7 Foam Production and Stability by Semi-Micro TK Method at 1.0 % Sample Concentration and 25°C. Black : 0 min, white: 5 min.

gradually hydrolyzed at pH 8 and quickly so at pH10 at 30°C.

3.5 Biodegradation

A quick and complete biodegradation after use is one of the indispensable factors for the next generation surfactants, because water-soluble surfactants are generally difficult to recover or recycle. The biodegradation of R'-RGlcN occurs by hydrolytic cleavage of ester bonds to liberate fatty acid and the corresponding RGlcN. A convenient way to predict aerobic biodegradability is to measure BOD. The biodegradability of R'-RGlcN was evaluated based on BOD. BOD was measured with a BOD tester, using activated sludge at substrate concentration of 15 mg/L. **Figure 10** shows the time course of the biodegradation (BOD/ThOD $\times$ 100) of R'-RGlcN based on BOD and theoretical oxygen demand (ThOD). All samples were equally biodegraded at more than 60% after 10 d, which can be considered as readily biodegradable. On the other hand, the biodegradation rate of a conventional ethoxylated alcohol, such as C₁₂EO₆, was less than that of R'-RGlcN under the same conditions.

3.6 Antimicrobial Activity

R'-RGlcNs were screened for antimicrobial activity toward gram-positive and gram-negative bacterial and

Fig. 8 Emulsion of 0.1% Aqueous Solution (5 mL) and Benzene (5 mL) by Shaking for 1 min at 25°C. Volumes of emulsification (grey), hexane (white) and aqueous layers (black) were measured after 5-minute stand.

Fig. 9 Hydrolysis of C8-MeGlcN in Aqueous Solution (3000 ppm) of Different pHs at 30°C.
○: pH 4.0, ●: pH 7.0, △: pH 8.0, ▲: pH 10.0.

Fig. 10 BOD Biodegradability of R'-RGlcN at 30°C.
Activated sludge: 30 ppm, sample: 15 ppm.
○: C12-MeGlcN, ●: C12-EtGlcN, △: C8-EtGlcN, ▲: C8-BuGlcN.

Table 4 Antimicrobial Activity of 6-O-acylated β -D-glucosaminides.

Compounds/ organism	MIC (mg/mL)					
	C8-MeGlcN	C12-MeGlcN	C8-EtGlcN	C12-EtGlcN	C ₁₂ EO ₆	C ₁₂ β Glc ^a
<i>S. aureus</i>	>400	50	400	20	50	25
<i>B. subtilis</i>	>400	25	400	10	50	50
<i>M. luteus</i>	>400	10	400	10	50	—
<i>E. coli</i>	>400	25	200	25	>400	>400
<i>S. typhimurium</i>	>400	25	400	25	>400	>400
<i>P. aeruginosa</i>	>400	>400	400	>400	>400	>400
<i>C. albicans</i>	>400	200	400	100	>400	50
<i>S. cerevisiae</i>	>400	200	200	100	200	25
<i>T. mentagrophytes</i>	>400	10	100	10	50	—
<i>P. chrysogenum</i>	>400	400	>400	400	100	200
<i>A. niger</i>	>400	>400	>400	>400	100	200

^a C₁₂ β Glc: Dodecyl β -D-glucopyranoside (18)

fungus strains. Minimum inhibitory concentrations (MIC) are given in **Table 4**. C12-RGlcN with a dodecanoyl group showed a broad spectrum of antimicrobial activity. Antimicrobial activity of C12-RGlcNs was greater than that of 1-dodecyl β -D-glucopyranoside (C₁₂ β Glc) (18) or conventional n-dodecyl tetra(oxyethylene) ether (C₁₂EO₄) (18) toward *Escherichia coli* and *Salmonella typhimurium*.

4 Conclusions

Methyl, ethyl and butyl β -D-glucosaminide 6-O-fatty acid esters were prepared by the novel direct transglycosylation reaction of chitosan with methanol, ethanol and butanol using resting cells of the chitosan-assimilating strain, *Penicillium* sp. KS018 followed by the transesterification reaction with the fatty acid methyl ester using Lipozyme RM IM under reduced pressure. The methyl, ethyl and butyl 6-O-acyl- β -D-glucosaminides exhibited excellent surface activity and

biodegradability.

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References

1. K. ADELHORST, F. BIORKLING, S.E. GODTFREDSEN and O. KIRK, Enzyme Catalyzed Preparation of 6-*O*-Acylglucopyranosides, *Synthesis*, 112-115 (1990).
2. R.T. OTTO, U.T. BORNSCHEUER, C. SYLDATK and R.D. SCHMID, Synthesis of Aromatic *n*-Alkyl-Glucoside Esters in a Coupled β -Glucosidase and Lipase Reaction, *Biotechnol. Lett.*, Vol. **20**, 437-440 (1998).
3. I.J.A. BAKER, R.B. MATTHEWS, H. SUARES, I. KRODKIEWSKA, D.N. FURLONG, F. GRIESER and C.J. DRUMMOND, Sugar Fatty Acid Ester Surfactants: Structure and Ultimate Aerobic Biodegradability, *J. Surf. Deterg.*, Vol. **3**, 1-11 (2000).
4. I.J.A. BAKER, R.I. WILLING, D.N. FURLONG, F. GRIESER and C.J. DRUMMOND, Sugar Fatty Acid Ester Surfactants: Biodegradation Pathways, *J. Surf. Deterg.*, Vol. **3**, 13-27 (2000).
5. S. MATSUMURA, K. SAKIYAMA and K. TOSHIMA, Preparation of Octyl β -D-Xylobioside and Xyloside by Xylanase-Catalyzed Direct Transglycosylation Reaction of Xylan and Octanol, *Biotech. Lett.*, Vol. **21**, 17-22 (1999).
6. S. MATSUMURA, K. SAKIYAMA and K. TOSHIMA, One-Pot Synthesis of Alkyl β -D-Xylobioside from Xylan and Alcohol by Acetone Powder of *Aureobasidium pullulans*, *Biotech. Lett.*, Vol. **19**, 1249-1253 (1997).
7. S. MATSUMURA, T. NAKAMURA, E. YAO and T. TOSHIMA, Biocatalytic One-Step synthesis of *n*-Octyl β -D-Xylotrioside and Xylobioside from Xylan and *n*-Octanol in Supercritical Carbon Dioxide, *Chem. Lett.*, 581-582 (1999).
8. S. MATSUMURA, E. YAO and K. TOSHIMA, One-Pot Preparation of Alkyl β -D-Glucosaminide by the Transglycosylation of Chitosan and Alcohol Using Purified *exo*- β -D-glucosaminidase, *Biotech. Lett.*, Vol. **21**, 451-456 (1999).
9. S. MATSUMURA, E. YAO, K. SAKIYAMA and K. TOSHIMA, Novel Direct Preparation of *n*-Butyl 2-Amino-2-deoxy- β -D-Glucopyranoside from Chitosan and *n*-Butanol Using Biocatalyst, *Chem. Lett.*, 373-374 (1999).
10. S. MATSUMURA, H. TSURUTA and K. TOSHIMA, One-Pot Synthesis of 1-Octyl β -D-Glucoside from Cellulose and 1-Octanol in Combination of Cellulase and β -Glucosidase, *J. Jpn Oil Chem. Soc.*, Vol. **48**, 15-19 (1999).
11. F. NANJO, R. KATSUMI and K. SAKAI, Purification and Characterization of an *Exo*- β -D-glucosaminidase, a Novel Type of Enzyme, from *Nocardia orientalis*, *J. Biol. Chem.*, Vol. **265**, 10088-10094 (1990).
12. C.-C. KWAN and M.J. ROSEN, Relationship of Structure to Properties in Surfactants. 9. Syntheses and Properties of 1,2- and 1,3-Alkanediols, *J. Phys. Chem.*, Vol. **84**, 547-551 (1980).
13. W. YANO and W. KIMURA, Studies on the Evaluation Methods of Surface Active Agents. II. Foam Test: Semimicro Improved TK-Method, *Yukagaku*, Vol. **11**, 138-144 (1962).
14. R.G. BRISTLINE, JR., E.W. MAURER, F.D. SMITH and W.M. LINFIELD, Fatty Acid Amides and Anilides, Syntheses and Antimicrobial Properties, *J. Am. Oil Chem. Soc.*, Vol. **57**, 98-103 (1980).
15. OECD Guidelines for Testing of Chemicals, 301C, Modified MITI Test, Organization for Economic Cooperation and Development (OECD), Paris, 1981.
16. Y.-P. ZHU, A. MASUYAMA, Y. NAKATSUJI and M. OKAHARA, Synthesis and Properties of Bis(sulfonate) Types of Double-Chain Surfactants Bearing a Sulfur Atom in the Connecting Part, *Yukagaku*, Vol. **42**, 86-94 (1993).
17. Y.-P. ZHU, K. ISHIHARA, A. MASUYAMA, Y. NAKATSUJI and M. OKAHARA, Preparation and Properties of Double-Chain Bis(quaternary ammonium) Compounds, *Yukagaku*, Vol. **42**, 161-167 (1993).
18. S. MATSUMURA, K. IMAI, S. YOSHIKAWA, K. KAWADA and T. UCHIBOIRI, Surface Activities, Biodegradability and Antimicrobial Properties of *n*-Alkyl Glucosides, Mannosides and Galactosides, *J. Am. Oil Chem. Soc.*, Vol. **67**, 996-1001 (1990).