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3   Efficient synthesis of 6-*O*-palmitoyl-1,2-*O*-isopropylidene-

4   α-D-glucofuranose in an organic solvent system by

5   lipase-catalyzed esterification

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Abstract

In order to synthesize a sugar ester at high concentration, 1,2-*O*-isopropylidene-α-D-glucofuranose

(IpGlc), which is one of the sugar acetals and is more hydrophobic than unmodified glucose, was

esterified with palmitic acid at 40ºC using immobilized lipase from *Candida antarctica* in some

organic solvents or their mixtures. A mixture of acetone and *t*-butyl alcohol as a reaction medium

greatly improved both the initial reaction rate and yield at 80 h. The product concentration reached

the maximum value (240 mmol/kg-solvent; *ca*. 110 g/kg-solvent) when 400 mmol/kg-solvent IpGlc

and 1200 mmol/kg-solvent palmitic acid were used in acetone/*t*-butyl alcohol at 75/25 (v/v).

11

1   Introduction

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3   Sugar esters have been used as emulsifiers. They have been produced so far by chemical

4   synthetic methods, in which many kinds of products (regioisomers with different acylation degree)

5   are formed. Lipase-catalyzed esterification (dehydrative condensation) of sugar with fatty acid is one

6   of the most favorable ways to synthesize sugar esters acylated at a specific position. Although there

7   are many studies dealing with lipase-catalyzed syntheses of various kinds of sugar esters in organic

8   solvents (Cramer et al. 2007; Ferrer et al. 2005; Šabeder et al. 2006; Walsh et al. 2009), their

9   industrialization has not been realized yet. One of the reasons is low productivity or product

10   concentration due to the low solubility of sugars in an organic solvent (Flores et al. 2005). This

11   drawback may be overcome by using a less polar sugar derivative as a hydroxyl donor instead of an

12   unmodified sugar.

13   There are many sugar derivatives such as alkyl glycosides, boronic acid esters, and sugar

14   acetals. Although alkyl glycosides are one of the popular sugar derivatives, selective dealkylation is

15   relatively difficult after esterification. On the other hand, sugar acetals or boronic acid esters are

16   easily deprotected. There are some studies dealing with esterification of these derivatives (Fregapane

17   et al. 1991; Sarney et al. 1994; Skagerlind et al. 1997). Because an isopropylidene derivative, one of

18   the sugar acetals, is more popular than others, its use is appropriate for esterification. However,

19   optimization of reaction conditions, such as substrate concentrations and selection of a reaction

20   medium, has been insufficient. Therefore, in this study, we performed esterification of

1 1,2-*O*-isopropylidene-α-D-glucofuranose (IpGlc) with palmitic acid to form

2 6-*O*-palmitoyl-1,2-*O*-isopropylidene-α-D-glucofuranose (C16-IpGlc), and high product

3 concentration could be achieved in a mixture of acetone and *t*-butyl alcohol (TBA) with

4 improvements in the reaction rate and maximum yield.

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6 **Materials and Methods**

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8 Materials

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10   Immobilized lipase from *Candida antarctica* (fraction B, CHIRAZYME® L-2, cf.-C2) was

11 obtained from Roche Diagnostics, Mannheim, Germany. IpGlc was purchased from Sigma-Aldrich,

12 MO, USA, and palmitic acid and other reagents were from Wako Pure Chemical Industries, Osaka,

13 Japan.

14

15 Synthesis of 6-*O*-palmitoyl-1,2-*O*-isopropylidene-α-D-glucofuranose

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17   Reaction media were acetone, acetonitrile, TBA, hexane, toluene, diisopropyl ether (DIPE),

18 *N,N*-dimethyl formamide (DMF), and dimethyl sulfoxide (DMSO). Prior to esterification, these

19 organic solvents were dehydrated over 4A molecular sieve for at least 3 days. Given amounts of

20 IpGlc (0.25 mmol is typical) and palmitic acid (0.75 mmol is typical) were weighed into a glass vial,

1 and an organic solvent (5 mL) was added. The vial was tightly screw-capped and pre-incubated at

2 40ºC for 10 min to solubilize the substrates. Immobilized lipase (50 mg) was then added. The vial

3 was vigorously shaken at 40ºC and 130 rpm. At appropriate intervals, the reaction mixture was

4 analyzed with an HPLC.

5   Synthesis of C16-IpGlc without any organic solvent was also performed. A mixture of IpGlc

6 (0.25 mmol) and palmitic acid (0.25 mmol) was heated at 70ºC to melt palmitic acid and was then

7 cooled down to 40ºC prior to addition of 50 mg immobilized lipase. The reaction conditions were the

8 same as those in an organic solvent system.

9

10 Analysis of the product

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12   The concentration of C16-IpGlc was determined by an HPLC equipped with a pump

13 (LC-10AD*VP*, Shimadzu, Kyoto, Japan) and a UV detector (SPD-10AV*VP*, Shimadzu) at 220 nm.

14 The reaction mixture (5 μL) was injected and eluted through an ODS column (J'sphere ODS-M80, 3

15 × 150 mm, YMC, Kyoto, Japan) with methanol/water at 90/10 (v/v) at 0.4 mL/min.

16

17 Product purification

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19   The reaction mixture containing C16-IpGlc, unreacted substrates (IpGlc, palmitic acid), and a

20 small amount of by-product was concentrated by evaporation after removing immobilized lipase by

1 filtration. The evaporation residue was dissolved in ethyl acetate, and palmitic acid and IpGlc were

2 removed by extraction with 0.1 mol/L sodium hydroxide aqueous solution. After separation of the

3 organic and aqueous phases, the organic phase was dehydrated over anhydrous sodium sulfate

4 followed by solvent evaporation. The residue was recrystallized twice in hexane to obtain C16-IpGlc.

5 The product was identified by ^{1}H-NMR (ECP-500, JEOL, Tokyo, Japan).

6   The by-product was recovered from the supernatant obtained during recrystallization. The

7 supernatant was condensed by evaporation, and the residue was applied on a column (20 × 300 mm)

8 packed with silica gel (Wakogel C-200, Wako). Elution was performed by hexane/ethyl acetate at

9 40/60 (v/v). The crude by-product was further purified by a semi-preparative HPLC equipped with a

10 pump (LC-10AD*VP*) and a UV detector (SPD-10AV*VP*) at 220 nm. The column was J'sphere

11 ODS-M80 (10 × 250 mm, YMC), and the eluent was methanol/water=85/15 (v/v) at 2 mL/min.

12

13 **Results and Discussion**

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15 Screening of the reaction medium

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17   Esterification yields at 20 h using substrates at equimolar concentrations (66 mmol/kg-solvent

18 (*ca.* 50 mmol/L)) were 62%±2.1% (mean ± SD, *n*=3) in acetonitrile, 46%±1.5% in acetone, and

19 35%±1.4% in TBA, where yields were defined based on a molar basis of IpGlc, a limiting reactant.

20 Because standard deviations were not large, following experiments were performed in *n*=1. Although

1 esterification proceeded the most in acetonitrile, the reaction mixture was opaque due to low IpGlc

2 solubility. Therefore, it is difficult to increase the product concentration in acetonitrile. Low or trace

3 yields could be achieved in toluene, hexane, DIPE, DMSO, and DMF. This may be due to the very

4 low IpGlc solubility in toluene, hexane, and DIPE, while in DMF and DMSO, lipase may be

5 inactivated by removing water which is essential to maintain its activity (Krishna et al. 2001).

6   In the reaction without any solvent, esterification proceeded only slightly at 40ºC. The main

7 reason would be low immiscibility between IpGlc and palmitic acid. Fregapane et al. (1991) reported

8 that the reaction proceeded using immobilized lipase from *Rhizomucor miehei* without any solvent at

9 high temperature (75ºC). Addition of an organic solvent would therefore contribute to lower the

10 reaction temperature to moderate level. Based on these results, following studies were performed

11 only in acetone, TBA, or their mixtures.

12

13 Esterification in acetone or TBA

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15   The   main   product   was   identified   to   be

16 6-*O*-palmitoyl-1,2-*O*-isopropylidene-α-D-glucofuranose (**1**) by NMR (^{1}H and ^{1}H-^{1}H COSY, Scheme ←Scheme 1

17 1); ^{1}H NMR: δ(ppm, CDCl$_3$, TMS, 500 MHz, 298K) 0.88 (3H, t, *J*=7.1 Hz, 1'-CH$_3$), 1.25 (27H, m,

18 acetal-CH$_3$, 2'~13'-CH$_2$), 1.49 (3H, s, acetal-CH$_3$), 1.63 (2H, m, 14'-CH$_2$), 2.37 (2H, t, *J*=7.8 Hz,

19 15'-CH$_2$), 4.08 (1H, m, 4-CH), 4.23 (2H, m, 6-CH$_2$), 4.36 (1H, d, *J*=2.5 Hz, 3-CH), 4.43 (1H, m,

20 5-CH), 4.54 (1H, d, *J*=3.7 Hz, 2-CH), 5.96 (1H, d, *J*=3.7 Hz, 1-CH). The results indicate that the

1   6-*O*-position of IpGlc is esterified and that this structure is identical to the product obtained by

2   esterification using lipase from *Rhizomucor miehei* (Fregapane et al. 1991). In this study, however, a

3   small amount of by-product (<8% of product (**1**)) was detected (Fig. 1). The by-product, the   ←Fig. 1

4   formation of which has not been reported, was a regioisomer that was esterified at the 5-*O*-position

5   of IpGlc. The structure of the regioisomer (5-*O*-palmitoyl-1,2-*O*-isopropylidene-α-D-glucofuranose

6   (**2**)) was identified; ^{1}H NMR: δ(ppm, CDCl$_3$, TMS, 500 MHz, 298K) 0.87 (3H, t, *J*=7.1 Hz, 1'-CH$_3$),

7   1.24 (27H, m, acetal-CH$_3$, 2'~13'-CH$_2$), 1.49 (3H, s, acetal-CH$_3$), 1.62 (2H, m, 14'-CH$_2$), 2.39 (2H,

8   m, 15'-CH$_2$), 3.95 (2H, m, 6-CH$_2$), 3.99 (1H, d, *J*=1.9 Hz, 3-CH), 4.11 (1H, dd, J_1= 1.9 Hz, J_2=9.3

9   Hz, 4-CH), 4.56 (1H, d, *J*=3.6 Hz, 2-CH), 4.95 (1H, m, 5-CH), 5.91 (1H, d, *J*=3.6 Hz, 1-CH).

10   Figure 2 shows the time courses for the syntheses of C16-IpGlc at different molar ratios of   ←Fig. 2

11   palmitic acid and IpGlc in acetone and TBA. In TBA, esterification proceeded faster and reached

12   almost the maximum yield within 8 h. However, slight decreases in the yield were observed after

13   long-term reaction. This would be due to the formations of diester (Zhang et al. 2003) and/or

14   regioisomer (**2**) by transesterification from the 6-*O*- to the 5-*O*-position. Meanwhile, it took 1-2 days

15   to reach the maximum yield in acetone. However, the maximum yield in acetone was higher than

16   that in TBA at any molar ratio. The low yield in TBA would be attributed to the strong interaction

17   between palmitic acid and TBA, which shifts the reaction equilibrium toward hydrolysis (Kobayashi

18   et al. 2003). Conversely, it indicates that the interaction of palmitic acid with acetone is relatively

19   weaker than that with TBA. Because the maximum yield leveled off when a 3 times or greater

20   amount of palmitic acid was used both in acetone and TBA, the molar ratio of palmitic acid to IpGlc

1   was adjusted to be 3:1.

2

3   Reaction in the mixed solvent

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5   The maximum yield was higher in acetone, although esterification was faster in TBA.

6   Therefore, the use of a mixture of these two solvents may improve both the reaction rate and

7   maximum yield. Figure 3 shows the dependence of the initial reaction rate and yield at 80 h on the   ←Fig. 3

8   volumetric fraction of TBA, f_{TBA}, in the mixed solvent. When a small amount of TBA ($f_{TBA} \leq 0.25$)

9   was added to acetone, the initial reaction rate steeply increased, and it increased gradually at f_{TBA}

10   greater than 0.25. The initial rate was 8.4 μmol/min/(g-immobilized lipase) at $f_{TBA} \approx 0.25$ and was 5.9

11   times higher than that in acetone (1.4 μmol/min/(g-immobilized lipase)). These results suggest the

12   activation of lipase in TBA.

13   Meanwhile, the yield at 80 h became lower with an increase in the volumetric fraction of TBA.

14   When the reaction was performed in TBA, the yield was *ca.* 42%, although it was *ca.* 70% in

15   acetone. Mixed solvents gave 50-70% yields. These results may indicate that interaction between

16   TBA and palmitic acid is gradually weakened by the addition of acetone as a diluent. The mixture of

17   TBA and acetone at 25/75 (v/v) is a promising solvent to improve both the reaction rate and yield at

18   80 h.

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20   Optimization of the product concentration

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2   To improve the product concentration, esterification was performed at different initial

3 concentrations of IpGlc (66-400 mmol/kg-solvent) at a constant molar ratio of palmitic acid to IpGlc

4 (3:1) in acetone/TBA (75/25, v/v). The initial rate steeply decreased with an increase in initial IpGlc

5 concentrations lower than 200 mmol/kg-solvent (Fig. 4a). Although this reason is not clear, a ←Fig. 4

6 possible one may be a change in the nature of the reaction mixture as a solvent because substrates

7 themselves can act as solvents at high substrate concentration. Meanwhile, the initial rate was almost

8 constant at the initial IpGlc concentrations higher than 200 mmol/kg-solvent because the IpGlc

9 concentration reached saturation.

10   Although the yield at 24 h, which may be an indication of lipase activity (Fig. 4b), showed the

11 same tendency as the result in Fig. 4a, the maximum yield during the 20-day reaction was 61-72%.

12 The maximum product concentration was calculated to be 240 mmol/kg-solvent (*ca.* 110

13 g/kg-solvent) when 400 mmol/kg-solvent IpGlc and 1200 mmol/kg-solvent palmitic acid were used,

14 and the maximum yield gradually decreased with the increase in initial IpGlc concentration.

15 Previously, a change in the equilibrium yield was observed in a reaction system without any organic

16 solvent for the synthesis of L-menthyl oleate (Kobayashi et al. 2007). The phenomenon observed in

17 this study resembles the previous one and suggests that the reaction shifted slightly toward

18 hydrolysis.

19   In conclusion, lipase-catalyzed synthesis of C16-IpGlc in a mixture of acetone and TBA was

20 successfully performed with good yield at moderate temperature. Use of the solvent mixture

1 improved both the reaction rate and maximum yield. Although the reaction rate was slower at higher

2 substrate concentration, high product concentration could be achieved at high IpGlc concentration.

3

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5

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1   **Figure captions**

2

3   **Scheme 1** Structures of the main product **1** and by-product **2**

4

5   **Fig. 1** HPLC chromatogram for the formations of C16-IpGlc and by-product in TBA at 80 h

6   (absorbance at 220 nm). Peak 1, by-product; 2, C16-IpGlc; 3, palmitic acid

7

8   **Fig. 2** Time courses for the formation of C16-IpGlc in acetone or TBA at different molar ratios of

9   palmitic acid and IpGlc. Palmitic acid:IpGlc=1:1 (○, ●), 3:1 (△, ▲), 5:1 (□, ■). Open

10   and closed symbols represent the results in acetone and TBA, respectively

11

12   **Fig. 3** Effects of volumetric fraction of TBA on the initial reaction rate and yield at 80 h for

13   esterification in the mixture of acetone and TBA

14

15   **Fig. 4** Effect of the initial IpGlc concentration on (a) the initial reaction rate and (b) yield: yield at 24

16   h (◆), maximum yield during 20-day reaction (△)

17
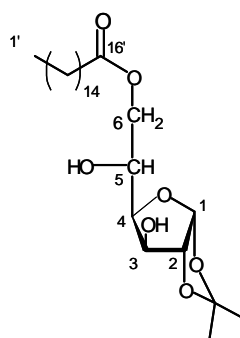
6-*O*-Palmitoyl-1,2-*O*-isopropylidene-
α-D-glucofuranose **1**
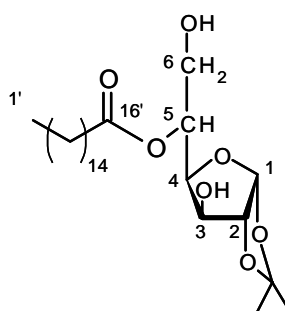
5-*O*-Palmitoyl-1,2-*O*-isopropylidene-
α-D-glucofuranose **2**

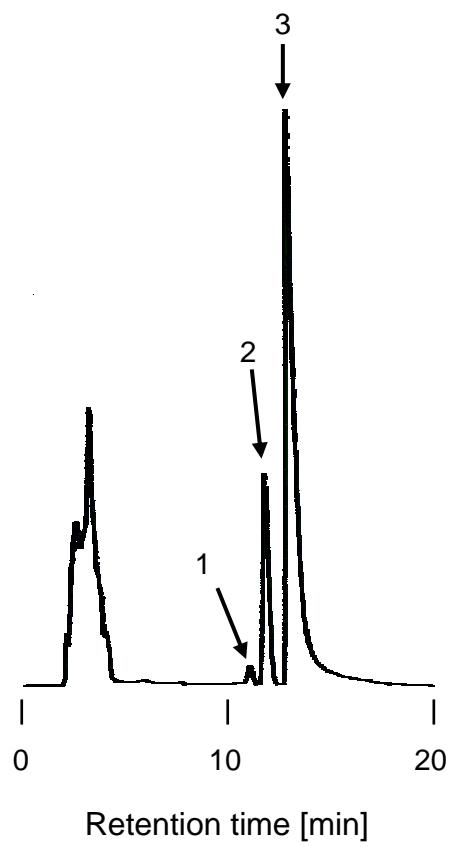Scheme 1 T. Kobayashi et al.

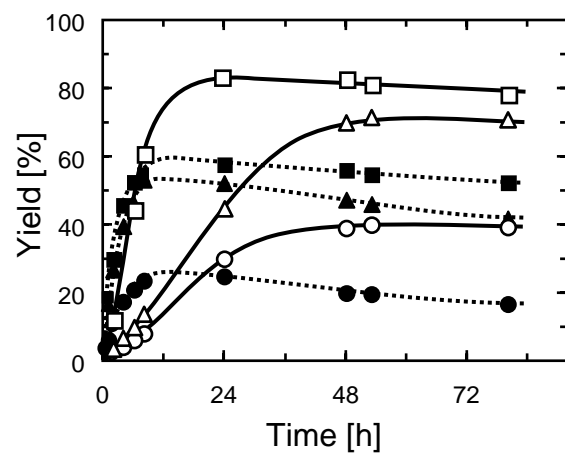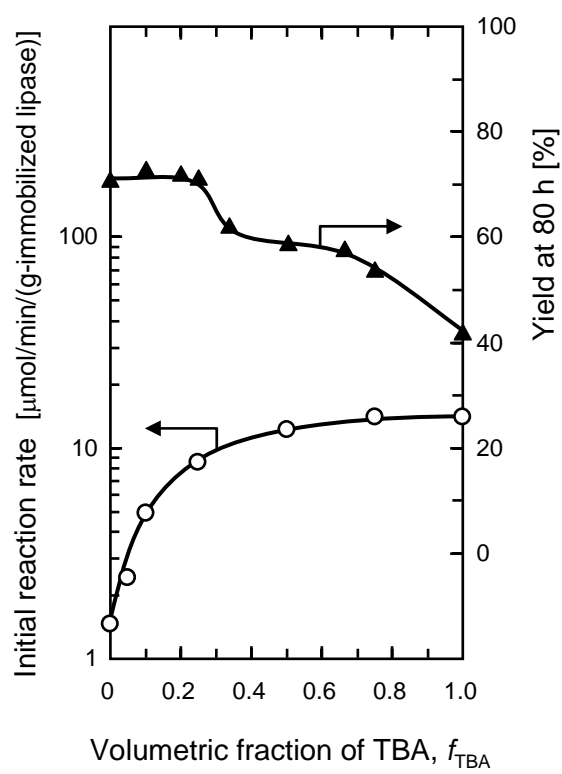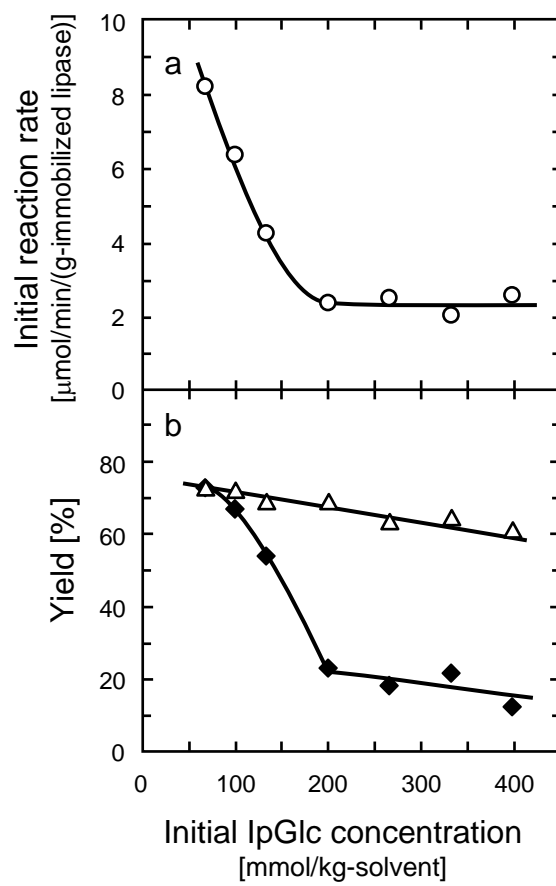Fig. 1 T. Kobayashi et al.

Fig. 2 T. Kobayashi et al.

Fig. 3 T. Kobayashi et al.

Fig. 4 T. Kobayashi et al.