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Synthesis of lauroyl saccharides through lipase-catalyzed condensation in microaqueous water-miscible solvents

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Abstract

Lauroyl saccharides were synthesized at 50°C through condensation in acetonitrile, acetone and *tert*-butanol with various water contents using the immobilized lipase Novozym® 435. Saccharides used were glucose, galactose, mannose and fructose. The equilibrium yields of the monoesters in acetonitrile were significantly different among the saccharides tested. The apparent equilibrium constants based on the concentrations of substrates and products in acetonitrile could be correlated to the dynamic hydration numbers of the saccharides, indicating that the water activity played an important role during the condensation in the microaqueous water-miscible solvent. However, a significant correlation between the equilibrium constant for lauroyl fructose formation and parameters characterizing the solvent property could not be found.

Keywords: Saccharide–fatty acid ester; Lipase; Condensation; Microaqueous solvent

1. Introduction

Saccharide–fatty acid esters are surfactants with good emulsifying properties [1–3], and have been of much interest for use in food, cosmetics, and pharmaceutical industries [4]. Compared to the conventional chemical synthesis, enzymatic synthesis using lipase has some benefits: the direct use of unmodified substrates, moderate reaction conditions, and high regiospecificity of the enzyme.

The lipase-catalyzed synthesis of the esters through both transesterification [5] and reverse hydrolysis (condensation reaction) [3,5–10] has been reported. Since the lipase-catalyzed reaction in conventional aqueous system thermodynamically favors the hydrolysis, organic medium with a low water content [6–10] or solvent-free system [5,11] is usually used to shift the reaction toward synthesis. Furthermore, the reaction under reduced pressure and/or in the presence of a desiccant [3,6,10] has been adopted to remove water, one of the condensation products, from the reaction system.

The equilibrium constant, which is an important parameter to predict the equilibrium yield

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under any condition, has scarcely been reported for the synthesis of saccharide–fatty acid esters in the organic solvents with low water contents. We have previously reported [12] that the equilibrium yield for lauroyl erythritol in the lipase-catalyzed synthesis in acetonitrile depended on the water content of the solvent with maximum yield at about 1% (v/v) water. The apparent equilibrium constant K_C also depended on the water content.

In this paper, we estimated the K_C values for the lipase-catalyzed synthesis of some lauroyl saccharides (hexoses) in acetonitrile with different water contents. The constants for lauroyl fructose in some water-miscible solvents with various water contents were also evaluated.

2. Materials and methods

2.1. Materials

Immobilized lipase from *Candida antarctica*, Novozym® 435, was a gift from Novo Nordisk Bioindustry Japan, Chiba, Japan. D(+)-Galactose, D(+)-glucose, D(−)-fructose, D(+)-mannose, *tert*-butanol, and 5A molecular sieves were purchased from Wako Pure Chemical Industries, Osaka, Japan. Lauric acid, acetonitrile, acetone, and silica gel 60 (spherical, 70–230 mesh) were purchased from Nacalai Tesque, Kyoto, Japan. The chemicals were of analytical grade. *n*-Octyl and *n*-decyl β-D-glucosides with purity of more than 98%, either of which was used as an internal standard for the HPLC analysis, were obtained from Sigma, St. Louis, MO.

2.2. Lipase-catalyzed condensation

The condensation reaction was carried out almost similarly to that described in our previous studies [12,13]. A solvent was dehydrated over the 5A molecular sieves. A specified amount of water was added to the dehydrated solvent to adjust the water content of the solvent to the desired level, which was determined by Karl Fischer titration using a Kyoto Electronics MKS-1s. A hexose (fructose, galactose, glucose or mannose; 0.25 mmol) and lauric acid (1.25 mmol) were put in a vial, and 5 ml of the solvent was then added there to dissolve or disperse the hexose and the fatty acid. The immobilized lipase (100 mg) was added to the vial. The vial was tightly screw-capped, and then immersed into a thermoregulated water bath at 50°C. In some cases, the amounts of hexose and lauric acid were doubled with 250 mg of the enzyme for the solvent volume of 5 ml.

The transient changes in yield were observed for some condensations. A portion of the reaction mixture was removed and mixed with the same volume of either 10 mmol/l decyl β-D-glucoside or 20 mmol/l octyl β-D-glucoside, which was used as the internal standard in the HPLC analysis. The concentration of the product was then determined. All the observed condensations reached equilibrium within 3 days as shown later. Therefore, the yield after four or more days was regarded as equilibrium when the transient changes were not measured. The equilibrium water content in the solvent was also measured by Karl Fischer titration.

2.3. Solubility of hexose in organic solvent with various water contents

Five hundred milligrams of a hexose was put into a vial together with 5 ml of solvent with a known water content, then the vial was tightly screwed shut. The vial was held at 50°C with occasional stirring for about 3–4 days. A portion of solvent was carefully sampled so as not to include the undissolved hexose, and was diluted twice by adding the same volume of eluent used for the HPLC analysis as quickly as possible. The concentration of the dissolved hexose was determined.

2.4. Estimation of apparent equilibrium constant

The equilibrium constant is an important parameter for predicting the equilibrium yield of

the desired product under any conditions. The apparent equilibrium constant, K_C, is defined by Eq. (1), based on the concentrations of the substrates and products:

$$K_C = C_{Pe}C_{We}/C_{Se}C_{Fe} \quad (1)$$

where C is the concentration in units of moles per liter, and the subscripts S, F, P, W represent saccharide (hexose), fatty acid (lauric acid), product (lauroyl saccharide) and water, respectively. The subscript e indicates equilibrium.

The concentrations of product and water at equilibrium, C_{Pe} and C_{We}, were experimentally observed. The water content in units of percent (v/v) was converted to the concentration in units of moles per liter, assuming roughly the volume additivity among substrates, products and solvent. The molar volumes used were (in l/mol): 0.114 for all hexoses [14], 0.231 for lauric acid, 0.0182 for water, 0.0548 for acetonitrile, 0.0740 for acetone, and 0.0942 for *tert*-butanol. The molar volumes expect for the hexoses were calculated from the density and molecular mass of each compound. The molar volume of lauroyl hexose was assumed to be 0.326 l/mol, which was obtained by $v_S + v_F - v_W$ (v: molar volume). The concentration of lauric acid at equilibrium, C_{Fe}, was estimated by $C_{F0} - C_{Pe}$, where C_{F0} is the initial concentration of lauric acid. In the present reaction system, hexose was not fully dissolved in the solvent, and only the hexose dissolved in the solvent would be effective as a substrate for the condensation. The smaller of either the solubility C_S at C_{We} or $C_{S0} - C_{Pe}$ (C_{S0} : the overall initial concentration of hexose) was regarded as the concentration of hexose at equilibrium C_{Se}. By substituting into Eq. (1) the concentrations of substrates and products at equilibrium estimated above, the K_C value was determined.

2.5. Adsorption of water onto immobilized lipase

Novozym®435 particles were packed in a cylindrical glass column with a 1.0 cm I.D. and 15 cm height. The bed was washed with acetonitrile dehydrated with molecular sieves, the water content of which was about 0.04 mol/l (C_0). The acetonitrile of a given water concentration C_{Wf} was fed to the bed at a flow rate of 0.5 ml/min. The effluent was fractionated at appropriate intervals, and the water concentration in the effluent was determined. Thus, the breakthrough curve of water was obtained. The amount of water adsorbed on the immobilized enzyme q was estimated by the following equation:

$$(1 - \varepsilon_b)V_t(q - q_0) = QC_{Wf}t_E - \varepsilon_b V_t(C_{Wf} - C_0) - Q\int_0^{t_E} C\,dt \quad (2)$$

where C is the concentration of water in the effluent, Q is the volumetric flow rate, V_t is the total volume of the bed, t is the elution time, t_E is the time at the end point, and ε_b is the bed voidage. q_0 is the amount of water adsorbed at C_0. Since we could not directly measure q_0, the value of $q - q_0$ was obtained. The integration of Eq. (2) was numerically carried out. The bed voidage ε_b was estimated to be 0.406 by dividing the volume of solvent withdrawn from the void of the bed by the total bed volume, although such an estimation was somewhat rough. All measurements were carried out at room temperature (ca. 25°C).

The apparent density of the wet immobilized enzyme was pycnometrically determined to be 1.044 g/ml. A rough estimation of the immobilized-enzyme particle porosity was made from the difference in weight between the wet and dry immobilized-enzyme particles, and the porosity was 0.588.

2.6. Analysis

A produced lauroyl hexose was analyzed using HPLC (LC-10AS, Shimadzu Seisakusho, Kyoto) with an octadecyl-bonded silica gel (ODS) column (4.6 mm ∅ × 300 mm, Chemco Scientific, Osaka) and the YRU-880 refractome-

ter. The eluent used was a mixture of acetonitrile and water (80/20 (v/v)) for lauroyl hexoses synthesized in acetonitrile (the flow rate: 0.8 ml/min), or of acetonitrile and water (70/30 (v/v)) for lauroyl fructose synthesized in acetone and *tert*-butanol (flow rate: 1.0 ml/min). The calibration curves were prepared using the products isolated from the reaction mixtures according to the reported methods [6] with a slight modification.

For determination of hexose solubilized in a solvent, a Cosmosil $5NH_2$ column (4.6 mm $\varnothing \times 250$ mm) was used. The eluent used was a mixture of acetonitrile, methanol, and water (70/15/15 (v/v/v)) for fructose, glucose and galactose in acetonitrile, of acetonitrile and water (80/20 (v/v)) for fructose in acetone and *tert*-butanol, or of acetonitrile and water (60/40 (v/v)) for mannose in acetonitrile.

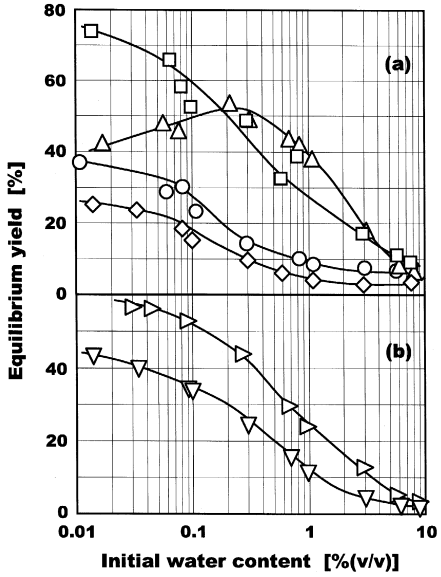

Fig. 2. Effect of initial water content on the equilibrium yield of lauroyl glucose (○), galactose (◇), mannose (□), and fructose (△) in (a) acetonitrile, and (b) lauroyl fructose in acetone (▷) and *tert*-butanol (▽).

3. Results and discussion

3.1. Dependence of equilibrium yield on the water content of solvent

Fig. 1 shows the transient changes in the yield of lauroyl glucose or galactose (monoester) in acetonitrile with various water contents. Di and higher esters were not detected for galactose, glucose and mannose under the analytical conditions. HPLC chromatograms of the product from fructose and lauric acid, observed under various conditions, revealed that it consisted of at least three components. Judging from their retention times, they seemed to be positional or conformational isomers of monoesters, although the possibility of formation of di- or higher esters still remained. In this study, the yield in the condensation was calculated under the assumption that only monoester(s) was formed.

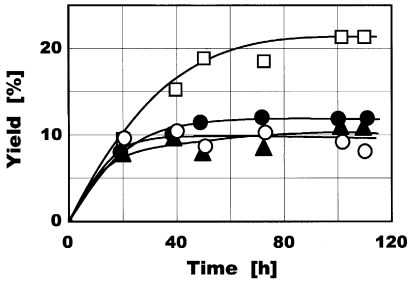

Fig. 1. Transient changes in yield of lauroyl glucose and galactose in acetonitrile with different initial water contents. The initial water contents were 0.32% (v/v) (●) and 0.50% (▲) for synthesis of lauroyl glucose, and were 0.059% (□) and 0.32% (○) for synthesis of lauroyl galactose. The curves were empirically drawn.

The yield of each ester reached equilibrium within 3 days. The equilibrium yield was higher with lower water content for the synthesis of both lauroyl glucose and galactose. It also depended on the kind of hexose used as a substrate even if the initial water content of acetonitrile was the same (0.32% (v/v)).

Fig. 2(a) shows the effect of initial water content on the equilibrium yield of lauroyl glucose, galactose, mannose and fructose. Except for lauroyl fructose, the equilibrium yields were higher at the lower initial water content. The synthesis of lauroyl fructose showed a peculiar dependence on the water content, and a maximum yield appeared at about 0.3% (v/v) water

content. A similar dependence had been observed during the synthesis of the lauroyl erythritol, and it was suggested that the activity coefficients of the substrates and products depended on the water content [12]. Fructose and erythritol have two primary hydroxyl groups, while other hexoses have only a primary hydroxyl group. Although Acros et al. [9] reported that fructose was quantitatively converted into its diester at a low temperature (5°C) in acetonitrile, a significant amount of the diester could not be observed under our experimental and analytical conditions. Therefore, further study is required to clarify the reason why only fructose showed a different dependence.

Fig. 2(b) shows the equilibrium yields of lauroyl fructose in acetone and *tert*-butanol with various initial water contents. The yields were higher at lower water contents. At the higher water contents, the yield was the highest in acetonitrile among the solvents tested, while the yield in acetone became the highest at lower water contents.

3.2. Solubility of hexoses in organic solvents

Fig. 3 shows the solubilities of the hexoses at 50°C in the solvents with different water contents. The solubility depended on both the kind of hexose and solvent, and it could empirically be expressed as an exponential function of the water content w for all the cases:

$$C_S = \alpha \exp(\beta w) \quad (3)$$

where α and β are the constants, and the values are listed in Table 1. This indicated that a certain amount of hexose could be solubilized even in a completely dehydrated solvent. Mannose was the most soluble in acetonitrile, followed by fructose, galactose and glucose. *tert*-Butanol possessed the highest ability to solubilize fructose among the solvents tested.

Table 1
Parameter in Eq. (3) for solubility of hexoses in organic solvents (50°C)

Hexose	Solvent	α [mol/l]	β [% (v/v)$^{-1}$]
Galactose	Acetonitrile	2.11×10^{-4}	0.415
Glucose	Acetonitrile	9.27×10^{-5}	0.398
Mannose	Acetonitrile	5.46×10^{-3}	0.251
Fructose	Acetonitrile	9.78×10^{-4}	0.353
Fructose	Acetone	3.17×10^{-2}	0.233
Fructose	*tert*-Butanol	1.67×10^{-1}	0.238

3.3. Equilibrium constant at various water contents of solvent

Fig. 4 shows the apparent equilibrium constant, K_C, for lauroyl hexoses at various equi-

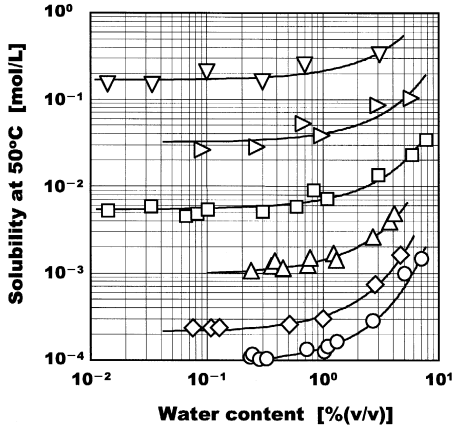

Fig. 3. Solubility at 50°C of (○) glucose, (◇) galactose, (□) mannose and (△) fructose in acetonitrile, and of fructose in (▷) acetone and in (▽) *tert*-butanol.

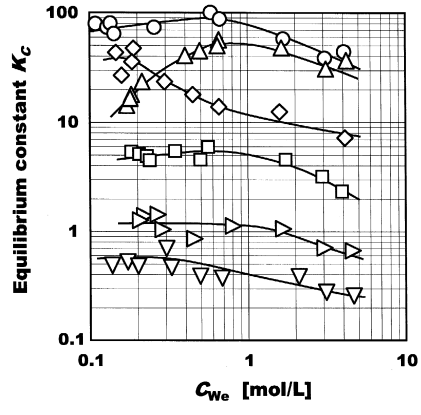

Fig. 4. Dependence of equilibrium constants for lauroyl hexose synthesis on equilibrium water concentration. Symbols are lauroyl glucose (○), galactose (◇), mannose (□), and fructose (△) in acetonitrile, and lauroyl fructose in (▷) acetone and (▽) *tert*-butanol. The curves were empirically drawn.

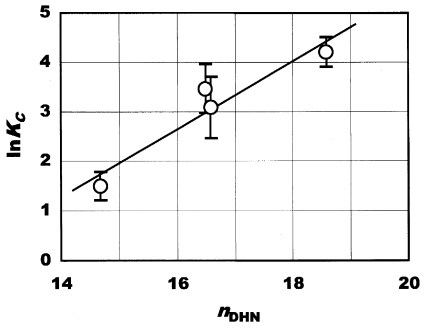

Fig. 5. Relationship between the apparent equilibrium constant K_C for lauroyl hexose formation in acetonitrile and the dynamic hydration number n_{DHN} of hexoses. Symbols represent mean ± standard deviation.

librium water concentrations. The K_C value largely depended on the kind of hexose. Except for lauroyl fructose, there was a tendency that the K_C was slightly higher at the lower C_{We}. The kind of solvent also significantly affected the K_C value. The K_C values were different by two orders of magnitude among the solvents.

3.4. Relationship between apparent equilibrium constant and dynamic hydration number of saccharide

As shown above, the apparent equilibrium constants, K_C, significantly depended on the kind of hexose and the solvent. The equilibrium constant, K_a, should, in principle, be defined based on activities, a, of the substrates and products, and is truly constant. Unfortunately, it is impossible or very difficult to evaluate all the a values (or activity coefficients γ) of the substrates and products in the present reaction system. When a saccharide hydrates, the water activity decreases. Therefore, it would be supposed that a hexose with stronger binding of water gives a larger K_C value although the activities of other components would also be affected by the presence of the hexose. We chose the dynamic hydration number, n_{DHN}, of hexose as a measure of the extent of hydration, and examined the relationship between the K_C values observed in acetonitrile and the n_{DHN} of hexoses [15,16] used as substrates (Fig. 5). Since the K_C value depended on the C_{We}, the K_C value averaged over all the C_{We} values for each product is plotted in the figure. As expected, there was a positive correlation between ln K_C and n_{DHN}. This indicates that the water activity plays an important role for the condensation in microaqueous organic solvents.

The K_C value for the formation of lauroyl fructose also depended on the kind of solvent. Although the correlation between the K_C value and the solvent parameter such as log P (P: partition coefficient between 1-octanol and water phases) and the Dimroth–Reichardt parameter for polarity of solvents $E_T(30)$ [17] was examined, no significant correlation was found. Further efforts are required to elucidate the effect of the kind of organic solvent on the K_C value.

3.5. Adsorption isotherm of water onto immobilized enzyme

The K_C value was evaluated based on the concentrations of substrates and products in the bulk phase. However, the condensation proceeds in the immobilized-enzyme particle. Therefore, the concentrations in the immobilized-enzyme phase would be desirable to estimate the K_C value. In this study, the concentra-

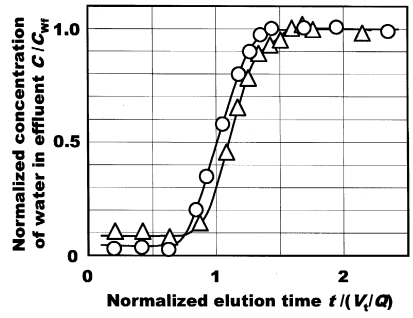

Fig. 6. Breakthrough curves of water in the bed packed with immobilized-lipase particles at room temperature. The water concentrations in feed were (△) 0.552 and (○) 1.61 mol/l.

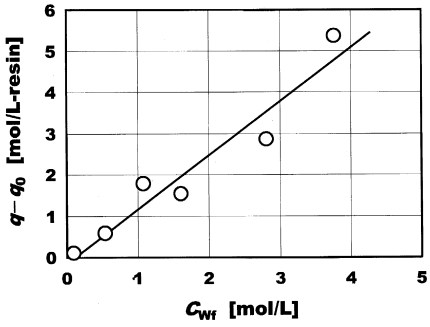

Fig. 7. Adsorption isotherms of water onto immobilized-lipase particle observed at room temperature.

tion of water in the phase was estimated because water played an important role in the condensation.

Fig. 6 shows examples of the breakthrough curves of water observed at different concentrations of water in the feed C_{Wf} using acetonitrile as a solvent. The curves were numerically integrated, and the $q - q_0$ values were calculated according to Eq. (2). Fig. 7 shows the adsorption isotherms of water onto the immobilized-enzyme. Although the condensation was conducted at 50°C, the isotherm was obtained at room temperature because of the experimental ease and to rough estimate of the extent of water adsorbability. The isotherm was almost linear. The slope of the line, which corresponds to the distribution coefficient of water onto the immobilized-enzyme phase, was 1.31. Since the porosity of the immobilized-enzyme was 0.588, the concentration of water in the pore was higher than that in the bulk phase by a factor of about 2. Thus, the possibility that the concentrations of substrates and products would be different from those in the bulk phase has been suggested. To more precisely discuss the K_C value, we need information about the adsorption isotherms of all the components. The isotherms should be observed in a multicomponent system because at least four components participate in the condensation.

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