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SYNTHESIS OF ESTERS BY LIPASE IMMOBILIZED ON POLY(VINYL ALCOHOL)-POLY(ETHYLENEIMINE) COPOLYMERS IN ORGANIC SOLVENTS

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ABSTRACT: The synthesis of lauric esters of mono- and polyhydric alcohols was studied in organic solvents using lipase from *Candida cylindracea* immobilized on poly(vinyl alcohol)-*co*-poly(ethyleneimine) gels. The conversion to *n*-hexyl laurate increased with increasing water content in the reaction system and levelled off above 3%. A similar result was obtained with wet-treated immobilized lipase. The synthesis of *n*-hexyl laurate in isooctane revealed the highest conversion and the highest rate of esterification among the organic solvents used. Fifty units of lipase was necessary to achieve the almost quantitative esterification in 22 h. Lauric esters of polyhydric alcohols, such as glycerol, ethylene glycol and 1,3-butanediol, were similarly synthesized with a relatively high conversion in isooctane. Esters prepared were characterized by gel permeation chromatography.

1. INTRODUCTION

The lipase-catalyzed esterification of alcohols has been extensively studied by many groups using free and immobilized lipases. Tsujisaka et al. showed that the reaction mechanism was different and depended on the origin of lipase for the synthesis of glycerides from various carboxylic acids[1]. Goderis et al. reported the interesterification of triolein and palmitic acid in *n*-hexane using lipase absorbed on celite and pointed out that the control of water content in the reaction system was of crucial importance[2]. Chopineau et al.[3] obtained optically active esters and alcohols by the transesterification of racemic alcohols with methyl propionate using lipase absorbed on Sepharose or Chromosorb. Sonnet[4] also synthesized optically active esters using Liposyme in *n*-hexane. Inada et al. synthesized pentyl pentanoate using polyethylene glycol-modified lipase in various organic solvents[5]. Esterification of carbohydrates such as glucose and sorbitol was investigated using fatty acids[6] or plant and animal oils[7]. Recently, the acetylation of methacrylic polymers containing hydroxyl groups was studied using vinyl acetate in tetrahydrofuran[8]. This method had an advantage that the rate of transesterification was higher than that with the corresponding saturated ester[9].

Bianchi used acid anhydrides as acylating agents on the esterification of alcohols catalyzed by lipase absorbed on celite[10]. This method had an advantage that the reverse reaction, the hydrolysis of esters, hardly occurred because water or alcohols were not produced unlike the esterification with acids or esters.

We have been studying the immobilization of enzymes such as α -amylase, invertase and lipase on cellulose-*co*-poly(ethyleneimine) (PEI) or poly(vinyl alcohol) (PVA)-*co*-PEI gels[11]. Glycerides were synthesized with lipase immobilized on PVA-*co*-PEI gels[12].

In this paper, *n*-hexyl laurate was synthesized in various organic solvents using lipase from *Candida cylindracea* immobilized covalently on PVA-*co*-PEI gels. Effects of the content of water in the reaction system, the kind of organic solvents and the concentration of lipase on the conversion and the rate of esterification were investigated. The esterification of polyhydric alcohols was also studied.

2. EXPERIMENTAL

2.1 Materials

PVA (Gousenol NH18, DP = 1800) was provided from Nippon Gousei Kagaku Kogyo Co. PEI (MW = 10000) was provided from Nippon Shokubai Kagaku

Kogyo Co. Lipase from *Candida cylindracea* (lipase-OF, 360 units/mg) was provided from Meito Sangyo Co. Glutaraldehyde (24 - 26% in water) was purchased from Tokyo Kasei Kogyo Co. Alcohols, lauric acid and organic solvents were commercial guaranteed reagents.

2.2 Synthesis of PVA-co-PEI Gels[11]

The bromoacetalization of PVA was performed by the reaction of PVA (18.5 g) dispersed in ethanol (180 ml) with bromoacetaldehyde diethylacetal (125 g) in the presence of sulfuric acid (1 ml) at 80 °C for 24 h. The product was precipitated in methanol and purified by Soxhlet extraction with methanol over 24 h. The bromoacetylated PVA (5 g) was then reacted with PEI (5 g) in DMF (200 ml) at 100 °C for 4 h. The product was precipitated in water and purified by Soxhlet extraction with water to exclude the unreacted PVA and PEI. The PEI content of PVA-co-PEI gels was calculated from the nitrogen content.

2.3 Immobilization of Lipase on PVA-co-PEI Gel

The swollen PVA-co-PEI gel (1 g at dry, PEI content = 48%) was added to 1% glutaraldehyde solution (50 ml) and stirred at 37 °C and 200 rpm for 2 h. After the free glutaraldehyde was washed away with 0.1 M phosphate buffer (pH 7.0), lipase-OF (100 mg) in the phosphate buffer (50 ml) was added to the activated gel and the mixture was stirred at 37 °C and 200 rpm for 2 h. The product was then filtered, washed with distilled water and lyophilized. The amount of lipase coupled on the gels was about 60 mg under these reaction conditions.

The activity of immobilized lipase was assayed by the hydrolysis of olive oil[13]. One unit of lipase was defined as the amount of the enzyme which was able to liberate 1 μ mol equivalent of fatty acid from olive oil per min. The activity of immobilized lipase was 880 units/g gel, the activity yield of lipase being about 4%.

2.4 Synthesis of Esters Catalyzed by Immobilized Lipase in Organic Solvents

In the synthesis of *n*-hexyl laurate, equimolar amounts of *n*-hexyl alcohol and lauric acid were dissolved in an organic solvent containing *n*-pentadecane as an internal standard for gas chromatography. The immobilized lipase was added to the solution and the reaction mixture was stirred at 25 °C and 300 rpm.

Aliquots were withdrawn from the mixture for the determination of the conversion by gas chromatography.

Lauric esters of polyhydric alcohols were also synthesized by a similar method. In this case, however, the conversion was calculated from the decrease in the peak area of lauric acid on the gas chromatogram.

2.5 Instrumental Methods

The nitrogen analysis was performed using a Yana-co MT500 CN Coder. Gas chromatography was performed using a Yanaco G180 gas chromatograph equipped with a 0.5 m glass column packed with Chromosorb W AW-DMCS containing 2% Dexil 300 GC (80/100 mesh). Gel permeation chromatography (GPC) was performed using a TOSOH HLC-803D liquid chromatograph equipped with a G1000H6 column or G1000H6 and G2000H8 columns at 40 °C. The eluate was THF.

3. RESULTS AND DISCUSSION

3.1 Effect of Water Content in the Reaction System on the Synthesis of *n*-Hexyl Laurate

The chemical reactions catalyzed by lipase in organic solvents are well known to be affected by the water content in the reaction system. For example, the

Table 1 Effect of Water Content on the Conversion to *n*-Hexyl Laurate in Benzene^a

Water content (%)	Conversion (%) ^b	
	Free	Immobilized
0.03	1.1	0.9
0.06	—	1.1
0.3	1.4	20.8
0.6	1.6	38.1
1.5	—	38.2
3.0	—	44.8
4.5	—	47.1
6.0	—	44.0
Wet-treated ^c	—	42.7
Wet carrier ^c	—	1.0

a) Reaction conditions. *n*-hexyl alcohol, 0.62 ml; lauric acid, 1 g; *n*-pentadecane, 0.5 ml; lipase, 50 units; dry benzene + water, 2.88 ml; temperature, 25 °C; stirring, 300 rpm; reaction time, 22 h.

b) Measured by gas chromatography.

c) Gels were dipped into water and filtered.

optimal water content for the synthesis of glycerides from glycerol and fatty acids was about 3 - 4% [14]. The synthesis of *n*-hexyl laurate from *n*-hexyl alcohol and lauric acid catalyzed by lipase from *Candida cylindracea* was slightly detectable in dry benzene at 20 °C. However, when water was added to the reaction mixture to raise the water content, water was separated from the solution above 0.05% due to the low solubility of water in benzene. The conversion did not increase even at relatively high water contents.

On the other hand, as shown in Table 1, the conversion increased with increasing water content, up to about 45%, using the immobilized lipase because the support used in this work was liable to absorb water. In fact water was not separated from the reaction mixture below 1.5% water content. This shows that water is necessary for lipase to catalyze the synthesis of esters in organic solvents and that the hydrophilic support contributes to make the environment more optimal for enzymatic activity [17]. It is well known that water has a dual effect in enzymatic systems: it is essential for the acquisition and maintenance of enzyme's catalytically active conformation [15].

The synthesis of *n*-hexyl laurate was also carried out with the wet-treated immobilized lipase in place of the addition of water to the reaction system. The wet-treatment of dry gels was performed by dipping them into water for a while and filtering them to exclude the free water. As shown in Table 1, the conversion was almost the same as those at higher water contents in the reaction system. In the following reactions, therefore, the wet-treated immobilized lipase was used.

3.2 Synthesis of *n*-Hexyl Laurate in Organic Solvents

The synthesis of *n*-hexyl laurate was carried out in toluene, *n*-heptane and isooctane in addition benzene. Table 2 shows that reaction in aliphatic solvents achieved a higher conversion than in aromatic solvents. Although the calibration method showed slight uncertainty, it can be considered that the ester was quantitatively synthesized in isooctane. Similar effectiveness of the catalytic action of lipase in isooctane was reported on the hydrolysis of fats in an organic

Table 2 Synthesis of *n*-Hexyl Laurate in Organic Solvents^a

Solvent	Conversion (%)
Benzene	42.7
Toluene	42.7
<i>n</i> -Heptane	63.9
Isooctane	99.9

a) Reaction conditions: *n*-hexyl alcohol, 0.62 ml; lauric acid, 1 g; *n*-pentadecane, 0.5 ml; immobilized lipase, 50 units; solvent, 2.88 ml; temperature, 25 °C; stirring, 300 rpm; reaction time, 22 h.

Fig. 1. Rate of formation of *n*-hexyl laurate in organic solvents. Reaction conditions are the same as shown in Table 2.

solvent-aqueous buffer system [16].

3.3 Rate of Formation of *n*-Hexyl Laurate

The rate of formation of *n*-hexyl laurate was studied in benzene and isooctane. Fig. 1 shows that the conversion increased with increasing reaction time in both solvents and the rate of formation in isooctane was higher than that in benzene. The conversion in benzene was 43% in 1 d and it continued to increase up to 83% in 3 d. Fig. 2 shows GPC curves for the reaction mixture in benzene as a function of reaction time. The peak of *n*-hexyl laurate appeared on the higher molecular-weight side of lauric acid and the peak area of *n*-hexyl laurate apparently increased with increasing reaction time.

3.4 Synthesis of *n*-Hexyl Laurate at Various Lipase Concentrations

The effect of lipase concentration on the conversion

Fig. 2. GPC curves for the reaction mixture in benzene as a function of reaction time.

Fig. 3. Effect of lipase concentration on the conversion to *n*-hexyl laurate. Reaction conditions are the same as shown in Table 2.

of *n*-hexyl laurate was studied in isooctane using various quantities of wet-treated immobilized lipase. Fig. 3 shows that the conversion increased with increasing lipase concentration. The figure also shows that fifty units of lipase was necessary to achieve the almost quantitative esterification within 22 h.

Table 3 Synthesis of Lauric Esters of Polyhydric Alcohols in Isooctane^a

Polyhydric alcohol	Conversion (%)
Glycerol	43.9
Ethylene glycol	77.3
1,3-Butanediol	91.0

a) Reaction conditions: polyhydric alcohol, 5 mmol; lauric acid, 1 g; *n*-pentadecane, 0.5 ml; immobilized lipase, 50 unit; solvent, isooctane; temperature, 25 °C; stirring, 300 rpm; reaction time, 22 h; total volume, 5 ml.

Fig. 4. GPC curves of lauric esters of polyhydric alcohols. EG, ethylene glycol, BD, 1, 3-butanediol; Gly, glycerol.

3.5 Synthesis of Lauric Esters of Polyhydric Alcohols

The esterification of polyhydric alcohols with lauric acid was carried out in isooctane. Polyhydric alcohols used were glycerol, ethylene glycol, and 1, 3-butanediol. The reaction proceeded heterogeneously due to their low solubility in isooctane. Nevertheless, the conversion was relatively high for three polyhydric alcohols as shown in Table 3. This was also ascertained from GPC curves of the reaction mixtures shown in Fig. 4. The ester peaks appeared on the higher molecular-weight side of lauric acid. Although several ester derivatives are expected for these

polyhydric alcohols, the esters synthesized under present reaction conditions are ascertained to be mono-laurate for ethylene glycol and 1,3-butanediol and mono- and dilaurates for glycerol from GPC analysis. The fact that the esters of polyhydric alcohols sparingly soluble in the organic solvents could be synthesized is probably explained in terms of the hydrophilicity of the support polymer. The local concentration of polyhydric alcohols near the immobilized lipase is considered to be relatively high due to their affinity for the hydrophilic support.

4. CONCLUSION

Lipase immobilized on PVA-co-PEI gels was used to synthesize esters in organic solvents. The water content in the reaction system significantly affected the conversion of *n*-hexyl laurate. Although the ester was not formed below 0.06% water content, the conversion increased with increasing water content above 0.3%. The reaction in aliphatic solvents achieved higher conversions than in aromatic solvents. Isooctane was the most appropriate solvent for the quantitative synthesis of the ester. Fifty units of lipase was necessary to achieve the almost quantitative esterification within 22 h. The lauric esters of polyhydric alcohols such as glycerol, ethylene glycol and 1, 3-butanediol were also successfully synthesized in isooctane by lipase immobilized on PVA-co-PEI gels.

The PVA-co-PEI gel is therefore a useful support for the ester synthesis by lipase in organic solvents. This is due to the high hydrophilicity of the gel resulting in the acquisition and maintenance of enzyme's catalytically active conformation.

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