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POLYCONDENSATION OF α-HYDROXY ACIDS BY ENZYMES OR PEG-MODIFIED ENZYMES IN ORGANIC MEDIA

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ABSTRACT

In order to provide biodegradable poly(hydroxy acid)s by a simple process, fundamental studies on the polycondensation of α-hydroxy acids by enzymes and polyethylene glycol (PEG)-modified enzymes in organic media were carried out. The oligomer formation by unmodified hog liver esterase proceeded well using glycolic acid as a substrate in cyclohexanone. Lipase from *Aspergillus niger* gave the best result for the polycondensation of glycolic acid among the unmodified enzymes used. The effects of the PEG-modification of enzyme on the polycondensation of α-hydroxy acids are also discussed. When the enzyme modified with a small amount of low-molecular-weight PEG was used, the conversion by polycondensation was relatively high. In particular, by using ethyl glycolate as a substrate, which allows an ester exchange reaction, and 1,4-dioxane as a solvent, polycondensation by PEG-modified enzyme proceeded effectively to give the pentamer of poly(glycolic acid).

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INTRODUCTION

Biodegradable polymers have become important for environmental and medical applications. Since poly(α-hydroxy acid)s, such as poly(lactic acid) and poly(glycolic acid), are biodegradable polyesters and have good biocompatibility, they are expected to be applied as biomaterials. Many studies about controlled release systems using poly(lactic acid) or poly(lactide-*co*-glycolide) as drug depots have been carried out [1-3]. We also reported that poly(α-malic acid) having pendant carboxylic acid groups is a useful polymer as a biodegradable drug carrier of anticancer agents [4, 5].

Some kinds of poly(hydroxy acid), such as poly(hydroxy butylic acid), can be produced by biological processes in microorganisms. Recently, it was reported that some hydrolases can catalyze condensation reactions in organic media. Many studies on the application of enzymes to organic synthesis have been carried out. Klibanov et al. demonstrated that some enzymes, such as lipases, proteases, and peroxidases, act as catalysts in organic media [6-12]. They emphasized that a trace amount of water strongly affects not only their catalytic activities but also their thermostabilities. Lipases catalyze the hydrolysis of triglycerides to produce 2-monoacylglycerols and fatty acids, and the hydrolysis of water-insoluble esters composed of fatty acids and alcohols with long alkyl chains. Esterase is a generic term for the enzymes which catalyze various esters. These hydrolases were used in ester synthesis by condensation or ester exchange reaction in organic media. Therefore, when hydroxy acids having both hydroxy and carboxylic acid groups are used as substrates, polycondensation of the hydroxy acids might be possible. In fact, Kobayashi et al. reported the enzymatic synthesis of polysaccharide [13], and Okumura et al. reported the enzymatic polycondensation of fatty dicarboxylic acids and diols [14].

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However, because enzymes are insoluble in organic media, the reactivity of enzymes is very restricted in such heterogeneous systems. Inada et al. reported that enzyme modified with amphiphilic polyethylene glycol (PEG) dissolves easily in organic solvents and catalyzes condensation reactions effectively [15-17].

In this paper, in order to provide biodegradable poly(hydroxy acid)s by a simple process, fundamental studies on the polycondensation of α-hydroxy acids by enzymes were carried out. Glycolic acid, D,L-lactic acid, 2-hydroxy isobutylic acid, L-malic acid, and ethyl glycolate were used as substrates (Fig. 1). The favorable conditions, substrates, solvents, and enzymes of the polycondensation of α-hydroxy acids were investigated using unmodified enzymes. Moreover, we prepared PEG-modified enzymes and investigated the effect of the degree of introduction of PEG enzymes and the chain length of PEG on the polycondensation of α-hydroxy acids in organic media.

EXPERIMENTAL

Materials

Lipase from *Aspergillus niger*, *Candida rugosa*, and hog pancreas, and protease from *Rhizopus niveus* and *Aspergillus oryzea* were obtained from Amano Pharmaceutical Ltd. Lipase from *Candida cylindracea* and esterase from hog liver were

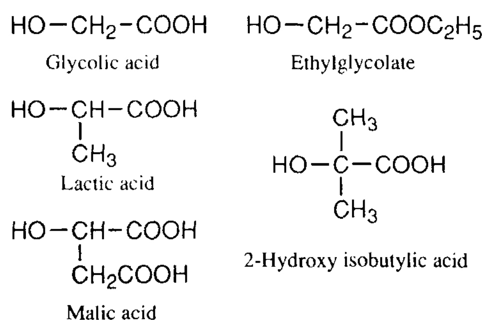

FIG. 1. Molecular structures of α-hydroxy acids and α-hydroxy acid ester used in this paper.

obtained from Seikagaku Kogyo Co., Ltd., and Boehringer Mannheim Yamanouchi Co., respectively. Lipase from *Chromobacterium viscosum* and monomethoxypolyethylene glycol (number-average molecular weight = 5000, 1000) were purchased from Sigma Chemical Co. L-Malic acid was provided by Fuso Chemical Ltd. Cyclohexanone, benzene, and the other organic solvents were purified by the usual distillation. Other reagents were commercial grade and used without further purification.

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Modification of Enzymes with PEG

2,4-Bis(*O*-methoxypolyethylene glycol)-6-chloro-*s*-triazine (activated PEG_2) was synthesized from monomethoxypolyethylene glycol and cyanuric chloride according to the procedure described in Reference 18. Preparation of PEG-modified enzyme is described as a typical example. Activated PEG_2 (200 mg) was gradually added to enzyme (3200 units) in 3.0 mL of 0.2 N borate buffer (pH 10.0). The reaction mixture was incubated for 1 hour at room temperature under stirring. Then cold phosphate buffer (pH 7.0, 70 mL) was added to the reaction mixture to stop the reaction. The unreacted polyethylene glycol derivatives were removed by ultrafiltration with a Daiaflo PM-30 membrane (Amicon, cut off = 30,000) and gel-filtration chromatography (Sephadex G-50, column: 1.1 × 25 cm). The degree of introduction of PEG per amino group (DPEG) of enzyme was determined by measuring the amount of free amino groups by trinitrobenzene sulfonate (TNBS) according to a method reported previously [19]. These procedures were carried out using various amount of activated PEG_2 to obtain PEG-modified enzyme having various DPEG values.

The relative activities of the PEG-modified enzymes were determined by measuring their hydrolysis efficiency against 4-methylumbelliferyl nonanate. 4-Methylumbelliferyl nonanate (10 mg) was dissolved in 10 mL of 1/30 M phosphate buffer (pH 7.0). This solution was kept at the optimum reaction temperature of each enzyme for 5 minutes. A small portion (308 μL) of PEG-modified enzyme dissolved in 1/30 M phosphate buffer (1300 units/mL) was added to the solution. The mixture was stirred vigorously at the optimum reaction temperature of each enzyme. After stirring for a certain time, the amount of hydrolysis product of 4-methylumbelliferyl nonanate was measured by fluorescence spectrophotometer with excita-

tion at 375 nm and emission at 450 nm. The ratio of the hydrolysis rate of 4-methylumbelliferyl nonanate by PEG-modified enzyme to that of unmodified enzyme was defined as the relative activity of the PEG-modified enzyme.

Polycondensation of α-Hydroxy Acids by Enzymes or PEG-Modified Enzymes in Organic Media

Glycolic acid, D,L-lactic acid, 2-hydroxy isobutylic acid, and L-malic acid were used as substrates, and cyclohexanone, 1,4-dioxane, benzene, and 2-butanone were used as reaction solvents for preliminary experiments to determine the conditions of polycondensation using unmodified enzymes. Substrate (0.6 mmol) was dissolved in 50 mL organic solvent containing 0.6 wt% water and kept at the optimum reaction temperature of each enzyme for 5 minutes. A small portion (308 μL) of 1/30 M phosphate buffer containing unmodified enzyme (1300 units/mL) was added to the solution. The reaction mixture was stirred vigorously at the optimum reaction temperature of each enzyme. After 72 hours the solution was quickly cooled to -70°C to stop the reaction. Then the solution containing the products was evaporated under reduced pressure and redissolved in 3.0 mL THF. The solution was filtered to remove any enzyme insoluble in THF. The crude products were confirmed to be a polyester by IR analysis. The progress of the polycondensation was evaluated by GPC analysis (column, Toso TSK-G2500H + G1000H; eluent, THF; detector, RI) of the products.

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After determination of the reaction conditions by such preliminary experiments, polycondensations using various unmodified enzymes and PEG-modified enzymes were carried out according to procedures similar to those described above. Eight kinds of enzyme (lipases from *Aspergillus niger*, from *Candida rugosa*, from hog pancreas, from *Candida cylindracea*, and from *Chromobacterium viscosum*; protease from *Rhizopus niveus* and from *Aspergillus oryzea*, and esterase from hog liver) were used for the screening of enzymes. Glycolic acid and ethyl glycolate were used as substrates for these experiments. The progress of the polycondensation was evaluated by GPC analysis (column, Toso TSK-G2500H + G1000H; eluent, THF; detector, RI) of the products to assign each oligomer's peak (Fig. 2). The area of each oligomer's peak was calculated. Then the proportion of the area of all the oligomer peaks which had a higher molecular weight than the monomer to the sum was determined as the conversion of the reaction.

The remaining activities of enzymes after incubation in organic solvents were measured by the same methods as the relative activities of PEG-modified enzymes described above.

RESULT AND DISCUSSION

Polycondensations of α-Hydroxy Acids by Unmodified Esterase

Preliminary studies of polycondensation by unmodified enzyme in organic solvent were carried out using the esterase from hog liver. Some kinds of α-hydroxy acid (glycolic acid, D,L-lactic acid, 2-hydroxy isobutylic acid, and L-malic acid) were reacted with the esterase from hog liver in cyclohexanone. Cyclohexanone was employed as the solvent because it dissolves these substrates. Table 1 shows the

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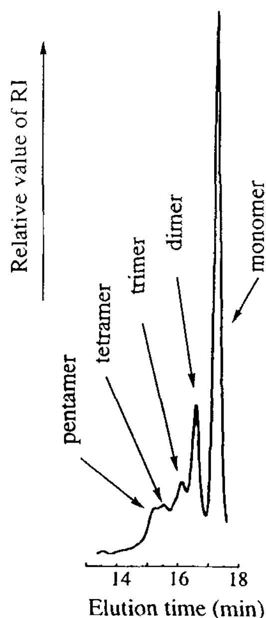

FIG. 2. Typical elution profile of GPC (column, Toso TSK-G2500H + G1000H; eluent, THF; detector, RI) of the products of polycondensation of α-hydroxy acid by enzyme.

conversions for polycondensation of these α-hydroxy acids by the esterase from hog liver in cyclohexanone. Among these four kinds of α-hydroxy acids, glycolic acid showed the highest conversion. This is because the esterase is likely to recognize glycolic acid as having low steric hindrance. Then we investigated what kinds of organic solvent are suitable for the polycondensation of α-hydroxy acid by unmodified esterase from hog liver using glycolic acid as a substrate. Cyclohexanone, benzene, 1,4-dioxane and 2-butanone were used as solvents. Table 2 shows the conversions of polycondensation of glycolic acid by the esterase from hog liver in various organic solvents, and the remaining activities of enzymes after incubation in

TABLE 1. Conversions for Polycondensation of Various α-Hydroxy Acids by Unmodified Esterase[a] in Cyclohexanone

Substrates	Conversion, %
Glycolic acid	33
D,L-Lactic acid	16
L-Malic acid	9
2-Hydroxyisobutylic acid	12

[a]Esterase from hog liver.

TABLE 2. Conversions for Polycondensation of Glycolic Acid by Unmodified Esterase[a] in Various Organic Solvents and the Remaining Activity of the Enzymes

Solvents	Conversion, %	Remaining activity of enzyme, %[b]
Cyclohexanone	33	40 ± 8
Benzene	20	51 ± 10
1,4-Dioxane	6	6 ± 3
2-Butanone	28	17 ± 5

[a]Esterase from hog liver.

[b]Remaining activities of enzymes after incubation in organic media were estimated by hydrolysis of 4-methylumbelliferyl nonanate in PBS (pH 7.0) after incubation in organic solvents for 72 hours and compared with untreated enzymes.

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such organic solvents. Cyclohexanone and 2-butanone gave good conversions, but 1,4-dioxane showed low conversion. The conversions depended on the activity of the enzyme in organic solvents. Cyclohexanone and 2-butanone had a relatively high remaining activity of the enzyme. Solvents miscible with water, such as 1,4-dioxane, should take water molecules away from enzymes and thus cause deactivation of the enzyme. Another factor is the solubility of the substrate. Benzene, which has no miscibility with water, showed a high remaining activity of enzyme; however, the conversion in benzene was not very high. This is because benzene scarcely dissolves glycolic acid. From these results we conclude that a solvent having no miscibility with water and having a high solubility of substrate is suitable for enzymatic condensation reactions. We therefore employed cyclohexanone as the reaction solvent in the following experiments.

Polycondensations of Glycolic Acid or Ethyl Glycolate by Various Unmodified Enzymes

The substrate specificity and stability of enzymes in organic media are dependent on the origins of the enzymes. Therefore, polycondensations of α-hydroxy acids with various enzymes in cyclohexanone were investigated. In these experiments we used glycolic acid and ethyl glycolate as substrates. Ethyl glycolate was employed to investigate the reactivities of the enzymes for the ester exchange reaction. The results of enzyme screening are shown in Table 3. Lipase from *Aspergillus niger* showed the highest conversions for these two substrates among all the enzymes used. This lipase has a wide substrate specificity and has been used in studies of ester synthesis [20, 21]. Most of the enzymes showed higher conversions with ethyl glycolate than with glycolic acid. These results suggest that polycondensation through the ester exchange reaction proceeded well in organic solvent when using such hydrolases. Based on these results, we employed lipase from *Aspergillus niger* in addition to the esterase from hog liver for the preparation of PEG-modified enzymes.

TABLE 3. Conversions for Polycondensation of Glycolic Acid or Ethyl Glycolate by Various Unmodified Enzymes in Cyclohexanone

Enzymes	Conversion (%) for glycolic acid	Conversion (%) for ethyl glycolate
A. niger lipase	53	51
C. rugosa lipase	47	46
C. cylindracea lipase	28	35
Hog pancreas lipase	44	49
C. viscosum lipase	52	4
R. niveus protease	32	36
A. oryzea protease	31	50
Hog liver esterase	33	34

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Preparation of PEG-Modified Enzymes

Esterase from hog liver and lipase from *Aspergillus niger* were modified with PEG (MW = 5000). Esterase from hog liver was also modified with low-molecular-weight PEG (MW = 1000) to investigate the effect of the chain length of PEG on the polycondensation of α-hydroxy acids. The results of our preparation of PEG-modified enzymes are shown in Table 4. The PEG-modified enzymes obtained showed a 50–80% degree of introduction of PEG per amino group of enzyme (DPEG). The relative activities of these PEG-modified enzymes tended to decrease with an increase in DPEG values. This suggests that the introduction of PEG leads to deactivation of enzymes. However, the PEG-modified enzymes with low DPEG values showed higher relative activities than unmodified enzymes (relative activity > 100%). This is because the affinity of the enzymes to hydrophobic 4-methylumbelliferyl nonanate was increased by the introduction of a small amount of

TABLE 4. Preparation of PEG-Modified Enzymes

Enzymes	MW of PEG	DPEG, %[a]	Relative activity, %[b]
Lipase[c]	5000	70.8	64.7
	5000	67.5	87.8
	5000	53.7	122.7
Esterase[d]	5000	77.4	51.4
	5000	58.7	63.0
	1000	55.3	148.7

[a]Degree of introduction of PEG per amino group of enzyme.

[b]Relative activities compared with unmodified enzyme were estimated by hydrolysis of 4-methylumbelliferyl nonanate in PBS (pH 7.0).

[c]Lipase from *Aspergillus niger*.

[d]Esterase from hog liver.

amphiphilic PEG chains. PEG-esterase with a PEG MW of 1000 (DPEG = 55.3%) showed a higher relative activity than PEG-esterase with a PEG MW of 5000 (DPEG = 58.7%), although they had similar DPEG values. This suggests that steric hindrance is also important for keeping enzyme activity.

Polycondensations of Glycolic Acid or Ethyl Glycolate by PEG-Modified Enzymes

The polycondensations of glycolic acid and ethyl glycolate by PEG-modified enzymes were carried out in cyclohexanone. Figure 3 shows conversions for the polycondensation of glycolic acid and ethyl glycolate by unmodified enzymes and PEG-modified enzymes (MW of PEG = 5000) with various DPEG values. The conversions of these α-hydroxy acids by the esterase were increased by modification with PEG. PEG-esterase (DPEG = 58.7%) showed about 2 times higher conversion of ethyl glycolate than did unmodified esterase. However, PEG-lipases did not show higher conversions compared with unmodified lipase. In both cases of esterase and lipase, PEG-modified enzymes with low DPEG values showed higher conversions than PEG-modified enzymes with high DPEG values. These results suggest that a large introduction of PEG leads to deactivation of enzymes and an increase in steric hindrance.

In order to discuss these results in detail, the molecular weight distribution of products from the polycondensation of glycolic acid and ethyl glycolate by PEG-modified enzymes with low DPEG values are shown in Fig. 4. When PEG-esterase (DPEG = 58.7%) was used, the content of oligomers having higher molecular weights than the dimer was increased compared with unmodified esterase for both glycolic acid and ethyl glycolate. These results suggest that PEG modification is effective for the polycondensation of ethyl glycolate by esterase in organic media. On the other hand, when PEG-lipase (DPEG = 53.7%) was used, the reactivity

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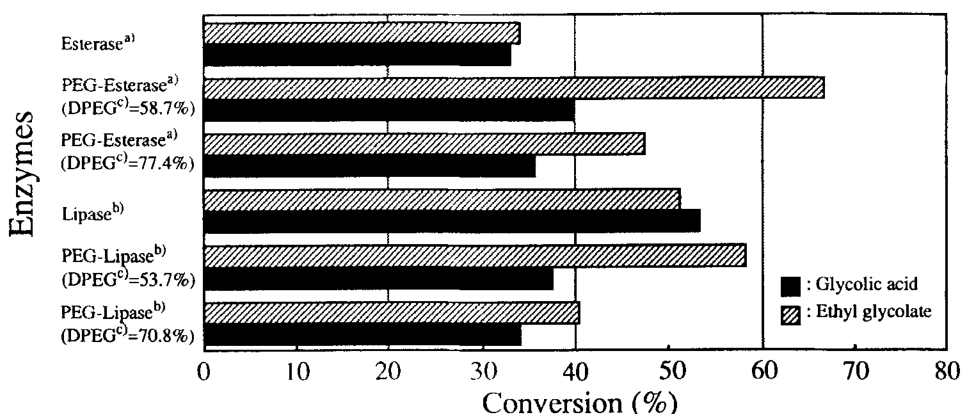

FIG. 3. Conversions for polycondensation of glycolic acid or ethyl glycolate for 72 hours by unmodified enzymes and PEG-modified enzymes in cyclohexanone. [a]Esterase from hog liver. [b]Lipase from *Aspergillus niger*. [c]Degree of introduction of PEG per amino group of enzyme.

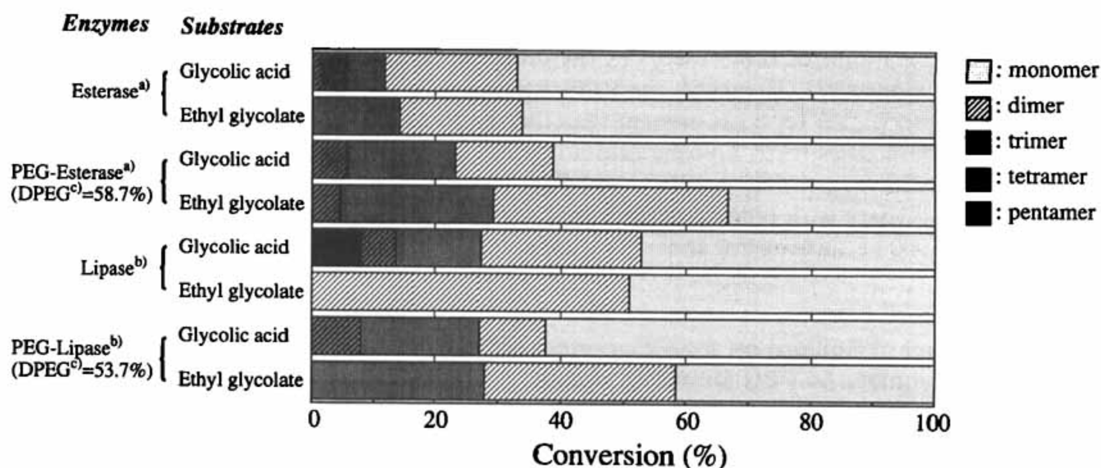

FIG. 4. Molecular weight distribution of the products for polycondensation of glycolic acid or ethyl glycolate for 72 hours by unmodified enzymes and PEG-modified enzymes in cyclohexanone. [a]Esterase from hog liver. [b]Lipase from *Aspergillus niger*. [c]Degree of introduction of PEG per amino group of enzyme.

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with ethyl glycolate slightly increased compared with unmodified lipase; however, the reactivity with glycolic acid decreased. Although unmodified lipase could produce the tetramer of glycolic acid, PEG-lipase could not. These results suggested that the reactivity of lipase with oligomers was decreased by modification with PEG because of the steric hindrance of the PEG chain.

The Effect of Chain Length of PEG

In order to investigate the steric effect of the PEG chain, we prepared PEG-modified esterase using two kinds of PEG (MW = 1000 and 5000). Figure 5 shows the molecular weight distribution of the products for the polycondensation of gly-

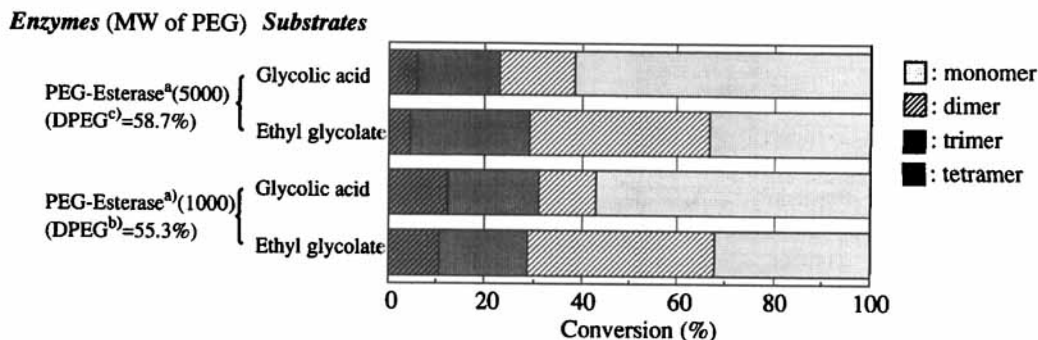

FIG. 5. The effect of chain length of PEG on the molecular weight distribution of the products for polycondensation of glycolic acid or ethyl glycolate for 72 hours by PEG-modified esterase in cyclohexanone. [a]Esterase from hog liver. [b]Degree of introduction of PEG per amino group of enzyme.

colic acid and ethyl glycolate. These two PEG-modified esterases showed similar conversions (sums of the contents of the products having higher molecular weight than the monomer). However, the PEG-esterase with a PEG MW of 1000 gave a significantly higher tetramer content than did the PEG-esterase with a PEG MW of 5000 in both cases when glycolic acid and ethyl glycolate were used as substrates. These results suggest that the PEG-modified esterase with a PEG MW of 1000 has a higher reactivity with oligomers than does the PEG-modified esterase with a PEG MW of 5000 because of low steric hindrance.

The Effect of Solvent on the Polycondensation of Ethyl Glycolate by PEG-Modified Lipase

The reaction solvent might affect the conformation of PEG chains and the solubility of PEG-modified enzyme. Therefore it was expected that the effect of solvent on polycondensation using PEG-modified enzymes would be different from the results when using unmodified enzymes. We also investigated the effect of solvent on the polycondensation of ethyl glycolate by PEG-modified enzymes using PEG-modified lipase (DPEG = 67.5%). 1,4-Dioxane, cyclohexanone, benzene, and toluene were used as solvents. Figure 6 shows the molecular weight distribution of the products in polycondensation by PEG-lipase (DPEG = 67.5%) in various organic solvents. The highest conversion was obtained when cyclohexanone was used as the solvent. However, when 1,4-dioxane was the solvent, the higher molecular weight content of the product increased, thus confirming the formation of pentamer. This tendency was very different from the results for unmodified enzymes. When benzene was used as the solvent, the formation of pentamer was confirmed, but the total conversion was low. These results suggest that the deactivation of PEG-modified enzyme by organic solvents is low compared to unmodified enzymes, and that the solubility of substrates is also important in the polycondensation of α-hydroxy acids in organic media.

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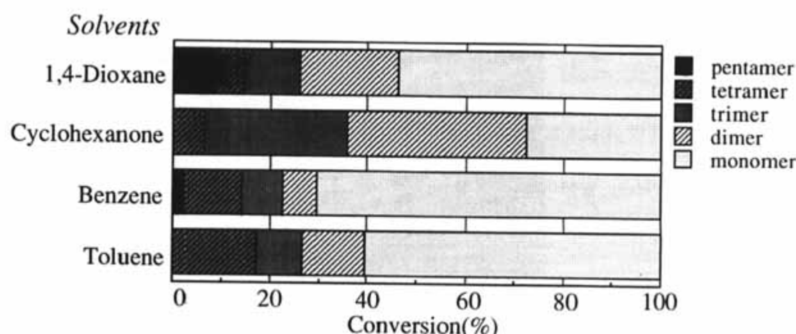

FIG. 6. The effect of solvent on the molecular weight distribution of the products for polycondensation of ethyl glycolate for 72 hours by PEG-modified lipase [from *Aspergillus niger*, DPEG (degree of introduction of PEG per amino group of enzyme) = 67.5%] in cyclohexanone.

CONCLUSION

Fundamental studies on the synthesis of α-hydroxy acid oligomers using enzymes or PEG-modified enzymes were carried out. When unmodified esterase from hog liver was used, glycolic acid, which has a low steric hindrance, was appropriate as a substrate, and cyclohexanone, which shows high substrate solubility and no miscibility with water, was appropriate as a solvent. We studied the reactivities of various unmodified hydrolases against glycolic acid and ethyl glycolate. Among the hydrolases tested, lipase from *Aspergillus niger* showed the highest conversions for these two substrates. In the case of PEG-modified enzyme, the reactivity of hog liver esterase for the substrates, especially for ethyl glycolate, was increased; however, that of *Aspergillus niger* lipase was not. Concerning the modification of enzyme with PEG, a low DPEG value (ca. 50%) and a low-molecular-weight PEG were more appropriate. From these results we concluded that the optimized conditions to obtain a high molecular weight oligomer and high conversion are as follows:

Unmodified enzyme:	Enzyme	Lipase from *Aspergillus niger*
	Substrate	Glycolic acid
	Solvent	Cyclohexanone
PEG-modified enzyme:	Enzyme	Esterase from hog liver
	Substrate	Ethyl glycolate
	Solvent	Cyclohexanone or dioxane
	DPEG	ca. 50% or lower
	MW of PEG	1000

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In order to obtain a higher molecular weight polymer, control of the steric hindrance of PEG is important. Moreover, because the PEG-modified enzyme showed higher reactivity for ethyl glycolate, we should consider elimination groups in the condensation reaction and removing them from the system to obtain higher molecular weight polymer.

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