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Enzymatic Formation of 13,26-Dihexyl-1,14-dioxacyclohexacosane-2,15-dione via Oligomerization of 12-Hydroxystearic Acid

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Abstract: The enzymatic polymerization of 12-hydroxystearic acid (12-HSA) was carried out with Lipase CA® in benzene to produce poly(12-hydroxystearate) (PHS) with a low molecular weight. When this polymerization was continued for a long reaction time, the PHS once formed was depolymerized into a cyclic diester, 13,16-dihexyl-1,14-dioxacyclohexacosane-2,15-dione (12-HSAD). Similar polymerization and depolymerization were observed when 12-hydroxyoleic acid (12-HOA) was treated with Lipase CA®, whereas only polymerization occurred when 12-hydroxydodecanoic acid (12-HDA) was treated in a similar manner. The preferential formation of cyclic diesters for 12-HSA was attributed to the structural requirements due to the bulky *n*-hexyl side groups stemming from the ring systems.

Keywords: lipase, 12-hydroxystearic acid, enzymatic polymerization, ring formation, 12-hydroxyoleic acid.

Introduction

Lipases are a class of enzymes which catalyze the hydrolysis of fatty acid esters in aqueous environment of living systems. Some lipases are also stable in non-aqueous organic solvents and catalyze the esterification and transesterification reactions.[1-5] The specific catalysis of the lipases enables production of useful polyesters by various polymerization modes.[6] Lactones, such as β-butyrolactone, ε-caprolactone and so on, can be the substrates of the lipase-catalyzed ring-opening polymerization,[7,8] while hydroxy fatty acids can be used as the substrates of another reation type lipase-catalyzed polycondensation.[9,10] However, the molecular weights of the polymers resulting from the lipase-catalyzed polycondensation are not generally high (<10,000).[11,12] In this study, 12-hydroxystearic acid (12-HSA) is examined as a substrate of the lipase-catalyzed polycondensation. Since 12-HSA can be obtained from naturally occurring caster oil, it is one of the natural renewable resources. The use of the substances from renewable resources as well as the use of enzymes in the reaction adheres the concept of "Green Sustainable Chemistry".[13] Here, we demonstrate that the lipase-catalyzed polycondensation of 12-HSA leads to the formation of a cyclic diester, 13,16-dihexyl-1,14-dioxacyclohexacosane-2,15-dione (12-HSAD) in high conversion via the formation of poly(12-hydroxystearate) (P(12HS)). 12-HSAD is a macrocyclic lactone (macrolide) consisting of a 26-membered ring involving two ester groups, and its simple formation by one-step process using biocatalysts is of particular interest as compared with the complicated multistep procedures that have previously been used in synthesizing such macrolides.[14-20] Similar lipase-catalyzed polycondensation of ricinoleic acid (12-HOA) is also demonstrated.

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Experimental

Materials. Highly pure 12-HSA (12-hydroxyoctadecanoic acid) and 12-HOA (*Z*-12-hydroxyoctadec-9-enoic acid) were kindly supplied by Ito Oil Chemicals Co., Ltd. (Yokkaichi, Japan) and used without further purification. 12-HDA (12-hydroxydodecanoic acid) was purchased from Aldrich Chemical Co. and purified by recrystallization from methanol. Their purity was over 99.5% as determined by gas chromatography (GC) utilizing (trimethylsilyl) diazomethane as the esterification agent. Novozym 435™, a lipase originated from *Candida antarctica* (Lipase CA®

having activities of triacylglycerol hydrolase and carboxyl esterase and immobilized on porous polystyrene gel) was kindly supplied by Novo Nordisk Bioindustry Ltd. (Chiba, Japan). Its activity expressed in propyl laurate units per gram enzyme (PLU/g) was declared to be 7000 PLU/g (from the supplier). Lipase PS®, Lipase A®, and Lipase AY® originated from *Pseudomonas cepacia*, *Aspergillus niger*, and *Candida rugosa*, respectively, were kindly supplied from Amano Pharmaceutical Co. (Nagoya, Japan). Lipase PPL (porcine pancreatic lipase: 190 unit/mg protein using olive oil according to the supplier) was purchased from Sigma Chemical Co. (St. Louis, MO). Lipase RD from *Rhizopus delemer* was purchased from Biocatalysts, Ltd (England). All of the lipases were dried in vacuum before use.

Measurements. ^{1}H and ^{13}C NMR spectra were measured on a Brucker ARX NMR spectrometer operated at 500 and 125 MHz, respectively, in chloroform-*d* with tetramethylsilane (TMS) as the internal standard. IR spectra were recorded on a Shimadzu FTIR 8200PC spectrometer. GC analysis was conducted by using a Shimadzu GC-6A apparatus equipped with a TCD detector and a silica OV-1 column (1 m in length and 5 mm ϕ, I.D.) in a temperature range of 70-300 °C at an increasing rate of 5 °C/min. Number-(M_n) and weight-average (M_w) molecular weights were determined by gel permeation chromatography (GPC). The analyzer was composed of a Shimadzu LC-10ADvp system consisting of a LC-10ADvp HPLC pump and a RID-6A refractive index detector. A combination of two polystyrene gel columns of Tosoh TSK gel G4000H_8 and G2500H_8 (7.5 mm I.D.×300 mm, each) was used with chloroform as the eluent at 35 °C. The molecular weights were calibrated according to polystyrene standards. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) was measured with a Bruker Reflex III™ spectrometer equipped with a nitrogen laser. The detection was done by the reflector mode with positive ionization by using 2,5-dihydroxybenzoic acid (DHB) as the matrix. High-resolution mass spectra (HRMS) were measured on a JEOL JMS-700 spectrometer with the conventional chemical ionization (CI) and electron ionization (EI) modes.

Enzymatic Reaction of 12-HAS. In a flask equipped with a Dean-Stark tube connected to a ball condenser and a three-way stopcock, 12-HSA (12.00 g, 0.04 mol) and Lipase CA® (5.00 g) were mixed with benzene (50 mL). Then the mixture was refluxed with stirring on a magnetic stirrer under a nitrogen atmosphere. With the refluxing, the condensed water was azeotropically distilled and collected in the depot of the Dean-Stark tube. After the predetermined reaction time, the mixture was diluted with toluene (100 mL), and the insoluble enzyme was filtered off. The filtrate was then evaporated at reduced pressure to obtain a reaction product, which was subjected to various analyses.

When this enzymatic reaction was continued for a week, the finally obtained filtrate was composed of a crude oligomeric product. It was subjected to recrystallization from ethanol to obtain pure 12-HSAD in a yield of 9.96 g (83%); mp=66 °C, IR (KBr); 2923, 2848 (CH_2), 1730 (C=O), 1471, 1377, 1363, 1259, 1238, 1218, 1193, 1168, 1105, 1093, 1070, 1033, 991 cm^{-1}. ^{1}H NMR (500 MHz in $CDCl_3$) δ(ppm) 0.87 (t, J = 6.75 Hz, 3H, CH_3), 1.2-1.4 (b, 22H, CH_2), 1.4-1.6 (d, 2H, COOCH(CH_2)$_2$), 1.6-1.8 (b, 2H, OCOCH_2CH_2), 2.27 (m, J = 7.31 Hz, J = 6.75 Hz, 2H, OCOCH_2), 4.90 (q, J = 4.43 Hz, 1H, COO*CH*). ^{13}C NMR (125 MHz in $CDCl_3$) δ (ppm) 14.1 (CH_3), 22.6 (CH_3CH_2), 25.3-25.6 (CH_2), 29.4-29.7 (CH_2), 31.7 ($CH_3CH_2CH_2$), 34.2 (COOCH(CH_2)$_2$), 34.6 (OCOCH_2), 73.9 (COO*CH*), 173.7 (C=O). HRMS, calcd 564.5117, found 564.5105. Anal. Calcd for $C_{36}H_{68}O_4$: C 12.13, H 76.54. Found: C 12.26, H 76.84.

Results and Discussion

Enzymatic Reaction of 12-HAS. Various commercially available lipases (Lipase PPL, RD, PS®, A®, AY®, and CA®) were screened for the enzymatic polycondensation of 12-HSA. Among them, only Lipase CA® showed the catalytic activity. Figure 1 shows a typical ^{1}H NMR spectrum of a product of the polycondensation of 12-HAS when the reaction was conducted in refluxing benzene for 24 h with Lipase CA® (40 wt% relative to 12-HSA). The signal assignments are shown in the molecular formula involved. The signal shift of the methyne proton from δ = 3.58 ppm (e) for the unreacted hydroxyls (12-HAS) to δ = 4.87 ppm (e') for the esterified hydroxyls (P(12HS)) corresponds to the polycondensation of 12-HSA. Therefore, the conversion can be calculated from the integral ratio of those two signals. Figure 2 shows the time-conversion curves plotted with different amounts of Lipase CA® (enzyme-to-substrate ratio) at a monomer concentration of 0.20 M. It is shown that the rate

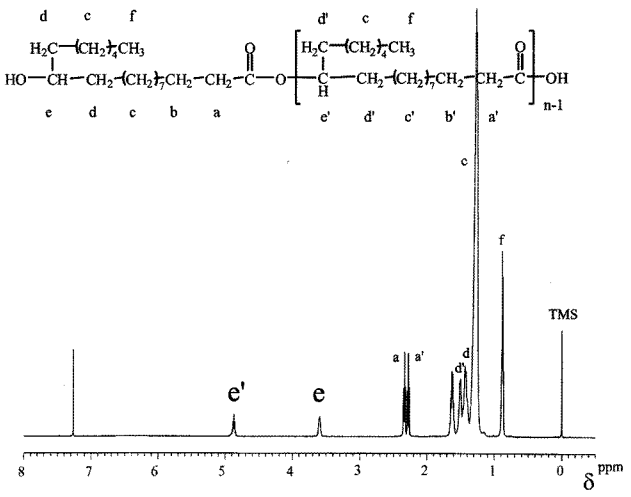


Figure 1. A typical ^{1}H NMR spectrum of a reaction product obtained after the polycondensation of 12-HAS (0.40 M in benzene) with Lipase CA® (40 wt%) for 24 h.

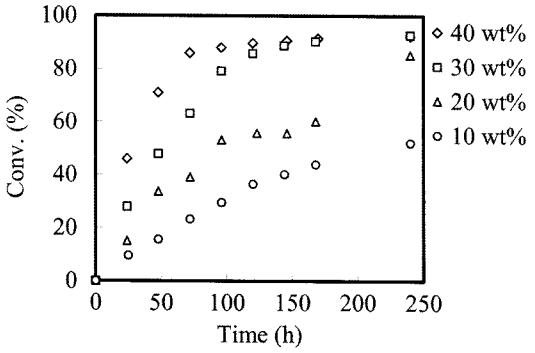


Figure 2. Effects of the amount of Lipase CA® on the monomer conversion in the enzymatic polycondensation of 12-HSA (0.2 M in benzene).

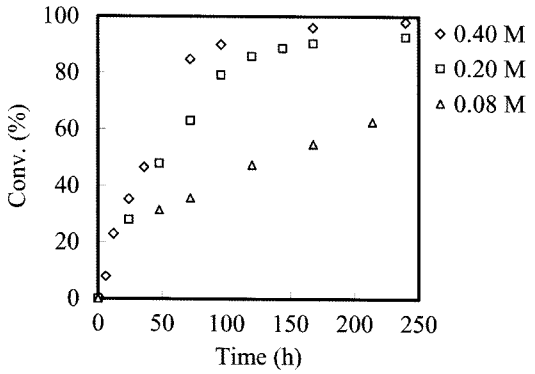


Figure 3. Effects of the monomer concentration on the conversion in the enzymatic polycondensation of 12-HSA using Lipase CA® (30 wt%) in benzene.

of monomer consumption increases with increasing the amount of Lipase CA®. A relatively large amount (over 30 wt% in enzyme-to-substrate ratio) is required for efficient polycondensation as reported in the related enzymatic polymerizations.[21] Since no reaction occurred in the absence of Lipase CA®, the dehydration/hydrolysis equilibrium in this enzymatic polycondensation was allowed to decline in the direction of dehydration by the azeotropic distillation of the condensed water. However, the reaction rate is very slow even in the presence of large amount of enzyme, because the real active sites that can work for the reaction should be limited in the surface area of the heterogeneously dispersed enzyme. Reuse of the Lipase CA® was possible with a slight loss of activity. For example, the conversion decreased to 18% with the recycled enzyme from 48% with the virgin enzyme at the identical reaction conditions (30 wt% in enzyme-to-substrate ratio). Figure 3 shows the time-conversion curves plotted with three different initial monomer concentrations. The rate of monomer consumption increases with increasing the monomer concentration, although it is almost identical above 0.20 M.

For further study of this enzymatic polycondensation, the reaction time was elongated with the optimum conditions:

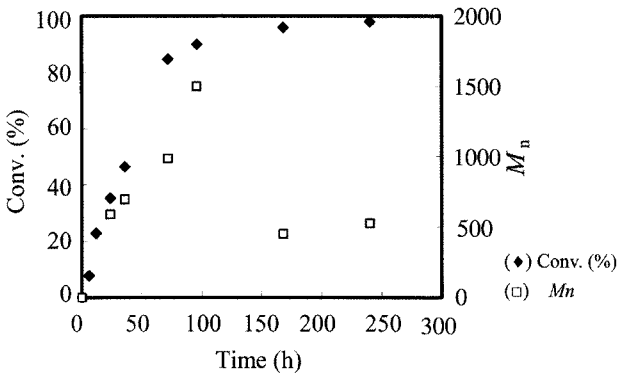


Figure 4. Time-courses of the conversion (♦) and M_n (□) of the polycondensate in the polymerization of 12-HSA (0.4 M) using 30 wt% Lipase CA® in benzene.

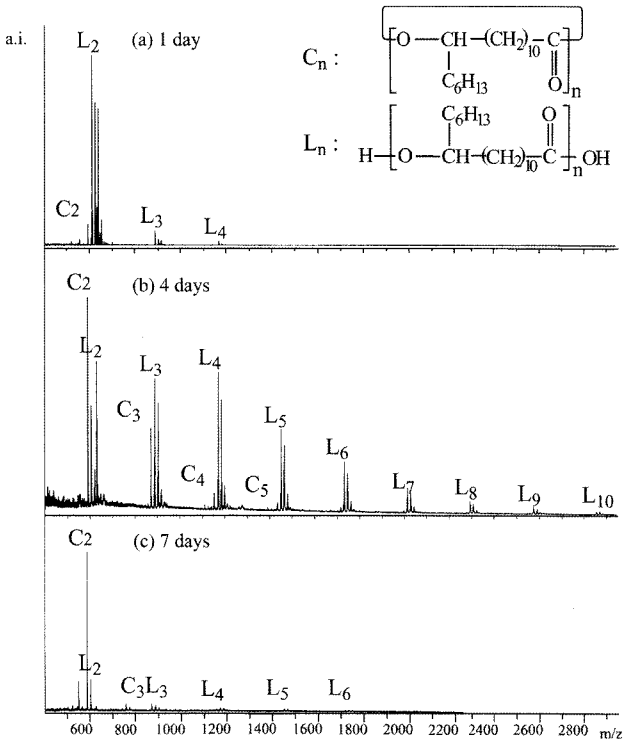


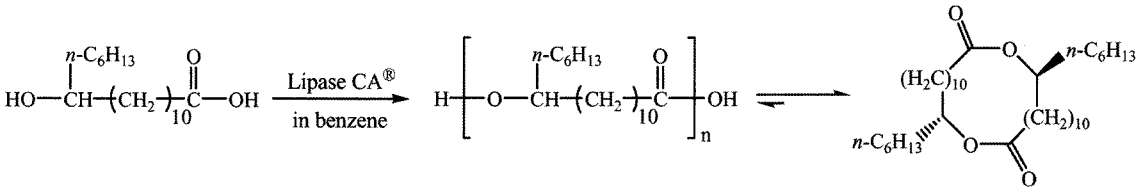
Figure 5. MALDI-TOF mass spectra of the products of the enzymatic polycondensation of 12-HSA (0.4 M) in benzene at 80 °C after (a) 1, (b) 4, and (c) 7 days.

0.20 M in initial monomer concentration, 30 wt% in enzyme-to-substrate ratio, and in refluxing benzene (80 °C). Figure 4 shows the time-dependent changes in the conversion and M_n of the polycondensates. It is noted that the conversion increases with the reaction time to reach almost 100% after 100 h. The M_n value of the resultant polycondensate also increases with the reaction time in the initial stage, but it decreases gradually down to several hundred over 100 h. The polydispersities of the polycondensates M_w/M_n were around 1.50 and 1.12 at 100 h and 168 h in reaction time, respectively. Figure 5 shows the representative MALDI-

TOF mass spectra of the polycondensates obtained at different reaction times. Two series of mass peaks denoted by L_n (linear oligomers with both carboxyl and hydroxyl terminals) and C_n (cyclic oligomers) are detected with a constant mass difference of m/z = 282 which corresponds to the mass of one 12-hydroxystearate unit. The mass difference between L_n and C_n in each set of the mass peaks is m/z=18, corresponding to the mass of one water molecule that has been lost by the intramolecular dehydration. It is therefore deduced that the linear P(12HS) are cyclized into their cyclic ester derivatives during the polycondensation. In the initial stage (1 day) of the reaction (Figure 5(a)), several peaks of linear oligomers are mainly shown with a small peak of a cyclic oligomer C_2. After 4 days (Figure 5(b)), in return, the peaks of cyclic oligomers with degrees of polymerization (DP) below 5 appear together with those of linear oligomers with DP up to 10. After 7 days (Figure 5(c)), a strong peak due to the cyclic dimer (C_2) is noted, telling that the final product of this enzymatic reaction is to be C_2, or 12-HSAD consisting of a 26-membered ring involving two ester groups. Actually, recrystallization of the final product gave highly pure crystals of 12-HSAD in high recovery (83%).

The above results reveal that the P(12HS) oligomers are first formed by the enzymatic polycondensation of 12-HSA and that these oligomers are allowed to depolymerize into 12-HSAD. When the monomer conversion reaches almost 100%, the cyclization by back-biting predominates over the chain extension as shown in Scheme I.

Isolation of 12-HSAD. 12-HSAD was readily isolated by recrystallization from ethanol. It is a white crystalline compound melting at 66 °C. Table I summarizes the results of synthesis of 12-HSAD with Lipase CA® at different conditions. The yield of 12-HSAD became the highest when the monomer concentration was 0.4 M (Entry 1-4). At the higher concentrations, the yield decreased because of the favored intermolecular reaction rather than the intramolecular reaction. The optimum amount of Lipase CA® was 30 wt% relative to 12-HAS (Entry 5-8). When the amount of Lipase CA® was 10 wt%, little12-HSAD was formed. In this case the monomer conversion did not reach 50% even after 240 h as mentioned above (Figure 2), and the unreacted 12-HAS and its low oligomers retarded the cyclization. When the amount of Lipase CA® was 40 wt%, the yield of 12-HSAD decreased significantly. At 110 °C in toluene, the yield of 12-HSAD was lower, while at its refluxing conditions under a reduced pressure the yield became comparable with that obtained



12-HSA P(12HS) [n = 2-10] 12-HSAD

Scheme I. Formation of 12-HSAD via oligomeric P(12HS).

Table I. Synthesis of 12-HSAD at Various Reaction Conditions

Entry	Monomer Conc. (M)	Enzyme (wt%)	Solvent	Time (h)	Temp. (°C)	Yield (%)[a]
1	0.2	30	benzene	168	80	40
2	0.4	30	benzene	168	80	72
3	0.8	30	benzene	168	80	50
4	2.0	30	benzene	168	80	43
5	0.2	10	benzene	240	80	1
6	0.2	20	benzene	240	80	45
7	0.2	30	benzene	240	80	55
8	0.2	40	benzene	240	80	40
9	0.4	30	benzene	168	65 (490 mmHg)	27
10	0.4	30	toluene	168	65 (90 mmHg)	54
11	0.4	30	toluene	168	110	23
12	0.4	30	$CHCl_3$	168	60	0

[a]After recrystallization from ethanol.

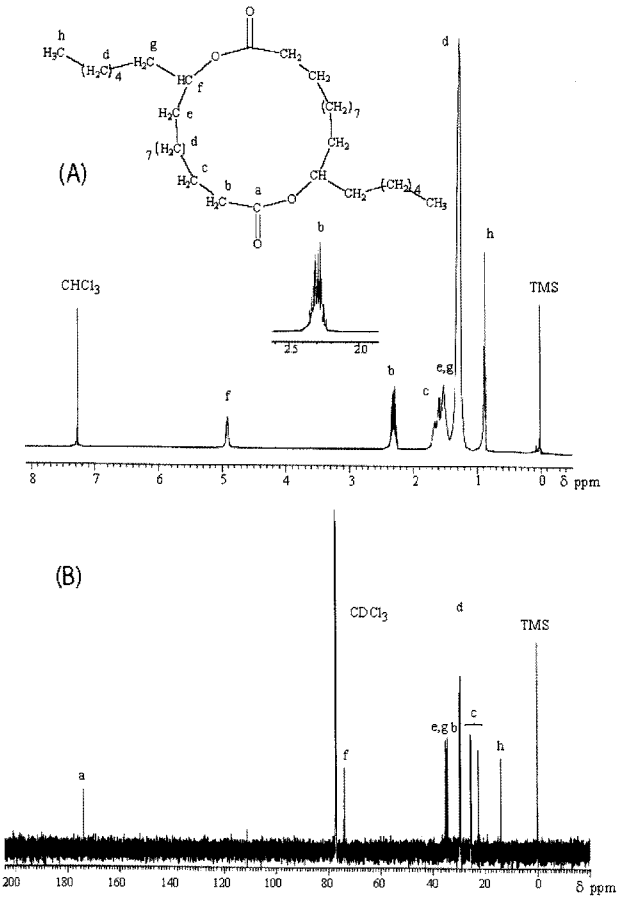
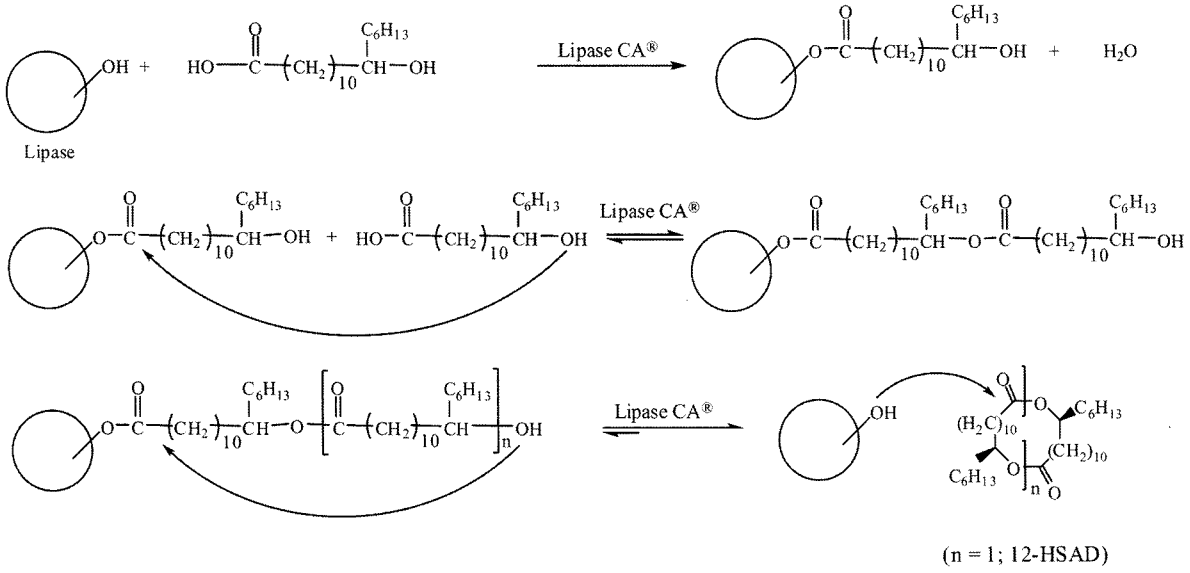


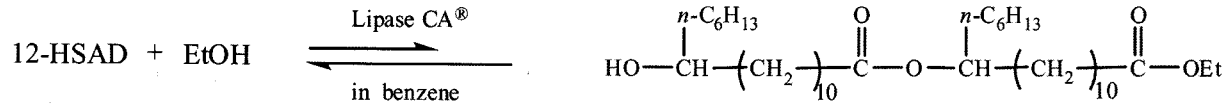
Figure 6. (A) 500 MHz ^{1}H and (B) 125 MHz ^{13}C NMR spectra of 12-HSAD ($CDCl_3$).

with benzene (Entry 9-12). Although Lipase CA® is a robust enzyme with a maximum activity in the temperature range of 60-80 °C, 110 °C is too high. With refluxing benzene at reduced pressure, the yield was not so high as that with toluene. With chloroform as the solvent, no reaction occurred, probably because of the unfavorable solvation effect of chloroform. Figure 6 shows the 500 MHz ^{1}H and 125 MHz ^{13}C NMR spectra of 12-HSAD. The appearance of the carbonyl-methylene protons as a multiplet at δ2.27 ppm (b) is characteristic of the cyclic structure of 12-HSAD, because the corresponding methylene signal for P(12HS) is shown as a triplet. The observation of a single carbonyl signal in the ^{13}C NMR spectrum also supports the symmetric cyclic structure. The IR spectrum of 12-HSAD (data not shown) exhibited an ester carbonyl vibration at 1730 cm^{-1}, being 4 cm^{-1} higher in wavenumber than that of the carbonyl stretching of 12-HSA.[22] The HRMS in the CI mode showed a parent peak at *m/z* = 565 in accordance with the $[M+1]^+$ of 12-HSAD (MW=564.5117). The ratios of the (M+1) and (M+2) isotopic peaks were 39.6 and 8.6%, respectively, to agree with the theoretical values (40.0 and 8.7%, each). The HRMS of 12-HSAD in the EI mode showed a peak at *m/z* = 197, which is due to a fragmentation into 12-HSAD. All these data well support the structure of 12-HSAD.

Proposed Reaction Mechanism. Lipase CA® is a typical serine-enzyme for ester hydrolysis in which the serine residue acts as the nucleophile to form an acyl adduct in the hydrolysis of ester bond. Therefore, the similar enzyme-acyl bond ought to be formed between the serine residue and the carboxyl group of 12-HSA in the first step of the polycondensation, which is a reverse reaction of the hydrolysis. In the polycondensation, the acyl group bound to the enzyme is intermolecularly transferred to the terminal hydroxyl groups of the oligomeric P(12HS) to form the polyester bonds, while it is intramolecularly transferred by back-biting mechanism to form the cyclics. Scheme II shows the whole pro-



Scheme II. Ring/chain equilibria in the enzymatic reaction of 12-HSA to form 12-HSAD as the final product.



Scheme III. Transesterification of 12-HSAD with ethanol.

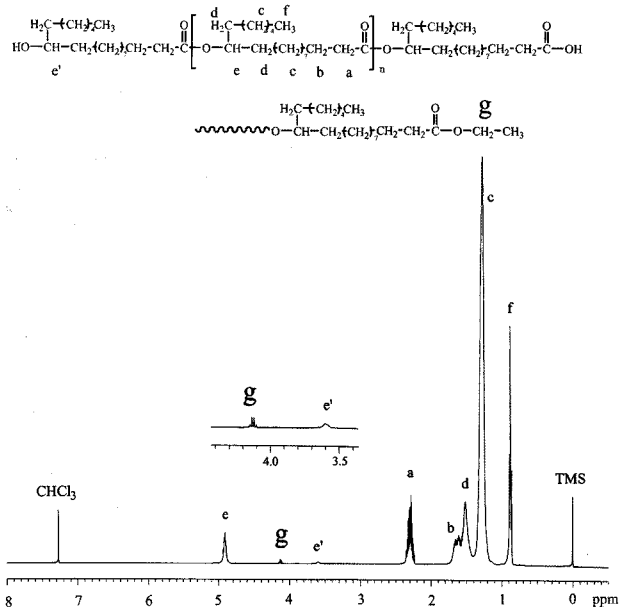


Figure 7. A typical ^{1}H NMR spectrum of the product of the Lipase CA®-catalyzed reaction of 12-HSAD (0.2 M) and ethanol (0.2 M) in benzene at 80 °C for 96 h.

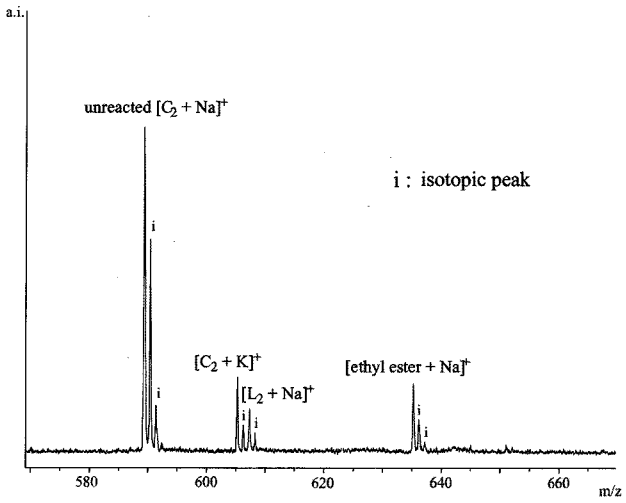


Figure 8. Molecular ion peaks from the MALDI-TOF mass spectrum of the products in the Lipase CA®-catalyzed reaction of 12-HSAD (0.2 M) and ethanol (0.2 M) in benzene at 80 °C for 96 h.

cess of this enzymatic polycondensation where the interconversions from the respective linear oligomers to the corresponding cyclic oligomers should be reversible. These reversible systems are allowed to decline to the formation of the structurally stable cyclic dimer in the final stage. As a consequence, 12-HSAD is concentrated in the system. To confirm the reversible enzymatic reactions, 12-HSAD (0.2 M) was reacted with an equimolar amount of ethanol in the presence of Lipase CA® (30 wt%) in refluxing benzene for 96 h (Scheme III). Figure 7 shows a ^{1}H NMR spectrum of the reaction products obtained after the filtration of Lipase CA®. The quartet signal at $\delta = 4.15$ ppm (g) is reasonably assigned to the ethyl groups introduced as ester while the signal at $\delta = 3.58$ ppm (e') is to the hydroxyl terminated methyne protons. Without Lipase CA®, such a spectrum change was not observed at all. Figure 8 shows a MALDI-TOF mass spectrum of the product. In addition to the molecular ion peaks of 12-HSAD (C_2), i.e., $[C_2 + Na]^+$ and $[C_2 + K]^+$, those of the ring-opened forms (L_2-ester) are shown as $[L_2 + Na]^+$ and $[L_2\text{-ester} + Na]^+$. Detection of the small peaks due to L_2 reveals that hydrolysis of 12-HSAD occurred together with ester-interchange reaction with ethanol. It is therefore confirmed that Lipase CA® can bind 12-HSAD as the sub-

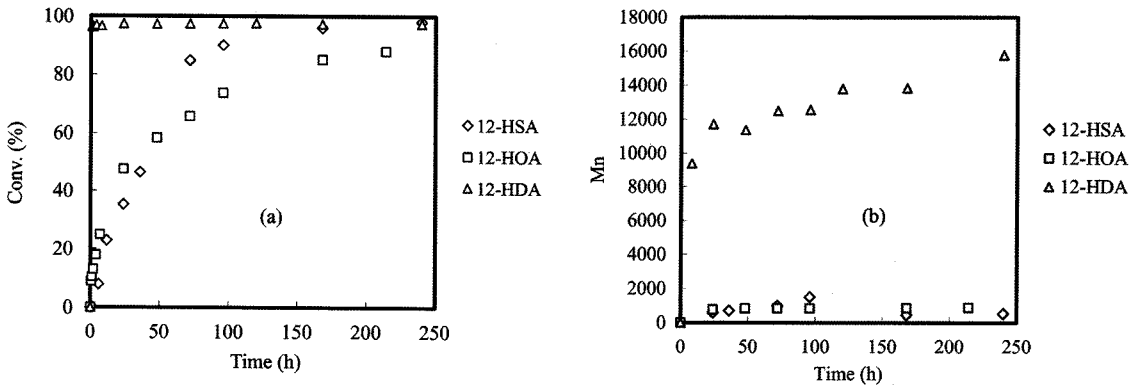


Figure 9. Time-courses of the (a) conversion and (b) M_n in the enzymatic polymerization of 12-HSA, 12-HOA, and 12-HDA (0.4 M, each) using 30 wt% Lipase CA® in benzene.

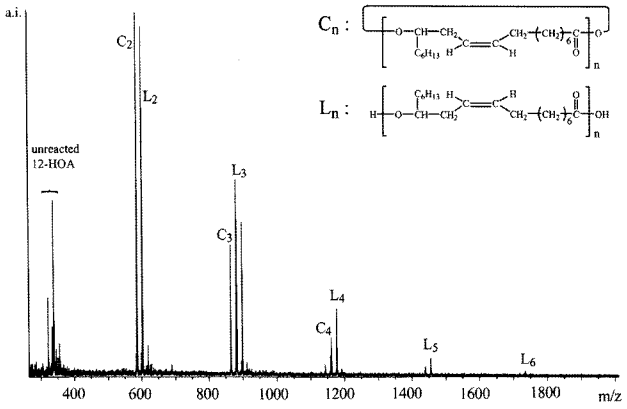


Figure 10. MALDI-TOF mass spectrum of the products in the polycondensation of 12-HOA (0.2 M) in benzene at 80 °C for 9 days.

strate to catalyze the transesterification where the enzyme-acyl intermediate is formed from 12-HSAD, transferring the acyl group to ethanol.

Enzymatic Reaction of Similar Substrates. Similar polycondensations were examined using 12-HDA and 12-HOA. Figure 9 compares the changes in (a) monomer conversion and (b) M_n of their polycondensates as a function of reaction time for the three different monomers, 12-HSA, 12-HOA, and 12-HDA. In the case of 12-HDA, the monomer conversion reached almost 100% within 1 h with predominant polymerization. This is because 12-HDA doesn't have a bulky *n*-hexyl group, and the ring-chain equilibrium ought to decline in the side of polymer formation to produce a high-molecular-weight polyester. In the reaction of 12-HOA, the monomer conversion and M_n were even lower than those observed for 12-HSA. Figure 10 shows a MALDI-TOF mass spectrum of the product isolated from the reaction system where 12-HOA was treated with 30 wt% of Lipase CA® in benzene for 9 days. Two series of mass peaks L_n and C_n are shown with a constant mass difference of m/z=280. It is therefore deduced that the linear oligomers are allowed to cyclize into their cyclic derivatives during the enzymatic polycondensation as observed for 12-HSA. However, the cyclic diester of 12-HOA could not be isolated in discrete form, probably because of its low yield.

Conclusions

12-HSA was polymerized into P(12HS) by the action of Lipase CA®. When this reaction was prolonged, P(12HS) was depolymerized into a cyclic dimer, 12-HSAD. The similar Lipase CA®-catalyzed polymerization of 12-HOA gave its linear and cyclic oligomers as 12-HSA while that of 12-HDA gave its polymer preferentially. The ring-chain equilibrium is allowed to decline in the direction of ring formation in the presence of the bulky *n*-hexyl side groups in the cases of 12-HAS and 12-HDA.

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