Lipase-Catalyzed Synthesis and Characterization of Biodegradable Polyester Containing L-Malic Acid Unit in Solvent System

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ABSTRACT: Lipase-catalyzed direct polycondensation of L-malic acid (L-MA), adipic acid, and 1,8-octanediol in organic media was achieved using Novozym 435 as the biocatalyst. ^{1}H-nuclear magnetic resonance spectroscopy indicated that the selectivity of Novozym 435 was unaffected by changes in the organic media. The molecular weight (M_w) of the copolymers was affected by the L-MA feed ratio in the diacids, hydrophobicity of the solvent, and solubility of the substrates in the solvents. The M_w reached a maximum of 17.4 kDa at 80°C in isooctane at a L-MA feed ratio in the diacids of 40 mol %. The M_w increased from 3.2 to 16.6 kDa when the reaction time was extended from 6 to 48 hr at 70°C, and remained relatively constant with further increases in reaction time from 48 to 72 hr. The hydrophilicity, thermal stability, and crystallizability of the copolymer were also investigated. © 2010 Wiley Periodicals, Inc. J Appl Polym Sci 120: 1114–1120, 2011

Key words: polyesters; biodegradable; polycondensation; enzymes

INTRODUCTION

As one of the most important classes of synthetic biomaterials, many aliphatic polyesters have good biocompatibility and biodegradability and have attracted increasing attention in biomedical fields, such as tissue engineering, surgical sutures, gene therapy, and controlled drug delivery.[1] However, the applications of conventional aliphatic polyesters are limited because of their hydrophobicity and absence of functional groups that could be used to tailor the physical properties and introduce bioactive substances. Compared with conventional aliphatic polyesters, poly(malic acid) and polyesters containing L-malic acid (L-MA) units contain many pendant carboxyl- or hydroxyl-functional groups along the macromolecular chains and have attracted considerable attention.[2–6] The chemical properties of copolymers, such as their hydrophobic/hydrophilic balance,[7,8] degradation rate,[9–11] modifiability, etc.,[12,13] can be adjusted for different applications[14–16] by changing the L-MA content or varying the chemical structures of the pendant groups.

There are many reports on chemical routes to synthesize functional polyesters containing L-MA units by ring-opening polymerization (ROP) and polycondensation.[17,18] Amphiphilic poly[(R,S)-β-malic acid-*b*-ε-caprolactone] diblock copolymers were synthesized by the ROP of (R,S)-β-benzyl malolactonate with ε-caprolactone, followed by removing the benzyl ester protecting group by catalytic hydrogenolysis.[7,8] Wang and coworkers[9,10] synthesized a functional polylactide copolymer with carboxyl acid side chains using a similar method. Zhang et al.[18] synthesized poly(butylenes

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succinate-*co*-butylene malate) with free hydroxyl pendant groups by the polycondensation of dimethyl malate, 1,4-butanediol, and succinic acid. The entire process involves four steps, including protection and deprotection steps of hydroxyl of dimethyl malate and repeated purification of intermediates.

Common difficulties encountered during the synthesis of functional polyesters containing L-MA using conventional methods include the following: (1) protection–deprotection steps lead to a lower yield, (2) repeated purification of intermediate consumes a large amount of organic solvents, resulting in environmental problems, (3) difficulty in achieving complete deprotection of the functional groups, and (4) toxicity of the residual organometallic compounds used as polymerization catalysts.

In vitro enzyme-catalyzed synthesis of polyesters is a new and eco-friendly process.[19–22] Functional polyesters with different functional pendant groups can be synthesized simply by enzyme-catalyzed synthesis because of its high selectivity.[23–25] The difficulties encountered in synthesizing functional polyesters containing L-MA by conventional methods can be resolved by enzyme-catalyzed synthesis. Previous work[26,27] investigated the lipase-catalyzed direct polycondensation of L-MA, adipic acid (ADA), and 1,8-octanediol (OC) for preparing polyesters containing L-MA units in a solvent-free system. However, for enzyme catalysis in nonaqueous medium, organic solvents play an important role.[19] The selectivity, catalytic activity, and stability of enzyme can be altered by proper selection of the organic solvent. The solvent also can regulate the partitioning of substrates and products between the solvent and the enzyme.[19] Therefore, this study examined the effect of different reaction conditions on the lipase-catalyzed direct polycondensation of comonomers L-MA, ADA, and OC in a solvent system.

EXPERIMENTAL

Materials

OC (98% purity) was purchased from Aldrich Chemical Co. (St. Louis, MO). L-MA (98% purity) was obtained from Guangzhou Huangkai Co. (Guangzhou, China). Isooctane, *n*-hexane, toluene, chloroform, *t*-butanol, tetrahydrofuran (THF), acetone, and ADA (Guangzhou Chemical Co.) were of analytical grade. All chemicals were used as received. Novozym 435 (immobilized lipase from *Candida antarctica*, type B, 10,000 U g^{-1}) was purchased from Novozymes (Denmark).

Enzyme-catalyzed polymerizations of L-MA with OC

OC (2.193 g, 15 mmol) was mixed with a mixture of total of 15 mmol of L-MA and ADA in a 100-mL round-bottom flask. The flask was capped with a rubber septum and placed into an oil bath maintained at 120°C for 3 hr with magnetic stirring. The temperature of the oil bath was then decreased to the prescribed temperature (90–50°C), and 4-Å molecular sieves (to absorb the water producing in the reaction), different organic solvents, and Novozym 435 (10% by weight based on total monomers, dried in vacuum desiccator under 10 mmHg at 25°C for 24 hr) were added to the flasks. The flask was sealed, and the magnetic stirring rate was set to 200 rpm. The reaction was quenched by adding an excess of chloroform with constant stirring for 15 min after 48 hr. The Novozym 435 and 4-Å molecular sieves were removed by filtration. The filtrate was added slowly into a flask containing an excess of cold *n*-hexane with rapid stirring, and the resulting product was isolated by centrifuging. The product was dried in vacuum at 50°C for 48 hr. The control synthesis was done without lipase in different organic solvents (the feed molar ratio is OC : ADA : L-MA = 15 : 9 : 6), and the results are shown in Table I.

TABLE I
Effect of Solvents and Temperature on the M_w and M_w/M_n without Lipase

Entry	Organic solvent	T (°C)	M_w ($\times 10^3$)	PDI
1	Isooctane	60	1.3	1.17
2	*n*-Hexane	60	1.5	1.19
3	Toluene	60	1.6	1.21
4	Toluene	70	1.9	1.29
5	Toluene	80	2.0	1.24
6	Chloroform	60	0.8	1.07
7	*t*-BuOH	60	0.9	1.10
8	THF	60	1.1	1.15
9	Acetone	60	0.8	1.04

The feed molar ratio was OC : ADA : L-MA =15 : 9 : 6. The reaction time was 48 hr. All the procedures are same with the procedure for the enzyme-catalyzed copolymerization described in the article. PDI, polydispersity index of the polymer.

Characterization

Structural characterization

The structures of the prepared products were characterized by nuclear magnetic resonance (NMR) spectroscopy. The ^{1}H-NMR spectra were recorded on a Bruker AV 300 spectrometer operating at 300.1 MHz. The polyesters were dissolved in deuterated chloroform. Tetramethylsilane was used as the internal standard ($\delta = 0.00$ ppm).

Molecular weight measurements

The molecular weight and polydispersity were determined by gel permeation chromatography using the basis of conventional calibration curve

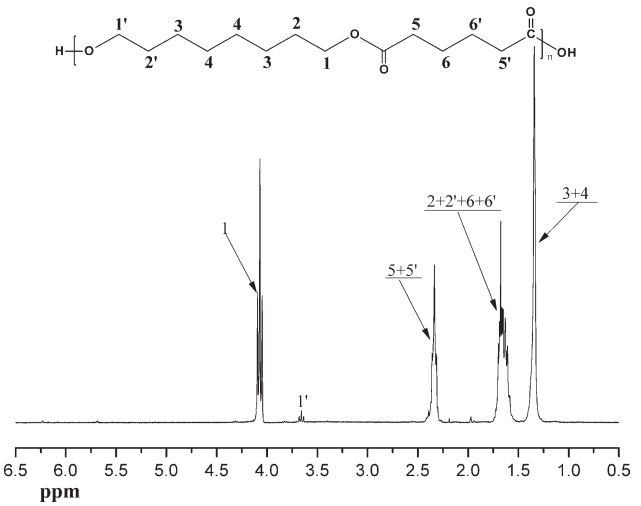

Figure 1 ^{1}H-NMR spectrum of poly(octylene adipate).

generated by narrow molecular weight polystyrene standards. Gel permeation chromatography analyses were performed at 35°C using a Shimadzu LC-10 HPLC System equipped with a LC-10AD pump, a SIL-10A auto sampler, a differential refractometer (RI detector), and a series of Agilent columns (three PLgel 5 μm MIXED-D and -E columns). THF was used as the eluent at a flow rate of 1.0 mL/min.

Thermal analysis

Differential scanning calorimetry (DSC) analyses were carried out using a DSC 204 F1 differential scanning calorimeter from Netzsch Instruments. The samples (8–14 mg) were heated from −70 to +100°C at a rate of 10°C/min with a nitrogen purge. Thermogravimetric analysis was carried out using a TG 209 thermogravimetric analyzer from Netzsch Instruments. The samples (12–16 mg) were heated from ambient temperature to 800°C at a rate of 20°C/min with a nitrogen purge.

Contact angle measurement

The contact angles were measured to determine the hydrophilicity of the copolymers at 20°C using the sessile drop method on a contact-angle measurement apparatus (JY-82; Harke Co., St. Charles, MO). The static contact angle was measured at a contact time of $t = 30$ sec. Drops of liquid were prepared using a microsyringe and dropped onto the surface of the polymer films.

RESULTS AND DISCUSSION

Organic solvents play an important role on enzyme catalysis in nonaqueous medium. The organic solvents change the molecular conformation of enzyme and regulate the partitioning of substrates and products between the solvent and the enzyme. Thus, the selectivity, catalytic activity, and stability of enzyme are altered by the organic solvents.

Structural characterization

Figure 1 shows the ^{1}H-NMR spectra of poly(octylene adipate), and Figure 2 shows the ^{1}H-NMR spectra of copolyesters containing the L-MA units synthesized in different organic media. The resolved signals for the OC units protons C**H$_2$**—O(C=O), **1**, and C**H$_2$**—OH, **1′**, were observed at 4.07 and 3.65 ppm from the ^{1}H-NMR spectrum of poly(octylene adipate), respectively. The signals attributed to the methylene protons C**H$_2$**—(C=O), 5, and C**H$_2$**—(C=OOH), **5′**, of the ADA units were not resolved and appear as a multiplet from 2.25 to 2.45 ppm. The signals for the protons C**H$_2$**CH$_2$—O(C=O), **2**, and C**H$_2$**CH$_2$—OH, **2′**, of the OC units, and ADA units protons C**H$_2$**CH$_2$—(C=O), **6**, and C**H$_2$**CH$_2$—(C=O)OH, **6′**, all appear as a broad multiplet from 1.57 to 1.78 ppm. Similarly, the signals corresponding to the methylene protons, C**H$_2$**CH$_2$CH$_2$—O(C=O), **3**, and C**H$_2$**CH$_2$CH$_2$CH$_2$—O(C=O), **4**, of the OC units were unresolved and observed between 1.24 and 1.45 ppm.[23,26,27] The ^{1}H-NMR spectra of the copolyesters containing the L-MA units synthesized in different solvents (Fig. 2) showed signals corresponding to the L-MA units protons C**H**OH(C=O), **7**, and C**H$_2$**(C=O), **8**, at 4.5 ∼ 4.6 and 2.7 ∼ 2.9 ppm.[18,26,27] There was no reaction between the hydroxyl groups of L-MA and the carboxyl groups of ADA or L-MA according to ^{1}H-NMR. The MA units were incorporated into the macromolecular chains exclusively through their carboxyl groups. The copolyesters obtained in different solvents were poly(octylene

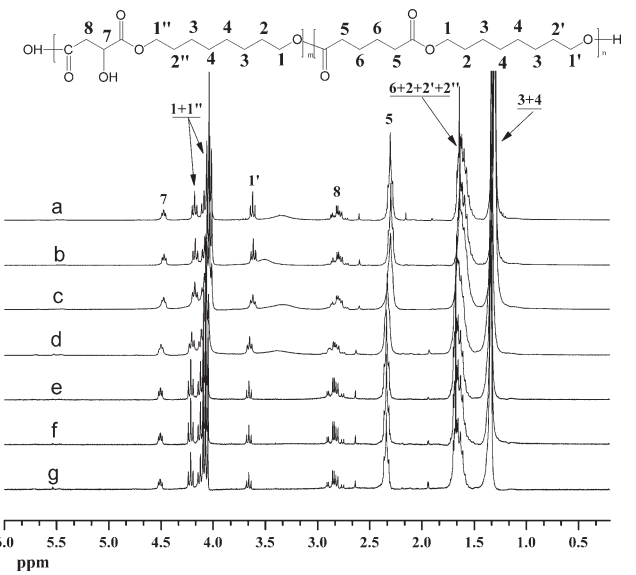

Figure 2 ^{1}H-NMR spectra of poly(octylene adipate-*co*-octylene malate) synthesized in different solvents: (a) acetone, (b) chloroform, (c) THF, (d) *t*-BuOH, (e) toluene, (f) *n*-hexane, and (g) isooctane.

adipate-*co*-octylene malate). The copolymers produced by Novozym 435-catalyzed direct polycondensation showed no branching through the esterification of L-MA hydroxyl groups. Instead, the copolyesters containing L-MA units contained pendant hydroxyl groups that were available for postproduct modification. The selectivity of Novozym 435 was not affected by the organic media.

Effects of solvents

The reaction medium is one of the most important factors in nonaqueous enzyme catalysis. The enzyme-catalyzed reaction can be manipulated in a nonaqueous phase by prudently choosing the appropriate solvent.[19,28–30] According to reports on the lipase-catalyzed polyesters by ROP and polycondensation,[28–30] the molecular weights of the polymer and conversion of the monomer in hydrophilic organic solvents were lower than those in hydrophobic solvents. Enzymatic polymerization was carried out in seven solvents with different hydrophobicity (expressed as log *P*, in which *P* was the partition coefficient between 1-octanol and water) to determine the effects of the organic solvents. The prepolymer and the copolymer all dissolve in *t*-BuOH, THF, acetone, chloroform, and toluene, and are insoluble in hexane and isooctane. The effect of different solvents on the molecular weight and polydispersity index without using lipase is summarized in Table I. As shown in Figure 3, using hydrophilic solvents (log $P < 2$, including *t*-BuOH, THF, and acetone) resulted in lower molecular weights compared with hydrophobic solvents (log $P > 2$, including toluene, hexane, and isooctane), which was in accordance with the results of the lipase-catalyzed ROP of ε-caprolactone. This is attributed to the deactivation of enzyme in hydrophilic media because of enzyme conformational changes. Compared with hydrophobic solvents, hydrophilic solvents tend to strip the essential hydration water from the enzyme, and result in the distortion of catalytic conformation and losing its catalytic activity partially or even completely.

However, the effects of highly hydrophobic solvents on the enzymatic polymerization are more complex compared with the lipase-catalyzed ROP of ε-caprolactone. Toluene, hexane, and isooctane are all benign media if the L-MA content in the diacids in the polymerization system is low [Fig. 3(a)]. If the L-MA content is high [Fig. 3(b)], hexane and isooctane are still benign solvents, but toluene is not. This could be due to the variation of hydrophilicity of the substrates (including prepolymer and copolymer) with the variation of monomers ratio. The substrates are amphiphilic and soluble in toluene, and their hydrophilicity increases with increasing L-MA content. The molecular chains of substrates can easily form aggregates in toluene-containing hydrophilic

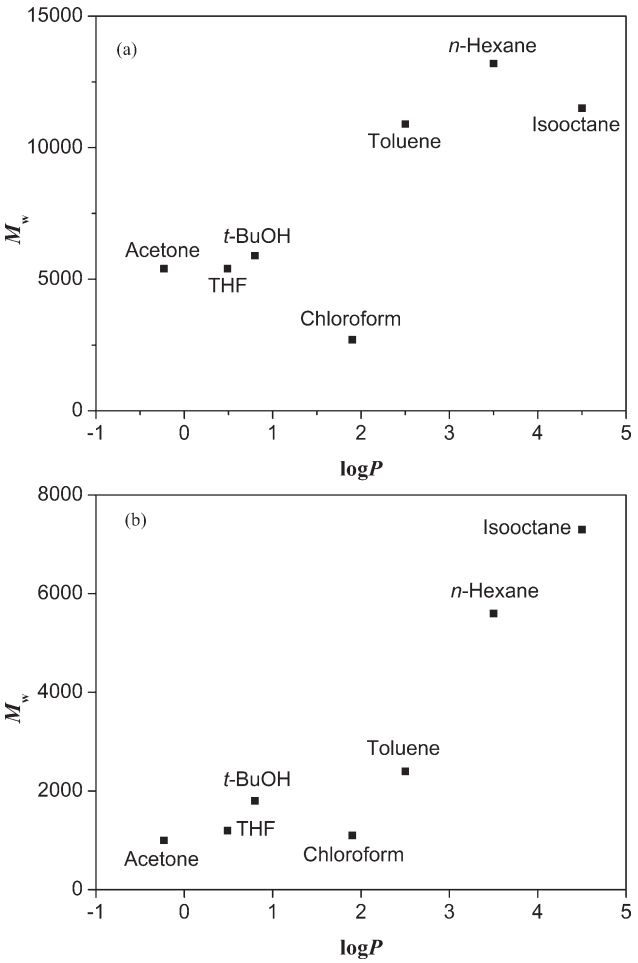

Figure 3 Effects of solvents on M_w for low content (a) and high content (b) of L-MA. The diacids feed molar ratios (ADA : L-MA) were 6 : 4 for (a) and 0 : 10 for (b). For all experiments, the reaction time was 48 hr, and the reaction temperature was 60°C.

cores of hydroxyl and carboxyl groups surrounded by hydrophobic molecular chains. The reactive terminal groups of the substrates were embedded in hydrophilic cores and show poor reactivity. This results in a lowering of the molecular weights of the copolymer at a higher L-MA content in toluene. On the other hand, the molecular weight of copolymer is higher in isooctane and *n*-hexane because the substrates are insoluble in these two solvents.

Effects of the L-MA content

Table II lists the molecular weight (M_w) and its distribution (M_w/M_n) of copolymers prepared by Novozym 435-catalyzed polycondensation with various L-MA contents in the diacids. The M_w of the copolymer decreased with increasing L-MA content in the diacids from 0 to 60 mol % and remained relatively constant with further increases in concentration. For example, M_w decreased from 24,200 to 11,500 when the L-MA content in the diacids was increased from

TABLE II
The Content of L-MA versus the Enzymatic Polycondensation

		Feed molar ratio	Molar ratio in polymer[a]			
Entry	Sample	OC : ADA : L-MA	ADA : L-MA	Yield (%)	M_w ($\times 10^3$)	M_w/M_n
1	B1	100 : 100 : 0	100 : 0	97	24.2	2.30
2	B2	100 : 90 : 10	91 : 9	94	21.5	2.10
3	B3	100 : 80 : 20	79 : 21	95	19.8	2.01
4	B4	100 : 70 : 30	73 : 27	92	17.2	2.50
5	B5	100 : 60 : 40	58 : 42	91	16.6	2.42
6	B6	100 : 40 : 60	42 : 58	89	11.5	2.29
7	B7	100 : 20 : 80	23 : 77	87	11.2	2.25
8	B8	100 : 0 : 100	0 : 100	90	10.6	2.18

The organic medium was isooctane, and the isooctane (volume, mL) to total monomer ratio (weight, g) was 2 : 1. The reaction temperature was 70°C.
[a] Determined by ^{1}H-NMR.

0 to 60 mol %, and changed from 11,500 to 10,600 with further increases in the L-MA content from 60 to 100 mol %. This suggests that the carboxylic groups of L-MA have lower activity than that of ADA. This is due to the higher hydrophilicity of L-MA, which makes it difficult to diffuse into the hydrophobic active sites of the enzyme and form a transition complex.[31,32] In addition, copolymers with free hydroxyl pendants can form hydrogen bonds, and the energy of hydrogen bond of the copolymer increases with increasing L-MA content in the copolymers. This results in a higher viscous reaction system and decreases the diffusion rate of the acyl donor to the hydrophobic active sites of the enzyme. This could restrict the molecular weight of the copolymers.[33,34]

Compared with the solvent-free system, M_w in isooctane is higher.[26,27] This can be explained by the strong negative effect of L-MA on the stability of Novozym 435.[32,35] Novozym 435 had higher stability and activity in isooctane system than those in solvent-free system.

Effects of the reaction temperature and time

Figures 4 and 5 show the effects of reaction temperature and time on the lipase-catalyzed polymerization, respectively. As shown in Figure 4, the rule of M_w changing with reaction temperature in isooctane is similar to that in a solvent-free system.[27] However, the M_w in isooctane was higher than that in the solvent-free system. As shown in Figure 4, the M_w of the copolymers increased from 8600 to 17,400, with increasing temperature from 50°C to 80°C. However, further increases in reaction temperature from 80°C to 90°C led to decrease in M_w from 17,400 to 12,800. The viscosity of the reaction system decreased with increasing temperature, which resulted in decreasing diffusion constraints existing in the lipase-catalyzed polymerization reaction.[33] Consequently, the M_w of the copolymer is increased with increasing temperature. On the other hand, lipase can be denatured, and it will partially or even completely lose its catalytic activity at higher temperature, which can result in the corresponding decrease in M_w. The change in the molecular weight distribution M_w/M_n

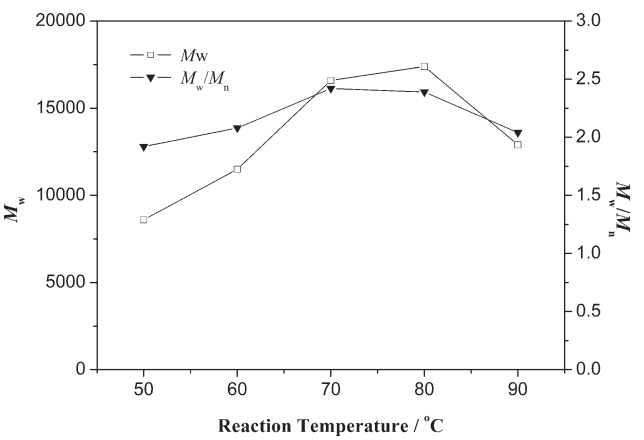

Figure 4 M_w and M_w/M_n versus reaction temperature in isooctane solvent. The diacids feed molar ratios were ADA : L-MA = 6 : 4, and the reaction time was 48 hr.

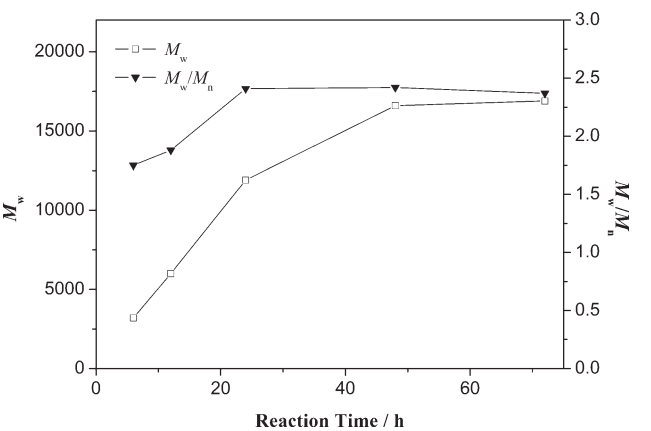

Figure 5 M_w and M_w/M_n versus reaction time in isooctane. The diacids feed molar ratios were ADA : L-MA = 6 : 4, and the reaction temperature was 70°C.

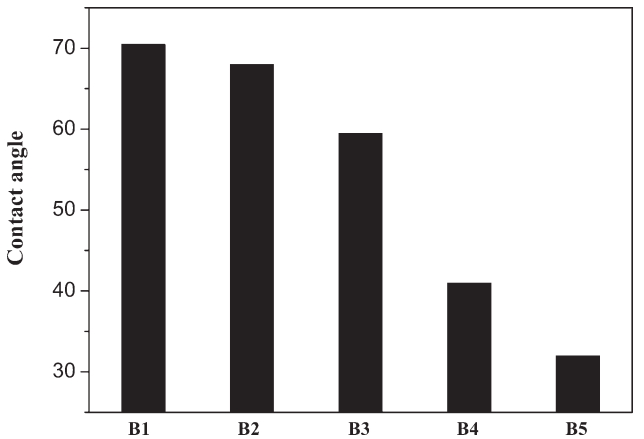

Figure 6 The contact angle of the copolymers with different L-MA content.

(polydispersity index) of the copolymers with temperature is similar to the change in M_w.

As shown in Figure 5, the M_w of the copolymers increased from 3200 to 11,900 and 16,600 with increasing reaction time from 6 to 24 hr and 48 hr. The M_w changed little when the reaction time was increased from 48 to 72 hr. This may be due to a side reaction, such as hydrolysis, enzyme specificity with the chain length, and deactivation.[19] The concentration of ester bonds increased with increasing molecular weight of the copolymers. At the same time, the concentration of reactive hydroxyl and carboxyl terminal groups decreased. The hydrolysis of ester bonds increased and esterification decreased accordingly. These would limit the increase in the molecular weight of the copolymers. Moreover, unlike the common chemical esterifying catalyst, the molecular chain length had an enormous effect on lipase-catalyzed polymerization. In other words, the lipase reacts at different rates according to the substrate chain length.[22] The enzyme has higher activity for short- and medium-chain substrates, with less

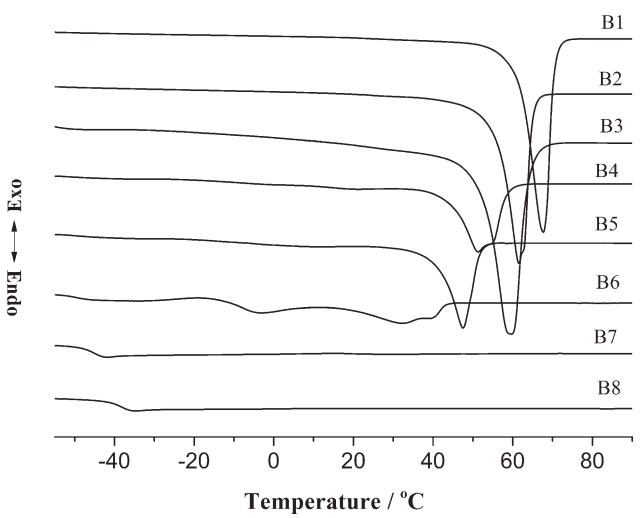

Figure 7 DSC melting endotherms of the copolymers with different L-MA content.

TABLE III
Thermal Properties Obtained from DSC Analyses

Sample	T_m (°C)	ΔH_f (J/g)
B1	69.3	111.8
B2	63.8	107.5
B3	61.8	77.8
B4	53.5	62.7
B5	48.3	53.6
B6	32.4	32.5
B7	–	–
B8	–	–

steric hindrance than for long-chain substrates because of the specific structure of the enzyme binding site.[31] As a type of protein, lipase can be denatured with prolongation of reaction time, which would also limit the increase in molecular weight of the copolymers.

Contact angle

Figure 6 shows the relationship between the static water contact angle and the L-MA content in the monomer feed ratios. The contact angle decreased with increasing L-MA content. For example, a decrease in contact angle from 70.5° to 32° was observed when the L-MA content in the diacids was increased from 0 to 40 mol %, suggesting that the hydrophilicity of the copolymer increases with increasing L-MA content. This is due to the increase in the density of the pendant hydroxyl groups in copolymers with increasing L-MA content. The results show that the hydrophobic/hydrophilic balance can be adjusted for different applications by varying the L-MA ratios in the monomer feed.

Thermal properties

Figure 7 shows the DSC curves of the samples from B1 to B8. Table III summarizes the results of DSC analyses. The melting point (T_m) and melting fusion (ΔH_f) of the polymers decreased with increasing L-MA content in the monomer feed, and the melting range of the polymers become broader. Beyond 60 mol % L-MA in the diacids, no melting transition peaks appeared in polymers. This indicates that the crystallinity of the polymer decreases with increasing L-MA content in the copolymers and forms amorphous polymers as the molar ratios of L-MA in the diacids exceeds 60 mol %. Among the polymers, the pure homopolymer B1 [poly(octylene adipate)] had the highest ability to crystallize, and A8 [poly (octylene malate)[could not crystallize. By increasing the L-MA content in the copolymers, the crystal structure of copolymers was disrupted and the average length of the crystallizable sequence was shortened. All these contributed to a decrease in melting temperature (T_m) and a broader melting range. Accordingly, melting fusion (ΔH_f) decreased from 111.8 to 32.5 J/g with increasing molar ratio of L-MA

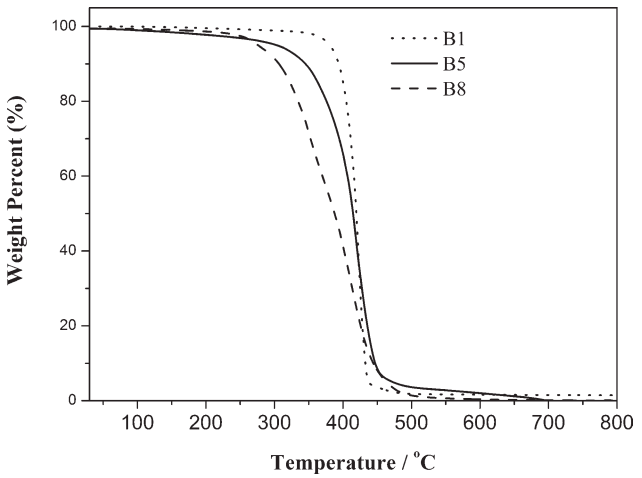

Figure 8 Thermogravimetric analysis curves of the copolymers with different L-MA content.

in the diacids feed from 0 to 60 mol %. However, when the molar ratios of L-MA in the diacids exceeded 60 mol %, the average length of the crystallizable sequence [poly(octylene adipate)] was so short that it could not form a crystal.

Figure 8 shows the TG thermograms of B1, B5, and B8. The thermal stability of the polymer decreased with increasing L-MA content in the polymer. The temperatures at which 5% weight losses of B1, B5, and B8 occurred were 380.8°C, 302.8°C, and 278.1°C, respectively. This might be due to the lower thermal stability of the L-MA units in the copolymers. The L-MA units can be decomposed easily by intramolecular dehydration at high temperatures and form a fumaric acid structure.[36]

CONCLUSION

Linear copolymers with L-MA repeating units along the chain providing a hydroxyl group were prepared using a simple one-pot biocatalytic route. The selectivity of Novozym 435 led to the exclusive esterification of the L-MA carboxylic groups while leaving the hydroxyl groups unreacted. In contrast to conventional methods of synthesizing linear copolymers containing L-MA units, the high selectivity of this biocatalytic process avoided the need for a series of complicated protection–deprotection steps. The hydroxyl groups of the functional copolymers can be converted to many other functional copolymers via simple chemical transformations or directly by conjugation bioactive molecules. The selectivity of Novozym 435 was not affected by the organic solvents used. The effects of the organic solvent on polymerization were more complex compared with the lipase-catalyzed polymerization of hydrophobic polyesters. The hydrophobicity of the solvents and the solubility of the substrates (including oligomers and copolymers) in the solvents have significantly influenced the lipase-catalyzed polymerization.

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