Biodegradation **13:** 141–147, 2002.

Degradation of aliphatic polyester films by commercially available lipases with special reference to rapid and complete degradation of poly(L-lactide) film by lipase PL derived from *Alcaligenes sp.*

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Accepted 17 May 2002

Key words: aliphatic polyester, biodegradation, lipase, poly(L-lactide)

Abstract

Commercial lipases were examined for their degradation efficiency of aliphatic polyester films. In 100 days immersion of polyester films in lipase solutions at 37 °C at pH 7.0, Lipase Asahi derived from *Chromobacterium viscosum* degraded polybutylene succinate-*co*-adipate (PBSA), poly (ε-caprolactone) (PCL) and polybutylene succinate (PBS), and Lipase F derived from *Rhizopus niveus* degraded PBSA and PCL during 4–17 days. Lipase F-AP15 derived from *Rhizopus orizae* could degrade PBSA in 22 days. In these cases, PBS and PBSA were mainly degraded to dimers, whereas PCL was mainly degraded to monomers. Only poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHB/V) and poly (L-lactide) (PLA) were not degraded in the experiments. However, PLA degraded completely at 55 °C, pH 8.5 with Lipase PL during 20 days. This result could be explained with the sequential reactions of the chemical hydrolysis of the polymer to oligomers at higher pH and temperature, and the succeeding enzymatic hydrolysis of oligomers to the monomers.

Abbreviations: PBS – polybutylene succinate, PBSA – polybutylene succinate-co-adipate, PCL – poly (ε-caprolactone), PHB/V – poly (3-hydroxybutyrate-co-3-hydroxyvalerate), PHB – poly (3-hydroxybutyrate), PHV – poly (3-hydroxyvalerate), PLA – poly (L-lactide).

Introduction

Along with the increase in the quantity of plastics production, plastic disposal has been regarded as a serious problem, therefore biodegradable polymers have received much attention in recent years as one of the approaches to solve the problem (Doi 1997; Shirai 1999). Simultaneously, investigations of the polyester degradation mechanism have been carried out in soil, in compost and in activated sludge (Sawada 1994; Hoshino et al. 2001). Interestingly, reports about PBS, PBSA and PLA are relatively rare compared with studies on PCL and PHB. Research of biodegradation of PLA seems to be especially inactive, although several types of PLA are commercially available. This is probably due to the low degradation rate of PLA. Several studies concerning PLA degradability of enzymes or microorganisms have been confirmed by clear zone creation and /or little weight loss (Williams 1981; Torres et al. 1996). Only limited reports showed that the complete degradation of PLA in soil takes about 1 year (Shirai 1999), and 60–100 days in compost (Kimura 1999).

We report here the enzymatic degradation of aliphatic polyester films using commercially available lipases. Rapid and complete degradation of PLA by lipase is our ultimate goal in this investigation.

Materials and methods

Polyesters

Five kinds of polyesters were used in the investigations: PCL (Celgreen P-H4, Daicel Chemical Industries, Japan), PLA (LACTY 1012, Shimadzu Corporation, Japan), PBS (BIONOLLE 1003, Showa

Highpolymer Co., Ltd., Japan), PBSA (BIONOLLE 3003, Showa Highpolymer Co., Ltd., Japan), and PHB/V (Biopol, Zeneca K. K., Japan). Physicochemical properties of polyesters used in this study are summarized in Table 1.

Enzymes

Commercial lipases used in this study and their properties are listed in Table 2. They were used in the experiments without further purification.

Lipase-catalyzed degradation of polyesters

The polyester films were cut to approximately 2 cm $\times$ 2 cm, and weighed. The film was then placed in a screw cap vial containing 20 ml of enzyme solution (10.0 g / L of enzyme in 0.1 M McIlvaine buffer (0.1 M citric acid : 0.2 M Na_2HPO_4 = 3.53 : 16.47) adjusted to pH 7.0) and incubated at 37 °C. To prevent contamination, 2.0 g/L of sodium azide was added to the solution. After 100 days incubation the films were gently washed with diluted water and their dry weight was measured. Biodegradability was evaluated as the ratio of the weight loss of film after 100 days reaction to the initial weight of film. In addition the solution, after 100 days of polymer immersion, was filtrated with Samprep-LCR13-LH (Millipore Corp., USA; pore size, 0.5 μm), and then analyzed with gel permeation chromatography (GPC) to detect the molecular weight distribution of water soluble oligomers produced by enzymatic degradation of polymers. GPC analysis was performed on a high performance liquid chromatograph (HPLC) system equipped with Waters M600 multi-solvent delivery system (Waters Corporation, USA) and Waters 410 Differential refractometer (Waters Corporation, USA). Samples were analyzed on two jointed hydrophilic GPC columns TSK gel G2000PW (21.5 mm $\times$ 30 cm; exclusion size, 5 kDa; Tosoh Corporation, Japan) at a flow rate of 0.5 ml/min of water. Pullulan standards (Showa Denko K. K., Japan) were used to generate a molecular weight calibration curve.

Lipase-catalyzed degradation of PLA at higher pH and temperature

PLA film was soaked in 20 mL of boric acid/NaOH buffer (pH 8.5) containing 20.0 g/L of lipase, and incubated at 55 °C with stirring. Biodegradability was determined as the ratio of film weight after incubation to that prior to incubation. GPC analysis to measure the molecular weight of PLA was performed using the same HPLC equipment described above with a hydrophobic GPC column Shodex K-80M (21.5 mm $\times$ 30 cm; exclusion size, 20000 kDa; Showa Denko K. K., Japan) at a flow rate of 1.0 ml/min of chloroform. Polystyrene standards (Waters Corporation, USA) were used to generate a molecular weight calibration curve. The solution, after the 50 days immersion, was filtered with Samprep-LCR13-LH, and then analyzed with HPLC to detect the aqueous soluble products. Samples were analyzed on an ODS column Finepak SIL C18S (Japan Spectroscopic Co., Ltd., Japan) at a flow rate of 1.0 ml/min of 0.01 M phosphate buffer (pH 2.4).

Experimental results

Degradation of polyesters

Table 3 shows the biodegradability of the polyester films after 100 days incubation in lipase solutions. PBSA and PCL films were rapidly degraded during 4–17 days when either Lipase Asahi, Lipase F, or Lipase QL was used. Lipase Asahi could also degrade PBS film within 17 days. Lipase F-AP15 could degrade PBSA after 22 days. In other cases, the polyester films were not out of shape. The biodegradability of PBSA when either Lipase AY30 or Lipase M10 was used, and that of PCL when Lipase M10 was used, showed relatively high values which were more than 20%. In other conditions, enzymes showed little degradability value. The weights of PLA and PHB/V films after 100 days incubation scarcely decreased in all experiments.

Figures 1(a) and 1(b) show the molecular weight distributions of oligomers derived from PCL and from PBSA by enzymatic degradation, respectively. As shown in Figure 1(a), the peaks of monomer derived from PCL (molecular weight = 113 Da) were mainly observed when either Lipase Asahi or Lipase F was used. This result means that PCL films were completely decomposed to the monomer by these enzymes. Relative to the decrease in the biodegradability value, the content of large oligomers increased. A similar tendency could be observed in Figure 1(b), but the main products were considered to be dimers because of their molecular weight (180–220 Da). The molecular distribution of oligomers derived from PBS using Lipase Asahi was similar to that derived from PBSA using Lipase Asahi (data not shown). Oligomers derived from PLA and PHB/V were not observed in any case.

Table 1. Properties of biodegradable polymers

Polymer	MW[1] (kDa)	Tm[2] (°C)	Tg[3] (°C)	Tensile strength (MPa)	Flexural modulus (MPa)
PBS	180–190	114	−28	56	60–70
$[–O–(CH_2)_4–O–CO–(CH_2)_2–CO–]_n$					
PBSA	180–190	95	−45	54	320–380
$[–\{O–(CH_2)_4–O\}_1–\{CO–(CH_2)_2–CO–\}_x–\{CO–(CH_2)_4–CO–\}_y–]_n$ (x + y = 1)					
PCL	40	60	−60	45–60	15–20
$[–O–(CH_2)_5–CO–]_n$					
PLA	200	175	58	90	–
$[–O–CH(CH_3)–CO–]_n$					
PHB/V	400	144	–	25	1000
$[–\{O–CH(CH_3)–CH_2–CO\}_x–\{O–CH(C_2H_5)–CH_2–CO–\}_y–]_n$					

[1] Molecular weight.
[2] Melting point.
[3] Glass transition point.
The values in this table were quoted from the suppliers' catalogues.

Table 2. Properties of lipases

Enzyme	Supplier	Source	Specificity[1]
Lipase F	a	*Rhizopus niveus*	1,3
Lipase AY30	a	*Candida rugosa*	N
Lipase F-AP15	a	*Rhizopus oryzae*	1,3
Lipase A-6	a	*Aspergills niger*	1,3
Lipase M10	a	*Mucor miehei*	1,3
Lipase-MY	b	*Candida cylindracea*	N
Lipase-OF	b	*Candida cylindracea*	N
Lipase-PL	b	*Alcaligenes sp.*	1,3
Lipase-QL	b	*Alcaligenes sp.*	1,3
Lipase-AL	b	*Achromobacter sp.*	1,3
Lipase Saiken100	c	*Rhizopus japonicus*	U
Lipase A-5	c	*Rhizopus japonicus*	U
Lipase B-4	c	*Rhizopus japonicus*	U
Roosepase FD	c	*Rhizopus japonicus*	U
Talipase	d	*Rhizopus delemar*	1,3
Lipase Asahi	e	*Chromobacterium viscosum*	N
Lipase Type7	f	*Candida cylindracea*	U
Lipase USB	g	*Porcine pancreas*	U

a, Amano Pharmaceutical (Tokyo, Japan); b, Meito Sangyo (Tokyo, Japan); c, Nagase Biochemicals (Kyoto, Japan); d, Tanabe Seiyaku (Tokyo, Japan); e, Asahi Chemical Industry (Tokyo, Japan); f, Aldrich (USA); g, US Biochemical Corp.
[1] Specificity; 1,3, 1,3-specificity; N, non-specificity; U, unknown.

Degradation of PLA at 55 °C at pH 8.5

Among 8 commercial lipases, Lipase PL showed remarkable degradability of the PLA film at 55 °C at pH 8.5 as shown in Table 4. When Lipase PL was used, the surface of the PLA film became rough (Phase I) after 5 days of incubation. The film was visibly broken down (Phase II) after 10 days of incubation, and finally disappeared (Phase III) only after 20 days. Visible changes of the films were not observed using the other enzymes during 20 days of incubation. On the other hand, the surfaces of some films became rough, and fragments of film were observed after 15–60 days of incubation in other cases, even the experimental blank.

Table 3. Biodegradability (%) of polyester film after 100 days incubation at 37°C, pH 7.0

Enzyme	Polyester				
	PBS	PBSA	PCL	PLA	PHV/B
Lipase F	*	100.0 (6)	100.0 (11)	*	*
Lipase AY30	*	21.9	*	*	*
Lipase F-AP15	*	100.0 (22)	*	*	*
Lipase A-6	*	*	*	*	*
Lipase M10	*	36.1	23.8	*	*
Lipase-MY	*	*	*	*	*
Lipase-OF	*	*	*	*	*
Lipase-PL	*	*	*	*	*
Lipase-QL	*	100.0 (11)	100.0 (14)	*	*
Lipase-AL	*	*	*	*	*
Lipase Saiken100	*	*	*	*	*
Lipase A-5	*	*	*	*	*
Lipase B-4	*	*	*	*	*
Roosepase FD	*	*	*	*	*
Talipase	*	*	*	*	*
Lipase Asahi	100.0 (17)	100.0 (4)	100.0 (6)	*	*
Lipase Type7	*	*	*	*	*
Lipase USB	*	*	*	*	*
Blank	*	*	*	*	*

The figure in parentheses presents the day when the complete degradation of film was performed.
The asterisk means that the value of biodegradability was less than 5.0%.

Table 4. Biodegradation of PLA film at 55 °C, pH 8.5

	Incubation time (days)										
	5	10	15	20	25	30	35	40	45	50	60
Lipase F	*	*	*	*	*	I	I	I	I	I	
Lipase AY30	*	*	*	*	*	I	I	I	I	I	
Lipase M10	*	*	*	*	*	*	I	I	I	I	
Lipase PL	I	II	II	III							
Lipase AL	*	*	*	*	*	*	I	I	I	I	
Lipase Saiken100	*	*	I	I	I	I	II	II	II	II	
Talipase	*	*	*	*	*	I	I	I	I	I	
Lipase Asahi	*	*	*	*	*	*	I	I	I	I	
Blank	*	*	*	*	*	*	I	I	I	I	

*, No change; I, film surface became rough; II, film decomposed into fragments; III, film disintegrated.

Generally, polyesters have a tendency to decompose at higher temperatures and/or higher pH conditions. Therefore, PLA film could be chemically broken down at 55 °C at pH 8.5 even without lipases. In this case, the PLA film transformed into water insoluble small particles, without complete degradation. In order to examine a change in the molecular weight of PLA before and after 50 days of incubation without lipase, a comparison of GPC curves between the original PLA film and the PLA particles was performed. As shown in Figure 2, the main peak of 147 kDa decreased and a new peak of 6.5 kDa appeared after incubation. However, no aqueous soluble products such as lactic acid and its oligomers were detected by HPLC in the solution incubated for 60 days. Therefore, it could be estimated that the PLA film was degraded only into

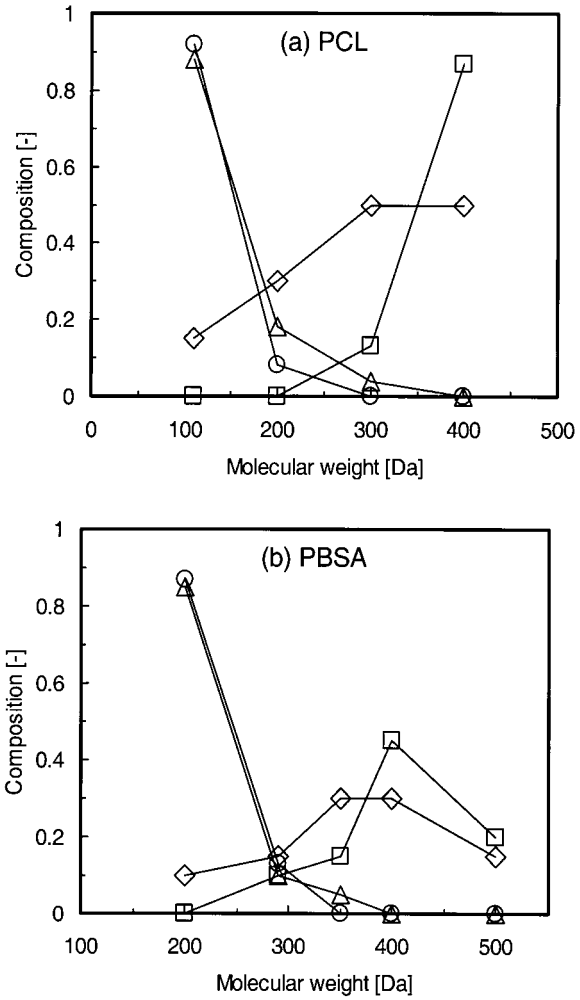


Figure 1. Molecular weight distributions of oligomers derived from PCL (a) and PBSA (b) by enzymatic degradation at 37 °C, after 100 days incubation. Symbols; ○, Lipase Ashahi; △, Lipase F; □, Lipase M10; ◊, Lipase AY30. Column, TSK gel G2000PW (21.5 mm × 30 cm × 2; exclusion size, 5 kDa; Toso Co.), eluent, 0.5 ml/min of water, detector, RI; molecular weight standard, pullulan.

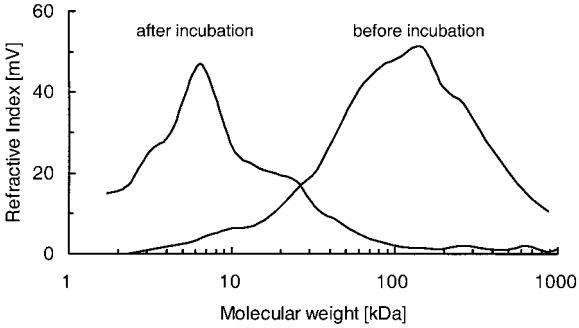


Figure 2. GPC curves for the PLA before and after 50 days incubation at 55 °C at pH 8.5 without lipases. Column, Shodex K-80M (21.5 mm × 30 cm; exclusion size, 20000 kDa; Showa Denko Co.), eluent, 1.0 ml/min of chloroform, detector, RI; molecular weight standard, polystyrene.

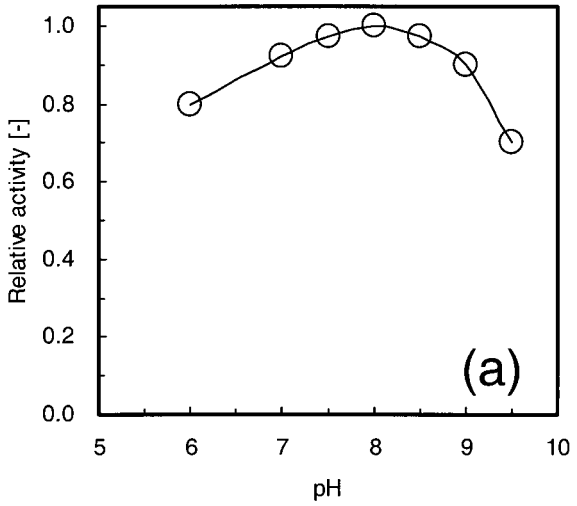
the water insoluble oligomers. In contrast, Lipase PL produced lactic acid within 50 days, as detected by HPLC. Moreover, Lipase PL shortened the period of Phase I and Phase II in the degradation process of PLA as shown in Table 4.

The optimum reaction pH and temperature values of Lipase PL for hydrolysis of olive oil are shown in Figure 3(a) and Figure 3(b), respectively. The detail of reaction conditions was described in our previous report (Isono et al. 1996). The optimum pH for Lipase PL was around 8.0, and the enzyme was active in a wide pH range of 6.0- 9.0. Lipase PL had the optimum reaction temperature at 50 °C, and about 70% of the activity remained at 60 °C.

Figure 4 shows the effect of the pH of the reaction mixture on the degradability of PLA film by Lipase PL. The degradability values were calculated using the lactic acid production rate. A higher pH of solution gave higher degradability, although the optimum pH value of Lipase PL is 8.0. As described above, this phenomenon could be explained by the integration of chemical hydrolysis at the higher pH and enzymatic hydrolysis by Lipase PL. At pH 9.0, the chemical hydrolysis was accelerated and the enzyme activity did not dramatically decrease.

Discussion

According to an increasing public concern about environmental pollution, development of biodegradable polymers has been promoted as one of the approaches to solve the problem. At present, various kinds of biodegradable plastics have been commercially manufactured such as PBSA, PCL, PLA, PHB/V, and PBS. The biodegradability of these polyesters by lipases has tended to be PBSA > PCL > PBA ≥ PHB/V = PLA (Table 3). This tendency is similar to the results obtained for the soil burial conditions (Sawada et al. 1994). Additionally, the degradation process of PCL and PBSA was investigated with GPC analysis (Figure 1(a) and 1(b)). Generally, polymer chains were randomly hydrolyzed by lipases to high- molecular weight oligomers and finally to monomers and dimers. On the other hand, the relationship between the enzyme properties (source and specificity) and the



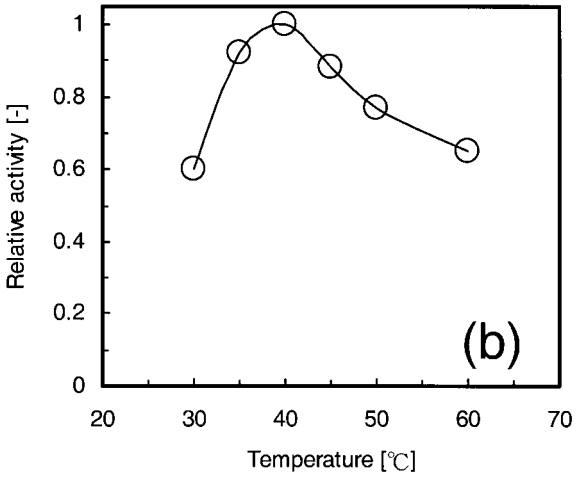


Figure 3. Optimum reaction pH and temperature of Lipase PL for hydrolysis of olive oil. The assay of lipase activity was carried out by automatic titration method using pH-stat. The details of the experimental method was described in our previous paper (Y. Isono et al, 1996).

experimental results was not clear in this study. This point requires further investigation

Lipase PL has revealed its higher degradation activity of PLA film in the present study. The enzyme was isolated from *Alcaligenes* sp. by Kokusho et al. (1982a, b) and has been commercialized. Some kinds of enzymes derived from *Alcaligenes* sp. were reported to degrade PHB (Tanio et al. 1982; Jendrossek et al. 1996), however, the degradability of PLA has never been reported with this type of enzyme.

Based on the results of GPC and HPLC analyses, it could be concluded that complete degradation of

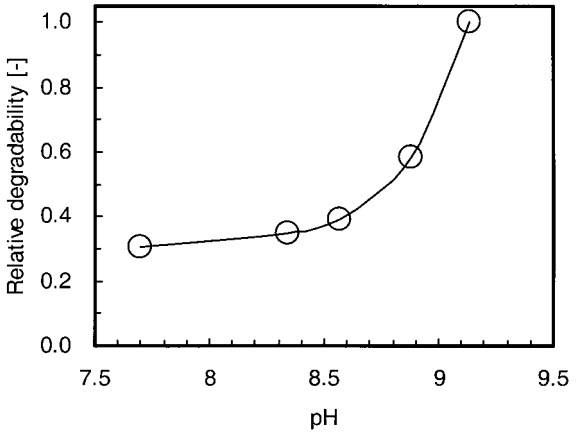


Figure 4. Effect of pH of reaction mixture on the degradability of PLA film by Lipase PL at 55 °C. The degradability was estimated by the rate of lactic acid production measured with HPLC.

PLA resulted from two processes. Firstly, the chemical hydrolysis from PLA into oligomers at higher pH and/or under higher temperature conditions, because polyesters are generally not stable under such conditions. Secondly, the enzymatic hydrolysis from the oligomers to the monomer. Moreover, Lipase PL should be suitable to the hydrolysis reaction of polyesters at higher pH and at higher temperature (Figure 4). This would be supported by the results shown in Figures 3(a) and 3(b). Lipase PL catalyzed the hydrolysis reaction from PLA polymer to its oligomers (Table 4). Further studies concerning the catalysis mechanism of lipase on the hydrolysis of polymers will contribute to the design of PLA waste treatment (Shirai 1999).

Acknowledgements

The authors would like to thank Daicel Chemical Industries, Shimadzu Corporation, Showa Highpolymer Co. Ltd., and Zeneca K. K. for supplying polyester films. We are also grateful to Meito Sangyo Co., Ltd. for supplying lipases. We sincerely thank professor Makoto Kimura of Nagoya University for his help in the preparation of this manuscript.

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