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Different enantioselectivity of two types of poly(lactic acid) depolymerases toward poly(L-lactic acid) and poly(D-lactic acid)

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ABSTRACT

Poly(lactic acid) (PLA) depolymerases are categorized into protease-type and lipase-type. Protease-types can hydrolyze poly(L-lactic acid) (PLLA) but not poly(D-lactic acid) (PDLA). Lipase-types, including cutinase-like enzyme (CLE) from *Cryptococcus* sp. strain S-2 preferentially hydrolyze PDLA. Both enzymes degraded not only PLA emulsion but also PLA film, in which amorphous region is preferentially attacked, but crystalline region can be also attacked. Stereocomplex PLA (sc-PLA) formed by 50:50 blending of PLLA and PDLA included no homo crystals, but a tiny homo crystallization peak appeared and crystallinity increased by 5% when attacked by CLE, although no significant change of molecular weight and crystalline size was found. Enantioselective degradation must occur in amorphous region of PLLA/PDLA film and preferentially hydrolyzed PDLA, resulting in a slightly excess amount of PLLA remained, which must be crystallized.

1. Introduction

The start of chemical synthesis of poly(lactic acid) (PLA) dates back to 1932 when Carothers first synthesized PLA of approximately 3000 Da [1]. In the 1960s, PLA found its use in medical fields as a bio-absorbable material. Since the latter half of the 1980s, plastic waste caused public concern as an environmental issue. Very recently, rising cost and limited resources of crude oil has turned more attention toward alternative sources. This trend shed light on PLA again as a bio-based material capable of replacing oil-based materials. PLA is chemically synthesized from lactic acid, a representative fermentation product from plant resources and is thereby defined as a biomass plastic. The biodegradability of biomass plastics is not a main focus, since the CO_2 released from PLA by combustion is thought to equal the CO_2 absorbed by plants, thereby yielding zero-emission of CO_2 and designation as "carbon-neutral". However, the biodegradability of PLA has been well known since the first report on enzymatic hydrolysis of PLA by William in 1981 [2] that described the feasibility for proteases and unfeasibility for esterases. Later, some lipases and esterases were reported to be able to hydrolyze PLA [3,4], but they seem to be active only for low molecular weights [5] or poly(DL-lactic acid) [6]. Among acid, neutral and alkaline proteases, only alkaline proteases showed appreciable activity (14 positive results among a total of 22) for high molecular weight PLA [7], but all the commercial lipases were negative [8], Lim et al. showed that all the six mammalian and microbial serine proteases hydrolyzed PLA well [9]. There are also many reports on the microbial assimilation of PLA, since Pranamuda et al. first isolated the PLA-assimilating *Amycolatopsis* sp. strain HT-32 [10]. Tokiwa and Calabia concluded that most of the PLA-degrading microorganisms phylogenetically belong to the family of *Pseudonocardiaceae* and related genera such as *Amycolatopsis*, *Lentzea*, etc. in which proteinous materials promote the production of the PLA-degrading enzyme [11]. PLA-degrading enzymes from PLA-assimilating microorganisms were purified from different strains of *Amycolatopsis* at the same time by two groups [12,13] and were characterized as proteases. Later, both groups cloned the genes [14,15]. It is notable that almost all the degradation tests have been carried out using poly(L-lactic acid) (PLLA) and that no information regarding the biodegradability of poly(D-lactic acid) (PDLA) is available, except that proteinase K hydrolyzed PLLA but not PDLA [16] and that Tomita et al. isolated a thermophilic *Bacillus stearothermophilus* able to grow at 60 °C on PDLA as a sole carbon source, although they did not mention

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enzyme activity contributing to the degradation of PDLA [17]. As PLA is hydrolyzed at a relatively high rate at high temperatures (>50 °C), whether the strain excretes PDLA-degrading enzyme or utilizes hydrolyzed products depends on future characterization of PDLA-degrading enzyme. Thus, no information has been available on the enzymatic hydrolysis of PDLA until now. The present study describes the biodegradability of PLLA and PDLA (either emulsions or films) by proteases, lipases and PLA-degrading enzymes from PLA-assimilating bacteria. Based on these results, we first report the enzymatic degradation of PDLA and we propose two different types of PLA depolymerases that have different enantioselectivity toward PLLA and PDLA and can be categorized into protease-type and lipase (cutinase)-type. The enantioselectivity of two types of PLA depolymerases will be useful for the biological recycling of PLA and the recovery of lactic acid (especially expensive D-lactic acid). The enantioselectivity of crude enzymes can be a good indicator for prediction of the enzyme category, upon which enzyme characterization and induction are based and with which the cloning of enzyme genes would become easier. Recently, stereocomplex PLA (sc-PLA) has been extensively studied to improve the mechanical and thermal durability for engineering usages. It is widely known that melting temperature (T_m) of sc-PLA reaches 230 °C at maximum, while T_m of conventional homo crystal of PLLA and PDLA is around 180 °C. A thermal degradation of sc-PLA was well studied and its degradation mechanism was suggested [28]. The degradation is dependent on trans-esterification and back-biting which are mainly caused by nucleophilic attack of free chain ends such as hydroxyl group and carboxylic acid. On the other hand, biodegradability or enzymatic hydrolysis of sc-PLA has not been reported yet. We confirmed the enzymatic hydrolysis of sc-PLA containing sample. The result indicated that a cutinase-like enzyme of *Cryptococcus* sp. strain S-2 (CLE) can attack amorphous regions in PLLA/PDLA blend with PDLA-preferential enantioselectivity, leaving a slightly excess amount of PLLA with which homo crystallization proceed.

2. Materials and methods

2.1. Materials

Lipozyme CALBL, Novozym 435 and 42044 and Savinase 16L type EX were kindly donated by Novozymes Japan, Ltd. Savinase CLEA (*Bacillus clausii* protease, a product of CLEA Technologies BV., The Netherlands) was donated by Shigematsu & Co. Ltd, Osaka, Japan. Lipases G, AS, PS, and AYS and Proleather FG-F are products of Amano Enzyme Co. Ltd. (Nagoya, Japan), which were kindly provided. Recombinant CLE was purified according to the same procedure as previously described [18]. Purified recombinant PLA-degrading enzyme from *Amycolatopsis* sp. strain K104-1 [14] was kindly provided by Dr. N. Abe (Graduate School of Agricultural Science, Tohoku Univ). Culture supernatants of PLA-degrading microorganisms T16-1 and B12-1 grown on PLA were kindly provided by Dr. S. Tokuyama (Graduate School of Shizuoka Univ), which included extracellular PLA-degrading enzymes. PLLA and PDLA used in this research were synthesized from L-lactide and D-lactide, respectively, by applying conventional ring opening polymerization with tin octoate ($Sn(Oct)_2$) as catalyst and n-hexanol as initiator. $Sn(Oct)_2$ and n-hexanol were distilled and dried before use. Other enzymes and reagents were purchased through commercial routes. The PLA emulsion was prepared following the method of Nishida and Tokiwa [19], filtered to remove precipitates and used for biodegradation tests. PLLA and PDLA cast films were prepared as follows: PLLA or PDLA (each 1.0 g) was dissolved in 15 ml of dichloromethane, which was then cast on a glass Petri dish (diameter of 90 mm) and dried at 60 °C overnight. sc-PLA samples were prepared as follows: Equal weights of PLLA and PDLA were dissolved in a mixture of chloroform and 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) (5:5, v/v), then casted on a glass Petri dish (diameter of 90 mm) and dried at room temperature for 2 days. The obtained cast film was dried at 60 °C overnight.

2.2. Degradability tests

A reaction mixture of various enzymes included approximately 1 mg/ml PLA, an appropriate buffer at 25 mM and an appropriate amount of each enzyme to total 50 ml, and then this mixture was incubated at 30 °C with slow shaking. As a control, a reaction mixture without enzyme was incubated at 30 °C with slow shaking. At certain time intervals, an aliquot of the reaction mixture was

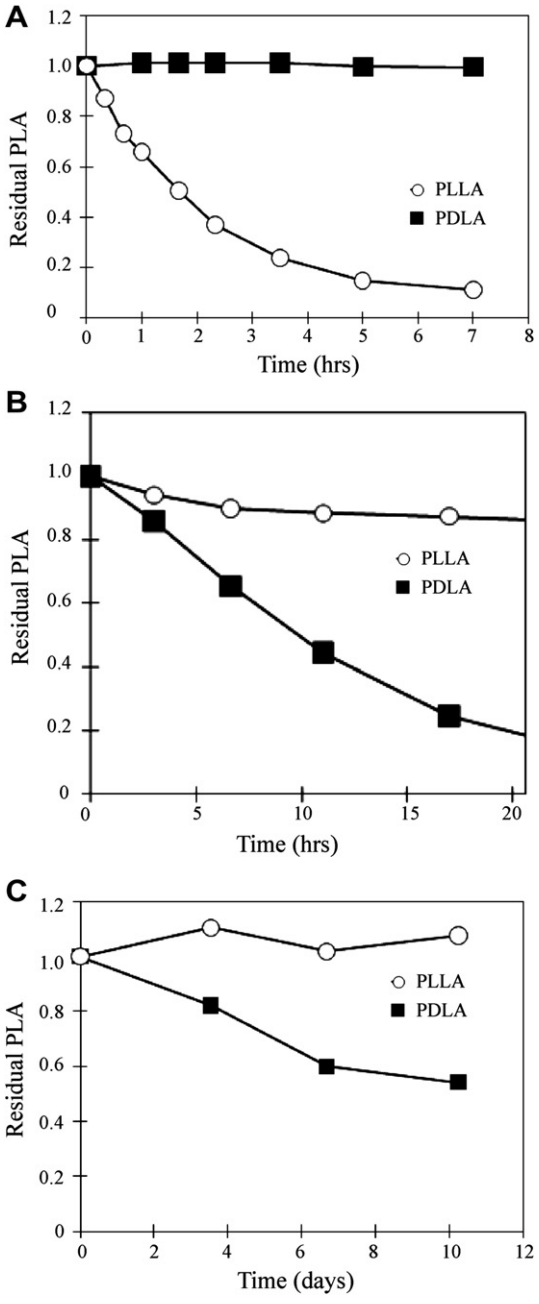

Fig. 1. Enzymatic degradation of PLLA and PDLA emulsion. A) Proteinase K; B) CLE; C) lipase PS. Degradation was measured by the decrease of absorbance at 600 nm.

withdrawn, to which an equal volume of chloroform was added to stop the reaction, and at the same time PLA was extracted. The chloroform layer was concentrated to remove chloroform, solved in pure water and subjected to measurement of optical density (OD) at 600 nm and gel permeation chromatography (GPC). Degradation rate was calculated by the decrease of OD. On the other hand, the reaction mixture was added to acetonitrile at the concentration of below 0.1 mg/ml as lactic acid and the acetonitrile solution was filtered with hydrophilic membrane of 0.45 μm pore size and subjected to mass spectrometry (ESI–TOF–MS analysis). The degradation of PLA film was examined using films measuring 15 mm × 5 mm. A PLLA film was incubated with proteinase K (200 μg) in 2 ml of 25 mM Tris–HCl buffer (pH 8.6) at 37 °C with slow shaking. PDLA films were also incubated with CLE (50 μg) in 2 ml of 25 mM potassium phosphate buffer (pH 7.0) at 37 °C with slow shaking. The degradation of PLA films was confirmed by GPC analysis. Reaction mixtures of proteinase K and CLE with PLLA and PDLA emulsions, respectively, were subjected to GPC analyses. GPC conditions were as follows: a GL Sciences Chromatograph GL7410 equipped with RI detector GL7454, degasser DG 660B and column oven CO 6310A; Shimpack GPC-800CP, GPC-802C, GPC-804C and GPC-806C connected in this order; eluent, chloroform; elution speed, 1.0 ml/min; and column temperature, 40 °C. Mass spectrometry (ESI–TOF–MS analysis) was performed, using a micrOTOF instrument (ESI–TOF–MS; Bruker Daltronics GmbH, Germany).

2.3. Measurements

Droplet sizes of PLA emulsion were measured by drying 5 ml of PLA emulsion *in vacuo* and pasting the obtained powder particles on carbon tape to use as a sample and subjected to scanning electron microscopy (SEM). SEM was measured using an S-3000N scanning electron microscope (Hitachi Co., Japan). The PLA film in a reaction mixture was picked up, washed once with distilled water, washed with 70 vol/vol % ethanol twice and then dried at room temperature *in vacuo*. Dried films were subjected to differential scanning calorimetry (DSC) and measurement of wide angle X-ray scattering (WAXS). DSC was performed using a DSC-50 differential scanning calorimeter (Shimadzu Co., Japan) at 10 °C/min under nitrogen flow rate at 20 ml/min. The WAXS measurements were performed at 25 °C using a RINT-2500 diffractometer equipped with a Cu-Kα source ($\lambda = 0.154$ nm) that was operated at 40 kV and 100 mA (Rigaku Co., Tokyo, Japan). The degrees of crystallinity of homo crystal and sc-PLA crystal were determined from the DSC and WAXS results. Namely, for a 2θ range of 5–30°, the crystalline peak areas for the stereocomplex crystal at 2θ values of 12°, 21° and 24° and for the homo crystal at 2θ values of 17° and 19° relative to the total area between a diffraction profile and a halo baseline were used to estimate degree of crystallization.

3. Results and discussion

3.1. Degradation of PLLA and PDLA by proteases and lipases

Using a PLA emulsion, the biodegradability of PLLA and PDLA were examined with commercially available enzymes, CLE and PLLA-degrading enzymes from actinomycetes. The droplet size of the PLA emulsion was measured by SEM to be in the range of 10–30 μm. From the relationship between dry weight and the OD of the PLA emulsion, 1.0 of OD at 600 nm corresponded to approximately 0.1 wt/vol.-%. No substantial difference was detected between PLLA and PDLA emulsions. Examples of degradation patterns are shown in Fig. 1 and the results are summarized in Table 1. Proteases were all PLLA-specific and lipases were non-specific toward PLLA and PDLA, but preferential to PDLA, compared with activity toward PLLA. Masaki et al. found that CLE had a higher hydrolytic activity toward PLLA emulsion than proteinase K [20]. In this study, we confirmed that CLE and commercial lipase preferentially act on PDLA over PLLA. Lipase PS specifically degraded PDLA but not PLLA at all. However, we could not compare the biodegradability of PLLA and PDLA under

Table 1
Enantioselectivity of various commercial lipases, proteases and PLA-degrading enzymes.

Enzyme	Source	Reaction conditions			Activity on PLA	
		pH	Temp (°C)	Concentration	PLLA	PDLA
Lipases						
CLE[a]	*Cryptococcus* sp. S-2	7.0	37	11 μg/ml	+	+
Lipase	Porcin Pancreas	6.0	37	25 μg/ml	–	–
R.d. Lipase	*Rhizopus delemer*	5.6	40	25 μg/ml	–	–
Lipase G	*Penicillium camemberti*	5.0	40	25 μg/ml	–	–
Lipase AS	*Aspergillus niger*	6.0	45	25 μg/ml	–	–
Lipase PS	*Burkholderia cepacia*	7.0	50	25 μg/ml	–	+
Lipase AYS	*Candida rugosa*	7.0	45	300 μg/ml	–	–
Novozym 435[b]	*Candida antarctica*	7.0	40	20 wt/vol.-%	–	–
Lipozyme CALBL	*Candida antarctica*	7.0	50	5 vol/vol.-%	–	–
Novozym 42044	No information	7.0	30	5 vol/vol.-%	+	+
Proteases						
Savinase 16L[c]	*Bacillus lentus*	7.0	30	5 vol/vol.-%	+	–
Savinase CLEA[c]	*Bacillus clausii*	7.0	40	3 mg/ml	+	–
Proteinase K	*Tritirachium album*	8.6	37	50 μg/ml	+	–
Proleather FG-F	*Bacillus subtilis*	8.0	50	3 mg/ml	+	–
PLA-degrading enzymes						
PLA-degrading enzyme[d]	*Amycolatopsis* sp.	9.0	40	28 μg/ml	+	–
PLA-degrading enzyme[e]	*Amycolatopsis* sp.	–	–	–	+	–
T16-1[f]	*Actinomadura* sp.	7.0	40	5 vol/vol.-%	+	–
B12-1[f]	Unidentified actinomycete	7.0	40	5 vol/vol.-%	+	–

[a] Purified recombinant CLE [20].
[b] Novozym 434 and Lipozyme CALBL is the same enzyme, but the former is the immobilized form of the latter.
[c] Protease (Subtilisin) CLEA is an immobilized form of Savinase 16L.
[d] Purified recombinant enzyme [14].
[e] Personal communications from Dr. Y. Tokiwa [15].
[f] Culture supernatant of PLA-degrading actinomycetes grown on PLLA. Most probably proteases.

elevated concentrations, as solubility of the enzyme preparation were low (approximately up to 25 μg/ml) and degradation rates even at a concentration of 25 μg/ml are far lower than other positive lipases such as CLE and Novozym 42044. Therefore, at the moment, we cannot conclude whether the enantioselectivity of lipase PS is absolutely PDLA-specific or if activity toward PLLA is below the detection level. However, the latter case is most probable. The lack of enantioselectivity of the lipases (including CLE) toward PLLA and PDLA is probably due to the fact that substrates for lipases have no enantiomers and therefore lipases do not have to acquire strict enantioselectivity. The PLA-degrading enzymes from *Amycolatopsis* species were PLLA-specific. Culture supernatants from PLA-utilizing actinomycetes were also PLLA-specific, suggesting that these enzymes are most probably protease-type enzymes. Thus, we can conclude that protease-type PLA depolymerase is enantioselective toward the L-isomer of PLA. This is quite natural since proteases must have evolved to be specific to proteins (polymer of L-amino acids). PLA depolymerase from *Amycolatopsis* sp. strain-41 [12] is able to degrade silk fibroin, and the degradability of PLA is explained by the analogy of lactic acid to the L-alanine abundant in silk fibroins. Proteases in general must recognize PLA as a protein homolog.

Proteases and lipases are members of serine hydrolases, which have a catalytic triad consisting of serine, histidine and aspartic acid: the former is serine proteases and the latter is α/β-hydrolase fold enzymes. However, the topology of a catalytic triad in α/β-hydrolase fold enzymes is the mirror image of that in serine proteases [25], which probably causes the different preference toward PLLA and PDLA, as illustrated in Fig. 2. Proteases can hydrolyze *n*-butyl or ethyl-D- and L-lactate (data not shown), but cannot PDLA. No hydrolysis of PDLA is probably due to that PDLA cannot be accommodated in the active site or that PDLA cannot make an acyl-enzyme intermediate. On the other hand, lipases can accommodate either PDLA or PLLA in the active site, although PDLA is recognized well than PLLA. It is already reported that immobilized lipase CA (Novozym 434) hydrolyzes D-lactate faster than L-lactate [27].

3.2. Degradation rate of PLLA and PDLA emulsion by proteinase K and CLE

Proteinase K and CLE were subjected to further degradation tests as they showed the highest degradation rates toward PLLA and PDLA, respectively. The changes of M_w and M_n were confirmed with GPC and summarized in Table 2. Rapid depolymerization suggested the endogenous random scission of ester bonds in PLLA and PDLA by two enzymes. The degradation products were analyzed by ESI–TOF–MS. The degradation product of both PLLA and PDLA showed negative and positive peaks corresponding to linear oligomers ($n = 2$–24), respectively. Besides, peaks corresponding to cyclic L-lactic acid oligomers were detected by positive mode at m/z 455, 527, 599, 671, 743, 815, 887, and 959 ($n = 6$–13), while cyclic D-lactic acid oligomers were detected by positive mode at m/z 455, 527, and 599 ($n = 6$–8). Matsumura's group reported lipase-catalyzed transformation of PLA (poly(D,L-lactic acid) and PLLA) into cyclic oligomers in organic solvent [26]. The evidence for cyclic oligomers of D-lactide has not been reported. In this paper, we newly found that PLLA is degraded by proteinase K into oligomers including cyclic ones in an aqueous reaction mixture. In addition, PDLA is degraded by CLE into oligomers including cyclic ones. It remains to be confirmed whether cyclic oligomers are derived from the PLA used or not. Velocities of PLA-degrading rates by CLE and proteinase K were measured with PLLA and PDLA, as shown in Fig. 3 and both V_{max} and K_m values are summarized in Table 3. When the concentration of CLE was too high, both of PDLA and PLLA were completely hydrolyzed in less than one hour and difference in hydrolysis rates toward PDLA and PLLA could not be measured.

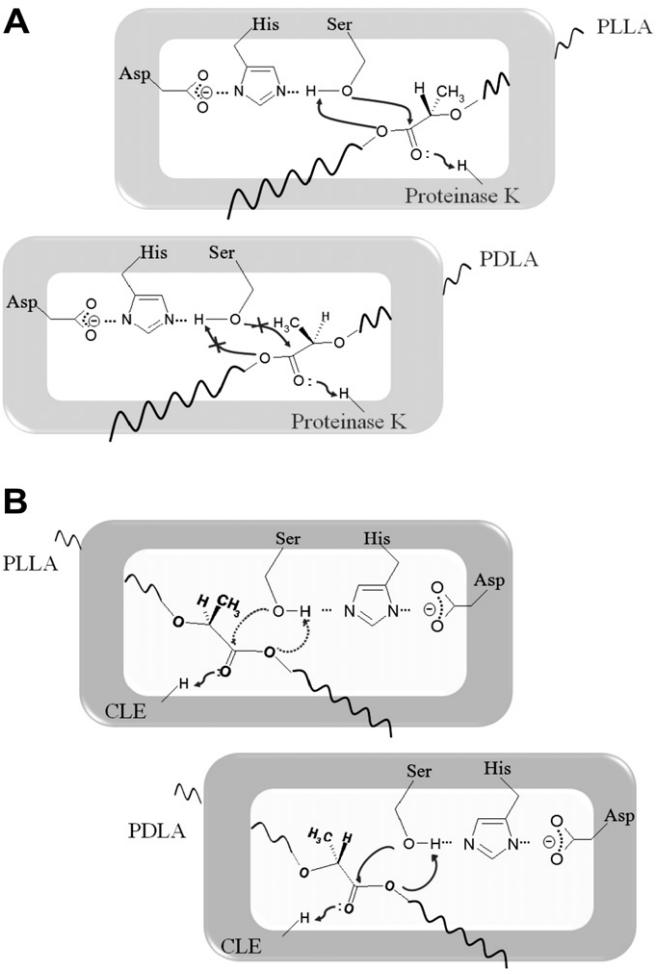

Fig. 2. Different enantioselectivity of proteinase K and CLE toward PLLA and PDLA.

Measurements were performed with appropriate concentrations of CLE and proteinase K in the range that activity/min/mg is approximately constant during the incubation. The V_{max} and K_m values of CLE for PDLA are approximately twice and half, respectively, of those for PLLA, supporting the preferential hydrolysis of PDLA. Both PLLA and PDLA caused substrate inhibition at higher than 1.6 and 1.8 mg/ml, respectively. Proteinase K showed lower V_{max} for PLLA than that of CLE and no substrate inhibition was found. From these results, CLE is the best PLA-degrading enzyme among the proteases and lipases.

3.3. Degradation of PLLA and PDLA films by proteinase K and CLE

PLLA and PDLA films (15 mm × 5 mm) were incubated with proteinase K and CLE at 37 °C with shaking for 4 days. Weight loss, molecular weight shift and polydispersity change of PLLA and PDLA were found after incubation with proteinase K and CLE respectively,

Table 2
The change of M_n and M_w of PLLA and PDLA emulsions after incubation with proteinase K and CLE.

	Incubation time	
	0 h	2 h
PLLA (37 C, proteinase K)	M_n: 117,000 M_w: 169,000	M_n: 3800 M_w: 44,600
PDLA (37 C, CLE)	M_n: 102,000 M_w: 163,000	M_n: 18,600 M_w: 157,000

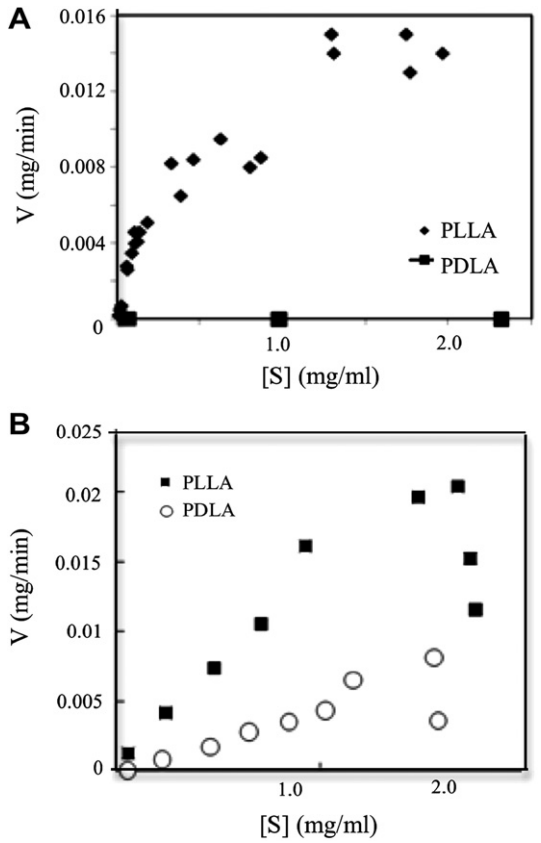

Fig. 3. Degradation rates of PLLA and PDLA emulsions with proteinase K and CLE. A) Proteinase K; B) CLE. Reactions were performed under the same conditions as described in experimental part.

as summarized in Table 4. Incubation of both PLLA and PDLA without enzymes (controls) caused no substantial weight loss, but change of their molecular weight and polydispersity suggested the partial hydrolysis of them. Weight loss and change of molecular weight and polydispersity by incubation with both enzymes clearly showed that both enzymes promoted degradation, compared with those of controls. The degree of crystallization (X_c) of PLLA and PDLA samples were determined by equation (1).

$$X_c(\%) = 100 \times (\Delta H_m - \Delta H_c)/\Delta H_m^0 \quad (1)$$

Here, ΔH_c and ΔH_m denote the heat of crystallization and crystal fusion, respectively, which were measured by DSC in the 1st heating scan. ΔH_m^0 is the specific heat of crystal fusion of PLLA and PDLA, which is defined as 93.7 J/g [21]. The crystallinity of PDLA film incubated with CLE increased once in 1–2 days, but later decreased, as shown in Fig. 4. Hence we can conclude that the amorphous regions are degraded faster to show the increased

Table 3
V_{max} and K_m values for PLLA and PDLA.

Enzyme	Substrate	V_{max}[a]	K_m[b]
Proteinase K	PLLA	0.03	8
	PDLA	–	–
CLE	PLLA	0.04	10
	PDLA	0.09	5
Novozyme 42044	PLLA	0.02	10
	PDLA	0.04	10

[a] mg/min.
[b] mg/ml.

Table 4
Change of molecular weight and crystallinity of PLLA and PDLA film after incubation with proteinase K and CLE.

Reaction	Weight loss (%)	Molecular weight		Polydispersity	X_c[a] (%)
		M_w	M_n	M_w/M_n	
PLLA, not incubated	0	1.9×10^5	1.2×10^5	1.6	51
PLLA incubated	0	1.9×10^5	8.5×10^4	2.2	48
PLLA + Proteinase K	7	1.9×10^5	4.0×10^4	4.8	40
PDLA, not incubated	0	1.8×10^5	1.2×10^5	1.5	41
PDLA incubated	0	1.8×10^5	9.0×10^4	2.0	42
PDLA + CLE	14	1.8×10^5	6.0×10^4	3.0	41

Note: reaction was performed at 37 °C for 4 days under the conditions described in experimental part.
[a] Degree of crystallization.

crystallinity in 1–2 days, but the crystalline regions are also susceptible to enzymatic attack, resulting in later decrease of crystallinity. This result is coincident with the previous report that enzymatic hydrolysis is faster in the amorphous region than in the crystalline region [22].

3.4. Crystal structure of degraded PLLA and PDLA

WAXS analysis of PLLA and PDLA films indicated that the diffraction patterns of PLLA and PDLA films before and after incubation with proteinase K and CLE respectively, correspond to homo crystallization of PLA in α-form crystal, as shown in Fig. 5. We calculated the size of crystallites by applying Scherrer's equation (2).

$$D_{hkl} = k\lambda/\beta\cos\theta \quad (2)$$

Here λ, β, θ, and k are the wave length (0.154 nm) of the incident beam, peak width at half height, Bragg angle, and Scherrer's constant (0.9), respectively. During the incubation, the crystalline size in (200) plane increased from 19.6 nm (PLLA) and 19.1 nm (PDLA) to 21.7 and 21.0 nm, respectively. The interfacial amorphous edge between crystallites in spherulite is first attacked and small crystallites can be released. As the degradation of small crystals is considered faster than that of larger ones, the average size of the remaining crystals becomes bigger and bigger, which results in the increased average crystalline size.

3.5. Biodegradability of PLLA/PDLA blend film consisting of sc-PLA

Recently, sc-PLA has been extensively studied to improve the mechanical and thermal durability for engineering usages. It is widely known that melting temperature (T_m) of sc-PLA reaches 230 °C at maximum, while T_m of conventional homo crystal of PLLA

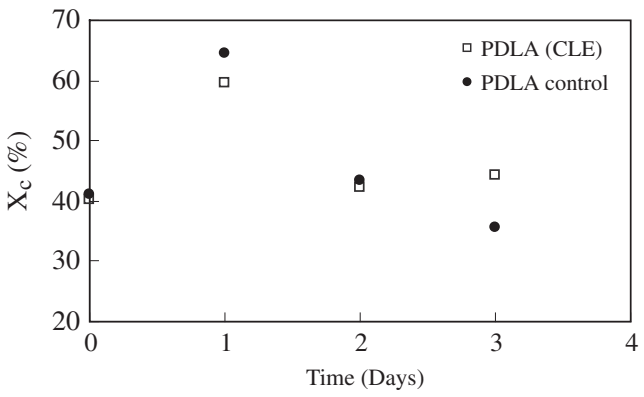

Fig. 4. The change in crystallinity of PDLA film by incubation with CLE.

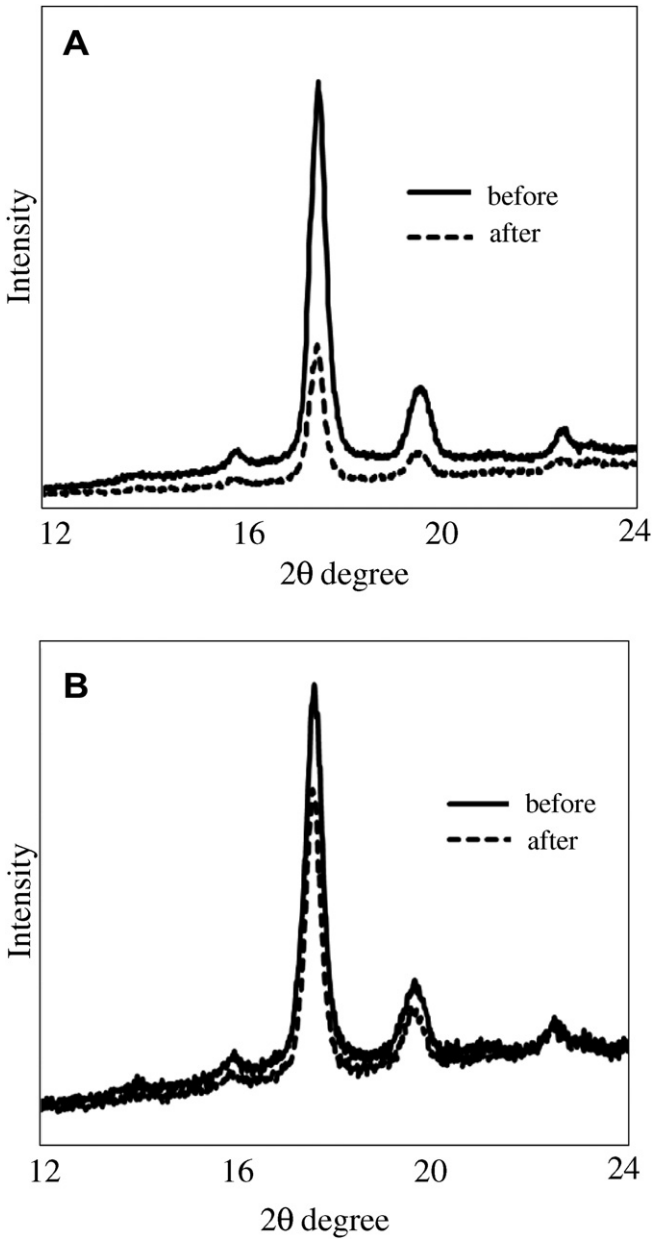

Fig. 5. WAXS profiles of PLLA and PDLA films before and after incubation with proteinase K and CLE, A) PLLA film with proteinase K; B) PDLA film with CLE. A solid line is before reaction and a broken line is after reaction, respectively.

and PDLA is 180 °C. In this study, 0.50 g of PLLA ($M_n = 1.2 \times 10^5$; polydispersity = 1.60, optical purity over 99%) and 0.50 g of PDLA ($M_n = 1.2 \times 10^5$; polydispersity = 1.50, optical purity over 99%) were dissolved in 10-ml mixed solution of equal volumes of chloroform and HFIP and casted on a Petri dish in hood. Remaining solvent in prepared film was completely removed by vacuuming at 60 °C overnight. WAXS and DSC analyses indicated that the resultant cast film consisted of sc-PLA (29% in degree of crystallization) and didn't contain homo crystals of PLLA and PDLA. As HFIP that we used is a strong solvent for both of homo crystal and sc-PLA, amorphous region probably remained in the cast film as gel state, resulting in a low crystallinity of the cast film. The crystallinity was determined by equation (1) (ΔH_m^0 for sc-PLA is 124 J/g) [22]. A cast film having 29% crystallinity was incubated with CLE under the same conditions as PLLA and PDLA film degradation. No significant weight loss,

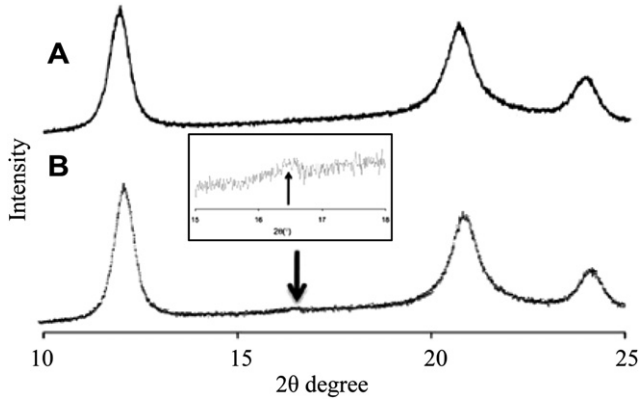

Fig. 6. WAXS profiles of sc-PLA containing PLLA/PDLA blend film before and after incubation with CLE. A) Before incubation, B) after incubation.

decrease of molecular weight and change of sc-PLA crystallite size were found, but degree of crystallinity increased approximately by 5%. These results indicate that sc-PLA domain consisting of β-form was unchanged during incubation, while amorphous PLLA and PDLA were degraded. WAXS profile in Fig. 6 shows an interesting tiny peak at a 2θq value of around 17° corresponding to homo crystallization (200) plane in α-form) [23] appeared after incubation. The same results were repeatedly obtained by the same procedure. This homo crystallization was not confirmed in DSC measurement, because melting peak of the homo crystal is contaminated with broad crystallization peak of sc-PLA. Degradation rate is very slow or only at trace level probably due to the strong interaction between D- and L-lactyl unit sequence preventing the penetration of water or enzyme into the sc-PLA bulk, as suggested by Lee et al. in 2005 [24]. Degradation of sc-PLA crystal at higher temperatures under composting or by thermostable enzymes must await further work.

4. Conclusions

From the aforementioned results, the following conclusions can be obtained for the enzymatic degradation of PLLA, PDLA and sc-PLA by proteases, lipases/cutinases and PLA-degrading enzymes. Both PLLA and PDLA are enzymatically hydrolyzed by two different classes of enzymes: proteases and lipases. The former is PLLA-specific and the latter is PDLA-preferential. Based on their different enantioselectivity toward PLLA and PDLA, we propose two classes of PLA depolymerases. The different enantioselectivity of active proteases and lipases toward PLLA and PDLA would be a good indicator for categorizing them and molecularly cloning their genes, based on the conserved regions of proteases and lipases.

1) The biodegradation ability of proteases and lipases for PLA is dependent on the geometry of an active site being wide and large enough to accommodate macromolecules and the absence of a lid covering the active site usually found in true lipases. Active lipases are rather designated as cutinases.
2) Both CLE and proteinase K degraded amorphous and homo crystalline regions of PLA films to produce finally oligo(lactic acid)s consisting mainly of linear ones and including small amounts of cyclic forms.
3) sc-PLA (29% crystallized) prepared by a blending of the same weight amount of PLLA and PDLA was only slightly degraded by CLE at the amorphous interface among crystallites and the remaining excess PLLA chains formed homo crystal in α-form.

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