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Enzymatic degradation of polyester films by a cutinase-like enzyme from *Pseudozyma antarctica*: surface plasmon resonance and atomic force microscopy study

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Abstract Enzymatic degradation of polyester films by a cutinase-like enzyme from *Pseudozyma antarctica* JCM10317 (PaE) was analyzed by surface plasmon resonance (SPR). The adsorption of PaE and the degradation rate for polyester films were quantitatively monitored by a positive and negative SPR signal shifts, respectively. The decrease in SPR signal and the erosion depth of amorphous poly(L-lactide) (a-PLLA) film measured by atomic force microscopy (AFM) had a linear relationship, and the weight loss was estimated from the AFM data combined with a density of a-PLLA film. Furthermore, SPR sensorgrams for various polyester films showed that degradation rate of poly (ε-caprolactone) and poly(butylene succinate-*co*-adipate) which contain C6 units was higher than that of other polyesters such as poly(butylene succinate) and a-PLLA. These results suggest that C6 is the preferred chain length as substrates for PaE.

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Introduction

Biodegradable polyesters (BPs) such as poly(butylene succinate) (PBS), poly(butylene succinate-*co*-adipate) (PBSA), poly(ε-caprolactone) (PCL), and poly(lactide) (PLA) are promising environmentally friendly materials because they are degraded and converted into water and CO_2 by the enzymatic reaction of microorganisms in natural environments (Gross and Kalra 2002).

Characterization of BP-degrading enzymes is expected to contribute to the control of BP degradation and for effective recycling of BP materials. We previously reported that phyllosphere yeasts of *Pseudozyma* spp. have strong degradation activity on agricultural mulch films composed of PBS and PBSA (Kitamoto et al. 2011). A cutinase-like enzyme named PaE was purified from *Pseudozyma antarctica* JCM10317, and its basic properties have been characterized. The degradation activity for several BP films (PBS, PBSA, PCL, PLA, and polyurethanes) has been confirmed on the basis of qualitative analyses (Shinozaki et al. 2012a, b). However, the detailed mechanisms of the degradation of BP films, including the adsorption behavior of PaE onto the surface of BP films and the degradation rates, remain to be clarified.

Surface plasmon resonance (SPR) is a label-free and real-time analytical method that can be applied to study the interaction of complementary partners such as antibodies, antigens, enzymes, membranes, cells, etc. (Karlsson 2004). Only a few studies have analyzed the enzymatic degradation of polymer thin films by SPR. Sumner et al. (2000) showed the degradation of poly(ester amide), dextran hydrogel, and poly(trimethylene) succinate by α-chymotrypsin, dextranase,

and lipase, respectively. Adsorption of the enzymes onto the BP film coated on the sensor surface and degradation of the film could be monitored by SPR as positive and negative signal shifts, respectively. Recently, enzymatic degradation of poly[(*R*)-3-hydroxybutyrate] (PHB) by a PHB depolymerase was also analyzed by SPR (Phithakrotchanakoon et al. 2009; Sinsereekul et al. 2010). They described that SPR was highly sensitive for the enzymatic degradation of BP films in the range of 10^{-6} to 10^{-9} g, which cannot be efficiently detected by most conventional detection techniques. They also observed the surface morphologies of the degraded PHB films by atomic force microscopy (AFM) and confirmed the film degradation visually (Phithakrotchanakoon et al. 2009). However, conversion of the SPR signal to the weight loss of BP films was based on the calibration of the SPR system, which was not specified for BP films (vide infra). Since it is unclear whether conversion of the SPR signal to the weight loss of BP films by applying a common calibration rate is correct or not, the enzymatic degradation rate could not be compared to previous bulk studies.

In this study, we investigated the BP film-degrading characteristics of PaE by using SPR and AFM. From the SPR sensorgram obtained using a sensor chip coated with an amorphous poly(L-lactide) (a-PLLA) film, the amount of PaE adsorption and degradation rate of the film were analyzed, and the values were compared to those obtained from a commercially available enzyme, proteinase K, which is commonly used for enzymatic degradation of a-PLLA (Williams 1981; Iwata et al. 2010). Furthermore, erosion depth of the a-PLLA film on the SPR sensor chip was measured by AFM. Based on combination of the SPR and the AFM data, weight loss of the a-PLLA film was calculated. In addition, the degradation behavior of PaE on several BP films, i.e., PBS, PBSA, PCL, and poly(D,L-lactide) (PDLLA), was analyzed by SPR.

Materials and methods

Materials

PBS (Bionolle 1020) and PBSA (Bionolle 3020) were purchased from Showa Denko K. K. (Tokyo, Japan); PCL and PDLLA were from Wako Pure Chemical Industries Ltd. (Tokyo, Japan); PLLA was supplied by Toyota Motor Co. Ltd. (Aichi, Japan). These substances were stored in sealed containers at 4 °C and used without further purification. The weight-average molecular weight (M_w), number-average molecular weight (M_n), and polydispersity (M_w/M_n) of these BPs were determined by gel permeation chromatography (GPC) in chloroform (0.5 ml/min) at 40 °C, using a Tosoh GPC-8020 system and refractive index detector RI-8020 with a TSKgel G4000H_{HR} column. Low-polydispersity polystyrene standards (Fluka, Sigma-Aldrich, St. Louis, MO, USA) were used to generate a molecular weight calibration curve. The molecular weight data are shown in Table 1.

Preparation of cutinase-like enzyme (PaE)

PaE was produced by *P. antarctica* JCM10317 and purified as described previously (Kitamoto et al. 2011). The concentration of the purified PaE was determined on the basis of its amino acid sequence and the A_{280} value of the enzyme solution (Shinozaki et al. 2012a).

Assay for esterase activity

Esterase activity was assayed as described previously (Kitamoto et al. 2011), with the following modifications: PaE was added at a concentration of 10 nM and the reaction was carried out at 30 °C for 3 min. The *p*-nitrophenyl (*p*NP) esters, *p*NP-butyrate (C4), or *p*NP-caproate (C6; Sigma-Aldrich) were used as substrates. One unit of esterase activity was defined as the release of 1 μmol of *p*-nitrophenol per minute.

Sensor chip preparation

The BP thin films were prepared on SPR sensor chips (SIA Kit Au, GE Healthcare, Little Chalfont, UK) by a spin-cast method. Each BP was dissolved in chloroform at a final concentration of 0.5 wt%. Then, 20 μl of the chloroform solution was dropped on the SPR sensor chip with a rotation speed of 3,000 rpm (Kyowa Riken, K-359 S-1, Tokyo, Japan). The sensor chips coated with PLLA were heated at 220 °C for 30 s on a hot plate, and then quenched immediately at 0 °C to form completely amorphous PLLA (a-PLLA) films, or heated at 220 °C followed by 160 °C for 24 h to form crystallized PLLA (c-PLLA) films (Kikkawa et al. 2004). The sensor chips coated with BPs, except for PLLA, were stored in glass laboratory dishes at room temperature over 1 week to evaporate the chloroform completely.

SPR measurement and data analysis

SPR measurement was performed with BIACORE X (GE Healthcare). For the standard analysis, HBS-N buffer

Table 1 Molecular weight of BP samples used in this study

Sample	$M_w \times 10^{-4}$	$M_n \times 10^{-4}$	M_w/M_n
PBS	11.1	4.2	2.7
PBSA	6.0	2.6	2.3
PCL	25.4	13.2	1.9
PDLLA	1.1	0.4	2.6
PLLA	18.6	11.3	1.7

(10 mM HEPES, 150 mM NaCl, pH 7.4, GE Healthcare) or 50 mM Tris–HCl buffer (pH 8.5) was used as running buffer, at 25 °C under continuous flow at 20 μl/min. Enzyme solution diluted with running buffer was injected at 20 μl/min for 180 s. Then, after 3 min of buffer flow, the running buffer containing 20 % ethanol (20 % EtOH-buffer) was injected for 1 min at 1-min intervals until the SPR signal was stable. Complete dissociation of the enzyme from the BP film surface by this treatment was confirmed by the stability of the SPR signal. The SPR sensorgram obtained with PaE was compared to that of proteinase K from *Engyodontium album* (formerly *Tritirachium album*; Sigma-Aldrich), which is commonly used for degradation of a-PLLA.

The amount of adsorbed and dissociated enzyme on the sensor chip was calculated from the measured SPR response unit (RU) on the basis of reported data, i.e., protein adsorption of 1 ng/mm^2 resulted in an increase in the SPR signal by 1,000 RU (Stenberg et al. 1991). The BP film degradation rate was evaluated from the decrease in the SPR response unit (ΔRU) after enzyme injection and dissociation of the enzyme from the film surface, compared to RU before enzyme injection (baseline). Note that the relation of 1 ng/mm^2=1,000 RU is applicable to protein adsorption, and that the weight loss of BP films cannot be estimated directly from the SPR signal.

AFM observation

To investigate the correlation between ΔRU and erosion depth of a-PLLA films, surface morphologies of the a-PLLA films on the SPR sensor chips were observed by AFM in a dynamic force (tapping) mode (SII Nanotechnology Inc., Japan). After SPR measurement, the sensor chip was washed by injection of 20 % EtOH-buffer and MilliQ water for at least 2 min each, undocked from BIACORE X, and air dried at room temperature. AFM observation of these pretreated SPR sensor chips was carried out at 25 °C in air. The erosion depth of the film was measured at degraded and nondegraded regions, which correspond to the inside and outside of the SPR flow cell.

Results

Enzyme adsorption and a-PLLA film degradation analyzed by SPR

The degradation of a-PLLA films by PaE and proteinase K was investigated by SPR. SPR signal shifts below baseline were possibly due to the decrease in the film thickness caused by enzymatic degradation (Sumner et al. 2000). As shown in Fig. 1, the SPR sensorgram of the control (without enzyme) was stable for at least 600 s (dashed line (a) in Fig. 1a), indicating that autohydrolysis of the a-PLLA film did not occur under the present experimental condition. During the injection of PaE with HBS-N buffer, the SPR signal increased up to 1,140 RU (solid black line (b) in Fig. 1a) and after 100 s, the SPR signal gradually decreased. Three minutes after stopping the injection of PaE, the film surface was washed with 20 % EtOH-buffer for 1 min. As the result, the SPR signal decreased below baseline (−800 RU) and reached a plateau. These results indicate that [(adsorption)>(degradation)] was observed during PaE injection. At least 1.14 ng/mm^2 of PaE adsorbed onto the film surface, almost all of the adsorbed PaE was dissociated by washing with 20 % EtOH-buffer, and the surface of the a-

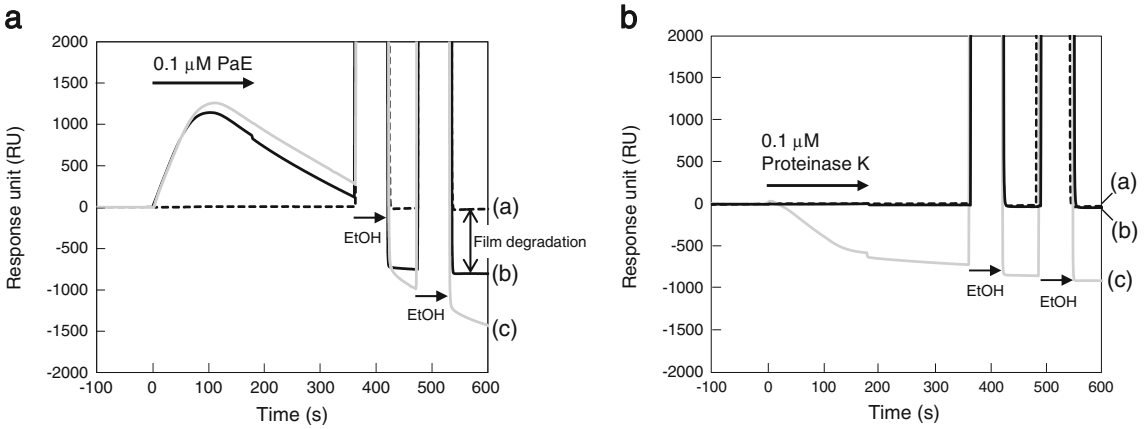

Fig. 1 SPR sensorgrams of enzymatic degradation of a-PLLA films by PaE (**a**) and proteinase K (**b**). The curve (*a*) *dashed line* is a control (without enzyme). The curves (*b*) *solid black line* and (*c*) *solid gray line* were measured in HBS-N or 50 mM Tris–HCl (pH 8.5) as running buffer, respectively, at 25 °C under continuous flow of 20 μl/min. Enzyme solution diluted with the running buffer at 0.1 μM was injected for 180 s. Then, after 3 min of buffer flow, running buffer containing 20 % ethanol was injected for 1 min, which was done twice for dissociation of the enzyme from the BP film

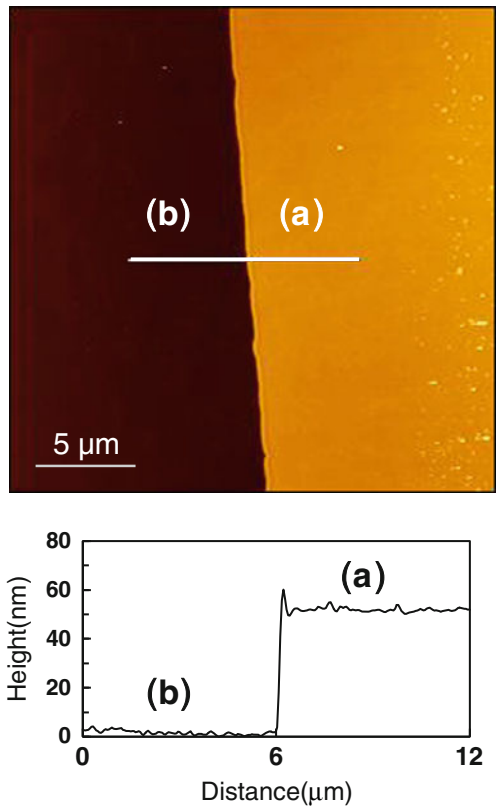

Fig. 2 AFM height image of the a-PLLA film coated on the SPR sensor chip, which completely degraded during SPR measurement following injection of proteinase K [30 μM in 50 mM Tris–HCl (pH 8.5) at 25 °C for 15 min]. **a** The outside of the SPR flow cell (nondegraded region), **b** inside of the SPR flow cell (degraded region). The graph under the AFM image represents cross-sectional data at the *white line* region

PLLA film was degraded by PaE. On the other hand, when 50 mM Tris–HCl (pH 8.5) was used as running buffer with PaE, a slightly different sensorgram was observed. After the maximum adsorption of PaE, the slope of ΔRU was almost constant throughout the whole process, even after repeated washing with 20 % EtOH-buffer (solid gray line (c) in Fig. 1a). This means that the adsorbed enzymes were still active and degraded the a-PLLA film or dissociated gradually from the a-PLLA surface, even after washing with 20 % EtOH-buffer. These results indicate that PaE adsorption is strongly dependent on the buffer condition.

The SRP experiment was also performed for proteinase K under the same reaction conditions. Injection of proteinase K with HBS-N buffer resulted in a very slight decrease in the SPR signal (ΔRU=40; solid black line (b) in Fig. 1b). On the other hand, during injection of proteinase K in 50 mM Tris–HCl (pH 8.5), the SPR signal immediately decreased to below the baseline (solid gray line (c) in Fig. 1b). Therefore, the binding affinity of proteinase K onto the a-PLLA surface was considered to be higher in 50 mM Tris–HCl (pH 8.5) than in HBS-N buffer. These results also indicate that [(adsorption)<(degradation)] is observed during injection of proteinase K in 50 mM Tris–HCl (pH 8.5).

Relationship between ΔRU and erosion depth of a-PLLA films

To confirm the degradation of a-PLLA films evaluated by the ΔRU, surface morphologies of the enzyme-treated a-PLLA films on SPR sensor chips were analyzed by AFM. Because a-PLLA film degradation (ΔRU) by proteinase K was higher than by PaE at relatively higher concentration, complete degradation of a-PLLA film by proteinase K was confirmed in the SPR analysis [30 μM in 50 mM Tris–HCl (pH 8.5) at 25 °C for 15 min; ΔRU=29,350; sensorgram data are not shown], then the surface of the film on the sensor chip was observed by AFM (Fig. 2). The erosion depth at enzyme-injected areas was approximately 50 nm. Regardless of the different enzymes and buffer conditions (HBS-N and 50 mM Tris–HCl), ΔRU and erosion depth of a-PLLA films showed a linear correlation (Fig. 3).

The effect of enzyme concentration on PLA film degradation

Degradation of PLA films (a-PLLA and PDLLA) was evaluated with various concentrations of PaE and proteinase K during 180-s enzyme injections. The ΔRU on the degradation of PDLLA was proportional to PaE concentrations in the tested range of 0.1–1.5 μM (ΔRU=18,000 at 1.5 μM

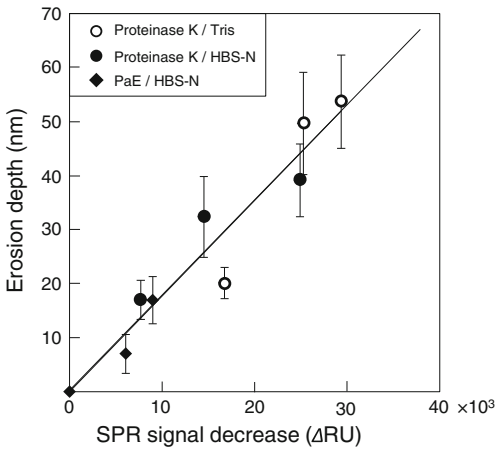

Fig. 3 Relationship between the SPR signal decrease (ΔRU) and the erosion depth of a-PLLA film measured by AFM. SPR was measured with 30 μM proteinase K in 50 mM Tris–HCl (pH 8.5) (*open circle*) or in HBS-N (*closed circle*), and 0.1 μM PaE in HBS-N (*closed diamond*), at 25 °C for different periods. The ΔRU values were obtained after enzyme injection and washing by 20 % EtOH-buffer solution for 1 min, which was done twice

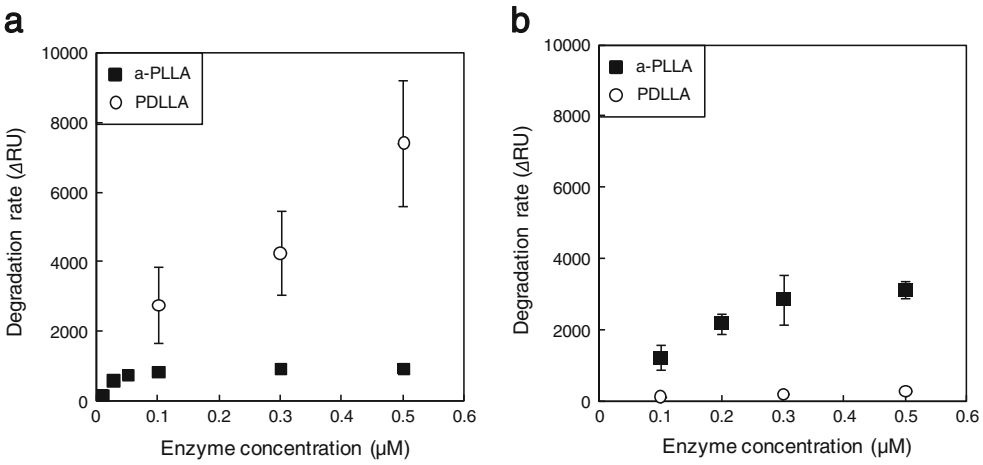

Fig. 4 Dependence of PLA film (a-PLLA and PDLLA) degradation rate on the concentration of **a** PaE in HBS-N buffer and **b** proteinase K in 50 mM Tris–HCl (pH 8.5). Degradation rate (ΔRU) for a-PLLA (*closed square*) and PDLLA (*open circle*) was obtained after 180 s of enzyme injection and washing by 20 % EtOH-buffer solution for 1 min (twice). Values are expressed as mean (SD) at $n=3$

PaE). The data up to 0.5 μM are shown in Fig. 4a. The ΔRU on the degradation of a-PLLA was also proportional at lower concentrations of PaE (0.01–0.05 μM; Fig. 4a); however, at PaE concentrations >0.1 μM, ΔRU was almost constant. This result indicates that PaE preferentially degrades PDLLA compared to a-PLLA. In contrast, the ΔRU of proteinase K for a-PLLA was concentration-dependent (0.1–0.3 μM), and it was much higher than that for PDLLA (Fig. 4b). This result agreed with studies reported by Reeve et al., namely, proteinase K specifically degrades L-lactyl units rather than D-lactyl ones in PLA films (Reeve et al. 1994).

Degradation of various BP films by PaE, monitored by SPR

The adsorption and degradation behavior of PaE against various BP films (PBS, PBSA, PCL, PDLLA, a-PLLA, and c-PLLA) were further analyzed by SPR. Among the tested polyesters, the degradation profiles were classified into two groups. One group comprises PBS, PDLLA, a-

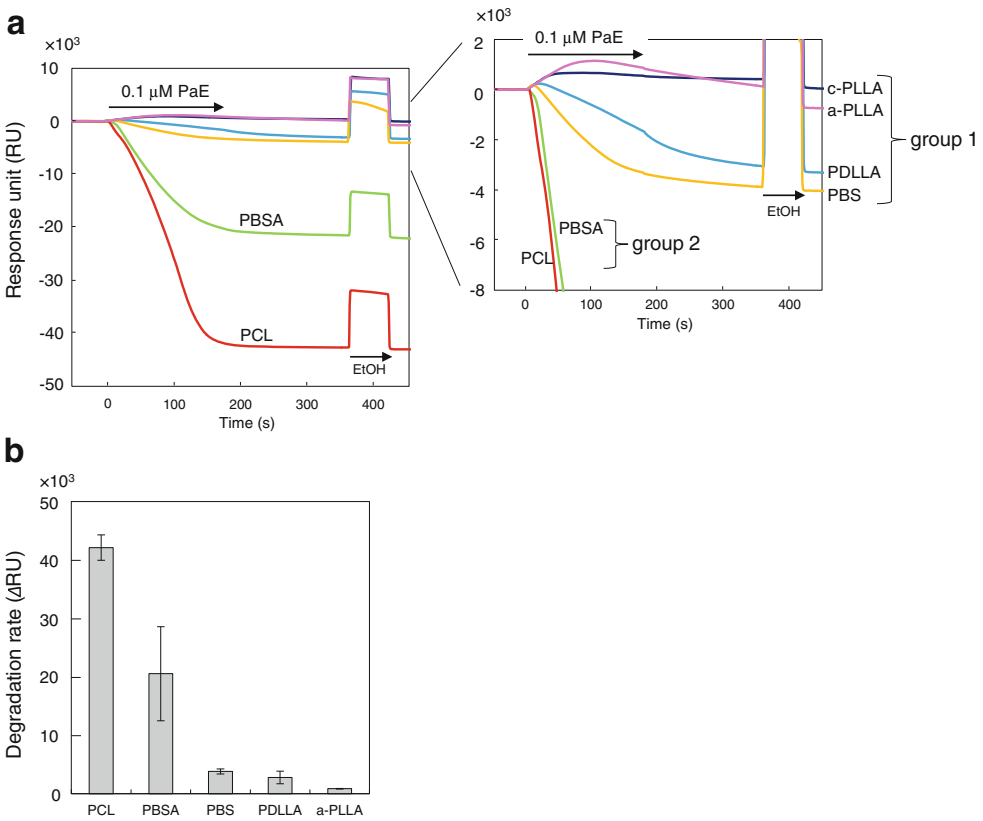

Fig. 5 **a** SPR sensorgrams of degradation of BP films by PaE. PaE was injected at a concentration of 0.1 μM in HBS-N for 180 s. Then, after 3 min of buffer flow, the running buffer containing 20 % ethanol was injected for 1 min. **b** Comparison of the degradation rate (ΔRU) of PaE for BP films. The ΔRU was obtained after 180 s of enzyme injection and washing by 20 % EtOH-buffer solution for 1 min. Values are expressed as mean (SD) at $n=3$

PLLA, and c-PLLA, in which an initial increase in the SPR signal due to PaE adsorption was observed (Fig. 5a, indicated as group 1 polyesters). The other group comprises PCL and PBSA in which the SPR signal decreased immediately during the injection of PaE (Fig. 5a, indicated as group 2 polyesters). On the surface of group 1 polyester films, the initially absorbed amount of PaE was greater than that of film degradation [(adsorption)>(degradation)]; the opposite was the case of group 2 polyesters [(adsorption)< (degradation)].

The signal in the SPR sensorgram for c-PLLA films decreased on the first injection of PaE; however, it remained almost unchanged after the second injection of PaE (Fig. 6). Several groups have reported preferential erosion of amorphous regions compared to crystalline ones (Reeve et al. 1994; Tsuji and Miyauchi 2001; Kikkawa et al. 2004). Therefore, the decrease in the SPR signal on the first injection of PaE could be due to the degradation of amorphous regions existing interspherulite and interlamellae. Since only slight erosion occurs in crystalline regions, the increase in the SPR signal at the second injection of PaE is accounted for by the adsorption of PaE onto the surface of the remaining c-PLLA film.

Kinetic analysis for PaE adsorption on c-PLLA film by SPR

The kinetics for PaE adsorption onto the BP film surface was analyzed. The c-PLLA film was used as substrate because association or dissociation of PaE can be monitored without degradation. Curve fitting of the SPR sensorgrams was performed with a 1:1 Langmuir binding model for both association and dissociation phases. As the results, the kinetic parameters, k_a, k_d, and K_A were 8.4 ($10^5 M^{-1} s^{-1}$), 1.4 ($10^{-3} s^{-1}$), and 6.2 ($10^8 M^{-1}$), respectively. These kinetic parameters were comparable to those of the substrate-binding domain of PHB depolymerase from *Ralstonia pickettii* T1 adsorbed on the PHB surface (Hiraishi et al. 2010).

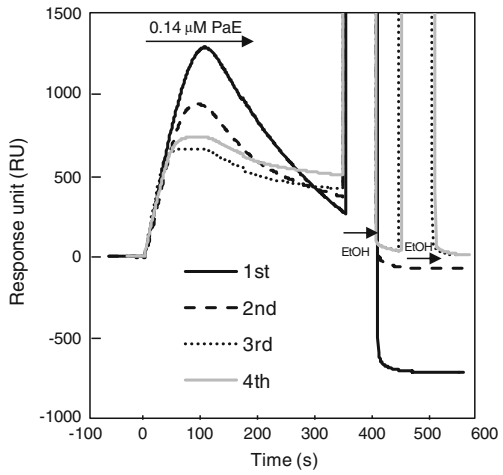

Fig. 6 SPR sensorgrams with c-PLLA film. PaE diluted with HBS-N at 0.14 μM was injected for 180 s. Then, after 3 min buffer flow, 20 % EtOH-buffer solution was injected for 1 min, one or two times. PaE injection and washing with 20 % EtOH-buffer solution was repeated several times

Discussion

We studied the adsorption and degradation behavior of a cutinase-like enzyme from *P. antarctica* JCM10317 (PaE) with various BP films by SPR. The analysis on a-PLLA films revealed that PaE strongly adsorbed onto the film surface and did not dissociate spontaneously under the applied reaction conditions. After removal of PaE by washing with 20 % EtOH-buffer solution, the degradation rate of a-PLLA films was evaluated through the decrease in the SPR signal (ΔRU; curve (b) in Fig. 1a).

Relationship between the ΔRU and the erosion depth (Fig. 3) indicates that ΔRU=10,000 corresponds to an erosion depth of 18±3 nm of a-PLLA films. The weight loss of a-PLLA films per unit area (W) has been represented as follows:

$$W = d \times V = d \times E \times S \quad (1)$$

where d is the density of a-PLLA (1.248 g/cm^3), V is the volume reduction due to degradation per unit area, E is the erosion depth, and S is the surface area fraction (Kikkawa et al. 2009). According to Eq. (1), the weight loss of a-PLLA film degradation corresponding to ΔRU=10,000 was calculated to be 22±4 ng/mm^2. The mass change was different from that calculated according to reported data, which were specified for protein adsorption (1,000 RU=1 ng/mm^2). Based on the relationship between the weight loss and ΔRU, the degradation rate for a-PLLA films during 180 s injection of 0.1 μM PaE in HBS-N (ΔRU=829±47, typical data are shown in curve (b) in Fig. 1a) was calculated to be 61 ng/cm^2 per minute.

The degradation rate at 0.3 μM proteinase K (ΔRU= 2,863±705, Fig. 4b) was calculated to be 210±52 ng/cm^2 per minute. Yamashita et al. (2005) analyzed a-PLLA film degradation by proteinase K using quartz crystal microbalance (QCM). They reported that the degradation rate was 120±24 ng/cm^2 per minute at 10 μg/ml of proteinase K [0.35 μM in 50 mM Tris–HCl (pH 8.5)]. Considering the error bars, these degradation rates were almost the same, in spite of the differences in batch (QCM) and flow (SPR).

The degradation rates of several BP films were evaluated on the basis of ΔRU after dissociation of the enzyme by washing with 20 % EtOH-buffer (Fig. 5a, b). They were found to be in the order of PCL>PBSA>PBS>PDLLA>a-

PLLA, and 50, 24, 11, 4.5, and 3.3 times higher than that of a-PLLA, respectively. Although molecular weight effects should be taken into account for the comparison of enzymatic degradation rates, the present results are discussed with regard to the chemical structures of the molecular components. The component of PCL, which shows the highest degradation rate, is $[-O-(CH_2)_5-CO-]_n$. PBSA also shows higher degradation rates, contains an adipate unit $[-CO-(CH_2)_4-CO-]$; thus, group 2 but not group 1 polyesters contains a C6 unit. The esterase activity of PaE was 320 ±13 and 197±16 U/mg for *p*NP-caproate (C6) and *p*NP-butyrate (C4), respectively. It has been known that the activity for *p*NP esters with longer chains such as *p*NP-octanoate (C8) was lower than that for *p*NP-butyrate (C4) (Shinozaki et al. 2012a). Therefore, it can be concluded that C6 is the preferred chain length as substrates for PaE.

Yamashita et al. (2003) analyzed the density of adsorbed PHB depolymerase on the PHB film surface by QCM using mutated PHB depolymerase, which lacked hydrolytic activity of the catalytic domain. They reported that the adsorption density increased linearly with enzyme concentration under the condition of [enzyme]≤2.5 μg/ml (0.05 μM) and then attained a maximum value of 182±44 ng/cm^2. They estimated the apparent cross-area per one molecule of enzyme bound to the film surface as 44±11 nm^2 assuming a molecular mass of 48 kDa of the enzyme. The calculated cross-area agreed with that of 30–50 nm^2, which was estimated from the hydrodynamic radius (3–4 nm) of a globular protein of a molecular weight of 48 kDa.

In the present study, the degradation rate for a-PLLA by PaE was almost constant under the condition of PaE≥ 0.1 μM (Fig. 4a). The amount of PaE adsorption on a-PLLA films during injection of 0.1 μM PaE was at least 1.14 ng/mm^2 as described above (curve (b) in Fig. 1a); thus, the apparent cross-area per one molecule of PaE bound to the film surface is estimated to be 30 nm^2, according to the molecular mass of PaE (20 kDa; Shinozaki et al. 2012a). These results indicate that most parts of the a-PLLA film surface is covered with PaE after injection of 0.1 μM PaE, and that there is little space for further PaE adsorption. Therefore, a-PLLA film degradation did not increase by injection of higher concentrations of PaE. Though PaE shows high affinity for a-PLLA films, the degradation rate of PaE for a-PLLA and PBS was lower than that for PCL and PBSA (Fig. 5b), possibly due to the high catalytic activity for C6 units.

The present SPR and AFM analyses could evaluate both enzyme adsorption and film degradation. These analyses could be very useful for further characterization of several BP-degrading enzymes.

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