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Degradation profiles of biodegradable plastic films by biodegradable plastic-degrading enzymes from the yeast *Pseudozyma antarctica* and the fungus *Paraphoma* sp. B47-9

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Abstract

Esterases from the yeast *Pseudozyma antarctica* (PaE) and the fungus *Paraphoma* sp. B47-9 (PCLE) can degrade biodegradable plastics (Shinozaki *et al*., 2013; Suzuki *et al*., 2014). The degradation profiles of plastic films composed of poly(butylene succinate), poly(butylene succinate-*co*-adipate), or poly(butylene adipate) by these enzymes were characterized by liquid chromatography-mass spectroscopy in terms of the molecular structures and molecular weights of the degradation products. Monomers and oligomers with molecular weights corresponding to dimers to octamers were identified as products of degradation by PaE in an aqueous reaction solution, irrespective of the type of biodegradable plastic film. Size-exclusion chromatography indicated that the number-average molecular weight of degraded films decreased with reaction time, suggesting that PaE degraded polyester films randomly into monomer units (endo-type degradation). PCLE also degraded polyester films randomly into monomer units, albeit more slowly than did PaE.

Key words: Biodegradable, biodegradable plastics, *Pseudozyma antarctica*, polyester hydrolase

1. Introduction

Much attention has focused on biodegradable plastics (BPs), which can be degraded into carbon dioxide and water by the activity of environmental microbes. BPs are used in the agriculture and fishery industries, because non-degradable plastics are associated with waste management problems, which are costly to overcome. In fact, agricultural mulch films composed of BPs are used to reduce the quantity of plastic waste. Such mulch films are used to cover soil surfaces to retain warmth and prevent the growth of weeds; however, these films may undergo faster or slower degradation than expected, because the rate of degradation depends on the environmental conditions, and is difficult to control [1-3]. Such properties of BP mulch films may limit their applications; therefore, technological developments are required to facilitate expanded use of BPs. The aliphatic polyesters are representative BPs. Of these, poly(butylene succinate) (PBS), poly(butylene succinate-*co*-adipate) (PBSA), poly(*ε*-caprolactone) (PCL) and poly(3-hydroxybutyrate) can be degraded by ester hydrolases, *i.e.*, lipases [3, 4], esterases [5], and cutinases [6] produced by microbes. Treatment of these enzymes will facilitate on-demand and on-site control of degradation of BPs (*e.g.*, rate, ratio, timing, *etc.*).

The phyllosphere yeasts *Pseudozyma* spp., classified as basidiomycetous can degrade agricultural mulch films composed of PBS and PBSA. PaE, a 20.4 kDa BP-degrading enzyme, was isolated from *Pseudozyma antarctica* JCM10317 and shares 61% identity with a BP-degrading enzyme from the yeast *Cryptococcus* sp. strain S-2 (AB102945) [8]. Studies of the substrate specificity of the PaE using *p*-nitrophenyl esters have shown that PaE prefers C6 acyl chains (range, C4 to C18 acyl chains) [9, 10]. PaE degrades polyester films composed of carboxylic acid units with C6 acyl chains, such as PBSA and PCL, more rapidly than those of other chain lengths, such as PBS and

poly(lactide) (PLA) [8-10]. These characteristics are distinct from the commercially available *P. antarctica* lipase B, which does not degrade these polyester films with the exception of PCL [10]. However, the ascomycetous fungus *Paraphoma* sp. B47–9, isolated from barley, can degrade PBS and PBSA films [11,12]. This fungus secretes the 19.9 kDa BP-degrading enzyme PCLE, a protein similar to filamentous fungi cutinases (57.3% similarity with *Fusarium solani* and 54.2% similarity with *Glomerella cingulata*), while it shares only 21.3% similarity with PaE [13]. Although the overall amino acid sequence identity between PaE and PCLE is low, the sequence of seven residues including a serine, which is presumed to be the active center, was identical. Although cutinase activity has not yet been analyzed, three residues (serine, aspartic acid, and histidine), which form a catalytic triad, and four cysteine residues, which form intramolecular disulfide bonds in cutinases, were also conserved in both enzymes. PCLE also shows rapid degradation on PBSA and PCL among capable of degrading various BP films. We recently demonstrated the acceleration of BP mulch film degradation in agricultural settings by treating the film with B47-9 culture filtrate that contained PCLE [14]. Elucidation of the mechanisms of BP film degradation by these enzymes will lead to improved techniques for efficiently degrading BP film.

In this study, two BP-degrading enzymes, PaE and PCLE, were used to degrade BP films for 1–4 h, and the water-soluble products were analyzed by liquid chromatography-mass spectroscopy (LC-MS). Comparison of the molecular structures of BPs, degradation products, and molecular weights of polymers in degraded films provided information on the BP degradation mechanisms of PaE and PCLE.

2. Materials and Methods

1 2.1 Materials

2 The biodegradable polymers used in this study were PBSA (Bionolle #3020,
3 Showa Denko K.K., Tokyo, Japan), PBS (Bionolle #1001, Showa Denko K.K.) and
4 poly(butylene adipate) (PBA, Sigma, St. Louis, MO, USA) (Fig. 1). Polymer pellets were
5 used as received. The average molecular weights (M_n, number-average molecular weight;
6 M_w, weight-average molecular weight; and M_w/M_n, polydispersity) of the polymers
7 determined in this study are shown in Table 1. Teflon-coated slide glasses (10 wells, φ 7
8 mm, Immuno-Cell, Mechelen, Belgium) were used to prepare cast BP films. Other
9 reagents and solvents were of analytical grade and used without further purification.

10

11 2.2 Enzymatic degradation of cast BP films

12 Cast BP films were prepared on Teflon-coated diagnostic slide glasses by casting
13 20 μl 1 wt% BP chloroform solution per well, followed by drying in air at room
14 temperature for ≥24 h [10]. Prior to enzymatic degradation, the surface of the cast film
15 was rinsed with Milli-Q water and then dried. PaE and PCLE were purified by the affinity
16 method as described previously [13, 15]. The degradation reaction was initiated by
17 placing 50 μl 100 nM enzyme solution on the film surface. PaE activity was analyzed in
18 10 mM Tris-HCl, pH 8.5. PCLE was analyzed in 25 mM HEPES-NaOH (pH 7.2) and 1
19 mM $CaCl_2$, since the activity was markedly enhanced by the presence of Ca^{2+} [13].
20 Enzymatic degradation was allowed to proceed at room temperature (25°C) in a humidity-
21 controlled incubator (humidity >95%, CO_2 incubator without CO_2 injection, Yamato,
22 Tokyo, Japan). The reaction was stopped by removing the enzyme solution from the film
23 surface, and the reaction mixture was mixed with an identical volume of 10 vol%

acetonitrile aqueous solution. The obtained solution was filtered through a 0.45 μm pore PTFE membrane (Milex-LH, Millipore, Darmstadt, Germany), and 5 μl of the filtrates were subjected to LC-MS assay.

Residual films on the slide glasses after enzymatic treatment were rinsed with Milli-Q water and dried overnight in air. To obtain sufficient polymer materials for molecular weight analysis by size-exclusion chromatography (SEC), films from 30 wells were dissolved in excess chloroform and then dried by evaporation. The obtained dry solids were dissolved in chloroform to 0.3 wt%, and 50 μl aliquots were subjected to molecular weight analysis.

2.3 LC-MS measurement

LC-MS measurements were performed using the Shimadzu LCMS-2020 system (Shimadzu, Kyoto, Japan) equipped with a reverse-phase Synergi Fusion-RP 4 u column (150 × 2.0 mm, Phenomenex, Torrance, CA, USA) and a security guard cartridge (for Synergi Fusion-RP, Phenomenex). Samples were eluted using the following gradient of 0.1% formic acid (A) and acetonitrile (B): 95/5% A/B for 2 min, then 20/80% for 25 min, followed by 0/100% A/B for 5 min. The flow rate was 0.2 ml/min, and the column temperature was maintained at 40°C during the analysis. Effluents were ionized by electrospray ionization using nitrogen at 10 ml/min as the drying gas. The detection mass range was m/z = 50–2000, and positive and negative ions were detected simultaneously. The adduct ions detected in this analysis were a parental molecule plus H^+ (+1), Na^+ (+22), K^+ (+39), $CH_3CN^+H^+$ (+42), and $HCOO^-$ (+45).

1 2.4 Molecular weight analysis

2 The average molecular weights of BPs were determined by SEC. Samples were
3 applied to the Tosoh 8020 GPC system (Tosoh, Tokyo, Japan) equipped with two
4 tandemly linked GPC LF-804 columns (Shodex, Tokyo, Japan), a guard column LF-G
5 (Shodex), and a refractive index detector RI-8020 (Tosoh). Chloroform was used as the
6 eluent at 1.0 ml/min, and the column temperature was maintained at 40°C during the
7 analysis. Polystyrenes with a narrow polydispersity (Shodex) were used to generate a
8 calibration curve. The absolute molecular weights of BPs were determined by SEC
9 analysis using a multi-angle light scattering (MALS) detector, operated by Mitsui
10 Chemical Analysis & Consulting Service, Inc. (Chiba, Japan).

11

12 2.5 Preparation of low-molecular-weight BPs

13 To prepare low-molecular-weight BPs, polyester pellets were hydrolyzed
14 overnight in the presence of KOH at room temperature. In a glass sample vial, 500 mg
15 BP pellets were dissolved in 5 ml chloroform, and then 0.5 ml 1 M potassium hydroxide
16 (Wako Pure Chemical, Osaka, Japan) and 0.5 mmol 15-crown-5 (Tokyo Chemical
17 Industry, Tokyo, Japan) were added. The reaction proceeded overnight at room
18 temperature with stirring. Hydrolyzed polyesters were precipitated in a 10-fold volume
19 of methanol, and the precipitates were collected by filtration, followed by drying under
20 reduced pressure. The molecular weights of the hydrolyzed samples were confirmed by
21 SEC and SEC-MALS measurements as described above.

22

23 **3. Results and Discussion**

3.1 LC-MS analysis of water-soluble products after degradation of PBSA film by PaE

To investigate the mechanism of BP film degradation by PaE, cast PBSA films were subjected to enzymatic degradation. The reaction proceeded at the interface between the enzyme solution and PBSA film surface; thus, the degradation products were expected to be water soluble and of low molecular weight. After incubation of a PaE-containing solution on the film surface for 1 to 4 h, LC-MS analysis was performed, and peaks were assigned by matching the mass spectra and molecular weights of oligomers derived from ester cleavage of PBSA. Figure 2 shows the total ion current chromatogram (TIC) of the degradation products detected after incubation for 1 and 4 h. Several peaks of molecular weights ranging from 190 (SB) to 806 (SBSBSBSBS) were detected, of which 318 (SBA), 362 (SBSB) and 534 (SBSBSB) were the major products. Oligomers with carboxylic acid ends were detected as deprotonated negative ions in TIC(-) (Fig. 2A), whereas oligomers with butandiols at both ends were detected in TIC(+) as H+, Na+, or K+ adduct ions (Fig. 2B). Note that long-chain oligomers were also detected as the formate addition formed. After 4 h of degradation, the intensities of the peaks corresponding to degradation products increased compared with those corresponding to buffer components (Tris, in Fig. 2). Additionally, monomers of butandiol and adipic acid were detected (3.1 min in Fig. 2B and 8.4 min in Fig. 2A, respectively). These results suggested that PaE liberated oligomers from the PBSA film surface, which were degraded into monomer units.

Low-molecular-weight PBSA obtained by alkaline-hydrolysis (L_{Mw}-PBSA) was also subjected to PaE degradation. After 1 h of degradation, oligomers with molecular weights of 190 (SB) to 534 (SBSBSB) were detected as negative ions in the TIC (Fig. 3). Monomers (S and A) were also detected (3.1 and 6.8 min, respectively) after 1 h of degradation. In addition, degradation for 4 h resulted in detection of oligomers with

molecular weights of 662 (SBSBSBA) or less, together with monomers. These results suggested that PaE can degrade low-molecular-weight PBSA chains into monomers more rapidly than those of high molecular weight.

3.2 Effect of BP monomer structures on film degradation by PaE

To evaluate the substrate preference of PaE in terms of monomer structures, cast PBS and PBA films were degraded by PaE, and the degradation products were analyzed by LC-MS. Figure 4A shows the elution pattern of the products of PBS film degradation by PaE for 1 and 4 h. Oligomers with molecular weights of 190 (SB) to 534 (SBSBSB) were detected after 1 h of degradation; the dominant peaks were 362 (SBSB) and 534 (SBSBSB). Oligomers with molecular weights up to 806 as well as monomers (B and S; data not shown) were detected after 24 h of degradation. Also, degradation of a low-molecular-weight PBS (L_{Mw}-PBS) film (Fig. 4B) for 4 h resulted in production of primarily oligomers with a molecular weight of 290 (SBS) together with monomers. These results suggest that PaE degrades low-molecular-weight PBS chains into monomers faster than those of high molecular weight.

PaE degraded PBA film into oligomers with molecular weights up to 346 (ABA) after 1 h of degradation (Fig. 4C). PBA monomers, adipic acid (A) and butandiol, were also detected after 1 h of degradation: butandiol was confirmed in TIC(+) (data not shown). The intensity of the dominant peak [molecular weight 218 (AB)] increased after 4 h of degradation (Fig. 4C), and oligomers with a molecular weight of 418 (ABAB) were also detected. The fact that the molecular weight of PBA was similar to that of L_{Mw}-PBSA and L_{Mw}-PBS samples (Table 1) suggests that PaE preferentially degraded polyester

chains composed of adipate (C6 dicarboxylate) rather than succinate (C4 dicarboxylate), and that the molecular weight of BPs influences the degradation rate. Shinozaki *et al.* [9] reported that PaE degraded PBSA more rapidly than PBS by surface plasmon resonance, which is in agreement with the LC-MS results in this study.

Previous studies have detailed the degradation of PBSA by lipases. Lipases from *Chromobacterium viscosum* and *Pseudomonas* sp. degrade PBSA into oligomers of 2- and 3-mers [3], although these oligomers were not further degraded into monomers. Another study reported liquid chromatography mass spectrometric (LC-MS) analyses of PBSA degradation by a lipase from *Mucor miehei* [4]. Their results demonstrated that oligomers resulting from the degradation of PBSA were detected in the total ion current chromatogram of the LC-MS, whereas monomers were barely detectable. Because lipases require hydrophobic interactions with their substrate for activation, they are preferentially activated by hydrophobic substrates. This may explain why the shorter and less hydrophobic oligomers, such as the 2- or 3-mers of PBSA, are not suitable lipase substrates. The degradation of polyesters into oligomers and not monomers was also observed in lipase-dependent PBST degradation in *P. cepacia* [16]. However, PaE degraded PBSA into monomers in 4 h and degraded PBS and PBA into monomers. Unlike lipases, PaE recognizes short-chain length acylester substrates [8], resulting in high degradation activity of PaE toward a variety of polyesters.

3.3 LC-MS analysis of the water-soluble products of BP film degradation by PCLE

PCLE can degrade PBSA and PBS [13]. To compare the mechanism of BP film degradation by PCLE with that by PaE, five cast BP films (PBSA, L_{Mw}-PBSA, PBS, L_{Mw}-

PBS, and PBA) were subjected to enzymatic degradation by PCLE, and the water-soluble products were analyzed by LC-MS. As shown in Fig. 5A, oligomers with molecular weights up to 534 (SBSBSB) were detected after degradation of the PBSA films for 1 h, but degradation did not proceed further in 4 h. In contrast, degradation of the L_{Mw}-PBSA films yielded numerous peaks corresponding to oligomers with molecular weights of 190 (SB) to 706 (SBSBSBSB) after 1 h of degradation. Moreover, the intensities of the dominant peaks corresponding to SBSB, SBA and SBSBS increased after 4 h of degradation (Fig. 5B).

Degradation of PBS films for 4 h resulted in production of oligomers, although the levels of degradation products after 1 h were negligible (Fig. 5C). In comparison with degradation of PBSA, PBS degradation for 1 h resulted in fewer peaks, suggesting that PCLE preferentially degrades PBSA. L_{Mw}-PBS film degradation by PCLE for 1 and 4 h resulted in production of longer oligomers as the dominant peaks (SBSBS, Fig. 5D) than did degradation by PaE (SBS, Fig. 4B). These results suggested that longer oligomers are produced via degradation of polyesters by PCLE than by PaE.

The degradation products of PBA films by PCLE were also analyzed. As shown in Fig. 5E, oligomers with molecular weights of 218 (AB) to 746 (ABABABA) were detected, and the dominant peak was ABAB after 1 h of degradation. After 4 h of degradation, the peak intensities increased, and adipate was also detected.

Both enzymes degraded polyesters composed of adipate (C6) more rapidly than those composed of succinate (C4), as reported previously [8, 9, 13]. In contrast, the molecular weight profiles of oligomers produced by PaE were slightly different from those produced by PCLE, as shown for L_{Mw}-PBS degradation (Figs. 4B and 5D). This difference was likely due to differences in the rate-limiting step of film degradation.

Degradation by PaE resulted in production of 3-mers (SBS) after 1 h, followed by production of monomers. In contrast, degradation by PCLE resulted in production of 4-mers (SBSB) to 5-mers (SBSBS) after 1 h, after which their proportions in the reaction solution increased. These results suggest that the oligomer-to-monomer degradation activity of PaE is greater than that of PCLE. We previously quantitatively monitored the enzymatic degradation of BP films by surface plasmon resonance (SPR) [9]. From the SPR sensorgram obtained with a sensor chip coated with BP film, erosion depth of the enzyme-treated film was correlated with a decrease in SPR response unit (ΔRU). PaE solution diluted to 100 nM in running HBS-N buffer (10 mM HEPES, 140 mM NaCl, pH 7.4) was injected for 180 s on the PBSA film (Bionolle #3020). After 3 min of buffer flow, the running buffer containing 20% ethanol was injected for 1 min to remove the adsorbed PaE from the PBSA film surface and ΔRU = 20,523 ± 8,037 was obtained [9]. The SPR sensorgram reproducibility with PaE (ΔRU = 22,190) was confirmed in this study (Supplemental Fig. 1). However, SPR analyses with PCLE in the same running buffer and the same buffer with 1 mM $CaCl_2$ was ΔRU = 570 and 1,520, respectively (Supplemental Fig. 1). These results also indicate that the rate of PBSA degradation by PaE is nearly 10 times greater than that of PCLE.

3.4 Molecular weight of residual films after enzymatic degradation

LC-MS analyses revealed that PaE and PCLE degraded BP films by releasing oligomers from the film surface, followed by their degradation into monomers. However, the composition of the films remaining on the slide glass after enzymatic treatment has not been determined. Thus, we used SEC to evaluate the molecular weights of the

polymers remaining on the slide glass.

Because a greater quantity of polymer materials is required for SEC analysis compared with LC-MS analysis, films from 30 wells were pooled (~6 mg before degradation). PBSA and PBS films remaining on the slide glass after degradation by PaE were collected by dissolving in chloroform and then subjected to SEC analysis. The molecular weight distributions of PBSA and PBS after degradation by PaE were compared with those of non-degraded films. As shown in Fig. 6, changes in the molecular weight distributions of PBSA (Fig. 6A) and PBS (Fig. 6B) were detected. In particular, the proportion of products with a molecular weight less than 10^4 Da increased with the reaction time. Therefore, PaE degraded polyester chains with a molecular weight of ~10^5 into water-insoluble polymers with molecular weights of 10^3–10^4, suggesting endo-type degradation by PaE. In contrast, no changes of molecular weight at peak top over time were observed. This indicated that degradation proceeded only at the film surface, and thus the majority of high-molecular-weight polyester chains within the film were not degraded. Moreover, oligomers with a molecular weight <10^3 were present at negligible levels. LC-MS indicated the presence of water-soluble oligomers with molecular weights up to 10^3 in the enzyme solution, suggesting that PaE can recognize ester linkages irrespectively of the molecular weight. Thus, PaE degraded polyester chains in PBSA and PBS films into oligomers and monomers.

Table 2 shows the changes in the average molecular weights and polydispersities due to degradation of polyester films by PaE and PCLE Degradation of PBSA and PBS by PaE resulted in M_ns values of 5.9×10^4 and 4.5×10^4 before degradation, respectively. The M_ns values rapidly decreased to 1.9–2.0×10^4 after 4 h of degradation and 1.4–1.5×10^4 after 24 h of degradation. In contrast, M_ws values decreased from 8.9×10^4 to $8.0\times$

10^4 for PBSA and from 6.7×10^4 to 5.8×10^4 for PBS after 24 h of degradation, which resulted in an increase in M_w/M_n values from 1.5 to 5.3 for PBSA and from 1.5 to 4.1 for PBS after 24 h of degradation. These results demonstrated that PaE is an endo-type enzyme. Similarly, degradation by PCLE resulted in decreases in the M_ns values of PBSA and PBS from 5.9×10^4 to 1.4×10^4 for PBSA and from 4.5×10^4 to 1.7×10^4 for PBS after 24 h of degradation, whereas the M_ws values decreased from 8.9×10^4 to 7.4×10^4 for PBSA and from 6.7×10^4 to 6.3×10^4 for PBS after 24 h. These data also indicated that degradation by PCLE resulted in random production of oligomers from the surface of the polyester films, a characteristic of endo-type enzymes. Kawai *et al*. reported that the cutinase-like enzyme CLE in *Cryptococcus* sp. S-2 randomly cleaves endogenous ester bonds in PLA randomly [17], suggesting that BP-degrading enzymes in yeast degrade polyesters via endo-type scission, and that the degradation characteristics of PCLE are similar to those of yeast BP-degrading enzymes. Ando *et al*. used SEC analysis to demonstrate that lipases from bacteria degrade PBSA powder in an endo-type degradation [3]. These enzymes showed a rapid decrease in the M_n of residual PBSA powder but little change in M_w during analyses. This trend is consistent with the average molecular weight changes in residual PBSA films degraded by PaE and PCLE (Table 2 and Fig. 6). Thus, the initial degradation pattern of BP chains by lipases and BP-degrading enzymes are the same, but further degradation of water-soluble oligomers by PaE and PCLE may occur faster than lipase-dependent degradation due to the difference in substrate specificity among these enzymes. These properties will help in the development of strategies for efficient and on-site degradation of BPs by multi-enzymatic systems.

4. Conclusion

Degradation of PBSA, PBS, and PBA films by two BP-degrading enzymes, PaE and PCLE, was characterized by LC-MS and SEC. LC-MS analysis suggested that polyester film degradation by these enzymes resulted in the release of oligomers and monomers, irrespective of film composition, and that PaE degraded polyester films into monomers more rapidly than did PCLE under the conditions used. SEC analysis revealed that polyester chains were randomly degraded into oligomers by both enzymes. These results indicate that PaE and PCLE are endo-type enzymes that degrade polyester films randomly into monomer units, and that their characteristics lead to efficient BP film degradation. The use of these enzymes for on-site and on-demand BP degradation may expand the use of BP materials in the agricultural industry, thereby saving labor and reducing plastic waste.

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4 **Figure captions**

5 Fig. 1. Chemical structures of the polyesters used in this study. (A) Poly(butylene
6 succinate-*co*-adipate) (PBSA), (B) poly(butylene succinate) (PBS), and (C)
7 poly(butylene adipate) (PBA).

8 Fig. 2. Total ion current chromatograms (TIC) of the water-soluble products of PaE-
9 catalyzed degradation of a PBSA film for 1 and 4 h. (A) Negative ion chromatograms
10 [TIC(-)] and (B) positive ion chromatograms [TIC(+)]. A, adipate; B, butandiol; S,
11 succinate; and Tris, trishydroxyaminomethane.

12 Fig. 3. TIC(-) of the water-soluble products of PaE-catalyzed degradation of a low-
13 molecular-weight PBSA film by alkaline hydrolysis (L_{Mw}-PBSA) for 1 and 4 h. A,
14 adipate; B, butandiol; and S, succinate.

15 Fig. 4. TIC(-) of the water-soluble products of PaE-catalyzed degradation of (A) PBS, (B)
16 L_{Mw}-PBS, and (C) PBA films for 1 and 4 h. A, adipate; B, butandiol; and S, succinate.

17 Fig. 5. TIC(-) of the water-soluble products of PCLE-catalyzed degradation of (A) PBSA,
18 (B) L_{Mw}-PBSA, (C) PBS, (D) L_{Mw}-PBS, and (E) PBA films for 1 and 4 h. A, adipate; B,
19 butandiol; and S, succinate.

20 Fig. 6. Molecular weight distribution of polyesters in films after PaE-catalyzed

1 degradation of (A) PBSA and (B) PBS films for 4 h (gray lines) and 24 h (dashed lines).

2 Black lines indicate the molecular weight distributions of residual films after incubation

3 without enzyme (buffer only) for 24 h.

4

5

Table 1. Molecular weights of BPs used in this study

	SEC			SEC-MALS		
	M_n ($\times 10^4$)	M_w ($\times 10^4$)	M_w/M_n	M_n ($\times 10^4$)	M_w ($\times 10^4$)	M_w/M_n
PBSA	5.9	8.9	1.5	3.1	5.5	1.7
PBS	4.5	6.7	1.5	2.7	4.5	1.7
PBA	0.87	1.5	1.7	nd	nd	nd
Hydrolyzed sample						
L_{Mw}-PBSA	1.3	2.2	1.7	0.78	0.95	1.2
L_{Mw}-PBS	1.2	1.9	1.5	0.69	0.86	1.2

nd, not determined. M_n, number-average molecular weight; M_w, weight-average molecular weight; M_w/M_n, polyeispersity

1

1

Table 2. Molecular weight of BP films after degradation by PaE or PCLE

Reaction time (h)	PBSA			PBS		
	M_n ($\times 10^4$)	M_w ($\times 10^4$)	M_w/M_n	M_n ($\times 10^4$)	M_w ($\times 10^4$)	M_w/M_n
PaE						
0	5.9	8.9	1.5	4.5	6.7	1.5
4	1.9	8.4	4.4	2.0	6.1	3.0
24	1.5	8.0	5.3	1.4	5.8	4.1
PCLE						
0	5.9	8.9	1.5	4.5	6.7	1.5
4	2.6	8.1	3.1	2.0	6.1	3.0
24	1.4	7.4	5.2	1.7	6.3	3.6

M_n, number-average molecular weight; M_w, weight-average molecular weight; M_w/M_n, polyeispersity

Supplemental data

Journal: Polymer degradation and stability

Electronic supplementary materials

Degradation profiles of biodegradable plastic films by biodegradable plastic-degrading enzymes from the yeast *Pseudozyma antarctica* and the fungus *Paraphoma* sp. B47-9

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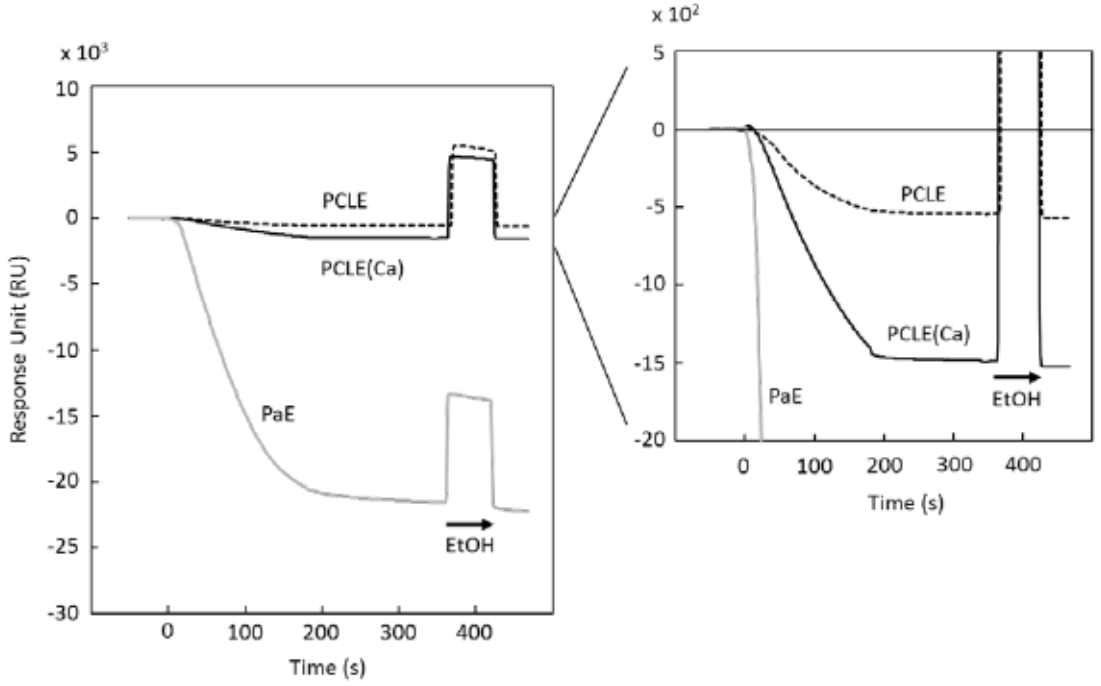
Supplemental materials and methods

PBSA (Bionolle 3020) films were prepared on SPR sensor chips (SIA Kit Au, GE Healthcare, Little Chalfont, UK) by a spin-cast method. PBSA 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 PBSA were stored in glass laboratory dishes at room temperature over 1 week to evaporate the chloroform completely. SPR measurement was performed with BIACORE X (GE Healthcare). For the analysis, HBS-N buffer (10 mM HEPES, 150 mM NaCl, pH 7.4, GE Healthcare) with or without 1 mM $CaCl_2$ was used as running buffer, at 25°C under continuous flow at 20 μl/min. Enzyme solution (PaE or PCLE) diluted at 100 nM 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

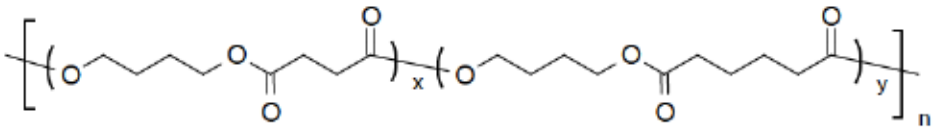
film surface by this treatment was confirmed by the stability of the SPR signal.

Supplemental Data: A SPR sensorgram of degradation of PBSA (Bionolle#3020) film by PaE and PCLE. Each enzyme was injected at a concentration of 100 nM in HBS-N for 180s. Then, after 3 min of buffer flow, the running buffer containing 20 % ethanol was injected for 1 min. The data for PCLE was obtained in HBS-N buffer with and without 1mM $CaCl_2$.



Figures

(A)



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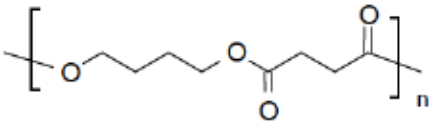

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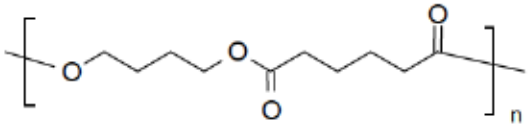


Fig. 1. S. Sato et al.

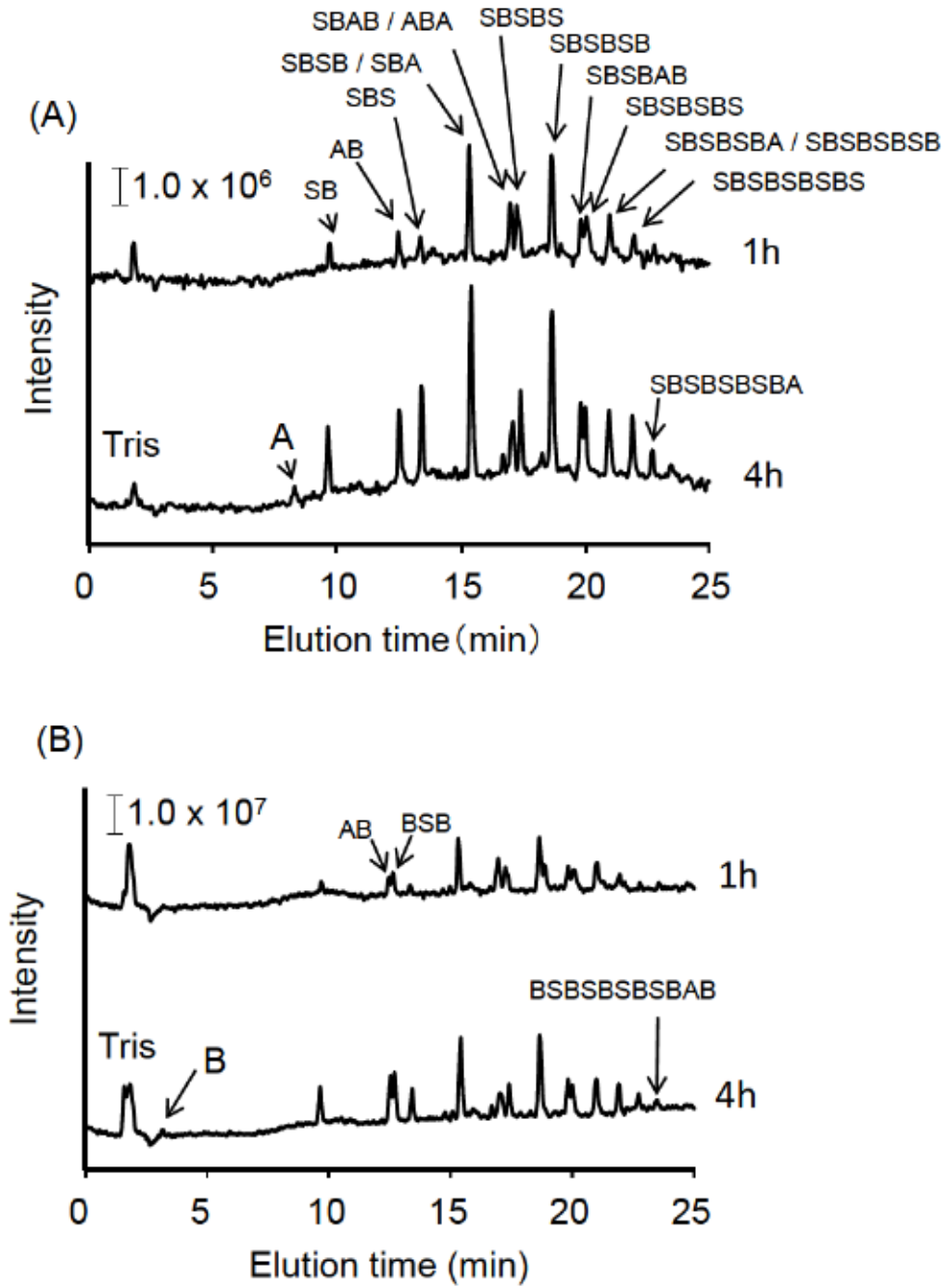
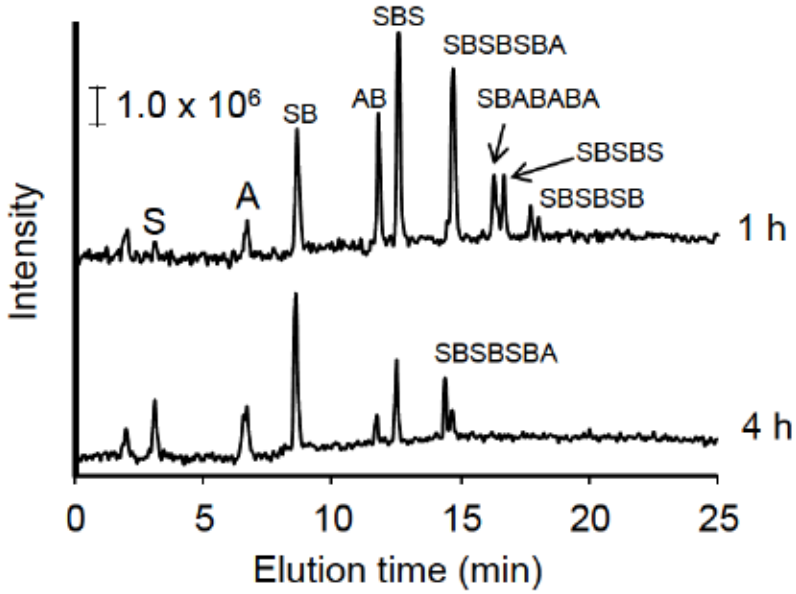


Fig. 2. S. Sato et al.



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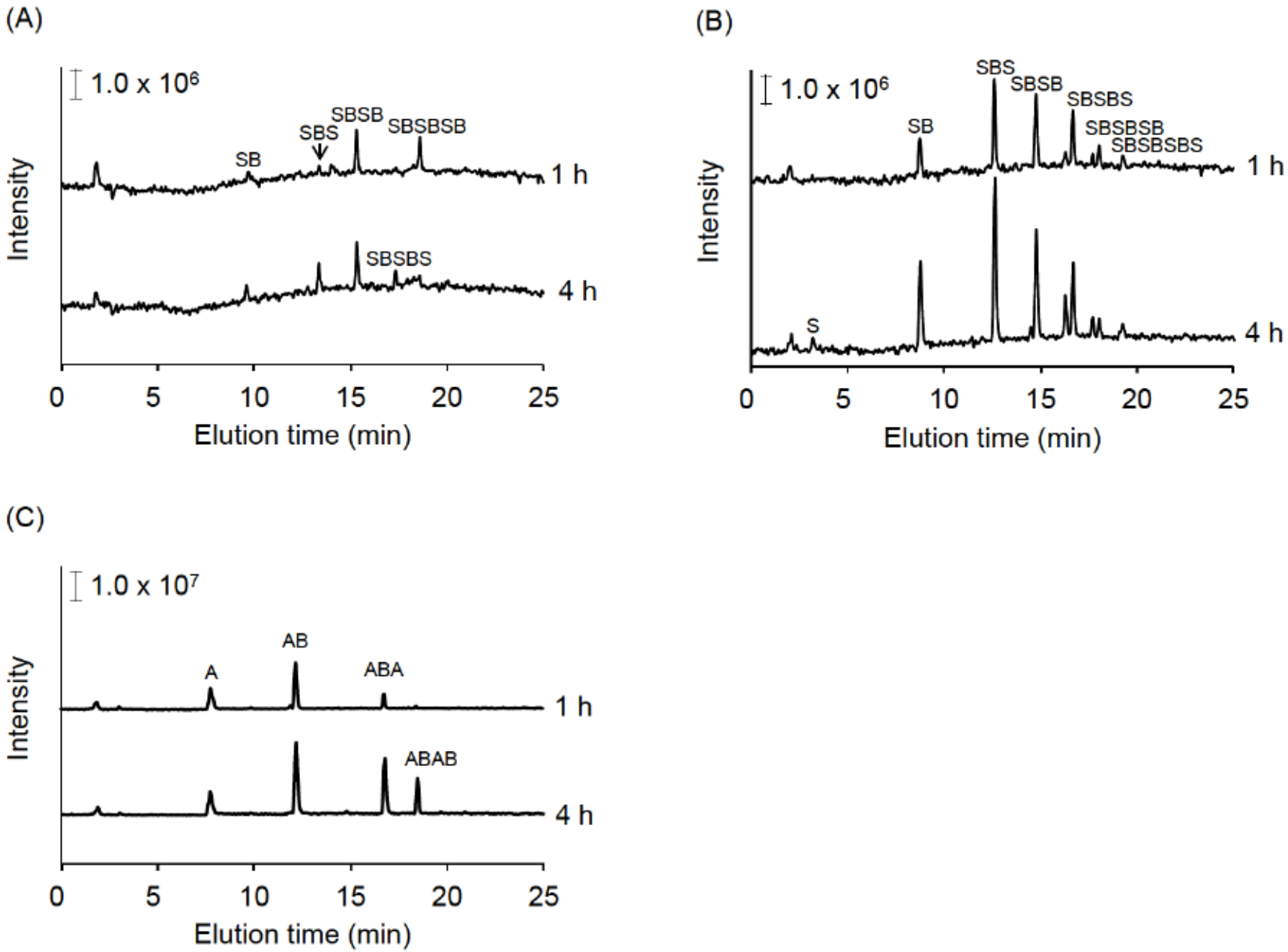

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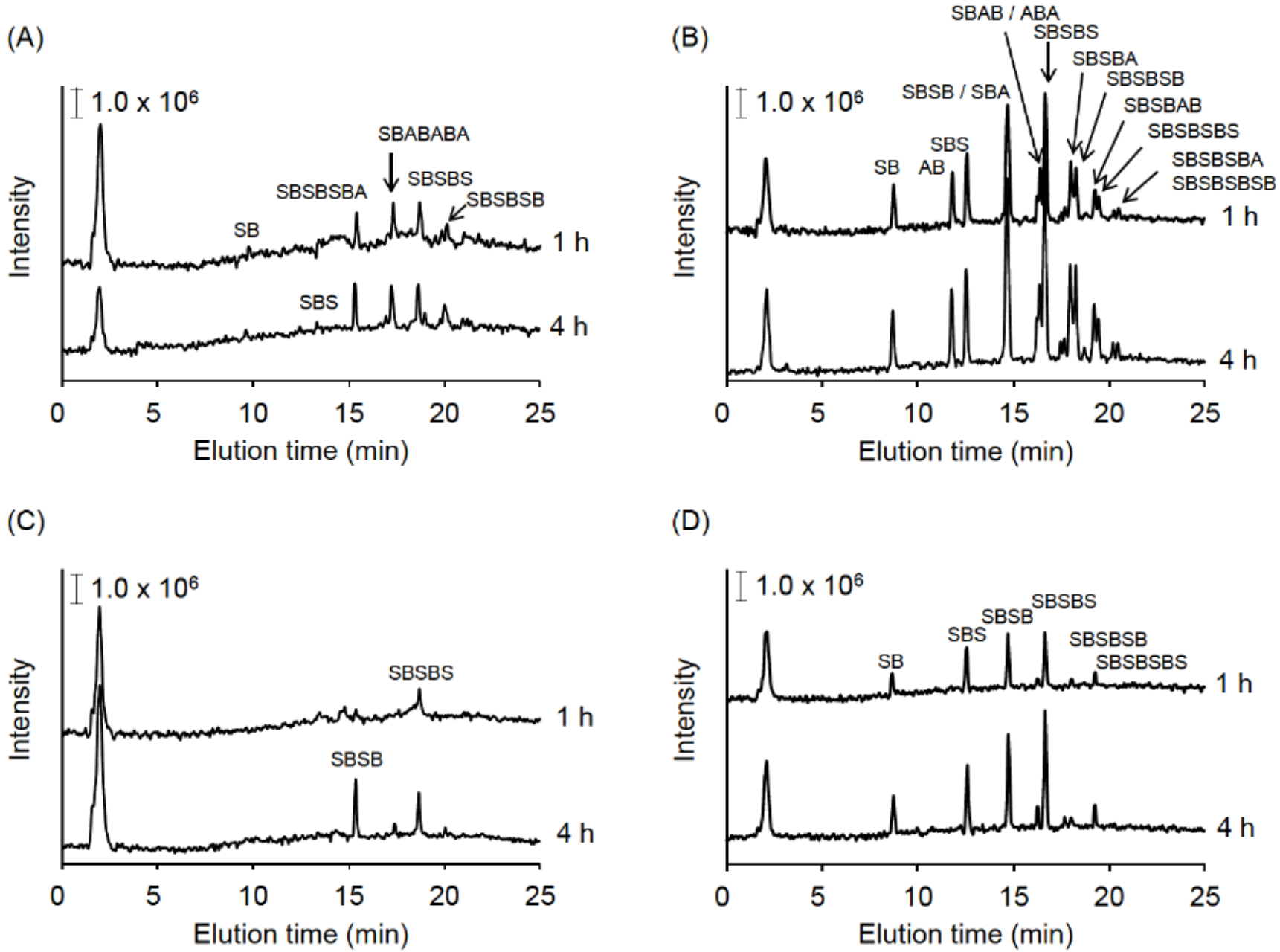

Fig. 5. S. Sato et al.

(E)

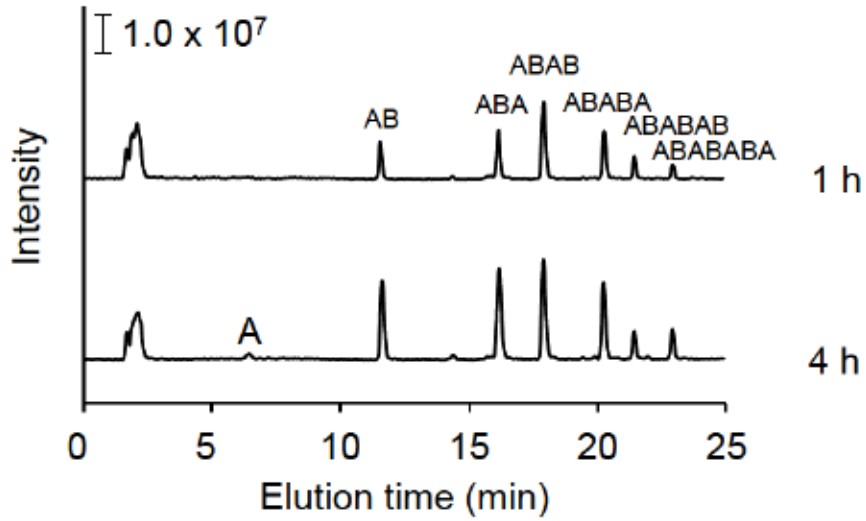

Fig. 5 (continued). S. Sato et al.

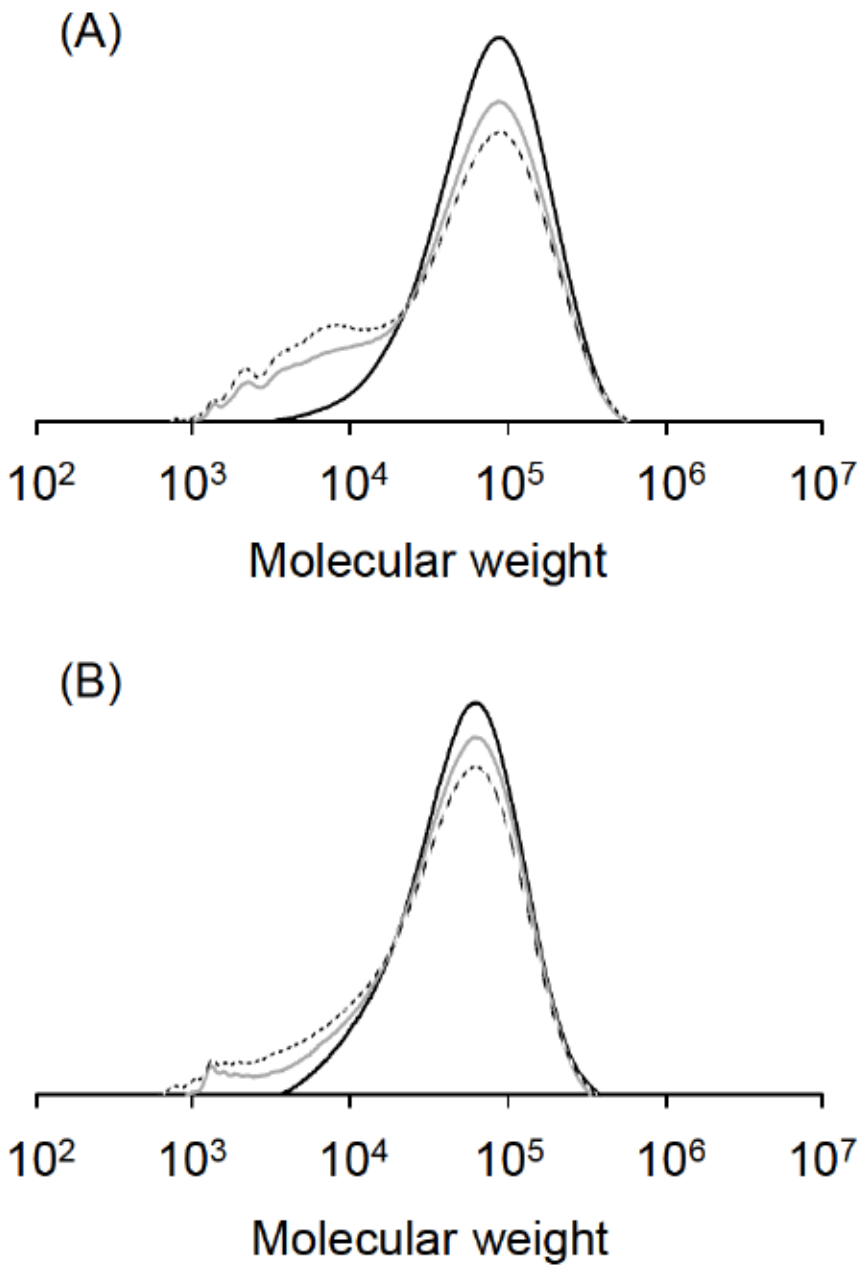

Fig. 6. S. Sato et al.