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Biodegradable plastic-degrading enzyme from *Pseudozyma antarctica*: cloning, sequencing, and characterization

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Abstract *Pseudozyma antarctica* JCM 10317 exhibits a strong degradation activity for biodegradable plastics (BPs) such as agricultural mulch films composed of poly (butylene succinate) (PBS) and poly(butylene succinate-*co*-adipate) (PBSA). An enzyme named PaE was isolated and the gene encoding PaE was cloned from the strain by functional complementation in *Saccharomyces cerevisiae*. The deduced amino acid sequence of PaE contains 198 amino acids with a predicted molecular weight of 20,362.41. High identity was observed between this sequence and that of cutinase-like enzymes (CLEs) (61–68 %); therefore, the gene encoding PaE was named *PaCLE1*. The specific activity of PaE against emulsified PBSA was 54.8±6.3 U/mg. In addition to emulsified BPs, PaE degraded solid films of PBS, PBSA, poly(ε-caprolactone), and poly(lactic acid).

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Introduction

In recent times, synthetic plastics have been widely used as basic materials in various industries. However, they have also caused severe environmental problems because most of these plastics persist for many years after disposal. These synthetic plastics seem inappropriate for applications in which they are used for short periods and then disposed (Gross and Kalra 2002).

Biodegradable plastics (BPs) such as poly(butylene succinate) (PBS), poly(butylene succinate-*co*-adipate) (PBSA), poly(ε-caprolactone) (PCL), and poly(lactic acid) (PLA) have received much attention for their potential to provide solutions for the problems caused by the use of synthetic plastics. BPs can be degraded into water and CO_2 by microorganisms in bioactive environments (Gross and Kalra 2002; Song et al. 2009).

Several microorganisms or enzymes that can degrade BPs have been reported to date (Pranamuda et al. 1997; Nakajima-Kambe et al. 1999; Nakamura et al. 2001; Hoshino and Isono 2002; Akutsu-Shigeno et al. 2003; Hayase et al. 2004; Masaki et al. 2005; Maeda et al. 2005; Seo et al. 2007; Li et al. 2008). However, there are only a few enzymes that can degrade solid BP films and for which the crystal structure and DNA sequence have been analyzed.

Proteinase K is a commercially available enzyme that can degrade PLA film (Iwata and Doi 1998; Oda et al. 2000); its crystal structure has already been analyzed and reported

(Betzel et al. 1988). Cutinase from *Aspergillus oryzae* has been reported to degrade PBS and PBSA films (Maeda et al. 2005). Recombinant cutinases of *Fusarium solani* and *A. oryzae* were expressed in *Pichia pastoris*, and the relationship between their PCL film degradation activities and the structures has been analyzed (Liu et al. 2009).

Another well-studied enzyme is the cutinase-like enzyme (CLE) from the yeast *Cryptococcus* sp. strain S-2, which can degrade emulsified PBS, PCL, PLA (Masaki et al. 2005) and PLA in solid films (Kawai et al. 2011). The cDNA sequence and crystal structure of CLE have been analyzed by Masaki et al. (2005) and Kodama et al. (2009). Furthermore, Masaki et al. (2005) reported that CLE was more effective than proteinase K in degrading emulsified PLA. Kawai et al. (2011) showed the degradation of PLA in a solid film by CLE by analyzing weight loss, molecular weight shifts, and polydispersity changes. However, to date, no studies have compared the specific degradation activities of this enzyme for various BP films.

We have previously reported that *Pseudozyma* spp., common yeasts in the phyllosphere that are easily isolated from plant surfaces, displayed strong degradation activity on agricultural mulch films composed of PBS and PBSA (Kitamoto et al. 2011). The BP-degrading enzyme named PaE was isolated from *Pseudozyma antarctica* JCM10317, which showed the strongest degradation activity among the tested strains. Enzymes that were immunologically identical to PaE were detected in the culture broth of various *Pseudozyma* spp. strains but not in the culture broth of *Cryptococcus* spp. strains despite their BP-degrading activity (Kitamoto et al. 2011), indicating that the structure of PaE may be different from that of enzymes produced by *Cryptococcus* spp. strains. To use PaE for applications such as degrading agricultural mulch films in crop fields, it is important to clarify its characteristics so that users can specify suitable operational conditions. In this study, the basic characteristics of PaE were analyzed, and the PaE-encoding gene of *P. antarctica* JCM10317 was cloned by functional complementation in *Saccharomyces cerevisiae* to analyze its structural characteristics. In addition, the ability of PaE to degrade several solid BP films (PBS, PBSA, PCL, and PLA) was evaluated visually and by measuring the amount of total organic carbon (TOC) eluted from the films.

Materials and methods

Substrate and chemicals

The BPs used in this study are listed in Table 1. To prepare PLA cast films, 0.1 g PLA pellet (M_w, 1.3×10^5) was dissolved in 5 ml dichloromethane, and the solution was then cast on a 9-cm diameter glass laboratory dish and dried at room temperature overnight.

Table 1 Biodegradable plastics used in this study

Plastic	Characteristics and/or source
Emulsified plastic	
PBSA	Bionolle[a] EM-301, M_w 1.2–1.5×10^5
Plastic sources (pellet) for hand-made cast film	
PBSA	Bionolle[a]#3020, M_w 1.4×10^5
PCL	M_w 0.7–1.0×10^5
	Wako Chemicals, Osaka, Japan
PLA	PLLA: poly(L-lactic acid), M_w 1.3×10^5
	Toyota Motor Co. Ltd. (Aichi, Japan)
	PLA-0020: poly(DL-lactic acid), M_w 2×10^4
	Wako Chemicals, Osaka, Japan
Agricultural mulch film	
PBS film	Bionolle[a] 1001 G, M_w 2.0-2.5×10^5, thickness 20 μm
PBSA film	Bionolle[a] 3001 G, M_w 2.0–2.5×10^5, thickness 20 μm

[a] All the Bionolle materials were supplied by Showa Denko K.K. (Tokyo, Japan)

M_w: weight-average molecular weight

p-Nitrophenyl (pNP) esters (pNP-acetate, pNP-butyrate, pNP-valerate, pNP-octanoate, pNP-decanoate, pNP-dodecanoate, pNP-palmitate, and pNP-stearate; Sigma-Aldrich, St. Louis, MO) were used to assay esterase activity.

Strains and media

P. antarctica JCM10317 was obtained from the culture collection of the Japan Collection of Microorganisms (JCM) of the Riken BioResource Center in Wako, Japan. *Ustilago maydis* MAFF 236374, 236375, 236376, 236377, and 236378 were obtained from the NIAS Gene Bank (Tsukuba, Japan). Laboratory yeast strains of *S. cerevisiae* BY4741 were obtained from EUROSCARF (Frankfurt, Germany). These strains were incubated at 30 °C for 3 days on malt-yeast-glucose-peptone (YM) agar medium containing 1 % glucose, 0.5 % peptone, 0.3 % yeast extract, 0.3 % malt extract, and 1.5 % agar. Seed cultures were prepared by inoculating cells grown on YM agar plates into flasks containing fungal minimum medium (FMM) with 4 % glucose and incubating them at 30 °C in a rotary shaker at 220 rpm for 4 days. FMM was composed of 0.2 % $NaNO_3$, 0.02 % $MgSO_4$, 0.02 % KH_2SO_4, and 0.1 % yeast extract, which were dissolved in tap water before being autoclaved.

Purification of PaE

PaE was produced from *P. antarctica* JCM10317 as described previously (Kitamoto et al. 2011). Crude PaE solution was prepared by ammonium sulfate precipitation. The crude PaE was further purified by ion-exchange

chromatography and gel filtration as described previously (Kitamoto et al. 2011). The purified PaE was stored in 20 mM Tris–HCl (pH 7) at 4°C until use.

Determination of protein concentration

The purified PaE solution was appropriately diluted, and its absorbance was measured at 280 nm (A_{280}). The concentration of PaE was calculated according to the following equation:

A_{280} (M^{-1} cm^{-1})=5,500 n_W+1,490 n_Y+125 n_C (Pace et al. 1995; Kelly et al. 2005), where n_W, n_Y, and n_C are the number of Trp (=1), Tyr (=7), and Cys (disulfide bond=2) residues per polypeptide chain and M is the molecular mass (=20,362.41). These numbers were based on the deduced amino acid sequence of PaE described in the results (Fig. 2), and M was the average molecular weight calculated using GENETYX software ver. 9 (GENETYX, Tokyo, Japan).

Protein analysis of PaE

Purified PaE was applied for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method described by Laemmli (1970) by using a 14.1 % polyacrylamide slab gel. The gel was stained with Coomassie brilliant blue (CBB; PhastGel Blue R, GE Healthcare, Little Chalfont, UK). A single CBB-stained protein spot was subjected to internal peptide sequencing by the method described by Shevchenko et al. (1996). The protein separated by gel electrophoresis was electroblotted onto a polyvinylidene difluoride (PVDF) membrane (Pall, Port Washington, NY) and detected by CBB staining. The stained protein was excised from the PVDF membrane and directly subjected to Edman degradation on a PPSQ-23A gas-phase protein sequencer (Shimadzu, Kyoto, Japan). The N-terminal amino acid sequence was analyzed using an ABI Procise 491HT (Applied Biosystems, Foster City, CA).

Isolation and identification of the gene encoding PaE

P. antarctica JCM 10317 was cultivated in FMM-6 % glycerol liquid medium at 30 °C for 2 days and total RNA was extracted by the procedure described by Schmitt et al. (1990). The mRNA was purified from total RNA by using an Oligotex-dT30 (Super) mRNA purification kit (Takara, Shiga, Japan). The internal DNA fragments coding for PaE was synthesized from mRNA using a PrimeScript reverse transcriptase PCR (RT-PCR) kit (Takara) with primers designed on the basis of internal amino acid sequences of purified PaE (Fig. 2, underlined): PaE3F (5′-tacgtSatcatcaacacVcg-3′), PaE4F (5′-atcggcaacccNgagcac-3′), PaE-4R (5′-cttgtgctcNgggttgcc-3′) and PaE-6R (5′-gacgccctgBgtgaaNgc-3′). PCR was performed in 35 cycles; 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min. The amplified DNA fragments were purified using a GeneElute PCR Clean-Up Kit (Sigma-Aldrich), and the fragments were sequenced with the same primers using a 3100 Genetic Analyzer (Applied Biosystems) with a BigDye Terminator v.3.1 cycle sequencing kit (Applied Biosystems). From the sequences, primers PaE134R (5′-atgcgggtgttcatggtgcg-3′) and PaE308F (5′-atgcccttcctcagcttactg-3′) were designed for the subsequent inverse PCR (Ochman et al. 1988).

Genomic DNA of *P. antarctica* JCM 10317 was isolated with a genomic DNA isolation kit (GenTLE; Takara) from freezed and destroyed cell. The locus number in the genome was estimated by a general Southern blot transfer and hybridization after digestion of the genomic DNA with *Bam*HI, *Eco*RI or *Hin*dIII. The probe was prepared from a PCR fragment amplified with the primer pair PaE4F/PaE6R and was detected using the AlkPhos Direct Labeling and Detection System (GE Healthcare).

Self-circularization of the 1–4 kbp of the *Bam*HI digested genomic DNA was obtained using Ligation High (TOYOBO, Osaka, Japan). Inverse PCR was performed using a standard reaction mixture containing 2 μl of ligated DNA as the template and PaE134R and PaE308F as primers. The PCR products were cloned into pGEM-T vectors (Promega, Madison, WI) and the sequence was analyzed. Based on the sequence, primers PaE-20 F (5′-cccttccgatctatctcaaga-3′) and PaE+16R (5′-atggacgaggctacgatctg-3′) were designed upstream and downstream of the predicted open reading frame (ORF). With these primers, the full length of the ORF gene from genomic DNA and cDNA synthesized from mRNA were obtained.

Identification of the BP-degrading enzyme by functional complementation in *S. cerevisiae*

The PCR products harboring the complete PaE-encoding gene were cloned under the *GAL1* promoter of expression vector for *S. cerevisiae* (pYES2.1; Invitrogen, Carlsbad, CA) and were transformed into *S. cerevisiae* BY4741. The PBSA emulsion-degrading activity of the transformant was detected using a synthetic minimum medium (yeast nitrogen base without amino acids; Difco, BD Diagnostics, Sparks, MD) containing 1 % PBSA emulsion and 2 % glucose or galactose. The degradation activities of the liquid culture of the transformants with and without the PaE-encoding gene were analyzed in the medium after 17 h of cultivation at 30 °C and 220 rpm.

Assay of BP-degrading enzyme activity

The emulsified PBSA-degrading activity was assayed as described previously (Kitamoto et al. 2011) with the following modifications: emulsified PBSA (Bionolle EM-301) at a

concentration of 0.02 % (*w*/*v*) in 20 mM Tris–HCl buffer (pH 8.8), and purified PaE (final concentration, 7–10 nM) were mixed to a volume of 2 ml in a glass test tube (10 mm internal diameter) and incubated at 30°C for 15 min. The percentage transmittance of the BP emulsion was measured at a wavelength of 660 nm as the reduction of absorbance against a blank (emulsified PBSA without enzyme). One unit of PBSA emulsion-degrading activity was defined as a 1 U/min decrease in A_{660}.

The optimal pH of PaE was determined using the same conditions as the standard assay excluding the buffer. The following buffers (10 mM) were used: sodium acetate (pH 4.0–6.0), potassium phosphate (pH 6.0–8.0), and Tris–HCl (pH 7.5–10.0).

The optimal temperature of the enzyme was determined using the same condition as the standard assay excluding the temperature (20–60 °C). The effect of temperature on PaE stability was determined using the residual activity after 60 min of pre-incubation in 20 mM Tris–HCl buffer (pH 8.8) at various temperatures.

Assay for esterase activity

Esterase activity was assayed as described previously (Kitamoto et al. 2011), and pNP esters with different alkyl chain lengths were used as substrates. One unit of esterase activity was defined as the release of 1 μmol of *p*-nitrophenol per minute.

Observation of enzymatic BP film degradation

Degradation of PLA cast film and commercially available agricultural mulch films (PBS and PBSA) by PaE were assessed using the modified method described by Maeda et al. (2005). Paper filters (6-mm diameter, Advantec Toyo Kaisha, Ltd., Tokyo, Japan) were wetted with 30 μl PaE solution, placed on top of the film, and incubated at 30 °C.

To observe the degradation of PLA film, a crude enzyme solution (20 mM Tris–HCl, pH 8.8) containing 0.26 μM PaE (estimated from the degradation activity for PBSA emulsion) was placed on the PLA cast film and incubated at 30 °C for 72 h. To compare the extent of PLA degradation, proteinase K solution (8 μg/ml, 0.28 μM) was also used. After incubation, the paper filter was removed, and the PLA film was washed with Milli-Q water and dried at room temperature. The PLA film was coated with a gold-palladium layer in an ion sputter (E-1010, Hitachi, Tokyo, Japan) and was observed by scanning electron microscopy (SEM) using a JSM-5610LV (JEOL Ltd., Tokyo, Japan) with a voltage of 10 kV. To observe PBS and PBSA film degradation, 0.9–7 μM purified enzyme in 20 mM Tris–HCl buffer (pH 8.8) was used. After incubation at 30 °C for 24 h, the paper filters were removed, and the black mulch films were scanned using a transparency scanner. The whiteness of the resulting image in the film indicated the extent of degradation.

Evaluation of enzymatic BP film degradation

Pellets of the test BPs, namely PBSA, PCL, and PLA (M_w, 2×10^4), were each dissolved at 1 wt% concentration in dichloromethane, and the mixtures were applied on a water-repellent printing glass slide (Matsunami Glass Ind. Ltd., Osaka, Japan) at 20 μl in each well (7-mm diameter) and air-dried at room temperature for 24 h. The BP films on the glass slide were washed twice with 80 μl of distilled water. Next, 50 μl of purified PaE (130 nM in 20 mM Tris–HCl, pH 8.8) was placed onto the BP films. To avoid drying, the glass slide was put into a small container lined with wet paper, sealed, and incubated at 30 °C for 24 h. The enzyme solutions were collected, diluted with 20 ml Milli-Q water, and then filtered through a 0.45-μm membrane (DISMIC-25, PTFE; Toyo Roshi Kaisha, Ltd.). The water-soluble TOC was measured using a TOC-V CSH analyzer (Shimadzu Co., Kyoto, Japan). The substrate and enzyme controls were prepared by omitting the enzyme or the substrate from the reaction, respectively. The TOC released by BP film degradation was obtained after subtracting the TOC of the enzyme control (without substrate).

To confirm the products obtained from PLA film degradation by PaE, PLA cast films were prepared and treated with the purified PaE solution, as described above. After PLA cast film-degradation, the D,L-lactic acid concentrations in the reaction solutions were determined using an F-kit (Cat. No. 1112821; R-Biopharm AG, Darmstadt, Germany).

Molar ratio of succinate and adipate in PBSA was determined by the liquid-state nuclear magnetic resonance (NMR) technique. PBSA pellet was dissolved in chloroform-*d* ($CDCl_3$), and ^1H and ^{13}C NMR signals were determined at 600 and 151 MHz by using a NMR spectrometer (Alpha 600, JEOL Ltd.). Chemical shifts were quoted with respect to tetramethylsilane but were determined by referring to the residual ^1H and ^{13}C signals of the solvent (chloroform). The ^{13}C NMR spectrum was used for assignment of the signals, and the ^1H NMR spectrum was used for determining the molar ratio of the monomers.

Results

Degradation of PLA cast film by crude PaE solution

To analyze the possibility that PaE can degrade various BPs, crude PaE solution was prepared by ammonium sulfate precipitation, and its PLA cast film-degrading activity was

evaluated. After treatment of the PLA film with crude PaE solution, the surface of the transparent film became frosted (data not shown). This result indicated that PaE degraded the surface of PLA film; therefore, SEM observation of the film was performed. The results showed that the surface of the PLA film remained smooth in the control (without enzyme) (Fig. 1a). In contrast, after treatment with crude PaE solution, the film surface became rough, and many holes were formed (Fig. 1b). The same result was obtained with proteinase K (Fig. 1c), which is already known to degrade PLA film (Iwata and Doi 1998; Oda et al. 2000). PaE treatment resulted in the formation of many long holes that were scattered along the surface of the film, whereas proteinase K treatment resulted in irregularly shaped holes.

Isolation and identification of the BP-degrading enzyme-encoding gene from *P. antarctica*

PaE was purified to electrophoretic homogeneity by ion-exchange chromatography and gel filtration (Kitamoto et al. 2011). A single CBB-stained protein spot was subjected to Edman degradation, and the N-terminal and internal peptide sequences of PaE were analyzed (Fig. 2, underlined). By using degenerate primers designed on the basis of these amino acid sequences, the DNA fragment containing the gene encoding PaE was isolated from the genomic DNA of *P. antarctica* JCM10317, cloned, sequenced (GenBank accession number DM067526), and single locus in the genome was detected as PaE gene by Southern hybridization (data not shown). The cDNA sequence showed 100 % identity with the gene fragment isolated from the genomic DNA. A yeast strain (*S. cerevisiae*) with a plasmid pYES2.1 harboring the predicted ORF of the PaE-encoding gene degraded PBSA emulsions on agarose medium under induction but not under suppression conditions (data not shown). The degradation activity of the liquid culture broth of the transformant under induction conditions was 6.8 mU/ml, whereas the transformant without the gene fragment showed a degradation activity of only 0.2 mU/ml. These results indicated that the gene required for PaE activity had been successfully isolated.

The predicted amino acid sequence of PaE was compared with those in the DNA Data Bank of Japan (DDBJ; on 14 April 2011, by tblastn program). The sequences of two cutinase-like genes of *U. maydis*—Q4PBK7_USTMA and Q4PHG8_USTMA—showed the highest identity to PaE (68 % and 63 %, respectively). *U. maydis* is a pathogenic basidiomycete fungus that infects maize; the entire genome of this fungus has been sequenced and registered with the DDBJ (Kämper et al. 2006). All five strains of *U. maydis* obtained from a culture collection (strain Nos. MAFF236374-MAFF236378) degraded the PBSA emulsion, but not the PBSA cast film, within 7 days at 25 °C (data not shown). In the same tblastn search of the genes with confirmed cutinase activity, a cDNA sequence of CLE from *Cryptococcus* sp. strain S-2 (*CLE1:* GenBank accession number AB102945) (Fig. 2) showed the highest identity (61 %) to the predicted amino acid sequence of the gene encoding PaE, therefore the gene was named as *PaCLE1*.

Characterization of purified PaE

The concentration of purified PaE was determined based on A_{280} and the number of Trp, Tyr, and Cys (disulfide bond) residues in the sequence. The concentration was approximately 3.3-fold higher than that determined in the previous report by using a protein assay kit (Bio-Rad Laboratories, Hercules, CA) (Kitamoto et al. 2011).

For emulsified PBSA, purified PaE showed a specific activity of 54.8±6.3 U/mg; maximum degradation activity was observed at pH 9.5 (Fig. 3a). The optimal temperature was 40°C; however, its activity declined to 44.8 % after incubation at 40°C for 60 min (Fig. 3b).

Short-chain-length substrates such as pNP-valerate (C5) were the preferred substrates for PaE; however, even with long-chain substrates such as pNP-stearate (C18), approximately 28 % of the maximal activity still remained (Fig. 4).

Agricultural mulch film degradation by purified PaE

The agricultural mulch film (PBS and PBSA) degradation activity of the purified PaE was analyzed using the modified method described by Maeda et al. (2005) (Fig. 5a). The extent of decomposition differed based on the enzyme concentration used. The surface of the PBS film became rough when treated with 0.9–1.8 μM PaE for 24 h. Higher PaE concentrations (3.5–7 μM PaE) caused the PBS film to be pierced with holes and partly dissolved. In the case of PBSA film, similar results were observed, and treatment with 3.5–7 μM PaE resulted in complete dissolution.

Evaluation of BP cast film degradation by purified PaE

To evaluate the BP cast film-degrading activity of PaE, water-soluble TOC released from the BP cast film was measured after treatment with purified PaE solution. In the substrate controls (without PaE), TOC increased only slightly (<2 μg/50 μl) after incubation at 30 °C for 24 h. In contrast, a significant increase in TOC was observed for all of the tested BP films. The amount of TOC released from BP films of PBSA, PCL, and PLA was 72.3±5.0, 77.7±6.9, and 50.4±13.8 μg/50 μl, respectively (Fig. 5b).

The amount of D,L-lactic acid released from PLA cast films after degradation by PaE was measured with the F-kit. The results showed an increased amount of D,L-lactic acid (26.0±1.2 and 18.4±0.7 μg/50 μl, respectively) compared

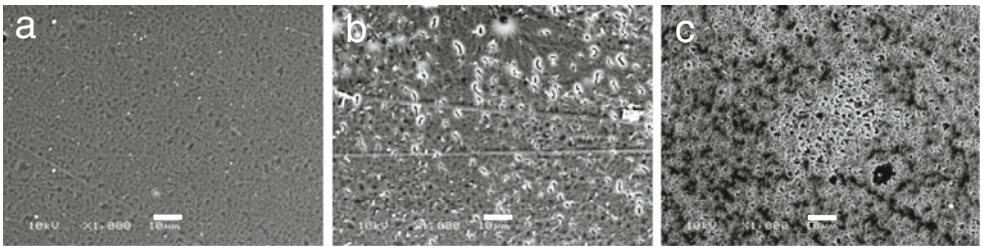

Fig. 1 Surface structures of PLA cast films after treatment with **a** 20 mM Tris–HCl buffer, pH 8.8 (control, without enzyme), **b** crude PaE solution (0.26 μM), and **c** proteinase K (8 μg/ml, 0.28 μM) (bar, 10 μm)

to the control (without PaE; <0.12 μg/50 μl). Values are expressed as the mean±SD, at n=3.

Discussion

Studying the enzymatic degradation of commercial BPs such as agricultural mulch films is important for the application of BPs. In our previous study, a BP-degrading enzyme named PaE was obtained from *P. antarctica* JCM10317. In the present study, we characterized PaE and the PaE-encoding gene. We found that purified PaE could degrade solid films of PBS, PBSA, PCL, and PLA. We visually confirmed the degradation of materials and evaluated the amount of degradation by measuring the incremental amount of TOC content in the eluate.

Because PLA film is less susceptible to degradation at moderate temperatures (Hoshino and Isono 2002), we first examined whether the treatment of crude PaE visibly frosted the clear PLA cast film (high-molecular weight, 130,000) at 30°C. The SEM observation showed that the surface of the film was etched by treatment with crude PaE solution (Fig. 1b) in a manner similar to that observed for proteinase K (Fig. 1c), which is already known to degrade PLA films. We then carried out a detailed analysis of the degradation of PLA films and other BP films by purified PaE.

To date, the only known PLA-degrading enzymes are protease-type enzymes such as proteinase K, and lipase (cutinase)-type enzymes such as CLE from *Cryptococcus* sp. strain S-2 (Kawai et al. 2011). In addition to PLA, CLE is known to degrade other BPs such as emulsified PBS and PCL (Masaki et al. 2005). However, studies comparing the specific degradation activities of lipase (cutinase)-type enzymes against various BP films have not yet been conducted.

The deduced amino acid sequence of PaE contains 198 amino acids with a predicted molecular weight of 20,362.41. The amino acid sequence showed 61 % identity with CLE, as determined from the cDNA of the yeast *Cryptococcus* sp. strain S-2. Therefore, the gene encoding PaE was named *PaCLE1*.

Five cysteine residues are present in CLE, and they are known to form disulfide bonds (Cys6–Cys78 and Cys161–Cys168, Kodama et al. 2009) that correspond to Cys3–Cys75 and Cys156–Cys163 in PaE (Fig. 2). The catalytic site in CLE, which contains a typical triad, corresponds to Ser82, Asp160, and His175 in PaE.

Kodama et al. (2009) compared the specific activity of *F. solani* cutinase with CLE and reported that *F. solani* cutinase exhibits catalytic activity against short-chain triglycerides and a low activity against long-chain substrates. In contrast, CLE, which exhibits homology to cutinase, has a broad substrate specificity for long-chain substrates as

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                 *
PaE   1  AGCSSYVIINTRGTSEPQGPSVGFRTMNTRIRSAVSGGSEYDTVYPAGIDQNSAQGTANI  60
         + C  YV+INTRGT EPQG S GFRTMN++I +A+SGG+ Y+TVY A   QNSA GTA+I
CLE   4  SACPQYVLINTRGTGEPQGQSAGFRTMNSQITAALSGGTIYNTVYTADFSQNSAAGTADI  63

                    *   ▼
PaE  61  VAQVKAGLARNPNTCFLLEGYSQGAAATCNALPQL--TGAAFDAVKGVILIGNPEHKPNL  118
         + ++ +GLA NPN C++L+GYSQGAAAT  AL QL  +GAAF+AVKGV LIGNP+HK  L
CLE  64  IRRINSGLAANPNVCYILQGYSQGAAATVVALQQLGTSGAAFNAVKGVFLIGNPDHKSGL  123

                                                *  ▼  *          ▼
PaE 119  ACNVDGNGGKTTFSARGISAAFTQGVPSNWVSKTLDICIYGDGVCDVSSGFGITPQHLTY  178
          CNVD NGG TT +  G+S A+    VPS WVSKTLD+C YGDGVCD + GFGI  QHL+Y
CLE 124  TCNVDSNGGTTTRNVNGLSVAYQGSVPSGWVSKTLDVCAYGDGVCDTAHGFGINAQHLSY  183

PaE 179  GYNTNVQTMGANFGIKALQG  198
           +  VQTMG  F +  L G
CLE 184  PSDQGVQTMGYKFAVNKLGG  203
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Fig. 2 Alignment of the amino acid sequences of PaE and cutinase-like enzyme (CLE) without the signal peptide. The three residues comprising the catalytic triad are indicated by *arrows*. The *boxes* show the hydrophobic residues important for the hydrolytic activity of the enzymes against long-chain substrates. The conserved cysteine residues that form disulfide bonds are indicated by *asterisk*. The amino acid sequences of purified PaE, analyzed by Edman degradation, are underlined. The origins of the enzyme and their GenBank accession numbers are as follows: PaE (*P. antarctica* JCM 10317, DM067526); CLE (*Cryptococcus* sp. strain S-2, AB102945)

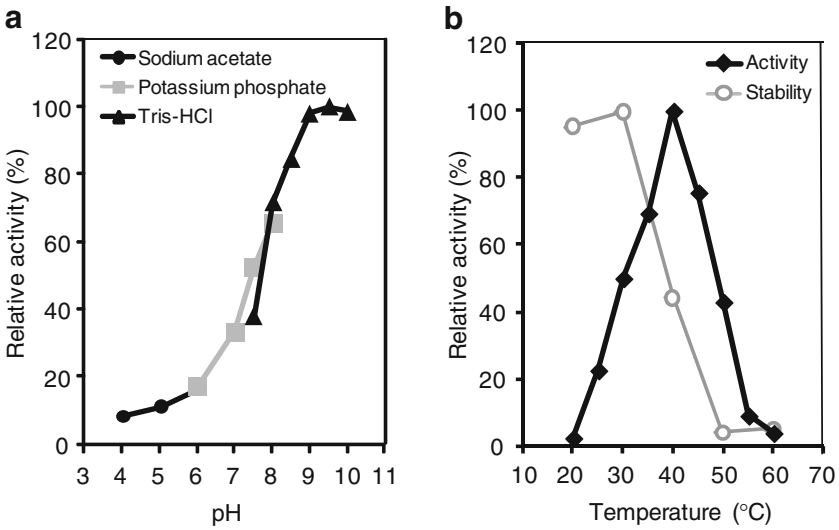

Fig. 3 **a** Effects of pH on the degradation of emulsified PBSA by PaE. **b** Effects of temperature on the degradation of emulsified PBSA by PaE. The optimal temperature was evaluated by measuring the activity at different temperatures and pH 8.8. Thermostability was measured at 30 °C and pH 8.8 after incubating the enzyme for 60 min at various temperatures at pH 8.8. The values are the means of duplicate experiments

compared with *F. solani* cutinase (2009). They also reported that residues Phe52, Tyr145, and Phe174 in CLE, located close to the catalytic triad and exposed to the solvent despite their hydrophobic properties, are important for the hydrolytic activity of CLE against long-chain substrates. The ratio (pNP-palmitate/pNP-butyrate) of hydrolysis activity decreased when Phe52, Tyr145, and Phe174 were substituted with less hydrophobic amino acids (Kodama et al. 2009). The amino acids Ile49, Phe140, and Phe169 in PaE correspond to Phe52, Tyr145, and Phe174, respectively, in CLE and have similar or greater hydrophobic properties. In fact, similar to CLE, PaE also showed relatively higher activity against long-chain substrates (Fig. 4). Because of these hydrophobic amino acids, PaE also had relatively high activity against long-chain substrates. Further structural research is needed to demonstrate whether the differences in specific activity arise from the sequences conserved in yeasts.

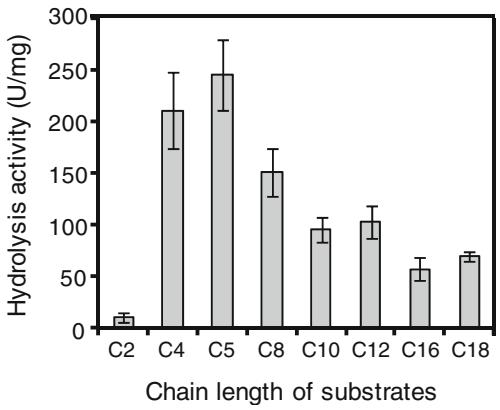

Fig. 4 Hydrolysis of pNP-acetate (C2), pNP-butyrate (C4), pNP-valerate (C5), pNP-octanoate (C8), pNP-decanoate (C10), pNP-dodecanoate (C12), pNP-palmitate (C16), and pNP-stearate (C18) by PaE. The average and SD of hydrolysis activities were obtained from three independent experiments

The structural characteristics of PaE were similar to those of CLE, as described above; however, its optimal pH was different from that of CLE, which exhibited maximal activity at pH 7.0 (olive oil as the substrate) (Kamini et al. 2000). Under the same buffer conditions, PaE displayed maximal activity at pH 9.5 (emulsified PBSA as the substrate); only 33 % of its relative activity remained at pH 7.0 as compared to that at pH 9.5 (Fig. 3a).

Liu et al. (2009) investigated the thermostability of cutinases from *A. oryzae* and *F. solani* and reported that the level of activity was maintained up to 40°C in the case of *A. oryzae* cutinase; in contrast, *F. solani* enzyme activity revealed a 40 % drop after incubation at 30°C for 60 min. In the present study, PaE activity was maintained at 30°C; however, its activity decreased to less than half after incubation at 40°C for 60 min (Fig. 3b). These results indicate that thermostability of PaE was higher than that of the *F.*

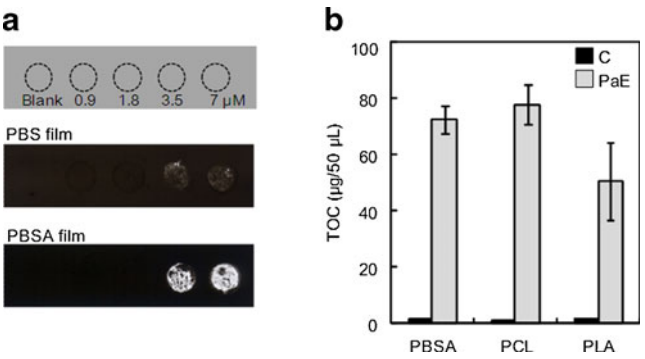

Fig. 5 **a** Degradation of agricultural mulch films (PBS and PBSA) by purified PaE. PBS and PBSA films (20-μm thick) were treated with the purified enzyme at concentrations of 0.9–7 μM in 20 mM Tris–HCl buffer (pH 8.8). After treatment, the films were scanned using a transparency scanner. **b** The eluted TOC from BP cast films after treatment with 20 mM Tris–HCl buffer, pH 8.8 (control, without enzyme; indicated by the *black column*) or purified PaE solution (130 nM in 20 mM Tris–HCl, pH 8.8; indicated by the *gray column*). Values are expressed as the mean (SD) at $n=3$

solani enzyme, but lower than that of *A. oryzae* cutinase. They also reported that the improved thermostability of *A. oryzae* cutinase is attributed to a unique disulfide bond (Cys63–Cys76), which is present in addition to two well-conserved disulfide bonds in previously published cutinase structures (Liu et al. 2009; Prompers et al. 1999), Cys6–Cys78 and Cys161–Cys168 in CLE, also found in PaE (Fig. 2, indicated by an asterisk). An additional cysteine residue is also present in both CLE and PaE (Cys125 in CLE and Cys120 in PaE), but only PaE contains additional Cys89, suggesting the possibility of formation of another disulfide bond. Further study is needed to detect the presence of a unique disulfide bond and to discuss the relationship between the structure and thermostability of PaE.

Evaluation of the degradation of several BP cast films by purified PaE solution showed that PBSA, PCL, and low-molecular-weight (20,000) PLA were solubilized as water-soluble compounds (Fig. 5b). The approximate weight of each BP film used in the analysis was 200 μg. The carbon contents of PBSA, PCL, and PLA films were 56.6 %, 63.2 %, and 50.0 %, respectively, based on each structure (the molar ratio of succinate and adipate in PBSA film was 81.7 % and 18.3 %, respectively, by the ^{1}H NMR spectrum). If these films were completely degraded, the TOC released from PBSA, PCL, and PLA films would be 113.2, 126.3, and 100 μg, respectively. Based on the actual TOC values measured after treatment with PaE (72.3, 77.7, and 50.4 μg (Fig. 5b)), the rate of degradation to water-soluble compounds from PBSA, PCL, and PLA films were calculated to be 63.9 %, 61.5 %, and 50.4 %, respectively. Because the BPs used in this study have different molecular weights, further comparative studies using BPs with similar molecular weights should be conducted to clarify the relationship between the specificity of PaE and the chemical structure of the substrates.

The mechanism of polymer degradation of PaE is not known. After treatment of PaE on the PLA cast film, we measured and compared the carbon contents in the eluted monomer lactic acid and actual TOC content in the reaction solution. The F-kit was used to detect the monomer D,L-lactic acid amount based on enzymatic reaction. This comparison may suggest the mode of PaE action. After treatment with purified PaE, 44.3 μg of the total D,L-lactic acid was released from the PLA film in 50 μL of reaction solution. The carbon content in the detected total amount of D,L-lactic acid was calculated to be 17.7 μg/50 μL because the carbon content of D,L-lactic acid is 40 %. This amount of carbon in D,L-lactic acid corresponds to 35.1 % of water-soluble TOC content (50.4 μg). The amount of carbon released as the monomer was lower than that of water-soluble TOC in the eluate, suggesting that PaE degrades the PLA film with a random endo-type scission. This is in agreement with the properties of CLE described by Kawai et al. (2011) on the basis of the rapid changes of polydispersity (M_w/M_n) after degradation of PLA emulsion by CLE, confirmed with gel-permeation chromatography.

We have previously reported various strains of *Pseudozyma* spp. and *Cryptococcus* spp. isolated from the phyllosphere of various crops, including vegetables, degraded PBS, and PBSA films (Kitamoto et al. 2011). The strong degradation activity and broad specificity of the enzymes against solid BP films may be peculiar to the phyllosphere basidiomycetous yeasts.

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