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Purification and characterization of a biodegradable plastic-degrading enzyme from *Aspergillus oryzae*

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Abstract We used biodegradable plastics as fermentation substrates for the filamentous fungus *Aspergillus oryzae*. This fungus could grow under culture conditions that contained emulsified poly-(butylene succinate) (PBS) and emulsified poly-(butylene succinate-*co*-adipate) (PBSA) as the sole carbon source, and could digest PBS and PBSA, as indicated by clearing of the culture supernatant. We purified the PBS-degrading enzyme from the culture supernatant, and its molecular mass was determined as 21.6 kDa. The enzyme was identified as cutinase based on internal amino acid sequences. Specific activities against PBS, PBSA and poly-(lactic acid) (PLA) were determined as 0.42 U/mg, 11 U/mg and 0.067 U/mg, respectively. To obtain a better understanding of how the enzyme recognizes and hydrolyzes PBS/PBSA, we investigated the environment of the catalytic pocket, which is divided into carboxylic acid and alcohol recognition sites. The affinities for different substrates depended on the carbon chain length of the carboxylic acid in the substrate. Competitive inhibition modes were exhibited by carboxylic acids and alcohols that consisted of C_4-C_6 and C_3-C_8 chain lengths, respectively. Determination of the affinities for different chemicals indicated that the most preferred substrate for the enzyme would consist of butyric acid and *n*-hexanol.

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Introduction

In recent times, the excessive consumption of synthetic plastics derived from petroleum has had an adverse impact on the environment because the majority of these synthetic plastics do not degrade in the environment, and incineration of plastics generates CO_2 and dioxin (Huang 1995; Potts et al. 1973). These molecules increase the warming of the Earth and environmental pollution. In view of this, some aliphatic polymer types have been developed as biodegradable plastics (Mayer and Kaplin 1994). Biodegradable plastics have several excellent properties and may provide solutions to global environmental problems. First, biodegradable plastics are degraded by microorganisms in the natural environment (Ishigaki et al. 2000; Suyama et al. 1998a,b). Second, they can be composted, and burn with a lower calorific value than that of synthetic plastic materials. To date, some aliphatic polyester types have been developed. Poly-(lactic acid) (PLA), poly-(butylene succinate) (PBS), and poly-(butylene succinate-*co*-adipate) (PBSA) are the most promising materials among commercially available synthetic biodegradable plastics.

PBS/PBSA is produced through the copolymerization of a glycol, such as 1,4-butanediol, with an aliphatic dicarboxylic acid, such as succinate. It is a white crystalline thermoplastic, with melting points in the range of 90–120°C, and has excellent processability; therefore, it can be processed into injection molded products, films, paper laminates, and sheets (Fujimaki 1998). PBS/PBSA also biodegrades in compost, moist soil, fresh water with activated sludge, and seawater (Ando et al. 1998; Kitakuni et al. 2001). Further, various microorganisms have been isolated as PBS-degraders (Kleeberg et al. 1998; Suyama et al. 1998; Uchida et al. 2000; Teeraphatpornchai et al. 2003).

Biodegradable plastics, including PBS and PBSA, which have excellent properties as mentioned above, have not yet been commonly used. From an industrial point of view, the production cost of biodegradable plastics is higher than that of synthetic plastics. Further, current disposal systems cannot recover the production cost of biodegradable plastics because almost all the biodegradable plastics used are incinerated with other garbage, and recycling of biodegradable plastics, such as synthetic plastics, has not been contrived, even though several biodegradable plastic degradation studies have been performed. Therefore, in order to recover plastic hydrolysates, we have undertaken research to develop a cost-effective biodegradable plastic recycling system using the filamentous fungus *Aspergillus oryzae*. Both *A. oryzae* and *Aspergillus sojae*, the so-called *koji* molds, have been extensively used for traditional Japanese fermentation products, such as *sake* (rice wine), *shoyu* (soy sauce), and *miso* (soybean paste), for more than 1,000 years (Machida 2002; Yu et al. 2004). The long history of the use of these fungi in food industries prompted the United States Food and Drug Administration (FDA) to include *A. oryzae* in the list of generally regarded as safe (GRAS) organisms. The safe use of *A. oryzae* is also supported by the World Health Organization (WHO) (Barbesgaard et al. 1992; Machida 2002; Takahashi et al. 2002). In another current application, *koji* molds are used as host cells for enzyme production by recombinant DNA technology (Christensen et al. 1988; Hatamoto et al. 1999; Shibuya et al. 1992). The beneficial effect of *koji* molds has driven research and development activities in diverse fields including academia, industry, pharmacology, and agriculture (Maeda et al. 2004). Since filamentous fungi, including *A. oryzae*, can secrete large amounts of proteins, the fermentation industry has developed several recombinant protein production systems in these fungi (Gouka et al. 1997; Tsuchiya et al. 1992, 1994). These features of *A. oryzae* are well suited for its use in biodegradable plastic recycling systems. First, we confirmed that *A. oryzae* could degrade PBSA and grow under culture conditions that contained PBSA as the sole carbon source, through the production of a PBS-degrading enzyme in the medium. Second, we purified the enzyme and identified it as an *A. oryzae* cutinase (L1) (GenBank accession number P52956) (Ohnishi et al. 1995) by comparing its amino acid sequence with other sequences in databanks. Cutinases are well known enzymes produced by phytopathogenic and insect-pathogenic fungi, such as *Fusarium solani pisi* and *Magnaporthe grisea* (Purdy and Kolattukudy 1975a,b; Sweigard et al. 1992a,b). They hydrolyze the natural polyester material, cutin, which is present in the plant cuticle. Cutinases allow pathogenic fungi to penetrate the cuticular barrier on the surface of the host plant during the initial stage of the fungal infection (Hawthorne et al. 2001; Kolattukudy et al. 1995; Schafer 1993). These enzymes are also able to hydrolyze a variety of synthetic esters; in particular, cutinases from *F. solani pisi* degrade a biodegradable plastic, poly-(caprolactone) (PCL) (Murphy et al. 1996).

In this study, we clarify the substrate specificity of the enzyme by determining the kinetic parameters for *p*-nitrophenyl-substrates and the inhibition constants (K_I) for various carboxylic acids and alcohols. We confirm that the substrate specificity depends on the hydrocarbon chain length of the carboxylic acid portion in the substrate. Unlike previous studies, in which screening was performed to identify a suitable depolymerase for biodegradable plastics, this study is the first step of a new approach that chemically designs novel plastics that are degraded only by a specifically engineered enzyme.

Materials and methods

Substrate and chemicals

PBS film (Bionolle #1001; weight-average molecular weight, 1.0×10^5; thickness, 20-μm), PBSA film (Bionolle #3001; weight-average molecular weight, 2.2×10^5; thickness, 50-μm), emulsified PBS (Bionolle EM-150; weight-average molecular weight, 1.0×10^5) and PBSA (Bionolle EM-301; weight-average molecular weight, 1.0×10^5) were obtained from Showa Highpolymer (Tokyo, Japan). Emulsified PLA (Plasema L110) was obtained from Dai-Ichi kogyo seiyaku (Kyoto, Japan) and *p*-nitrophenyl (pNP)-substrates were purchased from Nacalai Tesque (Kyoto, Japan). Unless stated otherwise, all chemicals were of certified reagent grade.

Strain

Aspergillus oryzae RIB40 (ATCC-42149) (Machida 2002) was used as the enzyme source.

Purification of PBS-degrading enzyme

All purification procedures were performed at 4°C. Conidiospores of *A. oryzae* RIB40 were prepared from colonies on Czapek Dox agar plate containing 1% (w/v) PBSA [0.3% $NaNO_3$, 0.2% KCl, 0.1% KH_2PO_4, 2 mM $MgSO_4$, 34 μg/ml chloramphenicol, 0.5% agarose, and 2% (v/v) emulsified PBSA, pH 6.5] (CD-PBSA). The conidiospore suspension (1×10^9 conidiospores in 0.025% Tween 80 and 0.8% NaCl) was inoculated into 100 ml CD-PBSA medium (0.3% $NaNO_3$, 0.2% KCl, 0.1% KH_2PO_4, 2 mM $MgSO_4$,

34 μg/ml chloramphenicol, 2% (v/v) emulsified PBSA, pH 6.5), and the final concentration of conidiospores was 1×10^6 spore/ml. After aerobic cultivation at 30°C for 5 days, the crude enzyme solution was obtained by filtration through Miracloth (Calbiochem, Darmstadt, Germany). Ammonium sulfate was added to the crude enzyme solution (650 ml), to 20% saturation, and the mixture was centrifuged at 12,000 *g* for 20 min. The supernatant was loaded onto an Octyl-cellulofine column (3 cm diameter ×8 cm; Seikagaku ko-gyo, Tokyo, Japan) equilibrated with 10 mM Tris-HCl buffer (pH 8.0) containing 20% saturated ammonium sulfate. The column was washed extensively with the same buffer; subsequently, the activity was eluted with a linear gradient (from 20 to 0%) of saturated ammonium sulfate. The active fractions were pooled and dialyzed against 10 mM Tris-HCl buffer (pH 8.0). The enzyme solution was then applied to a DEAE-Toyopearl 650S column (3 cm diameter ×8 cm; Tosoh, Tokyo, Japan) equilibrated with 10 mM Tris-HCl buffer (pH 8.0). The column was washed with the same buffer, and the active fractions were eluted with a 0–0.3 M NaCl linear gradient. The active fractions were pooled, dialyzed against 10 mM MES buffer (pH 5.5), and then loaded onto a Hitrap SP column (1 ml; Amersham Pharmacia Biotech, Piscataway, N.J.) equilibrated with 10 mM MES buffer (pH 5.5). The column was washed with the same buffer and eluted with a linear gradient of 0–0.3 M NaCl.

Determination of protein concentration

Protein concentration was measured by the Lowry method using BSA fraction V (Seikagaku ko-gyo, Tokyo, Japan) as the standard (Lowry et al. 1951).

Assay for PBS-degrading enzyme activity

The substrate used was emulsified PBS, PBSA, or PLA, 0.2% (w/v) in 10 mM Tris-HCl buffer (pH 8.0). Adequate volumes of the enzyme solution (5–100 μl), 250 μl PBS, PBSA, or PLA emulsion, and 10 mM Tris-HCl buffer (pH 8.0) were mixed to 500 μl volume. The mixture was incubated at 37°C for 1–12 h, and then the absorbance at 630 nm was measured. One unit (U) of degrading activity was defined as a 1-U/min decrease in absorbance at 630 nm, in a 10-mm path length cell, under the assay conditions described above (Nakamura et al. 2001).

Degradation of PBS film and PBSA film by the enzyme was also observed. Glass fiber filters (9 mm diameter) were wetted with 30 μl purified enzyme solution (10–80 μg/ml; in 10 mM Tris-HCl buffer, pH 8.0), and placed on PBS film (20-μm thick) or PBSA film (50-μm thick). After incubation at 37°C for 6 h, the filter was removed and the film was washed with water. Degradation of the film surface was estimated visually.

Assay for esterase activity

Esterase activity was assayed at 37°C; 16 μl 50 mM pNP-butyrate in DMSO was added to 1,568 μl 100 mM Tris-HCl buffer (pH 8.0). The enzyme reaction was started by the addition of 16 μl enzyme solution. The color of *p*-NP produced was monitored at 410 nm using a Shimadzu UV-visual recording spectrophotometer (model UV-265).

Electrophoresis of proteins

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (1970) using a 15.0% polyacrylamide slab gel. The molecular mass standards included lactate dehydrogenase (36.5 kDa), triosephosphate isomerase (26.6 kDa), trypsin inhibitor (20.1 kDa), and lysozyme (14.3 kDa) (New England Biolabs, Beverly, Mass.)

N-terminal amino acid sequence

The purified enzyme was subjected to SDS-PAGE and transferred onto a PVDF membrane using a Trans-Blot SD semi-dry transfer cell (Bio-Rad, Tokyo, Japan). The protein was stained with Coomasie Brilliant Blue R-250 and excised from the blot. This piece of the blot was analyzed using an Applied Biosystems 473A protein sequencer with a 610A data analysis system (Matsudaira 1987).

Internal amino acid sequence

To identify internal amino acid sequences, limited proteolysis and gel electrophoresis analysis were performed using a modified Cleveland procedure (Hirano and Watanabe 1990; Saito et al. 1992). Purified enzyme (50 μg) was dissolved in 500 μl 10 mM Tris-HCl buffer (pH 8.0) to prepare the digests. Trichloroacetic acid (TCA; 100%. 250 μl) was added to the enzyme solution and the mixture was left on ice for 2 h; subsequently, it was centrifuged at 18,500 *g* for 20 min. The denatured enzyme precipitate was suspended in 100 μl SDS-sample buffer (0.12 M Tris-HCl buffer, pH 8.8 containing 5% SDS, 5% 2-mercaptoethanol, 10% glycerol, and 0.05% bromophenol blue) and boiled for 5 min. Tricine-SDS-PAGE was performed on a 16% polyacrylamide slab gel (16×16×0.1 cm), as reported previously (Schagger and von Jagow 1987). Denatured enzyme (50 μl) and 25 μl V8-protease (252 μg/ml, Sigma-Aldrich Japan, Tokyo, Japan) dissolved in SDS-sample buffer were applied to the same well of the gel and electrophoresis was started. When the dye front was in the middle of the stacking gel, the electrophoresis was paused for 1 h to allow limited proteolysis by the V8-protease. Subsequently, electrophoresis was resumed. The *N*-terminal amino acid sequences of these limited-digestion peptides were analyzed by the method described above.

Preparation of antibody and immunodetection of the PBS-degrading enzyme

We entrusted the preparation of an antibody against the PBS-degrading enzyme to T. K. Craft (Gunma, Japan). This antibody was produced in mice, against the PBS-degrading enzyme purified from *A. oryzae*. Immunoblotting was performed by the method described by Coligan et al. (1991).

Expression of the PBS-degrading enzyme from *A. oryzae*

To demonstrate the effect of the carbon source on PBS-degrading enzyme production, *A. oryzae* RIB40 was cultured in minimal medium containing glucose, succinate, 1,4-butanediol, emulsified PBS, or emulsified PBSA as the sole carbon source and inducer. Each carbon source was added to 5 ml minimal medium, to 1% final concentration, and conidiospores of *A. oryzae* RIB40 were inoculated into the medium. After aerobic cultivation of the fungus at 30°C for 2 days in the case of the medium containing glucose, or 5 days for all other media, each culture broth was obtained by filtration through Miracloth. Production of the PBS-degrading enzyme was detected by immunoblotting using anti-PBS-degrading enzyme antibody.

Characterization of PBS-degrading enzyme

In all these procedures, enzyme activity was measured using pNP-butyrate as the substrate. The pH optimum of the enzyme was determined as described below, and the purified PBS-degrading enzyme activity was measured in 100 mM borate-citrate-phosphate buffer (BCP buffer) at pH 4–12. For determination of pH stability, the residual activity of the enzyme was assayed at 37°C and pH 8.0, following incubation of 0.5 μg/ml enzyme solution in 100 mM BCP buffer (pH 4–10) at 37°C and each pH for 1 h.

The optimum temperature of the enzyme was evaluated by measuring its activity in 100 mM Tris-HCl buffer (pH 8.0) at each temperature (20–80°C). Thermostability of the enzyme was determined by measuring the residual activity at 37°C and pH 8.0, following enzyme incubation in 100 mM Tris-HCl buffer (pH 8.0) at a range of temperatures (20–80°C) for 30 min.

Kinetic characterization

Three pNP-substrates, pNP-propionate, -butyrate, and -valerate, were used as substrates for the kinetic study. Assays were performed using 50 ng/μl (2.3×10^{-9} M) enzyme with at least five concentrations of the substrates (pNP-propionate, [*S*]=20–100 mM; pNP-butyrate, [*S*]= 16.7–100 mM; pNP-valerate, [*S*]=3.3–100 mM) at pH 8.0 and 37°C, as described above. The kinetic constants, K_m and V_{max}, were determined from a Lineweaver-Burk plot (Rudolph 1979a,b). Values for k_{cat} were calculated according to $V_{max}= k_{cat}$ [*E*], where [*E*] is the enzyme concentration.

Inhibition study on carboxylic acids and alcohols

Carboxylic acids (acetic acid, 1-propionic acid, 1-butyric acid, 1-valeric acid, 1-hexanoic acid, 1-heptanoic acid, 1-octanoic acid, L-lactic acid, and succinic acid) and alcohols (ethanol, *n*-propanol, 2-propanol, *n*-butanol, *n*-hexanol, *n*-octanol, and 1, 4-butanediol) were used as inhibitors and pNP-butyrate was used as the substrate for kinetic inhibition analyses. Inhibition modes of these chemicals with respect to the enzyme were determined using 5 nM PBS-degrading enzyme. Assays were performed with at least four different concentrations of substrates ([*S*]=14.3–50 mM) in 100 mM Tris-HCl buffer (pH 8.0) at 37°C. The enzyme was co-incubated with each inhibitor ([*I*]=0.1 mM) for 5 min at 37°C, and the reactions were initiated by addition of the substrate. The K_I values were determined from Lineweaver-Burk plots (Fromm 1979).

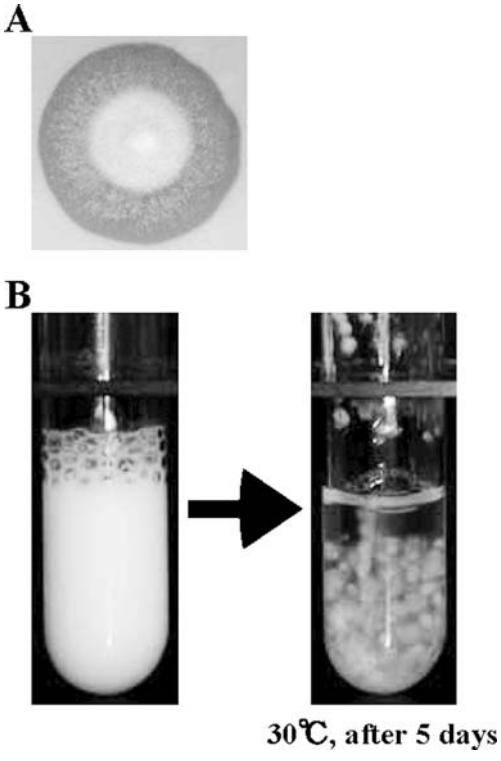


Fig. 1a, b Poly-(butylene succinate-*co*-adipate) (PBSA) degradation by *Aspergillus oryzae* RIB40. **a** Conidiospores of *A. oryzae* RIB40 were inoculated on Czapek Dox-PBSA (CD-PBSA) agar containing 1% (w/v) emulsified PBSA as the sole carbon source. A clear zone indicating PBSA degradation was observed after 5 days of incubation at 30°C. **b** Conidiospores of *A. oryzae* RIB40 were inoculated in CD-PBSA medium containing 1% (w/v) emulsified PBSA. Based on the clearing of the culture supernatant, it was proposed that *A. oryzae* RIB40 secreted a PBSA-degrading enzyme

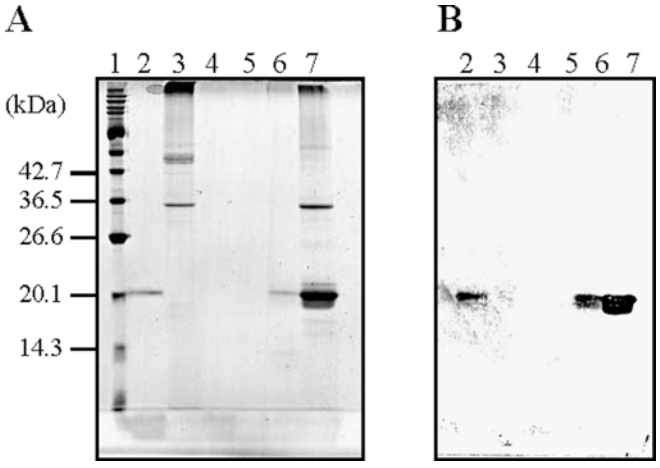


Fig. 2 SDS-PAGE and immunoblot analysis of the effect of carbon source on poly-(butylene succinate) (PBS)-degrading enzyme production. SDS-PAGE was carried out as described in Materials and methods. **a** Expressed proteins in culture broth stained with Coomassie Brilliant Blue R-250. **b** Proteins transferred to PVDF membrane and detected using anti-PBS-degrading enzyme antibody. Lanes: *1* Molecular mass standards; *2* purified PBS-degrading enzyme; *3–7* culture broth of *A. oryzae* grown with glucose (*3*); succinate (*4*); 1,4-butanediol (*5*); emulsified PBS (*6*); emulsified PBSA (*7*)

Results

Purification of PBS-degrading enzyme

We found that *A. oryzae* RIB40 could digest PBS and PBSA. A clear zone was observed on CD-PBSA agar plates and clarification of CD-PBSA liquid medium occurred after 5 days of incubation at 30°C (Fig. 1). The same results were obtained using PBS instead of PBSA (data not shown). A PBS-degrading enzyme was purified from the culture broth of *A. oryzae* by three different chromatographic steps, as described in Materials and methods. The yield and purification of the enzyme were 13% and 22-fold, respectively, and 250 μg purified PBS-degrading enzyme was obtained from 650 ml culture broth. The specific activities for PBS, PBSA, and PLA were 0.42, 11 and 0.067 U/mg, respectively. The purified enzyme appeared as a single band on SDS-PAGE with a molecular mass of 21.6 kDa. The N-terminal amino acid sequence of the enzyme could not be determined. Limited proteolysis with V8-protease revealed three fragments of estimated molecular masses of 11.9, 10.3, and 7.9 kDa on SDS-PAGE. Two fragments of 10.3 and 7.9 kDa had the same N-terminal sequence of Ala-Gln-Gly-Leu-Phe-Glu-Gln-Ala-Val-Ser. However, the N-terminal sequence of the 11.9 kDa fragment was not detected, therefore this fragment might have the same N-terminal sequence as the intact enzyme. The amino acid sequence of the PBS-degrading enzyme was compared with sequences in databanks, using the BLASTP program (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov/BLAST/). The protein was identified as cutinase (L1) from *A. oryzae* (GenBank accession number P52956). Thus, the PBS-degrading enzyme from *A. oryzae* RIB40 in this study is a cutinase (CutL1) (Ohnishi et al. 1995).

Effect of carbon source on PBS-degrading enzyme production

Aspergillus oryzae was inoculated into minimal medium containing emulsified PBS, emulsified PBSA, glucose,

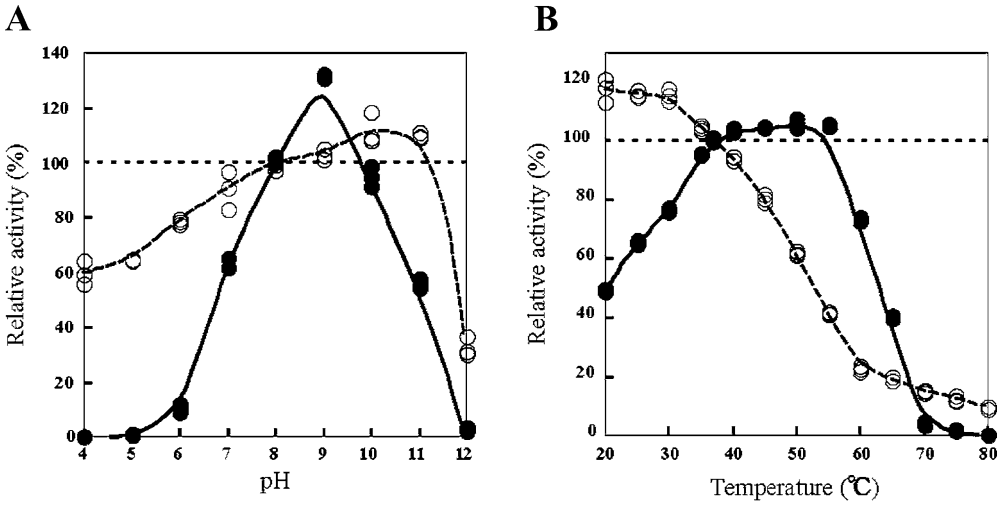


Fig. 3 Effects of pH (**a**) and temperature (**b**) on the esterase activity of the PBS-degrading enzyme. **a** The optimal pH (*solid line/filled circles*) of the enzyme was measured at 37°C using pNP-butyrate as the substrate. Esterase activities were assayed spectrophotometrically as described in Materials and methods at the pH values indicated, and are shown relative to the activity at pH 8.0. pH stability (*dashed line/open circles*) was measured with pNP-butyrate as the substrate at 37°C and pH 8.0, after incubating the enzyme in 100 mM BCP buffer at each pH value for 30 min at 37°C. Residual esterase activities were assayed at pH 8.0. **b** The optimal temperature (*solid line/filled circles*) was evaluated by measuring the esterase activity with pNP-butyrate as the substrate at different temperatures (20–80°C) and pH 8.0. Thermostability (*dashed line/open circles*) of the enzyme was measured at 37°C and pH 8.0 using pNP-butyrate as the substrate, after incubating the enzyme for 30 min at various temperatures (20–80°C) at pH 8.0. Activities are shown relative to the activity at 37°C

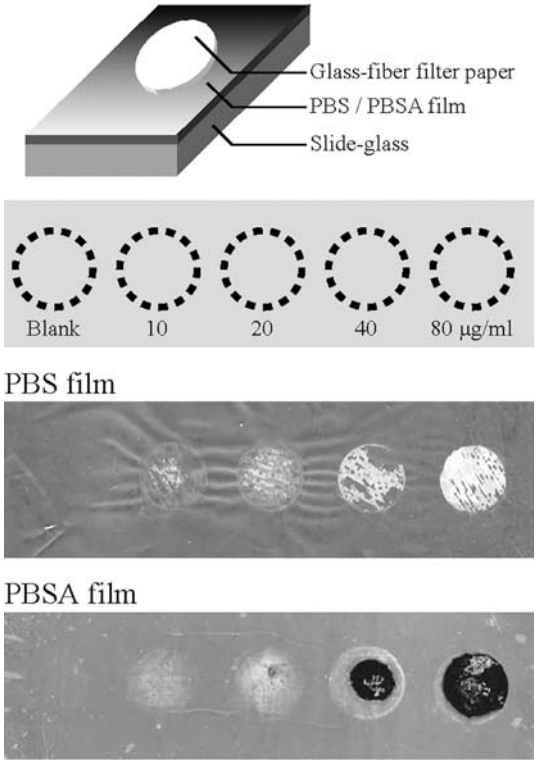


Fig. 4 Degradation of PBS film by purified PBS-degrading enzyme. PBS and PBSA films (20- or 50-μm-thick, respectively) were treated with the purified enzyme (0–80 μg/ml in 10 mM Tris-HCl buffer, pH 8.0) as described in Materials and methods. After treatment, the films were scanned using a flatbed scanner

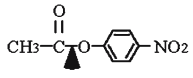
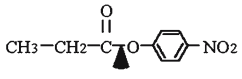
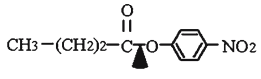
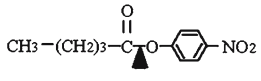
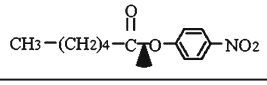
succinic acid or 1,4-butanediol, as the sole carbon source and inducer, and incubated at 30°C for 2 days (glucose) or 5 days (all other substrates). Esterase activity with respect to pNP-butyrate was observed (data not shown) in the culture broth when emulsified PBS or emulsified PBSA was added to the medium. The PBS-degrading enzyme was also detected in broth containing PBS or PBSA using anti-PBS-degrading enzyme antibody (Fig. 2b). Addition of succinic acid and 1,4-butanediol, which are constitutional units of PBS/PBSA, had no effect on production of the PBS-degrading enzyme.

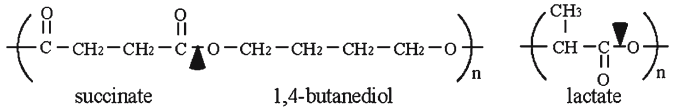
Enzymatic profiles of the PBS-degrading enzyme

The purified enzyme exhibited maximum esterase activity at pH 9.0 and was stable within the pH range 6.0–11.0 (Fig. 3a). The optimum temperature was between 35 and 55°C and enzyme activity was thermostable up to 45°C (Fig. 3b).

We confirmed that the enzyme was able to decompose solid PBS and PBSA film, and that the extent of decomposition depended on the enzyme concentration (Fig. 4). After 6 h of incubation, 10–80 μg/ml enzyme dissolved the surface of PBS film, and the surface of the treated film appeared rough. The same results were obtained with PBSA film with 10 and 20 μg/ml enzyme, and 40 and 80 μg/ml enzyme completely dissolved the PBSA film.

Table 1 Substrate specificity of the purified PBS degrading enzyme

Substrate		Specific activity (mkat / kg)[a]	Relative activity (%)[b]
*p*NP-Acetate		46 ± 2	6.7
*p*NP-Propionate		235± 8	34
*p*NP-Butyrate		694 ± 3	100
*p*NP-Valerate		459 ± 5	66
*p*NP-Hexanoate		192 ± 2	28



PBS **PLA**

[a]One katal of esterase activity was defined as the amount of enzyme required to liberate 1 mol of *p*-nitrophenol in 1 sec under standard condition (37°C, pH 8.0). The specific activity (value±standard error) was calculated with three times independent experiments

[b]The values were relatively shown as compared with the activity against *p*NP-butyrate as a substrate

	K_m (mM)	V_{max} (nM / sec)	k_{cat} (sec^{-1})
*p*NP-propionate	1.1 ± 0.01	26 ± 0.5	11 ± 0.2
*p*NP-butyrate	0.22 ± 0.001	42 ± 0.1	18 ± 0.03
*p*NP-valerate	0.044 ± 0.001	32 ± 0.1	14 ± 0.05

The kinetic parameters (value ± standard error) was calculated with three times independent experiments.

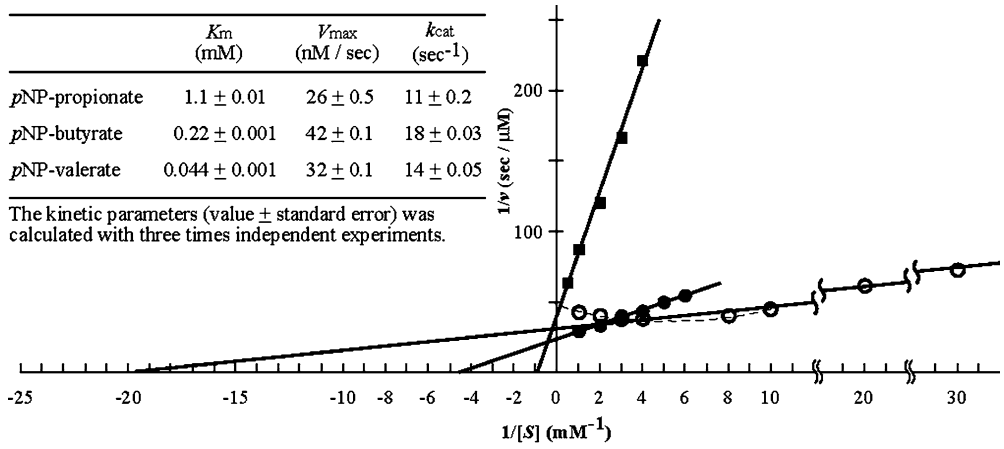


Fig. 5 Lineweaver-Burk plots to determine the kinetic constants of esterase activity for different pNP-substrates. Esterase activity was assayed with each substrate (pNP-propionate, [*S*]=20–100 mM; pNP-butyrate, [*S*]=16.7–100 mM; pNP-valerate, [*S*]=3.3–100 mM). The enzyme concentration [*E*] was 2.3 nM. Esterase activity was measured spectrophotometrically at 37°C, pH 8.0 as described in Materials and methods. *Filled squares*, *filled circles*, and *open circles* represent data for pNP-propionate, pNP-butyrate, and pNP-valerate, respectively. The *dashed line* represents the actual plot for pNP-valerate. *Inset* Kinetic parameters of PBS-degrading enzyme toward each pNP-substrate

The substrate specificity of the enzyme toward pNP substrates is shown in Table 1. The most preferred substrate for the enzyme was pNP-butyrate, whereas enzyme activity with pNP-valerate was 66% of that with pNP-butyrate. When the chain length of the carboxylate was increased beyond that of butyrate, a lower activity was observed. Therefore, according to these results, when the substrate consisted of a shorter carbon chain carboxylate group, enzymatic activity was also decreased.

Kinetic characterization

In order to establish the relationship between the chain length of carboxylate in the substrate and the activity of the enzyme, we conducted a kinetic study of the enzyme using pNP-propionate, pNP-butyrate, and pNP-valerate as pNP substrates. The kinetic constants K_m and V_{max} were calculated from a Lineweaver-Burk plot (Fig. 5). The catalytic constant (k_{cat}) of the enzyme was derived from $k_{cat}=V_{max}/[E]$ (see inset in Fig. 5). The carbon chain lengths of the carboxylic acids in the substrates did not affect the k_{cat} of the enzyme. However, the values of K_m depended on the carbon chain length of the carboxylic acid in the substrates. Substrates that consisted of longer carboxylic acids displayed a lower K_m value. The plot of pNP-valerate values was not linear at high substrate concentration (33.3–100 mM) and it appeared to be subject to either substrate inhibition or product inhibition (open circles and dashed line in Fig. 5).

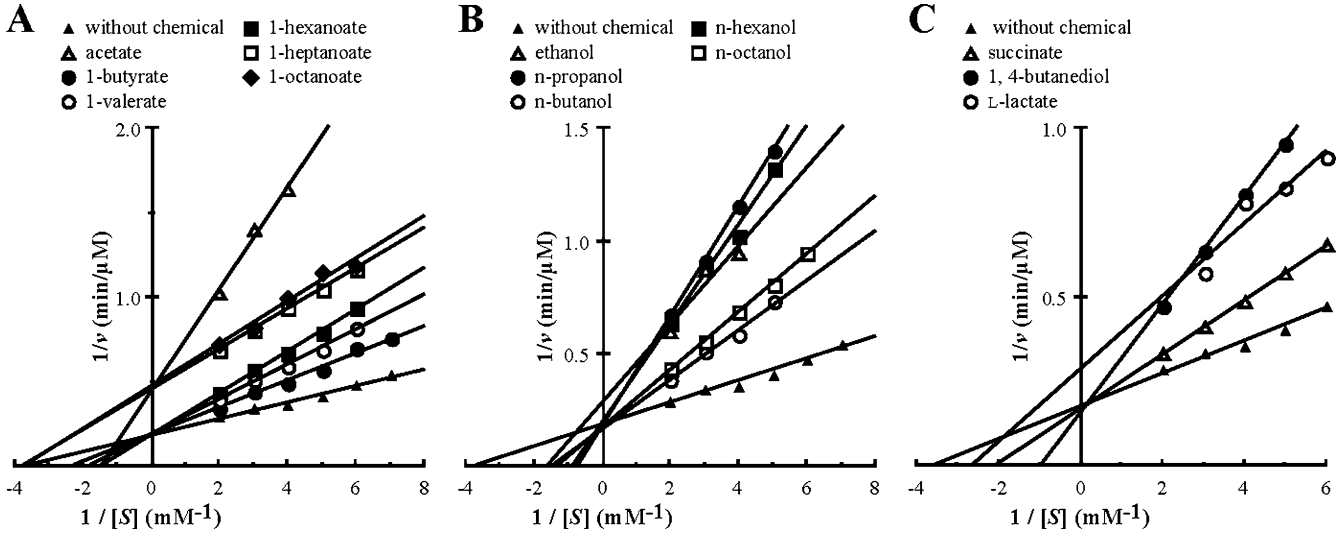


Fig. 6 Lineweaver-Burk plots of esterase activity to determine the inhibition constants of various carboxylic acids and alcohols. Enzyme inhibition was assayed in the presence of pNP-butyrate as the substrate ([*S*]=14.3–50 mM). The enzyme concentration [*E*] and each chemical concentration [*I*] were fixed at 5 nM and 0.1 mM, respectively. The enzyme solution was pre-incubated at pH 8.0, 37°C for 5 min, after which substrate solution was added to initiate the enzyme reaction. The effects of the carboxylic acids, alcohols, and components of PBS/PBSA (succinate and 1,4-butanediol) are indicated in **a**, **b**, and **c**, respectively, as indicated

Effect of chemicals on enzymatic activity

In order to understand the recognition mechanism of the enzyme for the carboxylic acid (Fig. 6a) and the alcohol (Fig. 6b) in the ester, we estimated the inhibition modes of the carboxylic acid and alcohols and determined their K_I values. Butyric acid, valeric acid, and hexanoic acid exhibited competitive inhibition modes. The K_I values of 1-butyric acid, 1-valeric acid and 1-hexanoic acid were 171 μM, 93 μM and 66 μM, respectively. The inhibition mode of 1-heptanoic acid and 1-octanoic acid was non-competitive, with K_I values of 67 μM and 62 μM, respectively. Acetic acid and 1-propionic acid (data not shown) exhibited mixed inhibition modes.

n-Propanol, *n*-butanol, *n*-hexanol, and *n*-octanol acted as competitive inhibitors of the enzyme, with K_I values of 36 μM, 67 μM, 31 μM, and 58 μM, respectively. On the other hand, ethanol demonstrated a mixed inhibition mode.

Succinic acid and 1,4-butanediol behaved as competitive inhibitors (Fig. 6c). The K_I values of succinate and 1,4-butanediol were determined as 138 and 36 μM, respectively. L-Lactate exhibited mixed inhibition of the enzyme (Fig. 6c).

Discussion

We found that *A. oryzae* could grow in medium containing only PBS or PBSA as a carbon source by producing cutinase to digest and incept the polymers. Cutinases from phytopathogenic fungi, such as *F. solani pisi* and *M. grisea*, have been extensively studied with respect to their structure and function (Egmond and de Vlieg 2000; Ferreira et al. 2003; Longhi et al. 1997a,b; Martinez et al. 1992; Prompers et al. 1999; Sweigard et al. 1992a,b). However, there have been few studies on biodegradable plastic degradation by cutinases. In this study, we focused on the correlation between the substrate specificity of the cutinase from *A. oryzae* and its PBS-degrading ability.

We purified a PBS-degrading enzyme from culture broth containing PBSA as the sole carbon source. The purified PBS-degrading enzyme was a small protein of 21.6 kDa and its N-terminal amino acid sequence could not be determined by peptide sequencing. However, the internal amino acid sequences obtained by limited V8-protease proteolysis revealed that the PBS-degrading enzyme was the cutinase (L1) of *A. oryzae* (GenBank accession number P52956) (Ohnishi et al. 1995). Information regarding the properties of the cutinase from *A. oryzae* is limited and there is no previous report on the degradation of biodegradable plastic by *A. oryzae* cutinase (Ohnishi et al. 1995). It has been reported that the N-terminal amino acid of the cutinase from *F. solani pisi* was modified with glucuronic acid (Kolattukudy 1984; Lin and Kolattukudy 1977, 1980); therefore, we propose that the N-terminal amino acid of *A. oryzae* cutinase might also be blocked by glucuronisylation.

We confirmed that *A. oryzae* produced the cutinase inductively when PBS or PBSA stimulated the production of the PBS-degrading enzyme. We observed clearing of culture medium containing emulsified PBS or PBSA as the sole carbon source (Fig. 1), and could detect cutinase by immunoblotting (Fig. 2b). On the other hand, the constitutional units of PBS—succinic acid and 1,4-butanediol—could not induce the cutinase whereas PBS and PBSA polymers could. It was reported that some triacylglycerols, such as olive oil or soybean oil, play a role as inducers for lipases and esterases, including the cutinase from *A. oryzae* (Ohnishi et al. 1994); however, our finding differed from known induction mechanisms. Succinic acid and 1,4-butanediol might be the final hydrolysates of the enzymes from *A. oryzae* and these two molecules could be ingested by the fungal cell since *A. oryzae* can grow in medium containing only succinic acid or 1,4-butanediol as the carbon source. However, these two substances could not induce cutinase production. PBS and PBSA polymer could induce the enzyme, but the fungi would be unable to incept the intact polymer. Thus, we consider that PBS/PBSA-mediated induction of the enzyme from *A. oryzae* could occur by physical contact and recognition of hydrophobic materials around the cells. In another example, *Fusarium* cutinase is induced with PCL (Murphy et al. 1996). The cutinases produced by phytopathogenic fungi play an important role in degrading the hydrophobic cuticular barriers on the surface of the host plants during the initial stages of infection (Hawthorne et al. 2001; Kolattukudy et al. 1995; Schafer 1993). Thus, we suggest that exposure to a hydrophobic environment or hydrophobic surface would be one of the important signals that induces fungi to produce enzymes able to degrade biodegradable plastics.

The amount of enzyme produced by *A. oryzae* cultivated with PBS was less than that with PBSA (Fig. 2). PBSA polymer should be more suitable for degradation by the enzyme (Fig. 4), because the crystallinity and the glass transition temperature of PBSA polymer are lower than those of PBS polymer (Fujimaki 1998). *A. oryzae* grew faster with PBSA than with PBS, and growing fungus should produce the enzyme more vigorously.

During purification, the enzyme adsorbed to the hydrophobic chromatography column at 20% ammonium sulfate saturation and was eluted in buffer without ammonium sulfate; therefore, we considered the possibility that the PBS-degrading enzyme molecule might have a large hydrophobic area on its molecular surface. Prompers et al. (1999) reported that the cutinase from *F. solani* possesses hydrophobic areas on the molecular surface, such as a hydrophobic binding loop and a flap close to the active site. The cutinase from *A. oryzae* could have a similar hydrophobic surface, because the amino acid sequence of the enzyme exhibited 73% similarity (51% identity) to that of *F. solani* cutinase.

The cutinase from *A. oryzae* was stable over a high pH range (pH 6.0–11.0; Fig. 3a), and the enzyme displayed full activity even at 55°C (Fig. 3b). The enzyme could also digest commercial PBS/PBSA film (Fig. 4); 80 μg/ml cutinase completely dissolved a 50-μm thick PBSA film in only 6 h. Consequently, the facts indicate that the enzyme has potential for practical applications.

We estimated the substrate specificity of the cutinase using pNP-carboxylate substrates (Table 1). The cutinase preferred pNP-butyrate to other pNP-substrates and the enzyme appeared to recognize the length of carboxylate in the substrate: butyrate was the most preferred substrate, followed by valerate and propionate. pNP-acetate, which had the shortest carboxylate group, was a poor substrate. However, a very long carboxylate group was also not preferred.

Further, to prove the effect of carboxylate chain length of the substrate on the enzymatic reaction of cutinase, we determined kinetic parameters using pNP-propionate, pNP-butyrate, and pNP-valerate (Fig. 5). There was no significant change in the catalytic constant, k_{cat}; however, the K_m decreased by 20% with each increment of the methylene unit (inset in Fig. 5) in the carboxylate region. The results indicate that affinity for the substrate increases according to the chain length of the carboxylic ester in these substrates.

To better understand substrate recognition by the cutinase, we separately studied the carboxylate region and the alcohol region of the substrate. Thus, we determined the inhibition modes of the carboxylic acids and alcohols using pNP-butyrate as a substrate. All the chemicals studied were constitutional units of the probable substrate. First, the inhibition modes of the normal carboxylic acids were estimated, and acetic acid and 1-propionic acid displayed mixed inhibition. 1-Butyric acid, 1-valeric acid, and 1-hexanoic acid exhibited competitive inhibition, and 1-heptanoic acid and 1-octanoic acid demonstrated noncompetitive inhibition (Fig. 6a; data for propionic acid is not shown). These results indicated that carboxylic acids shorter than butyric acid or longer than hexanoic acid could not bind to the substrate-binding site of the cutinase. Further, only butyric acid, valeric acid, and hexanoic acid could interact with the substrate-binding site of the cutinase as competitive inhibitors instead of the substrate.

The K_I values of 1-butyric acid, 1-valeric acid and 1-hexanoic acid were determined as 171 μM, 93 μM and 66 μM, respectively. The affinity of hexanoic acid was higher than that of butyric acid and valeric acid. Based on the results of X-ray crystallography, NMR, and enzymatic studies of *F. solani pisi* cutinase (Longhi et al. 1997a,b; Mannesse et al. 1995; Prompers et al. 1999), it was reported that the cutinase from *F. solani* recognized the hydrophobicity of the substrate and that this enzyme had a hydrophobic environment in the fatty acid binding site. We consider the *A. oryzae* cutinase also to have a hydrophobic binding pocket. This is because the amino acid residues, which form the hydrophobic bottom of the carboxylic acid binding pocket, the hydrophobic binding loop, and the hydrophobic flap close to the active site, were conserved in the enzymes from both *A. oryzae* and *F. solani pisi*. Thus, we suggest that the affinity of the enzyme toward carboxylic acid would depend on the hydrophobic interaction between the hydrophobic bottom of the enzyme and the linear hydrophobic hydrocarbon (alkane) portion of the carboxylic acid. Further, it was observed that the affinity for carboxylic acid increased as the length of the alkane increased, i.e., the degree of hydrophobicity increased from butyric acid to hexanoic acid. We observed the same tendency in kinetic studies in terms of the enzyme-substrate affinities (K_m) (Fig. 5). In conclusion, this enzyme precisely recognized the alkane chain length of carboxylic acid, and binding should be due mainly to the hydrophobic interaction between the substrate and the binding pocket. Longer or shorter carboxylic acids, such as propionic acid and heptanoic acid, should be immediately eliminated from the carboxylic acid binding site in the binding pocket of the cutinase because the hydrophobic interaction would not be stable enough for enzymatic activity.

In the apparent activity study, pNP-valerate exhibited lower activity than pNP-butyrate. On the contrary, the cutinase from *A. oryzae* exhibited the highest affinity (smallest K_m value) for pNP-valerate in the kinetic study. The value of the catalytic efficiency (k_{cat}/K_m) was also highest at low substrate concentrations. However, at high substrate concentration ([*S*]>33.3 mM; dashed line in Fig. 5) the plots did not conform to the Michaelis-Menten rule. These results suggested that substrate inhibition or product inhibition could have occurred (Cleland 1979; Rudolph 1979). Since valeric acid demonstrated a competitive inhibition mode, the inhibition of the cutinase at high concentrations of pNP-valerate could be product inhibition. *p*-NP valerate should be an adequate substrate for the cutinase; however, the catalytic activity might be inhibited by the resulting 1-valeric acid. Consequently, the apparent activity of pNP-valerate should be lower than that of pNP-butyrate.

Further, the inhibition mode of ethanol was judged to be mixed inhibition, whereas *n*-propanol, *n*-butanol, *n*-hexanol and *n*-octanol exhibited competitive inhibition of the cutinase (Fig. 6b). This suggested that alcohols other than ethanol could attach to the binding pocket as the substrate analog (Fromm 1979). Further, the K_I values of *n*-propanol, *n*-butanol, *n*-hexanol, and *n*-octanol were determined as 36 μM, 67 μM, 31 μM, and 58 μM, respectively. The affinity for the *n*-alcohol and the chain length of the alcohol did not have a distinct correlation like that for carboxylic acids. We used pNP-carboxylate as the substrate and *p*-NP as one of the alcohols. The results indicated that a non-linear and bulky alcohol could bind to the alcohol binding site of the cutinase. The structure of *F. solani pisi* cutinase reveals that the alcohol recognition site of the cutinase is exposed to the solvent and is wider than the carboxylic acid recognition site (Longhi et al. 1997a,b; Prompers et al. 1999). We propose that the cutinase from *A. oryzae* would have a similar alcohol binding site and would not precisely recognize the size of the alcohol.

The inhibition modes of succinic acid, 1,4-butanediol (the constitutional units of PBS/PBSA), and L-lactate (which is a unit of PLA) were estimated. Succinic acid and 1,4-butanediol exhibited competitive inhibition and L-lactate displayed mixed inhibition (Fig. 6c). The K_I values of succinic acid and 1,4-butanediol were determined as 138 μM and 36 μM, respectively. We compared the K_I values of *n*-butanol and 1,4-butanediol. Both alcohols have the same number of carbon atoms; however, the former has one hydroxyl group whereas the latter has two. The K_I

values of *n*-butanol and 1,4-butanediol were 67 μM and 36 μM, respectively. The results demonstrated that the affinity toward 1,4-butanediol was twice that for *n*-butanol. We consider that the hydroxyl group plays an important role in enzyme recognition. Therefore, mono-alcohol might bind to the binding site of the enzyme uni-directionally against the active center; however, the diol could bind in both directions. On the other hand, the K_I value for butyric acid (171 μM) was slightly higher than that for succinic acid (138 μM). Both molecules have the same number of carbon atoms (Fujimaki 1998), which is different in 1,4-butanediol, and the carbons at both ends are carboxyl groups. Succinic acid has only two hydrophobic methylenes (-CH_2-CH_2-) involved in binding to the binding pocket. Therefore, if the affinity for the carboxylic acid depends on the length of the hydrophobic alkane, the affinity for succinate should correspond to that of propionic acid, which has CH_3-CH_2-. Succinic acid might bind to the binding pocket bi-directionally, along with 1,4-butanediol. Thus, succinic acid could have an apparent affinity twice that of the actual affinity. We did not estimate the K_I of propionic acid; however, the kinetic study using pNP-substrates indicated that the affinity of propionic acid was weaker than that of butyric acid. Thus, the succinic acid in polyester material should be recognized mainly as -CH_2-CH_2-, in the same manner as the alkane portion of propionic acid (CH_3-CH_2-).

L-Lactate, acetate, ethanol, and 2-propanol displayed mixed inhibition. It was difficult for the cutinase to bind small molecules, regardless of whether they were carboxylic acids or alcohols. L-Lactic acid behaves as both a carboxylic acid and an alcohol. The alpha carbon of lactic acid not only has a carboxyl group but also a hydroxyl group. The alpha carbon might be unable to bind to the carboxylic acid binding pocket and the alcohol binding pocket due to steric hindrance by the carboxyl or hydroxyl group and the lack of hydrophobicity. Therefore, the PLA-degrading activity of the enzyme should be lower than that for PBS/PBSA.

This study on the recognition mechanism of carboxylic acids and alcohols suggested that the cutinase from *A. oryzae* could accept a carboxylic acid that has a hydrophobic linear alkane consisting of 2–7 carbon atoms in the carboxylic acid binding site and an alcohol that consists of more than 3 carbon atoms (longer than *n*-propanol) in the alcohol binding site. However, a carboxylic acid longer than butyric acid may result in product inhibition. We suggest that the most suitable combination of the monoester substrate for the enzyme would be butyric acid (CH_3-$(CH_2)_2$-COOH) and *n*-hexanol (CH_3-$(CH_2)_5$-OH), and the most suitable combination for polyester would be glutaric acid (HOOC-$(CH_2)_3$-COOH) and 1,6-hexanediol (HO-$(CH_2)_6$-OH). However, considering the profile of the plastic, the polyester might not be suitable for practical use. At present, the most digestible biodegradable-plastic for the cutinase from *A. oryzae* was PBS/PBSA, which consists of succinic acid and 1,4-butanediol. We described the degradation of PBS/PBSA in the present report; however, it is possible to adapt the cutinase to degrade other biodegradable plastics, such as PLA and others. Since the cutinase could bind only C_4-C_6 carboxylic acids, it is necessary to reinforce the affinity for short carboxylic acids in the binding pocket. In order to clarify the details of the interaction between the binding pocket of the enzyme and the substrate, we are currently investigating crystallization conditions. This will enable analysis of the crystal structure of the enzyme bound to the substrate analog.

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