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Cutinase-like biodegradable plastic-degrading enzymes from phylloplane yeasts have cutinase activity

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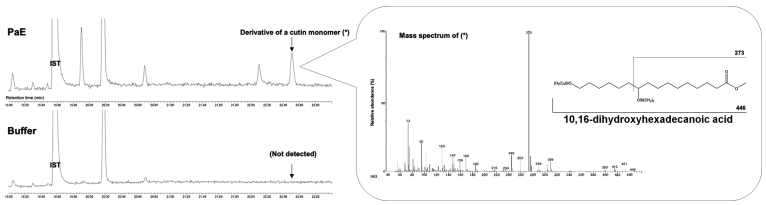
ABSTRACT

Phylloplane yeast genera *Pseudozyma* and *Cryptococcus* secrete biodegradable plastic (BP)-degrading enzymes, termed cutinase-like enzymes (CLEs). Although CLEs contain highly conserved catalytic sites, the whole protein exhibits ≤30% amino acid sequence homology with cutinase. In this study, we analyzed whether CLEs exhibit cutinase activity. Seventeen *Cryptococcus magnus* strains, which degrade BP at 15 °C, were isolated from leaves and identified the DNA sequence of the CLE in one of the strains. Cutin was prepared from tomato leaves and treated with CLEs from 3 *Cryptococcus* species (*C. magnus*, *Cryptococcus flavus*, and *Cryptococcus laurentii*) and *Pseudozyma antarctia* (PaE). A typical cutin monomer, 10,16-dihydroxyhexadecanoic acid, was detected in extracts of the reaction solution via gas chromatography–mass spectrometry, showing that cutin was indeed degraded by CLEs. In addition to the aforementioned monomer, separation analysis via thin-layer chromatography detected high-molecular-weight products resulting from the breakdown of cutin by PaE, indicating that PaE acts as an endo-type enzyme.

Graphical Abstract



The cutinase-like enzymes of phylloplane yeasts degrade cutin, a polyester component of the cuticle layer, producing cutin monomer 10,16-dihydroxhexadecan acid.

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Keywords: cutin monomer, cutinase, cutinase-like enzyme, phylloplane yeast

Abbreviations: CLE: cutinase-like enzyme; BP: biodegradable plastic

The leaf surface is covered by a cuticular layer, which is mainly consisting of a cutin–polymer matrix of ester-linked ω-hydroxylated fatty acids, covered by epicuticular waxes and infiltrated by intracuticular waxes (Martin *et al.* 2017). Thus, the cuticular layer of the leaf surface serves as a barrier against permeation by gases, water, and other substances (van den Ende and Linskens 1974; Trouvelot *et al.* 2014). This suggests that the leaf surface, otherwise termed the phylloplane is thought to limit the availability of nutrients to resident microorganisms (Martin 1964).

Cutinases (E.C. 3.1.1.74) are esterases belonging to the α/β-hydrolase superfamily. The cutin-hydrolyzing activity of cutinase, derived from phytopathogenic fungi, was previously evaluated via detection of radioactive products released by a substrate made of radiolabeled pericarp cutin (Kolattukudy, Purdy and Maiti 1976; Sebastian *et al.* 1987; Fett *et al.* 1992; Kontkanen *et al.* 2009; Inglis, Yanke and Selinger 2011).

A biodegradable plastic (BP)-degrading enzyme isolated from the basidiomycetous yeast, *Cryptococcus* sp. S-2, has a cutinase consensus sequence, and therefore, it was termed a cutinase-like enzyme (CLE); (Masaki *et al.* 2005). Although relatively less attention has been paid to the activities of microorganisms inhabiting leaves, basidiomycetous yeasts, such as *Pseudozyma* and *Cryptococcus*, which are phylloplane residents (found at low densities), produce CLEs that efficiently degrade biodegradable plastics (Kitamoto *et al.* 2011). *Pseudozayma antarctica* and *Cryptococcus flavus* isolated from rice husks secrete the approximately 21-kDa enzymes PaE and CfCLE, respectively. They show broad specificity for degrading synthetic aliphatic polyesters, such as poly(butylene succinate-*co*-adipate) (PBSA), poly(butylene succinate) (PBS), and polylactic acid. The ester hydrolysis activities of PaE and CfCLE were also identified using *para*-nitrophenyl 2-18 carbon chain fatty acids as substrates (Shinozaki *et al.* 2013; Watanabe *et al.* 2015a). We previously generated *P. antarctica* strains and optimized culture conditions for the production of highly concentrated enzymes in order to develop a method for the accelerated degradation of used BP mulch films via enzymatic treatment (Watanabe *et al.* 2014a; Sameshima-Yamashita *et al.* 2019).

We were also interested in the ecological role of CLEs. We hypothesized that phylloplane yeasts may secrete enzymes on leaf surfaces and examined the effects of such secretions. When leaves were immersed in a highly concentrated BP-degrading enzyme solution secreted by *P. antarctica*, the lipid layer on the leaf surface grew thinner due to the release of C16 and C18 fatty acids from the leaves. This suggested that resident yeasts may be utilizing CLEs to extract fatty acids as nutrients (Ueda *et al.* 2015). It was further observed that leaf surfaces were heavily damaged by high concentrations of these enzymes, allowing plant pathogens to easily invade leaves (Ueda *et al.* 2018). However, it is unclear whether CLE can degrade leaf cutin. Therefore, in the current study, cutinase activity of purified CLEs was evaluated using cutin prepared from tomato leaves. The cutinase of *Fusarium solani*, which is known to exhibit radiolabeled apple cutin-degrading activity, was used as a control. In addition, the cutinase activity of the BP-degrading enzyme from the filamentous fungus *Paraphoma* sp. B47-9 isolated from barley (Koitabashi *et al.* 2012), was evaluated.

Materials and methods

Preparation of CLEs from BP-degrading microorganisms

The CLEs used in this study were produced by cultivating previously isolated BP-degrading microorganisms, as follows. *Pseudozyma antarctica* (*Moesziomyces antarcticus*) GB-4(0)-HPM7 [MAFF Genebank at the National Institute of Agrobiological Sciences, Japan (accession number: MAFF 307000)] was used to prepare PaE. The strain GB-4(0)-HPM7 is a low-foam-forming mutant of the rice husk-derived strain GB-4(0) (Ueda *et al.* 2015). *Cryptococcus flavus* (*Saitozyma flava*) GB-1, isolated from rice husks, was used to prepare CfCLE (Watanabe *et al.* 2015b). *Cryptococcus magnus* (*Filobasidium magnum*) BPD1A (MAFF 306841), isolated from the midgut of stag beetle larvae, was used to prepare CmCut1 (Suzuki *et al.* 2013). The name of each yeast based on recent reclassification was shown in parentheses (Liu et al. 2015; Wang *et al.* 2015). The filamentous fungus *Paraphoma* sp. B47-9, isolated from barley, was used to prepare PCLE (Koitabashi *et al.* 2012).

Each enzyme was purified from the culture filtrate using BP adsorption and degradation properties of BP-degrading enzymes, as described by Suzuki *et al.* (2013). In addition, a recombinant cutinase of *F. solani*, produced using genetically modified *Aspergillus oryzae*, was used as a control (Kodama *et al.* 2009). Purified enzymes, which were separated via SDS-PAGE, are shown (Figure S1).

Isolation of *C. magnus*, a BP-degrading yeast, from leaves

Isolation of phylloplane yeasts capable of degrading BP films at low temperatures was carried out at 15 °C according to the method described by Kitamoto *et al.* (2011). The isolated strains were identified as *C. magnus* based on the DNA sequence of the internal transcribed spacer (ITS) and the D1/D2 region of *18S rRNA*. The CLE gene sequences of the newly identified *C. magnus* strain were compared with those of *C. magnus* BPD1A (GenBank AB731475.1). A detailed explanation has been provided in the Supplemental Methods.

Homology analysis

For homology analysis, a BLAST search (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was performed using the amino acid sequence of PaE as input (August 1, 2019). The top 100 sequences with a similar homology were selected, and overlapping strain enzymes, hypothetical enzymes, as well as partial sequence enzymes, were excluded. A total of 38 amino acid sequences, including those selected putative enzyme sequences and the cutinase sequences of *Fusarium solani*, *Botrytis cinerea*, *Colletotricum gloeosporioides*, and *Pseudomonas putida*, were subjected to phylogenetic analysis via the neighbor-joining (NJ) method using the

GENETYX Tree Ver. 2.2.5 software (Genetyx, Tokyo, Japan), and a bootstrap analysis of 1000 replicates was performed (Felsenstein 1985).

Evaluation of enzyme BP-degrading activity

BP-degrading activity was evaluated via turbidity reduction of emulsified PBSA (Bionolle EM -301, average molecular weight (12-15) × 10^4; Showa Denko K.K., Tokyo, Japan). One unit of PBSA-degrading activity was defined as a reduction of 1 OD_{660} per minute in 30 °C, in 20 mM Tris-HCl buffer (pH 9.0), unless stated otherwise. The protein concentration of PaE in Tris-HCl buffer (pH 9.0) at 4 U was determined via the Bradford method (Bradford 1976).

Preparation of crude cutin from tomato leaves

Isolation of crude cutin from leaves was performed according to the method described by Osman *et al.* (1999), with minor modifications. Fresh tomato leaves (approximately 2.7 kg) were collected from a farmer's greenhouse and lyophilized (approximately 1.5 kg). An aliquot of lyophilized leaves (51 g) was stirred in 1000 mL of methanol (MeOH) for 1 d. MeOH was removed through a filter, and the residue was stirred in 500 mL chloroform ($CHCl_3$) for 1 d. The residue was then stirred in 500 mL of 1:1 $CHCl_3$/MeOH for 1 d, thoroughly washed with $CHCl_3$/MeOH, and dried completely. Finally, 38 g of crude cutin was obtained and used to evaluate cutinase activity.

Treatment of cutin with CLEs and cutinase

To measure cutinase activity, 200 mg of cutin was suspended in 2 mL of 25 mM HEPES buffer (pH 7.3) (Wako, Osaka, Japan), treated with enzymes, and subjected to shaking at 200 rpm (at 25 °C) on a rotary shaker (RS-2, AS-ONE, Osaka, Japan) for 48 h. The amount of each enzyme used was estimated as that required to achieve the same PBSA-degrading activity (0.8 U) in HEPES buffer. *F. solani* cutinase was used as the control. In addition, the activities of PaE and *F. solani* cutinase proteins at similar concentrations were evaluated in 20 mM Tris-HCl (pH 9.0) under alkaline conditions. To quantify the cutin monomer released from crude cutin following enzyme treatment, it was extracted from the reaction solution with 2% acetic acid, subjected to methyl esterification, and analyzed via gas chromatography-mass spectrometry (GC-MS). The buffer applied to the enzyme-free solution was used as a control. Standard cutin degradation products were prepared as follows: crude cutin (200 mg) was suspended in 2 mL of 1.5 M potassium hydroxide prepared by dissolved in methanol (KOMe) and incubated at 25 °C for 48 h with shaking at 200 rpm.

Identification of cutin degradation products using GC-MS

Free fatty acids, including cutin monomers, in the PaE-treated and KOMe-hydrolyzed cutin solutions described above, were extracted thrice with 2 mL of diethyl ether. Thereafter, 0.1 mg of 15-hydroxypentadecanoic acid (Tokyo Chemical Industry Co., Tokyo, Japan) was added to the extracts as an internal standard compound. The fatty acids in extracts were converted to methyl esters via a conventional method (Ichihara and Fukubayashi 2010). Following methyl ester conversion, trimethylsilylation using 0.2 mL of *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA; Wako, Tokyo, Japan) was performed at 60 °C overnight in order to derivatize the hydroxy groups of constituents in preparation for GC-MS analysis. The GC-MS conditions were as described in our previous report (Tabata *et al.* 2020).

Separation and detection of cutin degradation products using thin-layer chromatography

Thin-layer chromatography (TLC) analysis was performed as described by Bischoff *et al.* (2015), as well as Walton and Kolattukudy (1972), with some modifications. Crude cutin (500 mg) was suspended in 5 mL of PaE solution (4 U in 20 mM Tris-HCl at pH 9.0) and 0.1 M KOMe, or 1.5 M KOMe, followed by incubation for 16 h (overnight: o/n) with shaking at 200 rpm on an RS-2 rotary shaker at 25 °C. The 0.1 and 1.5 M KOMe-treated solutions were prepared in order to detect various oligomeric products. Following incubation, the reaction solution was acidified with HCl, and free fatty acids were released with chloroform, dried, and redissolved in 1 mL of chloroform. PaE-treated samples (50 and 100 µL) and KOMe-treated samples (20 µL) were spotted onto a silica gel 60 F_{254} TLC plate (Merck, Darmstadt, Germany). A mixture of diethyl ether, *n*-hexane and MeOH (80:20:10) was used as the separation solution. The spots were visualized using saturated iodine.

Results

Isolation of the BP-degrading yeast *C. magnus* from leaves

We have frequently isolated the biodegradable plastic (BP) film degraders, *Pseudozyma* and *Cryptococcus* yeasts, from plant leaves and seeds at 25-30 °C. In this study, the same selection was made at 15 °C to isolate microorganisms that are able to effectively degrade BP products in an open environment at relatively low temperatures. A total of 60 strains, obtained from 13 kinds of plants, were selected as emulsified PBSA degraders. Of these 60 isolates, 17 with remarkably high PBSA and PBS film-degrading activities were identified as *C. magnus* strains based on rDNA and ITS sequences (Table S1). The results indicated that phylloplane *C. magnus* strains possessed high BP film-degrading activity at low temperatures, when compared with other yeasts. The *C. magnus* strain, BPD1A, a BP-degrading yeast which was previously isolated by us from the midgut of stag beetle larvae at room temperature (Suzuki *et al.* 2013), also degraded PBSA and PBS films at 15 °C (Figure S2). While our previous study had failed to find *C. magnus* strains in other stag beetle larvae, we succeeded in isolating *C. magnus* from the leaves of various plant species in the current experiment (Table S1; Figures S2 and S3A). Since stag beetle larvae feed on wood, plant-derived *C. magnus* may enter their midgut via ingestion. The DNA sequence of the gene encoding the BP-degrading enzyme from the *C. magnus* strain (Table S1: No. 14) from sweet potato leaves was identical to that of the BP-degrading enzyme CmCut1. The specific PBSA-degrading enzyme activity of purified CmCut1 was higher at temperatures above 15 °C and reached a maximum at 40 °C (Suzuki *et al.* 2013). These results suggest that the ability of *C. magnus* to degrade BPs at 15 °C is due to the fact that this strain has adapted to growing and secreting CmCut1 at low temperatures. Thus, these strains may be able to degrade used BP products in an open environment at relatively low temperatures.

Table 1. Amino acid sequence identity (%) between cutinase and cutinase-like enzymes

Strain	Enzyme	Identity (%)		
		Fusarium solani	*Botrytis cinerea*	*Pseudomonas putida*
Pseudozyma antarctica	PaE	25.4	24.1	17.5
Cryptococcus magnus	CmCut1	24.7	27.5	34.2
Cryptococcus flavus	CfCLE	24.3	24.1	26.6
Cryptococcus sp. S-2	CLE	24.3	24.1	26.6
Paraphoma sp. B47-9	PCLE	56.6	34.0	19.2

Homology analysis of CLEs and cutinase

To identity the deduced amino acid sequences of *C. magnus* CmCut1 and phylloplane basidiomycete yeast CLEs (*P. antarctica* PaE, *C. flavus* CfCLE, and *Cryptococcus* sp. S-2 CLE), typical cutinases of plant pathogenic fungi *F. solani* and *B. cinerea,* as well as plant-interacting bacteria (*P. putida*) registered with the DNA databank of Japan (DDBJ), were compared (Table 1). A BP-degrading enzyme from the filamentous fungus, *Paraphoma* sp. B47-9 (PCLE), was also analyzed. Sequence homology between phylloplane yeast enzymes and pathogenic plant fungi cutinase was approximately 30% or less. By contrast, PCLE showed a relatively high level of identity to phytopathogenic fungal cutinases (56.6% identity with *F. solani* cutinase and 34.0% identity with *B. cinerea* cutinase), as reported by Suzuki *et al.* (2014). Eukaryotic (yeast and filamentous fungi) enzymes exhibited less than 20% identity with a bacterial cutinase derived from *P. putida*.

Multiple alignment analysis revealed a high degree of similarity between the amino acid sequences of the above-compared enzymes (Figure S3B and C). CLEs contain 3 amino acids, Ser, Asp and His, which constitute a typical triad, with a conserved GYSQG motif that is characteristic of cutinases (Masaki *et al.* 2005), and 4 cysteines that make up the 2 disulfide bonds (Kodama *et al.* 2009). Two cysteine residues corresponding to Cys195 and 202 of *Cryptococcus* sp. S-2 CLE, which are reported to form a disulfide bond to play an important role in the stabilization of the catalytic site structure were also preserved in PaE and CmCut1 (Suzuki *et al.* 2013). Amino acid motifs around catalytic sites were highly conserved between CmCut1, basidiomycete yeast CLEs, the ascomycete cutinase of F. solani and the PCLE of Paraphoma sp. B47-9 (Figure S3B and C), indicating that CmCut1 is a CLE.

Thus, we added *C. magnus* to the group of phylloplane yeasts that secrete CLEs, which includes *P. antarctica* and *C. flavus*. In this study, we compared the genes and enzymes associated with CLEs with those associated with cutinases, with particular reference to PaE, which we have studied in depth. In order to compare the homology between enzymes associated with CLEs, a molecular phylogenic tree of 38 amino acid sequences, including those of the enzymes compared above, several cutinases and sequences with high similarity to PaE, are shown (Figure 1). The enzymes of basidiomycete species were separated into those associated with clades of *Pseudozyma*, *Ustilago*, *Testicularia*, *Jaminaea*, and *Cryptococcus*. Ascomycetous enzymes from several saprotrophs as well as fungi not related to the plant were placed relatively close to the basidiomycete CLEs. PCLE and phytopathogenic fungal cutinases from *F. solani, B. cinerea*, and *C. gloeosporioides* were relatively close to each other and placed in a separate branch. Moreover, the bacterial cutinase of *P. putida* was evolutionarily closer to that of the pathogenic filamentous fungi cutinase than to yeast CLEs.

Cutin degradation products identified via GC-MS

Thirty-eight grams of crude cutin was prepared from 51 g of lyophilized tomato leaves. Crude cutin was treated with a high concentration of KOMe for a relatively long time (48 h) in order to induce hydrolysis and methyl esterification of the cutin polyester. GC-MS analysis detected a peak corresponding to that of 10,16-dihydroxyhexadecanoic acid (10,16-DHHA) in the NIST mass spectrum library, including characteristic ions at *m/z* 446 (M^+), $M^+ - 15$ (CH_3), and $M^+ - 173$ [$(CH_2)_6OSi(CH_3)_3$] (Figure 2). This dihydroxyl fatty acid is a typical cutin monomer found in tomato plants (Yeats *et al.* 2013; Martin *et al.* 2017). The 20.8 mg of 10,16-DHHA released from 200 mg of crude cutin was the highest amount obtained from the sample in our GC-MS analyses. Saturated and unsaturated C16 and C18 fatty acids were also discovered, although the amounts produced were relatively small. The residue presumably consisted of insoluble carbohydrates, including cellulose and lignin (Osman *et al.* 1999).

Treatment of cutin with CLEs and cutinase

First, phylloplane yeast CLEs, filamentous fungus *F. solani* cutinase and PCLE were prepared. Next, 200 mg of crude cutin was incubated with these enzymes in HEPES buffer (pH 7.3) for 48 h. In order to compare the cutin-degrading activity of each enzyme, the amount of enzyme added to the reaction system was adjusted, so that their PBSA emulsion-degrading activities were of the same value at pH 7.3. The reaction temperature was unified at 25 °C, based on the relationship between the stability of each enzyme and its activity (Suzuki et al. 2013, 2014; Bischoff *et al.* 2015). GC-MS analysis detected the 10,16-DHHA derivative in all enzyme-treated products, as well as in the alkaline hydrolysate of cutin (Figure 2). Enzymatic activities (i.e. the concentration of generated 10,16-DHHA released in the 48 h reaction solution in the reaction and per 1 M of enzyme) based on the amounts of 10,16-DHHA released in the 48 h reaction solution were as follows: 4.28 μg (2.8 g/M PaE), 0.71 μg (0.5 g/M CmCut1), 1.04 μg (0.7 g/M CfCLE), 8.38 μg (5.6 g/M *F. solani* cutinase), and 3.21 μg (1.7 g/M PCLE) (Table 2, upper panel). The 10,16-DHHA derivative was detected in the crude cutin for all 3 CLEs tested, indicating that they did exhibit cutinase activity. The intensity of cutinase activity between CLEs differed, with PaE exhibiting the highest activity, as indicated by the amount of cutin monomer produced per molar concentration of the enzyme. Using apple cutin as a substrate, Soliday and Kolattukudy (1976) demonstrated that the optimum pH of *F. solani* cutinase was alkaline, while Shinozaki *et al.* (2013) used emulsified PBSA as a substrate to demonstrate that the optimum pH for PaE was also alkaline. Therefore, the cutinase activity of both enzymes was tested in 20 mM Tris-HCl buffer (pH 9.0) at the same protein concentration. After 48 h of reaction, 16.9 μg (11.1 g/M PaE) and 6.94 μg (4.6 g/M *F. solani* cutinase) of the cutin monomer were detected in the reaction solution (Table 2; bottom panel). The amount of cleaved monomer corresponding to the *F. solani* enzyme was higher at pH 7.3 than at pH 9. The opposite was observed for PaE. Due to differences in the optimum pH, it is not possible to simply compare cutinase activity between these enzymes. Since the results obtained in this experiment

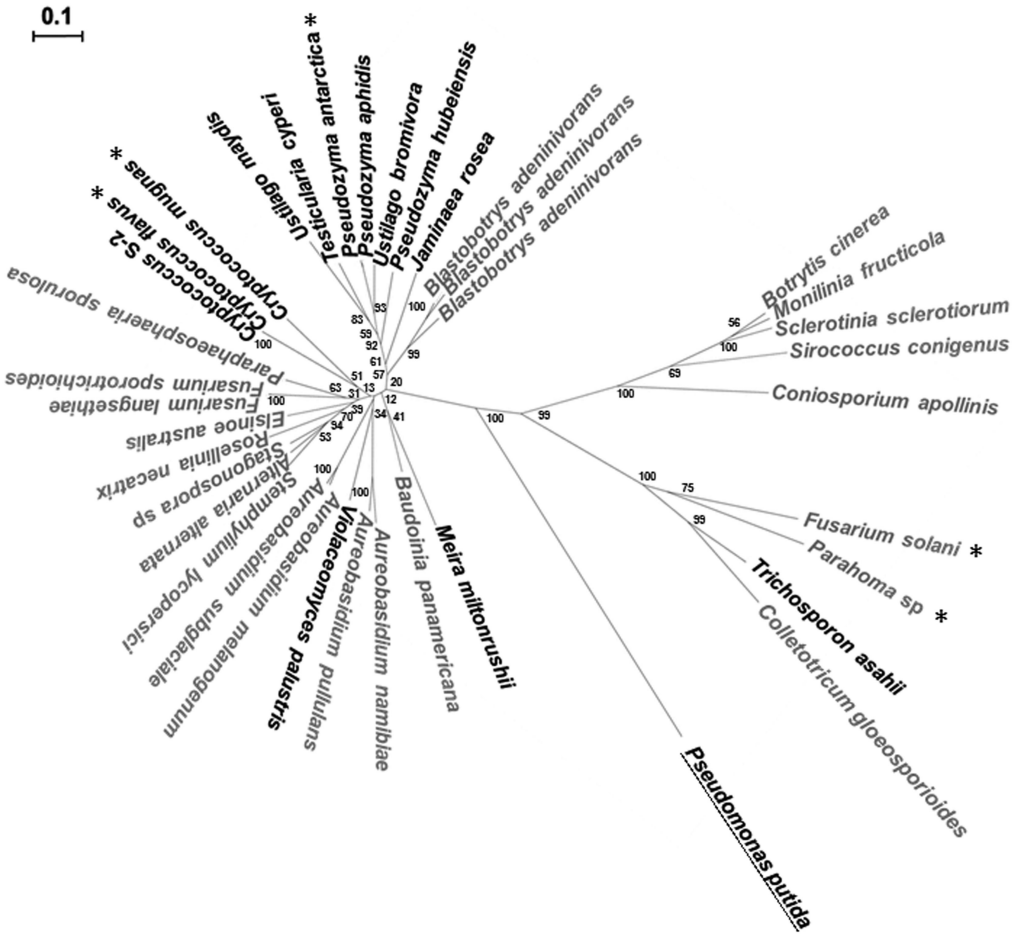

Figure 1. Molecular phylogenetic tree based on the amino acid sequence homology of cutinases and cutinase-like enzymes (CLEs). The results of homology analysis for each enzyme via the NJ method are presented in the rooted phylogenetic tree. Black letters indicate basidiomycetes, gray letters indicate ascospores, and black letters with underbar indicate bacteria. Asterisks indicate enzymes whose activity was measured in this study. Bootstrap value percentages (derived from 1000 replicates) are indicated at the branch point. The scale bar represents the unit of distance between sequence pairs.

Table 2. Cutinase activity of CLEs and cutinase derived from *F. solani*

pH	Enzyme	Protein conc. (μg/mL)	Unit	Cutin monomer (μg)	Cutin monomer/Enzyme molar conc. (g/m)
pH7.3	PaE	31.0	0.8 U	4.3	2.8
	CmCut1	37.0	0.8 U	0.7	0.5
	CfCLE	33.0	0.8 U	1.0	0.7
	PCLE	37.0	0.8 U	3.2	1.7
	FsCutinase	31.0	NT	8.4	5.6
	Buffer	–	–	ND	–
pH9.0	PaE	31.0	4 U	16.9	11.1
	FsCutinase	31.0	NT	6.9	4.6
	Buffer	–	–	ND	–

The amount of cutin monomer 10,16-dihydroxyhexadecanoic acid detected in the reaction solution after 48 h; NT, not tested; ND, not detected; FsCutinase, *F. solani* cutinase.

are of the same order, there may be no significant differences between the cutinase activities of these enzymes under optimal conditions. The crystal structure of strain S-2 CLE (Protein Data Bank ID: 2CZQ [10.2210/pdb2CZQ/pdb]) was very similar to that of PaE (Protein Data Bank ID: 7CW1 [10.2210/pdb7CW1/pdb]) and *F. solani* cutinase (Protein Data Bank ID: 1CUS [10.2210/pdb1CUS/pdb]) (Figure S4). Strain S-2 CLE and *F. solani* cutinase have a characteristic structure in which the catalytic site is directly exposed to the solvent, and the loops observed in most lipases are not located above the catalytic site (Longhi and Cambillau 1999). This structure around the catalytic site is said to be related to the ability to degrade BPs (Kodama *et al.* 2009). PaE also showed a similar structure, suggesting that this structure around the catalytic site plays an important role in the degradation activity of cutin and BPs.

Separation and detection of cutin degradation products via TLC

Cutin is a complex multimer, and if cutinase is hypothetically considered as an endo-type enzyme, monomers as well

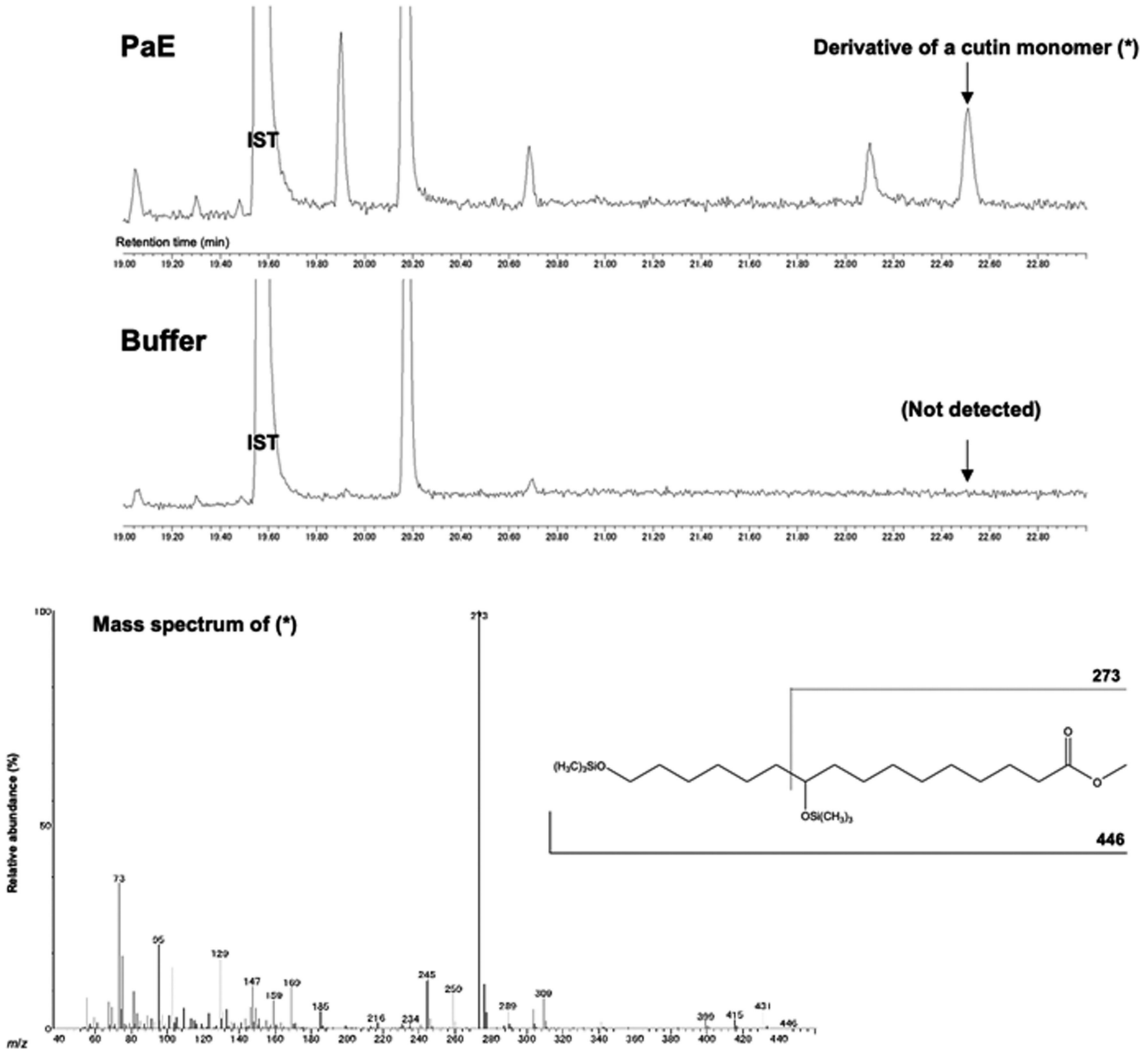

Figure 2. GC-MS analysis of cutin degradation products. Crude cutin prepared from tomato leaves was treated with PaE, and the degradation products were analyzed via GC-MS. The peak, indicated by an arrow, was detected only after PaE processing. Structural analysis of this peak via MS was in accordance with 10,16-DHHA (cutin monomer) in the NIST mass spectrum library. No peaks were detected in the buffer-treated samples. ITS: Internal standard substance

as oligomers should be produced following cutinase treatment. Cutin alkali hydrolysates and PaE-treated products were separated via TLC and detected using iodine. The most mobile spots (R_F: 0.96) detected in both reaction mixtures were considered to be caused by the cutin monomer, 10,16-DHHA. Although multiple spots with low mobility were detected for both decomposed substances, the mobility of these spots was different (Figure 3). This may be due to the fact that, structurally, cutin is a linear and/or dendritic polymer, comprising multiple organic compounds. Furthermore, cutin monomers are cross-linked and can be divided into different sizes depending on the site of cleavage (Fich, Segerson and Rose 2016). These data suggest that PaE exhibits endo-cutinase activity. Due to the insolubility of cutin oligomers in various solvents, the structure of these oligomers cannot be determined. Thus, these oligomers will expectedly be analyzed in the future.

Discussion

Cutin, the original substrate of cutinase, is not commercially viable and must be prepared in the laboratory. Thus, suitable and readily available cutinase specific substrates are required. On the other hand, cutinase came to the fore during the process of searching for a synthetic long chain polyester-degrading enzyme. Several filamentous fungal and bacterial cutinases that show both cutin and synthetic polyester degradation ability have been reported (Murphy *et al.* 1996; Nimchua, Punnapayak and Zimmermann 2007; Brueckner *et al.* 2008). Molecular taxonomy indicates that these fungal cutinases have low structural similarity with bacterial cutinases. Yeast CLEs, which showed only 30% similarity to filamentous fungal cutinases, were identified as synthetic polymer-degrading enzymes. Although the cutinase activity has not been determined, CLEs were classified as a third group of cutinases by Chen *et al.* (2013). Here, we demonstrated that CLEs secreted by phylloplane *Pseudozyma* and *Cryptococcus* yeasts display the same cutin-degrading activity as filamentous phytopathogenic fungus cutinases (Table 2). In addition, amino acid sequences of CLEs form a group that is separate from that of filamentous fungi and bacterial cutinases (Figure 1). This result substantiates Chen's classification and indicates that it is appropriate to use synthetic polyesters as substrates for cutinase. However, many enzymes that act on biodegradable plastics have evolved by microorganisms not to

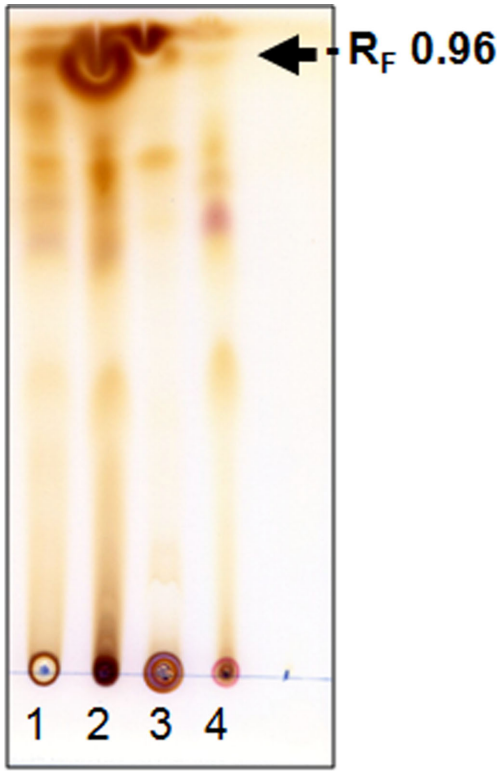

Figure 3. TLC analysis of cutin degradation. Degradation products of tomato leaf crude cutin treated with KOMe or PaE were subjected to TLC. Lane 1: Cutin degradation products used as standard, 100 µL (0.4 mg); Lane 2: 1.5 M KOMe o/n treatment, 20 µL; Lane 3: 0.1 M KOMe o/n treatment, 20 µL; and Lane 4: PaE o/n treatment, 100 µL. Spots with an R_F value of 0.96, indicated by an arrow, were considered as cutin monomers. RF: retardation factor.

break down artificially synthesized polyesters, but to catalyze the breakdown of naturally occurring biopolymers (Chen *et al.* 2013; SAPEA 2020).

The CLEs of phylloplane yeasts exhibit a wide range of esterase activities against alkyl chain length fatty acids, compared with the cutinases of phytopathogenic filamentous fungi (Kodama *et al.* 2009). Analyses comparing the crystal structures of CLE and PaE with that of *F. solani* cutinase have indicated that a larger space in the proximity of the active site (Figure S4) confers CLEs with an advantage in binding to long-chain fatty acids (Kodama *et al.* 2009), suggesting that CLEs may have been adapted for fatty acid extraction. In fact, saturated and unsaturated fatty acids with 16 and 18 carbon chains embedded in the cuticular layer of leaves have been extracted from leaves treated with PaE (Ueda *et al.* 2015). In addition, natural oils were used to induce the production of CLEs by phylloplane yeasts (Kamini *et al.* 2000; Watanabe *et al.* 2014b). The surface of the cuticular layer of leaves reportedly restricts the ability of microorganisms to obtain nutrients (Lindow and Brandl 2003). Although the densities of CLEs producing yeasts in healthy leaves were low, these results suggested that these yeasts were able to obtain sufficient carbon sources, in the form of fatty acids, from leaves (Franke *et al.* 2005; Kitamoto *et al.* 2011; Nadakuduti *et al.* 2012).

In previous experiments, no cutin monomers were detected in leaves treated with PaE (Ueda *et al.* 2015). In this study, when PaE was applied to cutin as a residue of lipid extraction from leaves with organic solvents, cutin monomers were detected. This suggests that PaE preferentially cleaves fatty acid ester bonds compared to cutin polymer ester bonds located on the surface of fresh leaves. During the time from seed to plant growth and death, resident microorganisms attached to the seed expand their habitat on the plant surface (Vorholt 2012; Saleem *et al.* 2017). But the biology of yeast cells remaining on the surface of dead plants is not yet known. In the laboratory, phylloplane yeasts produced CLEs when treated with natural oils but produced even more enzymes when treated with xylose (Watanabe *et al.* 2014a,b; Kamini *et al.* 2000). Xylose is contained in hemicellulose, which constitutes plant cell walls. This indicates that the yeast cells may obtain xylose from dead plants, produce CLEs, and degrade cutin. On the other hand, the cutin-degrading ability of CLEs is similar to that of cutinase. However, cutinase production by phytopathogenic filamentous fungi is induced by a cutin monomer and is tightly regulated to prevent production by other substrates (Li *et al.* 2002). Therefore, the role of phylloplane yeast CLEs and phytopathogenic filamentous cutinases may not be identical. The function of cutinase isolated from phytopathogenic filamentous fungi is yet to be confirmed experimentally, although some theories suggest that its function pertains to the infection of plants (Skamnioti and Gurr 2007; L'Haridon *et al.* 2011). There are some reports that it is produced during the saprophytic life (Stahl and Schafer 1992; Yao and Köller 1995). While living on the surface of plants, microorganisms might be present as either harmless residents or pathogens, but via their enzyme activity, they may contribute to the cooperative decomposition of cutin when the plant dies. However, information obtained by culturing isolated strains on laboratory media alone does not offer a full clarification of naturally occurring phenomena. Analysis of enzyme productivity of microorganisms capable of producing cutinase and the subsequent effects on plant components in the real environment will reveal the underlying mechanisms.

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Supplementary material

Supplementary material is available at *Bioscience, Biotechnology, and Biochemistry* online.

Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

Author contribution

H.U. and H.K. conceived and supervised the study; H.U. and H.K. designed the experiments; H.U., J.T., and Y.S. performed the experiments; K.M., and Y.S-Y. carried out expression and purification of recombinant enzymes; H.U. and H.K. wrote the manuscript; J.T., K.M., and Y.S-Y made manuscript revisions.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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