

Uniqueness of Microbial Cutinases in Hydrolysis of *p*-Nitrophenyl Esters

KIM, YANG-HOON^{1,2}, JEEWON LEE^{1*}, AND SEUNG-HYEON MOON²

¹Department of Chemical and Biological Engineering, Korea University, Sungbuk-Ku, Seoul 136-701, Korea

²Department of Environmental Science and Engineering, Kwangju Institute of Science and Technology, Kwangju 500-712, Korea

Received: June 10, 2002

Accepted: December 2, 2002

Abstract Using fungal (*Fusarium solani f. pisi*) and bacterial (*Pseudomonas mendocina*) cutinases, the initial hydrolysis rate of *p*-nitrophenyl esters was systematically estimated for a wide range of enzyme and substrate concentrations using a 96-well microplate reader. Both cutinases exhibited a high substrate specificity; i.e. a high hydrolytic activity on *p*-nitrophenyl butyrate (PNB), yet extremely low activity on *p*-nitrophenyl palmitate (PNP). When compared to the hydrolysis of PNB and PNP by other hydrolases [lipases and esterases derived from different microbial sources, such as bacteria (*Pseudomonas cepacia*, *Pseudomonas fluorescens*, *Bacillus stearothermophilus*), molds (*Aspergillus niger*, *Mucor miehei*), and yeasts (*Candida rugosa*, *Candida cylindracea*)], the above substrate specificity would seem to be a unique characteristic of cutinases. Secondly, the hydrolytic activity of the cutinases on PNB appeared much faster than that of the other hydrolytic enzymes mentioned above. Furthermore, the current study proved that even when the cutinases were mixed with large amounts of other hydrolases (lipases or esterases), the initial hydrolysis rate of PNB was determined only by the cutinase concentration for each PNB concentration. This property of cutinase activity would seem to result from a higher accessibility to the substrate PNB, compared with the other hydrolytic enzymes. Accordingly, these distinct properties of cutinases may be very useful in the rapid and easy isolation of various natural cutinases with different microbial sources, each of which may provide a novel industrial application with a specific enzymatic function.

Key words: Cutinase, lipase, esterase, hydrolysis, substrate specificity, accessibility

Cutinases are hydrolytic enzymes that degrade cutin, the cuticular polymer (i.e. a polyester composed of hydroxy

and epoxy fatty acids with usually *n*-C16 and *n*-C18) in higher plants. Certain microorganisms live on cutin as their sole carbon source, while producing extracellular cutinolytic enzymes, and several cutinases have already been isolated and characterized from different sources, mainly fungi (*Fusarium solani f. pisi*) and bacteria (a phyllospheric fluorescent *Pseudomonas putida* and *Pseudomonas mendocina*, cohabiting with a nitrogen-fixing bacteria, and *Corynebacterium* sp.) [2, 7, 10, 12, 14, 15, 17]. Based on its cutinolytic activity, an enzyme preparation containing cutinase has been developed to increase the pharmacological effect of agricultural chemicals [4]. In recent years, the esterification [20] and transesterification activities of cutinases have been extensively exploited and could be advantageously applied in chemical synthesis [5]. Cutinases have also been applied as a lipolytic enzyme in laundry or dishwashing detergent composition to efficiently remove immobilized fats [3, 9, 13, 18], while potential uses include application in the dairy industry for the hydrolysis of milk fat, the oleochemistry industry [1], and pollutant degradation [6, 12, 19]. Due to these potential applications of cutinases, this enzyme has been cloned and expressed in heterologous hosts, and more recently, an efficient production system for recombinant cutinase in *Saccharomyces cerevisiae* was developed, as the yeast is capable of carrying out post-translational modifications important for full protein biological activity [1].

Accordingly, the present study examined and compared the hydrolytic properties of cutinases, lipases, and esterases from different microbial sources in the hydrolysis of *p*-nitrophenyl esters with different fatty acid chains as the substrate. Rapid access to the substrate and chain length-specific hydrolysis were identified as intrinsic properties of just the cutinases, which were not found in the hydrolysis by the other lipases and esterases. As such, these unique characteristics of cutinases could be utilized as simple phenotype markers for selecting novel microorganisms producing cutinases.

*Corresponding author
Phone: 82-2-3290-3304; Fax: 82-2-926-6102;
E-mail: leejuw@korea.ac.kr

MATERIALS AND METHODS

Preparation of Enzyme Solutions (Cutinases, Lipases, and Esterases)

The purified cutinase from *Fusarium solani f. pisi* was kindly provided by Prof. C. M. J. Sagt at Utrecht University (The Netherlands) and kept at -21°C . The purified commercial lipases and esterases preparations were purchased from various manufacturers as follows: lipases (*Aspergillus niger*, 1.4 U/mg and *Pseudomonas cepacia*, 48 U/mg) from Fluka (Buchs City, Switzerland); lipase (*Candida rugosa*, 360 U/mg) from Meito Sangyo (Tokyo, Japan); cholesterol esterases (*Pseudomonas fluorescens*, 25 U/mg and *Candida cylindracea*, 26 U/mg) from Boehringer Mannheim GmbH

(Mannheim, Germany); and esterases (*Mucor miehei*, 1.2 U/mg and *Bacillus stearothermophilus*, 0.41 U/mg) from Fluka (Buchs City, Switzerland).

The purified enzymes listed above were dissolved in 10 mM Tris-HCl buffer (pH 8.00) and well mixed. The enzyme solution was centrifuged at 7,000 rpm for 15 min to remove any insoluble debris, then the dissolved enzyme concentrations were determined using a Bio-Rad (Hercules, CA, U.S.A.) protein assay kit with bovine serum albumin as the standard.

Cultivation of Cutinolytic Bacteria

The cutinase-producing bacteria, *Pseudomonas mendocina* (ATCC 55613), was cultivated on an NBY medium [0.2%

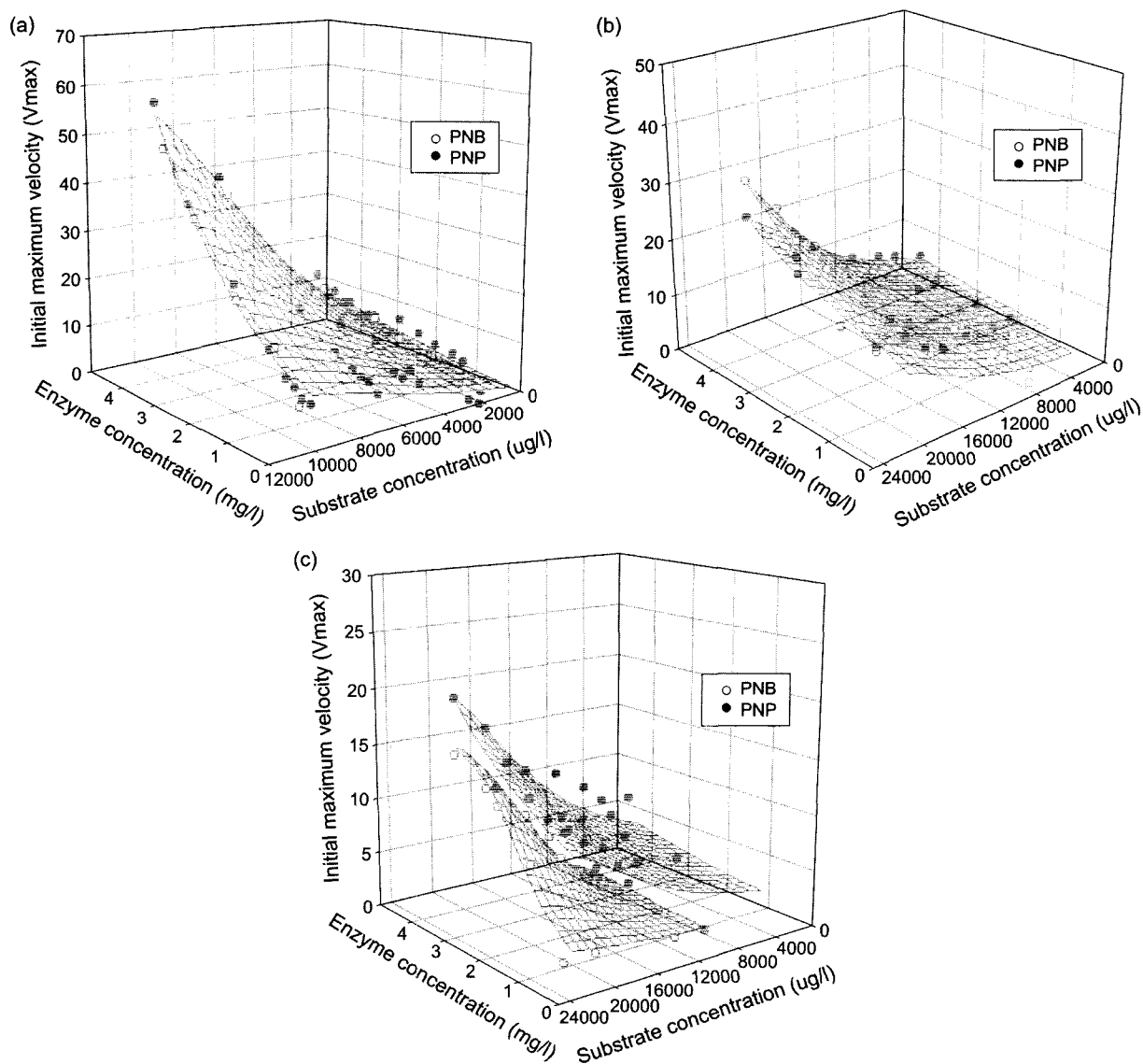


Fig. 1. Initial maximum rate of PNB and PNP hydrolysis by lipases from various microbial sources. (a) *Pseudomonas cepacia*; (b) *Candida rugosa*; (c) *Aspergillus niger*.

yeast extract; 0.8% nutrient broth (Difco)]. To enhance the production of the cutinases, apple cutin (1.0%) was added as an inducer. The apple cutin was prepared using the same procedure as previously described by Kolattukudy *et al.* [8].

Substrate Preparation, Enzymatic Hydrolysis, and Activity Estimation

Using *p*-nitrophenyl butyrate (PNB) and *p*-nitrophenyl palmitate (PNP) (Sigma, St. Louis, MO, U.S.A.) as the substrates, the substrate solutions were prepared by following a previously described procedure [8].

The hydrolysis reactions were carried out in a 96-well microplate at 30°C for 4 min, where each well contained 200 μ l of an enzyme/substrate solution (solution A), comprising 106.7 μ l of phosphate buffer (0.1 M, pH 8.0), 13.3 μ l of

Triton X-100 solution (4 g/l), 13.3 μ l of enzyme (lipase, esterase, or cutinase at variable concentrations), and 66.7 μ l of substrate (PNB or PNP at variable concentration).

The reaction was started by adding 66.7 μ l of the substrate solution to each well in the 96-well microplate. The initial velocity [i.e. initial maximum rate of absorbance change ($\Delta OD_{405\text{ nm}}$ per second)] was measured using a Bio-Rad microplate reader (Cat. No. 170-6850, Hercules, CA, U.S.A.), while the above solution A without any enzyme was used as a blank. Since the eight wells in each of the 12 columns of the 96-well microplate were under the same reaction conditions, i.e. contained an equal content of enzyme and substrate, the eight initial velocities measured for each column were used to calculate an average initial velocity for specific reaction conditions (the standard deviations were less than 5%).

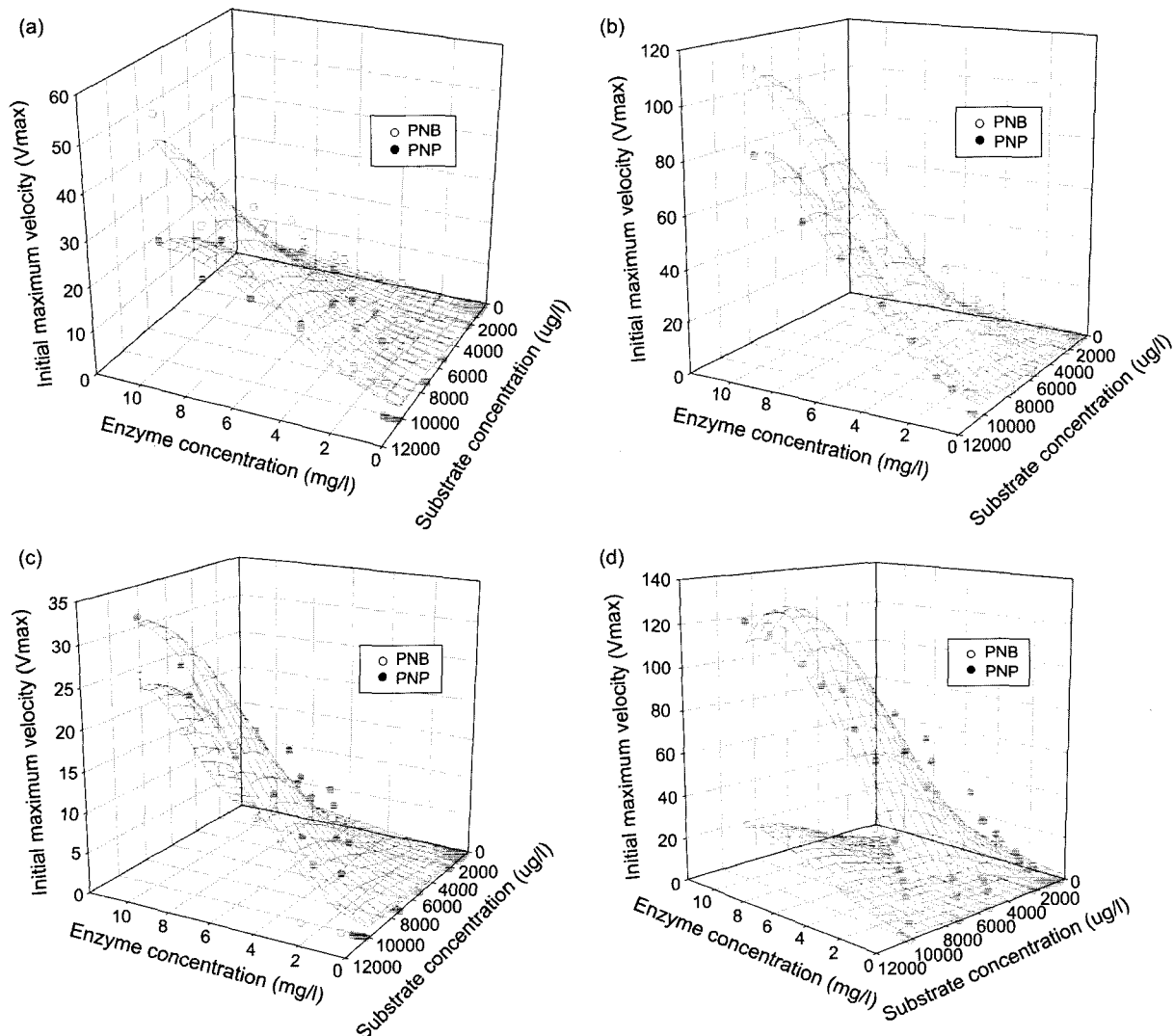


Fig. 2. Initial maximum rate of PNB and PNP hydrolysis by esterases from various microbial sources. (a) *Bacillus stearothermophilus*; (b) *Candida cylindracea*; (c) *Mucor miehei*; (d) *Pseudomonas fluorescens*.

RESULTS

Substrate-Specific Hydrolysis by Cutinases

On two *p*-nitrophenyl esters, PNB and PNP, the hydrolytic activities of various lipases and esterases (commercial purified products, see Materials and Methods) were estimated for a range of enzyme and substrate concentrations. The hydrolytic activities of the enzymes in the current study are defined as the initial maximum rate of hydrolysis at each substrate concentration. As presented in Figs. 1 and 2, the hydrolytic activities of all lipases and esterases were comparable on both PNB and PNP for the entire range of substrate and enzyme concentrations tested, except for the esterase from *P. fluorescens*, which exhibited a much higher activity on PNP than on PNB [Fig. 2(d)].

However, the hydrolysis of both *p*-nitrophenyl esters by cutinases revealed that the initial hydrolysis rate was significantly dependent on the fatty acid chain length of the *p*-nitrophenyl esters. The initial rate of PNB hydrolysis by the purified fungal cutinase (derived from *F. solani f. pisi*) was up to several hundred-fold higher than that by the lipases or esterases, whereas the cutinase showed an extremely low hydrolytic activity on PNP with the same range of enzyme and PNB concentrations (Fig. 3). The extracellular culture broth of the cutinolytic bacteria *P. mendocina* (ATCC 55613) exhibited exactly the same substrate-specificity in the hydrolysis of PNB and PNP as the fungal cutinase above; i.e. the supernatant of the bacterial culture hydrolyzed PNB at a high rate, but not PNP (Fig. 4a). Cutin (10 g/l) was used as an inducer for cutinase production in the cultivation of *P. mendocina*. Since the time-course of hydrolytic activity of the noninduced culture (where no cutin was added) was almost negligible (Fig. 4b), it would seem evident that the induced culture broth contained extracellular cutinase.

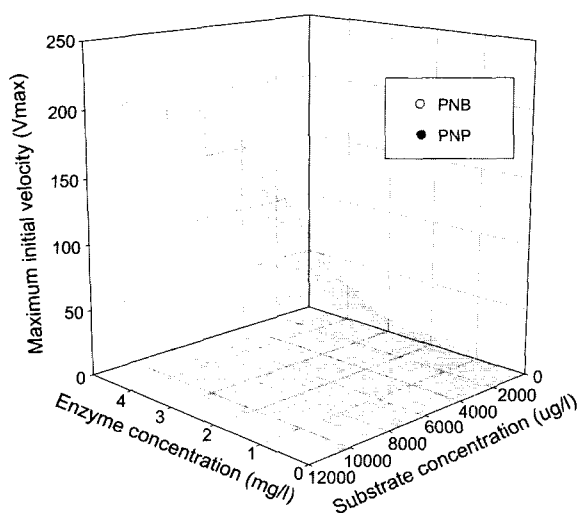


Fig. 3. Initial maximum rate of PNB and PNP hydrolysis by cutinase from *Fusarium solani f. pisi*.

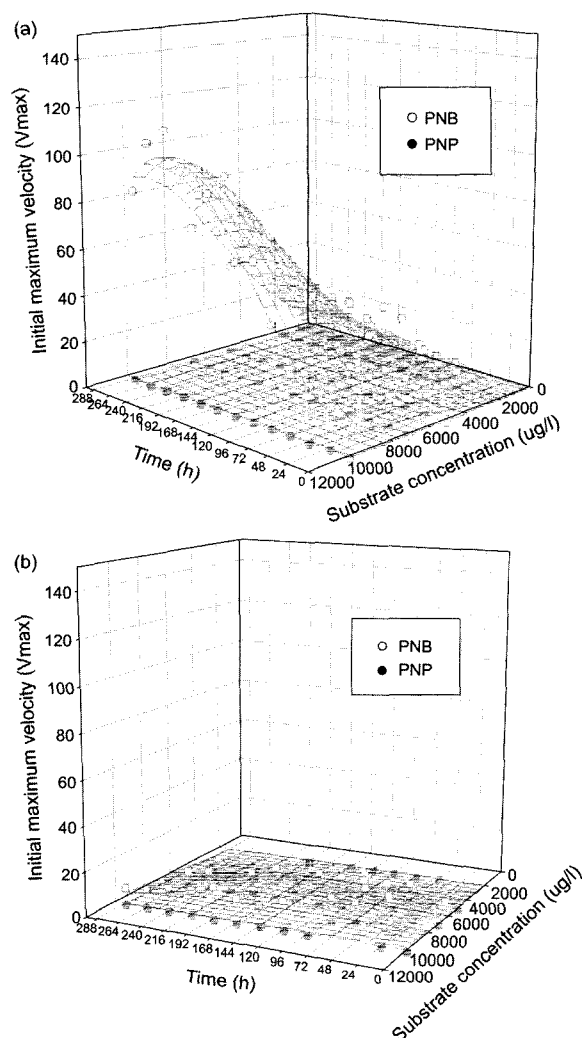


Fig. 4. Initial maximum rate of PNB and PNP hydrolysis by extracellular culture broth of *Pseudomonas mendocina* (ATCC 55613).

Cultivated (a) with cutin (10 g/l) as an inducer; (b) without cutin.

High Accessibility of Cutinases to Substrate PNB

The initial maximum rate of PNB hydrolysis by enzyme mixtures [(lipase+cutinase) or (esterase+cutinase) at various compositions] was also estimated and compared with that for pure hydrolases (lipase, esterase, or cutinase). The results are summarized in Tables 1 and 2. Although lipase or esterase was present, even in excess, with cutinase, the initial maximum rate of PNB hydrolysis was basically invariable as long as the cutinase concentration remained unchanged. For example (from Table 1), the initial maximum rate of PNB hydrolysis ($V_{0, \text{PNB}}$) by the pure lipase (from *P. cepacia*, 9.9 mg/l) was around 69.7 for PNB 10,000 $\mu\text{g/l}$; however, it drastically decreased to around 9.4 when the lipase was replaced by an enzyme mixture containing the *P. cepacia* lipase and the fungal cutinase at 9.9 and 0.1 mg/l, respectively. Therefore, based on the result that the $V_{0, \text{PNB}}$ (with PNB 10,000 $\mu\text{g/l}$) for

Table 1. Initial maximum rate of PNB hydrolysis ($V_{0, \text{PNB}}$) by cutinase (from *F. solani f. pisi*), lipases (from different sources), and different mixtures at various PNB and enzyme concentrations.

No.	Enzyme	Conc. (mg/l)	PNB concentration ($\mu\text{g/l}$)							
			300	500	700	1,000	2,000	5,000	7,000	10,000
1	CT	0.1	0	0	0	0	0	7.15 \pm 0.25	7.40 \pm 0.20	8.15 \pm 1.30
	LP ^p	9.9	0	0	8.05 \pm 1.38	10.6 \pm 0.58	24.4 \pm 2.20	38.05 \pm 0.47	53.45 \pm 1.48	69.73 \pm 1.13
	Mixture (1% CT)	0.1 (CT) 9.9 (LP/P)	0	0	0	0	0	7.30 \pm 0.23	7.05 \pm 0.10	9.35 \pm 2.14
2	CT	0.5	0	0	7.13 \pm 0.05	11.35 \pm 0.50	17.75 \pm 0.47	23.88 \pm 0.69	29.70 \pm 0.62	36.35 \pm 2.87
	LP ^p	9.5	0	0	7.13 \pm 0.05	11.68 \pm 1.05	22.03 \pm 0.63	30.58 \pm 1.92	48.93 \pm 3.02	67.45 \pm 1.56
	Mixture (5% CT)	0.5 (CT) 9.5 (LP/P)	0	0	7.40 \pm 0.20	12.35 \pm 0.30	16.15 \pm 0.75	23.18 \pm 0.67	30.83 \pm 2.24	37.55 \pm 1.12
3	CT	0.1	0	0	0	0	0	7.15 \pm 0.25	7.40 \pm 0.20	8.15 \pm 1.30
	LP ^c	9.9	0	0	0	7.15 \pm 0.25	9.05 \pm 1.84	11.70 \pm 0.58	17.20 \pm 0.58	21.85 \pm 1.44
	Mixture (1% CT)	0.1 (CT) 9.9 (LP/C)	0	0	0	0	0	7.15 \pm 0.06	8.01 \pm 1.36	8.70 \pm 1.62
4	CT	0.5	0	0	7.13 \pm 0.05	11.35 \pm 0.50	17.75 \pm 0.47	23.88 \pm 0.69	29.70 \pm 0.62	36.35 \pm 2.87
	LP ^c	9.5	0	0	0	7.10 \pm 0.00	7.33 \pm 0.21	10.88 \pm 0.72	14.68 \pm 0.51	20.90 \pm 3.21
	Mixture (5% CT)	0.5 (CT) 9.5 (LP/C)	0	0	7.10 \pm 0.28	11.10 \pm 0.82	17.00 \pm 0.52	23.93 \pm 1.43	28.05 \pm 3.33	36.93 \pm 3.36
5	CT	0.1	0	0	0	0	0	7.15 \pm 0.25	7.40 \pm 0.20	8.15 \pm 1.30
	LP ^a	9.9	0	0	0	0	8.80 \pm 1.50	7.30 \pm 0.23	11.65 \pm 0.64	12.83 \pm 0.46
	Mixture (1% CT)	0.1 (CT) 9.9 (LP/A)	0	0	0	0	0	7.00 \pm 0.12	8.80 \pm 1.50	11.63 \pm 0.67
6	CT	0.5	0	0	7.13 \pm 0.05	11.35 \pm 0.50	17.75 \pm 0.47	23.88 \pm 0.69	29.70 \pm 0.62	36.35 \pm 2.87
	LP ^a	9.5	0	0	0	0	8.05 \pm 1.38	7.30 \pm 0.23	10.45 \pm 0.44	12.05 \pm 0.19
	Mixture (5% CT)	0.5 (CT) 9.5 (LP/A)	0	0	7.50 \pm 0.00	13.15 \pm 0.68	15.63 \pm 1.20	24.20 \pm 1.04	31.75 \pm 0.52	39.60 \pm 1.10

CT: cutinase from *F. solani f. pisi*; LP^p: lipase from *P. cepacia*; LP^c: lipase from *Candida rugosa*; LP^a: lipase from *A. niger*.

the pure fungal cutinase (0.1 mg/l) was approximately 8.2, it is quite likely that the initial hydrolysis rate of PNB by the enzyme mixture containing cutinase was primarily determined by the cutinase. Also in Table 1, the $V_{0, \text{PNB}}$ (with PNB 10,000 $\mu\text{g/l}$) for another pure lipase (from *A. niger*, 9.5 mg/l) is around 12.1, yet increased to around 39.6 when the *A. niger* lipase was replaced by an enzyme mixture containing the same lipase (9.5 mg/l) and the fungal cutinase (0.5 mg/l). The $V_{0, \text{PNB}}$ (with PNB 10,000 $\mu\text{g/l}$) for the latter enzyme mixture was almost the same as that for the pure fungal cutinase (0.5 mg/l), \sim 36.4 (Table 1). Table 2 presents similar results when various esterases were used with cutinase. Consequently, these results would seem to suggest that the cutinases acted on the PNB with an apparently higher accessibility to PNB than the other lipases or esterases, hence the $V_{0, \text{PNB}}$ for the enzyme mixtures containing cutinase was determined by the cutinase activity, irrespective of the amount or concentration of lipases or esterases.

DISCUSSION

Cutinases are known to have a high hydrolytic activity for a variety of esters, ranging from soluble *p*-nitrophenyl esters

to insoluble long-chain triglycerides. However, as presented in the current report, the hydrolytic activity of cutinases on *p*-nitrophenyl esters of fatty acid was very sensitive to the chain length of the fatty acid. Previous reports have elucidated that a fungal cutinase (from *F. solani f. pisi*) lacks a large hydrophobic surface around the active site, in contrast to other lipases [11]. This structural feature of cutinases may be closely related to a high substrate-specificity, for example, an extremely low activity on the *p*-nitrophenyl ester of a long-chain fatty acid, like PNP. The initial maximum rate of PNB hydrolysis by cutinases was exactly reproduced, even when the cutinase was mixed with large amounts of lipases or esterases from diverse microbial sources. When using enzyme mixtures containing various cutinase concentrations, the $V_{0, \text{PNB}}$ was significantly dependent on the cutinase concentration. Unlike other lipases, since the catalytic serine of cutinases is not buried under surface loops, yet accessible to the solvent, cutinases do not exhibit interfacial activation [11]. Therefore, this structural difference may explain why the cutinases had easier access to the substrate and exerted hydrolytic activity before the other hydrolases. As such, this unique function of cutinases can provide a significant advantage when they are used as a

Table 2. Initial maximum rate of PNB hydrolysis ($V_{0, \text{PNB}}$) by cutinase (from *F. solani f. pisi*), esterases (from different sources), and different mixtures at various PNB and enzyme concentrations.

No.	Enzyme	Conc. (mg/l)	PNB concentration ($\mu\text{g/l}$)							
			300	500	700	1,000	2,000	5,000	7,000	10,000
1	CT	0.1	0	0	0	0	0	7.10±0.00	7.20±0.20	9.35±1.50
	ET ^c	9.9	7.10±0.00	7.40±0.20	10.35±0.50	14.25±0.17	35.40±0.20	72.40±0.23	92.13±0.10	115.43±0.10
	Mixture (1% CT)	0.1 (CT) 9.9 (LP/C)	0	0	0	7.20±0.20	7.33±0.21	10.40±0.20	15.38±0.25	19.95±1.33
2	CT	0.5	0	0		10.70±0.49	17.05±0.52	22.73±0.61	30.88±0.52	36.88±0.32
	ET ^c	9.5	7.10±0.00	7.20±0.20	10.10±0.00	13.18±0.15	33.18±0.15	68.70±0.14	92.13±0.10	111.43±0.17
	Mixture (5% CT)	0.5 (CT) 9.5 (LP/C)	0	0	7.23±0.19	11.05±0.06	19.08±1.13	28.45±0.40	39.45±0.75	55.23±0.05
3	CT	0.1	0	0	0	7.00±0.12	7.30±0.23	7.50±0.00	10.60±0.58	
	ET ^b	9.9	0	0	7.30±0.23	11.65±0.52	18.45±0.40	23.30±1.27	33.25±0.10	52.18±0.05
	Mixture (1% CT)	0.1 (CT) 9.9 (LP/C)	0	0	0	7.10±0.00	7.30±0.24	11.35±0.50	12.75±0.64	13.70±0.46
4	CT	0.5	0	0	0	8.60±1.73	13.78±0.56	18.63±0.35	32.65±0.52	39.45±0.75
	ET ^b	9.5	0	0	7.20±0.20	10.80±0.35	18.25±0.64	23.28±0.10	31.15±1.21	51.15±1.21
	Mixture (5% CT)	0.5 (CT) 9.5 (LP/C)	0	0	7.10±0.00	7.50±0.00	14.25±0.17	22.68±0.55	36.35±0.29	45.50±1.27
5	CT	0.1	0	0	0	7.00±0.12	7.30±0.23	7.50±0.00	10.60±0.58	
	ET ^m	9.9	0	0	0	7.00±0.12	10.05±0.06	17.10±0.00	25.08±0.05	
	Mixture (1% CT)	0.1 (CT) 9.9 (LP/C)	0	0	0	7.00±0.12	7.20±0.20	11.10±0.00	13.70±0.46	15.85±0.87
6	CT	0.5	0	0	0	8.60±1.73	13.78±0.56	18.63±0.35	32.65±0.52	39.45±0.75
	ET ^m	9.5	0	0	0	7.30±0.23	8.80±1.50	16.45±0.30	24.75±0.40	
	Mixture (5% CT)	0.5 (CT) 9.5 (LP/C)	0	0	0	7.40±0.20	14.73±0.38	19.45±0.75	38.25±0.37	46.85±0.29

CT: cutinase from *F. solani f. pisi*; ET^c: esterase from *C. cylindracea*; ET^b: esterase from *B. stearothermophilus*; ET^m: esterase from *M. miehei*.

biodetergent, since they facilitate hydrolysis and thereby effectively remove immobilized fat. Recently, recombinant cutinase mutants have been designed by modifying the hydrophobic surface around the active site to improve the wash performance, making them useful in detergents [16]. Because of the significant application potential of cutinases in various industries, new cutinolytic microorganisms and novel cutinases with specific functions are worthy of being isolated. Furthermore, the identification of unique cutinase properties in the hydrolysis of *p*-nitrophenyl esters may significantly contribute towards establishing a rapid and reliable method for screening and isolating novel natural cutinases.

Acknowledgment

This work was supported by a Korea University Grant.

REFERENCES

- Cristina, M. L. C., R. A. B. Maria, and M. S. C. Joaquim. 1999. Cutinase: From molecular level to bioprocess development. *Biotechnol. Bioeng.* **66**: 17–34.
- Dantzig, A. H., S. H. Zuckerman, and M. M. Andonov-Roland. 1986. Isolation of a *Fusarium* mutant reduced in cutinase activity and virulence. *J. Bacteriol.* **168**: 911–916.
- Flipsen, J. A. C., A. C. M. Appel, H. T. W. M Van der Hijden, and C. T. Verrips. 1998. Mechanism of removal of immobilized triacylglycerol by lipolytic enzymes in a sequential laundry wash process. *Enzyme Microb. Technol.* **23**: 274–280.
- Genencor. 1988. Increasing pharmacological effect of agricultural chemicals. US patent 88-08945.
- Gerard, H. C., W. F. Fett, S. F. Osman, and R. A. Moreau. 1993. Evaluation of cutinase activity of various industrial lipases. *Biotechnol. Appl. Biochem.* **17**: 181–189.
- Kim, Y. H., J. Lee, J. Y. Ahn, M. B. Gu, and S. H. Moon. 2002. Enhanced degradation of an endocrine-disrupting chemical, butyl benzyl phthalate, by *Fusarium oxysporum f. sp. pisi* cutinase. *Appl. Environ. Microbiol.* **68**: 4684–4688.
- Kolattukudy, P. E. 1984. Cutinase from fungi and pollen, pp. 471–504. In B. Borgstrom and T. Brockman (eds.), *Lipase*. Elsevier, Amsterdam.
- Kolattukudy, P. E., R. E. Purdy, and I. B. Maiti. 1981. Cutinase from fungi and pollen, pp. 652–664. In J. M. Lowenstein (ed.), *Methods in Enzymology*, Vol. **71**. Academic Press, New York, U.S.A.

9. Kwon, D. Y., W. J. Chung, D. Tlan, C. S. Jin, T. J. Lee, and H. K. Shon. 2002. Degradation of fat, oil, and grease (FOGs) by lipase-producing bacterium *Pseudomonas* sp. strain D2D3. *J. Microbiol. Biotechnol.* **12**: 583–591.
10. Lin, T.-S. and P. E. Kolattukudy. 1980. Structural studies on cutinase, a glycoprotein containing novel amino acids and glucuronic acid amide at the N terminus. *Eur. J. Biochem.* **106**: 341–351.
11. Martinez, C., P. De Geus, M. Lauwereys, G. Matthysens, and C. Cambillau. 1992. *Fusarium solani* cutinase is a lipolytic enzyme with a catalytic serine accessible to solvent. *Nature* **356**: 615–618.
12. Murphy, C. A., J. A. Cameron, S. J. Huang, and R. T. Vinopal. 1996. *Fusarium* polycaprolactone depolymerase is cutinase. *Appl. Environ. Microbiol.* **62**: 456–460.
13. Okkels, J. S., A. Svendsen, K. Borch, M. Thellersen, S. A. Patkar, D. A. Petersen, J. C. Royer, and T. Kretzschmar. 1997. New lipolytic enzymes with high capacity to remove lard in one wash cycle. US patent 97-05735.
14. Petersen, M. T. N., P. Martel, E. I. Petersen, F. Drablos, and S. B. Pertersen. 1997. Surface and electrostatics of cutinases, pp. 130–154. In B. Rubin and E. A. Dennis (eds.), *Methods in Enzymology*, Vol. **284**. Academic Press, New York, U.S.A.
15. Purdy, R. E. and P. E. Kolattukudy. 1975. Hydrolysis of plant cutin by plant pathogens purification, amino acids composition, and molecular weight of two isoenzymes of cutinase and a nonspecific esterase from *Fusarium solani* f. *pisi*. *Biochemistry* **14**: 2824–2831.
16. Sagt, C. M. J., W. H. Muller, J. Boonstra, A. J. Verkleij, and C. T. Verrips. 1998. Impaired secretion of a hydrophobic cutinase by *Saccharomyces cerevisiae* correlates with an increased association with immunoglobulin heavy-chain binding protein (BiP). *Appl. Environ. Microbiol.* **64**: 316–324.
17. Sebastian, J., A. K. Chandra, and P. E. Kolattukudy. 1987. Discovery of a cutinase-producing *Pseudomonas* sp. cohabiting with an apparently nitrogen-fixing *Corynebacterium* sp. in phyllosphere. *J. Bacteriol.* **169**: 131–136.
18. Unilever. 1994. Enzyme-containing surfactant compositions. US patent 94-04771.
19. Wariishi, H. 2000. Fungal metabolism of environmentally persistent compounds. *Biotechnol. Bioprocess Eng.* **5**: 422–430.
20. Xin, S., Y. B. Qu, D. H. Shin, and E. K. Kim. 2001. Purification and characterization of lipase from *Trichosporon* sp. Y-11 and its use in ester synthesis of unsaturated fatty acids and alcohols. *J. Microbiol. Biotechnol.* **11**: 951–956.