

Evaluation of cutinase activity of various industrial lipases¹

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Eight industrial lipases were tested for cutinase activity on apple (*Malus pumila* cv. Golden Delicious) cutin and compared with cutinase from the fungus *Fusarium solani* f. sp. *lisi*. The analysis of released cutin monomers was performed by normal-phase h.p.l.c. with an evaporative light-scattering detector. Calibration curves of cutin monomers were established for quantification of cutinolytic activity of the industrial lipases. The pH optimum for cutinase activity of the lipase preparations was approx. 8.0. A high concentration of detergent was necessary for expression of cutinase activity. The most active lipase was found to be Amano PS-800, which was capable of hydrolysing 16% of apple cutin.

Hydroxylated fatty acids such as ricinoleic acid are used to modify fats and oils via inter-esterification reactions. Ricinoleic acid has been generated from castor oil using ground-oat (*Avena sativa*)-seed lipase [1] or castor-bean (*Ricinus communis*) lipase [2]. Other natural hydroxy and epoxyhydroxy fatty acids occur as monomers linked with ester bonds in the cuticle of plants [3,4]. Fungal and bacterial cutinases are able to depolymerize this polymer by hydrolysing ester bonds [5,6]. Pancreatic lipase, a triacylglycerol hydrolase, is able to degrade apple cv. McIntosh cutin by cleaving ester bonds [7]. In the present study we have investigated the cutinolytic activity of industrial lipases as possible enzymes for generation of hydroxy fatty acids from apple (*Malus pumila*) cv. Golden Delicious cutin and compared this activity with cutinase of *Fusarium solani* f. sp. *lisi*.

Materials and methods

Chemicals

H.p.l.c.-grade solvents were obtained from Burdick and Jackson (Muskegon, MI, U.S.A.). Cellulase (EC 3.2.1.4) and pectinase (EC 3.2.1.15)

from *Aspergillus niger*, Triton X-100, n-octyl glucoside, sodium deoxycholate and sodium taurodeoxycholate were obtained from Sigma (St. Louis, MO, U.S.A.). Lipases GC-20, AK, CES, P, PS-30, PS-800 were kindly provided by Amano International Enzyme Co. (Troy, VA, U.S.A.). Lipase S and lipase R were obtained from Gist-Brocades (Charlotte, NC, U.S.A.) and Enzenco (Keyport, NJ, U.S.A.) respectively.

Cutin preparation

Cutin was prepared from mature apple (cv. Golden Delicious) fruits obtained from a local supermarket as previously described [8] with minor modifications. Discs of peel from fresh fruits of apple were removed and boiled in oxalate buffer [oxalic acid (4 g/litre)/ammonium oxalate (16 g/litre)]. Cutin discs were collected, washed several times with deionized water, dried and ground in a Wiley mill (20 mesh followed by 40 mesh). The powdered cutin was Soxhlet-extracted in chloroform for 24 h, dried under vacuum, washed and treated with a clarified solution of cellulase (5 g/litre) and pectinase (1 g/litre) in 0.05 M acetate buffer, pH 4.0, at room temperature for 14–16 h. The cutin powder was then washed thoroughly with deionized water and dried in a vacuum at over 40 °C.

Preparation of Fusarium cutinase and industrial lipases

Fusarium solani f. sp. *pisi* strain T8 was grown on potato dextrose agar (Difco, Detroit, MI, U.S.A.) contained in a culture dish (100 mm × 15 mm) at room temperature until the entire agar surface was covered by mycelia (13 days). The entire content of the dish was then homogenized in 20 ml of sterile water in a blender. This preparation (0.5 ml/bottle) was used to inoculate a basal mineral medium, pH 7.5 [9], supplemented with apple cv. Golden Delicious cutin (0.4%, w/v) contained in Roux culture bottles (100 ml media/bottle). Cultures were grown without shaking at 23 °C for approx. 2 weeks. The extracellular fluid was collected by filtration and freeze-dried. The freeze-dried enzyme preparation was dissolved in water and dialysed against 10 mM sodium phosphate buffer, pH 7.0. $(\text{NH}_4)_2\text{SO}_4$ was added to give 50% saturation. The precipitate was collected by centrifugation, dissolved in 50 mM sodium phosphate buffer, pH 7.0, and dialysed against the same buffer as that previously described [10].

Lipase solutions were dialysed against deionized water overnight before being assayed. The protein content of all enzyme preparations was determined by the method of Bradford [11] in triplicate with $\pm 10\%$ S.D.

Assays for lipase activity

Weighed amounts of industrial lipases were allowed to react in an emulsion of olive oil in 1 ml of 0.05 M potassium phosphate buffer, pH 8.0. The amount of non-esterified fatty acids released was determined by titration with 0.1 M NaOH by using a pH-stat (Radiometer, Copenhagen, Denmark) mode at pH 7.0 [12]. All samples were run in triplicate. The S.D. of the lipase assay was $\pm 7\%$.

Assays for cutinolytic activity

A typical reaction mixture (1 ml) contained 30 mg of cutin and various concentrations of dialysed industrial enzymes or cutinase preparations with or without detergent in the appropriate buffer at 28°C for 16 h. Assays utilizing 50 µg of *Fusarium* cutinase were performed in 0.1 M glycine/NaOH buffer at pH 9.0, the pH optimum for this cutinase [16]. The resulting solution was acidified with acetic acid, and cutin monomers were extracted with chloroform/methanol by the method of Bligh and Dyer [13]. The organic phase was removed and evaporated under a stream of nitrogen. The dry residue was weighed, dissolved in 1 ml of chloroform/methanol (17:3, v/v) and filtered through glass wool into auto-sampler vials. Cutin monomers released were analysed with a Hewlett-Packard model-1050 h.p.l.c. apparatus connected to a Chrompack ChromSep 7 µm LICHROSORB Si 60 silica cartridge system [10 cm long × 3.0 mm (int. diam.)] with a flow rate of 0.5 ml/min and a Varex Universal Evaporative Light-Scattering Detector [temperature, 40°C; nitrogen (172.5 kPa; 25 lbf/in²) as nebulizing gas]. The binary gradient used was a modification of a previously described gradient [14] that resulted in a reduced analysis time. The modified gradient started with 2% (v/v) of propan-2-ol and went linearly to 20% (v/v) propan-2-ol in acidified hexane over a 21 min period and returned to 2% propan-2-ol for 4 min. Calibration curves for 16-hydroxyhexadecanoic acid, 10,18-dihydroxyoctadecanoic acid and 9,10,18-trihydroxyoctadecanoic acid were generated using the modified gradient. Curves for all three monomers were linear up to 25 µg per injection.

Results and discussion

Optimization of reaction conditions

The effects of the amount of industrial lipases and the amount of cutin present in the assay mixture on release of cutin monomers was determined. These assays were done in the presence of 0.1 M potassium phosphate buffer, pH 8.0. Representative data for four of the eight industrial lipases is shown in Figures 1 and 2. In general, the amount of cutin monomers released showed a linear increase between 3 mg and 10 mg of lipase protein (Figure 1). Higher levels of lipases were not tested, owing to incomplete solubility. Results of experiments utilizing various levels of cutin (0.5–50 mg) in the assay mixture (Figure 2) revealed that the amount of monomers increased as the level of substrate was increased. Although the enzyme did not become 'saturated' with substrate in this concentration range, we chose to perform all subsequent assays with 30 mg of cutin as substrate, a convenient amount for chromatographic analysis of released monomers.

The optimum of cutinolytic activity for each industrial lipase was determined using 0.1 M citrate buffer, pH 4.0, 5.0 or 6.0, 0.1 M potassium

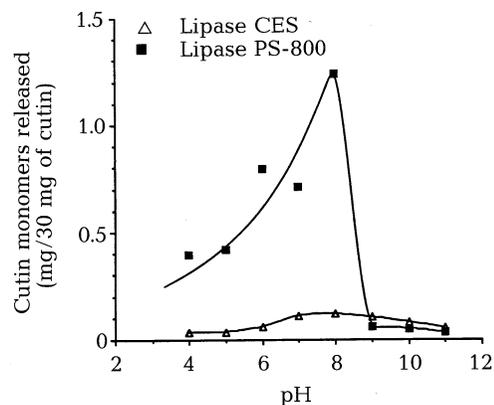


Figure 3

Effect of pH on cutinolytic activity of industrial lipases CES and PS-800. Buffers used were 0.1 M citrate buffer, pH 4.0, 5.0 and 6.0, 0.1 M potassium buffer, pH 7.0 and 8.0, and 0.1 M glycine/NaOH buffer, pH 9.0, 10.0 and 11.0. Incubations were conducted for 16 h at 28°C.

Table 1 Cutinase activity of industrial lipases

Lipase	Source	Protein content ^a	Lipase activity ^b	$10^{-4} \times$ Cutinase activity ^b	Cutinase activity ^c
GC-20	<i>Geotrichum candidum</i>	5.5	16.8	0.31	5.8
R	<i>Candida rugosa</i>	1.6	40.4	3.3	61.7
S	<i>Rhizomucor miehei</i>	5.0	24.2	11.9	221.2
AK	<i>Pseudomonas</i> sp.	1.4	18.6	33.3	615.7
CES	<i>Pseudomonas</i> sp.	9.1	5.0	4.2	76.9
P	<i>Pseudomonas fluorescens</i>	2.4	25.0	25.6	470.6
PS-30	<i>Pseudomonas fluorescens</i>	3.5	46.3	682.6	1260.6
PS-800	<i>Pseudomonas fluorescens</i>	3.3	170.0	2696.8	4980.5

^aExpressed in mg of protein/10 mg of dialysed lipase preparation.

^bExpressed in μ mol of fatty acids or hydroxy fatty acids liberated at 28°C/min per mg of protein (using an average M_r of 307.8 for a hydroxy fatty acid); lipase activity was determined as described in the Materials and methods section; cutinase activity was determined in the presence of 35 mM of n-octyl glucoside and 0.1 M potassium phosphate buffer, pH 8.0.

^cExpressed in μ g of hydroxy fatty acids liberated at 28°C/16 h per mg of protein.

The effect of detergent nature and concentration on cutinolytic activity of the industrial lipases was then determined. In general, the addition of Triton X-100 or deoxycholate at 0.5% or 1% led to decreased cutinolytic activity when compared with 1% (35 mM) n-octyl glucoside (Table 2). The addition of sodium taurodeoxycholate led to increased cutinolytic activity of several of the industrial lipases (Table 2), but it also led to the appearance of an interfering peak on the h.p.l.c. chromatograms, especially at the higher concentration. Interestingly, 1% n-octyl glucoside is above the critical micelle concentration for this detergent. The stimulatory effect of the detergent at this high concentration may be due to its binding to the lipase resulting in protein structure modification [15] or to facilitated release of cutin monomers or polymers.

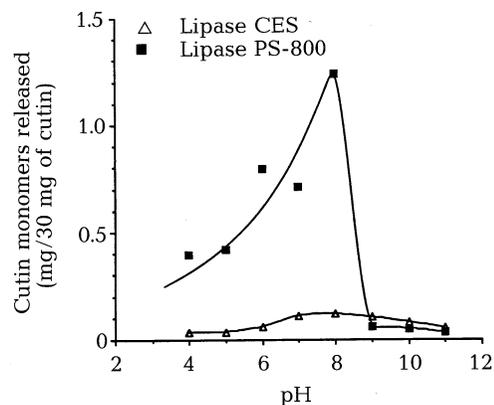


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Effect of pH on cutinolytic activity of industrial lipases CES and PS-800. Buffers used were 0.1 M citrate buffer, pH 4.0, 5.0 and 6.0, 0.1 M potassium buffer, pH 7.0 and 8.0, and 0.1 M glycine/NaOH buffer, pH 9.0, 10.0 and 11.0. Incubations were conducted for 16 h at 28°C.

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Table 2 Influence of the nature of detergent on the relative cutinolytic activity of industrial lipases compared with that obtained with 1% n-octyl glucoside

Industrial lipase	Detergent... Concn. (%)...	Relative activity													
		None	n-Octyl glucoside			Triton X-100			Deoxycholate			Taurodeoxycholate			
			1	0.5	1	0.5	1	0.5	1	0.5	1	0.5	1		
CG-20	5	100	55	25	95	110	77	38	100	70	35	42	89	97	115
R	12	100	70	35	28	49	20	57	100	66	67	85	94	100	120
S	5	100	20	28	50	49	50	20	100	35	39	44	26	83	99
AK	20	100	66	67	85	94	100	120	100	66	67	85	94	100	120
CES	18	100	35	39	44	26	83	99	100	15	15	60	39	95	115
P	15	100	85	59	60	39	95	115	100	30	30	100	69	100	110
PS-800	30	100	85	51	100	69	100	110	100	30	30	100	69	100	110

Comparison of cutin hydrolysis by treatment with alkali, cutinase from Fusarium solani f. sp. pisi or industrial lipases

The final percentage of hydrolysis and the initial rate of hydrolysis of apple cv. Golden Delicious cutin with the *Fusarium* cutinase and the PS-800 lipase were comparable under the conditions used, but both gave very low total hydrolysis when compared with hydrolysis with KOH, performed as previously described [14] (Table 3). Hydrolysis of apple cv. Golden Delicious cutin with all industrial lipases or the cutinase from *Fusarium solani* f. sp. *pisi* resulted in the release of the same cutin monomers (results not shown). The cutin monomers were identified as monohydroxy fatty acids (16-hydroxyhexadecanoic acid, 18-hydroxyoctadec-9-enoic acid and 18-hydroxyoctadecanoic acid), dihydroxy fatty acids (9,18-dihydroxyoctadecanoic acid and 10,18-dihydroxyoctadecanoic acid), epoxyhydroxy fatty acids (18-hydroxy-9,10-epoxyoctadecanoic acid and 18-hydroxy-9,10-epoxyoctadec-12-enoic acid) and trihydroxy fatty acids (9,10,18-trihydroxyoctadecanoic acid and 9,10,18-trihydroxyoctadec-12-enoic acid) as previously described [14]. A typical time course for monomers released by lipase PS-800 is presented in Figure 4. The rate of reaction was found to decrease after 14 h of incubation.

Table 3 Summary of properties of cutin hydrolysis by various methods

Property	Method...	KOH hydrolysis	<i>Fusarium cutinase</i>	Lipase PS-800
Extent of hydrolysis (%)		> 80	≈ 15	≈ 16
Initial rate ^a		—	1	0.5
pH optimum		—	9.0	8.0
Properties of monomer classes released ^b		25/40/15/16	15/50/10/20	18/55/10/16

^aExpressed in mg/h per mg of protein.
^bRatio of monohydroxy/epoxy hydroxy/dihydroxy/trihydroxy fatty acids.

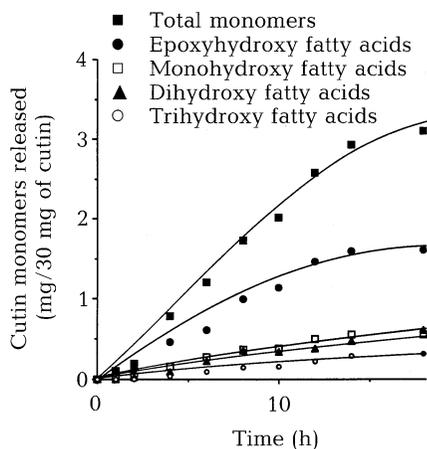


Figure 4

Time course of released cutin monomers during hydrolysis with 1.5 mg of lipase PS-800. The assay mixture contained 35 mM n-octyl glucoside and 0.1 M potassium phosphate buffer, pH 8.0, at 28 °C.

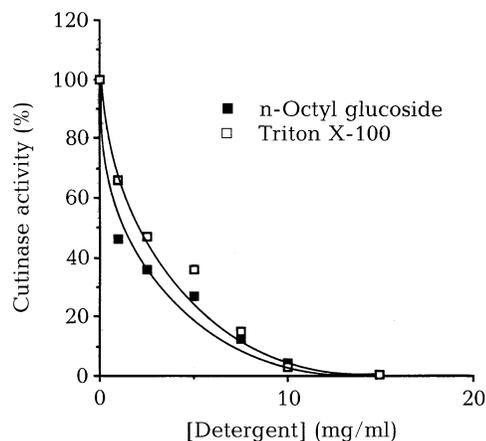


Figure 5

Effect of detergent on hydrolysis of apple cutin by *Fusarium* cutinase (50 μ g) in 0.1 M glycine/NaOH, pH 9.0, at 28 °C.

In contrast with the results obtained with the industrial lipases, the cutinase from *Fusarium solani* f. sp. *psii* was strongly inhibited by n-octyl glucoside and Triton X-100 (Figure 5). However, Purdy and Kolattukudy [5] reported that esterase activity of *Fusarium* cutinase against the artificial substrate *p*-nitrophenyl palmitate was stimulated by concentrations of Triton X-100 up to 3.7 mg/ml. Higher levels of Triton X-100 were reported to be inhibitory.

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