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SENSITIVITY OF MICROBIAL LIPASES TO ACETALDEHYDE FORMED BY ACYL-TRANSFER REACTIONS FROM VINYL ESTERS

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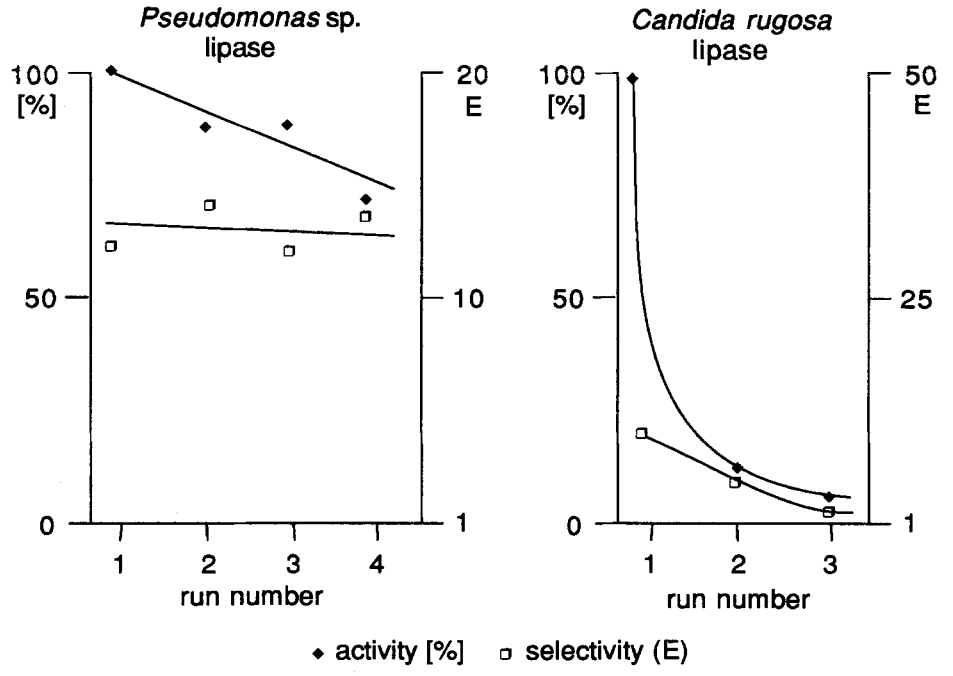
Summary: The sensitivity of twenty six microbial lipases towards acetaldehyde (an unavoidable by-product in lipase-catalysed acyl transfer reactions with vinyl esters) was investigated. The sensitivity of an individual enzyme strongly depends on its properties such as microbial source, molecular weight and relative lysine content. Whereas the majority of enzymes (from *Pseudomonas*, *Rhizopus*, *Chromobacterium*, *Mucor* and *Candida antarctica* sp.) proved to be remarkably stable, lipases from *Candida rugosa* and *Geotrichum candidum* lost most of their activity when exposed to acetaldehyde.

INTRODUCTION

Lipase-catalysed acyl transfer has become a standard technique for the biocatalytic resolution of racemic or asymmetrisation of prochiral alcohols (Faber and Riva, 1992; Carrea *et al.*, 1995; Santaniello *et al.*, 1993). From the numerous acyl donors employed, vinyl esters are most popular due to the irreversible nature of the reaction and the absence of low-volatile side-products which would hamper the work-up procedure (Degueil-Castaing *et al.*, 1987; Wang and Wong, 1988). On the other hand, the unavoidable generation of one molar equivalent of acetaldehyde may cause serious problems with respect to enzyme stability and -selectivity. Several observations on this topic have been reported during the past few years, but the messages are rather contradictory. For instance, possible reuse of the enzyme (albeit without data on activity and/or selectivity) was reported for *Candida rugosa* and *Pseudomonas* sp. lipases (Holla, 1989, 1990; Ader *et al.*, 1989). On the other hand, serious deactivation was also observed with lipases from *Pseudomonas* (Mitsuda and Nabeshima, 1991) and *Candida rugosa* (Berger and

Faber, 1991; Kaga *et al.*, 1994). The only available data on the reuse of lipases in acyl-transfer reactions are summarized in Figure 1. Thus, *Pseudomonas* sp. lipase gradually lost its *activity* in repeated batch reactions, but entirely preserved its *selectivity* (data from Mitsuda and Nabeshima, 1991), whereas *Candida rugosa* lipase was almost deactivated after the first run going in hand with a severe depletion of its selectivity (data from Berger and Faber, 1991). These data prompted us to perform a more detailed study.

Figure 1: Activity and selectivity of lipases in repeated batch reactions.



MATERIALS AND METHODS

General: Crude technical grade lipases were used as received from the producer without further purification. Toluene was saturated with aqueous phosphate buffer (0.05N, pH 7.0), acetaldehyde was distilled prior to use. Tributyrin (Aldrich 26,077-0), olive oil (Aldrich 24,816-9), gum arabic (Aldrich 26,077-0), *p*-nitrophenyl palmitate (Sigma N-2752) and sodium deoxycholate (Aldrich 23,839-2) were used as received.

Tributyrin assay (Battistel *et al.*, 1991): A crude lipase sample (25 - 100 mg, depending on the activity) was dissolved in sodium phosphate buffer (0.02M NaH_2PO_4, 0.9% (w/w) NaCl, pH 7.0) and shaken well. After centrifugation (10 min, 3000 rpm) an aliquot of the clear supernatant was transferred to the assay mixture. Tributyrin (0.5 mL) was added to phosphate buffer (0.02M NaH_2PO_4, 0.9% (w/w) NaCl, 0.02 M sodium deoxycholate, pH 7.0, 30 mL) in a thermostated glass-vessel at 37°C. (A beaker placed in a water bath may be used instead). The mixture was stirred magnetically at constant speed (950 rpm) and an aliquot of the sample solution was added. The release of butyric acid was titrated with sodium hydroxide solution (0.1 M) using a pH-stat for about 5-10

minutes. After a short equilibration period (~2 min) the constant slope of sodium hydroxide consumption was taken to calculate the activity (mmol / g • min).

Olive oil assay (Marks *et al.*, 1967): Stock solutions: (A) Gum arabic (Aldrich No. 26,077-0, 55 g) and $CaCl_2 \cdot H_2O$ (6.25 g) are dissolved in distilled water (500 mL) and then filtered. (B) Olive oil (Aldrich No. 24,816-9, 25 mL) and solution A (77 mL) are mixed on a magnetic stirrer (≥1000 rpm, 15 min) in an ice-water bath until a homogeneous yellow emulsion was obtained. This mixture should not be stored longer than one day. (C) Sodium deoxycholate (0.5 g) in distilled water (100 mL). Assay procedure: In a thermostated glass-vessel (37°C) solution B (20 mL) and C (1.7 mL) are added to water (7.5 mL) with magnetic stirring (950 rpm). The pH was adjusted to 7.0 by dropwise addition of sodium hydroxide solution (0.5 M). Then an aliquot of the lipase solution (prepared as described for the tributyrin-assay) was added and the release of oleic acid was titrated with sodium hydroxide solution (0.05 M) using a pH-stat for about 15 minutes. After an equilibration period of few minutes the constant slope of sodium hydroxide consumption was taken to calculate the activity (mmol / g • min).

p-Nitrophenyl palmitate assay (Winkler and Stuckmann, 1979): *p*-Nitrophenyl palmitate (30 mg) was dissolved in *i*-propanol (10 mL) with stirring (5 min). A 100 µL sample of this solution was then diluted with buffer (0.02M NaH_2PO_4, 0.9% (w/w) NaCl, 0.02 M sodium deoxycholate, pH 7.0) to a total volume of 1 mL. The reaction was started by addition of an aliquot of the lipase sample solution (prepared as described for the tributyrin-assay). The activity was measured by monitoring the increase of the absorbance at 410 nm against neat buffer for a period of about 5 minutes using a UV/VIS spectrophotometer. Under the conditions described, the extinction coefficient of *p*-nitrophenol is $\varepsilon_{410} = 15.000\ cm^2/mg$.

Toluene-treatment: A sample of lipase (100mg) was suspended in toluene (5ml) and was shaken at r.t. for 17 h. After centrifugation (1200•g), the liquids were decanted and the pellet was dried overnight (12 mm Hg, r.t.).

Acetaldehyde-treatment: The procedure was essentially the same as for the toluene-treatment with the exception that the toluene solution contained freshly distilled acetaldehyde (0.1M).

RESULTS AND DISCUSSION

Crude microbial lipases were exposed to acetaldehyde in toluene solution. The latter solvent is highly bio-compatible due to its logP of 2.5. The acetaldehyde concentration was set to 0.1M, which corresponds to the general reaction conditions of acyl transfer reactions at 50% conversion (Faber and Riva, 1992). After recovery, the lipase activity was measured again. To ensure that any deactivation was not caused by the organic solvent alone, lipase samples were shaken in toluene without aldehyde in separate experiments. The results are depicted in Table 1.

When lipases were treated with toluene alone, no significant deactivation was detected, the largest being -23% for *Candida rugosa* lipase (Amano AY-30). On the

contrary, lipases from other sources, such as *Aspergillus* (Amano AP6), *Humicola* (Amano CE) and *Rhizopus* (Amano D) lipases even showed enhanced activity. A similar activation by an organic-solvent treatment using 2-propanol was recently reported for *Candida rugosa* lipase (Colton *et al.*, 1995). However, the picture was totally different when lipases were treated with acetaldehyde. As depicted in the last column, the majority of lipases were quite stable under these conditions (values between +22 and -34%), only *Candida rugosa* and *Geotrichum* sp. lipases lost ~60-80% of their activity. The fact that both of the latter enzymes are highly homologous (Svendsen 1994; Cygler *et al.*, 1993), suggests that the stability of a lipase towards acetaldehyde is related to its molecular structure.

• Large lipases with an average molecular weight of ~60 kDa (from *Candida rugosa* and *Geotrichum* sp.) are sensitive, whereas small candidates with an average weight of 25-35 kDa are more stable.

• The mechanism of enzyme deactivation by aldehydes involves a Schiff-base formation on lysine residues with a concomitant loss of the positive charge (Donohue *et al.*, 1983). Thus, it might be assumed that the relative lysine content of a lipase determines its acetaldehyde-stability as well. As may be deduced from Table 1, this is true to some extent, e.g. *Mucor* and *Pseudomonas* lipases have a comparable low lysine content (2.2-2.6%) in comparison to the sensitive (large) *Candida rugosa* and *Geotrichum* lipases (3.7-4.2%). *Rhizopus* sp. lipases, however, do not fit into this pattern.

Figure 2: Activation/Deactivation of Lipases by Toluene or Acetaldehyde.

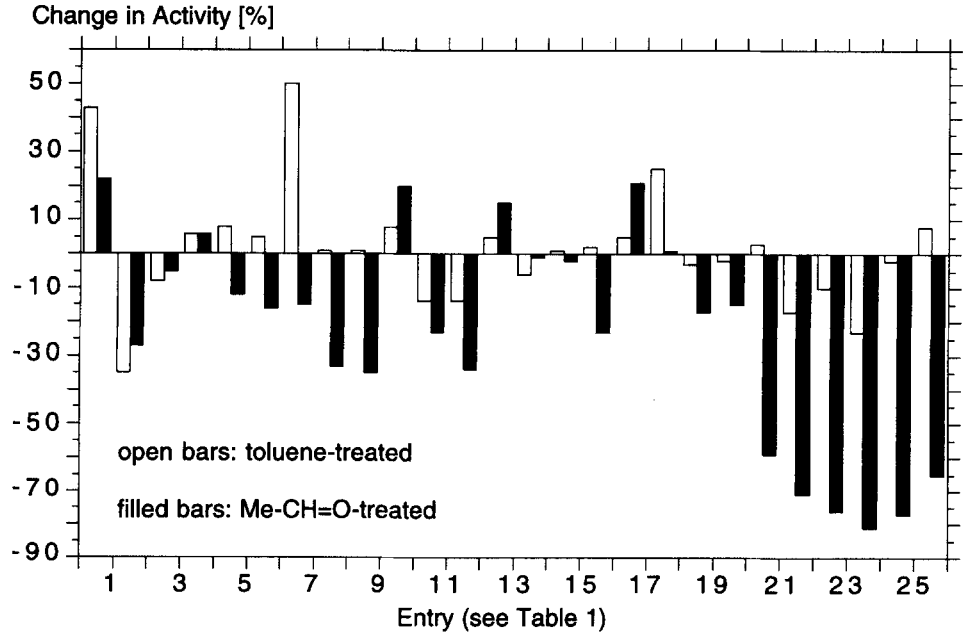


Table 1: Deactivation of lipases by acetaldehyde in toluene solution.

Entry	Lipase Source	Producer	Assay [a]	Mol. Weight [kDa][b]	Lysine Content [%][b]	Change in Activity [%][c] Treatment	
						Toluene	MeCHO
1	*Aspergillus niger*	Amano AP6	A	~35	–	+43	+22
2	*Aspergillus niger*	Röhm	A			-35	-27
3	*Candida antarctica* A	Novo SP526	B	~33	–	-8	-5
4	*Candida antarctica* B	Novo SP525	B		2.8	+6	+6
5	*Chromobacterium v.*[d]	Biocatalysts	B	25-30	–	+8	-12
6	*Chromobacterium v.* [d]	Asahi	B			+5	-16
7	*Humicola lanuginosa*	Amano CE	B	~30	–	+50	-15
8	*Mucor javanicus*	Biocatalysts	B	30-35	~2.6	~0	-33
9	*Mucor javanicus*	Amano M	B			~0	-35
10	*Mucor miehei*	Novo SP524	B			+8	+20
11	*Mucor miehei*	Biocatalysts	B			-14	-23
12	*Penicillium roqueforti*	Amano R	B	~30	–	-14	-34
13	*Pseudomonas* sp.	Boehringer LPL	B	29-33	2.2-2.5	+5	+15
14	*Pseudomonas* sp.	Amano P	B			-6	~0
15	*Pseudomonas* sp.	Amano AK	B			~0	-2
16	*Pseudomonas* sp.	Amano SAM II	B			+2	-23
17	*Rhizopus niveus*	Amano N	B	29-34	4.9-5.5	+5	+21
18	*Rhizopus delemar*	Amano D	B			+25	+1
19	*Rhizopus oryzae*	Amano F-AP	B			-3	-17
20	*Thermomyces* sp.	Novo SP523	B	~35	–	-2	-15
21	*Candida rugosa*	Biocatalysts	B	~60	3.7-4.2	+3	-59
22	*Candida rugosa*	Boehringer	B			-17	-71
23	*Candida rugosa*	Sigma (VII)	B			-10	-76
24	*Candida rugosa*	Amano AY-30	B			-23	-81
25	*Geotrichum candidum*	Amano GC-4	C	~60	4.0	-2	-77
26	*Geotrichum candidum*	Amano GC-20	C			+8	-65

[a] Assay methods: *p*-Nitrophenyl palmitate (A), tributyrin (B), olive oil (C). [b] Average literature values. [c] Increase (+) or decrease (-) in activity after treatment in relation to the native (untreated) enzyme (100%). [d] *Chromobacterium vicosum.*

The latter observation led us to study whether the position of a particular lysine in a lipase (for instance, near the active site) and/or its reactivity as a nucleophile (expressed in terms of pK-value, number of hydrogen bonds or steric accessibility) determines the stability of a lipase towards acetaldehyde. A detailed study on this topic will be reported in due course.

In conclusion, it has been shown that the sensitivity of microbial lipases towards acetaldehyde (emerging from acyl-transfer reactions from vinyl esters) depends on the individual enzyme properties such as the microbial source and its molecular weight.

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