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SHORT CONTRIBUTION

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Substrate selectivity of various lipases in the esterification of *cis*- and *trans*-9-octadecenoic acid*

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Abstract The substrate selectivity of numerous commercially available lipases from microorganisms, plants and animal tissue towards 9-octadecenoic acids with respect to the *cis*/*trans* configuration of the C=C double bond was examined by the esterification of *cis*- and *trans*-9-octadecanoic acid (oleic and elaidic acid respectively) with *n*-butanol in *n*-hexane. A great number of lipases studied, e.g. those from *Pseudomonas* sp., porcine pancreas or *Carica papaya*, were unable to discriminate between the isomeric 9-octadecenoic acids. However, lipases from *Candida cylindracea* and *Mucor miehei* catalysed the esterification of oleic acid 3–4 times faster than the corresponding reaction of elaidic acid and therefore have a high preference for the *cis* isomer. Of all biocatalysts examined, only recombinant lipases from *Candida antarctica* favoured elaidic acid as substrate. While the preference of *Candida antarctica* lipase B for the *trans* isomer was quite low, *Candida antarctica* lipase A had an extraordinary substrate selectivity and its immobilized enzyme preparation [Chirazyme L-5 (3) from Boehringer] esterified elaidic acid about 15 times faster than oleic acid.

Introduction

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are used as versatile biocatalysts in modern organic chemistry (Kazlauskas and Bornscheuer 1998; Schmid and Verger 1998), especially for modification of fats and other lipids via hydrolysis, esterification and interesterification (Mukherjee 1990). The utility of lipases is directly connected with their fatty acid selectivity, i.e. with their ability to discriminate between particular fatty acids or acyl moieties. Knowledge of the substrate specificity of lipases is therefore essential to their utilization.

There have been numerous studies dealing with selectivity of triacylglycerol acylhydrolases towards unsaturated fatty acids having specific positions of *cis* double bonds, e.g. in the esterification of long-chain mono- and polyenoic acids with *cis*-4, *cis*-5, *cis*-6, *cis*-8 and *cis*-9 unsaturation (Hills et al. 1990; Jachmanián et al. 1996; Mukherjee and Kiewitt 1996b; Mukherjee et al. 1993), in the alcoholysis of gondoic (*cis*-11-eicosenoic) acid, erucic (*cis*-13-docosenoic) acid and the corresponding ethyl esters (Pedersen and Hølmer 1995) or in the hydrolysis of high-erucic acid seed oils (Mukherjee and Kiewitt 1996a; Sonnet et al. 1993a).

On the other hand, the specificity of lipases towards *trans* fatty acids has been studied only for a limited number of substrates, such as *trans*-2- and *trans*-3-acid esters (Brockerhoff 1970; Kleimann et al. 1970), petroselaidic (*trans*-6-octadecenoic) acid (Hills et al. 1990), elaidic (*trans*-9-octadecenoic) acid (Charton and Macrae 1993; Hills et al. 1990; Sonnet et al. 1993b), elaidic acid esters (Labuschagne et al. 1997; Sidebottom et al. 1991) or triglycerides containing petroselaidic acid (Briand et al. 1995) or elaidic acid (Briand et al. 1995; Jensen et al. 1964; Jensen et al. 1965; Mozaffar and Weete 1993; Piazza et al. 1992).

However, there have been few systematic examinations of substrate selectivity towards positional isomers of unsaturated fatty acids. In the hydrolysis of synthetic triglycerides containing *cis*-octadecenoic acids of the Δ2–Δ16-range (Jensen 1974; Jensen et al. 1972) and in the hydrolysis of *cis*-2- to *cis*-17-octadecenoic acid methyl esters (Charton and Macrae 1992), the lipase from *Geotrichum candidum* showed a unique preference for substrates with *cis*-9-unsaturation. Furthermore, in the hydrolysis of the aforementioned synthetic triglycerides the lipase from porcine pancreas discriminated strongly

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against unsaturated substrates with C=C double bond position between Δ2 and Δ7 (Heimermann et al. 1973).

The aim of our research is the systematic determination of substrate selectivity of various lipases towards octadecenoic acids with respect to *cis*/*trans* configuration and position (Δ4–Δ13) of the C=C double bond. We report here the evaluation of 39 enzyme preparations from microorganisms, plants and animal tissue as biocatalysts in the esterification of oleic and elaidic acid with *n*-butanol in *n*-hexane.

Materials and methods

Materials

The activities of lipases supplied by Altus Biologics Inc., Cambridge, Mass., USA (A, see Table 1), Amano Pharmaceutical Co., Ltd., Nagoya, Japan (B), Biocatalysts Ltd., Pontypridd, Wales, (C), Boehringer Mannheim GmbH, Mannheim, Germany (D), Fluka Chemie, Neu-Ulm, Germany (E), Novo Nordisk Biotechnologie GmbH, Mainz, Germany (F) and Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany (G) are listed in Table 1. Lipozyme IM (immobilized lipase from *Rhizomucor miehei*) and Novozym 435 (immobilized *Candida antarctica* lipase B) are generous gifts from Novo. The Chirazyme lipases purchased from Boehringer are from the following sources: *Candida antarctica* lipase A (Chirazyme L-5) and lipase B (Chirazyme L-2), *Candida cylindracea* (synonym for *Candida rugosa*; Chirazyme L-3), *Humicola* sp. (Chirazyme L-8), porcine pancreas (Chirazyme L-7), *Pseudomonas cepacia* (synonym for *Burkholderia cep.*; Chirazyme L-1) and *Pseudomonas* sp. (Chirazyme L-4 and L-6). Latex from papaya (*Carica papaya*) is a commercially available crude powder from Sigma. Acetone powders were obtained from non-germinating and germinating seedlings of *Calendula officinalis* [*Calend. offic.* (1) and (2) in Table 1, respectively] as the residues after extraction with cold acetone, as described elsewhere (Hassanien and Mukherjee 1986). They were kindly prepared by Dr. Ludger Heiss (H) as a part of his postdoctoral research in our institute.

All pure fatty acids used were supplied by Sigma. All chemicals of analytical grade were purchased from Merck KGaA, Darmstadt, Germany. Distilled solvents were used throughout.

Esterification

For the determination of substrate selectivity reactions were carried out at 30 °C in sealed vials with magnetic stirring using 25 mM of each fatty acid (oleic or elaidic acid), individually, together with 25 mM of the reference standard myristic acid and 100 mM *n*-butanol in 250 µl *n*-hexane in the presence of different amounts of lipases. Aliquots of the reaction mixture were withdrawn at various intervals, taken up in *n*-hexane, centrifuged to separate the enzyme preparation, and the supernatants were treated with a solution of diazomethane in diethylether and a drop of methanol to convert the unesterified fatty acids to their methyl esters. Usually, the esterification reactions were stopped after about 50% of the myristic acid had been converted to butyl myristate.

Analysis of reaction products

The resulting mixtures of methyl esters and butyl esters were analysed by gas chromatography in a HP 5890 Series II instrument from Hewlett-Packard GmbH, Böblingen, Germany, equipped with a flame ionization detector. The separations were carried out on a 40 m × 0.18 mm, 0.20-µm fused silica capillary column DB-23 (J. & W. Scientific, Folsom, Calif., USA) using hydrogen as the carrier gas (linear velocity 20 cm/s) and a temperature programme from 170 °C to 230 °C with a linear heating rate of 2 °C/min. The split ratio was 1:10 and both the injector and the flame ionization detector temperature were 300 °C.

Peaks in gas chromatograms were assigned by comparison of their retention times with those of known standards. Peak areas and percentages were calculated using Hewlett-Packard PC integration software (HP 3365 Series II ChemStation Version A.03.21).

Kinetic analysis

The reaction rates were calculated from the composition of the reaction products as micromoles of butyl esters formed per gram of lipase per minute in the esterification of oleic acid and myristic acid.

Table 1 Sources and activities of lipases tested in the enzyme screening

Lipase[a]	Supplier[b]	Activity[c] (U/mg)	Substrate[d]
Aspergillus niger	E	1.0	IV
Aspergillus oryzae	E	52.9	IV
Calendula officinalis (1)	H	–	–
Calendula officinalis (2)	H	–	–
Candida antarctica (1)	E	3.2	IV
Candida antarctica (2)	E	9.2	III
Candida cylindracea (1)	A	–	–
Candida cylindracea (2)	E	2.2	IV
Candida cylindracea (3)	G	900.0	II
Candida cylindracea (4)	G	518.0	II
Candida lipolytica	E	1.0[e]	IV
Candida utilis	E	0.1	IV
Carica papaya	G	1.7	I
Chirazyme L-1	D	225.0	III
Chirazyme L-2	D	130.0	III
Chirazyme L-3	D	5.0	III
Chirazyme L-4	D	8.0	III
Chirazyme L-5 (1)	D	13.0	III
Chirazyme L-5 (2)	D	45.5	III
Chirazyme L-5 (3)	D	2.3	III
Chirazyme L-6	D	400.0	III
Chirazyme L-7	D	20.0	III
Chirazyme L-8	D	2500.0	III
Geotrichum candidum (1)	B	5.0	IV
Geotrichum candidum (2)	C	4.7	II
Lipozyme	E	48.3	IV
Lipozyme IM	F	–	–
Mucor javanicus	E	3.5[e]	IV
Mucor miehei	E	24.2	IV
Novozym 435	F	7000.0[f]	–[f]
Penicillium cyclopium	C	1.1	II
Penicillium roqueforti	E	1.8[e]	IV
Porcine pancreas	G	55.0	II
Pseudomonas cepacia	E	609.0	IV
Pseudomonas fluorescens	E	44.9	IV
Rhizomucor miehei	E	0.5	IV
Rhizopus arrhizus	E	1.5[e]	IV
Rhizopus delemar	E	45.6	II
Rhizopus niveus	E	2.6[e]	IV

[a] Numbers in parentheses indicate different enzyme preparations, even where the source is the same
[b] A: Altus; B: Amano; C: Biocatalysts; D: Boehringer; E: Fluka; F: Novo; G: Sigma; H: Dr. Heiss (see text)
[c] One unit per milligram (U/mg) is defined as 1 µmol fatty acid released per minute and milligram lipase
[d] I: BAEE (*N*α-benzoyl-L-arginine ethyl ester); II: olive oil; III: tributyrin; IV: triolein
[e] U/g
[f] PLU/g: ester synthesis activity expressed in *p*ropyl *l*aurate *u*nits

According to the method of Rangheard et al. (1989), specificity constants of each enzyme preparation for oleic and elaidic acid were obtained which quantitatively describe the substrate selectivity of the lipase towards the isomeric 9-octadecenoic acids.

If two substrates (Ac1X and Ac2X) with the same leaving group (X) and two different acyl groups (Ac1 and Ac2) compete for the enzyme, the ratio of the reaction rates for each substrate (v_1 and v_2) is given by:

$$v_1/v_2 = \alpha \cdot [\mathrm{Ac1X}]/[\mathrm{Ac2X}] \quad (1)$$

where [Ac1X] and [Ac2X] are the substrate concentrations and α is the competitive factor (Deleuze et al. 1987) defined as the ratio of specificity constants:

$$\alpha = (V_{\mathrm{Ac1X}}/K_{\mathrm{Ac1X}})/(V_{\mathrm{Ac2X}}/K_{\mathrm{Ac2X}}) \quad (2)$$

where V is the maximal velocity and K the Michaelis constant.

The competitive factor α was calculated by using the integrated form of Eq. 1:

$$\alpha = \log([\mathrm{Ac1X}]/[\mathrm{Ac1X}]_0)/\log([\mathrm{Ac2X}]/[\mathrm{Ac2X}]_0) \quad (3)$$

where $[\mathrm{Ac1X}]_0$ and $[\mathrm{Ac2X}]_0$ are the substrate concentrations at the beginning of the esterification.

From the competitive factor α the specificity constant (V/K) for each octadecenoic acid (Ac2X) can be calculated as $1/\alpha$, giving a value of 1 to the specificity constant for the reference standard myristic acid (Ac1X).

Results

Specificity constants of lipases for oleic and elaidic acid

The specificity constants of numerous lipases from microorganisms, plants and animal tissue for oleic and elaidic acid determined using the biocatalysts in the esterification of *cis*- and *trans*-9-octadecenoic acid, individually, together with myristic acid and *n*-butanol are shown in Fig. 1. Nine out of 39 enzyme preparations tested, among others the lipases from *Geotrichum candidum*, were unable to catalyse the esterification of the fatty acids under the reaction conditions chosen.

Most of the lipases esterify oleic and elaidic acid slower than or as fast as myristic acid, resulting in specificity constants equal to or less than 1; the exceptions are the commercially available biocatalysts from *Candida cylindracea* and *Rhizopus delemar*, which prefer *cis*-9-octadecenoic acid to tetradecanoic acid (Fig. 1).

The specificity constants of lipases from *Aspergillus niger* and *Aspergillus oryzae* are almost identical and both enzymes favour the *cis* isomer strongly (Fig. 1). The ability of an acetone powder from germinating rapeseed (*Brassica napus*) to catalyse esterification reactions of different fatty acids was described elsewhere (Jachmanián and Mukherjee 1996; Jachmanián et al. 1996). Because the seed oil of *Calendula officinalis* has a high content of the unusual calendic (*trans*-8,*trans*-10, *cis*-12-octadecatrienoic) acid (Röbbelen 1984), it was expected that enzyme preparations from this plant have preferences for *trans* fatty acids. For this reason, we evaluated lipases from non-germinating and germinating seedlings of *Calendula officinalis* for their ability to discrimate between *cis*- and *trans*-octadecenoic acids.

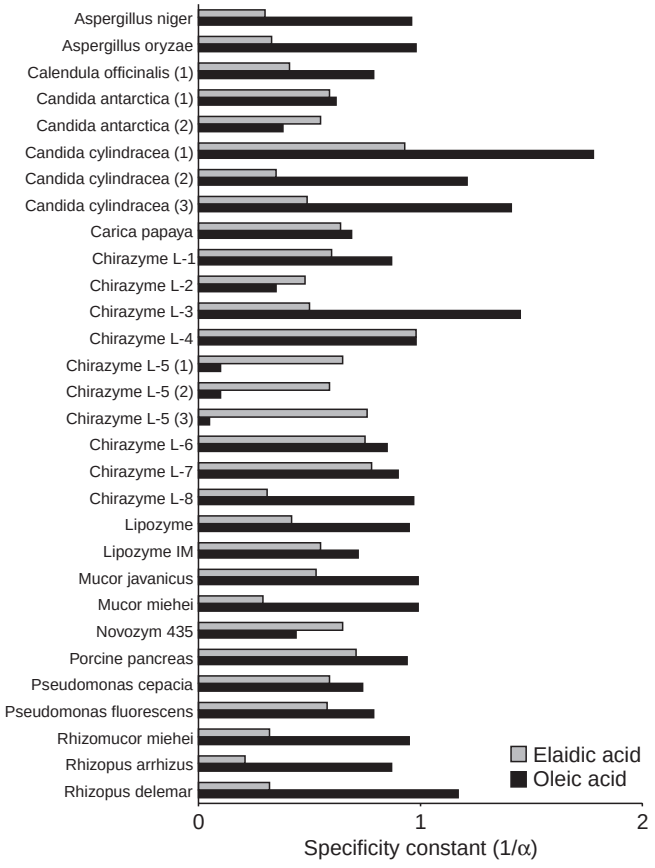

Fig. 1 Specificity constants of lipases for oleic and elaidic acid determined in their esterification with *n*-butanol in *n*-hexane

While the acetone powder from germinating seedlings does not catalyse the esterification reactions, lipase from non-germinating seedlings has a preference for oleic acid (Fig. 1). The lipase from *Candida antarctica*, i.e. *Candida antarctica* (1), has similar specificity constants for oleic and elaidic acid and does not discriminate between the isomers, whereas the recombinant *Candida antarctica* lipases B [*Candida antarctica* (2), Chirazyme L-2 and Novozym 435] have slightly higher specificity constants for elaidic acid and thus show some preference for the *trans* isomer (Fig. 1). While very low specificity constants are obtained in the esterification of oleic acid with *n*-butanol catalysed by the recombinant *Candida antarctica* lipases A [Chirazyme L-5 lipases (1)–(3) from Boehringer], these enzyme preparations accept elaidic acid as substrate with extraordinary selectivities for the *trans*-9-octadecenoic acid over *cis*-9-octadecenoic acid (Fig. 1). However, the reaction rates of these enzymes differ considerably. As part of a Boehringer Enzyme Screening Set, Chirazyme L-5 lipase (1) was tested at the beginning of these studies and a reaction rate of only 0.04 μmol/(g · min) was calculated. The reaction rate of Chirazyme L-5 lipase (2), a newer charge of the same enzyme source, is 0.94 μmol/(g · min) whereas Chirazyme L-5 lipase (3), an immobilized enzyme preparation, has a reaction rate of 5.28 μmol/(g · min).

Candida cylindracea (1), a cross-linked enzyme preparation from Altus sold under the brand name ChiroCLEC-CR, has the highest specificity constant for oleic acid of all lipases tested (Fig. 1). Moreover, this biocatalyst esterifies elaidic acid as rapidly as myristic acid. The *Candida cylindracea* lipases (2) and (3) also accept oleic acid very well as substrate, yielding specificity constants higher than 1, while they esterify elaidic acid to a much lesser extent (Fig. 1). Thus, the above enzyme preparations prefer oleic acid strongly. Surprisingly, *Candida cylindracea* (4), an immobilized form of the enzyme preparation *Candida cylindracea* (3), does not show any catalytic activity in the esterification reactions.

Mukherjee and Kiewitt (1996b) reported on the specificity of crude *Carica papaya* latex as biocatalyst in the esterification of several fatty acids with *n*-butanol. By using the same lipase we obtained a similar specificity constant for oleic acid as did Mukherjee and Kiewitt. However, the specificity constant of *Carica papaya* latex for elaidic acid is close to that of the *cis* isomer, and therefore the enzyme preparation from the plant material catalyses the esterification of *cis*- and *trans*-9-octadecenoic acid at similar rate (Fig. 1).

With the exception of Chirazyme L-4, the different commercially available pseudomonas lipases (Chirazyme L-1 and L-6, *Pseudomonas cepacia* and *Pseudomonas fluorescens*, respectively) have similar selectivities. Their specificity constants for oleic acid are only little higher than those obtained in the esterification of elaidic acid (Fig. 1), indicating that these enzyme preparations catalyse the test reactions rather unspecifically. Chirazyme L-4 exhibits a specificity constant for elaidic acid close to 1, which is the highest of all lipases used in our enzyme screening (Fig. 1). However, the specificity constant for oleic acid is exactly the same (Fig. 1), indicating that Chirazyme L-4 is not able to discriminate between the two isomers. Porcine pancreas lipases from Boehringer (Chirazyme L-7) and Sigma have similar specificity constants. The specificity constants for the *cis* isomer are only somewhat higher than those for the *trans* isomer, indicating that porcine pancreas lipases esterify the octadecenoic acids quite unspecifically (Fig. 1). Chirazyme L-8 (lipase from *Humicola* sp.) accepts oleic acid as readily as the reference standard myristic acid, resulting in a specificity constant close to 1, whereas elaidic acid is esterified at a much lower rate (Fig. 1). Thus, this lipase has a strong preference for *cis*-9-octadecenoic acid.

The lipase from *Mucor javanicus* catalyses the esterification of oleic acid as fast as the corresponding reaction of myristic acid and twice as fast as the esterification of elaidic acid, indicating a preference for the *cis*-isomer (Fig. 1). The Fluka lipases from *Mucor miehei* and *Rhizomucor miehei* (synonyms for each other) have similar specificity constants (Fig. 1); both biocatalysts favour the *cis* isomer considerably. The immobilized enzyme preparations of *Rhizomucor miehei* (Lipozyme and Lipozyme IM) also esterify oleic acid faster than elaidic acid, but their preferences for *cis*-9-octadecenoic acid are not as strong as in the case of the non-immobilized lipases (Fig. 1). For the lipases from *Rhizopus arrhizus* and *Rhizopus delemar* slightly different specificity constants are obtained, but their substrate selectivities are similar (Fig. 1); both lipases accept oleic acid much better than elaidic acid as substrate.

Ratio of specificity constants

To summarise the above results the lipases are classified with regard to the extent of their substrate selectivity, calculated as the ratios of their specificity constants for oleic and elaidic acid, as shown in Fig. 2.

Lipases listed at the top of the Fig. 2, i.e. those from *Rhizopus arrhizus* to *Candida cylindracea* (3), have a strong preference for *cis*-9-octadecenoic acid and they esterify oleic acid 3–4 times faster than elaidic acid. Since the reaction rates of *Rhizopus arrhizus* and *Rhizopus delemar* lipases are rather low (0.47 and 2.70 μmol/(g · min) respectively), lipases from *Candida cylindracea* and *Mucor miehei* with reaction rates of 7.51 and 7.85 μmol/(g · min), respectively, are found to be the best *cis*-9-specific biocatalysts (Fig. 2). A broad range of

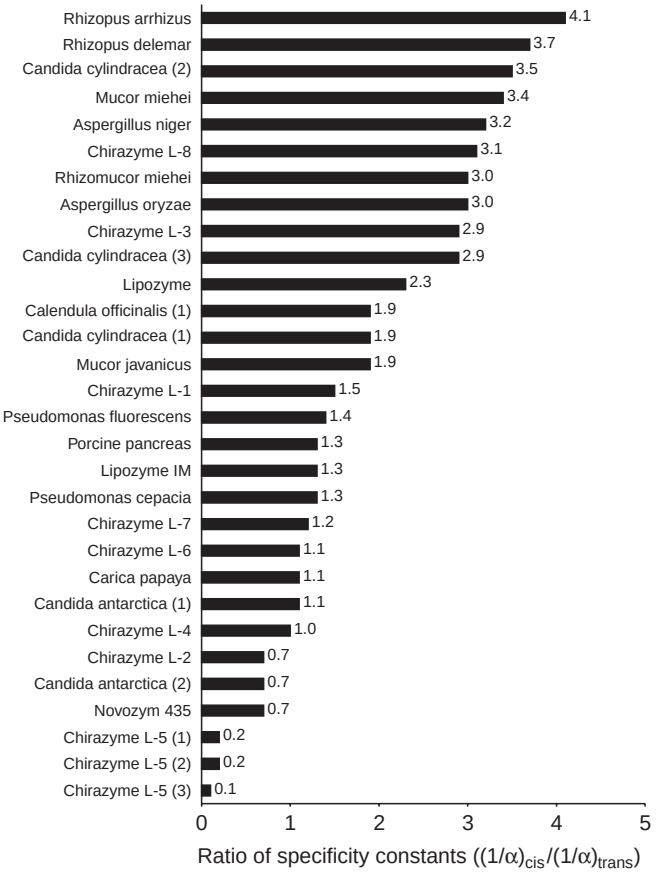

Fig. 2 Ratio of specificity constants for oleic and elaidic acid as an expression of the extent of substrate selectivity of lipases

enzyme preparations (Lipozyme IM to Chirazyme L-4) have lower preferences for oleic acid, indicating that they catalyse the esterification of *cis*- and *trans*-9-octadecenoic acid unspecifically (Fig. 2). Biocatalysts favouring elaidic acid are the recombinant lipases from *Candida antarctica* [Chirazyme L-2 to Chirazyme L-5 (3); Fig. 2]. While the preferences of *Candida antarctica* lipases B (Chirazyme L-2 to Novozym 435) for the *trans* isomer are quite low, the different *Candida antarctica* lipases A [Chirazyme L-5 (1)–(3) from Boehringer] have extraordinary selectivities for elaidic acid. For example, Chirazyme L-5 lipase (3) esterifies the *trans* isomer about 15 times faster than oleic acid (Fig. 2). Furthermore, the above lipase is the only one of all immobilized biocatalysts which has a higher preference than the corresponding non-immobilized lipases.

Esterification of a mixture of oleic and elaidic acid

To make sure of the remarkable selectivities of lipase from *Candida cylindracea* and *Candida antarctica* lipase A, we tested the enzyme preparations *Candida cylindracea* (2) and Chirazyme L-5 (3), respectively, in the esterification of a mixture of *cis*- and *trans*-9-octadecenoic acid using 25 mM oleic acid and 25 mM elaidic acid together with 100 mM *n*-butanol and about 10 mg of each lipase in 250 µl *n*-hexane. As shown in Fig. 3, the lipase from *Candida cylindracea* has a strong preference for the *cis*-9-octadecenoic acid (Fig. 3A), while the lipase A from *Candida antarctica* has a unique selectivity for the *trans* isomer (Fig. 3B).

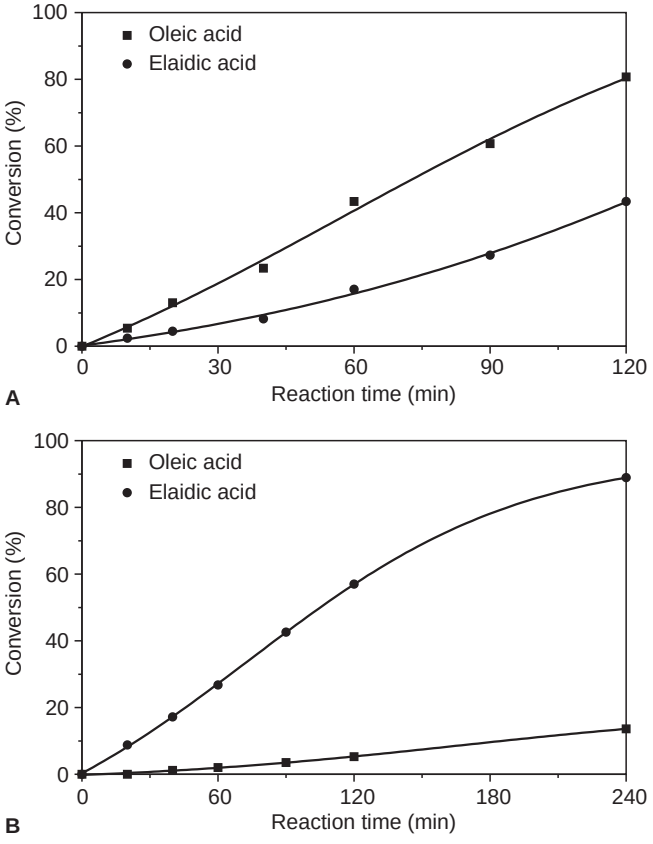

Fig. 3 Esterification of a mixture of oleic and elaidic acid catalysed by the lipase from **A** *Candida cylindracea* [*Candida cylindracea* (2) from Fluka] and **B** the lipase A from *Candida antarctica* [Chirazyme L-5 (3) from Boehringer]

Discussion

Our enzyme screening demonstrated that lipases from *Aspergillus niger*, *Aspergillus oryzae*, *Candida cylindracea*, *Humicola* sp., (*Rhizo-*)*Mucor miehei*, *Rhizopus arrhizus* and *Rhizopus delemar* are able to discriminate between *cis*- and *trans*-9-octadecenoic acid by preferring oleic acid over elaidic acid (Figs. 1, 2); thus, they are similar to the lipase from *Geotrichum candidum*, although the extents of preference are not comparable (Jensen 1974). Furthermore, by examining the substrate selectivity of *Candida antarctica* lipase A [Chirazyme L-5 (3) from Boehringer] in the esterification of oleic and elaidic acid, we succeeded for the first time in finding a lipase with a unique preference for *trans*-9-octadecenoic acid.

But what are the reasons for specific discrimination of biocatalysts between geometric isomers of an unsaturated fatty acid? The first should be the difference in acyl chain structure due to the direction of the twist of carbon chain after the first double bond. Such interpretations of experimental results have appeared in the literature (Lands 1979; Hills et al. 1990). Since we have determined lipases with opposite substrate selectivities, it is obvious that their preferences are influenced not only by the structure of the *key*, i.e. the substrate, but also by the structure of the *lock*, i.e. the enzyme.

In order to understand the specificity of lipases, insights into their molecular basis are necessary. Various enzymes have been examined by X-ray crystallography, computer-assisted modelling and protein engineering in recent studies (Schmid and Verger 1998). By analysing the shape and physico-chemical properties of the scissile fatty acid binding sites of several lipases, Pleiss et al. (1998) were able to explain the differences in fatty acid chain length specificity of acylhydrolases which are in accordance with differences in geometry of the lipase binding sites. So, if one considers that the lipases from *Rhizomucor miehei* and *Rhizopus delemar* are homologous proteins and have similar binding pockets, it is not surprising that they show the same preference for oleic acid. Furthermore, the *Candida antarctica* lipase B and pseudomonas lipases as well as pancreatic lipases have similar geometries of their fatty acid binding sites (Pleiss et al. 1998) and are rather unspecific in the esterification of *cis*- and *trans*-9-octadecenoic acid. The recent advances in understanding the molecular basis of substrate selectivity of lipases have also been utilized in studies of *Geotrichum candidum* and *Candida rugosa* lipases to explain their fatty acid selectivities (Holmquist 1998).

The competitive esterification reactions of isomeric octadecenoic acids in organic media used in this study are a simple practical method for the determination of fatty acid selectivity and should be useful for further theoretical examinations of lipases in combination with computer modelling.

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