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Enzymatic synthesis of fatty esters by alkylated lipase

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Abstract

Lipase from *Candida rugosa* was chemically modified by reductive alkylation with aldehydes of various chain lengths. The derivatised lipases showed a higher esterification activity compared to native enzyme. The degree of activity enhancement depended on the type and molecular weight of the modifiers used and the degree of modification of the enzyme. They exhibited higher activities in non-polar than in polar solvents. The optimum esterification temperature and preference of fatty acids as acyl donors of the derivatised lipases were very similar to those of native enzyme. Lipase derivatised with dodecyldehyde was more thermostable than those modified with acetaldehyde. Alkylated lipases are relatively more stable in organic solvents than the native enzyme.

Keywords: Reductive alkylation; Esterification; Selectivity; Stability

1. Introduction

The application of lipases for synthetic purposes is now well documented [1]. In the area of oil and fat research and technology, lipases were employed successfully in the interesterification of fats and oils for the production of high-value added products (e.g., cocoa butter), the alcoholysis of fats and oils, the production of partial glycerides and in the synthesis of fatty esters. These products have a wide range of uses in the food, pharmaceutic and cosmetic industries.

The benefits of employing lipase in synthetic reactions include its high selectivity and activity at relatively mild reaction conditions. However, lipases have inherent problems such as instability in organic solvents and high temperature, lack of dispersibility and narrow operational range when subjected to industrial process conditions. Research on redesigning the functional and physical properties of lipases so that they have properties suited to these conditions is a very promising field.

Enzymes have been chemically modified in order to alter and improve their native properties and endow them with useful new functions [2]. However, many of these enzymes derivatization methods involved laborious steps necessary for the activation purposes, low reactivity of the intermediates, easy cleavage of the modifier-protein adduct and sometimes uses toxic chemicals. A simple method which uses less toxic reagents for preparing hydrophobic enzyme derivatives is reductive alkylation of protein amino groups with aldehydes using cyanoborohydrides as the reductant [3]. In the

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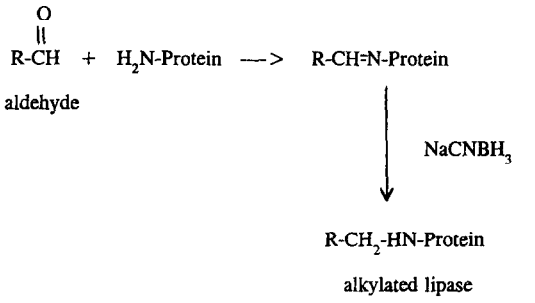
present study, a variety of hydrophobic aldehydes have been attached to lipase by reductive alkylation and the properties of the hydrophobic lipase derivatives with respect to their esterification reaction were investigated.



Scheme 1.

2. Experimental

2.1. Materials

Lipase from *Candida rugosa* (Type IV) and sodium cyanoborohydride were obtained from Sigma Chemical (St. Louis, MO). Aldehydes were obtained from Aldrich Chemical (Milwaukee, WI). All other reagents were of analytical grade. The organic solvents and substrates were dried over molecular sieve 3 Å before use.

2.2. Purification of lipase

Commercial lipase from *Candida rugosa* (2 g) was dissolved in distilled water (10 ml). The mixture was stirred at 4°C for 1 h and centrifuged at 12,000 rpm for 10 min. The supernatant (5 ml) was passed through a permeation chromatography column, Superose 6 on Pharmacia (Sweden) Fast Performance Liquid Chromatography systems. About 11–12 fold purification (calculated based on the specific activity of lipase after purification as compared to the specific activity before purification) and an overall activity yield of 60% was obtained.

2.3. Reductive alkylation of lipase

Reductive alkylation of lipase with aldehydes was as described by Ampon et al. [3] according to the reaction in Scheme 1. Aldehydes (about 40 μl) were added to lipase solution (0.25 g protein in 20 ml borate buffer, pH 9.0). Sodium cyanoborohydride ($NaCNBH_3$) (4–5 mg) was added as the reducing agent. The mixture was stirred at 4°C for 30 min. The reaction was terminated by adjusting the pH to pH 7.0 and passing the sample through a column (2.5 × 12.5 cm) of Sephadex G25. The active peak was collected and lyophilized in the cold and stored at −20°C prior to use. Lipase was derivatised to different degrees of modification by varying the molar ratio of the aldehyde with respect to the enzyme.

2.4. Protein assay

The amount of protein was determined by titration with trinitrobenzene sulfonate (TNBS) of the amino acids produced following the hydrolysis of the enzyme or its derivatives [4]. The extent of the protein modification was determined by comparing the number of amino acid groups that reacted with TNBS in the modified and unmodified protein [5].

2.5. Activity assay

The reaction system consisted of hexane (0.5 ml) (unless otherwise stated), propanol (2.67 mmol), oleic acid (0.35 mmol) (unless otherwise stated) and lipase (10–30 mg). The mixture was incubated at 28°C (unless otherwise stated) for 5 h with continuous shaking at 150 rpm. The reaction was terminated by dilution with 3.5 ml of ethanol:acetone (1:1 v/v) and the remaining free fatty acid in the reaction mixture was determined by titration with 0.05 M NaOH using an automatic titrator (ABU 90, Radiometer, Copenhagen) to an end point of pH 9.5. Specific activity of the enzyme was expressed as μmol min^{-1} mg^{-1} protein. Products

of the reaction were examined periodically on a Shimadzu 8A gas chromatograph using a 30 m polar capillary column Nukol TM (0.32 mm, i.d.) from Supelco (Australia). Helium was used as carrier gas. The split ratio used was 50:1. The injector and detector temperature were set at 250°C. The initial column temperature was 110°C. The temperature was increased at 8°C per min to 200°C.

2.6. Thermostability

The enzymes (10–30 mg) were incubated in benzene at various temperatures for 1 h in sealed vials. After the incubation, the enzyme mixtures were cooled to room temperature. The activities were then determined at 28°C. The residual activities were expressed as a percentage of the activity of the untreated enzyme.

2.7. Stability in organic solvents

The enzyme preparations were incubated in benzene for between 1 to 10 days at room temperature. After the incubation, their residual activities were determined at 28°C. The residual activities were expressed as a percentage of the activity of the enzyme at day 1.

3. Results and discussion

3.1. Activity of alkylated lipases

The effects of reductive alkylation of lipase with different aldehydes on its catalytic activity are summarized in Table 1. For the acetaldehyde lipase (AL), increasing the degree of modification of the enzyme from 0 to 83%, increased the activity of the enzyme by 10 fold. When homologs of aldehydes were used, increasing the molecular weight from 44 to 184 increased the enzyme activity by 2–3 fold. The increase in activity of the more hydrophobic enzyme may suggest that hydrophobic substrate moiety fits more effectively into the enzyme active site [6]. Remy et al. [7] suggested that the increase is a result of a change in the microenvironment at the active site, which caused an increase in the interaction of the substrate with the enzyme or in the solubilization of the product.

Table 1
Activities of alkylated lipases

Carbonyl compounds	MW [a]	Modification [b] (%)	Activity [c] (%)
—		0	100
Acetaldehyde (AL)	44	40	155
		56	360
		73	780
		83	1050
Propionaldehyde (PL)	54	56	182
Benzaldehyde (BL)	106	41	218
Octaldehyde (OL)	128	39	124
Dodecyldehyde (DL)	184	42	318

[a] Molecular weight of the modifier.
[b] Determined with TNBS [5].
[c] Activity is expressed as % of the unmodified lipase activity. The ester synthesis is followed by the rate of disappearance of oleic acid from the reaction mixture containing propanol and oleic acid.

3.2. Activity in organic solvents

Derivatised and native lipases were active in all the organic solvents tested (Table 2). Their activities were higher in the non-polar organic solvents ($\log P > 2$) compared with the more polar ones. These findings were in agreement with those of Laane et al. [8]. The lower activity

Table 2
Activities in various organic solvents

Solvent	Activity (μmol/min/mg protein)			
	$\log P$ [a]	NL	AL	DL
Hexane	3.5	0.43	1.28	0.60
CCl_4	3.0	0.27	1.23	0.60
Benzene	2.0	0.59	1.49	0.70
$CHCl_3$	2.0	0.39	1.12	0.58
CH_2CCl_3	na [b]	0.05	0.20	0.16
Pyridine	0.7	0.14	0.53	0.12
DMF	−1.0	0.06	0.76	0.13

NL native lipase. CCl_4 carbon tetrachloride. $CHCl_3$ chloroform. CH_2CCl_3 trichloroethane. DMF dimethylformamide.
[a] From Laane et al. [8].
[b] Not available.

of the enzymes in polar compared to the non-polar organic solvents was probably due to the ability of the more polar solvents to strip off the water layer from the enzyme molecules. The water layer around the enzyme molecule is essential to preserve the spatial conformation of the enzyme suitable for catalysis.

3.3. Optimum esterification temperature

The optimum esterification temperature of the derivatised lipases was very similar to that of the native enzyme (Fig. 1). Modification of lipase from *Candida rugosa* was through very stable amide bond linkages between the enzyme and the modifiers. These amino side chain groups of the protein were known not to be associated with the enzyme active sites [9]. Thus, the properties of the resulting modified lipase may not be grossly affected by the modification.

3.4. Substrate specificity

The effect of carbon chain length of the fatty acids used as acyl donors in the esterification reaction of the native and derivatised lipases

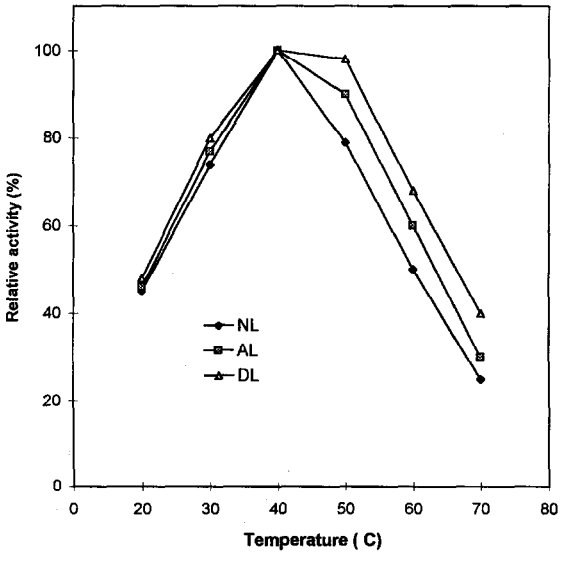


Fig. 1. The effect of temperature on the esterification by derivatised lipases. NL (native lipase), AL (acetaldehyde lipase), DL (dodecyldehyde lipase).

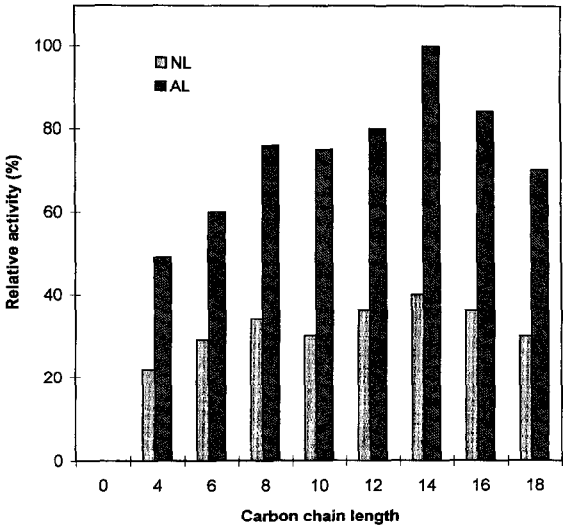


Fig. 2. The effect of chain length of fatty acids as acyl donors in the esterification reaction by derivatised lipase. NL (native lipase), AL (acetaldehyde lipase).

was similar (Fig. 2). As expected, the relative activity of the derivatised lipase was about two to three fold higher than the native lipase. It was generally found that the lipases favored the esterification of fatty acids with longer chain length. Similar studies by Kawamoto et al. [10] showed that fatty acids of longer chain length served as excellent acyl donors for esterification reactions compared to shorter chain fatty acids. However, the decrease in the activity of the enzyme with a relatively long chain of fatty acids may be due to the bulky chain moiety which may restrain the molecule from free rotation in the acyl binding site cavity of the enzyme active site or impose hindrance to attack by the nucleophile (alcohol) [11].

3.5. Thermostability

The derivatised lipase preparations were more thermostable when incubated for 1 h at temperatures from 40 to 70°C (Fig. 3) compared with the native lipase. The most thermostable modified lipase was dodecyl lipase (DL). The modifiers attached to the enzyme seemed to protect the enzyme against denaturation by heat. With

the increase in hydrophobic interaction at higher temperature, the enzyme molecules become more compact and rigid and less prone to denaturation by unfolding. Similar studies had shown that the chemical modification of lysine residues of ribonuclease increases its heat stability due to a decrease in the intermolecular crosslinking of the enzyme [12]. Unmodified lysines residues can undergo thermal aggregation which leads to a loss of enzymatic activity. Thus, modification of the lysine residues eliminates the tendency to form crosslinking and cause enzyme stabilization.

3.6. Solvent stability

The derivatised lipase preparations were more stable than the native lipase when incubated in benzene (Fig. 4). Basri et al. [2] had reported similar findings. The half-lives of the native lipase, AL and DL were 3.6, 5.75 and 6.00 days, respectively. The modifiers seemed to protect the enzyme from denaturation caused by the presence of the organic solvent. The modification may induce the surrounding water which was necessary for activity, to be tightly bound to the enzyme thus not be easily stripped by organic solvents.

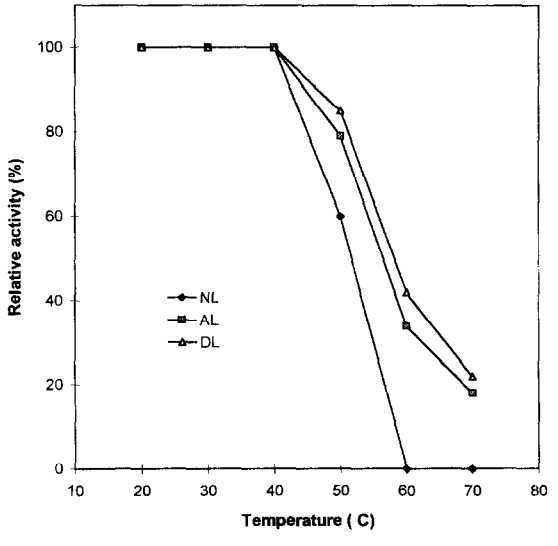


Fig. 3. Thermostability of alkylated lipases incubated for 1 h in benzene. NL (native lipase), AL (acetaldehyde lipase), DL (dodecyldehyde lipase).

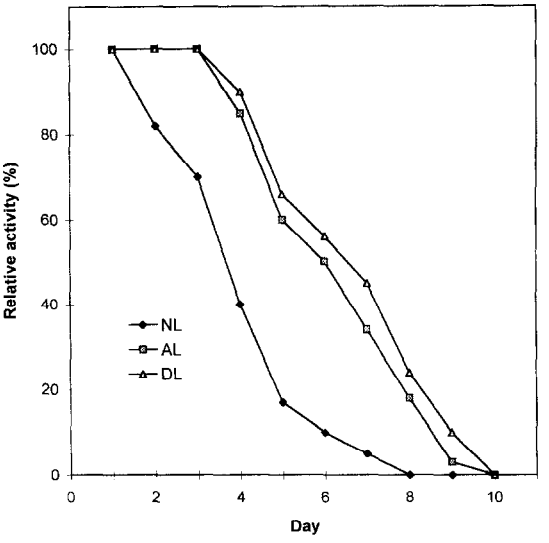


Fig. 4. Stability of alkylated lipases incubated in benzene for ten days at room temperature. NL (native lipase), AL (acetaldehyde lipase), DL (dodecyldehyde lipase).

In conclusion, alkylated lipases from *Candida rugosa* exhibited modified characteristics that may be suitable for industrial biotransformations. Its greatly increased activity in various organic solvents could lead to use in the increased esterification of fats and oils. The increased stability is very favorable in commercial applications.

Acknowledgements

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