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Fatty-acid-modified enzymes as enantioselective catalysts in microaqueous organic media

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Highly active lipase and protease complexes were prepared by non-covalent modification with stearic acid. The protein content and yield of the modified enzyme complexes depended on the enzymes' source. The increase in the transesterification activity of the modified enzymes was 15 fold for *Candida rugosa* lipase and porcine pancreatic lipase, with preservation of the enantioselectivity. *Pseudomonas sp*. lipase which showed no activity in its crude form, exhibited an activity of 38 μmol/h·mg protein in the modified form.

Introduction

Biocatalysis in organic media has become a field of increasing interest in recent years (Koskinen, 1996; Wong and Whitesides, 1994; Adlercreutz, 1994). The main advantages of non-aqueous enzymology are increased solubility of the substrates, favorable thermodynamic equilibrium shift for many reactions, and enhanced thermostability and ease of recovery of the insoluble enzymes. Despite the advantages of enzymatic catalysis, the problem of comparatively low reactivity of enzymes in organic solvents, is still a major drawback. (Klibanov, 1997). One means of improving enzymatic activity in organic media is the generation of an enzyme-surfactant complex (Okahata and Ijiro, 1988; Paradkar and Dordick, 1994; Basheer *et al*., 1996). Such soluble or dispersible enzyme preparations have been shown to possess higher activity in microaqueous environments, up to three orders of magnitude compared with the respective unmodified enzymes.

It is believed that the hydrophilic heads of the surfactant molecule form hydrogen bonds with the enzyme molecules, whereas the hydrophobic tails enhance the solubility of the enzyme in the organic solvent (Okahata and Ijiro, 1992). However, the exact structure of the enzyme-surfactant complex is not yet fully understood. In order to simplify the complex, we propose to use natural fatty acids as amphiphilic modifiers. Fatty acids resemble surfactants in their structure, and yet they are simpler molecules than surfactants such as dioleyl glutamate ribitol amide or sorbitan monostearate, which have been used by other research groups for enzyme modification (Okahata and Ijiro, 1992; Basheer *et al*., 1996). This paper describes the enhancement of enzymatic activity in microaqueous organic media, by modification with stearic acid as a model fatty acid.

Materials and methods

Materials

Candida rugosa (formerly *cylindracea*) lipase type VII (CCL) and porcine pancreatic lipase type II (PPL) were purchased from Sigma. Lipase from *Pseudomonas sp*. (Chirazyme L-6), *Candida antarctica* B (Chirazyme L-2) and Subtilisin Carlsberg (Chirazyme P-1) were purchased from Boehringer Mannheim. All enzymes were used without further purification. Stearic acid and sorbitan monostearate (Span 60) were purchased from Sigma. All the reagents and solvents used in this study were of analytical grade.

Modification of enzymes

The crude enzyme preparations contained different amounts of protein. For achieving comparative results, 150 mg protein of each lipase and 230 mg protein of protease powder were used for modification. The enzyme was dissolved in 200 ml 0.05M phosphate buffer, pH 6, and stirred for 20 min at 4°C. A solution containing 0.44 mmol of surfactant (stearic acid or Span 60) in 5 ml warm ethanol was added dropwise to the enzyme solution. After mixing for 15-30 min the dispersion was sonicated (Sonicor SC-52H, Sonicor Instrument Corp., Capiague NY) for 10 min. The enzyme-surfactant mixture was further stirred for 3 h and allowed to stand overnight at 4°C. The precipitated enzyme complex was collected by centrifuga-

tion (23,500 g, 4°C for 20 min) and lyophilized to yield a white powder. The protein content of the modified enzyme powders was determined by elemental analysis.

Enzymatic reaction

Enzymatic activity and stereoselectivity were determined by transesterification of 1-phenyl ethanol with vinyl acetate in diisopropyl ether. Modified enzyme containing 10 mg protein was dispersed in a solution of 50 mM 1-phenyl ethanol and 150 mM vinyl acetate in 10 ml diisopropyl ether. For Chirazyme L-6, 2 mg protein were used and, for Chirazyme L-2 and P-1, 5 mg protein were used. The ratio of enzyme to phenyl ethanol was kept constant at 1 mg protein per 50 μmol phenyl ethanol for a total reaction volume of 10 ml. The flasks were shaken in a water bath at 140 rpm, 35°C. Samples were withdrawn periodically, filtered, and examined by GC.

Analytical measurements

The conversion of 1-phenyl ethanol to the corresponding ester was monitored by gas chromatography, using a 15 m capillary column packed with 100% dimethyl polysiloxane (Restek Corp.) The temperature was programmed as follows: T1 = 90°C, 1 min; dT/dt = 20°C/min; T2 = 300°C, 1min. Under these conditions the retention times were: R_f = 1.4 min for 1-phenyl ethanol, and R_f = 2.1 min for 1-phenyl ethyl acetate. The conversion of the alcohol to its corresponding ester was calculated from the relative areas beneath the peaks, using a response factor obtained from calibration of standard solutions in the same organic solvent. The enantiomeric excess (ee) of the product and the substrate was detemined by HPLC using a Chiralcel OJ column from Daicel (5mm, 4 × 250 mm). Eluent composition was hexane:isopropanol 96:4, flow rate 0.9 ml/min, λ = 254 nm. The observed retention times were: R_f = 10.67 min for the (S)-ester, R_f = 11.21 min for the (R)-ester, R_f = 15.82 min for the (S)-alcohol and R_f = 18.2 min for the (R)-alcohol.

Results and discussion

Effect of enzyme source on the protein content and yield of the corresponding enzyme- stearic acid complex

The possibility of using fatty acids for modification of enzymes was tested on four lipases and one protease. Stearic acid was selected as a model fatty acid due to its resemblance to the hydrophobic chain of sorbitan monostearate, which was successfully used to modify enzymes in the past (Basheer *et al*., 1995; Okazaki *et al*., 1997). Upon the addition of stearic acid or Span 60 to the enzyme solutions, insoluble complexes were formed in the buffer. The weight and protein content of the complexes are presented in Table 1, and reflect a strong dependence on the origin of the enzyme. Similar observations were reported by other research groups, although different surfactants were used in their work (Okahata and Ijiro, 1992; Goto *et al*., 1994; Basheer *et al*., 1996).

Despite the initial protein concentration being equal for all enzymes, the protein content and yield of the complexes varied considerably. Moreover, for subtilisin, the initial protein concentration was higher, but the yield was nevertheless low. It is conceivable that individual protein entities will react differently with the surfactant, and subsequently the overall solvation of the formed complex will determine the extent of the resultant salting out of the enzyme.

Goto and co-workers (Goto *et al*., 1995) suggested that the protein content of the enzyme-surfactant complex depends on the nature of amino acid residues on the surface of the enzyme. It is also possible that other components present in the crude enzymes (stabilizers like lactose or salts) influence the interactions. That the two enzymes from Sigma resulted in complexes with a high yield of protein content compared to the enzymes obtained from Boehringer Mannheim, may bear relationship to the methods of enzyme preparation. In order to substantiate this possibility, one would need to modify (under the same

Table 1 Yield and protein content of the modified enzymes

Enzyme[a]	**CCL**		**PPL**		**L-6**		**L-2**		**P-1**	
Surfactant	*C18*[b]	*Span 60*	*C18*	*Span 60*	*C18*	*Span 60*	*C18*	*Span 60*	*C18*	*Span 60*
weight (mg)	160	236	160	170	126	88	125	100	78	160
protein (%)[c]	11.4	11.7	13.7	11.3	3.4	2.3	5.2	6.3	8.8	3.5
yield (%)[d]	12.2	18.4	14.7	12.8	2.9	1.3	4.3	4.2	3.0	2.4

a) CCL-*Candida rugosa* lipase, PPL-Porcine pancreatic lipase, L-6-*Pseudomonas sp.* lipase, L-2-*Candida antarctica* B lipase, P-1-Subtilisin Carlsberg. b) stearic acid. c) determined by elemental analysis, and represents an average of triplicates. d) calculated as ratio between the protein in the precipitate and the protein in the initial crude; for the lipases the initial amount of protein was 150 mg and for the protease – 230 mg. The modification of the various enzymes was carried out according to the procedure described in Materials and Methods.

Table 2 Initial activity and enantioselectivity of stearic acid and Span 60 modified enzymes

Enzyme[a]	*Modifier*	*Specific initial activity (μmol/h·mg protein)*	*Conversion (% at 25 h)*[b]	*ee of product (% at 25 h)*[c]
CCL	stearic acid	4.5	20	58
	Span 60	8.8	30	69
	crude	0.3	10	45
PPL	stearic acid	6	49	97
	Span 60	9.7	50	92
	crude	0.4	16	95
L-6	stearic acid	37.6	53	80
	Span 60	28	22	100
	crude	0	0	0
L-2	stearic acid	18	51	94
	Span 60	16	48	95
	crude	5	50	97
P-1	stearic acid	0.3	14	100
	Span 60	0.8	22	100
	crude	0	0	0

a) CCL-*Candida rugosa* lipase, PPL-Porcine pancreatic lipase, L-6-*Pseudomonas sp.* lipase, L-2-*Candida antarctica* B lipase, P-1-Subtilisin Carlsberg. Experimental conditions: 10 mg protein, 50 μmol 1-phenyl ethanol, 150 μmol vinyl acetate, 10 ml diisopropyl ether, 35°C
b) determined from GC analysis c) determined from HPLC using a chiral column.

conditions), a specific enzyme which is available from different suppliers (CCL for instance) and compare the results. There has been no report in the literature of such a comparison. Assuming that the interactions between the surfactant and enzyme are primarily hydrogen bonds, it was expected that Span 60-complexes would contain higher levels of protein due to the ability of Span 60 to form more hydrogen bonds per molecule. However, the protein content of the complexes obtained with stearic acid are similar to those obtained with Span 60 (with the exception of P-1). This indicates that steric effects, as well as the spatial of distribution of polar groups on the protein surface, also affects the mode of interaction. The pK of stearic acid is 5.05 (Fini *et al*., 1987). Therefore at pH 6, for which the modification was performed, this acid is largely ionized. Consequently, depending on the isoelectric pH and surface charge distribution of each protein, the interactions, at least in the initial state of complex formation, could be electrostatic as well.

Activity measurements of lipase-stearic acid complexes

The modified enzymes were utilized in the transesterification reaction of 1-phenyl ethanol and vinyl acetate, in diisopropyl ether as the solvent. The initial reaction rate of each enzyme is described in Table 2. In all of the experiments, the initial ratio of enzyme to substrate was constant at 1 mg protein per 50 μmol 1-phenyl ethanol. This compound is a good substrate for some enzymes, but a poor one for CCL or subtilisin. However, despite the differences in affinity, all of the modified enzymes exhibited a marked increase in activity compared with the crude forms. In the case of L-6 and P-1, the crude enzymes showed no activity in the reaction system, whereas the modified enzymes were active. Moreover, modification with stearic acid produced the most significant effect on lipase L-6, to give an initial acivity of 37.6 μmol/h·mg protein. Of the five enzymes studied, four were completely selective towards the (R)-ester, with CCL showing only a small preference towards this enantiomer (Table 2). In cases where the conversion exceeded 50%, the enantiomeric excess of the product slightly decreased (C 18-modified L-6 for example), but, at 45-49% conversion, the ee was greater than 96%. It is important to note that the modification with stearic acid did not affect the enantioselectivity of the enzymes, indicating that the active site remained unchanged.

The progress of the transesterification reaction with modified PPL is presented in Figure 1. The surfactant-modified PPL gave nearly full substrate conversion within 9 hours, during which time the crude enzyme attained a merely 7% conversion.

A control experiment was carried out to examine whether the existence of the fatty acid in the reaction mixture caused the marked increase in activity of the modified enzyme complex. Crude PPL and stearic acid (in powder form) were mixed together prior to the addition of the substrates. The concentrations of the modifier and

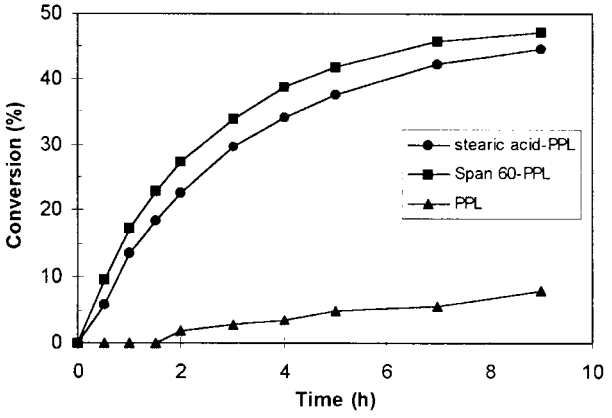

Figure 1 Formation of 1-phenylethyl acetate with time by modified PPL. Experimental conditions were 10 mg protein, 50 mM 1-phenyl ethanol, 150mM vinyl acetate, 10 ml diisopropyl ether, with shaking at 35°C.

the enzyme were adjusted to match their proportions in the modified complex (i.e. the protein comprising ~12% of the total amount of solids). The results showed that the addition of stearic acid in this manner did not increase the activity of the enzyme. Thus, the incorporation of stearic acid into the aqueous buffer system during the modification process itself, was necessary to generate the highly active enzymes.

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