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ESTER SYNTHESIS IN ORGANIC SOLVENT CATALYZED BY LIPASES IMMOBILIZED ON HYDROPHILIC SUPPORTS.

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Summary: Eight microbial lipases and one animal lipase were immobilized on hydrophilic supports either by adsorption or entrapment. All preparations catalyzed the synthesis of geranyl or menthyl butyrate or laurate using heptane as solvent. This is a simple and easy method for ester synthesis.

INTRODUCTION

It is well known that lipases (EC 3.1.1.3. triacylglycerol acylhydrolase) hydrolyze fatty acid ester bonds of triglycerides or other monoesters of primary and secondary alcohols. Glycerides synthesis was reported as early as 1964 by Iwai et al., using a lipase from **Aspergillus niger**, and further extended to three other fungal lipases (Tsujisaka et al. 1977). It was shown later that oleic acid esters of primary and secondary aliphatic or terpenic alcohols (Okumura et al. 1979; Iwai et al. 1980) were synthesized in a reaction catalyzed by lipases. When a racemic alcohol was used the reaction was stereospecific (Yamaguchi et al. 1977). In order to increase the yield of the reaction several authors used organic solvents such as heptane or diisopropyl ether (Bell et al. 1979; Patterson et al. 1979), hexane or benzene (Tanaka et al. 1981; Seo et al. 1982). In these conditions the crude lipase preparation was insoluble in the organic solvents giving a two-phase system. A lipase covalently bound to polyethylene glycol was recently reported (Yoshimoto et al. 1984; Tahahashi et al. 1984). This preparation is "soluble" and active in benzene allowing ester synthesis in a one-phase system. Although this catalyst can be recovered from the reaction mixture by precipitation it cannot be used in a continuous reactor.

This work describes the preparation of immobilized lipases by a simple method and their use in heptane for the synthesis of esters of terpene alcohols like menthol and geraniol.

MATERIALS AND METHODS.

Enzymes: the commercial lipase preparations were from **Geotricum candidum** (Rhone Poulenc, 89 u/g), **Aspergillus sp.** (Rhom, 2,000 u/g), **Mucor mieihi** (Gist-Brocades, 120,000 u/g), hog pancreas (Koch Light Laboratories, 15,000 u/g) **Alcaligenes** and **Candida cylindracea** (Meito Sangyo 4,470 and 8,600 u/g

respectively), **Rhizopus arrhizus** (Sigma, 546 u/g), **Rhizopus delamar** (Seppim, 16,000 u/g), **Pseudomonas**, (Serva, 2,600,000 u/g). Activity was assayed by hydrolysis of tributyrin (Lavayre and Baratti, 1982a) and the specific activity expressed as international units per g crude preparation.
Immobilization: in a typical experiment for adsorption, 1 g of lipase preparation was dissolved in 10 ml of water. When necessary the undissolved material (with no lipase activity) was discarded by centrifugation. Five g of support were then added and the water eliminated slowly under reduced pressure. The supports used were : Spherosil XOB 015, XOB 075 from Rhone Poulenc, Celite, porous glass, alumina and titania from Corning. Entrapment in polyurethane was done acording to a published procedure (Wood and Frisch, 1972) using a prepolymer from Chloe Chimie PE 203.
Ester synthesis: the reaction was carried out in 5 ml of heptane containing 0.125 M of butyric or lauric acid and 0.125 M of geraniol or (+/-) menthol. The immobilized lipase was then added (1 g of catalyst) and the mixture incubated at 33°C. Geranyl and menthyl esters were analyzed by GLC with a Carlo Erba apparatus equipped with a capillary SE 52 column (25 m), using helium (2 ml/min) and detection by flame ionization. Quantitative data were obtained with an integrator after calibration with standards.

RESULTS AND DISCUSSION.

Preparation of immobilized lipases:

The lipase from **Candida cylindracea** (trade name Lipase MY) has been used successfully for enantioselective ester synthesis of alpha-substituted cyclohexanols (Langrand et al. 1985). This preparation was used to compare immobilization on different hydrophilic supports (Table 1).

TABLE 1 Ester Synthesis by immobilized lipase preparations.

No:	Immobilization method	Support	Specific area m^2/g	Pore size A	Activity u/g	(a) %
0	free lipase	-	-	-	2.2	100
1	Adsorption	Glass XOB 075	123	375	0.9	41
2	Adsorption	Glass XOB 015	26	1170	1.5	70
3	Adsorption	Glass Corning	-	875	1.3	59
4	Adsorption	Alumina	-	1300	1.3	60
5	Adsorption	Titania	-	500	0.5	22
6	Adsorption	Celite	-	545	1.8	82
7	Entrapment	Polyurethane	-	-	1.1	52

(a) Specific activity of the immobilized lipase in /u mol of menthyl ester formed per minute per g of lipase.

Preparations 1-3 were obtained by adsorption on porous glass beads (Spherosils XOB 075, XOB 015 and Corning) and preparations 4-6 by adsorption on alumina, titania or Celite. Preparation 7 was obtained by entrapment in polyurethane.

Each preparation was incubated as described above with lauric acid and menthol (0.125 M each). All the immobilized lipase preparations were active with activity yields in the range 22-82%. The highest activity was observed for immobilization on Celite and porous glass beads of high specific area like Spherosil XOB 015. These values of activity yield are

high compared to the data (not higher than 1-3%) observed with emulsified substrate (Lavayre and Baratti 1982b). In that case the low activity was most probably the result of diffusional limitations of the substrate into the porous support and not of enzyme inactivation during immobilization. This is a general situation when lipases are acting on insoluble esters in emulsion. The use of an organic solvent resulted in solubilization of the substrates, so that the diffusional limitations in the porous supports were minimized and high retention of activity was obtained. This is a great advantage of the use of immobilized lipases for ester synthesis instead of hydrolysis. In addition, preliminary experiments done with preparations 2 and 8 showed high operational stability (half-life higher than 100 h) when used in a continuous reactor (unpublished results). Because of its simplicity and easy preparation adorption on glass beads was selected for further studies, using Spherosil XOB 075.

Synthesis of geranyl esters by immobilized lipases:

To check the generality of the immobilization procedure just described, eight microbial lipases and one animal lipase were immobilized by adsorption on porous glass beads and tested for their ability to catalyze the synthesis of geranyl esters of butyric and lauric acid. The results are shown in Table II.

TABLE II:Synthesis of geranyl butyrate and laurate with immobilized lipases

Lipase preparation	Geranyl butyrate			Geranyl laurate		
	Activity u/g[a]	u/u.10^6 [b]	yield %	Activity u/g[a]	u/u.10^6 [b]	yield[c] %
Alcaligenes sp.	0.21	47.1	14	-	-	37
Pseudomonas sp.	-	-	12	-	-	12
Geotricum candidum	-	-	-	0.13	1460	13
Candida cylindracea	1.47	172	74	18	2090	61
Aspergillus sp.	1.5	750	60	0.7	350	27
Rhizopus arrhizus	0.86	1593	6	0.23	421	0.5
Rhizopus delamar	5.12	320	51	-	-	29
Mucor mieihi	22.8	190	33	104	874	53
Hogpancreas	0.9	60	49	1.5	100	64

[a] specific acitivity of immobilized lipases in /u mole of geranyl ester formed per minute per g of lipase.
[b] ratio of activity (a) divided by the specific activity of non-immobilized lipase preparation, on tributyrin (see Materials and Methods for values).
[c] as percentage of ester formed in 48 hrs.

All the preparations were able to catalyze ester synthesis with initial rates ranging from 0.1 to 104 u/g. The highest activity was observed for the lipase from **Mucor mieihi**. Both butyric and lauric acid were esterified in our conditions with roughly similar rates. The crude lipase preparations showed very different specific activities when measured with the classical tributyrin assay (see Materials and Methods for values) due to the difference in enzyme purification. Comparison of the activity of the

nine immobilized lipase preparations is difficult on weight basis (u/g of lipase preparation). A more useful comparison is to use the ratio of activity in ester synthesis (u/g) to the activity in tributyrin hydrolysis. These results are also shown in Table II. The highest activity was obtained with the R. **arrhizus** lipase when butyric acid was used and with G. **candidum** lipase when lauric acid was used. The last data shown in Table II are the conversion yields for a 48 hrs reaction. The highest ones were observed for the lipase from **C. cylindracea** and hog pancrease despite the relatively low initial velocities.

It was recently reported (Cambou and Klibanov, 1984) that direct esterification of 2-(p-chlorophenoxy)-propionic acid by butanol, catalyzed by lipases, was not possible due to acidification of the water phase. The authors concluded that "this phenomenon will be a general one and probably (an) unsurmountable problem". The results reported in this paper clearly demonstrate that this is not the case, and immobilized lipases can catalyze ester synthesis starting from acid and alcohols in organic solvent. Further work is in progress to study the stereospecificity of the reaction under these conditions.

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