LIPASE IMMOBILIZED BY SOL–GEL TECHNIQUE IN LAYERS

Gabriela KUNCOVA[a], Massimo GUGLIELMI[b], Pavel DUBINA[c] and Bohuslav SAFAR[c]

[a] *Institute of Chemical Process Fundamentals, Academy of Sciences of the Czech Republic, 165 02 Prague 6-Suchdol, Czech Republic*
[b] *University of Padova, Via Marzolo 9, 35131 Padova, Italy*
[c] *Research Institute 070, 602 00 Brno, Czech Republic*

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Commercial lipase was immobilized into an organic–inorganic matrix formed by hydrolysis of silicon alkoxides (tetraethoxysilane, dimethyldiethoxysilane and methyltriethoxysilane) with (3-aminopropyl)triethoxysilane, (3-thiopropyl)trimethoxysilane and chlorodiisopropyloctylsilane. Hydrolytic activity of lipase was tested after addition of the enzyme to a prepolymer solution, after gelation, in xerogel particles and in thin layers deposited on glass slides by dip- or spin-coating. The prepolymer containing NH groups showed the higher activity then the native enzyme.

Lipases have been widely applied in clinical analysis and industry. Although their immobilization could be of advantage, the immobilized lipases prepared so far showed substantially lower activity than the other immobilized enzymes[1]. Recently, a novel method for their immobilization in hydrophobic sol–gel silica matrices has been reported, yielding the samples of high activity and enhanced stability[2,3]. Furthermore, transparent sol–gel glasses so prepared are suitable for preparing luminiscent and colorimetric enzymic sensors[4,5]. The limits of monolithic biogel sensors caused by diffusion controlled reaction rates can be improved by deposition of the active biomolecules in layers. However, a process of film deposition requires the stability of sol viscosity[6] which is decreased by addition of enzyme solution.

To overcome this problem we tried immobilization of lipase in thin layers. For this purpose we modified conventional sol–gel procedures by cohydrolysis of silicon alkoxides with (3-thiopropyl)trimethoxysilane and (3-aminopropyl)triethoxysilane, adding the enzyme at neutral conditions. Composition of precursor solutions is given in Table I.

The activity of the enzyme was determined by following the rate of sunflower oil or glyceryl tributyrate hydrolysis and was compared with the native enzyme (Table II). The activity of the sample prepared by addition of lipase to the solution containing 3-aminopropyl groups (solution I) exceeded that of the native enzyme. Increased activity of an immobilized lipase was already observed[3,7]. We suggest that lipase activity enhancement observed by us can be explained by more basic enzyme microenvironment and to

the formation of hydrophobic lipase-containing nanoparticles[8] formed in the water–oil emulsion.

The low activity of xerogel particles formed from solutions I–III (Table II) can be ascribed to a matrix shrinkage which reduces diffusion of the substrate and can also destroy lipase molecules. However, the xerogel formed from solution IV was soft and its particles disintegrated during activity tests.

Lipase deposited in layers on glass slides (Table II) showed nearly the same activity as the untreated enzyme. The substrate specificity of the enzyme, which is generally greater for glyceryl trioleate than for glyceryl tributyrate[9], was not changed by immobilization. Films prepared from solutions of lipase were less transparent and their thickness fluctuation was more than by one order of magnitude greater than the films obtained from siloxane solutions containing no enzyme (Table III). Moreover, lipase decreased markedly the film formation time (i.e. the time from enzyme addition to the gelation), which did not exceed 30 min, in contrast to several hours for the blank solutions. The adhesion of the enzyme layers to glass slides and their hardness was very good, except for the samples prepared from solution IV, which could be easily wiped off. Mechanical and catalytical properties of films formed from solutions I–III were not changed after one day immersion in a water bath at 30 °C, but they suffered destruction after 2 h at 40 °C. Lipase immobilized in particles or layers retained its activity even after its storage at 25 °C for two months.

TABLE I
Composition of precursor solutions

Solution	Molar ratio of components						
	EtOH	$MeSi(OEt)_3$ $Si(OEt)_4$	$Me_2Si(OEt)_2$	$(iPr)_2(C_8H_{14})SiCl$	H_2O	Trizma HCl	$H_2N(CH_2)_3Si(OEt)_3$ $HS(CH_2)_3Si(OMe)_3$
I[a]	3	1	0	0	3	0.001	1
		0					0
II	3	0	0	0	3	0.0001	0
		1					1
III[b]	1.73	0	0.1	0	5.3	0.001	0
		1					0.23
IV	0	0	0.15	0.001	0.62	0	0
		1					0

[a] Trizma hydrochloride was added as 0.05 M aqueous solution, of Trizma buffer pH (25 °C) 7.5. [b] 1,3-Pentanedione and methanol were added prior to addition of the thiopropylsilane.

EXPERIMENTAL

Materials

Lipase (EC 3.1.1.3) Lipolase 100 L was supplied by Novo Nordisk (Denmark) as an aqueous solution containing protein (6 mg in 1 ml). All reagents and solvents were of reagent grade or better. Sunflower oil was a commercial sample (Floriol, Cereol, Hungary).

Preparation of Precursor Solutions

The solutions are characterized in Table I, in which the components are arranged according to the sequence of their addition. The volume of all the solutions was 10 ml. An aqueous solution of 0.05 M Trizma hydrochloride (solutions I–III) or 0.05 M Trizma buffer of pH 7.5 at 25 °C (solution I) were

TABLE II
Relative activity of lipase in sol–gel process (A_{rel}, expressed as per cent of the activity of the native enzyme) for hydrolysis of sunflower oil (SFL) and glyceryl tributyrate (GTB)

Solution	A_{rel}, %	
	SFL	GTB
	In solution	
I	149.0	122.9
II	10.6	8.8
III	12.3	12.4
IV	39.0	42.2
	In gel	
I	26.0	26.1
II	2.2	1.6
III	3.8	3.2
IV	23.0	29.3
	In xerogel	
I	3.8	2.5
II	0.8	0.8
III	2.7	2.4
IV	7.2	6.0
	In layers	
I	144.7	100.4
II	9.0	8.0
III	10.8	8.0

added. Except for (3-thiopropyl)trimethoxysilane, the components of solutions I–III were mixed at 4 °C and stored at that temperature for 2 days. Then, the precipitate was removed by filtration. Solutions containing thiopropyl groups (II and III), thiopropylsilane were added as the last component. After standing at 25 °C for 2 days, solution viscosities increased from approximately 100 to 3 000 mPa s. The acidity of solutions II–IV was adjusted to pH 5–6 (an indicator paper) by 0.01 M NaOH and by a saturated aqueous Trizma base solution. Components of solution IV were mixed at 40 °C. Exothermic reaction was controlled by cooling with ice-water bath to 4 °C. The mixture was stirred for another 4 h, during which the temperature increased to 25 °C. The acidity of viscous solution (≈ 1 000 mPa s) was adjusted to pH 7 by slow addition of 0.01 M NaOH solution at 4 °C and the enzyme solution was added in the ratio 1 : 5 (v/v). After one minute stirring, the layers were deposited on glass slides. A prepolymerized solution (3 ml) containing lipase was placed in the test tube and allowed to stand at 20 °C for 30 min. A small sample was withdrawn and checked for hydrolytic activity. After standing at 20 °C for one week, the translucent pellets formed were disintegrated and sieved. The 75–300 μm fraction was dried at ambient temperature for 2 h under reduced pressure. Prior to activity tests, the particles (0.5 g) were suspended in water (10 ml) and allowed to stand overnight with intermittent shaking. This procedure was repeated three times to ensure complete removal of nonbonded enzyme (no activity was observed in the last eluate).

Film Formation

Prior to coating, microscopic glass slides were washed successively with a surfactant solution, 5% nitric acid, distilled water and dried at 120 °C. The coating was performed at 25 °C and 60% relative humidity.

Dip-coating. Microscopic glass slides were drawn out vertically from the precursor solutions containing lipase at a constant speed of 10 cm/min (ref.[10]).

Spin-coating. The precursor solution containing lipase was dropped on a glass slide[6] rotating at 4 000 s^{-1}.

Glass slides coated by the above procedures were kept at 20 °C and 70% humidity for one week when the thickness of the layers was measured (Alpha-Step 200, Tencor Instruments, U.S.A.) and the enzymatic activity was determined as described below.

TABLE III
Thickness of sol–gel films without lipase (A) and containing lipase (B) on glass slides prepared by dip-coating (DC) and spin-coating (SC)

Solution	Procedure	Thickness, μm	
		A	B
I	DC	2.4 ± 0.02	2.3 ± 0.4
II	DC	2.2 ± 0.02	1.1 ± 0.2
III	SC	1.9 ± 0.03	2.7 ± 0.3

Hydrolytic Activity of Immobilized Lipases

The content of proteins in the enzyme (Lipolase 100 L) was determined by the known method[11]. The enzyme had activity of 0.018 U/mg of protein for sunflower oil or 0.081 U/mg for glycerol tributyrate. The unit (U) was defined as the amount of the enzyme which liberates 1 μmol of a fatty acid from a triglyceride in 2 h at pH 7 and 40 °C. The commercial enzyme solution did not change its activity after standing at ambient temperature for 3 months or at 30 °C for 3 days.

The hydrolytic activity of lipase after its addition to precursor solutions was determined in the following way. An enzyme solution, a gel or a xerogel (0.1–1 g) was placed into a 50 ml Erlenmayer flask with distilled water (10 ml) and the substrate (0.011 mol of sunflower oil or 0.033 mol of glyceryl tributyrate, 10 ml each). The mixture was stirred (500 s^{-1}) at 40 °C for 30 min, using a 3 cm teflon covered magnetic stirring bar.

For activity measurement of the lipase immobilized on glass slides, 9 slides of 10^{-2} m^2 total area were fixed in a 50 ml cubic reactor, water and substrate were added and mixed by a magnetic stirring bar placed below the slides.

The background of free fatty acids was determined with samples not containing lipase under the same conditions. In determining the enzyme activity, the hydrolysis of sunflower oil or of glyceryl tributyrate was stopped by adding twofold volume of ethanol–acetone mixture (1 : 1, v/v), and free fatty acids were titrated with 0.05 M NaOH to the end point on phenolphthalein.

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