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Lipase enhanced catalytic efficiency in lactonisation reactions

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Abstract

We studied an enantioselective lipase-catalysed transesterification reaction for the production of the statin lactone moiety. It is well known that the chiral cyclic structure of statins is very important for their pharmaceutical activity in the prevention and treatment of coronary artery diseases.

We focused our attention on 3*R*,5*R*-6-(ethyl-2-phenyl)-4-hydroxytetrahydropyran-2-one (**2**); this was synthesised by employing an enantioselective lactonisation reaction catalysed by a commercial preparation of porcine pancreatic lipase (PPL) starting from a racemic mixture of *syn*-diol **rac-1**. Optimisation of the reaction conditions for the biocatalysed intramolecular transesterification reaction of the racemic diol **1** was carried out. It was seen that the maximal reaction rate could be greatly enhanced by the addition of solid matrices to the reaction medium.

Keywords: Lipase; Lactonisation; Statins; Organic solvent

1. Introduction

Statins have been shown to lower serum cholesterol levels in animal models and humans [1–3] and the presence of the chiral 3*R*,5*R*-3-hydroxy-5-substituted δ-lactone moiety in the statin structures (see Scheme 1) is of primary importance for their pharmacological mechanism [4,5]. For this reason, statins have been the target molecules of an increasing number of studies aimed at synthesising their enantiomerically pure form (for references on total synthesis of naturally occurring and synthetic analogous of statins) [6], with a major effort in the synthesising of the lactone moiety (for recent papers on the synthesis of lactone moiety) [7]. We initially used pancreatin-catalysed lactonisation of the racemic *syn*-diol **1** and other similar substrates in dry diethyl ether [8,9] (for the application of the same reaction to the synthesis of other naturally occurring lactones, see [9]), to obtain high enantiomeric excess but low chemical yields and conversions in the 3*R*,5*R*-lactone **2** (Scheme 1). After our first preparation, an improved procedure for the same reaction on a similar diol was published [10], involving the use of 4 Å molecular sieves, which gave lactone **2** in a yield of 33%, after 71 h reaction at 22°C and with an e.e. of 92%. Recently, only one report has appeared in literature on δ-lactonisation catalysed by PPL in Et_2O using 3 Å molecular sieves for the synthesis of a chiral intermediate for the multi-step synthesis of a tetrahydrolipstatin synthon [11].

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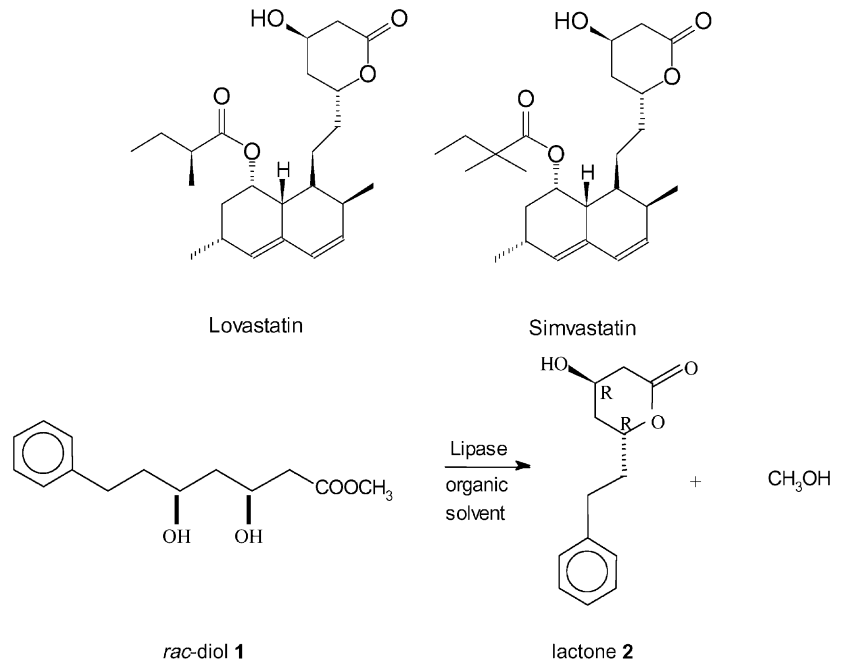

Scheme 1.

Since there is considerable industrial interest in the production of **2**, we tried to rationalise the process and to suggest further improvements in terms of yield and selectivity. A systematic study of biocatalysed[2] intermolecular transesterification of model compound **1**, was carried out: the reaction was tested in several organic solvents, at different temperatures and water content and with and without the addition of solid matrices.

The study of the reaction kinetics is also reported here.

2. Experimental

2.1. Enzyme and reagents

Crude porcine pancreatic lipase (PPL EC 3.1.1.2 type II, 11.4 U/mg solid) was obtained from Sigma Chemical Co. and used without further purification.

All the solvents (Aldrich) were redistilled and dried before use. All other chemicals used in this work were of analytical grade and were used as received.

The *syn*-3,5-dihydroxy-7-phenyl methyl eptanoate (compounds **1**) was synthesised according to [12–15]. Compound **1**: white solid; mp 33–34°C; ^{1}H NMR 1.4–1.6 (m, 2H), 1.6–1.8 (m, 2H), 2.38 (dd, CH_2CO, 2H), 2.5–2.8 (m, CH_2Ph), 3.56 (s, OCH_3, 3H), 3.73 (m, CHOH, 1H), 4.18 (m, CHOH, 1H), 4.4 (bs, OH, 2H), 7.0–7.2 ppm (m, 5H); IR (OH) 3500, (C=O) 1728 cm^{-1}.

Rac-6-(ethyl-2-phenyl)-4-hydroxytetrahydropyran-2-one (compound **2**) was synthesised from **1** in CH_2Cl_2 by acid catalysis (*p*-toluene sulphonic acid). Compound **2**: white solid; mp 74–76°C; $[\alpha]_D = +45^\circ$C (c = 0.22 g/100 ml) [8]; for the spectroscopic and analytical properties, see literature in [12–15].

2.2. Determination of water content

The water content of enzyme preparations and reaction media was measured with a Dosimat 665

[2] We have also used as catalysts *Candida rugosa* lipase and *Pseudomonas cepacea* lipase in different organic solvents with yields lower than 10%.

and Ti-Stand 703 (Metrohm Ltd., Switzerland) at room temperature. The measurement principle of the apparatus is the Karl–Fisher method. The initial water content of PPL was 2.5% (w/w) and solvent water content was from 0.005 to 0.03% (w/w).

2.3. General procedure for enzymatic lactonisation reaction

The transesterification reactions were carried out in duplicate in screw capped vessels in which all reagents were added in appropriate ratios. The racemic compound **1** (0.5 mmol) was added to 2 ml of organic solvent followed by the enzyme (PPL 200 mg). The sample was incubated in a thermostatic bath under magnetic stirring at 100 rpm, together with the respective control (sample with no enzyme). No reaction took place in the absence of enzyme. During the reaction we proceeded to take samples for analysis (50 μl diluted to 1 ml in isopropanol).

After 168 h the reaction reached plateau (40% of the conversion) and was finally stopped by filtering off the enzyme. Each reaction was repeated at least in duplicate and the data shown are the average of the experiments.

2.4. Analysis of substrates and products

Reaction products were analysed by HPLC, with a UV detector at 254 nm wavelength, employing a chiral column Chiracel OD-H (Diacel), with hexane/2-propanol (85:15, v/v) as eluent at a flow rate of 1.5 ml/min. The retention times of the two *syn*-diasteroisomeric diols were 18.8 and 33.9 min and the retention time of the lactone was 6.7 min. The quantitative lactone yield was determined on the basis of the amount of unreacted substrate and formed product by the percent area method, using a peak area integration on-line software. In order to calculate the enantiomeric excess (e.e.) of the product, the lactone was recovered from the first column elution and analysed by HPLC employing a chiral column Chiracel OB (Diacel), with hexane/2-propanol (80:20, v/v) as eluent at a flow rate of 1 ml/min. The retention times of the *R*,*R* and *S*,*S*-enantiomers were, respectively, 7.7 and 9.2 min. The quantitative lactone e.e. was determined by Chen et al. [16].

3. Results and discussion

The biocatalysed lactonisation reaction of racemic *syn*-diol **1** in organic solvent (diethylether) was carried out by using lipases from different sources (bacterial, fungal and mammalian), in particular lipases from *Pseudomonas cepacea* (PCL), *Candida rugosa* (CRL) and porcine pancreas (PPL). The reaction rates, optimised for lipase concentration, were very low in the conditions employed; the use of PPL as catalyst yielded 25% (e.e. >98%) of 2 after 168 h reaction time. The absolute configuration of the reaction product **2** was proved to be the 3*R*,5*R*-lactone as reported above in materials and methods and according to literature data [8].

The influence of the reaction temperature was also studied between 23 and 50°C. With respect to the reaction rate, enzyme stability (data not reported) and selectivity, 40°C was found to be optimal (Table 1).

The possibility of modulating the activity and selectivity of enzymes in anhydrous solvents with different physico-chemical properties is now well documented and in some cases valid correlations with enzyme performance have been found [17]. Taking this into account, different organic solvents were chosen on the basis of substrate and product solubility and employed as reaction media for the PPL catalysed lactonisation reaction of **1**. This study used the following solvents: THF, toluene, $CHCl_3$, CH_3CN, Et_2O, $N(Et)_3$. The results (Table 2) show the higher activity in toluene (35% yield) while in Et_2O (25% yield) we obtained the best enantioselectivity (>98% e.e.). No correlation between enzyme activity and solvent physico-chemical parameters (dielectric constant, solvent density, $\log P_{ow}$, solubility parameter) was found.

Table 1
Effect of temperature on lipase-catalysed lactonisation reaction[a]

Temperature (°C)	Yield[b] (%)	e.e. (%)	V_i[c] (μmol/min)
23	20	n.d.	1.6
30	42	>98	2.5
40	50	>98	2.5
50	52	73	1.9

[a] Reaction conditions: solvent Et_2O, PPL 200 mg, substrate concentration 0.05 M, water content 0.03%, reaction volume 2 ml.
[b] Reaction time 168 h.
[c] Calculated from kinetic curve according to [20].

Table 2
Lipase-catalysed lactonisation reaction in different organic solvents[a]

Solvents	C (%)[b]	e.e.$_p$ (%)	V_i[c] (μmol/min)
$CHCl_3$[d]	14	n.d.	2.1
CH_3CN[d]	22	n.d.	2.1
THF[d]	18	n.d.	2.2
Et_2O[d]	25	>98	2.5
Et_2O[e]	20	n.d.	1.9
$N(Et)_3$[d]	16	n.d.	2.2
Toluene[d]	35	88	3.1

[a] Reaction conditions: PPL 200 mg, substrate concentration 0.08 M, temperature 23°C, reaction volume 2 ml.
[b] Reaction time 168 h.
[c] Calculated from kinetic curve according to [20].
[d] 0.005% (w/w) water content.
[e] 0.03% (w/w) water content.

A further objective of this study was to investigate the effect of unreactive solid matrices on the yield and selectivity of the lactonisation reaction. It has been suggested that the effect of solid matrices on enzyme activity could be ascribed to different factors [18]. If the main function of solid matrices is to capture water, their positive effect on catalytic efficiency can be related to the decrease of hydrolytic reverse reaction, the reduction of enzyme particle agglomeration, the prevention of enzyme inactivation, the modulation of substrate partition between the different phases (organic solvent, water and solid matrices). Moreover, the use of inert solid matrices can lead to an improvement in the physical dispersion of enzyme particles in the reaction medium [18].

On this basis we tried to rationalise the influence of solid matrices on the yield and selectivity of the lactonisation reaction employing molecular sieves as powder (3 Å, particle 2–3 μm) or as beads (3 Å, particle of 8–12 mesh) and silica gel. Data reported in Table 3 show a positive effect of molecular sieves on enzymatic yield, while no substantial effect was observed in the presence of silica gel.

Moreover, since the water content of the commercial PPL preparations is 2.5% (w/w), in Et_2O is 0.03% (w/w) and the solubility of water in the solvent is 1.26% (w/w) it seems that most of the water present is expected to be associated with the enzyme and that the presence of molecular sieves can improve the water repartition equilibrium versus the bulk system.

Table 3
Influence of different solid matrices on the yield and selectivity of lipase lactonisation reaction[a]

	Yield (%)	e.e. (%)
Without solid matrices	25[b]	>98
	20[c]	n.d.
Molecular sieves as beads	50[b]	>98
	50[c]	>98
Molecular sieves as powder	50[b]	>98
Silica gel	25[b]	>98

[a] Reaction conditions: PPL 200 mg, substrate concentration 0.08 M, temperature 23°C, reaction volume 2 ml, reaction time 168 h.
[b] 0.005% water content.
[c] 0.03% water content.

On this basis, the rate improvement in the presence of porous matrices can be explained in terms of their entrapment power which reduces water content in the reaction system, especially on enzyme particles.

In addition, no hydrolysis of the product lactone was detected in the experimental conditions employed, both in the presence and the absence of solid matrices. Thus, the positive effect of molecular sieves cannot be ascribed to a reduction of collateral hydrolysis equilibria related to water presence.

Moreover, we considered immobilisation and/or adsorption phenomena of the enzyme on the matrices. In fact, ether solution (reaction medium) and solid matrices showed lipolytic activity, when recovered from the reaction mixture and separately tested (Table 4).

The best results (50% yield and >98% e.e.) were obtained using PPL at 40°C in Et_2O or using PPL at 23°C in Et_2O in the presence of molecular sieves (beads or powder).

Finally, the kinetic mechanism of the transesterification reactions was studied in ether solution at 40°C. The linear correlation between the reciprocals

Table 4
Recovery lipolytic activity from both ether solution and solid matrices in the reaction mixture

Solution	Lipolytic activity[a] (μeq. acid/min)
Aqueous solution	9.48
Ether solution[b]	5.03
Solid matrix[b]	4.22

[a] Tributyrin hydrolysis at 23°C.
[b] Reaction mixture.

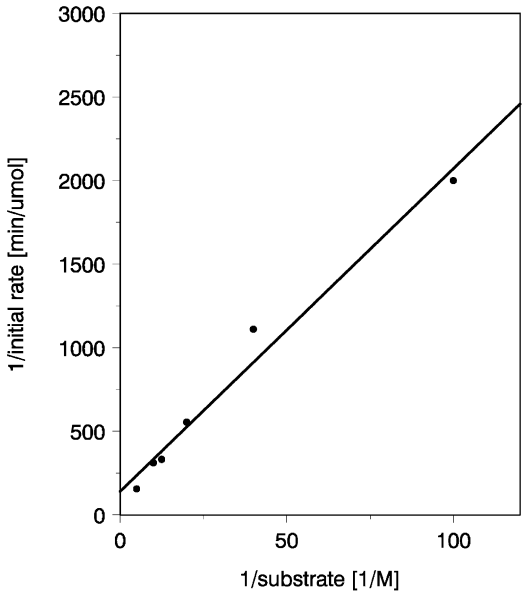

Fig. 1. The linear correlation between the reciprocals of initial rates and ester concentrations.

of initial rates and ester diol concentrations was then obtained and it suggested a Ping-Pong Bi-Bi model (Fig. 1), as is usual for lipase biocatalysed reactions [19]. According to this model the rate equation is

$$v = \frac{V_{\max} A}{K_M + A}$$

where A is the molar concentration of compound **1**. The kinetic constants were calculated as follows: $V_{\max}$ (maximum rate) $= 7.1\,\mu$mol/min; K_M (Michaelis–Menten constant) $= 137\,000\,\mu$M.

4. Conclusion

The PPL catalysed the lactonisation reaction of the *syn*-3,5-dihydroxy-7-phenyl methyl eptanoate to afford the lactone **2** with (5*R*) configuration. The activity and selectivity of the reaction can be modulated by the use of different reaction media, with different water contents, and also by the use of solid matrices. The results indicate that the major role of the molecular sieves in enhancing the catalytic efficiency of lipases in organic solvents could be ascribed to the water entrapment which causes an effective reduction of lipase agglomeration in the reaction system. Moreover, we think that the agglomeration of enzyme particles could be influenced by the surface properties of the enzyme, by its water content and also by the polarity of the solvent and substrate.

In terms of application, the use of molecular sieves can be considered a very attractive method to enhance the activity of free enzymes in organic media especially in the study of hydrolysis reaction in organic media where water acts as reactant.

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