Increase in catalytic activity of *Pseudomonas fluorescens* lipase upon its coprecipitation with hexadecane-1,2-diol suspension

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A simple method for the noncovalent modification of *Pseudomonas fluorescens* lipase resulting in an increase in its catalytic activity in aqueous and nonaqueous media was suggested.

Key words: lipase, hexadecane-1,2-diol, immobilization, ester synthesis

The use of immobilized enzymes is of considerable promise for fine organic synthesis because of the selectivity and mild conditions of the corresponding biocatalytic reactions.[1—4] Lipases (EC 3.1.1.3) occupy a specific place in the class of hydrolytic enzymes, as their activation occurs on the phase interface.[5,6] Lipases are used in the reactions of stereo- and site-specific hydrolysis of lipids and esters and also in the stereospecific esterification of alcohols in anhydrous or almost anhydrous media, when the equilibrium is shifted to the formation of the ester bond.[7—10]

Currently methods for immobilization of these enzymes by their application on hydrophobic sorbents from aqueous solutions[11—13] or by their incorporation into hydrophobic gels[14] are being intensely studied. It is known that modification of lipases with hydrophobic compounds in organic media in some cases results in an increase in their catalytic activity and enantiospecificity.[14,15] However, up to now the most effective methods for lipase immobilization and modification remain rather laborious.[14] In particular, they include treating the enzyme with lipid-like reagents, the synthesis of which is an independent complicated problem.[15]

We suggest here a simple method for the incorporation of *Pseudomonas fluorescens* lipase into hexadecane-1,2-diol (HDD) precipitates resulting in the increase of hydrolytic and, what is more important, esterifying activity of the enzyme.

Experimental

A commercially available preparation of *Pseudomonas fluorescens* lipase (PFL) with a specific activity for triacetin of 1200 U g^{-1} of enzyme preparation (U is μmol of AcOH min^{-1}) was purchased from Röhm Pharma Polymers (Germany). We also used Bu^{t}OMe, vinyl acetate, (*RS*)-**1**-phenylethanol ((*RS*)-**1**), and hexadecane-1,2-diol (Fluka Chemie AG, Switzerland); triacetylglycerol (triacetin) (Merck, Germany); and acetone, inorganic salts, and NaOH of reagent grade (Reakhim, Russia).

Lipase incorporation into HDD precipitates. *Samples 1—3.* A lipase preparation (0.2 g, protein content 0.5 mg) was dissolved in 10 mL of 0.01 *M* phosphate buffer solution (KH_2PO_4, pH 7.5) at 0 °C and separated from the insoluble impurities by filtration through filter paper with 1-μm pores. Hexadecane-1,2-diol (20, 40, and 60 mg for samples 1—3, respectively) was dissolved in acetone (0.7 mL) and added dropwise to the lipase solution on stirring with a magnetic stirrer. The resulting suspension was concentrated *in vacuo* to the starting volume to remove acetone and kept for 24 h at 6 °C. The solid phase was separated by centrifugation (8000 rpm) for 20 min, freeze-dried, and stored at 6 °C.

Sample 4. A lipase preparation (1 g, protein content 2.5 mg) was dissolved in 50 mL of 0.01 *M* phosphate buffer solution (KH_2PO_4, pH 7.5) at 0 °C and separated from insoluble impurities by filtration. Hexadecane-1,2-diol (100 mg) was dissolved in acetone (2 mL) and added dropwise to the lipase solution on stirring with a magnetic stirrer. The mixture was stirred for 5 min, then the catalyst was separated and dried as described for samples 1—3.

The immobilization efficiency (E) was estimated from the protein content in the precipitate calculated from the residual hydrolytic activity of the enzyme measured in the supernatant:

$$E\ (\%) = \left(1 - \frac{A_s}{A_0}\right) \cdot 100.$$

Here A_s and A_0 are the hydrolytic activity (U) of lipase in the supernatant and the initial activity of the lipase, respectively.

The determination of the enzyme hydrolytic activity. Triacetin (2 mL) was dissolved in 100 mL of NaCl (0.05 mol L^{-1}) and $CaCl_2$ (0.05 mol L^{-1}) aqueous solution, pH 7.0. The resulting substrate solution (5 mL) was placed in the cell of a Radiometer Copenhagen TTT80 titrator, and 50 μL of the enzyme solution or 0.5 mg of the dry precipitate was added. Acetic acid generated in the enzymatic reaction was titrated with 0.01 *M* NaOH to pH 7.0 in 10—15 min. The enzyme hydrolytic activity was calculated from the titration results in units (U, μmol AcOH min^{-1}) or in mmol h^{-1}.

The determination of the esterifying activity of the enzyme preparations (Scheme 1). The precipitate (sample 4, 2 mg, protein content = 19 μg) or 28 mg of the initial lipase prepara-

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tion (protein content 70 μg) was placed in an Erlenmeyer flask with 20 mL of Bu^{t}OMe containing 10 mmol of racemic substrate (*RS*)-**1** and 30 mmol of vinyl acetate. The suspension was shaken at a rate of 200 oscillations min^{-1} at 21—22 °C. Aliquots (0.5 mL) were sampled from the reaction mixture, the solid particles of the catalyst were separated by centrifugation, and the supernatant was analyzed by a FRAPTOVAR Series 4160 (Carlo Erba Strumentazione, Italy) instrument using a column with OV-1701. The retention times for (*RS*)-**1** and (*R*)-1-phenylethyl acetate ((*R*)-**2**) were 8.3 and 10.9 min, respectively, at a temperature gradient of 120—150 deg per 15 min. The product was identified by comparison with the retention times (t_R) of the individual substances. The amount of the reaction product (*R*)-**2** and the initial rates of the enzymatic reaction were determined according to the reported procedure.[10]

The determination of the reaction enantiospecificity. The esterification product (*R*)-**2** was isolated from the reaction mixture by reversed phase HPLC fractionation on a LiChroCART C18 250-4 (Merck, Germany) column in MeCN—water (1 : 1) with spectrophotometric detection (260 nm). Optical rotation was measured on a Digital Polarimeter DIP-360 (JASCO, Japan) instrument at 589 nm (Na D-line). The retention time t_R of (*R*)-**2** was 13.5 min. The concentration of (*R*)-**2** solution (0.02 mol L^{-1} in 1 : 1 MeCN—water) was determined by spectrophotometry at 260 nm (ε = 80 mol^{-1} L cm^{-1}).

Results and Discussion

The immobilization efficiency and the hydrolytic activity of the enzyme. The distribution of the enzymatic activity between the PFL/HDD precipitate and the supernatant is characterized by a concentration of more than 65% of the enzymatic activity in the solid phase (Table 1). The increase of the HDD amount in the precipitate results in the increase of the immobilization efficiency but causes the sharp drop of the enzymatic activity of immobilizates. The incorporation of nonprotein components of the lipase preparation into the precipitate can be inferred from the increase of its mass (220 mg of the precipitate is generated when 100 mg of HDD is used). The hydrolytic activity of the precipitate prepared under optimum conditions (samples 1 and 4) exceeds 1.2—1.5-fold the activity of the native lipase, which is evidence of some activation of the enzyme as a result of its coprecipitation with HDD.

Scheme 1

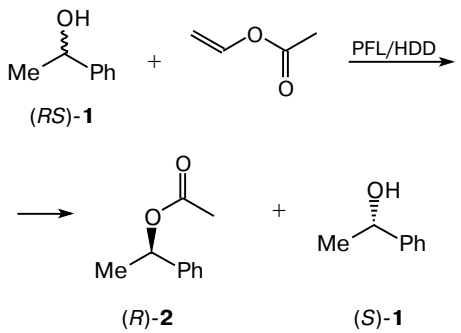

Table 1. The hydrolytic activity of the PFL/HDD precipitate (A_p) and of the supernatant (A_s)

Sample	A_s*	A_p*	E**
1	35	115	65
2	15	23	85
3	9	6	91
4	15	107	85

Note: The initial activity (A_0) of the PFL solution is 24 U mL^{-1}. * The values of activity are given in percent of the initial activity. ** E is the immobilization efficiency (%).

Esterifying activity. The activation of the coprecipitated enzyme becomes especially obvious if the catalyst obtained is used for the esterification of alcohol (*RS*)-**1** in the organic solvent. Various methods of PFL immobilization and its use as a catalyst of the enantiospecific esterification of alcohol (*RS*)-**1** with vinyl acetate have been previously studied.[13] The same reaction was also used for the study of the catalytic activity of the PFL/HDD precipitate obtained in the present study (see Scheme 1; Table. 1, sample 4). The enzymatic activity of the initial PFL and the PFL/HDD precipitate (for sample 4) measured in this study is given below:

Activity	PFL	PFL/HDD
Hydrolytic /mmol of AcOH mol^{-1} (mg of protein)$^{-1}$	6.4	8.1
Esterifying /mmol (*R*)-**2** h^{-1} (mg of protein)$^{-1}$	2.1	22.4

The esterifying activity of the PFL/HDD precipitate is 10.7-fold higher than that of the initial enzyme preparation, the reaction being started immediately after the catalyst addition to the reaction mixture, whereas the incubation period is observed in some cases when using commercial lipase preparations (Fig. 1). Interestingly, the esterifying activity of the immobilized PFL exceeds its hydrolytic activity in contrast to the initial enzyme preparation.

The enantiodirection of the reaction. The comparison of the optical activity of (*R*)-**2** samples obtained by the acylation of alcohol (*RS*)-**1** up to 42.5—43% substrate conversion in the presence of the native PFL (sample A)

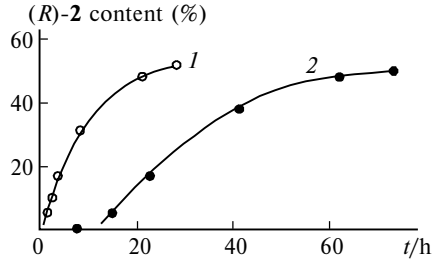

Fig. 1. The kinetic curves of (*R*)-**2** generation in the esterification catalyzed with (*1*) the PFL/HDD precipitate at the protein content of 19 μg and (*2*) the initial lipase at the protein content of 70 μg.

or its immobilized form PFL/HDD (sample B) showed that the reaction enantiodirection is not changed after the lipase immobilization and its enantioselectivity in both variants is practically the same. Thus, a solution of sample A is characterized with $[\alpha]_D$ 80.4 ($MeCN—H_2O$, 1:1), and a solution of sample B with the same concentration is characterized with $[\alpha]_D$ 80.0 ($MeCN—H_2O$, 1:1).

In the course of the reaction, the catalytic acetylation of racemic HDD with vinyl acetate accompanied by the gradual transfer of the reaction product to the solution cannot be ruled out. The control experiment without (*RS*)-**1** was found to result in the formation of the product absorbing at 210 nm and not absorbing at 260 nm. As the retention of the latter product (t_R = 14.1 min) in the HPLC regime (see the Experimental section) is noticeably stronger than that of (*R*)-**2**, there is no extraneous contribution to the optical activity of the chromatographic fraction of (*R*)-**2**.

The 7.5-fold increase in the catalytic activity of the lipase from *Candida cylindracea* has been observed previously on treating this enzyme with synthetic lipid-like reagents obtained on the basis of gluconateglutamic acid dodecyl ester.[15] For lipase incorporation into the hydrophobic precipitates, we have chosen HDD, which is characterized by a similar hydrophobic—hydrophilic balance, namely, contains the long-chain aliphatic radical and the diol moiety, but it is much more available and cheap. From the data obtained, it seems likely that hydrophobic coprecipitators of the type mentioned above have a similar effect on the lipase activation. The PFL/HDD precipitate that we obtained is insoluble in organic solvents (Bu^tOMe, hexane, benzene, acetone, etc.). This can be used for its separation from the reaction mixture and reuse in the chemical synthesis. It should be noted that suspending the precipitate in Bu^tOMe results in a fine suspension, whose separation from the reaction mixture is a technically complicated problem. Freeze-dried samples of the PFL/HDD precipitate retained ~95% of their catalytic activity for esterification on storage for more than 18 months at 6 °C.

Thus, we have suggested a new method for lipase immobilization by incorporation into hexadecane-1,2-diol precipitates and have defined the conditions for protein incorporation into hydrophobic precipitates, which permits us to increase the hydrolytic and esterifying activity of the enzyme by 1.14 and ~10 times, respectively, while retaining the enantiodirection of the reaction.

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