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Enantioselective hydrolysis of racemic esters using pig liver esterase

Jean-Michel Herdan *, Margareta Balulescu, Ovidiu Cira

Research Institute for Petroleum Processing and Petrochemistry, ICERP S.A., 291 A Republicii Bd., RO 2000 Ploiesti, Romania

Abstract

The utilization of enzymes is a recent achievement in organic synthesis. There are many good reasons for the organic chemist to consider this approach. Enzymatic reactions are very fast and specific. The same transformations carried out by classical means in several days or weeks, can be achieved in hours or days using enzymes as catalysts. Enzymes act in mild conditions with high yields, and do not require sophisticated equipment. As compared with conventional catalysts, enzymes have an excellent reaction, substrate and stereo chemical specificity. Many enzymes are now commercially available and their prices are not prohibitive. Moreover, the enzymes can be immobilized through simple techniques on inert supports. The immobilized enzymes can be easily recovered from the reaction medium and reused many times.

Enantioselective synthesis is an important area in which enzymes found large applications. Hydrolases, as lipase and pig liver esterase (PLE), have been successfully used to obtain optically pure alcohols, acids and esters. In this paper we report a new method for the immobilization of PLE on modified inorganic substrates and some applications of the immobilized PLE in synthesis of enantiomeric mono esters of dicarboxylic acids. As inorganic support we used silica with particle size of 40–80 mesh and average pore diameter of 550 Å. The surface isoelectric point of this material is relatively low. The support was derivatized with *p*-phenylenediamine and functionalized by diazotization to reaction with protein. The enzyme was covalently bonded to the support by reaction between diazonium salt and phenolic group of tyrosine residues. The immobilization yield was higher than 50% and the preparation was stored for one year at 4°C without significant loss of activity. Kinetic studies on immobilized PLE emphasized that Michaelis constant K_M was very close to that of free enzyme, that means that no conformational changes in active site occurred during immobilization.

Dimethyl 2-methyl-2-phenylmalonate was hydrolysed using immobilized PLE with slightly higher enatiomeric excess than reported earlier. The immobilized PLE can be removed from the reaction mixture by simple filtration and reused to hydrolyse other quantities of substrate without significant loss of enantioselectivity and with moderate reduction in chemical yield. Attempts to hydrolyse diethyl (3,5-di-*tert*-butyl-4-hydroxybenzyl)-malonate and ethyl (3,5-di-*tert*-butyl-4-hydroxybenzyl)cyanoacetate lead to complex mixtures of products that must be further investigated.

Keywords: Immobilized enzymes; Pig liver esterase; Enantioselectivity; Hydrolysis

1. Introduction

Enzymes have caused a significant change in organic synthesis. They are becoming increasingly important because of the multitude of

* Corresponding author. Tel.: (+40-44)135111; fax: (+40-44)117841.

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benefits emerging from using enzymes instead of chemical methods. For industry the future is more interesting because of the low cost of materials and simple procedures. We can mention only several advantages of using enzymes in organic synthesis:

1. Enzyme catalyzed reactions occur in mild conditions and results can be achieved in days, as compared with chemical reactions that usually required weeks or months.
2. Enzymes are commercially available and the possibility of immobilization makes them even more cost effective.
3. The equipment used in these processes is less complicated.
4. Enzymes are less harmful to the environment than traditional catalysts.
5. The high degree of stereoselectivity and of regioselectivity makes easier the preparation of chiral compounds, some of them virtually impossible to obtain by non-enzymic means.

Usually, enzymes are water soluble and that makes them too expensive to be used on large scale. If enzymes are to be used on an industrial scale, they must be reusable and so immobilized enzymes have been developed. An immobilized enzyme is unable to diffuse freely through reaction mixture because it is attached physically or chemically to an insoluble support or enclosed within a membrane. It can be used as a column packing or in stirred reactors without the need for separation process more elaborate than filtration.

Enantioselective synthesis of biologically active compounds is an important application where enzymes proved to be very useful. Chemical methods for obtaining pure chiral compounds are complicated and expensive and require special conditions. Hydrolases, as lipase and pig liver esterase (PLE) have been successfully used to obtain optically pure alcohols, acids and esters.

Pig liver esterase (EC 3.1.1.1) has been extensively studied in recent years. PLE has the advantage of a good stability, it is commercially available at relatively low cost ($0.005/U), requires no cofactors and can be used in aqueous or organic solvent media. It is a serine hydrolase and operates on a wide range of substrates with high stereoselectivity (relative substrate group specificity). Commercially available PLE preparations are mixtures of at least six isoenzymes that were found to exhibit essentially the same stereospecificity [1]. The enantioselectivity of PLE is due to the conformation of its active site. Recently, an active site model of PLE has been reported that can help to interpret its structural specificity and stereoselectivity [2]. According to this model, the substrate molecule is trapped in a tridimensional conformation consisting of two hydrophobic pockets and two polar pockets. The ester group to be hydrolyzed is located in nucleophilic region of the serine.

More than one hundred different esters, mainly meso- and prochiral diesters as malonates, glutarates, and also β-lactones were subjected to PLE treatment so far [3].

In this paper we report a new method for the immobilization of PLE on modified inorganic support. We also present some applications of the immobilized PLE in synthesis of enantiomeric pure malonates.

2. Experimental

2.1. Immobilization of PLE on modified silica

2.1.1. Preparation of PLE acetone powder

The PLE was obtained from fresh pig liver (still warm). Small pieces of pig liver were homogenized at 4°C with precooled acetone in a blender for 2 min. The mixtures were filtered and the residues were further washed with cold acetone to remove the fatty material. The acetone powder was dried at 4°C and the fibrous material was removed by sieving. About 200 g of fine powder was obtained from 1 kg pig liver. The enzyme activity and recovery yield were determined.

2.1.2. Derivatization of inorganic support

As inorganic carrier we used silica with particle size of 40–80 mesh and average pore diameter of 550 Å. This support had a low surface isoelectric point (less than 7), this being required to enhance the formation of strong electrostatic bonds between the acidic inorganic surface and the basic organic amine.

The first step of derivatization was the adsorption of *p*-phenylenediamine on silica in alcoholic medium. 30 g *p*-phenylenadiamine was dissolved in 300 ml ethanol and mixed with 20 g silica 30 min at 60°C. The excess of amine was removed and the amino groups were modified by diazotization (reaction with 2 N HCl and 4 N $NaNO_2$ at −4°C). The mixture was filtered and washed thoroughly with water.

2.1.3. Covalent binding of PLE to modified silica

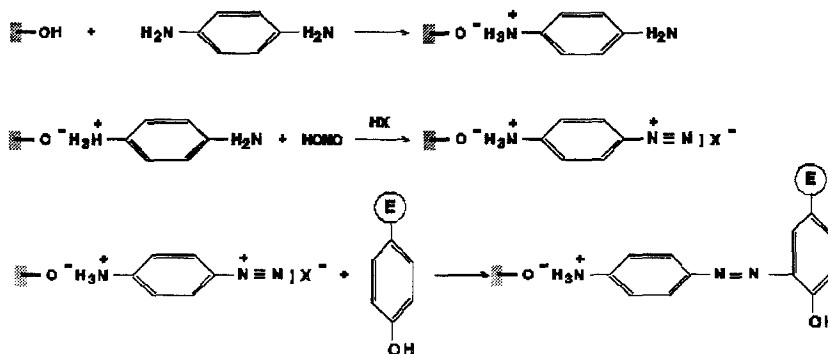
The immobilization of PLE was performed by mixing 20 g derivatized silica with 40 ml PLE acetone powder in phosphate buffer (pH 8.5) at 0°C for 2 h. The final preparation was filtered and washed with 0.5 M NaCl and water and stored in refrigerator under water. The immobilization yield was determined. In Scheme 1 the reactions involved in immobilization of PLE are presented.

Enzyme activity was determined spectrophotometrically as absorption at 540 nm. One unit hydrolyzed 0.01% β-naphthylacetate to β-naphthol in the presence of Fast Blue BB salt per minute at pH 8.0 at 25°C.

Kinetic parameters for free and immobilized enzyme were determined and compared. Several concentrations of substrate (β-naphthylacetate) were hydrolyzed with same concentration of enzyme (enzyme being in excess, according to Michaelis–Menton kinetic) and enzyme activities were determined. Michaelis constant (K_M) and maximum rate (V_{max}) was calculated as intersection with axes in Lineweaver–Burk plots.

2.2. Enantioselective hydrolysis with immobilized PLE

In order to assess the ability of immobilized PLE to catalyze enantioselectively, we used the dimethyl 2-methyl-2-phenylmalonate as substrate. Toone and Jones [2] found that this substrate is hydrolyzed by PLE to methyl hydrogen(+)-(*R*)-2-methyl-2-phenylmalonate with 90% chemical yield and 81% enantiomeric excess. The rotation angle $[\alpha]_D^{25}$ is +9.7 in $CHCl_3$. Hydrolyses with immobilized PLE were conducted using the basic procedure of Tone and Jones except we performed the reaction in phosphate buffer pH 8.0. The pH decrease was registered daily and the reaction was stopped when pH remained constant for 24 h. Enantiomeric excess was determined polarimetrically. The immobilized enzyme was separated



Scheme 1. Immobilization of PLE.

from the reaction mixture by filtration, thoroughly washed with diethylether and distilled water and reused in further experiments.

3. Results and discussions

3.1. Immobilization of PLE

For the first step of immobilization procedure, i.e., adsorption of amine on silica, we checked the efficiency by titration of three samples prepared with different concentrations of *p*-phenylenediamine solutions. Results are presented in Table 1.

As it is shown in the table, the best ratio bound amine per g support was obtained with 10 g% solution that was used in the next step.

The immobilization yield was calculated as measure of specific activity retained on the support. Enzyme activities of the crude liver extract, acetone powder and immobilized PLE were determined and the results are presented in Table 2.

Data shown in the Table 2 emphasize that PLE was immobilized without a significant loss of activity, meaning that the tyrosine residues that are supposed to bind covalently to diazonium salt of the silica, are not in the active site of PLE. Even after 1 year at 4°C under water the preparation still exhibits 20% of activity.

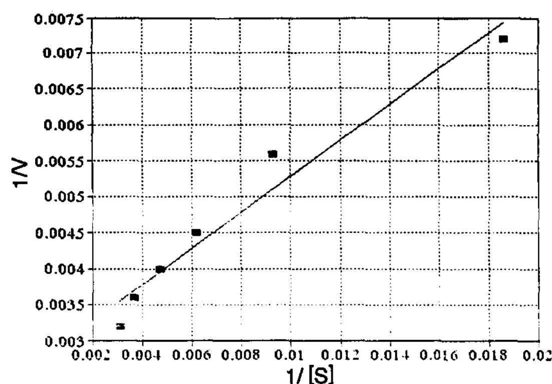


Fig. 1. Lineweaver–Burk plot for free PLE.

Further investigations were made on PLE acetone powder and immobilized preparation for determination of kinetic parameters (K_M and V_{max}). Values for K_M of 2.2×10^{-3} M for PLE acetone powder and 1.3×10^{-3} M for immobilized PLE led to the conclusion that the enzyme kept its affinity for the substrate after immobilization. Lineweaver–Burk type plots for free and immobilized PLE are presented in Figs. 1 and 2.

Table 1
Determination of amine adsorbed on silica

Sample	Solution of amine, g/100 ml	Nitrogen content, %	Amine bound, mequiv./g support
1	1	0.29	0.21
2	5	0.33	0.23
3	10	0.46	0.33

Table 2
Results of PLE extraction and purification

	Crude liver extract	PLE acetone powder	Immobilized PLE
Protein concentration, mg/g sample	1.27	0.01	0.16
Enzyme activity, U/g sample	23	20.4	19.1
Specific activity, U/mg protein/g sample	18.11	217.32	113
Extraction factor (fold)	–	12	–
Immobilization yield, %	–	–	52

Table 3
Hydrolysis with immobilized PLE

PLE, g/g substrate	Enzymic activity, U	Reaction time, h	Chemical yield, %	Enantiomeric excess, %
4	76.4	168	84	86
6	114.6	120	82	85
2	38.2	168	70	82
1	19.1	168	40	82

Table 4
Reuse of immobilized PLE on subsequent batches of substrate

Batch no.	Enzyme activity, U	Reaction time, h	Chemical yield, %	Enantiomeric excess, %
1	76.4	168	84	86
2	73.2	168	79	82
3	64.1	168	68	76
4	22.4	168	34	72

3.2. *Behavior of immobilized PLE in hydrolysis of dimethyl-2-methyl-2-phenylmalonate*

As shown above, the immobilization of PLE does not affect hydrolase activity of the enzyme. Obviously, the most important property of PLE is to enantioselectively catalyze the hydrolyses of diesters and related compounds.

It is known that dimethyl 2-methyl-2-phenylmalonate is hydrolyzed by free PLE to methyl hydrogen 2-methyl-2-phenylmalonate with an enantiomeric excess of 81%. The results of hydrolysis of the same substrate with immobilized PLE are summarized in Table 3. The enantiomeric excess was slightly higher than expected for free enzyme and the chemical yield was lower.

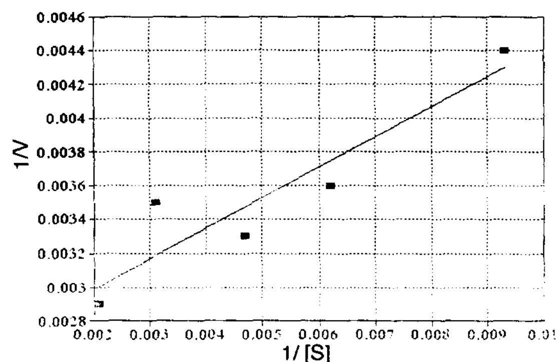


Fig. 2. Lineweaver plot for immobilized PLE.

The immobilized PLE was removed from reaction mixture and reused with another quantity of substrate without significant loss of enantioselectivity and enzyme activity, as shown in Table 4.

It was the authors' intention to use immobilized PLE in enatioselective hydrolysis of diethyl (3,5-di-*tert*-butyl-4-hydroxybenzyl)malonate and ethyl (3,5-di-*tert*-butyl-4-hydroxybenzyl)cyanoacetate, compounds which may be of high interest in cancer chemoprevention. Using these substrates in PLE catalysed hydrolysys, complex mixtures of products were obtained that require further investigations.

4. Conclusions

1. Crude PLE as acetone powder prepared from fresh pig liver was immobilized on derivatized silica with an immobilization yield of 52%.
2. The immobilization procedure does not af-

fect the enzymic activity and the enantioselectivity.

3. The preparation can be easily removed from the reaction mixture and reused several times without significant loss of activity and enantioselectivity.

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