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Journal of Molecular Catalysis B: Enzymatic 11 (2001) 757–763

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

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Enantioselective enzymatic hydrolysis of racemic glycidyl esters by using immobilized porcine pancreas lipase with improved catalytic properties

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Dedicated to Prof. Pietro Borgna on the occasion of his 65th birthday.

Abstract

The crude porcine pancreas lipase (PPL) extract is a mixture of several proteins (mainly lipases and esterases). In order to develop enzymatic catalysts with good catalytic properties for hydrolytic enantioselective reactions in aqueous homogeneous medium, we studied the immobilization of the different enzymes contained in the crude PPL extracts by selective sequential adsorption on hydrophobic supports bearing octyl, octadecyl and phenyl groups. Some minor proteins were selectively adsorbed on octyl and octadecyl supports while the most abundant lipase was adsorbed on the support bearing phenyl groups. The enantioselectivity of the different lipase derivatives were tested considering the hydrolysis of esters of 1,2-epoxi-1-propanol (glycidol). The most abundant lipase contained in the commercial crude PPL extract resulted almost inactive while some lipases contained in low concentrations displayed high activities and enantioselectivities. The most interesting results were obtained with a 28-kDa protein selectively adsorbed on octyl-agarose. With this enzyme derivative, the residual butyric ester of glycidol was recovered with 96% enantiomeric excess at 55% conversion.

Keywords: Lipases; Immobilization; Hydrophobic adsorption; Glycidyl butyrate; Enantioselective hydrolyses

1. Introduction

The resolution of raceme mixtures is an important target in pharmaceutical chemistry. In this context, the use of lipases for the "racemic switch" of chiral compounds has been reported as an important tool for the preparation of enantiopure chiral drugs [1].

Most lipase-catalyzed biotransformations reported in literature are carried out by using high concentrations of non-purified soluble lipases in organic medium or water. For example, the enzymatic resolution of 1,2-epoxi-1-propanol (glycidol), a useful

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1381-1177/01/$ - see front matter
PII: S1381-1177(00)00168-5

chiral synthon for several drugs [2], was carried out by Ladner and Whitesides using crude porcine pancreas lipase (PPL) extract through hydrolysis of racemic esters [3]. This process is now performed in a multitonne scale [4]. Free PPL provides a good enantioselectivity in the presence of over-saturated aqueous substrate solutions [5]. The hydrolysis of glycidyl esters in 25–30% yield with a 65% enantiomeric excess has also been reported when the lipase from *Rhizomucor miehei* was used [6].

The main problem in developing processes using free lipases is related to the catalytic mechanism of these enzymes. Molecules of free lipases in macroaqueous homogeneous systems mainly display low activity because their "closed inactive structure" is only in a partial equilibrium with the "open active one" [7,8]. To obtain good activities, an interfacial activation, derived from the presence of micelles or drops of insoluble substrates or unmixable organic solvents, is required. This interfacial activation occurs because the lipase is adsorbed on the hydrophobic surface of these drops through its hydrophobic external areas near to the active centre. In these conditions, the open active form of the molecule becomes much more stable and the catalytic activity improves.

Immobilized lipases can have quite different catalytic behaviours. In fact, these catalysts only act on the soluble substrate fraction, which penetrates inside the pores of the support where the lipase is present. Consequently, the drops of insoluble substrates are not in contact with the enzyme and the interfacial activation becomes impossible. In these conditions, the enzymatic activities of immobilized lipases are usually low.

Recently, the immobilization on octyl-agarose by hydrophobic adsorption was reported as a strategy for obtaining the simultaneous purification and hyperactivation of several microbial lipases [9]. Good activities were obtained with this immobilization support because when the lipases were adsorbed on the hydrophobic solid surface, the open active form

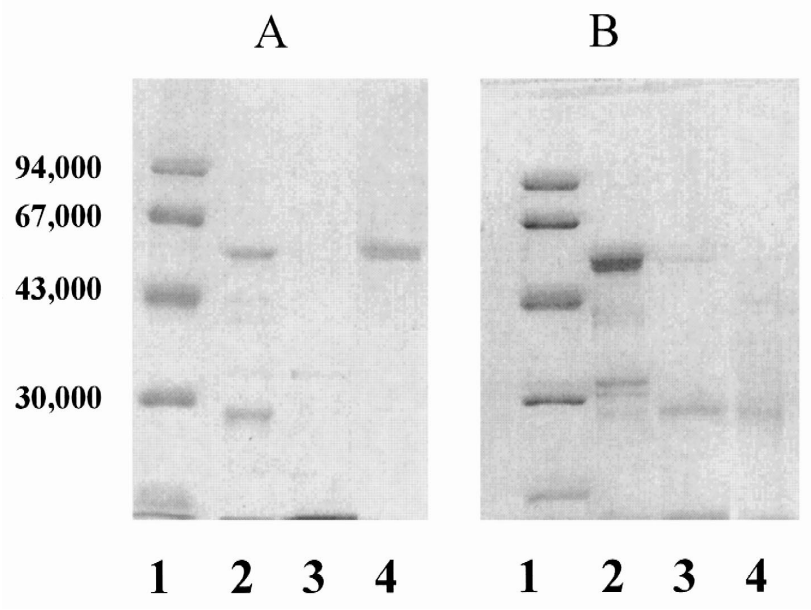
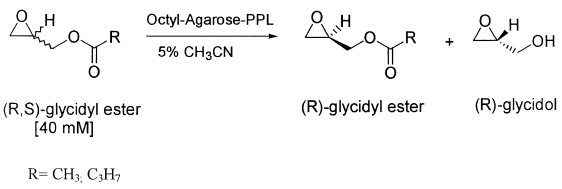


Fig. 1. Analysis of the adsorption of lipase extracts on solid supports bearing different hydrophobic groups by SDS-PAGE. Lane 1: Molecular weight markers. **Panel A:** Lane 2: Fraction of the extract from PPL which became adsorbed on octyl-agarose at 25°C. Lane 3: Fraction adsorbed on octadecyl-polymethacrilate at 25°C. Lane 4: Fraction adsorbed on phenyl-agarose at 25°C. **Panel B:** Lane 2: Soluble crude extract of PPL. Lanes 3 and 4: Fraction of the extract from PPL which became adsorbed on octyl-agarose at 25°C and 4°C, respectively.



Scheme 1.

of the enzyme becomes stabilized even in the absence of drops of insoluble substrates or solvents [9,10].

Further problems in the use of free lipases may be due to the presence of different proteins in the crude extracts. For example, the crude PPL extract is a mixture of several proteins (mainly lipases and esterases). The isolation and the study of some protein fractions demonstrated that the 33-kDa lipase is almost inactive in the hydrolysis of glycidyl butyrate [5].

A recent paper reported that the adsorption on different hydrophobic supports could be used for the separation of mixtures containing different lipases [11]. In the present work, we have used a similar approach for obtaining the simultaneous immobilization and separation of the different enzymes contained in the crude PPL extracts through selective sequential adsorption on hydrophobic supports bearing octyl, octadecyl and phenyl groups. The different enzyme derivatives prepared have been studied in order to select catalysts with remarkable properties (i.e. good activity and enantioselectivity) towards water-soluble substrates in homogeneous aqueous medium. The enantioselective hydrolysis of glycidyl esters was also studied comparing the different immobilized enzymes.

2. Results and discussion

2.1. Selective immobilization of different fractions of crude PPL extract

In attempt to fractionate and study the different enzymes contained in the commercial extract of PPL, a sequential interfacial adsorption on different hydrophobic supports has been studied. The porcine pancreas extract was, in fact, sequentially offered to three supports bearing different hydrophobic residues (octyl, octadecyl and phenyl).

Fig. 1 shows the SDS-PAGE analyses of the proteins adsorbed. The first incubation of the extracts with octyl-agarose at 25°C allowed the immobilization of only a part of the most abundant PPL (52 kDa) and a minor protein with a molecular weight of about 28 kDa (Fig. 1A, lane 2). The incubation of the supernatant with octadecyl-polymethacrilate (Sepabeads FP-RPOD) gave the selective immobilization of another protein of about 33 kDa (Fig. 1A, lane 3). The last incubation of the supernatant with phenyl-agarose allowed the immobilization of the most abundant PPL (Fig. 1A, lane 4). In order to obtain a higher selectivity for the immobilization of the 28-kDa protein on octyl-agarose, the incubation was performed at 4°C (Fig. 1B, lane 4). At 4°C, the most abundant enzyme in the crude extract was not adsorbed on octyl-agarose, showing an opposite behaviour from microbial lipases that are selectively immobilized on this support by hydrophobic adsorption [9].

Thus, by selecting the most adequate support and immobilization conditions, it has been possible to obtain three different enzyme derivatives: the 28-kDa

Table 1
Enantioselectivity of the different lipase derivatives toward glycidyl butyrate (40 mM)

Entry	Lipase derivative	Temperature (°C)	Activity (U/g) (%)	Conversion excess (%, R)	Enantiomeric	*E*
1	PPL-C8	4	3.2	55	96[a]	31
2	PPL-C18	4	1.1	54	90[a]	22
3	PPL-Ph	25	0.03	45	59[b]	6.2

[a] Enantiomeric excess evaluated on the residual glycidyl butyrate by chiral HPLC analysis.
[b] Enantiomeric excess evaluated on the obtained glycidol by chiral GC analysis.

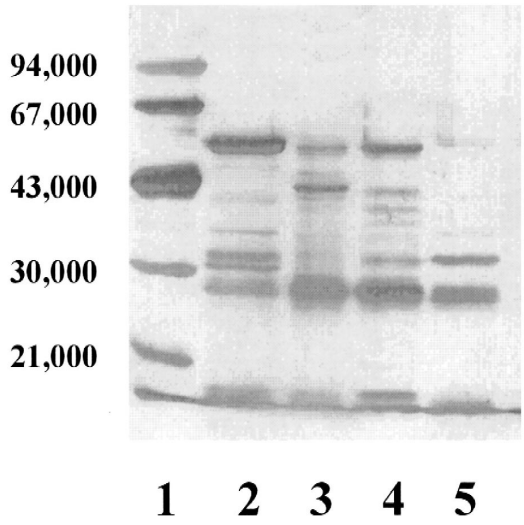


Fig. 2. Analysis of the adsorption of lipase extracts on solid supports bearing different hydrophobic groups by SDS-PAGE revealed by silver nitrate staining. Lane 2: Soluble crude extract of PPL . Lanes 3 and 4: Fraction of the extract from PPL which became adsorbed on octyl-agarose at 25°C and 4°C, respectively. Lane 5: Fraction adsorbed on octadecyl-polymethacrilate at 4°C.

protein adsorbed on octyl-agarose (PPL-C8), the 33-kDa protein adsorbed on octadecyl-polymethacrilate support (PPL-C18) and the most abundant lipase (52 kDa) adsorbed on phenyl-agarose (PPL-Ph).

2.2. Study of the enantioselectivity of different PPL enzymatic derivatives

All the different enzyme derivatives have been tested in the hydrolysis of racemic esters of glycidol (Scheme 1). At first, the enantioselectivity of the different fractions of PPL were compared in the hydrolysis of glycidyl butyrate (Table 1). The PPL-C8 derivative (entry 1) showed a very good enantioselectivity at 4°C ($E = 31$). In contrast, the PPL-Ph (entry 3) displayed a very low activity and very poor enantioselectivity. The activity of PPL-C18 (entry 2) was lower with respect to the PPL-C8 derivative (about 30%), while the enantioselectivity ($E = 22$) was similar.

These results seem to indicate that the most abundant protein contained in the crude extract of porcine pancreas, adsorbed onto phenyl hydrophobic support, is poorly active and enantioselective towards glycidyl esters. In contrast, a minor lipase of the crude extract is much more active and enantioselective. In particular, the 28-kDa protein selectively adsorbed on octyl-agarose seems to be the most adequate catalyst for the resolution of glycidol. The PPL-C18, which contains the 33-kDa protein, showed a good activity and enantioselectivity similar to the PPL-C8 derivative, although the isolation and the study of this fraction, reported in a previous work, demonstrated that the 33-kDa lipase is inactive in the hydrolysis of glycidyl butyrate [5]. This result is not surprising if the SDS-PAGE analysis of the proteins desorbed from the PPL-C18 and stained with silver nitrate (Fig. 2, lane 5) is considered. The high sensitivity obtained with this stain allows, in fact, the detection of a small amount of the 28-kDa lipase to which the catalytic properties of the PPL-C18 derivative could be ascribed.

To study the effect of the temperature on enantioselectivity, the hydrolyses of racemic glycidyl acetate and butyrate were evaluated at 25°C and 4°C (Table 2). The best enantioselectivity was observed at 4°C (entries 2 and 4) for both substrates. Furthermore, the enantioselectivity resulted higher for glycidyl butyrate (entries 3 and 4) with respect to

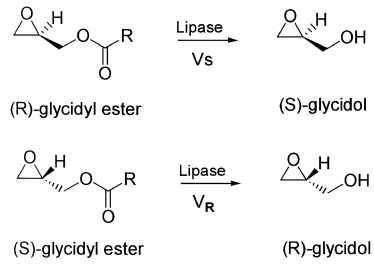
Table 2
Enantioselectivity of PPL-C8 derivatives toward glycidyl esters (40 mM)

Entry	R	Temperature (°C)	Activity (U/g)	Conversion (%)	Enantiomeric excess (%, R)	*E*
1	CH_3	25	6.8	42	56[a]	5.2
2	CH_3	4	3.3	44	69[a]	9.3
3	C_3H_7	25	8.9	54	83[a]	14
4	C_3H_7	4	3.2	55	96[b]	31

[a] Enantiomeric excess evaluated on the obtained glycidol by chiral GC analysis.
[b] Enantiomeric excess evaluated on the residual glycidyl butyrate by chiral HPLC analysis.

glycidyl acetate (entries 1 and 2). For example, in the hydrolysis of glycidyl butyrate at 4°C, the PPL-C8 derivative gave an E value of 31, while in the hydrolysis of glycidyl acetate, an E value of 9 was obtained. Thus, the PPL fraction immobilized on octyl-agarose resulted very active in the hydrolysis of racemic glycidyl butyrate even in mild conditions (4°C) and in the presence of fully soluble substrates, affording *R*-glycidyl butyrate in a very high enantiomeric excess (e.e. 96%) at the 55% of conversion.

The enantioselectivity obtained with the best enzyme derivative (PPL-C8) was compared with that of the crude PPL extract (Fig. 3). In this case, the enantioselectivities were evaluated by separately measuring the initial hydrolysis rate on each enantiomer of glycidyl acetate (Scheme 2). The enantioselectivity values were expressed as the ratio between the hydrolysis rates of the acetates derived from the *R* and *S* alcohol. The hydrolytic reactions were performed in fully aqueous medium with low substrate concentrations (5 mM), in order to ensure the correspondence between the E value measured and the effective enantioselectivity of the enzymatic catalysts. In this test, an enzyme derivative with very low protein charge (1 mg/g) and a free lipase at low concentration (1 mg/ml) have been used in order to avoid problems of substrate diffusion and protein–protein aggregation, respectively. The influence of the temperature on the enantioselectivity was also evaluated.



Scheme 2.

The PPL-C8 showed an improved enantioselectivity (Fig. 3) and activity (results not shown) with respect to the corresponding free lipase. The highest difference was observed at a low temperature, being the enantioselectivity of the immobilized enzyme ($E = 20$) at least 20-fold higher than the corresponding unimmobilized enzyme ($E = 1$). Good activities for PPL-C8 were also obtained both at 25°C (2.6 U/g) and 4°C (1.2 U/g) despite of the low substrate concentrations used (5 mmol). These results indicate that for this PPL fraction, good activity towards soluble substrates in homogeneous aqueous medium may be obtained by hydrophobic adsorption on solid support bearing octyl moieties.

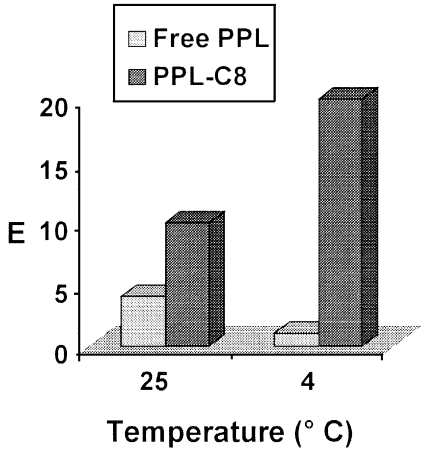


Fig. 3. Enantioselectivity of crude PPL (1 mg/ml) and PPL-C8 derivative (1 mg of protein/g of support) toward *R*- and *S*-glycidyl acetate (5 mmol).

3. Conclusion

In this work, the simultaneous immobilization and separation of the different proteins contained in commercial extract of porcine pancreas has been presented. The most abundant lipases contained in the commercial crude PPL extract resulted almost inactive and poorly enantioselective. In contrast, a minor fraction mainly composed by a protein of 28 kDa, showed the highest activity and enantioselectivity in the hydrolysis of glycidyl esters.

The immobilization of this PPL fraction on octyl-agarose gave an enzyme derivative with improved catalytic properties for the hydrolysis of raceme esters of glycidol in homogeneous aqueous medium in comparison with the free enzyme. Furthermore, this enzyme derivative displays enantioselectivity and ac-

tivity comparable with those previously observed using the free PPL in the presence of over-saturated concentration of substrates [3,5]. Probably, the interaction between the hydrophobic support surface and the enzyme produced a similar effect to that induced by the interaction between the free lipase and drops of insoluble substrates.

The good activity obtained with interfacial adsorption on octyl-agarose also suggests that this enzyme is a pancreatic lipase distinct from the most abundant proteins contained in the crude extract. Further studies are in progress in order to obtain a better separation and purification of the 28-kDa lipase to be used for significant biotransformations.

4. Experimentals

4.1. Materials

Lipases (E.C. 3.1.1.3) from porcine pancreas PPL was from Sigma. Octyl-agarose (sepharose CL-4B) and phenyl-agarose (phenyl-sepharose) were obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). Octadecyl-polymethacrilate (Sepabeads FP-RPOD) was from Resindion (Milano, Italy). Molecular weight markers (14,400–94,000 Da) for electrophoresis and Coomassie brilliant blue were purchased from Amersham Pharmacia Biotech. All other reagents were analytical grade. The pHs of the solutions during the enzymatic hydrolysis were kept constant by using an automatic titrator 718 Stat Tritino from Metrohm (Herisau, Switzerland).

4.2. Sequential adsorption of the different fraction of PPL crude extract on hydrophobic supports

4.2.1. Immobilization of the 28-kDa lipase on octyl-agarose

In 10-mM (500-ml) phosphate buffer, 4 mg of protein/g of support were dissolved at 25°C or 4°C. Then, 5 g of octyl-agarose were added and the mixture stirred for 30 min at pH 7. After the immobilization, the enzyme derivative was recovered by filtration and washed with water, while supernatant was used for the successive immobilization.

4.2.2. Immobilization of the 33-kDa lipase on octadecyl-polymethacrilate

To the supernatant derived from the immobilization of the crude PPL on octyl-agarose, 5 g of octadecyl-polymethacrilate was added and the mixture was stirred for 24 h at 25C° or 4°C. After filtration, the immobilized derivative was washed with water and the supernatant was used for the successive immobilization.

4.2.3. Immobilizazion of PPL on phenyl-agarose

To the supernatant derived from the previous immobilization of octadecyl-polymethacrilate, 5 g of phenyl-agarose was added and the mixture was stirred for 24 h at room temperature. After filtration, the immobilized derivative was washed with water.

4.3. SDS-PAGE analysis

The initial soluble crude extract and the enzymes desorbed from supports after boiling in SDS were analyzed by SDS-PAGE as previously reported [11] and stained with Coomassie or with silver nitrate [12].

4.4. Enzymatic hydrolysis of glycidyl esters

A suitable amount of the enzyme derivatives prepared with the different lipases was added to 20 ml of the substrate solution, at the desired concentration, in 10-mM phosphate buffer. The reaction mixture was maintained under magnetic stirring (200 rpm) at the desired temperature and at pH 7. During the reaction, the pH was kept constant by automatic titration and the hydrolytic activities were evaluated by measuring the initial hydrolysis rate (μmol/min $\times$ ml of enzyme derivative) calculated from the NaOH (50 mM) consumption.

In the hydrolysis, *R,S*-glycidyl esters (40 mM) in 5% CH_3CN medium were used. The reactions were monitored by HPLC analysis and the enantiomeric excess determined both by HPLC and GC analyses. The enantioselectivity *E* was calculated by the equation reported by Chen et al. [13]. HPLC (Merck-Hitachi L-7100) analyses of glycidyl butyrate for monitoring the conversion degree were carried out

on the aqueous phase. The column was a LiChroCART 250-4 RP select-B (Merck, Darmstad, Germany) and analysis was run at 25°C by using an L-7300 column oven. Eluent: 30% acetonitrile in water, flow rate 1.0 ml/min, UV detector L-7400 at 220 nm (acetate and butyrate, RT = 3.4 and 8.5 min, respectively).

The enantiomeric composition of glycidyl butyrate after hydrolysis was determined on the organic phase obtained by extracting 0.4 ml of aqueous phase with hexane (4×0.2 ml). Chromatographic conditions were: column Chiracel OD (Dyacell) maintained at 20°C; eluent: hexane/isopropanol 98:2, flow rate 0.8 ml/min, UV detector at 220 nm, *R*-glycidyl butyrate RT = 9.2 min, *S*-glycidyl butyrate RT = 10.0 min.

The enantioselectivity of the enzymatic reactions were also evaluated by gas chromatographic analysis performed on the glycidol produced during the reaction. GC (Varian 3800) analyses were carried out on organic phase obtained by extraction of 0.4 ml of aqueous phase with ethyl acetate (4×0.2 ml). The enantiomeric composition of glycidol was determined on a Cyclosilb 112-6632 chiral column (30 m, 0.25 mm, I&J Scientific). Chromatographic conditions were: injector temperature 230°C, initial oven temperature 60°C for 3 min to 76°C (1°C/min) and then to 240°C (25°C/min). Flame Ionization Detector was maintained at 200°C. Gas carrier: Helio (1.5 ml/min). *R*-glycidol RT = 9.8 min; *S*-glycidol RT = 10.1 min.

Acknowledgements

MURST (Progetti di ricerca di rilevante interesse nazionale-Area Chimica-Coord. Prof. Carlo Scolastico) is gratefully acknowledged. Part of the work has also been supported by CEE (BIO4-CT9-0005) and the Spanish CICYT (QUI97-0506-CO-02). We thank Ms. Paola Moro and Dr. Marta Alonso for experimental assistance.

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