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Effects of chemical modification on stereoselectivity of *Pseudomonas cepacia* lipase

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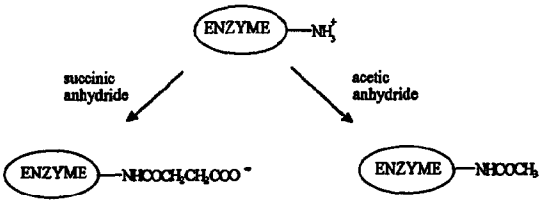
Abstract - Two chemically modified forms of lipase from *Pseudomonas cepacia* were prepared by acylation of the free amino groups of the protein with acetic and succinic anhydrides. The catalytic activity, the enantioselectivity and the thermal stability of the modified enzymes were compared with that of the native form. Succinylation determined an increase of stability without affecting the catalytical properties of the enzyme in the hydrolysis of chiral esters. Acetylation resulted in an enhanced catalytic activity coupled to a decreased stereoselectivity and thermal stability.

INTRODUCTION

In recent years many studies on remodelling enzymes have been undertaken in order to improve enzyme features or to induce new or additional catalytic functions[1]. Two main types of approach can be recognized: one utilizes the recombinant DNA technique, and the second is based on the use of group-specific chemical reagents[2]. The present paper describes the effect of the chemical modification with anhydrides on the lipase from *Pseudomonas cepacia*.

Figure 1. Protein modification using anhydrides.



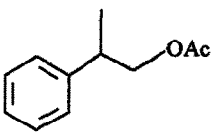
The microbial lipase Amano PS from *P. cepacia* has been selected as target enzyme because of its broad substrate specificity and because of the high stereoselectivity often displayed on chiral compounds[3]. Our aim was to understand whether a simple modification procedure was suitable for a significant improvement of the performances of such enzyme in the resolution of chiral molecules.

Acetic and succinic anhydrides were choosen as acylating agents because the small size of the introduced functions does not affect the solubility of the protein in water. Using acetic anhydride, the positive charge of the primary free amino groups of Lys was eliminated to give an uncharged acetamide (Figure 1). Using succinic anhydride, the positive charge was replaced by a negatively charged carboxylate group (Figure 1).

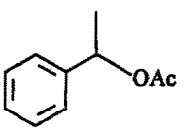
Since the anhydrides display a low chemical selectivity, the acylation of other functions, such as hydroxylic groups of Tyr, thiol groups of Cys and amino groups of His is also possible. However, the resulting esters and amides are highly unstable and rapidly hydrolyze even at neutral pH, therefore this type of modification can be neglected[4]. Ser and Thr hydroxyl groups, on the other hand, are not easily acylated in aqueous solution because they are too weak nucleophiles.

The effect of the modification on the catalytic properties of the enzymes was determined comparing the specific activity and the enantioselectivity displayed by the native and the acylated lipases in the hydrolysis of the chiral esters **1a-c** (Figure 2).

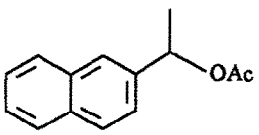
Figure 2. Lipase PS substrates



1a



1b



1c

RESULTS AND DISCUSSION

The modification with acetic anhydride was carried out at 20°C in saturated sodium acetate solution (pH 8.8) acting both as buffer and as catalyst for the acetylation reaction[5]. The modification with succinic anhydride was carried out in phosphate buffer solution maintaining the pH at 8 by addition of sodium hydroxide. The modification was carried out on commercial enzyme preparations and not on highly purified enzymes. The water-insoluble materials present in the raw lipase preparations were removed by centrifugation, before submitting the enzymes to the modification procedure.

The extent of modification was spectrophotometrically determined by titration of the residual free amino groups of the protein[6]. The number of free amino groups measured for the native enzyme was 8, close to the literature data[7]. An average of 30 % and 52 % of acylated groups were observed for the acetylation and the succinylation respectively. Electrophoresis analysis of the modified lipases in non-denaturating conditions showed the presence of various protein forms cointaining different numbers of modified groups. Nevertheless, in spite of their heterogeneous composition, the catalytic properties of different enzymatic samples were fully reproducible.

The hydrolytic reactions on the oily esters **1a-c** were carried out in phosphate buffer solution at 30° C, keeping the pH at 7 by using a pH-stat.

As shown in Table 1, the native enzyme displayed a very poor stereoselectivity on the primary ester **1a**, on the contrary the hydrolysis of the secondary esters **1b,c** proceeded with an almost complete enantioselectivity. The specific activity, expressed as initial rate, was comparable for **1a** and **1b** and was 5 times lower for the sterically hindered **1c**.

The succinylation of the lipase did not induce any significant change in both specific activity and stereoselectivity with respect to the native form.

A dramatically different behavior was instead observed with the acetylated lipase. In this case the low selectivity displayed by the native enzyme on **1a** was completely lost. The loss of stereoselectivity was confirmed by the decrease from more than 1000 to 64 of the enantiomeric ratio, (E)[8], in the hydrolysis of **1b**. Surprisingly the stereospecificity was fully retained with the naphtylic ester **1c** without any decrease in the specific activity, in spite of the larger size of this substrate. It is noteworthy that the specific activity of the acetylated lipase was higher than that of the native enzyme also in the hydrolysis of **1a,b**.

Table 1. Effect of chemical modification on the Enantioselectivity (Enantiomeric Ratio, E) and hydrolysis rate of Lipase Amano PS:

SUBSTRATE	LIPASE	INIT. RATE[b] μmol/min x mg	CONV.(%)	ALCOHOL e.e.%(CONF)	ESTER e.e.%(CONF)	E[a]
1a	native	7.3	55	16(S)	20(R)	2
1a	succinylated	7.4	55	16(S)	20(R)	2
1a	acetylated	9.9	60	0	0	1
1b	native	6.5	50	>99(R)	>99(S)	>1000
1b	succinylated	6.7	50	>99(R)	>99(S)	>1000
1b	acetylated	8.0	50	92(R)	92(S)	64
1c	native	1.3	50	>99(R)	>99(S)	>1000
1c	succinylated	1.3	50	>99(R)	>99(S)	>1000
1c	acetylated	6.8	50	>99(R)	>99(S)	>1000

(a) E values were calculated from the degree of conversion and the e.e. of the product according to Chen et al.[8]. Each value was the average of 3 E values calculated for different conversions. (b) Specific Initial Rate: μmol/min x lipase protein content (determined by the Bradford method)[9].

A possible explanation of these effects may be the increased flexibility of the protein molecule due to the introduction of non polar residues on its surface. The replacement of charged amino groups with uncharged hydrophobic residues may alter the protein-solvent and intramolecular elettrostatic interactions, with the concurrent lowering of the whole molecule rigidity. The change of the protein molecule mobility may influence the conformation of the active site allowing an easier access for all the substrates, and determining at the same time a relaxation of the enantiodiscrimination for the small-size substrates.

An indirect index of the enzyme rigidity is its thermostability. In order to support the above hypothesis the apparent inactivation constants of the native and modified lipases were calculated. The denaturation experiments were carried out in phosphate buffer (0.1 M, pH 7) at 60°C.

Since our samples were mixtures of enzymes forms with different degrees of modification, the inactivation constants calculated from the curves in Figure 3 can be considered only as average apparent constants. The curves were fitted with a single exponential function, and the calculated constants are $36 \cdot 10^{-2} \cdot hr^{-1}$, $0.18 \cdot 10^{-2} \cdot hr^{-1}$ and $57 \cdot 10^{-2} \cdot hr^{-1}$ for the native, succinylated and acetylated lipases respectively.

The expected lower stability of the acetylated enzyme experimentally confirmed the thesis of the increased flexibility of the enzyme structure.

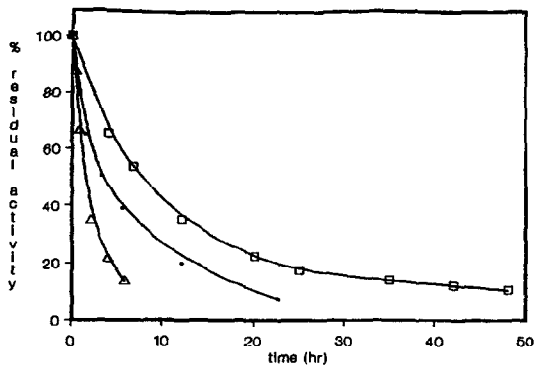


Figure 3. Stability of native and modified lipase PS.

• - native lipase; Δ - acetylated lipase; □ - succinylated lipase. The residual activity was measured on tributyrrin, after incubation of the lipases in phosphate buffer 0.1 M, pH 7 at 60° C.

The enhanced thermostability showed by the succinylated lipase was in good agreement with the literature data, reporting that the hydrophilic substitution of the protein surface groups causes a reduction of the surface area responsible for the unfavourable hydrophobic contact with water and consequently determines an increase of stability.[1e]

CONCLUSIONS

In conclusion the simple and cheap modification procedure described in this paper provided a versatile tool to manipulate the enzyme features in terms of stereoselectivity, substrate specificity and thermal stability.

The acylation technique appears to be a promising way to improve the catalytical properties of lipases in terms of stereoselectivity and protein thermostability. In fact, although an improvement of the lipase stereoselectivity is always desiderable, it is important to controll whether it is accompanied by an unfavourable change in some of the important enzyme properties, such as specific activity or thermostability. Acylation of enzyme surface may also have an effect on enzyme properties in anhydrous organic media, where, in absence of water, the enzyme conformation may be even more affected by changes in the protein-solvent interactions. The behavior of modified lipase PS in different organic solvents is currently under investigation.

EXPERIMENTAL SECTION

General: ^{1}H-NMR were recorded in $CDCl_3$ solution ($SiMe_4$ as internal standard) on a Bruker C 200 instrument. All the hydrolyses were performed with a Metrohm pH-stat. Lipase Amano PS from *Pseudomonas cepacia* (30 units mg^{-1}) was purchased from Amano Chemical Co. Optical rotations were measured with a Perkin Elmer 241 polarimeter using a 1 dm polarimeter cell. All the organic chemicals were of analytical grade.

Optical purities of the alcohols were determined by HPLC analysis, performed on a chiral column Chiralcel OB (Daicel Chemical Industries, Ltd). The eluant was hexane/propan-2-ol 98:2 (V/V), while the flow rate was 0.8 ml/min and readings were made at 220 nm.

Succinylation of lipase PS

5 g of crude lipase PS (150000 units) were dissolved in 100 ml of 0.1 M phosphate buffer, pH 8. The solution was stirred at 4 °C for 1 hour, then the insoluble material was separated by spinning down the suspension (15 minutes, 5000 rpm). 0.25 g (2.5 mmol) of solid succinic anhydride were added to the surnatant in small portions, under rapid stirring at 20 °C. The pH was kept at 8 in a pH-stat by adding 1 M sodium hydroxide solution. When the addition of succinic anhydride was complete, the mixture was allowed to react overnight. The enzymatic solution was then desalting by a dialysis against phosphate buffer (0.01 M, pH 7). Finally the resulting clear solution was lyophilized.

The extent of acylation was monitored by electrophoresis. Polyacrylammide discontinuos gel electrophoresis in non denaturating conditions was performed accorded to the method developed by Ornstein[10] and Davis[11]. The concentrations of acrylamide were 8 % in the resolving gel and 4 % in the stacking gel. The electrophoretic separations were run under constant corrent output (25 mA). The gel was stained with Coomassie Blue G-250.

The electrophoresis showed a complete modification and no native lipase was found in the lyophilized final powder.

Acetylation of lipase PS

Crude lipase PS (10 g, 300000 units) was dissolved in 200 ml of saturated sodium acetate solution stirring for 1 hour at 4 °C. The suspension was spun down (15 minutes, 5000 rpm) and the surnatant was stirred at 20 °C, slowly adding acetic anhydride (8 g, 7.8 mmol).

At the end of the addition, the pH of the solution was adjusted to 7.0 with sodium hydroxide. After dialysis against phosphate buffer (0.01 M, pH 7) the solution was lyophilized. The chemical modification of the enzyme preparation was confirmed by electrophoresis as described above.

Determination of degree of modification of lipase PS

The degree of chemical modification of the enzyme was determined by titration of the residual free amino groups of the protein with trinitrobenzene sulfonic acid[6] . The number of amino groups present in the native lipase was 9, while the NH_2 residues found for the succinylated and acetylated were 4.0 and 6.0 respectively.

Enzymatic hydrolysis of racemic 1a-c.

The following procedure is representative.

To a magnetically stirred suspension of (R,S)-1-phenylethanol acetate **1b** (0.2 g, 1.22 mmol) in 20 ml of phosphate buffer, 0.01 M, pH 7, at 30 °C was added the crude lipase PS (0.01 g, 300 units). The pH was kept constant at 7 by addition of aqueous sodium hydroxide by means of a pH-stat. Periodically aliquots (1 µl) of the liquid phase were withdrawn and analysed by HPLC. The hydrolysis was stopped at a conversion of 50 %, and the reaction mixture was extracted with ethyl acetate. The organic layer was washed with 5 % aqueous sodium hydroxide, dried with sodium sulphate and then evaporated to dryness. Chromatography on silica gel, with hexane-ethyl acetate (80:20 V/V) as eluant, afforded (R)-phenylethanol (0.07 g, 47 %); $[\alpha]^{D}_{25} = +41.0$ (neat) ; ee =99 %, δ_H:1.31-1.48 (3H, d), 2.38 (1H, s), 4.61-4.95 (1H, q), 7.30 (5H, s); and (S)-**1b** (0.092 g, 46 %); $[\alpha]^{D}_{25} = -106.0$ (c=1, ether); ee= 99%; δ_H : 1.45-1.60 (3H, d), 2.06 (3H, s), 5.71-6.08 (1H, q), 7.32 (5H, s). The optical purity of **1b** was determined after its chemical hydrolysis into the corresponding alcohol, whose e.e. was determined by HPLC.

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