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Porcine pancreatic lipase-catalized enantioselective hydrolysis of N-protected amino acid methyl-esters

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Summary. A preparative-scale enantioselective hydrolysis of racemic methyl esters of several N-protected amino acid has been carried out by using crude porcine pancreatic lipase (Triacylglycerol lipase, EC 3.1.1.3) PPL as a hydrolytic enzyme. In all cases 50% of the racemic methyl ester was hydrolysed to the N-protected L-amino acid with high yield and high optical purity.

Hydrolysis rates were very close related not only to the amino acid structure but also to the steric and/or electronic nature of the ester and N-protecting groups. Thus, the very convenient ester methyl group can be enantioselectively hydrolysed with PPL when N-protecting group is a carbonyl derivative, as it is the usual benzoyl group.

Keywords: Amino acids – Porcine pancreatic lipase – Asymetric resolution of amino acids – PPL-enantioselective methyl ester hydrolysis – PPL-resolution of amino acids

Introduction

The creation of chiral centres has become a major strategic concern in chemical synthesis, especially in drug synthesis. Thus, over the past ten years there has been a marked increase in the use of microorganisms and enzymes as catalysts (Klibanov, 1990; Wong, 1990; Yamada, 1988; Jones, 1986). In particular the synthetic value of several microbial lipases has been demonstrated (Chen, 1989). Furthermore, an inexpensive, commercially-available crude porcine pancreatic lipase (PPL: E.C. 3.1.1.3) has been used successfully as a chiral resolution agent in a wide range of reactions including the enantioselective hydrolysis of racemic alcohol esters (Cotterill, 1988), the monohydrolysis of prochiral diacetates of meso-diols (Guanti, 1989), or selective monoacylation of diols and triols (Ramasway, 1990) and several transesterification processes where not only racemic mixtures of alcohol (Kirchner, 1985; Hsu, 1990) but also oxime acetates (Ghogare, 1989) or chiral esters are resolved (Wallace, 1990).

In spite of the fact that natural substrates for lipase are triacylglycerol esters of long-chain fatty acids, only a few reports have dealt with enantioselective hydrolysis of esters from chiral carboxylic acids. Besides, in contrast to α-chymotrysin or liver esterasa, PPL hydrolyses α-substituted carboxylic esters with low chemical and optical yields (Guibé-Jampel, 1987).

In this respect, some active esters, such as 2,2,2-trifluoroethyl esters of the N-benzyloxycarbonyl derivatives of unusual amino acids (belonging to chiral α-substituted carboxylic esters), can be hydrolyzed by PPL with high enantioselectivity. However, methyl esters were not hydrolyzed (Miyazawa et al., 1989).

At the present time, according to the literature, only some proteases are able to carry out the enantioselective hydrolysis of N-protected amino acid methyl esters. Thus, chloramphenicol was synthesized through an enantioselective hydrolysis of racemic methyl N-Dichloroacetyl-β-(4-nitrophenyl) serinate by using Subtilisin Carlsberg, a type VIII protease (Chênevert, 1989). Besides, racemic t-butyloxycarbonyl-amino acid methyl esters can be resolved by asymetric hydrolysis with Thermitase (Lankiewicz, 1989). This is an alkaline serine protease from *Thermoactinomyces* characterized by a high esterase/peptidase activity ratio which previously was found to be a useful reagent in the selective stereospecific hydrolysis of C-terminal ester bonds in peptides (Hermann, 1983).

We now report on the enantioselective hydrolysis of racemic methyl esters of N-benzoyl amino acids by using PPL as a hydrolitic enzyme, according to the method previously claimed under Spanish patent (Bautista, 1989). In this respect, an efficient method for the preparative-scale enzyme hydrolysis of methyl esters of N-benzoyl-phenylalanine, N-benzoyl-phenylglycine and two other less common amino acids is described.

Material and methods

Instrumentation

Melting points were determined on a CTP mod. MP 300 capillary melting point apparatus and are uncorrected. Optical rotation was measured on a Bellingham & Stanley Ltd, mod. P20, digital polarimeter. Infrarred spectra were obtained using a Bomem, mod. MB-100 spectrophotometer. NMR studies on ^{1}H and ^{13}C were performed on a Bruker ACE 200 spectrometer at 4,6975 T in $CDCl_3$ solution using TMS as internal standard. Hydrogenation reactions were carried out in a conventional low-pressure hydrogenator (Parr Instrument Co., MD., 3911). The reaction temperature was controlled by pumping water from a thermostatic bath through the vessel jacket. An automatic titrator Crison mod. microTT 2050 was used to maintain a constant pH in the enzimatic resolution reactions. Merck silica gel 60 F_{254} analytical thin-layer chromatography plates (TLC) were used in this work.

N-Benzoylphenylglycine

Phenylglycine purchased from Merck (5 g.) was dissolved in 50 ml. of 10% sodium hydroxide solution contained in a conical flask. Then 5 ml of benzoyl chloride was added in five portions to the solution shaking after each addition until all the chloride was reacted. Finally, the solution was transfered to a beaker with a few grams of crushed ice and concentrated hydrochloric acid was slowly added until the mixture was acid to Congo red paper. Cooling in a refrigerator a precipitate was collected that after washing with carbon tetrachloride to

remove any benzoic acid and recrystallization from methanol yielded 6.3 g. (74%) of the product, mp 209–211°C.

N-Benzoyl-D,L-phenylglycine methyl ester (1E)

To a solution of 3.4 g of N-benzoylphenylglycine, previously obtained, in 50 ml of anhydrous methanol, cooled in ice, was added an ethereal solution of diazomethane in small portions until gas evolution ceases and the solution acquires a pale yellow colour. Total methylation was confirmed by TLC. Ethereal diazomethane solution was obtained from p-tolylsulphonylmethylnitrosamide (purchased from Merck) according to Vogel (1974). A precipitate that after dried yielded 2.4 g of (1E) (67%, mp 108–109°C), was obtained when the solvent was evaporated to a minor volume and cooled in a refrigerator.

Azlactones

A mixture of 67.5 g. of hippuric acid, 31.2 g. of anhydrous sodium acetate, 107.3 ml. of acetic anhydride and 0.38 mol of the corresponding carbonyl compound (39.7 ml of cyclohexanone or 39 ml of benzaldehyde) was placed in a 500 ml. conical flask and heated on an electric hot plate with constant shaking to liquefy completely the mixture. Then, the flask was heated in a water bath for 2 hours. Finally, 75 ml. of cool water (or ethanol in the case of benzaldehyde) was added to the contents of the flask, allowing the mixtures to stand overnight. The crystalline products precipitated after washed with aqueous sodium carbonate to remove benzoic acid, and recrystallized from ethanol, yield 17.2 g (19%) of 2-phenyl-4-cyclohexylidene-5-oxazolone, mp 128°C and 79.7 g (84%) of 2-phenyl-4-benzylidene-5-oxazolone, mp 167–168°C, respectively.

Methyl α-benzamido-cyclohexylidenacetate

In a 500 ml conical flask, 4.0 g of 2-Phenyl-4-cyclohexylidene-5-oxazolone and 0.8 g of anhydrous sodium acetate in 350 ml of methanol were held at reflux for 5 h. Then the solvent was evaporated to a minor volume (20 ml) and 10 ml of water was added. Cooling to 0–5°C overnight a precipitate was collected. Crystallization from methanol yielded 3.9 g (86%) of the product, mp 147–148°C.

Methyl α-benzamidocinnamate

This material was similarly prepared by refluxing 2.5 g of 2-phenyl-4-benzylidene-5-oxazolone with 0.5 g of anhydrous sodium acetate in 250 ml of methanol. After crystallization and dried 2.6 g (91%) of product, mp 142–143°C, was obtained.

N-Benzoyl-D,L-cyclohexylglycine methyl ester (2E)

To 500 ml pressure bottle were added 150 ml of methanol, 1.8 g of methyl α-benzamidocyclohexylideneacetate and 4.5 g of Ni-5132 P, a nickel supported catalyst (64 wt% Ni) from Harshaw Chemie B V. The solution after purged and filled with hydrogen was vigorously shaked in a hydrogenator at 50°C and 4.5 atm until gas uptake ceased. Time required for this hydrogenation reaction was 23 h. Then, the catalyst was filtrated and solvent evaporated at reduced pressure. The compound (2E) (mp 104°C) was quantitatively collected.

N-Benzoyl-D,L-phenylalanine methyl ester (3E)

This compound was obtained by hydrogenationof methyl α-benzamidocinnamate (2.87 g) with the same nickel catalyst and under above experimental conditions. Time required to carry out this hydrogenation reaction was 2 h. Mp of (3E) was 92–94°C.

N-Carbonylcyclohexyl-D,L-cyclohexylalanine (4E)

This compound was obtained by total hydrogenation of methyl α-benzamidocinnamate (including both aromatic rings) under above experimental conditions but using 0.5 g of a supported rhodium catalyst (5 wt % Rh) TypG 10 N/D from Degussa. Reaction was carried out in 8 h. A mp 96–98°C was obtained for (4E).

Enzymatic resolution of racemic methyl esters of N-protected amino acids

A suspension of 2 g of Lipase Type II crude from porcine pancreas, supplied by Sigma, in phosphate buffer (10 ml of 0.1 M KH_2PO_4) was added to another suspension of racemic substrate in the same phosphate buffer (10 mmol in 40 ml). Then, at 30°C constant temperature, pH was raised to 7.7 with the aid of an automatic titrator and the hydrolysis was followed by the NaOH 1 N solution volume expended to maintain this initial pH value. The reaction end point was obtained when pH was not changed for two or three hours. The unhydrolysed ester was removed from the aqueous layer by several extractions with chlorophorm. To confirm the absence of this compound in reaction mixture before the extraction of the hydrolysed amino acid it is very important to avoid its contamination. This was carried out by TLC, using diethyl ether-cyclohexane (1 : 1) as eluent system. Then, the aqueous phase was acidified with 1N HCl to pH 2, and the corresponding hydrolysed N-protected amino acid extracted with chloroform. Both the organic phases were separately dried with $MgSO_4$ and evaporated at reduced pressure to obtain the corresponding amino acid derivative enantiomers.

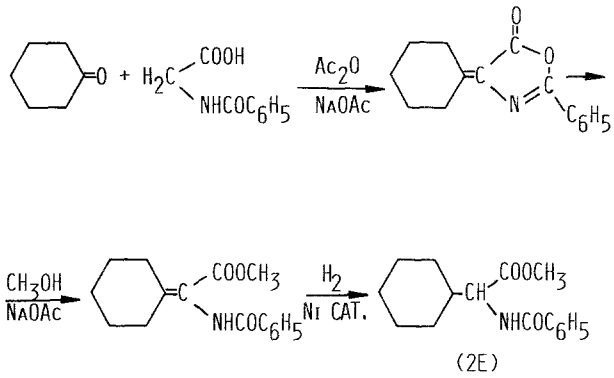
Determination of enantiomeric excess

The enantiomeric excess was determined from different unhydrolysed esters by ^1H NMR spectroscopy at 200 MHz in the presence of tris[3(2,2,2-trifluor-1-hydroxyethyliden)-d-camphoratol europium, $Eu(tfc)_3$, purchased from Merck. As solvent was used $CDCl_3$ (0.7 ml) with a substrate concentration of 0.08 M and a molar relation 3 : 1, respect to $Eu(tfc)_3$.

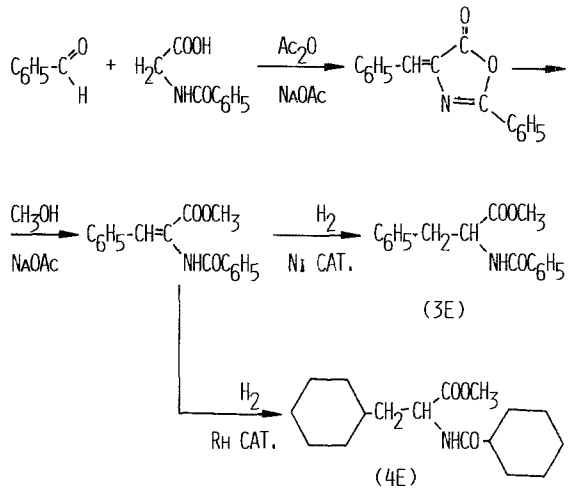
Results

Racemic N-benzoylphenylglycine methyl ester (1E) was prepared by methylation with diazomethane of the corresponding N-benzoyl-DL-phenylglycine previously obtained by standard Schotten-Bauman procedure through the reaction between benzoyl chloride and commerical racemic α-phenylglycine (Vogel, 1974).

Methyl ester racemics of N-benzoylphenylalanine and N-benzoylcyclohexylglycine were obtained from the corresponding oxazolones that were prepared according to the Erlenmeyer method by reacting hippuric acid and acetic anhydride with cyclohexanone (Ramage, 1935) (Scheme 1), or benzaldehyde (Furniss, 1989) (Scheme 2), respectively. Oxazolone rings are readily opened by reflux in methanol in the presence of sodium acetate giving the corresponding methyl esters of α-benzamido-cyclohexylidenacetic and α-benzamidocinnamic acids. Catalytic hydrogenation of both substrates carried out on a supported nickel catalyst yielded the corresponding methyl esters of N-protected amino acids cyclohexylglycine (2E) and phenylalanine (3E), respectively. By using a rhodium catalyst in the hydrogenation reaction of methyl α-benzamidocinnamate not only was saturated the olefinic double bond but also both aromatic rings,



Scheme 1



Scheme 2

so obtaining methyl ester of N-Carbonylcyclohexyl-D, L-cyclohexylalanine (4E). In all cases, infrared spectra, ^{1}H NMR and ^{13}C NMR confirmed the structures of the synthesized compounds.

The preparative-scale enzymatic hydrolysis of corresponding N-protected racemic methyl esters was carried out in a buffered aqueous solution at 30°C and pH = 7.7 by using an automatic titrator. Reaction rates were followed by the amount of NaOH 1M necessary to neutralize liberated amino acid maintaining pH at the indicated constant value. In all the cases studied, according to the results shown in Fig. 1, NaOH consumption versus time was linear. The hydrolysis rate obtained for different racemic methyl esters is collected in Table 1. The time required for hydrolysis reaction was very highly dependent on the nature of the amino acid side-chain. It varied from 6 h for the phenylalanine derivative up to 55 h for the cyclohexylglycine one.

In all cases 50% of the racemic methyl ester was hydrolysed to the N-protected L-amino acid with very high stereoselectivity. Thus, enantiomeric excess, which was determined from unhydrolysed esters by ^{1}H N.M.R. spectroscopy in the presence of $Eu(TFC)_3$, was always 100%, as can be seen in Fig. 2.

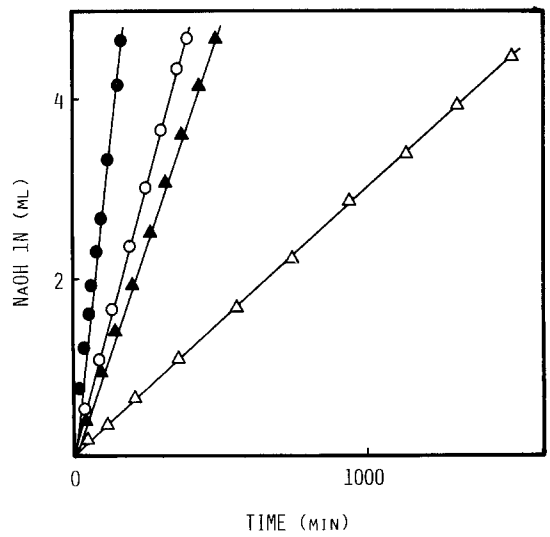


Fig. 1. NaOH 1N volume expended along the time to maintain pH at constant value in PPL-catalyzed hydrolysis of different racemic N-protected amino acid methyl esters: ● **3E**; ○ **4E**; ▲ **1E**, and △ **2E**

Table 1. PPL-catalized hydrolysis rates of different N-protected amino acid methyl esters

	Racemic methyl ester	hydrolysis rate 10^8 (mol/s g_{PPL})
1E	C_6H_5–CH(NHCO–C_6H_5)$COOCH_3$	8.0 ▲
2E	C_6H_{11}–CH(NHCO–C_6H_5)$COOCH_3$	2.5 △
3E	C_6H_5–CH_2–CH(NHCO–C_6H_5)$COOCH_3$	22.8 ●
4E	C_6H_{11}–CH_2–CH(NHCO–C_6H_{11})$COOCH_3$	10.1 ○

Both enantiomers were obtained by successive extraction with chloroform from the reaction mixture. First, the unhydrolysed D-methyl ester was directly extracted at initial pH under operation conditions. Then, the corresponding N-protected L-amino acid present in the aqueous phase as sodium salt, was obtained after acidification and extraction with chloroform. The corresponding rotatory power values of different extracted compounds as well as their characteristic spectral bands are shown in Table 2, where for every (1–4E) unhydrolysed ester, there is the corresponding (1–4A) N-protected L-amino acid obtained by hydrolysis.

Discussion

Taking into account that enantiomeric excess was always 100%, rotatory powers collected in Table 2 can be considered as the corresponding specific optical rotation values, $[\alpha]_D^{25}$, of different compounds. Thus the preferential PPL-hydrolysis of L-enantiomer can be concluded by comparing literature data on N-benzoyl-L-phenylalanine $[\alpha]_D^{25}$ values (Fryzuk, 1977; Ojima, 1980; Goodman,

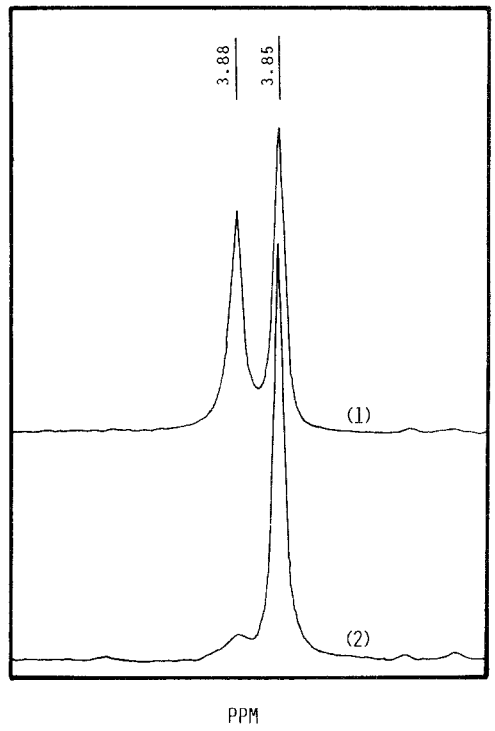


Fig. 2. (s, 3H, $COOCH_3$) Spectra of ^1H NMR at 200 MHz of **1E** compound (0.015 g) in the presence of 0.02 g of $Eu(tfc)_3$ in 0.7 ml of $CDCl_3$. *1* Racemic compound **1E**. *2* Unhydrolysed material obtained in the PPL-resolution process of racemic **1E**

1964) to the corresponding value in Table 2. These values were −40.3 and −38.7 at (c 1.0, MeOH), very close to that here obtained.

On the other hand, according to the results, PPL-catalyzed enantioselective hydrolysis of N-protected amino acid methyl esters can be considered a part of a general process shown in Scheme 3: where *R* and *R'* can be not only aromatic substituents but also aliphatic ones. However, according to the results of Miyazawa et al. (1989), when *R'* is a benzyloxy group ($-O-CH_2-C_6H_5$), the hydrolysis rate is negligible. This rate can be improved by the substituting the ester methyl group with another more active ester such as the 2,2,2-trifluoroethyl group.

Similarly, the structure of the *R* amino acid side-chain plays an important role in determining the PPL-hydrolysis rate. Thus, according to the results in Fig. 1 and Table 1, the hydrolysis rate decreases for different *R* substituents in the following order:

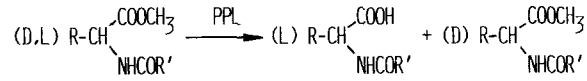
$$C_6H_5-CH_2- > C_6H_{11}-CH_2- > C_6H_5- > C_6H_{11}-$$

In this connection, aromatic sustituents are activating groups when compared to aliphatic ones. Similarly, a benzyl group is more activating than a phenyl one.

In conclusion, PPL is able to carry out ester hydrolysis of different N-protected amino acids with a very high degree of enantioselectivity. However, the hydrolysis rate is very closely related not only to the amino acid structure but also to the steric and/or electronic nature of the ester and N-protecting

Table 2. Specific optical rotation, $[\alpha]_D^{25}$, melting points, mp, and characteristic spectral bands of different amino acid derivatives obtained by PPL-catalyzed enantioselective hydrolysis

Comp.	mp (°C)	$[\alpha]_D^{25}$ (deg)	Infrarred (cm^{-1})	^1H NMR (δ, ppm)	^{13}C NMR (δ, ppm)
1E	108	−97(c, 0.05)	3283(N–H)	5.8(d, 1H, CH)	CH_3: 53
			1753(C–O)	7.5(m, 10 H arom)	CH: 57; 127–132
			1630(C=O)	3.8(s, 3H, CH_3)	C: 134, 137; 167, 172
			1530(N–H)	8.3(d, 1H, NH)	
1A	210	+90(c, 0.03)	3322(N–H)	5.7(d, 1H, CH)	CH: 55; 128–132
			1703(C=O)	7.5(m, 10 H arom)	C: 135, 140; 165, 175
			1640(C=O)	8.2(d, 1H, NH)	
			1532(N–H)		
2E	104	+5.3(c, 0.06)	3354(N–H)	4.5(dd, 1H, CH)	CH_3: 56
			1740(C=O)	7.5(m, 5H arom)	CH_2: 31, 33, 34
			1640(C=O)	3.7(s, 3H, CH_3)	CH: 56; 63; 133–137
			1516(N–H)	1.6(m, 10 H cycle)	C: 140; 172, 177
2A	179	+8.7(c, 0.03)	3293(N–H)	4.7(dd, 1H, CH)	CH_2: 26, 28, 30
			1703(C=O)	7.5(m, 5H arom)	CH: 41; 57; 127–132
			1640(C=O)	1.5(m, 10 H cycle)	C: 135; 165, 175
			1535(N–H)	6.6(d, 1H, NH)	
3E	92	+43.9(c, 0.04)	3360(N–H)	5.1(dt, 1H, CH)	CH_3: 52
			1745(C=O)	7.5(m, 10 H arom)	CH_2: 38
			1650(C=O)	3.8(s, 3H, CH_3)	CH: 54; 127–132
			1545(N–H)	3.3(t, 2H, CH_2)	C: 134, 136; 167, 172
3A	178	−38.6(c, 0.03)	3320(N–H)	5.2(dt, 1H, CH)	CH_2: 38
			1725(C=O)	7.5(m, 10 H arom)	CH: 54; 127–132
			1610(C=O)	3.1(t, 2H, CH_2)	C: 134, 137; 168, 174
			1500(N–H)	6.6(d, 1H, NH)	
4E	97	+19.7(c, 0.08)	3310(N–H)	4.7(dt, 1H, CH)	CH_3: 52
			1765(C=O)	3.7(s, 3H, CH_3)	CH_2: 26–34; 40
			1650(C=O)	2.2(t, 2H, CH_2)	CH: 34, 45; 50
			1570(N–H)	1.5(m, 22 H cycle)	C: 174, 176
4A	15	−7.8(c, 0.08)	3315(N–H)	4.6(dt, 1H, CH)	CH_2: 26–34; 40
			1740(C=O)	2.2(t, 2H, CH_2)	CH: 34, 45; 50
			1650(C=O)	1.5(m, 22 H cycle)	C: 175, 177
			1550(N–H)	6.5(d, 1H, NH)	



Scheme 3

groups. Thus, the very convenient ester methyl group can be enantioselectively hydrolysed with PPL when the N-protecting group is a carbonyl derivative, as is the usual benzoyl group. Consequently, the PPL-catalyzed enantioselective of N-protected amino acid methyl-esters, due to the low enzyme cost, can be a convenient synthetic method for preparation of both stereoisomers in many different amino acids.

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