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ENANTIOSELECTIVE HYDROLYSIS OF HYDROPHOBIC AMINO ACID DERIVATIVES BY LIPASES

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ABSTRACT : Microbial lipases preferentially cleave the L-isomers of N-benzyloxycarbonyl(Z)-hydrophobic D,L-amino acid methyl esters which is the same as that of subtilisin. This implies that lipases and proteases may share the same ancestor in the evolutionary point and lipases can be practically applied to resolve racemic hydrophobic amino acid derivatives.

INTRODUCTION

Lipases (triacylglycerol ester hydrolases, E. C. 3.1.1.3), because of their high enantioselectivity, have been widely used in the preparation of chiral synthons and optically active compounds through esterification, transesterification and hydrolysis (Ladner and Whitesides, 1984; Sonnet, 1987; Koshiro et al, 1985; Langrand et al, 1985; Kirchner et al, 1985; Sih and Wu, 1989). Recently, the tertiary structure of microbial lipases from Mucor miehei (Brady et al, 1990) and Geotrichum candidum (Schrag et al, 1991) had been refined to high resolution. It revealed that the catalytic triad of microbial lipases was Ser-His-Asp or Ser-His-Glu which were similar to those of the serine proteases such as chymotrypsin, trypsin and subtilisin. In order to compare the hydrolytic ability of lipases and proteases toward amino acid derivatives, the methyl ester of N-benzyloxy-carbonyl(Z)-hydrophobic D,L-amino acids were used as common substrates for the hydrolysis of lipases and subtilisin.

MATERIALS AND METHODS

Lipase AP6 (Aspergillus niger), lipase GC (Geotrichum candidum) and lipase PS (Pseudomonas sp) were obtained from Amano, Japan., and lipase Candida cylindracea OF was from Meito Sangyo Co., Ltd, Japan. Subtilisin (Carlsberg type VIII) was

purchased from Sigma, USA. TLC was performed on silica gel G.60 (E. Merck, FRG) precoated on a glass plate. All solvents were obtained from Alps Chem. Co., Taiwan. Chiralcel OD, Chiralpak WH and Crownpak CR (+) columns (25 cm x 4.6 mm I.D.) were products of Daicel Chemical Industries, LTD, Japan. A HPLC System of Waters Assoc.(Milford, MA, USA) was used for the analytical separations, which consisted of one M6000A solvent delivery unit and a U6K Universal liquid chromatograph injector, coupled to a M450 variable-wavelength UV spectrophotometer and HP 3394A integrator, Hewlett Packard, USA. N-benzyloxy- carbonyl(Z)-D,L-amino acids were synthesized by conventional methods and their methyl esters were prepared by diazomethane treatment.

General procedure for the resolution of Z-DL-amino acids by enzymatic hydrolysis : To 100 mg (about 0.35 mmole) of the racemic methyl ester, suspended in 2 ml of 0.2 M phosphate buffer, pH 7.1, was added 30 mg of crude lipase (3 mg of subtilisin). The reaction mixture was stirred at room temperature, and the progress of the reaction was roughly monitored by silica gel TLC with n-hexane/EtOAc/AcOH (15:5:1, by volume) as the developing system and visualized under UV lamp. Thye reaction was terminated by adjusting the pH of the solution to pH 2 with 1N HCl. The remaining ester substrate and acid product were was purified by silica gel column.

Determination of Enantiomeric Excess (ee) of remaining substrates and products

The ee of remaining substrate (ee_s) and acid product (ee_p) were analyzed on Chiralcel OD column at ambient temperature isocratically with n-hexane /isopropanol/formic acid (80:20:1, by volume) at a flow rate of 0.5 ml/min and detected at UV 254 nm or on Chiralpak WH column with 0.25 mM $CuSO_4$ aqueous solution as eluent at a flow rate of 1.0 ml/min. Some results were reconfirmed by Crownpak CR (+) column, after deprotecting Z-group by hydrogenolysis.

Calculation of E value

Based on the ee_s and ee_p, the E (enantiomeric ratio) of different enzymes to the desired substrate could be calculated based on the equation 1 and 2 (Chen et al, 1982).

$$\frac{\ln([1 - C][1 - ee_s])}{\ln([1 - C][1 + ee_s])} = E \quad \text{or} \quad \frac{\ln[1 - C(1 + ee_p)]}{\ln[1 - C(1 - ee_p)]} = E \qquad (1)$$

$$\text{conversion } (C) = \frac{ee_s}{ee_s + ee_p} \qquad (2)$$

RESULTS AND DISCUSSION

Racemic amino acids are usually resolved by three different kinds of enzymes : acylase, aminopeptidase and proteases. However, proteases and aminopeptidase have auto-digestive properties which would weaken their hydrolytic rate and resolution. Three enzymes show poor properties with water-

immiscible substrates. Although the hydrolytic rate would be enhanced by adding an aqueous-miscible organic solvent, enzymes are often unstable in aqueous-organic solution and reduce or change their hydrolytic ability. Lipases can hydrolyze aqueous-immiscible substrates in aqueous solution and have no auto-digestion properties, if not contaminated by proteases.

Four lipases and subtilisin were used to resolve the methyl ester of hydrophobic Z-D,L-amino acids. According to the results shown in Table 1., all enzymes tested cleave the L-isomers more effectively. However, compared with subtilisin, the lipases show

Table 1. Enantioselective hydrolysis of methyl esters of Z-D,L-amino acids by enzymes

racemic compounds	Enzymes[a]	reaction time (h)	ee_s (%)	ee_p (%)	C[b] (%)	E
Z-Ala-OMe	lipase PS	240	63	47	57	5
	lipase AP6	4	30	82	27	14
	lipase OF	4	22	51	30	4
	lipase GC	100	7	99	7	>100
	subtilisin	30	99	77	56	34
Z-Leu-OMe	lipase PS	240	24	84	22	15
	lipase AP6	240	21	85	20	15
	lipase OF	240	37	83	31	16
	lipase GC	120	3	100	3	>100
	subtilisin	6	97	96	50	>100
Z-Met-OMe	lipase PS	240	30	89	25	24
	lipase AP6	48	71	95	43	86
	lipase OF	120	54	74	42	12
	lipase GC	120	2	100	2	>100
	subtilisin	7	99	92	52	>100
Z-Phe-OMe	lipase PS	240	14	83	14	15
	lipase AP6	5	34	88	28	22
	lipase OF	5	92	73	56	21
	lipase GC	120	3	100	3	>100
	subtilisin	2	92	93	50	85
Z-Val-OMe	lipase PS	---	--	--	--	--[c]
	lipase AP6	200	19	99	16	>100
	lipase OF	216	7	97	7	53
	lipase GC	---	--	--	--	--[c]
	subtilisin	24	68	99	40	>100

[a] Pure lipase OF had similar results as those of crude lipase OF.
[b] C is defined as concentration of product/initial concentration of substrate.
[c] No reaction.

lower reaction rates and enantioselectivity toward the substrates; effectively, the substances are natural substrates for subtilisin, but not for lipases. Judging from the reaction rate and enantiomeric ratios (E), lipase AP6 is the most suitable for the resolution of amino acid derivatives. Lipase GC and PS have extremely low hydrolytic rate and are not suitable for these substrates, even though lipase GC shows excellent enantioselectivity. The results are quite similar to those of a previous report (Miyazawa et al, 1988) in which unusual amino acid derivatives were used as substrates. In our experiment, the enantiomeric excesses of remaining substrates and products were directly measured by chiral columns, and enantioselectivity was theoretically checked by enantiomeric ratio (E) which is recognized as the criterion of enantioselection (Wong, 1989).

In conclusion, two points deserve to be mentioned : (a) because of similar active sites and enantioselectivity, lipases and proteases probably shared the same ancestor in the evolutionary tree; (b) because of their cheapness, easy availability and stability in aqueous-organic solution, lipases can be extended to resolve practically some hydrophobic racemic amino acid derivatives instead of using proteases or acylases.

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