Resolution of Amino Acids in a Mixture of 2-Methyl-2-propanol/Water (19:1) Catalyzed by Alcalase via in Situ Racemization of One Antipode Mediated by Pyridoxal 5-Phosphate

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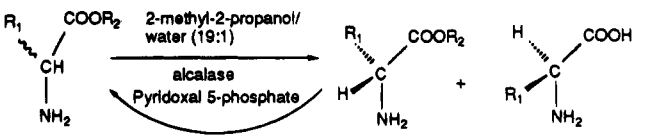
Summary: Procedures for the conversion of a racemic amino acid into the L-enantiomer by the alcalase-catalyzed resolution of the amino acid ester in 2-methyl-2-propanol/water (19:1) simultaneously with the pyridoxal 5-phosphate-catalyzed racemization of the unhydrolyzed antipode have been developed.

Proteases can be used as catalysts for peptide synthesis in organic solvents.[1,2] Resolution of amino acids in aqueous solution or in high concentrations of organic solvents catalyzed by stable proteases attracted the attention of many investigators.[3] One drawback of this reaction is recovery of unreacted antipode to increase the total yield. Here we describe a procedure that enables complete resolution of amino acid esters in a mixture of

Scheme 1



2-methyl-2-propanol/water (19:1) with high yield and optical purity via alcalase-catalyzed hydrolysis coupled with in situ racemization of unhydrolyzed substrate mediated by pyridoxal 5-phosphate. Alcalase is a proteolytic enzyme prepared from a selective strain of *Bacillus Licheniformis*. The major enzyme component in alcalase is subtilisin Carlsberg (alkaline protease A), which is a serine protease widely used as additive in detergents as a digesting enzyme. Alcalase exhibits high enzymatic activity and stability in organic solvents.[2d,4]

Scheme 1 shows the consecutive reactions of this process. The amino acid esters were hydrolyzed in a mixture of 2-methyl-2-propanol/water (19:1) at an apparent pH 8.5, and the hydrolyzed L-amino acid that was insoluble under these conditions precipitated during the course of hydrolysis. In the presence of pyridoxal 5-phosphate, the D-amino acid ester was racemized and the reaction continued until the substrate was consumed completely.

The substrate was prepared by esterification of the corresponding amino acid with alcohol catalyzed by acid (thionyl chloride or toluenesulfonic acid) according to an established method.[5] In a preliminary test, D-phenylalanine benzyl ester (D-Phe-OBzl, 1 mmol, 292 mg) and pyridoxal 5-phosphate (0.2 mmol, 125 mg) in a mixture of 2-methyl-2-propanol–water (19:1; 10 mL) was reacted at 40 °C. Racemization was followed by HPLC on a Chiral-CR column. Figure 1 shows the time course for the racemization of D-Phe-OBzl to D,L-Phe-OBzl. D-Tyr-OBzl and D-Leu-OBzl were similarly tested, and their courses of racemization appear also in Figure 1. Under the same conditions, the rates of racemization of amino acid propyl esters or amino acid butyl esters were similar. When amino acid methyl esters were used in the reaction, the methyl group was stripped and free amino acids precipitated after 5 h. Benzyl, butyl, and propyl groups of the amino acid esters are very stable during the racemization. For the conditions tested, racemization of free phenylalanine with pyridoxal 5-phosphate in aqueous solution (pH 8.5, 40 °C) was slow (<3%, 24 h), and the solubility of Phe in a mixture of 2-methyl-2-propanol/water (19:1) was negligible.

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(4). Alcalase was purchased from NOVO industrial (Denmark) as a brown liquid with a specific activity of 2.5 AU/mL (According to NOVO, one Anson-unit (AU) is the amount of enzyme which, under standard conditions, digests haemoglobin at an initial rate liberating per min an amount of trichloroacetic acid soluble product, which gives the same color of phenol reagents as 1 mequiv of tyrosine. Thus, 1 AU = 1000 U, 1 U = 1 mmol of L-Tyr-OMe hydrolyzed per min).

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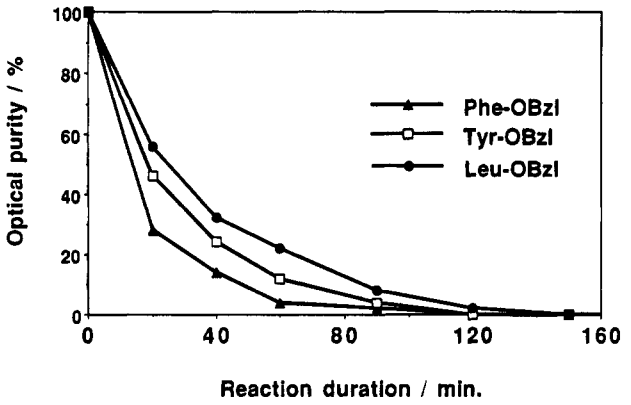

Figure 1. Racemization of D-Phe-OBzl, d-Tyr-OBzl, and D-Leu-OBzl in a mixture of 2-methyl-2-propanol/water (19:1) at 40 °C, pH 8.5, mediated with pyridoxal 5-phosphate. The decreased optical purity of the D-amino acid esters was measured on a Chiral Diacel CR(+) column.

Table 1. Alcalase-Catalyzed Resolution of Amino Acid Derivatives with in Situ Racemization Mediated by Pyridoxal 5-phosphate

substrate	product[a]	reaction time/h	yield/ %	ee/ %	$[\alpha]^{25}_D$ (c 5, 1 N HCl)	mp/°C
D,L-Phe-OBzl	L-Phe	3.5	92	98	35.4	189–191
D,L-Phe-OBun	L-Phe	4	92	98	35.4	188.5–190
D,L-Try-OBzl	L-Tyr	3	95	97	10.9	211–213
D,L-Tyr-OPron	L-Tyr	4	95	97	10.9	211–213
D,L-Leu-OBzl	L-Leu	3.5	87	93	+13.2	176–177
D,L-NorLeu-OBzl	L-NorLeu	4	87	90	+23.4	171–172
D,L-NorVal-OBzl	L-Norval	4	87	91	+24.9	177–179

[a]The structures and optical purities of all the products were confirmed by comparison with authentic samples.

A representative resolution of phenylalanine on a preparative scale was tested as follows: alcalase (10.0 mL), pyridoxal 5-phosphate (2.51 g, 4 mmol) and D,L-Phe-OBzl (5.84 g, 20 mmol) in a mixture of 2-methyl-2-propanol (190 mL)/water (10 mL) was reacted at 40 °C with the apparent pH controlled at 8.5 by addition of NaOH (5 N). L-Phe precipitated during the course of hydrolysis. After D,L-Phe-OBzl was consumed (about 4 h, HPLC), the reaction solution was chilled (ice bath, 15 min) and the precipitate collected by filtration to yield L-Phe (yield 92%, ee 98%). The ee of the product was determined with HPLC on a Chiral CR-(+) column and confirmed by optical rotation with an authentic sample. Figure 2 shows a typical chiral HPLC analysis of resolved L-Phe and L-Tyr. Several other amino acid esters were similarly resolved with high ee and yields; the results are shown in Table 1.

Comparing the resolution of D,L-Phe-OBzl with that conducted without pyridoxal 5-phosphate, the presence of pyridoxal 5-phosphate in the reaction solution does not inhibit hydrolysis with this alcalase catalyst. For all amino acids tested here, the ee based on this resolution exceed that obtained with that resolution without in situ racemizatione.[3g] Under these conditions, the ratio of both enantiomers is probably maintained at 1:1 during the resolution process, so the ee of the product in this process became increased.

The hydrolysis of hydantoins catalyzed by hydantoinase[6] and hydrolysis of oxazolin-5-ones catalyzed by the

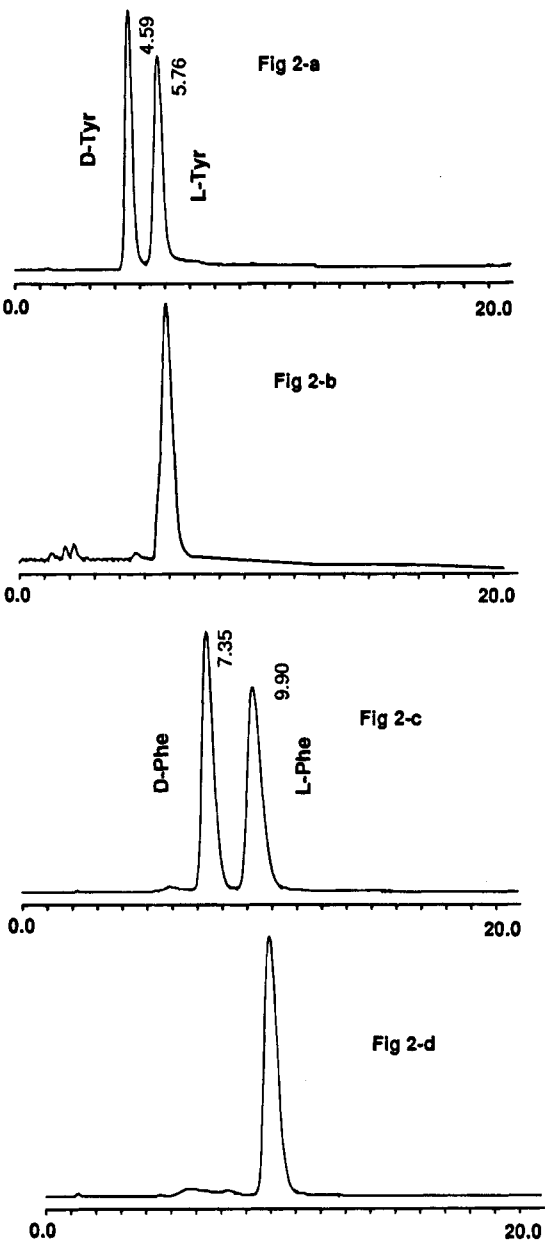

Figure 2. Optical purity of resolved L-Phe and L-Tyr measured on a CR(+) column. Eluent: aqueous perchloric acid pH 2.0 with methanol (15%). Flow rate: 0.7 mL/min. Detector: UV 200 nm. (a) Authentic D,L-Tyr. (b) Isolated L-Try. (c) Authentic D,L-Phe. (d) isolated L-Phe.

lipase or protease[3e,7] by in situ racemization of unreacted substrate were documented. One drawback of these processes is that one extra hydrolysis reaction is required to remove the N-terminal carbamoyl or benzoyl protecting group. As many enzymatic procedures to resolve chiral amino acids or derivatives in large concentration of organic solvents are known, the in situ racemization mediated with pyridoxal 5-phosphate may be useful for those purposes.

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