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Kinetic Resolution of Esters of Amino Acids in *t*-Butanol Containing 5% Water Catalyzed by a Stable Industrial Alkaline Protease

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ABSTRACT We developed a procedure for the resolution of esters of amino acids in 95% *t*-butanol, followed by saponification of the unreacted esters to afford both enantiomers with high yield and optical purity. The hydrolysis, catalyzed by alkaline protease, was conducted in a mixture of *t*-butanol (95%) and water (5%) at 25°C, with a pH controlled at pH 8.5 by the addition of NaOH (2 *M*). The hydrolyzed L-amino acid, which was insoluble under these conditions, precipitated during the course of hydrolysis. After separation of the precipitate, the pH of the filtrate was adjusted to 11.5 to saponify the unreacted ester. The D-antipode precipitated at pH 6.2–6.5. Both optically pure antipodes were obtained with high enantiomeric excesses and yields by simple filtration.

KEY WORDS: resolution, D,L-amino acid esters, alkaline protease, simple separation, high enantiomeric excess and yields

The search for proteases for peptide synthesis that are stable in organic solvents is the subject of extensive investigation.[1,2] Several studies have demonstrated the possibility of using proteases to catalyze peptide synthesis in organic solvents, but a drawback of those reactions is the poor stability of the enzyme in organic solvents. Many immobilization processes have been developed to overcome this. Here we describe the use of an inexpensive enzyme, alcalase, as a catalyst for resolution of amino acids, some of which have uncommon side chains and are found in many biologically and pharmaceutically important peptides.[3–5] Most of these unnatural amino acids cannot be obtained by fermentation or recombinant DNA technology. Resolution is an effective way to produce optically pure unnatural amino acids.

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 and which is widely used as additives in detergents as a digesting enzyme. This alcalase can maintain enzymatic activity and stability in organic solvents.[6,7] Application of alcalase in organic synthesis has been limited, although it is a potent and inexpensive catalyst.[8,9] Scheme I shows the reaction sequence of the resolution. The hydrolysis catalyzed by alkaline protease was conducted in a mixture of *t*-butanol (95%) and water (5%) at 25°C with a pH controller at pH 8.5. The hydrolyzed L-amino acid was insoluble under these conditions and precipitated during the course of hydrolysis. The precipitate was separated by filtration, and the pH of the filtrate was adjusted to 11.5 to saponify the unreacted ester of the D-amino acid. After the D-antipode was completely saponified, it was reprecipitated at pH 6.2 and isolated by filtration. Both optically pure antipodes were obtained by simple filtration.

MATERIALS AND METHODS

Alcalase was purchased from Novo industry (Denmark) as a brown liquid with a specific activity 2.5 AU ml^{-1}. It was used without further purification. The amino acids were purchased from Sigma USA. Thionyl chloride and benzyl alcohol were purchased from E. Merck, Germany. The substrates were prepared according to the established method.[10] Optical rotation was measured on a Universal Polarimeter (Schmidt & Haensch, Germany). The HPLC system consisted of two Waters model 6000 pumps, a Waters Model 450 UV detector, and an M-660 solvent programmer. The Chiral CR-(+) HPLC column was purchased from the Diacel Chemical Company (USA). A Suntex P.C. 303 Auto-pH Controller was used (Suntex Instruments Co., Taiwan). TLC was performed on silica gel G precoated plates (E. Merck, Germany). Methanol, ethyl acetate, methylene chloride, acetonitrile, dioxane, ether, and acetone (HPLC grade and reagent grade) were obtained from a local supplier (ALPS Chemical Co., Taiwan).

Enzyme Assay

Subtilisin activities were measured spectrophotometrically according to the absorbance increase at 500 nm (colored by

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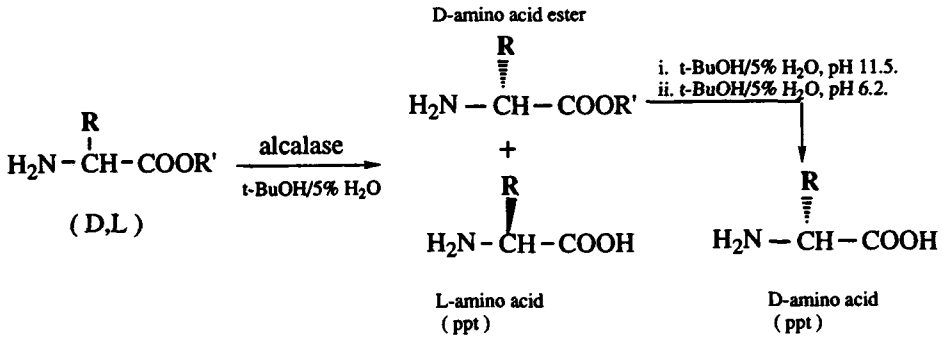


Scheme I. Simple resolution of D,L-amino acid esters catalyzed by alcalase in *t*-butanol/5% water.

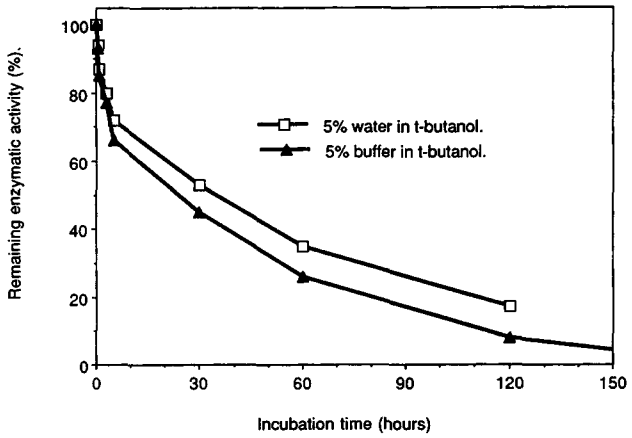


Fig. 1. Stability of alcalase in *t*-butanol containing 5% phosphate buffer or 5% water.

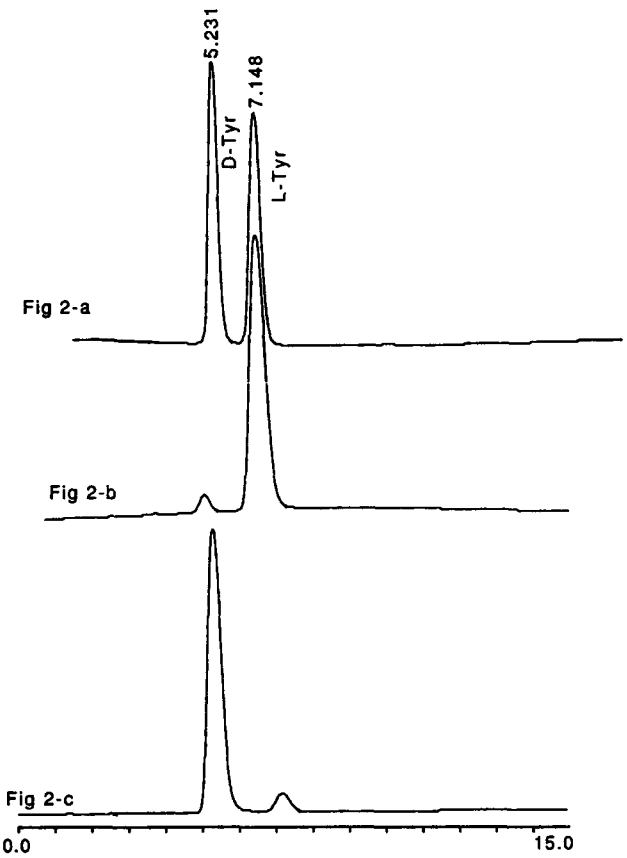


Fig. 2. Determination of enantiomeric excess of resolved tyrosine. **(a)** D,L-tyrosine standard; **(b)** isolated L-tyrosine; **(c)** isolated D-tyrosine on a Chiral CR-(+) column. Elution with aqueous $HClO_4$ (pH, 1.8), flow rate 0.8 ml/min, detected at UV 200 nm.

Folin–Ciocalteu's phenol reagent) due to release of trichloroacetic acid-soluble compounds from hydrolysis of hemoglobin. The assays were performed at pH 7.5 at 37°C in phosphate buffer. [According to Novo, one Anson-unit (AU) is the amount of enzyme that, under standard conditions, digests hemoglobin at an initial rate liberating per minute an amount of TCA-soluble product that gives the same color of phenol reagents as 1 meq of tyrosine. Thus, 1 AU = 1000 U, and 1 U = 1 mmol of L-Tyr-OMe hydrolyzed per min.]

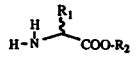
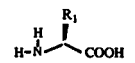
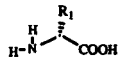
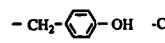
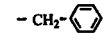
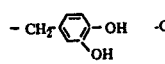
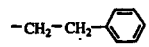
Stability in t-butabol(95%)/H_2O(5%)

The stability of alcalase in mixture of *t*-butabol (95%) and H_2O(5%) was determined by spectrophotometric measurement of the absorbance rise at 405 nm due to the release of *p*-nitrophenol (ϵ_{405} = 18.5 cm^2 μm^{-1}) from the substrate. In a typical reaction, alcalase (0.1 ml) in a mixture of *t*-butanol (9.5 ml) and water (0.5 ml) was stirred at 25°C to maintain homogeneity. Periodically, an aliquot (50 μl) was withdrawn and added to a cuvette containing benzoyl arginine *p*-nitrophenol (10.0 mmol) in phosphate buffer (0.20 *M*). The initial rates of reaction were determined from plots of absorbance at 405 nm with time. The point of 100% activity was taken to be 10 sec after addition of alcalase to the *t*-butanol solution.

Resolution in Aqueous Solution (D,L-Nol-OMe as Example)

A solution of D,L-Nol-OMe (0.10 mol, 17.1 g) and alcalase (5 ml) dissolved in a mixture of water (10 ml) and *t*-butanol (190 ml) was incubated at 25°C. The pH of the reaction solution was kept at 8.2 with a pH controller. During hydrolysis, L-amino acid precipitated. The reaction was continued until 25 ml of the NaOH (2.0 *M*) was consumed (about 4 h). The resulting solution was filtered to yield L-Nol (12.65 g, yield

TABLE 1. Resolution of D,L-amino acid esters in *t*-butanol/water (95/5)

Entry	Substrates				Products[a]			
								
	$-R_1$	$-R_2$	Reaction time (min)	Conversion (%)	e e[b]	Yield (%)	e e	Yield (%)
1		$-CH_3$	80	47	95	99	98	92
2		$-CH_3$	150	46	91	90	68	53
3	$-CH_2-CH-(CH_3)_2$	$-CH_3$	180	51	93	94	58	51
4	$-CH_2-(CH_2)_2-CH_3$	$-CH_3$	240	53	99	96	93	88
5		$-CH_3$	150	50	95	100	87	62
6	$-CH_2-CH_2-CH_3$	$-CH_3$	70	45	91	76	73	60
7	$-CH_3$		30	55	78	81	21	47
8	$-CH_2-CH_3$		150	46	92	65	56	79
9			18	43	41	85	37	76
10			18	43	66	73	47	58

[a]Physical data for the isolated amino acids are listed at amino acids; $[\alpha]^{25}_D$ (c, solvent); mp = °C.
L-1, −9.6 (c 4, 1 *N* HCl); >300.
L-2, −32.6 (c 2, 1 *N* HCl); 273.
L-3, +14.6 (c 2, 5 *N* HCl); >300.
L-4, +21.7 (c 5, 5 *N* HCl); >300.
L-5, −10.7 (c 5, 1 *N* HCl); 292.
L-6, +12.6 (c 10, 6 *N* HCl); >300.
L-7, +19.4 (c 2, 5 *N* HCl); >300.
L-8, +22.8 (c 2, 5 *N* HCl); >300.
L-9, +112 (c 1, 1 *N* HCl); >300.
L-10, +40.3 (c 1, 3 *N* HCl); >300.
D-1, +10.1 (c 4, 1 *N* HCl); >300.
D-2, +28.5 (c 2, 1 *N* HCl); 274.
D-3, −12.7 (c 2, 5 *N* HCl); 285.
D-4, −19.2 (c 5, 5 *N* HCl); >300.
D-5, +10.3 (c 5, 1 *N* HCl); >300.
D-6, −6.2 (c 6, 1 *N* HCl); 286.
D-7, −6.9 (c 4, 1 *N* H_2O); >300.
D-8, −18.6 (c 10, 5 *N* HCl); >300.
D-9, −108 (c 1, 1 *N* HCl); >300.
D-10, −34.9 (c 1, 3 *N* HCl); >300.
[b]e e, enantiomeric access.

96%) mp > 300°C, $[\alpha]_D^{25}$ + 21.7° (c 5, 5 *M* HCl); the pH of the filtrate was adjusted to 11.5 with NaOH (6 *M*) and left overnight. After the amino acid was saponified (monitored by TLC), the pH of the resulting solution was adjusted to 6.2 with citric acid (10%). The D-amino acid was precipitated after chilling for several hours. The precipitation was filtered to yield D-Nol-OH (11.52 g, yield 88%). mp > 300°C, $[\alpha]_D^{25}$ − 19.20° (c 5, 6 *M* HCl).

Chiral HPLC Analysis

The enantiomeric excess of both antipodes were measured on a chiral CR-(+) column using aqueous perchloric acid (pH 1.5–2.0) as eluent. The retention times of the solutes increased as the pH decreased. For hydrophobic amino acids 5–15% methanol was added to the eluent to adjust the retention period. Peaks were detected at 200 nm and identification of components was made by comparison of the retention times with those of authentic compounds prepared chemically.

RESULTS AND DISCUSSION

The enzymatic activity of alcalase and subtilisin Carlsberg in organic cosolvents[11,12] and the stability of alcalase in *t*-butanol

with phosphate buffer (5%, pH 8.2) was previously reported.[13] The alcalase had a half-life 12 h in *t*-butanol/phosphate buffer (5%) and was stable in organic solvents containing aqueous solution (0–20%). Under those reaction conditions, the alcalase not only retained the esterification activity, but also had slightly improved catalytic efficiency.[13,14] In order to avoid a salt effect during the isolation of the latter product, we conducted the resolution in a mixture of *t*-butanol (95%) and water (5%) at 25°C with the pH controlled at 8.5. The stability of alcalase in this case was similar to that in *t*-butanol with phosphate buffer (5%). Figure 1 shows the time course of alcalase inactivation under typical reaction conditions and comparison with the condition of *t*-butanol with phosphate buffer (5%).

Methyl esters of racemic amino acids were used in the resolution. The alcalase had greater esterase activity at pH 8.2 than at pH 7.0. Under these conditions the solubility of amino acid esters was also large. Using a large concentration of the substrate (>10%) in resolution is potentially useful for a large scale process. The free amino acid was less soluble in organic solvents containing water (5%). Precipitation of the product occurred during hydrolysis; the course of hydrolysis was monitored by reverse-phase HPLC. After half the substrate was consumed, the reaction solution was chilled to 0°C, and the precipitate was isolated with filtration to yield L-amino acid. The ester of D-amino acid in the filtrate was saponified when the pH of the solution was increased to 11.5 and maintained there overnight. The saponification of methyl esters of amino acid was rapid at this pH. Most amino acid was released within 1–2 h. After all of the ester was hydrolyzed, the pH of the solution was adjusted to 6.2–6.5, and the resulting solution was chilled to precipitate the D-amino acid. The precipitate was isolated by filtration. The optical purity of the amino acid was determined with a chiral-CR-(+) column and by measuring the optical rotation. Figure 2 shows a typical measurement of authentic D,L-tyrosine (Fig. 2a), the isolated L-tyrosine (Fig. 2b), and D-tyrosine (Fig. 2c) from a chiral CR-(+) column. The enantiomeric excess was determined directly from the chromatographic peak areas. In a similar manner, compounds **1**, **2**, **3**, **4**, **5**, and **6**, were resolved in high enantiomeric excess (ee) and yields. Table 1 shows the results.

Methyl esters of alanine **7**, α-aminobutyric acid **8**, phenylglycine **9**, and homophenylalanine **10** hydrolyzed slowly. According to our previous work, alcalase catalyst hydrolyzed the benzyl ester of Cbz-Ala five times faster than did the methyl ester of Cbz-Ala. Thus, **7**, **8**, **9**, and **10** were resolved via their benzyl esters. The hydrolysis of the benzyl ester was rapid. All racemic derivatives were hydrolyzed. The saponification of the benzyl ester of the D-antipode was slow, requiring 7–10 h to complete the hydrolysis. The ee obtained from hydrolysis of the benzyl esters was invariably smaller than that observed for methyl esters.

In order to ensure that racemization would not occur during saponification, we developed a chiral separation procedure to determine the ee of the D-amino acids before and after saponification. Baseline separation of the enantiomeric pairs of the amino acids and the esters of the amino acids was achieved with the HPLC chiral column. Figure 3 shows typical chromatographic results of the chiral HPLC analysis. Figure 3a shows the baseline separation of authentic D-tyrosine, L-tyrosine, D-tyrosine methyl ester, and L-tyrosine methyl ester. Figure 3b shows the analysis of D-tyrosine methyl ester in the solution, after L-tyrosine was separated by filtration; before saponification, the filtrate contained D-tyrosine methyl ester (94.5%), unhydrolyzed L-tyrosine methyl ester (3%), and L-tyrosine (2.5%). Figure 3c shows the analysis of D-amino acid after saponification; both the D-tyrosine methyl ester and unhydrolyzed L-tyrosine methyl ester were totally converted to the free amino acid. Under these conditions no racemization occurred in the base-catalyzed saponification of the unhydrolyzed residue of either methyl or benzyl ester.

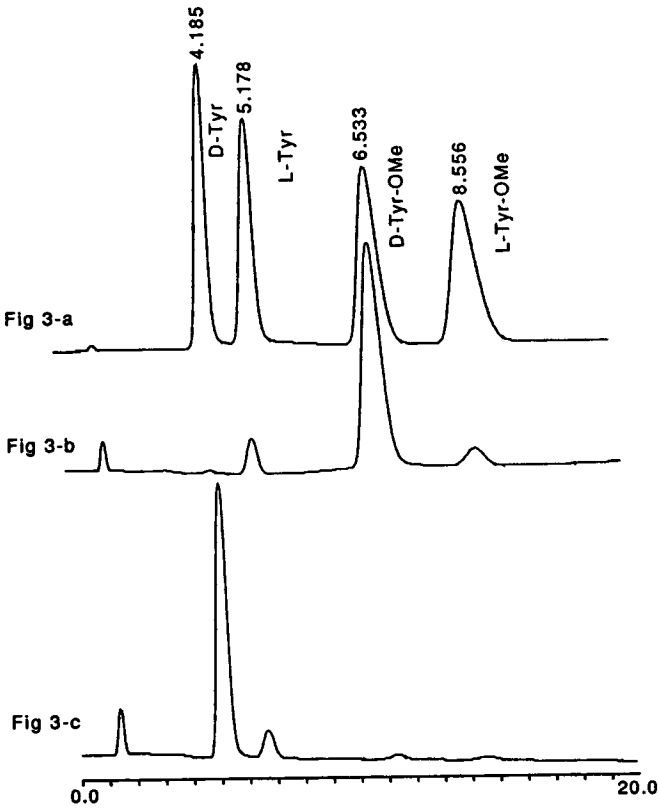


Fig. 3. Determination of enantiomeric excess of each product in resolution solution before isolation via the chiral separation of four components. (**a**) Standard of D,L-tyrosine and D,L-tyrosine methyl ester; (**b**) each of components in reaction solution after hydrolysis; (**c**) enantiomeric excess of D-tyrosine after saponification. Conditions for HPLC were the same as for Figure 2.

In summary, the procedure reported here describes a practical application of alcalase in amino acid resolution, particularly in the resolution of hydrophobic amino acid derivatives that are insoluble in aqueous solutions. The results demonstrate that a small amount of water in the reaction solution suffices for the purpose of resolution. The initial enzymatic activity is enhanced or at least not decreased in *t*-butanol when the molarity of water is reduced from 55 *M* in aqueous solution to 2.75 *M* (5%) in *t*-butanol. The catalytic properties and specificity of the alcalase used here are not limited in usage for resolution purposes.

ACKNOWLEDGMENT

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