Chirally Selective Hydrolysis of D,L-Amino Acid Esters by Alkaline Protease

Shui-Tein Chen,[a] Kung-Tsung Wang,*[a] and Chi-Huey Wong[b]

[a] *Institute of Biological Chemistry, Academia Sinica, P.O. Box 23-106, Taipei, Taiwan, Republic of China*
[b] *Department of Chemistry, Texas A & M University, College Station, TX 77843, U.S.A.*

Alcalase selectively catalyses the hydrolysis of D,L-amino acid methyl and benzyl ester to provide L-amino acid and D-amino acid ester with high optical purity.

We describe a new and practical enzymatic procedure for preparative enantioselective hydrolysis of racemic amino acid esters using the alkaline protease alcalase† as a catalyst.

The enzyme alcalase from *Bacillus licheniforms* is an endoproteinase of the serine type, the major component of which is subtilisin A (Subtilisin Carlsberg). It catalyses the hydrolysis of proteins and amino acid esters[1] and has been widely used as a detergent additive. It is stable and active at room and higher temperature (*e.g.* $t_{\frac{1}{2}}$ at 60 °C is *ca.* 5 h), and in the range pH 6—12. We have found that at pH 6—8, it catalyses the hydrolysis of racemic amino acid methyl esters selectively to give L-amino acids and D-amino acid esters with high enantiomeric excess (e.e.). Figure 1 shows the time course of the hydrolysis of some amino acid esters. In a

† Available from NOVO Industri A/S as a brown liquid. 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 TCA soluble product which gives the same colour with phenol reagent as one mequiv. of Tyr (1 AU ≃ 1000 U, 1 U = 1 μmol L-Tyr-OMe hydrolysed per min). Thus alcalase 0.6 l contains 0.6 AU/g.

DL-Amino acid ester	→ L-Amino acid	+ D-Amino acid ester
	Yield %, e.e.%, $[\alpha]_D^{25}$	Yield %, e.e. %, $[\alpha]_D^{25}$
DL-Phe-OMe·HCl (0.12 mol)	96, 90, −31.1 (*c* 1, H_2O) (0.058 mol)	85, 100, −37.0 (*c* 2, EtOH) (0.051 mol)
DL-Tyr-OMe·HCl (0.11 mol)	95, 91, −9.1 (*c* 2, 5 M HCl) (0.053 mol)	86, 100, −74.0 (*c* 1, pyridine) (0.047 mol)
DL-Ala-OBzl (0.14 mol)	98, 86, +12.5 (*c* 2, 5 M HCl) (0.069 mol)	75, 93, +5.6 (*c* 1, MeOH) (0.052 mol)

Scheme 1. *Conditions:* 25 g of the ester in a 500 ml $NaHCO_3$ buffer (0.2 M, pH 8.0) was added to 1 ml of alcalase 2.5 l. The mixture was then slowly stirred at 30 °C for *ca.* 20 min until 50% of the ester was hydrolysed. The products were isolated by extraction of the reaction mixture with dichloromethane to give D-amino acid esters and then the resultant aqueous solution was concentrated *in vacuo* to precipitate free L-amino acids at pH 6—6.2.

representative preparative scale reaction, when 25.8 g (0.12 mol) of DL-Phe-OMe·HCl‡ was incubated at 30 °C for 20 min in 0.4 l sodium hydrogen carbonate solution (0.2 M, pH 8.0) with 1 ml of alcalase 2.5 l,† 10.9 g (0.051 mol) of D-Phe-OMe·HCl (85% yield, 100% e.e.) and 9.5 g (0.058 mol) of L-Phe (96% yield, 90% e.e.) were obtained after a simple isolation. In a similar manner, DL-Ala-OBzl and DL-Tyr-OMe·HCl were enantioselectively hydrolysed on 25 g scales. The results are summarized in Scheme 1. The optical purity of the amino acids was determined by using a chiral plate[2] and by measuring the optical rotation.

Further studies on the kinetics of the hydrolysis and the effects of pH, organic solvents, and temperature on the reactions have been carried out. The course of the hydrolysis was monitored by measuring the decrease in amino acid ester using h.p.l.c. Details are given in the caption for Figure 1. The results indicate that a high enantioselectivity is observed in the hydrolysis of methyl esters. As shown in Figure 1, L-Tyr-OMe and L-Phe-OMe are hydrolysed 50 times faster than the corresponding D-isomers, while in the case of benzyl esters L-Ala-OBzl is hydrolysed ten times faster than the D-isomer under the conditions used. In general, benzyl esters are hydrolysed faster than methyl esters by a factor of three for the same amino acid. The study on the effects of organic solvents including dioxane, acetonitrile, and DMF indicates that addition of each of these solvents up to 30% does not lower the enzyme activity. This is useful for large-scale preparation because the organic solvents increase the solubility of the substrates. The enzyme also shows a broad substrate specificity. Of the L-amino acid methyl esters tested, Ala, Phe, Tyr, and Trp esters are hydrolysed faster than Asp, Leu, Glu, and Pro. Met, Thr, and Ser esters are hydrolysed slower and Val, Arg, Ile, Lys, Cys, and His cannot be hydrolysed. Diesters of Asp and Glu are hydrolysed only at the α-carboxy group. The interesting feature that the enzyme does not hydrolyse the amino acid with a branch at β-carbon may have some implication concerning its stereospecificity and may limit its general application in the resolution of amino acids.

In summary, this new enzymatic process has several advantages over the other existing processes currently used for

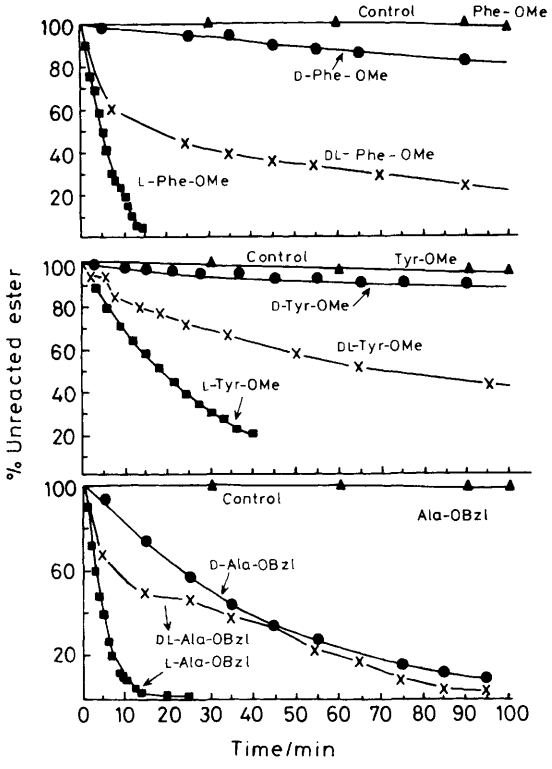

Figure 1. Time course for the alcalase catalysed hydrolysis of amino acid esters at 25 °C. For D- or L-amino acid esters, 0.1 ml of alcalase 2.5 l was added to a 30 ml $NaHCO_3$ solution (0.2 M, pH 8.2) containing 2 mmol of the substrate. For D,L-amino acid esters, the same amount of enzyme was added to a 60 ml $NaHCO_3$ solution containing 4 mmol of the substrate. To study the relative reactivity of different L-amino acid esters, the mixture was stirred slowly for 50 min at 25 °C and the amount of unreacted ester was then determined as follows (values in parentheses): L-Ala-OMe (<3%), L-Phe-OMe (<3%), L-Tyr-OMe (10%), L-Trp-OMe (10%), L-Asp-$(OMe)_2$ (20%), L-Leu-OMe (30%), L-Glu-$(OMe)_2$ (30%), L-Pro-OMe (40%), L-Met-OMe (60%), L-Thr-OMe (70%), L-Ser-OMe (85%), L-Val-OMe (>95%), L-Arg-OMe (>95%), L-Cys(Bzl)-OMe (>95%), L-His-OMe (>95%), L-Ile-OMe (>95%), L-Lys-OMe (>95%), D-Phe-OMe (90%), D-Tyr-OMe (>95%), L-Ala-OBzl (<3%), L-Leu-OBzl (<20%), L-Ser-OBzl (40%), D-Ala-OBzl (75%), L-Val-OBzl (90%), L-Ile-OBzl (100%). H.p.l.c. was used to measure the concentration of reacted and unreacted amino acid esters, using an RP-8 column with 0.1% trifluoroacetic acid in 10—30% MeCN (depending on the hydrophobicity of amino acid studied) as eluant, flow rate 1.5 ml min^{-1}, u.v. (214 nm) detection.

‡ *Abbreviations used:* Phe = phenylalanine, Ala = alanine, Tyr = tyrosine, Trp = tryptophan, Asp = aspartic acid, Leu = leucine, Glu = glutamic acid, Pro = proline, Met = methionine, Thr = threonine, Ser = serine, Val = valine, Arg = arginine, Ile = isoleucine, Lys = lysine, Cys = cysteine, His = histidine, Bzl = benzyl, DMF = dimethylformamide.

large-scale production of amino acids:[1,3] (i) the high turnover rate (*ca.* 10^2 g h^{-1} AU^{-1}) and low cost make the enzyme immobilization unnecessary; (ii) the substrates can be prepared easily; (iii) the enzyme is stable at high temperature and in the presence of organic solvents, which allows operation at high substrate concentration (*ca.* 0.5 M at 45 °C); (iv) the reaction is highly enantioselective for a number of amino acids; and (v) product isolation is simple.

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