Facile Amide Bond Formation From Esters of Amino Acids and Peptides Catalyzed by Alkaline Protease in Anhydrous *tert*-Butyl Alcohol Using Ammonium Chloride/Triethylamine as a Source of Nucleophilic Ammonia

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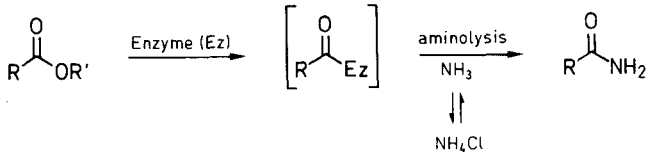
Received 10 November 1992; revised 19 January 1993

An industrial alkaline protease "Alcalase", stable and active in *tert*-butyl alcohol, was used to catalyze the synthesis of *N*-protected amino acids or peptide amides in anhydrous *tert*-butyl alcohol using ammonium chloride/triethylamine as source of nucleophilic ammonia

The C-terminals of many biologically active peptides are amidated. Many chemical amidation methods have been reported.[1] Recently, the search for proteases that are stable in organic solvents for peptide synthesis has been the subject of extensive investigation.[2] The industrial alkaline protease, "Alcalase", has been reported to be very stable and to be able to maintain enzymic activity in alcoholic solutions.[3] Here we report that amide bond formation from esters of *N*-protected amino acids and peptides can be conveniently accomplished by Alcalase-catalyzed amidation of the corresponding esters with a mixture of ammonium chloride and triethylamine in *tert*-butyl alcohol.

The substrates were simply prepared by the established method.[4] The reaction protocol was a kinetically controlled approach. As shown in the Scheme, the substrate was reacted with enzyme to form an intermediate "acyl-enzyme", which in turn was attacked by the nucleophile, in this case the ammonia, to produce the desired amino acid or peptide amide. The solubility of the substrates in *tert*-butyl alcohol was high, while the solubility of ammonium chloride was very low. In the presence of triethylamine, ammonia was liberated and dissolved in *tert*-butyl alcohol. The nucleophilicity of ammonia in *tert*-butyl alcohol/triethylamine solution was high enough to form the amide bond. The Alcalase was obtained as a brown liquid. The enzyme did not dissolve in *tert*-butyl alcohol, but it did disperse well in alcoholic solvents to make a transparent solution.



substrate		substrate	
1	Cbz-Phe-OMe	5	Cbz-Ala-OMe
2	Cbz-DL-Phe-OMe	6	Moz-Leu-OBzl
3	Moz-Asp(Bzl)-Bzl	7	Boc-Ala-Phe-OMe
4	Moz-Glu(Bzl)-Bzl	8	Cbz-Ala-Phe-Leu-OMe

Scheme

Since water can compete with the amine nucleophile in a kinetically controlled amide bond formation, the concentration of water in the reaction mixture will affect the yield of the reaction. The water can be removed from the alcalase solution by repeated washing with anhydrous *tert*-butyl alcohol. The pH value of the reaction mixture was difficult to measure. It was determined by diluting the reaction mixture with one equivalent of water and then measuring the pH directly using a pH meter. A mixture of Alcalase (2.0 mL) and ammonium chloride (3.0 mmol), which contained one, two and three equivalents of triethylamine, was diluted with water (20 mL), and the pH of the solution was found to be 10.6, 11.0, and 11.2, respectively. A preliminary test using Cbz–Phe–OMe in *tert*-butyl alcohol containing Alcalase, ammonium chloride and triethylamine, was reacted for 12 hours and worked up to give the crude product, which was further purified by either recrystallization (methanol/ether) or flash column chromatography. In a similar manner, esters of amino acids or peptides were reacted and isolated with yields between 50–70%. The results are summarized in the Table. All the products were confirmed either by comparison with the authentic sample[5] or ^{1}H NMR and FAB mass spectra.

A small amount (3–8%) of the hydrolysis byproduct (R–AA–CO_2H) was observed during the reaction as measured by HPLC analysis of the reaction mixture because the water molecules associated with the enzyme diffused into the reaction mixture. In aqueous solution, the alcalase with enantioselectivity has been used for DL-amino acid resolution.[6] In this reaction the enantioselectivity in amide bond formation catalyzed by alcalase in *tert*-butyl alcohol solution was also high. When using Cbz–DL–Phe–OMe as a substrate in reaction, only the L-form of the substrate was consumed, and the concentration of D-enantiomer did not change during the reaction. Figure 1 shows the HPLC analysis of Cbz–DL–Phe–OMe before the reaction (Fig 1,A) and the Cbz–D–Phe–OMe isolated after the end of the reaction (Fig 1,B).

The use of ammonium chloride/triethylamine as a source of nucleophilic ammonia is a new method. It may be used for other synthesis using ammonia as a nucleophile. Application of an inexpensive industrial enzyme to amide synthesis in anhydrous *tert*-butyl alcohol has not been reported before. In conclusion, the Alcalase-catalyzed amide formation was regioselective for the α-carboxyl of Asp and Glu residues, enantioselective for the L-amino acid substrates at the enzyme's s-1 sub-site. This new enzymatic process has several advantages: 1. the high turnover rate and low cost of the enzyme make enzyme immobilization unnecessary; 2. the enzyme is stable in alcoholic solvents, thus allowing operation at high substrate concentration; and 3. the reaction is highly selective for ester hydrolysis, and the peptide bonds remain intact.

Table. Enzymatic Synthesis of Amino Acid and Peptide Amides in *tert*-Butyl Alcohol

Substrate	Product	Reaction Time (h)	Conversion (%)[a]	Yield (%)[b]	mp (°C)	Lit. mp (°C)	$[\alpha]_D^{25}$ (c = 2, MeOH)	^{1}H NMR (DMSO-d_6/TMS) δ, J (Hz)
1	Cbz-Phe-NH_2	8	81	64	160–161	164–165[5a]	−5.15[c]	2.69–2.76 (m, 1H), 2.95–3.00 (m, 1H), 4.15–4.16 (m, 1H), 4.93 (s, 2H), 7.05 (s, 2H), 7.18–7.32 (m, 10H), 7.39 (d, 1H, J = 8.68)
2	Cbz-L-Phe-NH_2	10	100[c]	64	160–161	164–165[5a]	−5.15[d]	–
3	Moz-Asp(Bzl)-NH_2[e]	12	64	44	106–107	101[5b]	−2.86	2.48–2.76 (m, 2H), 3.73 (s, 3H), 4.34–4.36 (m, 1H), 4.93 (s, 1H), 5.06 (s, 1H), 7.12 (s, 2H), 7.26–7.34 (m, 9H)
4	Moz-Glu(Bzl)-NH_2[f]	12	88	75	128–129		−4.20	1.73–1.95 (m, 2H), 2.37 (t, 3H, J = 7.65), 3.73 (s, 3H), 3.91–3.97 (m, 1H), 4.92 (s, 2H), 5.06 (s, 2H), 6.89 (d, 2H, J = 8.50), 7.03 (s, 2H)
5	Cbz-Ala-NH_2[g]	10	93	41	129–130	130–131	−3.32[h]	1.18 (d, 3H, J = 7.31), 3.92–3.99 (m, 1H), 5.00 (s, 2H), 6.95 (s, 2H), 7.28– 7.40 (m, 6H)
6	Moz-Leu-NH_2[f]	11	73	67	139–140		−7.00	0.82–0.87 (m, 6H), 1.37–1.44 (m, 3H), 3.73 (s, 3H), 3.90–4.00 (m, 1H), 6.88 (d, 1H, J = 12.73), 7.18 (s, 2H), 7.23–7.30 (m, 4H)
7	Boc-Ala-Phe-NH_2[f]	12	68	43	158–160		−12.4	1.05 (d, 3H, J = 7.16), 1.35 (s, 9H), 2.83–2.99 (m, 2H), 3.80–3.87 (m, 1H), 4.35–4.45 (m, 1H), 6.95 (s, 2H), 7.09–7.34 (m, 6H), 7.68 (d, 1H, J = 8.24)
8	Cbz-Ala-Phe-Leu-NH_2[f,i]	10	–	47	106–108		−23.6	0.77–0.86 (m, 6H), 0.93–0.98 (d, 3H, J = 7.05), 1.05–1.09 (m, 1H), 1.35–1.51 (m, 2H), 2.75–3.08 (m, 2H), 3.62 (s, 2H), 3.92–4.44 (m, 2H), 4.50–4.64 (m, 1H), 6.81–6.83 (d, 1H, J = 7.04), 6.98 (s, 1H), 7.05–7.30 (m, 10H), 7.97–8.00 (d, 1H, J = 8.49), 8.39 (d, 1H, J = 8.00)

[a] The conversion is determined by the decreasing concentration of the substrate using HPLC analysis.
[b] The products from **1**–**3**, **5**, **7**, **8** were purified by column chromatography and the rest by recrystallization.
[c] Lit.[5a] $[\alpha]_D$ −4.9° (c = 1.5, 80% AcOH).
[d] Lit.[5b] $[\alpha]_D$ +1.5° (c = 2.5, 50% aq dioxane).
[e] MS: m/z = 386 (M^+).
[f] For new compounds satisfactory microanalyses obtained: C ± 0.30, H ± 0.10, N ± 0.25.
[g] MS: m/z = 223 (M^+).
[h] Lit $[\alpha]_D^{25}$ −4.5° (c = 2, MeOH).
[i] MS: m/z = 457 (M^+).

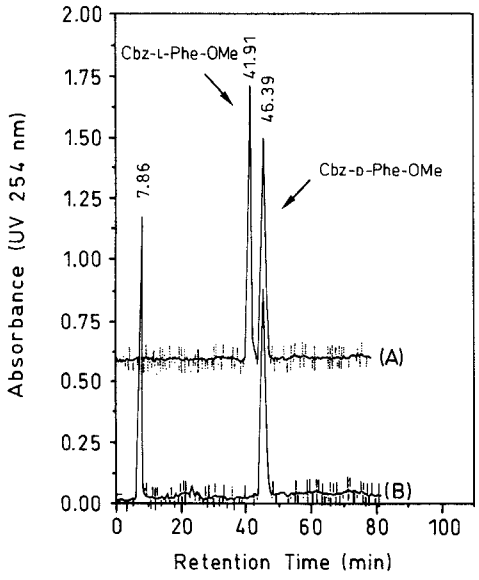


Figure 1. HPLC spectra of Cbz–DL–Phe–OMe (A) and of Cbz–D–Phe–OMe (B) Column: Diacel Chiral OD; Eluent: hexane/isopropyl alcohol (91 : 9); Flow Rate: 0.5 mL/min; Detector: UV 254 nm.

Alcalase was purchased from NOVO industrila (Denmark) as a brown liquid with a specific activity of 2.5 AU.mL^{-1}. It was used without further purification. The amino acids were purchased from Sigma USA. $SOCl_2$ and benzyloxycarbonyl chloride (Cbz–Cl) were purchased from E. Merck, Germany. The substrates were prepared by the established method.[4] Optical rotation was measured on a Universal Polarimeter (Schmidt & Haensch, Germany). NMR spectra was taken on Bruker AM-300 and chemical shifts of ^{1}H NMR spectra were referenced to solvent peaks. Kinetic data was measured on a Hitachi U-2000 spectrophotometer. HPLC was run on an Alcott 760-HPLC pump (Japan) with Soma 3701 UV-Detector (Japan) and data collected on a Hewlett-Packard HP 3394A Integrator (USA). Chiral OD-column was purchased from Diacel (USA). TLC was performed on silica gel G pre-coated plates (E. Merck, Germany). MeOH, EtOAc, CH_2Cl_2, dioxane, Et_2O and acetone (HPLC and reagent grade) were obtained from the ALPS Chem. Co. (Taiwan).

Removal of Water from the Alcalase Solution:

Alcalase 2.5 L (1.0 mL) and anhydrous *tert*-butyl alcohol (10 mL) were added to a centrifuge tube (20 mL), and the mixture was agitated on a super-mixer for 5 min. The resulting mixture was centrifuged (3000 rpm) for 15 min to spin down the enzyme, and the supernant was decanted. *tert*-Butyl alcohol (10 mL) was added again, and the same procedure was repeated three times until the water was removed completely.

Enzyme Assay:
The proteolytic activities of alcalase and subtilisin carlsberg were determined spectrophotometrically by measuring the absorbency increase at 500 nm (colored by Folin-Ciocalteu's phenol reagent) due to the release of trichloroacetic acid (TCA)-soluble compounds from the 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 which, under standard conditions, digests hemoglobin at an initial rate liberating the same amount of TCA-soluble product per min as 1 mequiv of tyrosine (again colored by Folin-Ciocalteu's phenol reagent). Thus 1 AU = 1000 U, and 1 U = 1 mmol of L-Tyr-OMe hydrolyzed per min.

Enzymatic Amide Bond Formation in *tert*-Butyl Alcohol; Typical Procedure for Cbz-Phe-NH_2:
Cbz-Phe-OMe (10 mmol, 3.16 g), NH_4Cl (30 mmol, 1.08 g), Et_3N (30 mmol, 4.2 mL), and Alcalase 2.5 L (2.0 mL, prewashed with *tert*-butyl alcohol three times) were added to anhydrous *tert*-butyl alcohol (50 mL). The mixture was shaken at 35°C for 12 h. The resulting mixture was then diluted with EtOAc (300 mL) and washed with 0.5 N $NaHCO_3$, 5% citric acid, and water. After the solvent was evaporated under reduced pressure, crude Cbz-Phe-NH_2 was obtained as a white amorphous solid. The crude product was further purified by silica gel column chromatography using CH_2Cl_2/MeOH (9:1) as eluent. The desired portion was collected and evaporated to yield pure Cbz-Phe-NH_2; yield: 1.92 g (64%); mp 160–161°C; $[\alpha]_D = -5.15°$ ($c = 2$, MeOH).

^{1}H NMR (DMSO-d_6): δ = 2.69–2.76 (m, 1 H), 2.95–3.00 (m, 1 H), 4.15–4.16 (m, 1 H), 4.93 (s, 2 H), 7.05 (s, 2 H), 7.18–7.32 (m, 10 H), 7.39 (d, 1 H, J = 8.68 Hz).

Synthesis of Cbz-L-Phe-NH_2 from Cbz-DL-Phe-OMe:
Cbz-DL-Phe-OMe (20 mmol, 4.77 g), NH_4Cl (30 mmol, 1.08 g), Et_3N (30 mmol, 4.2 mL), and Alcalase 2.5 L (2 mL, prewashed three times with *tert*-butyl alcohol) were added to anhydrous *tert*-butyl alcohol (50 mL). The mixture was shaken at 35°C for 10 h. The resulting mixture was diluted with water (150 mL) and extracted with EtOAc (3 × 100 mL). The combined EtOAc layers were washed with 0.5 N $NaHCO_3$ (3 × 50 mL), 5% citric acid (3 × 50 mL), and water (4 × 50 mL). The solvent was evaporated under reduced pressure, and the residue was further purified by column chromatography. The Cbz-D-Phe-OMe was eluted out first, collected and evaporated; yield: 1.96 g (82%); oil; $[\alpha]_D^{25} = +10.77°$ ($c = 2$, MeOH; ee 100% by chiral column).

^{1}H NMR: δ = 2.5–2.78 (m, 2 H), 2.92 (s, 3 H), 4.02–4.07 (m, 1 H), 4.64 (s, 2 H), 6.79–6.97 (m, 11 H)).

The next fraction was Cbz-L-Phe-NH_2; yield: 1.43 g (64%). The physical properties of Cbz-L-Phe-NH_2 are listed in the Table.

Support for this research provided by the National Science Council, Taiwan, (NSC 81-0208-N-001-70 & NSC82-0115-C001-01-078M) is gratefully acknowledged.

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