Kinetically Controlled Peptide Bond Formation in Anhydrous Alcohol Catalyzed by the Industrial Protease Alcalase

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The industrial alkaline protease alcalase has been found to be very stable (half life > 5 days in ethanol or 2-methyl-2-propanol) and active in alcoholic solvents (except methanol). Procedures have been developed for alcalase-catalyzed, kinetically controlled peptide bond formation in anhydrous alcohol (ethanol, 2-methyl-2-propanol). Studies of the selectivity of an alcalase-catalyzed reaction show that only L-amino acid acyl donors are substrates at the p-1 subsite of alcalase; at the p-1′ subsite both D- and L-amino acid nucleophiles are substrates. Other amino compounds such as benzylamine and phenylhydrazine are good nucleophiles. Studies of the effect of the water content of the reaction solution on the yield in the synthesis of Moz-Phe-Leu-NH_2 showed that the 95% yield obtained in anhydrous 2-methyl-2-propanol was decreased to 48% in 2-methyl-2-propanol containing 4.86% water.

Introduction

Recently, the search for proteases that are stable in organic solvents for peptide synthesis has been extensive.[1] Several studies have demonstrated that it is possible to use proteases to catalyse peptide synthesis in organic solvents.[2] We have found that the industrial alkaline protease alcalase maintains enzymatic activity in alcoholic solutions and catalyzes peptide bond formation in high yields by a kinetically controlled approach. Peptide bond formation catalyzed by alcalase in an organic solvent has not been reported before.

Alcalase is a proteolytical enzyme prepared from submerged formation of a selective strain of *Bacillus licheniformis*. The major enzyme component of alcalase is the serine protease subtilisin carlsberg (alkaline protease A), which is one of the most throughly characterized bacterial proteases. Much is understood about the kinetic properties and chemical modification behavior of this and other bacillus subtilisins.[3] Alcalase is widely used as an additive in detergents as a digesting enzyme. It has high protease activity and is inexpensive. The use of alcalase as a biocatalyst in organic synthesis has not been studied extensively. Very recently, we found that alcalase possesses regioselective esterase activity with a high turnover rate (ca. 10^2 g h^{-1} AU^{-1}).[4] We have also used alcalase as a catalyst for the preparation of amino acid derivatives[4] and for the selective hydrolysis of protected dipeptide esters.[4] In this study, we found that alcalase is very stable and active in alcoholic solvents and is suitable for catalysis of peptide bond formation in anhydrous alcohol via a kinetically controlled approach. We describe here the stability of alcalase in various alcohols, the reactivity of alcalase in catalyzing transesterification in alcohols, the kinetic constants of alcalase-catalyzed peptide bond formation in 2-methyl-2-propanol, the selectivity and stereospecificity of alcalase-catalyzed peptide bond formation at the p-1 and p-1′ subsite, and the effect of the water concentration in the reaction solution on the yield of the products.

Results and Discussion

Stability of Alcalase in Alcohols. Alcalase was obtained from Novo Industrial as a brown liquid with an enzymatic activity of 2.5 AU and was used without further purification. The enzyme did not dissolve in alcohols, but it did disperse well in alcoholic solvents to make a transparent solution. The enzyme can be precipitated from the alcohol solution by centrifugation. The stability of alcalase in organic solvents was measured spectrophotometrically on the basis of the activity of the enzyme remaining after incubation with the solvent (see Experimental Section). Figure 1 shows the time course of alcalase inactivation under typical reaction conditions. In methanol, nearly half of the original activity was lost within 35 min. In 2-methyl-2-propanol and ethanol, half of the original activity remained after about 5 days, and in *tert*-amyl alcohol, alcalase was stable for weeks. The stability increased as the polarity or dipole moment of the solvents decreased. In solvents such as acetone, dioxane, acetonitrile, and chloroform, the enzyme was insoluble and stuck to the wall of the reaction flask. Subtilisin carlsberg was found to have one-fourth the stability of alcalase in ethanol solution.

Removal of Water from Alcalase. It is generally accepted that enzymes have a high binding affinity for water molecules.[5] Bound water molecules can prevent denaturation of the enzymes and can maintain the enzymatic activity in solvent systems with high concentrations of organic solvents. An X-ray crystal structure of a complex of subtilisin carlsberg and a genetically engineered *N*-acetyl eglin-c was found to have 170 water molecules in the crystal lattices.[6] Since water can compete with the amine nucleophile in kinetically controlled peptide bond synthesis, the concentration of water in the reaction so-

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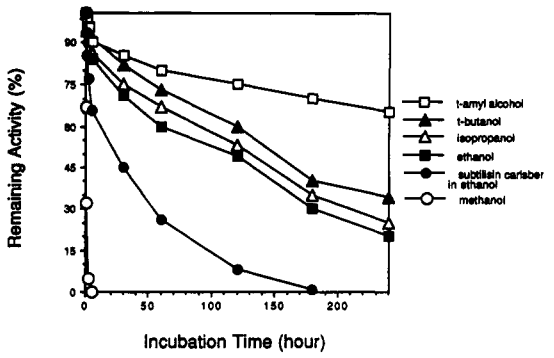


Figure 1. Stability of alcalase in various alcohols and subtilisin carlsberg in ethanol at room temperature.

Table I. Kinetic Constants for Alcalase-Catalyzed Transesterification in Ethanol and Ethanol/2-Methyl-2-propanol Mixtures at 25 °C

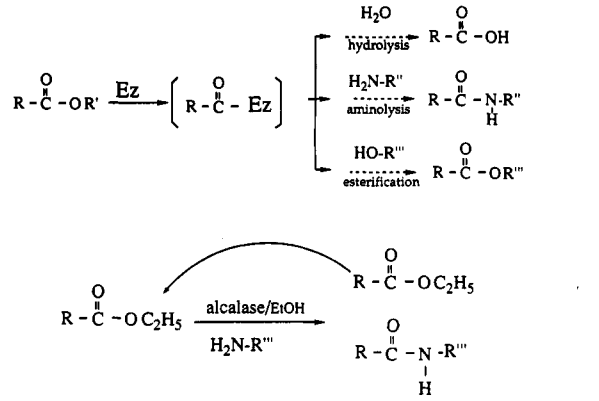
substrate	V_{init} (μmol/min)	K_{cat} (min^{-1})	K_m (mmol)	K_{cat}/K_m
Moz-Phe-OMe				
100% EtOH	300.48	3.732	54.58	68.38
80%, EtOH	400.96	9.638	124.3	77.73
50%, EtOH	252.21	4.511	61.19	73.83
25%, EtOH	198.24	3.862	69.43	55.64
25%, EtOH[a]	629.44	333.6	3081.0	108.28
10%, EtOH	168.67	3.254	63.27	51.43
10%, EtOH[a]	537.24	20.337	181.0	112.36
Moz-Leu-OBzl				
100% EtOH	221.4	76.751	483.2	158.84
Moz-Ala-Ala-Leu-OMe				
100% EtOH	427.2	4.41	34.93	126.3
100% EtOH[a]	2751.52	295.79	503.7	587.29
Moz-Ala-Ala-Leu-pNA				
100% EtOH	0.56	19.8×10^{-3}	5.67	3.50

[a] Kinetic data were determined at 40 °C.

lution will affect the yield of the reaction. The water in the alcalase solution will not affect the reaction since it can be removed before the alcalase is added to the reaction solution, but the water bound to the enzyme will affect the yield. The water can be removed from the alcalase solution by repeated washing with anhydrous alcohol. A typical procedure for removing water from the alcalase solution was as follows: the enzyme solution was suspended in an anhydrous alcohol by agitation, the resulting mixture was centrifuged to separate the enzyme from the solvent, and the alcohol was removed by decantation. The procedure was repeated several times, and the enzyme was then transferred to a reaction flask for peptide synthesis. The concentration of water in the alcalase solution can be measured by a Karl-Fisher titration method. The sensitivity of this method in determining the water content in organic solvents under our conditions was approximately 0.02% (v/v).[7] By using the Karl-Fisher titration, we determined that the water concentration in the alcalase solution can be reduced to 0.1% by repeated washings with anhydrous alcohol and that the water concentration will no longer decrease after the alcalase has been washed three times.

Activity of Alcalase in Alcohols. The decrease of enzymatic activity in organic cosolvents has been documented.[4,8] In anhydrous alcohol, enzymatic trans-

Scheme I. Kinetically Controlled Peptide Synthesis in Ethanol



esterification of esters will occur instead of hydrolysis. Table I shows the results of the investigation of the kinetics of the alcalase-catalyzed transesterification of Moz-Leu-OBzl, Moz-Phe-OMe, Boc-Ala-Ala-Leu-OMe, and Boc-Ala-Ala-Leu-pNA in ethanol and in a mixture of 2-methyl-2-propanol/ethanol. Kinetic data for alkaline protease-catalyzed transesterifications in anhydrous alcoholic solvents are limited. Comparison of the alcalase-catalyzed transesterifications in Table I with the subtilisin carlsberg-catalyzed transesterification in ethanol/water (9.5:0.5) shows that alcalase in anhydrous solution not only retains the tansesterification activity but also has slightly improved catalytic efficiency.[9] In general, the second-order rate constant (K_{cat}/K_m) and the first-order rate constant (K_{cat}) indicate that the presence of 2-methyl-2-propanol in the ethanol solution does not affect the catalytic efficiency. We believe this results from the higher polarity of ethanol versus 2-methyl-2-propanol and the higher concentration of ethanol. No 2-methyl-2-propanol transesterification product was observed. The fact that, in 80% ethanol solution with Moz-Phe-OMe as substrate, the catalytic efficiency of alcalase is higher than that in the other ethanol solutions is very interesting. The same effect also observed when Moz-Leu-OBzl is used as a substrate.

Peptide Bond Formation in Organic Solvent. Kinetically controlled peptide synthesis catalyzed by serine or cysteine protease is widely used, since short reaction times and low enzyme concentrations are required.[1,2,10] Scheme I shows a typical reaction path for this approach. The initially formed acyl enzyme intermediate [RC(=O)Ez] can be deacylated by water or by an amine nucleophile [:NH_2R'']. The yield of the peptide bond formation depends on two factors (1) the relative rate of hydrolysis and aminolysis, which is determined by the nucleophilicity of water vs that of the amine nucleophile, and (2) the molar ratio of the nucleophiles, water, and the amine. If the kinetically controlled peptide bond formation takes place under anhydrous conditions, the hydrolysis will not occur. The following study of alcalase-catalyzed peptide synthesis was conducted in ethanol and 2-methyl-2-propanol.

When ethanol is used as the solvent in the reaction, esterification of the acyl enzyme by ethanol will occur competitively with aminolysis. Esterification results in the formation of an ethyl ester [RCOOEt], which in turn serves as a substrate. The time required for the reaction is short.

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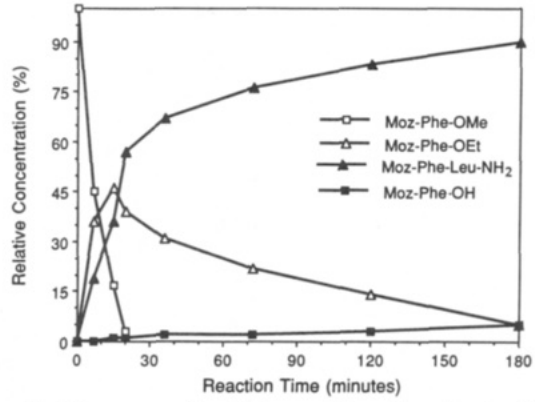

Figure 2. Time course for alcalase-catalyzed synthesis of Moz-Phe-Ala-NH_2 in ethanol at room temperature.

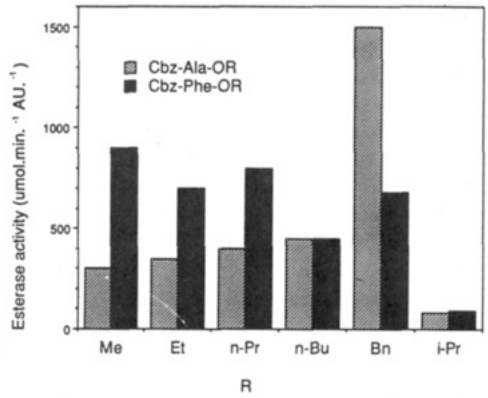

Figure 3. Esterase activity of alcalase in the hydrolysis of Cbz-Ala- and Cbz-Phe- esters in phosphate buffer at room temperature pH 8.2.

Figure 2 shows the time course of a typical reaction, the formation of Moz-Phe-Leu-NH_2 from Moz-Phe-OMe and Leu-NH_2. Moz-Phe-OMe disappeared within half an hour while Moz-Phe-OEt and Moz-Phe-Leu-NH_2 were formed, and the concentration of Moz-Phe-Leu-NH_2 increased constantly until the end of the reaction. This reaction profile agrees with the proposed formation of an acyl-Ez [Moz-Phe-Ez], which is then attacked by the nucleophile (Leu-NH_2 or ethanol) to form the peptide and a new amino ester (Moz-Phe-OEt). A small amount of the hydrolysis byproduct (Moz-Phe-OH) was observed during the reaction because of the water molecules that associated with the enzyme into the reaction solution. The same manner, we have used methyl, ethyl, and benzyl esters of N-protected amino acids or peptides as acyl donors. The results are shown in Table II.

The esterase activity of alcalase on esters of N-protected amino acids, which are made from N-protected amino acids and primary, secondary, or tertiary alcohols, is different for each ester. The esterase activity was measured in phosphate buffer (pH = 8.2) with 30% acetone as a co-solvent. Figure 3 shows the results of alcalase-catalyzed hydrolysis of amino acid esters of Cbz-Phe and Cbz-Ala. The esters made from primary alcohols are hydrolyzed faster than those made from secondary alcohols, and the esters which are made from tertiary alcohols are not hydrolyzed at all. A similar result is also obtained in alcalase-catalyzed transesterification in primary, secondary, or tertiary alcohols. Cbz-Phe-OMe was not transesterified in 2-methyl-2-propanol. This means that in kinetically controlled peptide synthesis catalyzed by alcalase, 2-methyl-2-propanol will not act as a nucleophile. Thus, in a 2-methyl-2-propanol solution, the amino group is the only available nucleophile, and only peptide bond formation

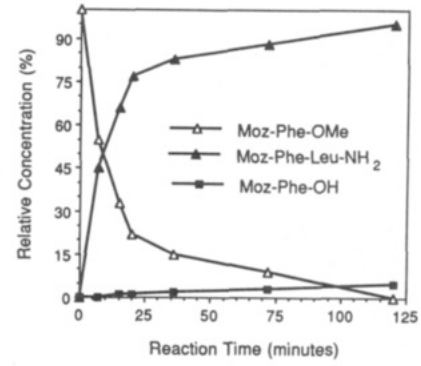

Figure 4. Time course for alcalase-catalyzed synthesis of Moz-Phe-Ala-NH_2 in 2-methyl-2-propanol at room temperature.

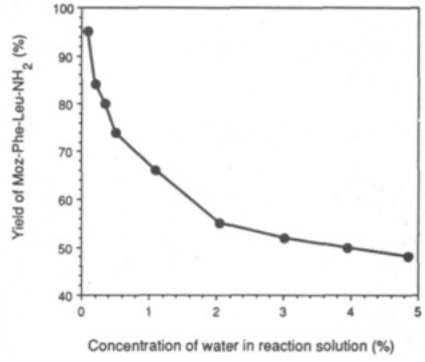

Figure 5. Effect of water concentration on the yield of Moz-Phe-Leu-NH_2 in a kinetically controlled peptide bond formation catalyzed by alcalase in 2-methyl-2-propanol.

occurs. Figure 4 shows the time course of the alcalase-catalyzed synthesis of Moz-Phe-Leu-NH_2 in 2-methyl-2-propanol with Moz-Phe-OMe as an acyl donor and Leu-NH_2 as a nucleophile. Comparing this result with peptide bond formation in ethanol shows that a shorter reaction time is required, and equal or higher yields are obtained when 2-methyl-2-propanol rather than ethanol is used as the solvent. (See Table II).

Effects of Water on the Yield. The effect of water on the yield of kinetically controlled peptide synthesis was determined by the study of the reaction of Moz-Phe-OMe and Leu-NH_2 in 2-methyl-2-propanol containing varying amounts of water. The alcalase solution was first washed three times with anhydrous 2-methyl-2-propanol, and then the enzyme was added to a mixture of 2-methyl-2-propanol containing a known concentration of water, 1 equiv of the acyl donor, and 3 equiv of the nucleophile. The mixture was stirred at room temperature for 3 h, and the product was isolated by diluting the reaction mixture with ethyl acetate, washing with 5% citric acid and 0.5 N bicarbonate, and evaporating the solvent. In Figure 5, the yield of Moz-Phe-Ala-NH_2 is plotted against the concentration of water in the 2-methyl-2-propanol. Because the water concentration could not be decreased any more after three washes with anhydrous 2-methyl-2-propanol, a maximum yield was obtained when the reaction solution contained a minimum amount of water (0.1%) and the ratio of Moz-Phe-OH/Moz-Phe-Leu-NH_2 was 6:94. Extending the reaction time to 6 h did not increase the yield. The reaction in 2-methyl-2-propanol containing more than 5% water was not studied because the yield in a 5% water solution was lower than 50%. In all the reaction conditions tested above, using 2-methyl-2-propanol as a solvent and prewashing the enzyme with 2-methyl-2-propanol at least three times gave the best results. This protocol was used for all the peptide synthesis in the following study.

Table II. Kinetic Controlled Peptide Bond Formation in Alcohol Catalyzed by Alcalase

	acyl donor#	nucleophile#	solvent@	product#	reaction time (h)	yield (%)
1	Moz-Phe-OMe	Ala-NH_2	E	Moz-Phe-Ala-NH_2	4	79
	Moz-Phe-OMe	Ala-NH_2	B	Moz-Phe-Ala-NH_2	2	86
	Moz-Phe-OMe	D-Ala-NH_2	B	Moz-Phe-D-Ala-NH_2	2	87
	Moz-Phe-OMe	D-Ala-NH_2	B	Moz-Phe-D-Ala-NH_2	2	85+
2	Moz-Phe-OBzl	Leu-NH_2	E	Moz-Phe-Leu-NH_2	4	82
	Moz-Phe-OBzl	Leu-NH_2	B	Moz-Phe-Leu-NH_2	3	94
	Moz-Phe-OBzl	D-Leu-NH_2	B	Moz-Phe-D-Leu-NH_2	2.5	89
	Moz-Phe-OBzl	D-Leu-OMe	B	Moz-Phe-D-Leu-OMe	2.5	88
3	Moz-Phe-OMe	Phe-NH_2	B	Moz-Phe-Phe-NH_2	3.5	82
	Moz-Phe-OMe	D-Phe-NH_2	B	Moz-Phe-D-Phe-NH_2	3.5	91
	Moz-Phe-OMe	Phe-O^{t}Bu	B	Moz-Phe-D-Phe-O^{t}Bu	2.5	63
4	Moz-Leu-OBzl	D-Ala-NH_2	B	Moz-Leu-D-Ala-NH_2[a]	2	91
	Boc-Leu-OBzl	Met-NH_2	E	Boc-Leu-Met-NH_2[b]	4	76
	Boc-Leu-OBzl	p-NH_2-C_6H_4-NO_2	E	Boc-Leu-p-NA	12	25
5	Moz-Asp(Bzl)-OBzl	D-Ala-NH_2	B	Moz-Asp(Bzl)-D-Ala-NH_2	2	86
	Moz-Asp(Bzl)-OBzl	D-Ala-O^{i}Pr	B	Moz-Asp(Bzl)-D-Ala-O^{i}Pr	2.5	82
6	Cbz-Asp-α-OBzl	D-Ala-O^{i}Pr	B	Z-Asp-D-Ala-O^{i}Pr	2.5	69
7	Moz-Thr-Leu-OMe	Ala-NH_2	E	Moz-Thr-Leu-Ala-NH_2[c]	4	82
8	Cbz-Ala-Phe-OMe	Ala-NH_2	E	Cbz-Ala-Phe-Ala-NH_2[d]	4	79
9	Boc-Met-Leu-OBzl	Phe-$NHCH_2Ph$	E	Boc-Met-Leu-Phe-$NHCH_2Ph$[e]	6	85
10	Boc-D,L-Asp-OBzl	NH_2-NH-Ph	E	Boc-Asp-NH_2-NH-Ph[f]	5	95
11	Cbz-D,L-Leu-OBzl	Ala-NH_2	B	Cbz-Leu-Ala-NH_2[g]	2	71
12	Cbz-D,L-Ala-OBzl	Thr-$NHNH_2$	B	Cbz-Ala-Thr-$NHNH_2$[h]	2	65
13	Moz-Ala-OEt	Phe-NH_2	E	Moz-Ala-Phe-NH_2[i]	6	76
14	Boc-Glu(Bzl)-OBzl	NH_2-NH-Ph	B	Boc-Glu(Bzl)-NH_2-NH-Ph[j]	2	90
15	Cbz-Tyr-OMe	D-Arg-OMe	B	Cbz-Tyr-D-Arg-OMe[k]	2	81
16	Cbz-Ala-Phe-OMe	Ala-pNA	B	Cbz-Ala-Phe-Ala-pNA[l]	2	92
17	Cbz-D-Phe-OMe	Ala-NH_2	E	Cbz-D-Phe-Ala-NH_2	12	0

All the amino acids used are of L-configurations except where specified. @ E, ethanol; B, t-butanol. + 500 mg of subtilisin carlsberg was used. [a] Mp 182–184 °C, $[\alpha]_D$ −3.52° (c 2.5, DMF). [b] Mp 149–151 °C, $[\alpha]_D$ −35.6° (c 2.5, DMF) (lit.[20] $[\alpha]_D$ −35° (DMF), mp 158–159 °C). [c] Mp 125–129 °C, $[\alpha]_D$ −8.9° (c 2.5, DMF). Amino acid analysis: Thr:leu:Ala = 0.89:0.99:1.00. [d] Mp 194–197 °C, $[\alpha]_D$ −6.52° (c 2.5, DMF) (lit.[21] mp 244.5–247 °C). [e] Mp 197–201 °C, $[\alpha]_D$ −49.5° (c 2.5, DMF) (lit.[22] $[\alpha]_D$ −49.2° (c 1, MeOH), mp 208–210 °C). [f] Mp 154–156 °C, $[\alpha]_D$ −3.65° (c 2.5, DMF). [g] Mp 174–176 °C, $[\alpha]_D$ −26.8° (c 2.5, DMF) (lit.[23] $[\alpha]_D$ −26°, mp 185–190 °C. [h] Mp 211–213 °C, $[\alpha]_D$ −36.85° (c 2.5, DMF) (lit.[24] $[\alpha]_D$ −37° (c 0.2 M HCl), mp 208–210 °C). [i] Mp 172–176 °C; $[\alpha]_D$ −31.52° (c = 2.5, DMF). Amino acid analysis: Ala:Phe = 1.00:1.02. [j] Mp 134–136 °C, $[\alpha]_D$ −13.65° (c 2.5, DMF). [k] Mp 146–148 °C, $[\alpha]_D$ −15.6 (lit.[8a] mp 121–124 °C). [l] Mp 225–227 °C, $[\alpha]_D$ −26.48° (c 2, DMF). Amino acid Analysis: Ala:Phe = 2.00:1.01.

Selectivity. Two different reaction scales were used for peptide synthesis. For the studies of the time courses of the reactions, 0.1 mmol of the acyl donor, 3 equiv of the nucleophiles, and enzyme (0.1 mL, 0.25 AU) were used. In representative preparative-scale synthesis, 10 mmol of the acyl donor, 3 equiv of the nucleophiles, and enzyme (2.0 mL, 5 AU) were used. Table II shows the results for all the peptides tested using a kinetically controlled synthesis in absolute ethanol or anhydrous 2-methyl-2-propanol. The yields ranged from 80% to 95%, and the reaction times were relatively short. In terms of enantioselectivity, only the esters of the L-amino acid are substrates for the alcalase-catalyzed acylation of the acyl donor. In terms of regioselectivity in the alcalase-catalyzed acylation of diesters of L-amino acids such as aspartate or glutamate, only the ester α to the amino group can form the acyl donor.

For the selectivity of the nucleophile, hydrophobic amino acid amides nucleophiles were found to be suitable substrates. We reasoned that a more hydrophobic alkyl side chain causes the nucleophile to bind for a longer time in the enzyme's active site. D-Amino acid amides are also good nucleophilic substrates. The time course for the alcalase-catalyzed synthesis of Moz-Phe-Ala-NH_2 from D-Ala-NH_2 and from L-Ala-NH_2 shows that the profiles of both reactions nearly coincide. A preparative-scale reaction with the same substrates gave an 86% yield. Other compounds with an amino group, such as phenylhydrazine and 4-nitroaniline, also react. The former was a good nucleophile (yield 90%) and the latter was poor (yield <25%; see Table II).

Reactivities of Alcalase and Subtilisin Carlsberg. According to NOVO, alcalase contains no significant side activities. An enzyme assay using hemoglobin as substrate in a phosphate buffer showed that the proteolytic activity of alcalase is 2.35 AU/mL (i.e., 2.35 unit/μL liquid) and that of subtilisin carlsberg is 8.96 unit/mg (obtained with an activity of 11.6 unit/mg). The proteolytic activity of alcalase was completely inhibited when alcalase was pretreated with (*N*-tosyl-L-phenylalaninyl)chloromethane (TPCK).[11] A procedure for purification of alcalase by affinity column chromatography has been reported.[12] The purification resulted in a 211-fold increase in the activity. Alcalase has the same retention behavior as subtilisin carlsberg. We have studied the reactivity and the enantiomeric selectivity of both alcalase and subtilisin carlsberg. An activity test in phosphate buffer at 25 °C with Cbz-Lys-SBzl as a substrate showed that the same amount of hydrolysis product was produced by both enzyme-catalyzed reactions, as determined by the increment of the UV absorbance at 324 nm.[13] In another study, with Moz-Leu-OBzl as substrate in absolute ethanol at 25 °C, HPLC measurement of the concentration increase of the transesterification product, Moz-Leu-OEt, showed that, with the same reaction time and the same amount of enzyme, both alcalase and subtilisin carlsberg produced equal amounts of Moz-Leu-OEt. As far as enantioselectivity, both alcalase and subtilisin carlsberg have the same enantioselectivity in the synthesis of Moz-Phe-Ala-NH_2 by peptide bond formation with Moz-Phe-OMe as the acyl donor and D- or L-Ala-NH_2 as the nucleophile in 2-methyl-2-propanol. The kinetic profiles of subtilisin carlsberg-catalyzed synthesis of Moz-Phe-L-Ala-NH_2 and

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Moz-Phe-D-Ala-NH_2 coincide. In a preparative-scale (10 mmol) synthesis of Moz-Phe-D-Ala-NH_2, 500 mg of subtilisin carlsberg yielded the same amount of product within the same reaction time as did the alcalase-catalyzed synthesis.

The procedure given here is a new practical application of alcalase to peptide synthesis. The method is not limited to peptide bond formation. The regioselectivity of alcalase for the α-carboxyl of Asp and Glu can be used to prepare dicarboxylic acid synthons, and the enantioselectivity of the alcalase p-1 subsite makes the enzyme a good catalyst for the resolution of racemic amino acids in organic solvents. Several of the peptides prepared are biologically active (see Table II). For example, Tyr-D-Arg is an enkephalin-releasing factor[14] and Met-Leu-Phe-$NHCH_2C_6H_5$ is a chemotactic peptide.[15] Asp-D-Ala-NH_2 and Asp-D-Ala-O^{i}pr are artificial sweetners.[16,17] Ala-Phe-Ala-pNA is a chromogenic substrate for papain. The subtilisin protease has very broad specificity at its p-1′ subsite. Both D- and L-peptides and amino acids are accepted as nucleophiles at the p-1′ subsite. Perhaps many biologically active peptide derivatives, particularly those containing unusual amino acids, can be made by this enzymatic method.

The use of alkaline proteases to catalyze peptide bond formation in anhydrous solvents was limited. Recently, the application of an engineered subtilisin BPN′ variant that is stable in anhydrous dimethylformamide as catalyst for peptide bond formation was reported.[18] A comparison of the transesterification reaction in ethanol containing 5.0% water catalyzed by wild-type subtilisin BPN′ and that catalyzed by subtilisin carlsberg showed that the two enzymes have very different catalytic properties.[9] Application of an inexpensive industrial enzyme to peptide synthesis in anhydrous alcohol had not been reported before. In conclusion, the alcalase-catalyzed peptide bond formation is regioselective for the α-carboxyl of Asp and Glu residues, enantioselective for the L-amino acid substrates at the enzyme's p-1 subsite and nonselective for the amine nucleophiles. 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 solvent, thus allowing operation at high substrate concentration, (3) the reaction is highly selective for ester hydrolysis, and the peptide bonds remain intact, and (4) with hydrolysis under mild conditions, no side reactions occur during peptide bond formation, and the product is easy to isolate.

Experimental Section

Materials and Methods. Alcalase was purchased from NOVO Industrial (Denmark) as a brown liquid with a specific activity of 2.5 AU·mL^{-1}. The proteolytic activities of alcalase and subtilisin carlsberg were determined spectrophotometrically using hemoglobin as substrate and were found to be 2.35 AU/mL for alcalase and 8.96 unit/mg for subtilisin carlsberg. Alcalase was used without further purification. The amino acids were purchased from Sigma USA. L-Amino acids were used, except where specified. Thionyl chloride and benzyloxycarbonyl chloride (Cbz-Cl) were purchased from E. Merck, Germany. The substrates were prepared by the established method.[19] Optical rotation was measured on a Universal Polarimeter (Schmidt & Haensch, Germany). NMR spectra were taken on a Bruker AM-300, and chemical shifts of ^{1}H NMR and ^{13}C NMR spectra were referenced to solvent peaks. Kinetic data was measured on a Hitachi U-2000 spectrophotometer. 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, dioxane, ether, 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 ethanol (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 decantated. Ethanol (10 mL) was added again, and the same procedure was repeated three times until the water was removed completely.

Enzyme Assays. (i) Proteolytic Activities of Alcalase and Subtilisin Carlsberg. The proteolytic activities of alcalase and subtilisin carlsberg were measured spectrophotometrically by measuring the absorbency increase at 500 nm (colored by Folin-Ciocalteu's phenol reagent) due to the release of trichloroacetic acid-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 μmol of L-Tyr-OMe hydrolyzed per min.

(ii) Thio-esterase Activity. Alcalase (100 mL) or subtilisin carlsberg (30 mg) was dissolved in phosphate buffer (pH = 6.0, 5.0 mL) at room temperature. Periodically, 100-mL aliquots were removed and added to a cuvette containing 0.20 mmol of Cbz-Lys-SBzl, 0.40 mmol of 4,4′-dithiodipyridine, and 900 mL of 0.2 M phosphate buffer containing 10% v/v of DMF (pH = 8.2). The initial reaction rates were determined from time-dependent plots of the increasing absorbance at 324 nm.

(iii) Transesterification Activity. Moz-Phe-OBzl (0.25 mmol, 114 mg) dissolved in absolute ethanol (5 mL) was added alcalase (100 mL, prewashed with ethanol three times) or subtilisin carlsberg (30 mg). The resulting solution was stirred at room temperature for 25 min and quenched by the addition of enough HCl (0.10 N, 4.8–4.9 mL) to make the final volume 10 mL. The solution was centrifuged for 5 min at 3000 rpm, and aliquots (20 μL) of the supernatant were analyzed by HPLC using a RP-18 column, a UV detector at 254 nm, and 15% (v/v) acetonitrile as the eluent. The peak area corresponding to Moz-Leu-OEt was determined, and the reaction rates of transesterification were measured by fitting the area to a calibration curve for Moz-Leu-OEt.

Kinetics of Alcalase. The kinetic values of the alcalase-catalyzed reaction were determined by varying the substrate concentration and measuring the initial rates using HPLC. These initial rates were fitted to the Michaelis–Menten equation by using a nonlinear least-squares computer program. The concentration of substrates ranged from 0.005 to 0.050 M, and the amount of alcalase was increased by 10-, 100-, or 1000-folds.

Stability of Alcalase. The stability of alcalase in various cosolvents was determined by spectrophotometric measurement of the absorbency rise at 405 nm due to the release of *p*-nitrophenol (ϵ_{405} = 18.5 nm^{-1} cm^{-1}) from the substrate. In a typical reaction, 0.1 mL of alcalase 2.5L in alcohol (25 mL) was stirred

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at 35 °C to maintain homogeneity. Periodically, 50-mL aliquots were taken and added to a cuvette containing benzoylarginine *p*-nitrophenol (10.0 mmol) in 0.2 M phosphate buffer. The initial reaction rates were determined from time-dependent plots of the increasing absorbency at 405 nm. The 100% activity point was taken to be 10 s after the addition of alcalase to the alcoholic solution.

Enzymatic Peptide Bond Formation in Ethanol. Moz-Phe-Ala-NH_2. A kinetically controlled approach was utilized for peptide bond formation. Moz-Phe-OMe (10 mmol, 3.43 g), Ala-NH_2·HCl (30 mmol, 3.66 g), triethylamine (30 mmol, 4.2 mL), and alcalase 2.5L (2.0 mL, prewashed three times with ethanol) were added to absolute ethanol (50 mL). The mixture was shaken at 35 °C for 3–4 h. The resulting mixture was then diluted with ethyl acetate (300 mL) and washed with 0.5 N $NaHCO_3$, 5% citric acid, and water. After the organic solvent was evaporated under reduced pressure, Moz-Phe-Ala-NH_2 was obtained as a white, amorphous solid (3.56 g, 86%): mp 201–202 °C; $[\alpha]_D$ = +14.12° (*c* 2, DMSO); ^{1}H NMR (DMSO-d_6) δ 1.19 (d, 3 H), 2.62–3.05 (m, 2 H), 3.72 (s, 3 H), 4.16–4.26 (m, 2 H), 4.84 (s, 2 H), 6.84–7.26 (m, 9 H), 7.01, (s, 2 H), 7.43 (d, 1 H), 8.03 (d, 1 H).

Enzymatic Peptide Bond Formation in 2-Methyl-2-propanol. Moz-Phe-D-Ala-NH_2. Moz-Phe-OMe (10 mmol, 3.43 g), D-Ala-NH_2·HCl (30 mmol, 3.66 g), triethylamine (30 mmol, 4.2 mL), and alcalase 2.5L (2.0 mL, prewashed with 2-methyl-2-propanol three times) were added to anhydrous 2-methyl-2-propanol (50 mL). The mixture was shaken at 35 °C for 3–4 h. The resulting mixture was then diluted with ethyl acetate (300 mL) and washed with 0.5 N $NaHCO_3$, 5% citric acid, and water. After the organic solvent was evaporated under reduced pressure, Moz-Phe-Ala-NH_2 was obtained as a white amorphous solid (3.60 g, 87%): mp 205–206 °C; $[\alpha]_D$ = +2.13° (*c* 2, DMSO); ^{1}H NMR (DMSO-d_6) δ 1.10 (d, 3 H), 2.67–2.96 (m, 2 H), 3.72 (s, 3 H), 4.13–4.27 (m, 2 H), 4.85 (s, 2 H), 6.85–7.23 (m, 9 H), 7.07, (s, 2 H), 7.48 (d, 1 H), 8.12 (d, 1 H).

Moz-Phe-Leu-NH_2. In a similar manner, using Moz-Phe-OMe (10 mmol, 3.43 g), Leu-NH_2·HCl (30 mmol, 4.92 g), triethylamine (30 mmol, 4.2 mL), and alcalase 2.5L, Moz-Phe-Leu-NH_2 was obtained as a white, amorphous solid (4.29 g, 94%): mp 198–199 °C; $[\alpha]_D$ = −17.59° (*c* 2, MeOH); ^{1}H NMR (DMSO-d_6) δ 0.82 (d, 3 H), 0.87 (d, 3 H), 1.41–1.48 (m, 2 H), 1.52–1.61 (m, 1 H), 2.64–3.03 (m, 2 H), 3.72 (s, 3 H), 4.18–4.28 (m, 2 H), 4.84 (s, 2 H), 6.84–7.25 (m, 9 H), 6.98 (s, 2 H), 7.44 (d, 1 H), 7.95 (d, 1 H).

Moz-Phe-D-Leu-NH_2. In a similar manner, using Moz-Phe-OMe (10 mmol, 3.43 g), D-Leu-NH_2·HCl (30 mmol, 4.92 g), triethylamine (30 mmol, 4.2 mL), and alcalase 2.5L, Moz-Phe-D-Leu-NH_2 was obtained as a white, amorphous solid (4.06 g, 89%): mp 133–134 °C, $[\alpha]_D$ = +14.39° (*c* 2, DMSO); ^{1}H NMR (DMSO-d_6) δ 0.76 (d, 3 H), 0.82 (d, 3 H), 1.41–1.54 (m, 3 H), 2.68–2.92 (m, 2 H), 3.61 (s, 3 H), 3.72 (s, 3 H), 4.25–4.36 (m, 2 H), 4.84 (s, 2 H), 6.85–7.25 (m, 9 H), 7.37 (d, 1 H), 8.42 (d, 1 H).

Moz-Phe-D-Leu-OMe. In a similar manner, using Moz-Phe-OMe (10 mmol, 3.43 g), D-Leu-OMe·HCl (30 mmol, 5.41 g), triethylamine (30 mmol, 4.2 mL), and alcalase 2.5L, Moz-Phe-D-Leu-OMe was obtained as a white amorphous solid (3.56 g, 78%): mp 133–134 °C; $[\alpha]_D$ = +16.25° (*c* 1, MeOH); ^{1}H NMR (DMSO-d_6) δ 0.71 (d, 3 H), 0.78 (d, 3 H), 1.25 (m, 1 H), 1.35 (m, 2 H), 1.35 (m, 2 H), 2.69–2.94 (m, 2 H), 3.72 (s, 3 H), 4.08–4.26 (m, 2 H), 4.85 (s, 1 H), 6.85–7.31 (m, 9 H), 7.03 (s, 2 H), 7.51 (d, 1 H), 8.16 (d, 1 H).

Moz-Phe-Phe-NH_2. In a similar manner, using Moz-Phe-OMe (10 mmol, 3.43 g), Phe-NH_2·HCl (30 mmol, 5.94 g), triethylamine (30 mmol, 4.2 mL), and alcalase 2.5L, Moz-Phe-Phe-NH_2 was obtained (4.02 g, 82%): mp 240–241 °C; $[\alpha]_D$ = −25.50° (*c* 2, DMF); ^{1}H NMR (DMSO-d_6) δ 2.55–3.05 (m, 4 H), 3.72 (s, 3 H), 4.08–4.56 (m, 2 H), 4.83 (s, 2 H), 6.84–7.37 (m, 14 H), 7.14 (s, 2 H), 7.39, (d, 1 H), 7.80 (d, 1 H).

Moz-Phe-D-Phe-NH_2. In a similar manner, using Moz-Phe-OMe (10 mmol, 3.43 g), D-Phe-NH_2·HCl (30 mmol, 5.94 g), triethylamine (30 mmol, 4.2 mL), and alcalase 2.5L, Moz-Phe-D-Phe-NH_2 was obtained (4.46 g, 91%): mp 198–199 °C; $[\alpha]_D$ = 8.18° (*c* 2, DMSO); ^{1}H NMR (DMSO-d_6) δ 2.55–3.07 (m, 4 H), 3.72 (s, 3 H), 4.13–4.23 (m, 1 H), 4.41–4.52 (m, 1 H), 4.82 (s, 2 H), 6.83–7.30 (m, 14 H), 8.31 (d, 1 H).

Moz-Phe-Phe-O^{t}Bu. In a similar manner, using Moz-Phe-OMe (10 mmol, 3.43 g), Ala-NH_2·HCl (30 mmol, 7.71 g), triethylamine (30 mmol, 4.2 mL), and alcalase 2.5L, Moz-Phe-Phe-O^{t}Bu was obtained (3.47 g, 63%): mp 92–94 °C; $[\alpha]_D$ = +5.83° (*c* 2, DMSO); ^{1}H NMR (DMSO-d_6) δ 1.40 (s, 9 H), 2.77–3.14 (m, 4 H), 3.76 (s, 3 H), 4.40–4.45 (m, 1 H), 4.54–4.59 (m, 1 H), 4.95 (s, 2 H), 6.86–7.31 (m, 9 H).

Cbz-Asp-D-Ala-O^{i}Pr. Cbz-Asp-OBzl (8 mmol, 2.84 g), D-Ala-O^{i}Pr·HCl (24 mmol, 4.02 g), triethylamine (24 mmol, 3.36 mL), and alcalase 2.5L (5 mL, prewashed three times with 2-methyl-2-propanol) were added to anhydrous 2-methyl-2-propanol. The mixture was shaken at 35 °C for 2 h, diluted with ethyl acetate (300 mL), and washed with 5% citric acid (3 × 20 mL) and water (3 × 20 mL). After the organic solvent was evaporated, Cbz-Asp-D-Ala-O^{i}Pr was obtained as a white, amorphous solid (2.29 g, 81%): mp 201–202 °C; $[\alpha]_D$ = +13.2° (*c* 2, dichloromethane); ^{1}H NMR (DMSO-d_6) δ 1.07–1.18 (m, 9 H), 2.54–2.76 (m, 2 H), 4.04–4.11 (m, 2 H), 4.40–4.35 (m, 1 H), 4.40 (s, 2 H), 7.30–7.36–7.26 (m, 5 H), 7.74, (d, 1 H), 8.03 (d, 1 H).

Moz-Asp(Bzl)-D-Ala-NH_2. Moz-Asp(Bzl)-OBzl (10 mmol, 4.77 g), D-Ala-NH_2·HCl (30 mmol, 5.03 g), triethylamine (30 mmol, 4.2 mL), and alcalase 2.5L (2 mL, prewashed three times with 2-methyl-2-propanol) were added to anhydrous 2-methyl-2-propanol (50 mL). The mixture was shaken at 35 °C for 2.5 h. The resulting mixture was then diluted with ethyl acetate (300 mL) and washed with 0.5 N $NaHCO_3$, 5% citric acid, and water. After the organic solvent was evaporated under reduced pressure, Moz-Asp(Bzl)-D-Ala-NH_2 was obtained as a white, amorhous solid (3.94 g, 86%): mp 154–156 °C; $[\alpha]_D$ +11.88° (*c* 2, DMSO); ^{1}H NMR (DMSO-d_6) δ 1.16 (d, 3 H), 2.64–2.79 (m, 2 H), 3.73 (s, 3 H), 4.15–4.26 (m, 1 H), 4.41–4.49 (m, 2 H), 4.94 (s, 2 H), 5.06 (s, 2 H), 6.88–7.33 (m, 9 H), 7.06 (s, 2 H), 7.60 (d, 1 H), 8.02 (d, 1 H).

Cbz-Ala-Phe-Ala-pNA. Cbz-Ala-Phe-OMe (10 mmol, 3.72 g), Ala-pNA (30 mmol, 6.39 g), and alcalase 2.5L (2 mL, prewashed three times with ethanol) were added to absolute ethanol (50 mL). The mixture was shaken at 45 °C for 2 h. The resulting mixture was then diluted with ethyl acetate (300 mL) and recrystallized from methanol/ether (1:3, 50 mL) to give Cbz-Ala-Phe-Ala-pNA (3.14 g, 92%): mp 225–227 °C; $[\alpha]_D$ −26.48° (*c* 2, DMF); ^{1}H NMR (DMSO-d_6) δ 1.09–1.11 (d, 3 H), 1.32–1.34 (d, 3 H), 2.81–3.07 (m, 2 H), 3.95–4.00 (m, 1 H), 4.37–4.42 (m, 1 H), 4.51–4.54 (m, 1 H), 4.94–4.99 (m, 2 H), 7.13–7.41 (m, 10 H), 7.84–8.33 (m, 4 H).

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Supplementary Material Available: Plot of yield of Moz-Phe-Ala-NH_2 vs time and ^{1}H NMR spectra for all compounds (13 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.