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PROLINE AS NUCLEOPHILE IN KINETICALLY CONTROLLED PEPTIDE SYNTHESIS CATALYZED BY ALCALASE IN 2-METHYL-2-PROPANOL.

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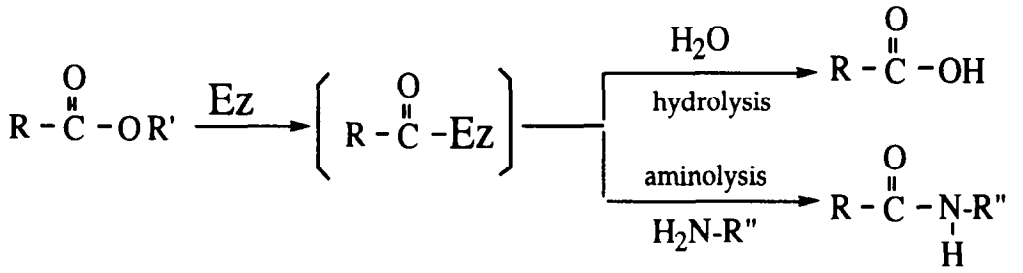
Summary

Procedures have been developed to synthesize proline-containing peptides in good yields and high purities via a kinetically controlled approach using an industrial alkaline protease, alcalase, as a catalyst in anhydrous 2-methyl-2-propanol. The yield of the reaction was dependent on the structure of the acyl-donor and the water content in the solvent. Using tripeptide as an acyl donor, the yield was higher than that using dipeptide, and when amino acid derivatives were used the yield was the lowest. The yield was also dependent on the concentration of water present in the solvent. The higher the water concentration in the solvent, the lower was the yield in the reaction. Both L-proline or D-proline derivatives could be used as nucleophiles in the reaction.

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 catalyze peptide synthesis in organic solvents.[2,3] That an alkaline protease, alcalase, can maintain activity, stability, and catalyzed peptide bond formation in anhydrous alcohol has been documented.[3c,d] We have found that the enzyme can accept proline as a nucleophile in kinetically controlled peptide bond formation in 2-methyl-2-propanol. This report describes our study of the alcalase-catalyzed synthesis of proline containing peptides in anhydrous 2-methyl-2-propanol.

Alcalase is a proteolytic enzyme prepared from the submerged formation of a selective strain of *Bacillus licheniformis*. The major enzyme component of alcalase is the subtilisin Carlsberg (alkaline protease A), which is an extracellular protease[4] and is commercially available as a brown liquid.[5] The alcalase is

obtained as a brown liquid, and the water can be removed from the alcalase solution by repeated washing with anhydrous alcohol.[6] The kinetically controlled reaction was carried out in 2-methyl-2-propanol. In a typical reaction, Moz-Phe-OMe (3.63 g, 10 mmol), Pro-NH_2 (3.93 g, 30 mmol) in 2-methyl-2-propanol (20 mL) and alcalase 2.5L (2mL, pretreated with anhydrous 2-methyl-2-propanol) was stirred at 25°C. After all the Moz-Phe-OMe disappeared (after about 48 hours, monitored by hplc), the mixture was diluted with ethyl acetate (200 mL). The resulting solution was washed with 5% citric acid (3x 25 mL), water (3x 25), 5% sodium bicarbonate (3x25 mL), dried over anhydrous sodium sulfate, and evaporated to offer crude Moz-Phe-Pro-NH_2, which was purified via silica gel flash column chromatography eluted with MeOH:CH_2Cl_2 (4:1, v/v) to yield pure Moz-Phe-Pro-NH_2 (1.96g, 46% yield) mp:174-176°C. The results of ^{1}H-NMR spectra and amino acid composition analysis are shown onTable 1.



Scheme 1

The kinetically controlled synthesis catalyzed by serine and cysteine proteases is widely used due to the short reaction times and low enzyme concentrations required.[7] Scheme 1 shows a typical scheme for this approach. In the presence of nucleophiles, an acyl-enzyme intermediate [R-C(=O)-Ez] can be deacylated competitively by water or by amino-nucleophile [:NH_2-R"]. The yield of the reaction is determined by both the relative rate of hydrolysis and aminolysis, and the ratio of the concentration of each nucleophile (i.e., water and amine). In order to study the effect of water concentration on the yield, a reaction of Moz-Phe-OMe and Pro-NH_2 in 2-methyl-2-propanol containing 0-10% of water was carried out at room temperature, and the conversion of the product was measured by hplc. Figure 1 shows the percentage of the product present in solvent with various amounts of water. In anhydrous solvent, the yield was 59%, and in 0.5% of water the yield wa still 52%. When the concentration of water in the reaction solution was increased to 1%, the yield decreased to 18%, and in 10% of water the yield was 0%. The water present in the reaction solution

affects the yield when the concentration of water is higher than 1%.

The substrates were simply prepared using the established method.[8] The solubility of the substrates in 2-methyl-2-propanol was high. All the following reactions were carried out in anhydrous 2-methyl-2-propanol. In the same manner, many proline containing peptides were prepared using esters of N-protected amino acid esters or peptide as acyl donors and L- or D-Pro derivatives as nucleophiles. Table 1 shows the reaction conditions and the results of each synthesis. The physical properties of the products were confirmed by mp, amino acid analysis of peptide hydrolysate, and ^{1}H-NMR spectra. The yield of the reaction was dependent on the structure of the acyl-donor. When the acyl donor had Phe at the S_1-subsite, the yield of the product obtained was higher than when the acyl donor had Ala at the S_1-subsite. Using peptides as acyl donors the yield was higher than when using amino acid derivatives. The reaction was not affected by the stereochemistry of the nucleophiles; using either derivatives of L-proline or D-proline as a nucleophile in reaction, the yields of the products were the same. The C-terminal protecting group of the proline could affect the reaction yield. The nucleophile with OBzl as C-terminal protecting group, the yield was higher than the nucleophile with amide at C-terminal. We reasoned that the hydrophobic substrate was more acceptable as a nucleophile than the hydrophilic one. We also found that the contaminants in the alcalase solution would affect the yield of the reaction. When the alcalase was prepared by pre-dialysis in phosphate buffer (pH 6.2) containing 0.1% of calcium chloride for 48 hous and then precipitation with 2-methyl-2-propanol, most of the contaminants in the nutrition broth were removed by this process. With the same substrates, using the prepared enzyme in the

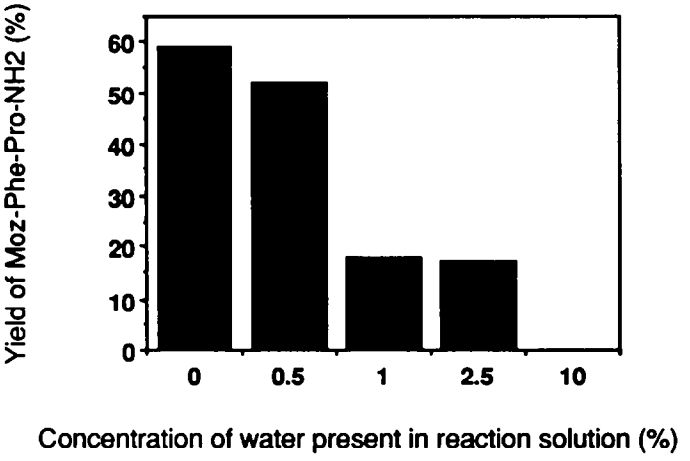


Figure 1. The yields of Moz-Phe-Pro-NH v.s. various concentration of water in an alcalase-catalyzed peptide-bond formation in 2-methyl-2-propanol at 25 C.

Table 1. Alcalase catalyzed synthesis of proline-containing peptides.

acyl-donor[a]	nucleophile	Product	reaction time[b] days	yield[b] %	MP: °C	amino acid analysis[c]
Moz-Phe-OMe	Pro-NH_2	1 Moz-Phe-Pro-NH_2[d]	2	46	174-176	Phe:Pro=1.00:1.03
Moz-Phe-OMe	D-Pro-NH_2	2 Moz-Phe-D-Pro-NH_2	2	45	oil	Phe:pro=1.00:1.04
Cbz-Ala-OMe	D-Pro-OBzl	3 Cbz-Ala-D-Pro-OBzl	4	39	oil	Ala:Pro=1.00:1.07
Cbz-Ala-OMe	Pro-OBzl	4 Cbz-Ala-Pro-OBzl	4	30	oil	Ala:Pro=1.00:1.05
Moz-Ala-OBzl	Pro-NH_2	5 Moz-Ala-Pro-NH_2	4	38	112-116	Ala:Pro=1.00:1.05
Cbz-Ala-Ala-OMe	Pro-NH_2	6 Cbz-Ala-Ala-Pro-NH_2	4	30	oil	Ala:Pro=2.00:1.20
Cbz-Ala-Ala-OMe	Pro-OBzl	7 Cbz-Ala-Ala-Pro-OBzl	4	54	oil	Ala:Pro=2.00:0.82
Cbz-Ala-Phe-OMe	Pro-NH_2	8 Cbz-Ala-Phe-Pro-NH_2	4	59	138-142	Ala:Phe:Pro=1.05:1.00:1.01
Cbz-Ala-Phe-OMe	D-Pro-OBzl	9 Cbz-Ala-Phe-D-Pro-OBzl	4	86[e]	oil	Ala:Phe:Pro=1.07:1.00:0.96
Cbz-Ala-Pro-Phe-OBzl	Pro-NH_2	10 Cbz-Ala-Pro-Phe-Pro-NH_2	5	55	157-159	Ala:Phe:Pro=1.2:1.0:1.9
Cbz-Lys(TFA)-OMe	Pro-OBzl	11 Cbz-Lys(TFA)-Pro-OBzl	4	30	oil	Lys:Pro=1.00:1.09
Cbz-Lys(TFA)-OMe	Pro-OBzl	11 Cbz-Lys(TFA)-Pro-OBzl	4	83[e]	oil	Lys:Pro=1.00:1.09

a. All amino acids used were of L-configuration except specialized. abbreviation. Moz: methoxylbenzyloxycarbonyl; Cbz: benzyloxycarbonyl.

b. The reaction was stopped after the acyl-donor was disappeared and the yield was not optimal.

c. peptides were hydrolyzed in 12N HCl/TFA 1:1 at 140°C for 3 hours and the dried-hydrolysates were analyzed on Beckman 6300 high performance amino acid analyzer.

d. ^{1}H-NMR of 1: $CDCl_3$ δ 1.16-1.19 (d 3H), 1.20-1.22 (d 3H), 1.37-1.41 (d 3H), 2.87-3.11(m 2H), 3.60 (S 3H), 3.89-3.99 (q 1H), 4.06-4.21(m 2H), 4.52-4.59(q 1H), 7.09-7.23(m 5H); 2: $CDCl_3$ δ 1.01-1.05 (d 3H), 1.20-1.24 (d 3H), 1.35-1.39 (d 3H), 2.80-3.20 (m 2H), 3.60 (S,3H), 3.88-3.98(q,1H), 4.04-4.23 (m,2H), 4.53-4.58 (q,1H), 7.08-7.25 (m, 5H); 3: $CDCl_3$ δ 1.14-1.25 (m, 2H), 1.30-1.44 (m, 4H), 2.49 (m,2H), 3.37-3.71 (m 1H), 3.94-4.14 (m 3H), 4.23-4.38 (m 1H), 4.64 (s 2H), 5.00-5.45 (m 2H), 5.45-5.53 (m 2H), 7.23-7.55 (m 10H); 4: $CDCl_3$ δ 1.73-1.93 (m 3H), 2.10-2.16 (m 2H), 2.89-2.95 (m 1H), 3.30-3.31 (m 1H), 3.49-3.51 (m 2H), 3.53-3.87 (m 2H), 4.66 (s 1H), 5.40 (q 3H),7.05-7.35 (m 10H); 5: DMSO-d_6 d 1.18-1.21 (d 3H), 1.27-1.30 (d 3H), 1.30-2.07 (m 6H), 2.92-3.06 (m 2H), 3.77-3.93 (m 1H), 4.30-4.56 (m 3H), 7.16-7.30 (m 5H), 8.04 (S 2H), 8.15-8.19 (d 1H), 8.56-8.60 (d 1H); 6: DMSO-d_6 δ 0.84 (d 1H), 1.04 (d 4H), 1.66-1.75 (m 3H), 1.80-1.88 (m 1H), 2.06-2.14 (m 1H), 2.88-3.00 (m 3H), 4.12 (d 1H), 4.90-5.05 (m 5H), 5.95 (d 1H), 6.42 (s 1H), 7.02-7.60 (m 10H); 7: $CDCl_3$:δ 1.08-1.23 (m 6H), 1.68-1.93 (m 5H), 2.11-2.20 (m 1H), 3.06-3.12 (m 2H), 3.35-3.43 (m 2H), 4.02-4.20 (m 2H), 4.90-5.10 (m 3H), 7.10-7.27 (m 10H).; 8: $CDCl_3$ δ 0.89-1.23 (m 5H), 1.66-1.88 (m 4H), 2.07-2.14 (m 1H), 2.89-3.00(m 3H), 4.64 (s 1H), 4.90-5.06(m 5H), 5.90 (s 1H), 6.42 (s 1H), 7.02-7.38 (m 10H); 9 $CDCl_3$ δ 1.07-1.24 (m 5H), 1.71-1.77(m 3H), 1.82-1.89 (m 1H), 2.10-2.16 (m 1H), 2.94-3.08 (m 3H), 3.86-3.91 (q 1H), 5.09-5.17(m 5H), 7.24-7.35 (m 10H); 10: $CDCl_3$ δ 0.99-1.23 (m 4H), 1.72-1.76 (m 4H), 1.88-2.09 (m 9H), 3.00-3.13 (m 5H), 4.35-4.39 (m 3H), 4.89-5.06 (m 2H), 6.97-7.49 (m 10H); 11: $CDCl_3$:δ 1.72-2.26 (m 9H), 3.23-3.24 (m 1H), 3.44-3.48 (m 1H), 3.45 (q 3H), 3.69-3.75 (s 4H), 3.89-4.01 (m 3H), 5.11 (q 1H), 7.22-7.34 (m 10H).

e. Pre-dialysed alcalase was used in the reaction.

reaction, the yield was increased from 30-40% to 83-86%. (entry 8, & 10). Cbz-Ala-Pro-OBzl and Cbz-Lys(TFA)-Pro-OBzl are precusors of the designed inhibitors of angiotensin converting enzyme that currently widely used.[9]

In conclusion, proline derivatives have been used as acyl donors to synthesize proline containing peptides catalyzed by thermolysin.[10] This report is the first case that proline was used as a nucleophile to synthesize proline containing peptides. Using alcalase that was pre-dialysised to removed contaminants gave the best yield. The yield of the reaction was dependent on the concentration of the water present in the solvent. The higher the water concentration was in the solvent, the lower was the yield. Both L-proline and D-proline derivatives could be used as nucleophiles in the reaction. Using peptides as acyl donors gave higher yields than using amino acids as acyl donors. The enzyme has high esterase activity in an organic solvent and can be obtained inexpensively.

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References.

1). a) C. H. Wong, K. T. Wang, *Experientia*, **1991**, *47*, 1123.
b) V. Schellenberger, H. D. Jakubke, *Angew Chem, Int. Ed. Engl.* **1991**, *30*, 1437.
c) A. M. Klibanov. *Chemtech.* **1986**, *16*. 354.
d) L. E. S. Brink, J. Tramper, K. Ch. A. M. Luyben, K. V. Riet, *Enzyme Microb. Technol.* **1988**, *10*, 736.
e) Y. L. Khmelnitsky, A. V. Levashov. N. L. Klyachko, K. Martinek, *Enzyme Microb. Technol.* **1988**, *10*, 710.
f) M. N. Gupta, *Eur. J. Biochem.* **1992**, *203*, 25.
g) M. Waks, *Proteins: Structure, Function, and Genetics*, **1986**, *1*, 4.
h) J.S. Dordick, *Biotechnol. Prog.* **1992**, *8*, 259.; i) S. H. M.V. Erp, E.O. Kamenskaya, Y. L. Khmelnitsky, *Eur. J. Biochem.* **1991**, *202*, 379.

2). a) P. Kuhl, P. J. Halling, H. D. Jakubke, *Tetrahydron Letters* **1990**, *31*, 5213.
b) R. Affleck, Z .F. Xu, V. Suzawa, K. Focht, D. S. Clark, J.S. Dordick, *Proc. Natl. Acad. Sci.USA*, **1992**, *89*, 1100.
c) H. Kitaguchi, A. M. Klibanov, *J. Amer. Chem. Soc.* **1989**, *111*, 9272.
d) M. Stahl, M. O. Mansson, K. Mosbach, *Biotech. Letters*, **1990**, *12(3)*, 161.
e) H. Kise, *Bioorg. Chem.* **1990**, *18*, 107.
f) H. Kise, Y. Tomiuchi, *Biotech. Letters* **1991**, *13*, 317.
g) T. Nagashima, A. Watanabe, H. Kise, *Enzyme Microb. Technol.*, **1992**, *14*. 842.
h) Z. Zhong, J. J. C. Liu, L. M. Dinterman, M. A. J. Finkelman, T.W. Mueller, M. L. Rollence,M. Whitlow, C.H. Wong, *J. Amer. Chem. Soc.* **1991**, *113*, 683.

i) C. H. Wong, S. T. Chen, W. J. Hennen, J. A. Bibbs, Y. F. Wang, J. L. C. Liu, M. W. Pantoliano, M. Whitlow, P. N. Bryan, *J. Amer. Chem. Soc.* **1990**, *112*, 945.
j) R.S., Phillips, M.S. Matthews, E. Olson, R.L.V. Tersch, *Enzyme microb. Technol.* **1990**, *12*, 731.
k) K. Chen, F.H. Arnold, *Biotechnology*, **1991**, *9*, 1073.

3). a) T. Sakurai, A. L. Margolin, A. J. Russell, A. M. Klibanov, *J. Amer. Chem. Soc.* **1988**, *110*, 7236.
b) P.A. Fitzpatrick, A. M. Klibanov, *J. Amer. Chem. Soc.* **1991**, *113*, 3166.
c) S. T. Chen, S. C. Hsiao, K. T. Wang, *Bioorg. & Med. Chem. Letters.* **1991**, *1(9)*, 445.
d) S. T. Chen, S. Y. Chen, K. T. Wang, *J. Org. Chem.* **1992**, *57*, 6960.
e) A.M. Klibanov, *TIBS* **1989**, **April**, 141.
f) S. Riva, J. Chopineau, A.P.G. Kieboom, A.M. Klibanov, *J. Amer. Chem. Soc.* **1988**, *110*, 584.
g) A.L. Margolin, D.F. Tai, A.M. Klibanov, *J. Amer. Chem. Soc.* **1987**, *109*, 7885.

4). Philipp, M., Bender, M. L., *Molecular & Cellular Biochem.* **1983**, *51*, 5.& references, cited herein.

5). 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 TCA-soluble product, which gives the same color of phenol reagents as 1 mequiv of tyrosine. Thus, 1AU=1000U, 1U= 1 mmol of L-Tyr-OMe hydrolyzed per min).

6). 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 (3000-4000 rpm) 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 further use. see reference 3-d.

7). a) M. L. Bender, G. E. Clement, C. R. Gunter, F. J. Kezdy, *J. Amer. Chem. Soc.* **1964**, *86*, 3097.;
b) L. J. Brubacher, M. L. Bender, *Biochem. Biophys. Res. Commu.* **1967**, *27*, 176.;
c) J. Fastrez, A. R. Fersht, *Biochem.* **1973**, *12*, 2025.;
d) A. R. Fersht, D. M. Blow, J. Fastrez, ibid, **1973**, *12*, 2035.

8). a) M. Bodanszky, "Principle of Peptide Synthesis", Springer-Verlag, Berlin, 1984.
b) E. Wunsch (1974) Houben-Weyl, Vol. XV/2. Georg Thieme Verlag, Stuttgart.

9) S. C. Stinson, *C.& E News*, **1988, October 3**. p-35.

10) a) T. Oka, K. Morihara, *J. Biochem.* **1980**, *88*, 807.
b) E. Cheng, M. T. M. Miranda, M. Toninaga *Int. J. Peptide Protein Res.* **1988**, *31*, 116.
c) J. M. Ricca, D.H.G. Crout, *J. Chem. Soc. Perkin Trans. I.* **1989**, 2126.

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