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IMPROVED YIELD BY CRYOEFFECT IN KINETICALLY CONTROLLED PEPTIDE SYNTHESIS CATALYZED WITH ALCALASE

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The yields of a kinetically controlled enzymatic reaction catalyzed by alcalase in alcohols increased 15%~40% when the reaction temperature was 5°C instead of 25-30°C. High yields of biologically active peptide intermediates were obtained from reaction at 5°C.

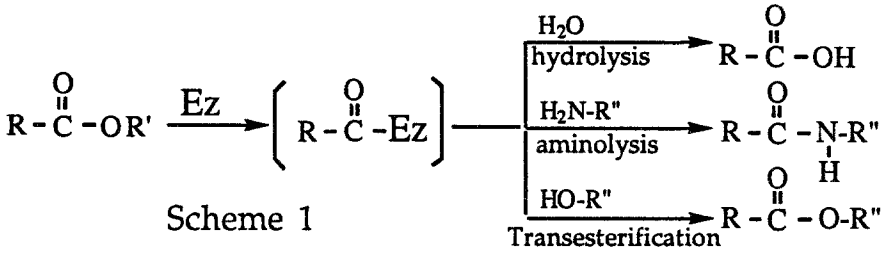
In the search for proteases that are stable in organic solvents for peptide synthesis [Wong & Wang 1991; Gupta 1992; Waks, 1986; Dordick, 1992.], several authors demonstrated that it is possible to use proteases to catalyze peptide synthesis in organic solvents [Affleck, et al., Kise & Tomiuchi, 1991; Nagashima et al., 1992.]. That the alkaline protease alcalase can maintain activity and stability, and can catalyze peptide bond formation in anhydrous alcohol, is documented [Chen et al., 1992]. We found that in these reaction conditions the rate of hydrolysis decreased as the temperature of reaction decreased and that yields increased 15%~40% with reaction at 5°C instead of 25-30°C. Here we report use of this temperature effect to systhesize peptides formerly prepared in low to moderate yield at 25-30°C. This cryoeffect of protease-catalyzed peptide-bond formation in a solvent of much organic content is not reported previously.

Alcalase is a proteolytic enzyme prepared from submerged formation of a selected strain of *Bacillus licheniformis*. The major enzyme component of alcalase is the subtilisin Carlsberg (alkaline protease A), which is an extracellular protease [Philipp & Bender, 1983] and is commercially available as a brown liquid. The kinetically controlled synthesis catalyzed by serine and cystein proteases is widely used due to the brief reaction durations and small concentrations of enzyme required [Bender et al., 1964; Fersht et al., 1973]. Scheme 1 is a typical for this approach. In the presence of nucleophiles, an acyl-enzyme intermediate [R-C(=O)-Ez] is deacylated competitively by water or by amino-nucleophile [:NH_2-R"]. The yield of the

Abbreviation:

Moz-, p-methoxybenzyloxycarbonyl- ; Cbz-, Benzyloxycarbonyl-; OBzl, benzyl ester; OMe, methyl ester; Bu^t-, t-butyl ester.

reaction is determined by the relative rates of hydrolysis and aminolysis, and the ratio of concentrations of the nucleophiles (i.e., water and amine).



Scheme 1

Experimental part.

Alcalase was purchased from NOVO industrial (Denmark) as a brown liquid with a specific activity 2.5 AU/mL. (According to NOVO, one Anson-unit (AU) is the amount of enzyme that, 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 μmol of L-Tyr-OMe hydrolyzed per min). The enzyme was pretreated according to the reported procedure [Chen et al., 1992]. The amino acids were purchased (Sigma USA). The substrates were prepared using the established method. Optical rotation was measured on a Universal Polarimeter (Schmidt & Haensch, Germany). A pH controller (Suntex P.C. 303 Auto-pH Controller Suntex Instruments Co., Taiwan) was used. Tlc was performed on silica gel G pre-coated plates (E. Merck., Germany). Methanol, ethyl acetate, dichloromethane, acetonitrile, dioxane, ether, and acetone (hplc grade and reagent grade) were obtained from a local supplier (ALPS Chem. Co. Taiwan).

Preparation of alcalase solution. A typical procedure to remove water and contaminants from the alcalase solution follows: the enzyme solution (5 mL) was dialysized in phosphate buffer (10 mM, pH 6.2, containing 1% $CaCl_2$) for 36 h; the resulting solution was precipitated with t-butanol (50 mL); the precipitate was suspended in anhydrous t-butanol by agitation, the resulting mixture was centrifuged (5000 rpm) to separate the enzyme from the solvent, and the t-butanol was removed by decantation. The agitation and centrifugation were repeated several times, and the enzyme was then transferred to a reaction flask for further use.

Transesterification activity of Moz-Leu-OBzl (as example). To Moz-Leu-OBzl (0.25 mmol, 114 mg) dissolved in absolute ethanol (5 mL) was added alcalase (100 μL, prewashed with ethanol three times). The resulting solution was stirred at the desired temperature for 25 min and quenched on the addition of enough HCl (0.10 M, 4.8-4.9 mL) to make the final volume 10 mL . The solution was centrifuged for 5 minutes at 3000 rpm, and aliquots (20 mL) of the supernatant were analyzed by HPLC with a RP-18 column, a uv detector at 254 nm, and 15% (v/v) acetonitrile as 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.

Synthesis of Cbz-Ala-Pro-OBzl (as example),

To the solution of Pro-OBzl.HCl (1.548 mg, 6.4mmol) dissolved in t-butanol (10 mL) was added triethylamine (1.1 mL, 7.5 mmol) at 5°C and the resulting mixture was stirred until the Pro-OBzl completely dissolved. To the solution was added Cbz-Ala-OMe (848 mg, 3.6 mmol) and alcalase (4 mL, 10 AU) at 5°C and stirred for another 60 h. The resulting solution was partitioned between ethyl acetate (250 mL) and saturated citric acid solution (20 mL). The organic layer was separated and washed with saturated $NaHCO_3$ solution (2x30 mL), water (2x20 mL), and dried over anhydrous sodium sulfate. Concentration of the organic solvent in vacuo yielded Cbz-Ala-Pro-OBzl (360 mg). ^{1}H-NMR: δ ppm 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).

Results & Discussion

That alcalase maintained enzymatic activity in tertiary alcohols such as t-butanol or t-amyl alcohol is documented [Chen et al, 1992.]. Here t-amyl alcohol was used because it does not solidify at low temperature (t-butanol, mp 24°C). In order to find the best conditions for using alcalase in peptide synthesis at various temperatures, the stability of alcalase incubated in t-amyl alcohol was examined. Figure 1 shows the stability of the alcalase at various temperatures. At 5°C the enzyme maintained 95% of its transesterification activity for more than 100 h. The decrease of enzymatic activity in organic cosolvents is documented [Barbas et al. 1988.]. In t-amyl alcohol containing ethanol (10%), the initial rate of transesterification of Moz-Phe-OBzl to Moz-Phe-OEt catalyzed by alcalase at 43°C, 25°C and 5°C was found to be 314.2, 189.4 and 105.9 μmol/minAU, respectively. Results show that alcalase with a transesterification activity 105.9 μmol/minAU at 5°C is efficient for kinetically controlled peptide synthesis.

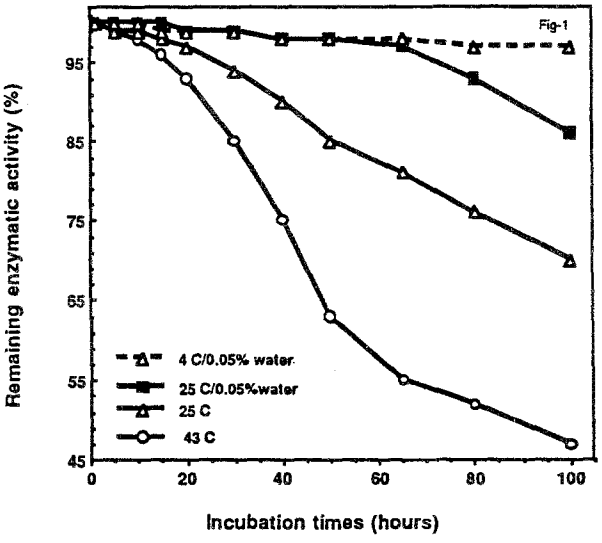


Figure 1. The remaining activity of alcalase after incubated in t-amyl alcohol at various temperature.

Because water on small proportions in the reaction solution maintains the enzyme at a high reactivity

and stability, the cryoeffect on hydrolysis was examined in ethanol containing water (0.05%). In a preliminary test, Moz-Phe-OMe in ethanol was reacted at 43, 35, 25 and 5°C, respectively. The reaction was followed by the disappearance of the substrate using HPLC; concentrations of the hydrolysis product, Moz-Phe-OH, and the transesterification product, Moz-Phe-OEt, were measured. Table 1 shows the percentage conversion of each product present in each reaction solution at various temperatures. In anhydrous ethanol, the yield of Moz-Phe-OEt increased as the temperature decreased, whereas at 43°C, the concentration of Moz-Phe-OH was six times that at 5°C. The yield of Moz-Phe-OEt was similar when 0.05% of water was present in the reaction solution. Small amounts may not affect the yield.

Table I. Yield of Moz-Phe-OH and Moz-Phe-OEt in reaction of Moz-Phe-OMe in ethanol.

Solvents	temperature (°C)	reaction time (hrs)	Moz-Phe-OEt (%)	Moz-Phe-OH (%)
EtOH	43	4	78.1	21.9
"	35	5	85.5	14.5
"	25	7	91.0	9.0
"	5	24	96.6	3.4
H_2O(0.05%) in EtOH	35	5	77.2	22.8
"	25	7	84.3	15.7
"	5	24	96.5	3.5

Preparative scale peptide synthesis was carried out at 43, 25 and 5°C. In a typical reaction, Moz-Phe-OMe (3.63 g, 10 mmol), Ala-NH_2HCl (3.93 g, 30 mmol) in t-amyl alcohol (20 mL) and alcalase (2mL, pretreated by dialysis with phosphate buffer and precipitate, with t-amyl alcohol) was stirred at the each desired temperature. After all Moz-Phe-OMe disappeared (monitored by HPLC), the mixture was diluted with ethyl acetate (200 mL). The resulting solution was washed with citric acid (5%, 3x25 mL), water (3x25 mL), and sodium bicarbonate (5%, 3x25 mL), dried over anhydrous sodium sulfate, and evaporated to offer crude Moz-Phe-Ala-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-Ala-NH_2. In a similar manner, many peptides were obtained in high yield. Results show that significantly increased yields were obtained when the reaction was conducted at 5°C rather than 25-30°C, compared with previously report small or poor yields of alcalase-catalyzed reactions. The results and physical data are shown in Table II.

The product yield of enzymatic peptide synthesis in a frozen aqueous system increased using papain, V8 protease, Chymotrypsin at -13°C ~ -25°C [Schuster et al. 1991; Schuter et al. 1990] and at -22°C using Chymotrypsin in N,N-dimethyl formamide as cosolvent has been documanted [Kurt et al. 1992]. A suitable

reaction temperature to increase the yield of trypsin-catalyzed semisynthesis of human insulin must be below 12°C and preferably at 4°C [Morihara et al. 1986] All those reactions were carried out in aqueous solution containing organic cosolvents (10-40%). In our present work the reactions were carried out in amyl alcohol with water (0.05%) at 5°C. The results demonstrate that with this large propartion of organic solvent, the temperature effect is still valid; this effect improved the yields of peptides that are formed in poor yields in normal synthetic conditions. In conclusion, this report is the first about the cryoeffect found for a large proportion of organic solvent. The reaction temperature depressed the rate of hydrolysis, thus increasing the yield of peptide bond formation catalyzed by alcalase in t-amyl alcohol. At 5°C, the reactivity of alcalase-catalyzed transesterification was decreased, but the catalytic efficiency still resulted in peptide bond formation with high yields.

Table II Comparison of alcalase-catalyzed peptide bond formation at various temperatures

			Reaction conditions		
Acyl donor	nucleophile	product[a]	time (hr)	temp.(°C)	Yield (%)
Moz-Phe-OMe	Ala-NH_2	Moz-Phe-Ala-NH_2	48	5	90
"	"	"	4	25	79
Moz-Phe-OMe	Phe-OBu^t	Moz-Phe-Phe-OBu^t	60	5	80
"	"	"	20	30	45
Cbz-Ala-Phe-OMe	Ala-NH_2	Cbz-Ala-Phe-Ala-NH_2	72	5	87
"	"	"	24	30	48
Cbz-Ala-OEt	Phe-NH_2	Cbz-Ala-Phe-NH_2	96	5	95
"	"	"	2	25	71
Cbz-Leu-OBzl	Ala-NH_2	Cbz-Leu-Ala-NH_2	96	5	96
"	"	"	2	25	71
Moz-Phe-OMe	Leu-Leu-NH_2	Moz-Phe-Leu-Leu-NH_2	22	5	80
"	"	"	3	30	64
Cbz-Ala-OMe	Pro-OBzl	Cbz-Ala-Pro-OBzl[@]	72	5	47
"	"	"	96	25	30

a), Moz-Phe-Ala-NH_2: $[\alpha]^{25}_D$=+14.12 (c 2, DMSO); mp: 201-202° ; ^{1}H-nmr (D_6-DMSO): δ 1.19 (d, 3H), 2.62-3.05 (m, 2H), 3.72 (s, 3H), 4.16-4.26 (m, 2H), 4.84 (s, 2H), 6.86-7.26 (m, 9H), 7.01 (s, 2H), 7.43 (d, 1H), 8.03 (d, 1H); Moz-Phe-Phe-OBu^t: $[\alpha]^{25}_D$=+5.83 (c 2, DMSO); mp: 92-94° ; ^{1}H-nmr (D_6-DMSO): δ1.40 (s, 9H), 2.77-3.14 (m, 4H), 3.76 (s, 3H), 4.40-4.45 (m, 1H), 4.54-4.59 (m, 1H), 4.95 (s, 2H), 6.86-7.31 (m, 9H); Cbz-Ala-Phe-Ala-NH_2: $[\alpha]^{25}_D$=-6.52 (c 2.5, DMF); mp:194-197°; ^{1}H-nmr ($CDCl_3$): δ 1.36 (m, 6H), 2.76-2.81 (m, 1H), 2.94-2.99 (m, 1H), 3.53-3.57 (m, 3H), 5.05 (s, 2H), 7.30-7.48 (m, 10H); Cbz-Ala-Phe-NH_2: $[\alpha]^{25}_D$=+18.1 (c 4.13, MeOH); mp:Oil; ^{1}H-nmr ($CDCl_3$): δ 1.56 (m, 3H), 2.76-2.81 (m, 1H), 2.94-2.99 (m, 1H), 3.53-3.65 (m, 2H), 5.05 (s, 2H), 7.18-7.30 (m, 10H); Cbz-Leu-Ala-NH_2: $[\alpha]^{25}_D$=-26.8 (c 2.5, DMF); mp: 174-176°; ^{1}H-nmr ($CDCl_3$): δ 1.21-1.38 (m, 9H), 1.82 (b, 2H), 2.77-2.83 (m, 1H), 2.96-3.01 (m, 1H), 3.55-3.58 (m, 1H), 7.15-7.31 (m, 5H); Moz-Phe-Leu-Leu-NH_2: $[\alpha]^{25}_D$=+4.84 (c 3.1, MeOH); mp: 192-194°; ^{1}H-nmr ($CDCl_3$): δ 0.85 (b, 12H), 1.58-1.70 (m, 2H), 2.93 (b,3H), 3.12 (b, 3H), 3.73 (s, 3H), 4.26-4.38 (m, 3H), 4.94 (s, 2H), 7.10-7.24 (m, 9H);

@) Cbz-Ala-Pro-OBzl is a precusor of a ranin inhibitor currently used clinically.

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