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# Stable Industrial Protease Catalyzed Peptide Bond Formation in Organic Solvent.

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Procedures have been developed for peptide bond formation in ethanol using the industrial enzyme "Alcalase" as a catalyst.

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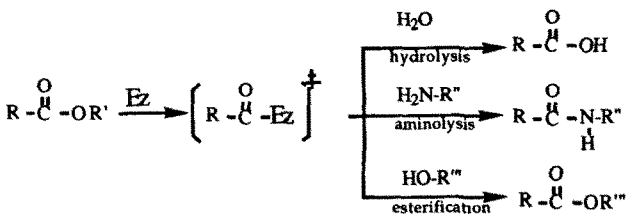
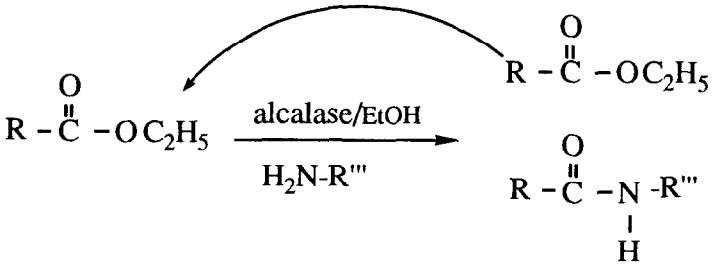
Recently, the search for proteases that are stable in organic solvents for peptide synthesis has been the subject of extensive investigation.[1] Several studies have demonstrated the possibility of using proteases to catalyse peptide synthesis in organic solvents.[2] We have found that an industrial alkaline protease, "alcalase", can maintain enzymic activity in ethanol solution and catalyse peptide bond formation in high yield using a kinetically controlled approach. The peptide bond formation catalyzed by alcalase in an organic solvent has not been reported before.

The kinetically controlled synthesis catalysed by serine and cysteine proteases is widely used. Since only short reaction times and low enzyme concentrations are required.[3] 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). Using ethanol as the solvent in the reaction, esterification of the acyl-enzyme by alcohol will occur instead of hydrolysis by water. This will result in the formation of product, or transesterification to form an new ester [R-COOEt]. The ester in turn will quickly form an acyl-enzyme intermediate for further deacylation.




Scheme 1

"Alcalase" is a proteolytic enzyme from a selective strain of *Bacillus Licheniformis*. The major enzyme component in alcalase is Subtilisin Carlsberg (alkaline protease A), which is a serine protease.[4] The esterolytic activity of alcalase is broad and in general, the bulky aliphatic or aromatic amino acid esters are preferred as acyl donors. This requirement is easily met with the use of a common amino protecting group such as benzyloxycarbonyl (CBZ), t-butyloxycarbonyl (Boc), and p-methoxybenzyloxycarbonyl (Moz). We have used methyl, ethyl, and benzyl esters of N-protected amino acids or peptides as acyl donors. With regard to nucleophile, unprotected peptide can be used as well as C-terminally protected amino acids. The time required for the reaction is short, because the alkaline protease has a very high esterolytic activity, and formation of an acyl-enzyme intermediate is fast.




The stability of alcalase[5] in ethanol and in aqueous solution at pH=8.2 were studied respectively. The rate of inactivation of the enzyme was measured on the basis of the remaining activity.[1] Figure 1 shows the results; the alcalase has a half-life time of 24 h in absolute ethanol and 6 h in aqueous solution. In a preliminary test, alcalase (0.5 μL, 1.25 AU),[5] Moz-Ala-OMe (0.274 g, 1.0 mmol), and ethanol (10 mL) were stirred at 35°C. Periodically, 50 ml aliquots were taken and analysed on hplc. The initial rates of transesterification was determined from time dependent plots of the increase of concentration of Moz-Ala-OEt or the decrease of concentration of Moz-Ala-OMe. The rate of transesterification is about 66.6 $mmol.min^{-1}.AU^{-1}$. In a similar test with the same reaction condition but containing Phe-$NH_2$ (0.84 g, 5 mmol) as a nucleophile, the appearance of product and disappearance of each substrate were measured. Figure 2 shows the time course of this reaction. The Moz-Ala-OMe disappeared within half an hour while Moz-Ala-OEt and Moz-Ala-Phe-$NH_2$ were formed and the concentration of Moz-Ala-Phe-$NH_2$ increased constantly until the end of reaction. This reaction profile coincides with the proposed reaction that Moz-Ala-OMe forms an Acyl-Ez [Moz-Ala-Ez] and is then attacked by

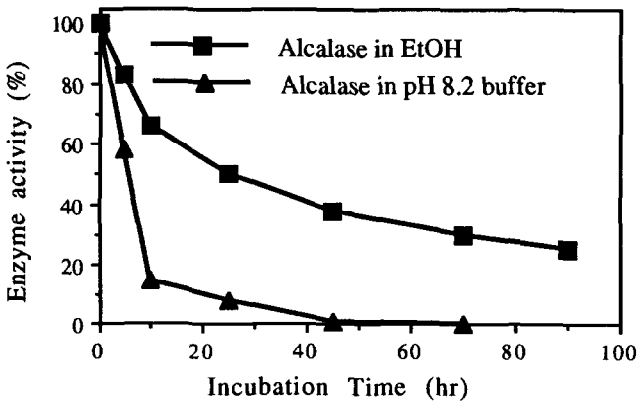



Figure 1: Stability of alcalase in phosphate buffer (0.2 M, pH 8.2), and ethanol, respectively. 100% activity was defined as the esterolytic activity of an aliquot of the mixture towards the Bz-Lys-SBzl as monitored at 324 nm ($\varepsilon$=15,000 $M^{-1}cm^{-1}$), 10 s after addition of the incubated enzyme solution to the reaction mixture.

the nucleophile (Phe-$NH_2$ and ethanol) to form the product and new amino ester (Moz-Ala-OEt). A small amount of hydrolysis by-product (Moz-Ala-OH) was observed at the reaction. This is due to the water molecules that come with the enzyme solution. In a representive preparative scale reaction, alcalase (3 mL, 7.5 AU), Moz-Ala-OMe (1.37 g, 5.0 mmol), Phe-$NH_2$ (4.21 g, 25. mmol), and ethanol (50 mL) were stirred at 35°C, until all the acyl-donors had been consumed (tlc, about 6 h). To the reaction mixture was added ethyl acetate (250 mL), and the resulting mixture was washed with 0.1 M HCl (3x30 mL), 0.5 M $NaHCO_3$ (3x30 mL), and then water (3x30 mL). The organic solution was concentrated and ether was added to it to precipitate the crude product. It was recrystallized with ethyl acetate/hexane (3:1) to give pure Moz-Ala-Phe-$NH_2$ (1.67 g, 76% yield). In a similar manner, amino acids or peptide esters, which have commonly used the N-protecting group in peptide synthesis, were used as acyl-donor and reacted. The results are summarized in table 1. The physical properties of the products were confirmed by NMR, amino acid analysis of peptide hydrolysate or with authentic samples. The D-amino acid of each synthetic peptide was measured by gc analysis of the TFA-derivatives of the peptide hydrolysates using a chiral column.[6]

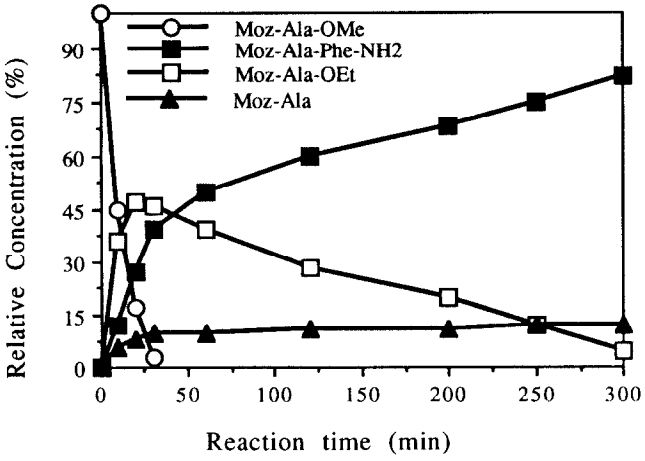



Figure 2: The time course of Moz-Ala, Moz-Ala-OEt, Moz-Ala-Phe-$NH_2$, formation and of Moz-Ala-OMe disappearance. The concentration of each component was monitored by hplc using an RP-18 column.

Hydrophobic amino acid amides as nucleophile were found to be suitable substrates. The dipeptide without the C-terminal protecting group was also acceptable. The nucleophile with the hydrophobic C-terminal protecting group such as Met-OTMB (entry 4), or Phe-$NHCH_2Ph$ (entry 5,8,10), were the most favorable substrates tested. We reasoned that a substrate with a hydrophobic ester group makes the nucleophile more hydrophobic and bind for a longer time in the enzyme's active site to form the peptide bond. In enantioselectivity of nucleophiles, the D-amino acid amide could be reacted, But the yield is low. Using D-Ala-$NH_2$ as nucleophile resulted in a yield (57%), whereas no reaction occurred in using D-Phe-OBzl or D-Tyr-OTMB as nucleophile. Compounds with an amino group such as Phenylhydrazine and 4-nitroaniline, could be reacted. The former was a good nucleophile (yield. 90%) and latter was poor (yield$<$25%). In selectivity of the acyl-donor, only the esters of L-amino acid could be reacted, and amount the esters of amino di-acids, only $\alpha$-position could react.

In summary, the procedure illustrated here describes the practical application of alcalase in peptide synthesis using a new approach. The method is not limited to peptide bond formation. The regioselectivity of alcalase at the $\alpha$-position of Asp or Glu could be used to prepare dicarboxylic acid synthons, and the enantioselectivity of the alcalase makes the enzyme a good catalyst for the resolution of racemic amino acids in an organic solvent. The amino acid hydrazide is interesting, for it has been employed as a C-terminal protecting group in enzyme-catalyzed peptide synthesis.[7] The amino acid 4-nitroanilide is a chromogenic substrate[8] which is widely used in enzyme assay. The enzyme has high esterolytic activity in an organic solvent and can be obtained inexpensively.

Table 1. Alcalase catalyzed synthesis of peptides.

| Acyl doner | Nucleophile | product | rxn time (hr) | yield (%) |
|---|---|---|---|---|
| 1. Moz-Ala-OEt | Phe-$NH_2$ (2.0 mmol) | Moz-Ala-Phe-$NH_2$[a] | 6 | 36 |
| " | (4.0 mmol) | " " | 6 | 45 |
| " | (6.0 mmol) | " " | 6 | 66 |
| " | (10. mmol) | " " | 10 | 68 |
| 2. Cbz-Tyr-OBzl | Gly-Gly | Cbz-Tyr-Gly-Gly-OH[b] | 8 | 60 |
| 3. Moz-Thr-Leu-OMe | Ala-$NH_2$ | Moz-Thr-Leu-Ala-$NH_2$[c] | 10 | 69 |
| 4. Moz-Trp-OBzl | Met-OTMB | Moz-Trp-Met-OTMB[d] | 4 | 90 |
| 5. Boc-Met-Leu-OBzl | Phe-$NHCH_2Ph$ | Boc-Met-Leu-Phe-$NHCH_2Ph$[e] | 6 | 85 |
| 6. Moz-Phe-OBzl | Leu-$NH_2$ | Moz-Phe-Leu-$NH_2$[f] | 6 | 68 |
| 7. Cbz-Ala-Phe-OMe | Ala-$NH_2$ | Cbz-Ala-Phe-Ala-$NH_2$[g] | 10 | 69 |
| 8. Boc-D,L-Asp-OBzl | $NH_2$-NH-Ph | Boc-L-Asp-$NH_2$-NH-Ph[h] | 5 | 95 |
| 9. Boc-Leu-OBzl | Met-$NH_2$ | Boc-Leu-Met-$NH_2$[i] | 4 | 76 |
| 10. Boc-Glu(Bzl)-OBzl | $NH_2$-NH-Ph | Boc-Glu(Bzl)-$NH_2$-NH-Ph[j] | 4 | 90 |
| 11. Cbz-D,L-Leu-OBzl | Ala-$NH_2$ | Cbz-L-Leu-Ala-$NH_2$[k] | 6 | 71 |
| 12. Cbz-D,L-Ala-OBzl | Thr-$NHNH_2$ | Cbz-L-Ala-Thr-$NHNH_2$[l] | 6 | 65 |
| 13. Moz-Leu-OBzl | D-Ala-$NH_2$ | Moz-Leu-D-Ala-$NH_2$[m] | 10 | 55 |
| 14. Moz-Leu-OBzl | D-Phe-OBzl | Moz-Leu-D-Phe-OBzl | 10 | trace |

a) mp: 172-176°C, $[\alpha]_D$: -31.52 (c=2.5, DMF), amino acid analysis: Ala:Phe=1.00:1.02; b) mp:146-149°C, $[\alpha]_D$: -12.67, (c=2.5, DMF); 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: 127-131°C, $[\alpha]_D$: + 3.57 (c=2.5, DMF); e) mp: 197-201°C, $[\alpha]_D$: -49.5 (c=2.5, DMF), Lit[9] $[\alpha]_D$: -49.2 (c=1, MeOH), mp: 208-210°C; f) mp: 105-109°C, $[\alpha]_D$: -2.05 (c=2.5, DMF), Lit[10] $[\alpha]_D$:-2.0 (c=2 DMF), mp: 101-103°C; g) mp: 194-197°C, $[\alpha]_D$: -6.52 (c=2.5, DMF); Lit[11] mp: 244.5-247°C; h) mp: 152-154°C, $[\alpha]_D$: 11.34 (c=2.5, DMF); i) mp: 149-151°C, $[\alpha]_D$:-35.6 (c=2.5, DMF); Lit[12] $[\alpha]_D$: -35, (DMF), mp: 158-159°C; j) mp:134-136°C $[\alpha]_D$:-13.65, (c=2.5, DMF); k) mp: 174-176°C, $[\alpha]_D$: -26.8 (c=2.5, DMF); Lit[13] $[\alpha]_D$:-26°, mp: 185-190°C; l) mp: 211-213°C, $[\alpha]_D$: -36.85 (c=2.5, DMF); Lit[14] $[\alpha]_D$: -37 (c 0.2M HCl); mp: 208-210°C; m) mp: 182-184°C, $[\alpha]_D$: -24.53 (c=2.5, DMF).

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5) Alcalase was purchased from NOVO industrial as a brown liquid with a specific activity of 2.5 AU/mL. It was used without further purification. 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.

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