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Trypsin-Catalyzed Peptide Synthesis in Acetonitrile with Low Water Content

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Trypsin was immobilized to alumina by adsorption from its aqueous solutions. The activity of trypsin was increased by immobilization for peptide synthesis. With these immobilized enzymes Bz-Arg-Leu-NH_2 dipeptide synthesis was carried out. Iso amylalcohol, tetrahydrofuran and acetonitrile were used as solvents giving the highest yield in the peptide formation reaction. The yield of the peptide was strongly dependent on water content in the reaction medium. The stability of alumina-immobilized trypsin is described.

Key Words: Peptide synthesis, trypsin, immobilization, organic solvent, alumina.

Introduction

Enzymatic reactions in organic solvents are very common in recent studies, due to the high substrate and stereoselectivities of enzymes. However, in general, enzymes are liable to be deactivated by direct contact with organic solvents. In order to overcome this problem, biphasic reaction systems consisting of water and water-immiscible organic solvents have frequently been employed; examples are peptide synthesis by lipase or proteases. In these reaction systems, enzymes were solubilized in aqueous phase or immobilized on hydrophilic supports and were therefore considered to be protected from direct contact with organic solvents[1−3].

Proteases catalyzed peptide bond formation either by condensation (acid substrates) or substitution (ester or amide substrates) reactions. In both cases, in organic solvents, peptide yield is thermodynamically controlled. From previous studies, it is known that the activities of immobilized proteases in organic solvents depend on the structure of the support material[4−14].

Some authors reported that free trypsin has the ability to catalyze peptide formation in acetonitrile or aliphatic alcohols. Trypsin was chosen as the model protein for the protein immobilization since the enzymatic activity of immobilized trypsin is readily measurable[15,16].

In this study, enzyme preparations that are easy to use and have high activity were obtained. It was found that derivatives of stabilized trypsin can be used as a catalyst for peptide bond synthesis.

Experimental

Bovine pancreatic trypsin (E.C. 3.4.21.4), activity 200 Umg^{-1}, was purchased from Merck, Darmstadt, Germany. N-α-benzoyl arginine (BA), DL-leucinamide hydrochloride (LeuNH_2 HCL), glycinamide hydrochloride (Gly-NH_2 HCL), L-alaninamide hydrochloride (Ala-NH_2 HCL) and casein were obtained from Sigma Chemical Co. Other chemicals and organic solvents were also obtained from E. Merck.

Immobilization of trypsin on alumina

The enzyme trypsin (200mg) was dissolved in 3mL of phosphate buffer (0.1M pH 6). One gram alumina and 5mL cold acetone were added to the solution consecutively and then left still after stirring for ½ h at 4°C. The mixture was separated by vacuum filtration. Then the solid was washed with 10mL of cold acetone until the filtrate was free from any unbound enzyme and finally it was lyophilized.

The amount of enzyme bound on the support was found by the method described by Lowry et al.[17]. From the standard plot, the amount of enzyme present in the enzyme solution used for the immobilization reaction and that of the remaining unbound filtrate were estimated.

Determination of Enzymatic Activity of Free and Immobilized Trypsin Derivatives

This activity was determined according to the procedure described by Northrop and Kunitz[18]. The reaction with the free enzyme was carried out in the mixture of 1mL enzyme solution containing a given amount of enzyme, 2mL of 1% casein solution, and 2mL of 0.1M phosphate buffer (pH 7.6).

After incubating the reaction mixture by shaking for 20min at 35°C, the reaction was stopped by adding 1% trichloroacetic acid. The alumina was removed by filtration and the absorbance of the supernatant, read at 280 nm, was used to calculate the activity of trypsin based on calibration curves.

Peptide Synthesis

A standard reaction for peptide synthesis was carried out as follows. Organic solvent (9mL), phosphate buffer (1mL, 0.1M pH 7), Bz-Arg (28mg, 10mM), Leu-NH_2 (33mg, 20mM) and immobilized trypsin (200mg) were added to the reaction vessel. The mixture was incubated with constant reciprocal shaking (200 cycles per min) at 37°C for 24h. After the reaction, enzyme was taken from the reaction mixture. The peptide product obtained in each reaction was investigated by TLC and HPLC.

TLC was performed on glass plates coated with Silica Gel G (Merck, Darmstadt, Germany) and developed in a solvent system of n-butanol/acetic acid/water (24:6:10), vol/vol/vol). Spots were detected by spraying ninhidrin reagent.

HPLC was performed on a Waters 484 instrument equipped with a UV detector, a 3392 A Hewlett-Packard Integrator and a Hichrom RP-C_{18} column (250/4.6 mm), flow rate 1mL/min, detection at 254 nm, mobile phase with a mixture of 40% ethanol-60% water containing 0.1% phosphoric acid. The identities of the products isolated were verified by mass spectra.

Results and Discussion

The activity of free trypsin was calculated as 9.16×10^{-3}Umg^{-1} by Northrop and Kunitz's method. Optimum substrate concentration (mg/mL), enzyme concentration (mg/mL), K_m value, and V_{max} value of trypsin in free form were 1.5mg/mL, 1.5mg/mL, 2.5×10^4 M and 1.95×10^{-2} U mg^{-1}min^{-1} respectively.

After immobilization with trypsin enzyme on alumina, protein and triptic activity determinate were studied according to Lowry et al. and Northrop and Kunitz respectively. Total amount of protein bound to immobilized enzyme and its activity were determined to be 91% and 9.96×10^{-2}U g^{-1} respectively. After spectrophotometric measurements were obtained, it was found that immobilized trypsin keeps its activity for a long time. Therefore, trypsin immobilized on alumina was used as a catalyst in peptide formation reactions, because better protection of triptic activity lower time means a stronger bond of enzyme on support and better protection of peptide formation activity.

Peptide Synthesis from Bz-Arg and Amino Acid Amides

Immobilized trypsin was mixed with a solution of Bz-arg and Leu-NH_2hydrochloride in an organic solvent: iso amylalcohol, tetrahydrofuran or acetonitrile. In these mixtures, trypsin catalyzed the reaction of Bz-arg with Leu-NH_2 and the yields of Bz-arg-Leu-NH_2 after 24h at 37°C are listed in Table 1. Among the solvents used, acetonitrile gave the highest yield of the peptide. The following studies were performed by using acetonitrile as the solvent in order to avoid complicated side reactions.

Table 1. Effect of organic solvents on peptide synthesis.

Solvent	Bz-Arg-Leu-NH_2 yield, %
Iso amylalcohol	25
Tetrahydrophuran	34
Acetonitrile	48

Bz-Arg 28 mg, Leu-NH_2 33 mg, trypsin 200 mg, phosphate buffer (0.1M, pH 7) 1mL, solvent 9mL, 37°C, 24h. Immobilization was carried out with 1g of alumina.

The effect of water content in acetonitrile on the trypsin-catalyzed synthesis of Bz-Arg-Leu-NH_2 from Bz-Arg and Leu-NH_2 is shown in Figure 1. In these experiments, water content was varied by changing the amount of buffer in solution. The peptide yield exhibits strong dependency on water content for alumina-immobilized trypsin. Under anhydrous conditions, peptide formation was totally inhibited, indicating that minimal amounts of water are essential for the activation of trypsin. At higher concentrations of water, however, peptide yield decreases, probably due to the shift of equilibrium to hydrolysis. The maximum yield of the peptide by alumina-immobilized trypsin was obtained at around 5% water.

The effect of the reaction temperature on the peptide yield is shown in Figure 2. The optimum temperature was 25°C in the peptidation reaction with alumina-immobilized trypsin. Above 30°C, however, the peptide yield sharply decreased, although a 20% yield was obtained at 35°C with alumina-immobilized trypsin.

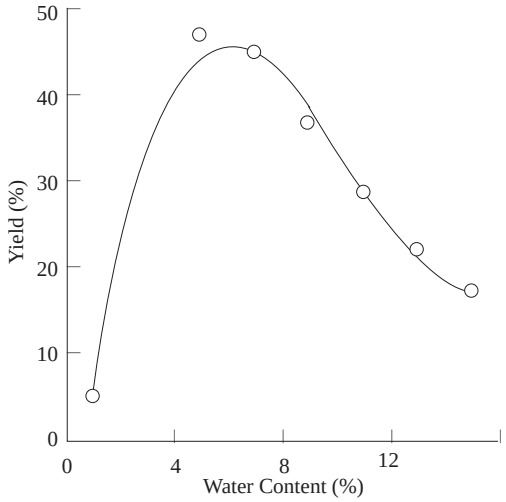

Figure 1. Effect of water content on trypsin-catalyzed peptide synthesis. Alumina-immobilized trypsin 200 mg, BA 28 mg, Leu-NH_2 33 mg, acetonitrile 9 mL, 37°C, 24 h.

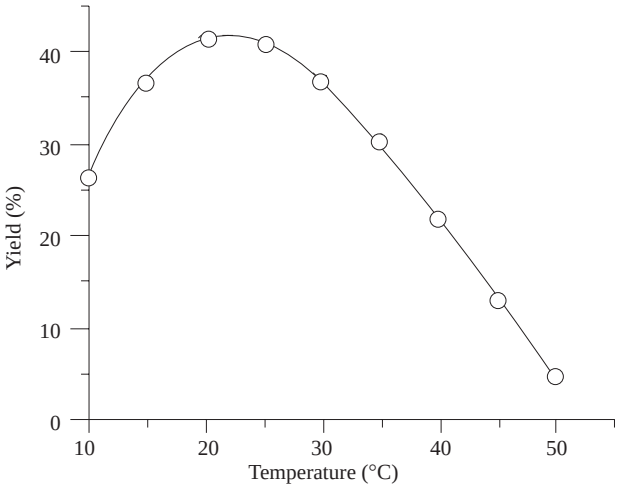

Figure 2. Effect of reaction temperature on peptide synthesis. Alumina-immobilized trypsin 200 mg, BA 28 mg, Leu-NH_2 33 mg, acetonitrile 9 mL, phosphate buffer 1mL, 24 h.

Figure 3 shows plots of the peptide yields against the pH of the buffer solution. The peptide yield was affected by pH because of immobilized trypsin. The peptide yields increased with pH. pH 7.0 seems to be the most suitable pH at which to run these syntheses.

The results of the reactions of Bz-Arg with several amino components are summarized in Table 2. Only a slight increase in the peptide yield was demonstrated in going from glycinamide to leucinamide for the reactions with immobilized trypsin. The values for these three amino acid amides were almost identical.

Table 2. Trypsin-catalyzed synthesis of peptides from BA and amino acid amides in acetonitrile.

Amino acid amide (A-NH_2)	Yield of BA-A-NH_2 / %
Glycinamide	53
L-Tyrosinamide	57
DL-Leucinamide	48

BA 28 mg, A-NH_2 33 mg, alumina-immobilized trypsin 200 mg, acetonitrile 9 mL, phosphate buffer (0.1M, pH 7) 1 mL, 25°C, 24 h.

To determine the rate of synthesis (formation of the dipeptide BA-leucinamide), the effect of BA concentration varying between 0.5 and 10mM was tested; 15mM Leu-NH_2 and 200mg alumina immobilized trypsin with BA in acetonitrile were added to the mixture. After incubating the reaction mixture under shaking for different lengths of time at 25°C, the reaction rate was calculated from the graph of dipeptide efficiency for each reaction vessel versus time.

As seen in Figure 4, synthetic rate increases with BA concentration. From the 1/synthetic rate versus 1/ [benzoyl arginine] graph, V_{max} and K_m were calculated as 8.38 mM h^{-1} and 20x10^{-3}M respectively (Figure 5).

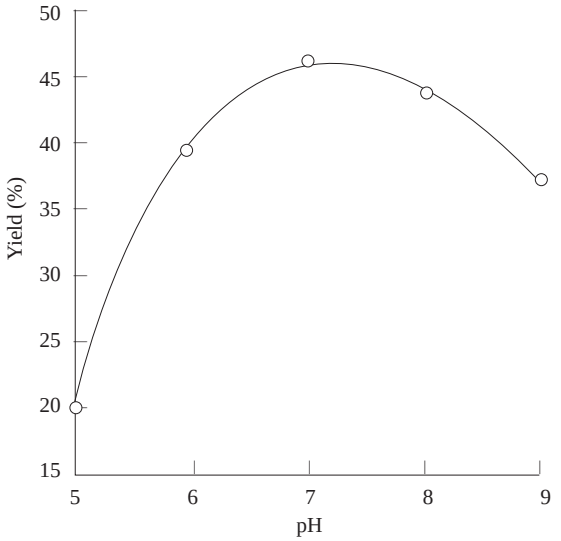

Figure 3. Effect of pH on peptide synthesis. Alumina-immobilized trypsin 200 mg, BA 28 mg, Leu-NH_2 33 mg, acetonitrile 9 mL, phosphate buffer 1 mL, 37°C, 24 h.

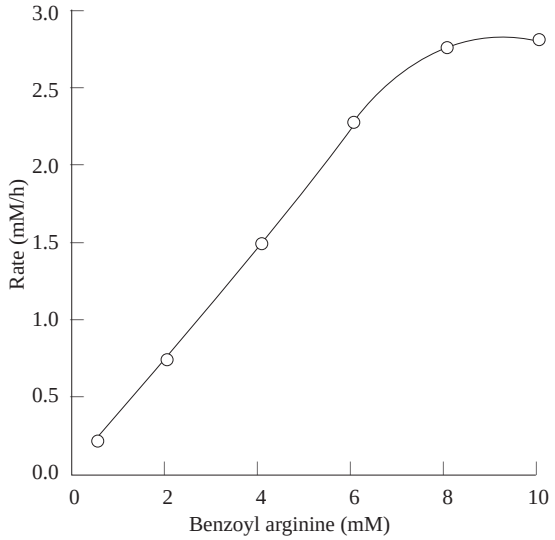

Figure 4. Effect of the concentration of BA on the synthesis of BA-Leu-NH_2. Reaction temperature 25°C, pH 7. Concentration of Leu-NH_2=15 mM.

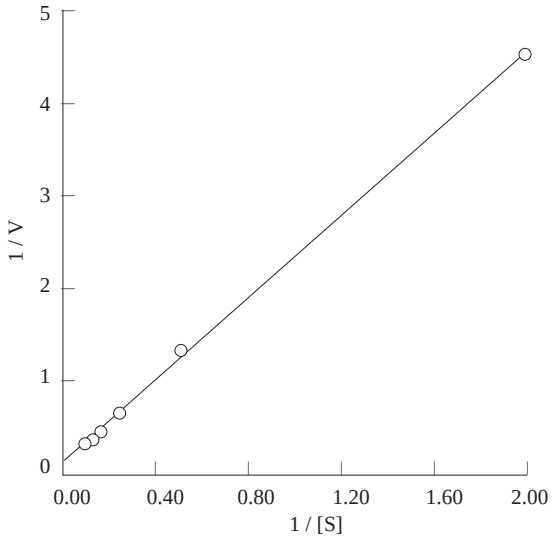

Figure 5. Lineweaver-Burk plots for the synthesis reaction of BA-Leu-NH_2. Reactions performed at pH 7, 25°C. Concentration of Leu-NH_2=15 mM.

In conclusion, trypsin can be immobilized onto alumina by a simple adsorption method, and the immobilized trypsin is a stable and efficient catalyst for peptide synthesis from Bz-Arg in hydrophilic organic solvents. No covalent binding of trypsin to alumina is required, and this may be an easy and versatile method for the immobilization of enzymes in organic solvents. For peptide synthesis, water content was found to be a primary factor influencing the reaction rate and product yields.

Acknowledgment

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