Peptide Synthesis Catalyzed by Immobilized Trypsin in Organic Solvents

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Summary: Trypsin was immobilized to poly vinyl alcohol (PVA) by adsorption from its aqueous solutions. The catalytic activity of trypsin increased markedly by immobilization for peptide synthesis from N-α-benzoyl L-arginine in hydrophilic organic solvents such as tetrahydrofuran or ethanol. The yield of Bz-Arg-Leu-NH_2 is strongly dependent on the PVA/trypsin ratio and water content in the reaction medium. The optimum pH and temperature were determined for immobilized trypsin as 7.0 and 40°C, respectively.

Introduction

Utilization of proteases as catalysts for peptide bond formation in organic solvents is still an area of active research. Peptide synthesis catalyzed by proteases is a good example of an industrial enzyme process. This enzymatic approach offers several important advantages over more classical and conventional chemical methods: no risk of racemization, the absence of side reactions, the use of milder experimental conditions, and so on [1-8].

In general, enzymes are liable to be deactivated by direct contact with organic solvents. In order to overcome this problem, biphasic reaction systems which consist of water and water-immiscible organic solvents have frequently been employed for peptide synthesis by proteases. In these reaction systems, enzymes were solubilized in aqueous phase or immobilized on hydrophilic supports and therefore considered to be protected from direct contact with organic solvents [9-18].

Enzymatic reactions in aqueous solutions with water-miscible organic cosolvents have also been applied to peptide synthesis, but reactions in high concentrations of hydrophilic organic solvents have mostly been unsuccessful due to deactivation of the enzymes [19-28]. The peptide synthesis by immobilized trypsin in various solvents have been extensively studied. Because enzymes are useful tools for many special synthetic applications in peptide and protein chemistry. Furthermore, a preliminary study on the peptide synthesis by trypsin immobilized to alumina was reported elsewhere [29].

In this paper, the details of peptide synthesis catalyzed by free or PVA-immobilized trypsin in hydrophilic organic solvents were described. Optimal conditions (range of pH values, temperature, water content and the rate of synthesis) for performing the synthesis of peptide bonds were defined. However, it was found that also native non-modified trypsin is very potent to catalyze peptide bond formation in hydrophilic organic solvents.

Results and Discussion

For future applications of the enzymatic peptide synthesis, the use of immobilized proteases will probably be of technological and economical importance.

A phosphate buffer solution of trypsin was mixed with a solution of Bz-Arg and Leu-NH_2 hydrochloride in an organic solvent, such as ethanol or tetrahydrofuran. In these mixtures, trypsin catalyzed the reaction of Bz-Arg with Leu-NH_2 and the yields of Bz-Arg-Leu-NH_2 after 24 h at 37°C were listed in Table 1. Among the solvents used, ethanol gave the highest yield of the peptide.

Table 1. Peptide Synthesis from Bz-Arg and Leu NH_2 by Free Trypsin and PVA-immobilized Trypsin.

Solvent	Yield of Bz-Arg-Leu-NH_2 (%)	
	Free trypsin	PVA-trypsin
DMF	35	16
Acetone	24	43
Acetonitrile	32	73
THF	18	68
Ethanol	40	51
	17	16

Bz-Arg 28 mg, Leu NH_2 hydrochloride 33 mg, trypsin 10 mg, phosphate buffer (0.1 M, pH 7.0) 1 mL, solvent 20 mL, 37°C, 24 h, Immobilization was done with 1 g of PVA.

When a buffer solution was added to a mixture of trypsin and PVA and then the substrate

solution was added the resulting mixture consisted of a clear solution and a gellous PVA. The change in the amount of trypsin in solution was negligible after 24 h incubation at 37°C. These results indicate that most of trypsin was immobilized through non-covalent binding to PVA matrix probably due to hydrophilic interaction between PVA and trypsin. The results of the peptide synthesis by PVA-immobilized trypsin were also shown in Table 1. In all the solvents used, especially in acetonitrile and tetrahydrofuran (THF), peptide yields were much higher than those for the reactions without PVA. It may be considered that in the PVA matrix trypsin was hydrated enough to maintain its native conformation and catalytic activity. Since in ethanol the peptide formation is competitive with ester formation, the following studies were made using tetrahydrofuran as a solvent in order to avoid complicated side reactions.

Figure 1 shows plots of the yields of the peptide against water content. In these experiments, water content was changed by changing the amount of buffer solution in which trypsin was dissolved. The peptide yield exhibits strong dependency on water content both for trypsin-phosphate and PVA-immobilized trypsin. Under anhydrous conditions, peptide formation was totally inhibited, indicating that minimum 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 PVA-immobilized trypsin was obtained at around 6 % water.

It was found that a PVA/trypsin ratio larger than 10 was required to enhance the catalytic activity of trypsin. At lower PVA/trypsin ratios, a part of trypsin was suspended in the organic phase. The peptide yield increased with the PVA/trypsin ratio up to 200.

The effect of the reaction temperature on the peptide yield was shown in Figure 2. The optimum temperatures were 40 and 35°C for reactions with and without PVA, respectively, and in the whole range of temperature studied PVA-immobilized trypsin gave higher yields of the peptide than free trypsin. Above 45°C, however, the peptide yield sharply decreased for free and immobilized trypsin, although 40% yield was obtained at 50°C by PVA-immobilized trysin.

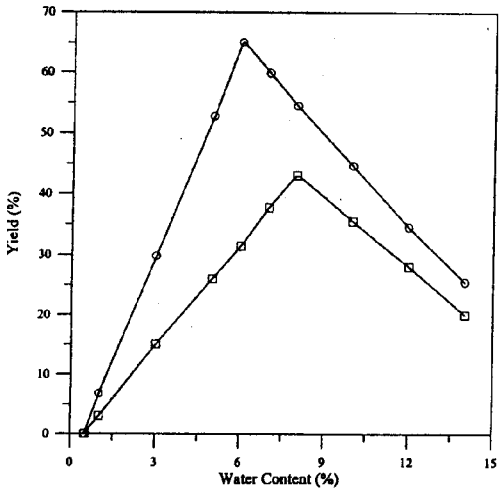

Fig. 1: Effect of water content on trypsin-catalyzed peptide synthesis. Trypsin 10 mg, Bz-Arg 28 mg, Leu-NH_2 HCl 33 mg, tetrahydrofuran 20 mL, 37°C, 24 h.o: PVA (1 g)-immobilized trypsin, □ : Free trypsin

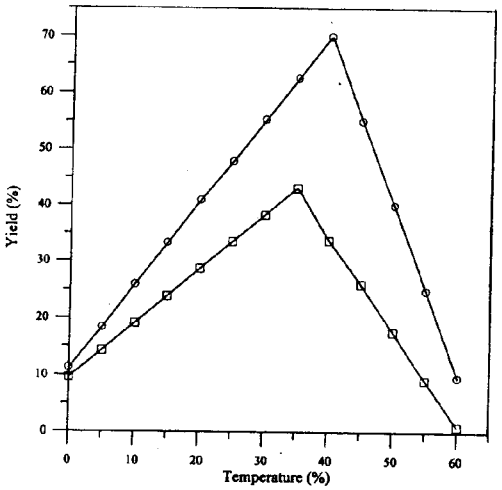

Fig. 2: Effect of reaction temperature on peptide synthesis. Trypsin 10 mg, Bz-Arg 28 mg, Leu-NH_2 HCl 33 mg, tetrahydrofuran 20 mL, phosphate buffer 1.5 mL, 24 h. o : PVA (1 g)-immobilized trypsin, □ : Free trypsin.

Figure 3 shows plots of the peptide yields against the pH of the buffer solution. In the presence of a large excess of tetrahydrofuran, the solution was

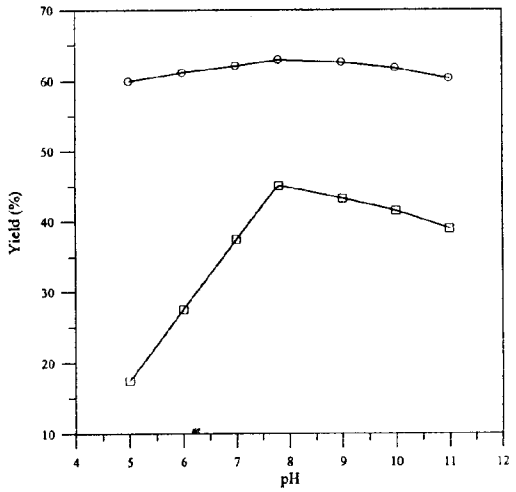

Fig. 3: Effect of pH on peptide synthesis. Trypsin 10 mg, Bz-Arg 28 mg, Leu-NH_2 HCl 33 mg, tetrahydrofuran 20 mL, phosphate buffer 1.5 mL, 37°C, 24 h. o : PVA (1 g)-immobilized trypsin, □ : Free trypsin

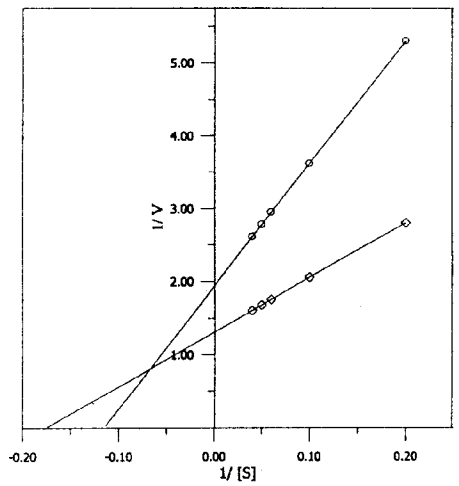

Fig. 4: Lineweaver-Burk plots for the synthesis of Bz-Arg-Leu NH_2. Leu-NH_2 HCl 33 mg, tetrahydrofuran 20 mL, phosphate buffer 1.5 mL, 37°C, pH 7.0.

considered not to be buffered, but a certain effect of salt composition in PVA matrix on the peptide formation was expected. Actually, however, the peptide yield by immobilized trypsin was scarcely affected by pH (Figure 3). Blanco *et al.*, reported the pH optimum of peptide synthesis catalyzed by immobilized trypsin to be 7 [22]. When pure water was used instead of the buffer solution, the peptide yield decreased markedly. These results were not shown. But it is known that contrary to a peptide synthesis, pure water is a good co-solvent for trypsin in the esterification of Bz-Arg by PVA-immobilized trypsin.

Three amino acid amides were tested as nucleophiles for this reaction: leucinamide, tyrosinamide and glycinamide. The results of the reactions of Bz-Arg with several amine components were summarized in Table 2. Only a slight decrease in the peptide yield was demonstrated in going from leucinamide to glycinamide for the reactions by immobilized trypsin, whereas an opposite tendency was found for the reactions by free trypsin.

The kinetic properties of free and immobilized trypsin were shown in Figure 4. We tested the effect of nucleophile concentration, varying between 0 and 25 mM. The synthetic rate greatly increase with

Table-2:Trypsin-catalyzed Synthesis of Peptides from Bz-Arg and Amino Acid Amides in Tetrahydrofuran.

Amino acid amide (A-NH_2)	Yield of Bz-Arg-A-NH_2 (%)	
	Free trypsin	PVA-trypsin
DL-Leucinamide	36	81
L-Tyrosinamide	39	78
Glycinamide	40	77

Bz-Arg 28 mg, equimolar A-NH_2 to Bz-Arg, trypsin 10 mg, phosphate buffer (0.1 M, pH 7.0) 1.5 mL, tetrahydrofuran 20 mL, 37°C, 24 h. Immobilization was done with 1 g of PVA.

nucleophile concentration for both the trypsins. In fact, reciprocal plots (1/synthetic rate vs. 1/ [leucinamide] are fairly linear (Figure 4) and allow us to calculate the maximal synthetic rates. As shown in Figure 4, the Michaelis constants (K_m) for both the trypsins were determined at pH 7.0, 37°C. The K_m value of PVA-immobilized trypsin was 5.71 mM as calculated from the Lineweaver-Burk plots, while that of free trypsin was 8.69 mM, respectively. Our findings showed that the PVA-immobilized trypsin has a good reactivity towards Bz-Arg and Leu NH_2 substrates for peptide synthesis.

In conclusion, trypsin can be immobilized into PVA matrix 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. Non covalent binding of trypsin to PVA 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 to influence the product yields. The ratio of PVA to trypsin was also an important factor for controling the reaction.

Experimental

Bovine pancreatic trypsin (E.C. 3.4.21.4) was obtained from Merck, Darmstadt, FRG. N-α-benzoyl arginine (BA), DL-leucinamide hydrochloride (Leu-NH_2 HCl), glycinamide hydrochloride (Gly-NH_2 HCl) and L-tyrosinamide hydrochloride (Tyr-NH_2 HCl) were purchased from Sigma Chemical Co. (St. Louis, MO). Poly vinyl alcohol (PVA) (degree of polimerization 1500, degree of saponification 86-89%) was supplied from Wako Pure Chemical Ind., Ltd. Other chemicals and organic solvents were also obtained from E. Merck.

Peptide Synthesis

The reaction mixture for peptide synthesis was as follows: Trypsin (10 mg) was thoroughly mixed with PVA powder (1.00 g), and then a certain amount of phosphate buffer solution (0.1 M, pH 7.0) was added to the mixture. After keeping the mixture standing for about 10 min. BA (28 mg, 10 mM) and Leu-NH_2 (33 mg, 20 mM) in tetrahydrofuran (20 mL) were added. The mixture was incubated with constant reciprocal shaking (150 cycle per min) at 37°C for 24 h. After the reaction, an aliquot was taken from the reaction mixture and, if necessary, filtered. The reaction components were determined with HPLC.

HPLC was performed on a Waters 484 instrument equipped with UV detector, 3392 A Hewlett-packard Integrator and Hichrom RP-C_{18} column (250\4.6 mm) eluted with water-acetonitrile (50:50 by volume). Acetanilide was used as the internal standard.

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