Communications to the Editor

Enzymic Peptide Synthesis in Microaqueous, Solvent-Free Systems

P. Kuhl*
Technical University of Dresden, Institute of Biochemistry, Mommsenstraße 13, D-01062 Dresden, Germany

U. Eichhorn and H.-D. Jakubke
University of Leipzig, Biosciences Division, Department of Biochemistry, Talstrasse 33, D-04103 Leipzig, Germany

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Thermolysin-catalyzed (EC 3.4.24.4) and chymotrypsin-catalyzed (EC 3.4.21.1) peptide synthesis reactions were accomplished without any organic solvent in the presence of low amounts of water under sonication and fluidization. The systems used are considered to be microaqueous solvent-free ones. The influence of several reaction parameters, such as time, the amount of enzyme, the amount of water in free form or bound as hydration water, and the N/C component ratio, on the yield of the thermolysin-catalyzed synthesis of Z–Phe–Leu–NH_2 (up to 87% yield) was investigated in a sonicated system. Besides Z–Phe–Leu–NH_2, the tripeptide derivatives Ac–Xaa–Trp–Leu–NH_2 (Xaa = Gly, Ala) were also obtained in good yields of 79 and 71%, respectively. In the latter case, no hydrolytic side reactions were observed. Using a fluidized-bed reactor, chymotrypsin- and thermolysin-catalyzed syntheses of N-protected di- and tripeptide amides could be performed with yields in the range of 10 to 40%. © 1995 John Wiley & Sons, Inc.
Key words: enzymic peptide synthesis • solvent free system, chymotrypsin • thermolysin • peptides

INTRODUCTION

Enzymic peptide synthesis has seen a tremendous development over the last two decades.[4,7,8] One important research field is the investigation of suitable methods to shift equilibria toward the desired peptide product. Among other means to meet this target, different reaction media have been used, ranging from purely aqueous through aqueous–organic (homogeneous as well as heterogeneous) to microaqueous systems. Several previous experiments revealed that it is not necessary to dissolve the reactants completely.[1,3,5,6] Even aqueous–organic heterogeneous systems containing such hydrophobic, scarcely solubilizing solvents as hexane with low amounts of water can be successfully used for enzymic coupling of amino acid and peptide derivatives in good yields. However, under these conditions effective mixing of the reactants has to provide good transport and contact of the reaction partners.

* To whom all correspondence should be addressed.

Because of the very low solubilizing properties of solvents like hexane, the question arises whether one can dispense with any organic solvent at all. This would be of great interest from the economical and environmental point of view and abolish the problem of any unfavorable solvent–enzyme interaction. In a previous paper we described the thermolysin-catalyzed synthesis of Z–Phe–Leu–NH_2 (**1**) from Z–Phe–OH and Leu–NH_2 in hexane in the presence of $Na_2SO_4 \cdot 10H_2O$.[5] We want now to report on enzymatic peptide synthesis in the presence of low amounts of water and without any organic solvent.

MATERIALS AND METHODS

Experiments with Sonication

In a typical sonication experiment 0.1 mmol of the starting materials was placed in a flat-bottomed vial of 14 mm internal diameter and 0.5 mmol $Na_2SO_4 \cdot 10H_2O$ was added. The reaction mixture was sonicated at 33 kHz, 50 W (USG 50, Meßgerätewerk, Ballenstedt, Germany), in a water bath at 40°C. The reaction was started by adding 5 mg thermolysin. After the indicated time 0.4 mL of 5% (v/v) aqueous trifluoroacetic acid and 10 mL acetonitrile were added to stop the enzyme reaction. Samples were filtered and analyzed by a Shimadzu high-performance liquid chromatography (HPLC) system consisting of an integrator (C-R4AX), a UV spectrophotometric detector (SPD-6A), two pumps (LC-6A), a system controller (SCL-6B), and an autoinjector (SIL-6B) using a Merck LICHROCART RP 18 column (250 × 4 mm^2, 5 μm). The column was eluted isocratically. The eluent contained different amounts of acetonitrile for analysis. To the aqueous phase of the eluent 0.1% of the trifluoroacetic acid was added. The peptide products were identified by comparison with authentic samples.

Experiments with Fluidized-Bed Reactor

Starting materials and the enzyme were introduced into the fluidized-bed reactor in amounts given in Table I. Air was

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blown through the reactor to such an extent that a good fluidization was attained. Additionally the whole reactor was shaken with the help of a laboratory shaker S 421 (MLW Prüfgeräte-Werk Medingen, Germany). After the indicated time the reactants were dissolved in 0.4 mL of 5% (v/v) aqueous trifluoroacetic acid and 10 mL acetonitrile. HPLC analysis was carried out as described above.

The X–Ala–Phe–OMe (X = Z, Boc) and Leu–NH_2 were purchased from BACHEM Feinchemikalien AG, Switzerland. The Z–Phe–OH and Ac–Xaa–Trp–OH (Xaa = Ala, Gly) were a generous gift by DEGUSSA AG, Germany. Thermolysin was delivered as protease type X by Sigma Chemical Company. The other chemicals were from our laboratory stock.

RESULTS AND DISCUSSION

Studying the thermolysin-catalyzed synthesis of **1** as indicated above, but omitting hexane, we found that nearly the same time course of this reaction can be observed, provided that ultrasound is used for an efficient mixing of the reactants (Fig. 1).

The positive result prompted us further to investigate the following reaction parameters: N/C component ratio, amount of thermolysin used, and amount of water applied either in free form or bound as hydration water in $Na_2SO_4 \cdot 10H_2O$.

The amount of Leu–NH_2 was varied from 0.5 to 1 mmol, whereas that of Z–Phe–OH was kept constant at 0.1 mmol. The variation of the N/C component ratio did not significantly influence the yield of **1**.

On increasing the thermolysin amount from 1 to 5 mg the yield of **1** increased from 25 to 77%. A further increase in the amount of thermolysin did not lead to higher yields within a reaction time of 30 min.

In a heterogeneous system as we have presented it here, one or both reactants may form compact particles which cannot be completely dissolved. During the reaction progress these reactant particles will be covered with precipitating product. Thus, the necessary contact with the enzyme's active site is no longer possible, and the addition of neither enzyme nor nucleophile leads to better yields.

No peptide coupling took place without any water added

Table I. Yields of thermolysin- and chymotrypsin-catalyzed peptide synthesis in a fluidized-bed reactor using Leu–NH_2 as nucleophile.

Enzyme (amount)	C component (amount; N/C ratio)	Product yield (time)
Thermolysin (5 mg)	Z–Phe–OH (0.1 mmol; 1)	**1** 34% (2h)
Chymotrypsin (10 mg)	Z–Ala–Phe–OMe (0.1 mmol; 2)	Z–Ala–Phe–Leu–NH_2 32–46% (3h)
Chymotrypsin (10 mg)	Boc–Ala–Phe–OMe (0.1 mmol; 2)	Boc–Ala–Phe–Leu–NH_2 7–20% (3h)

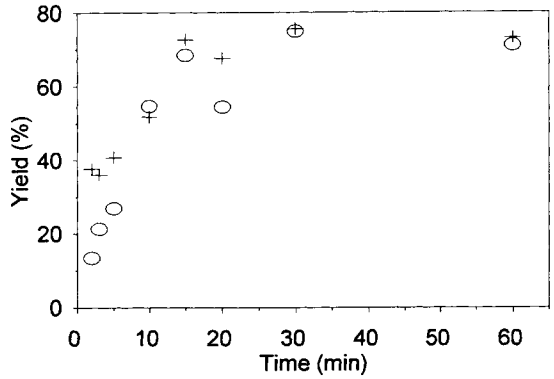

Figure 1. Time course of thermolysin-catalyzed synthesis of **1** from Z–Phe–OH and Leu–NH_2 in presence of $Na_2SO_4 \cdot 10H_2O$ with and without hexane. Reaction conditions: 0.1 mmol Z–Phe–OH and Leu–NH_2, 0.5 mmol $Na_2SO_4 \cdot 10H_2O$, 5 mg thermolysin, 40°C, sonicated with USG 50 (33 kHz, 50 W). (+) Without hexane. (○) In 2 mL hexane.

to the reaction mixture. The yields of **1** in reactions with varying amounts of water added either in free or bound form are summarized in Figure 2. As can be seen, there is a minimum amount of 1 mmol water necessary to assure a conversion of up to 80%. This water can also be added in the form of a salt hydrate. Although the yields obtained in this case were slightly lower, the use of salt hydrates may be advantageous for fragment couplings, in which minimization of hydrolytic side reactions is very important.

Hence we compared this reaction system with a pure aqueous one using Z–Ala–Phe–OH as substrate, which has a preformed Ala–Phe bond sensitive to side reactions. The desired product Z–Ala–Phe–Leu–NH_2 was obtained in yields of 65%, 74%, and 56% when the reaction was performed in the presence of 5 mmol water bound in $Na_2SO_4 \cdot 10H_2O$ in free form and in the presence of 0.5 mL water, respectively. Only 5% side products resulted, when water was applied bound in $Na_2SO_4 \cdot 10H_2O$ and increased from 19% to 28% in the case of 90 μL and 500 μL water added. Side products were identified as Z–Ala–OH,

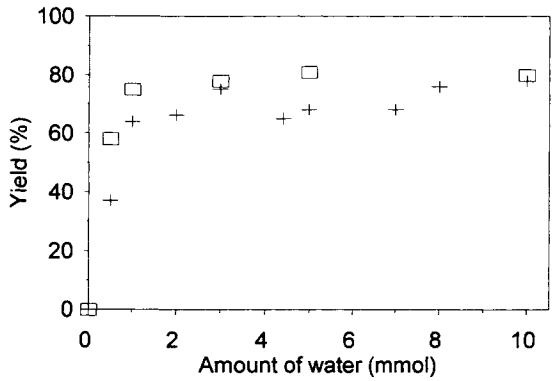

Figure 2. Yield dependence of thermolysin-catalyzed synthesis of **1** on amount of water added (in free form and bound as hydration water). Reaction conditions: 0.1 mmol Z–Phe–OH and Leu–NH_2, 5 mg thermolysin, 40°C, 20 min sonicated with USG 50 (33 kHz, 50 W). Water added: (+) bound in $Na_2SO_4 \cdot 10H_2O$; (□) in free form.

Z–Ala–Leu–NH_2, and H–Phe–Leu–NH_2 and one unidentified product occurred, with less than 1% peak area in HPLC. Further we used this reaction system to synthesize Ac–Xaa–Trp–Leu–NH_2 (Xaa = Gly, Ala) from Ac–Xaa–Trp–OH and Leu–NH_2 and obtained yields of 79% and 71%, respectively. No side reactions were observed.

The foregoing examples give evidence that enzyme-catalyzed peptide coupling can be accomplished in the presence of only low amounts of water without any other solvent. As an alternative to ultrasound for an efficient mixing of the reactants the application of a homogenizer is possible as recently published by Čeřovský.[2]

To ensure substrate and product transport, we also considered the fluidized solids technique a promising approach. For this purpose enzyme and reactants were placed into a reactor consisting of a glass tube equipped with sinter glass filters on both ends (see Fig. 3, 1a). The particles were suspended and mixed by an air stream, which had been moisturized by passing through buffer in a Dreschel bottle. Without moisturizing the reaction mixture no peptide coupling took place. A sodium bicarbonate solution was used for chymotrypsin-catalyzed reactions and a neutral buffer in the case of thermolysin. The pH of these solutions roughly corresponds to that used normally for peptide synthesis with these two proteases in aqueous media.

In the first experiments with chymotrypsin as catalyst the air stream passed a bottle with distilled water. Although the obtained yield of Z–Ala–Phe–Leu–NH_2 was 32% in the first run, it decreased to 5% in repeating experiments. Because the pH of the water in the Dreschel bottle had fallen to 5.7, we substituted it for the sodium bicarbonate solution and then obtained a yield of 46% after a reaction time of 3 h. Additionally, the whole reactor was shaken to prevent sticking of enzyme and substrate particles on the wall of the reactor. Vibrating alone, however, was not sufficient for successful peptide synthesis. At high gas velocities, separation of particles depending on their densities was observed. In order to avoid this, the velocity of the gas stream was controlled or a heart-shaped reactor was used (see Fig. 3, 1b).

Results obtained in experiments with the fluidized-bed reactor are summarized in Table I. They demonstrate that this new technique of using reactants and enzyme as solids is practicable on principle. Although the yields of peptide products are only moderate so far, one has to consider that they have been obtained in a rather simple procedure not yet being optimized. The relatively large variations in the product yield reflect the necessity of further improvements of the mixing process and may be explained by the phenomenon of compact particle formation as discussed above.

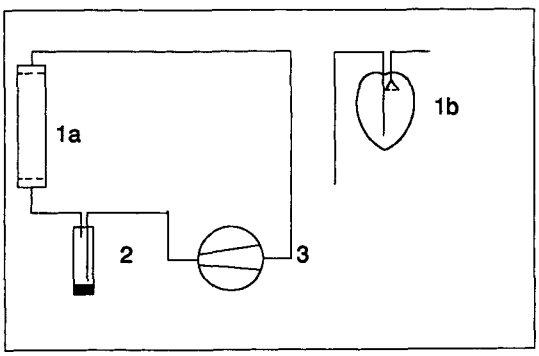

Figure 3. Schematic drawing of fluidized-bed reactor: (1a) fluidized-bed reactor; (1b) heart-shaped reactor; (2) washing flask containing buffer solution; (3) pump.

CONCLUSIONS

In this article we have demonstrated that chymotrypsin- and thermolysin-catalyzed peptide syntheses can be carried out in a micro-aqueous, solvent-free system if ultrasound or fluidization is applied.

One of the advantages of such a procedure is a relatively low water content in the reaction system, thus diminishing hydrolysis side products and favoring synthesis in water forming condensation reactions. The water necessary for maintaining protease activity can be supplied (i) by direct addition to the reaction mixture from a syringe, (ii) in the form of hydration water of a salt hydrate, or (iii) by a wet air stream.

As a further advantage, dispensing with any kind of organic solvent can be desirable in some respects, e.g., concerning enzyme stability, ensuring easy workup procedures, and for economic reasons. Although the yields obtained so far are very different, the proposed techniques may broaden the spectrum of methods in enzymic peptide synthesis and deserve attention for special applications.

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