

Studies on Enzymatic Peptide Synthesis in Biphasic Aqueous-Organic Systems with Product Extraction

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The dipeptide derivatives $Z\text{-Tyr-Leu-NH}_2$ and $Mca\text{-Tyr-Leu-NH}_2$ were synthesized by α -chymotrypsin-catalyzed coupling reactions in solvent systems consisting of buffer and ethyl acetate. In comparison to a pure aqueous medium, in which only insignificant synthesis takes place, the product formation is greatly enhanced in a biphasic medium due to extraction of the dipeptide into the organic phase. The influence of several reaction parameters, such as buffer concentration, reaction time, volume ratio of organic and aqueous phase, and reagent concentration on the yield of $Z\text{-Tyr-Leu-NH}_2$ was investigated. Replacement of the hydrophobic Z -group by the more hydrophilic chloroacetyl group resulted in better dipeptide yields at higher reaction rates.

(Keywords: Enzymatic synthesis in biphasic systems; Peptide synthesis; α -Chymotrypsin)

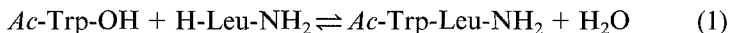
Untersuchungen zur enzymatischen Peptidsynthese in biphasischen wäßrig-organischen Systemen mit Produktextraktion

Die Dipeptidderivate $Z\text{-Tyr-Leu-NH}_2$ und $Mca\text{-Tyr-Leu-NH}_2$ wurden durch α -chymotrypsin-katalysierte Kupplung in biphasischen Systemen aus Puffer und Essigsäureethylester hergestellt. Im Vergleich zu einem rein wäßrigen Medium, in dem nahezu keine Synthese erfolgt, wird in einem biphasischen System die Synthesereaktion durch Extraktion des Dipeptids in die organische Phase wesentlich begünstigt. Es wurde der Einfluß verschiedener Reaktionsparameter, wie Pufferkonzentration, Reaktionszeit, Volumenverhältnis von organischer zu wäßriger Phase und Reaktandenkonzentration auf die Ausbeute an $Z\text{-Tyr-Leu-NH}_2$ untersucht. Ein Austausch der hydrophoben Z -Gruppe gegen die hydrophilere Chloroacetylgruppe führte zu besseren Dipeptidausbeuten bei höheren Reaktionsgeschwindigkeiten.

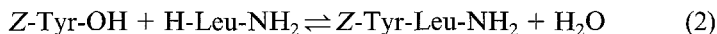
Abbreviations: IUPAC-IUB rules for peptides are followed, see Eur J Biochem 27: 201 (1972). *Ac* = acetyl, *Glt* = 4-carboxybutyryl (glutaryl), *Mca* = mono-chloroacetyl, *Z* = benzyloxycarbonyl, *-OMe* = methyl ester, *-Nan* = 4-nitroanilide, *TLC* = thin layer chromatography. All amino acids are of *L*-configuration.

Introduction

During the last decade enzymatic peptide synthesis has drawn the attention of peptide chemists as well as enzymologists due to some outstanding features of theoretical and practical importance involved with it. One of this has been the convincing demonstration, that proteases are a very useful tool in the biotechnological synthesis of biologically active peptides and proteins on a preparative scale (for some late reviews see [1–4]). When we started our studies in this field in 1979 we substituted an aqueous-organic two-phase system for the usual homogeneous aqueous-organic reaction medium. This not only enabled a better protection of the protease from unfavourable influence of the solvent, but made it also possible to control concentration of reactants, product precipitation and product extraction, resp., in a positive manner (cf. Refs. in [1]). A systematic search in the literature revealed that two-phase media had already been used successfully for various enzyme-catalyzed organic reactions (e.g. [5–7]). The application of biphasic solvent systems for peptide synthesis was first described nearly at the same time by *Martinek et al.* [8–10] and our group [11]. The results of the former in optimization of the α -chymotrypsin-catalyzed reaction [12, 13]:



confirmed our intention to determine reaction parameters of a protease-catalyzed equilibrium controlled peptide synthesis reaction under biphasic conditions using a carboxyl component with an easily cleavable N-protecting group. Therefore we began with the α -chymotrypsin-catalyzed reaction



1

and included later on *Z-Tyr-OMe* and *Mca-Tyr-OH* as further carboxyl components.

We varied the buffer concentration and *pH*, the volume ratio of solvents, the substrate/nucleophile ratio, the starting concentration of reactants and determined the equilibrium constant of the reaction as well as the change in α -chymotrypsin activity.

Results and Discussion

First we studied the kinetics of reaction (2) in biphasic systems consisting of 50 ml ethyl acetate and 1 ml buffer, corresponding to a volume ratio $\alpha = V_{\text{org}}/V_{\text{aq}} = 50$. The reactant concentration was 5 mM (referring to the total volume), and 20 mg α -chymotrypsin as catalyst were used. The solutions were gently agitated on a laboratory shaker for several

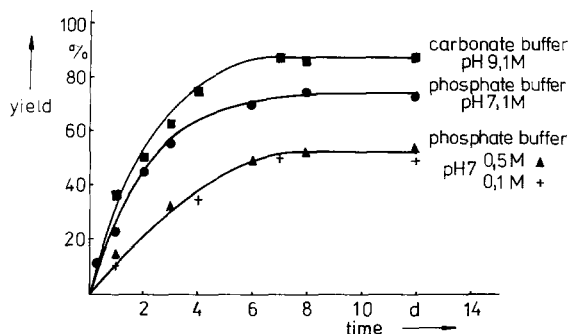


Fig. 1. Kinetics of the synthesis of **1** in ethyl acetate/buffer, $\alpha = 50$, total volume 51 ml, reactant concentration 5 mM each, 20 mg α -chymotrypsin

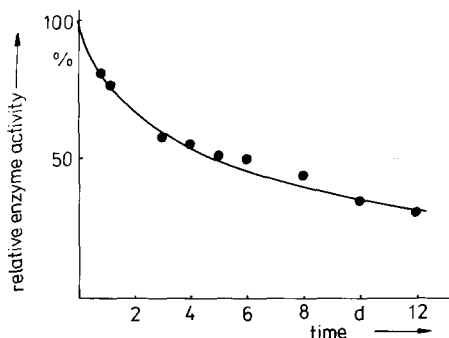


Fig. 2. Change of the α -chymotrypsin activity during the synthesis of **1** in ethyl acetate/phosphate buffer (1 M, pH 7) under the conditions given in Fig. 1 (for activity measurement see Exp.)

days until the reaction equilibrium had been attained. Figure 1 shows the progress of the peptide formation at varying buffer concentration. In all cases equilibrium was reached only between 7–8 days. When phosphate buffer of pH 7 in concentrations of 0.1 and 0.5 M was used, the equilibrium yield amounted only to about 50% and the buffer pH decreased to 5.5–6.0, although it had been adjusted at the beginning of the reaction. Using 1 M buffer the pH remained constant and the product yield increased up to 75%. An even better result could be obtained with 1 M carbonate buffer of pH 9.

Although the α -chymotrypsin activity continuously decreased with reaction time, it was sufficiently high yet during equilibrium attainment,

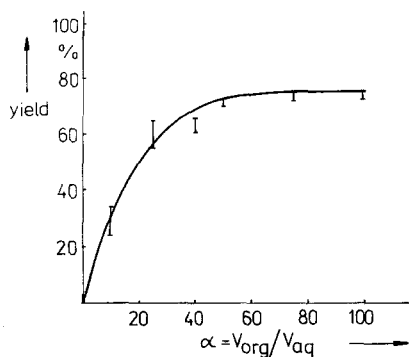


Fig. 3. The yield of **1** as a function of the volume ratio of organic and aqueous phase (α), aqueous phase 1 ml phosphate buffer (1 M, pH 7), variable total volume, reactant concentration 5 mM each, 20 mg α -chymotrypsin

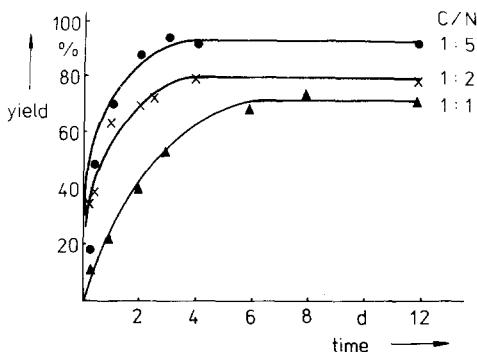


Fig. 4. Kinetics of the synthesis of **1** at different nucleophile content in ethyl acetate/phosphate buffer (1 M, pH 7), $\alpha = 50$, total volume 51 ml, [Z-Tyr-OH] = 5 mM, 20 mg α -chymotrypsin

as it can be seen from Fig. 2. Since the protease is localized in the aqueous phase, its activity is only influenced by the saturation concentration of ethyl acetate in water, and the enzyme is therefore inhibited far less than by solvents miscible with water.

The result of a synthesis reaction in a biphasic system is decisively determined by the ratio of the partition coefficients of the reactants and products, and furthermore greatly influenced by the volume ratio of the phases (for theory see [14]). Under ideal conditions, the reactants diffuse from the organic into the aqueous phase, where they undergo the enzyme-catalyzed reaction, and the product diffuses into the organic phase. Unfortunately, partition coefficients of amino acids or peptide derivatives

are scarcely known in the literature, and it must be kept in mind that they depend on the particular solvent which has to be compatible with the applied protease. Hence, it can be much easier to vary the solvent ratio in optimizing the peptide yield, provided that the reactants and the produced peptide derivative are sufficiently soluble in the aqueous and the organic phase, respectively.

In Fig. 3 the dependence of the dipeptide yield on α is represented. It follows from the experimental data that a drastic increase in the synthesis

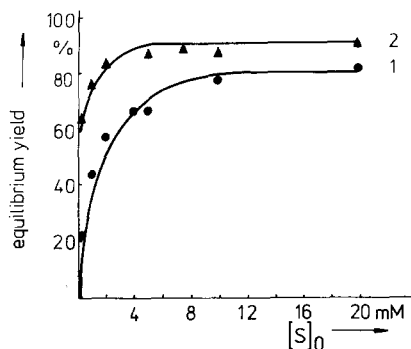


Fig. 5. The equilibrium yield of **1** and **2** as a function of reactant starting concentration $[S]_0$ (C/N-ratio 1 : 1) in ethyl acetate/phosphate buffer (1 *M*, *pH* 7), $\alpha = 25$, total volume 52 ml, α -chymotrypsin 20 mg/ml buffer

of **1** can be attained augmenting the ethyl acetate content up to 98% ($\alpha = 60$). This is in good agreement with the results obtained for model reaction (1) [13].

Figure 4 shows the effect of an increased nucleophile content on the kinetics of the product formation. As expected from the law of mass action and the rate law, both the dipeptide yield and the reaction rate are markedly influenced in favour of a more economical result. Obviously due to the less favourable partition coefficient(s) of *Z*-Tyr-OH and/or **1** the final equilibrium yield did not reach the high value as in reaction (1), although the other reaction conditions, apart from the buffer, had nearly been the same.

The concentration of the starting reactants has an important influence on the equilibrium yield of **1**. This can be seen from Fig. 5, curve 1, which demonstrates that equal concentrations of *Z*-Tyr-OH and H-Leu-NH₂ ≥ 5 mM give reasonably good yields. These were determined after 8 days to be sure that equilibrium had been attained.

Applying the law of mass action the obtained data can serve to determine the equilibrium constant *K* of reaction (2) in the aqueous-

organic system [13]. If two substrates of equal starting concentration $[S]_0$ react to form the peptide P , K is given by Eq. (3):

$$K = \frac{[P]_{\text{eq}} [H_2O]_{\text{eq}}}{([S]_0 - [P]_{\text{eq}})^2} \quad (3)$$

Denoting the reaction yield with x and substituting $x [S]_0$ for $[P]$ results in Eq. (4):

$$K = \frac{x [H_2O]_{\text{eq}}}{[S]_0 (1 - x)^2} \quad (4)$$

Because a change in the water concentration during the reaction can be neglected, it holds $[H_2O]_{\text{eq}} \approx [H_2O]_0$.

If Eq. (4) is rearranged and brought into logarithmic form, one obtains the equation of a straight line (5):

$$\lg \frac{x [H_2O]_0}{(1 - x)^2} = \lg K + \lg [S]_0 \quad (5)$$

from which K can be determined by the intercept with the y -axis in the corresponding plot. The result is shown in Fig. 6, line 1. The equilibrium constant thus obtained amounts to $5.7 \cdot 10^3$.

For comparison we also studied reaction (2) in 1 *M* phosphate buffer, *pH* 7, without addition of organic solvent, all the other conditions being the same as given in Fig. 1. After 7 days the reaction mixture had remained absolutely clear and no **1** could be detected by tlc. Hence it follows that the yield of **1** did not exceed 0.5%.

Figure 7 shows the kinetics of the formation of **1** using *Z*-Tyr-OH, *Z*-Tyr-OH in the presence of 50 mM CH_3OH and *Z*-Tyr-OMe, resp. In all cases the same progress of dipeptide synthesis was noticed, and no differences in yield or reaction rates within the experimental error were found.

As mentioned above the partition coefficients of all reactants play an important part in reactions in biphasic systems. Because the protease-catalyzed coupling step occurs in the aqueous phase, hydrophilic properties of the starting components are to be desired. Unfortunately, the most used *N*-protecting groups in classic peptide chemistry, *Z* and *Boc*, are unfavourable. On the other hand, the much more hydrophilic acetyl group cannot be cleaved without undesired side reactions implicating a considerable racemization risk. However, substitution by a single chlorine atom preserves the hydrophilic properties and enables the removal of this protecting group after the coupling by several known procedures [16].

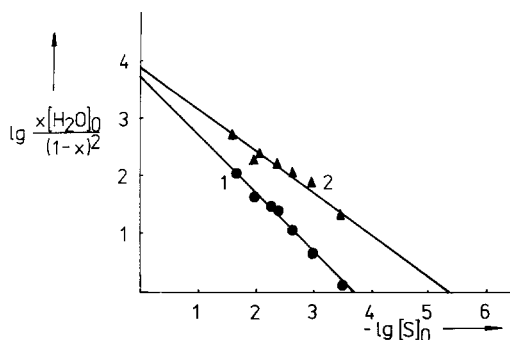


Fig. 6. Graphical representation of the experimental data of Fig. 5 using Eq. (5) for the determination of the equilibrium constants of reactions (2) and (6). x equilibrium yield of **1** and **2**, resp., $[S]_0$ starting concentration of reactants

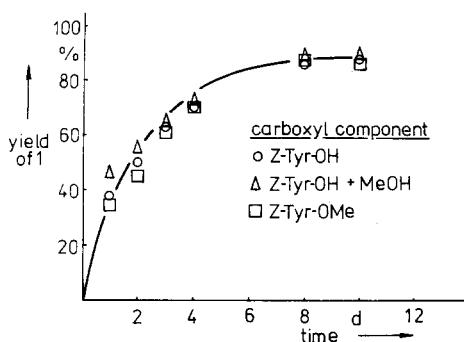
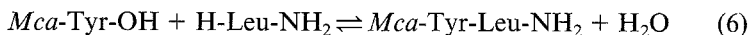


Fig. 7. Kinetics of the synthesis of **1** using *Z*-Tyr-OH, *Z*-Tyr-OH in the presence of 50 mM CH_3OH and *Z*-Tyr-OMe, resp., as carboxyl component. Solvent system ethyl acetate/carbonate buffer (1 *M*, *pH* 9), other conditions as given in Fig. 1.

On the basis of the above considerations we also investigated the kinetics of the α -chymotrypsin-catalyzed reaction (6):



2

The results are shown in Fig. 8 and compared to those for the synthesis of **1**. As we had expected, the equilibrium yield of **2** exceeded that of **1**, and probably because of higher concentrations of *Mca*-Tyr-OH in the aqueous phase the reaction proceeded faster so that the equilibrium was already attained after 5–6 days. This corresponds very well to the data obtained for reaction (1) [14].

The influence of the starting concentration of *Mca*-Tyr-OH and H-Leu-NH₂ on the equilibrium yield of **2**, as it was determined after 6 days, is shown in Fig. 5, curve 2. Reactant concentrations of 1–5 mM were sufficient to get reasonably good yields. These data have been used for the calculation of the equilibrium constant of reaction (6) according equation (5), as presented in Fig. 6, line 2. From the intercept of the straight line with the *y*-axis follows $K = 8,2 \cdot 10^3$.

The presented experimental results allow the conclusion that a biphasic system consisting of ethyl acetate/buffer is a suitable reaction medium

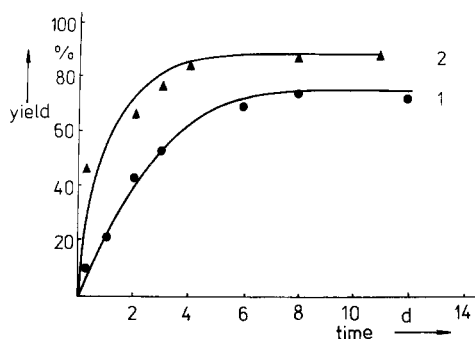


Fig. 8. Kinetics of the synthesis of **2** compared to that of **1**. Reaction conditions as given in Fig. 4

for the α -chymotrypsin-catalyzed coupling of *Z*-Tyr-OH with H-Leu-NH₂. In comparison to a pure aqueous medium, in which only insignificant dipeptide synthesis takes place, the biphasic reaction conditions due to product extraction into the organic phase give rise to an increase of the equilibrium constant by several orders of magnitude. The use of *Z*-Tyr-OMe or *Z*-Tyr-OH in the presence of little amounts of CH₃OH did not bring any advantages, but replacement of the hydrophobic *Z*-group by the more hydrophilic chloroacetyl group resulted in better dipeptide yields and greater reaction rates. Nevertheless, in all cases the reaction equilibrium was attained only after several days, which from a practical point of view is disadvantageous. Although improvements in this line by an intensified phase contact may be possible (cf. also [13]), biphasic reaction conditions, under which the synthesized peptide derivative precipitates, represent a valuable alternative (see [11] and literature cited in [1]).

Acknowledgement

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Experimental

α -Chymotrypsin from bovine pancreas (EC 3.4.21.1) (3 times crystallized and lyophilized) was commercially available from Lečiva Narodni Podnik (Prague, Czechoslovakia). Amino acids were obtained from Reanal (Budapest, Hungary). Z-Tyr-OMe [17] (yield 75%, m.p. 90–91 °C, $[\alpha]_D^{20} = -3.5^\circ$, $c = 2$ in EtOH), Z-Tyr-OH [18] from Z-Tyr-OMe (yield 85%, m.p. 98–99 °C, $[\alpha]_D^{20} = +10.4^\circ$, $c = 2$ in AcOH), Mca-Tyr-OH [19] (yield 42%, m.p. 155–157 °C, $[\alpha]_D^{20} = +56.6^\circ$, $c = 2$ in EtOH) and H-Leu-NH₂ [20] (yield 70%, m.p. 97–100 °C, $[\alpha]_D^{20} = +8.2^\circ$, $c = 5$ in H₂O) were synthesized according to known procedures. Compounds **1** (yield 72%, m.p. 209–211 °C, $[\alpha]_D^{20} = -15.1^\circ$, $c = 1$ in DMF) and **2** (yield 77%, m.p. 214–216 °C, $[\alpha]_D^{20} = -5.5^\circ$, $c = 1$ in DMF) were prepared by α -chymotrypsin-catalyzed coupling using Z-Tyr-OMe and Mca-Tyr-OH, resp., as carboxyl components. For the determination of molar absorbances a Specord UV VIS of VEB Carl Zeiss Jena was used (**1** in ethanol, $\lambda_{\max} = 280$, $\epsilon = 1\,700\,M^{-1}\,cm^{-1}$, **2** in ethanol, $\lambda_{\max} = 280$, $\epsilon = 1\,850\,M^{-1}\,cm^{-1}$). Melting points were obtained with a Boëtius apparatus and are corrected. Optical rotations are determined with a Polamat A instrument of VEB Carl Zeiss Jena using 1 dm cells. Ethyl acetate was purified and distilled prior to use.

α -Chymotrypsin-catalyzed Reactions

If not stated otherwise, 5 mM carboxyl component in a minimal volume of ethyl acetate was mixed with 1 M phosphate buffer, pH 7, which contained an equal amount of H-Leu-NH₂ and 20 mg/ml aqueous phase α -chymotrypsin. The concentrations of the reactants are referred to the total volume of the biphasic system. After adjusting the pH of the resulting mixture in cases where it was necessary, it was given into a flask, and then water-saturated ethyl acetate was added corresponding to the needed volume ratio. The closed flask was gently agitated at 20 °C on a laboratory shaker with 200 oscillations per minute.

Determination of the Reaction Yield

50–500 μ l of the organic phase were taken in certain time intervals and placed on a 8 $\times$ 12 cm silica-precoated foil (Kavalier, Czechoslovakia). TLC was performed using CHCl₃/EtOH/NH₃ (25%) (10 : 2 : 0.1) and the expected dipeptide derivative as a marker. After drying, a 3 $\times$ 12 cm strip, which contained the marker, was cut off and developed by successive treatment with chlorine and KI/starch solution. Corresponding to the position of the peptide product a 2 $\times$ 5 cm strip was cut off, reduced to small pieces and the peptide eluted with 3 ml EtOH (absolute for UV-spectroscopy) by allowing to stand for 3 h. Then the ethanolic solution was separated by centrifugation and the concentration of the product determined spectrophotometrically at 280 nm using a VSU 2 instrument of VEB Carl Zeiss Jena.

Determination of the α -Chymotrypsin Activity

At time intervals indicated in Fig. 2 5 μ l of the aqueous phase of the reaction mixture were withdrawn and given to 195 μ l water in a cuvette. Then 1.8 ml of 1 mM Glt-Leu-Phe-Nan [21] in 0.05 M Tris buffer, pH 8, were added. The rate of the α -chymotrypsin-catalyzed hydrolysis of the substrate was measured at 405 nm by determination of the liberated *p*-nitroaniline. The obtained data were related to the enzyme activity at the beginning of the synthesis reaction.

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