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OPTIMIZATION OF ENZYME CATALYZED PEPTIDE SYNTHESIS IN A "WATER - WATER-IMMISCIBLE ORGANIC SOLVENT" BIPHASIC SYSTEM

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Abstract - Optimal conditions were found for enzymatic synthesis of the dipeptide, N-acetyl-L-tryptophanyl-L-leucine amide in the biphasic system water - ethyl acetate. The synthesis was carried out using both free and immobilized α-chymotrypsin. Optimization was performed by such parameters as the "organic phase/aqueous phase" volume ratio, the pH of aqueous phase, and the concentration of starting reactants. Under most favourable conditions the dipeptide was synthesized on the preparative scale in ca. 100% yield. As a result of immobilization (adsorption on the Sorsilen terephtalate support) the enzyme practically did not inactivate and may be used repeatedly.

INTRODUCTION

Application of enzymes in fine organic synthesis appears very promising and in some instances is highly advantageous compared to conventional methods of chemical synthesis.[1-6] In particular, certain progress has been achieved in applying enzymes for the synthesis of peptides, see reviews.[3,7-14]

Unfortunately, a broader application of enzymes in preparative organic synthesis is somewhat limited because of the fact that enzymes retain their catalytic properties only in a rather narrow range of conditions, viz. in aqueous solutions at pH close to neutral and at moderate temperatures. This hinders the enzymatic synthesis of compounds for which the thermodynamic equilibrium in aqueous solution is shifted in the unfavourable direction. Typical examples of such processes are syntheses of esters and peptides, polymerization of sugars, dehydration reactions, etc.

This variance could be successfully settled by using biphasic reaction media of the type "water - water-immiscible organic solvent" proposed to shift chemical

equilibria.[15,16] The thermodynamics of the biphasic method has been analyzed in detail by our Moscow group.[17,18] There are a few reviews on enzymatic syntheses carried out using the biphasic technique.[3,19-24] Some peptides have been synthesized under mild conditions in a high yield.[25-28] Among the proteases used for peptide synthesis so far, carboxypeptidase Y from yeast has been a subject of special interest.[28-30] The biphasic method has undoubtedly started a novel stage[31] in the development of synthesis of peptides.

Recently, the possibility of 100% yield in the biphasic enzymatic reaction has been questioned.[32,33] We believe that this aim is well attainable if the conditions of synthesis are optimized. So far this problem had been settled only for the enzymatic synthesis of amino acid esters where the nature of organic solvent[17], pH,[34] reagent (alcohol) concentration,[35] and some parameters of biocatalyst[36] were varied. The present work endeavours to optimize the peptide synthesis. As an example[27] we have chosen the reaction:

$$\text{N-Ac-L-Trp} + \text{L-LeuNH}_2 \rightleftharpoons \text{N-Ac-L-Trp-L-LeuNH}_2 + H_2O \qquad (1)$$

catalyzed by α-chymotrypsin in the biphasic system water - ethyl acetate. We varied the "organic phase/aqueous phase" volume ratio, the pH value of aqueous phase, and the concentrations of starting reactants. The experiments were carried out using both free and immobilized enzyme.

RESULTS AND DISCUSSION

Kinetics of the product formation and stability of α-chymotrypsin under the reaction conditions. For free α-chymotrypsin at a concentration in the aqueous phase of 20 mg/ml, the dipeptide synthesis reached equilibrium only in 6-7 days. Fig. 1 shows, as an example, the kinetic curves 1-3 for product accumulation (1) at various values of pH. The broken lines show the concentrations corresponding to the limiting yield of the dipeptide.

While determining the yield of the reaction product, one should be sure that the horizontal part of the kinetic curve does correspond to the equilibrium of reaction (1) and is not caused by other factors, e.g., enzyme inactivation. In order to clarify this, we added a new portion of the enzyme to the aqueous phase and found no change in the content of the reaction product. Thus the horizontal part on curves 1-3 does correspond to the true equilibrium of reaction (1).

The reaction time shown in Fig. 1 corresponds to that for the synthesis catalyzed by free enzyme in the simplest biphasic system (see Experimental) consisting of two mildly stirred macrolayers (water and ethyl acetate). The reaction time can evidently be altered by varying the catalyst concentration, the area of interface, and the stirring rate. To increase significantly the interface, an emulsion of an aqueous enzyme solution in ethyl acetate should be used. However, in this case the free enzyme becomes rather quickly inactivated because of adsorption on the interface.

Therefore, the enzyme should be immobilized on or inside a support. To this end α-chymotrypsin adsorbed on terephtalate granules (Sorsilen) was used. After immoblization the enzyme becomes more stable, i.e. it essentially does not inactivate during the synthesis and can be used repeatedly (Fig. 2). Furthermore, intensive stirring (emulsifying) reduced the reaction time from a few days (for the free enzyme) to a few hours. However, when the immobilized enzyme preparation is used the synthesis time (rate of enzymatic reaction) is significantly dependent on the carrier porosity and the surface concentration of catalytic sites. The results found for α-chymotrypsin adsorbed on Sorsilen are completely in line with earlier experimental data obtained by Monsan's group for the same enzyme covalently immobilized on Spherosil,[36] and with theoretical conceptions.[37,38]

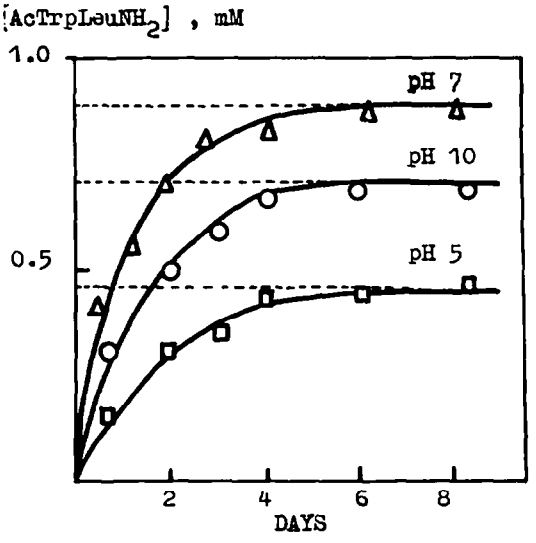

Fig. 1. Kinetics of the dipeptide accumulation in reaction (1) proceeding in the biphasic system water - ethyl acetate. Conditions: V_{org}/V_{aq} = 50, [AcTrp] = [$LeuNH_2$] = = 10^{-3} M, enzyme concentration 20 mg per ml of aqueous phase.

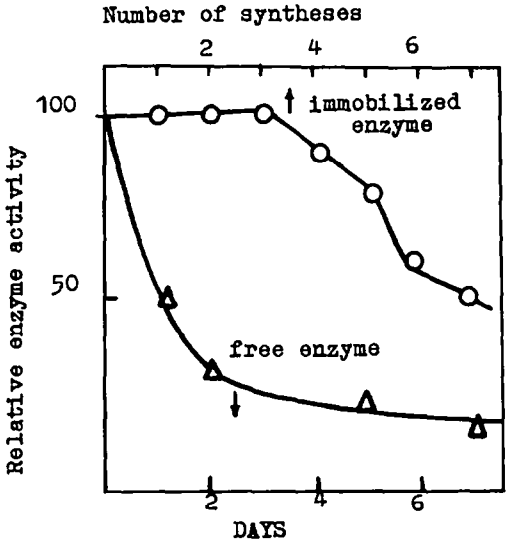

Fig. 2. Comparison of stability of free and immobilized α-chymotrypsin. The results for free enzyme (lower abscissa) reflect its inactivation under the synthesis conditions given in Fig. 1 (pH 7). Immobilized enzyme (adsorbed on Sorsilen) is much more stable and may be used repeatedly (upper abscissa); for the synthesis conditions, see Experimental.

Effect of the "organic phase/aqueous phase" volume ratio on the product yield. It follows from the theory of biphasic method[17,18] that the reaction yield is dependent on the organic phase/aqueous phase volume ratio (V_{org}/V_{aq}). The experimental data are presented in Fig. 3. The increase in the product yield with increasing V_{org}/V_{aq} ratio is accounted for by differences in partition coefficients of starting reactants and reaction products between organic and aqueous phases. More precisely, the solubility of the dipeptide in water is much lower than that of the starting amine and acid. Thus, the dipeptide formed in the course of enzymatic reaction is removed from the reaction medium (i.e. from aqueous phase) by being extracted into the organic solvent. Therefore, the equilibrium of the reaction is shifted toward the side of product formation, the shift being the greater the better extraction, i.e., the greater the "organic phase/aqueous phase" volume ratio (Fig. 3).

Effect of the pH of aqueous phase on the product yield. The dipeptide yield as a function of pH of the aqueous phase is shown in Fig. 4. The curve has a broad maximum in agreement with the data reported earlier.[18]

Effect of the concentrations of starting reactants on the product yield. The product yield of reaction (1) as a function of the concentrations of the starting reactants (N-acetyl-L-tryptophane and L-leucine amide) is shown in Fig. 5; the dipeptide yield increases with increasing concentrations of starting reactants.

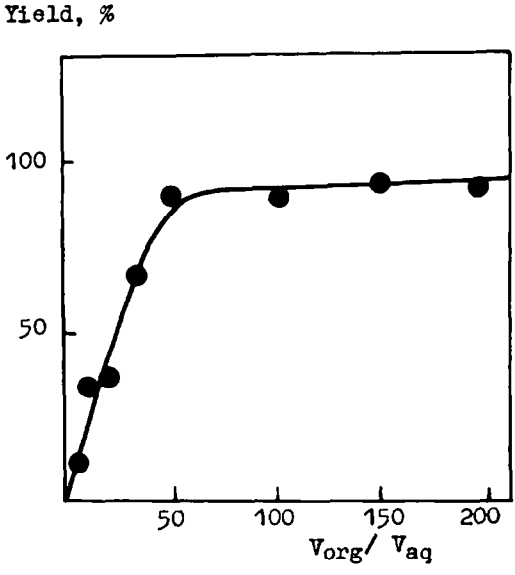

Fig. 3. The dipeptide yield as a function of the "organic phase/aqueous phase" volume ratio. Conditions: [AcTrp] = [$LeuNH_2$] = 10^{-3} M, concentration of free α-chymotrypsin is 20 mg per ml of aqueous phase, pH 7.

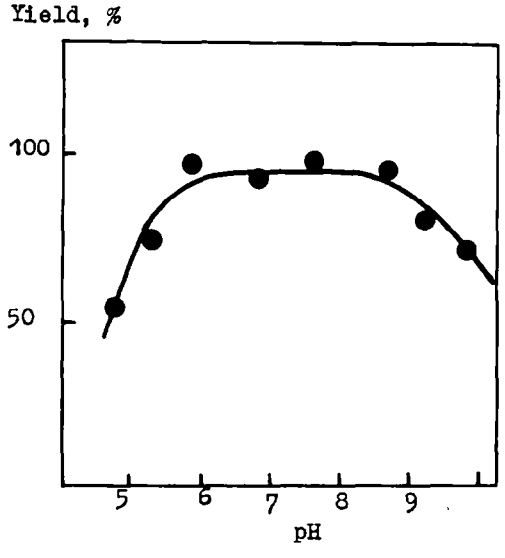

Fig. 4. The dipeptide yield as a function of the pH value in aqueous phase. Conditions: [AcTrp] = [$LeuNH_2$] = 10^{-3} M, concentration of free α-chymotrypsin is 20 mg per ml of aqueous phase, V_{org}/V_{aq} = 50.

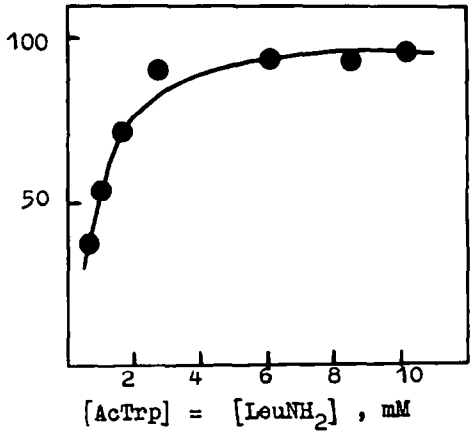

Fig. 5. The dipeptide yield as a function of the concentration of starting reactants. Solid curve is calculated from equation (4) using the experimental value $K = 3 \times 10^4$. Conditions: $V_{org}/V_{aq} = 25$, pH 7, concentration of free α-chymotrypsin is 20 mg per ml of aqueous phase.

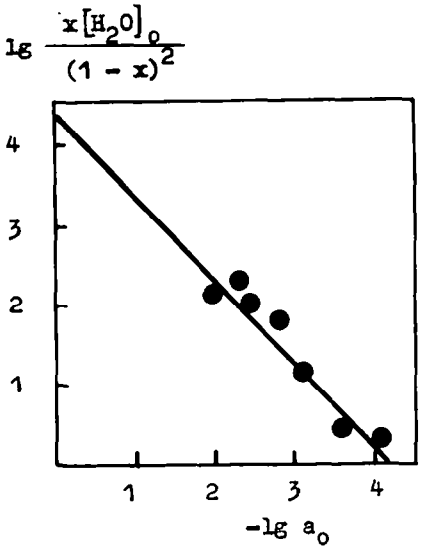

Fig. 6. Linearization of experimental data of Fig. 5 in coordinates of equation (4).

Equilibrium constant. Analysis of the dependence presented in Fig. 5 allows one to determine the equilibrium constant for reaction (1):

$$K = \frac{[\text{AcTrp - LeuNH}_2]_{eq}\,[H_2O]_{eq}}{[\text{AcTrp}]_{eq}\,[\text{LeuNH}_2]_{eq}} . \qquad (2)$$

Using designations $[\text{AcTrp - LeuNH}_2] = P$, $[\text{AcTrp}] = [\text{LeuNH}_2] = \underline{a}$ we obtain:

$$K = \frac{P_{eq}\,[H_2O]_{eq}}{a^2_{eq}} = \frac{P_{eq}\,[H_2O]_o}{(a_o - P_{eq})^2} \qquad (3)$$

where the index "o" denotes initial concentrations. The assumption $[H_2O]_{eq} \approx [H_2O]_o$ is based on the fact that $[H_2O]_o \gg P_{eq}$, implying that a change in water concentration during the reaction can be neglected. Equation (3) may be rewritten as

$$\lg \frac{x\,[H_2O]_o}{(1 - x)^2} = \lg K + \lg a_o \qquad (4)$$

where $x = P_{eq}/a_o$ is the reaction yield. According to equation (4), the intercept of the plot $\lg x[H_2O]_o/(1 - x)^2$ vs $\lg \underline{a}$ gives $\lg K$ (Fig. 6). The equilibrium constant thus determined is 3×10^4; the K value in water is only about 10, see Ref. 37. The plot of the product yield as a function of the concentrations of starting reactants calculated from equation (4) using the determined value $K = 3 \times 10^4$, is shown in Fig. 5 as a solid line.

Preparative enzymatic synthesis. The experimental results obtained (Figs. 3-5) enable us to formulate the conditions optimal for reaching the maximum product yield of reaction (1) in the biphasic system water - ethyl acetate: the "organic phase/aqueous phase" volume ratio (V_{org}/V_{aq}) is more than 50, the pH value of aqueous phase is from 6 to 9 and the concentrations of starting reactants are higher than 3×10^{-3} M (referred to the total volume of the system).

Under these conditions the preparative yield of N-acetyl-L-tryptophanyl-L-leucine amide in the biphasic system was practically 100%. In fact, 274 mg dipeptide were obtained (from the organic phase only), which coincides, within the experimental error, with theoretical yield. The purity of the product was established by means of thin-layer chromatography. The amount of the dipeptide in the spot was determined spectrophotometrically (see Experimental). Furthermore, the product was shown to be chromatographically homogeneous; spots on a chromatographic plate corresponding to starting reactants were not detected at the end of the reaction.

Conclusion. Beyond any doubt, more experiments must be carried out to obtain detailed physicochemical description of the particular reaction system employed in the present study. For example, it would be necessary to determine partition coefficients of both substrates and the product between the two phases, to take into account the effect of pH and substrates concentrations on the ionization degree of both substrates and the product, the possibility of pH variation in the course of the reaction, etc. However, the main goal of the present study was just to outline the general way or procedure for searching optimal conditions for enzyme-catalyzed syntheses in biphasic systems, and this goal was achieved quite successfully.

EXPERIMENTAL

Materials. α-Chymotrypsin from bovine pancreas was product of Biokhimreaktiv (Olaine, Latvia). The content of the active enzyme in the preparation, determined as described by Bender's group[40], was 66%. N-Acetyl-L-tryptophane, L-leucine amide hydrochloride and N-acetyl-L-tyrptophanyl-L-leucine amide was obtained from Reanal (Hungary). Ethyl acetate was a product of Reakhim (USSR).

Enzyme immobilization. To 0.5 ml 0.5 M phosphate buffer (pH 7), 0.5 g Sorsilen were added. The mixture was deaerated under low pressure (10 mm Hg) and 10 mg α-chymotrypsin added. The mixture was agitated for 30 min, the time sufficient for a complete adsorption of the enzyme. Characteristics of the terephtalate support (Sorsilen), produced at the Polymer Division of the Institute of Chemical Technology (Prague, Czechoslovakia) were reported earlier.[41]

Reaction catalyzed by the free enzyme. α-Chymotrypsin (20-40 mg/ml) was added to the solution of N-acetyl-L-tryptophane and L-leucine amide hydrochloride, taken in equimolar concentrations (6×10^{-4} to 2 M), in 0.1 M acetate/phosphate/borate buffer, pH 7. The pH value of the solution changed, since commercial leucine amide was in a hydrochloride form and because of buffer properties of α-chymotrypsin. Therefore, pH of the solution was adjusted to the needed value every time after dissolving the reactants.

The buffer solution of reactants with adjusted pH was placed in a flask and water-saturated ethyl acetate was added to obtain the needed "organic phase/aqueous phase" volume ratio. The total volume was 10 to 100 ml. The reaction mixture was carefully agitated using a laboratory shaker so that not to allow emulsion to form.

It is to be noted that, if it is not indicated otherwise, everywhere in the text all concentrations are referred to the total volume of the reaction system, i.e. including both aqueous and organic phases.

Reaction catalyzed by immobilized α-chymotrypsin. Water-saturated ethyl acetate (8 ml) containing 0.008 M of both starting reactants was added to the buffer suspension of polymeric granules with adsorbed enzyme (see above). The reaction mixture was stirred at more than 160 r.p.m.

Determination of the reaction yield using thin-layer chromatography. Aliquotes of organic phase were taken from the biphasic reaction mixture in certain time intervals. To determine the product yield, the aliquote of organic phase (0.05 to 4.5 ml) was placed on a 5 x 15 cm Silufol chromatographic plate (Czechoslovakia) which was placed into elution camera containing a mixture of propyl alcohol and aqueous ammonia (7 : 3 v/v). The chromatography was performed using the ascending elution technique and employing commercial N-acetyl-L-tryptophanyl-L-leucine amide as a marker. After drying the chromatographic plate a 2 x 5 cm strip was cut out off at the level corresponding to the R_f value of the reaction product (0.87). The strip was cut to small pieces which were placed into 4 ml distilled water to wash off the dipeptide. The aqueous solution was separated in a day by centrifugation and the concentration of the product was measured spectrophotometrically at 280 nm (molar absorbance being 6.5×10^3 M^{-1} cm^{-1}, see Ref. 42).

Determination of the relative catalytic activity of α-chymotrypsin in the course of reaction. In certain time intervals aliquotes (0.01 to 0.1 ml) were withdrawn from the aqueous phase of the reaction mixture and placed into a spectrophotometer cell containing 2 ml 0.1 M acetate/phosphate/borate buffer at pH 7 and 0.02 ml 1.3×10^{-2} M N-3-carboxypropionyl-L-phenylalanine-p-nitroanilide in the acetonitrile-dioxane (1 : 1 v/v) mixture. The α-chymotryptic hydrolysis of this specific substrate[43] was followed at 380 nm by the rate of appearance of p-nitroaniline.

Preparative synthesis of N-acetyl-L-tryptophanyl-L-leucine amide. The preparative synthesis of the dipeptide was performed in the biphasic system containing 1 ml of 0.1 M acetate/phosphate/borate buffer (pH 7) and 100 ml of water-saturated ethyl acetate. The aqueous phase contained 0.01 M of both N-acetyl-L-tryptophane and L-leucine amide hydrochloride and 20 mg/ml α-chymotrypsin. The synthesis was carried out with careful agitation of the reaction mixture in a laboratory shaker. After the reaction completed, the organic phase was separated and ethyl acetate removed in vacuo. The preparation should be dried under reduced pressure over P_2O_5 at room temperature for three days to obtain satisfactory results of elemental analysis.

Spectrophotometric measurements were made on a Beckman-25 instrument.

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