PEPTIDE SYNTHESIS BY STABILIZED TRYPSIN

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STABİLİZE TRİPSİNLE PEPTİD SENTEZLERİ

ÖZET

Tripsin adsorpsiyonla alümina'ya immobilize edildi. İmmobilize enzim amino asit peptidasyonu için serbest enzimden daha yüksek katalitik aktivite gösterdi. Tripsin katalizörlüğünde peptid sentezlerinin kinetik çalışmaları yapıldı. Stabilitesi arttırılmış tripsin türevleri hazırlandı ve dipeptid sentezlerinde kullanıldı.

Substrat olarak benzoil arginin ve lösin amid kullanarak denge kontrollü sentezleri çalışıldı, pH ve sıcaklık gibi başlıca birkaç değişken test edildi. Endüstriyel parametrelerin hepsi için iyi değerler elde etmek amacıyla bu sentetik yaklaşım için optimum deney şartları tayin edildi.

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SUMMARY

Trypsin was immobilized onto alumina (Al_2O_3) by the adsorption. The immobilized enzyme showed higher catalytic activities than free enzyme for amino acid peptidation. Studies of the kinetics of peptide synthesis catalyzed by trypsin have been carried out. We have studied on and prepared the trypsin derivatives with the enhanced stabilities and have used these successfully for initial dipeptide synthesis.

We have studied on the equilibrium controlled synthesis(ECS) by using benzoyl arginine and leucinamide as substrates and mainly tested a number of variables, e.g. the temperature and pH. Optimal experimental conditions for that synthetic approach was established in order to simultaneously obtain good values for all industrial parameters.

Keywords: Immobilization, organic synthesis, peptide synthesis.

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INTRODUCTION

Peptides have an increasing importance in medicine and biotechnology. Enzymatic synthesis (and semisynthesis) can usefully complement chemical methods of peptide synthesis. Immobilization will likely be undertaken to permit easy enzyme removal and reuse. Peptide synthesis catalyzed by proteases is a good example of an interesting 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, and the use of milder experimental conditions 1-4. Moreover proteases present extraordinary opportunities to be used as catalysts of peptide synthesis because of their impressive specificity (towards substrate, stereospecificity, regiospecificity) 5-7.

Some enzymatic reactions of industrial interest, such as peptide synthesis by proteases, can be performed by following different synthetic strategies (kinetically controlled synthesis in aqueous media, equilibrium-controlled synthesis in biphasic systems). However many important drawbacks must be overcome when we try to progress from discovering these interesting processess (e.g., peptide synthesis catalyzed by trypsin) to the scaling up to industrial levels8-10.

In this paper we present studies of the synthesis of the model peptide benzoyl argininеleucinamide using these stabilized trypsin derivative as catalyst. We have mainly tested the integrated effect of different variables that define the reaction medium on various parameters of industrial interest. Optimal conditions (temperature, range of pH values) for performing the industrial synthesis of peptide bonds were defined.

We have studied the synthesis of dipeptide of benzoylargininеleucinamide by means of equilibrium controlled synthesis (direct condensation of benzoyl arginine with leucinamide in the presence of acetonitrile as co-solvent).

Thermodynamically controlled synthesis

From different points of view theoretical, practical, and economic 'thermodynamically controlled synthesis' in monophasic water-cosolvent systems is a prior the easiest strategy to synthesize peptide bonds by proteases:

1. This is a direct reaction between an acid and an amine group to yield an amide bond.
2. The product of the reaction is fully stable.
3. The activation of the acyl donor becomes unnecessary.
4. The pH values and water activities are easily controllable.
5. The synthetic yield corresponding to the most expensive component can be increased to nearly 100% by using an excess of the most inexpensive, most soluble, most easily separable reactant.
6. The subsequent purification steps may be quite simple: we only need to separate the synthetic product from the component in excess. Since only the non-ionic forms of both acid and amine are involved in the synthetic reaction, the equilibrium constant, Kth, may be represented as:

$$\begin{array}{ccc} & + & \\ R-COOH & NH_2R' & \xleftrightarrow{Trypsin} R-CONH-R' \\ \updownarrow & \updownarrow & \\ R-COO^- & +H_3N^+-R' & \end{array}$$

$$K_{th}/a_w = \frac{[R-CONH-R']}{[R-COOH][NH_2-R']}$$

From this equation, the peptide concentration in the equilibrium may also be represented as a function of the total concentrations of acid and amine:

$$[\text{Peptid}] = \frac{K_{th} K_{nonion} [\text{acid}][\text{amine}]}{a_w}$$

where $K_{non\text{-}ion}$ is the ratio between the product of the concentrations of the non-ionic forms and the product of the total concentrations of acid and amine. Equilibrium controlled synthesis in water-cosolvent systems must be very carefully designed. The main parameters to be tested are as follows:

1. Thermodynamic synthetic yield
2. Temperature
3. pH

Enzyme Loading and Activity Determination

The amount of protein bound onto alumina was determined by the Lowry method11 indirectly from the difference between the initial total protein exposed to the support and the amount of protein recovered in the washes. Various parameters were used to characterize the support-immobilized enzyme system according to Imai et. al.12.

$$\text{Immobilization yield(\%)} = \frac{(A - B)}{A} \times 100$$

where A is the amount of enzyme added in the initial immobilization solution; B, the amount of the residual enzyme in the immobilization and washing solutions after the immobilization procedure.

The proteolytic activity of the support-immobilized and free trypsin, which was used to compare the characteristics of the enzyme between the to states, was determined by the trichloroacetic acid precipitate method. Casein was used as the substrate as described by Kunitz13.

EXPERIMENTAL

Materials.

Alumina G-60, the enzyme trypsin(E.C. 3.4.21.4) from bovine pancreas, other chemicals and solvents were obtained from Merck, Darmstadt, FRG. Casein, BA(benzoylarginine), and LeuNH2 hydrochlorid(leucinamide hydrochloride) were purchased from Sigma Chemical Co., St. Louis, MO.

Preparation of Trypsin-alumina Derivative

Trypsin (200 mg) dissolved in 1 mL of a 0.1M phosphate buffer pH 6 was kept at 4°C for 1/2h. This mixture was stirred with 1 g of alumina and added to 1.5 mL of cold acetone. The mixture was kept standing for 1h at 4°C and the resultant mixture was seperated by vacuum filtration. Then the solid was washed with 3mL of cold acetone till the filtrate was free from any unbound enzyme and finally lyophilized.

Determination of the Amount Bound on the Supports: Lowry Protein Measurements

Sodium carbonate (2% in 50 mL 0.1N NaOH) and copper sulfate solution (1% in 1 mL of 2% sodium tartarate) were mixed well. To 4 mL of this reagent were added various amounts of trypsin(mg/mL) dissolved in bidistilled water. After 10 minute 0.3 mL of 2N Folin Phenol Reagent was added, and the system was allowed to stand for minute.

The absorbance at 500 nm was measured spectrophotometrically in each case. From the standart plot, the amount of enzyme present in the enzyme solution used for immobilization reaction and that remaining unbounding the filtrate was estimated. Finally, the amount the enzyme bound on the support was found by difference.

Determination of Enzymatic Activity of Free and Immobilized Trypsin Derivatives

It was determined according to the procedure purposed by Kunitz13 The reaction with the free enzyme was carried out in the mixture of 1 mL enzyme solution containing a given amount of enzyme, 2 mL of 1% casein solution, and 2 mL of 0.1M phosphate buffer (pH 7.6).

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

Thermodynamic Controlled Synthesis

Reaction mixture was composed of 10 mL of 10mM BA and 20mM LeuNH2 dissolved in a mixture of acetonitrile-buffer (9:1) at various pH values. For equilibrium analysis, the reaction mixture was fluxed through the column at a constant flow rate (0.7 mL min-1), and the first 50 mL eluted out from the column were discarded. Then the reaction mixture was recirculated through the column at the same flow rate.

HPLC Analysis

Substrate (benzoyl arginine) and product (peptide) were seperated and quantified by reverse phase HPLC. At different times, aliquots of the supernatants in thermodynamically controlled synthesis (TCS) were withdrawn and diluted with 4 volumes of mobile phase, filtered through a Millipore filter (0.45mm), and injected. An instrument with Waters 484. Tunable Absorbance Detector Millipore and 250\4.6mm RP-C18 (5mm) column (Spherisorb) was used.

Samples were eluted isocratically with a mixture of 40% ethanol, 60% water containing 0.1% phosphoric acid as the mobile phase in the thermodynamically controlled synthesis. The flow rate was 1mL min-1, and amounts of reactant and products were determined from the calibration curves using stock solutions.

RESULTS AND DISCUSSION

Direct condensation of benzoyl arginine and leucinamide were studied in the presence of 90% of an organic solvent to yield the dipeptide benzoyl arginine leucinamide.

Effect of the storage time

The storage stability of the immobilized preparation was measured by storing immobilized trypsin in 0.1M phosphate buffer, pH 7.0, at 35°C for different periods of time. The residual activity was measured at these different storage times. Figure I shows the results for immobilized enzyme.

Effect of pH

In Fig. II we compared the very different patterns for yields-pH. On the one hand, synthetic yields practically constant within a fairly wide range of pH values (between 7-9). The concentrations of non-ionized forms of the acyl donor and the nucleophile are influenced by pH in opposite ways:

From a technological point of view, the performance of these synthetic processes at pH 7.0 indicates that this is the best solution for obtaining good activity and equilibrium values simultaneously.

Effect of temperature

Figure III shows the effect of temperature on the yields of this reaction. These equilibrium yields rise as temperature decreases. Indicating that this synthetic process is an exothermic one. This is consistent with data reported in the literature. From data represented in Figure III, 25°C seems to be the most favorable temperature. Synthetic yields are reduced only slightly over those obtained at lower temperatures.

Concluding remarks

We have performed on integrated study of the performance of equilibrium controlled synthesis of BALeuNH2 catalyzed by stabilized trypsin.

Optimal conditions for this synthetic reaction were pH: 7.0, 25°C. In these conditions and using 10mM BA and 20mM LeuNH2 we have obtained the following result:

Synthetic yield= 47%

We have studied the role of some valuables which are very poorly controlled in the scientific literature. These variables (temperature, pH) proved to be essential for a correct design of both synthetic approaches.

Stabilization of trypsin by adsorption method has been very useful for the performance of the reaction engineering of that synthetic approach.

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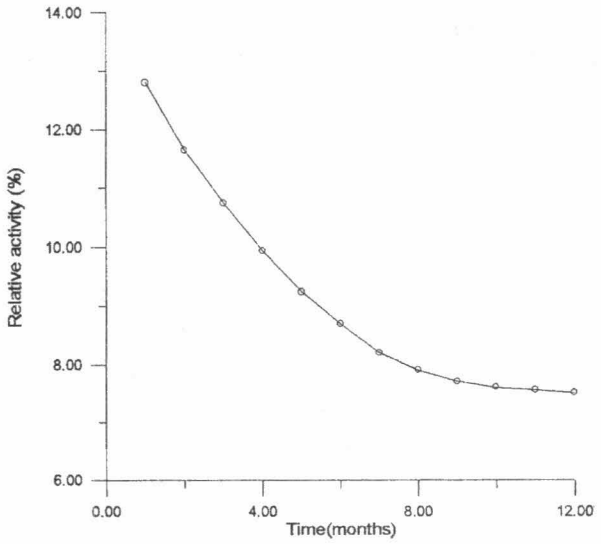

Figure I. Effect of the storage time on immobilized trypsin-alumina. Activity was measured in 0.1M phosphate buffer (at pH 7.0 , 35°C)

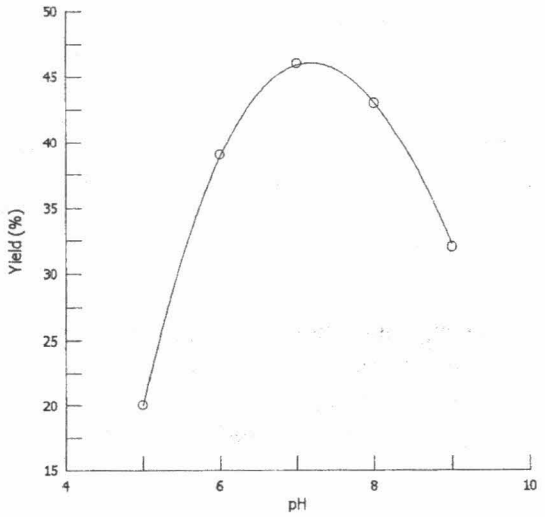

Figure II. Effect of pH on yields of thermodynamically controlled synthesis reaction of BA-Leu-NH2. (o): yields given in percent conversion of acyl donor (BA). Concentration of Leu-NH2 = 20mM, concentration of BA = 10mM, acetonitrile-buffer(9:1), reaction temperature 25°C.

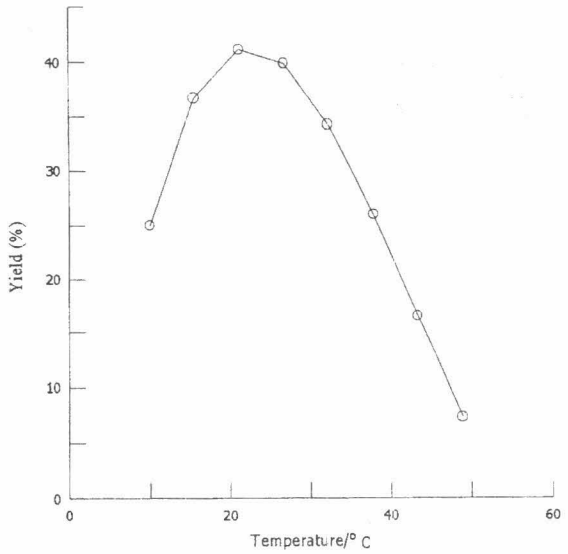

Figure III. Effect of temperature on thermodynamically controlled synthesis of $BALeuNH_2$ catalyzed by stabilized trypsin [pH 7.0, acetonitrile-buffer(9:1)].

REFERENCES

1 Jakubke H.D., Kuhl P., and Könnecke A., Basic principles of protease-catalyzed peptide bond formation., Angew. Chem. Int. Ed. Engl., 24, 85, 1985.

2 Kise H. and Hayakawa A., Immobilization of proteases to porous chitosan beads and their catalysis for ester and peptide synthesis in organic solvents., Enzyme Microb. Technol., 13, 584, 1991.

3 Sears P.S. and Clark D.S., Comparison of soluble and immobilized trypsin kinetics:Implications for peptide synthesis., Biotechnology and Bioengineering, 2,118,1993.

4 Simon L.M., Kotorman M., Garab G. et al., Structure and activity of alpha-chymotrypsin and trypsin in aqueous organic media, Biochem. Bioph.Res.Co., 280, 5, 1367-1371, 2001.

5 Zhuang P. and Butterfield A.D., Optimization of covalently coupling enzymes to polymeric membranes:EPR studies of papain., Journal of Applied Polymer Science, 47, 1329, 1993.

6 Kulik E.A., Kato K., Ivanchenko M.I and Ikada Y., Trypsin immobilization on to polymer surface through grafted layer and its reaction with inhibitors., Biomaterials, 4, 763, 1993.

7 Klein Ju., Prykhodzka A., Cerovsky V., The applicality of subtilisin carlsberg in peptide synthesis, J. Pept.Sci., 6, 11, 541-549, 2000.

8 Blanco R.M., Alvaro G. and Guisan J.M., Enzyme reaction engineering: design of peptide synthesis by stabilized trypsin., Enzyme Microb. Technol., 13, 573, 1991.

9 Blanco R.M., Alvaro G., Tercero J.C. and Guisan J.M., Peptide synthesis by stabilized trypsin:Industrial kinetic studies under extreme experimental conditions., Journal of Molecular Catalysis, 73, 97, 1992.

10 Braun K., Mitin Y.V., Salchert K., v.d., Synthesis and use of new semispecific substrates for trypsin-catalyzed peptide bond formation, Biocatal. Biotransfor., 18, 6, 427-441, 2000.

11 Lowry O.H., Rosenbough N.J., Farr A.L. and Randall R.J., J. Biol. Chem. 193, 265, 1951.

12 Imai K., Shiomi T., Uchida K. and Miya M., Biotechnol. Bioeng. 28, 198, 1986.

13 Northrop J.M. and Kunitz M., Trypsinogen and trypsin, Science, 73, 262, 1931.