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Immobilization strategy of accessible transmission for trypsin to catalyze synthesis of dipeptide in mesoporous support

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Abstract–Immobilized trypsin in mesoporous silica foams was used to catalyze dipeptide synthesis in hydrophilic organic solvent instead of soluble form. The area surface of nano support was measured. The catalytic activity, coupled yield and kinetic characterization of immobilized trypsin were examined. Bz-Arg-OEt was chosen as the acyl donor with Lys-OH as the nucleophile. The trypsin-catalyzed synthesis condition was optimized, such as catalytic temperature, pH, reaction time, physical properties and content of organic solvents, together with the added enzyme amount. The immobilized trypsin showed 112.8% of residual activity with 91.9% of coupled yield, and the kinetic parameters exhibited accessibility for transmission. The product yield of 5.8% was reached at the optimum conditions for enzymatic synthesis of dipeptide: 800 mg of wet immobilized trypsin (200 mg/g support) was used in Tris-HCl buffer (0.1 mol/L, pH 8.0) containing 80% (v/v) ethanol solvents for 6 h of reaction time at 35 °C. This attempt of immobilized strategy for trypsin in nanopores renders the possibility of wide application of inorganic nano-sized support in catalytic synthesis process, which can avoid usage of large amounts of organic solvents in washing steps by chemical methods and reduce the tedious purification process of its soluble form.

Key words: Immobilized Trypsin, Enzymatic Synthesis, Mesoporous Silica Foams, Hydrophilic Organic Solvents

INTRODUCTION

Peptides, which show promising immuno-modulating properties, are molecules of paramount importance in the fields of health care and nutrition. Thymopentin (TP5) is a synthetic pentapeptide (Arg-Lys-Asp-Val-Tyr), which can reproduce biological activity of thymopoietins, and influences the immune system by promoting the differentiation of thymocytes and affecting the function of mature T-cells [1]. TP5 was chemically synthesized in our team by solid-phase condensation [2], using repeated side-chain protection (Boc or Fmoc) and deprotection of amino acids and washing by kinds of deleterious organic solvents.

The peptide synthesis catalyzed by proteases (such as trypsin, papain, subtilisin, and chymotrypsin), which is racemization-free and requires minimal side-chain protection, is superior to the chemical coupling method. Biocatalysis in non-conventional media has expanded the spectrum of application of proteases to those reactions that cannot proceed effectively in aqueous environments [3]. That is the synthesis of peptide bonds instead of hydrolysis. There are several obstacles for enzymatic catalytic synthesis. The substrate encountered the solubility problem in hydrophobic organic solvents [4]. Thus, the hydrophilic organic solvents are more suitable which offer better property for dissolving amino acids [5,6]. However, they cause deactivation to enzyme solutions. In existence of aqueous medium, the disadvantage comes in that the activity of trypsin causes secondary hydrolysis of the growing peptide chain, consistent with the normal recognition properties of proteases. Another problem is that the soluble enzymes in reaction medium need purification in a subsequent process. Circumventing these problems is immobilization strategy, so it is usual that enzymes are used in immobilized form when performing in homogeneous liquid medium [7].

There are many inorganic supports and immobilization strategies for trypsin, which are focused on the interaction between support and enzyme and the environment established for catalysis [8]. The nano-support mesoporous cellular foams (MCFs) with high surface area and accessible mass transfer showed potentials for enzyme immobilization [9]. Accessibility is essentially a problem encountered with porous solid supports that are commonly used in solid-phase synthesis. Porous supports generally have a high loading of functional groups, which are distributed over surfaces inside the pores so that enzymes need to penetrate the pores for successful catalysis. Thus, support material with suitable pore parameters for enzyme can present as offering better enzyme accessibility [10]. The confined channels established suitable microenvironment for trypsin, preventing self-hydrolysis.

In our research, the adsorptively immobilized trypsin in MCFs was used to catalyze dipeptide precursor of TP5 in hydrophilic organic solvents. The catalytic condition of enzymatic synthesis was optimized to increase the dipeptide yield, such as the added amount of immobilized trypsin, time course, catalytic temperature and pH, organic solvent property and its content. The immobilized trypsin in MCFs offers accessible mass transfer for synthesis of dipeptide.

MATERIALS AND METHODS

1. Materials

Trypsin (E.C.3.4.21.4) was purchased from Sinopharm Chemi-

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cal Reagent Co., Ltd. (Shanghai, China). The substrate of Bz-Arg-OEt·HCl and the pure dipeptide of Bz-Arg-Lys-OH were obtained from GL Biochem (Shanghai, China). The other substrate Lys-OH was bought from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Other chemicals were all of analytical grade. Casein and trichloroacetic acid were provided by Lingfeng Chemical Reagent Co., Ltd. (Shanghai, China). The organic solvents were all HPLC grade. Deionized water with a resistance greater than 18 MQ was obtained from a Millipore-Q Plus water purifier.

2. Preparation of Support and Immobilized Trypsin

Mesoporous silica foams (MCFs) were synthesized by Schmidt-Winkel et al. [11] and Wang et al. [12]. 20 mg of MCFs was dispersed in 2 mL phosphate buffer solutions (PBS, pH 7.0, 0.1 mol/L) containing certain amount of trypsin. The hydroxyl supports were adsorptively assembled with trypsin. The immobilization was processed in ice-water system and magnetic stirring. The mixture was centrifuged and washed by 0.01 mol/L of PBS (pH 7.0) for 3 times. The protein in the supernatant phase was determined to estimate the coupled yield of immobilized trypsin by Bradford methods [13].

3. Catalytic Activity Assay of Immobilized Trypsin Preparations

Activity of trypsin preparation was determined using casein as the substrate. 10 mg/mL of casein was dissolved in borax-boric acid buffers (0.1 mol/L, pH 8.0) at 90 °C and pre-incubated at 40 °C. 0.1 mg of native trypsin or 5-10 mg of wet immobilized trypsin was introduced into 5 ml substrate solutions. Reactions were carried out for 30 min (modified Kunitz's method) [14] and stopped by addition of 5 ml of trichloroacetic acid solution (5%, w/v). After centrifugation, the supernatant solution was measured spectrophotometrically at 275 nm compared with the adjusted sample containing no trypsin. One unit of enzymatic activity was defined as the amount of enzyme producing 1 μmol of L-tyrosine in 1 minute from casein at 40 °C.

4. Enzymatic Synthesis of Dipeptide in Organic-aqueous Solvent

Enzymatic synthesis process was carried out in organic solvent and Tris-HCl buffer (0.1 mol/L, pH 8.0) mixtures in different ratios. Lys-OH (0.5 mol/L), Bz-Arg-OEt·HCl (0.5 mol/L) and triethylamine (209 μL) were dissolved in 3 mL reaction medium. A certain amount of wet immobilized trypsin was used as the catalyst at 35 °C for more than 6 h. The reaction was terminated and separated by centrifugation. The product was taken for HPLC analysis and confirmed by MS.

5. Analysis of Peptide Synthesis

Quantitative analysis of the dipeptide product was performed by HPLC with a reverse phase C_{18} column (100-5 C_{18} Kromasil, 250 mm*4.6 mm, Sweden). The HPLC analysis conditions were set gradient elution as follows: 0.1 min, 90% A, 10% B; 25 min, 65% A, 35% B; 25.01 min, 100% B; 30 min, 100% B. Here, A contains 0.1 % (v/v) TFA in 100% water and B contains 0.1% (v/v) TFA in 100% acetonitrile; flow rate, 1.0 ml/min; wavelength, 220 nm; volume, 20 μl. The product concentration (Y) was calculated by the data of peak areas (X, Mau*min) according to the equation $Y=-0.01871+0.00402*X$ ($R^2=0.999803$). The reaction product was identified by MS analysis. MS conditions: spray voltage of 5 kV, capillary temperature of 350 °C, positive ion detection.

6. Optimization of Condition for Dipeptide Synthesis

The synthesis condition of dipeptide was optimized to increase the product yield. The synthesis process was scheduled at different amounts of wet immobilized enzyme added into the reaction, while the amount of trypsin was 400 mg/g support. The time profile was carried out by using enzymatic reaction from 2 h to 12 h. The optimum pH and temperature were determined under the ranges from pH 6-9 and 25 °C to 50 °C, respectively. In organic-aqueous medium, the selection of organic solvents (hydrophilic and hydrophobic) and its content was investigated carefully. All the experiments were done in triplicate, and standard error was never over 5%.

RESULTS AND DISCUSSIONS

1. Preparation of Immobilized Trypsin in MCFs with Accessible Mass Transfer

Trypsin was immobilized in MCFs through electrostatic adsorption, which covered plenty of hydroxyl groups. Immobilization process was optimized for 6 h stirring in ice-incubator. The characterization of MCFs (Fig. 1) showed 15 nm of pore diameter and 252 m^2/g of surface area, presenting good potential for trypsin immobilization, while the diameter of trypsin molecules was about 4.8 nm [15]. The immobilized trypsin in MCFs showed 112.8% of residual activity and 91.9% of coupled yield. These corroborated the successful immobilization strategy for trypsin in mesoporous support. Trypsin is a serine protease found in the digestive system, where it hydrolyzes proteins along with itself. The immobilization strategy was beneficial for inhibition of protease autolysis and promoted the reaction equilibrium to synthesis. The stability at 50 °C of immobilized preparations was incubating in 20% (v/v) ethanol medium

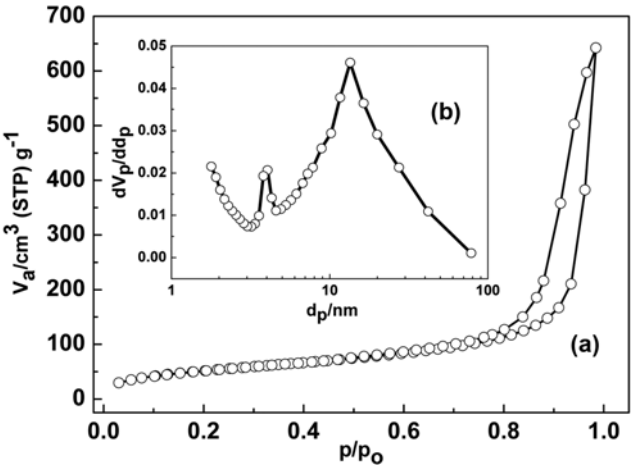

Fig. 1. N_2 adsorption–desorption isotherms (a) and corresponding pore size distribution curve (b) of MCFs support.

Table 1. Kinetic parameters analysis of soluble and immobilized trypsin

Trypsin preparation	K_m [mol/L, 10^{-3}]	V_{max} [mol/(L·min), 10^{-4}]	K_{cal}/K_m [L/(mol·min), 10^5]
Soluble	1.70	2.23	4.04
MCFs	1.63	1.73	2.39

Soluble, soluble trypsin; MCFs, trypsin that was adsorptively immobilized in MCFs

for 6 h, showed more stable than the soluble trypsin in thermal stability. The immobilized trypsin retained 60% of relative activity while the initial activity was 85%, whereas the activity of soluble trypsin was below 40% of relative activity at zero time and decreased to 20% after 6-h treatment.

The analysis of kinetic parameter for trypsin is investigated in Table 1. The K_m of the immobilized preparation was 95.9% of that of the soluble counterpart, together with the decrease of V_{max} and K_m/V_{max}. This decrease demonstrated the successful immobilization of enzyme in confined nanopores compared to its soluble state. Nevertheless, the data were in the same order of magnitude. Thus there existed better mass transfer in the nanopores than other reported results [16]. The established accessible mass transfer of trypsin in MCFs was also corroborated by other immobilized enzyme of larger sized in our previous work [17].

2. Synthesis of Precursor Dipeptide of TP5

Bz-Arg-Lys-OH was synthesized by enzymatic method under kinetic control. Trypsin was chosen as the candidate enzyme to synthesize this precursor dipeptide. The substrate specificity of trypsin requires that the carbonyl component of a peptide bond to be catalyzed must be contributed by Lys or Arg in an aqueous system. The synthesis process was carried out using Bz-Arg-OEt as the acyl donor with Lys-OH as the nucleophile. The addition of triethylamine was used to ensure an alkaline pH. The trypsin as the catalyst conferred the possibility of combining the peptide bond between amino and carboxyl of these suitable substrates. The synthesis can be completed by acyl transfer reaction by trypsin, while the yield of the peptide bond formation may depend on the catalytic properties of trypsin and the rate and mole ratio of the water and amine nucleophile. The formed acyl-enzyme intermediate shown in Fig. 2 can be deacylated by water or nucleophile. The target product was analyzed by HPLC and confirmed by MS-ESI m/z: 407.3 $[M+H]^+$. To establish suitable catalytic conditions, pivotal parameters such as temperature, pH, reaction time, enzyme amount and organic solvents were optimized in the following work.

3. Effect of Enzyme Amount on the Catalytic Yield of Dipeptide

The amount of immobilized trypsin into reaction medium can be observed in Fig. 3. As the added amount of immobilized trypsin increased, the product yield increased to 194.1%. The increased amount of added enzyme enhanced the probability of forming the intermediate reacted by Bz-Arg-OEt and enzyme. The synthesis process in nanopores was hardly affected by transfer mass resistance, which was confirmed by lower yield catalyzed by the same amount of soluble trypsin (3.3%) in the same condition. Compared with free trypsin, product can be more easily collected by easy centrifugation to remove enzyme protein. According to the practical manipulation, transfer mass accessibility and stability of the immobilized enzyme, 800 mg was chosen as the suitable amount of immobilized trypsin in the subsequent investigation.

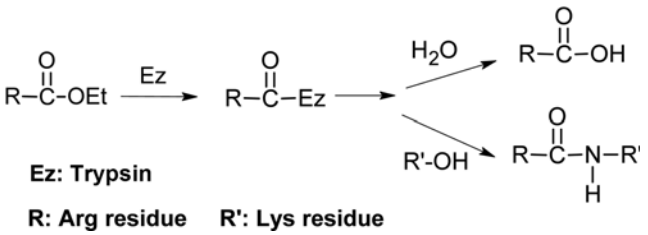

Fig. 2. Scheme of kinetic control of dipeptide under enzymatic catalysis of trypsin. The synthesis process conditions: Bz-Arg-OEt (0.5 mol/L) used as the acyl donor, Lys-OH used as the nucleophile, in organic solvents and Tris-HCl buffers (pH 8.0, 0.1 mol/L).

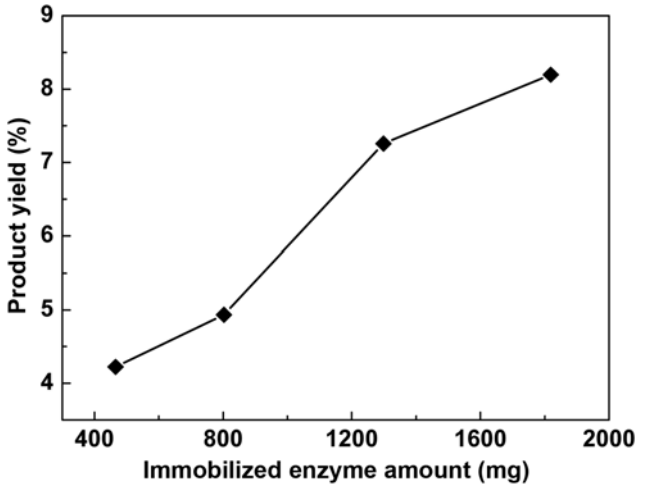

Fig. 3. Effect of immobilized enzyme amount on the yield of dipeptide. The amount of immobilized trypsin into reaction medium was observed in organic solvent and Tris-HCl buffers (0.1 mol/L, pH 8). Lys-OH (0.5 mol/L), Bz-Arg-OEt·HCl (0.5 mol/L) and triethylamine (209 μL) were reacted, and separated by centrifugation after termination.

4. Effect of Reaction Time on Enzymatic Synthesis of Dipeptide

Trypsin can easily self-degrade after long-time treatment in due temperature, for its essence of protein. The examination of reaction time for catalyzing synthesis is presented in Fig. 4. The product yield increases dramatically in the first 6 h, and trends smooth from 6 h.

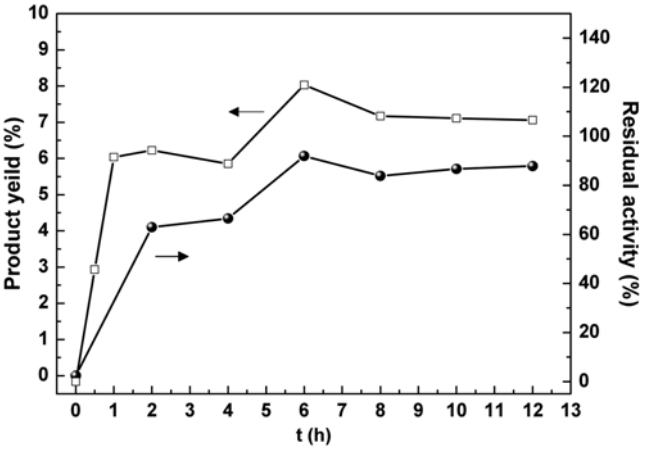

Fig. 4. Catalytic time profile of immobilized trypsin. Lys-OH (0.5 mol/L) and Bz-Arg-OEt·HCl (0.5 mol/L) were reacted for more than 6 h in organic solvent and Tris-HCl buffers (0.1 mol/L, pH 8) at 35 °C. The reaction was terminated and separated by centrifugation.

In the process of trypsin catalysis, the gradual inactivation of trypsin may cause its leak from the nano mesopores and autolysis of enzyme protein. The long-term catalysis by enzyme may cause break process of dipeptide chain, which exhibited a tiny decrease of yield from 6 h to 12 h. The most suitable time for dipeptide synthesis and catalytic properties of immobilized trypsin was 6 h in general, with shared reaction hour of immobilization condition in ice-incubator.

5. Effect of Reaction Temperature and pH on Enzymatic Synthesis of Dipeptide

Advantages of employing high temperature are high process rates, fewer diffusion limitation, and decreased bacterial contamination. However, trypsin undergoes severe denaturation reaction during high temperature, which showed 20% of residual activity after 50 °C treatment for 90 min [18]. The temperatures of reaction medium in enzymatic synthesis affected both the enzyme activity and the dipeptide stability. In Fig. 5(a), the yield of dipeptide is investigated at temperature ranges from 25 °C to 50 °C. Trypsin showed stable activity during this temperature range, and presented highest yield at 35 °C, 1.44 times of that at 50 °C. The hydrolysis activity of trypsin at 50 °C, which may exhibit highest activity, showed low product yield. The optimum temperature for synthesis was lower than that for hydrolysis, which may be attributed to the autolysis of enzyme and the preference to hydrolysis of dipeptide under high temperature.

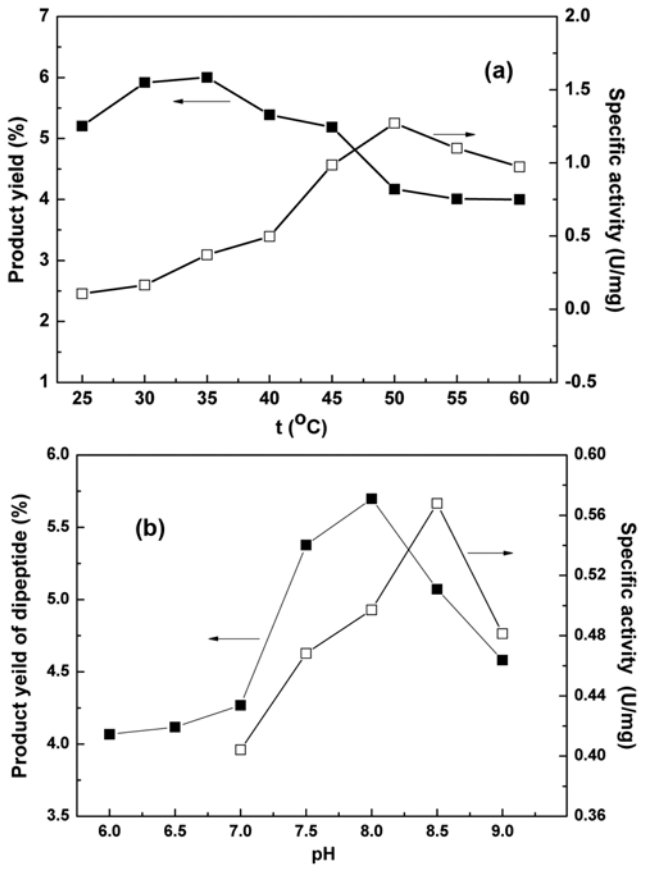

Fig. 5. Effect of temperature (a) and pH (b) on the catalytic yield of dipeptide. Lys-OH (0.5 mol/L) and Bz-Arg-OEt·HCl (0.5 mol/L) were reacted in organic solvent and Tris-HCl buffers. The optimum pH and temperature were determined under the ranges from 25 °C to 50 °C (A) and pH 6-9 (b), respectively. The reaction was terminated and separated by centrifugation.

Together with temperature, pH is another basic factor investigated in enzymatic catalysis. The low-percent aqueous phase played an important role in enzymatic synthesis. Variation in product yield with respect to pH is presented in Fig. 5(b). The optimum pH for synthesis was 8.0, more acid compared with catalysis condition for hydrolysis. With change of two pH units in acid direction, the product yield dropped to 71.4% of that at pH 8.0. The advantage of addition of immobilized trypsin was that there was no previous dissolution in aqueous phase or organic solvent compared with soluble form [19]. There was plenty of essential water surrounded with enzyme in immobilized preparation. The catalytic property of trypsin in organic solvents was dependent on this essential water and buffers. The optimum pH for synthesis was codetermined by the plenty groups in the nano channels of supports and enzyme together with ionic strength of buffers.

6. Effect of Organic Solvents and Content on Catalytic Yield of Dipeptide

The organic-aqueous solvents confer better catalytic properties of immobilized trypsin. It permits the advantage of using organic solvents as reaction medium such as increased solubility of substrates, shifts the equilibrium to favor synthesis over hydrolysis, and suppression of many water-dependent side reactions [20]. The highest yield of dipeptide was obtained in ethanol-aqueous phase. The physical properties of organic solvents, dielectric constants (ε) and log P are compared in Table 2. Here, the log P parameter can be called a measure of solvent 'hydrophobicity', which is the logarithm of the ratio of the un-ionized solute concentrations in the solvents. This contrasts with another parameter dielectric, which measures the bulk 'polarity'. Log P and ε showed no extinct relationship with catalytic activity, while the physical property may be one among those affected parameters [21]. Hydrophilic organic solvent favored the solubility of hydrophilic substrate, and increased the chance to form Bz-Arg-Ez at the first stage of synthesis. Taking consideration of the solubility of substrate, ethanol was chosen as the suitable organic solvent.

The volume ratio between aqueous and organic solvents was essential for enzyme. It is recognized that addition of water molecules increases biological activity. However, beyond a threshold limit of water concentration, synthetic activity starts effectively competing with hydrolytic activity. The suitable amount of water molecules affected the whole catalytic process. In organic-aqueous medium,

Table 2. Selection of organic solvents for enzymatic synthesis in organic-aqueous biphase medium

Organic solvents	Dielectric constants (ε)[a]	Log P[b]	Catalytic yield (%)
Petroleum ether	1.84	3.51	3.84
Ethyl acetate	6.02	0.68	4.93
Ethanol	24.3	−0.24	6.08
Methanol	32.7	−0.33	5.90

[a]Dielectric constants (ε) measures the polarity of solvents

[b]log P parameter can be called a measure of solvent 'hydrophobicity', which is the logarithm of the ratio of the un-ionized solute concentrations in the solvents

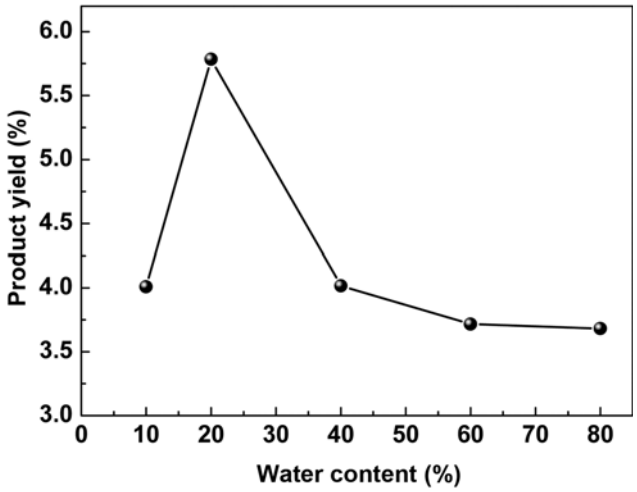

Fig. 6. Effect of water content on the catalytic yield of dipeptide. Lys-OH (0.5 mol/L) and Bz-Arg-OEt·HCl (0.5 mol/L) were reacted for more than 6 h in organic solvent and Tris-HCl buffers (0.1 mol/L, pH 8) at 35 °C. The reaction was terminated and separated by centrifugation.

water partitions among enzyme, solvent, headspace of the reaction vessel and the immobilization matrix, and was assigned by Halling et al. [22] in establishing water activity as a valid and useful parameter for carrying out biocatalysis in anhydrous media. When the ratio of water in reaction medium was 20% (v/v), the product yield increased to 1.57 times of that in 80% medium (shown in Fig. 6). The sharp decrease of product yield with respect to increased water content was due to the undesired hydrolysis of dipeptide. In low percent aqueous medium, trypsin prevented the essential water being deprived by alcohols because of the surrounding hydrated layer in immobilized preparation, which was mainly caused by abundant hydroxyl and amino groups on the surface of MCFs and enzyme, respectively.

By investigation of catalytic parameters, such as pH, temperature, time course, organic solvents and content, enzyme amounts, the optimized dipeptide concentration of 12.5 mg/mL was obtained with the yield of 5.8%. The product yield of dipeptide was low, which may be attributed to the actual low amount of pure trypsin (less than 20 mg) in immobilized form during catalytic process and the weak catalytic property of this kind of trypsin. The enzymatic synthesis yields partially depended on the origin and producer of enzyme, which showed great deal of difference between distinct origins [23]. Immobilization strategy for enzymatic synthesis offers the possibility of biological catalyst in peptide synthesis, and provides accessible mass transfer and catalytic efficiency in existence of confined environment in nano-channel support.

CONCLUSIONS

The adsorptively immobilized trypsin on the wall in MCFs was used as the catalyst. The highest product concentration of 12.5 mg/mL and yield of 5.8% were reached at the optimum conditions for enzymatic synthesis of dipeptide: 800 mg of wet immobilized trypsin (200 mg/g support) was used in Tris-HCl buffer (0.1 mol/L, pH 8.0) containing 80% (v/v) ethanol solvent for 6 h of reaction time at 35 °C. The application of immobilized enzyme preparations in synthesis of precursor peptide segment shows great potential to partially replace chemical methods which cause damage to environment, instead of tedious purification process of its soluble form. This attempt of immobilized strategy in nanopores renders the possibility of wide application of inorganic support for enzyme immobilization in catalytic synthesis process. More effort should be made for technical reform of enzyme and establishment of catalytic environment for high yield. A better understanding of microenvironment of enzyme immobilization and interaction involved in catalysis will greatly facilitate ongoing enzymatic catalysis in peptide synthesis.

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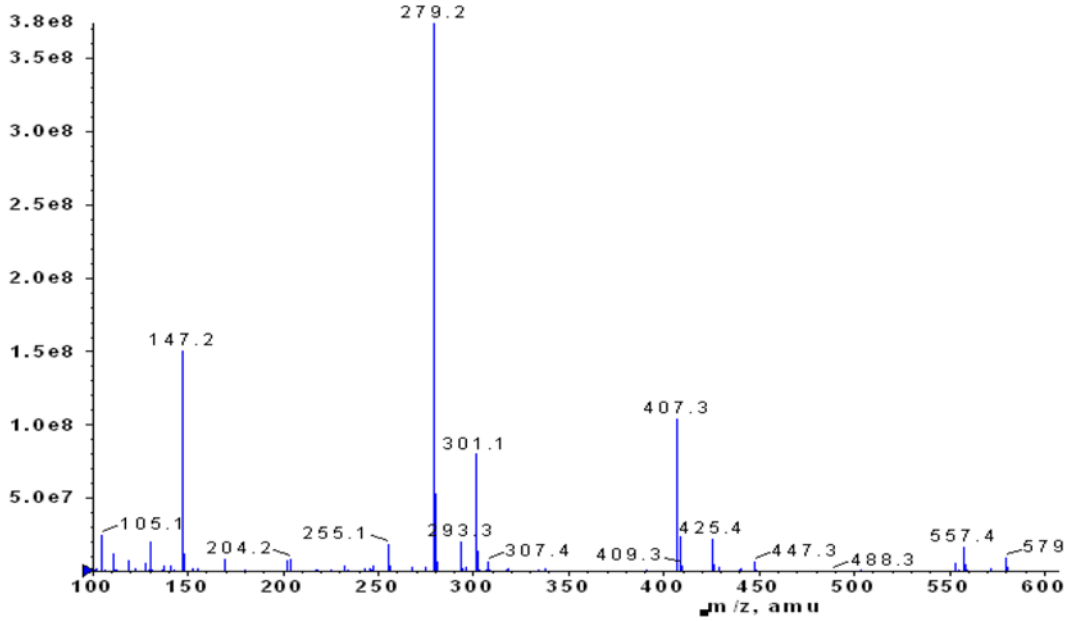

Dipeptide was confirmed by MS analysis.