Korean J. Chem. Eng., **28**(3), 853-859 (2011)
DOI: 10.1007/s11814-010-0412-3

Covalent crowding strategy for trypsin confined in accessible mesopores with enhanced catalytic property and stability

Cheng Zhou*,, Bo Jiang*,**, Zecui Sheng*,**, Shemin Zhu*,***, and Shubao Shen*,**,†**

*National Engineering Research Center for Biotechnology, Nanjing 210009, P. R. China
**College of Biotechnology and Pharmaceutical Engineering, Nanjing University of Technology, Nanjing 210009, P. R. China
***College of Material Science and Engineering, Nanjing University of Technology, Nanjing 210009, P. R. China
(*Received 28 April 2010 • accepted 26 August 2010*)

Abstract−Chemically modified macromolecules were assembled with adsorptive trypsin in mesoporous silica foams (MCFs) to establish covalent linkage. Effects of catalytic properties and stability of immobilized trypsin were examined. The addition of chemically modified protein (BSA) and polysaccharide (ficoll) to the immobilized trypsin exhibited high coupled yield (above 90%) and relative activities (174.5% and 175.9%, respectively), showing no protein leaching after incubating for 10 h in buffers. They showed broader pH and temperature profiles, while the half life of thermal stability of BSA-modified preparation at 50 °C increased to 1.3 and 2.3 times of unmodified and free trypsin, respectively. The modified trypsin in aqueous-organic solvents exhibited 100% activity after 6 h at 50 °C. The kinetic parameters of trypsin preparations and suitable pore diameter of MCFs warranted compatibility of covalent modification for substrate transmission. The covalent crowding modification for immobilized trypsin in nanopores establishes suitable and accessible microenvironment and renders possibility of biological application.

Key words: Immobilized Trypsin, Covalent Modification, Crowding Environment, Accessible Transmission

INTRODUCTION

Enzymes are catalysts bearing excellent properties and permitting sustainable application in an immobilized form, which provide facile separation from the product, thereby minimizing or eliminating protein contamination [1-3]. Trypsin is one of the serine proteases commonly used as the immobilized form, which substrate specificity requires that the carbonyl component to be catalyzed must be contributed by Lys or Arg. Immobilization of enzyme inside porous support may have the enzyme molecules fully dispersed and prevent it from interacting with any external interface. Mesoporous supports with high surface area and accessible transmission offer high potentials for enzyme immobilization, with respect to the adjustable pore diameter (4-30 nm) compared with that of the commonly used enzyme [4]. The stable structure and accessible transmission for enzyme in mesopores are determined by the interaction between enzyme and support in surrounded microenvironment [5,6].

Numerous techniques have been used to immobilize trypsin and focused on the modulation of the microenvironment [7]. In real living cells, proteins exist in crowded cytoplasm environment with synthetic or natural macromolecules called crowding agents (occupying large percentage of cell volume, typically from 5% to 40%) instead of dilute solution, which function through electrostatic adsorption and affect the intrinsic catalytic efficiency [8-10]. Moreover, the surface of macromolecules can be modified to form stronger covalent bonds with enzyme, preventing protein leaching from supports [11].

Trypsin suffers sharp inactivation during shaking or storage conditions in soluble form. The MCFs supports have short spacer arms compared with functionalized support and a high density of hydroxyl groups, thereby providing rigidity to enzyme [12]. The random interactions between macromolecules and enzyme molecules through electrostatic adsorption show enhanced activity, but exhibit low stability and easy leaching. To achieve more stabilization of adsorptive enzyme in MCFs, ameliorated immobilization strategy of chemical cross-linking with poly-aldehyde reagents was applied. In this research, poly-functional BSA and ficoll were introduced to the previously adsorbed trypsin in mesoporous silica foams (MCFs) to establish biomimic environment and establish covalent linkage. Effects of buffer sorts, temperature and pH profiles, kinetic characteristics, thermal stability in organic solvents were investigated carefully. The immobilized trypsin was used to catalyze precursor dipeptide of TP5 in aqueous-organic solvents.

MATERIALS AND METHODS

1. Materials

Trypsin (E.C.3.4.21.4) was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Casein and trichloroacetic acid were provided by Lingfeng Chemical Reagent Co., Ltd. (Shanghai, China). Bovine serum albumins (BSA, 67,000) and ficoll (70,000) were purchased from Sigma-Aldrich (Steinheim, Germany) of the highest grade commercially available. Other chemicals were all of analytical 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

The supports were prepared by Schmidt-Winkel et al. [13]. Then MCFs support was obtained by removing the organic templates, using hydrogen peroxide and nitric acid in closed vessels for 12 h.

†To whom correspondence should be addressed.
E-mail: zsbshen@gmail.com

Nitrogen adsorption and desorption measurement of nano-support was carried out by TriStar II 3020 surface area and porosity analyzer. 20 mg of MCFs were dispersed in 2 mL phosphate buffer solutions (PBS, pH 7.0, 0.1 mol/L) containing certain amount of trypsin. The hydroxyl support was 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 [14]. Aliquots of immobilized trypsin were investigated in PBS, borate and Tris-HCl buffers.

3. Co-assembly of Modified Macromolecules with Trypsin in MCFs

A portion of 100 mg of macromolecules were oxidized in 25 mg/mL of solid sodium periodate at 25 ºC for 3 h. The whole mixture was dialyzed four times against 100 mL of distilled water at 4 ºC. The aldehyded crowding reagents assembled with trypsin in MCFs through covalent linkage in presence of amino and aldehyde groups. The sodium borohydride was used for reduction of reversible imino linkages that existed between aldehyde and amino groups.

4. 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 ºC. 0.1 mg of native trypsin or 5-10 mg of wet immobilized trypsin was introduced into 5 ml substrate solutions and pre-incubated at 40 ºC. Reactions were carried out for 30 min and stopped by addition of 5 ml of trichloroacetic acid solution (5%, w/v). The supernatant solution after filtration was measured colorimetrically 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 ºC. As for relative activity, 100% was considered as the activity of soluble trypsin, 0.29 U/mg. Coupled yield meant the percentage of protein attached to support among the total added trypsin. All the experiments were performed in triplicate, and standard error was never over 4%. The coupled yield and relative activity were calculated as follows:

$$\text{Coupled yield } (\%) = \frac{(A-B)}{A} \times 100 \quad (1)$$

$$\text{Relative activity } (\%) = \frac{C}{(A-B)} \times 100 \quad (2)$$

Here, A represents the total activity of enzyme that added before immobilization; B is the activity of the same amount enzyme that remains in the supernatant after immobilization; C expresses the activity of immobilized enzyme.

5. The pH and Temperature Profiles of Immobilized Trypsin Preparations

For monitoring stability at extreme pH ranges, trypsin preparations were incubated from pH 5.0 to 9.0 in the presence and absence of additive. Aliquots were removed after 1 h of incubation and checked by hydrolyzing substrate. The optimum temperature of trypsin preparation was monitored by incubating trypsin preparation ranging from 25 ºC to 65 ºC in borate buffers at pH 8.0, 0.1 mol/L. Aliquots were removed at the scheduled time, chilled quickly at room temperature, and assayed for enzyme activity.

6. Thermal Stability Assays of Trypsin Preparations

The stability of trypsin preparations defined as $t_{1/2}$ was exhibited by using the half life of activities under thermal treatment. The parameter represents the time trypsin takes for undergoing decay by half, and can be calculated as the formula,

$$t_{1/2}=0.693*t/(2.303*\lg(E_0/E)) \quad (3)$$

Here $t_{1/2}$ is the half-life parameter of trypsin stability, E_0 shows the original activity of trypsin at the zero time and E is the activity measured at the scheduled point. Thermal stability of trypsin preparations was investigated at 50 ºC and 60 ºC for hours. Samples were quickly removed and determined colorimetrically at the specified time in standard assay.

7. Determination of Kinetic Characterization of Trypsin Preparations

Kinetic parameters of trypsin preparations were determined by assaying the rates of producing L-tyrosine at different substrate concentrations ranging from 2.5 to 15 mg/mL, which were calculated by plotting the Lineweaver Burk plots. The turnover number K_{cat}, represents the maximum number of substrate molecules that enzyme can turn over to produce in a set time, calculated by V_{max}/[E], where [E] is the enzyme concentration at zero time. The specificity constant K_{cat}/K_m represents the relative rate of reaction at low substrate concentration, which is much lower than K_m.

8. Catalytic Stability of Trypsin Preparations in Organic-aqueous Solvents

The stabilities of trypsin preparations in organic solvents were analyzed in 20% and 80% (v/v) aqueous-ethanol miscible solvents. Trypsin preparations were incubated at 50 ºC for 0-6 h. The residual activity of each sample was determined as mentioned above.

9. Enzymatic Synthesis and Analysis of Dipeptide

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. The wet immobilized trypsin was used as the catalyst at 35 ºC for 6 h. The reaction was terminated and separated by centrifugation.

Quantitative analysis of the dipeptide product was carried out by HPLC with a reverse phase C18 column (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; sample size, 20 μl. The reaction product was identified by mass spectrum (MS) analysis, with the condition of spray voltage of 5 kV, capillary temperature of 350 ºC, positive ion detection.

RESULTS AND DISCUSSIONS

1. Preparation of Immobilized Trypsin in MCFs

Trypsin was immobilized in MCFs by electrostatic adsorption through hydroxyl groups. Immobilization process was optimized for 6-h stirring in ice-incubator. The characterization of MCFs support (16.5 nm of pore diameter and 386.91 m^2/g of surface area) presented potentials for trypsin immobilization, while the diameter of

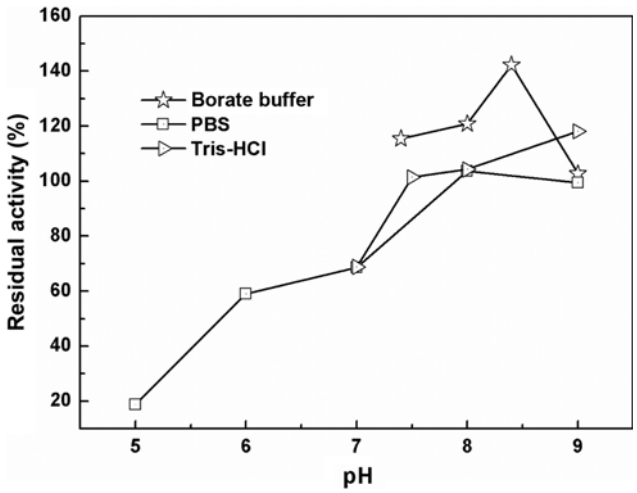

Fig. 1. Optimization of buffers for immobilized trypsin in MCFs. The borate, PBS and Tris-HCl buffers were all of pH 8.0, 0.1 mol/L.

trypsin molecule was about 4.8 nm [15] When the amount of trypsin offered to MCFs was 200 mg/g, the relative activity (120.9%) and coupled yield (91.9%) of trypsin corroborated its successful immobilization in MCFs. The adsorbed trypsin showed better catalytic activity than covalently assembled trypsin, without any chemical modification for enzyme surface and active centre by cross-linkers such as glutaraldehyde [16]. The buffer types and ionic strength were compared in Fig. 1. Buffers are vital parameters which maintain adequate ionic strength for enzyme catalysis, and are convenient for reaching a high enzyme-support reaction [17]. The immobilized trypsin catalyzing in borate buffers showed highest peak in alkaline environment. The borate buffers can offer a buffer buffering at pK_a of the first dissociation equilibrium, but interfere in the aldehyde-amine reaction. Tris-HCl buffers are physiologically inert but easily changed with temperature, and also contain toxic polyamine. PBS with broad ranges (pH 4.9 to 9.2) are the most commonly prepared buffers and mimic certain components of extracellular fluids. But they are likely to form precipitates, slowly contaminated with micro-organisms. As a result, the borate buffers were used as the suitable buffers for hydrolysis, while PBS are applied in immobilization process, accompany with the Tris-HCl used in enzymatic synthesis.

Table 1. Structural characterization of trypsin preparations and support by N_2 adsorption-desorption measurement

Sample	S_{BET}[a] (m^2/g)	Pore diameter[b] (nm)	Pore volume (cm^3/g)
MCFs-OH	386.91	17.75	1.78
MCFs-Trypsin	276.14	16.42	1.47
BSA-MCFs-Trypsin	275.86	16.54	1.48
Ficoll-MCFs-Trypsin	275.69	16.49	1.48

[a]S_{BET}, the surface area measured by BET methods
[b]Pore diameter, the most probable pore diameter
BSA-MCFs-Trypsin, trypsin co-immobilized with BSA in MCFs; Ficoll-MCFs-Trypsin, trypsin co-immobilized with ficoll in MCFs; MCFs-Trypsin, trypsin solely immobilized in MCFs; MCFs-OH, MCFs support with plenty of hydroxyl groups on surface. The concentration of macromolecules was 5% (w/w) of content of enzyme

2. Immobilization of Trypsin in Covalently Modified Environment

The adsorptive trypsin suffered inactivation for its weak linkage via electrostatic interaction. To retain high catalytic activity together with high stability in extreme environment, aldehyded crowding reagents were co-assembled with immobilized trypsin to establish covalent linkage. The modification of this previously adsorbed trypsin was completed by the interaction between the aldehyde groups of crowding reagents and the amino groups of trypsin. The overall immobilization strategy is presented in Fig. 2. First, trypsin was immobilized adsorptively in MCFs. Then the aldehyde groups of oxidized macromolecules reacted with amino groups of trypsin, forming reversible imino linkages which can be reduced by sodium borohydride. The BSA and ficoll preparations showed relative activities of 174.5% and 175.9% compared with the pristinely immobilized one (120.9%), respectively, while their coupled yield reached 92.5% and 93.8%. These may be attributed to the rebuilding of the enzyme configuration and establishment of the suitable microenvironment in confined nanopores, created by biomimic reagents. The biological reagents appeared to provide protection against the irreversible

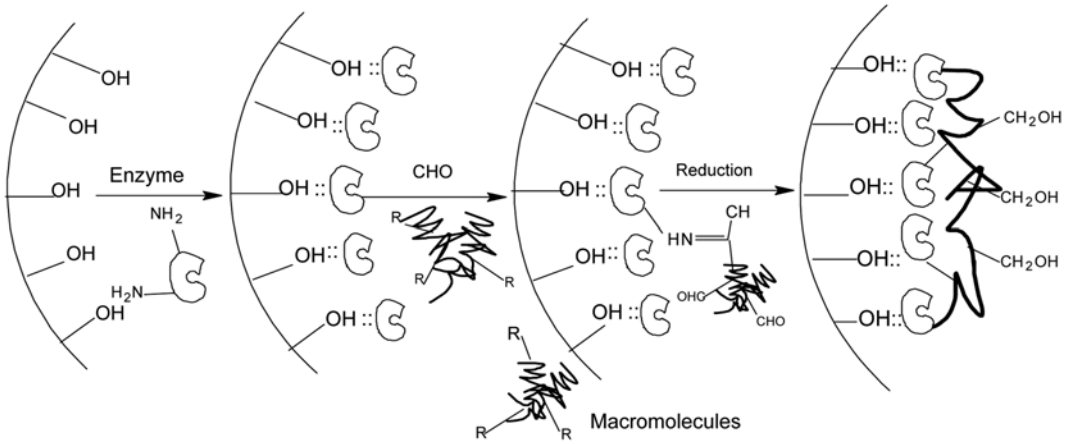

Fig. 2. Schematic for overall immobilization strategy for trypsin in MCFs.

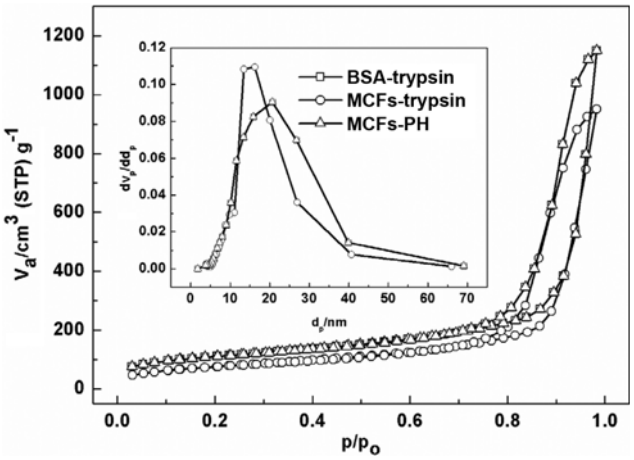

Fig. 3. N_2 adsorption-desorption isotherms and corresponding pore size distribution curve of trypsin preparations and MCFs support. BSA-Trypsin, trypsin co-immobilized with BSA in MCFs; MCFs-Trypsin, trypsin solely immobilized in MCFs; MCFs-OH, MCFs support with plenty of hydroxyl groups on surface.

aggregation of unfolded polypeptides, thus inducing the forming of enzyme-substrate complex [18].

The surface area, pore diameter and volume of trypsin preparations are shown in Table 1. The decrease of pore volume and surface area of solely immobilized trypsin compared with MCFs support corroborated successful assembly of enzyme to the nanopore walls. The sharp decline was also caused by the disordered distribution and occupation of enzyme in nanopore in a great measure, resulting in the decrease of pore diameter for the immobilized enzyme in Fig. 3. Trypsin modified with BSA and ficoll in MCFs showed the similar pore volume and surface area with the pristine one, which were attributed to the rebuilding of a well-distributed attachment for enzyme assembly under biomimic affinity effect. The trypsin preparation with BSA showing similar geometry property demonstrated the successful establishment for enzyme immobilization, reflecting no serve blockage in nanopores.

3. Catalytic pH and Temperature Profiles of Biomimic Trypsin Preparations

Variations of catalytic activities of trypsin preparations with respect to pH under biomimic strategy are presented in Fig. 4(a). The BSA and ficoll preparations showed broader profiles over the pH ranges. They shared the same optimum pH value of 8.5 with other preparations, compared with unmodified macromolecules in our previous work (shifted to neutral pH) [19]. This unchanged optimum pH may be caused by the part substitution of hydroxyl groups of macromolecules with aldehyde groups through oxidation, resulting in decrease of negative charges in microenvironment and the increase of pH surrounding, thus consequently retaining the optimum pH. It was codetermined by plenty of groups provided by macromolecules, enzyme and nano-channels of supports [20]. The intensive electrostatic adsorption and covalent crowding effects between enzyme and functional reagents established the stable acting forces for enzyme catalysis.

The temperature profiles of trypsin preparations are investigated ranging from 25 ºC to 65 ºC in Fig. 4(b). Biomimically immobilized

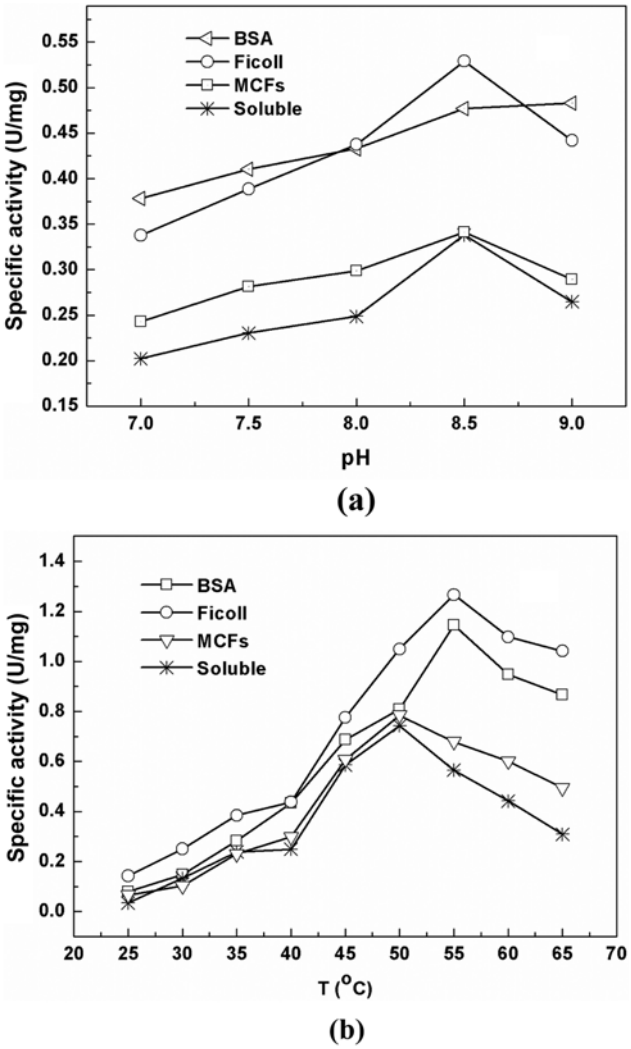

Fig. 4. The catalytic pH (a) and temperature (b) profiles of trypsin preparations. BSA, trypsin co-immobilized with BSA in MCFs; Ficoll, trypsin co-immobilized with ficoll in MCFs; MCFs, trypsin solely immobilized in MCFs; Soluble, soluble trypsin.

trypsin showed stable activity during this temperature range, especially from 50 ºC, when solely immobilized trypsin and its soluble counterpart lost their activities sharply. Increased temperature may cause enzyme autolysis, which can be efficiently prevented by the confinement in MCFs under crowding environment. Instead of sharp decrease, trypsin modified with BSA and ficoll exhibited broader and higher peaks with respect to the tolerant temperature, while their optimum temperature reached 55 ºC, 5 ºC higher than solely immobilized and soluble trypsin. BSA and ficoll favored stable configuration of enzyme in confined pores, which could alleviate the deactivation of enzyme to temperature [21,22].

4. Accessible Mass Transfer of Immobilized Trypsin Preparations

The kinetic parameters for trypsin preparations are investigated in Table 2. It was found that the K_m parameters of the modified trypsin preparations were lower than the solely immobilized and soluble ones. The decreases in K_m of modified preparations by BSA and ficoll

Table 2. Kinetic parameters of covalently biomimic trypsin preparations

Trypsin preparation	K_m (mol/L, 10^{-3})	V_{max} (mol/(L·min), 10^{-4})	K_{cat}/K_m (L/(mol·min), 10^8)
Soluble	1.70	2.23	4.03
MCFs	1.63	1.73	2.38
BSA	0.31	1.23	6.79
Ficoll	0.51	1.29	4.49

BSA, trypsin co-immobilized with BSA in MCFs; Ficoll, trypsin co-immobilized with ficoll in MCFs; MCFs, trypsin solely immobilized in MCFs; Soluble, soluble trypsin. The concentration of macromolecules was 5% (w/w) of content of enzyme

indicated that they had higher apparent affinity for their substrate than unmodified ones. The increases in the catalytic efficiency of K_{cat}/K_m were mainly attributed to the decrease in K_m values, i.e., the preferential formation of the enzyme-substrate complex. The well structured mesoporous support and the long-chain of macromolecules established the stable framework, which may maintain enzyme configuration and provide accessible channels for substrate. The microenvironment offered flexibility for enzyme, which was necessary for substrate binding. Thus, there existed no severe diffusion resistance in the confined immobilized particles for enzyme and substrate induced by biomimic reagents [23]. The diameter of MCFs channels was more than three times of that of trypsin, which exhibited the instinct structure being potential for accessible transmission. This accessible transmission by crowding reagents in MCFs channels was also corroborated by other immobilized enzyme of larger size in our previous work [19].

5. Thermal Stability of Trypsin Preparations

Advantages of employing high temperature are high process rates, lesser diffusion limitation, and decreased bacterial contamination. However, as a kind of protease, trypsin left terrible residual activity when it underwent severe denaturation reaction during high temperature. BSA and ficoll were co-assembled with trypsin to enhance the tolerance to thermal treatment at 50 °C and 60 °C (in Table 3). The half life of the BSA-modified trypsin at 50 °C was 58.8 min, increased to 1.3 and 2.3 times of solely immobilized and soluble trypsin, respectively. Ficoll, a markedly branched and cross-linked polymer of sucrose and epichlorohydrine having a rather open and

Table 3. Thermal stability of biomimic trypsin preparations at 50 °C and 60 °C

Half-life ($t_{1/2}$) of trypsin preparations (min)	BSA	Ficoll	MCFs	Soluble
50 °C	58.8	47.6	47.1	25.6
60 °C	12.7	14.8	12.6	9.9

The concentration of macromolecules was 5% (w/w) of content of enzyme. The half-time of trypsin preparations at high temperature was determined at the time when half of activity was reached. BSA, trypsin co-immobilized with BSA in MCFs; Ficoll, trypsin co-immobilized with ficoll in MCFs; MCFs, trypsin solely immobilized in MCFs; Soluble, soluble trypsin

deformable structure, can effectively confine the enzyme configuration owing to its compact and quasi-spherical shape. As a kind of globulin, BSA shows similar geometry property with trypsin, offering more compatibility of enzyme configuration in nanopores. The decreased rate of thermal denaturation may be ascribed to the aldehyded crowding reagents that covalently reacted with enzyme and protected the protein structure, which was also supported by molecular modeling computation analysis [24]. The enhanced stability was reached by covalent crowding theory and the confinement of nano-support. This was demonstrated by investigating the thermal stabilities of soluble preparations with and without existence of aldehyded reagents, showing similar residual activity and inactivation profiles (data not shown).

6. The Structure Stability of Immobilized Trypsin in Continual Process and its Enzymatic Synthesis of Precursor Dipeptide of TP 5

The trypsin preparations were incubated in 20% and 80% (v/v) ethanol medium at 50 °C. The residual activities of BSA-modified trypsin and its pristine one are plotted in Fig. 5. The BSA preparation retained about 100% of its original relative activity after incubating in 20% (v/v) ethanol medium for 6 h at 50 °C, more stable than the unmodified trypsin both in original activity and thermal stability. The enzyme preparations gained stable stability in 80% (v/v) ethanol medium instead of high activity. That was attributed to the high percentage of hydrophilic organic solvent, on account of the displacement of bound water by organic solvent resulting in dramatic change of protein structure and activity loss. In this procedure, water-organic miscible solvents stripped away of essential water, and then partially dehydrated protein binding of organic co-solvent; thus conformational transition in protein molecule formed denatured situation [25]. To investigate structural stability, the modified trypsin and its pristine one were incubated in buffers for 10-h shaking, with leaching protein profiles checked in Fig. 6. The pristinely immobilized trypsin suffered severe leaching ever since 2 h, while covalent modification effectively retained stable structure even at 10 h, showing the same profiles in 20% (v/v) ethanol (data not

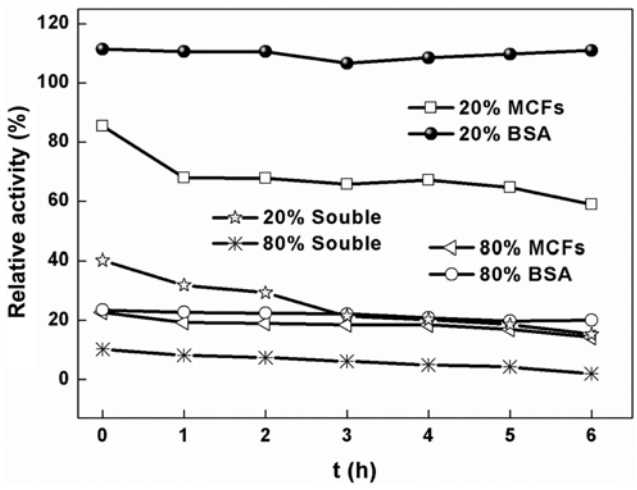

Fig. 5. Stability of trypsin preparations in 20% and 80% (v/v) aqueous-ethanol medium after thermal treatment at 50 °C. BSA, trypsin co-immobilized with BSA in MCFs; MCFs, trypsin solely immobilized in MCFs; Soluble, soluble trypsin.

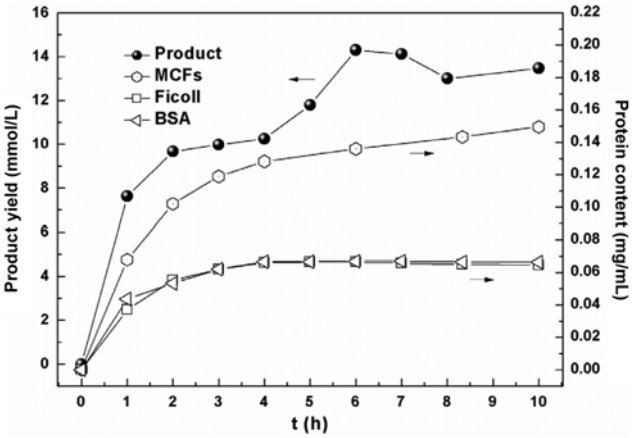

Fig. 6. The structural stability of immobilized trypsin in continual process and its enzymatic synthesis of precursor dipeptide of TP 5. BSA, trypsin co-immobilized with BSA in MCFs; Ficoll, trypsin co-immobilized with ficoll in MCFs; MCFs, trypsin solely immobilized in MCFs; Product, precursor dipeptide of TP5. Trypsin preparations were incubated for 10 h, and aliquots of leached protein and produced product samples were determined.

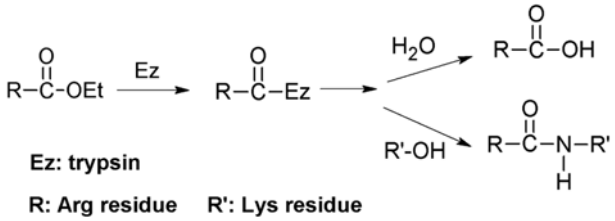

Fig. 7. Scheme of kinetic control of dipeptide under enzymatic catalysis of trypsin. The synthesis process was carried out in buffers of 20% (v/v) ethanol, using Bz-Arg-OEt as the acyl donor with Lys-OH as the nucleophile.

shown).

Immobilized trypsin was used to synthesize Bz-Arg-Lys-OH under kinetic control, which substrate specificity requires the carbonyl component of the peptide bond contributed by Lys or Arg. This dipeptide was the precursor of TP5 pentapeptide (Arg-Lys-Asp-Val-Tyr), commonly synthesized by solid-phase condensation via side-chain protection and deprotection process and repeated washing by deleterious organic solvents [26]. The synthesis process was completed by acyl transfer reaction in 20% (v/v) ethanol, using Bz-Arg-OEt as the acyl donor and Lys-OH as the nucleophile. The peptide bond formation may depend on the reaction rate and mole ratio of water and nucleophile, and the formed acyl-enzyme intermediate can be deacylated by water or nucleophile (in Fig. 7). The target product analyzed by HPLC using reference standard was confirmed by MS-ESI m/z: 407.3 $[M+H]^+$. The peak of MS-ESI m/z: 279.3 $[M+H]^+$ was taken for Bz-Arg-OH in this reaction system, which was formed by hydrolysis of reactant of Bz-Arg-OEt•HCl. The peak of 147.2 $[M+H]^+$ was identified as Lys-OH.

CONCLUSIONS

Chemically modified macromolecules with poly-aldehyde groups were co-assembled with the adsorbed trypsin in MCFs. The catalytic activity and coupled yield exhibited potentials for enzyme immobilization in MCFs, in possession of high surface area and suitable pore diameter. PBS and borate buffers were used for immobilization and reaction, respectively, while Tris-HCl was used for enzymatic synthesis. The addition of polysaccharide (ficoll) and stable protein (BSA) to trypsin exhibited relative activities of 175.9% and 174.5%, respectively. They showed broader catalytic pH and temperature profiles compared with the pristine preparations. This modification theory offers accessible mass transfer for enzyme and substrate in nano-channels and enhancement of thermal stability and structure stability of enzyme preventing protein leaching. A better understanding of microenvironment and interactions between MCFs and enzyme involved in immobilization will greatly facilitate ongoing use of immobilized enzymes in biological applications.

ACKNOWLEDGEMENTS

This research was supported by the National High Technology Research and Development Program of China (863 Program, No. 2009AA05Z313), National High Technology Research and Development Program of China (863 Program, No. 2006AA02Z211), National Natural Science Foundation of China (50872052), National Natural Science Foundation of China (20376034), Natural Science Foundation of Jiangsu Province of China (BK2006181), Foundation of Jiangsu Province of China for College Postgraduate Students in Innovation Engineering (2007), Foundation of Nanjing University of technology of China for College Postgraduate Students in Innovation Engineering (BSCX200910).

REFERENCES

1. D. Brady and J. Jordan, *Biotechnol. Lett.*, **31**, 1639 (2009).
2. R. A. Sheldon, *Adv. Synth. Catal.*, **349**(8-9), 1289 (2007).
3. S.-K. Lee, S.-W. Park, Y.-I. Kim, K.-H. Chung, S.-I. Hong and S.-W. Kim, *Korean J. Chem. Eng.*, **19**, 261 (2002).
4. Y. J. Wang and F. Caruso, *Chem. Mater.*, **17**, 953 (2005).
5. C. Mateo, J. M. Palomo, G. Fernandez-Lorente, J. M. Guisan, and R. Fernandez-Lafuente, *Enzyme Microb. Technol.*, **40**, 1451 (2007).
6. A. M. Wang, C. Zhou, Z. Q. Du, M. Q. Liu, S. M. Zhu and S. B. Shen, *J. Biosci. Bioeng.*, **107**, 219 (2009).
7. T. Liu, S. Wang and G. Chen, *Talanta*, **77**, 1767 (2009).
8. M. Jiang and Z. H. Guo, *J. Am. Chem. Soc.*, **129**, 730 (2007).
9. R. J. Ellis, *Curr. Opin. Struc. Biol.*, **11**, 114 (2001).
10. S. Zorrilla, G. Rivas, A. U. Acuna and M. P. Lillo, *Protein Sci.*, **13**, 2960 (2004).
11. B. C. Pessela, C. Mateo, M. Filho, A. V. Carrascosa, R. Fernandez-Lafuente and J. M. Guisan, *Process Biochem.*, **43**, 193 (2008).
12. C. Mateo, J. M. Palomo, G. Fernandez-Lorente, J. M. Guisan and R. Fernandez-Lafuente, *Enzyme Microb. Technol.*, **40**, 1451 (2007).
13. P. Schmid-winkel, W. W. Lukens, D. Y. Zhao, P. D. Yang, B. F. Chmelka and G. D. Stucky, *J. Am. Chem. Soc.*, **121**, 254 (1999).
14. M. M. Bradford, *Anal. Biochem.*, **72**, 248 (1987).
15. G. V. Lyubinskii, E. A. Kalinichenko and V. A. Tertykh, *Theor. Exp. Chem.*, **28**, 216 (1993).
16. F. Lopez-Gallego, L. Betancor, C. Mateo, A. Hidalgo, N. Alonso-Morales, G. Dellamora-Ortiz, J. M. Guisan and R. Fernandez-Lafu-

ente, *J. Biotechnol.*, **119**, 70 (2005).
17. C. Mateo, J. M. Palomo, M. Fuentes, L. Betancor, V. Grazu, F. Lopez-Gallego, B. C. C. Pessela, A. Hidalgo, G. Fernandez-Lorente, R. Fernandez-Lafuente and J. M. Guisan, *Enzyme Microb. Technol.*, **39**, 274 (2006).
18. A. R. Kinjo and S. Takada, *Phys. Rev.*, **66**, 031911 (2002).
19. C. Zhou, A. M. Wang, Z. Q. Du, S. M. Zhu and S. B. Shen, *Korean J. Chem. Eng.*, **26**, 1065 (2009).
20. R. J. Ellis, *Curr. Opin. Struc. Biol.*, **11**, 114 (2001).
21. C. M. Soares, H. F. De Castro, M. H. Santana and G. M. Zanin, *Appl. Biochem. Biotechnol.*, **91-93**, 703 (2001).
22. M. S. Cheung and D. Thirumalai, *J. Mol. Biol.*, **357**, 632 (2006).
23. A. P. Minton, *Curr. Opin. Struc. Biol.*, **10**, 34 (2000).
24. Z. D. Zhang, Z. M. He and M. X. He, *J. Mol. Catal. B-Enzym.*, **14**, 85 (2001).
25. B. B. Boonyaratanakornkit, C. B. Park and D. S. Clark, *Biochem. Biophys. Acta*, **1595**, 235 (2002).
26. W. G. Wang, P. H. Li, S. B. Shen, H. J. Ying and P. K. Ouyang, *Chinese J. Org. Chem.*, **26**, 826 (2006).