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Immobilized trypsin on hydrophobic cellulose decorated nanoparticles shows good stability and reusability for protein digestion

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ABSTRACT

The preparation of biocatalysts based on immobilized trypsin is of great importance for both proteomic research and industrial applications. Here, we have developed a facile method to immobilize trypsin on hydrophobic cellulose-coated silica nanoparticles by surface adsorption. The immobilization conditions for the trypsin enzyme were optimized. The as-prepared biocatalyst was characterized by Fourier transform infrared spectroscopy, transmission electron microscopy, and elemental analysis. In comparison with free enzyme, the immobilized trypsin exhibited greater resistances against thermal inactivation and denaturants. In addition, the immobilized trypsin showed good durability for multiple recycling. The general applicability of the immobilized trypsin for proteomic studies was confirmed by enzymatic digestion of two widely used protein substrates: bovine serum albumin (BSA) and cytochrome *c*. The surface adsorption protocols for trypsin immobilization may provide a promising strategy for enzyme immobilization in general, with great potential for a range of applications in proteomic studies.

Trypsin digestion has been one of the most widely used biological reactions for both research and industrial applications [1–3]. The in vitro reaction conditions for trypsin often differ significantly from physiological conditions, with involvements of organic solvents and incubation at extremes of temperature and pH [4,5]. Therefore, stabilization of trypsin is of great importance. The major strategy for physical stabilization of trypsin is by immobilizing trypsin onto a heterogeneous support [6–8]. There are typically three methods for trypsin immobilization: cross-linking trypsin by glutaraldehyde, entrapment of trypsin in mesoporous materials, and immobilization of trypsin onto the surface of a support [4,9,10]. Among the trypsin immobilization methods, surface adsorption has advantages because the active sites of the enzyme are more accessible. Moreover, this method avoids reactive chemicals, which are unfavorable toward the stability of trypsin [9]. In addition, surface adsorption methods can be further optimized for development of practical bioreactors because the amount of enzyme can be easily controlled [11].

The cellulose matrix has been widely employed for enzyme immobilization [12–14]. However, most cellulose materials are functionalized by hydrophilic moieties such as —NH_2, —COOH, and —DEAE [13]. Compared with hydrophobic cellulose, the utility of hydrophilic cellulose may be compromised due to its tendency to be washed away in aqueous solutions during the reaction of enzymatic digestion. However, the adsorption of trypsin onto hydrophobic cellulose has rarely been investigated. One major concern is that the hydrophobic active sites of enzymes on the hydrophobic carrier would likely be irreversibly open, thereby reducing their activities [6]. On the other hand, cellulose could provide a large surface and rigid framework, which may lack the mobility and could efficiently bind to the loaded trypsin [15]. Moreover, trypsin can be tightly adsorbed onto a hydrophobic support in aqueous solutions, thereby avoiding leaching. Thus, it is envisaged that the active sites of the enzyme could be partially preserved because both the mobility of trypsin and cellulose framework are restricted in the aqueous digestion solution. Recently, fabrication of silica nanoparticles has attracted increasing interest for enzyme immobilization due to their uniform morphology, high stability, permeability, and good biocompatibility [16–18]. In addition, functionalized silica nanoparticles have advantages over bulky materials due to their better dispersion in solution during the enzyme immobilization process.

Here, we have developed a method for preparation of trypsin biocatalysts by adsorption onto the surface of hydrophobic cellulose-coated silica nanoparticles. The conditions for trypsin immobilization, such as enzyme concentration, pH, and temperature, were

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optimized. The enzymatic activity and reusability of the immobilized trypsin were evaluated by digestions of casein, cytochrome *c*, bovine serum albumin (BSA),[1] and collagen.

Materials and methods

Reagents

Trypsin (bovine pancreas) was purchased from Sangon Biotech (Shanghai, China). Cellulose powder was purchased from Aladdin (Shanghai, China). Anhydrous sodium carbonate, urea, sodium hydroxide, trifluoroacetic acid (TFA), trichloroacetic acid (TCA), and tetrahydrofuran (THF) were obtained from Guangfu Fine Chemical (Tianjin, China). Toluene, acetonitrile (ACN), and anhydrous ethanol were obtained from Lianlong Bohua Pharmaceutical Chemicals (Tianjin, China). Phenyl isocyanate and pyridine were purchased from National Pharmaceutical Group Chemical Reagents (Beijing, China). Folin and casein were of biological grade and obtained from Yuanye Biotechnology (Shanghai, China). Tetraethoxysilane (TEOS) was purchased from Kelong (Chengdu, China). Ammonia was obtained from Shuangshuang Development (Yantai, China).

Analytics

Fourier transform infrared (FTIR) spectra were recorded on a Nicolet NEXUS 670 infrared spectrophotometer. Optical density measurements were performed on a Shimadzu UV-1760 spectrophotometer. Liquid chromatography–mass spectrometry (LC–MS) analysis was carried out on an AB SCIEX API 2000 mass spectrometer equipped with an electrospray interface (Turbo Ion Spray) coupled with a Shimadzu Prominence LC-20A chromatography system consisting of a CTO-20A column oven and an SIL-20AC autosampler. Transmission electron microscopy (TEM) micrographs were recorded on an FEI TECNAI G2 transmission electron microscope. Elemental analyses were obtained on an Elementar Vario EL analyzer. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed using a 15% gel on a Liuyi Electrophoresis apparatus. Dynamic light scattering (DLS) assay was carried out on a Brookhaven Instruments BI-200SM system.

Synthesis of silica nanoparticles

Silica nanoparticles were prepared according to the sol–gel method. Specifically, TEOS (4.5 ml) was dissolved in anhydrous ethanol (45.5 ml). Thereafter, the solution was added to a mixture of aqueous ammonia (28%, 1.5 ml), anhydrous ethanol (16.25 ml), and deionized water (24.75 ml). The mixture was ultrasonicated for 10 min and stirred mildly for 2 h. The clear reaction solution slowly turned turbid due to the formation of silica. The silica nanoparticles were collected by centrifugation and dried overnight at 100 °C. The product yield was 61.84%.

Synthesis of tris-O-phenylcarbamoyl cellulose

Cellulose (2 g) and pyridine (30 ml) were added to a 100-ml, three-necked round-bottom flask and slowly heated to 85 °C with stirring for 30 min. Phenyl isocyanate (13.69 g) was then added dropwisely into the flask. The reaction was completed in 10 h, and the raw product was obtained after removing the solvent. The raw product was washed by ultrasonication in methanol (100 ml) for 40 min and collected by centrifugation (6790*g*, 10 min). The washing process was repeated three times. The product was dried in an oven at 70 °C. The purified product is 7.86 g (yield: 50.07%). Elemental analysis results: C (62.885%), H (4.572%), N (8.775%). Accordingly, the cellulose has been fully modified by *O*-phenylcarbamate groups.

Adsorption of modified cellulose on silica nanoparticles

The silica nanoparticles were coated with tris-*O*-phenylcarbamoyl cellulose, and such hydrophobic nanoparticles (SilCel) were employed as support for trypsin immobilization in the following experiments. Tris-*O*-phenylcarbamoyl cellulose (0.10 g) was dissolved in THF (1.2 ml), and silica nanoparticles (0.50 g) were added. The suspension was mixed by vortex and then placed in a shaker at 220 rpm for 12 h at 25 °C. The product was dried in an oven at 70 °C for 7 h. The collected raw material was then washed with deionized water in a Soxhlet extractor for 7 h and dried at 53 °C. The cleaned product SilCel is 0.34 g. Elemental analysis results: C, 15.85%; H, 0.67%; N, 1.12%. Accordingly, 12.77 wt% of modified cellulose was added onto the silica nanoparticles.

Preparation of immobilized trypsin

During the trypsin immobilization process, the hydrophobic support (SilCel 3 mg) was immersed in the solution of trypsin (200 μl) and incubated in a shaker at 220 rpm at 25 °C. The trypsin was immobilized through surface adsorption onto the support after incubation. Several variables, such as the concentration and pH of trypsin solution and incubation time, were optimized to obtain the highest enzymatic activity of immobilized trypsin (Tr-SilCel). The concentration of trypsin solution was optimized in a range from 250 to 1500 μg/ml. The pH of the trypsin solution was varied between 5.0 and 10.0. The incubation time was varied between 0.5 and 10 h. The Tr-SilCel was separated by centrifugation (6790*g*, 10 min) and washed by 50 mM sodium phosphate (pH 7.4) three times.

FTIR and TEM characterization of samples

FTIR and TEM studies were performed on silica nanoparticles, SilCel, and Tr-SilCel. The FTIR spectra of pristine silica nanoparticles and those coated with SilCel are illustrated in Fig. 1A. The spectrum of SilCel has additional vibration bands of C=O (1739 cm^{-1}) and C=C (1600 cm^{-1}) of the *O*-phenylcarbamoyl group. Thus, the modified cellulose had been successfully coated onto the surface of silica nanoparticles. As illustrated in Fig. 1B, the FTIR spectrum of trypsin showed the C—N bond (1531 cm^{-1}), whereas the benzene ring (1643 cm^{-1}) and the C—H bond (2931 cm^{-1}) indicated its hydrophobic characters. Tr-SilCel maintained the vibrations of the C—H bond (2927 cm^{-1}) and C—N bond (1517 cm^{-1}) of trypsin in Fig. 1B. Therefore, the FTIR results confirmed the adsorption of trypsin onto the hydrophobic support SilCel.

To prepare TEM samples, the powder was dispersed in anhydrous ethanol and a drop of the solution was placed on a copper grid coated with carbon. The nanoparticles were left on the grid after evaporation of the solvent at room temperature. The TEM micrograph of silica nanoparticles exhibited a spherical shape with a diameter of approximately 200 nm (Fig. 2A). SilCel and Tr-SilCel maintained the morphology of the spherical nanoparticles (Fig. 2B and C). The prepared nanoparticles were analyzed by DLS, indicating that the sizes of the nanoparticles were reasonably homogeneous (see Fig. S1 in the online supplementary material). Modified cellulose is hydrophobic in nature, increasing its ability

[1] *Abbreviations used:* BSA, bovine serum albumin; TCA, trichloroacetic acid; TFA, trifluoroacetic acid; THF, tetrahydrofuran; ACN, acetonitrile; TEOS, tetraethoxysilane; FTIR, Fourier transform infrared; LC–MS, liquid chromatography–mass spectrometry; TEM, transmission electron microscopy; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; DLS, dynamic light scattering; SilCel, silica nanoparticles coated with tris-*O*-phenylcarbamoyl cellulose; Tr-SilCel, immobilized trypsin.

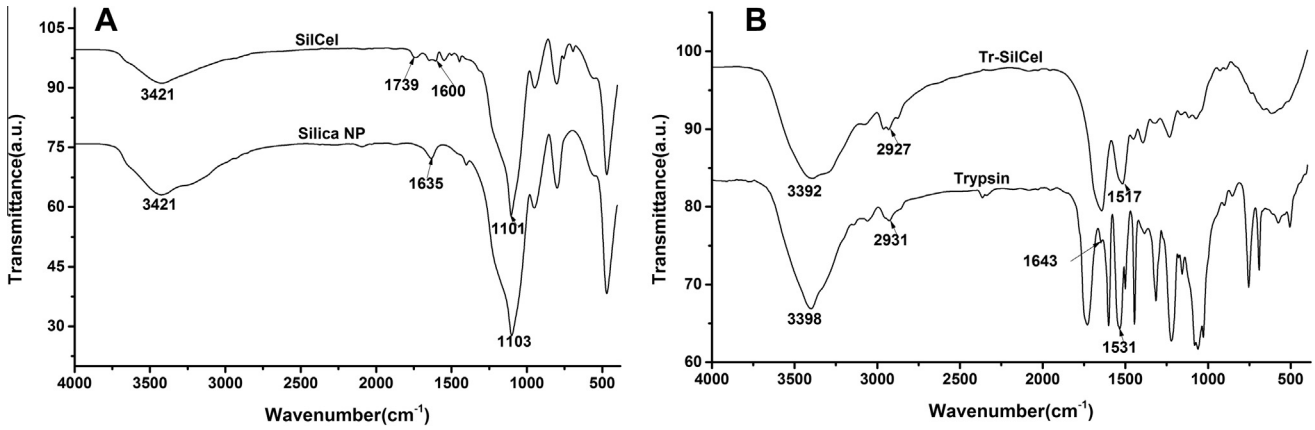

Fig.1. FTIR spectra of silica nanoparticles (NP) and SilCel (A) and trypsin and Tr-SilCel (B). NP, nanoparticles. SilCel displays the additional vibration band of C=O at 1739 cm^{-1} when compared with silica nanoparticles. Tr-SilCel maintains the vibrations of C—H (2927 cm^{-1}) and the C—N bond (1517 cm^{-1}) of trypsin.

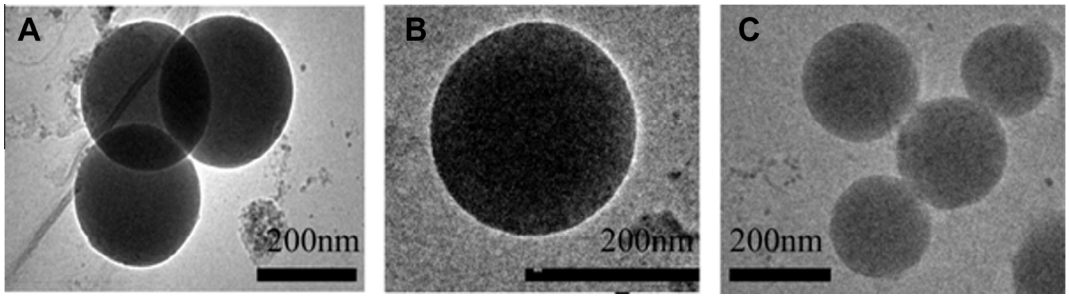

Fig.2. TEM of silica nanoparticles (A), SilCel (B), and Tr-SilCel (C). Silica nanoparticles exhibit a spherical shape with a diameter of approximately 200 nm. SilCel and Tr-SilCel maintain the morphology of the spherical nanoparticles.

to absorb enzyme through hydrophobic adsorption, which could be even stronger in aqueous solvent. Thus, the adsorbed trypsin is unlikely to be washed away in aqueous solutions in the following enzymatic digestion applications.

Enzymatic activity of free and immobilized trypsin

The activities of free and immobilized trypsin were determined with a Folin assay using casein as substrate [19]. Briefly, immobilized trypsin was incubated in 1 ml of 10 g/L casein solution (50 mM sodium phosphate, pH 7.4), which was placed in a shaker at 200 rpm for 30 min at 30 °C. The supernatant (800 μl) was collected after centrifugation at 10,000*g* for 5 min. Trichloroacetic acid (TCA) solution (2 ml, 0.4 M) was added to the supernatant, mixed for 2 min, and kept still for 8 min to stop the enzymatic reaction. The supernatant was collected after centrifugation. Casein hydrolysis performance was then determined by optical density measurement at 763 nm using Folin and Ciocalteu's phenol reagent. Specifically, the supernatant (1 ml) was added to 5 ml of Na_2CO_3 solution (0.4 M), and then Folin phenol reagent (1 ml) was added. The color development reaction took 20 min at 40 °C, and then its optical density was measured. The activity of free trypsin was determined by the same method using the corresponding amount of immobilized trypsin (7 μg). The enzymatic activities of both free and immobilized trypsin under denatured conditions were evaluated by adding various amounts of urea or ACN.

LC–MS characterization of trypsin digestion products of BSA and cytochrome c

In-solution digestion of cytochrome *c* and BSA was performed using a common protocol [20,21]. Briefly, cytochrome *c* was dissolved in 100 μl of 100 mM sodium bicarbonate buffer at a concentration of 40 μM. Such solution was first mixed with 200 μl of 0.4 M ammonium bicarbonate and 50 μl of 8 M urea. Thereafter, 700 μl of water was added to the solution. BSA solution (100 μl, 10 μM) was prepared in ammonium bicarbonate (200 μl, 0.4 M) and urea (50 μl, 8 M). Disulfide bridges in BSA were reduced with 5 μl of DTT (45 mM in 0.4 M ammonium bicarbonate buffer) at 50 °C for 15 min. After that, free sulfhydryl groups were alkylated with 5 μl of iodoacetamide (0.1 M in 0.4 M ammonium bicarbonate buffer) at room temperature in the dark for 15 min. Then, 600 μl of water was added to dilute the solution. Two mixed protein substrates were also prepared by mixing BSA, cytochrome *c*, V-CL, and lysozyme in 1 ml of 100 mM sodium bicarbonate buffer at a total concentration of 1 μM. One mixture (PM-1) contained BSA, cytochrome *c*, V-CL, and lysozyme at equal molar ratio, and the other mixture (PM-2) contained proteins at a molar ratio of 5:1:1:1. Each of the prepared substrates was then mixed with 1 mg of Tr-SilCel or free trypsin of the same amount (7 μg), respectively. The enzymatic digestions with free trypsin and Tr-SilCel were performed at 37 °C for 20 h. After digestion, the protein digests were collected and desalted using C18 ZipTips according to the procedure suggested by the manufacturer (Millipore, Bedford, MA, USA). Peptides were eluted by solvent of ACN/TFA/H_2O (50 μl, 50:0.1:49.9, v/v/v). Then, the solvent were removed using a rotary evaporator, and the product was lyophilized. After that, LC–MS samples were prepared in H_2O (with 0.1 vol% TFA) at 1 μg/ml.

Typical chromatographic separation of produced peptides was performed on a Promosil C18 analytical column. A gradient program was applied for analysis of produced peptides. The eluents were as follows: A, 99.9% H_2O with 0.1% TFA; B, ACN/TFA/H_2O (90:0.1:9.9, v/v/v). The applied elution conditions were as follows:

0 to 10 min, linear gradient from 0 to 1% B; 10 to 40 min, linear gradient from 1 to 55% B; 40 to 50 min, linear gradient from 55 to 90% B; 50 to 60 min, 90% B isocratic; 60 to 70 min, linear gradient from 90 to 1% B; 70 to 75 min, linear gradient from 1 to 0% B. The flow rate was 0.3 ml/min. All spectra were processed by Analyst software (AB SCIEX), and the sequence coverage was calculated using the software ProteinProspector (http://prospector.ucsf.edu/prospector/mshome.htm).

Thermal stability and reusability of Tr-SilCel

The temperature profile of free and immobilized trypsin was determined by incubating trypsin with casein solution at different temperatures (20–70 °C). The thermal stability of free and immobilized trypsin was determined by incubating trypsin with casein solution for various periods (1–10 h) at 55 °C. The relative activities of free and immobilized trypsin at different temperatures and time were normalized to the highest value of each set.

The reusability of Tr-SilCel was evaluated by collagen digestion using SDS–PAGE, a widely used electrophoretic technique for separating proteins [22]. Gel was prepared immediately before use by polymerization of acrylamide and *N,N′*-methylene-bis-acrylamide, which was initiated by the addition of ammonium persulfate and tetramethylethylenediamine (TEMED). Here, 15% separating gel and 5% stacking gel were used. Bacterial collagen V-CL (100 µl, 2.25 mg/ml, 50 mM glycine buffer, pH 8.6) was incubated with 1 mg of the support with immobilized trypsin for approximately 12 h at 25 °C. When one reaction was finished, the same immobilized enzyme was removed from the medium by centrifugation and the supernatant was collected and stored at −20 °C for further SDS–PAGE analysis. The immobilized enzyme was reused after thorough rinsing with glycine buffer (50 mM, pH 8.6) three times. The experiments were repeated for 15 cycles.

Results and discussion

Optimal immobilization conditions for Tr-SilCel

Because the adsorption–desorption equilibrium was highly dependent on the pH of the trypsin solution and the ratio between trypsin and SilCel, the concentration and pH of trypsin solution were optimized to obtain the highest enzymatic activity of immobilized trypsin [5,23]. It was observed that the enzymatic activity of the prepared biocatalyst gradually increased with higher concentrations of free trypsin at consistent temperature until the amount of free trypsin accounted for 13 wt% of the support. Further addition of free trypsin into the solution could not increase the activity of the prepared biocatalyst (Fig. 3A). Meanwhile, the activity of Tr-SilCel was proportional to the concentration of free trypsin in dilute solutions, whereas it reached a plateau in concentrated trypsin solutions. This likely indicated a single layer adsorption of trypsin onto the surface of the support [8].

The pH of trypsin solution was also optimized in the range from 5.0 to 10.0 during the immobilization process. The adsorption of trypsin in acid solution of pH below 5.0 was unachievable due to the poor solubility and instability of trypsin under such conditions. Although bovine pancreas trypsin is a basic protein with a p*I* value of 8.0, it was found that the enzymatic activity of Tr-SilCel was optimal at pH 6.1, indicating that the p*I* value of trypsin may be lowered in the presence of a hydrophobic support (Fig. 3B). Thus, it was envisaged that basic groups of trypsin may be involved during the immobilization process [24]. It is probable that amino groups of trypsin formed hydrogen bonds with the carbonyl groups of the phenylcarbamoyl moieties on the support. Meanwhile, the hydrophobic domain of trypsin may be directed toward the hydrophobic surface of the support in aqueous solutions that resulted in the single-layer adsorption model. In conclusion, the addition of 13 wt% trypsin onto the nanoparticle support at pH 6.1 is the optimal preparation protocol for Tr-SilCel. In comparison between elemental analysis results of lyophilized free trypsin (C: 46.55%; H: 7.00%; N: 14.82%) and immobilized trypsin (C: 16.70%; H: 0.76%; N: 1.22%), the content of trypsin on Tr-SilCel is calculated as 0.71 wt%.

High tolerance of Tr-SilCel to denaturing conditions

Trypsin is one of the most important enzymes in proteomic studies, where urea and ACN are widely used in typical experiments to denature native proteins for their further cleavage [3]. To evaluate the performance of free and immobilized trypsin under these extreme but necessary conditions, urea and ACN were added to the casein solution at different ratios (Fig. 4). Tr-SilCel exhibited higher tolerance toward high content of ACN and urea in solution. Tr-SilCel kept most of its activity when the concentration of urea was no more than 4 M, whereas the activity of free enzyme was dramatically reduced toward 30% under the same conditions. Furthermore, Tr-SilCel maintained around 60% of its activity when the ratio of ACN was beyond 10% in solution. In contrast, free trypsin retained only 20% of its original activity under the same conditions. These data indicate that the denaturants of urea or ACN exerted only marginal suppression on the enzymatic activity of Tr-SilCel (Fig. 4).

Thermal stability

The temperature profile of free and immobilized trypsin was determined by incubating trypsin with casein solution at different temperatures (20–70 °C). The optimal temperatures of free and immobilized trypsin were similar (35 °C for both). The relative activities of free and immobilized enzyme at different temperatures were normalized to that of 35 °C. In comparison with free enzyme, the immobilized trypsin showed a higher relative activity beyond 45 °C, suggesting that Tr-SilCel is more tolerant to inactivation at high temperature (Fig. 5). The increased stability of immobilized trypsin may be due to the noncovalent interactions of the amino groups of trypsin with the carrier, which reduced the conformational flexibility of the enzyme.

Thermal stabilities of both free and immobilized trypsin were further investigated by casein hydrolysis after incubating the free trypsin and Tr-SilCel at 55 °C (Fig. 6). After 3 h of incubation, the free trypsin maintained only 65% of its initial activity, whereas Tr-SilCel kept 92% of its activity. As the incubation time increased, the catalytic activities of both free and immobilized trypsin decreased. However, it was obvious that Tr-SilCel always exhibited much stronger resistance toward thermal inactivation than free enzyme throughout the whole time range evaluated. Conclusively, the experimental results implied that the immobilization process may help to stabilize the conformation of trypsin.

Reusability of Tr-SilCel

Reusability is one of the best advantages of enzyme immobilization. Bacterial collagen V-CL is a good model system to visualize the enzymatic cleavage activity of trypsin [25,26]. At temperatures lower than the melting temperature of 37 °C, the V domain of collagen V-CL can be completely degraded into small fragments that cannot be observed by SDS–PAGE analysis. The CL domain, instead, is resistant to trypsin cleavage and is left as a single band after the cleavage on the SDS–PAGE. This provides a convenient method to test the activity and reusability of trypsin. As shown in Fig. 7, the control sample was the V-CL protein (MW = 33 kDa) in the absence

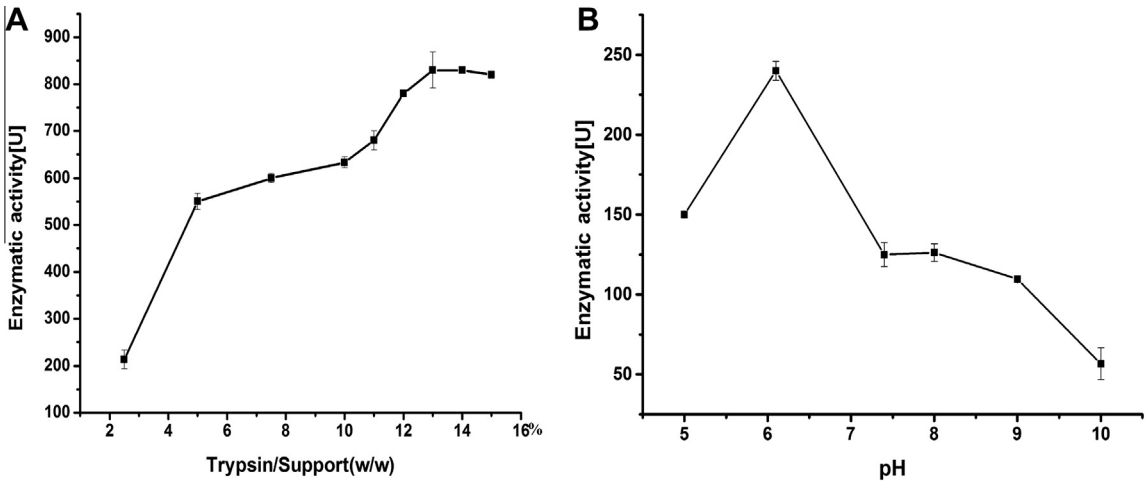

Fig.3. Effect of enzyme concentration (A) and pH (B) on trypsin immobilization. The concentration and pH of trypsin solution were optimized to obtain the highest enzymatic activity of immobilized trypsin (Tr-SilCel). The addition of trypsin solution at pH 6.1 onto the nanoparticle support (trypsin/support [w/w] = 13%) provides the optimal immobilization condition for Tr-SilCel.

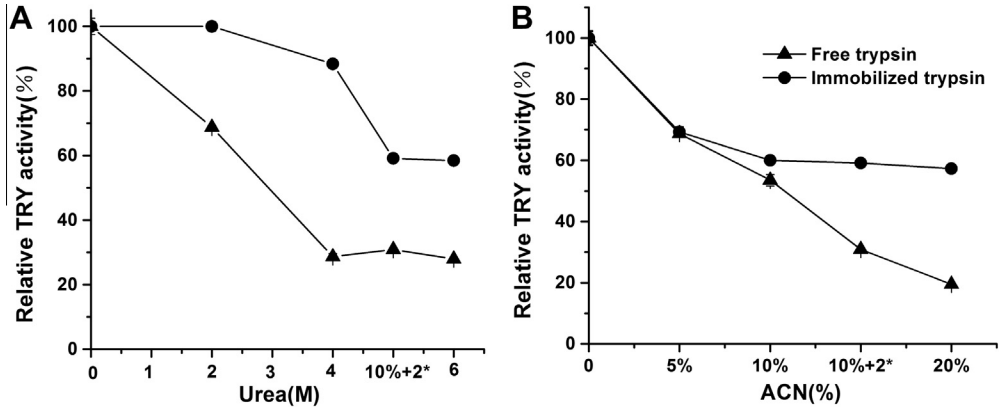

Fig.4. Enzymatic activity of free trypsin (▲) and Tr-SilCel (●) under denaturing conditions: (A) urea; (B) ACN. TRY, trypsin. On the *x*-axis of both panels, "10%+2*" indicates 10% ACN plus 2 M urea. When 4 M or less urea is used, Tr-SilCel retains most of its activity, which is much higher than that of free enzyme. When 10% or more ACN is used, Tr-SilCel retains approximately 60% of its activity, whereas with free trypsin only approximately 20% of its activity is observed.

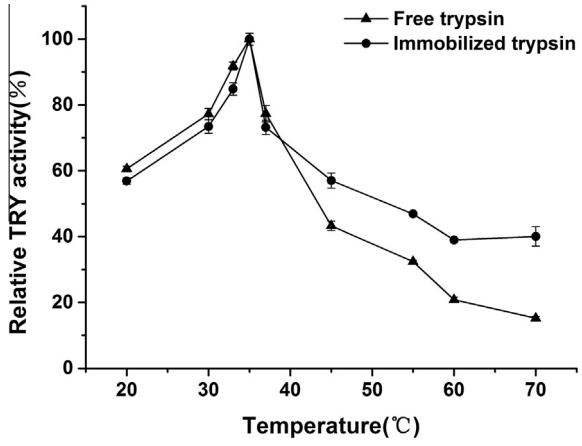

Fig.5. Temperature profiles of free trypsin (▲) and Tr-SilCel (●). TRY, trypsin. The optimal temperatures of free trypsin and Tr-SilCel are both 35 °C. In comparison with free enzyme, the immobilized trypsin shows a higher relative activity above 45 °C.

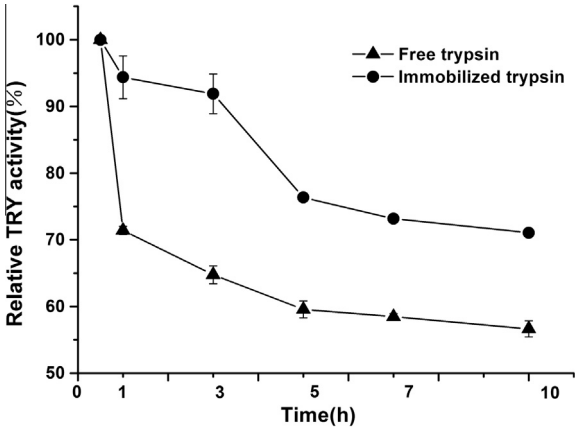

Fig.6. Thermal stability of free trypsin (▲) and immobilized trypsin (●). TRY, trypsin. The immobilized trypsin retains consistently much higher relative activity than free enzyme.

of any trypsin, and it migrated as a single band near 35 kDa. After treatment with immobilized trypsin, collagen V-CL was completely cleaved into the smaller CL fragment (MW = 22 kDa), migrating as a single band below 25 kDa. Thus, the degradation of V-CL into CL could be easily monitored as two totally different bands on SDS–PAGE gels. Tr-SilCel was reused for 15 cycles, and it was treated

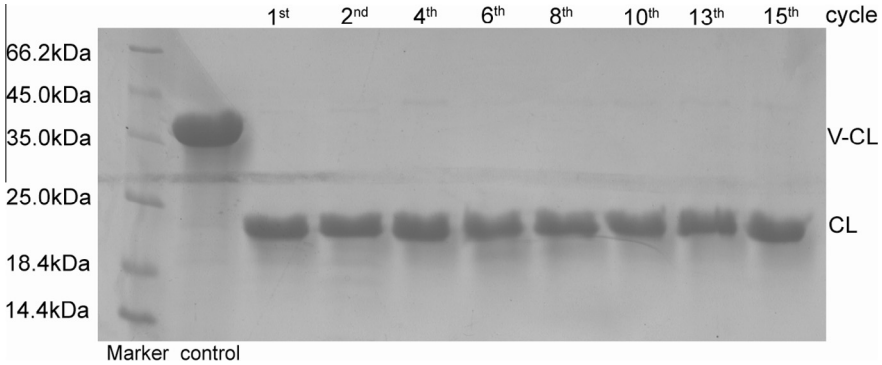

Fig.7. SDS–PAGE of digestion products of bacterial collagen V-CL by reused Tr-SilCel. The control sample V-CL protein (MW = 33 kDa), in the absence of any trypsin, migrates as a single band near 35 kDa. After the treatment with immobilized trypsin, V-CL is completely cleaved into a smaller fragment CL (MW = 22 kDa) and CL migrates as a single band below 25 kDa. Tr-SilCel was reused for 15 cycles, retaining similar activity to be able to completely cleave V-CL in each cycle.

with the same amount of V-CL for each cycle. V-CL was completely cleaved every time, suggesting that the immobilized enzyme kept similar activity to be able to completely cleave V-CL even after repeated use of 15 cycles. Three different samples of Tr-SilCel were repeatedly used at least 10 times, and they all kept similarly high activity during such use. All of the results suggest that the immobilized trypsin displayed very good reusability.

Proteomic digestion efficiency of Tr-SilCel

The performance of Tr-SilCel was further evaluated with respect to its general applicability to proteomic studies. Sequence coverage was used to compare the digestion efficiency of two widely used proteins, BSA and cytochrome *c*, as well as two protein mixtures, PM1 and PM2. It was observed that both Tr-SilCel and free trypsin resulted in similar sequence coverage for BSA (~86%) and cytochrome *c* (~64%; Table 1). For two more complex protein mixture samples, the immobilized trypsin exhibited higher sequence coverage than the free enzyme (Table 1). These results showed the advantage of immobilized trypsin over free enzyme for proteomic studies in that the immobilization eliminated the need of enzyme removal and, therefore, reduced the possible interference of the enzyme with the mass peaks of produced peptides.

To investigate whether there is any trend in the peptides found in the digestion products using immobilized and free trypsin, those detected peptide sequences are mapped to the X-ray crystal structures of BSA (see Fig. S2 in supplementary material) and cytochrome *c* (Fig. S3) [27,28]. Both the immobilized and free enzyme showed high digestion efficiency given that they could digest most part of the protein. However, differences were also observed between different protein substrates. For BSA with a more complex three-dimensional structure, the undetected peptides were distributed more evenly over the structure using the free enzyme than the immobilized trypsin (Fig. S2); however, those undetected peptides in cytochrome *c* were located at similar positions in the structure with both immobilized and free trypsin (Fig. S3). Those undetected peptides may provide useful information about the accessibility of protein substrates to the enzyme prepared by different methods.

Table 1
Sequence coverage of proteomic digestion by free and immobilized trypsin.

Protein	Enzyme condition	Sequence coverage (%)
BSA	Free trypsin	84.7
	Immobilized trypsin	88.3
Cytochrome *c*	Free trypsin	66.3
	Immobilized trypsin	62.5
Protein mixture PM-1[a]	Free trypsin	66.3
	Immobilized trypsin	83.7
Protein mixture PM-2[a]	Free trypsin	51.9
	Immobilized trypsin	81.7

[a] Protein mixture PM-1: BSA/cytochrome *c*/V-CL/lysozyme (molar ratio 1:1:1:1). Protein mixture PM-2: BSA/cytochrome *c*/V-CL/lysozyme (molar ratio 5:1:1:1). Sequence coverage is calculated for cytochrome *c* in protein mixtures PM-1 and PM-2.

Conclusions

A facile method has been developed to immobilize trypsin on the surface of hydrophobic silica nanoparticles. The TEM micrographs indicated that the as-prepared biocatalyst maintained the uniform morphology of the spherical nanoparticles, with the modified nanoparticle support providing a large surface area for enzyme absorption. The absorption of trypsin enzyme onto the hydrophobic silica gel was confirmed by the FTIR spectra. The concentration and pH of trypsin solutions were optimized for the enzyme immobilization, and the optimal enzyme activity was achieved when 13 wt% of trypsin was added onto the nanoparticle support at pH 6.1.

Compared with free enzyme, the immobilized trypsin showed much higher thermal stability as revealed by better tolerance to higher temperature and longer thermal inactivation time. Tr-SilCel also displayed very good reusability and maintained high activity after repeated use (15 times). In addition, the immobilized enzyme showed good resistance to the denaturants ACN and urea, which are widely used in proteomic studies. The general applicability of the immobilized trypsin to proteomic analyses was further confirmed by LC–MS studies of two widely used proteins: BSA and cytochrome *c*. The sequence coverage measurements indicated that Tr-SilCel showed digestion efficiency comparable to the free enzyme. To conclude, our newly developed Tr-SilCel on hydrophobic SilCel may provide a promising strategy for enzyme immobilization with great potential in applications for proteomic analyses.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ab.2015.02.009.

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