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Covalent Immobilization of Mixed Proteases, Trypsin and Chymotrypsin, onto Modified Polyvinyl Chloride Microspheres

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Supporting Information

ABSTRACT: A commercially available trypsin−chymotrypsin mixture was covalently immobilized onto modified polyvinyl chloride (PVC) microspheres, which were activated by the subsequent treatment of PVC microspheres with ethylenediamine and glutaraldehyde. The immobilized mixed protease was characterized by FT-IR and SEM analyses. Immobilization conditions were optimized by Box−Behnken design and the response surface method. The activity of the immobilized mixed protease prepared under optimal conditions (pH 6.6, 23 °C, 2 h) reached 1341 U/g. Compared with the free form, the immobilized enzyme possesses a slightly higher optimal pH value and a wider pH-activity profile, superior thermal stability, and a higher K_m value. Reusability of the immobilized mixed protease indicated that >70% of the original activity was retained after having been recycled six times.

KEYWORDS: *immobilized protease, functionalized PVC microsphere, trypsin, chymotrypsin, covalent immobilization*

INTRODUCTION

Both trypsin (EC 3.4.21.4)[1] and chymotrypsin (EC 3.4.21.1)[2,3] are members of the serine protease family, which plays a major role in protein digestion. Trypsin differs from chymotrypsin in that it specifically cleaves peptide bonds on the carboxyl side of arginine and lysine residues, whereas chymotrypsin specifically hydrolyzes proteins and peptides at the carboxyl side of phenylalanine, tyrosine, and tryptophan residues. Degradation of protein is more efficient when these two enzymes are simultaneously present in solution compared with their sequential addition. Thus, the trypsin−chymotrypsin mixture has been widely applied in the pharmaceutical, chemical, and bioengineering fields and found to be beneficial in the manufacture of protein hydrolysates and the preparation of functional polypeptides.[4−6] However, their application is limited due to their relative instability in solution and rapid loss of catalytic activity due to autolysis during both operation and storage.

Immobilized enzyme provides many important advantages over the native enzyme, such as ready isolation, reusability, and improved stability.[7,8] The immobilization of enzymes onto insoluble carriers has been an active research topic in enzyme technology for many years.[9,10] Xi et al.[11] immobilized trypsin on silica gel and reported that the immobilized enzyme could tolerate relatively tough environmental conditions, such as high temperature and a wide pH range. Such preparations were found to possess long storage times and to be reusable. Katchalski-Katzir[12] also demonstrated that bound enzymes possess marked stability, which renders them more applicable for industrial production. These findings encouraged us to develop novel approach to immobilize trypsin−chymotrypsin onto a suitable support. Polyvinyl chloride (PVC), as an inert support for the immobilization of enzymes, has many advantages such as being chemically inert, tough, lightweight, and corrosion free.[13] However, it is essential to introduce suitable functional groups on PVC to render it possible to be used as a support for immobilized trypsin−chymotrypsin.[14,15]

In the present study, a commercially available trypsin−chymotrypsin mixture was immobilized onto functionalized PVC microspheres. The immobilized trypsin−chymotrypsin preparations were characterized by Fourier transform infrared spectroscopy (FT-IR) and scanning electron microscopy (SEM) analysis. The kinetic behavior, pH, and temperature profiles and thermal stability of the immobilized trypsin−chymotrypsin are also reported.

MATERIALS AND METHODS

Materials. PVC, ethylenediamine (EDA), glutaraldehyde (GA), and trypsin−chymotrypsin (2400:400) were purchased from Aladdin (Shanghai, China). Casein was obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals used were of analytical reagent grade. Buffer solutions were prepared with distilled water.

Preparation of Amino-Functionalized PVC Microspheres. PVC microspheres with terminal amine groups were prepared by the reaction of PVC microspheres with EDA using a previously reported method[16] with slight modifications. PVC microspheres (1 g) were incubated with 20 mL of 3% (v/v) EDA aqueous solution at 50 °C for 1 h. After completion of the reaction, the microspheres were filtered and washed with distilled water to remove unreacted EDA and then dried in a vacuum oven at 45 °C. The nitrogen content was measured by Kjeldahl analysis apparatus (Buchi B-324, Flawil, Switzerland) according to the method of Mohy Eldin;[17] the HCl solution (0.05 M) was used in titration.

Immobilization of Trypsin−Chymotrypsin on Modified PVC. One gram of aminated PVC was activated with 20 mL of glutaraldehyde 1% (w/v) solution at pH 3.0 for 60 min at 40 °C. This activated support was rinsed with water and then transferred to 10 mL of phosphate buffer (20 mM), which contained 0.02−0.03 g of

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trypsin−chymotrypsin. The mixture was stirred at room temperature for 2 h to complete the immobilization process. Excess enzyme was decanted off, and then the microspheres were washed with distilled water until no enzyme was detected in the washing liquid. The immobilized enzyme was lyophilized.

Measurement of Trypsin−Chymotrypsin Activity. *Determination of the Activity of Native Trypsin−Chymotrypsin.* The activity of soluble trypsin−chymotrypsin was determined by the trichloroacetic acid (TCA)−Lowry assay[18] at 37 °C using casein as substrate. One milliliter of trypsin−chymotrypsin solution (0.2 mg/mL) in phosphate-buffered solution (PBS, 20 mM, pH 8.0) was added to casein solution (20 g/L, 5 mL) in PBS buffer (20 mM). The mixture was incubated constantly at 37 °C for 3 min in a temperature-controlled water bath, and then the reaction was quenched by the addition of 5 mL of TCA; the resulting precipitate was removed by filtration and centrifugation. The tyrosine concentration was determined using the Lowry method.[19] Briefly, 1 mL of filtrate was mixed vigorously with 5 mL of 0.5 M Na_2CO_3 solution and 1 mL of 2-fold diluted Folin reagent. The absorbance of the solution was recorded at 680 nm after incubation at 37 °C for 30 min. One unit of activity of mixed protease is defined as the amount of trypsin−chymotrypsin required to liberate 1 μmol of tyrosine per minute under the following conditions: pH 8.0 and 37 °C. A UV-2100 UV−vis spectrophotometer (Unico, USA) was used to monitor the optical absorption.

Determination of the Activity of Immobilized Trypsin−Chymotrypsin. The assay of immobilized enzyme was performed as described for its free form except that 1 mL of free enzyme solution (0.2 mg/mL) in PBS was replaced by 0.2 g of PVC microsphere-bound enzyme in 1mL of PBS. One unit of activity of immobilized enzyme is the amount of immobilized enzyme required to liberate 1 μmol of tyrosine per minute under the conditions previously described. The percentage of immobilization was calculated as

$$\text{immobilization (\%)} = \frac{\text{amount of enzyme immobilized on support (mg)}}{\text{amount of added enzyme (mg)}} \times 100$$

where the amount of enzyme immobilized on support was calculated on the basis of the nitrogen content measured by Kjeldahl analysis.

Matrix Characterization. PVC, amino-PVC, and immobilized enzyme were characterized by FT-IR spectra (Thermo Fisher, USA). FT-IR spectra in the absorbance mode were recorded on an FT-IR spectrometer, connected to a PC, and the data were analyzed by IR solution software (omnic). Samples were mixed with KBr to make pellets. The surface morphology of PVC, aminated PVC, and enzyme-immobilized PVC was analyzed with a scanning electron microscopy (SEM) (S-4700, Hitachi Ltd., Japan) up to 15 kV. The fracture surfaces were coated with gold under vacuum and then imaged and photographed by SEM.

Determination of Kinetic Parameters. The apparent Michaelis constants (K_m) of free and immobilized enzyme were determined by measuring the reaction rates (at 37 °C, pH 8.0) using casein as a substrate with various initial concentrations ranging from 10 to 40 g/L. The apparent K_m value was obtained by analyzing the data according to the Hanes−Woolf equation. Turnover number (k_{cat}) and specific constant (k_{cat}/K_m) have not been calculated because trypsin−chymotrypsin is not a pure enzyme.

Effect of pH and Temperature on Enzyme Activity. The effect of pH value on the activity of free and immobilized enzyme for hydrolysis of casein (20 g/L) was investigated at 37 °C by varying the pH value (6.0−9.0). Different buffer systems were used within their effective pH ranges: 20 mM PBS (pH 6.0−8.0) and 20 mM borate (pH 8.0−9.0). Free enzyme (1 mg) or immobilized enzyme (1 g) was individually added to a test tube containing 3 mL of buffer, and the measurement of activity was performed 3 min later. The effect of temperature for hydrolysis of casein (20 g/L) on the activity of free enzyme (pH 7.5) and immobilized enzyme (pH 8.2) was determined by varying the temperature over the range of 30−80 °C.

Thermal Stability. The activity of free and immobilized enzymes was determined after incubation at varying temperatures (from 30 to 80 °C) for 1 h in buffer (pH 7.5 and 8.2, respectively). After incubation, the samples were quickly cooled and assayed for catalytic activity as described above. The residual activities were calculated as the ratio of the activity of the enzyme after incubation at a particular temperature to the maximum activity.

Reusability of Immobilized Trypsin−Chymotrypsin. The operational stability of the immobilized trypsin−chymotrypsin was assessed by the repeated use of enzyme-conjugated microspheres. An activity assay was performed by hydrolyzing the casein solution at pH 8.2 and 25 °C for 30 min. Immobilized trypsin−chymotrypsin was washed by submersion in a series of four test tubes containing 1 mL of sodium phosphate buffer (pH 8.2, 20 mM) and then resuspended in the same buffer to start a new run. The activity of the immobilized trypsin−chymotrypsin was expressed as the percentage of residual activity when compared to the initial activity.

Statistical Methods. Box−Behnken design[20] and response surface method (RSM) were employed to evaluate the effects of immobilization parameters, optimized conditions, and response surfaces being calculated and drawn, respectively. The model was simplified by dropping terms that were not statistically significant ($p > 0.05$) and by analysis of variance (ANOVA). GraphPad Prism 5.0 was employed for kinetic parameter data analysis.

RESULTS AND DISCUSSION

Functionalization (Amination) of PVC. Surface functionalization of PVC microspheres was achieved by treatment with EDA. The effects of EDA concentration on the nitrogen content of aminated PVC and the catalytic activity of immobilized enzyme were investigated (Figure 1). The

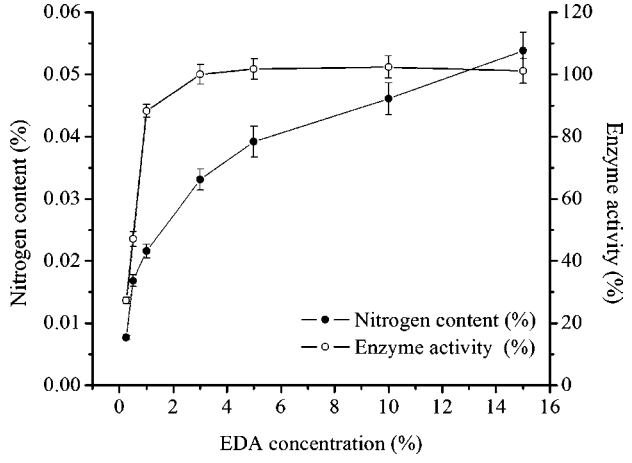

Figure 1. Effect of EDA on the nitrogen content and catalytic activity of immobilized enzyme.

resulting aminated PVC was found to contain higher nitrogen content with increasing EDA concentration. To ascertain a suitable nitrogen content for enzyme immobilization, the range of aminated PVC microspheres (containing different nitrogen contents) was activated with GA, on which trypsin−chymotrypsin was then immobilized. When the nitrogen content is <0.03%, the activity of the immobilized enzymes increased with the increase of nitrogen content. Maximum activity was obtained at about 0.03% nitrogen.

Activation of Aminated PVC. Generally, enzymes are deposited on the surface of aminated PVC when conjugated using GA cross-linking reactions.[16,21] In many cases, the treatment of enzyme adsorbed in supports bearing primary amino groups with glutaraldehyde offers a shorter spacer arm

(monomer), which could maintain the stability of the enzyme for a longer duration[13,17,22] and give a higher rigidity.[23,24] Thus, GA was used to activate aminated PVC microspheres in this study. The effects of GA concentration, reaction pH, reaction temperature, and contact time on the activity of immobilized enzyme conjugated to the corresponding activated PVC microspheres were investigated. The independent variables, including GA concentration (0.5−1.5%), reaction pH (2.5−3.5), reaction temperature (30−50 °C), and contact time (60−90 min) at three levels in the activation process, were employed. On the basis of the single-factor experiment, an orthogonal experiment ($L_9(3)^4$) was used to evaluate the combination effects of the four parameters on the activation of enzyme. (The details are presented in Supporting Information Table S1.) The optimal conditions for activation of aminated PVC microspheres were determined as follows: GA concentration, 1.0%; reaction pH, 3.0; contact time, 60 min; and reaction temperature, 40 °C. This result is in agreement with the papers of Silva et al.[25] and Monsan,[26] who reported that high GA concentration and alkaline pH values yield an uncontrolled reaction and result in the polymerization of GA. It has also been demonstrated that higher temperatures may promote polymerization and yield dimeric and cyclic forms, which are less reactive toward amine groups.[24,26] Thus, the inclusion of a spacer, GA, was essential to improve conformational flexibility,[27] to restrict interaction among immobilized enzyme molecules,[29] and to enhance enzymatic activity.

Immobilization of Enzyme on Modified PVC. Exposure of a mixture of trypsin−chymotrypsin with activated PVC microspheres in the presence of phosphate buffer leads to effective interaction between the amine groups on the enzymes and the aldehyde groups of activated PVC microspheres. Thus, the activity of the yielded immobilized enzyme is typically determined by the soluble enzyme concentration in the reaction system. According to Tischer et al.,[4] the resulting activity of the immobilized enzymes depends not only on losses caused by the binding procedure but can be further reduced as a result of the diminished availability of enzyme molecules within pores or by slowly diffusing substrate molecules. Such limitations lead to lowered efficiency. Therefore, to obtain immobilized enzyme with high activity, it is essential to establish a well-balanced relationship between reaction system and carrier by adjusting the amount of added enzyme, reaction pH, and immobilization temperature. Evaluation of the effect of immobilization variables, amount of added enzyme (X_3), immobilization pH (X_1), and reaction temperature (X_2), is presented in Table 1. Table 1 shows the designed experiment matrix, together with the experimental results. The activity of immobilized enzyme varied significantly (from 900 to 1300 U/g). High activities of immobilized enzymes (>1300 U/g) were obtained when an intermediate level of trypsin−chymotrypsin amount was used at intermediate levels of both immobilization pH and temperature (runs 8, 9, and 10). Xiao et al.[29] prepared the immobilized chymotrypsin with chitosan−arginine resin, the activity of which reached 850 U/g, whereas the immobilized trypsin prepared with chitosan microspheres by Tan et al.[30] had a low activity of 42.02 U/g. Compared with other immobilized proteases, the immobilized trypsin−chymotrypsin developed in the present study possesses much higher activity. However, the different solid support and immobilization type would both affect the percentage of immobilization.[31] Because the main objective of this study is to prepare immobilized enzyme with high activity, a relatively high concentration of enzyme was employed, resulting in a relatively low percentage of immobilization (5.78−9.63%). The filtrate of the reaction mixture still contains high catalytic activity and can be reused in subsequent immobilization runs with the supplement of a corresponding amount of enzyme immobilized on the support. Comprehensive analysis of the overall data indicates that the immobilization pH had the most significant impact on response, whereas the temperature exerted a statistically significant effect. (The details are presented in Supporting Information Table S2.)

Table 1. Experimental Design and Results According to Box−Behnken Design[a]

	variable level[b]			immobilization enzyme activity[c]	
run	X_1	X_2	X_3	exptl	predicted
1	6.5	20	20	1205.56 ± 39.27	1195.35
2	6.5	25	25	1283.89 ± 49.93	1317.492
3	7.0	25	20	1060.56 ± 31.29	1063.825
4	6.0	20	25	989.44 ± 38.29	966.525
5	7.0	30	25	945.00 ± 29.45	967.915
6	7.0	20	25	1106.11 ± 41.23	1113.055
7	6.5	30	20	1182.78 ± 43.26	1156.6
8	6.5	25	25	1324.44 ± 46.01	1317.492
9	6.5	25	25	1345.56 ± 40.18	1317.492
10	6.5	25	25	1340.00 ± 44.78	1317.492
11	7.0	25	30	1102.78 ± 32.17	1069.655
12	6.0	30	25	914.44 ± 28.14	907.495
13	6.5	30	30	1102.22 ± 37.84	1112.43
14	6.5	20	30	1251.67 ± 40.59	1277.85
15	6.5	25	25	1293.57 ± 36.94	1317.492
16	6.0	25	30	982.78 ± 32.63	979.515
17	6.0	25	20	913.89 ± 27.66	947.015

[a]Contact time: 2 h; the activity of the free enzyme is 732 ± 34 U/mg. [b]Numbers in parentheses represent actual experimental amounts. X_1, X_2, and X_3 are immobilization pH, reaction temperature (°C), and addition of enzyme (mg/g), respectively. [c]In U/g of support.

ANOVA was used to determine the adequacy and significance of the quadratic model. This model presented high adjusted R^2 and low coefficients of variation (CV), which were obtained as $R^2 = 0.9788$ and CV = 2.97%, indicating that good precision and reliability were associated with these experiments. The adjusted R^2 value of 0.9516 implied that the model was significant. The values of "Prob > F" <0.05 indicated model terms were significant. Therefore, three factors on the immobilization process were determined by the multiple-regression model as follows:

$$Y = 1317.49 + 51.47X_1 - 51.04X_2 - 31.67X_2X_3 - 249.65X_1^2 - 79.09X_2^2 - 52.84X_3^2$$

The three-dimensional response surface plots and contour plots of the response are shown in Figure 2. As can be seen from Figure 2-I, the activity of immobilized mixed protease was low when the immoblization was carried out at the lowest pH and lowest enzyme concentration at a fixed temperature; thereafter, the response value increased quickly at the beginning and then decreased with the increase of pH at a fixed enzyme concentration. Usually, a neutral pH value is used to prevent support inactivation; this reaction should only involve the most reactive amino groups in the protein.[24] The mixed protease in this case will be immobilized by the most reactive amino (in most cases the terminal amino group); therefore, a suitable

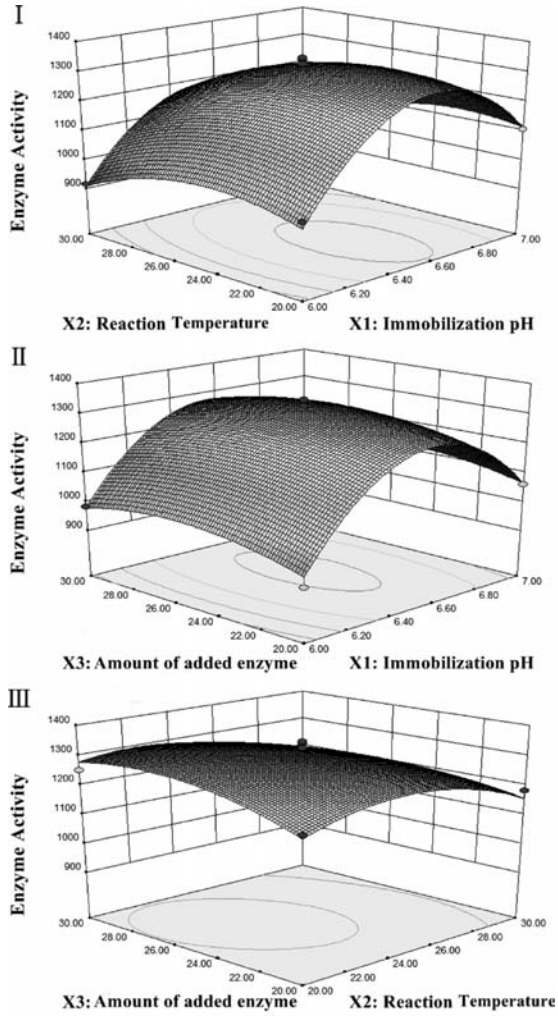

Figure 2. Three-dimensional response surface plots and contour plots of immobilization conditions (immobilization pH, reaction temperature, and amount of added enzyme) on catalytic activity of immobilized trypsin−chymotrypsin: (I) interactive effects of immobilization pH and reaction temperature on enzyme activity; (II) interactive effects of immobilization pH and amount of added enzyme on enzyme activity; (III) interactive effects of reaction temperature and amount of added enzyme on enzyme activity.

reaction pH is necessary to get high activity of immobilized enzyme, because under lower pH values immobilization may not occur, and under high pH values the enzymes may become inactivated.[28] Figure 2-II demonstrates the immobilization increased and then remained unchanged with increasing enzyme concentration at a fixed pH and temperature, indicating that the effect of enzyme concentration is not significant, which is in keeping with the analysis of variance in the Supporting Information (Table S2) ($P > 0.05$). Figure 2-III shows the dependence of immobilization on temperature and addition of enzyme at a fixed immobilization pH. Moderately high temperatures may increase the thermal vibration in the enzymes and support, increasing the possibility of enzyme−support linkage formation.[28]

The optimized immobilization conditions were found to be pH 6.56, addition of enzyme at 26 mg, and temperature at 23 °C. Using this model under these conditions, the catalytic activity of immobilized enzyme was predicted to be 1331 U/g. A validation assay was conducted using the above optimum conditions to confirm the catalytic activity, and this was determined as 1340.78 ± 50.13 U/g.

FT-IR Analysis. The formation of aminated PVC, activated PVC, and enzyme-conjugated PVC was confirmed by comparison of FT-IR spectra with those of the initial PVC microspheres from 400 to 4000 cm^{-1} (Figure 3). Curve I shows the IR spectrum of PVC microspheres. In curve II, a new broad characteristic peak at 3392 cm^{-1} indicates the existence of amine groups ($-NH_2$) on the surface of the microspheres. Activated microspheres (curve III) show two new peaks at 1731 and 1695 cm^{-1}, which correspond to carbonyl groups (C═O) and the carbon−nitrogen double bond (C═N−), respectively. In curve IV, a strong and broad absorption band around 3427 cm^{-1} appears, which may attributed to hydroxyl groups in the enzyme. The decreased intensity of the peak at 1731 cm^{-1} and the increased intensity of the peak at 1632 cm^{-1} indicate that the aldehyde groups on the surface of activated PVC reacted with amine groups on the enzyme, forming Schiff base linkages, as previously reported.[16]

Morphological Characterization. The SEM analyses of PVC microspheres (Figure 4), aminated microspheres, and immobilized enzyme were made at ×500 and ×5000 magnification, to demonstrate the change on the surface of

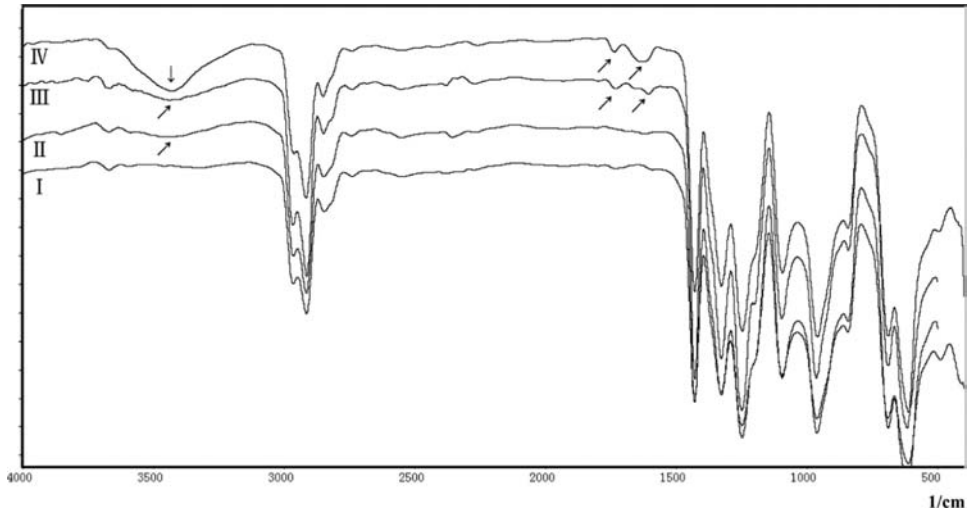

Figure 3. FT-IR spectra: PVC (I); aminated PVC (II); activated PVC (III); immobilization enzyme on PVC (IV).

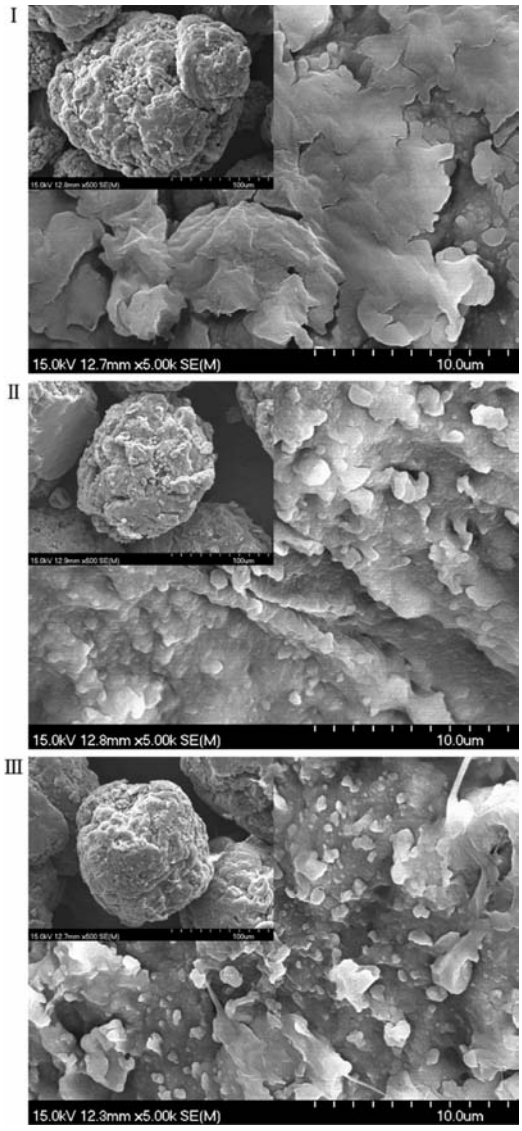

Figure 4. SEM morphology analysis: PVC (I); aminated PVC (II); enzyme immobilized PVC (III). Bar = 500/5000 μm.

PVC microspheres. As can be seen from Figure 4-I, the initial PVC consists of nonporous microspheres; thus, the functionalization process occurs on the surface. Comparison of the surface morphology of the modified matrix (Figure 4-II,-III) with the unmodified one (Figure 4-I) indicated that the surface roughness increases.

Determination of Kinetic Parameters. Parameters of soluble and immobilized enzyme were assessed using casein as the substrate (Table 2). Both free and immobilized trypsin−chymotrypsin were found to follow Michaelis−Menten kinetics (Figure 5). A Hanes−Woolf plot was employed for the calculation of K_m and V_{max} values. The immobilized enzyme has a larger Michaelis constant than the soluble one. This increase in K_m of immobilized enzyme indicates the lower affinity for its substrate, which may be attributed to the diffusional limits, steric effects, structural changes of the active site by the support, or loss of enzyme flexibility necessary for optimal substrate binding.[25,32] The V_{max} value of immobilized enzyme was determined as 1.36 U, which was lower relative to that of free enzyme (1.91 U). This finding suggests the introduction of some limited conformational changes in the enzyme molecule on immobilization.[32]

Table 2. Enzymatic Properties of Free and Immobilized Trypsin−Chymotrypsin

	trypsin−chymotrypsin	
property	free	immobilized
K_m (g/L)	19.33 ± 3.65	23.65 ± 4.51
V_{max} (U)	1.91 ± 0.05	1.36 ± 0.04
optimum pH	7.5	8.2
optimum temperature (°C)	55	55

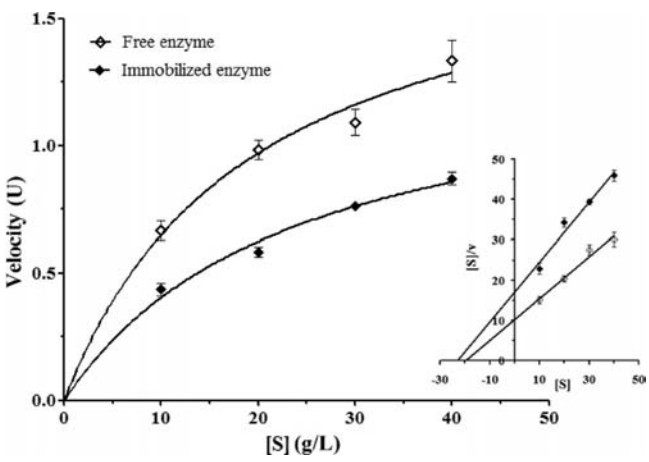

Figure 5. Effect of substrate concentration on reaction velocity of free and immobilized enzyme; Hanes−Woolf plots (inset) for free and immobilized enzyme with R^2 = 0.9715 and 0.9687, respectively.

Effect of pH and Temperature on Enzyme Activity. The free and immobilized trypsin−chymotrypsin mixtures were found to have slightly different optimal pH values; the value for the free enzymes is pH 7.5 and that for the immobilized enzymes, pH 8.2 (Figure 6a). The shift of optimal pH is most probably attributed to the loss of $-NH_2$ groups on the surface of enzyme after coupling to the modified PVC microspheres and the resulting change in the microenvironment of the immobilized enzyme. The immobilized enzyme was found to possess a wider active pH range when compared to the native enzyme. The immobilized enzyme achieved 75% of the optimum effect of hydrolysis of casein in pH range 7.5−8.6. Both free and immobilized enzymes have an optimum temperature of 55 °C (Figure 6b). At 70 °C, the immobilized enzyme exhibited higher activity than the native enzyme, suggesting that it has a higher thermal stability.

Thermal Stability. The immobilized mixed protease was found to possess a higher thermal stability than the free enzyme (Figure 7). Both the free and immobilized enzymes had this value decreased 42.1 and 19.6%, respectively, at 40 °C. At 70 °C, the immobilized preparation retained 30.9% of the initial activity, whereas the nonconjugated form had only 8.2% of the initial activity after the same treatment. The higher stability of the immobilized mixed protease could be caused by the diminished autolysis, as the functional groups of enzyme molecules on microsphere have greatly restricted contact with each other. In addition, Mozhaev[33,34] assumed that one of the main causes involving the irreversible inactivation of enzyme was the unfolding of the protein structure. Ortega et al.[18] proposed that the variations in protease conformational

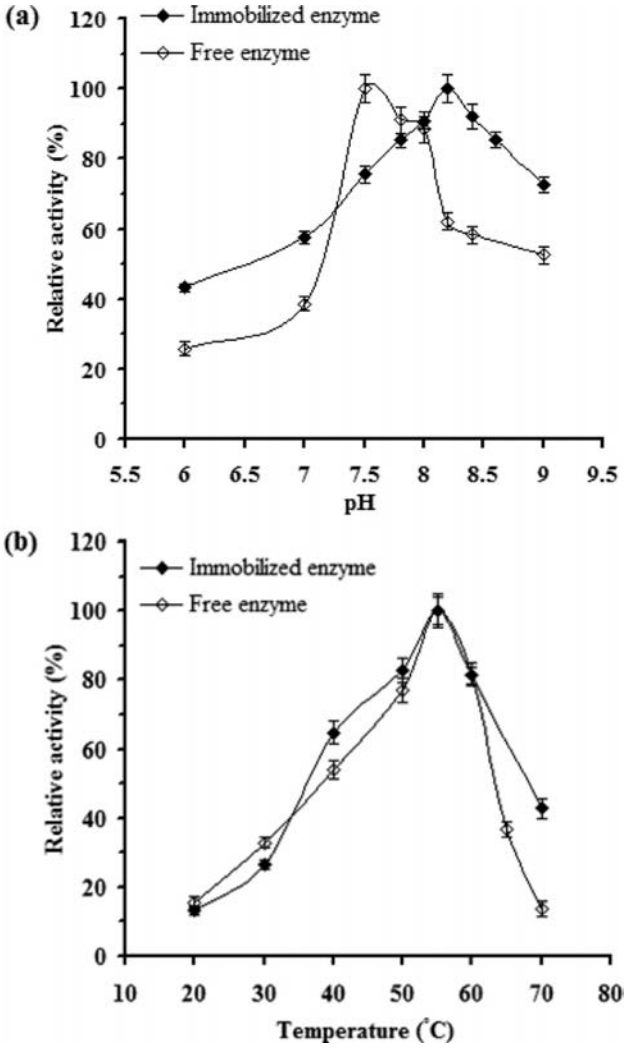

Figure 6. Relative activity of free and immobilized enzyme at various (a) pH values and (b) temperatures.

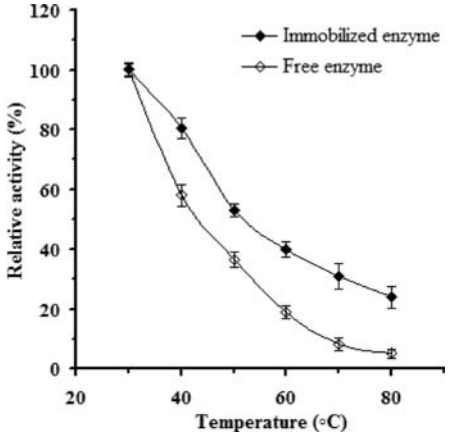

Figure 7. Thermal stability of free (pH 7.5) and immobilized (pH 8.2) trypsin–chymotrypsin at different temperatures in buffer for 1 h.

structure, particularly in the rigidity of the secondary and tertiary structures that reduce the unfolding rate, were closely connected to the protein stability. Thus, any decrease of conformational change involved in enzyme inactivation may also increase the enzyme stability.[34]

Reusability. Increased operational stability of immobilized enzyme is essential to achieve the highest benefit. Reusability of immobilized trypsin–chymotrypsin was tested, as this is important for practical application of the preparation. The immobilized mixed protease retained >70% of initial activity after having been reused six times for the hydrolysis of casein at pH 8.2 and 25 °C (30 min each time) (Figure 8).

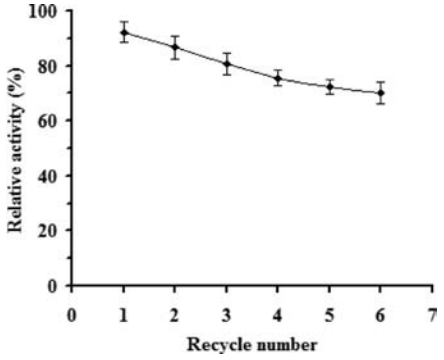

Figure 8. Reusability of immobilized trypsin–chymotrypsin at 25 °C in buffer (pH 8.2) for 0.5 h each time using casein as substrate.

In conclusion, a mixed protease (trypsin–chymotrypsin) was innovatively coupled to modified PVC microspheres. The resulting immobilized enzyme possesses high activity because of the excellent composite enzyme systems of trypsin–chymotrypsin. The immobilization conditions were effectively optimized by Box–Behnken design and response surface method. It was feasible to get immobilized mixed protease with higher activity at mild reaction conditions. Compared with the free protease, the immobilized mixed protease possesses wider optimal pH and superior thermal stability; furthermore, it has advantages of reusability and easy separation from the hydrolyzed reactant. These properties give the immobilized mixed protease better control of the hydrolytic process and avoid the adverse effects of a pyroprocess. Thus, the immobilized mixed protease developed in this study may find application in the protein–food industry and the environmental protection field, for instance, the treatment and utilization of wastewater with abundant protein derived from food production and the processing of protein–polypeptide beverages and nutritious oral liquids.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Supplementary Tables S1 and S2. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

PVC, polyvinyl chloride; EDA, ethylenediamine; GA, glutaraldehyde; TCA, trichloroacetic acid; PBS, phosphate-buffered solution; FT-IR, Fourier transform infrared spectroscopy; SEM, scanning electron microscopy; RSM, response surface method

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