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Optimal covalent immobilization of α-chymotrypsin on Fe_3O_4-chitosan nanoparticles

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ABSTRACT

This study investigated the immobilization of α-chymotrypsin onto magnetic Fe_3O_4-chitosan (α-chymotrypsin-Fe_3O_4-CS) nanoparticles by covalent binding. The response surface methodology (RSM) with a 3-factor-3-level Box–Behnken experimental design was employed to evaluate the effects of the manipulated variables, including the immobilization time, temperature, and pH, on the enzyme activity. The results indicate that the immobilized temperature and pH significantly affected enzyme activity. In a ridge max analysis, the optimal condition for α-chymotrypsin immobilization included a reaction temperature of 21.7 °C, a pH of 7.6, and an incubation time of 1.1 h. The predicted and the experimental immobilized enzyme activities were 354 and 347 ± 46.5 U/g-support, respectively, under the optimal condition. Besides, the synthesis reactions of the dipeptide derivative using the free and immobilized α-chymotrypsin were compared. The yields of the dipeptide derivative via the free or immobilized α-chymotrypsin catalyzed were almost the same. The α-chymotrypsin-Fe_3O_4-CS nanoparticles exhibited a good acid-resisting ability and the less reaction time was required for dipeptide synthesis. After twelve repeated uses in dipeptide synthesis, the immobilized α-chymotrypsin still retained over 60% of its original activity. The magnetic α-chymotrypsin-Fe_3O_4-CS nanoparticles can be easily recovered by magnetic field will have potential application in industry.

1. Introduction

α-Chymotrypsin (EC 3.4.21.1) is an endoprotease, which cleaves protein chains at the C-terminal position with aromatic amino acids (tryptophan, tyrosine, or phenylalanine) and yielding the mixture of peptides of different molecular sizes or free amino acids [1–3]. Food protein hydrolysis is frequently used to improve their functional and nutritional properties. The protein hydrolysates are mainly applied in geriatric products, high-energy supplements, and hypoallergenic foods [4,5]. α-Chymotrypsin also can use as a catalyst to catalyze dipeptide derivatives synthesis [6–8]. Enzymatic peptide synthesis has several advantages, such as mild reaction conditions, stereoselectivity, no racemization, and minimal side chain protection requirements. Therefore, enzymatic synthesis of peptides is considered to be better than chemical synthesis, especially in di- or tri-peptide production [9].

Magnetic iron (II, III) oxide (ferrous–ferric oxide, Fe_3O_4) nanoparticles are non-toxic to human beings and are found naturally in the environment. They have been widely used in biotechnology, pharmacology, and biochemistry, including applications such as nucleic acid detection [10], cell separation [11], targeted drug [12], biosensors [13], and magnetic resonance imaging contrast agent fields [14]. Methods for preparing magnetic nanoparticles reported in the literature include solid state reaction [15], sol–gel [16], co-precipitation [17], hydrothermal processes [18], and the ultrasonic method [19]. Currently, several studies have immobilized various enzymes or proteins, such as lipase [20], protease [21,22], laccase [23], and β-D-galactosidase [24] using magnetic nanoparticles or chitosan.

Chitosan (2-amino-2-deoxy-(1→4)-β-D-glucopyranan; CS) is a biopolymer carrying amino and hydroxy groups, which can be chemically modified for uses in many fields [25]. Recently, some researchers have proposed the use of these methods for preparing magnetic Fe_3O_4-CS nanoparticles and established the applications of those particles, such as protein absorption, enzyme immobilization [26], and release of drugs [27]. Generally, the application of magnetic Fe_3O_4-CS nanoparticles in the immobilization of protein engineering has attracted an extensive attention. The use of magnetic Fe_3O_4-CS nanoparticles as supports for enzyme immobilization has various advantages: (1) selective separation

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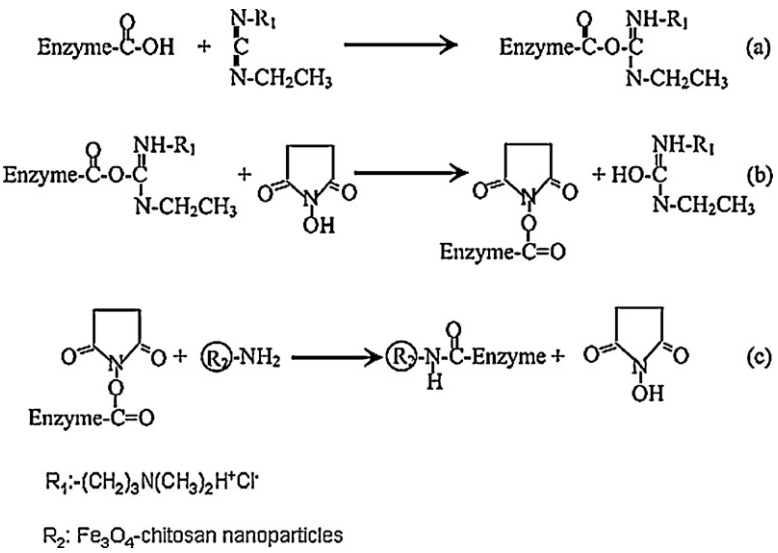

Fig. 1. Schematic presentation of α-chymotrypsin immobilized on Fe_3O_4-chitosan nanoparticles. (a) Carboxyl groups of α-chymotrypsin were activated by EDC; (b) in the present of NHS, an amine-reactive sulfo-NHS ester was formed; (c) sulfo-NHS esters is reacted with amines group from Fe_3O_4-chitosan nanoparticles and form an amide bond.

of immobilized enzymes under the magnetic field to decrease the operation cost [28], (2) high binding efficiency attributed to a higher specific surface area [29], (3) non-toxic, biocompatible, biodegradable and anti-bacterial characteristics, and (4) the application in a continuous biocatalysis system [30]. Covalent binding of enzyme immobilization has been used to improve enzyme stability. However, if α-chymotrypsin is intended to be used in large-scale processes, as it is the case of production of protein hydrolysates or synthesis of dipeptide derivatives, it is very important to reduce biocatalyst costs. If the enzyme is immobilized on magnetic Fe_3O_4-CS nanoparticles, the immobilized enzyme can be recovered under a magnetic field after reaction; thereby decreasing production costs.

This study focused on the immobilization of protease of α-chymotrypsin, which was conjugated to 1-3-dimethyl-aminopropyl-3-ethyl-carbodiimide-hydrochloride (EDC·HCl) and *N*-hydroxysuccinimide (NHS) on Fe_3O_4-CS nanoparticles. The goals of this study were to establish the relationships among the manipulated variables (incubation time, reaction temperature, and pH) and their outcomes (enzyme activity of α-chymotrypsin-Fe_3O_4-CS), as well as to search for an optimal condition for enzyme immobilization. Finally, the immobilized α-chymotrypsin properties were tested, including its characteristics, hydrolytic activity, and catalytic capability for peptide synthesis.

2. Materials and methods

2.1. Materials

The chemicals used in this study included *N*-acetyl-phenylalanine ethyl ester (*N*-Ac-Phe-OEt), glycinamide hydrochloride (Gly-NH_2·HCl), ethyl acetate (99.9%), acetonitrile (99.9%), trifluoroacetic acid (TFA, 99.9%), Trizma® base buffer (Tris buffer), CBZ-aspartic acid (CBZ-Asp, used as an internal standard), methanol (99.5%), *N*-hydroxysuccinimide (NHS), *N*-benzoyl-tyrosine ethyl ester (BTEE), 1-3-dimethyl-aminopropyl-3-ethyl-carbodiimide-hydrochloride (EDC·HCl), and α-chymotrypsin (from bovine pancreas type II, EC 3.4.21.1, 40–60 U/mg), all of which were purchased from Sigma–Aldrich (St. Louis, MO, USA). Potassium dihydrogen phosphate (KH_2PO_4), sodium chloride (NaCl), and sodium hydroxide (NaOH) were purchased from Katayama Chemical Co. (Tokyo, Japan). Fe_3O_4-CS nanoparticles (containing 1% chitosan and particle sizes ranging 200–300 nm) prepared by spray-drying were provided by Huang et al. [31].

2.2. Method for covalent binding

Two milligram of α-chymotrypsin were added to the 4 mL of KH_2PO_4 buffer solution (containing 2 mg of EDC) and placed at room temperature in a shaking bath with a rotational speed of 100 rpm for 2 h to form the enzyme-EDC complex (Fig. 1a). Then 3 mg of NHS was added to the above reaction solution and the incubation continued for another 2 h to form the enzyme-NHS complex (Fig. 1b). At the same time, 0.2 g of Fe_3O_4-CS nanoparticles was added to 4 mL of buffer solution (pH 5.5 and containing 3 mM KH_2PO_4 and 0.1 M NaCl) for chitosan swelling. After 2 h, the swelling magnetic nanoparticles were collected by magnet, the solution containing the enzyme-NHS complex was poured into the triangle bottle for covalent binding with swelling Fe_3O_4-CS nanoparticles at room temperature, at a speed of 100 rpm, at the pre-determined incubation time (0.5, 1, 3, 6, or 12 h). The reaction is depicted in Fig. 1c. After the completion of this reaction, magnetic nanoparticles were collected by the magnet. The supernatant was discarded, and the nanoparticles were washed several times with double distilled water (ddH_2O) to remove free α-chymotrypsin until the enzyme activity in the supernatant was not detected. The surface morphology of the immobilized α-chymotrypsin of Fe_3O_4-CS particles was measured from the enlarged field emission scanning electron microscopy (FESEM, JEOL JSM-1200EX II) micrographs by counting at least 200 individual particles from different regions on a film. Fourier transform infrared spectroscopy (FTIR, Shimadzu 8400S) was used to reassess the changes of Fe_3O_4-CS particles after immobilization.

2.3. Determination of the enzyme activity

The α-chymotrypsin activity is determined according to Laskowski [32] method by measuring an increase of pH resulting from *N*-benzoyl-L-tyrosine, which is the hydrolysis product of BTEE. The auto titration was performed by a pH-stat (Mettler-Toledo DL50, Switzerland) equipment. A solution of 10 mL 250 mM $CaCl_2$ was combined with 30 mL ddH_2O in a 200 mL plastic cup. Ten milliliters of 25 mM BTEE (previously dissolved in 50% methanol)

was then added and mixed by swirling at 25 °C. The pH of the mixture was adjusted to 8.0 by adding 0.05 M NaOH, and then the immobilized enzyme was added into the solution. For the first 3 min, the NaOH (0.05 M) solution was titrated into the reaction mixture to maintain the pH at 8.0, and the volume of the NaOH solution needed to maintain a pH stat was recorded. One unit (U) of the enzyme activity was defined as the amount of enzyme required to hydrolyze 1.0 μmol of BTEE/min at room temperature and pH 8.0. The immobilized enzyme activity could be calculated as follows:

$$\text{U/g support} = \frac{0.05\ \text{M} \times \text{titration volume(mL)} \times 1000}{\text{gram of support(g)} \times \text{time(min)}}$$

2.4. Experimental design and statistical analysis

A 3-level-3-factor Box–Behnken design, requiring 15 experiments, was employed in this study. To avoid bias, 15 runs were performed in a totally random order. The variables and their levels selected for the study of enzyme immobilization included the incubation time (0.5–1.5 h), reaction temperature (25–45 °C), and pH (7.0–9.0), as shown in Table 1. All experiments were performed in 4 mL of KH_2PO_4 buffer solution. The experimental data were then analyzed by the response surface regression (RSREG) procedure using SAS software [33] to fit the following second-order polynomial equation:

$$Y = \beta_0 + \sum_{i=1}^{3}\beta_i x_i + \sum_{i=1}^{3}\beta_{ii}x_i^2 + \sum_{i=1}^{2}\sum_{j=i+1}^{3}\beta_{ij}x_i x_j = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{11}x_1^2 + \beta_{22}x_2^2 + \beta_{33}x_3^2 + \beta_{12}x_1x_2 + \beta_{13}x_1x_3 + \beta_{23}x_2x_3 \quad (1)$$

where Y (the response) denotes the enzyme activity of Fe_3O_4-CS-chymotrypsin; β_0, β_i, β_{ii}, and β_{ij} are regression coefficients; x_i and x_j represents the un-coded independent variable. The suffixes i, j, and k in the above equation denote the three independent variables: x_1 for reaction time, x_2 for temperature, and x_3 for pH. The option of RIDGE MAX in the SAS software was employed to compute the estimated ridge of maximum response for increasing the radius from the center of the original design.

2.5. Catalytic capability of α-chymotrypsin Fe_3O_4-CS nanoparticles

2.5.1. Comparison between the free and immobilized enzyme

The catalytic capability of α-chymotrypsin Fe_3O_4-CS nanoparticles was examined with a reaction of *N*-Ac-Phe-Gly-NH_2 (a dipeptide derivative) synthesis, which was performed in a biphasic system containing 80 mM Tris buffer and ethyl acetate (96:4, v/v). The 10 mM of acyl donor (*N*-Ac-Phe-OEt) and 15 mM of nucleophile (Gly-NH_2·HCl), in a molar ratio of 1:1.5, were placed in 10 mL of biphasic buffer in a triangle bottle. To compare the catalytic capability between the free and immobilized enzyme, α-chymotrypsin Fe_3O_4-CS nanoparticles (350 U/g × 0.2 g = 70 U) and free chymotrypsin (55 U/mg × 1.3 mg = 71.5 U) were separately used to catalyze the synthesis at various pH, temperatures, and reaction times. After the reaction was completed, the product (*N*-Ac-Phe-Gly-NH_2) was analyzed by HPLC (Hewlett Packard 1100 series, Avondale, PA, USA) equipped with an ultraviolet detector and Spherisorb ODS-2 column (250 mm × 4.6 mm (i.d.); film thickness, 5 μm; Restek, Bellefonte, PA, USA). The elution solvents included 0.1% TFA of water and acetonitrile. The flow rate was set to 1.0 mL/min, and the oven temperature was maintained at 40 °C. Gradient elution was performed as follows: acetonitrile was set at 30% for the first 7 min, gradually increased to 35% between 7 and 9 min, and then hold at 35% for the last 6 min. The UV detector was set at a wavelength of 254 nm. The yield of dipeptide derivative could be calculated as follows:

$$\text{Yield}(\%) = \frac{\text{initial Phe} - \text{residual Phe}}{\text{initial Phe}}$$

where the concentration of Phe is in mmol.

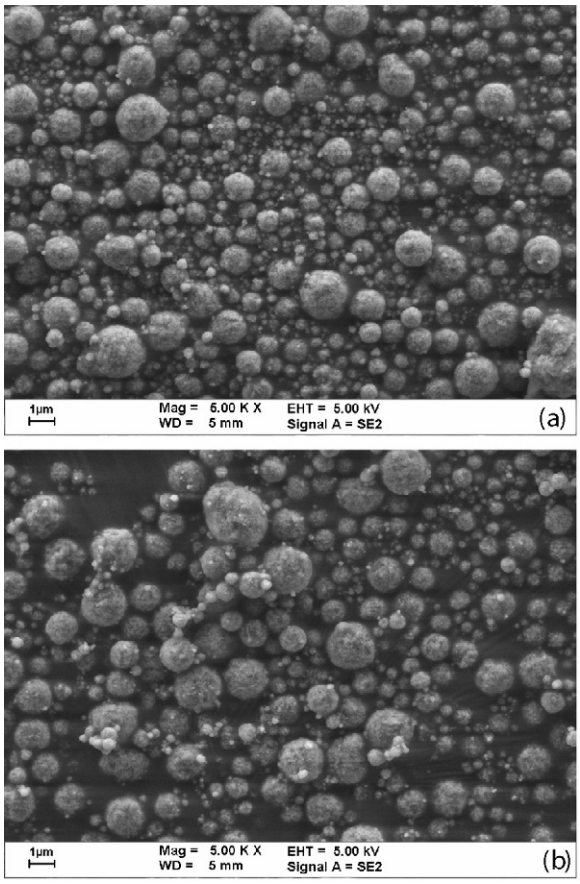

Fig. 2. The characterization of surface morphology on FESEM images (a) Fe_3O_4-CS and (b) α-chymotrypsin-Fe_3O_4-CS nanoparticles.

2.5.2. Reusability of the immobilized enzyme

To examine the reusability of the immobilized enzyme, α-chymotrypsin Fe_3O_4-CS nanoparticles (350 U/g × 0.2 g = 70 U) were reused to catalyze the above synthesis. In this study, the immobilized enzyme was reused twelve times under a fixed operating condition of 35 °C, pH 9.0, 180 rpm and 10 min of the reaction time. The above experiment was repeated twice to reduce the experimental errors.

3. Results and discussion

3.1. Characterization of magnetic α-chymotrypsin-Fe_3O_4-CS nanoparticles

Fig. 2a shows a field emission scanning electron microscope (FESEM) image of the magnetic Fe_3O_4-CS nanoparticles. Each particle was smooth and nearly spherical, and the average diameter of nanoparticles was approximately 300 nm. However, the surface morphology of the magnetic α-chymotrypsin-Fe_3O_4-CS nanoparticles (Fig. 2b) had irregular pores of varying dimensions. The average particle size of nanoparticles with enzyme immobilization was approximately 450 nm. This indicates that the enzyme was immobilized on the surface and the pore space.

Table 1
A 3-factor-3-level Box–Behnken experimental design and experimental results of immobilized enzyme activity and enzyme activity retention ratio for response surface analysis.

Treatment[a] no.	Factor			Enzyme activity (U/g-support) $Y \pm SD$	Enzyme activity retention ratio (%) $Y \pm SD$
	Time (h) x_1	Temperature (°C) x_2	pH (–) x_3		
1	−1 (0.5)[b]	−1 (25)	0 (8.0)	250 ± 15.1 [c]	45.4 ± 2.8 [c]
2	−1 (0.5)	+1 (45)	0 (8.0)	63.7 ± 0.2	11.6 ± 0.0
3	+1 (1.5)	−1 (25)	0 (8.0)	310 ± 29.7	56.3 ± 5.4
4	+1 (1.5)	+1 (45)	0 (8.0)	86.8 ± 1.9	15.8 ± 0.3
5	0 (1.0)	−1 (25)	−1 (7.0)	328 ± 10.6	59.6 ± 1.9
6	0 (1.0)	+1 (45)	−1 (7.0)	248 ± 8.0	45.0 ± 1.5
7	0 (1.0)	−1 (25)	+1 (9.0)	267 ± 4.4	48.5 ± 0.8
8	0 (1.0)	+1 (45)	+1 (9.0)	44.2 ± 0.5	8.0 ± 0.1
9	−1 (0.5)	0 (35)	−1 (7.0)	228 ± 7.5	41.5 ± 1.4
10	+1 (1.5)	0 (35)	−1 (7.0)	270 ± 12.1	49.1 ± 2.2
11	−1 (0.5)	0 (35)	+1 (9.0)	99.8 ± 3.3	18.1 ± 0.6
12	+1 (1.5)	0 (35)	+1 (9.0)	96.7 ± 0.3	17.6 ± 0.1
13	0 (1.0)	0 (35)	0 (8.0)	62.5 ± 0.1	11.4 ± 0.0
14	0 (1.0)	0 (35)	0 (8.0)	85.0 ± 0.4	15.5 ± 0.1
15	0 (1.0)	0 (35)	0 (8.0)	81.4 ± 0.2	14.8 ± 0.0

[a] Treatments were run in a random order.
[b] Numbers in parentheses represent actual experimental values.
[c] Each run was performed twice, and the immobilized enzyme activity shown here is the average ± standard deviation (SD) of the duplicated experiments.

The magnetic α-chymotrypsin-Fe_3O_4-CS nanoparticles were lyophilized before Fourier transform infrared (FTIR) spectroscopy measurement. The linkage groups of free α-chymotrypsin and immobilized α-chymotrypsin were determined by FTIR spectrum, and the results were given in Fig. 3. The three characteristic peaks of α-chymotrypsin appeared at 1646, 1532, and 1425 cm^{-1} in the IR spectrum as shown in Fig. 3a [21]. The strong absorption band at 579 cm^{-1} was found in α-chymotrypsin-Fe_3O_4-CS nanoparticles (Fig. 3b), which indicates the presence of the Fe—O bond in the naked Fe_3O_4. The C—O stretching vibration of polymer backbone (chitosan) was observed at 1082 cm^{-1}. The broad band due to the stretching vibration of amino (—NH_2) and hydroxy (—OH) group could be observed at 3400–3500 cm^{-1} [34]. The IR spectrum of Fig. 3b indicates that α-chymotrypsin was successfully bound to the magnetic Fe_3O_4-CS nanoparticles.

3.2. Preliminary study

The enzyme immobilization was carried out in 4 mL of the KH_2PO_4 solution (pH 7.5, 50 mM), containing 2 mg of α-chymotrypsin and 0.2 g of Fe_3O_4-CS nanoparticles at room temperature. Fig. 4 shows the time course of α-chymotrypsin immobilized on the Fe_3O_4-CS nanoparticles. The enzyme activity retention ratio could be calculated as follows:

$$\text{Enzyme activity retention ratio (\%)} = \frac{\text{immobilized activity(U/g support)} \times 0.2\ \text{g}}{\text{added enzyme}\ \ 2\ \ \text{mg} \times \text{average unit}\ \ 55\ \ \text{U/mg}} \times 100\%$$

The result indicates that the immobilized enzyme activity increased proportionally to the immobilized time due to the increase of enzyme binding capacity, but remained almost constant after 3 h. However, the enzyme activity retention ratio was only 30–38% in this immobilization condition.

3.3. Model fitting

The major objective of this work was to establish a statistical approach for understanding the relationship between the variables of the immobilization reaction and the response (enzyme activity of the α-chymotrypsin-Fe_3O_4-CS). Compared with the

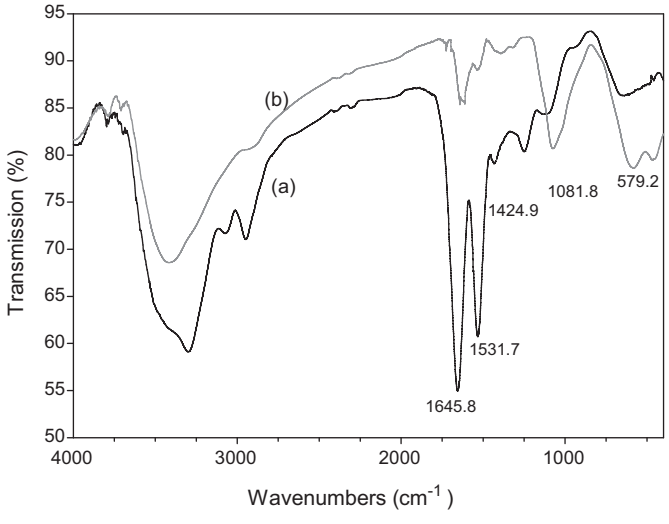

Fig. 3. FTIR spectra analysis of (a) free α-chymotrypsin and (b) α-chymotrypsin-Fe_3O_4-CS nanoparticles.

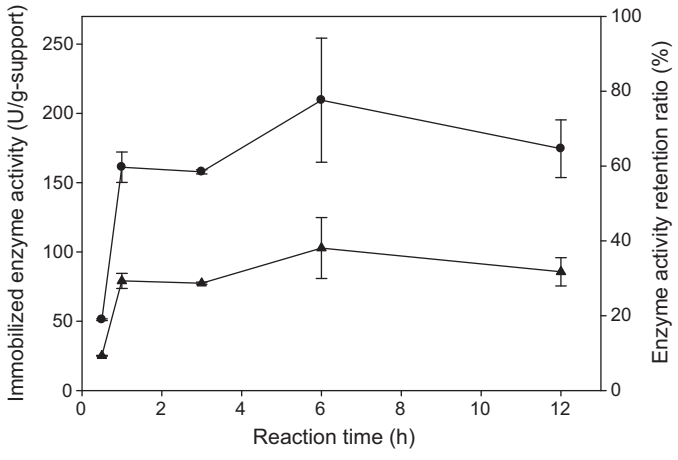

Fig. 4. The time courses of immobilized enzyme activity (●) and enzyme activity retention ratio (▲) of α-chymotrypsin-Fe_3O_4-CS nanoparticles. The immobilization was carried out in 4 mL KH_2PO_4 solution (pH 7.5, 50 mM) containing 2 mg α-chymotrypsin and 0.2 g Fe_3O_4-chitosan nanoparticles at room temperature.

Table 2
Analysis for joint test of all independent variables.

Factor	df	Sum of squares	Mean square	*F*-value	Prob. > *F*[a]
Time (x_1)	4	5321	1330	3.1	0.12
Temperature (x_2)	4	89115	22279	52.3	<0.01
pH (x_3)	4	64194	16049	37.7	<0.01

[a] Level of significance.

one-factor-at-a-time method, the response surface methodology (RSM) was more efficient in reducing the experimental runs and time for optimizing the immobilization process [35]. The experimental results (enzyme activities) of a 3-level-3-factor design were given in Table 1. The highest enzyme activity (328 U/g-support) and enzyme activity retention (59.6%) was obtained in treatment #5 (1 h, 25 °C, and pH 7.0); the lowest activity (44.2 U/g-support) and enzyme activity retention (8.0%) in treatment #8 (1 h, 45 °C, pH 9.0). The RSREG procedure in SAS software was employed to fit the second-order polynomial equation as follows:

$$Y = 5231.988 + 60.873x_1 - 30.675x_2 - 1056.436x_3 + 106.645x_1^2 + 0.745x_2^2 + 70.796x_3^2 - 1.842x_1x_2 - 22.405x_1x_3 - 3.563x_2x_3 \quad (2)$$

The analysis of variance (ANOVA) gave a very small p-value (<0.01) for the total model and a very high coefficient of determination ($R^2 = 0.99$). This revealed that the second-order polynomial model was highly significant and adequate to represent the actual relationship between the response (enzyme activity) and the significant variables. Furthermore, the overall effects of the three manipulated variables on the enzyme activity were also analyzed by the joint test, and the results (Table 2) revealed that the reaction temperature (x_2) and pH value (x_3) were the most important factors, and exerted a statistically significant overall effect ($p < 0.01$) on the enzyme activity.

3.4. Mutual effects of variables

The relationships between reaction factors and response can be better understood by examining the planned series of three dimensional mesh plots generated from the predicted model [Eq. (2)] by keeping the pH level constant. Fig. 5a shows that, at a steady pH 7.0, the enzyme activity of α-chymotrypsin-Fe_3O_4-CS was high at 25 °C and reduced significantly at 45 °C. This result was expected because the active sites of α-chymotrypsin were exposed to combine with the linkage reagents (EDC and NHS), and therefore the enzyme activity was lower at a higher temperature. In this study, the incubation time exhibited no significant effect on the enzyme activity of α-chymotrypsin-Fe_3O_4-CS from 0.5 to 1.5 h. A similar result was observed when using pH 8.0 (Fig. 5b) and pH 9.0 (Fig. 5c). However, the enzyme activity immobilized at pH 7.0 (Fig. 5a) was higher than the other two pH levels since the EDC and NHS were less active in alkaline solution. Thus, a less amount of α-chymotrypsin loading onto Fe_3O_4-CS nanoparticles lead to a lower immobilized α-chymotrypsin activity was obtained at pH 8 and 9.

3.5. Attaining the optimal immobilized condition

The ridge max analysis can be used to determine the optimal operating condition by computing the estimated ridge of the maximum response for increasing radius from the center of the original design. The result (Table 3) obtained from the ridge max analysis indicates that enzyme immobilization at an incubation time of 1.1 h, a reaction temperature of 21.7 °C, and a pH level of 7.6 obtained a maximum enzyme activity of 354 U/g-support.

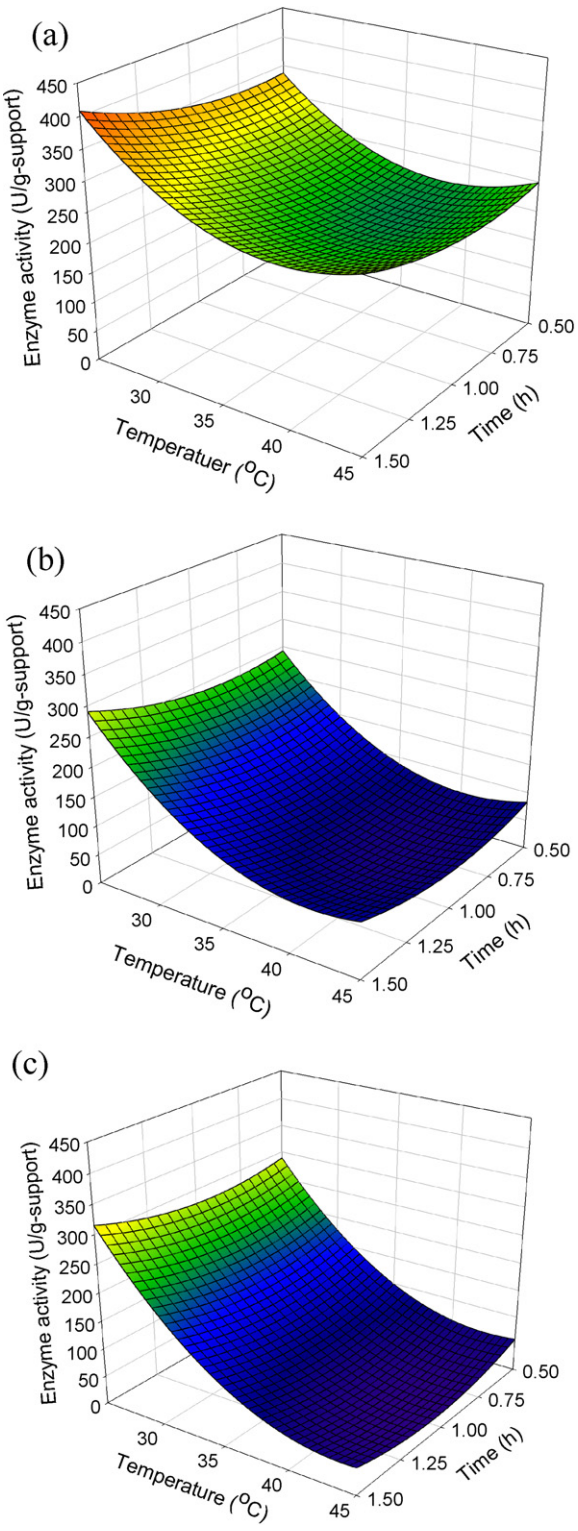

Fig. 5. Response surface plots for enzyme activity of α-chymotrypsin-Fe_3O_4-CS nanoparticles. Effects of reaction temperature and incubation time on enzyme activity (a) pH 7.0, (b) pH 8.0, and (c) pH 9.0.

The validity of the predicted model was examined by repeating the experiments three times at the suggested optimal immobilized condition. The average enzyme activity of the three batches was 347 ± 46.5 U/g-support, which was compatible with the predicted value (354 U/g-support) obtained from the ridge max analysis. Moreover, approximate 63% of enzyme activity retention ratio was

Table 3
Estimated ridge of maximum response of enzyme activity.

Coded radius	Actual (un-coded) factor values			
	Estimated response	Time x_1 (h)	Temperature x_2 (°C)	pH x_3 (–)
0.0	76.3	1.00	35.0	8.00
0.2	101	1.01	33.4	7.88
0.4	131	1.03	31.8	7.77
0.6	166	1.05	30.1	7.67
0.8	205	1.06	28.2	7.60
1.0	249	1.08	26.2	7.55
1.2	299	1.09	24.0	7.55
1.4	354	1.10	21.7	7.60

reached. Thus, the optimization of the covalent binding condition for α-chymotrypsin to be immobilized on Fe_3O_4-CS-nanoparticles was successfully developed by the RSM.

3.6. Catalytic capability of α-chymotrypsin-Fe_3O_4-chitosan nanoparticles

Fig. 6 shows that the dipeptide derivative (*N*-Ac-Phe-Gly-NH_2) synthesized via free and immobilized α-chymotrypsin catalyzed at 35 °C, 180 rpm, and 30 min in media with various pH levels (pH 4.0–10.0). The free α-chymotrypsin had the best yield (90%) at pH 9.0 and 10.0, but the yield substantially decreased at a pH lower than 8.0. However, the best yield (95%) of the dipeptide derivative catalyzed with α-chymotrypsin-Fe_3O_4-CS occurred at a pH range of 8.0–10.0, and the yield still remained higher than 55% at pH 4.0. This result indicated that the α-chymotrypsin, once immobilized on Fe_3O_4-chitosan nanoparticles, increase its acid-resisting capacity.

The catalytic capability of free and immobilized α-chymotrypsin was evaluated at 35 °C, pH 9 and 150 rpm condition. The kinetic curves obtained at this step are presented in Fig. 7. When using free enzyme, it can be observed that slight high initial reaction rates and high conversions were obtained in short reaction times. On the other hand, the immobilized α-chymotrypsin exhibited a similar kinetics curve. It indicates the catalytic capability of immobilized α-chymotrypsin is the same as free enzyme.

To examine the enzyme reusability, the immobilized enzyme was reused twelve times each with a reaction time of 10 min at 35 °C, pH 9.0, and 180 rpm. The results are given in Fig. 8, each point in this figure was the mean yield from the two runs of the experiment. As shown in the figure, the immobilized enzyme catalytic capability declined with the number of reused times. The yield remained about 60% after the immobilized enzyme had been used over ten times. In previous reports, the enzymes immobilized on the chitosan beads still possessed about 60% of their original activities after the immobilized enzymes had been reused five times [36]. The immobilized enzyme on magnetic Fe_3O_4-CS nanoparticles is advantageous over the free enzyme because the enzyme is easy recycled and reused, and this advantage can be demonstrated by estimating the productivity. In this study, productivity is defined as the amount of product yielded per unit activity of enzyme used in the reaction. Fig. 8 shows the productivities of immobilized and free enzyme. For the free enzyme, the productivity was a constant at 0.14 mM product/U because the enzyme unable reuse. However, the productivity of the immobilized enzyme increased with the number of reused times. After twelve times of operation, the productivity was increased to 1.21 mM product/U. In the other words, the immobilized enzyme increases at least 8.6 folds of production than the free enzyme. Besides, the free enzyme had to be heated to inactivate the enzyme and stop the reaction after reaction. The heating made the solution turbid or formation of undesired byproduct. In contrast, the heating process was not necessary for immobilized enzyme because the enzyme was easy separated from the reaction mixture by a magnet. These results demonstrated that

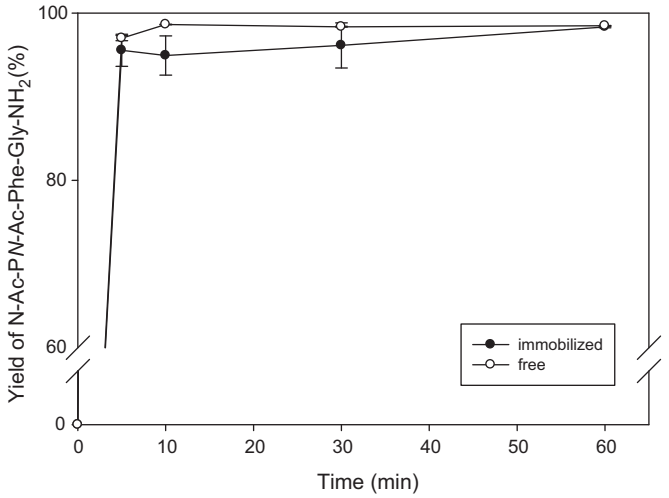

Fig. 7. Kinetics of yields of N-Ac-Phe-Gly-NH2 synthesis catalyzed with α-chymotrypsin-Fe_3O_4-CS (●) and free α-chymotrypsin (○).

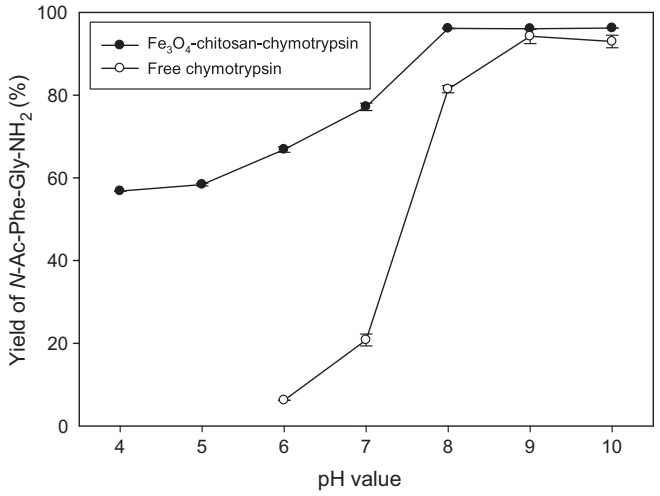

Fig. 6. Comparison of yields of *N*-Ac-Phe-Gly-NH_2 synthesis catalyzed with α-chymotrypsin-Fe_3O_4-CS (●) and free α-chymotrypsin (○) in media of various pH.

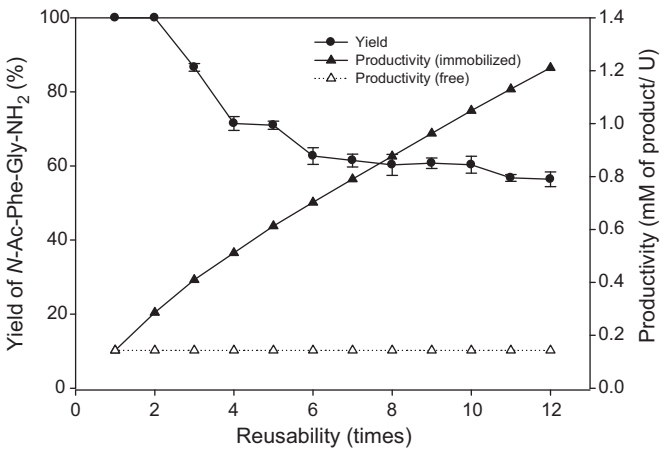

Fig. 8. Reusability and productivity of immobilized enzyme (α-chymotrypsin-Fe_3O_4-CS) to catalyze *N*-Ac-Phe-Gly-NH_2 synthesis under an operating condition of 35 °C, pH 9.0, 180 rpm, and 10 min. For free enzyme case, 70 U of α-chymotrypsin was used.

the immobilized enzyme could be effectively reused for the synthesis of the dipeptide derivative.

4. Conclusion

In this study, α-chymotrypsin was successfully immobilized through covalent-bonding onto the Fe_3O_4-CS nanoparticles via EDC and NHS activation. The immobilization efficiency can be further enhanced by central composite design and response surface methodology. A second-order model was obtained to describe the relationship between the immobilized α-chymotrypsin activity and the parameters of the immobilized time, pH, and temperature. The results indicate the immobilized temperature and pH were affected the enzyme activity significantly. The optimal immobilized conditions were at 21.7 °C, pH 7.6, and 1.1 h. The predicted and experimental enzyme activities were 354 and 347 ± 46.5 U/g-support, respectively. Besides, the Fe_3O_4-CS-chymotrypsin nanoparticles exhibited a high acid-resisting capacity in different pH for catalyze *N*-Ac-Phe-Gly-NH_2 synthesis reaction. At least 95% of yield of *N*-Ac-Phe-Gly-NH_2 catalyzed by immobilized α-chymotrypsin was achieved. On the other hands, this immobilized α-chymotrypsin retained 60% of the original catalytic capability activity after 12 repeated recovery and uses. It was also noticed that the α-chymotrypsin-Fe_3O_4-CS nanoparticles not only used in hydrolysis protein, but also applied to catalysis *N*-Ac-Phe-Gly-NH_2 synthesized. Taking the above results into consideration, Fe_3O_4-CS nanoparticles have been proving to be an efficient support for α-chymotrypsin immobilization. The developed immobilization model provides a simple, effective and inexpensive process for industry application.

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