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Immobilization of *Candida rugosa* lipase on magnetic poly(allyl glycidyl ether-co-ethylene glycol dimethacrylate) polymer microsphere for synthesis of phytosterol esters of unsaturated fatty acids

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ABSTRACT

In this article, magnetic microspheres were prepared by suspension polymerization of allyl glycidyl and ethylene glycol dimethacrylate in the presence of vinylphosphonic acid-coated Fe_3O_4 nanoparticle. *Candida rugosa* lipase (CRL) was immobilized on the hydrophobic magnetic microspheres via the active epoxy groups. The resulting immobilized CRL had better resistance to pH and temperature inactivation in comparison to free CRL, the adaptive pH and temperature ranges of lipase were widened, and it exhibited good thermal stability and reusability. The immobilized CRL was used as biocatalyst for enzymatic esterification of phytosterols with unsaturated fatty acids (UFAs) to produce the corresponding phytosterol esters. The phytosterols linolenate esterification degree of 93.5% was obtained under the optimized condition: 100 μmol/mL phytosterols, 200 μmol/mL linolenic acid, 15 mg/mL immobilized CRL at 160 rpm and 55 °C for 15 h in 10 mL of isooctane. Phytosterols esters could also be converted in high yields to the corresponding long-chain acyl esters via transesterification with methyl esters of fatty acids (55.3%) or triacylglycerols (above 78.1%) using magnetic immobilized CRL as biocatalyst.

1. Introduction

Fatty acid esters of sterols are advantageous in applications to a wide range of food products because of their better lipid-solubility and more significant cholesterol-lowering activity compared with the free sterols. The kinds of fatty acids that linked with sterol ester could influence the activity of sterol ester. Compared with sterol esters of saturated fatty acid, the sterol esters of unsaturated fatty acids (UFAs) possessed more significant hypocholesterolemic effect [1,2]. On the other hand, the health benefits of UFAs are now well known. UFA serves a role as precursors of a wide variety of metabolites (prostagladins, leukotrienes and hydroxyl fatty acids) regulating critical biological functions. In addition their anti-tumor properties have recently been acknowledged [3,4]. Esterification of phytosterols with beneficial UFA such as linolenic acid (LNA), conjugated linoleic acid (CLA), could lead to even higher amount of valuable phytosterol esters [5].

Although fatty acid esters of phytosterols can be synthesized by chemical reaction, the chemical method involves problems such as high energy consumption, formation of a 3,5-diene steroid derivative as a side product and staining. In addition, UFA are very sensitive to heat and oxidation during chemical reaction. Enzymatic catalysis, which proceeds efficiently under mild conditions and produce fewer by-products benefit from the biocatalyst's high specificity, is attractive for the synthesis of phytosterol esters of UFAs. Compared to the current chemical methodologies, they provide for an environmentally friendlier, more energy efficient and potentially more cost-effective techniques due to low-energy demanding operation and easier downstream processing. Lipase-catalyzed synthesis of phytosteryl ester in the presence of organic solvent has been previously reported [6,7]. Moreover, a method for the preparation of sterol esters with unsaturated fatty acids has firstly been described by Shimada et al. [8].

In most of the methods mentioned above, the biocatalysts for phytosterol esters formation are free enzymes. However, the industrial applications of the biocatalysts have not yet reached a significant level because of the high cost of the enzymes and the inconvenience in their separation, recycling, and reusing [9]. As an alternative, the use of immobilized enzyme not only provides enzyme reusability and hence reduces operational costs, but also reduces enzyme contamination and facilitates easy separation of products.

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Among the lipase from various source, *Candida rugosa* lipase (CRL) is extensively utilized for exterification of sterol with successful result Among the wide array of lipases available from various sources, *Candida rugosa* lipase (CRL) has been extensively utilized for esterifications at the C-3 position of steroids with successful results [5,10,11], and has been immobilized on different carriers [9,12–15]. In recent years, magnetic carrier technology has showed significant attractive for the preparation of immobilized enzymes. Used as the support material, magnetic carriers can be quickly separated from the reaction medium and effectively controlled by applying a magnetic field, thus the catalytic efficiency and stability properties of enzyme can be greatly improved [16]. Recently, Yang et al. reported the immobilized of CRL on magnetic microperes for olive oils hydrolysis, however the application in esterification was not involved has not been reported yet [9,17].

In this article, magnetic microspheres were prepared by suspension polymerization of allyl glycidyl and ethylene glycol dimethacrylate in the presence of vinylphosphonic acid (VPA)-coated Fe_3O_4 nanoparticle. Compared with conventional modification method by silanization, the VPA modification via Lewis acid/base interaction is simply carried out in mild aqueous conditions and is reported to yield a much stable product with higher surface coverage [18,19]. CRL was immobilized on the hydrophobic magnetic microspheres via the active epoxy groups. The properties of the immobilized lipase were characterized and compared with those of the free lipase. Then, based on this immobilized lipase, a method was proposed for the esterification of phytosterols with UFAs. To our knowledge, there have been no published methods for the esterification of phytosterols using this technique.

2. Materials and methods

2.1. Materials and reagents

Allyl glycidyl ether (AGE), ethylene glycol dimethacrylate (EDMA), *Candida rugosa* lipase (lyophilized powder, Type VII, 700 U/mg solid), and p-nitrophenyl palmitate (p-NPP) were purchased from Sigma–Aldrich (St. Louis, USA). Fe_3O_4 nanoparticle (20 nm) was purchased from Beijing Nachen S & T Ltd. (Beijing China). Vinylphosphonic acid (VPA) was purchased from TCI (Shanghai) Development Co. Ltd. (Shanghai, China); phytosterols (β-sitosterol 77%, campesterol 17%, stigmasterol 5%) was purchased from Xian Bluesky Biological Enineering Co. Ltd. (Xi'an, China); linolenic acid (80%), conjugated linoleic acid (90%), oleic acid (90%) were purchased from Henan Linuo Biochemical Co. Ltd. (Anyang, China). Refined and bleached reapseed oil, linseed oil, teaseed oil, which was used as the source of mixed UFAs, were purchased from the supermarket. Azobisisobutyronitrile (AIBN) and other reagents were purchased from Sinopharm Chemical Reagent (Shanghai, China).

2.2. Preparation of Fe_3O_4/poly(AGE-co-EDMA) magnetic microspheres

The Fe_3O_4 nanoparticles were modified with vinylphosphonic acid (VPA) as follows. The VPA solution was prepared by dissolving VPA (2.0 g) in 50 mL phosphate buffer (50 mM), and then was adjusted at pH of 6.0 with NaOH. Then Fe_3O_4 nanoparticles (5.0 g) were added into the solution. The mixture was refluxed at 105 °C for 12 h. The final product was magnetically collected and washed by water/ethanol successively and repeatedly, then vacuum-dried at 60 °C for 6 h.

The Fe_3O_4/poly(AGE-co-EDMA) composite was synthesized by the distillation–precipitation polymerization method as proposed by Gao et al. [20]. VPA-modified Fe_3O_4 (2.0 g), AGE, EDMA, AIBN, and acetonitrile (400 mL) were successively added into a 500 mL three-necked round bottom flask equipped with a distillation apparatus and a stirring device. The flask was submerged in a water bath, and the reaction mixture was heated from room temperature to boiling state within 30 min. Then, the mixture was kept at boiling state till about half of the acetonitrile was distilled out within 2 h. After the mixture was cooled to room temperature, the resultant Fe_3O_4/poly(AGE-co-EDMA) composite was separated by means of external magnetic filed, and then washed several times by re-dispersion in acetonitrile and magnetic collection. The composite was dried under vacuum at 60 °C overnight. Fig. 1 shows the reaction schemes for the preparation of Fe_3O_4/poly(AGE-co-EDMA) microspheres and immobilization of lipase.

2.3. Characterization of magnetic polymer microspheres

Surface structure and particle size of the materials were observed by Quanta 200 scanning electron microscopy (SEM, FEI, Holland). FT-IR spectra were obtained with a TENSOR 27 FT-IR instrument (Bruker, Germany). Nitrogen sorption experiments were carried out at 77 K using Gemini V2380 surface area and pore size analyzer (Micromeritics, Norcross, USA).

2.4. Immobilization of lipase on magnetic polymer microspheres

Due to the active epoxy groups on the magnetic microspheres, lipase immobilization was carried out by the treatment of the enzyme solution with the microspheres directly [9]. The microspheres (1.0 g) were equilibrated in 50 mL phosphate buffer (0.1 M, pH 7.0) for 12 h. It was then transferred to the same fresh buffer (50 mL) containing CRL (0.1 g). The mixture was placed in a shaking incubator at 30 °C and 150 rpm, while continuously shaken for 8 h to finish immobilization of CRL. The immobilized CRL was recovered by magnetic separation, and washed with phosphate buffer (0.1 M, pH 7.0) three times to remove excess lipase. The washed solution was collected to assay the amount of residual enzyme. The resulting immobilized CRL was lyophilized and stored at 4 °C prior to use.

2.5. Measurement of the immobilized CRL activity

The enzymatic activities of free and immobilized CRL were measured by the detection of the p-nitrophenol which comes from the hydrolysis of p-nitrophenyl palmitate (p-NPP) [14]. 0.5 g of p-NPP dissolved in 100 mL of ethanol was used as substrate. The increase in absorbance at 410 nm caused by the release of p-nitrophenol in the hydrolysis of p-NPP was measured spectrophotometrically. Free lipase of 0.1 g (or 200 mg of immobilized lipase) was added to a mixture of 1 mL of 0.5% (w/v) p-NPP solution and 1 ml of 0.05 M PBS (pH 7.0) and incubate for 5 min at 30 °C. The reaction was terminated by adding 2 mL of 0.5 N Na_2CO_3 followed by centrifuging for 10 min (10,000 rpm). The supernatant of 0.5 mL was diluted 10-folds with distilled water, and measured at 410 nm in a UV/VIS spectrophotometer (Beckman DU-800, Fullerton, USA). One unit (U) of enzyme activity was defined as the amount of enzyme which catalyzed the production of 1 mmol p-nitrophenol per minute under the experimental conditions. The relative activity (%) was the ratio between the activity of every sample and the maximum activity of sample.

The amount of immobilized CRL on the magnetic beads was determined by measuring the initial and final concentration of protein in the immobilization medium using the Bradford protein assay method [21].

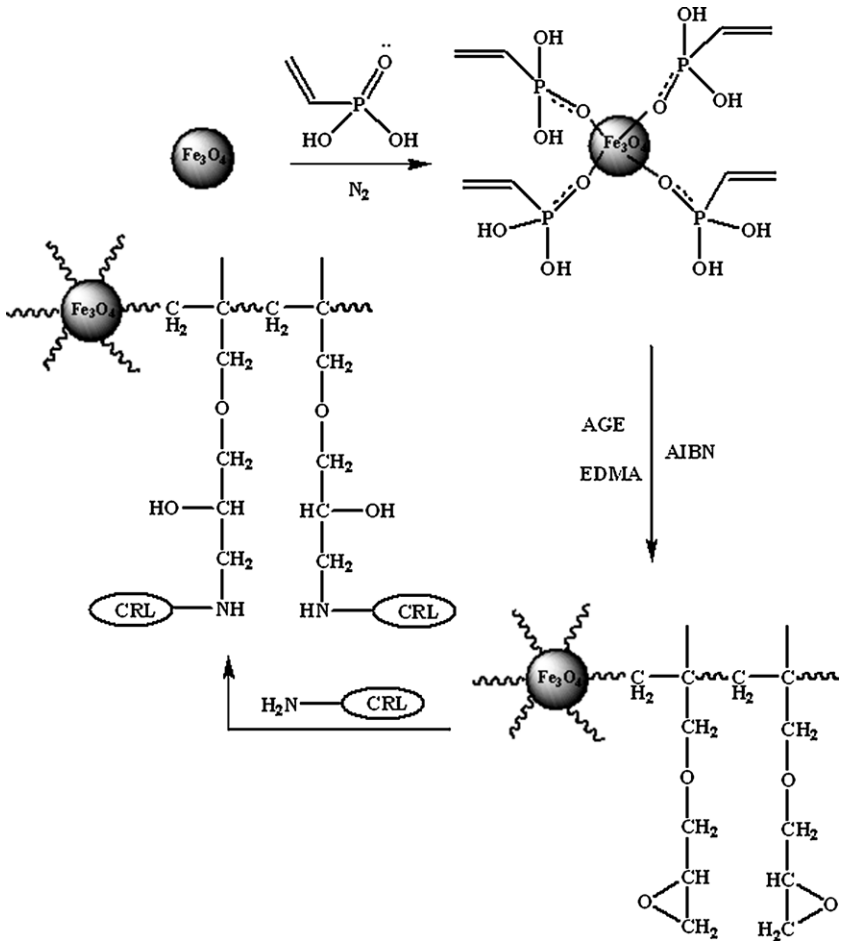

Fig. 1. Preparation scheme of Fe_3O_4/poly(AGE-co-EDMA) magnetic microsphere immobilized CRL.

2.6. Lipase-catalyzed esterification or transesterification of phytosterols

Esterification condition: phytosterols (0.25–2 mmol), UFAs such as oleic acid, linolenic acid or conjugated linoleic acid (0.5–5 mmol), immobilized CRL (50–400 mg), solvent (10 mL) and molecular sieves (3 Å) were added into a 25 mL Erlenmeyer flask. The solvent had, in advance, been dehydrated with molecular sieves 4 Å for at least 24 h. The vial was placed in a water bath at 35–65 °C and the reaction mixtures were shaken at 160 rpm. Over the time course of the reactions, a portion of the reaction mixture (0.1 mL) was periodically removed from the reaction for GC analysis.

For phytosterols transesterified with fatty acid methyl esters (obtained by the methyl esterification of linseed oil): phytosterols, fatty acid methyl esters, in the presence of immobilized CRL preparation by mechanical stirring in a flask with three necks in vacuo at 55 °C for various periods. The vacuum used was 20–40 mbar, measured at room temperature.

For phytosterols transesterified with triacylglycerols: phytosterols, triacylglycerols, immobilized CRL, solvent (10 mL) were added into a 25 mL Erlenmeyer flask. The vial was placed in a water bath at 55 °C and the reaction mixtures were shaken at 160 rpm. Fig. 2 shows the scheme for lipase-catalyzed synthesis of phytosterol esters.

2.7. Qualitative and quantitative analysis of phytosterols esters

The composition of the crude products was analyzed by GC. An Agilent 6890 Series II gas chromatograph (Hewlett-Packard Co., Avondale, PA, USA), equipped with a FID and a fused silica capillary column (DB-5 HT, 15.0 m × 320 μm × 0.10 μm, Agilent Technologies, Deerfield, IL, USA) was used. The carrier gas was nitrogen and the total gas flow rate was 3.5 mL/min. The injector and detector temperatures were maintained at 320 °C and 350 °C, respectively. The oven temperature was held at 210 °C for 2.0 min, then increased to320 °C at a rate of 10 °C/min and held for 15 min, then increased

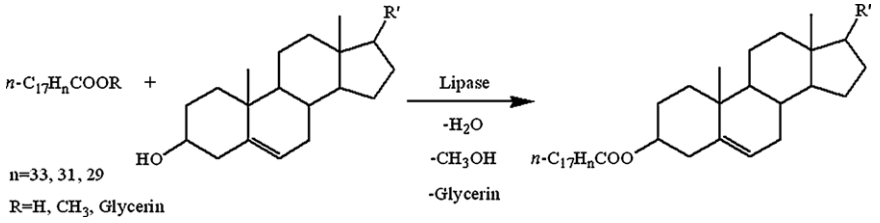

Fig. 2. Scheme for lipase-catalyzed synthesis of phytosterol esters.

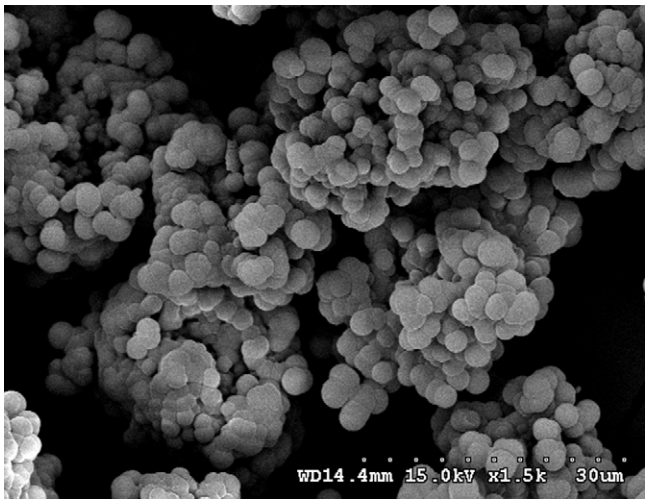

Fig. 3. SEM micrographs of the Fe_3O_4/poly(AGE-co-EDMA) magnetic microspheres.

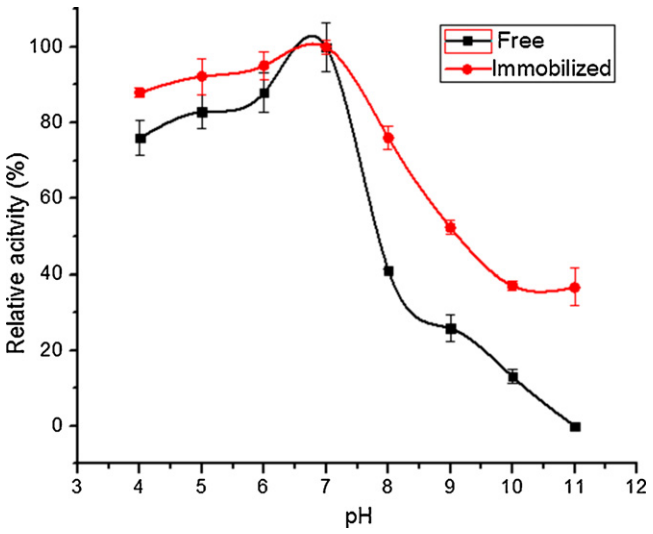

Fig. 4. pH profile of free and immobilized CRL.

to 380 °C at a rate of 10 °C/min, finally it was held at 380 °C for another 5 min. The injection volume was 1 μL. With the GC operation conditions above, the retention times of each compound were as follows: UFAs (before 2.00 min), campesterol (6.23 min), stigmasterol (6.44 min), β-sitosterol (6.78 min), and phytosterols esters (16.45–18.21 min).

The degree of esterification (%) of phytosterols with UFAs to form phytosterol esters was calculated from the GC profile of reactants using the following Eq. (1):

$$\text{degree of esterification(DE, \%)} = \frac{B}{B + 1.63 \times A} \times 100 \quad (1)$$

where A = peak area of total phytosterols (campesterol + stigmasterol + β-sitosterol); B = peak area of total phytosterol esters of UFAs. 1.63 = ratio of average molecular weight of total phytosterol esters of UFAs to average molecular weight of total phytosterols.

3. Result and discussion

3.1. Characterization of the Fe_3O_4/poly (AGE-co-EDMA) magnetic microspheres

The Fe_3O_4/poly (AGE-co-EDMA) magnetic microspheres were characterized by Fourier-transform IR. The absorption spectrum of the magnetic polymer microspheres displays readily identifiable peaks at 1736 and 1153 cm^{-1} due to stretching vibrations of C=O and C–O of carboxyl and ester groups from AGE and EDMA links, respectively. The absorption peaks of the stretching vibration of the epoxy groups from AGE link in the magnetic microspheres were at 1269 cm^{-1}. The characteristic adsorption band at 580 cm^{-1} proves the existence of Fe_3O_4 in the magnetic microspheres.

The specific surface areas for Fe_3O_4/poly (AGE-co-EDMA) magnetic microspheres from nitrogen adsorption–desorption experiments were 27 m^2/g, which was higher than Arıca's report [17]. The spherical nature of Fe_3O_4/poly (AGE-co-EDMA) magnetic microspheres was confirmed by the SEM micrographs. The SEM image (Fig. 3) clearly indicates that the Fe_3O_4/poly (AGE-co-EDMA) microparticles are fairly uniform in size and shape, with a mean diameter around 2–3 μm.

3.2. Immobilization of CRL on Fe_3O_4/poly (AGE-co-EDMA) magnetic microspheres

A comparative study between free and immobilized lipase is provided in terms of pH, temperature, and thermal stability. The activity recovery up to 75.2% and enzyme loading of 32.5 mg/g carrier when CRL was immobilized on the Fe_3O_4/poly (AGE-co-EDMA) magnetic microspheres.

3.2.1. Effext of pH and temperature on free and immobilized CRL activity

The effect of pH on the activity of free and immobilized CRL in NPP hydrolysis was determined in the pH range 4.0–11.0 and the results are presented in Fig. 4. Both the free and immobilized CRL exhibited maximal activity at pH 7.0. Immobilization of CRL resulted in stabilization of enzyme over a broader pH range. The free CRL retained 41.2% and 13.1% residual activity at pH 8 and pH 10 whereas at same pH conditions immobilized CRL retained 76.4% and 37.3% residual activity, respectively. The results indicate that the immobilization procedure improved the stability of CRL appreciably particularly in the alkaline region, which was similar to Arıca's study [15].

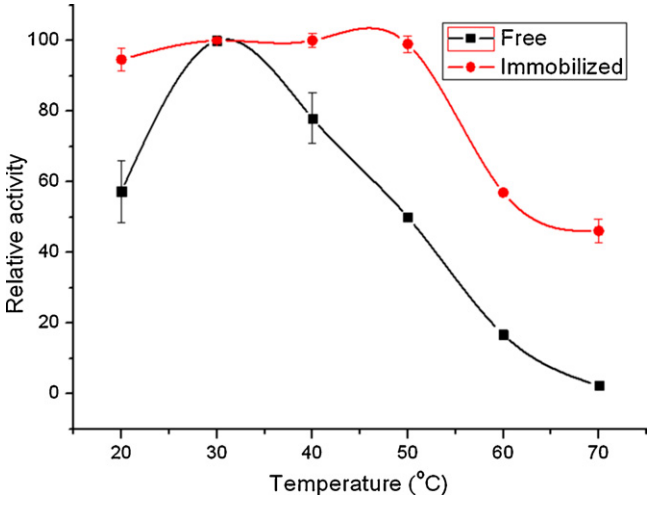

Fig. 5. Effect of temperature on the activity of free and immobilized CRL.

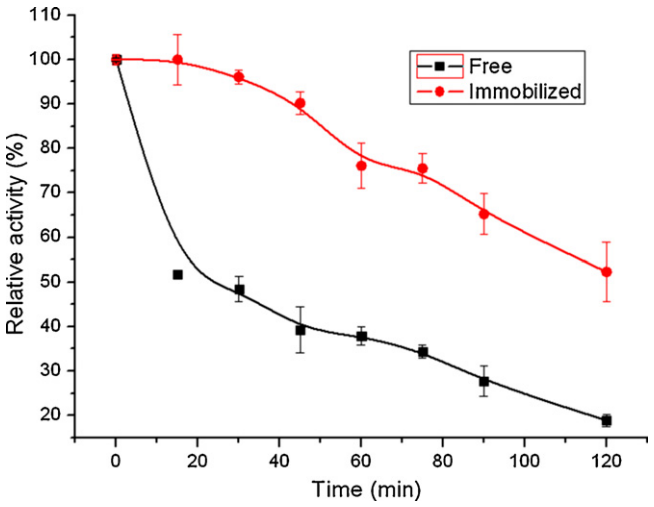

Fig. 6. Thermal stability of the free and immobilized CRL.

The effect of temperature on enzyme activity was expressed as percentage of the maximum activity, which was investigated in phosphate buffer (0.1 M, pH 7.0) in the range of 20–70 °C after 5 min of reaction. The activity profiles of free and immobilized CRL at different temperatures are represented in Fig. 5. As the temperature increases above 30 °C, the relative activity of the free lipase decreased greater than the immobilized one. At 50 °C the free CRL retained only 50.2% residual activity while immobilized lipase retained 96.1% of its initial activity. Similarly at 70 °C the free CRL retained only 5.3% residual activity while immobilized CRL was found to retain 46.1% of its initial activity. It might be due to the creation of conformational limitation on the lipase movement as a result of covalent bonds formation between the lipase and the Fe_3O_4/poly (AGE-co-EDMA) carriers [17].

3.2.2. Thermal stability of free and immobilized CRL

Thermal stability experiments were carried out with free and immobilized CRL, which were incubated in the absence of substrate at 50 °C. As shown in Fig. 6, the activity of immobilized CRL decreased less and more slowly than that of the free form. The free lipase lost half of its activity within only 20 min. However, the immobilized lipase retained its initial activity of 52.3% after 120 min of heat treatment. These results demonstrated that the thermal stability of immobilized lipase was much better than the free one. The increase in the thermal stability of the immobilized CRL may arise from the conformational integrity of the immobilized enzyme structure after covalent binding to the carriers. These results suggest that thermostability of immobilized lipase increased remarkably because of multi-point covalent immobilization of CRL onto Fe_3O_4/poly(AGE-co-EDMA) magnetic microspheres.

3.3. Optimization of esterification conditions of phytosterols with UFAs

3.3.1. Effect of reaction solvent

Generally speaking, the kind of organic solvent is a key factor for esterification reaction, for it affect not only the activity and stability of lipases, but also the solubility of substrates. Taking the whole into consideration, n-hexane, isooctane, cyclohexane and tert-amyl alcohol, which show different Log *P* values, were chose as solvents for esterification reaction. As shown in Table 1, the reaction in isooctane which is the most hydrophobic solvent exhibits the highest conversion after 24 h among the four solvent. It might be attributed to the fact that the more hydrophobic solvent used, the more activity and stability of the lipase exhibited. Therefore, isooctane was selected as the reaction solvent.

Table 1
Effect of organic solvents on esterification of phytosterols in lipase-catalyzed reaction.

Solvent	Log *P*	Esterification (%)
Cyclohexane	3.5	53.2
Isooctane	4.5	92.7
n-Hexane	3.2	38.0
Tert-amyl alcohol	1.2	13.2

Reaction condition: 50 μmol/mL phytosterols, 1:2 of the molar ratio of phytosterols to linolenic acid, 20 mg/mL immobilized CRL in 10 mL solvent, 50 °C, 160 rpm.

3.3.2. Effect of water or molecular sieves content

It is well-known that a small amount of water is necessary for the enzyme to preserve its optimal active. On the contrary, an excess of water decreases the lipase's activity both from kinetic and thermodynamic points of view in esterification reaction [22]. Therefore, controlling of water in the reaction system was particular important to obtain stable and relatively high conversion.

The effect of the water content and molecular sieves amount on esterification of phytosterols was examined. As shown in Fig. 7, with the addition of water, the esterification rate of phytosterol raises gradually after 6 h of reaction. However, the conversion of phytostanyl laurate decreased obviously with adding 3 Å molecular sieves. The higher amount of molecular sieves added, the lower esterification degree obtained. The highest conversion of phytostanyl linolenate was obtained when no water and molecular sieves was added. It might be ascribed to the fact that the loss of necessary water absorbed by molecular sieves led to the decrease of immobilized CRL activity.

3.3.3. Effect of substrate concentration

The effect of phytosterols concentration on esterification of phytosterols was investigated (Fig. 8). The substrate molar ratio of phytosterols to linolenic acid was kept at 1:2. The esterification rate increase with the prolonging of reaction time, and the phenomenon was more obvious at higher concentration. The esterification rate increased with increasing of the phytosterols concentration till 100 μmol/mL, but decreased with further increase of phytosterols

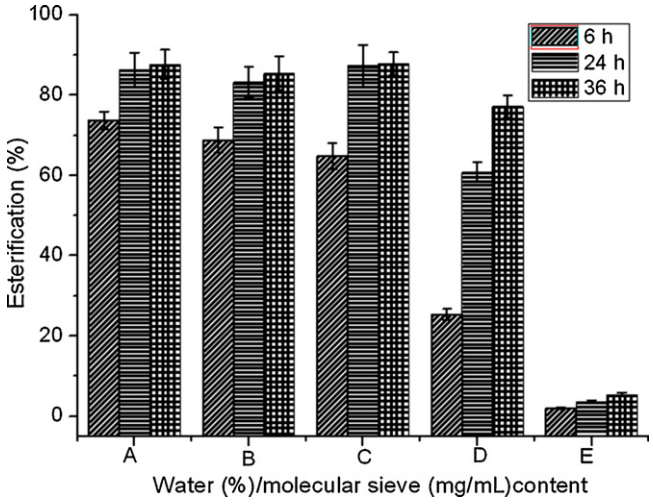

Fig. 7. Effect of water/molecular sieve content on esterification of phytosterols in lipase-catalyzed reaction. Reaction conditions: 50 μmol/mL phytosterols, 1:2 of the molar ratio of phytosterols to linolenic acid, 20 mg/mL immobilized CRL in 10 mL isooctane, 160 rpm, 50 °C. The water content of A and B was 0.1% and 0.3%, respectively; the molecular sieve content of C and D was 20 mg/mL and 40 mg/mL, respectively.

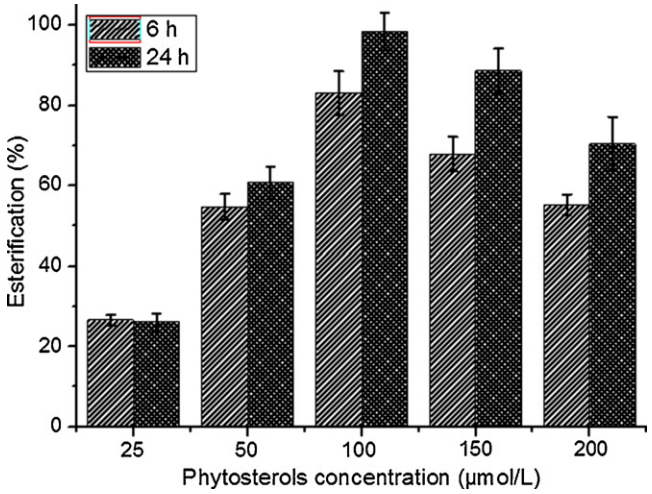

Fig. 8. Effect of phytosterols concentration on esterification of phytosterols in lipase-catalyzed reaction. Reaction conditions: 1:2 of the molar ratio of phytosterols to linolenic acid, 20 mg/mL immobilized CRL in 10 mL isooctane, 160 rpm and 50 °C.

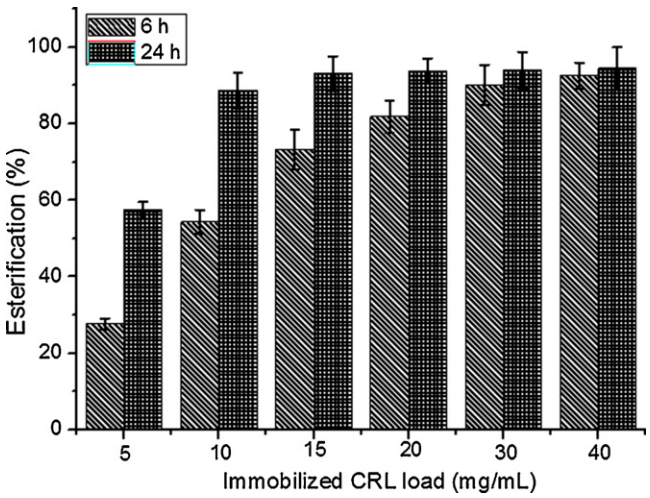

Fig. 10. Effect of immobilized CRL load on esterification of phytosterols in lipase-catalyzed reaction. Reaction conditions: 100 μmol/mL phytosterols, 1:2 of the molar ratio of phytosterols to linolenic acid, 10 mL isooctane, 160 rpm and 50 °C.

concentration. Most of the phytosterols were not soluble in the solvent at higher concentration, which was responsible for the lower esterification rate at higher phytosterols concentration. In addition, an increase in phytosterols and fatty acid concentration may change the catalytic environment and the active site of immobilized CRL to some extent [23]. Thus, 100 μmol/mL phytosterols were chosen for the follow experiments.

3.3.4. Effect of substrate molar ratio

The effect of molar ratio of phytosterols to linolenic acid on the conversion of phytosterols was evaluated. As shown in Fig. 9, it was observed that increasing the molar ratio of phytosterols to linolenic acid from 1:1 to 1:2 led to a sharply increase in the rate of esterification. With 1:1 molar amounts of phytosterols to linolenic acid, the conversion of phytosterols only reached 64.6% and 84.1% after 6 h and 24 h, respectively. At the fixed phytosterols concentration of 100 μmol/mL, the relative high conversion (over 90% after 24 h) was achieved when the molar ratio was above 1:2, and it kept almost unchanged with further increase of the molar ratio. Considering the extent of esterification, economical aspect of the process and further purification of the crude products, the mole ratio of 1:2 was select for the subsequent experiment.

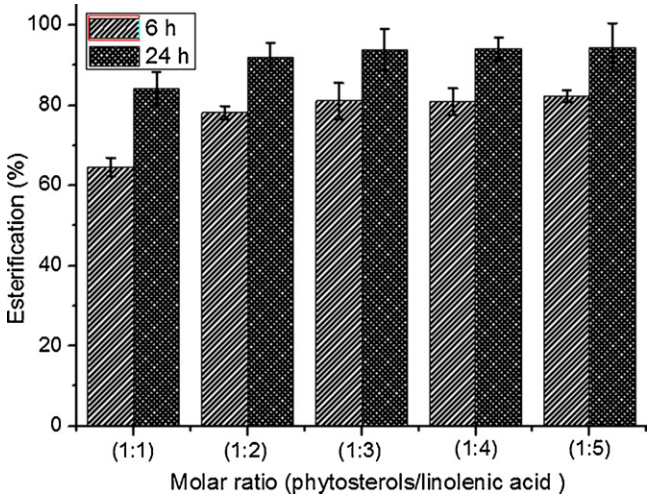

Fig. 9. Effect of molar ratio of linolenic acid to phytosterols on esterification of phytosterols in lipase-catalyzed reaction. Reaction conditions: 100 μmol/mL phytosterols, 20 mg/mL immobilized CRL in 10 mL isooctane, 160 rpm and 50 °C.

3.3.5. Effect of enzyme load amount

The influence of the enzyme load was evaluated with a 1:2 phytosterols/linolenic acid molar ratio using varying amounts of immobilized CRL from 0 to 40 mg/mL (Fig. 10). Almost no formation of the phytosterols linolenate occurred in the absence of CRL. It was observed that the phytosterols linolenate formation increases with the increasing of immobilized CRL loaded. A good synthesis method should consider the esterification rate and economical interest of the reaction, in other words, using less amount of CRL to obtain satisfactory production of phytostanyl linolenate. Using minimal amount of immobilized CRL such as 5 mg/mL would be economically attractive, but conversion of phytosterols only reached 27.6% and 57.4% after 6 h and 24 h, respectively. Increasing of immobilized CRL loaded lead to better production of phytostanyl linolenate. The formation of the phytostanyl linolenate was much higher with 15 mg/mL enzyme load and resulted in a 93.2% conversion after 24 h of reaction. It is also observed that there was almost no obvious difference in esterification rate when the CRL load beyond 15 mg/mL.

3.3.6. Effect of reaction temperature

The effect of reaction temperature on esterification of phytosterols in CRL-catalyzed reaction was investigated at different temperatures ranging from 35 °C to 65 °C. The esterification rate, solubility of the phytosterols and the stability as well as activity of the CRL are strongly related to the temperature of lipase-catalyzed reaction. As shown in Fig. 11, the esterification rate express as the conversion after 6 h increase with the rising of the temperature from 35 °C to 55 °C. With temperature further increased from 55 °C to 65 °C, an obvious decrease in esterification rate was observed. It might be ascribed to the loss of lipase activity at such high temperature. When the reactions were carried on at 55 °C, the highest conversions of 87.3% and 94.2 after 6 h and 24 h were obtained. In order to obtain higher conversion in shorter time, 55 °C was selected as the optimized temperature.

3.3.7. Time course on esterification of phytosterols

The effect of reaction time on the esterification of phytosterols with linolenic acid catalyzed by the magnetic immobilized CRL was also investigated. As shown in Fig. 12, the conversion of phytostanyl linolenate increases sharply during the first 10 h, further prolonging reaction time beyond 15 h could not lead to a significant

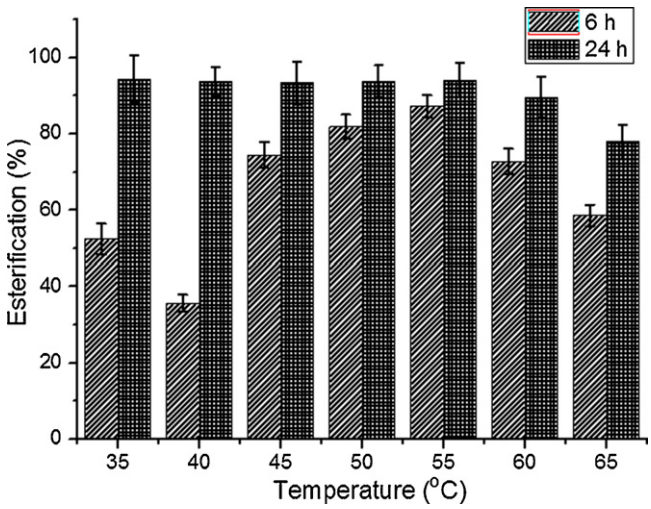

Fig. 11. Effect of reaction temperature on esterification of phytosterols in lipase-catalyzed reaction. Reaction conditions: 100 μmol/mL phytosterols, 1:2 of the molar ratio of phytosterols to linolenic acid, 15 mg/mL immobilized CRL in 10 mL isooctane, 160 rpm.

increase in phytostanyl linolenate formation, whit a conversion into phytostanyl linolenate of 93.5% at 15 h. The result indicating the reaction had nearly reached the equilibrium after 15 h, which was consistent with the findings of Weber et al. [10]. Weber et al. reported that the conversion of sitostanyl oleate was rapidly increased with the first 4–8 h, and then tended to equilibrium using CRL as the catalyst.

3.4. Esterification and transesterification of phytosterols with different acyl donors

Under the optimized condition, the esterifications of phytosterols with other acyl donors were investigated. Table 2 summarizes the data on conversion in the esterification and transesterification of phytosterols with different acyl donors including free fatty acid (FFA), triglyceride and fatty acid methyl ester. As shown in Table 2, the conversions were above 85.8% and 78.1% for FFA and triglycerides (three kinds of edible oil), respectively. However, the conversion was only 55.3% using fatty acid methyl ester as the acyl donors, which ascribe to the by-product (methanol)

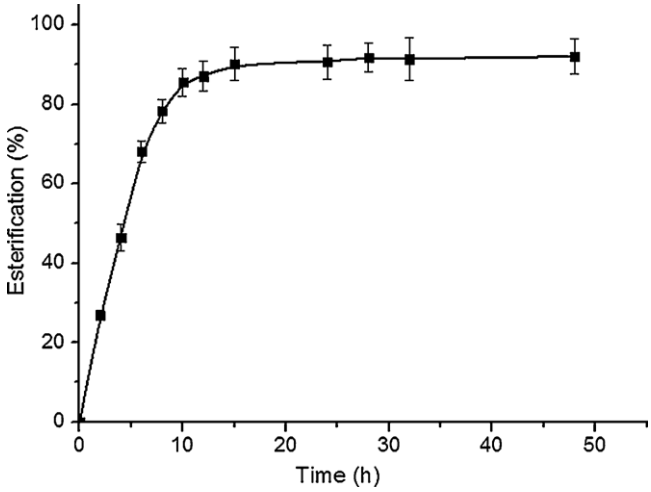

Fig. 12. Reaction time on esterification of phytosterols in lipase-catalyzed reaction. Reaction conditions: 100 μmol/mL phytosterols, 1:2 of the molar ratio of phytosterols to linolenic acid, 15 mg/mL immobilized CRL in 10 mL isooctane, 160 rpm, 55 °C.

Table 2
Esterification or transesterification of phytosterols with different acyl donors.

Acyl donors		Esterification (%)	
		15 h	24 h
Triglyceride	Rapeseed oil	73.9	78.1
	Teaseed oil	68.8	80.0
	Linseed oil	78.4	78.2
Fatty acid	Oleic acid	88.6	91.3
	Linoleic acid	86.2	85.8
	Linolenic acid	93.5	94.2
Fatty acid methyl ester[a]	–	48.2	55.3

[a] The fatty acid methyl esters were obtained from methyl esterification of linseed oil. Reaction condition: 100 μmol/mL phytosterols, 1:2 of the molar ratio of phytosterols to fatty acids (triglyceride), 1:8 of molar ratio of phytosterols to fatty acid methyl ester, 15 mg/mL immobilized CRL in 10 mL solvent, 50 °C, 160 rpm.

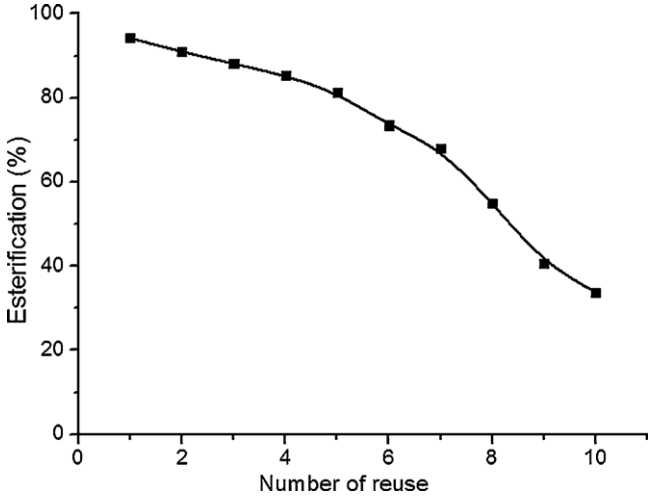

Fig. 13. Reuse of the magnetic immobilized CRL for esterification of phytosterols. Reaction conditions: 100 μmol/mL phytosterols, 1:2 of the molar ratio of phytosterols to linolenic acid, 15 mg/mL immobilized CRL in 10 mL isooctane, 160 rpm, 55 °C.

may influence the activity of immobilized CRL to some extent. The results suggest that the magnetic immobilized CRL could be used to catalyze the esterification of phytosterol with different kinds of acyl donor.

3.5. Reuse of the immobilized CRL

The reusability of immobilized enzyme is rather important for its practical application. To investigate the reusability of the magnetic immobilized CRL, the immobilized CRL was first washed with tertiary butyl alcohol and then isooctane after one catalysis cycle and reintroduced into a fresh esterification reaction. Fig. 13 shows the effect of repeated immobilized CRL use on the extent of esterification of phytostanyl with linolenic acid by magnetic separation. It was observed that the conversion of phytostanyl linolenate was still retained 80% after the 5 reuses. This result confirmed that the immobilized CRL on hydrophobic magnetic microspheres has a good durability and magnetic recovery. The decrease of esterification rate might be caused by the denaturation of the enzyme on the carriers during use. The half-life of CRL activity was estimated from the graph to be 160 h.

4. Conclusions

In the present study, the magnetic polymer coated microspheres (2–3 μm) were prepared and employed in immobilizing *Candida rugosa* lipase. The magnetic immobilized lipase exhibited high

activity, better resistance to pH and temperature inactivation, superior thermal stability and reusability. An esterification reaction to synthesize phytosterol esters with unsaturated fatty acids under mild conditions was carried out with the magnetic immobilized CRL. The effect of temperature, reaction time, substrate molar ratio, and enzyme amount was studied in order to attain optimal reaction conditions. In addition, phytosterols esters could also been converted in high yields to the corresponding long-chain acyl esters via transesterification with methyl esters of fatty acids (55.3%) or triacylglycerols (above 78.1%) using immobilized CRL as biocatalyst.

Acknowledgements

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