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Lipase Immobilization on Hyper-Cross-Linked Polymer-Coated Silica for Biocatalytic Synthesis of Phytosterol Esters with Controllable Fatty Acid Composition

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ABSTRACT: In this study, a novel mixed-mode composite material, SiO_2@P(MAA-*co*-VBC-*co*-DVB), was prepared via the hyper-cross-linking of its precursor, which was produced via suspension polymerization in the presence of SiO_2 particles. *Candida rugosa* lipase (CRL) was immobilized on the SiO_2@P(MAA-*co*-VBC-*co*-DVB) particles via hydrophobic and weak cation-exchange interaction. The resulting immobilized CRL showed much better thermal stability and reusability in comparison to free CRL. On the basis of the excellent biocatalyst prepared, a method for high-efficiency enzymatic esterification of phytosterols with different fatty acids to produce the corresponding phytosterol esters was developed. Six phytosterol esters with conversions above 92.1% and controllable fatty acid composition were obtained under the optimized conditions: 80 μmol/mL phytosterols, 160 μmol/mL linolenic acid, and 15 mg/mL CRL@HPCS at 300 rpm and 50 °C for 7 h in 30 mL of isooctane. The prepared phytosterol esters possessed a low acid value (≤0.86 mg of KOH/g), peroxide value (≤3.3 mequiv/kg), and conjugated diene value (≤1.74 mmol/kg) and high purity (≥97.8%) and fatty solubility (≥28.9 g/100 mL). All the characteristics favored the wide application of phytosterol esters with controllable fatty acid composition in different fields of functional food.

KEYWORDS: *phytosterol ester, biocatalysis, esterification, hyper-cross-linked polymer, fatty acid composition*

INTRODUCTION

Phytosterols are derived from vegetable oils and have been proved to be a kind of important natural active compound. Due to their cholesterol-lowering, anti-inflammatory, antioxidation, and anticancer functions, phytosterols have a wide application in pharmaceuticals, nutrition, and cosmetics.[1−3] Fatty acid esters of phytosterols are advantageous in applications to a wide range of food products because of their better lipid solubility, low melting point, and more significant cholesterol-lowering activity compared to the free phytosterols.[4,5] The fatty acid composition linked with phytosterol esters could influence the activity of sterol esters significantly and has gained much attention. For example, phytosterol esters of polyunsaturated fatty acid (PUFA) possess a more significant hypocholesterolemic effect compared with sterol esters of saturated fatty acid (SFA) and monounsaturated fatty acid (MUFA).[6,7] On the other hand, some fatty acids, especially unsaturated fatty acids (UFAs), not only serve a role as precursors of metabolites regulating critical biological functions, but also possess many physiological functions such as antitumor activity, cholesterol reduction, growth promotion, and blood microcirculation improvement.[8,9] Therefore, esterification of phytosterols with beneficial fatty acids such as linolenic acid and conjugated linoleic acid could lead to an even higher amount of valuable phytosterol esters.[10] However, little attention has been paid to the synthesis of phytosterol esters with controllable fatty acid (FA) composition until now.

Technical processes presently used for the preparation of phytosterol esters involve chemical and enzymatic methods. The chemical method involves problems such as high energy consumption and formation of a 3,5-diene steroid derivative as a side product. Furthermore, FAs, especially UFAs, are susceptible to thermal oxidation during chemical reaction.

Enzymatic catalysis is quite favorable for the synthesis of phytosterol esters due to excellent specificity and high efficiency. Lipase-catalyzed synthesis of phytosteryl ester in the presence of an organic solvent has been previously reported.[11,12] In most of the methods mentioned above, the biocatalysts for phytosterol ester 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 of their separation, recycling, and reuse.[13] As an alternative, the use of an immobilized enzyme not only provides enzyme reusability and hence reduces operational costs, but also reduces enzyme contamination and facilitates easy separation of products.

Carrier materials which play an important part in the usefulness of an immobilized enzyme should be low-cost and possess an adequately large surface area together with the least diffusion limitation in the transport of substrate and

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product.[14,15] Recently, lipase has been immobilized on several attractive carriers, including magnetic microspheres, microporous polymers, chitosan beads, and so on.[13−15,19] However, most of the immobilized lipase is used for oil hydrolysis or biodiesel production. Successful application in other esterification fields is seldom. Furthermore, due to the low surface area and the low activity yield, the esterification activity and reusability were not satisfactory. As a new generation of highly porous polymers, hyper-cross-linked polymers (HCPs) possess high micropore contents and correspondingly high specific surface areas, suggesting their great application potential in sorption processes.[16] HCPs are now being developed into a new branch of solid materials with unique applications in gas storage materials,[16] heterogeneous catalysts,[17] and solid-phase extraction.[18] Meanwhile, the merger of HCPs with silica beads can extend the utilization scope of these materials to enzyme immobilization. However, the conjunction of HCPs to other solid materials to produce hybrid materials is barely studied.[18]

The present study describes the synthesis of hyper-cross-linked polymer-coated silica (HPCS) particles with hydrophobic and weak cation-exchange character and the application of these carriers in immobilization of *Candida rugosa* lipase (CRL). The properties of the immobilized lipase were characterized and compared with those of the free lipase. Then, on the basis of this immobilized lipase, a method was proposed for the esterification of phytosterols with different FAs to produce phytosterol esters with controllable FA composition. The physiochemical properties of phytosterol esters with different FA compositions were also determined. As far as we are aware, a method related to synthesis of phytosterol esters with controllable FA composition has not been reported.

MATERIALS AND METHODS

Reagents and Chemicals. The irregular silica (300−400 mesh) for preparing the HPCS was obtained from the Qingdao Haiyang Chemical Plant (Qingdao, China). Ammonia−water (25 wt %), anhydrous disodium phosphate, dichloroethane, anhydrous ferric chloride, acetonitrile (ACN), 2-propanol, and 2,2-azobis(2-methylpropionitrile) (AIBN) were all purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). Tetraethyl orthosilicate (TEOS) and [3-(methacryloxy)propyl]trimethoxysilane (MPS) were obtained from the Chemical Plant of Wuhan University (Wuhan, China). Methacrylic acid (MAA; 98%), vinylbenzyl chloride (VBC; 90%), and divinylbenzene (DVB; 80%) were purchased from Sigma-Aldrich (St. Louis, MO). AIBN was recrystallized from ethanol, and other reagents were of analytical grade. Purified water was obtained with Millipore water purification equipment (Boston, MA).

CRL (lyophilized powder, type VII, 700 U/mg of solid), and *p*-nitrophenyl palmitate (*p*-NPP) were purchased from Sigma-Aldrich. Phytosterols (*β*-sitosterol (77%), campesterol (17%), stigmasterol (5%)) were purchased from Xian Bluesky Biological Enineering Co. Ltd. (Xi'an, China). Linolenic acid (80%), linoleic acid (90%), and oleic acid (90%) were purchased from Henan Linuo Biochemical Co. Ltd. (Anyang, China). Refined and bleached teaseed oil, sunflower oil, and linseed oil, which were used as the source of mixed FAs, were purchased from the supermarket.

Preparation and Characterization of HPCS Particles. First, silica particles with abundant reactive double bonds on the surface (i.e., MPS-modified silica) were prepared. Typically, silica particles (10 g) were added to a solution containing 2-propanol (200 mL), Milli-Q water (18 mL), and ammonia (25 wt %, 12 mL). After the solution was stirred at 300 rpm for 30 min, MPS (2.0 mL) was introduced, and the mixture was stirred for 24 h at room temperature. The final product, MPS-modified silica beads, was collected by centrifugation and then dried in a vacuum oven at 60 °C until constant weight.

Then the precursor material was prepared by a suspension polymerization method. MPS-modified SiO_2 (5.0 g), MAA (1.0 g), DVB (5.0 g), VBC (4.0 g), AIBN (200 mg), and ACN (400 mL) were successively added into a 500 mL three-necked round-bottom flask equipped with a condenser pipe and a stirring device. After deoxygenation by N_2 for several minutes, the flask was submerged in a water bath and heated from room temperature to boiling within 30 min. After half of the ACN was distilled out within 2 h, the mixture was cooled to room temperature, and then the resulting precursor particles were separated by centrifugation, washed several times by redispersion in ACN, and dried overnight in vacuo at 60 °C.

At last, the hyper-cross-linked reaction (i.e., Friedel−Crafts reaction) was carried out similarly to the reported method.[18] Briefly, anhydrous $FeCl_3$ (5.3 g) was dissolved in dichloromethane (150 mL). After filtration, the filtrate (i.e., $FeCl_3$-saturated solution) was introduced into a 200 mL round-bottom flask. Then, as-prepared precursor particles (5.0 g) were added. The hyper-cross-linked reaction was allowed to proceed at 80 °C for 18 h under nitrogen. After the mixture was cooled to room temperature, the resulting product HPCS was separated by means of centrifugation and washed successively by methanol and water. The product was dried under reduced pressure at 60 °C until constant weight. Figure 1 shows the reaction scheme for the preparation of HPCS and immobilization of CRL.

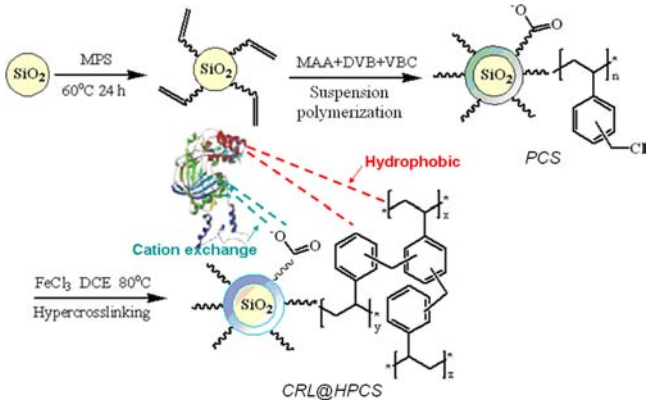

Figure 1. Preparation scheme of SiO_2@P(MAA-*co*-VBC-*co*-DVB) (HPCS)-immobilized CRL.

The surface structure and particle size of the materials were observed with a Hitachi S-3000N scanning electron microscope (Tokyo, Japan). Fourier transform infrared (FT-IR) spectra were obtained with a TENSOR 27 FTIR instrument (Bruker, Germany). Nitrogen sorption experiments were carried out at 77 K using Gemini V2380 surface area and pore size analyzer (Micromeritics, Norcross, GA).

Immobilization of Lipase on the HPCS Particles. First, 1.0 g of HPCS particles was prewetted with 1.5 mL of ethanol. Then immobilization of lipase on the HPCS particles (1.0 g) via adsorption was studied in phosphate buffer (15 mL, 25 mM, pH 7.0). The initial concentration of lipase was kept at 0.5−6.5 mg/mL in each corresponding buffer solution. The immobilization experiments were conducted at 20 °C for 4 h with continuous stirring. After immobilization, the HPCS particles were separated from the lipase solution and washed with buffer solutions. To investigate the properties of the HPCS particles as the carrier for lipase, lipase was also immobilized on the polymer-coated silica (PCS) as a control. The immobilized lipases were designated as CRL@HPCS and CRL@PCS using HPCS and PCS as the carrier, respectively. The amount of immobilized lipase was obtained by using the following equation:

$$Q = [(C_0 - C)V]/M \quad (1)$$

where Q is the amount of lipase immobilized onto HPCS (mg/g), C_0 and C are the concentrations of the lipase in the initial and final

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solutions (after combination with the wash solution), before and after immobilization, respectively (mg/mL), V is the volume of the aqueous solution (mL), and M is the mass of the particles (g). The amount of protein in the medium and wash solutions was determined by the Bradford method.[19]

Measurement of the Immobilized CRL Activity and Stability. The enzymatic activities of free and immobilized CRL were measured by the detection of *p*-nitrophenol, which comes from the hydrolysis of *p*-NPP.[20] One unit (U) of enzyme activity is defined as the amount of enzyme which catalyzes the production of 1 mmol of *p*-nitrophenol per minute under the experimental conditions. The relative activity (%) is the ratio between the activity of every sample and the maximum activity of a sample. The activity yield after immobilization is defined by the following equation:

$$\text{activity yield}\ (\%) = (B \times 100)/A \tag{2}$$

where A is the total activity of CRL added to the initial immobilization solution and B is the activity of immobilized CRL.

The thermal stability assays were performed by the incubation for different times (0−100 min) of CRL@HPCS, CRL@PCS, and free CRL at 50 °C. After the enzyme was cooled to room temperature, its activity was measured under standard conditions (pH 7.0, 37 °C) as described above. Residual activities were calculated as the ratio of the activity of the lipase measured after incubation to the maximal activity of the lipase.

Lipase-Catalyzed Esterification of Phytosterols. The following esterification conditions were used: Phytosterols (80 μmol/mL), an FA such as oleic acid, linolenic acid, or conjugated linoleic acid (80−320 μmol/mL), immobilized CRL (5−40 mg/mL), and solvent (isooctane, 30 mL) were added into an Erlenmeyer flask. The solvent had, in advance, been dehydrated with 15% (w/w) molecular sieves 4 Å for at least 24 h. The vial was placed in a water bath with stirring at 300 rpm for a certain time. A parallel reactor with six mechanical stirrer units and heat units (Radleys, Essex, U,K.) was used for optimization of the esterification conditions. Over the time course of the reactions, a portion of the reaction mixture (50 μL) was periodically removed from the reaction for GC analysis.

Qualitative and Quantitative Analysis of Phytosterol Esters and FA Composition. The composition of the crude products was analyzed by GC. An Agilent 6890 series II gas chromatograph (Hewlett-Packard Co., Avondale, PA), equipped with a flame ionization detector (FID) and a fused silica capillary column (DB-5 HT, 15.0 m × 320 μm × 0.10 μm, Agilent Technologies, Palo Alto, CA) 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 and 350 °C, respectively. The oven temperature was held at 210 °C for 2.0 min, then increased to 320 °C at a rate of 10 °C/mi, held at 320 °C for 15 min, then increased to 380 °C at a rate of 10 °C/min, and finally held at 380 °C for 5 min. The injection volume was 1 μL in split mode. The split ratio was 50:1. The degree of esterification (%) of phytosterols with FAs to form phytosterol esters was calculated from the GC profile of reactants using the following equation:

$$\text{degree of esterification (DE, \%)} = \frac{B}{B + 1.63 \times A} \times 100 \tag{3}$$

where A is the peak area of total phytosterols (campesterol + stigmasterol + β-sitosterol), B is the peak area of total phytosterol esters, and 1.63 is the ratio of the average molecular weight of total phytosteryl esters to the average molecular weight of total phytosterols.

The FA compositions of the phytosterol esters and acyl donors were further confirmed by GC analysis. First, the fatty acid methyl esters (FAMEs) were prepared from FAs and phytosterol esters according to "Animal and Vegetable Fats and Oils—Preparation of FAMEs" (GB/T 17376, ISO 5509:2000, IDT) with some modification. Briefly, 1 mL of a NaOH solution (0.5 M in methanol) and 5 mL of hexane were added to 0.2 g of free FAME or phytosterol esters; after reaction for 10 min in the shaker at 55 °C, 20 mL of hexane was added to extract the generated methyl ester. After 10 min of centrifugation, the supernatant was removed for GC analysis as described previously.[21]

Physiochemical Properties of Phytosterol Esters with Different FA Compositions. The conjugated diene (CD) value was determined as reported previously.[22] Briefly, the sample was dissolved in 50 mL of cyclohexane and the optical density (1 cm light path) recorded at 234 nm against a cyclohexane blank. The conjugated diene value was calculated according to the equation

$$\text{CD (mmol/kg of oil)} = \frac{AV}{\varepsilon bm} \times 100 \tag{4}$$

where A is the absorbance at 234 nm of the test sample, V the constant volume, ε the molar absorption coefficient, 26 000 L/(mol·cm), b the thickness of the absorption cell, and m the sample mass.

The peroxide value (PV) and acid value (AV) were determined according to the National Standard of the People's Republic of China (PRC) (GB/T 5538, ISO 3960:2001, IDT; GB/T 5530, ISO 660:1996, IDT).[23,24] The melting temperature was determined using the method described in the National Standards of the PRC (GB/T 5536-85).

The differential scanning calorimetry (DSC) melting properties were determined with a modulated differential scanning calorimeter, Q2000 (TA Instruments, New Castle, DE). A sample (6 ± 0.1 mg) was accurately weighed for DSC analysis. The instrument temperature was increased to 70 °C, and after 5 min at this temperature, the cooling curve was obtained by cooling at 5 °C/min to −65 °C. After being held for 5 min at −65 °C, the temperature was returned to 70 °C at 5 °C/min.[25]

The solubility of phytosterol esters in vegetable oil was measured according to the method described by Deng et al.[26]

RESULTS AND DISCUSSION

Characterization of the HPCS Particles and the Immobilized CRL. The morphologies of HPCS were investigated by scanning electron microscopy (SEM; Figure 2). It could be seen that the hyper-cross-linked silica particle possesses a spherical polymer nanoparticle agglomeration on its surface with a size distribution range from 200 to 400 nm (Figure 2B), demonstrating the successful encapsulation of P(MAA-*co*-VBC-*co*-DVB) on the surface of the SiO_2 particle.

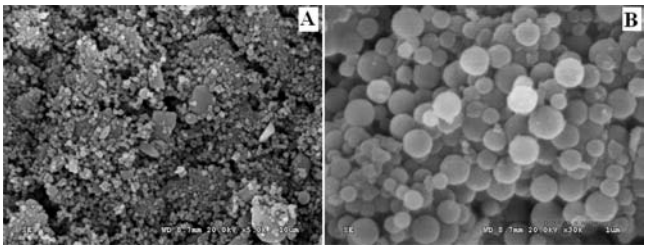

Figure 2. SEM images of SiO_2@P(MAA-*co*-VBC-*co*-DVB) (HPCS): wide view (5000×, A) and close-up view (30000×, B).

FT-IR spectroscopy was employed to further verify the P(MAA-*co*-VBC-*co*-DVB) formation on the surface of the SiO_2 core. As shown in Figure 3A, the MPS component of MPS-modified SiO_2 can be confirmed by the peaks at 1640 and 1718 cm^{-1} corresponding to the stretching vibration of the vinyl groups and carbonyl units of MPS, respectively. The successful coating of P(MAA-*co*-VBC-*co*-DVB) onto the surface of SiO_2 is also proven by the FT-IR spectrum in Figure 3B with the presence of peaks at 1614, 1511, and 1452 cm^{-1} assigned to the carbon−carbon stretching vibrations in the aromatic ring. In addition, it can be found that the absorption peaks of the vinyl groups of MPS (1640 cm^{-1}) disappeared after P(MAA-*co*-VBC-*co*-DVB) formation (Figure 3B). This may be due to an in-

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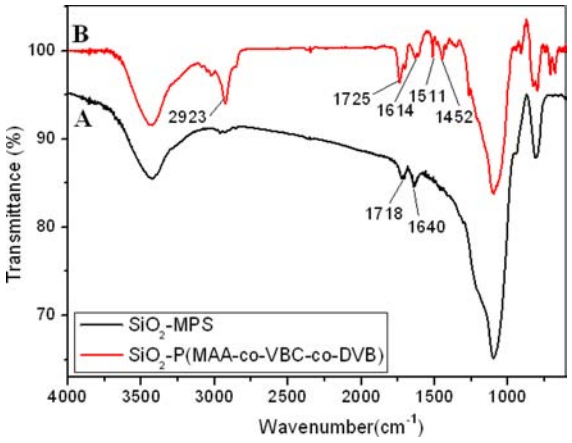

Figure 3. FT-IR spectra of MPS-modified SiO_2 (A) and SiO_2@P(MAA-*co*-VBC-*co*-DVB) (HPCS) (B).

depth reaction of the reactive surface MPS groups with the polymer monomers.

The highly porous polymer shell of HPCS was also characterized by measuring the texture parameters of samples using a Brunauer–Emmett–Teller (BET) treatment of the N_2 sorption isotherm data (Table 1). It can be found that the surface area and pore volume of MPS-modified SiO_2 are 340 m^2/g and 0.27 cm^3/g, respectively. After precipitation polymerization, the as-prepared PCS particles have a much lower surface area (141 m^2/g) and lower pore volume (0.12 cm^3/g). Interestingly, the two parameters of SiO_2@P(MAA-*co*-VBC-*co*-DVB) reach 487 m^2/g and 0.32 cm^3/g after Friedel–Crafts reaction, indicating the hyper-cross-linked network of P(MAA-*co*-VBC-*co*-DVB) has been formed on the SiO_2 core.

Table 1. Surface Area and Pore Volume Parameters of Materials Used in This Study

sample	surface area (m^2/g)	pore volume (cm^3/g)
MPS-modified SiO_2	340	0.27
PCS[a]	141	0.12
HPCS	487	0.32

[a]Polymer-coated silica (PCS) is the precursor of hyper-cross-linked polymer-coated silica (HPCS).

To evaluate the loading capacity of the carrier, 1.0 g of HPCS particles was loaded with 15 mL of different initial protein concentrations ranging from 0.5 to 6.5 mg/mL. The adsorbed CRL amount increases with increasing initial CRL amount until 5.0 mg/mL and reaches a maximum value of 65.4 mg/g, which is higher than that of CRL immobilized on magnetic microspheres (48.1 mg/g) and a fibrous polymer (44.7 mg/g).[13,19] The activity yields of CRL to HPCS particles reach 70.2%, which is also higher than that in Arica and Yong's reports.[13,19] The result shows that HPCS particles have excellent adsorption properties due to their highly porous polymer shell which allow the adsorption of a high amount of CRL with high activity.

Thermal Stability of Free and Immobilized CRL. To evaluate the suitability of HPCS particles as stable platforms for lipase immobilization, the thermal stability, which is one of the most important properties of the enzymes, of different applications was examined. Thermal stability investigation was carried out with CRL@HPCS, CRL@PCS, and free CRL, which were incubated in the absence of substrate at 50 °C. As shown in Figure 4, it was observed that the activity of immobilized CRL decreased less and more slowly than that of the free form at two different temperatures. The free CRL and CRL@PCS lost 54.4% and 42.7% of their activity within 60 min at 50 °C, respectively. In contrast, immobilized CRL retained most of its initial activity (92.2%) under the same conditions, proving enhanced thermostability for this composite material. This is attributed to the stabilizing effect of the hyper-cross-linked network structure of the HCPS matrix, which prevents extensive structure changes typical of thermal denaturation.[27] The ability to retain enzyme activity at high temperatures provides several processing advantages, such as improved reaction rate and substrate solubility.

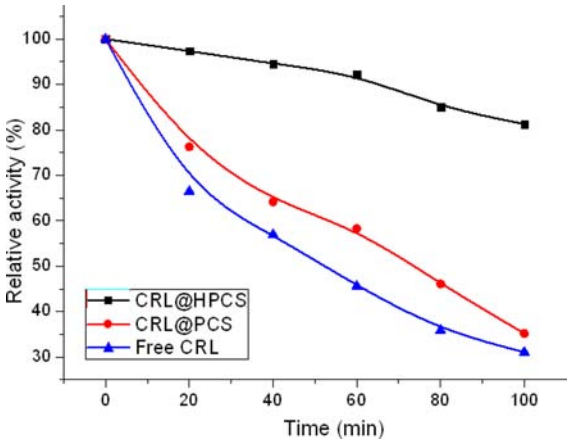

Figure 4. Thermal stability of CRL@HPCS, CRL@PCS, and free CRL. The incubation temperature was kept at 50 °C.

Optimization of Lipase-Catalyzed Esterification Conditions. The effect of the molar ratio of phytosterols to linolenic acid on the conversion of phytosterol linolenate was evaluated. As shown in Figure 5, it was observed that increasing the molar ratio of phytosterols to linolenic acid from 1:1 to 1:2 led to a sharp increase in the conversion. With 1:1 molar amounts of phytosterols to linolenic acid, the conversion of phytosterol linolenate only reached 65.2% and 90.7% after 5

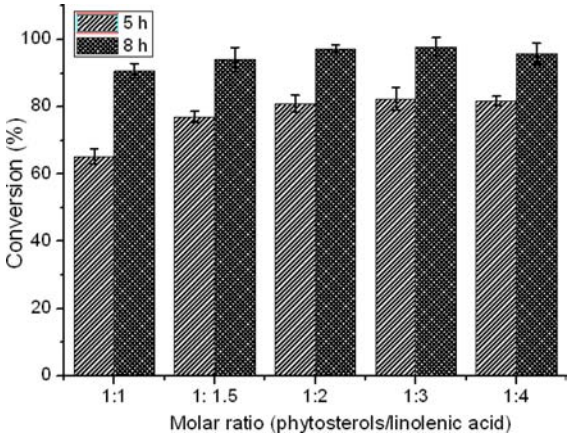

Figure 5. Effect of the molar ratio of linolenic acid to phytosterols on esterification of phytosterols in the lipase-catalyzed reaction. Reaction conditions: 80 μmol/mL phytosterols and 15 mg/mL CRL@HPCS in 30 mL of isooctane at 300 rpm and 50 °C.

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and 8 h, respectively. At the fixed phytosterol concentration of 80 mM, a relatively high conversion (over 95% after 8 h) was achieved when the molar ratio was above 1:2, and it remained almost unchanged with a further increase of the molar ratio. Considering the conversion, the economical aspect of the process, and further purification of the crude products, the mole ratio of 1:2 was selected for the subsequent experiment.

The influence of the enzyme load was evaluated with a 1:2 phytosterols:linolenic acid molar ratio using varying amounts of HPCS-immobilized CRL from 0 to 40 mg/mL. As shown in Figure 6, almost no formation of the phytosterol linolenate

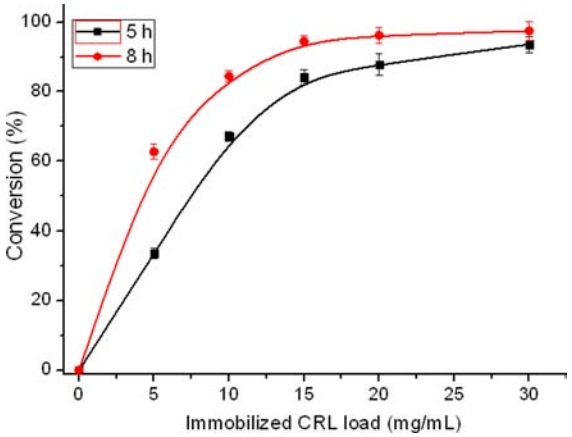

Figure 6. Effect of the CRL@HPCS load on esterification of phytosterols in the lipase-catalyzed reaction. Reaction conditions: 80 μmol/mL phytosterols, 1:2 molar ratio of phytosterols to linolenic acid, 30 mL of isooctane, 300 rpm, 50 °C.

occurred in the absence of CRL. It was observed that the phytosterol linolenate formation increased with increasing amount of immobilized CRL loaded. A good synthesis method should consider the conversion and economical interest of the reaction, in other words, using less amount of CRL to obtain satisfactory production of phytostanyl linolenate. Using a minimal amount of immobilized CRL such as 5 mg/mL would be economically attractive, but conversion of phytosterols only reached 33.6% and 62.7% after 5 and 8 h, respectively. Increasing the amount of of immobilized CRL loaded led to better production of phytostanyl linolenate. The formation of the phytostanyl linolenate was much higher with a 15 mg/mL enzyme load and resulted in a 96.6% conversion after 8 h of reaction. It was also observed that there was almost no obvious difference in conversion when the CRL load was beyond 15 mg/mL.

The effect of the reaction time on the esterification of phytosterols with linolenic acid catalyzed by CRL@HPCS and CRL@PCS was also investigated. As shown in Figure 7, the conversion of phytostanyl linolenate increased sharply during the first 5 h (further prolonging the reaction time beyond 7 h does not lead to a significant increase in phytostanyl linolenate formation), with a conversion into phytostanyl linolenate of 95.6%. This result indicates the reaction had nearly reached equilibrium after 7 h, which was consistent with the findings of Weber et al.[4] Weber et al. reported that the conversion of sitostanyl oleate rapidly increased in the first 4−8 h and then tended to equilibrium using CRL as the catalyst. In contrast, the conversion of phytostanyl linolenate was quite slow using CRL@PCS as the catalyst, with a conversion of only 46.5% after 8 h of reaction.

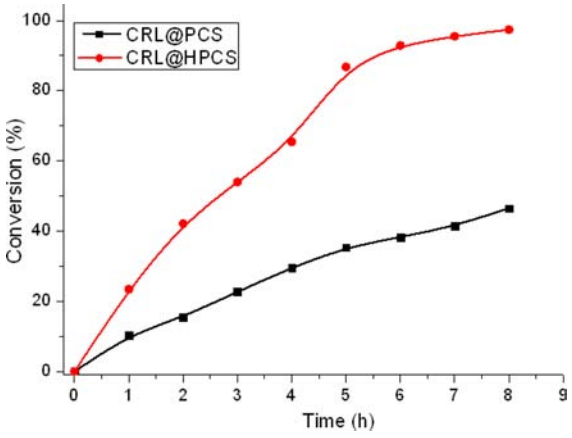

Figure 7. Effect of the reaction time on esterification of phytosterols using CRL@HPCS or CRL@PCS as the catalyst. Reaction conditions: 80 μmol/mL phytosterols, 1:2 molar ratio of phytosterols to linolenic acid, and 15 mg/mL CRL@HPCS in 30 mL of isooctane at 300 rpm and 50 °C.

Synthesis of Phytosterol Esters with Controllable FA Composition. Under the optimized conditions, the esterification of phytosterols with three common UFAs and three mixed FAs from hydrolyzed vegetable oils were investigated. As listed in Table 2, the eaterification ratios of phytosterols with

Table 2. Fatty Acid Profile of the Phytosterol Esters Corresponding to Different FAs as the Acyl Donors[a]

	fatty acid composition (%, w/w)					
product	C16:0	C18:0	C18:1	C18:2	C18:3	conversion (%)
oleic acid	1.9	2.0	**83.4**	12.7	0	92.1
phytosterol oleate **1**	2.5	1.8	**82.6**	13.1	0	
linoleic acid	7.6	1.9	8.9	**81.6**	0	93.2
phytosterol linoleate **2**	7.9	2.1	8.9	**81.1**	0	
linolenic acid	0	0	10.1	10.6	**79.3**	95.1
phytosterol linolenate **3**	0	0	8.3	14.6	**77.2**	
tea seed fatty acid	8.2	2.7	**80.9**	8.6	0	92.8
phytosterol ester **4**	5.3	1.8	**78.2**	14.7	0	
sunflower fatty acid	6.5	5.3	26.7	**61.5**	0	95.4
phytosterol ester **5**	5.8	3.9	28.0	**62.3**	0	
linseed fatty acid	5.2	3.9	19.7	16.9	**54.4**	96.2
phytosterol ester **6**	4.9	2.7	21.6	16.5	**54.3**	

[a]Values are the average of analysis from triplicate sets. Bold values indicate the amount of significant fatty acids. Phytosterol esters **1**−**6** were obtained by esterification of phytosterol with fatty acid from oleic acid, linoleic acid, linolenic acid, tea seed oil, sunflower oil, and linseed oil, respectively. Reaction conditions: 80 μmol/mL phytosterols, 1:2 molar ratio of phytosterols to linolenic acid, 30 mL of isooctane, 300 rpm, 50 °C.

six different acyl donors were between 92.1% and 96.2%. The results suggest that CRL@HPCS could be used to catalyze the esterification of phytosterol with different kinds of acyl donors with relatively high conversion. This might be ascribe to the fact that lipases prefer to adsorb on hydrophobic supports, involving the adsorption of the hydrophobic areas surrounding the active center and leaving the active site fully exposed to the reaction medium. Mateo et al. have shown that immobilization

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of lipases on hydrophobic sol–gels yields a higher activity recovery.[28]

Table 2 also lists the FA profile of the original acyl donors (six kinds of FAs) and the phytosterol esters produced. In the first set of reactions, three common UFAs, including oleic acid, linoleic acid, and linolenic acid, were used as acyl donors; in the second set of reactions, three hydrolyzed vegetable oil FAs, respectively rich in oleic acid, linoleic acid, and linoleic acid, were used as the acyl donors. Analysis of the FA composition of phytosterol esters by GC showed that almost all the FAs present in the acyl donors were incorporated into the corresponding phytosterol esters. In other words, the developed method could produce phytosterol esters with controllable FA composition by modulating the initial acyl donors with the wanted FA composition.

The reusability of immobilized enzyme is rather important for its practical application. At the end of each reaction batch, the immobilized CRL was washed with isooctane to remove any substrate or product retained on the support. Then the immobilized CRL was consecutively reused after each reaction cycle (8 h). The effect of repeated CRL@HPCS and CRL@PCS use on the conversion of phytostanyl linolenate was investigated (Figure 8). It was observed that the conversion of phytostanyl linolenate still remained 80.6% after 10 reuses, whereas CRL@PCS lost almost its whole activity after 5 cycles. This result confirmed that the immobilized CRL on HPCS particles allowed not only excellent activity in the presence of organic solvent but also satisfactory reusability in several reaction cycles. The retention of enzymatic activity and fine reproducibility can be attributed to the mild synthetic conditions and the robust immobilization ability of the HPCS matrix without affecting the active sites and disruption of subunits, respectively. In comparison, CRL@PCS obtained by conventional suspension polymerization showed a quite poor reaction rate (46.5% conversion after 8 h) and a significant decrease in enzymatic activity during repeated use due to enzyme denaturation and substantial leaching to the reaction medium.

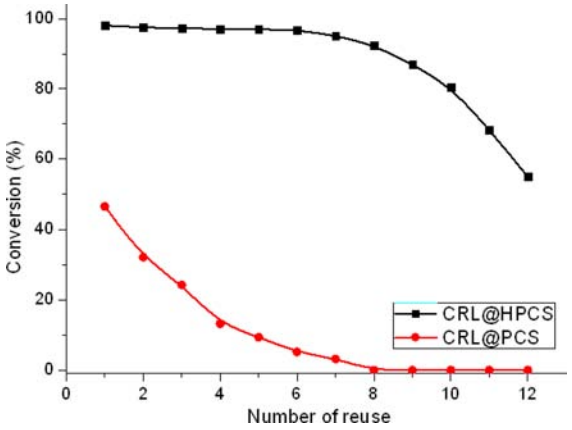

Figure 8. Reuse of CRL@HPCS and CRL@PCS for esterification of phytosterols. The reaction time was 8 h for each reaction cycle. Other reaction conditions were the same as those listed in Figure 7.

Physiochemical Properties of Phytosterol Ester with Different FFA Compositions. The physiochemical properties of phytosterol esters with different FFA compositions are shown in Table 3. The PV and CD value were low, which showed that few products of peroxidation were produced, a benefit of the mild reaction conditions. The AV and GC analysis results showed that the excess FFAs and phytosterol could be removed by purification and the content of phytosterol esters in the final product was above 97.8%. The basic physiochemical indexes have reached the "Chinese New Food Resource Quality Requirement" of phytosterol esters.

The melting points, which are mainly affected by the saturation of FAs, were in the range of 2.3–26.1 °C for phytosterol esters with different FA compositions. The solubilities of phytosterol esters in rapeseed oil were in the range of 28.9–36.2 g/100 mL of rapeseed oil, which was above 20 times the solubility of phytosterol in the rapeseed oil. The high solubility in the oil and low crystallization temperature favored its wide application in different fields and different types of food.

In conclusion, a novel hyper-cross-linked silica particle was prepared and used as a carrier for CRL immobilization. Due to the advantages of a large surface area, a special surface structure, and a desirable chemical composition, CRL@HPCS showed high catalytic performance with increased thermal stability and reusability. On the basis of CRL@HPCS, an esterification method was developed to synthesize phytosterol esters with controllable FA composition in high yield under mild conditions. The physiochemical properties of six prepared phytosterol esters were found to be closely related to their FA composition. These findings could promote the wide application of phytosterol esters with controllable FA composition produced by the food grade process in different formulations of functional foods.

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Table 3. Physiochemical Properties of Phytosterol Esters with Different FA Compositions

phytosterol ester	PV[a] (mequiv/kg)	AV[a] (mg of KOH/g)	CD[a] (mmol/kg)	melting piont (°C)	solubility (g/100 mL)	purity (%)
1	2.1	0.76	1.04	25.6	32.1	98.9
2	2.4	0.67	1.33	12.5	36.2	98.6
3	1.9	0.75	1.61	2.3	35.8	98.2
4	3.3	0.51	1.12	26.1	30.7	99.1
5	2.6	0.86	1.74	23.6	28.9	97.8
6	2.8	0.81	1.53	22.7	31.5	98.0
standard[b]	≤5.0	≤1.00				≥90

[a]PV, AV, and CD are abbreviations for peroxide value, acid value, and conjugated diene value, respectively. [b]The standard is the "Chinese New Food Resource Quality Requirement" of phytosterol esters. Phytosterol esters **1**–**6** are the same as those described in Table 2.

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Notes

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