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Immobilization of lipase onto aminopropyl-functionalized MSU-H type mesoporous silica and esterification

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Abstract−*Candida rugosa* lipase (CRL) was immobilized on an aminopropyl-functionalized MSU-H type mesoporous silica (AFMS) through physical adsorption and a covalent cross-linking. It was evaluated as a class of biocatalysts in the esterification of conjugated linoleic acid (CLA) isomers with ethanol. AFMS materials with varied content of aminopropyl were prepared by a simple co-condensation at near neutral pH condition. Introduction of aminopropyl chains and CRL molecules onto the AFMS supports was confirmed by Fourier transform infrared (FT-IR) spectra. CRL was immobilized on the AFMS through electrostatic and covalent interactions. The covalently cross-linked CRL gave a loading amount of 34.3 mg CRL/g-support and a hydrolytic activity of 2471.5 U/g-catalyst. It exhibited high operational stability and remained 23.9-27.5% of total esterification in 32 h consecutive four runs in the esterification of CLA with ethanol. Moreover, the immobilized CRLs catalyzed 2.8-3.8 times of esterification of *cis*-(*c*)9, *trans*-(*t*)11-CLA faster than that of *t*10, *c*12-CLA.

Keywords: Lipase, Immobilization, Mesoporous Silica, Conjugated Linoleic Acid, Esterification

INTRODUCTION

Conjugated linoleic acid (CLA) is a mixture of positional and geometric isomers of octadecadienoic acid with a pair of conjugated double bonds [1]. Due to its functions such as anticarcinogenic, antiatherogenic and antiobesity and improvement of immunity, CLA has received much attention [2]. However, its applications are limited in food and feed additives, drugs and cosmetics because CLA is easily oxidized and has unpleasant taste [3]. The ethyl ester of CLA (CLAEE) is more stable, nontoxic and readily absorbed by living bodies [4]. Therefore, CLAEE can be a substitute for CLA. The industrial production of CLAEE is mainly by chemical esterification of CLA in presence of catalysts such as sulfuric acid, sodium or potassium hydroxide. These catalysts have environmental and processing disadvantages and lead to poor quality of the CLAEE product due to peroxidation of the unsaturated bonds in CLA [5]. Hence, it is desirable to develop an eco-friendly biocatalytic process for this conversion. Due to its mild reaction conditions and no pollution, an enzymatic process for producing CLAEE is more attractive than the chemical one.

In addition, the most commonly used commercial CLA is a mixture of more than seven positional and geometric isomers [6]. The *cis*-(*c*)9, *trans*-(*t*)11- and *t*10, *c*12-CLA isomers are believed to have beneficial physiological effects in anticancer activity and decreasing fat in the body, respectively, whereas a very small amount of the *trans*-isomers of *t*9, *t*11- and *t*10, *t*12-CLA is harmful to health [7]. Methylation of CLA under acid catalysis is commonly used in separation and identification of CLA isomers [8]. However, the acid-catalyzed methylation alters the CLA isomer composition to form *t*, *t* isomers or methoxy and hydroxy artifacts [5]. Ethyl esterification of CLA with ethanol under lipase catalysis provides an alternative to separate and identify CLA isomers because this method not only prevents CLA isomerization but also is safe in food supplements [9].

Candida rugosa lipase (CRL) is an inexpensive and available biocatalyst for food production that can catalyze esterification, transesterification and interesterification reactions in non-aqueous media [9]. However, its use as a catalyst is hindered due to the lack of long-term stability under process conditions and difficulties in recovery and recycling. Immobilization of free CRL onto solid supports can overcome such problems. Various solids such as mica [10], magnetic nanoparticles [11] and mesoporous silicas [12] have been used as supports for immobilization of CRL. The CRL attached on mica gave an activity of 679.3 U/g and a loading amount of 8.2 mg CRL/g-support [10], while immobilization of CRL onto mesoporous SBA-15 silica reached a maximal loading amount of 343.6 mg/g-support [12]. Mesoporous silicas are a promising family of materials as supports for immobilizing enzyme because of their high surface area, large pore volume and nontoxicity [13,14]. MSU-H is a two-dimensional hexagonal ordered mesoporous silica which can be prepared by using a low-cost and convenient reagent of sodium silicate as a silica source and a nonionic surfactant of triblock copolymer Pluronic P123 as template at near neutral pH condition [15]. It has an open porous system with interconnected channels

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which favors diffusion of enzyme and bulky substrate. However, to date, there is no report on preparation of the aminopropyl-functionalized MSU-H mesoporous silica (AFMS) and its application in immobilization of CRL for esterification.

In this work, the AFMS materials with different contents of aminopropyl chains were prepared via co-condensation and were used as support for CRL immobilization by physical adsorption and a covalent cross-linkage. The ability and the operational stability of the resultant catalysts were evaluated in esterification of CLA with ethanol in isooctane as a solvent. The interactions between CRL molecules and supports were also discussed.

MATERIALS AND METHODS

1. Materials

Pluronic P123 triblock copolymer ($EO_{20}PO_{70}EO_{20}$, M_w=5800) and APTES (98%) and crude CRL type VII (7,030 U/g-solid) were purchased from Sigma-Aldrich. CLA (78-84%) with almost equal amounts of *c*9, *t*11- and *t*10, *c*12-CLA was obtained from Puritan's Pride (USA). Coomassie brilliant blue G-250 was produced by Sinopharm Chemical Reagent Co., Inc. (Shanghai, China). Bovine serum albumin (BSA) was purchased from Shanghai Bio Life Science & Technology Co., Ltd. (Shanghai, China). All other chemicals used were AR grade and obtained locally.

2. Synthesis of Support

The AFMS materials were synthesized via direct co-condensation by the following procedure: 2.35 g of P123 was dissolved in a solution of acetic acid (pH 5.5). 11.0 g of sodium silicate was added dropwise to a vigorously stirred solution above an ambient temperature. After stirring for 1 h, APTES was added to this solution, and the resulting mixture was stirred for 20 h at 60 °C. The pH of reaction mixture was adjusted to about 6.5 by addition of acetic acid to it. The overall molar ratio of the reactant was $1.00SiO_2 : 0.0081P123 : x/100APTES : 0.5CH_3COOH : 115H_2O$. The solid products were filtered and washed with water, and then dried at 80 °C. The surfactant P123 was removed by ethanol extraction at 76 °C for 12 h. This was repeated three times to ensure complete removal of the surfactant, which was confirmed by thermogravimetry. For preparation of different samples, x equal to 7, 15 and 18 was used individually. The obtained samples were designated as x%NH_2-Si. By contract, pristine silica MSU-H was prepared without addition of APTES and calcined at 550 °C for 4 h to remove the surfactant.

3. Immobilization of CRL

Immobilization of CRL was performed by physical adsorption and a covalent cross-linking method. CRL solution was prepared by adding an appropriate amount of crude CRL powder to phosphate buffer (0.025 M, pH 7.0). The experiment of physical adsorption was carried out by mixing 400 mg of support and 25 ml of CRL solution (16 mg/ml). The mixture was stirred at 37 °C and 200 rpm for 12 h. The immobilized-CRL was collected by centrifugation at 12,000 rpm, and washed with a pH 7.0 phosphate buffer solution, then dried at 30 °C. The immobilized CRL, which was prepared using the x%NH_2-Si as support by physical adsorption, was designated as CRL/x%NH_2-Si(p).

To obtain high activity and operational stability, the effect of cross-linker concentration (0.25-1.25 wt%) and cross-linking time (30-120 min) on activity of immobilized CRL was studied. CRL/15%NH_2-Si(c) and CRL/MSU-H(c) were prepared by covalent cross-linking with 5 ml of 0.5 wt% glutaraldehyde for 2 h stirred at 200 rpm using the 15%NH_2-Si and the pristine MSU-H silica as supports after the physical adsorption, respectively. For CRL/15%NH_2-Si(cs), 5 ml of 0.75 wt% glutaraldehyde and 45 min of cross-linking time were used in preparation. Its preparation process and support were the same as CRL/15%NH_2-Si(c).

4. Protein Assay

The protein of CRL was measured by the Bradford method using bovine serum albumin as a standard [16]. The loading amount of CRL on the supports was determined indirectly from the difference between the total protein amount of CRL for initial immobilization (added CRL, mg) and the amount of protein both in the filtrate and in the washing solution after immobilization (unimmobilized CRL, mg). The CRL loading amount (q) and immobilization mass percentage (Y) were calculated according to Eq. (1) and Eq. (2).

$$q\ (\text{mg/g-support}) = \frac{m_1}{m_2} \tag{1}$$

$$Y\ (\%) = \frac{m_1}{m_1 + 1000 m_2} \times 100\% = \frac{100q}{q + 1000} \tag{2}$$

where m_1 was the mass of CRL protein loaded on support (mg), i.e., m_1=added CRL (mg)−unimmobilized CRL (mg); m_2 was the mass of support (g).

5. Activity Assay

Hydrolytic activity of immobilized CRL was assayed titrimetrically by using an olive oil emulsion method [17]. The substrate was prepared by mixing 50 ml of olive oil with 150 ml of polyvinyl alcohol solution (2% w/v) to obtain emulsion. The reaction mixture consisting of 5.0 ml of phosphate buffer (0.025 M, pH 7.0) and 5 ml of the substrate emulsion above and a certain amount of immobilized CRL was incubated for 15 min at 37 °C in a vessel. The reaction was terminated by adding 15 ml of 95 wt% ethanol. The liberated fatty acids were titrated with 0.05 M of sodium hydroxide solution in presence of phenolphthalein indicator. In addition, a blank experiment for comparison without adding the immobilized CRL was done by the above assay procedure. One unit of activity (U) was defined as the amount of immobilized CRL (g) which produced 1 μmol of free fatty acids per minute under the standard assay conditions described above. The hydrolytic activity (HA) and specific activity (SA) of the catalyst were calculated according to Eq. (3) and Eq. (4), respectively.

$$\text{HA (U/g-catalyst)} = \frac{(V_2 - V_1) \times 0.05 \times 1000}{m \times 15} \tag{3}$$

$$\text{SA (U/mg-protein)} = \frac{\text{HA}}{1000Y} \tag{4}$$

where V_2 and V_1 are the volumes of aqueous NaOH solution consumed in titration for the hydrolysis reaction and the blank experiments (ml), respectively; 0.05 is the concentration of aqueous NaOH solution used in the titration (M); 15 is the reaction time (min); m is the mass of immobilized CRL after drying (g), i.e., $m=0.001m_1+m_2$; m_1, m_2 and Y are the same as those in Eq. (1) and Eq. (2).

6. Esterification Reaction and Reuse Test

The performance of the prepared biocatalyst was evaluated in esterification of CLA with ethanol using isooctane as a solvent. The esterification reaction was in a 25 ml glass reactor in a shaking water bath at 120 rpm at 45 °C for 8 h. The reaction mixture contained 2.35 mmol of CLA, 4.7 mmol of ethanol and 50 mg of biocatalyst in 2 ml isooctane. The reaction products were collected after centrifugation and analyzed by gas chromatography coupled to mass spectrometry (GC-MS). The products were quantified by a GC equipped with a DB-5ms capillary column and a flame ionization detector. For recycling the biocatalyst, the used biocatalyst was separated from the reaction mixture by centrifugation and washed with a phosphate buffer solution. After drying at 30 °C, the biocatalyst was introduced into a fresh medium for the next run under the same reaction conditions. This process was repeated for four cycles.

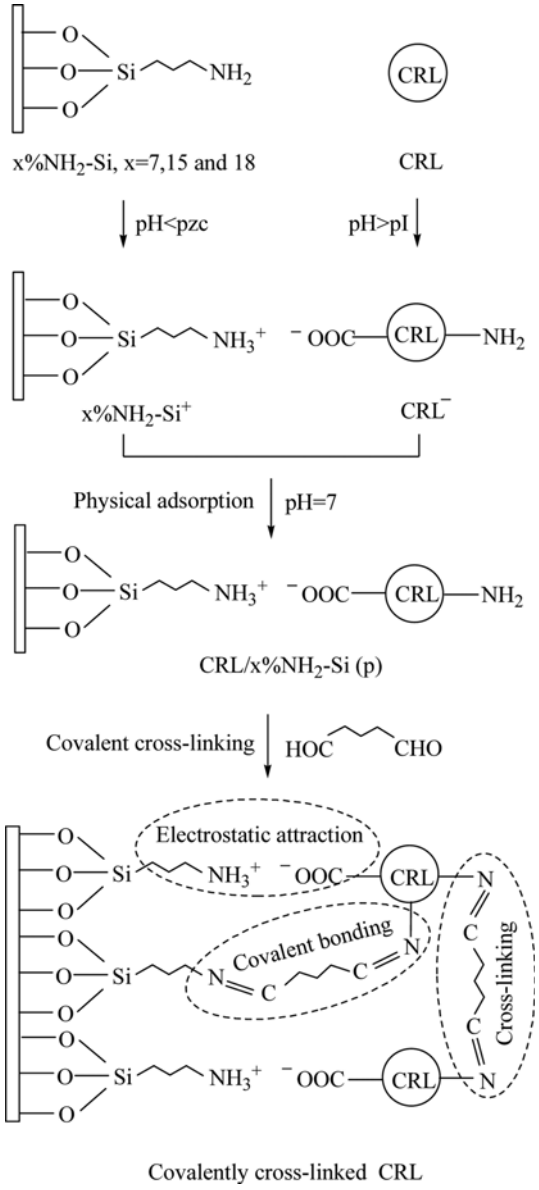
7. Characterization of Support and Immobilized CRL

Powder X-ray diffraction (XRD) patterns were recorded on a Thermo ARL X'TRA powder diffractometer equipped with a rotating anode and Cu K_α radiation (λ=0.1542 nm). The nitrogen adsorption-desorption isotherms were obtained at −196 °C on a Micromeritics ASAP 2020 sorptometer. Prior to analysis, the samples were outgassed at 150 °C and 10^{-6} Torr for a minimum of 12 h. BET surface areas were calculated from the linear part of the adsorption curve. Pore size distributions were calculated by using the desorption branches of the N_2 isotherms and the Barret-Joyner-Halenda (BJH) method. Thermogravimetry was performed on a Shimadzu TG60 from room temperature to 800 °C at a rate of 5 °C/min. FT-IR spectra of the samples were measured by using a Nicolet 6700 FT-IR spectrometer (Thermo-Nicolet, USA) and the KBr-sample pellet was used. The spectra were collected in the spectral range of 400-4,000 cm^{-1} with a resolution of 4 cm^{-1}.

RESULTS AND DISCUSSION

1. Interaction Mechanisms between CRL Molecules and Supports

The immobilized-CRLs were prepared by physical adsorption and covalent cross-linking at pH 7.0, respectively. Scheme 1 displays the interaction mechanisms between CRL and support in preparation of the immobilized-CRLs. Physical adsorption of enzyme into solid support depends on major factors such as the charge feature and hydrophobicity/hydrophilicity and pore size of support, isoelectric point (pI) of enzyme and pH of adsorption solution. The pI of CRL is about 4.5 [18], and the point of zero charge (pzc) of amino-functionalized silica is around 7.9-10.3 [19,20]. Thus, in the buffer used for immobilizing CRL at pH 7.0, the CRL molecule carried a negative net charge due to the presence of COO^- and NH_2 groups, while the AFMS particles carried a positive net charge because the amino groups fixed on the AFMS supports could absorb H^+ ions to form -NH_3^+ groups [21]. Therefore, it could be concluded that physical adsorption of CRL molecules onto the AFMS supports went through mainly via electrostatic attractions. As shown in Scheme 1, in the covalent cross-linking of CRL, there existed covalent bonds besides electrostatic attractions between the AFMS supports and CRL molecules. The -CHO groups of glutaraldehyde



Scheme 1. Interaction mechanisms between of CRL molecules and supports.

reacted with the -NH_2 groups of both of the AFMS supports and CRL molecules through formation of Schiff base (-CH=N-) [22]. Thus, glutaraldehyde not only coupled CRL molecules covalently to the AFMS supports but also made many CRL molecules cross-link together [23]. This multipoint covalent cross-linking inhibited enzyme leaching from the AFMS supports effectively [24]; however, it caused conformational rigidity of enzyme molecules or hindered enzyme active sites, thus hampering their activities [25]. Hence, the optimization of cross-linking step is crucial for immobilizing CRL to avoid insufficient or excessive cross-linking, which causes loss of activity and low operational stability.

2. Characterization of Supports and Immobilized CRL Biocatalysts

XRD patterns of the supports and the immobilized-CRLs are presented in Fig. 1. All the samples exhibited well-defined [100]

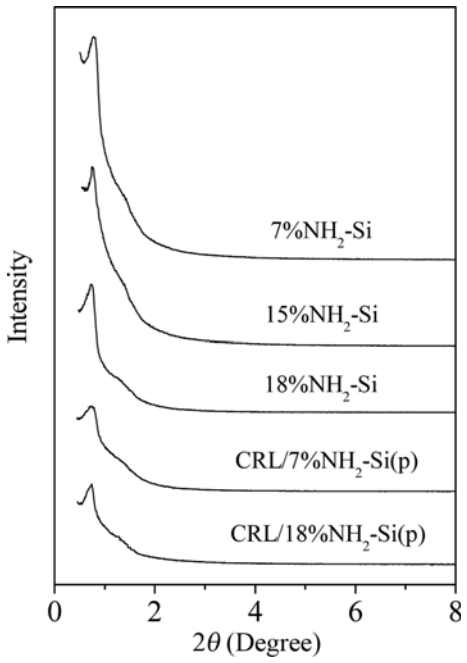


Fig. 1. XRD patterns of support and immobilized CRL.

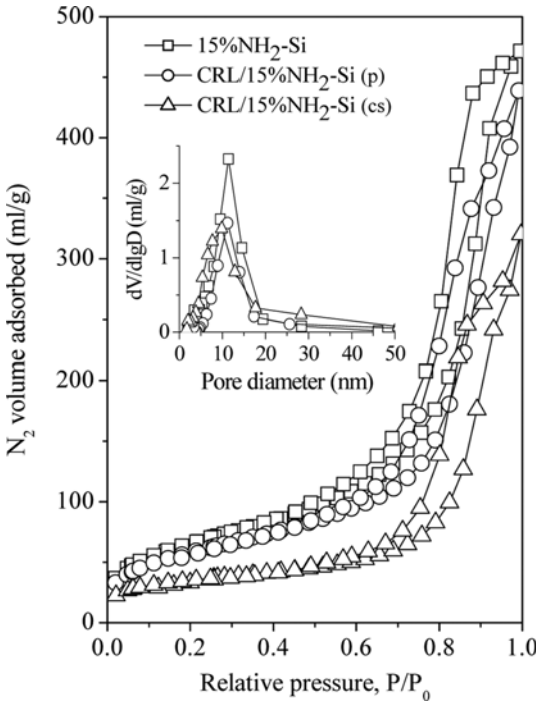


Fig. 2. N_2 adsorption-desorption isotherms and BJH pore size distribution plots.

reflections at 2 theta of 0.6-0.8° characteristic of mesoporous silica materials with highly ordered 2-D hexagonal structure [15]. The intensity of [100] reflections of the AFMS materials decreased slightly with increasing of the molar ratios of APTES to SiO_2 from 7 to 18% in the synthesis gel, while the lattice spacing did not change dramatically. The results were similar to the findings reported by Rosenholm and Lindén [20] and Chong and Zhao [26], which occurred on the amino-functionalized SBA-15 silicas prepared at strong acid conditions when the molar ratios of APTES to SiO_2 increased from 11 to 25% and from 5 to 17%, respectively. This means that the increase of APTES content in the synthesis gel led to the reducing of long-range order of mesostructure. The phenomenon was explained by postulating that APTES would perturb the self-assembly of surfactant micelles and the silica precursor [27]. Compared to the supports, the [100] reflections of the immobilized-CRLs shifted to the lower angles and their intensity reduced, probably because of loss of the small partial mesostructure in CRL adsorption process [28].

N_2 adsorption-desorption isotherms and BJH pore size distributions of the support 15%NH_2-Si and its corresponding immobilized CRL are illustrated in Fig. 2. The isotherms, which could be classified as type IV, exhibit clear hysteresis loops of H1-type [29]. The results revealed that they all had distinct mesoporosity. Table 1 summarizes the textural properties of the immobilized CRLs on 15%NH_2-Si and the parent. The adsorption of CRL molecules onto 15%NH_2-Si caused the decreasing of BET surface area and pore volume of the resultant catalysts, which could be ascribed to the fact that CRL molecules occupied pore channels or blocked pore openings [30,31]. After 15%NH_2-Si adsorbed CRL physically, the pore volume decreased from 0.73 to 0.68 cm^3/g and the decrease value was about the same volume of CRL adsorption. CRL is a globular protein with a molecular weight of 45-60 kDa and a molecular volume of 5 nm×4.2 nm×3.3 nm [12]. The BJH pore size of 15%NH_2-Si was about 11.3 nm, which is bigger than the molecular dimension of CRL. It was possible for CRL molecules to be adsorbed into the pore of 15%NH_2-Si. Similar results were reported by Zhang et al. [31] that the pore volume of the amino-functionalized mesostructured cellular silica foam decreased from 1.38 to 1.32 cm^3/g after adsorption of enzyme, and the decrease value was also the same as the volume of enzyme adsorption. The findings revealed the possible fact that the enzyme molecules entered the mesoporous channels not on the external surface of the support [31].

Table 1. Textural properties of 15%NH_2-Si and the immobilized CRLs

Sample	BJH pore size (nm)	Surface area (m^2/g)	Pore volume (ml/g)
15%NH_2-Si	11.3	240.0	0.73
CRL/15%NH_2-Si (p)	11.1	212.5	0.68
CRL/15%NH_2-Si (cs)	9.8	119.0	0.45

The introduction of CRL molecules and aminopropyl chains to the silica framework can be qualitatively confirmed by the FT-IR spectra (Fig. 3). The bands of crude CRL are centered at *ca.* 1,540 cm^{-1}, which was ascribed to symmetrical -NH_3^+ bending vibration indicative of existence of amino groups and at *ca.* 1,650 cm^{-1} characteristic of C=O stretching vibration of enzyme molecule [32,33].

Compared to the pristine silica, the 7%NH_2-Si, 15%NH_2-Si and 18%NH_2-Si supports exhibited two new peaks at around 676 and 1,540 cm^{-1}. The weak peak at 676 cm^{-1} was attributed to N-H bending vibration [34], and its intensity increased slightly with an increase in the molar ratio of APTES to SiO_2 from 7 to 15% used in

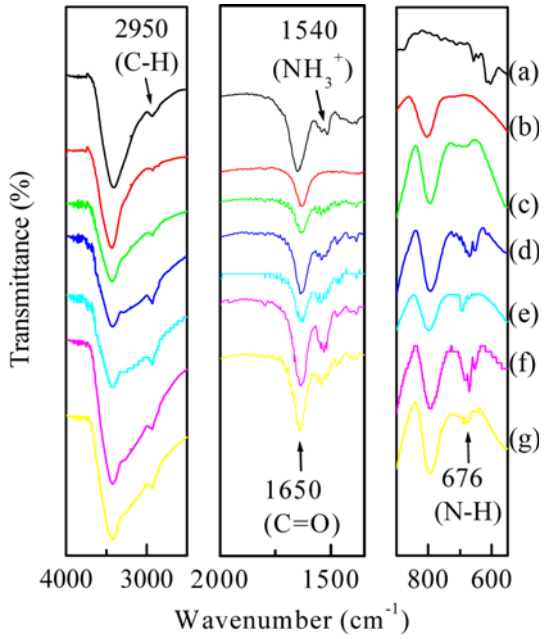


Fig. 3. FT-IR spectra of free CRL, supports and immobilized CRLs. (a) CRL. (b) Pristine silica. (c) 7%NH_2-Si. (d) 15%NH_2-Si. (e) 18%NH_2-Si. (f) CRL/15%NH_2-Si(p). (g) CRL/15%NH_2-Si(cs).

the initial reaction mixture for preparation and then decreased when it increased up to 18%. The results suggested that amino groups had been modified onto the AFMS supports and 15%NH_2-Si loaded more amino groups than 7%NH_2-Si. Compared to 7%NH_2-Si, 15%NH_2-Si and 18%NH_2-Si have similar stronger intensity of the symmetrical -NH_3^+ bending at 1,540 cm^{-1}. The results indicated that the content of -NH_2 groups loaded on 15%NH_2-Si and 18%NH_2-Si reached nearly saturation. The presence of organosilane on the supports was identified by the absorption band at *ca.* 2,950 cm^{-1} for C-H stretching vibrations of the propyl chains from APTES [35]. Moreover, the bands in the region of *ca.* 3,305-3,500 cm^{-1} for the pristine silica were caused by the adsorbed or hydrogen-bonded water molecules [22]. For the APTES-functionalized silicas, the peaks at *ca.* 3,305-3,500 cm^{-1} broadened due to the effect of symmetric N-H stretching [26]. The results confirmed further that aminopropyl chains were introduced onto the framework of the silica supports by co-condensation.

CRL/15%NH_2-Si(p) exhibited stronger peaks at *ca.* 676, 1,540 and 1,650 cm^{-1} in comparison of the parent 15%NH_2-Si. These bands indicated the presence of CRL on the 15%NH_2-Si support by physical adsorption. For CRL/15%NH_2-Si(cs), the absorption bands at *ca.* 676 and 1,540 cm^{-1} weakened because the reaction of glutaraldehyde with amino groups of CRL and 15%NH_2-Si caused the decreasing of amount of amino groups on CRL/15%NH_2-Si(cs). The results suggested that CRL molecules were successfully immobilized on the 15%NH_2-Si through the covalent cross-linking with glutaraldehyde.

3. Hydrolytic Activity and CRL Loading Amount

The hydrolytic activity and the amount of CRL immobilized onto the supports are listed in Table 2. Hydrolytic activity characterizes potential of the prepared biocatalysts. Among the immobilized CRLs which were prepared by physical adsorption, CRL/15%NH_2-Si(p) afforded the highest 2496.6 U/g-catalyst of activity and 75.1 U/mg-protein of specific activity. Therefore, the molar ratio of APTES to SiO_2 at 15% in the initial preparation reaction mixture was proper for obtaining high activity and specific activity of the immobilized CRL. It might be explained by the hydrophilic/hydrophobic balance of the surface functional aminopropyl chains in the nanopores of the support [34,36].

The increasing of the molar ratios of APTES to SiO_2 used in the synthesis gel from 7% to 15% caused an increase in loading amount of CRL. The pzc value of aminopropyl-functionalized silica was in the range of 7.9-10.3, and it increased with the increasing of APTES content in the synthesis gel [19,20]. Thus, 15%NH_2-Si has probably higher net positive charges than 7%NH_2-Si in the buffer used for immobilizing CRL at pH 7.0, which led to stronger electrostatic attractions between the high positively charged silica particles of the 15%NH_2-Si sample and the negatively charged CRL molecules. The strong electrostatic attractions enhanced the amount of CRL adsorbed on the 15%NH_2-Si support [25]. Moreover, the amount of CRL adsorbed on 15%NH_2-Si was close to that on 18%NH_2-Si probably because they loaded the close amounts of amino groups as confirmed by the IR spectra above thereafter close electrostatic forces.

In preparation of the covalently cross-linked CRLs, cross-linker amount and cross-linking time are crucial for obtaining high activity and operational stability. CRL/15%NH_2-Si(c) showed lower activity hydrolytic activity and specific activity than CRL/15%NH_2-Si(cs) presumably due to the insufficient cross-linker amount and the long cross-linking time. Different cross-linking time and glutaraldehyde concentration were tested in preparing the covalently cross-linked CRLs. The results indicated that 0.75 wt% of glutaraldehyde concentration and 45 min of cross-linking time were suitable for immobilizing CRL with less loss of the enzyme activity.

Table 2. The activity and the loading amount of CRL immobilized on the supports

Sample	Hydrolytic activity (U/g-catalyst)	CRL loading amount (mg/g-support)	Specific activity (U/mg-protein)
CRL/7%NH_2-Si (p)	1966.7	30.9	65.6
CRL/15%NH_2-Si (p)	2496.6	34.4	75.1
CRL/18%NH_2-Si (p)	2050.8	35.0	60.6
CRL/MSU-H (c)	1000.0	41.3	25.2
CRL/15%NH_2-Si (c)	1983.3	34.1	60.1
CRL/15%NH_2-Si (cs)	2471.5	34.3	74.5

Table 3. Catalytic activity of the immobilized CRLs in esterification of CLA with ethanol

Sample	THA[a] (U)	TE[b] (mol%)	*c*9, *t*11-CLA[c] (mol%)	*t*10, *c*12-CLA[c] (mol%)	Esterification ratio[d]
CRL/7%NH_2-Si (p)	98.3	20.2	39.1	11.6	3.4
CRL/15%NH_2-Si (p)	124.8	27.8	55.5	17.9	3.1
CRL/18%NH_2-Si (p)	102.5	25.6	49.3	17.5	2.8
CRL/MSU-H (c)	50.0	20.7	42.0	11.2	3.8
CRL/15%NH_2-Si (c)	99.2	24.1	48.0	14.7	3.3
CRL/15%NH2-Si (cs)	123.6	27.5	58.4	16.1	3.4

[a]THA was total hydrolytic activity which was added in CLA esterification reaction system and measured using olive oil emulsion
[b]TE was total esterification, i.e., the esterification degree of total CLA fatty acids
[c]*c*9, *t*11- and *t*10, *c*12-CLA were the esterification degrees of *c*9, *t*11- and *t*10, *c*12-CLA, respectively
[d]Esterification ratio was the ratio of esterification degree of *c*9, *t*11- to *t*10, *c*12-CLA
Reaction conditions: CLA, 2.35 mmol; ethanol, 4.70 mmol; biocatalyst, 50 mg; isooctane, 2.0 ml; reaction time, 8 h; temperature 45 °C; shaking rate, 120 rpm

CRL/15%NH_2-Si (cs) exhibited much higher hydrolytic activity and specific activity than CRL/MSU-H(c). The results indicated that the aminopropyl groups on the surface of the 15%NH_2-Si could activate the catalytic center of CRL. Similar results were reported by Xu et al. [32] and Wang et al. [37].

4. Synthetic Activity of Immobilized CRL in Esterification

Table 3 summarizes the esterification degrees of total fatty acids and *c*9, *t*11- and 10*t*, 12*c*-CLA isomers. The immobilized CRLs gave 20.2-27.8% of total esterification, suggesting they were efficient for the conversion of CLA to CLAEE in isooctane. CRL/MSU-H(c) afforded lower total esterification than the CRLs immobilized on 15%NH_2-Si and 18%NH_2-Si. This indicated that the aminopropyl chains on the AFMS supports had better interaction with the hydrophilic/hydrophobic sites on the lipase molecules [34]. Total esterification over the physically adsorbed CRLs increased with the increasing of total activity added in the reaction mixture. CRL/15%NH_2-Si(p) afforded 27.8% of total esterification, which was higher than CRL/7%NH_2-Si(p) and CRL/18%NH_2-Si(p). It could be probably due to their difference in the physical structures and the surface hydrophilic/hydrophobic properties, which were caused by the different molar ratios of APTES to SiO_2 used in synthesis gel [38]. In addition, the synthetic activity of CRL/15%NH_2-Si(cs) was higher than CRL/15%NH_2-Si(c) according to their total esterification degrees. The optimization of cross-linking step in preparation of CRL/15%NH_2-Si(cs) obviously played a crucial role in enhancing the enzymatic synthetic activity.

From the results in Table 3 note that esterification of *c*9, *t*11-CLA over the immobilized CRLs was 2.8-3.8 times faster than that of *t*10, *c*12-CLA, suggesting that the immobilized CRLs were very effective on esterification of *c*9, *t*11-CLA to its ester. The result was consistent with the findings reported by Wang et al. [9]. Based on this property, the immobilized-CRLs can be used in separation of two main isomers with different functions in a mixture of CLA.

5. Operational Stability of Immobilized CRL in Esterification

For immobilized enzyme, the future application in industry depends on operational stability because of inevitable inactivation in inadequate environment. The immobilized CRLs were reused four times in the esterification reaction (Fig. 4). The total esterification over the immobilized CRLs reduced during four cycles in the successive 32 h esterification reactions. CRL/15%NH_2-Si(p) inactivated almost after four cycles. This indicated that only the electrostatic attractions between CRL and 15%NH_2-Si support were weak. Hence, for the physically adsorbed CRL, APTES functionalities did not show great improvement in the operational stability. The result was in agreement with the report [39]. CRL/15%NH_2-Si(cs) maintained 23.9-27.5% of total esterification in four cycles of reuse without significant loss of activity. This result indicated the possibility to reuse CRL immobilized on the 15%NH_2-Si by optimization of covalent cross-linking process with proper cross-linking time and glutaraldehyde concentration. The proper cross-linking time and glutaraldehyde concentration resulted in not only cross-linking among CRL molecules but also covalent binding of CRL molecules onto the support. As a result, CRL leaching from the support was minimized. CRL/15%NH_2-Si(c) was less stable than CRL/15%NH_2-Si(cs), presumably because of insufficient cross-linking. CRL/MSU-H(c) inactivated more easily than CRL/15%NH_2-Si(c) and CRL/15%NH_2-Si(cs). The reason might be that the hydrogen bonds between CRL

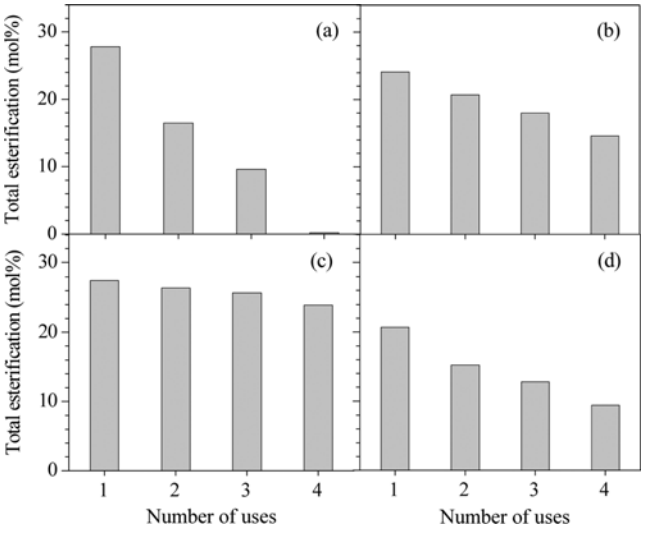


Fig. 4. Operational stability of the immobilized CRLs in esterification of CLA with ethanol. (a) CRL/15%NH_2-Si(p). (b) CRL/15%NH_2-Si(c). (c) CRL/15%NH_2-Si(cs). (d) CRL/MSU-H(c).

molecules and silica particles of the pristine MSU-H silica were weaker than the covalent bonds and the electrostatic attractions between CRL molecules and the AFMS supports.

CONCLUSIONS

The AFMS supports with APTES/SiO_2 molar ratios at 7, 15 and 18% used in synthesis gel were successfully prepared by co-condensation under a neutral pH condition, and can be used as support for immobilization of soluble CRL by physical adsorption and covalent cross-linking method. APTES/SiO_2 molar ratio at 15% used in synthesis gel was in favor of adsorbing CRL molecules onto the support. The multipoint covalent cross-linking of CRL onto the AFMS support by proper cross-linking process achieved high activity and improved operational performance in the esterification of CLA with ethanol in isooctane. Moreover, the immobilized CRLs catalyzed 2.8-3.8 times of esterification of *cis*-(*c*)9, *trans*-(*t*)11-CLA faster than that of *t*10, *c*12-CLA. These findings provide an eco-friendly catalytic process to produce CLAEE and a viable alternative to separating two isomers of *c*9, *t*11- and *t*10, *c*12-CLA with different beneficial bioactivities from CLA mixture.

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