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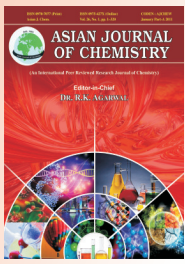

Immobilization of α-Amylase by SBA-15

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SBA (Santa Barbara Amorphous)-15 mesoporous material was prepared by hydrothermal synthesis and the sample was characterized by X-ray diffraction and scanning electron microscopy. The immobilization of α-amylase by SBA-15 was made by physical adsorption method and the optimal conditions of immobilization of α-amylase by SBA-15 were obtained as follows: pH = 5.4, α-amylase concentration:2 mg/mL, immobilization time:27 h, temperature:30 °C. At the same time, it is concluded that the maximum amount of α-amylase immobilized by SBA-15 was 87.5 mg/g. By studying of chemical analysis, scanning electron microscopy, infrared spectroscopy, X-ray diffraction, low temperature N_2 adsorption-desorption analysis characterization, the α-amylase was immobilized on the SBA-15 carrier was proved. Catalytic experimental results indicated that (SBA-15)-(α-amylase) composite material has the advantages of high pH value resistance and high temperature resistance compared with the free enzyme and has a higher potential applied value.

Keywords: α-Amylase, SBA-15, Immobilization.

INTRODUCTION

In 1998, the scientists of the University of California of America, Professor Stucky *et al.*[1,2] used tri-block non-ionic surfactant to synthesize a new type silica base mesoporous SBA (Santa Barbara Amorphous)-$_n$ systematic materials, among which especially the SBA-15 mesoporous materials most attract people's attention. The mesoporous material not only has the advantages of large specific surface area, narrower pore size distribution, tunable pore size, high mechanical strength, good hydrothermal stability and higher thermal stability than 900 °C, but also has long use life and non-toxic to biology. Enzyme is a protein that has activity and it has very important applied value in biology, food and chemical engineering fields, *etc*, as biological catalyst. Unfortunately, many of free enzymes have the disadvantages that the enzymes are easily dissolved in water, the stability is poor, enzyme purification is difficult after reaction and the recovery is difficult and repetition cycle use is not easy, *etc*. These disadvantages often made limited free enzymes application. Through the physical/chemical methods the immobilization of enzyme intrigued scientists.

As the immobilized enzyme has better stability and higher use efficiency than the free enzyme, thus the immobilized enzyme is widely used in industrial production. Mesoporous materials have larger specific surface area, pore volume, uniform pore size, stronger adsorption performance, surface easy functionalization and can be adjusted in nanometer size, thus they can become good carriers for assembly of the enzyme protein[3]. In the limited space, immobilization of protease enzyme can reduce the self decomposition of enzyme, can also reduce protein enzyme aggregation, can improve the separation performance of the enzyme molecules adsorbed on the molecular sieve. Under usual situations, enzyme immobilization can improve the stability of the immobilized enzyme and the immobilized enzyme obtained will have good application prospect in the catalysis, biosensors[3,4]. In 1996, Balkus *et al.*[5] first explored nanoporous silica based mesoporous material for enzyme immobilization, finding that after the enzyme was encapsulated in mesoporous material channels, the activity of enzyme was still retained. Yiu *et al.*[6] studied the immobilization of insulin in MCM-41. Gustafsson *et al.*[7] studied the effect of isoelectric points of lipases from *Mucor miehei* and *Rhizopus orzae* on immobilization efficiencies by mesoporous materials. Laszloa *et al.*[8] utilized SBA-15 to immobilize *Candida antarctica* lipase for the hydrolysis of butyrin and found that SBA-15 can efficiently keep lipase's active conformation not be destroyed. Shakeri and Kawakami[9] studied the immobilization of *Rhizopus oryzae* lipase by SBA-15 and found that the immobilized enzyme can improve lipase-catalyzed *trans*-esterification reaction production butyric acid glyceride vitality.

Amylase is an enzyme widely existing in natural world and can be divided into α-amylase and β-amylase. α-Amylase exists in saliva, pancreas, barley and many microorganism

bodies. The use of amylase is extensive. The amylase from the animal and microorganism can be used as digestive medicine component in medicine industry. In the food industry, amylase is used in the locality where starch needs saccharification. In the candy industry, amylase can be used to manufacture syrup. In the textile industry, amylase can be used to make a slurry containing starch and it can also be used to remove the pulp layer containing starch. This paper uses SBA-15 as host material and studied immobilization α-amylase optimum conditions and characterized the prepared composite material using a series of characterization techniques.

EXPERIMENTAL

Reagents for the preparation of SBA-15: Tetraethyl-orthoxysilicate (TEOS) and hydrochloric acid were purchased from Shanghai Chemical Reagent Company, Chinese Pharmaceutical Group. Poly (ethylene glycol)-block-poly-(propylene)-block-poly (ethylene glycol) (average molecular weight 5800) was obtained from Sigma-Aldrich, USA.

α-Amylase was the product of Sinopharm Chemical Reagent Co., Ltd., China.

Experimental reagents for α-amylase enzymatic activity: Indicator solution:1 g of 3,5-dinitro salicylic acid ($C_7H_4N_2O_7$) was taken and mixed with a little distilled water. 20 mL of 2 mol/L sodium hydroxide solution were added for dissolution. 30 g of tetrahydrated sodium potassium tartrate was taken, dissolved in the solution and diluted to 100 mL by distilled water. The indicator solution was kept at a refrigerator at 4 °C and could be used for 4 weeks. Substrate solution:1 g of soluble starch and 0.039 g sodium chloride were weighed and dissolved in a 100 mL of pH 6.9 KH_2PO_4-Na_2HPO_4 buffer solution. Maltose($C_{12}H_{22}O_{11}·H_2O$) solution:1 mg/mL. The above used reagents were purchased from Sinopharm Chemical Reagent Co., Ltd., China.

Unless otherwise specified, all reagents were of analytical grade. Deionized water was used throughout all the experiments.

Synthesis of material

Synthesis of SBA-15: SBA-15 mesoporous material was synthesized using hydrothermal method according to the procedure[10]. 2 g of tri-block copolymer poly(ethylene glycol)-block-poly(propylene glycol)-block-poly(ethylene glycol) was dissolved in 15 g of deionized water and 60 g of 2 mol/L hydrochloric acid solution, stirred up to complete dissolution. At 40 °C 4.25 g of TEOS was added and stirred for 24 h and then transferred into autoclave. At 100 °C, crystallization was made for 48 h at the constant temperature. After the crystallization finished, filtration was made and the product was washed using deionized water. The product was dried at room temperature. The above product was placed in a ceramic crucible and put into a muffle oven and calcined at 550 °C for 24 h to eliminate the tri-block copolymer template. A mesoporous SBA-15 molecular sieve white powder was obtained.

Immobilization of α-amylase on SBA-15: In a 25 mL beaker, 0.20 g of calcined SBA-15, 5 mL of 2 mg/mL α-amylase solution, 5 mL of pH = 5.4 CH_3COOH-CH_3COONa buffer solution were in turn added. The mixture was shaken for adsorption for 27 h at a room temperature of 30 °C. The mixed solution was centrifugated at a speed of 6000 r/min. The solid was washed with water and the supernatant clear solution was retained. The operation was repeated until α-amylase could not be detected in the supernatant clear solution. And the supernatant clear solution and solid material were obtained and the supernatant clear solutions were merged. The solid substance was dried at room temperature and a required composite material was obtained. The sample was marked as (SBA-15)-(α-amylase).

Calculative method for the immobilization amount of α-amylase: Determination method for α-amylase used dibromo-*p*-chloro-chlorophosphonazo spectrophotometry[11]. The pepsin amount adsorbed in SBA-15 is equals to the total amount of α-amylase minus the α-amylase amount in supernatant, which can be calculated by the following formula:

$$Q_t = (C_i - C_t) \times V/W \quad (1)$$

where Q_t is the amount of immobilized α-amylase on SBA-15 at the time of *t* (mg/g, mg enzyme per gram support); C_i and C_t are the concentration of α-amylase in the solution (mg/mL) at the initial time and at the time of *t*, respectively; V is the volume of the solution (mL); W is the weight of the SBA-15 carrier (g).

Determination of enzymatic activity: The reduction materials are decomposed from starch by amylase. These reduction materials can be determined from their reduction ability with 3,5-dinitrosalicylic acid and calculation is made by monohydrate maltose. One amylase unit corresponds to the amount of enzyme required at the time of releasing 1 mg of reduction carbohydrate (by monohydrate maltose calculation). 3,5-dinitrosalicylic acid can be used to determine the reduction ability of the decomposed carbohydrate[12].

A 1 mL of 10 mg/mL soluble starch substrate solution was transferred into a 25 mL test tube by using suction pipette, which was heated to 25 °C. 1 mL of the enzyme, which was also preheated to 25 °C, was added, shaken well. After heat preservation for 3 min, 2 mL of 3,5-dinitrosalicylic acid indicator solution was added to terminate the reaction. This solution was placed in a boiling water bath for heating for 5 min, taken out and placed in running water for rapid cooling. Subsequently, deionized water was used to dilute to a constant volume of 20 mL. By using a spectrophotometer at 490 nm with 1 cm cells absorbance was determined against blank reagent[12].

For blank value test procedure, only one point was different. 3,5-Dinitrosalicylic acid indicator solution was firstly added and then the enzyme solution was added.

By formula (3) the results of enzymatic activity can be obtained:

$$1.428 \times A_{490\,nm} = \text{Maltose (mg)/test solution} \quad (2)$$

$$[\text{Maltose (mg)/test solution}]/E_w = U/g \quad (3)$$

In the formula:

E_w -content containing enzyme in a mL enzyme solution used (g); $A_{490\,nm}$ - absorbance value at 490 nm.

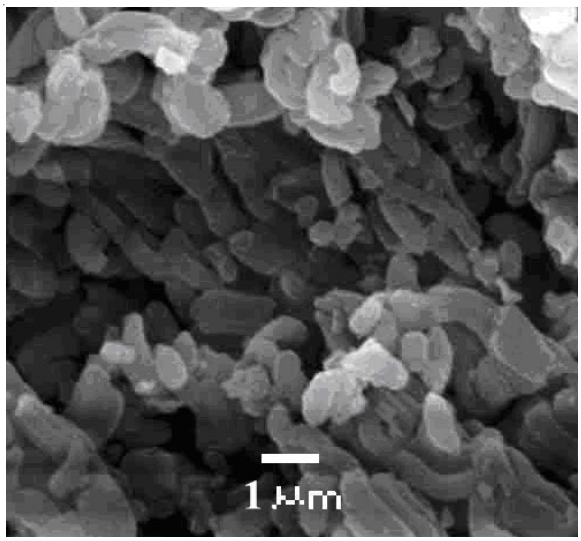
A XL 30 ESEM FEG (American FEI Company) scanning electron microscope was used to characterize the surface topography and particle size of sample with an accelerating voltage of 20 kV. A Vertex-70 (Bruker Company, Germany) spectrometer was employed to measure Fourier transform infrared (FT-IR) spectra to analyze the characteristic groups of sample using KBr compression slice technique. A D8/

ADVANCE (Bruker, Germany) X-ray diffractometer (XRD) was adopted to analyze the phase structure of sample using CuK_α (λ = 1.5406Å) ray for 2θ ranging from 0.6° to 80° with a scan speed of 2.0°/min at 40 kV and 30 mA. Small-angle and wide-angle XRD patterns were, respectively recorded from 0.6° to 10° of 2θ, from 10° to 80°. An ASAP 2020 surface and porosity analyzer (Micromeritics Instrument Corporation, USA) was used to determine the N_2 adsorption-desorption isotherm curve of sample at 77 K. By means of BET (Brunauer-Emmett-Teller) method[13] and BJH (Barrett-Joyner-Halenda) method[14], the specific surface area and average pore size of sample were calculate, respectively. α-Amylase component content determination in (SBA-15)-(α-amylase) was obtained by the spectrophotometry[11] with subtraction method with a 722S type spectrophotometer (Shanghai Lengguang Technology Co., Ltd., China).

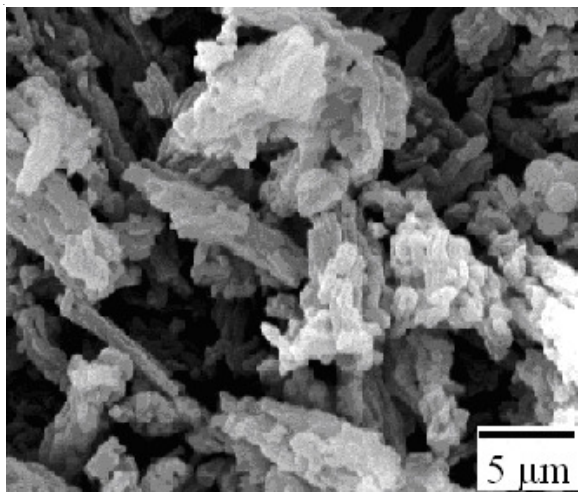
RESULTS AND DISCUSSION

Preparation conditions of (SBA-15)-(α-amylase) composite material and immobilization amount of enzyme: Influence of between pH 3.5-6.9 was studied on SBA-15 immobilization α-amylase for preparation of (SBA-15)-(α-amylase) composite material. The results showed that when the pH value was in range 3.5-5.4, the adsorption amount gradually increased with the increase of pH value. When the pH value was between 5.4 and 6.9, the adsorption quantity decreased gradually with the increase of pH value. When the pH value was equals to 5.4, the maximum adsorption amount appeared. This result is consistent with the isoelectric point of α-amylase and at this time the electrostatic repulsive force existed between SBA 15 surface and the α-amylase was the smallest. This adsorptive process was completed by means of the electrostatic force of the SBA 15 and α-amylase, hydrogen bonding and hydrophobic effect. Adsorptive effect results of different initial concentration α-amylase on the SBA-15 amount showed that when the initial concentration of α-amylase was 0-2 mg/mL, with the increase in the concentration of α-amylase its adsorptive amount gradually increased. Over 2-5 mg/mL, the adsorption achieved equilibrium. After it, the adsorptive amount declined. 2 mg/mL was selected. When the adsorption of α-amylase on SBA-15 was over 0-27 h, the adsorption amount increased as the time increased. At 27 h, the adsorption achieved equilibrium. The experimental results of temperature on adsorptive effect revealed that with the increase in temperature (4-40 °C) the adsorptive amount of α-amylase on SBA-15 gradually increased, indicating that the adsorption reaction was an endothermic process. For operation convenience, the study was made at room temperature (30 ± 2 °C). The optimal conditions of immobilization of α-amylase by SBA-15 were obtained in this paper as follows:pH = 5.4, α-amylase concentration:2 mg/mL, immobilization time: 27 h, temperature:30 °C. At the same time, it is concluded that the maximum amount of α-amylase immobilized by SBA-15 was 87.5 mg/g.

SEM: By using scanning electron microscope, the SBA-15 and (SBA-15)-(α-amylase) samples were analyzed. From the results it can be known (Fig. 1) that the SBA-15 and (SBA-15)-(α-amylase) presented orderly fibrous state and their average particle diameter was 330 ± 10, 335 ± 10 nm, respectively. A slight increase in (SBA-15)-(α-amylase) particle average diameter is the result that the α-amylase was introduced into the host material.



(a)



(b)

Fig. 1. Morphological images of samples: (A):SBA-15;(B):(SBA-15)-(α-amylase)

FT-IR spectra: Fig. 2 is infrared spectra of SBA-15, (SBA-15)-(α-amylase) and α-amylase. Compared composite material with SBA-15, it can be seen that during the immobilization of α-amylase by SBA-15 the framework structure of host pore channels did not subside due to loading of α-amylase and it was still maintained. This shows that destructive action of α-amylase guest material was very small to framework of SBA-15 during the process of immobilization. It can be seen from infrared spectra of each sample that for SBA-15 sample at 464 cm^{-1} there is an absorption peak and for composite material sample at 466 cm^{-1} there is an absorption peak. They can be assigned to the absorption peak caused by T-O bend vibration. The absorption peak located at 801 cm^{-1} for SBA-15 sample and the absorption peak located at 798 cm^{-1} for (SBA-15)-(α-amylase) sample can be assigned to the absorption peaks caused by TO_4 symmetrical expansion vibration of

Si-O-Si. The absorption peaks located at 965 and 1084 cm^{-1} for SBA-15 sample and the absorption peaks located at 968, 1086 cm^{-1} for(SBA-15)-(α-amylase) sample can be assigned to the absorption peak caused by TO_4 asymmetrical expansion vibration of Si-O-Si. The presence of these absorption peaks in the composite material can fully prove the existence of SBA-15 framework. After immobilization of α-amylase by SBA-15, the characteristic peaks of SBA-15 emerged for the (SBA-15)-(α-amylase), showing that integrity of the framework of SBA-15 in the composite material was retained. The 1400,1630 cm^{-1} in SBA-15 and 1400,1636 cm^{-1} in composite material are the flexural vibrations of H-O-H groups adsorbed onto the surface and channels of SBA-15 silica material. The 3444 cm^{-1} in SBA-15 and 3443 cm^{-1} in composite material are assigned to a non-symmetric stretching vibration mode resulting from the Si-OH groups observed from the surface of SBA-15. This vibration may also be assigned to the vibrational modes of Si-OH unreacted hydroxyl groups on the SBA-15 silica surface or may be assigned to isolated and geminal hydroxyl groups, respectively. The characteristic peaks 2359, 2928 cm^{-1} of α-amylase appeared in (SBA-15)-(α-amylase) composite material, indicating that α-amylase has already entered into SBA-15 host material.

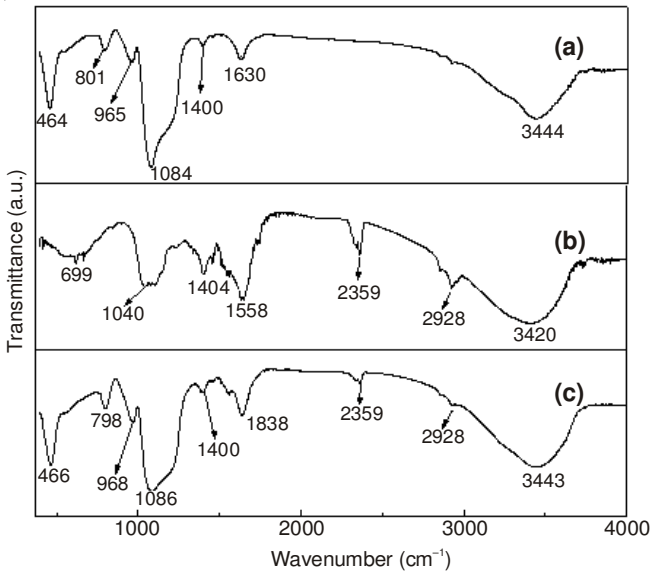


Fig. 2. Infrared spectra of samples: (a) SBA-15; (b) α-Amylase; (c) (SBA-15)-(α-amylase)

Powder XRD analysis: The XRD results of samples are shown in Fig. 3. From Fig. 3a it can be seen that α-amylase is an amorphous phase and no characteristic peaks appeared. Fig. 3b, over the low angle region, displays three clear visible peaks, which respectively represent the characteristic diffraction peak (100) of mesoporous SBA-15 and the hexagonal symmetric structure (110, 200) peaks, showing that the synthesized SBA-15 had long-range order[1,2,10]. In contrast to the SBA-15 sample, (SBA-15)-(α-amylase) sample showed (Fig. 3c) the (100), (110), (200) characteristic peaks of the host, indicating that after SBA-15 immobilized α-amylase, its framework structure still existed and retained a highly order and the framework integrity of the host SBA-15 was not effected. However, the diffraction peak intensity decreased slightly after α-amylase immobilization, suggesting that the immobilization of enzyme slightly had an effect on the ordered nature of host material and the result can be attributed to larger contrast in density between the silica wall and the empty pore than that between the silica walls and the pores filled with enzyme molecules. The calculated results of interplanar spacing (d_{100}) and unit cell parameter (a_0) for the samples are listed in Table-1.

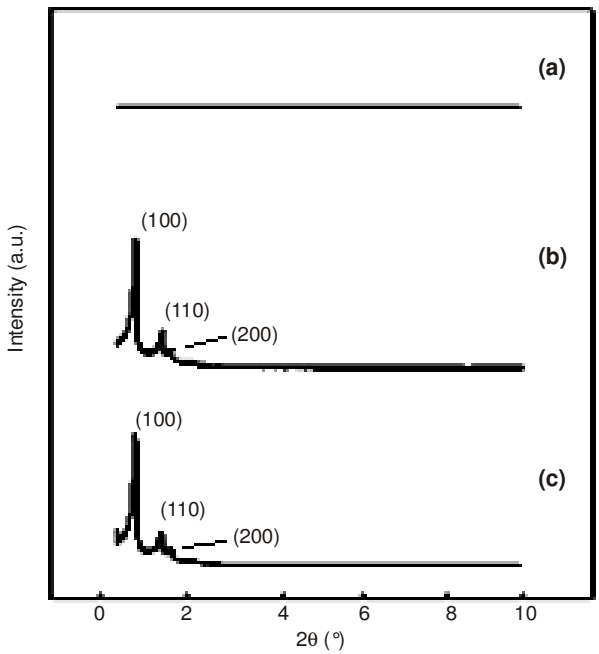


Fig. 3. Small angle XRD pattern of sample:(a) α-Amylase; (b) SBA-15; (c) (SBA-15)-(α-amylase).

Porosity and surface area: The low temperature nitrogen gas absorption-desorption isotherms of SBA-15 and (SBA-15)-(α-amylase) composite material at 77 K is revealed in Fig. 4. Isotherms of SBA-15 and (SBA-15)-(α-amylase) present typical irreversible Langmuir IV type and belong to the typical adsorption type of mesoporous substance. This indicates that after α-amylase was immobilized in SBA-15, this kind of mesoposous channel structure characteristic still existed. That is to say that the introduction of α-amylase did not destroy the SBA-15 mesoporous structure but its surface area was lowered. Analysis of the mesoporous diameter distribution curve of SBA-15 and (SBA-15)-(α-amylase) showed that both have narrower mesoporous diameter distribution, but the composite material pore diameter decreased a little. The nitrogen gas adsorption-desorption results of SBA-15 and (SBA-15)-(α-amylase) and the XRD results are summarized in Table-1. From these data it can be seen that compared with SBA-15 host due to the immobilization of α-amylase, the BET specific surface area, average pore diameter and mesoporous volume of (SBA-15)-(α-amylase) were diminished.

Enzymatic activity: The experimental results indicated that for the α-amylase at pH = 5.5 enzymatic activity reached maximum value, while for the (SAB-15)-(α-amylase) the maximum value of enzymatic activity appeared at pH = 6.4 and relative to the free enzyme the enzymatic activity increased by 22.4 %. The enzymatic activity of post-immobilization enzyme appeared in the higher pH, this is due to that SBA-15

TABLE-1
PORE STRUCTURE PARAMETERS OF SAMPLES

Sample	Interplanar spacing, d_{100} (nm)	Unit cell parameter, a_0[a] (nm)	BET specific surface area (m^2/g)	Pore volume[b] (cm^3/g)	Pore size[c] (nm)	Pore wall thickness[d] (nm)	Immobilized amount of α-amylase (α-Amylase/SBA-15) (mg/g)
SBA-15	10.50	12.12	594.7	1.059	8.14	3.98	0
Composite material (SBA-15)-(α- amylase)	10.45	11.59	453.4	0.854	7.69	3.90	87.5

(a)-Unit cell parameter, $a_0 = \frac{2}{\sqrt{3}} d_{100}$; (b) -BJH adsorption cumulative volume of pores; (c) -Pore size calculated from the adsorption branch; (d) -Wall thickness calculated by (a_0-pore size)

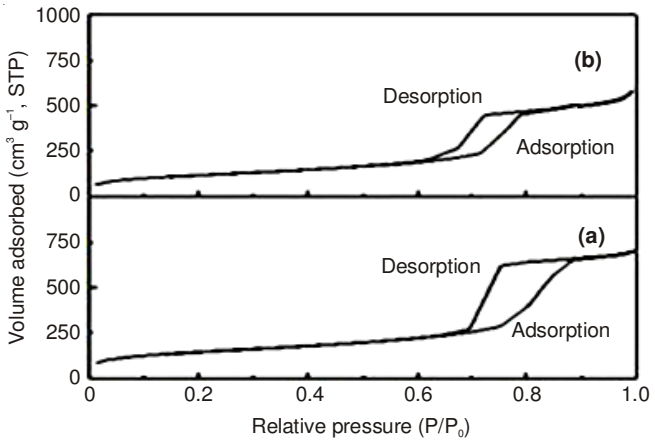


Fig. 4. Low temperature nitrogen adsorption-desorption isotherm of sample:(a) SBA-15; (b) (SBA-15)-(α-amylase)

surface hydroxyl and α-amylase surface hydrophobic layer combine to make the internal active point of α-amylase get the full contact with the substrate, which improved the activity of the enzyme. The shielding effect of mesoporous SBA-15 structure made the composite material maximum enzymatic activity appearance pH value increase. When pH value was 7.4, the activities of free enzyme and post-immobilization enzyme significantly reduced and the enzymatic conformation might be changed. In addition, the experiments found that the optimum temperature for the highest activity of free α-amylase was 35 °C, while the optimum temperature for the highest activity of post-immobilization enzyme was 50 °C. The immobilized enzyme had the characteristic of high temperature resistant properties. The post-immobilization of α-amylase showed more stable properties to both pH and temperature environment, which can be attributed to the mesostructure of SBA-15 and the rigidity of the SiO_2 matrix. This reduced the freedom of peptide-chain refolding molecular motions that occur in protein denaturation processes.

Conclusions

(1) α-Amylase was adsorbed on SBA-15 material by physical adsorption method and (SBA-15)-(α-amylase) composite material was prepared. By means of chemical analysis, scanning electron microscopy, infrared spectroscopy, X-ray diffraction, low temperature N_2 adsorption-desorption analysis characterization, the α-amylase was immobilized on the SBA-15 carrier was proved.

(2) The catalytic results of relevant materials showed that (SBA-15)-(α-amylase) composite material has the advantages of high pH value resistance and high temperature resistance compared with the free enzyme and has a higher potential use value.

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