The Neutral Protease Immobilization: Physical Characterization of Sodium Alginate-chitosan Gel Beads

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The neutral protease immobilization：Physical characterization of sodium alginate-chitosan gel beads

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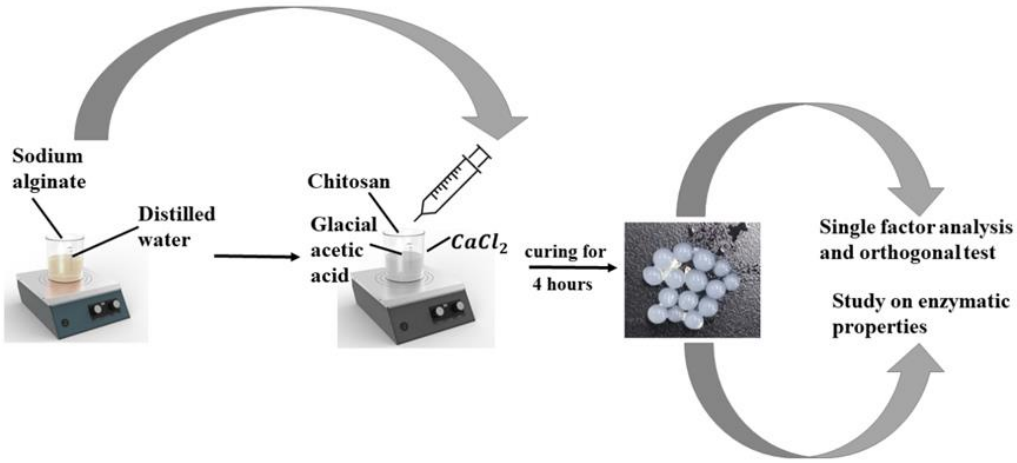
Abstract：Sodium alginate and chitosan were cross-linked to form composite gel spheres, which were entrapped to immobilize the free neutral protease. The matrix of the immobilized neutral protease was detected and characterized using Fourier transform infrared spectroscopy, Energy Dispersive X-Ray. The optimum immobilization conditions were determined by orthogonal test, the sodium alginate was 3.5%, $CaCl_2$ was 2.5%, chitosan was 2.5%, and immobilizing time was 1.5h. Meanwhile, the activities of immobilized neutral protease and free enzyme were compared. The results showed that the pH value of immobilized enzyme was 5-8, the relative activity was above 90%, the free enzyme was above 80%, the relative activity of immobilized enzyme was above 80% in 30 °C -80 °C, the free enzyme was above 64% in 40-80 °C. The immobilized enzyme is better than the free enzyme in the stability of pH and thermal. The relative activity of immobilized enzyme was 50% after six hydrolysis cycles, and 80% after 11 days of storage.

Key words：chitosan ; immobilized; orthogonal test; relative enzyme activity ；sodium alginate；neutral protease

Graphical abstract



HIGHLIGHTS：

- Neutral protease was immobilized on sodium alginate-chitosan beads.
- The optimal conditions for the preparation of immobilized neutral protease were obtained by single factor optimization and orthogonal methods.
- The relative enzyme activity remained 50% after 6 cycles.

1. Introduction

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As biocatalysts, enzymes are used to speed up biochemical and chemical reactions. It has been widely used in various fields because of its high catalytic efficiency and substrate specificity under appropriate reaction conditions (suitable pH, temperature, etc.) [1]. However, due to the disadvantages of high cost, poor stability and difficult to reuse, their use is limited and cannot play a maximum role. Immobilized enzymes can not only overcome these problems, but also has some advantages including repeated application in the same catalysis reaction, increased reaction control of the catalytic process, rapid termination of reactions with ease by removal of enzyme from the reaction mixture. Furthermore, the immobilized enzyme can remain active in more widely range of pH and temperature condition due to the increased conformation stability[2]. Enzyme immobilization restricts the enzyme to the carrier through simple adsorption, covalent binding, embedding or different combinations of methods, which not only does not significantly affect the activity of the enzyme, but also improves the stability[3-4]. Since there is no covalent interaction between the enzyme molecule and the carrier, embedding and adsorption can be called physical immobilization methods[5], while covalent and cross-linking are chemical immobilization methods because the enzyme molecule is fixed to the carrier through covalent bonds[6]. In addition, the immobilized enzyme carrier is also an important factor affecting the immobilization of enzymes. In order to achieve the reusability of enzymes, the enzyme carrier should have good stability and mechanical strength. In addition, the enzyme carrier should be characterized by low cost, abundant sources and environmental friendliness[7].

Natural polymer carriers are mainly the structural proteins of plants and animals, and are natural polymer products. In addition to chitosan, chitin and sodium alginate[8], conventional carriers commonly used also include agarose aggregation, ovalbumin, agarose beads and lignin fiber[9].For example, chitin and chitosan can be extracted from the shell of shrimp[10]. Sodium alginate can be extracted from seaweed[11].

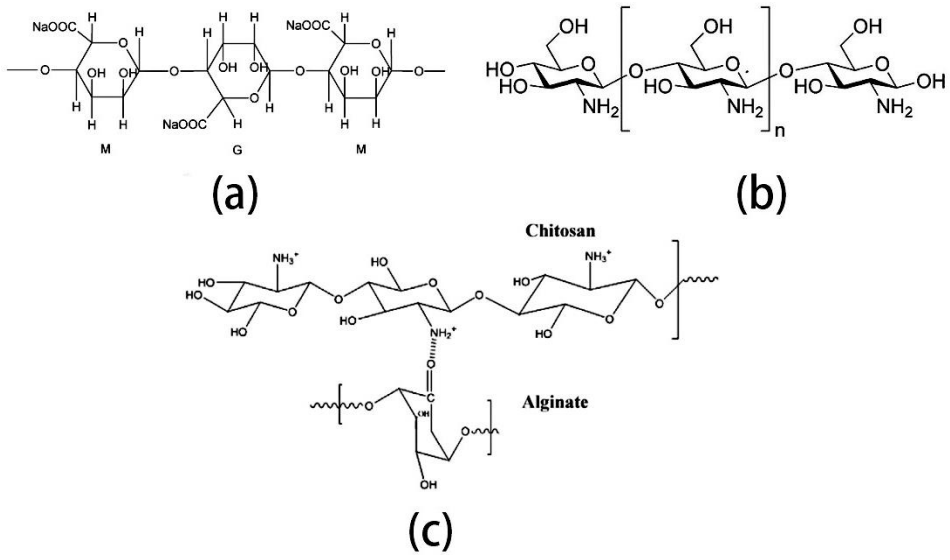
Sodium alginate is a kind of natural polymer material extracted from plants. Compared with synthetic polymer materials, it has better biocompatibility. In addition, among natural polymer materials, sodium alginate is cheap and easy to obtain. Sodium alginate molecular chain is formed by copolymerization of arroturonic acid and mannouronic acid, and the carboxyl groups on the molecular chain enable sodium alginate to quickly undergo ion crosslinking reactions with divalent (polyvalent) cations or other polycations[12] . Sodium alginate can be cross-linked with calcium ions to form sodium alginate gel spheres. The crosslinking of sodium alginate and calcium ion is physical crosslinking, and the reaction conditions are mild and the reaction speed is fast. The gel spheres obtained by crosslinking sodium alginate with calcium ions also have some disadvantages which limit their application. Calcium belongs to small molecules, ions with sodium alginate is usually an interaction between calcium ion and sodium alginate on the molecular chain of two carboxyl formed "egg" structure, the integration between the three-dimensional network structure of the crosslinking density compared to the molecular chain of the interpenetrating network structure is low, therefore, sodium alginate and calcium ion crosslinking gel beads usually low mechanical strength, if for enzyme fixation, may lead to leakage of enzyme. However, sodium alginate is a polyanion, which can be used to prepare gel beads by ionic crosslinking between sodium alginate and polycations. When ionic crosslinking occurs between polyanions and polycations, there are many reaction sites on each molecular chain, so the crosslinking density can be improved, and the mechanical strength of hydrogels can be improved[13].

Polysaccharide is a kind of natural polymer with unique structure and properties. It has broad application prospects in different fields. Although polysaccharide is a carbohydrate and is abundant on

Earth, chitin is the second most readily available polysaccharide in nature[14]. Chitin, which is structurally similar to cellulose, is also considered to be a similar structural material. Chitin is found in the skin of crabs and shrimps and forms tightly bound compounds with other substances, making it insoluble in common solvents. Chitin, a polysaccharide that occurs abundantly in nature after cellulose, has attracted the interest of the scientific community due to its plenty of availability and low cost[15]. After chitin is deacetylated, it is converted to chitosan, a polysaccharide soluble in acid medium. This occurs when the NH_2 group of the D-glucosamine repeat unit in the chitosan is protonated, while in the same medium the polysaccharide is converted to a polyelectrolyte[16].

Chitosan is a kind of natural alkaline straight chain cationic polymerization polysaccharide, which is made from chitin after the removal of more than half of the acetyl group. It is chemically named as poly (1,4) -2-amino-2-deoxy-β-D-glucan. Chitosan is insoluble in water due to the existence of hydrogen bond between molecular chains, but in acid solution, the amino group on the molecular chain of chitosan is converted to ammonium ion. Therefore, chitosan can be soluble in acid solution and belongs to polycation[17]. The molecular chain of chitosan contains two structural units, N-aminoglucan and N-ethylphthalamide glucan, and the proportions of the two are different with the degree of deacetylation. There are a lot of primary amino groups in the side chain structure of chitosan which can be complexed with anion polyelectrolytes such as sodium alginate in aqueous solution. Chitosan has the advantages of good biocompatibility, biodegradation by a variety of enzymes in vivo, non-toxicity, non-antigenicity, abundant resources, low cost and so on, and at the same time it has unique molecular structure characteristics, chemical properties and biological functions[18].Therefore, chitosan is considered as an effective biodegradable polymer and widely used as a functional material due to its excellent properties such as biocompatibility, biodegradability, adsorbability and non-toxicity [19]. Chitosan is a promising organic compound for immobilization of enzyme carriers.

Sodium alginate gel beads have problems such as low mechanical strength and easy leakage of enzymes from the gel beads[20]. In order to reduce this problem, sodium alginate can be mixed with chitosan to prepare sodium alginate and chitosan composite gel beads to improve the stability of the gel beads. The interaction between sodium alginate and chitosan is shown in the figure1.



(a).Sodium alginate structure formula;(b).Chitosan structure formula;(c).A schematic representation of the intermolecular interaction between chitosan and alginate in sodium alginate- chitosan composite beads.

Fig.1. Molecular structural formulas

Chitosan, a polysaccharide obtained from chitin by alkaline deacetylation, is mixed with sodium alginate to produce a reinforced composite gel sphere[21]. The strengthening is based on the electrostatic interaction between the carboxylate alginate group and the ammonium chitosan group[22-23].Chitosan molecules contain a large number of primary amino and carboxyl groups, the primary amino group has a positive charge, carboxyl has a negative charge. Through electrostatic interactions, amino and carboxyl complexion reaction, form polyelectrolyte complex microspheres, drip when sodium alginate sol containing calcium ion and the mixture of chitosan, under the effect of static electricity, the molecular chain of free carboxyl sodium alginate and chitosan molecules on free amino complexion reaction, and form polyelectrolyte chitosan - sodium alginate microspheres. Because the molecular weight of calcium ions is significantly lower than that of chitosan, calcium ions can pass through the polyelectrolyte microsphere formed by chitosan-sodium alginate and diffuse to the molecular core of sodium alginate, and finally form the composite gel sphere of sodium alginate and chitosan（figure1）.

In the study, neutral protease was immobilized on sodium alginate-chitosan beads. The optimal conditions for the preparation of immobilized neutral protease were obtained by single factor optimization and orthogonal methods. The effects of immobilization the conditions such as the concentration of sodium alginate, calcium chloride, and chitosan-sodium alginate were evaluated. The characterizations of the beads were performed using Fourier transform infrared spectroscopy (FTIR), scanning electron microscopy (SEM), and X-ray diffraction (XRD). The reusability of the immobilized neutral protease was also studied.

2.Materials and Methods

2.1 Materials

Unico uv2100 ultraviolet spectrophotometer was purchased from Beijing Boya Innovation Technology Co., Ltd to determine the concentration of protein. High-resolution X-ray D8Discover25 Cuk α radiation source, tube voltage 40KV, tube current 500mA, scanning range 3 °-40 °to determine the crystal structure of materials. Fourier transform infrared spectrometer VERTEX70, KBr compression method, scanning range 4000-500$cm^{-1}$was studied the structure features of sodium alginate (SA), chitosan（CA），and sodium alginate – chitosan(SA-CA). Scanning electron microscope JSM-6710f of cold field emission type, image resolution: 1.0nm (15KV), 2.2nm (1KV), magnification: X25-65000, accelerating voltage: 0.5KV-30KV was used to observe the morphology of materials.

The chemicals used in this study were of analytical grade. SA, chitosan, calcium chloride, casein, tyrosine were purchased from the Sinopharm Chemical Reagent Co, Ltd, China.

2.2 Experimental method

2.2.1 Determination of free enzyme activity

The neutral protease activity was determined with reference to Folin-Ciocalteu method.

Firstly, L-tyrosine standard solutions of 0，10ug/mL，20ug/mL，30ug/mL，40ug/mL，50ug/mL，and 60ug/mL were prepared, then 5mL sodium carbonate solution and 1mL formaldehyde reagent was added, rested 20 min at 40℃, tested the optical density(OD) value at 680 nm after cooling, adjusted the OD value of the test tube without tyrosine to 0, drawn the curve of the concentration versus OD value, and obtained the standard curve.

Furthermore, three test tubes were added with protease solution of 1 mL each at 40℃ in water bath for 5min. 1 mL casein solution was preheated for 5 min, the reaction time was 10 min, and two

mL trichloroacetic acid solution was added to stop the reaction. After holding for 10 Min, the reaction was filtered with medium speed filter paper. 1mL of each filtrate was sucked into the other three test tubes, 5 mL sodium carbonate solution and 1 ml folin reagent were added. The filtrate was heated at 40℃ for 20 min, then, rapidly cooling to room temperature, OD value at 680 nm was measured and quantified by calibration curve. Added 2 mL trichloroacetic acid and 1mL casein to the blank tube. The enzyme activity was calculated as follows formula (1).

Protease activity (U/g)=$(C \times n \times N)/t$ (1)

Where,

C--Tyrosine produced by enzymatic hydrolysis,ug/mL(namely the microgram number of tyrosine in the extracted 1mL supernatant, was calculated according to the absorption value and substituted into the tyrosine standard curve)；

n--Take out 1mL of 4ml reaction solution for determination (n=4)；

N--Dilution ratio of enzyme solution；

t--The reaction of time,min.

2.2.2 Preparation of composite gel beads

3g sodium alginate was mixed with 100mL distilled water, heated at 50 °C, then stirred evenly for standby. The dilution of 1 mg/L neutral protease was 5000 times, and the mixture of diluted protease solution and sodium alginate was oscillated at a volume of 1:3 for 40 min at room temperature. The mixture of 3g chitosan and 2% glacial acetic acid stirred evenly, and then 3g calcium chloride was added into the mixed solution of chitosan, and stirred again for uniform use. The oscillating sodium alginate was dropped into the mixed solution of chitosan with a syringe to form sodium alginate-chitosan composite gel beads. The composite gel beads were washed several times with distilled water, the composite gel beads are placed in the calcium chloride solution in the refrigerator to harden for 3h after washing. The gel beads are filtered out, wash with distilled water until neutral. The immobilized enzyme was preserved at 4℃ for further experiment. All experiments were carried out three times and took the average to plot graphs.

The relative activity was 100% of the maximum absorbance in each group, and the remaining value was as a percentage of the maximum value

3. Results and discussion

3.1 Single factor analysis

3.1.1Effect of SA concentration

In figure 2(a), the concentration of SA was 1% and 2%, the relative enzyme activity was 92.7% and 96.7%, respectively. During the preparation process, the gel beads were shaped quickly and regularly, However, the effect of cross-linked gel spheres was not good, and the gel spheres had adhesion phenomenon, this is because the concentration of SA was too low, resulting in gel beads viscosity was low, physical strength was small, couldn't effectively form a stable structure. While the concentration of SA was 3%, the gel-forming effect was better, the relative enzyme activity was 88.5%, which was lower than that of other concentration. Concentration of SA was 4%, the gel bead forming effect was better, and the relative enzyme activity was the highest When the concentration of SA was 5%, the forming effect of the composite gel beads was poor, due to the excessive concentration of sodium alginate, the gel bead has a high viscosity, which led to the slow forming and irregular shape of the gel bead. Another reason may be that as the concentration of SA increased, the strength of the gel beads increased, the surface became denser, the enzyme was not easy to be better embedded, so the

effect of enzymatic hydrolysis was not good. So the concentration of SA was 4% for the follow-up experiment.

3.1.2 Effect of $CaCl_2$ concentration

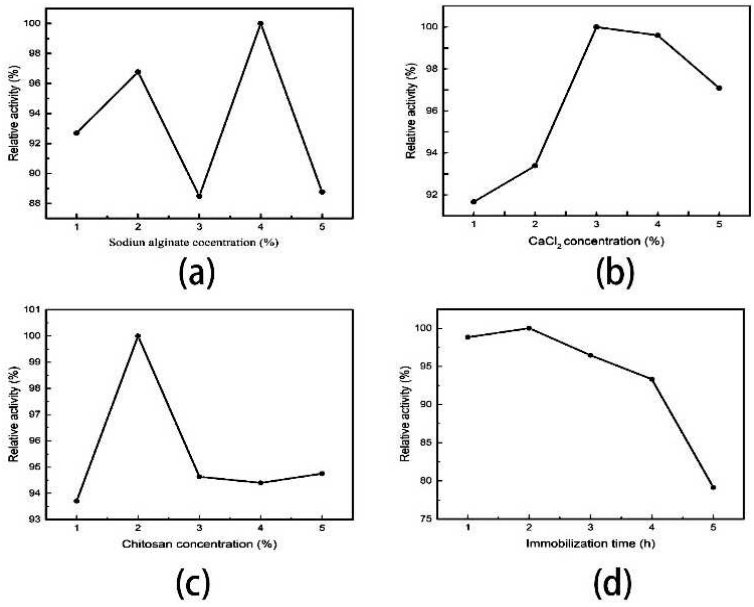
In figure 2(b), the concentration of $CaCl_2$ was 1% and 2%, the relative enzyme activity was 91.7% and 93.4% respectively. During the preparation, the gel beads easy formed to rupture and the enzyme was leaked out. The concentration of $CaCl_2$ reached 4% and 5%, the relative enzyme activity values were 99.6% and 97.15%, respectively, which was due to the release of $CaCl_2$ dissolved in the aqueous phase, resulting in high value of determination and no reference value. In the egg-box model, the gel strength was proportional to the concentration of calcium ion, which protected the enzyme and improved thermal stability of the enzyme, but the concentration of $CaCl_2$ is too high, Ca^{2+} will react with the enzyme and decrease its activity. The concentration of $CaCl_2$ was 3%, the relative activity was the highest, the shape of the gel sphere formed was fixed for a long time and the relative activity was the highest. So the concentration of $CaCl_2$ concentration was 3% for the follow-up experiment.

3.1.3 Effect of CA concentration

In figure 2(c), the concentration of CA was 1% , the shaping effect was better, but the relative enzyme activity was 93.8%, which was lower than others. The concentration of CA was 3%, 4%, and 5%, the relative enzyme activity values were 94.6%, 94.4% and 94.7%, respectively. However, when the concentration of chitosan was higher, the formation of gel spheres was slow and irregular, and the adhesion of most gel spheres was serious. Excessive concentration of CA makes the polyelectrolyte membrane on the surface of gel sphere more compact, which makes the diffusion of substrate difficult and the effect of enzymatic hydrolysis less effective. The concentration of CA was 2%, the molding effect was better, and the relative enzyme activity was the highest. CA concentration of 2% was selected for subsequent experiments

3.1.4 Effect of immobilization time

In figure 2(d), the immobilized time was 1 h, the relative enzyme activity was 98.9%, and the immobilized time was 2 h, the relative enzyme activity reached the greatest. This is because the longer the time of immobilization, the more completed the reaction, the denser the structure of the carrier, which can reduce the loss of enzyme molecules due to the large pore size on the surface and inside of the carrier. However, the relative activity was 96.5%, 93.1% and 79.1%, at 3h, 4h and 5h respectively. The relative activity decreased because the pore structure of the carrier surface was too compact with the further extension of time, to some extent, it will prevent the substrate from entering into the carrier to contact with the enzyme molecules sufficiently, and the mass transfer resistance increases, which makes the enzyme activity of the neutral protease decrease. Another reason may be that the immobilization time is too long, resulting in some of the structural changes of the enzyme, reduced activity. 2h was selected to the immobilizition to continue the subsequent experiments.



(a)Effect of sodium alginate concentration on the relative activity of immobilized enzyme

(b)Effect of $CaCl_2$ concentration on the relative activity of immobilized enzyme

(c)Effect of chitosan concentration on relative activity of immobilized enzyme

(d)Effect of immobilization time on the relative activity of immobilized enzyme

Fig.2. Single factor analysis

3.2 Orthogonal experimental analysis

3.2.1 Orthogonal Test

According to the results of single-factor test, the factor level of orthogonal test was showed in Table 1.

Table 1. Factor level of composite gel beads orthogonal test

Levels	Factors			
	A- Sodium-alginate concentration （%）	B- $CaCl_2$ concentration （%）	C- Immobilization time （h）	D- Chitosan concentration （%）
1	3.5	2.5	1.5	1.5
2	4	3	2	2
3	4.5	3.5	2.5	2.5

The L9(3^4) orthogonal test scheme was established according to the design standard of orthogonal table, and the preparation scheme of composite gel beads was further improved by referring the results.

3.2.2 Orthogonal experimental analysis

In table 2, the optimal values of each factor were as follows: 3.5% SA, 2.5% $CaCl_2$, 1.5 h immobilized time and 2.5% CA, respectively. Finally, the optimal combination was determined as 3.5% SA, 2.5% $CaCl_2$, 1.5h immobilized time, 2.5% CA.

The rank of mean response results showed in table 3, the order of the effect of single factor on the relative enzyme activity was SA concentration > immobilized time > $CaCl_2$ concentration > CA concentration.

Table 2. The results of orthogonal test for SA-CA

Test series	Factors				Relative activity (%)
	Sodium-alginate concentration (%)	$CaCl_2$ concentration (%)	Immobilization time (h)	Chitosan concentration (%)	
1	3.5	2.5	1.5	1.5	100.000
2	3.5	3.0	2	2	85.714
3	3.5	3.5	2.5	2.5	88.407
4	4	2.5	2	2.5	86.066
5	4	3.0	2.5	1.5	76.464
6	4	3.5	1.5	2	87.354
7	4.5	2.5	2.5	2	85.246
8	4.5	3.0	1.5	2.5	84.075
9	4.5	3.5	2	1.5	52.459

Table 3. Mean response

Levels	Sodium-alginate concentration	$CaCl_2$ concentration	Immobilization time	Chitosan concentration
1	91.37	90.44	90.48	76.31
2	83.29	82.08	74.75	86.10
3	73.93	76.07	83.37	86.18
Delta	17.45	14.36	15.73	9.88
Row rank	1	3	2	4

3.3 Study on enzymatic properties

3.3.1. pH stability

pH value can affect the dissociation and complexion of substrate and enzyme, the conformation of enzyme active center and the stability of enzyme. The results showed in figure 3(a), the relative enzyme activity of immobilized enzyme was obviously lower than that of free enzyme at pH 3 and pH 4. On the one hand, there were a lot of —COO— structure in SA under strong acid condition, which can be transformed into —COOH under strong acid condition, thus inhibited the activity of enzyme proteins. When pH tends to be neutral, the relative activity of immobilized enzyme increased gradually, and in the range of pH5-8, the relative activity of immobilized enzyme was above 90%, but the free enzyme was 87%. The optimum pH value of free enzyme was 6, the optimum pH value of immobilized enzyme was 7. The reason may be that sodium alginate has a large number of functional groups in the reaction system and can combine with H^+ to form carboxylic acid, in order to maintain the optimal pH value of the enzyme molecules, the apparent pH value of the immobilized enzyme moves towards alkalinity. In addition, chitosan contains a large number of amino and hydroxyl groups, which can reduce the destruction of lactase by external solution in alkaline solution. In the optimal pH range, the immobilized enzyme has a wider pH range than the free enzyme, indicating that the immobilized enzyme has better pH stability.

3.3.2. Analysis of thermal stability

The activities of free enzyme and immobilized enzyme were measured at the gradient of 30℃-80℃. The results showed in figure 3(b), the activity of immobilized enzyme increased at first and then decreased. The reason may be that raising the temperature of the reaction system can increase the molecular kinetic energy of the substrate and the enzyme, and help to accelerate the adsorption,

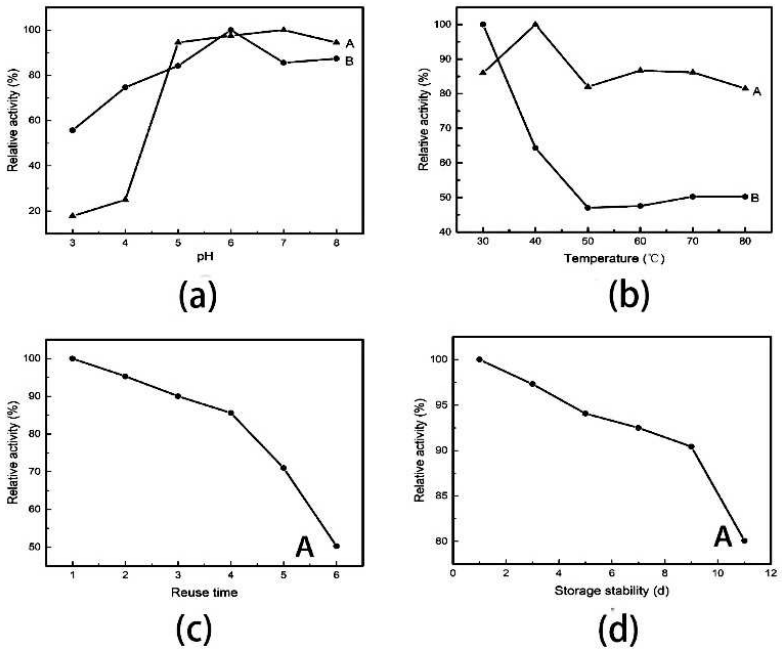
dissociation and complexion, and accelerate the process of enzymatic reaction. The relative activity of free enzyme reached the maximum at 30℃, but with the increase of temperature, the relative activity of free enzyme was in a decreasing trend. At 40℃, the relative activity of free enzyme was only 64% , this was likely due to high temperature that broke down the protein structure, and inactivate enzymes. The relative enzyme activity of immobilized enzyme was kept above 80% at 30℃-80℃. The combination between the immobilized enzyme and the carrier can effectively improved its thermal stability, and improved the ability of the enzyme to adapt to environmental temperature changes, which is conducive to the enzyme activity in the higher temperature environment. The thermal stability of the immobilized enzyme is improved because of the interaction between the enzyme and the carrier after immobilization, which makes the molecular structure of the enzyme more stable and makes the immobilized neutral protease have better thermal stability.

3.3.3. Reusability of immobilized neutral protease

The immobilized enzyme can be used for many times. After the initial use, some of the shallow free enzyme molecules which were not completely fixed by the carrier were lost, which led to the decrease of the enzyme activity. In the process of reuse, the pH value and temperature of the reaction system will affect the stability of the carrier and the enzyme, which may lead to the leakage of the enzyme and the decrease of the enzyme activity. The results showed in figure 3(c), the immobilized enzyme was reused for 6 times, the relative activity of the immobilized enzyme remained above 50% at the 6th times, which fully indicated the high reuse efficiency of the immobilized enzyme. This is due to the introduction of CA, chitosan itself has a good stability, unique physical and chemical properties to improve the stability of the immobilized enzyme carriers. As previously reported, enzyme activity was retained by 18% after immobilized cellulase-polymethacrylate particles were used during four hydrolysis cycles[24], and in another study, the fixed nanohybrids showed 51% relative activity in the fifth cycle[25]. In this study, SA-CA-immobilized neutral protease was more reusable.

3.3.4. Storage stability of immobilized neutral protease

The immobilized enzyme could maintain 80% relative enzyme activity when stored at -4℃ for 11 days. The results showed in figure 3(d), at 9d, the relative activity decreased from 90.4% to 80.1% , which was probably due to the rapid decrease of storage stability caused by $CaCl_2$ and the release of free enzyme from the breakdown of gel beads. The immobilized enzyme itself has consumed most of the enzyme activity, and the stability of the immobilized enzyme retained above 80% after 11 days storage.



(a) pH stability of free and immobilized enzymes

(b)Thermal stability of free and immobilized enzymes

(c)Determination of reuse time of immobilized enzymes

(d)Storage stability of immobilized enzymes

Fig.3. Enzymatic properties

3.3.5. Infrared spectrum analysis

The chemical structure of SA-CA composite gel beads was studied by Fourier infrared spectrum analysis. The infrared spectrum was showed in figure4.

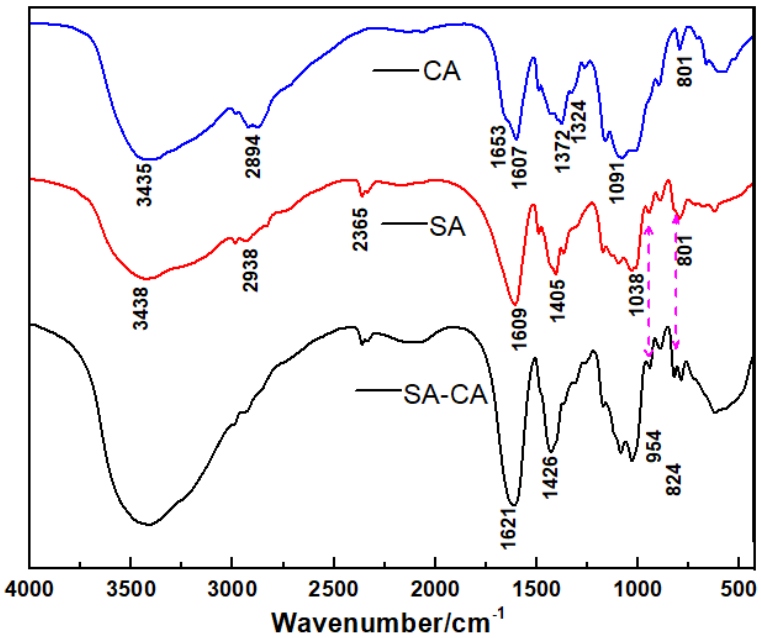


Fig.4. FT-IR of composite gel beads

In the vicinity of 3435cm^{-1}, CA had a relatively wide absorption peak, which represented the overlapping of -OH stretching vibration of phenol, alcohol, carboxylic acid and so on with that of -NH, and the antisymmetric stretching vibration of methyl group 2894cm^{-1} .The stretching vibration

absorption peak of C=O in 1653 cm^{-1} amide I, the bending vibration absorption peak of N-H at 1607 cm^{-1}, the bending vibration absorption peak of CH_2 and CH_3 near 1372 cm^{-1}, the stretching vibration absorption peak of 1653 cm^{-1} amide I, the bending vibration absorption peak of N-H at 1607 cm^{-1}, and the bending vibration absorption peak of CH_2 and CH_3 respectively .The absorption peaks of 1324cm^{-1} and 1091cm^{-1} represent the asymmetric stretching vibration of C-O-C bond of glycosidic bond.

The absorption peaks of SA near wave number 3438cm^{-1} represent the stretching vibration of -OH in the free carboxyl group, the stretching vibration peaks at 2938cm^{-1} are C-H, the stretching vibration peaks at 2365cm^{-1}, the stretching vibration peaks at 2938cm^{-1} and the stretching vibration peaks at 2365cm^{-1}, in addition, the absorption peaks at 1606cm^{-1} and 1405cm^{-1} are due to the formation of carboxyl anion, and 1038cm^{-1} is due to the asymmetric stretching vibration of something .954cm^{-1}, 801cm^{-1} is produced by asymmetric and symmetric stretching vibration of C-O-C skeleton.

The Ir absorption peaks of SA-CA were compared with those of CA and SA alone. The absorption peaks of CA at 1653 cm^{-1} and 1607 cm^{-1} were shifted to those of amide at 1621 cm^{-1}, probably due to the interaction between SA carboxyl anion and CA amino group, the asymmetric vibration of 954cm^{-1}C-O-C skeleton of SA was produced, the results showed that SA and CA had interaction.

3.3.6. XRD analysis

The figure 5 showed X-ray diffraction pattern of SA-CA gel beads during added CA to form the composite gel beads, the crystal peak of SA around 13.7 °disappears and the scattering peak of SA in the amorphous region at 2 is 40.3°became narrow, CA had interaction with SA and destroyed the crystal form of SA.

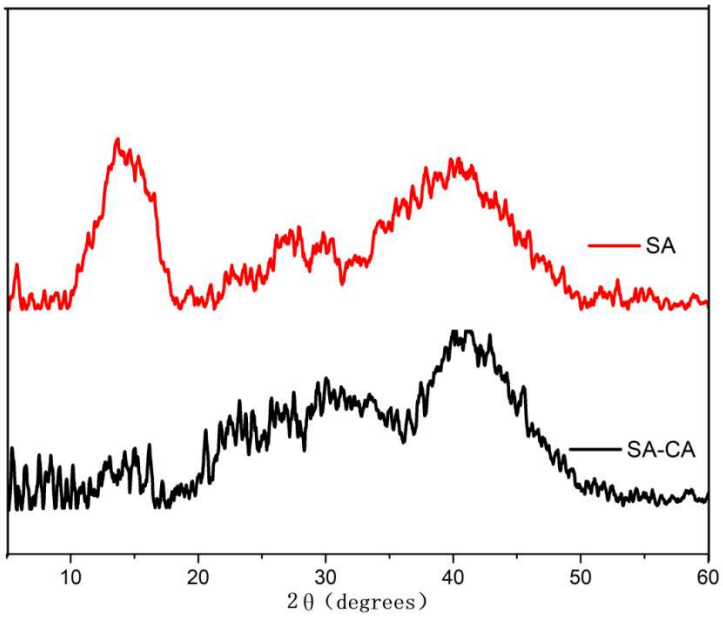


Fig.5. XRD of composite gel beads

3.3.6. SEM characterization analysis

The composite gel beads formed by SA-CA had a certain void structure, with compact, smooth and uniform cross-section, and no separation occurs, which showed in figure 6.

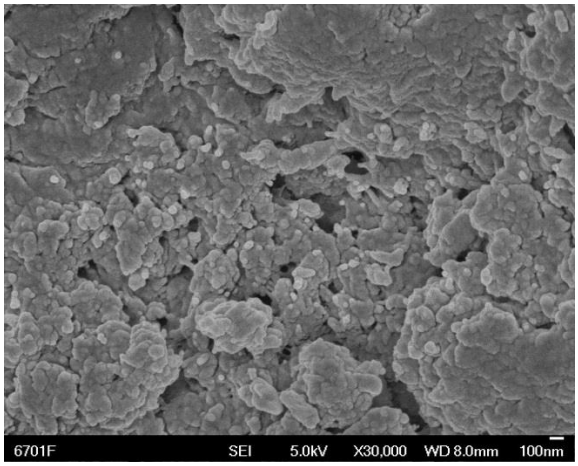


Fig.6. SEM of composite gel beads

4. Conclusion

Chitosan/ sodium alginate composite were prepared with chitosan and sodium alginate as raw materials. The morphology of the prepared microspheres was regular, and the dispersion between the microspheres was better, and there is no adhesion. The effects of immobilized conditions on the enzymological properties of immobilized neutral protease were investigated by using enzyme activity as an index.

(1) Optimum preparation conditions of chitosan/ sodium alginate composite: the optimum combination was 3.5% SA, 2.5% $CaCl_2$, 1.5 h immobilization time and 2.5% CA.

(2) Compared with the free enzyme, the thermal stability and pH stability of immobilized neutral protease were improved, especially the alkaline resistance. Meanwhile, Fourier transform infrared spectroscopy showed that the shift of chitosan absorption peak might be due to the interaction between SA carboxylate anion and CA amino group. It showed that SA and CA better combined. XRD results showed that the scattering peak in the amorphous region of sodium alginate was narrowed, which indicated that the crystal form of the sodium alginate powder was destroyed, and the chitosan alginate sodium had an interaction. The immobilized enzyme also had better operation stability. After repeated use for 6 times, the enzyme activity still retained more than 50%. And after 11 days storages, the relative activity remained above 80%.

Ethical Approval

Not applicable

Consent to Participate

Not applicable

Consent to Publish

Not applicable

Authors Contributions

Yuan Bai: Conceptualization, Supervision, Writing - review& editing. **Wei Wu:** Software, Data curation, Writing- Original draft preparation.

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Competing Interests statement

No competing financial interests exist.

Availability of data and materials statement

Some or all data, models, or code that support the findings of this study are available from the

corresponding author upon reasonable request.

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