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Use of Chitosan Gel for the Purification of Protein

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ABSTRACT

The aim of this study was to optimize the preparation of chitosan gel and to use it for protein purification. The optimized preparation parameters were chitosan concentration 2.0%, glutaraldehyde concentration 0.6%, low cross-linking rate, NaOH concentration 1.6%, amount of $NaBH_4$ 0.4 g. In order to use the chitosan gel, the elution conditions were optimized as follows. NaCl concentration 0.05 mol/L in the tris-HCl (pH 9.05) at the flow rate of 2.03.0 mL/min. Particle size of chitosan gel was 120-140 μm. Neutral protease could be separated into four ingredients through chitosan gel column. The yield of enzyme was more than 90%. Albumin bovine serum could be separated into two ingredients through gel column and the total yield of albumin bovine serum was more than 70%.

Key words: Chitosan, Gel, Preparation, Application

INTRODUCTION

Gels have been widely used for protein/enzyme separation in food and pharmaceutical industry (Braun et al., 1989 and Louis et al., 1979). At present, most of the gel carriers are sepharose such as sepharose CL-4B and sepharose CL-6B. However, sepharose has some drawbacks. Firstly, it is very expensive. Secondly, the preparation and regeneration of the gel are very difficult. Thus, it would be worth to make a new and low cost gel, which offers relative ease on the preparation and regeneration.

Chitin is an economical material and it represents part of marine resources (Muzzareli et al., 1997), which is widely distributed in fungal biomass, crustacean shells, etc (Robert et al., 1992).Since chitosan is soluble in acidic solutions, it is necessary to increase its chemical stability in acidic solutions for the sorption of several anions whose recovery is only effective at pH lower than 4–5 (Inoue et al., 1993; Guibal et al., 1999 and Larkin et al., 1999). The crosslinking treatment can be carried out using several chemical reagents such as glutaraldehyde (Guibal et al., 1999 and Larkin et al., 1999). Glutaraldehyde crosslinking treatment may affect the sorption efficiency, involving a decrease in the number of free amine groups and a decrease in the accessibility to internal sites. This restrictive effect may be decreased using chitosan whose structure has been physically modified. A gel bead formation procedure was proposed and investigated by several authors (Guibal et al., 1999 and Rorrer et al., 1993). This physical modification allows: (1) the polymer network to be expanded: the increase in the polymer network opening enhances the diffusion of large size molecules, and (2) the crystalline state of the polymer to be reduced. Piron et al (Piron et al., 1997) had shown that the dissolving of chitosan followed by a freeze–drying

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of the resulting solution resulted in a strong decrease in the crystalline structure of the polymer, which in turn could be correlated to the improvement of sorption kinetics.

Chitosan has many excellent chemical properties similar to sepharose, such as chemical stability and compatibility with bioactive compounds. Hence, it could be used as gel carrier. Holme (Holme et al., 1988) studied chitosan grafted 1-β-D-furan, using this chitosan gel to separate protein, but the effect of separation was not good. Stenstad (Sentsad et al., 1989) used chitosan as the gel, but chitosan and the objective protein were difficult to be separated. Shi Y C et al prepared two kinds of chitosan affinity gel (Shi et al., 1996). The separation results were not effective too. The aim of this study was to optimize the preparation of chitosan gel and to use it for protein purification.

MATERIALS AND METHODS

Materials

Chitosan (deacetylation percentage 87%) was provided by Yuhuan Ocean Biochemical Co. Lit (Zhejiang, China), Glutaraldehyde (25%) was purchased from Wulian Chemical Factory (Shanghai, China); Albumin Bovine Serum was purchased from Huamei Bio-Chemical Factory (Zhejiang, China); Neutral protease was a gift from Wuxi Enzymatic Preparation Factory (Jiangsu, China); All the other used reagents were analytical-grade quality and purchased from the local suppliers.

Preparation of chitosan gel

Chitosan (1.0 g) was dissolved in 2.0% acetic acid and filtered to remove insoluble materials. The dissolved chitosan was extruded into the 2.0% NaOH solution with a thin nozzle. Then chitosan was washed with distilled water repeatedly until it was neutral. Chitosan, distilled water and glutaraldehyde (25%) were added to the flask for cross-linking. This procedure was carried out for 6 h at room temperature. Then 20 mL $NaBH_4$ (1.0%) was added and stirred for 24 h to block any residual aldehyde functional groups. This gel was filtered and thoroughly rinsed with distilled water until it was neutral. After the gel was sifted by the sieve, different sizes of chitosan gel were gotten.

Orthogonal optimization of chitosan gel preparation parameters

On the basis of single factor, five factors were selected to carry out orthogonal optimization experiments. The factors and the levels of the preparation of chitosan gel were listed in Table 1.

Table 1 - Factors and levels of the preparation of chitosan gel

Factors	Level 1	Level 2	Level 3	Level 4
Chitosan concentration / %	1.3	1.6	2.0	2.4
Glutaraldehyde concentration / %	0.20	0.40	0.60	0.80
The rate of cross-linking stirring / (r/min)	50	150	200	600
NaOH concentration / %	1.0	1.5	1.8	2.0
The amount of reducing agent / g	0.20	0.30	0.40	0.50

Stability of chitosan gel assay method

Chitosan gel (1.0 g) was added to some acid solution. The mixture was shook for 1 h at room temperature, filtered with glass fibers. And then the aqueous sample was analyzed with UV/visible Spectrometer 751 (Shanghai, China) the wavelength was 540 nm.

Adsorption ability of chitosan gel assay method

Chitosan gel was packed into a column (10 mm × 300 mm).The settled chitosan gel was equilibrated and eluted with distilled water. Albumin bovine serum was dissolved in the equilibrating buffer, whose protein concentration was 20 mg/mL. Albumin bovine serum sample (1 mL) was loaded. The progress of elution was examined all the time by nucleic acid and protein detecting machine (Shanghai China), the eluate was collected by fractional collector machine (SBS−100 Shanghai China). The area of penetrating peak was calculated.

Determination of the elution system of chitosan gel

Chitosan gel to be examined was packed into a column (10 mm × 300 mm). The settled chitosan gel was equilibrated with distilled water. Neutral protease was dissolved in distilled water, whose concentration was 20 mg/mL. 1 mL neutral protease sample was loaded. The elution system was NaCl (0–0.5 mol/L), tris-HCl (pH 9.05), $(NH_4)_2SO_4$ (0–0.5 mol/L), phosphate buffer (pH 8.0), baritone-HCl (pH 9.0), respectively. The progress of elution was examined all the time by nucleic acid and protein detecting machine (Shanghai China), the eluate was collected by fractional collector machine (Shanghai China).

Optimization of elution conditions of chitosan gel

Chitosan gel was packed and equilibrated (as above). Albumin bovine serum sample (20 mg/mL, 1 mL) was loaded. Under the optimized system (tris–HCl pH 9.05), different Cl^-concentrations, different pH and different sizes of chitosan gel were selected to investigate their effect on the elution conditions respectively.

Application of chitosan gel

Chitosan gel was packed and equilibrated (as above). Albumin bovine serum sample (20 mg/mL, 1 mL) or neutral protease (20 mg/mL, 1 mL) was loaded. Under the optimized elution conditions, albumin bovine serum and neutral protease were purified with chitosan gel respectively.

Protein determination

Protein determination was determined by the method of Folin-Lowry.

Neutral protease assay

Neutral protease activity was measured by the method of Jadwiga et al. (1998).

RESULUTS AND DISCUSSION

Preparation of chitosan gel

In order to separate out chitosan from the acid solution, the effect of alkali concentration on the amount of chitosan precipitated from the acid solution was examined.

Table 2 - Effect of alkali concentration on the mount of chitosan precipitated from the acid solution

The alkali mass concentration / %	The mount of the chitosan precipitated from the acid solution / g
2.5	0.395
2.0	0.390
1.0	0.383
0.5	Little
PH=9.0	Minim

Table 3- Effect of glutaraldehyde concentration on the stability of chitosan gel

Glutaraldehyde concentration / %	**The stability in pH 1.5 solution**
0.5	Partial dissolution
0.6	Dissolution
1.0	Insoluble
1.5	Insoluble
2.0	Insoluble

Table 4 - Effect of glutaraldehyde concentration on the adsorption of chitosan gel

Glutaraldehyde concentration / %	**The penetrating peak area / cm^2**
0.6	0.6±0.10
1.0	6.3±0.30
1.5	12.0±0.10
2.0	24.4±0.20
2.5	46.1±0.50

Table 2 showed when NaOH concentration was over 1.0%, the amount of the sedimentation of chitosan was high. But when NaOH concentration was below 1.0%, the amount of the sedimentation of chitosan decreased. Hence, 1.0% alkali concentration was selected for subsequent experiments.

In order to investigate the effect of cross-linking solution on chitosan adsorption ability, the experiments were carried out about the stability and adsorption of chitosan gel at different cross-linking solution concentration. The results in Table 3 showed that when glutaraldehyde concentration was less than 0.6%, chitosan gel was partially dissolved when solution pH was 1.5; when glutaraldehyde concentration was more than 0.6%, chitosan gel was stable. With the increase of glutaraldehyde concentration, the degree of cross-linking was increased. The adsorption ability of chitosan gel was increasingly step-down. From this conclusion, we could know if the mount of glutaraldehyde concentration was more than the critical point, more amino groups in chitosan were reacted and less free amino groups were left. So the eluate peak area was large. Under the premise of stability of chitosan gel, the mount of cross-linking solution was as little as possible.

The effect of different degree of the deacetylation of chitosan on the adsorption ability was examined.

Table 5- Effect of the degree of deaectylation on the adsorption ability of chitosan gel

The degree of deacetylation / %	The penetrating peak area / cm^2
90.24	40.5
87.52	77.5

Table 6- Effect of chitosan concentration on the adsorption ability of chitosan gel

Chitosan concentration / %	The penetrating peak area/ cm^2
1.0	42.3±0.50
1.5	12.0±0.10
2.0	0.60±0.10
2.5	3.70±0.20
3.0	21.3±0.40

The results in the Table 5 indicated higher the degree of the deacetylation of chitosan, the more was the amount of free amino groups in chitosan, resulting bigger adsorption ability of chitosan gel. Thus, the degree of the deacetylation of chitosan was >90%.

The experiment was carried out under different chitosan concentration. The results in Table 6 indicated that with the enhancement of chitosan concentration, the adsorption ability of chitosan gel was increased.

When chitosan concentration was 2.0%, the adsorption ability of chitosan gel reached the highest; but when chitosan concentration was increased further, the adsorption ability of chitosan gel began to decrease. Hence, chitosan concentration 2.0% was selected for further studies. From the results in Tables 7 and.8, it could be seen that chitosan concentration was the most important factor, among all the factors affecting the preparation of chitosan gel. The second important factor was glutaraldehyde concentration. The optimization preparation parameter of chitosan gel was $A_3B_2C_1D_2E_2$., which contained (%) chitosan 2.0, glutaraldehyde 0.6, NaOH 1.6, cross-linking rate 50 r/min, and the amount of $NaBH_4$ 0.4g.

From the results in Table 9, it could be seen that under the optimized situation the penetrating peak area was small and the adsorption ability of chitosan gel was big. Thus, the optimized preparation parameters were chitosan concentration 2.0%, glutaraldehyde concentration 0.6%, cross-linking rate 50 r/min.

Determination of the elution system of chitosan gel

In order to get a good separation of protein and enzyme, the experiments were carried out under different elution systems.

The results in Fig. 1a showed that when the eluent peak reached the highest, it was no longer

decreased. The eluent volume was high in the system of baritone-HCl (pH 9.0). It might have UV adsorption in baritone-HCl (pH 9.0). The results in Fig. 1b-1 e showed that the peak shape was similar, but it had the phenomenon of trailing in the upper elution, which caused the enhancement of the eluent volume. The results in Fig. 1f showed that the peak shape was symmetrical and the mount of the eluent was small. Thus, tris-HCl system was selected the elution system.

Table 7-Experiments results of orthogonal test of chitosan gel's synthesis

Number	Chitosan concentration / %	Glutaraldehyde concentration / %	The rate of cross-linking stirring / (r/min)	NaOH concentration / %	The amount of reducing agent / g	The penetrating peak area / cm^2
1	1	1	1	1	1	0.60±0.22
2	1	2	2	2	2	32.3±1.80
3	1	3	3	3	3	39.0±1.00
4	1	4	4	4	4	54.6±1.20
5	2	1	2	3	4	25.6±2.10
6	2	2	1	4	3	104±1.40
7	2	3	4	1	2	40.0±0.80
8	2	4	3	2	1	89.7±1.50
9	3	1	3	4	2	5.00±1.20
10	3	2	4	3	1	16.8±3.60
11	3	3	1	2	4	0.30±0.12
12	3	4	2	1	3	24.8±4.80
13	4	1	4	2	3	3.60±2.10
14	4	3	2	4	1	21.6±5.50
15	4	4	1	3	2	23.4±2.30
16	4	2	3	1	4	4.50±1.70
k_1	31.63	8.70	36.20	15.63	32.18	–
k_2	62.83	37.40	19.95	37.60	25.18	–
k_3	11.73	31.35	34.55	26.20	34.73	–
k_4	13.28	42.00	28.75	44.30	27.38	–
R	51.00	33.30	16.05	28.38	6.83	–

The symbol k_1, k_2, k_3 and k_4 means the average at each of a factor.

Table 8-The repetitive experiment results under the optimized situation

Source of variation	SS	Degree of freedom	Means square	significance P<0.05	significance P<0.1
Chitosan concentration/ %	7314.387	3	18.656	*	*
Glutaraldehyde concentration / %	3353.742	3	8.554		*
The rate of cross-linking stirring / (r/min)	392.062	3	1.000		
NaOH concentration / %	1691.802	3	4.315		
The amount of reducing agent / g	1130.862	3	2.884		
Error	392.06	3			

The critical F value is 5.390(P<0.1), 9.280(P<0.05).

Table 9-The repetitive experiment results under the optimized situation

Number	The penetrating peak area / cm^2
1	0.41±0.01
2	0.44±0.02

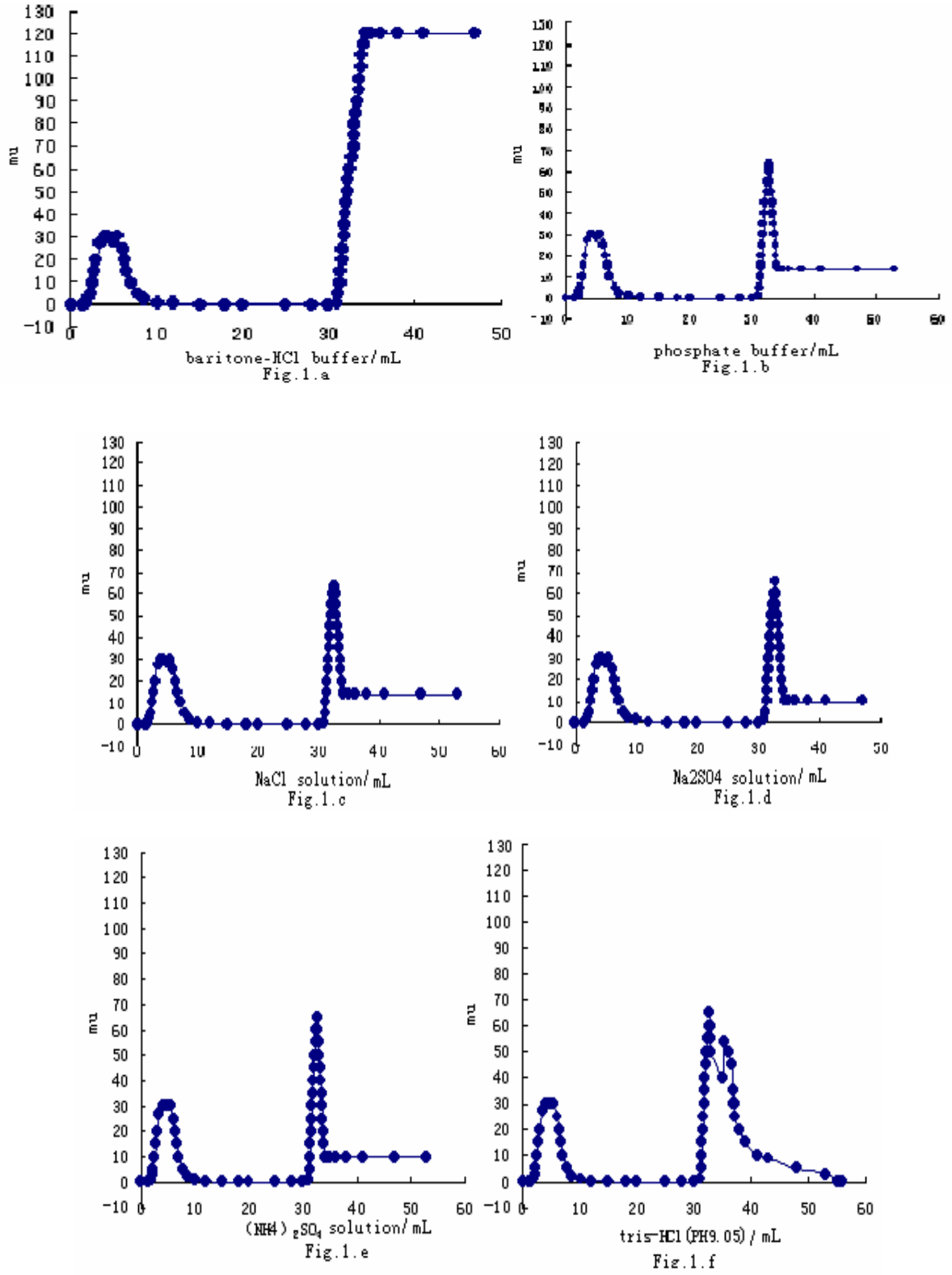

Figure 1- Elution curve under different elution systems
The symbol mu means electric current. The second peak is enzyme peak.

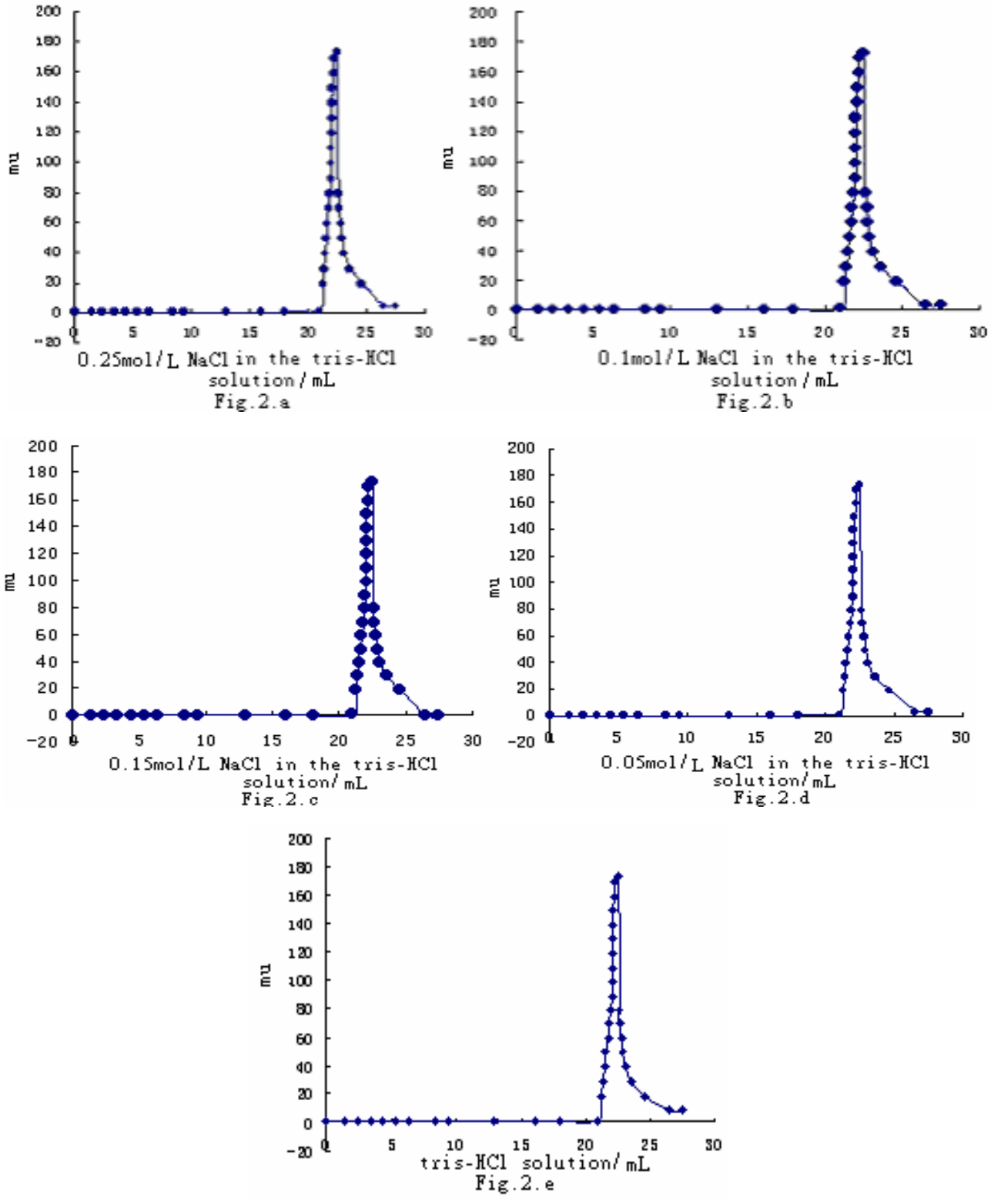

Figure 2 - Effect of ion strength on the elution condition of chitosan gel
The symbol mu means electric current

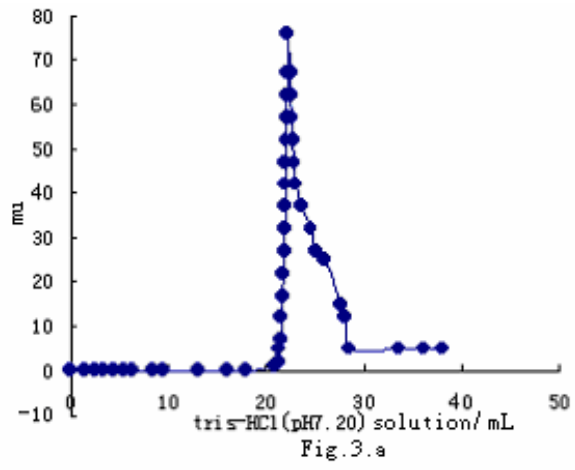

Fig.3.a

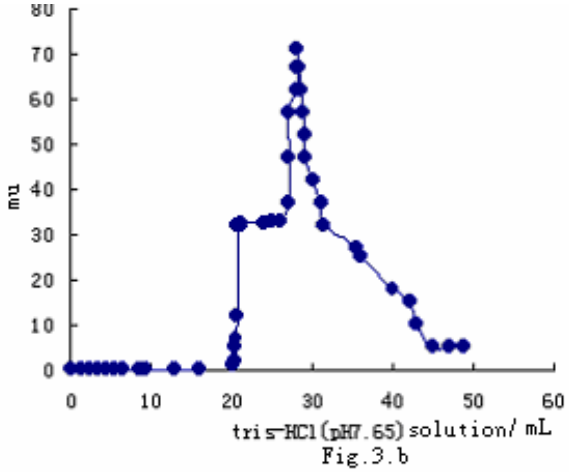

Fig.3.b

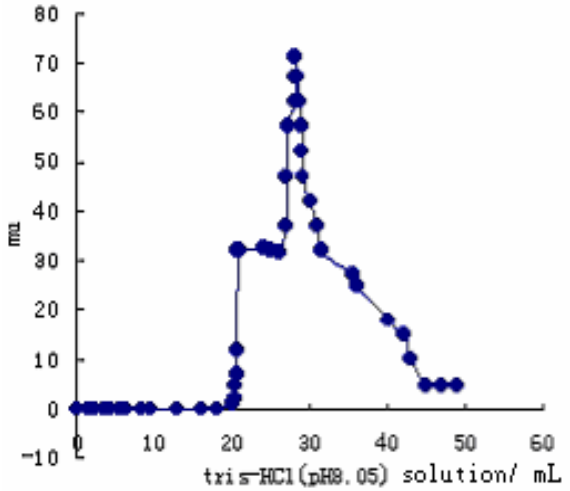

Fig.3.c

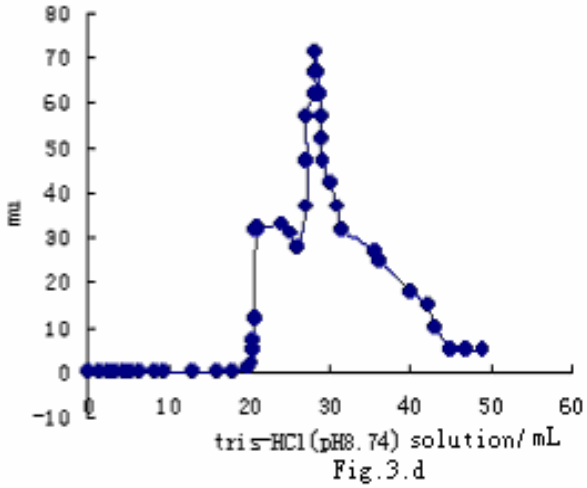

Fig.3.d

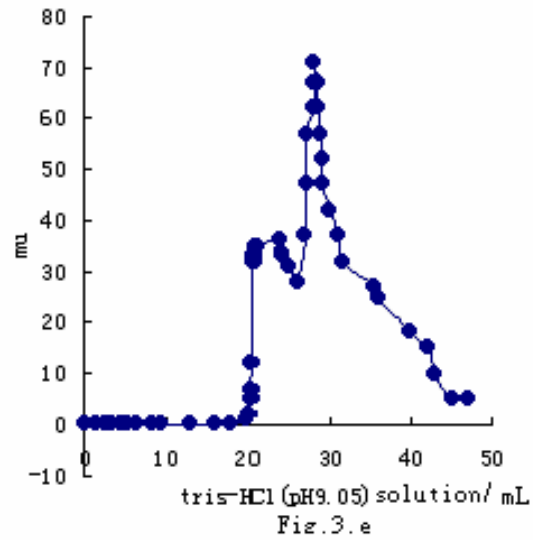

Fig.3.e

Figure 3 - Effect of pH on the eluent conditions
The symbol mu means electric current

Optimization of elution conditions of chitosan gel

When the ion strength was increased in the eluent solution, the competition to the absorbent site in the gel was enhanced too.

The results in Fig. 2 showed that with the enhancement of NaCl concentration, the elution peak had a trailing in separating albumin bovine serum. It might be that the hydrophobicity of the carrier was enhanced when Cl^- concentration was increased. But when NaCl concentration = 0.05 mol/L in tris-HCl (pH 9.05) solution, there was almost no trailing. It had a good peak shape. NaCl concentration = 0.05 mol/L in tris-HCl (pH 9.05) was selected as the eluent condition.

The exchange ability had a good relationship with solution pH. In order to investigate the effect of pH on the elution condition, the experiments were carried out at different pH in tris-HCl solution.

With the increase of solution pH, the selectivity of albumin bovine serum was increased too. Albumin bovine serum could be divided into two peaks. With the increase of pH, the ionization of chitosan was enhanced. The distance between pI and pH was bigger than before. Thus, tris-HCl (pH 8.0–9.0) was selected.

If the surface of the carrier was different, the exchange ability was different. In order to investigate the effect of different sizes of chitosan gel on the eluent condition, the experiments were carried out using different sizes of chitosan gel.

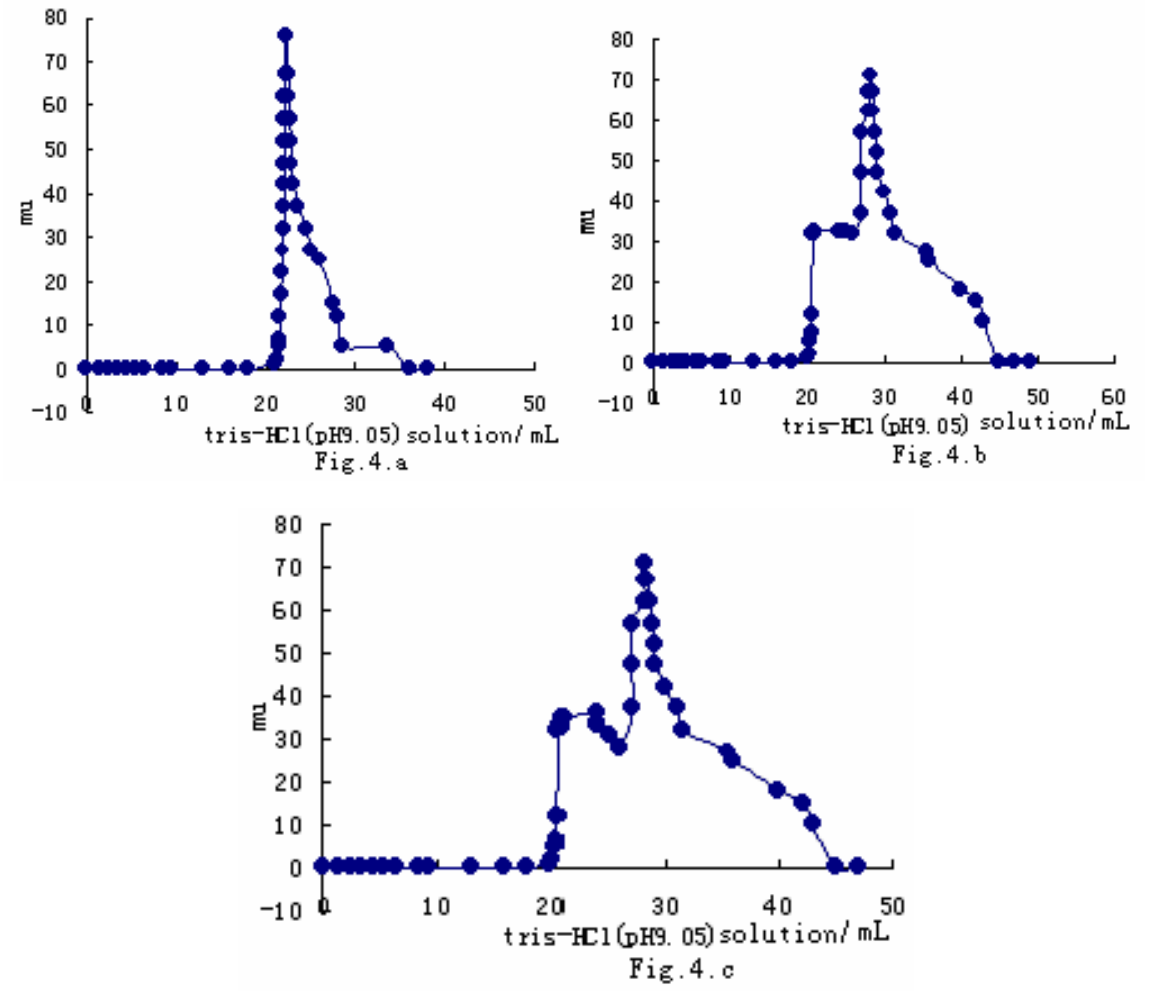

Figure 4- Effect of the different sizes of the gel on the eluent condition
The symbol mu means electric current

With the decrease of the size of chitosan gel, the separating power to albumin bovine serum was increased. But when the size of chitosan gel (120−140 μm) was used, the eluent flow rate was too slow. From the angle of the application, chitosan gel (<120 μm) was used.

Application of chitosan gel

Albumin bovine serum and neutral protease were purified with chitosan gel. The results were shown in Tables 9 and 10.

The results in Table 9 indicated that four ingredients peaks were obtained through chitosan gel. The activity peak mainly occurred in the peak 1 and activity yield was over 90%. Results in Table 10 showed that two ingredients were gotten, and the protein recovery was over 70%.

Table 9- The purification of neutral protease with chitosan gel

Number	Volume / mL	Total protein / mg	Total activity /U	Specific activity /(U/mg)	Purification factors	Enzyme activity /%	Protein yield / %
Sample	1.0	20.00	109.10	5.46	——	——	——
Peak 1	12.0	2.74	29.4	10.7	1.97	27.0	13.7
Peak 2	12.0	2.40	21.7	9.0	0.84	19.9	12.0
Peak 3	11.0	2.51	27.0	11.5	1.28	24.7	12.6
Peak 4	8.5	1.94	20.8	10.7	0.93	19.1	9.7
Total	42.5	9.59	98.9	——	——	90.7	48.0

Table 10 - The purification of albumin bovine serum with chitosan gel

Number	Volume /mL	Total protein /mg	Protein yield /%
Sample	1.0	20	—
Peak1	21.5	5.6	28.2
Peak2	25.0	8.4	42.0
Total	46.5	14.0	70.2

CONCLUSION

Chitosan has many excellent chemical properties of sepharose, such as chemical stability and compatibility with bioactive compounds. Results of the study showed that chitosan could be used as gel carrier. Neutral protease could be separated into four ingredients through chitosan gel column with total activity above 90% under the optimized elution conditions. Albumin Bovine Serum could be separated into two ingredients through chitosan gel column, the total yield of which reached above 70% under the optimized elution conditions.

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