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Affinity chromatography of trypsin using chitosan as ligand support

Yong-Chang Shi[a,*], Yong-Ming Jiang[a], De-Xin Sui[a], Yan-Li Li[a], Tian Chen[a], Lin Ma[b], Zhong-Tian Ding[b]

[a]*Laboratory of Enzyme Engineering, Agricultural College, Yangzhou University, Yangzhou, 225009 China*
[b]*National Laboratory of Enzyme Engineering, Jilin University, Changchun, 130023 China*

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Abstract

Chitosan beads were prepared for use as affinity adsorbent carrier. The affinity ligand, chicken ovomucoid, was immobilized on the chitosan via a cross-linker, glutaraldehyde. The results showed that 60 mg chicken ovomucoid could be immobilized on 1 g chitosan, and the maximum binding capacity for trypsin was about $8 \cdot 10^4$ U/g dry adsorbent. The procedure for preparing the chitosan-based affinity adsorbents was much safer and simpler than when a Sepharose-based matrix was the support. Columns packed with the affinity adsorbents were employed for trypsin chromatography. The experimental results revealed that the affinity adsorbents possessed good mechanical strength and storage stability and could be also operated repeatedly. Chitosan was suitable for use as an affinity adsorbent support for laboratory-scale and large-scale purification.

Keywords: Affinity adsorbents; Stationary phases, LC; Trypsin; Chitosan; Ovomucoid; Proteins

1. Introduction

Of all the purification techniques currently used, affinity chromatography is considered to be an ideal one, since it can preserve the biological activity of the isolated protein, it can also isolate protein from extreme values and in very dilute solution. For these reasons, affinity chromatography is a useful procedure for protein purification. In affinity chromatography, the main affinity support materials which are commercially available are agarose, silica, porous glass and synthetic organic polymers such as polyacrylamide [1]. Chitosan is a high-molecular-mass linear polymer, which consists of glucosamine in a 1→4 linkage (Fig. 1). It is formed by the reaction of chitin with concentrated alkali. Because of the presence of many free amino groups, the applications of chitosan to bio-medical, pharmaceutical and membrane materials have been investigated widely. Chitosan is a useful support of immobilized enzymes and cells [2], porous chitosan beads have been used as a protease immobilized support [3], but the application of chitosan in affinity chromatography

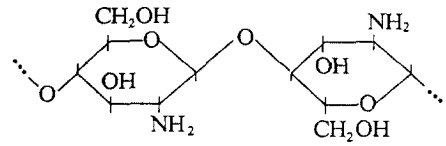

Fig. 1. Chemical structure of chitosan.

*Corresponding author.

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has not been frequently explored. These studies were concerned with affinity precipitation using chitosan as carrier [4] and chitosan derivatives as affinity chromatography materials for β-D-glucosidases [5]. In this work, we report a procedure for the preparation of chitosan beads with an affinity ligand, ovomucoid, immobilized on it to form an affinity adsorbent. The use of this adsorbent for the affinity chromatography of trypsin is described.

2. Experimental

2.1. Reagents and materials

Trypsin was purchased from Difco (Detroit, MI, USA). DEAE-cellulose 52 was from Whatman (Clifton, NJ, USA). Glutaraldehyde and $NaHB_4$ were obtained from Merck (Darmstadt, Germany). N-Benzyl-L-arginine ethyl ester (BAEE) was purchased from Dongfeng Biochemical Tech (Shanghai, China). Other solvents and reagents were of analytical-reagent grade and were used without further purification.

2.2. Preparation of chitosan from shrimp shell

Chitosan was prepared according to the method reported by Stanley et al. [6] with some modifications. After washing and removing the entrails adhering to the shrimp shell, the shell was treated with 1 *M* HCl for 12 h to decompose the $CaCO_3$ contained in the shell. The shell was then collected by filtering and thoroughly washed with water until neutral. Then it was put in 2 *M* NaOH for 6 h to remove pigments and protein followed by washing with water until neutral. This process was repeated three times to obtain the chitin. Chitosan was formed by the reaction of chitin with 50% NaOH for 8 h at 95°C, followed by washing with water until neutral. The degree of deacetylation of chitosan was estimated by titrimetry [7] to be greater than 85% and its molecular mass, determined by viscometry [8], was ca. 500 000. Before use, chitosan was powdered to 500 μm or so with a grinder.

2.3. Preparation of chicken ovomucoid (OMCHI)

OMCHI is a glycoprotein with p*I*=3.9–4.5 and M_r=28 000. It is a natural trypsin inhibitor with high specificity and is usually used as the affinity ligand for affinity chromatography of trypsin [9].

OMCHI was prepared from chicken egg white according to Lineweaver and Murray's procedure [10] with slight modifications. A 280-ml volume of egg white was added to 280 ml 0.5 *M* trichloroacetic acid–acetone (1:2, v/v). The mixture was stirred for 30 min and then stored overnight at 4°C in a refrigerator. The solution was filtered and then 1200 ml cold acetone was added to the supernatant. Thus the crude OMCHI was obtained after centrifugation. The product was further purified on a DEAE column (25×2 cm) that was equilibrated with 0.02 *M* phosphate buffer (pH 6.5) at a flow-rate of 40 ml/h, the eluent was equilibrated buffer containing 0.3 *M* NaCl, The result is shown in Fig. 2. The eluate was collected and lyophilized. The trypsin inhibitory activity of the OMCHI was measured by the following procedure: 0.2 ml OMCHI (protein concentration=1.75 mg/ml) was mixed with 2 ml trypsin (protein concentration=0.5 mg/ml). The mixture was allowed to stand for 15 min at 30°C, and then the residual trypsin activity of the mixture was measured. The result showed the trypsin inhibitory activity of the OMCHI was 9430 U/mg.

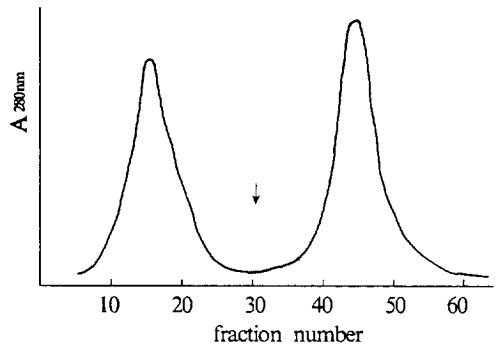

Fig. 2. Chromatography of OMCHI on DEAE-cellulose column. The arrow indicates replacement of the buffer by one which includes 0.3 *M* NaCl. Column, 25 cm×2 cm I.D.; flow-rate, 40 ml/h; fraction, 5 ml.

2.4. Trypsin assay method

The assay was done by the method of Rick [11] with slight modifications. Enzyme activity was determined by hydrolysis of BAEE solution (1 m*M* BAEE with 0.05 *M* $CaCl_2$ and 0.05 *M* Tris–HCl, pH 7.5) at 25°C. The reaction rate was measured on a Beckman DU-7 spectrophotometer at 253 nm. An increase of A_{253} by 0.001/min was defined as 1 unit of trypsin.

2.5. Protein measurement

Protein concentrations in solution were determined by the Lowry method [12].

2.6. Preparation of affinity adsorbents

A 3-g amount of chitosan was dissolved in 300 ml 1% (v/v) acetic acid and filtered to remove insoluble materials. The dissolved chitosan was extruded into basic solution [NaOH–ethanol–water (4:30:66, v/v)] according to the method of Vorlop and Klein [2] with a thin nozzle having an orifice 0.3 mm in diameter. Then the chitosan was thoroughly washed with distilled water until neutral. The chitosan beads were observed on the micrograph (Fig. 3). The diameter of a bead was about 1 mm.

In 100 ml of 0.1 *M* pH 5.5 acetate buffer (with 0.02 *M* $CaCl_2$), the chitosan beads and 220 mg OMCHI were added to adsorption. After 4 h, 3 ml glutaraldehyde (25%, v/v) was added for cross-linking. This procedure was carried out for 6 h. Then 10 ml $NaHB_4$ (1%, v/v) were added and stirred for 1 h to block any residual aldehyde functions. Matrices were filtered and thoroughly rinsed with distilled water until no more absorbance was recorded at 280 nm (absence of protein and glutaraldehyde). Thus, the affinity adsorbents were prepared. The color of the adsorbent is white.

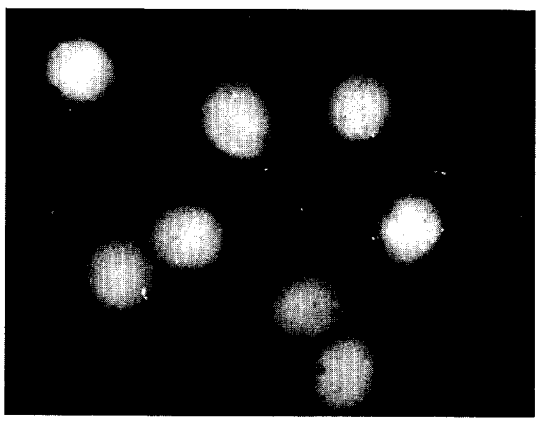

Fig. 3. Micrograph of chitosan beads.

2.7. FPLC

The automated fast protein liquid chromatography (FPLC) system (Pharmacia Biotech, Uppsala, Sweden) consisted of one motor valve MV-7 for sample application, two motor valves MV-8 to buffer selection, two high precision pumps P-500, one peristaltic pump P-1, one monitor, fraction collector, recorder and liquid chromatography controller LCC-500. A schematic diagram of the instrumental configuration is given in Fig. 4. Separation was achieved using a column (8.0×1.0 cm I.D.) packed with the affinity adsorbent.

3. Results and discussion

3.1. Adsorption capacity of affinity adsorbent

In the chitosan bead solution, different amounts of OMCHI were added. After adsorption and cross-linking, the amount of ligand immobilized on chitosan was measured. Protein immobilized on chitosan was determined by measuring the loss of solution protein in the prepared procedures. The results showed that 60 mg OMCHI could be immobilized on 1 g chitosan.

For measuring the adsorption capacity of the affinity adsorbent for trypsin, OMCHI–immobilized chitosan (0.1 g) was placed in each tube with 5 ml trypsin solution (0.05 *M* Tris–HCl, 0.02 *M* $CaCl_2$, pH 7.5) with different initial concentrations. The tube was gently stirred at 30°C for 15 min. The amount of trypsin bound to the affinity adsorbent was plotted against the trypsin concentration in the

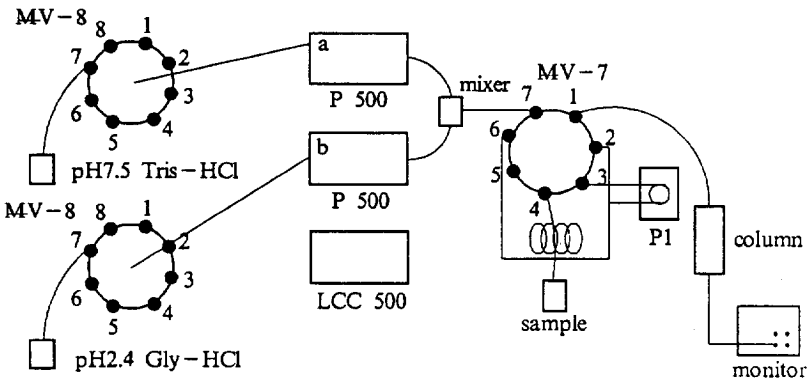

Fig. 4. Instrumental configuration of the automated FPLC system.

solution, the result is shown in Fig. 5. It was observed that the maximum binding capacity was ca. 8×10^4 U/g dry adsorbent. The adsorption capacity was very high.

3.2. Affinity chromatography of trypsin

The affinity adsorbents were packed into an 8.0 cm×1.0 cm I.D. column for FPLC and an 18.0 cm×2.0 cm I.D. column for conventional trypsin affinity chromatography, respectively. The results are demonstrated in Figs. 6 and 7. Elution was achieved by a stepwise change of buffers from 0.05 *M* Tris–HCl (pH 7.5, with 0.2 *M* $CaCl_2$) to 0.1 *M* glycine–HCl (pH 2.4). Eluted peaks were detected by the UV absorbance at 280 nm.

In FPLC, two elution peaks were observed and the second one showed trypsin activity. In conventional affinity chromatography, one elution peak was observed.

In order to demonstrate whether chitosan could be used as carrier of affinity adsorbents in large-scale protein purification, the affinity columns were en-

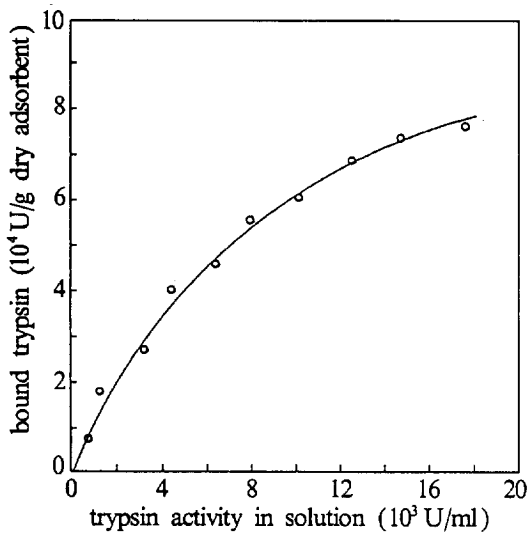

Fig. 5. Adsorption capacity for trypsin of OMCHI-immobilized chitosan. A 0.1-g amount of adsorbent was placed in each tube with 5 ml trypsin solution (0.05 *M* Tris–HCl, 0.02 *M* $CaCl_2$, pH 7.5) with different initial concentrations. The tubes were gently stirred at 30°C for 15 min. Trypsin adsorption was determined by measuring the loss in activity of the trypsin solution.

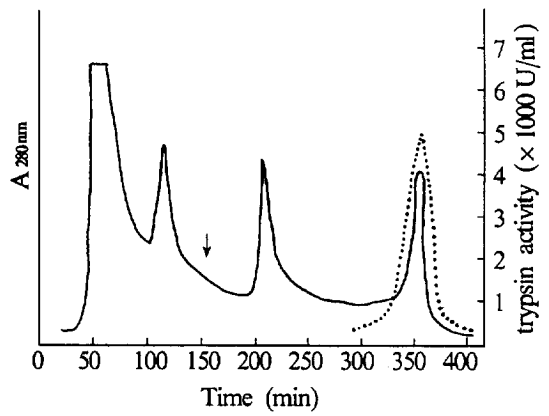

Fig. 6. Chromatogram of trypsin by FPLC, using the column packed with chitosan-based affinity adsorbents. The arrow indicates the stepwise change in the eluent solution pH from 7.5 to 2.4. Column, 8.0 cm×1.0 cm I.D.; flow-rate, 0.3 ml/min. The solid line represents the protein concentration while the dotted line represents the activity of trypsin.

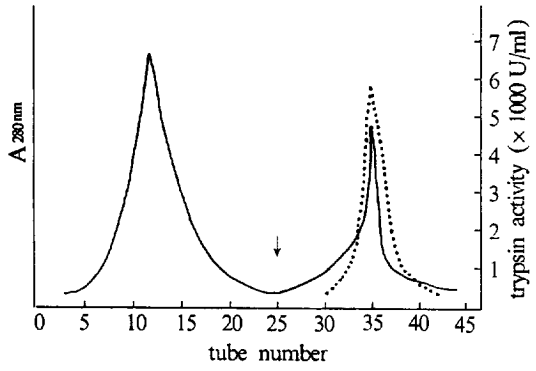

Fig. 7. Chromatogram of trypsin by affinity chromatography. The arrow indicates the stepwise change in the eluent solution pH from 7.5 to 2.4. Column: 18 cm×2 cm I.D.; flow-rate: 15 ml/h; tube fraction; 5 ml. The solid line represents the protein concentration while the dotted line represents the activity of trypsin.

larged to 21 cm×3 cm I.D. and 36 cm×4 cm I.D. The results showed that their chromatograms were similar to the one shown in Fig. 7. Their purification results are given in Table 1. The experimental results demonstrated that the specific activity of purified trypsin was high (it could reach to 7500 BAEE U/mg) and that the recovery of trypsin was 70%. Furthermore, the affinity adsorbents were not compressed in the column and liquid flow-rate did not change after the columns were enlarged. These results demonstrated that chitosan-based affinity adsorbents possessed good mechanical strength.

3.3. Non-specific interaction of chitosan

The basis of affinity chromatography is the specific interaction between the immobilized ligand and the molecule to be isolated. The affinity supports should not produce non-specific interaction, so the non-specific interaction of chitosan was studied. The chitosan was treated by using the same procedures including glutaraldehyde and $NaHB_4$, as described above only without OMCHI. It was demonstrated that there was no protein peak after pH 2.4 buffer elution on the column for trypsin chromatography that was packed with the treated chitosan. This result showed that chitosan had no non-specific interaction.

3.4. Stability of affinity adsorbents

The column packed with the affinity adsorbent was recycled four times under the above-mentioned conditions, with one use every seven days. The results showed that the purification and recovery of trypsin did not decrease and that its flow-rate did not change either.

The column which had been stored for one month at room temperature before a trypsin purification showed no change in its purification ability. The affinity column in an 0.05 *M* pH 7.5 Tris–HCl (with 0.02 *M* $CaCl_2$) buffer which had been stored for 1.5 years at 4°C before a trypsin purification could provide a 15.2% recovery also. These results indicated that the chitosan-based affinity adsorbent had good storage stability.

The currently available affinity supports for the preparation of affinity adsorbents, e.g. Sepharose 4B, are expensive and are usually activated using cyanogen bromide, which is highly toxic, whereas chitosan does not require CNBr activation. Ligands could be immobilized on chitosan by a crosslinker, glutaraldehyde, which is a much simpler and safer operation than that used where Sepharose was carrier. The affinity adsorbents prepared with chitosan as support have characteristics of high adsorption

Table 1
Purification of the trypsin by affinity chromatography with various columns

Results	Column					
	18×2 cm I.D.		21×3 cm I.D.		36×4 cm I.D.	
	Sample	Elution	Sample	Elution	Sample	Elution
Total activity (U)	$1.8 \cdot 10^5$	$1.26 \cdot 10^5$	$4.5 \cdot 10^5$	$3.16 \cdot 10^5$	$1.26 \cdot 10^6$	$8.8 \cdot 10^5$
Total protein (mg)	62.4	16.8	156	42.2	436.8	117.2
Specific activity (U/mg)	2885	7500	2885	7488	2885	7509
Purification factor		2.60		2.60		2.60
Yield (%)		70.0		70.2		69.8

capacity, good mechanical strength and no non-specific adsorption. In addition to the above properties, the purification results did not change after the affinity column was enlarged. These results indicated that chitosan is suitable as a carrier of affinity adsorbents.

Acknowledgments

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