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# Evaluation of *n*-valeraldehyde modified chitosan as a matrix for hydrophobic interaction chromatography ☆

Yun Wang[b,*], Mingliang Guo[a], Yongming Jiang[a]

[a]*College of Bioscience and Biotechnology, Yangzhou University, 225009 Yangzhou, China*

[b]*Department of Biology and Chemistry, Changshu College, 215500 Changshu, China*

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**Abstract**

The *n*-valeraldehyde modified Chitosan (pentyl-Chitosan CL) was prepared by Schiff-base formation and hydrogenation. By studying the IR spectra of Chitosan and pentyl-Chitosan CL, it is suggested that a pentyl group is linked to 2′-$NH_2$ by a C–N bond. The influence of temperature and ionic strength on the adsorption of protein on pentyl-Chitosan CL were studied, and it was found that the behavior of adsorption met with the theory of hydrophobic interaction. The storage stability of these packing materials was also investigated, the results show storage in 20% ethanol at 4 °C is the most suitable condition. α-Amylase was purified successfully by hydrophobic interaction chromatography, using pentyl-Chitosan CL as hydrophobic matrix. The purification factor is about 2.5 and the recovery is over 82%. © 2002 Elsevier Science B.V. All rights reserved.

*Keywords:* Hydrophobic interaction chromatography; Stationary phases, LC; Chitosan stationary phases; Proteins; Amylase; Enzymes

## 1. Introduction

Hydrophobic interaction chromatography (HIC) is widely used for protein separation because it sorts protein based on the differences in surface hydrophobicity of proteins under mild conditions which reduces losses due to protein denaturation [1,2]. Separation takes place by differential interaction with hydrophobic substitutes on gels and the strength of the binding depends not only on type ligand and matrix but also on type and concentration of salt, pH value and temperature [3–7]. Polysaccharide (i.e., agarose) is usually used as support in preparation of HIC matrix [8,9].

Chitosan is an economic material, which has many of the excellent chemical properties of agarose, such as chemical stability and compatibility with bioactive compounds. This work describes the evaluation of *n*-valderaldehyde modified Chitosan as a matrix for hydrophobic interaction chromatography.

## 2. Experimental

### 2.1. Materials

Chitosan prepared by the Laboratory of Enzyme Engineering, Yangzhou University, egg white lysozyme was purchased from the Institute of Biochemis-

☆This work was carried out in the Laboratory of Enzyme Engineering, Yangzhou University.

*Corresponding author. Tel.: +86-520-2771-696.

*E-mail address:* wangy1974@263.net (Y. Wang).

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try (Shanghai, China), *n*-valeraldehyde was purchased from Merck (Darmstadt, Germany). All other chemicals used were of analytical-grade quality.

### *2.2. Preparation of pentyl-Chitosan CL*

Chitosan was dissolved in 100 ml HAc (1%, v/v) solution. All insoluble materials left were removed by filtration. The pH of the above solution was adjusted to 5.0 using 3 *M* NaOH. Gluteraldehyde (3 ml, 10%) was added to the Chitosan-HAc solution and stirred at room temperature for 1 h. The cross-linked Chitosan (Chitosan CL) thus obtained was then treated with 10 ml $NaBH_4$ overnight for the purpose of quenching all aldehyde groups and hydrogenating C=N bonds. After extensive washing, the cross-linked Chitosan was suspended in 100 ml distilled water (pH 6.0), and 2.0 ml valderaldehyde was then added and stirred at room temperature for 6 h to obtain the *n*-valeraldehyde modified chitosan (pentyl-Chitosan CL). In order to hydrogenate the products of the Schiff-base reaction, pentyl-Chitosan CL was treated with 10 ml 5 *M* $NaBH_4$.

### *2.3. Spectroscopic measurement*

The infrared spectrum was measured on a Nicolet 170SX Fourier transform (FT) IR spectrophotometer. The spectrum was recorded with a KBr pressed disk.

### *2.4. Adsorption of protein on pentyl-Chitosan CL*

The binding of protein on pentyl-Chitosan CL was determined according to Agarwal and Gupta [10]. A 5-mg amount of bovine serum albumin (BSA) was dissolved in 5 ml of phosphate buffer. The above solution was added to pentyl-Chitosan CL or Chitosan CL or Chitosan under different conditions (i.e., different temperature and salt concentration). In all cases the mixture was incubated at 30 °C for 1 h and then centrifuged at 6000 *g* for 5 min. The protein in the supernatant was measured to calculate the total bound protein.

### *2.5. α-Amylase assay*

α-Amylase activity was measured spectrophotometrically at 620 nm as previously described [11].

### *2.6. Chromatographic conditions*

Pentyl-Chitosan CL was packed into a 10 mm diameter glass column to a bed height of 150 mm, the settled gel was equilibrated with 5 m*M* phosphate buffer (pH 8.1) containing 15% (w/w) sodium sulfate. The crude α-amylase was dissolved with the equilibrating buffer to obtain a protein concentration of 3.8 mg/ml. A 1.6-ml volume of α-amylase sample was loaded. The profile of the descending gradient used for the separation is given on the chromatogram.

### *2.7. Protein determination*

Protein concentration was determined spectrophotometrically at 280 nm, using BSA as standard protein.

## 3. Results and discussion

### *3.1. The effect of salt concentration on the adsorption of protein*

It is well known from other studies, that the hydrophobic interaction is promoted by high salt concentration [12]. The effect of salt concentration on the adsorption of protein on pentyl-Chitosan CL was determined. A control experiment was carried out using Chitosan and Chitosan CL.

Results are shown in Fig. 1. Here, with increasing salt concentration, the adsorption of protein on pentyl-Chitosan increased dramatically. However, the adsorption of protein on Chitosan or Chitosan CL varied gently with increasing salt concentration. The results show the binding of protein to pentyl-Chitosan CL was mostly due to hydrophobic interaction.

### *3.2. The effect of temperature on the adsorption of protein*

The previous studies show that the temperature is one of the most important parameters for retention in HIC [13,14]. In order to investigate the effect of temperature on hydrophobic interaction, the experiment was carried out at different temperatures (Fig.

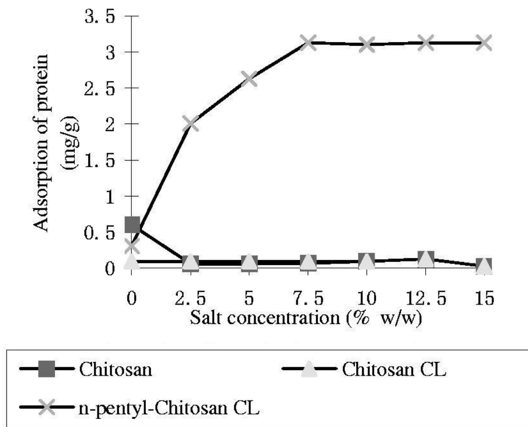


Fig. 1. The effect of salt concentration on the adsorption of protein. ■=Chitosan; ▲=Chitosan CL; ×=pentyl-Chitosan CL.

2). The results indicate that the nature of temperature plays multiple roles in the adsorption of protein. Increasing temperature enhanced not only the hydrophobic interaction (caused adsorption) but also the molecular motion (caused desorption). During 4 to 60°C, with increasing temperature, the enhancement of hydrophobic interaction is higher than that of molecular motion and the adsorption of protein increased. When the temperature is above 60°C, the increasing temperature enhanced molecular motion far more than hydrophobic interaction and the adsorption of protein decreased.

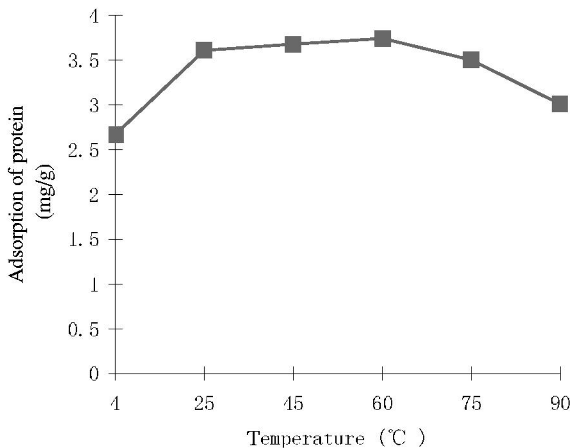


Fig. 2. The effect of temperature on the adsorption of protein.

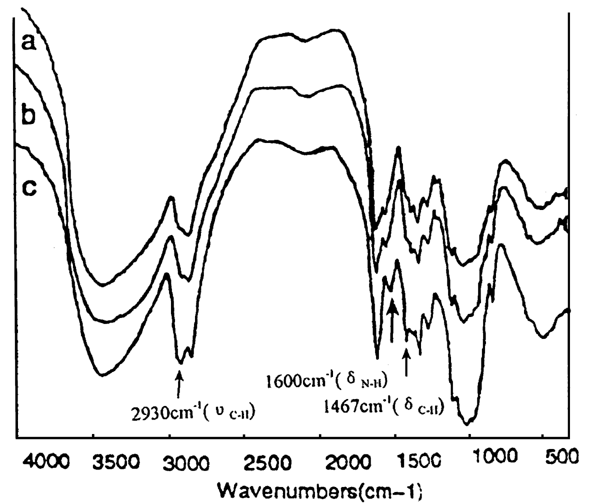


Fig. 3. IR spectra of three types of Chitosan: a=Chitosan; b=Chitosan CL; c=pentyl-Chitosan CL.

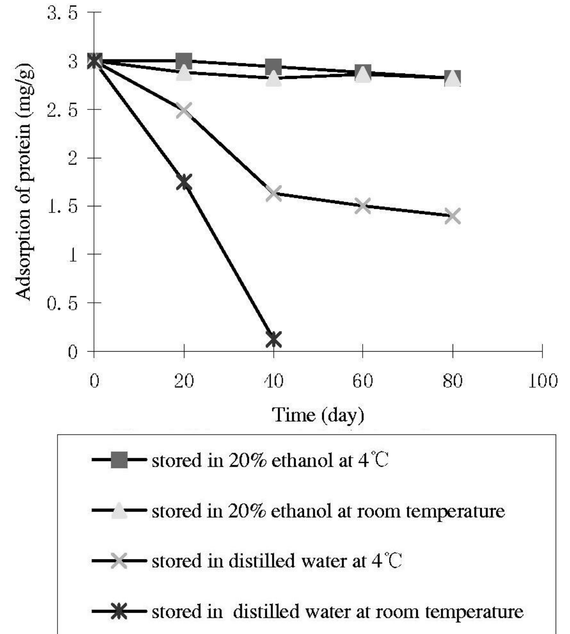


Fig. 4. Storage stability of pentyl-Chitosan CL. ■ Stored in 20% ethanol at 4°C; ▲ stored in 20% ethanol at room temperature; ×=stored in distilled water at 4°C; *=stored in distilled water at room temperature.

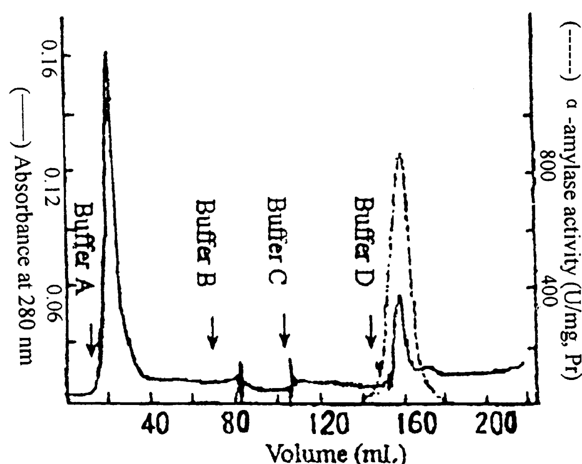


Fig. 5. Purification of α-amylase by HIC. Sample: 1.6 ml (6.0 mg) of crude α-amylase. Column size: 150×10 mm; flow-rate: 1 ml/min. Gradient elution: 0–70 min, buffer A; 70–105 min, buffer B; 105–150 min, buffer C; 150–220 min, buffer D. Buffer A: 5 m*M* phosphate buffer containing 15% sodium sulfate; buffer B: 5 m*M* phosphate buffer containing 10% sodium sulfate; buffer C: 5 m*M* phosphate buffer containing 5% sodium sulfate; buffer D: 5 m*M* phosphate buffer.

### *3.3. IR spectrum of pentyl-Chitosan CL*

The infrared spectrum of pentyl-Chitosan CL was investigated, and the control experiment was carried out with Chitosan and Chitosan CL (Fig. 3). The results indicate that an obvious change takes place after modification with *n*-valeraldehyde. Two strengthening adsorption bands at about 2930 $cm^{-1}$ ($\nu_{C-H}$ of $CH_2$ group) and 1467 $cm^{-1}$ ($\delta_{C-H}$ of $CH_2$ group) were observed. That means an alkyl group was linked to Chitosan CL during the modification with *n*-valderaldehyde. A weakened band in the region of 1600 $cm^{-1}$ ($\delta_{N-H}$ of NH group) was also observed, which indicates the 2′-$NH_2$ has been changed into secondary amine after the modification. And there is no obvious adsorption at about 2120~2185 $cm^{-1}$ (due to $\nu_{C=N}$). All these results show that the alkyl group was linked to the –$NH_2$ group by a C–N bond.

### *3.4. Storage stability of pentyl-Chitosan CL*

Pentyl-Chitosan CL was stored under four different conditions. Fig. 4 shows that compared with the other three storage conditions, after 80-day storage in 20% ethanol at 4°C, the adsorption quantity decreased a little. The most possible reason was the ethanol and low temperature restraining the microbiological activity. So 20% ethanol and 4 °C was the most suitable condition.

### *3.5. Purification of α-amylase*

Fig. 5 shows the purification of α-amylase by HIC. The purification factor was 2.5 and activity yield was over 82% (Table 1).

## 4. Conclusion

Over about the last decade, hydrophobic interaction chromatography has been widely employed for the separation of bioactive compounds [15–18]. Unlike other chromatographic methods, fewer separation media are available for this chromatography and it is largely dominated by matrices modified by attaching alkyl or aryl groups. The present work explores the behavior of a different matrix, i.e., Chitosan with a novel modification viz. valeraldehyde. The IR spectrum analysis shows a pentyl group was linked to –$NH_2$ by a C–N bond. The behavior of the binding protein to pentyl-Chitoan CL was investigated. The binding was clearly mediated by hydrophobic interaction; this was indicated by the observation that increasing salt concentration in-

Table 1
Purification of α-amylase by HIC

| Analyte | Total activity (U) | Total protein (mg) | Specific activity (U/mg, protein) | Yield (%) | Purification factor |
|---|---|---|---|---|---|
| Sample | 2073.6 | 6.0 | 345.6 | 100.0 | 1.0 |
| Peak I | 0.0 | 3.1 | 0.0 | 0.0 | 0.0 |
| Peak II | 1717.0 | 2.0 | 858.5 | 82.4 | 2.5 |

creased the binding. This met with the theory of hydrophobic interaction.

An attempt was made to purify α-amylase by HIC, using pentyl-Chitosan CL as packing material. The eluted α-amylase showed a 2.5-fold purification and the recovery was over 82%. Thus, pentyl-Chitosan CL described here appears to be a useful packing material for hydrophobic interaction chromatography. The most serious disadvantage of the packing material is its low mechanical strength. We are currently trying to improve the mechanical strength by cross-linking reactions and by coating Chitosan on silica gel.

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