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Silica-supported Macroporous Chitosan Bead for Affinity Purification of Trypsin Inhibitor

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Abstract: Macroporous cross-linking chitosan layer coated on silica gel (CTS-SiO_2) was prepared by phase inversion and polyethylene glycol (PEG) molecular imprinting methods. Formation of macroporous surface was investigated by scanning electron microscopy (SEM) and BET analysis. The prepared bead was activated by reacting with 1,2-ethylene diglycidyl ether for introducing epoxy groups, and trypsin could be efficiently immobilized on the bead as a biospecific ligand. The bead bearing trypsin was employed to purify trypsin inhibitor (TIs) from egg white as affinity adsorbent.

Keywords: Chitosan, silica, trypsin inhibitor, trypsin, affinity purification.

Recently, interests for trypsin inhibitors (TIs) had been raised in the fields of pharmacology and medicine based on their biological activities to prevent or suppress carcinogen-induced transformation, as detected in various in *vitro* and in *vivo* model systems. Various procedures for the purification of TIs had been proposed including different chromatographic approaches or preparative electrophoresis[1,2]. Amongst, affinity chromatography was recognized as the powerful tool owing to its high specificity, and usually employed affinity chromatographic adsorbent was sepharose bead immobilized with affinity ligands. However, such adsorbents were too expensive for large scale preparation[3]. Moreover, toxic reagent CNBr was needed to be an activator for coupling bio-ligands on the sepharose beads. An effective and inexpensive adsorbent for the purification of the TIs would help both studies on the biological properties and their applications in various fields. Chitosan was considered as one of the potential chromatographic matrices considering its promising characteristics including biocompatibility, hydrophilicity, biodegradability and low cost. Besides, amino groups on its polyglucosamine chains made it a suitable affinity chromatographic matrix for coupling bioligands. However, pure chitosan gel prepared in the form of beads exhibited low mechanical strength and large operation pressure[4-7]. In this work, we had prepared a new silica-supported macroporous chitosan bead coupled with trypsin, and the TIs purification on this affinity adsorbent was also reported.

Trypsin coupled bead was obtained through three steps: preparation of CTS-SiO_2 with macroporous surface, epoxy activating and trypsin coupling.

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0.2 g chitosan and 1 g PEG 20000 were dissolved in 10 mL 1 mol/L aqueous acetic acid. 10 g silica gel was immersed in the solution overnight before drying in vacuum**.** After chitosan-coated silica gel was dissipated in DMSO with stirring, small amount of concentrated NaOH solution was added till pH reached 9.0. Stirring the mixture was continued for 30 min to extract the PEG in order to form a macroporous surface. The solid was filtered and re-suspended in DMSO containing 0.1 mol/L NaOH and 1 mol/L epichlorohydrin at 60℃ for 8 hours with stirring. Then the cross-linking matrix was shaken in 0.85 mol/L ammonia solution at 60℃ for 4 hours[6, 7]. After the residual ammonia was removed with pure water, the CTS-SiO_2 was obtained. **Figure 1** presented the morphologies of SiO_2 and CTS-SiO_2, which was acquired by gold plating the dried powers and scanning them on a SIRION (FEI, USA) scanning electron microscopy (SEM). It could be found that a macroporous chitosan layer was formed on the silica bead after coating, PEG removing and crossing-linking step. BET analysis using Omnisorp 100CX (Coulter Omnisorp, USA) showed that specific surface area was 96.89 m^2/g and pore volume was 0.4063 mL/g with macroporous structure (pore diameter≥50 nm, 67.8%). The low pore volume might be ascribed to the special interconnected porous structure formed by the chitosan layer on the surface. Such porous surface might favor the accessibility for the target macromolecular binding. Moreover, distorting of the enzyme conformation usually occurring in non-porous matrix might be avoided with sufficient interaction sites at porous surface [8].

A diagram of the matrix activation and trypsin immobilization process was showed in **Figure 2**. In the surface modification procedure, 1,2-ethylene diglycidyl ether was used as activator to introduce epoxy groups and spacer arm. Activating of the matrix was achieved by instillation of 100 mL 1,2-ethylene diglycidyl ether in 2 h into a mixture of 100 g CTS-SiO_2, 100 mL DMSO and 100 mL 0.3 mol/L NaOH. The reaction lasted for 3 hours with stirring. After extensively washed with water, 1g unactivated CTS-SiO_2 or epoxy-activated bead was incubated in trypsin solution (*Bovine pancreas*, tissue culture, ≥250 U/mg, dissolved in 50 mmol/L Tris-HCl plus 10 mmol/L mol/L $CaCl_2$, pH 8.5) with total activity of 1250 U. The immobilization proceeded overnight at 4℃ before the remained epoxy groups was blocked with mercaptoethanol[9]. The residual activity in solution after incubation and the final activity on per gram dry bead was assayed using N-benzoylarginine-P-nitroanilide (BAPNA) as substrate according to the reference method[10]**.** The corresponding values were 380U and 594U for activated

Figure 1 SEM of the surface of SiO_2 and CTS-SiO_2

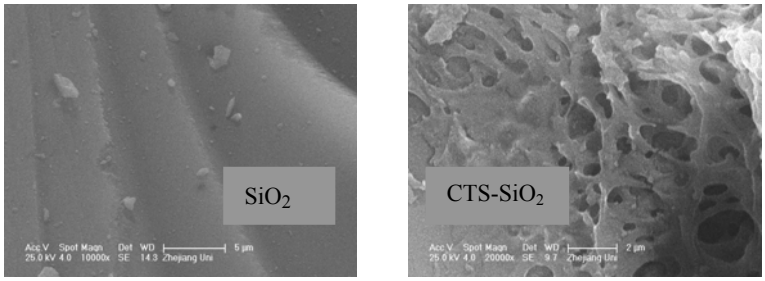
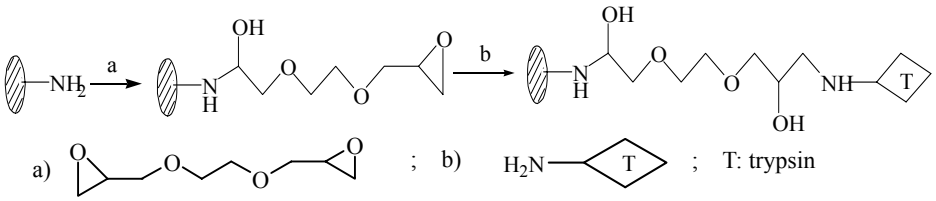


Figure 2 Activation of CTS-SiO_2 *via* bis-epoxide reagent and trypsin immobilization

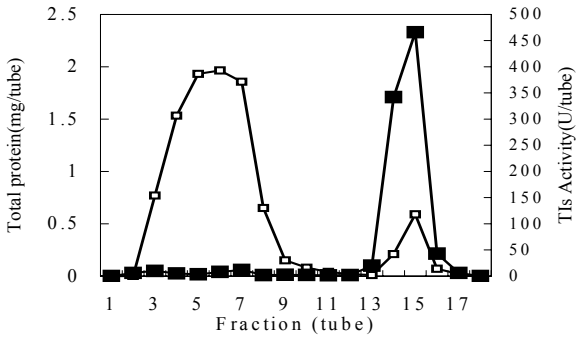


bead, while 1230 U and 8 U for unactivated bead. 8 U only accounted for 0.64% of the initial 1250U, which indicated physical adsorption of the trypsin on the unactivated matrix could be almost neglected. Unactivated CTS-SiO_2 bead was also incubated with prepared egg white solution overnight. The result showed that the unfavorable non-specific adsorption for target molecular could be completely neglected (figure was not shown for brief), which was different from the situation of strong nonspecific adsorption existing between porous silica and proteins[11]. The phenomenon might be ascribed to the coating of hydrophilic chitosan layer on porous silica support.

Chemical stability of the prepared CTS-SiO_2 was investigated by incubation the bead in 0.1 mol/L NaOH solution and the enzyme immobilization on the alkaline-treated beads was evaluated. The results showed that drawback of pH instability of SiO_2 could be overcome by the chitosan coating, which was in accordance with results reported by Shi Q. H[5]. Meanwhile, no obvious volume expanding or shrink of the matrix was observed in various conditions due to the rigid support of silica gel and the high dense cross-linking of the chitosan.

Trypsin inhibitory ability of ovomucoids from avian egg white had long been known and widely studied[1]. The treatment of egg white and activity measurement of TIs were according to the methods of Ruckenstein E.[9] Column packed with the prepared trypsin-coupled CTS-SiO_2 was used to purify TIs from egg white in the following way: After the column（1.0×20 cm, bed volumn 10 mL）was equilibrated with 50 mmol/L Tris-HCl buffer containing 10 mmol/L $CaCl_2$ and 0.1 mol/L NaCl (pH 7.4), 10 mL crude egg white sample was loaded by peristaltic pump at flow rate of 0.5 mL/min (1-3 tube). Then the column was washed with equilibrating buffer for two-column volume (4-10 tube) before elution was carried out with a 4 mol/L urea aqueous solution (11-18 tube) at the flow rate of 1 mL/min. The eluted fraction (3 mL/tube) was collected for the determination of protein content and TIs activity. **Figure 3** showed that a large protein peak with little inhibition activity was found in the sample loading and washing steps, while a large activity peak with little protein content was observed in the elution step. The specific inhibitory activity of the product was 1572U per mg total protein with 54.4% activity recovery. Purification fold could reach 10.2 after one-step affinity purification compared with the initial inhibitory activity of 154U per mg total protein in the loading solution. The results indicated that the affinity column had the high specificity for the target molecule. We also found that the affinity ability of the column did not decrease significantly after the re-equilibrium or long terms of storage. The results might be ascribed to the high stability of the immobilized enzyme coupled on the

Figure 3 Chromatogram of TIs separation from egg white on trypsin coupled CTS-SiO_2



Hollow rectangle: total protein content; Solid rectangle: TIs activity

high dense epoxy activated matrix with a multi-point attachment mode[12]. So, the affinity adsorbent could be reused.

In conclusion, the prepared adsorbent combined the advantages of traditional soft gel and the excellent mechanical property of the inorganic material. The preparation route was simple as well as the performance was satisfactory.

Acknowledgments

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