Non-porous monodisperse silica (NPS), 2 µm in diameter, was modified with 3-aminopropyltriethoxyxilane for immobilization of Cibacron Blue F3GA, a packing of NPS-ACB for high performance affinity chromatography was obtained. Up to 2 mg of Cibacron Blue F3GA could be attached to 1 ml of NPS beads. There was no obvious leakage of dye from NPS-ACB. The adsorption of lysozyme varied with pH value and ionic strength. Furthermore, the column could be used for separation and preparation of lysozyme from hen egg white. In addition, rapid separation of α-, β- , γ-globulin is also described.

**Key words**: high performance affinity chromatography, non-porous monodisperse silica, Cibacron Blue F3GA, lysozyme, α-globulin, β-globulin, γ-globulin

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**Abstract**

Non-porous monodisperse silica (NPS), 2µm in diameter, was modified with 3-aminopropyltriethoxyxilane for immobilization of Cibacron Blue F3GA, a packing of NPS-ACB for high performance affinity chromatography was obtained. Up to 2 mg of Cibacron Blue F3GA could be attached to 1 ml of NPS beads. There was no obvious leakage of dye from NPS-ACB. The adsorption of lysozyme varied with pH value and ionic strength. Furthermore, the column could be used for separation and preparation of lysozyme from hen egg white. In addition, rapid separation of α-, β-, γ-globulin is also described.

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Affinity chromatography is a well known technique for the purification of biopolymers. Conventional soft gel affinity media, owing to its unstable mechanical character, can not be used for rapid separation, which causes low mass and bioactivity recovery in separation process. With the development of high performance affinity chromatography (HPAC), silica[1], metal-oxides [2] and polymers [3,4] have been widely used as affinity media. Since the successful application of non-porous monodisperse silica in affinity chromatography in 1988[5], the use in reversed-phase chromatography[6], non-exchange chromatography[7] and hydrophobic chromatography[8] has been described.

In this study we used 2µm non-porous monodisperse silica (NPS) as matrix, Cibacron Blue F3GA as ligand, to produce an affinity packing. Experiments have been carried out to determine the interaction between Cibacron Blue F3GA and several proteins and enzymes.
aminopropyltriethoxysilane was added. The reaction mixture was heated under reflux for 24h. The modified silica was extracted in toluene and trichloromethane for 2 days in order to remove the reaction components. The modified silica was stored under anhydrous conditions until use.

The modified silica was suspended in 0.1 mol/L sodium phosphate, at pH 8.0, containing 0.5 mol/L KCl and Cibacron Blue F3GA. The reaction was carried out at 60°C for 3h. The immobilized dye matrix was washed in turn with 0.1 mol/L sodium phosphate, methyl alcohol-water (1:1), methyl alcohol, and water until the washing supernatants were colorless.

Fig. 1
Scanning electron micrograph of 2 µm non-porous silica beads

The amount of APS bonded was 56.5µmol/g from elementary analysis. The amount of dye immobilized on the modified silica was 2mg/ml by measuring the UV absorption at 620nm. This value was higher than 1.2mg/ml which was obtained by Unger[4].

Cibacron Blue F3GA leakage would interfere the assay of protein because of its UV absorption. The pH stability test showed that there was no significant dye leakage from pH3.0~6.0(Fig.2). In spite of the higher Cibacron Blue F3GA concentration at higher or lower pH value, the highest dye leakage was less than 25mg/L, which would not interfere the assay of protein.

Fig. 2
pH stability test

The interaction of protein and triazine dye is very complex. Ion exchange and hydrophobic interaction were expected owing to the presence of amino, sulphonic groups and polyyclic aromatic rings in the dye molecule. We studied the effect of different experimental conditions on the chromatography of lysozyme. The adsorption of lysozyme increased with the pH value (Fig.3). Lysozyme is positive at pH 5.0~8.0 because of its high pI (11.0). In case of higher pH values, dye bears higher density of negative charges and ionic interaction is enhanced. Fig.4 shows that the adsorption of lysozyme decreases with the increased of ionic strengths. It could be said that ion exchange was the dominant force between lysozyme and Cibacron Blue F3GA. So the elution could be carried out by increasing the ionic strength.

Fig. 3
The amount of lysozyme adsorbed at different pH value

Ovalbumin (Oval) and lysozyme from hen egg white showed different affinity to Cibacron Blue F3GA. Oval could not be retained. Its retention time did not vary with injection amount. Lysozyme was adsorbed on the column. Its mass concentration was proportional to the peak area(Fig.5). The mass recovery of lysozyme was more than 95%.

Fig. 4
The amount of lysozyme adsorbed at different ionic strength

Lysozyme was monitored using the non-porous silica diethylylamine(NPS-DEA) column. The retention time of the crude lysozyme with and without dialysis was accordant to that of lysozyme(Fig.8).

Fig. 5
Chromatogram of Oval and lysozyme Chromatographic conditions as in Fig.4 a: Oval; b: lysozyme

In addition, α-globulin could not be retained by the packing, while β- and γ-globulin could be adsorbed on the column. γ-Globulin could be eluted by 1 mol/L KCl and 20% glycol (v/v), while β-globulin was not able to be eluted by the same eluent(Fig.9). The difference in affinity interaction could be used to separate the three

Fig. 6
Chromatogram of Oval and lysozyme Chromatographic conditions as in Fig.5

Lysozyme was monitored using the non-porous silica diethylylamine(NPS-DEA) column. The retention time of the crude lysozyme with and without dialysis was accordant to that of lysozyme(Fig.8).
aminopropyltriethoxysilane was added. The reaction mixture was heated under reflux for 24h. The modified silica was extracted in toluene and trichloromethane for 2 days in order to remove the reaction components. The modified silica was stored under anhydrous conditions until use.

The modified silica was suspended in 0.1 mol/L sodium phosphate, at pH 8.0, containing 0.5 mol/L KCl and Cibacron Blue F3GA. The reaction was carried out at 60°C for 3h. The immobilized dye matrix was washed in turn with 0.1 mol/L sodium phosphate, methyl alcohol-water (1:1), methyl alcohol, and water until the washing supernatants were colorless.

**Scanning electron micrograph of 2**

The interaction of protein and triazine dye is very complex. Ion exchange and hydrophobic interaction were expected owing to the presence of amino, sulphonic groups and polycyclic aromatic rings in the dye molecule. We studied the effect of different experimental conditions on the chromatography of lysozyme. The adsorption of lysozyme increased with the pH value (Fig.3). Lysozyme is positive at pH 5.0~8.0 because of its high pI (11.0). In case of higher pH values, the highest dye leakage was less than 25mg/L, which would not interfere the assay of protein.


The amount of APS bonded was 56.5µmol from elementary analysis. The amount of dye immobilized on the modified silica was 2mg/ml by measuring the UV absorption at 620nm. This value was higher than 1.2mg/ml which was obtained by Unger[5]. Cibacron Blue F3GA leakage would interfere the assay of protein because of its UV absorption. The pH stability test showed that there was no significant dye leakage from pH3.0~6.0(Fig.2). In spite of the higher Cibacron Blue F3GA concentration at higher or lower pH value, the highest dye leakage was less than 25mg/L, which would not interfere the assay of protein.


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Oval could not bind on the column alone but could bind with Zn²⁺ or Mg²⁺. So the column could be used to remove most of the oval in the when preparation of lysozyme from hen egg white. Fig.7 shows that most component of hen egg white does not bind to this packing and eluted in the breakthrough peak. Lysozyme was bound and eluted by 1 mol/L KCl and 20% glycol (v/v). The purity of crude lysozyme was monitored using the non-porous silica-diethylamine(NPS-DEA) column. The retention time of the crude lysozyme with and without dialysis was accordant to that of lysozyme(Fig.8).

In addition, γ-globulin could not be retained by the packing, while β- and γ-globulin could be adsorbed on the column. γ-Globulin could be eluted by 1 mol/L KCl and 20% glycol (v/v), while β-globulin was not able to be eluted by the same eluent(Fig.9). The difference in affinity interaction could be used to separate the three proteins.
In this paper, non-porous silica was used as matrix, Cibacron Blue F3GA as ligand to attain an affinity packing NPS-ACB. The amount of ligand attached reached 2mg/ml. The stability test of pH indicated that this packing is stable. It could be used to preparation of lysozyme from hen egg white in a small scale and rapid separation of α-, β-, γ-globulin.


[Originally published in Sepu, 17, 427-430 (1999).]