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Evaluation and applications of a new dye affinity adsorbent

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Abstract

The basic properties of a new dye affinity adsorbent Toyopearl AF-Blue HC-650M and its applications to the purification of proteins were studied. The binding capacity for human serum albumin (HSA) was greater than 18 mg per ml gel. The dye leakage from Toyopearl AF-Blue HC-650M in 0.5 *M* NaOH and 0.5 *M* HCl was less compared with an agarose adsorbent. Caustic stability study also demonstrated this material withstood exposure to 0.1 *M* NaOH for 1 month with no significant loss of binding capacity for HSA. We purified human albumin from human serum and lactate dehydrogenase (LDH) from rabbit muscle extract in a single step. Sodium dodecylsulfate–polyacrylamide gel electrophoresis indicates that human albumin and LDH were highly purified.

Keywords: Affinity adsorbents; Stationary phases, LC; Albumin; Lactate dehydrogenase; Proteins

1. Introduction

Since its introduction in the early 1970s, the technique of dye ligand affinity chromatography has been widely used for protein purification [1–3]. In particular, immobilized Cibacron Blue 3GA appears to be an effective adsorbent for the purification of wide variety of proteins [4–9]. Today, the literature abounds with examples of the use of this dye in conventional low-pressure chromatography (for a review, see Ref. [1]) and in high-performance affinity chromatography [10–12] by using a variety of supports including agarose, cellulose, polyacrylamide, polyacrylate and silica (for a review, see Ref. [2]). Most of these are for analytical use, while only a few are for preparative. Among these supports, one of the first to be used for preparative use was agarose [13,14] or silica [15]. However, the use of agarose was limited [12,15], because it cannot withstand high pressure and high flow-rates and may suffer microbial degradation of its backbone resulting in ligand leakage. On the other hand, silica has much higher mechanical stability than agarose but is unstable against alkaline solution, which is used for cleaning-in-place of chromatographic columns.

An ideal adsorbent for preparative use should have the following properties: good mechanical stability so as to withstand high flow-rate; high binding capacity for proteins and protein recoveries from the adsorbent so as to increase productivity; withstanding the use of alkaline solution for cleaning-in-place; low dye-leakage.

Recently, Toyopearl AF-Blue HC-650M has been commercialized by Tosoh. According to the manufacturer, this new adsorbent was improved on the protein binding capacity and the dye leakage for

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preparative use. In this paper, we describe its basic properties and applications to the purification of proteins.

2. Experimental

2.1. Materials

Toyopearl AF-Blue HC-650M was obtained from Tosoh (Tokyo, Japan). Agarose-based adsorbent, Blue Sepharose 6 Fast Flow was obtained from Amersham Bioscience (Uppsala, Sweden). All chemicals were of the highest purity available unless otherwise stated. Sodium pyruvate, sodium phosphate monobasic and dibasic, potassium phosphate monobasic and dibasic, sodium acetate, acetic acid, sodium chloride and potassium chloride were obtained from Kishida Chemicals (Osaka, Japan). Crude lactate dehydrogenase (LDH) (Type I), pure LDH (Type II), NADH and human serum albumin (HSA; fraction V powder) were obtained from Sigma (St Louis, MO, USA). Human serum was obtained from Bio Whittaker (Walkersville, MD, USA). A molecular mass marker kit (Broad Range) and a Protein assay kit were obtained from Bio-Rad Labs. (Hercules, CA, USA). Coomassie Brilliant Blue R-250 was purchased from ICN Biomedicals (Aurola, OH, USA).

2.2. Determination of immobilized dye concentration

Immobilized dye concentration was measured by titration with a Hiranuma autotitrator COM-450 (Ibaragi, Japan). A 10-ml volume each of Toyopearl AF-Blue HC-650M or Blue Sepharose 6 Fast Flow in the swollen state was titrated in 100 ml of 0.5 *M* sodium chloride with 0.5 *M* NaOH.

2.3. Determination of adsorption capacity for HSA

A 1.0-ml swollen volume each of Toyopearl AF-Blue HC-650M or Blue Sepharose 6 Fast Flow was packed into a 12 ml Bond Elut empty reservoir column (GL Sciences, Tokyo, Japan). It was washed with 10 ml of equilibration buffer (pH 4.0 and 5.0; 0.1 *M* sodium acetate buffer, pH 6.0 and 7.0; 0.1 *M* sodium phosphate buffer). A 5.0-ml aliquot of 1% solution of HSA was charged onto the column and was equilibrated for 10 min. After 10 min, unbound HSA was eluted and the column was washed with 10 ml of equilibration buffer. Adsorbed HSA was removed with 0.1 *M* sodium phosphate buffer of pH 7.0 containing 2.0 *M* sodium chloride and 10-ml fractions were collected. HSA content was measured spectrophotometrically using the absorbance of 1.0 mg/ml at 280 nm of 0.53 [16].

2.4. Determination of dye leakage

A 200-mg aliquot of each of Toyopearl AF-Blue HC-650M and Blue Sepharose 6 Fast Flow was suspended in 4 ml of different solvents (0.1, 0.5 M NaOH or 0.1, 0.5 M HCl) and incubated at 25 °C with shaking for 24 h. The adsorbent was allowed to settle, and the supernatant was removed. The absorption at 620 nm of the supernatants was measured after appropriate adjustment to neutrality with a known volume of acid or alkali. Dye concentrations in the supernatant were estimated assuming a molar extinction coefficient of 12 750 M^{-1} cm^{-1} [17].

2.5. Caustic stability study

The caustic stability of Toyopearl AF-Blue HC-650M was tested in 0.1 and 0.5 *M* sodium hydroxide solutions at 25 °C for 1 month. One milliliter of the adsorbent was suspended with 5 ml of each solution. After treatment for an appropriate period, the stability was evaluated by measuring HSA binding capacity.

2.6. Affinity chromatography

Affinity chromatography was carried out on a column (4×0.6 cm) at 25 °C with a CCPM pump (Tosoh, Tokyo, Japan) equipped with a variable-wavelength UV detector Model UV-8000 (Tosoh, Tokyo, Japan) operated at 280 nm.

2.7. Determination of enzyme activity and protein content

The activity of LDH was determined spectrophotometrically at 340 nm using the following

conditions: 0.1 *M* potassium phosphate buffer (pH 7.0), 0.8 m*M* pyruvate and 0.2 m*M* NADH [18]. Protein was determined by the Bradford assay [19].

2.8. Sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

SDS–PAGE was carried out by the Laemmli method [20] using 8–16% polyacrylamide gel (Tefco, Tokyo, Japan). Electrophoresis was performed for 120 min at 18 mV. Detection was done by Coomassie Brilliant Blue R-250.

3. Results and discussion

3.1. Basic properties

We evaluated Toyopearl AF-Blue HC-650M compared to Blue Sepharose 6 Fast Flow which seems to be a representative among agarose adsorbents.

The immobilized dye concentrations of Toyopearl AF-Blue HC-650M and Blue Sepharose 6 Fast Flow by titration method were 16 and 6.2 μmol/ml gel, respectively. The immobilized dye concentration is usually determined by acid hydrolysis in the case of the agarose gel [2]. We applied the method to Toyopearl AF-Blue HC-650M, but it was not hydrolyzed probably because of the difference in chemical structures between Sepharose and Toyopearl. The titration method seems reasonable because the dye concentration of Blue Sepharose 6 Fast Flow is 7.3±0.6 μmol/ml gel according to the manufacturer.

The protein binding capacity at various pH values was measured with the static method using HSA. Table 1 summarizes the results. Both adsorbents have similar binding capacities for HSA, although Toyopearl AF-Blue HC-650M has higher immobilized dye concentrations. We think this phenomenon is caused by the difference in polymer network between Toyopearl and Sepharose. In the case of Toyopearl, many ligands may be immobilized on smaller pores where HSA cannot diffuse.

Table 1
Human albumin binding capacity at various pH values

pH	HSA binding capacity (mg/ml gel)	
	Toyopearl AF-Blue HC-650M	Agarose
4.0	41	39
5.0	41	37
6.0	22	23
7.0	18	18

Table 2
Comparative dye leakage study of AF-Blue HC-650M and agarose

Solvent	Concentration of Cibacron Blue 3GA (μ*M*)	
	Toyopearl AF-Blue HC-650M	Agarose
0.1 *M* NaOH	1.1	1.9
0.5 *M* NaOH	3.1	11.7
0.1 *M* HCl	4.8	6.7
0.5 *M* HCl	4.1	11.5

Lowering pH caused higher binding capacity for HSA with both adsorbents. This tendency was found by some authors [21,22], and is presumably because of ionic interactions favored by increasing positive charges on interacting protein molecules.

We studied the comparative dye leakage from Toyopearl AF-Blue HC-650M and Blue Sepharose 6 Fast Flow in acidic and alkaline solutions. Although immobilized dye adsorbents have a great potential for the preparative purification of a variety of biomolecules, it is said that a major problem is dye leakage from the adsorbents [23]. Table 2 summarizes the results. The dye leakage from Toyopearl AF-Blue HC-650M was less compared to Blue Sepharose 6 Fast Flow.

Fig. 1 shows the results of the caustic stability study of Toyopearl AF-Blue HC-650M for 1 month. Repeated use of immobilized dye adsorbents with crude protein extracts might lead to fouling them with denatured proteins, lipids, lipoproteins and other

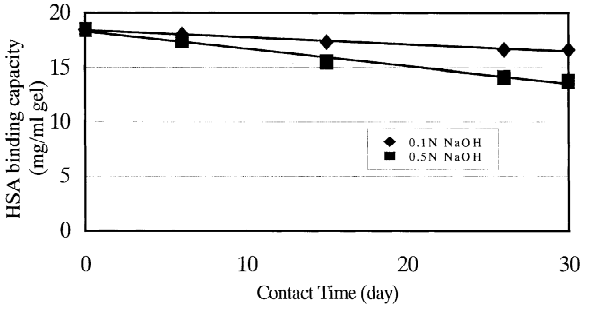

Fig. 1. Caustic stability study of Toyopearl AF-Blue HC-650M.

lipophilic materials. Usually in such cases, 0.1–0.5 *M* NaOH, 6–8 *M* urea, detergents or several organic solvents are used for cleaning of chromatographic adsorbents. We investigated the stability in 0.1–0.5 *M* NaOH. The stability of the adsorbent was evaluated by the determination of the remaining binding capacity for HSA. Fig. 1 shows that Toyopearl AF-Blue HC-650M withstood exposure to 0.1 *M* NaOH for long periods (30 days) with no significant loss of binding capacity for HSA; 0.5 *M* NaOH can be used for a short time, however, longer contact time decreases the binding capacity for HSA.

With regard to the mechanical stability, we did not study this because it is well known that the base material of Toyopearl AF-Blue HC-650M, that is Toyopearl HW-65M (40–90 μm), is a hydrophilic polymer-based material of large pore size and has high physical stability and excellent flow characteristics [24].

3.2. Applications to protein purification

Fig. 2 shows the purification of HSA from human serum on Toyopearl AF-Blue HC-650M. HSA was eluted stepwise with 1.5 *M* potassium chloride. Fig. 3 shows SDS–PAGE of the fractions indicating that the purity of HSA was nearly the same as that of HSA fraction V.

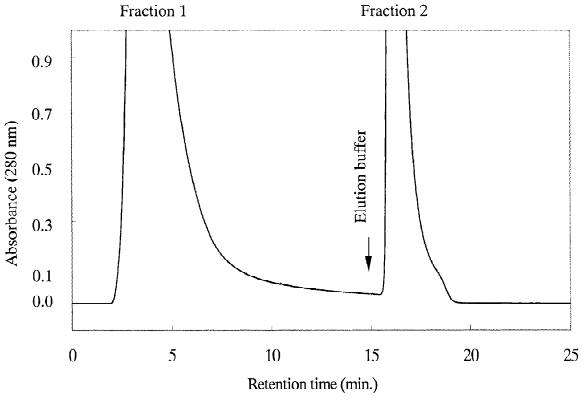

Fig. 2. Chromatogram of human serum on Toyopearl AF-Blue HC-650M. A column (4×0.6 cm) was equilibrated with potassium phosphate buffer (0.05 *M*, pH 7.0) at a flow-rate of 1.0 ml/min at 25 °C. Twofold diluted human serum (0.4 ml) was applied and the column washed with potassium phosphate buffer (0.05 *M*, pH 7.5) until the absorbance at 280 nm was <0.05, whence bound protein was eluted with potassium phosphate buffer (0.05 *M*, pH 7.5) containing 1.5 *M* potassium chloride (elution buffer).

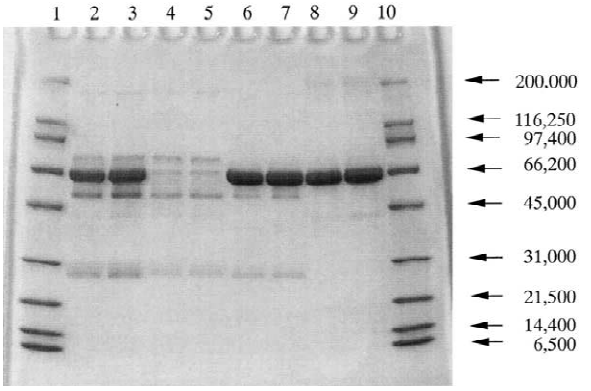

Fig. 3. SDS–PAGE analysis of the purity of each fraction in Fig. 2 eluted from the Toyopearl AF-Blue HC-650M and stained with Coomassie Brilliant Blue G-250. Lanes 1 and 10, molecular mass markers; 2 and 3, applied sample; 4 and 5, fraction 1; 6 and 7, fraction 2; 8 and 9, human albumin fraction V.

Fig. 4 shows the purification of LDH from a crude rabbit muscle on Toyopearl AF-Blue HC-650M. LDH was eluted stepwise with 1.5 *M* potassium chloride. Fig. 5 shows SDS–PAGE of the fractions indicating that the purity of the eluted protein is

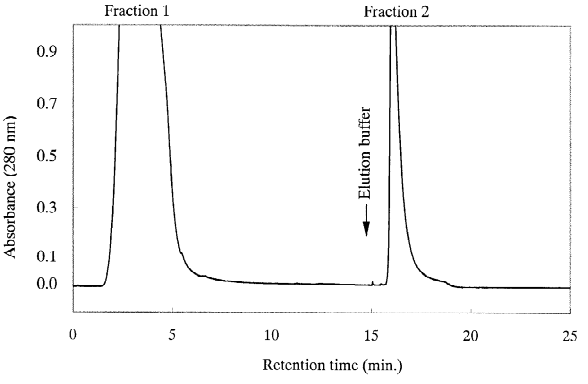

Fig. 4. Chromatogram of crude lactate dehydrogenase on Toyopearl AF-Blue HC-650M. A column (4×0.6 cm) was equilibrated with potassium phosphate buffer (0.05 *M*, pH 7.0) containing 0.225 *M* potassium chloride at a flow-rate of 1.0 ml/min at 25 °C. Fourfold diluted crude lactate dehydrogenase (1.2 ml) was applied and the column washed with potassium phosphate buffer (0.05 *M*, pH 7.5) containing 0.225 *M* potassium chloride until the absorbance at 280 nm was <0.01, whence bound protein was eluted with potassium phosphate buffer (0.05 *M*, pH 7.5) containing 1.5 *M* potassium chloride (elution buffer).

Table 3
Purification of lactate dehydrogenase from crude rabbit muscle using Toyopearl AF-Blue HC-650M

Stage	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude sample	7.5	502.1	67.0	100	1
Eluted fraction 2	0.5	441.0	868.2	87.8	13.0

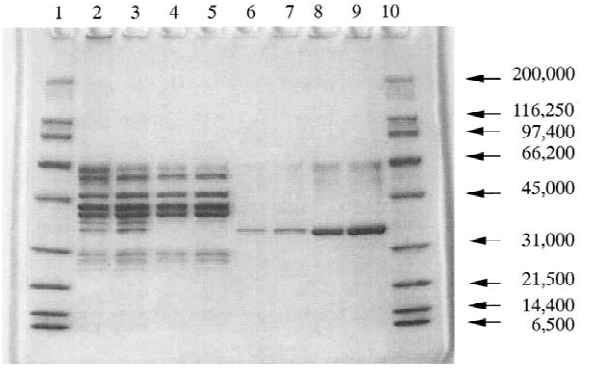

Fig. 5. SDS–PAGE analysis of the purity of each peak in Fig. 4 eluted from the Toyopearl AF-Blue HC-650M and stained with Coomassie Brilliant Blue G-250. Lanes 1 and 10, molecular mass markers; 2 and 3, applied sample; 4 and 5, fraction 1; 6 and 7, fraction 2; 8 and 9, pure lactate dehydrogenase.

comparable to that of the commercially available LDH sample. Table 3 shows the data for the purification of LDH. Eluted fraction with 1.5 *M* potassium chloride shows a 13-fold increase in the specific activity in 88% recovery yield. We also applied the specific elution using 5 m*M* NADH to the purification of LDH but the efficiency was similar compared with potassium chloride elution (data not shown).

4. Conclusion

It was demonstrated that Toyopearl AF-Blue HC-650M provides high binding capacity for human albumin, higher stability against acidic or alkaline solution and efficient separation of human albumin and LDH. Toyopearl AF-Blue HC-650M is a suitable adsorbent for preparative use because of its high mechanical stability, high binding capacity, high chemical stability, high protein recovery and easy regeneration.

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