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Trace adsorption of positively charged proteins onto Sepharose FF and Sepharose FF-based anion exchangers

Lin-Ling Yu, Yan Sun*

Department of Biochemical Engineering and Key Laboratory of Systems Bioengineering of Ministry of Education, School of Chemical Engineering and Technology, Tianjin University, Tianjin 300072, China

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ABSTRACT

Agarose-based matrices have been widely used in ion exchange chromatography (IEC). We have herein observed that positively charged proteins (lysozyme and cytochrome *c*) are adsorbed on the agarose-based anion-exchangers (Q and DEAE Sepharose FF gels) in a capacity of 10–40 μg/mL. In contrast, negatively charged protein (bovine serum albumin) is not adsorbed to Sepharose FF and SP Sepharose FF gels. Elemental analysis of the gel indicated that the residual anionic sulfate groups in agarose would have worked as the cation exchange groups for the positively charged proteins. The trace adsorption behavior of lysozyme onto Sepharose FF and Sepharose FF-based anion exchangers was studied and the effects of NaCl concentration and cation group density on the adsorption were examined for better understanding of the trace adsorption in chromatographic processes. At NaCl concentrations less than 0.05 mol/L, which is the normal adsorption condition in IEC, the trace adsorption kept at a high level, so this trace adsorption cannot be avoided in the ionic strength range of routine IEC operations. Grafting poly(ethylenimine) (PEI) chain of 60 kDa to a cation group density of 700 mmol/L could reduce the adsorption capacity to about 20 μg/mL, but further reduction was not possible by increasing the cation group density to 1200 mmol/L. Therefore, attentions need to be paid to the phenomenon in protein purification practice using agarose-based matrices. The research is expected to call attentions to the trace adsorption on agarose-based matrices and to the importance in the selection of the suitable solid matrices in the production of high-purity protein products in large-scale bioprocesses.

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1. Introduction

Nowadays agarose-based matrices have achieved increasing importance in biochemical and biotechnology research, such as immunodiffusion, electrophoresis, and especially chromatography, because their physical and chemical properties approach those of an ideal supporting medium for diffusion and electrokinetic movement of biopolymers. Commercial representatives of agarose-based chromatographic matrices are Sepharose and Superose from GE Healthcare, Bio-Gel A from Bio-Rad Laboratories, Ultrogel A form Pall Corporation, etc. However, agarose-based matrix also has a disadvantage in its more or less residual negative charges. The negative charges root in the production of agarose from agar and may cause undesirable adsorption of basic substances.

Agar is a heterogeneous mixture of two polysaccharides, agarose and agaropectin, as determined by Araki [1]. Agarose is a completely neutral polysaccharide with a linear structure of repeated units of disaccharide agarobiose, 1,3-linked-β-D-galactose and 1,4-linked-3,6-anhydro-α-L-galactose (Fig. 1). Agaropectin is negatively charged polysaccharides containing the above sugars substituted with acid groups, such as sulfate, pyruvic, and glucuronic acid, in varying amounts. Therefore, most attempts for obtaining neutral agarose were done by fractionating agar, such as precipitation of agaropectin with cetylpyridinium chloride [2], precipitation of agarose with ammonium sulfate and acetone [3], and adsorption of agaropectin on DEAE-Sephadex A-50 [4]. However, there may be still considerable amount of charged groups left in the fractionation product, and it is impossible to obtain completely neutral agarose by merely fractionating agar [4,5]. So, a practical definition for commercial agarose was proposed as the mixture of agar molecules with lowest charge content, less than 0.6% sulfate and 0.05% pyruvic acid [6]. In 1970s, a method, which circumvented the cumbersome isolation of agarose from agar, for preparing agar and agarose with very low adsorption capacity and cross-linking simultaneously, was developed [5,7]. After desulfation and reduction, sulfur content of agar and agarose could reduce to 0.004% and 0.014%, respectively [5]. Nevertheless, the micro-amount of sulfur still cause micro adsorption of positively charged proteins, such as cytochrome *c* [7] and histone [5].

* Corresponding author. Tel.: +86 22 27404981; fax: +86 22 27406590.
E-mail address: ysun@tju.edu.cn (Y. Sun).

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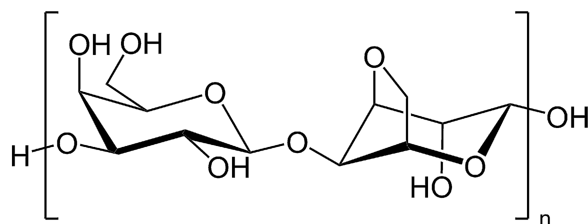

Fig. 1. Repeated units of agarose.

In 1984, Pharmacia Fine Chemicals AB (now GE Healthcare) introduced Sepharose FF on the market. Sepharose FF series of agarose-based matrices are supposed to have very low capacities of undesirable adsorption, as the cross-linked agarose discussed previously [7], and they have been widely used in protein chromatography, such as size exclusion chromatography (Sepharose FF) [8], ion exchange chromatography (IEC) (Q and DEAE Sepharose FF) [9,10], hydrophobic interaction chromatography (Phenyl Sepharose FF) [11,12], and affinity chromatography (rProtein A Sepharose FF) [13,14]. However, we have herein found that a trace adsorption of positively charged proteins on the anion-exchangers, caused by the residual charged polysaccharides, still exists, especially in low ionic strength, which is the normal loading condition in IEC operations. Because the trace adsorption is usually ignored in IEC practices, it may to some extent degrade the product purity and/or recovery in large scale processing.

In this paper, positively charged lysozyme was used as the model protein to investigate the trace adsorption of positively charged proteins on Sepharose FF and Sepharose FF-based anion exchangers. Cytochrome *c* (positively charged) and bovine serum albumin (negatively charged) were used as the positive and negative controls, respectively. Comparisons of the lysozyme adsorption at different NaCl concentrations and those on different resins are performed, which would benefit in understanding the effect of the trace adsorption in practical chromatographic processes. Moreover, the experimental data reported herein are expected to call attention to the trace adsorption in ion exchange chromatographic operations and to benefit the selection and design of suitable chromatographic matrices for the large-scale production of high-purity therapeutic proteins.

2. Materials and methods

2.1. Materials

Sepharose FF, DEAE Sepharose FF, Q Sepharose FF and SP Sepharose FF were purchased from GE Healthcare (Uppsala, Sweden). Poly(ethylenimine) (PEI) (molecular weight, 60 kDa) grafted resins (FF-PEI-L700 and FF-PEI-L1200) used in this work were prepared with Sepharose FF as described earlier [15]. The physical properties of the solid matrices are listed in Table 1.

Table 1
Physical properties of the resins.[a]

Resin	Cation group density (mmol/L)	ε_0	ε_p
Sepharose FF	0	0.80 ± 0.02	0.71 ± 0.03
DEAE Sepharose FF	160 ± 6	0.78 ± 0.01	0.60 ± 0.03
Q Sepharose FF	269 ± 10	0.79 ± 0.02	0.56 ± 0.04
FF-PEI-L700	699 ± 14	0.78 ± 0.04	0.21 ± 0.05
FF-PEI-L1200	1229 ± 62	0.73 ± 0.06	−0.06 ± 0.05

ε_0: total porosity of a resin; ε_p: effective porosity of a resin for lysozyme in equilibration buffer (20 mmol/L Tris–HCl, 1 mmol/L EDTA, pH 8.5).
[a] Data from Yu et al. [15].

Chicken egg white lysozyme (M_W ~14 300, pI ~11.4), horse heart cytochrome *c* (M_W ~12 384, pI ~10.0), and bovine serum albumin (BSA) (M_W ~66 400, pI ~4.9) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Tris(hydroxymethyl)aminomethane (Tris) was obtained from Genview (Houston, TX, USA). Ethylenediaminetetraacetic acid (EDTA) disodium, sodium chloride (NaCl) and other reagents were of analytical grade from Guangfu Fine Chemical Research Institute (Tianjin, China). Protein solutions were prepared by dissolving in equilibration buffer (20 mmol/L Tris–HCl, 1 mmol/L EDTA, pH 8.5). The protein content was adjusted photometrically with a Lamda 35 UV/VIS spectrophotometer (Shelton, CT, USA) at 280 nm or 550 nm, using the extinction coefficients $E^{1\%}$ (280 nm) = 26.3 for lysozyme [16], E^{mM} (550 nm) = 8.4 for cytochrome *c* [17], and E^{mM} (280 nm) = 45.5 for BSA [18].

2.2. Chromatographic experiments

Tricorn™ 5/50 column and a 25-μL sample loop were from GE Healthcare (Uppsala, Sweden). Chromatographic experiments were performed on an ÄKTA Purifier™ 10 (GE Healthcare, Uppsala, Sweden) at 25 °C.

The resins listed in Table 1 were used in the chromatographic experiments. Before packing, each resin was washed three times with the elution buffer (equilibration buffer plus 1 mol/L NaCl). The suspension was loaded to the Tricorn™ 5/50 column and the particles were packed by gravity settling. The column was then packed at 1.5 mL/min (450 cm/h) for at least three column volumes (CVs) and a stable packed-bed of 5.5 ± 0.4 cm in length (1.08 ± 0.08 mL in volume) was obtained.

Isocratic retention volumes (the system dead volume has been taken off) and the protein adsorbed (Q) on column for lysozyme were obtained at varying NaCl concentrations in the equilibration buffer. The column was equilibrated with 15 CVs of the solution and the detector was set to zero when the UV signal became stable. Thereafter, 25 μL of 1 mg/mL protein sample was injected at 0.1 mL/min and then washed with the same solution for 4 CVs. Finally, the column was eluted with the elution buffer for 4 CVs to get the elution peak. The adsorbed protein was calculated from the elution peak area and the total peak area according to the following equation:

$$Q = \frac{A_E}{A_T}\frac{C_0 V_0}{V_B}$$

where Q is the adsorbed protein on column, A_E is the elution peak area, A_T is the total peak area (sum of the areas of the flowthrough and elution peaks), C_0 is the protein loading concentration, V_0 is the loading volume, and V_B is the packed-bed volume.

Protein adsorption onto different anion exchanger columns was examined by a similar method as described above. The flow rate was 0.1 or 0.5 mL/min, and protein loading was 25 μL at different concentrations. In some cases, cytochrome *c* was used as a positive control, while BSA was used as a negative control.

In the above studies, each chromatographic experiment was conducted in triplicate and the average value is reported.

2.3. Analysis

Sulfur content of dried Sepharose FF gel was determined using a vario MACRO cube elemental analyzer (Elementar, Hanau, Germany).

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