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Chromatographic behavior of mouse serum immunoglobulin G in protein G perfusion affinity chromatography

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Abstract

In this study chromatographic behavior of mouse serum immunoglobulin G (IgG) on a protein G perfusion affinity chromatographic column was investigated experimentally. The results indicate that the protein G column has no non-specific binding to the other proteins in mouse serum but an irreversible adsorption to IgG under the conditions investigated. It was found that variations of the elution solution composition, ionic strength and pH played to some extent an essential effect on the chromatographic behavior of IgG. The influence of the mobile phase flow-rate on the chromatographic behavior of IgG was also researched. These results show that the dissociation of IgG from protein G affinity packings becomes the rate-limiting step in the perfusion affinity chromatographic separation process. © 2000 Published by Elsevier Science B.V. All rights reserved.

Keywords: Immunoglobulin G; Protein G

1. Introduction

Affinity chromatography (AC) has been widely used as an efficient method for the analysis, as well as for the large-scale separation, of biological macromolecules. The development of high-performance affinity chromatography (HPAC) makes broaden its applications in biotechnology. For the typical stationary phase, the first serious constraint to be considered is the problem of mass transfer, especially at high flow-rate. To improve mass transfer and speed up the separation process, in recent years perfusion chromatographic packings have been developed. The intraparticle convection in this technique enhances pore diffusivity and minimizes the mass transport effect in the stationary phase, so perfusion chromatography can achieve both the purification and rapid separation of proteins [1]. These packings have been successfully used in the affinity chromatography of proteins using immobilized ligands. For example, IgG was quickly determined by the protein A or protein G affinity packing [2]. The rapid characterization of the interaction between the human growth factor and its monoclonal antibody was conducted by protein G perfusion affinity chromatography [3]. Shigeo et al. [4] reported that a perfusion-type support in AC could be effectively used for the quick purification of bioactive materials secreted from cells. These investigations have mainly been concerned with studying the special features of these packings, such as short separation time, high accuracy, repeatability of analysis and operation at high flow-rate without reducing capacity.

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Non-specific adsorption, irreversible adsorption and flow-rate effects are the main factors affecting the separation efficiency of AC. In this paper, the specificity of the protein G perfusion affinity column to mouse serum IgG in the loading solutions of various ionic strengths, the irreversible adsorption of IgG on the surface of protein G packings and the effects of elution solutions and flow-rate on affinity chromatographic behavior of IgG are discussed.

2. Experimental

2.1. Apparatus and materials

The TSP liquid chromatograph (TSP, San Jose, CA, USA) consisted of a P4000 pump, an AS3000 autosampler and a Spectra FOCUS diode array detector. Chromatographic system control, data acquisition and chromatographic analysis were exerted with TSP PC 1000 Chromatography Manager software (3.0 version).

HPLC separations in high ionic strength solutions were carried out on an Alliance Waters 2690 Separation Module liquid chromatograph (Waters, Milford, MA, USA) with a Waters 996 PDA detector. Chromatographic system control, data acquisition and chromatographic analysis were controlled with Waters Millennium Chromatography Manager software.

The column used was a POROS G/M (PerSeptive Biosystems, Cambridge, MA, USA) with dimension of 30×2.1 mm I.D. (100 μl bed volume).

Mouse serum IgG, bovine serum albumin (BSA), transferrin and insulin were purchased from Sigma (St. Louis, MO, USA). The other chemicals are of analytical pure grade or were biological reagents, and the water was obtained from a Milli-Q system (Millipore, Bedford, MA, USA). All chemicals and reagents were used from commercial products without further purifications.

Mouse sera were obtained from Beijing Anapure Bioscientific (Beijing, China), filtered through 0.22-μm polysulfone filters, diluted 1:10 (v/v) with loading solution and injected in 20-μl aliquots into the protein G affinity column.

2.2. Experimental procedure

The chromatography was conducted at room temperature. All separations without the special description were carried out through the column by passing the loading solution for 3 min, the elution solution for 7 min and then loading solution again for 4 min at a flow-rate of 1 ml/min. The chromatographic profiles were monitored at 280 nm. Other conditions used were as described in Section 3.

To explore the effect of the mobile phase on the chromatographic behavior of IgG, the following loading and elution solutions were chosen in the investigation: The loading solutions were 10 mmol/l sodium phosphate buffer, pH 7.2, containing different concentrations of NaCl: (a) 0.15 mol/l, (b) 0.3 mol/l and (c) 0.6 mol/l.The elution solution compositions are shown in Table 1.

IgG purity analysis was carried out on a BIO-RAD

Table 1
Effect of different elution solutions on the perfusion affinity chromatographic behavior of IgG

Elution solution	Composition	t_R (min)	$W_{1/2}$ (min)	As[a]
A	0.15 mol/l NaCl, pH 2.5[b]	7.6	0.13	1.56
B	A+0.15 mol/l NaCl	7.8	0.18	1.70
C	A+0.45 mol/l NaCl	8.0	0.24	2.0
D	0.1 mol/l glycine, pH 2.5[b]	4.5	0.62	2.86
E	D+0.15 mol/l NaCl	4.5	0.62	2.88
F	D+0.30 mol/l NaCl	4.5	0.63	2.86
G	0.1 mol/l sodium phosphate buffer, pH 2.5	8.1	1.33	4.00
H	G+0.15 mol/l NaCl	8.1	0.88	3.81
I	G+0.30 mol/l NaCl	8.1	0.80	3.88
J	2% HAc, 0.3 mol/l $MgCl_2$	5.2	0.69	2.86

[a] As as peak asymmetrical factor.
[b] Adjusting pH by HCl.

electrophoresis system (Richmond, CA, USA) with Mini-PROTEAN II electrophoresis cell (gel size: 7×8 cm) and PAC 300 power. Samples were treated with SDS solution (containing DTT) and heated at 100°C for 5 min. The gels were stained with Coomassie Brilliant Blue R-250.

3. Results and discussion

3.1. Non-specific binding and effect of loading solution

Some common contaminant proteins (BSA, transferrin and insulin) in serum as standard proteins were injected into the POROS G/M perfusion affinity column and eluted under the elution conditions studied. The results have indicated that these proteins have not been retained on the column at all and the column has exhibited no any non-specific bindings to these contaminant proteins. It is coincident with the results in the literature [2].

Protein G has a very high affinity for the Fc region of IgG, and the driving force mainly comes from ionic interaction. The association constant (K_A) of protein G to mouse IgG is $4.1\times10^{10}\ M^{-1}$ and is not affected by the ionic strength in the loading solution at pHs above 4.0 [5]. However, non-specific binding may be caused by ionic interactions or hydrophobic interactions and can be controlled to a certain degree by adjusting the ionic strength of the loading solution. An increase of the salt concentration in solutions can decrease the non-specific binding on the POROS G/M column, as recommended by the manufacturer of the column [6]. Therefore, the separation of IgG in mouse serum was conducted with three loading solutions of different ionic strengths, a, b and c, and elution solution, A. It is found that the elution peak areas of IgG loaded with the loading solutions a, b and c were 6.31 E+5, 6.29 E+5 and 6.31 E+5, respectively. It means that changing the ionic strength of the loading solutions could not affect the binding of IgG on the protein G affinity column, and it proves alternatively there is no non-specific binding under our experimental conditions.

The central fraction of the IgG peak was collected and concentrated, and then analyzed by SDS–PAGE for purity assessment. The electropherogram in Fig. 1 has shown that IgG (150 kD) has always appeared two major bands on 55 and 24 kD due to DTT cleaving IgG into two identical heavy chains (55 kD) and light chains (24 kD), and that no any other contaminant proteins were found in IgG fraction. From the results it can be concluded that non-specific binding of proteins in mouse serum on the protein G column can be negligible during the process of affinity separation in our experimental conditions.

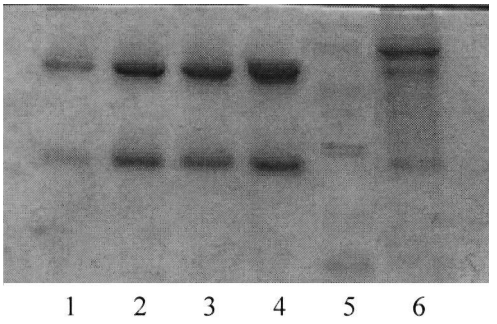

Fig. 1. SDS–PAGE analysis of IgG purity in different chromatographic fractions. Lane 1: standard IgG; lanes 2, 3 and 4 are IgG fractions from the perfusion protein G affinity chromatographic column when loading solutions were a, b and c, respectively; lane 5: molecular mass markers; lane 6: mouse serum The mass of standard proteins from top to bottom are 97.4, 68.0, 42.7, 30.1, 21.5, 14.4 KD, respectively.

3.2. Effect of elution solution

The proteins bound on an affinity column can be eluted by reducing their association constant (K_A) or increasing the dissociation constant (K_D) via alteration of the pH value or ionic strength of the mobile phases. IgG bound on the protein G column can be eluted effectively by decreasing pH [7]; however, the impact of the ionic strength of the elution solution on the retention behavior of IgG has been seldom investigated.

We have used different types of elution solutions A, D and G, which were used usually in AC, as listed in Table 1, and the same loading solution a. The results shown in Table 1 indicate that the chromatographic behavior of IgG in terms of t_R, $W_{1/2}$ and A*s* was different in the elution solutions of A, D and G at the same pH. This is due to the different K_D from different elution solutions. The composition of elution solutions determines the K_D through chang-

ing the conformation and the interactions between ligands and proteins [8].

As shown in Table 1, with increasing concentrations of NaCl in the elution solutions of the NaCl–HCl system, the t_R, $W_{1/2}$ and A*s* of the IgG-eluted peak was increased, and hence the chromatographic behavior of IgG became worse. In contrast, the chromatographic behavior of IgG was little affected by raising the concentration of NaCl in the elution solutions of the glycine–HCl system. In the elution solutions of phosphate system, t_R was not influenced, but the peak of the eluted IgG became sharper and more symmetrical with the increase in the concentration of NaCl. Increasing the ionic strength (by increasing the eluting salt concentration) is usually successful in desorbing bound proteins from many types of affinity column [9]. However, the case became complex in our study. The effect of ionic strength was different in different elution solution systems, e.g., NaCl–HCl, glycine–HCl and phosphate. In the NaCl–HCl system, hydrophobic interactions may have been involved in the interaction between IgG and protein G. These results show that increasing the ionic strength can either increase or reduce the binding, depending on conditions and the nature of the system. It has been also reported by Lyklema [10].

The chromatograms shown in Fig. 2 have explored the effect of elution solution A on chromatographic behavior of IgG at the different pHs of 1.5, 2.0 and 2.5, respectively. It was observed that with lowering pH values, the IgG peak shape was getting sharper and shaper; peak asymmetry factor was decreased from 1.56 to 1.33, and t_R of IgG was reduced from 7.6 to 5.7 min. This means that the lower the pH of the elution solutions and the higher the elution strength, the greater the increase of the K_D.

Fulton et al., [2] reported that IgG could be quickly analyzed on this column using elution solution J . However, in our experiments it led to a larger shift on the baseline and could not improve anything of the elution peak of IgG compared to elution solutions A and D.

3.3. Irreversible adsorption

As a standard IgG was injected into the POROS G/M column, which was equilibrated enough with loading solution before it was found that the signal of the IgG peak eluted was enhanced by increasing the number of continuous injections of the sample by 10 μl each at the concentration of 0.6 mg/ml, and got to be stable after the fifth injection, as shown in Fig. 3. This result indicated that the column has irreversible adsorption to IgG. Furthermore, under the same chromatographic conditions, if the sample

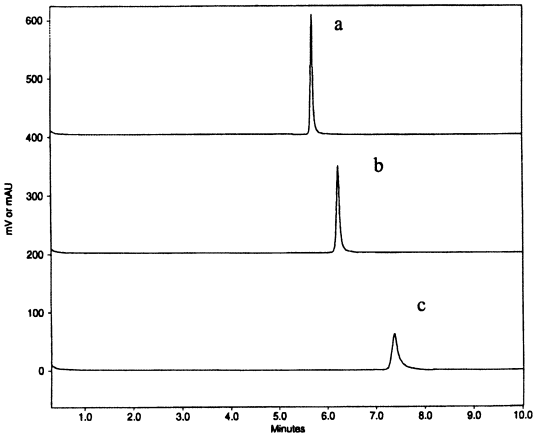

Fig. 2. Effect of pH values on the separation of IgG by affinity perfusion chromatography Loading solution: pH 7.2, 0.01 mol/l PB, 0.15 mol/l NaCl. Elution solution: (a) pH 1.5, 0.15 mol/l NaCl; (b) pH 2.0, 0.15 mol/l NaCl; (c) pH 2.5, 0.15 mol/l NaCl. Sample: IgG (1 mg/ml) 10 μl.

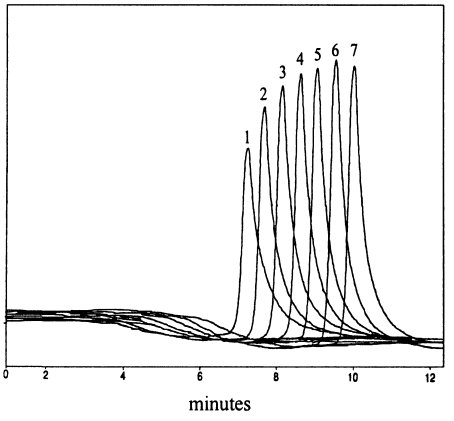

Fig. 3. Process of IgG irreversible adsorption equilibration on perfusion affinity chromatographic column. Loading solution: pH 7.2, 0.01 mol/l PB, 0.15 mol/l NaCl. Elution solution: pH 2.5 HCl, 0.15 mol/l NaCl. Sample: IgG (0.6 mg/ml) 10 μl. Peaks 1–7 were obtained from the first to seventh injections of IgG, respectively.

concentration was increased to 1 mg/ml and 1.5 mg/ml, respectively, the number of injections needed to get a stable peak signal still was five. One explanation for this phenomenon is that since the interaction of IgG between the mobile phase and the solid-phase is in the linear range of the isotherm at low concentration, the retention time or breakthrough point in frontal analysis should be constant and cannot be varied with the sample concentration [11,12].

In addition, it was found that the irreversible adsorption of IgG on the column was not changed with the other proteins present. To prove it, BSA was injected continuously for five times (once 10 μl, 1 mg/ml BSA) before the standard IgG injection (once 10 μl, 0.6 mg/ml IgG). The same as in Fig. 3 show that the irreversible adsorption of IgG on the column was not changed. The phenomenon has provided an alternative proof that BSA was not retained definitely on POROS G/M column by non-specific binding, otherwise, BSA should influence the irreversible adsorption of IgG. This is totally different from other types of chromatographic separations, such as ion-exchange, reversed-phase, hydrophobic interaction, etc., in which the adsorption behavior of the main protein of IgG has been influenced by the presence of other proteins in sample [12]. And hence this irreversible adsorption is specific to IgG.

Furthermore, the irreversible adsorption of IgG onto the column was measured using elution solution A at different pHs of 2.5–2.0 and 1.5, respectively. Aliquots of 10 μl of each of the samples were continuously injected at the concentration of 0.6 mg/ml. It was observed that with lowering pH values, the number of injections needed to get a stable peak signal was reduced from five to two. And the peak areas in the stable state were 2.38 E+5, 2.88 E+5 and 3.01 E+5 at corresponding pH values of 2.5, 2.0 and 1.5, respectively. The results have proved that the irreversible adsorption of IgG on the AC column was dependent on the elution conditions, and the lower the pH of the elution solutions, the less the irreversible adsorption.

A possible explanation for the irreversible adsorption of IgG on the column is that the interaction between the packings and the different IgG molecules is microheterogeneous. When eluted with a certain elution solution, various IgG molecules may show different K_D to the packings, k' will be different between them, and therefore the IgG molecule with a larger k' value will not be eluted from the packing in the elution time. The elution solutions with lower pHs can lead to the gradual dissociation of the various protein–ligand complexes in the elution time, then reduce the irreversible adsorption.

The heterogeneous surface of the packings may come from steric effects, which are very important for protein G since it has only two binding sites for IgG [13]. Another possibility is that protein G exhibits interactions with non-Fc regions of IgG [14]. In addition, IgG molecules themselves also display heterogeneity due to their subclass and hydrohydrate, introducing the differences of conformation and surface charge [15].

3.4. Effect of flow-rate

The effects of the flow-rate of both the loading and elution solutions on separation were investigated at the flow-rate of 1 ml/min for the corresponding elution and loading solution, respectively. The results in Fig. 4 have illustrated that the peak area of IgG was reduced with increasing the flow-rate. Additionally, comparing the slopes of the two curves in the figure, it was found that the effect of flow-rate on the

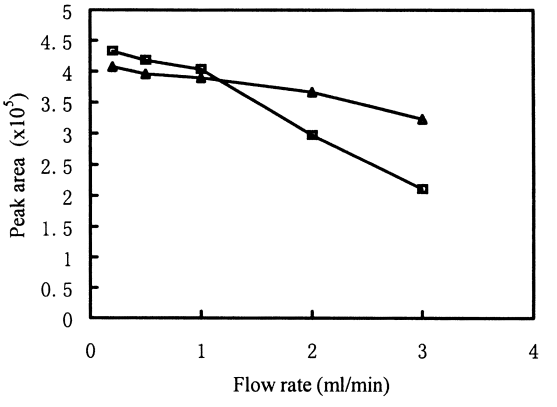

Fig. 4. Effect of flow-rate on the perfusion affinity chromatographic behavior of IgG. Loading solution: pH 7.2, 0.01 mol/l PB; 0.15 mol/l NaCl. Elution solution: pH 2.5 HCl. 0.15 mol/l NaCl. Sample: IgG (1 mg/ml) 10 μl. Curve —▲— illustrates the effect of the flow-rate of the loading solution with the flow-rate of the elution solution as 1 ml/min. Curve —□— illustrates the effect of the flow-rate of the elution solution with the flow-rate of the loading solution as 1 ml/min.

separation was much greater for the elution solution than for the loading solution, especially when flow-rate was above 1 ml/min.

The distribution of matrix itself to the mass transfer can be little affected by the flow-rate in perfusion chromatography [16]. However, our work shows that the flow-rate of both the loading and elution solutions influences the chromatographic behavior of IgG. It means that mass transfer in perfusion affinity chromatography may be affected by flow-rate through the interactions between ligand and IgG. Since mouse serum IgG has much larger affinity to protein G, the K_D of IgG on the protein G column in most cases is smaller than K_A, consequently the smaller K_D influences the flow-rate of mass transfer much more in the dissociation step than in the association step.

4. Conclusions

Data presented in this paper indicate firstly that the protein G column showed no non-specific binding to the other proteins in serum but an irreversible adsorption to IgG under the investigation conditions. Secondly, variations of the elution solution composition and pH played an essential effect on the retention time and the peak shape of IgG to some extent. In different elution solution systems, ionic strength may have different effects on the chromatographic behavior of IgG. Thirdly, the flow-rate of mobile phase notably influenced the chromatographic behavior of IgG on a POROS G/M column. Finally, the dissociation of IgG from protein G affinity packings became the rate-limiting step in the separation process of perfusion affinity chromatography.

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References

[1] N.B. Afeyan, S.P. Fulton, F. Regnier, J. Chromatogr. 544 (1991) 267.
[2] S.P. Fulton, M. Meys, L. Varady, R. Jansen, N.B. Afeyan, Biochromatography 11 (1991) 226.
[3] H. Zou, Y. Zhang, P. Lu, I. Krull, Biomed. Chromatogr. 10 (1996) 78.
[4] K. Shigeo, T. Masaaki, S. Eizo, I. Hisashi, K. Yoshihiro, Y. Kazunari, M. Tsuyoshi, J. Ferment. Bioeng. 78 (1994) 246.
[5] B. Akerstrom, L. Bjorck, J. Biol. Chem. 261 (1986) 10 240.
[6] Operating Instruction for POROS® G/M, PG ImmunoDetection™ Cartridge, Framingham, MA, USA: PerSeptive Biosystems.
[7] B. Akertrom, T. Brodin, L. Bjorck, J. Immunol. 135 (1985) 2589.
[8] F.H. Arnold, H.W. Blanch, J. Chromatogr. 355 (1986) 13.
[9] P.D.G. Dean, W.S. Johnson, F.A. Middle (Eds.), Affinity chromatography: a practical approach, IRL, Oxford, 1985, p. 75.
[10] J. Lyklema, in: T.C.J. Gribnau, J. Visser, R.J.F. Nivard (Eds.), Affinity chromatography and related techniques, Elsevier, Amsterdam, 1982, p. 11.
[11] J.X. Huang, Cs. Harvath, J. Chromatogr. 406 (1987) 285.
[12] J.X. Huang, J. Schudel, G. Guiochon, J. Chromatogr. Sci. 29 (1991) 122.
[13] M.N. Widjojoatmadia, A.C. Fluit, R. Torensma, J. Verhoof, J. Immunol. Methods 165 (1993) 11.
[14] L. Bjorck, G. Kronvall, J. Immunol. 133 (1984) 969.
[15] M. Carlsson, A. Hedin, M. Inganas, B. Harfast, F. Blomberg, J. Immunol. Methods 79 (1985) 89.
[16] A.E. Rodrigues, J. Loureiro, C. Chenou, J. Chromatogr. B 664 (1995) 233.