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Purification of monoclonal antibodies on dextran-coated silica support grafted by thiophilic ligand

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Abstract

Coated silica beads are promising supports for high-performance liquid chromatography (HPLC) of proteins; they combine the excellent mechanical properties of silica with minimal non-specific interactions with proteins in solution due to the presence of hydrophilic dextran polymers adsorbed at the silica surface. So, dextran-coated porous silica beads can be grafted with β-mercaptoethanol by using divinylsulfone as coupling reagent to obtain new thiophilic supports usable in HPLC. The affinity of monoclonal IgG subclasses from mouse ascitic fluids for the active phases can be analysed. These dextran-coated silica supports grafted with thiophilic ligands allow a one-step purification of these antibodies. Moreover, the chromatographic separation of two subclasses, immunoglobulin G1 (IgG1) and IgG3, is observed and can be correlated to the high resolution of these new HPLC thiophilic supports.

1. Introduction

Monoclonal antibodies are essential tools especially for many domains in immunology. The discovery of the production technique of monoclonal antibodies has led to a very important development of their applications in fundamental research, biological diagnosis, therapeutic applications and industry. However, in order to use monoclonal antibodies in the applications mentioned above, it is necessary to develop high-resolution techniques for their purification. Different methods exist for the purification of antibodies. Ion-exchange chromatography and affinity chromatography on immobilized protein A or G are the most used. Nevertheless, both the weak specificity of ion-exchange interaction and the high costs of protein A- or protein G-immobilized phases make the research of new kinds of supports important.

In 1985, Porath et al. [1] introduced a new chromatographic phase, the 'T-gel', constituted of agarose beads activated by divinylsulfone and grafted with β-mercaptoethanol. These authors demonstrated that some serum proteins, and in particular IgG, IgA and IgM, can be adsorbed on these new phases in the presence of antichaotropic salts, such as potassium sulfate, and selectively desorbed. They called this new adsorption concept 'thiophilic adsorption', and the corresponding chromatographic application 'thiophilic interaction chromatography' (TIC). Since the introduction of this new concept, several studies on the purification of Ig [2,3] or only IgG [4] have been realized by using this new thiophilic ligand grafted on a polysaccharidic gel. Moreover, HPLC thiophilic supports, based on silan-

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ized silica, have been synthesized for the purification of IgG [5].

In this study, we try to combine the properties of the affinity of IgG for the thiophilic ligand and the properties of the HPLC supports developed in our laboratory, based on dextran-coated porous silica. Because of its mechanical properties, silica is actually the most commonly used material for HPLC separations. However, the silica beads have to be modified in order to decrease the ionic adsorption of proteins due to the presence of charged silanol functions on the inorganic surface. Consequently, as previously reported, porous silica beads are coated with dextran bearing a calculated amount of positively charged diethylaminoethyl (DEAE) functions in order to neutralize the negatively charged silanols groups in aqueous media [6]. These coated supports (SID) keep the mechanical properties of silica. Moreover, they are easily activated in order to graft specific ligands by using the conventional coupling reagents [7]. Their performances in high-performance affinity chromatography (HPAC) have already been demonstrated for the purification of several biological compounds [8–11]. The dextran-coated silica beads can also be grafted with β-mercaptoethanol after activation by divinylsulfone using the procedure described by Porath et al. [1].

The affinities of the monoclonal IgG subclasses IgG1, IgG2a, IgG2b and IgG3 from mouse ascitic fluid for these new HPLC thiophilic supports are studied. First, we have decided to focus our study on the IgG3 subclass because of its structural differences. The IgG3 present an hinge region between Fab and Fc fragments much longer than the hinge region of the other subclasses, i.e. IgG1, IgG2a and IgG2b. This region is constituted of 62 amino acids and 11 disulfide bonds for IgG3, whereas for the other subclasses the number of amino acids and disulfide bonds is five times less [12]. As it is demonstrated in this study, the stronger adsorption of IgG3 antibodies suggests that the numerous disulfide bonds in these antibodies would involve stronger interactions with the thiophilic ligand. The thiophilic adsorption mechanisms are still unclear and several studies have been conducted in order to elucidate the type of interactions involved in the affinity mechanism. The tertiary structure of the adsorbed protein is essential in the adsorption process. For example, the Fab and the Fc fragments of the IgG molecule do not adsorb on a thiophilic support at the opposite of the whole molecule [13]. According to Porath and co-workers [13,14], it seems that the proximity of the sulfone group and a nucleophile atom (sulfur in the case of β-mercaptoethanol) is necessary to allow a thiophilic adsorption. The nature of the ligand terminal group is also an important parameter [15,16]. For example, the influence of the presence of an aromatic ligand on the purification of human and mouse Ig has been analyzed [17]. A comparative study of affinity chromatography on dextran-coated silica supports grafted by several ligands, with sulfone group or not, demonstrates the importance of this chemical function for the total IgG adsorption [18].

After analyzing the interactions of the IgG3 with this new thiophilic support by HPLC, we present the results obtained on the affinity of the other IgG subclasses, i.e. IgG1, IgG2a and IgG2b, under the same experimental conditions.

2. Experimental

The synthesis of dextran-coated silica grafted with thiophilic ligand is realized in three steps. First, the dextran polymers are substituted by DEAE groups through the formation of an ether bond with a hydroxyl function of the dextran glucosidic unit, probably in position 2 [6]. Secondly, the substituted polymer is adsorbed at the silica surface by a batch method and then cross-linked using a diepoxide. Finally, the ligand, β-mercaptoethanol, is coupled to the coated silica support previously activated by divinylsulfone.

2.1. Synthesis of DEAE-substituted dextrans

The mean molecular mass of the dextran polymers used (Pharmacia, Bois d'Arcy, France)

is 70 000 g mol^{-1}. A 40-g amount of dextran and 79 g of sodium hydroxide in a 120-ml solution of doubly distilled water are cooled and mixed at 4°C. The mixture is stirred for 20 min and 42.7 g of hydrochloride of 2-chloro-N,N-diethylaminoethane (Janssen Chimica, Pantin, France) are added. The mixture is heated to 55°C and the reaction proceeds at this temperature for 30 min. Then, the mixture is rapidly cooled and the pH adjusted to 9 using concentrated hydrochloric acid. DEAE-dextran polymers are precipitated with methanol. The solid product is filtered, washed with ethanol and dried under vacuum at 40°C overnight.

2.2. Preparation of DEAE-dextran-coated silica supports (SID)

The porous silica beads are kindly provided by Biosepra (Villeneuve-la-Garenne, France). The pore size of silica particles is about 1250 Å and two different bead sizes, 40–100 and 15–25 μm, are used in this study. The specific surface of these two kinds of beads is 25 $m^2 g^{-1}$.

A 10-g amount of DEAE-dextran is dissolved in 100 ml of doubly distilled water. The pH of the solution is adjusted to 11.5 by the addition of 1 *M* sodium hydroxide. A 50-g amount of silica, degassed under vacuum for 1 h, is gently added to this solution. After 30 min of impregnation, the packing is dried for 15 h at 80°C under vacuum. Then the dextran layer is cross-linked around the silica particle. A 50-g amount of coated silica is added to 100 ml of dry diethyl ether containing 0.3% (v/v) 1,4-butanediol diglycidyl ether (Sigma, La Verpillière, France). The mixture is gently stirred for 1 h at 40°C. After evaporation of the solvent, the silica is dried for 15 h at 80°C under vacuum and kept in the dry state.

2.3. Activation of coated silica by divinylsulfone

A 2.5-ml volume of divinylsulfone (Janssen Chimica) is added to 5 ml of 0.5 *M* carbonate buffer pH 11. A 1.5-g amount of coated silica is added to the activation solution, and the suspension is gently stirred for 2 h at room temperature. The support, activated by divinylsulfone, is then washed with 200 ml of doubly distilled water and 200 ml of 0.5 *M* carbonate buffer pH 10 and directly used for the immobilization of the active ligand.

2.4. Immobilization of β-mercaptoethanol

A 5-ml volume of β-mercaptoethanol is dissolved in 5 ml of 0.5 *M* carbonate buffer pH 10. A 1.5-g amount of activated support is added to this solution and the suspension is gently stirred for 48 h at room temperature. After the reaction, the support, functionalized by β-mercaptoethanol, is filtered and washed with 200 ml of 0.1 *M* Tris–HCl buffer pH 8.5. The support is deactivated by shaking the solid product in 10 ml of this latter buffer for 3 h at room temperature. The solid-phase is washed with 200 ml of the deactivation solution and 200 ml of 0.1 *M* Tris–HCl buffer pH 7.6 successively. The active support is kept at 4°C in this buffer or dried under vacuum at 60°C for 15 h. The quantity of grafted ligand is determined by elemental analysis of sulfur.

2.5. Immunoglobulin G and immunotest

The different monoclonal antibodies extracted from mouse ascitic fluid as well as the pure IgG1 fraction (PY057) are provided by CisBioindusties (Lapam, Bagnols sur Cèze, France). The protein quantities are determined by a Bradford protein assay kit (BioRad, Ivry sur Seine, France). The quantity of mouse monoclonal antibodies is determined by an enzyme-linked immunosorbent assay (ELISA) from Boehringer Mannheim (Meylan, France). The ascitic fluids are diluted in the chromatographic initial buffer and the solutions are filtered through 0.2-μm filters before injection onto the column.

2.6. HPLC apparatus and chromatographic elution conditions

The HPLC apparatus consists of a Merck-Hitachi 655 A-12 gradient system from Labs

Merck-Clevenot (Nogent sur Marne, France) with a Rheodyne 7125 injection valve, connected to an LMC variable-wavelength monitor and a D-2000 integrator.

The thiophilic supports are put in suspension in the chromatographic initial buffer. The columns (12.5 × 0.4 cm) are filled with the suspension under mechanical vibrations using a slurry technique in order to get an homogenous fill.

After equilibration of the column, the samples are injected and washed at a flow-rate of 1 ml/min in a 50 m*M* Tris–HCl buffer pH 7.4, 0.6 *M* K_2SO_4 for 10 min. The desorption of antibodies is obtained by a decreasing gradient of K_2SO_4 concentration in the eluent for 20 min in the same buffer. The eluted fractions are collected and the quantity of IgG and proteins in the different fractions determined. Then the column is washed with the initial buffer before injection of the next sample.

3. Results and discussion

Preliminary studies have demonstrated that β-mercaptoethanol has to be grafted on the dextran-coated silica beads in a sufficient quantity in order to obtain good chromatographic performances. Moreover, the optimum pH values of the two steps of the synthesis are 11 for the activation reaction and 10 for the coupling reaction to obtain an active support. Two kinds of supports constituted of silica with different bead sizes are synthesized following the conditions described in the Experimental section. Their characteristics are presented in Table 1.

The result of the elution of IgG3 antibodies on the support with the higher bead size is presented in the first part of this study. The elution profile of ascitic fluid containing antibodies of the IgG3 subclass on the SID1-T support (40–100 μm) is presented in Fig. 1. The analyses of F0- and F1-eluted fractions by sodium dodecylsulfate (SDS-PAGE) and the control by immunoblotting indicate the presence of IgG3 essentially in the F1-retained fraction. The amounts of injected and collected proteins are determined by the Bradford assay. When 1000 μg of proteins are injected, 700 μg are collected in the non-retained fraction and 200 μg in the retained fraction. In fact, this support presents an adsorption capacity of about 230 μg IgG3 per gram of dry support. However, the peak corresponding to the retained fraction is very broad and the adsorption capacity of the support is too low. Thus, in order to increase the resolution and the capacity, a silica phase with a smaller bead size (SID2-T) is chosen.

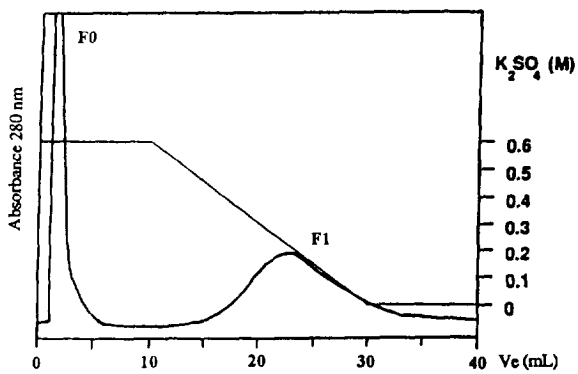

Fig. 1. Elution profile of 100 μl of ascitic fluid containing IgG3 on SID1-T support. Eluent A: 0.6 *M* K2SO4, 50 m*M* Tris–HCl pH 7.4. Eluent B: 50 m*M* Tris–HCl pH 7.4.

Table 1
Characteristics of dextran-coated silica supports grafted by a thiophilic ligand

Support	Silica bead size (μm)	Pore size (Å)	Adsorbed quantity of DEAE-dextran		Quantity of divinylsulfone (μmol/g)	Quantity of β-mercaptoethanol (μmol/g)
			(mg/m^2)	(mg/g)		
SID1-T	40–100	1250	2.6	65	410	389
SID2-T	15–25	1250	1.8	45	613	568

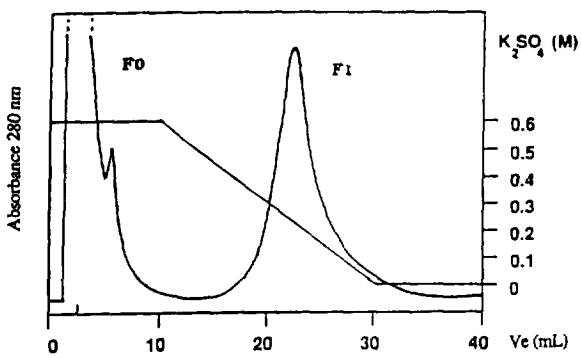

Fig. 2. Elution profile of 100 μl of ascitic fluid containing IgG3 on SID2-T support.

The elution profile of IgG3 antibodies on SID2-T support is presented in Fig. 2. In contrast to the chromatogram obtained on the SID1-T support, the shape of the peak of the retained fraction is finer indicating a better resolution. The analysis of the different eluted fractions by SDS-PAGE reveals only one band corresponding to the molecular mass of IgG3. This suggests that, in the sensibility limits of the electrophoretic method, a pure fraction of antibodies is obtained in the retained fraction. Under similar experimental conditions, the other three subclasses (IgG1, IgG2a and IgG2b) are injected and eluted on the same support. The different chromatograms are presented in Fig. 3. The peaks corresponding to the retained fractions are similar to the profile obtained for IgG3 antibodies. However, it can be observed that IgG2a are eluted in a very broad peak. The two other IgG subclasses seem to be eluted normally. The quantities of each antibody, injected on the column and collected in the different fractions, are determined by ELISA and reported in Table 2. The adsorption capacity of the support for the different IgG is about 1 mg per gram of dry support. Under non-overloading conditions, the recovery of antibodies in the collected fractions is about 55%. However, the adsorption capacity of this support for the IgG2a subclass is noticeably weaker than those of the other subclasses.

It appears that the reduction of the bead size and the increase of β-mercaptoethanol grafted on the dextran layer lead to an improvement of the chromatographic performances of these thiophilic supports. The adsorption capacity is

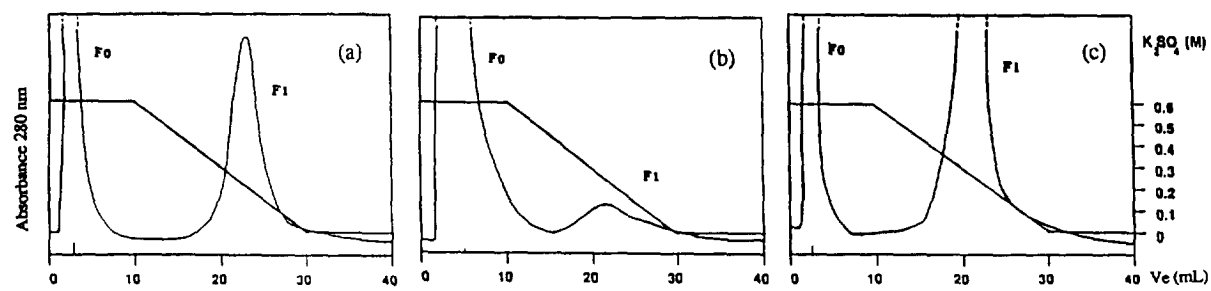

Fig. 3. Elution profile of 100 μl of ascitic fluids containing IgG1 (a), IgG2a (b) and IgG2b (c) on SID2-T support.

Table 2
Antibody quantities injected on SID2-T support in overloading conditions and in the eluted fractions

Antibody	Injected quantity quantity (μg)	Collected quantity in F0 (μg)	Collected quantity in F1 (μg)
IgG1	2730	148	1102
IgG2a	3125	108	648
IgG2b	4408	107	988
IgG3	1650	76	812

about five times higher. The better resolution of the SID2-T support can be used to separate different subclasses of IgG from a mixture (Fig. 4). This result implies a high resolution of the chromatographic separation and has to be correlated with some differences in the affinities of IgG1 and IgG3 for this thiophilic support. The longer retention time for the IgG3 suggests that the interactions between this subclass and the thiophilic dextran-coated silica support are stronger than those developed with IgG1. According to the hypothesis proposed in the introduction, these structural differences between these two antibodies could explain the differences in the retention times. It will be interesting to determine the affinity constants of each subclass for this support. One could then verify whether the immobilized thiophilic ligand on dextran-coated silica really develops a different affinity for each subclass, or if dynamic phenomena are involved in the differences observed in the elution times of the different IgG subclasses.

The understanding of adsorption IgG subclasses on thiophilic supports would require more studies. However, this preliminary work indicates that the dextran-coated silica supports grafted by a thiophilic ligand interact specifically with monoclonal antibodies in solution. This effect allows the one-step purification of each mouse IgG subclass from mouse ascitic fluid in a yield of about 55%. These new thiophilic supports have demonstrated their high mechanical stability and therefore the possibility to use them under HPLC conditions with a high resolution of the chromatographic separations. The optimization of the synthesis conditions of the thiophilic phases would allow the improvement of the capacity of these supports, which is still weaker as compared to the commercial affinity supports.

The development of selective IgG purification methods is a very essential research domain since the improvement of numerous medical applications depends on it. To improve these methods, the understanding of the separation mechanism is a relevant point. For this purpose, the knowledge of the correlations between the structure of the chromatographic phases and their separation properties is of major importance. Some structural aspects of the dextran-coated silica supports have already been reported [19–21]. In the present study, we have demonstrated that the particle size and the grafting rate are parameters influencing the separation performances of these new thiophilic phases. Moreover, coated silica supports are very interesting models because the presence of the dextran layer allows the minimization of the non-specific interactions between the silica surface and proteins in solution and, therefore, increases the importance of the affinity process in the interactions between the functionalized phase and the biological compounds.

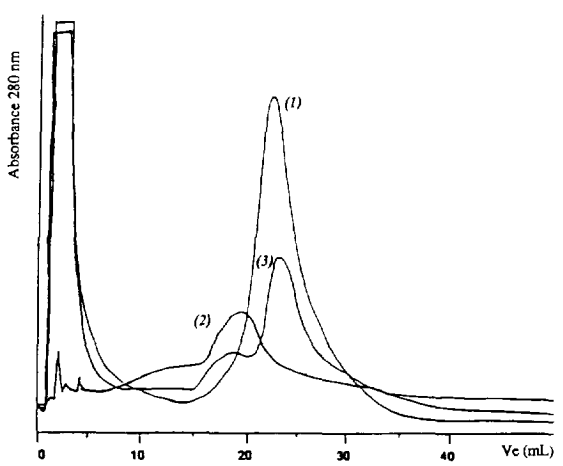

Fig. 4. Elution profile of ascitic fluid containing IgG3 (1), pure IgG1 (2) and the mixture of both (3) on SID2-T support.

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