Increase in IgG-binding Capacity of Recombinant Protein a Immobilized on Heterofunctional Amino and Epoxy Agarose

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Abstract. Protein affinity materials were widely used in purification of monoclonal antibodies and treatment of autoantibody-associated diseases. Due to the low binding capacity of protein A affinity materials compared to other ion exchange materials, protein A affinity materials are often discussed to be the bottleneck among current purification processes and diseases treatment. In this study, the heterofunctional amino and epoxy agarose was used to immobilize the recombinant protein A (rSpA). The immobilization courses of rSpA and the effect of pH and ionic strength and amino density of the heterofunctional supports on the coupling efficiencies of rSpA were investigated. The optimum conditions for rSpA immobilized on the heterofunctional supports were determined as pH 8.0, the ionic strength of 5 mM borate buffer, and the amino density of 15 \textmu mol g\textsuperscript{-1}. Moreover, the 3D structure of domain B of protein A was analyzed by PyMol software and the web-based program MemBrain. The most likely immobilization sites of B domain were located at the Lys-49 and Lys-50, exposing the IgG-binding pocket to the medium. After immobilization, the remaining epoxy groups were blocked with the ethanolamine to obtain the rSpA affinity materials with different ligand density for antibody adsorption from the human plasma. The static and dynamic IgG-binding capacities were determined to be 42 and 32 mg g\textsuperscript{-1}, respectively. The high IgG-binding capacities could be attributed to the oriented attachment of rSpA on the heterofunctional supports, and these results demonstrate that the heterofunctional amino and epoxy agarose is very promising to permit the oriented covalent attachment of rSpA, and that way to prepare affinity materials with high binding capacity.

1. Introduction

\textit{Staphylococcus aureus} protein A (SpA) is a type I membrane protein which contains five highly homologous Fc binding domains designated E, D, A, B, and C, proceeding from the N to the C terminus [1]. Due to its high affinity for the Fc domain of Immunoglobulin G (IgG), immobilized SpA has been extensively used for the purification of antibodies [2-6] and the treatment of autoantibody-associated diseases such as systemic lupus erythematosus, antiphospholipid syndrome [7], and sensitized renal transplant [8, 9]. A high IgG-binding capacity for immobilized SpA is crucial for reducing costs and increasing the elimination efficiency of antibodies on its application.
The recombinant protein A (rSpA) by the construction of multimere B-domain mutants, instead of natural protein A, has been frequently used as the protein ligand to prepare the affinity materials [10, 11]. The B domain contains three α-helices in the polypeptide segments Lys7 to His18 (α1), Glu25 to Asp36 (α2), and Ser41 to Ala54 (α3) [12]. The IgG-binding pocket of B domain is located at the region of α1 and α2 helices, not involving the α3 helix [13]. Therefore, the immobilization protocols which achieve the area-specific attachment through the α3 helix region of the B-domain onto the solid support, exposing the IgG-binding pocket to the medium, have positive effects on improving the IgG-binding capacity for SpA affinity materials.

Epoxy-activated supports seem to be almost ideal system to develop very easy protocols for protein immobilization [14-16]. Epoxy groups are very stable at neutral pH condition to be stored for long periods. Meanwhile, epoxy supports are able to react with different nucleophiles on the protein surface (e.g., amino, hydroxy, or thiol moieties) to form extremely strong covalent linkages with minimal chemical modification of the protein. In fact, epoxy supports are scarcely reactive for proteins immobilization even under slightly alkaline conditions because of its stability [17]. Successful immobilization of proteins at neutral pH values have been achieved by using the heterofunctional epoxy supports containing the adoptive groups and reactive groups [18-21]. Proteins are first physically adsorbed on the supports, and then a covalent attachment between nucleophile groups of the protein and a high density of nearby epoxy groups in the same support is strongly favored. Thus, this extremely low intermolecular reactivity between epoxy supports and proteins does open new exciting possibilities to dictate orientation of proteins on the surface of heterofunctional epoxy supports.

In this study, we report the oriented immobilization of rSpA (three tandem B domains) [11] on the heterofunctional amino and epoxy agarose. As support for rSpA immobilization Sepharose 6FF was used. The hydroxyl groups of Sepharose 6FF were activated with epichlorohydrin to prepare epoxy agarose supports, and then partial epoxy groups reacted with ethylenediamine to obtain the heterofunctional amino and epoxy supports. Using the supports, it is expected that the oriented covalent immobilization of rSpA will be produced after anion adsorption caused by interaction between the amino groups on the supports and the rSpA at the low ionic strength. Several studies of the heterofunctional supports used for the immobilization of rSpA as well as the IgG-binding capacity of the affinity materials prepared are presented.

2. Results and discussion

2.1. Preparation of heterofunctional amino and epoxy supports

Sepharose 6FF was chosen as a base matrix for the construction of heterofunctional amino and epoxy supports. Briefly, Sepharose 6FF was activated with epichlorohydrin, a bifunctional reagent used to obtain epoxy groups with the density of approximately 120 μmol g⁻¹ [22]. The activation protocol in the water-free system could obtain high density of epoxy groups, which was prone to multipoint covalent attachment of the protein on the supports [23]. Afterward, the epoxy supports reacted with ethylenediamine under slightly alkaline conditions of pH 8.5 for different time to obtain heterofunctional amino and epoxy supports bearing different amino density [19, 21]. The preparation protocol was shown in Figure 1.
2.2. Oriented immobilization of rSpA

Figure 2 shows the immobilization courses of rSpA on heterofunctional supports with the amino density of 15 μmol g⁻¹ at 5 mM borate buffer under different pH conditions. The results indicated that the coupling efficiency of rSpA on the supports increased with the increase of pH from 6 to 8, while decreased with the increase of pH from 8 to 9. The coupling efficiency of rSpA on the supports was higher than 90% when the pH value was 8. The main reason was that the low pH was not conducive to electronegativity for rSpA while the high pH was not conducive to electropositivity for amino groups on the supports. After immobilization, the reagent of NaCl was added to the suspension for releasing the physically adsorbed rSpA on the supports, to check that the immobilization was actually covalent. The results indicated that rSpA was covalently attached on the supports by the interaction of amino groups on the rSpA surface with the epoxy groups on the supports. In practice, the immobilization of rSpA on the epoxy agarose would hardly take place under neutral conditions (the results were not shown). The “first” adsorption cause was a necessity for the covalent immobilization of rSpA on the heterofunctional amino and epoxy agarose (Figure 1).

Figure 3 shows the immobilization courses of rSpA on heterofunctional supports with the amino density of 15 μmol g⁻¹ at several ionic strengths and pH 8. The results indicated that the presence of amino groups allows a very rapid ionic adsorption of the rSpA onto the support at low ionic strength (5 mM borate buffer) and the coupling efficiency of rSpA was higher than 90%. It is worth noting that approximately 20% of rSpA was immobilized on the supports even at higher ionic strength of 500 mM borate buffer, which was likely due to the co-operative effect of physical and covalent protein adsorption [19].

![Figure 1. Activation and immobilization mechanism of heterofunctional amino and epoxy agarose.](image1)

![Figure 2. Immobilization courses of rSpA on heterofunctional supports with the amino density of 15 μmol g⁻¹ at 5 mM borate buffer under different pH conditions. Circles: pH 6. Squares: pH 7. Triangles: pH 8. Rhombus: pH 9.](image2)
The density of amino groups on the heterofunctional supports could be tailored in a relatively simple way by controlling the reaction time of epoxy agarose with the ethylenediamine. Figure 4 shows the effect of the amino density on the surface of supports on the coupling efficiency of rSpA at 5 mM borate buffer and pH 8 after 30 hours of incubation. The coupling efficiency of rSpA on supports was calculated according to Eq. 1.

\[ CE = \frac{c_0 - c}{c_0} \times 100\% \]  

(1)

where \( CE \) is the coupling efficiency of rSpA on supports and \( c_0 \) and \( c \) are the concentration of rSpA in the supernatant in the case of inert agarose as a reference and heterofunctional agarose, respectively. When the density of amino groups on the heterofunctional supports reached 15 \( \mu \text{mol g}^{-1} \), the coupling efficiency of rSpA was higher than 90%. The higher density of amino groups on the heterofunctional agarose reasonably resulted in the decrease of epoxy density on the supports which is detrimental to multipoint intense covalent attachment of rSpA. Considering the balance of the coupling efficiency and the multipoint intense covalent attachment, the heterofunctional supports with the amino density of 15 \( \mu \text{mol g}^{-1} \) and the epoxy density of 105 \( \mu \text{mol g}^{-1} \) were the optimum systems for oriented covalent immobilization of rSpA.
2.3. Overview of B domain structure

To clarify the adsorptive and reactive sites of rSpA with the heterofunctional supports, the structures of B domain from rSpA were modeled using PyMol software version 1.7.4. The 3D structure of B domain was obtained from the Protein Data Bank (PDB). For B domain pdb code is 4NPF. Surface accessibility (ASA) values of residues were calculated by the web-based program MemBrain [24-26]. Table 1 shows the amount of anion adsorptive groups (aspartic acid and glutamate) and the reactive groups (lysine) of B domain and their ASA values. From Table 1, the 6 Lys residues that the B domain has are reasonably exposed to the medium, while Lys-4, Lys-7 and Lys-35 are involved in the IgG-binding pocket (α1 and α2 helices). Figure 5 shows the structure of B domain with the anion adsorptive groups, the reactive groups and the IgG-binding pocket marked. The most likely immobilization sites of B domain are located at the Lys-49 and Lys-50 according to the distribution of the anion adsorptive groups and the reactive groups and the spatial structure of B domain. From the 3D structure of B domain, the IgG-binding pocket would be exposed to the medium when rSpA was covalently attached on the heterofunctional amino and epoxy agarose by the immobilization sites described above, which likely lead to high IgG-binding capacity for the prepared affinity materials.

Table 1. List of anion adsorptive groups and reactive groups of B domain and their medium accessibilities (ASA). ASA values of residues from 4NPF were calculated by the web-based program MemBrain.

| Amino acid | Asp 2 | Asp 36 | Asp 37 | Asp 53 | Glu 8 | Glu 15 | Glu 24 | Glu 25 |
|------------|-------|--------|--------|--------|-------|--------|--------|--------|
| %ASA       | 70    | 49     | 32.9   | 81.2   | 71.5  | 49.5   | 63.5   | 81     |
| Amino acid | Glu 47| Lys 4  | Lys 7  | Lys 35 | Lys 49| Lys 50 | Lys 58 |
| %ASA       | 51.4  | 62.5   | 70.1   | 27.5   | 58.5  | 79.3   | 100    |

Figure 5. 3D surface structure model of B domain. The 3D surface structural model of B domain indicates lysine (blue), anion areas of acidic amino acid (red), and IgG-binding pocket (green).

2.4. IgG-binding capacity

The heterofunctional supports bearing the amino density of 15 μmol g⁻¹ and the epoxy density of 105 μmol g⁻¹ were chosen to immobilize the different amounts of rSpA at 5 mM borate buffer pH 8 for 30 hours, subsequently blocked with the ethanolamine to obtain the rSpA affinity materials. Afterwards, the rSpA affinity materials were used for IgG-binding from the human plasma using static and dynamic adsorption experiments [11]. For the static adsorption experiment, 1 g of rSpA affinity material was incubated with the 10 mL of human plasma at room temperature for 2 hours, and then was washed with the phosphate buffer pH 7.4. Afterward, the bonded IgG was eluted with citrate buffer pH 2.5 and the IgG concentration in the eluent was determined by measuring the UV absorption at 280 nm with an extinction coefficient of 1.36 mL mg⁻¹ cm⁻¹. The IgG-binding capacity of rSpA affinity material was calculated according to Eq. 2.

\[
Q_{\text{static}} = \frac{c \times V}{m}
\]
where $Q_{\text{static}}$ is the static IgG-binding capacity of rSpA affinity materials from the human plasma and $m$ is the suction-dried weight of rSpA affinity materials and $c$ and $V$ is the IgG concentration and solution volume of the eluent, respectively.

Figure 6 shows the influence of the rSpA density on the surface of agarose on the IgG-binding capacity. As discussed in previous publications [11], the IgG-binding capacity of the affinity materials depends on the amount of immobilized ligands. The results indicated that in the case of rSpA density ranging from 1.8 to 7 mg g\(^{-1}\), the static IgG-binding capacity from the human plasma for the rSpA affinity materials increased notably with the increase of the rSpA density on the surface of agarose, but the increase began to slow when the rSpA density is higher than 7 mg g\(^{-1}\). This was likely attributed to the steric hindrance of IgG during the adsorption process [27]. Due to the oriented immobilization of rSpA on the heterofunctional agarose, the static IgG-binding capacity of approximately 42 mg g\(^{-1}\) in the case of the rSpA density of 7 mg g\(^{-1}\) is higher compared to values reported in the literature [10], where the B3 was immobilized on epoxide-activated acrylamido-based resins even with the density of approximately 14 mg g\(^{-1}\). Therefore it can be concluded that oriented immobilization technique is a useful tool for the generation of high capacity affinity materials. Hence when exposing the binding pocket to the medium by the oriented immobilization, the rSpA affinity materials could adsorb IgG with weak steric hindrance between the supports and IgG molecules, and thereby also improve the binding capacity.

The dynamic binding capacity from the human plasma, which is more important for the development of antibody purification processes and the treatment of autoantibody-associated diseases, was analyzed by means reported in the literature [11]. 10 mL of human plasma were loaded onto the 1 mL of rSpA affinity materials to evaluate the dynamic IgG-binding capacity. As shown in Figure 6, the dynamic IgG-binding capacity also notably increased with the increase of rSpA density ranging from 1.8 to 7 mg g\(^{-1}\), while the increase began to slow when the density is higher than 7 mg g\(^{-1}\). The dynamic IgG-binding capacity from the human plasma was approximately 32 mg g\(^{-1}\) when the density of immobilized rSpA on the supports was 7 mg g\(^{-1}\). Obviously, only approximately 80% of $Q_{\text{static}}$ was reached for the dynamic IgG-binding capacity. To our knowledge, it was likely attributed to the slow diffusion because of the small effective porosities of approximately 0.5 determined for Sepharose 6FF [10].

![Figure 6](image_url)

**Figure 6.** Effect of the rSpA density on the surface of supports on the static and dynamic IgG-binding capacities from the human plasma. Circles: static. Squares: dynamic.

### 3. Summary

In this work, we presented a study for the oriented covalent immobilization of rSpA on the heterofunctional amino and epoxy agarose. The first immobilization of carrying out rSpA ionic adsorption at low ionic strength is advantages that allowed us to obtain both better coupling efficiency and exposing the IgG-binding pocket. Moreover, the present of amino groups on the heterofunctional supports can dramatically improve immobilization rates. Moreover, the oriented covalent
immobilization of rSpA on the heterofunctional supports can result in the increase of the IgG-binding capacity from human plasma for the rSpA affinity materials prepared.

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