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Capto MMC mixed-mode chromatography of murine and rabbit antibodies

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ABSTRACT

Murine antibodies have weak affinity for Protein-A. Here, we have tested binding of murine monoclonal antibody (mAb) to Protein-A or Protein-A/Protein-G mixture under salting-out conditions. The addition of ammonium sulfate to HEK conditioned medium (CM) expressing murine mAb resulted in complete binding, leading to its elution by low pH or neutral arginine solution. Alternatively, a mixed-mode chromatography using Capto MMC resin was developed as a capture step. Binding of murine mAb occurred at neutral pH. The bound mAb was eluted with a gradient from 0.3 M NaCl to 0.3 M arginine/0.3 M NaCl at pH 7.0. The Capto MMC-purified murine mAb was further purified by hydroxyl apatite chromatography. Similarly, rabbit mAb was processed with some modifications. Binding of rabbit mAb to Capto MMC required a lower pH. Elution of the bound rabbit mAb was achieved by a gradient to 0.3 M NaCl, pH 7.0.

1. Introduction

Protein-A affinity chromatography offers one step purification of antibodies based on the specificity of their Fc-domains for Protein-A. However, murine antibodies have weaker affinity for Protein-A than do antibodies from other species [1,2]. Such weak binding was overcome by the addition of salt or titration to alkaline pH during antibody binding [3,4]. Alternatively, Fc-portion of murine IgG1 was mutated to enhance Protein-A binding [1]. Here, we have explored three approaches for murine antibody purification: Protein-A/Protein-G mixture, binding under salting-out conditions, and mixed-mode chromatography.

Many different resins are commercially available for mixed-mode chromatography. Among them, MEP (4-MercaptoEthyl Pyridine) has been used as an alternative to Protein-A resin for purification of antibodies [5–11]. It belongs to a class so-called “charge induction hydrophobic interaction chromatography (CIHC)”, in which protein binding occurs near neutral pH essentially through hydrophobic interaction to the electrostatically neutral MEP resin. Elution of the bound antibodies is induced by a low pH solvent, which converts the electrostatically neutral MEP to the positively charged state, leading to charge repulsion of positively charged antibodies. Binding of proteins and antibodies to the MEP resin is, however, rather unpredictable and thus we have explored another mixed-mode resin, i.e., a cation exchange Capto MMC chromatography as a capture column in place of Protein-A for murine and rabbit monoclonal antibodies (mAb). In this class of mixed-mode resins, protein binding is mediated by both hydrophobic and electrostatic interactions. Capto MMC has a phenyl group and six methylene groups for hydrophobic interaction, a carboxyl group for ionic interaction and two hydroxyl groups for potential hydrogen bonding. In this paper, chromatographic conditions were developed for murine antibody and then applied with modifications to rabbit antibody.

2. Materials and methods

HEK and CHO cell conditioned media (CM) expressing murine or rabbit mAb were generated using shaker flasks. The murine mAb used here is IgG2a (*kappa* light chain), while the rabbit mAb is IgG (*kappa* light chain). The CM was filtered to remove cell debris and subjected to chromatographic purification. Protein-A, Protein-G and Capto MMC were obtained from GE Healthcare. CHT

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hydroxyapatite column (1 ml) was obtained from Bio-Rad.

HiTrap Capto MMC (1 ml) was equilibrated with phosphate-buffered saline (PBS) or 20 mM Na phosphate, pH 7.0. For the murine mAb tested here, binding and elution properties were identical whether the Capto MMC column was equilibrated with PBS or 20 mM phosphate. Flow rate was set at 0.5 ml/min. Detail of binding and elution conditions is described in the "Results and Discussion" section. Protein recovery was estimated by UV absorbance measurements. Non-reducing SDS-PAGE was carried out using 10% gel and non-reducing SDS-PAGE sample and tank buffers, all from Life Technologies. Gel was stained with SimplyBlue Safe-Stain from Life Technologies.

3. Results and discussion

3.1. Protein-A/Protein-G mixture

Protein-G is known to have higher affinity for certain subtypes of murine antibodies. Protein-A and Protein-G resins were mixed at equal volume and used to capture a murine monoclonal antibody (mAb). The above column was equilibrated with PBS. Loading of CM containing the murine mAb showed a portion of the mAb in the flow-through (FT)(data not shown). Elution of the mAb by low pH showed protein recovery far less than the loaded amount due to the observed FT. Addition of ammonium sulfate to the CM at 1 M prior to loading resulted in complete binding to the Protein-A/Protein-G mixed column equilibrated with 1 M ammonium sulfate, PBS (data not shown). The bound mAb was recovered with high purity by low pH elution. It thus appears that the specificity of antibody binding to Protein-A or Protein-G was not significantly altered by 1 M ammonium sulfate.

3.2. Protein-A chromatography

Similarly, binding of murine mAb occurred to Protein-A in PBS in the presence of 0.85 M ammonium sulfate (data not shown). After extensive wash with 0.85 M ammonium sulfate, the column was washed with 20 mM phosphate at pH 7.0, resulting in elution of small amounts of the murine mAb (~20% of the load). The remaining bound mAb was eluted with 1 M arginine, 20 mM citrate, pH 4.8 with high purity, indicating specificity of Protein-A for murine antibodies in the presence of 0.85 M ammonium sulfate. This elution pH in the presence of 1 M arginine is much higher than the pH required to elute human antibodies from Protein-A column. The highest elution pH with 1 M arginine for human mAb in Protein-A chromatography is ~pH 4.1 [12]. This is consistent with weaker affinity of murine antibodies for Protein-A.

3.3. Capto MMC chromatography

The above approach may effectively work to purify murine antibodies, but may suffer from complicated process due to the addition of ammonium sulfate at high concentrations. Addition of ammonium sulfate would increase the loading volume and solution viscosity. Furthermore, there is a possibility that the addition of ammonium sulfate causes protein precipitation. Here, we have attempted to use Capto MMC as a capture step.

3.3.1. Murine mAb

HEK CM containing the murine mAb was first tested. HEK CM (25 ml, ~2 mg mAb) was loaded onto a 1 ml Capto MMC column equilibrated with PBS. Fig. 1 shows SDS-PAGE profile of the load that contains various contaminating proteins. It is clear that the murine mAb bound to the column and many contaminants flowed through the column. A gradient elution was attempted from PBS to

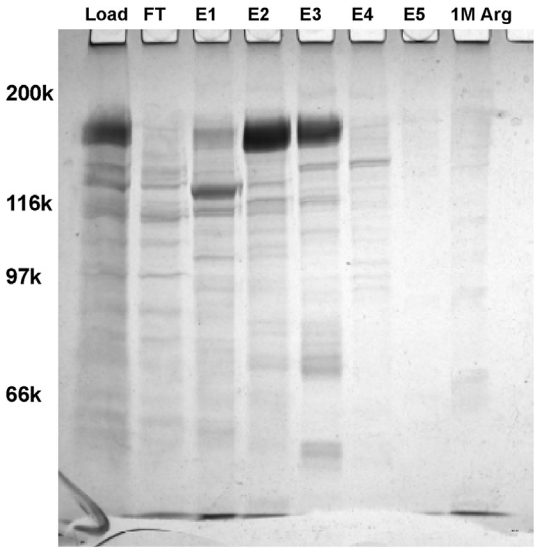

Fig. 1. Non-reducing SDS-PAGE profile of Capto MMC chromatography. All the figures below are non-reducing gels. HEK CM of murine mAb was loaded onto a 1 ml Capto MMC column in PBS. A flow rate of 0.5 ml/min was used here and in the following experiments. The bound proteins were eluted with a gradient from PBS to 1 M arginine, 20 mM phosphate, pH 7.0. The column was then washed with 1 M arginine, pH 7.0.

1 M arginine, 10 mM phosphate, pH 7.0. E1 showed elution of many contaminants. A majority of the murine mAb was eluted in E2 and E3 with some co-eluting contaminants. E4 and E5 and final 1 M arginine wash showed only contaminants.

A similar experiment was carried out with a different elution conditions. HEK CM (25 ml) was similarly loaded onto the 1 ml

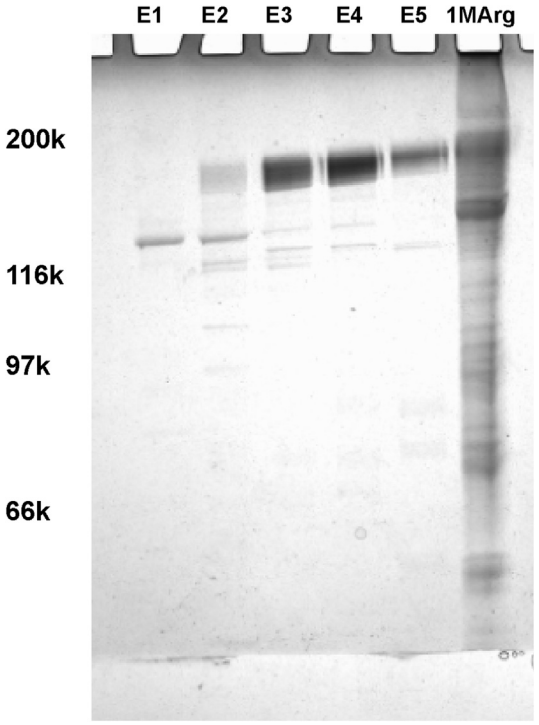

Fig. 2. SDS-PAGE profile of Capto MMC chromatography. HEK CM of murine mAb was loaded onto a 1 ml Capto MMC column in PBS. The bound proteins were eluted with a gradient from PBS to 1 M NaCl, 20 mM phosphate, pH 7.0, followed by a wash with 1 M arginine, pH 7.0.