Journal of Immunological Methods

Manuscript reference number: JIM-D-08-00300

Submitted: October 9, 2008

Revised: November 20, 2008

Accepted for publication: November 24, 2008

Reverse Calcium Affinity Purification of Fab with Calcium Derivatized Hydroxyapatite

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Abstract

This study introduces the application of calcium-derivatized hydroxyapatite for purification of Fab. Fab binds to native hydroxyapatite but fails to bind to the calcium derivatized form. IgG, Fc, and most other protein contaminants bind to the calcium form. This supports Fab purification by a simple flow-through method that achieves greater than 95% purity from papain digests and mammalian cell culture supernatants. Alternatively, Fab can be concentrated on native hydroxyapatite then eluted selectively by conversion to the calcium-derivatized form.

Keywords: Fab purification, hydroxyapatite, IgG, Fab, Fc

1. Introduction

Hydroxyapatite (HA) is a mineral of calcium and phosphate with the structural formula $(Ca_5(PO_4)_3OH)_2$. It has the ability to bind proteins by mixed mode interactions, principally phosphoryl cation exchange with protein amino residues, and calcium chelation with clusters of carboxyl residues (Gorbunoff, 1984a; 1984b; Gorbunoff and Timasheff, 1984). In the absence of phosphate, soluble calcium forms coordination complexes with HA phosphate groups, converting them into secondary calcium groups (Gorbunoff, 1984a, 1984b; Gorbunoff and Timasheff, 1984). This abolishes phosphoryl cation exchange while increasing the surface availability of calcium. The conversion to Ca-HA remains stable in the absence of phosphate, and can be reversed by washing the column with phosphate. Purification of Fab with phosphate gradients on native HA has been known for at least 20 years (Bowles et al, 1988). In this study we demonstrate that Fab flows through Ca-HA while IgG, Fc, and most mammalian cell culture supernatant (CCS) proteins bind by calcium affinity.

2. Materials and methods

2.1. Materials

The antibody used for these studies was Rituxan® (rituximab), an anti-CD20 chimeric monoclonal IgG used for the treatment of lymphoma. Immobilized papain was obtained from Thermo Fisher Scientific, Rockford, IL. Cell culture supernatant (CCS) depleted of IgG by protein A affinity chromatography, was obtained from Avid BioServices, Tustin,

CA. Buffer components were purchased from Sigma/Aldrich, St. Louis, MO. Ceramic hydroxyapatite CHT™ Type I, 20 µm, was obtained from Bio-Rad Laboratories, Hercules, CA. HA was packed into 1 mL (5 x 50 mm) and 2.5 mL (7.8 x 50 mm) MediaScout® columns by ATOLL GmbH, Weingarten, Germany. All chromatography experiments were performed on an AKTA® Explorer 100, GE Healthcare, Piscataway, NJ. Ready Gel™ Tris-HCl (10 % acrylamide) was purchased from Bio-Rad Laboratories. SeeBlue® Plus2 Pre-stained molecular weight standard was obtained from Invitrogen, Carlsbad, CA.

2.2. Procedures

2.2.1. Papain digestion of IgG

Purified IgG was digested with immobilized papain following the manufacturer's instructions. Briefly, the antibody and immobilized enzyme gel were equilibrated separately to 20 mM sodium phosphate, 10 mM EDTA, 20 mM L-cysteine, pH 7.0. The antibody (100 mg) was added to 5 mL of immobilized enzyme gel slurry and incubated at 37˚C for 16 hr with end-to-end mixing. The reaction was stopped by raising the pH to 7.5, and the immobilized papain was removed by centrifugation. After digestion, the preparation was dialyzed into 25 mM Tris, 50 mM sodium chloride pH 7.5, sterile filtered, and stored at -20°C.

2.2.2. Chromatography on Ca-HA

A 2.5 mL HA column was converted to Ca-HA by equilibration with 2.5 mM calcium chloride, 25 mM Hepes, pH 7.0, at a linear flow rate of 300 cm/hr (2.5 mL/min). 2 mL of

papain-digested antibody was equilibrated by addition of calcium chloride to a final concentration of 2.5 mM, and loaded onto the column. The column was restored to native HA by washing with 10 mM sodium phosphate, pH 7.0, eluted with a 20 column volume (CV) linear gradient to 200 mM phosphate, then cleaned with 500 mM phosphate, pH 7.0. The column was stored between runs in 20% ethanol, 10 mM phosphate, pH 7.0. In a second run, sodium chloride was added to the sample to a final concentration of 1 M. All other conditions were identical. In a third run, sample was equilibrated by addition of sodium phosphate to a final concentration of 5 mM, pH 7.0, and loaded onto a 2.5 mL native HA column equilibrated to the same conditions. The column was converted to Ca-HA and eluted with 2.5 mM calcium chloride, 25 mM Hepes, pH 7.0. It was then restored to native HA by washing with 10 mM sodium phosphate, pH 7.0, eluted with a 20 column volume (CV) linear gradient to 200 mM phosphate, and cleaned with 500 mM phosphate, pH 7.0. In a fourth run, one part sample was diluted in 9 parts IgG-depleted mammalian cell culture supernatant and equilibrated to 2.5 mM calcium chloride. It was applied to Ca-HA equilibrated to the same conditions. The column was restored to native HA, eluted, and cleaned with a step to 500 mM phosphate, pH 7.0.

2.2.3. SDS-Polyacrylamide gel electrophoresis (PAGE).

Fractions from Fab purifications were analyzed by SDS-PAGE under non-reducing conditions with a molecular weight standard. The proteins were stained with Coomassie blue. Fab purity was estimated by densitometry.

3. Results

Purified human monoclonal IgG was digested with immobilized papain, the immobilized enzyme removed, and the fragment-containing supernatant applied to Ca-HA. Fab was not retained, except for a fraction constituting less than 5% of the applied Fab. Fc and residual IgG were retained and subsequently eluted in a phosphate gradient (Fig. 1). The same results were observed when the sample was applied in 1 M sodium chloride. Fab bound to native HA and was eluted selectively when the column was converted to Ca-HA (Fig.2). Fc and residual IgG were also retained by native HA, but remained bound when the column was converted to Ca-HA, and were eluted in a phosphate gradient after the column was restored to native HA. Note the transient pH decrease that coincides with the introduction of calcium, and the transient pH increase that accompanies re-introduction of phosphate (arrows, Fig. 2). These pH transients provide a convenient indicator for conversion of native HA to Ca-HA, and for restoration of Ca-HA to native HA. Equilibration is complete when the transient passes. Fab diluted in mammalian CCS passes through Ca-HA with an amber color, indicating the presence of small-molecule media components, but most of the CCS proteins bound to Ca-HA along with Fc (chromatogram not shown). Analytical PAGE indicated that the purity of the Fab was 96% from both the papain digest and from the digest diluted in CCS (Fig. 3).

4. Discussion

In contrast to native HA, Ca-HA is devoid of surface phosphate groups for cation exchange retention of proteins (Gorbunoff, 1984a, 1984b; Gorbunoff and Timasheff, 1984). Retention of IgG and Fc fragments thus illustrates binding exclusively by calcium

affinity. The lack of ion exchange involvement is confirmed by the fact that IgG and Fc were retained with equal efficiency by Ca-HA in the presence of 1.0 M sodium chloride. Fab was retarded slightly by Ca-HA, indicated by trailing after injection (Fig. 1). Retardation suggests the existence of weak calcium interactions but insufficient affinity to achieve retention. The fact that Fab was unretained on Ca-HA indicates that Fab retention on native hydroxyapatite occurs dominantly through phosphoryl cation exchange.

These behaviors enable a simple purification procedure, in which a papain digest or Fab-containing mammalian CCS is applied to Ca-HA. Fab flows through the column at about 96% purity while other proteins are captured and disposed of in a subsequent cleaning step. The process requires only two buffers and process development is essentially limited to determining the Fc binding capacity of the column. Since the calcium affinity of Fc species in the absence of phosphate is unaffected by conductivity, sodium chloride can be included in the sample. This overcomes the solubility limitation reported as a problem by Bowles et al (1988) when equilibrating large sample volumes to low-phosphate binding conditions. Alternatively, the sample can be applied to native HA, and the Fab eluted selectively by conversion to Ca-HA (Fig. 2). This approach allows the Fab to be concentrated from a dilute source.

Fab purification with Ca-HA is reminiscent of Fab purification by protein A affinity chromatography (Ng and Osawa, 1997; Guerrier et al, 2001) to the extent that Fc and intact IgG are retained selectively on both supports. Protein A is simpler since it does not

require pre-equilibration of the sample, but HA offers several advantages: It is more economical. It is stable for thousands of hours in 1.0 M sodium hydroxide (Bio-Rad, 2007), allowing more rigorous sanitization than biological affinity ligands. There are no regulatory issues with potentially immunotoxic leachates. HA avoids the retention of Fab that can occur with antibodies that have variable-region affinity for protein A (Sasso et al, 1991; Åkerström et al. 1994; Ghose et al 2005). The greatest advantage over protein A affinity was that Ca-HA removed nearly all of the protein contaminants, in addition to Fc-containing species. Method performance with Fab produced in bacterial or yeast cell cultures remains to be evaluated. Additional studies will also help to determine what proportion of Fabs and other fragmentary constructs are served by this approach.

Acknowledgements

Some parts of this research were supported by NCI grant CA43904.

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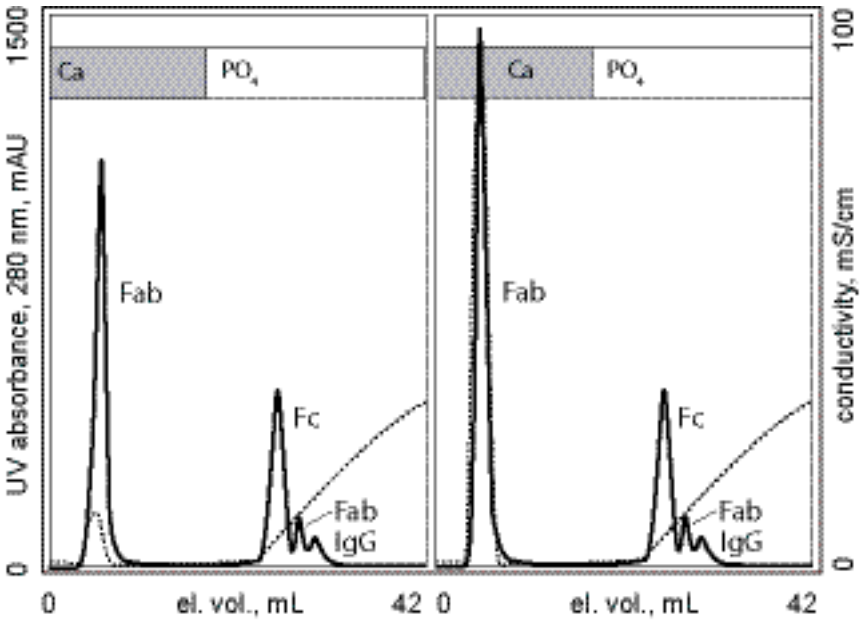

Figure 1. Purification of Fab with Ca-HA. The sample in the chromatogram on the left contained 50 mM sodium chloride. The sample in the chromatogram on the right contained 1.0 M sodium chloride. Refer to section 2.2.2 for experimental details.

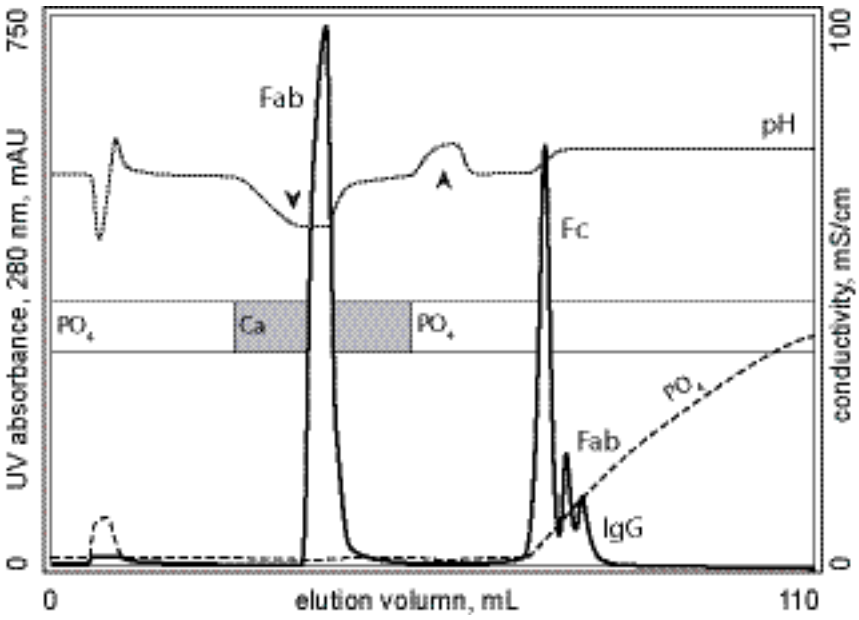

Figure 2. Binding of papain digest to native HA and selective Fab elution by conversion to Ca-HA, followed by restoration of native HA and elution in a phosphate gradient. The amplitude of the pH reduction associated with the introduction of 2.5 mM calcium was about 0.6 pH units. Please refer to sections 2.2.2 and 3 for additional discussion.

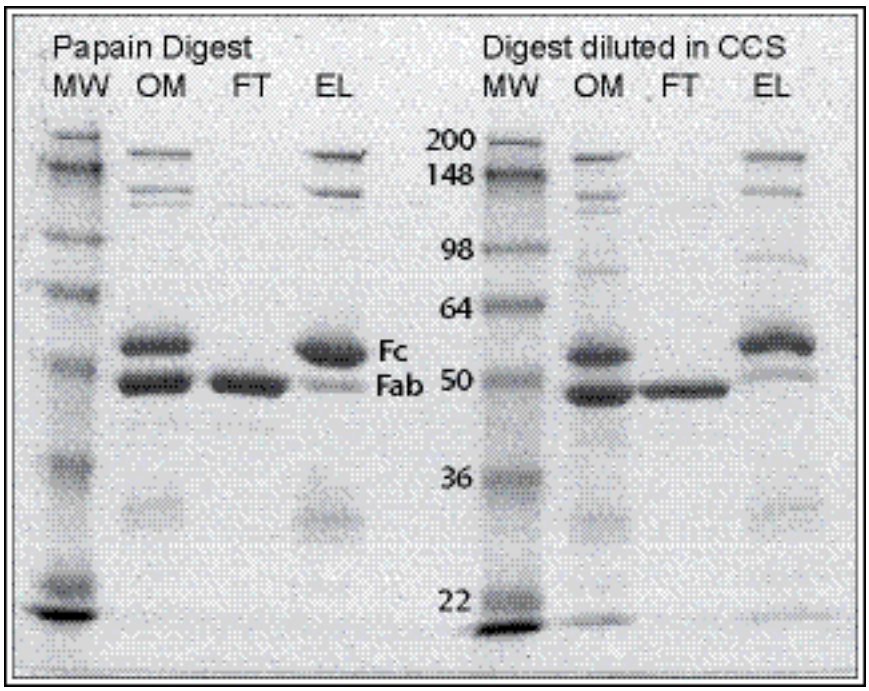

Figure 3. Non-reduced SDS-PAGE of Fab purification from papain digest and from a papain digest diluted in CCS. MW = molecular weight standards. OM = original material. FT = flow-through fraction. EL = elution fraction. Molecular weights of standards in kDa as indicated. For additional experimental details, please see section 2.2.3.