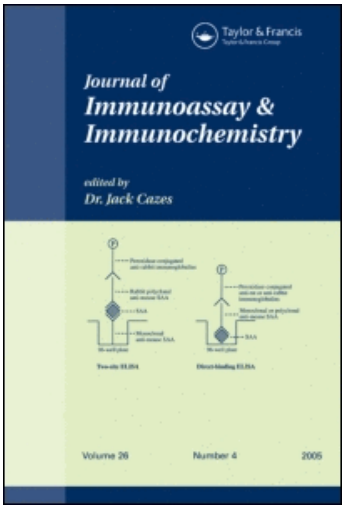

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Absence of Protein G-Fc Interaction in Ficin-Derived Mouse IgG_1 Digests

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ABSTRACT

Typical procedure for IgG fragmentation is based on proteolytic cleavage at the hinge region and usually involves a post-digestion purification step. In mice, IgG_1 has been found to bind poorly to protein A. As a result, protein G chromatography could be considered as an alternative for Fc removal. Protein G is generally expected to bind specifically to the Fc region of IgG, but applying protein G for the purification of Fab_2 fragment of mouse monoclonal IgG_1 under standard physiological conditions, we obtained reproducible clone-independent negligible protein G–Fc reactivity and strong protein G–Fab_2 interaction.

Key Words: Protein G; Mouse IgG_1 fragments; Ficin IgG digest; SDS-PAGE; Immunoblotting.

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INTRODUCTION

Protein G is widely used for isolation and purification of immunoglobulins. Native protein G is a 30–35 kDa molecule derived from the cell wall of the C or G strains of β-hemolytic streptococci. It has natural non-immune reactivity for immunoglobulin G.[1] The action of protein G is usually compared to protein A, but protein G offers wider host specificity. Unlike protein A, however, it doesn't usually interact with immunoglobulin classes other than IgG.[2] Like protein A, protein G is generally described as a protein that binds specifically to the Fc region of IgG.[3,4] Its affinity to the Fab region has been noticed previously, but was defined as poor or low,[5,6] and it's binding ability for Fab was estimated at only about 10% of its affinity for Fc.[5]

X-ray crystallography of protein G-mouse IgG_1 Fab fragment complex (using papain digest) suggested interaction of protein G with conserved regions of the Fab molecule.[7] More specifically, a majority of the hydrogen bonds between two proteins involves main-chain atoms from the highly conserved CH1 domain of IgG.[8] Erntell and colleagues showed the presence of two independent and separate binding sites for Fab- and Fc-fragments on protein G using human IgG.[9]

The use of mouse monoclonal F(ab′)2 fragments in immunochemical applications offers several advantages over intact antibodies. Fragments provide reduced non-specific interactions caused by the Fc region, decreased steric hindrance, and lower immunogenicity. A typical practical approach to antibody fragmentation is based on proteolytic cleavage of an immunoglobulin monomer (IgG) at the hinge region. The most frequently used enzymes for this purpose are papain, pepsin, elastase,[2] ficin, and bromelain.[10]

Preparation of the IgG fragments usually involves a post-enzymatic digestion purification step. In mice, IgG_1 has been found to bind poorly to protein A[11,12] and protein G chromatography could be considered as a reasonable, practical, option for Fc separation.

EXPERIMENTAL

Four clones of mouse IgG_1 were used in this study; they included two human thyroid stimulating hormone (hTSH) specific antibodies (Bayer Diagnostics, Elkhart, IN, USA) and two antibodies raised against human B-type natriuretic peptide (BNP32) produced by Shionogi and Co., Ltd. (Osaka, Japan). Fragmentation of antibodies was performed using ficin

according to a protocol outlined by Mariani et al.[10] Ficin was acquired from Sigma (St. Louis, MO, USA).

Enzymatic digests were fractionated using HiTrap Protein A and HiTrap Protein G columns (Amersham Pharmacia Biotech., Piscataway, NJ, USA). Chromatography was performed with a Waters multi-component HPLC system consisting of a model 717 autosampler, a 600E multisolvent delivery system, and a 490E multiwavelength detector (Waters Corp., Milford, MA, USA). Both columns were equilibrated using a loading buffer consisting of 0.1 M Tris/0.25 M NaCl (pH 7.3) at a flow rate of 0.5 mL/minute. The first fraction (void peak) on both protein A and G columns was eluted in the loading buffer and the second fraction (retained peak) was eluted using 0.1 M glycine buffer, pH 2.5. Data collection was carried out using TotalChrom Client Server system software by PE Nelson (Version 6.2.0). The functional activities of all Fab_2 fragments were tested in hTSH and BNP reagent systems developed for Bayer automated immuno analyzers (results not presented).

Electrophoresis[13] was performed with the Pharmacia Phast System using 4–15% polyacrylamide gradient gels (Amersham Pharmacia Biotech., Piscataway, NJ, USA). Immunoblotting[14] has been carried out using semi-dry transfer to a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA) followed by membranes' incubation with goat fragment-specific anti-mouse antibody conjugated to horse radish peroxidase (HRP) acquired from Accurate Chemicals (Westbury, NY, USA).

RESULTS AND DISCUSSION

As stated previously, four clones of mouse IgG_1 were used in this study. Figures 1–5, presented in this section, show the results obtained for one clone. All the other clones were tested and the results were identical to those presented below.

SDS-PAGE

Non-reducing SDS-PAGE (see Fig. 1) indicated that the first fraction (void peak, lane 3) collected from the protein A column contains the Fab_2 fragment and the second fraction (retained peak, lane 4) contained the Fc fragment. Electrophoretic profiles of the fractions obtained from the protein G (lanes 5 and 6) demonstrated a reversed pattern compared to results obtained with the protein A column.

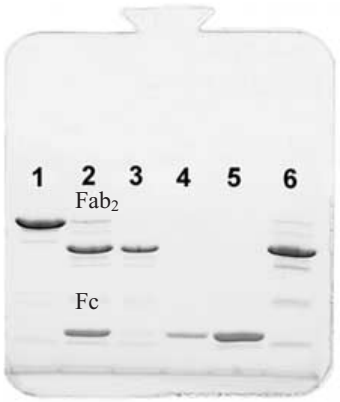

Figure 1. Non-reducing SDS-polyacrylamide gel electrophoresis analysis of protein A and protein G fractions. 1. Intact IgG; 2. Ficin digest of IgG; 3. Protein A-void fraction; 4. Protein A-retained fraction; 5. Protein G-void fraction; 6. Protein G-retained fraction.

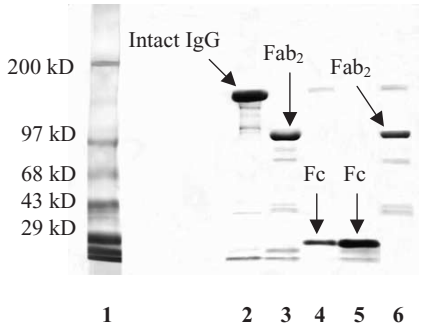

Figure 2. SDS polyacrylamide Western blot transfer. Nitrocellulose was stained with amido black. 1. High molecular weight standard; 2. Intact IgG; 3. Protein A-void fraction (Fab_2); 4. Protein A-retained fraction (Fc); 5. Protein G-void fraction (Fc); 6. Protein G-retained fraction (Fab_2).

Immunoblotting

Staining with amido black after Western transfer to a nitrocellulose membrane (see Fig. 2) has demonstrated that the protein profile is similar to SDS-gels. Main components of the protein A fractions 1

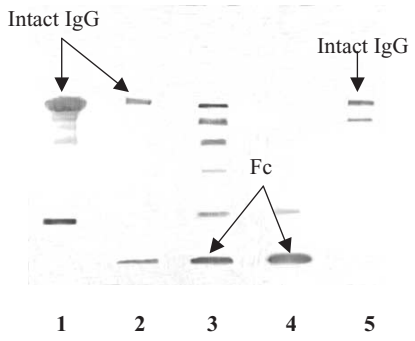

Figure 3. Fc specific Western blot analysis of protein A and G fractions. 1. Intact IgG; 2. Protein A-void fraction; 3. Protein A-retained fraction; 4. Protein G-void fraction; 5. Protein G-retained fraction.

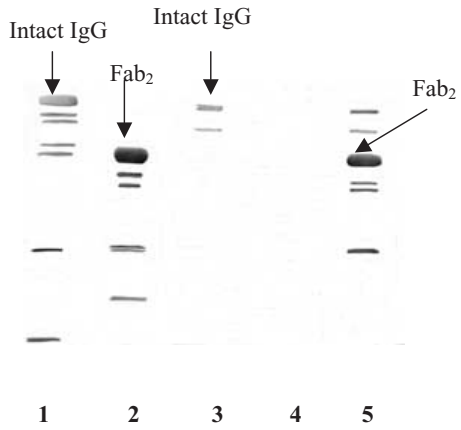

Figure 4. Fab specific Western blot analysis of protein A and G fractions. 1. Intact IgG; 2. Protein A-void fraction; 3. Protein A-retained fraction; 4. Protein G-void fraction; 5. Protein G-retained fraction.

(void, lane 2) and 2 (retained, lane 3) are Fab_2 and Fc bands, respectively. Conversely, protein G fraction 1 (void, lane 4) is dominated by the Fc fragment and the major element of the second fraction (retained, lane 5) is the Fab_2 band.

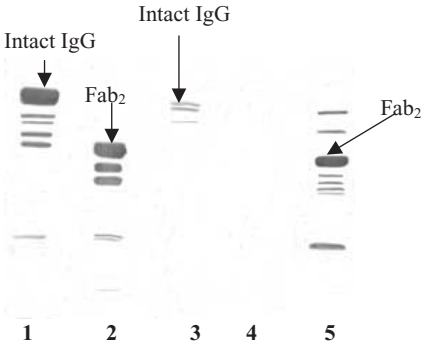

Figure 5. Fab_2 specific Western blot analysis of protein A and G fractions. 1. Intact IgG; 2. Protein A-void fraction; 3. Protein A-retained fraction; 4. Protein G-void fraction; 5. Protein G-retained fraction.

A membrane incubated with Fc specific antibody (see Fig. 3) has detected intact IgG (lane 1), and some minor IgG-components of both protein A fractions (lanes 2 and 3). Lane 4 shows that the Fc fragment is not retained on a protein G column. This observation, together with the electrophoretic pattern shown in lane 5, suggests that protein G has a stronger affinity for Fab_2. The presence of trace amounts of the Fc fragment in the void fraction of protein A separation is due to relative weakness of IgG_1 Fc binding to this column.

Immuno-staining with Fab and Fab_2 specific antibodies (see Figs. 4 and 5) have developed essentially the same patterns on both membranes. These reagents stained a major band in the first fraction of protein A (void, Figs. 4 and 5, lane 2) and second fraction of protein G column, and intact IgG (Figs. 4 and 5, lane 3). It has also detected a minor band in the second fraction of the protein A column. These results show the protein G column preferentially retains the Fab_2 fragment produced after ficin digestion.

CONCLUSION

Purification of ficin digests on a protein G column shows that the Fab_2 fragment is largely retained while the Fc fragment elutes

in the void. No noticeable interaction between protein G and mouse IgG_1 Fc fragment was detected under the applied conditions.

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