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Short communication

Use of thiophilic adsorption in the purification of biotinylated Fab fragments

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Abstract

A method for the purification and biotinylation of Fab fragments, using thiophilic adsorption (T-gel), is described. The T-gel was used to purify an IgG fraction directly in the buffer suitable for biotinylation, and to adsorb intact IgGs and papain after enzymatic digestion. For the final step, Fc fragments were removed with a protein A column.

1. Introduction

Although procedures for the purification of IgG fragments from human plasma have been reported [1,2] these methods suffer from various limitations, i.e. they are time consuming and consist of several tedious steps. Recently, an improved purification scheme has been proposed [3] using immobilized papain in a method that involved numerous steps.

Our interest in the improvement of the preparation of labelled (biotinylated) human Fab fragments to be used in specific binding experiments [4] led us to develop a simple procedure essentially based on salt-dependent chromatography on T-gel [5]. A major advantage of the proposed method is that the IgG and Fab fragments do not get into contact with denaturing buffers and hence retain their antigen-binding ability.

2. Experimental

2.1. Preparation of IgG fraction

From a pool of sera from healthy donors the IgG fraction was purified by salt dependent chromatography [5,6] on a support obtained by reaction of β-mercaptoethanol with divinylsulfone-activated agarose (the so-called "T-gel") prepared according to the procedure described in Ref. [7]. Pooled sera (35 ml) were mixed with 10% solid ammonium sulfate for one hour at 4°C, centrifuged for 10 min at 10 000 *g*, and the supernatant passed through a T-gel column (7 × 2.5 cm I.D.) equilibrated with 50 m*M* Trisacetate buffer (pH 7.4) containing 10% ammonium

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sulfate. Under these conditions, all IgG isotypes were bound [8] and could be eluted with 0.1 *M* borate buffer (pH 8.5). Albumin, IgG, IgA and IgM concentrations were determined in the eluted fractions by nephelometry (BNA, Behring, Rueil-Malmaison, France), and the protein concentration of the IgG fraction was determined by measurement of the absorbance at 280 nm.

2.2. Biotinylation and preparation of Fab fragments

Biotinylated IgGs were prepared by adding 3 ml of d-biotin-N-hydroxysuccinimide ester in dimethylsulfoxide (1 mg/ml) to the T-gel eluate (304 mg of protein in 68 ml). After 4 h at 20°C, the mixture was dialyzed twice against 10 m*M* Tris–20 m*M* NaCl (pH 7.0) at 4°C.

Fab fragments were prepared by 2% (w/w) papain digestion (Sigma Chimie, Saint Quentin Fallavier, France) according to the procedure described in Ref. [9], and iodoacetamide (100 m*M*) was used to terminate digestion. The digest was then dialyzed against 50 m*M* Tris buffer.

2.3. Separation of intact IgGs, papain, Fab and Fc fragments

The remaining intact IgG and papain were removed from the mixture using their thiophilic properties. The mixture was supplemented with 10% ammonium sulfate and passed through a column containing 6 ml of T-gel. Then, the effluent containing both the Fab and Fc fragments was dialyzed against PBS (pH 8) and loaded onto an immobilized protein A column (2.5 × 2 cm I.D.) (Pierce, Rockford, IL, USA). The separation of the fragments was controlled using anti-IgG light-chain antibodies.

2.4. Electrophoresis

The separated proteins were analyzed during the different steps by SDS-PAGE under reducing or non-reducing conditions, on Phastgels 10–15% (Pharmacia-Biotech, St. Quentin Yvelines, France) and visualized by silver staining.

3. Results and discussion

The human IgG fraction was purified as described in the Experimental section. The eluent for the T-gel column (0.1 *M* borate buffer, pH 8.5) was chosen to allow the biotinylation reaction without prior dialysis or desalting step. Table 1 shows that about 300 mg of purified protein was obtained from the T-gel, as measured spectrophotometrically at 280 nm using a specific absorbance of 14 for a 1% solution of IgG at 280 nm. By immunonephelometry IgGs were found to constitute $\geq 99\%$ of the total protein.

After biotinylation and papain digestion, a smaller T-gel column was used to separate intact IgGs and papain from the generated fragments. Using 10% ammonium sulfate, IgG and papain bound to the thiophilic gel, while the fragments were only weakly retained and found in the flow-through fraction (Fig. 1A). The gel was then washed in the absence of ammonium sulfate to elute the bound proteins (Fig. 1A). To remove any contaminant from the flow-through, it was chromatographed a second time on the same column (Fig. 1B). Analysis of the separation was carried out using SDS-PAGE. Only IgG fragments were found in the flow-through fraction (Fig. 2, lanes D,E).

Finally, Fab and Fc fragments were separated on protein A. The purity of the sample excluded from the column was assessed by SDS-PAGE and by its reactivity against anti-IgG light-chain antibodies (data not shown). Only one band was visible, corresponding to a polypeptide of about 50 000 Da. This polypeptide was recognized by anti-light chain antibodies, while peptides eluted from protein A were not recognized at all. To check the efficiency of its biotinylation, it was spotted onto nitrocellulose strips and tested using horse radish peroxidase-labelled streptavidin [10]. The different tests performed led us to conclude that the polypeptide was pure biotinylated-Fab.

In conclusion, salt-dependent (thiophilic) chromatography enables the preparation of labelled IgG fragments from plasma in a limited number of steps, with a high purity and in a

Table 1
Purification of biotinylated Fab fragments from human plasma

Step	Volume (ml)	Total protein (mg)	Identified proteins[a]
Starting plasma	35	2100	plasma proteins
Ammonium sulfate-treated plasma	36	2088	
T-gel (eluate)	68	304	IgG (≫ 99%)
Biotinylation	68	304	
Digestion with papain[b] and dialysis	37	67	Fab, Fc, undigested IgGs and papain
T-gel (flow-through)	40	27	Fab, Fc
Protein A[c] (flow-through)	8	1.4	Fab

[a] Proteins were identified by their molecular masses in PAGE, and their recognition by specific antibodies.
[b] The digestion was performed on 34 ml (150 mg) of biotinylated IgGs.
[c] Purification performed on a sample of 5 ml at 405 μg/ml.

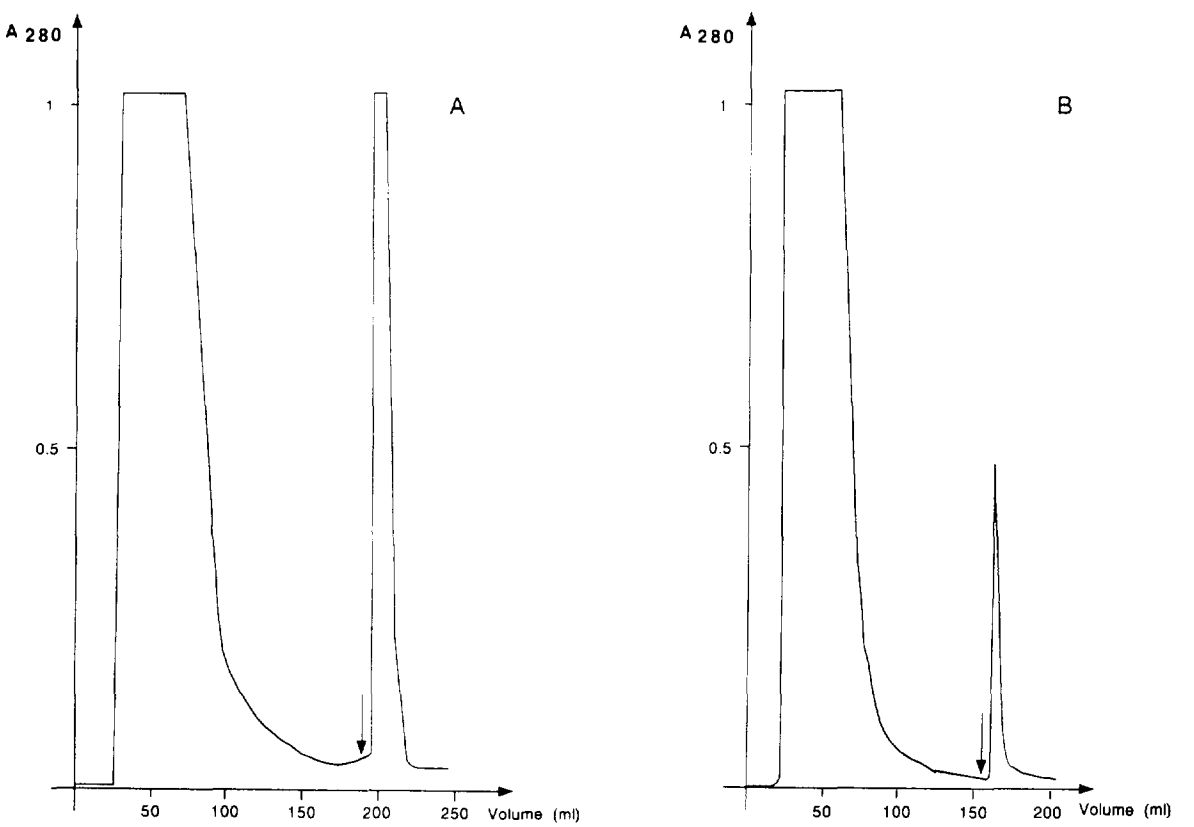

Fig. 1. Elution profiles obtained after chromatography of biotinylated-IgG digest on a 6-ml T-gel column. Flow-rate: 0.5 ml/min. Buffer 1: 50 m*M* Tris-acetate, pH 7.4 + 10% ammonium sulfate. Buffer 2: buffer 1 without ammonium sulfate. Arrow indicates change from buffer 1 to buffer 2. (A) Incorporation of 37 ml of digest. (B) Incorporation of the flow-through (peak 1) of chromatography A.

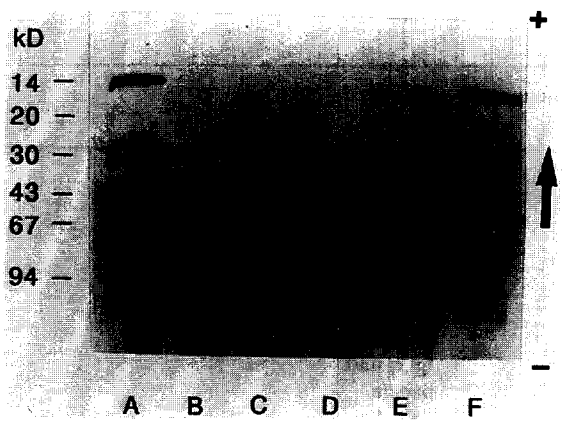

Fig. 2. Analysis by SDS-PAGE in non-reducing conditions of biotinylated-IgG digest before and after T-gel chromatography. Lanes: A and F, standards; B, human IgG; C, biotinylated-IgG digest; D and E, flow-through of the T-gel chromatography shown in Fig. 1A and B respectively, containing IgG fragments. In the right margin, vertical arrow indicates direction of electrophoresis.

bioactive form. For example, we have used biotinylated-Fab fragments prepared by this method in the study of the autoreactivity of natural antibodies with a human brain antigen [11].

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