Journal of Chromatography, 597 (1992) 257–262
Elsevier Science Publishers B.V., Amsterdam

CHROM. 23 898

Affinity purification of antibodies using immobilized FB domain of protein A

Beka Solomon*, Osnat Raviv, Efrat Leibman and Gideon Fleminger

Department of Molecular Microbiology and Biotechnology, George Wise Faculty of Life Sciences, Tel-Aviv University, Tel-Aviv 69978 (Israel)

ABSTRACT

A continuous method for the efficient digestion of protein A into active fragments (FB, M_r = 7000) using immobilized trypsin was developed. These fragments originate from almost identical five-repeated monovalent Fc-binding units of 58 residues each. The fragments obtained were found to be similar to the recently described genetically engineered fragment B. Antibody-binding characteristics of the FB domain and also of intact protein A, immobilized on to adipic dihydrazide-modified Eupergit CB6200 beads, were investigated. Based on the experimental data obtained, a high-performance liquid chromatographic column containing C30N Eupergit C-immobilized FB domain was prepared and its performance in antibody purification was compared with that of Eupergit C-immobilized intact protein A.

INTRODUCTION

Affinity chromatography using protein A is a well known one-step procedure for the purification of immunoglobulins [1]. Protein A contains a tandem of five similar domains, designated E, D, A, B and C, each capable of binding the Fc region of immunoglobulin G (IgG) from various mammals [2]. As has been demonstrated [3], steric hindrance prevents the binding of more than one or two IgG molecules to the immobilized protein A molecule. Tryptic digestion of protein A leads to formation of fragments with a molecular mass (M_r) of 7000 [4], capable of binding to IgG, and named fragment FB. The amino acid residues involved in the interaction between the FB fragment and the Fc region of IgG were identified by a X-ray study of the complex. These residues do not comprise a continuous sequence and the minimum number of amino acids which are required for binding of the IgG was found to be at least 25 [5].

It has recently been reported [6] that a synthetic gene coding for a single IgG binding domain B of protein A was constructed based on the three-dimensional structure of the crystallized complex between the FB domain and the Fc fragment. The recombinant domain B is a single-chain polypeptide of 60 amino acid.residues (M_r = 6770) [6].

In this paper, we describe a continuous process for the proteolytic hydrolysis of protein A by immobilized trypsin. The potential of the immobilized fragments obtained for purification of immunoglobulins, based on the antibody recognition, was investigated. These fragments, obtained by enzymic cleavage or by recombinant DNA technology, immobilized on to insoluble supports, may be useful in the general-purpose purification of monoclonal antibodies (mAbs).

EXPERIMENTAL

Protein A purchased from Repligen (Cambridge, MA, USA), immobilized trypsin from Sigma (St. Louis, MO, USA), Eupergit C beads C30N and CB6200 from Rohm Pharma (Darmstadt, Germany) and horseradish peroxidase (HRP)-labelled anti-mouse IgG antibodies from Jackson Immunoresearch Labs. (West Grove, PA, USA). Anti-CPA

0021-9673/92/$05.00 © 1992 Elsevier Science Publishers B.V. All rights reserved

mAb (CP100) was prepared as described previously [7].

Preparation of FB fragments by continuous proteolytic cleavage of protein A

The reaction mixture containing 10 mg of protein A in 2.0 ml of 0.1 *M* phosphate buffer (pH 8) and trypsin–agarose beads (1 mg) was incubated at room temperature in an Amicon filtration cell (10 ml) equipped with a PM 10 membrane. A constant volume of the reaction mixture was maintained by addition of the above buffer. The filtrate was concentrated in an Amicon cell equipped with a UM-2 membrane (M_r cut-off 2000). The concentrated filtrate containing the digested protein A fractions was loaded on to a gel permeation column of Superose HR-12 (Pharmacia, Uppsala, Sweden) in 0.1 *M* Tris buffer (pH 8.0). The elution of peptides was followed by measuring the absorbance ar 230 nm at a flow-rate of 0.4 ml/min. The amount of peptides released from the column was measured by the BCA method (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. The main peak (fractions 39–45) was collected and subjected to polyacrylamide gel electrophoresss and amino acid analysis. As a reference the B fragment obtained by genetic engineering [6], generously provided by Dr. Mori Sato (Tokyo Research Labs., Tokyo, Japan), was used for comparison.

Determination of biological activity of the protein A fragments

Antibody binding activity in each fraction was determined using an enzyme-linked immunosorbent assay (ELISA) procedure as follows. ELISA plates were coated with 100 μl of each fraction by overnight incubation at 4°C. After repeated washings with phosphate-buffered saline (PBS) normal mouse serum diluted 1:10 000 in 0.1 *M* Na_2PO_4 (pH 8.0) containing 2% bovine serum albumin (BSA) was added and the mixture was incubated for 1 h at 37°C. The amount of antibody bound to peptides was measured by reaction with goat anti-mouse antibodies labelled with HRP (1:5000).

In another set of experiments, peptide contained in the concentrated main peak (fractions 39–45) was bound covalently to CB6200 beads, either directly to the oxirane groups of the beads or via glutaraldehyde after treatment of the beads with adipic dihydrazide (ADH) as follows. Thirty beads were incubated with 0.1 *M* ADH in 0.2 *M* sodium carbonate buffer (pH 9) overnight at room temperature. After repeated washings with PBS and blocking of the unreacted oxirane groups with thioglycerol (0.2 *M*), glutaraldehyde (1% in PBS) was added. After incubation for 1 h the peptide, as well as the whole protein A in PBS containing 0.02 *M* $NaCNBH_3$, were added with gentle shaking for another 1 h at 37°C. The remaining aldehyde groups were blocked by treatment with 0.2 *M* Tris–HCl buffer (pH 8.0). Carboxypeptidase A (CPA) as a complex with anti-CPA antibody (CP100) was added to each bead (2 μg per bead) and incubated for 1 h at 37°C. The amount of the antibody bound by the immobilized fragments was determined by measuring the activity of the immunocomplexed CPA [8].

HPLC affinity purification of mAb using immobilized FB fragment

An affinity column was prepared by coupling of the FB fragment or of protein A to beads of C30N Eupergit C as follows. Protein A or FB fragment (1 mg in 1 ml of PBS) was coupled to 250 mg of Eupergit C-ADH via reaction with glutaradehyde, as described above. Each of the preparations was packed in a high-performance liquid chromatographic (HPLC) column (25 mm × 4 mm I.D.) and their performance in antibody purification was measured as follows.: 2 mg of anti-CPA (CP100) in 0.1 *M* sodium phosphate buffer (pH 8.0) were loaded on to each column, and the columns were eluted with 0.2 *M* citrate buffer (pH 3.0). The amount of eluted protein was determined by measuring the absorbance of the eluent at 280 nm.

Determination of the apparent binding constant of FB domain and protein A to the monoclonal antibodies

Immobilized protein A and/or FB fragments (20 μl containing 15 μg of bound ligand) were incubated with increasing amounts of fluorescein isothiocyanate (FITC)-labelled mAb (CP100, 15–75 μg) in PBS (pH 7.4), for 30 min. The amount of antibody bound was determined by measuring the fluorescence of the reaction mixture supernatant at $\lambda_{ex} = 475$ nm and $\lambda_{em} = 515$ nm at zero time and at the end of the incubation period using a Perkin-Elmer Model LC-50 spectrofluorimeter. The appar-

ent binding constant of the immobilized FB and protein A to the FITC-labelled antibody (CP100) was determined by a Scatchard plot [9].

RESULTS AND DISCUSSION

Immobilized protein A has wide application in the isolation of whole IgG fractions or individual IgG subclasses, the separation of cells and the removal of immunocomplexes from plasma as a means of immunotherapy [1]. In spite of the high selectivity of protein A affinity chromatography by the common commercially available immobilized protein A preparations has considerable drawbacks, *e.g.*, leakage from the matirx, relatively low IgG binding capacity and, particularly, high cost of commercially available preparations. In contrast, Eupergit C–protein A possesses a very low degree of protein leakage [10] but has a low antibody-binding capacity.

Protein A, in solution, possesses at least four sites for IgG binding. However, in the immobilized form, only one or two sites are available for antibody binding [3]. Immobilization of an isolated single active fragment is expected to increase the specific IgG-binding activity of the matrix. In order to obtain such active fragments in high yield, a procedure for digestion of protein A to its active monomers FB(s) by continuous treatment with trypsin was developed. The fragments formed were removed from the reaction mixture immediately by ultrafiltration, avoiding excessive digestion. Separation of the fragments obtained by gel filtration on a Superose HR-12 column yielded several peaks of similar molecular masses, all of them posessing antibody-binding activity (Fig. 1). The main peak (fractions 39–45), containing essentially the FB monomer, was collected and subjected to amino acid analysis. As shown in Table I, the FB and B (genetically engineered) fragments are very similar in their amino acid compositions.

Protein A and the FB fragment were immobilized on to Eupergit C-based carriers. The carriers used in this study were Eupergit C-coated, 6 mm polymethylmethacrylamide beads (designated CB6200), especially suitable for application in immunodiagnostic assays, and C30N Eupergit C beads, which are suitable for affinity chromatographic separation. Coupling was performed either directly via re-

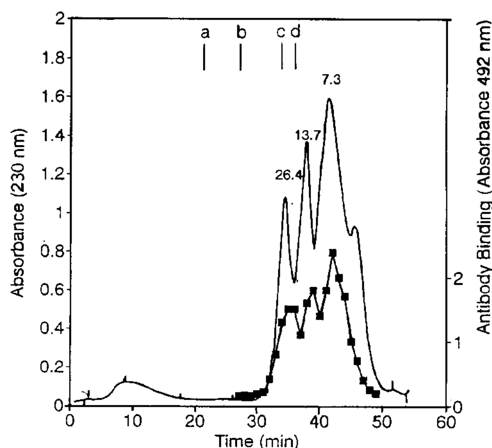

Fig. 1. Separation of protein A fragments obtained by tryptic digestion by gel filtration. Proteolytic digest of protein A (0.5 ml) was applied on a Superose HR-12 column (25 × 0.8 cm I.D.). Elution of the peptides with 0.1 *M* Tris–HCl (pH 8.0) at 0.4 ml/min was followed by measuring the absorbance at 230 nm (top trace). The antibody-binding capacity of the eluted peptides was determined by an ELISA test (see Experimental for details) (■). The following molecular mass standards were used for calibration of the column: (a) IgG (150 000); (b) BSA (64 000); (c) chymotrypsinogen (24 000); (d) cytochrome *c* (14 000). Numbers on top of the peaks indicate the calculated molecular masses (in kilodalton) of the respective peptides.

TABLE I

AMINO ACID ANALYSIS OF FB DOMAIN OBTAINED BY PROTEOLYTIC DIGESTION OF PROTEIN A WITH IMMOBILIZED TRYPSIN COMPARED WITH FRAGMENT B OBTAINED BY GENETIC ENGINEERING [6]

Amino residue	No. of residues	
	FB domain	Fragment B
His	–	1
Pro	–	3
Asp	10.1	12
Thr	2.9	0
Ser	3.6	3
Glu	10.5	11
Gly	7.6	1
Ala	4.1	7
Ile	3.2	2
Leu	6.1	7
Tyr	1.9	1
Phe	2.4	3
Lys	6.0	6

action with the oxirane groups of the matrix or via glutaraldehyde to ADH-treated beads. The antibody-binding activities of these preparations were determined by their capability to bind anti-CPA antibody CP100. This antibody has been shown in the past to react with regions of CPA remote from its active site, so that the activity of the enzyme is not subject to interference by reaction with the antibody [7]. The amount of antibody bound to each of the immobilized protein A preparations was thus determined by incubation with CPA and measurement of the bead-associated CPA activity. As shown in Table II, the FB fragment binds mAb to a similar extent as intact protein A, when immobilized by the direct procedure.

As reported [11], the dynamic capacity of solid matrices are strongly diffusion controlled. Introduction of a spacer between the matrix and the ligand is expected to improve the accessibility of IgG molecules to more flexible FB or protein A molecules. Indeed, an increase of 2–3 fold in the antibody-binding capacity was obtained for FB domain and protein A bound via the ADH–glutaraldehyde binding procedure (Table II).

TABLE II

ANTIBODY-BINDING CAPACITY OF INTACT PROTEIN A, FB DOMAIN, OBTAINED BY ENZYMATIC CLEAVAGE OF PROTEIN A AND GENETICALLY ENGINEERED FRAGMENT B IMMOBILIZED ON EUPERGIT C (CB6200) BEADS

Ligand	Binding method[a]	
	Via adipic dihydrazide	Via oxirane groups
Protein A	0.24	0.09
Fragment B	0.16	0.11
FB domain	0.28	0.10
Blank[b]	0.03	0.03

[a] Numbers represent enzymic activity of CPA bound to the antibodies immobilized via the respective ligands.
[b] Without bound ligand.

Binding studies showed that the apparent association constant of FB domain with the FITC-labelled mAb ($2 \cdot 10^6$ l/mol) is slightly lower than the respective value obtained for intact protein A ($6 \cdot 10^6$

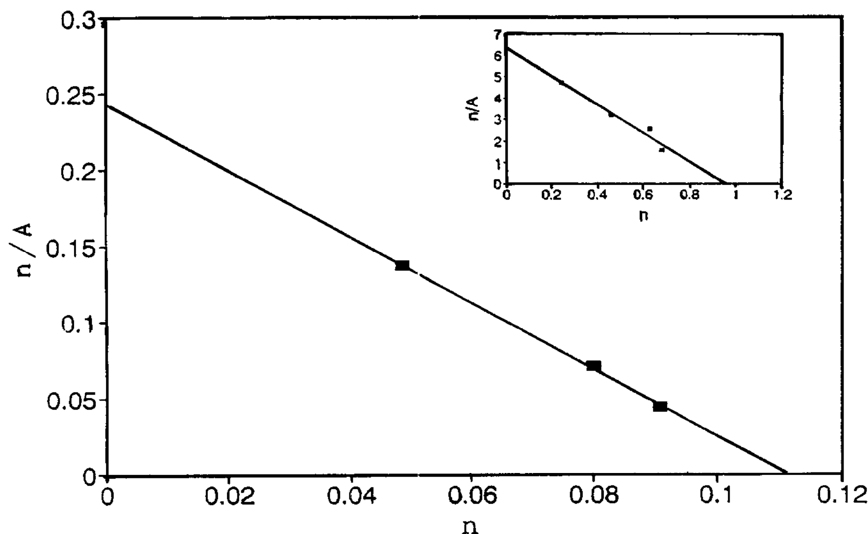

Fig. 2. Scatchard plot for the binding of antibody (CP100) to immobilized FB domain. Inset: corresponding Scatchard plot for immobilized intact protein A. Binding of fluorescently labelled antibody to the immobilized ligands was determined by measuring the fluorescence of the supernatants at zero time and after incubation for 30 min in PBS (pH 7.4) (see Experimental for details). The experimental binding parameter n is defined as the molar ratio between the bound antibody and the total ligand concentrations. The molar concentration of the free antibody is represented by A.

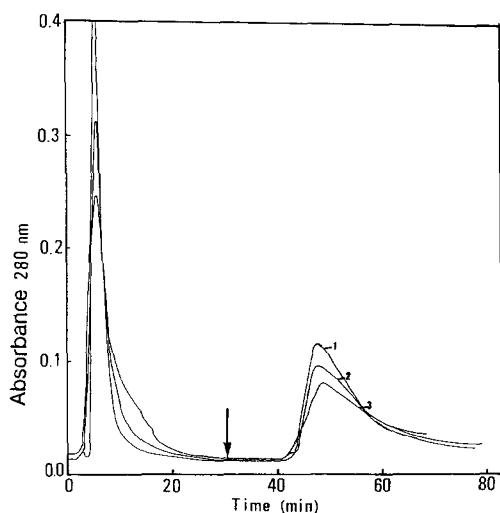

Fig. 3. Elution profiles of antibodies eluted from affinity columns of immobilized FB domain (1) and protein A (2) immobilized via ADH–glutaraldehyde and protein A (3) immobilized by direct reaction with oxirane groups of the matrix. A 2-mg amount of antibody in 0.1 *M* phosphate buffer (pH 7.4) was loaded on each column. Elution was carried out with 0.2 *M* citrate buffer (pH 3.0) (marked by the arrow).

l/mol), but is still high enough to maintain the high specificity towards antibodies to be purified (Fig. 2).

The experimental results were applied to the preparation of an affinity column and used in HPLC for the purification of mAbs. The carrier used (Eupergit C30N) has previously been shown to be suitable for HPLC and very stable with respect to leakage of the ligands [12]. Moreover, a detailed study of the leakage of protein A from Eupergit C as well as other commercially available matrices, performed by Fuglistaller [10], showed that Eupergit C–protein A has the lowest ligand leakage values. Eupergit C also possesses very good flow properties compared with the other gels and high stability at elevated pressure [10]. The columns (25 mm × 4 mm I.D.) were packed with the immobilized FB domain and intact protein A bound via ADH–glutaraldehyde to Eupergit C-C30N. The performance of the columns in binding and elution of CP100 is shown in Fig. 3. The antibody-binding capacity of the FB fragment was higher than intact protein A under similar conditions, as shown in Table III. FB monomer (1 mg) immobilized on ADH–Eupergit C was capable of binding 2.4 mg of mAb, compared with a value of 1.6 obtained for intact protein A. The same amount of protein A directly immobilized on to Eupergit C binds only 0.93 mg of mAb. These results are in accordance with those obtained with the CB6200 beads presented above (Table II).

TABLE III

ANTIBODY-BINDING CAPACITY OF EUPERGIT C30N-IMMOBILIZED PROTEIN A AND FB DOMAIN DETERMINED BY AFFINITY CHROMATOGRAPHY

Ligand	Binding method	Antibody bound (mg/mg ligand)
Protein A	ADH[a]	1.6
Protein A	Direct[b]	0.93
FB domain	ADH	2.4

[a] Binding of the protein via adipic dihydrazide groups of the matrix.
[b] Binding of the protein via oxirane groups of the matrix.

The data presented show that the immobilized FB domain possesses the same antibody-binding specificity as the Eupergit C-immobilized protein A but has increased binding capacity. Recently, an affinity chromatographic procedure, based on a non-peptide, low-molecular-mass ligand which mimics proteins A and G, was described [13]. This procedure seems to be efficient for antibody purification but lacks the sub-class specificity of protein A or FB domain.

CONCLUSIONS

Immobilized FB domain shows a higher antibody-binding capacity than intact protein A, measured under the same experimental conditions. Binding of FB domain or intact protein A to Eupergit C via ADH as a spacer results in a considerable improvement in the antibody binding capacity of the immobilized ligands. The immobilized FB domain or smaller fragments, containing the binding site of the Fc region of IgG, obtained by enzymatic or chemical cleavage or recombinant DNA technology, may be of general use in the purification of monoclonal antibodies in an efficient and economical manner.

REFERENCES

1 J. J. Langone, *J. Immunol. Methods*, 55 (1982) 277.
2 J. Sjodahl, *Eur. J. Biochem.*, 73 (1977) 343.
3 H. Hjelm, K. Hjelm and J. Sjoquist, *FEBS Lett.*, 28 (1977) 73.
4 H. Hjelm, J. Sjodahl and J. Sjoquist, *Eur. J. Biochem.*, 57 (1975) 395.
5 J. Deisenhofer, *Biochemistry*, 20 (1981) 2361.
6 A. Saito, S. Honda, T. Nishi, M. Koike, K. Okazaki, S. Itoh and M. Sato, *Protein Eng.*, 2 (1989) 481.
7 B. Solomon, R. Koppel, D. Kennet and G. Fleminger, *Biochemistry*, 28 (1989) 1235.
8 B. Solomon, N. Moav, G. Pines and E. Katchalski-Katzir, *Mol. Immunol.*, 21 (1984) 1.
9 G. Scatchard, *Ann. N.Y. Acad. Sci.*, 51 (1949) 660.
10 P. Fuglistaller, *J. Immunol. Methods*, 124 (1989) 171.
11 K. C. Hou, R. Zaniewski and S. Roy, *Biotechnol. Appl. Biochem.*, 13 (1991) 257.
12 G. Fleminger, E. Hadas, T. Wolf and B. Solomon, *J. Chromatogr.*, 510 (1990) 271.
13 T. T. Ngo, presented at the *9th International Symposium on Affinity Chromatography and Biological Recognition, Yokohama, September 24–28*, Abstract No. L31.