ELSEVIER

Journal of Chromatography B, 664 (1995) 175–183

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Affinity purification of polyclonal antibodies using immobilized multimeric peptides

Antonio Verdoliva, Giovanni Cassani, Giorgio Fassina*

Protein Engineering, TECNOGEN S.C. p. A., Parco Scientifico, 81015 Piana di Monte Verna (CE), Italy

Abstract

The possibility of using multiple antigenic peptides (MAP) not only for the production and characterisation of antibodies but also for their purification by affinity chromatography, has been explored with two different tetrameric MAPs synthesised starting from a tetradentate lysine core. Recognition selectivity and specificity of the multimeric antigens were retained after immobilization on preactivated affinity supports, allowing convenient antibody purification directly from crude sera in a single chromatographic step. Since antibodies raised against MAPs recognise very frequently the N-terminal portion of the peptide antigen, results suggest that only a limited number of peptide chains remains covalently linked to the solid phase, leaving the others uncoupled and free to interact fully with the antibody. Recovery of antibody immunoreactivity from affinity purifications on MAP-columns was much higher than that obtained from columns prepared by immobilizing at the same density the corresponding linear peptide antigen. The purity of thus obtained antibodies is also far superior, as detected by SDS-PAGE analysis. Retention of the multimeric peptide recognition properties for the corresponding antibodies after immobilization on solid supports suggests that production, characterization, and even the affinity purification of anti-peptide antibodies, could be carried out simply and conveniently via the synthesis of a single multimeric antigen, without additional steps.

1. Introduction

Production of antibodies to synthetic peptides has been extensively used to define the structure and function of proteins [1], to localize the antigenic determinants contributing to idiotypic systems [2], and to obtain sequence-specific ligands useful for the development of affinity purification strategies [3]. Synthetic peptides have also been suggested as potential vaccines, and used successfully for protection of cattle against foot-and-mouth disease [4] and protection of monkeys against experimental malaria infection [5]. Anti-peptide antibodies are conventionally obtained by immunization with synthetic peptides covalently coupled to a large carrier molecule capable of imparting immunogenicity to the hapten [6]. The most widely used carriers are proteins such as KLH (keyhole limpet hemocyanin) and BSA (bovine serum albumin). In addition, peptide antigen polymerization by chemical means has also been proposed [7,8]. These methods, while effective in producing antibodies, suffer from the ambiguity of the structure and composition of the resulting antigenic material, require several synthetic steps, and lead to modified antigens not usable for the development of purification strategies.

* Corresponding author.

0378-4347/95/$09.50
SSDI 0378-4347(94)00407-2

Recently, a new method has been proposed to produce synthetic peptides usable directly for immunization without the need of coupling to carriers or chemical polymerization, allowing the preparation of multiple antigenic peptides (MAPs) on a branching lysine core by a single solid-phase synthesis [9]. MAPs are macromolecules composed of 2^n copies of the same antigen peptide synthesised on a small immunogenically inert polylysine core. These structures are highly immunogenic in mice and rabbits, resulting in production of polyclonal anti-peptide antibodies with a high titer and a high degree of specificity. Multimeric antigens offer the advantage of being chemically well defined, and display a much higher adsorbance to plastic surfaces, used in the development of ELISA assays, than the corresponding linear, monomeric peptide antigens [10]. In the present study, we examined the possibility of extending the applicability of multiple antigenic peptides from the production and characterization of antibodies to the development of affinity purification media for their isolation from crude sera. Two tetrameric peptides, denoted as MAP-AV15 and MAP-AV13, corresponding to tetramers of 15 and 13 residue peptides, were synthesised, purified, characterized, and both used to raise antibodies in rabbits. The corresponding sera were first characterized by solid-phase assays on microtiter plates coated with the multimeric peptides for their ability to recognize the corresponding multimeric as well as the linear antigens. Antibody purification from crude sera was then attempted by affinity chromatography on columns prepared by immobilizing the corresponding MAPs on preactivated solid supports. Results were compared in terms of recovery of antibody immunoreactivity and purity with those obtained with affinity columns prepared by immobilizing the corresponding monomeric linear peptide.

2. Experimental

2.1. Reagents and chemicals

9-Fluorenylmethoxycarbonyl (Fmoc)-derivatized amino acids and 4-hydroxymethylphenoxyacetic acid (HMP) resin for solid-phase peptide synthesis were purchased from Novabiochem (Laufelfingen, Switzerland). HPLC-grade dichloromethane (DCM), N-methylpyrrolidone (NMP), methanol, water and acetonitrile were purchased from Merck (Darmstadt, Germany) and trifluoroacetic acid (TFA) from Pierce (Rockford, IL, USA). Reagents used as scavengers during cleavage of peptide from resins, such as phenol, thioanisol and ethanedithiol, were obtained from Aldrich (Milan, Italy). Eupergit C30N, an acrylic support for affinity chromatography preactivated with epoxy groups, was obtained from Rohm (Weiterstadt, Germany). Glass columns used for packing peptide-derivatized support were purchased from Omni (Cambridge, UK). PVC microtiter plates used to perform the solid-phase assays were from Becton Dickinson (Oxnard, CA, USA). Goat anti-rabbit IgG–peroxidase conjugate, streptavidine–HRP conjugate, bovine serum albumin, *o*-phenylendiamine and biotin-ε-aminocaproyl-N-hydroxysuccinimide ester were from Sigma (St. Louis, MO, USA). 9-Fluorenylmethyl chloroformate (Fmoc-Cl) and 6 *M* hydrochloric acid were from Pierce (Rockford, IL, USA). Glycine, Tris-(hydroxymethyl)aminomethane, acrylamide, N,N′-methylene-bis-acrylamide, and sodium dodecyl sulphate (SDS) were obtained from Bio-Rad (Richmond, CA, USA).

2.2. Solid-phase synthesis

Linear peptides were synthesised by solid-phase peptide synthesis following the Fmoc methodology on a fully automated Model 431A Applied Biosystems peptide synthesizer, using software version 1.2. For multimeric peptides synthesis, two subsequent couplings of Fmoc–Lys(Fmoc) were performed on a Gly-HMP resin, dividing the amount of resin in half after each step. At completion, on a 0.1-mmol scale, the corresponding peptide sequences AV15 [VRLGWLLAPADLDAR] and AV13 [GFRKYLHFRRHLL] were assembled. Final products were denoted 4AV15 and 4AV13 respectively (see Fig. 1). After completion of the synthesis cycles, peptide resins were dried overnight under vacuum. Peptides were cleaved from resin (100 mg)

using 5 ml of TFA–phenol–thioanisole–water–ethanedithiol (83:6:5:4:2, v/v), and incubated at room temperature for 3.5 h. The mixtures were then filtered, vacuum concentrated to 0.5 ml, and the peptidic material was precipitated by adding 10 ml of cold diethyl ether. Collected MAP material was then redissolved in 20 ml of 0.1 *M* acetic acid, extensively dialysed against 0.1 *M* acetic acid and lyophilised, whereas linear peptide was redissolved in water–acetonitrile (50:50, v/v) and purified by RP-HPLC on a LiChrospher RP-8 (100 × 10 mm I.D.) column. The amino acid composition of synthetic peptides, as evaluated by RP-HPLC analysis of Fmoc-derivatized acid hydrolysates, confirmed the intended chemical nature of the products, as well as the determination of their molecular mass by laser desorption mass spectrometry.

2.3. Affinity column preparation

Multimeric antigenic peptides and linear peptide AV15 were covalently immobilized on an epoxy-activated affinity support (Eupergit C30N). The coupling process was performed by incubating 5 mg of peptide dissolved in 10 ml of 0.1 *M* $NaHCO_3$ (pH 8.5), with 1.0 g of resin with stirring for 24 h. The extent of peptide incorporation, monitored by RP-HPLC, indicated that 90% of the MAPs and 60% of the linear peptide AV15 were support bound. Finally, the resins were washed repeatedly with 0.1 *M* Tris (pH 8.5) and then slurry packed in a 80 × 6.6 mm I.D. glass column. A control column was similarly prepared by incubating 1.0 g of resin with 10 ml of 0.5 *M* Tris (pH 8.5).

2.4. Preparation of anti-MAP antisera

Rabbit anti-4AV13 and anti-4AV15 antisera were prepared using the peptides without carrier conjugation. Six New Zealand rabbits were immunized with peptide (50–200 μg) dissolved in 0.15 *M* sodium chloride/0.05 *M* sodium phosphate buffer (pH 7.3), and emulsified with complete Freund's adjuvant containing H37RV (2 mg/ml) by injections in the rear foot pads. After two weeks the same immunogen was administered subcutaneously. After two subsequent boosts, blood samples were collected from each animal for monitoring antibody titers. One week after the last boost, animals were bled and sera stored at −80°C.

2.5. Peptide conjugation to carrier protein

Conjugation of linear peptides AV15 and AV13 on BSA was carried out by mixing 5 mg of peptide dissolved in 1 ml of 50 m*M* PBS (pH 7.0) with 15 mg of bovine serum albumin dissolved in 1 ml of the same buffer, and incubating the mixture with 2 ml of 0.2% glutaraldehyde solution in the same buffer. After 1 h of incubation at room temperature, 1 ml of 1 *M* Gly solution was added and after another hour the conjugates were extensively dialysed against 50 m*M* sodium phosphate (pH 7.0).

2.6. Antibody biotinylation

After affinity purification, anti-MAP antibodies were biotinylated by incubating 1.0 mg of antibodies with 3 mg of biotin-6-amino-caproate-N-hydroxysuccinimide ester dissolved in 0.5 ml of water–ethanol (1:1, v/v). Twenty four hours later the biotinylated antibody was extensively dialysed against 50 m*M* sodium phosphate (pH 7.5).

2.7. Enzyme-linked immunosorbent assay (ELISA)

Polystyrene microtiter plates were incubated overnight at 4°C or for 2 h at room temperature with a 50 μg/ml MAP solution and a 100 μg/ml peptide conjugated solution (100 μl/cell) in 0.1 *M* sodium carbonate buffer (pH 8.5).

After washing the microtiter plates five times with PBS, the wells were saturated with 200 μl of PBS containing 3% BSA, for 1 h at room temperature, to block the uncoated plastic surface. Plates were then washed again with PBS and filled with samples containing anti-MAP antibodies at varying concentrations, previously diluted with PBS containing 0.5% BSA (PBS-B).

For competition experiments, various concentrations of inhibitor (multiple and linear conju-

gated peptides) were incubated for 3 h at 37°C with anti-MAP antibodies. The mixture was then added to MAP coated wells.

After 1 h of incubation the plates were washed five times with PBS. For anti-MAP antibody detection, wells were filled with 100 μl of a horseradish peroxidase labelled goat anti-rabbit (IgG) immunoglobulin solution diluted 1:1000 with PBS-B. The plates were then left to stand for 1 h at room temperature in a humid covered box, washed with PBS five times, an then filled with a chromogenic substrate solution consisting of 1 mg/ml *o*-phenylenediamine in 0.1 *M* sodium citrate buffer (pH 5.0), containing 5 m*M* hydrogen peroxide. The absorbance at 450 nm was measured with a Model 2250 EIA Reader (Biorad).

For biotinylated antibody detection, wells were filled with 100 μl of a streptavidin–peroxidase solution diluted 1:1000 with PBS-B. After 1 h of incubation, development of the chromogenic reaction was carried out as described above for anti-MAP antibodies.

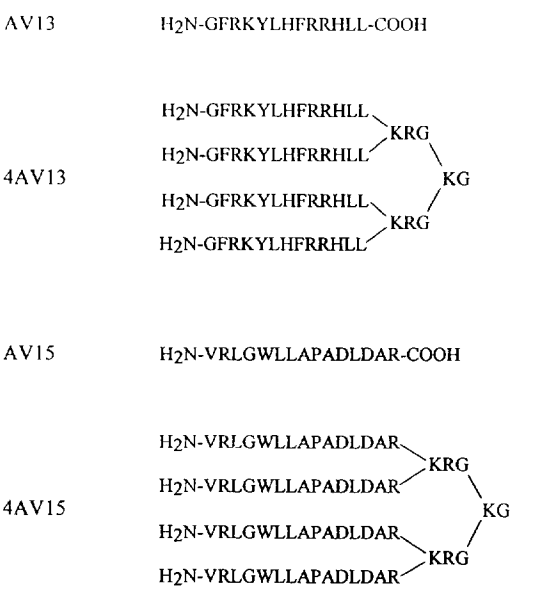

Fig. 1. Structure of the multimeric peptides 4AV13 and 4AV15, and the corresponding monomeric forms AV13 and AV15.

3. Results

3.1. Sera production and characterization

Sequences of the multimeric as well as of the monomeric peptide antigens used in this study are shown in Fig. 1. Immunization of rabbits with the multimeric peptides 4AV13 and 4AV15 produced antibodies reacting strongly with the corresponding antigens even after the first immunization, as detected by ELISA on microtiter plates coated with the multimeric antigens (Table 1). The signal was proportional to the amount of crude sera added to each well in a dose dependent manner and showed saturation, while preimmune serum gave very low absorbance values. Plates coated with unrelated MAPs, having the same core structure, did not provide appreciable signals (below 0.1 A.U.). Binding was specific since presence of soluble competing MAP reduced the signal in a dose dependent fashion (not shown). In addition, crude sera were able to recognise also the corresponding linear antigen immobilized on the microtiter plates after coupling to BSA (not shown). Also in this case interaction was reduced almost completely in the presence of soluble competing linear peptide (not shown), thus indicating that immunization with the multimeric peptides produced antibodies recognizing the linear peptide sequence, while the central polylysine core did not give an immunogenic reaction.

3.2. Sera purification on MAP-affinity columns

Antibodies were purified from crude sera by affinity chromatography on a column prepared by immobilizing the corresponding multimeric antigen on a preactivated solid support.

Coupling yields were very high for both MAPs (90%), while immobilization yields for the corresponding linear antigens were considerably lower (60%) under the same experimental conditions. After extensive washing with 0.1 *M* Tris (pH 8.5) to deactivate residual epoxy groups, derivatized supports were packed in 2.3-ml glass columns and connected to the HPLC system.

Table 1
Titers of sera as determined by ELISA assays

Dilution	Absorbance at 405 nm					
	Anti-4AV13 crude serum			Anti-4AV15 crude serum		
	Preimmune	1st Boost	2nd Boost	Preimmune	1st Boost	2nd Boost
1:100	0.34	2.71	>3	0.70	>3	>3
1:300	0.19	2.18	2.68	0.57	2.27	>3
1:900	0.10	1.37	1.94	0.60	1.66	>3
1:2700	–	0.69	0.98	–	0.94	2.06
1:8100	–	0.29	0.93	–	0.41	1.07
1:24 300	–	–	0.17	–	–	0.47
1:72 900	–	–	0.07	–	–	0.22

As an example, the affinity chromatography profile of the purification of the anti-4AV13 antiserum is reported in Fig. 2. The column, denoted (4AV13)Eupergit, was equilibrated at a flow-rate of 1 ml/min with 25 m*M* Tris (pH 7.2) (starting buffer), continuously monitoring the UV absorbance of the effluent at 280 nm. Crude serum (1 ml) was diluted 1:1 with starting buffer, filtered through a 0.45-μm Nalgene filter and then passed through the column. After complete elution of unbound material, denoted peak 1, the column was extensively washed until a stable baseline was attained, and then the eluent was changed to 0.1 *M* acetic acid. Material eluted following the acid wash, denoted peak 2, was collected and quickly neutralised for further analysis. Total purification time was close to 2 h. The same procedure was performed to purify anti-4AV15 antibodies on a (4AV15)Eupergit affinity column. The chromatographic profile was very similar to that obtained with the other column (not shown). As calculated from absorbance measurements at 280 nm, proteins recovered in the bound fractions constituted in both cases approximately 5–7% of the total protein applied to the column. Purification of serum samples was performed also on affinity columns prepared by immobilizing the corresponding linear peptides at roughly the same density as used for the preparation of the MAP columns, and unrelated molecules such as Tris (not shown). Bound and unbound fractions were

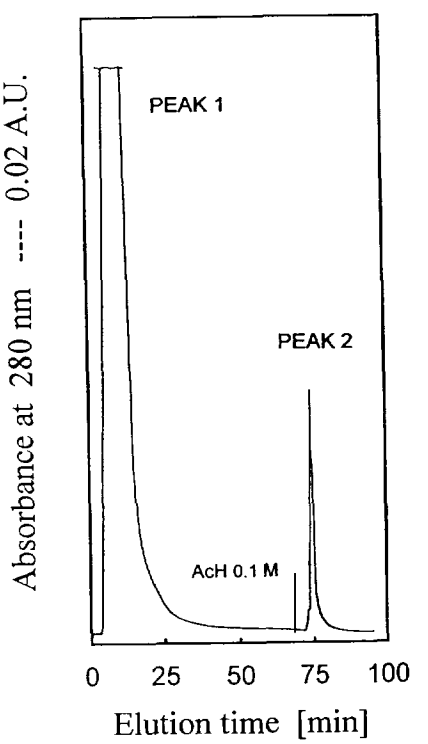

Fig. 2. Purification of anti-4AV13 antibodies by affinity chromatography on the (4AV13)Eupergit column, equilibrated at a flow-rate of 1.0 ml/min with 25 m*M* Tris (pH 7.2), and loaded with 1.0 ml of serum. After elution of unbound material (peak 1), the eluent was changed to 0.1 *M* acetic acid. Bound material (peak 2) was collected for ELISA and SDS-PAGE analysis.

collected and neutralized for ELISA and SDS-PAGE characterization. Purity of the bound materials from the different affinity columns was then checked by SDS-PAGE analysis performed under non-reducing conditions. Detection of the protein bands was performed with the silver staining method. As shown in Fig. 3, bound material from the 4AV13 (lane C) and 4AV15 (lane E) affinity columns contained almost exclusively a band at 150 kDa corresponding to the expected antibody molecular mass, and a faint band around 65 kDa, probably corresponding to a contamination of rabbit serum albumin. On the other hand, SDS-PAGE analysis of the bound fraction from the affinity column prepared with the monomeric antigen AV15 peptide, indicated the presence of many bands corresponding to contaminating proteins in the crude serum (lane F). The major contaminating protein band detected corresponds also in this case to rabbit serum albumin. Analysis of the fractions obtained from the purification on the Tris column of the anti-4AV13 antiserum indicated that the majority of the material applied to the column was not retained and was recovered in the column void volume (lane B). Similar results were obtained with the anti-4AV15 antiserum (not shown).

Fractions corresponding to the bound and unbound material from the different affinity chromatography experiments were then analyzed by ELISA assays in order to determine the recovery of the antibody immunoreactivity applied to each column. As shown in Fig. 4, the majority of the immunoreactivity applied was recovered from the affinity purifications with the multimeric peptide columns (close to 80% in both cases). On the other hand, antibody purification on the monomeric AV15 peptide affinity column resulted in a substantial loss of immunoreactivity (22% of the initial immunoreactivity in the bound fraction), which could not be directly attributed to the lower coupling yield (60%) of the monomeric peptide compared to that of the multimers (90%) during column preparation. A lower recovery of immunoreactivity was also found in the purification with the monomeric AV13 affinity column (not shown). No residual immunoreactivity was detected in the bound fraction from the serum purification on the (Tris)Eupergit column, since all the immunoreactivity was found in the column void volume. In the purifications on the MAP-affinity col-

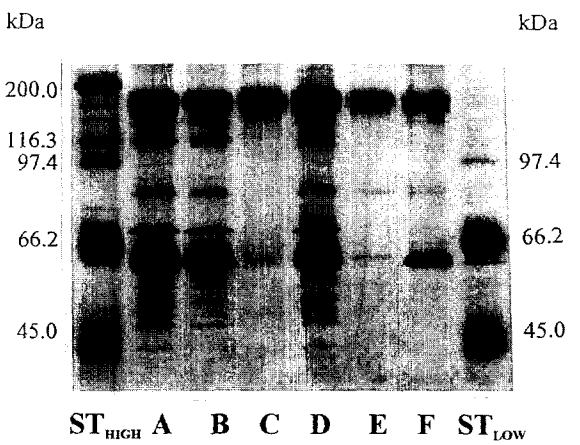

Fig. 3. SDS-PAGE analysis of (MAP)Eupergit affinity-purified antibodies and crude sera on a 8% polyacrylamide gel, under non-reducing conditions. Lane A: crude anti-4AV13 antiserum, lane B: unbound material eluting from the (Tris)Eupergit affinity column, lane C: anti-4AV13 antibody purified on the 4AV13 affinity column, lane D: crude anti-4AV15 antiserum, lane E: anti-4AV15 antibody purified on the 4AV15 affinity column, lane F: anti-4AV15 antibody purified on the AV15 affinity column. ST: molecular mass standards.

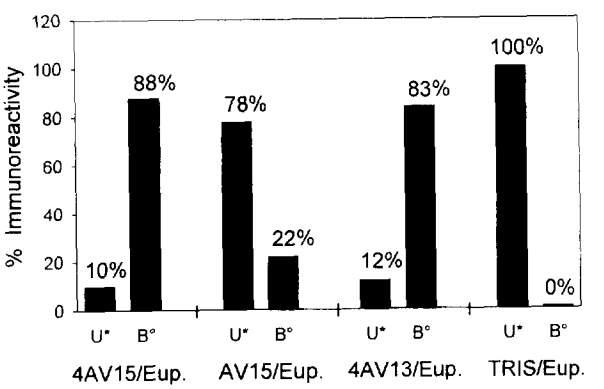

Fig. 4. Immunoreactivity recovered from the purification of anti-MAP antibodies on the corresponding affinity columns, as measured by ELISA. U* = unbound material, B° = bound material.

umns, 3–4 mg of purified antibodies were recovered in both cases from 1 ml of crude serum.

3.3. ELISA characterization of affinity purified antibodies

Purified antibodies were then quantitatively characterised with respect to their ability to recognize the multimeric as well as the monomeric peptide antigens, in order to clarify the difference in the recovery of immunoreactivity observed previously. Affinity-purified antibodies anti-MAP-AV15 and anti-MAP-AV13 were then biotinylated and tested for their reactivity against the corresponding immobilized multimeric antigens. As shown in Fig. 5, for both cases binding was found to be directly proportional to the amount of labelled antibody added and to the amount of multimeric peptide used in the coating procedure, as seen previously with crude serum. Interaction was reduced in the presence of soluble multimeric peptides in a dose-dependent manner, while presence of unrelated peptides did not affect binding (Fig. 6). Purified antibodies retained binding properties also for the corresponding linear peptides, conjugated to BSA to facilitate immobilization on the microtiter plates. Binding was also affected by the presence of soluble peptide–BSA conjugates, in a dose-dependent manner (not shown). More important, binding between multimeric antigens immobilized on microtiter plates and the corresponding purified antibodies was also affected by the presence of peptide–BSA conjugates, and at high competitor concentrations interaction was almost completely inhibited (Fig. 7). This strongly suggests that all the antigenic determinants in the multimeric peptides are present also in the monomeric form. Thus, all the antibody immunoreactivity is directed against the linear peptide sequences, and not to conformational determinants particular to the multimeric structure of the peptide.

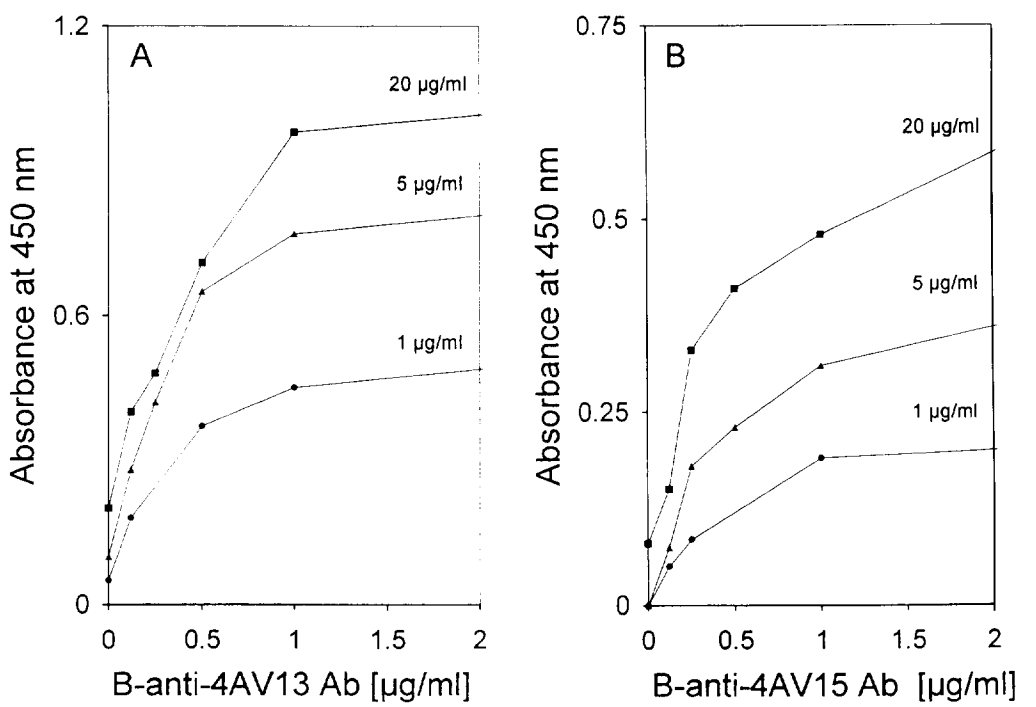

Fig. 5. Binding of affinity-purified biotinylated anti-4AV13 (A) and anti-4AV15 (B) antibodies to the corresponding multimeric antigens immobilized onto microtiter plates at different concentrations.

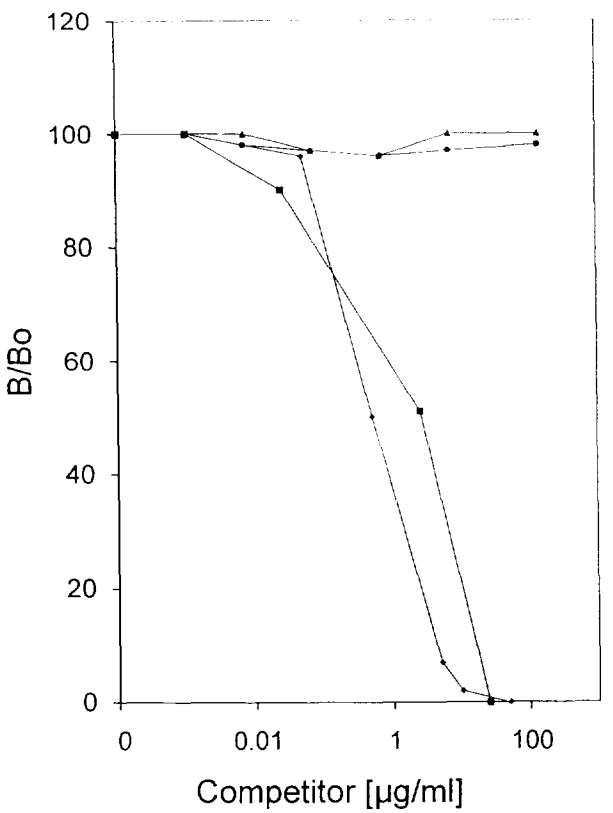

Fig. 6. Competitive binding of biotinylated antibodies to immobilized MAPs in the presence of increasing concentrations of the corresponding soluble multimeric antigens 4AV15 (■), 4AV13 (◆), and two unrelated multimeric peptides (▲,●).

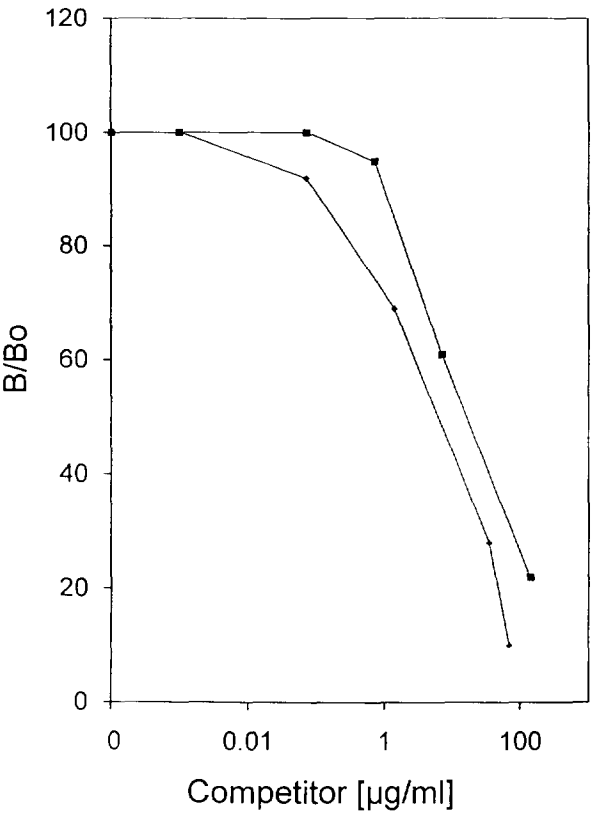

Fig. 7. Competitive binding of biotinylated antibodies to their corresponding immobilised MAPs in the presence of increasing concentrations of monomeric peptide AV15 (■) and AV13 (◆), conjugated to BSA.

4. Discussion

While different strategies for the preparation of peptides suitable for direct immunization without the need for carrier conjugation have been reported, only the synthesis of multimeric peptides allows purification and characterization of the corresponding antibodies without additional synthetic steps. The synthesis of antigenic peptides attached to solid-phase resins is extremely useful for eliciting high titer antibodies [11,12], while the peptide–resin conjugate can also be used for their purification [13]. However, accurate determination of the binding characteristics of the purified antibodies requires additional peptide cleavage from the resin and its purification [14]. Other drawbacks in the use of peptide antigens still attached to solid-phase resin include the resulting very high peptide density on the resin, the limited number of resins usable, and the need to confirm the chemical identity of the desired antigen after cleavage. In addition, after synthesis peptide–resin conjugates need to be sonicated to crack the beads prior to immunisation. Multimeric peptides can be easily synthesised, their structural characterization by chemical means does not present difficulties, and can be used for direct immunization without any further steps. On the other hand, since the coating capacity of multimeric peptides on microtiter plates is very high, they allow convenient characterization of the specificity and selectivity of the corresponding antibo-

dies. In addition, multimeric antigens can be used for direct immobilization on preactivated solid supports with a very high yield. In both cases evaluated in this study, MAPs preserved their antigenicity after immobilization on preactivated solid supports, allowing single-step purification of the corresponding antibodies directly from crude sera. Immunochemical characterization of the purified antibodies clearly demonstrated their ability to recognize the peptide antigen both in the linear as well as in the multimeric form. On the other hand, purification on affinity columns prepared by immobilizing linear antigens did not give results as good as those obtained with multimeric peptides. This is probably due to the limited availability of the linear antigen immobilized on the solid phase for antibody recognition. In the detailed investigation of the immunogenicity and antigenicity of multimeric peptides, it was found that immunization with MAPs induced the production of antibodies able to recognize the multimeric as well as the monomeric antigen, and that the main antigenic epitopes were located in the N-terminal region of the multimers [15]. Thus, preservation of the antigenicity of immobilized MAP suggests that only a limited number of peptide chains in the multimer are covalently linked to the solid phase, leaving the other uncomplexed and free to interact fully with the large antibody molecule. This suggestion is also supported by the evidence that immobilization on the same type of preactivated supports of multimeric peptides requiring a free α-amino group to recognize the corresponding protein ligand does not alter their recognition properties [16]. In addition, in other studies it has been shown that preparation of peptide ligands in a multimeric form and their subsequent immobilization on solid supports allowed the preparation of affinity columns with enhanced binding capacity compared with that of monomeric peptide columns [17]. Coupling of multimeric peptides to solid supports leads to antigenic peptides sufficiently spaced from the solid phase through the entire length of the chains involved in the covalent linkage. Since current automated instrumentation for solid-phase peptide synthesis allows an easy and convenient preparation of multimeric peptides, and since preactivated supports for affinity chromatography are also widely available, preparation of multimeric peptides may constitute a very versatile and global approach for the production, characterization, and purification of antibodies in a simple way and in high yield, with only one single synthesis.

Acknowledgements

The authors wish to thank Dr. Menotti Ruvo for peptide synthesis and characterization, Simona Germani for her excellent technical assistance, and Dr. Anna Maria Isetta for the help in antisera production.

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