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Eupergit C as a carrier for high-performance liquid chromatographic-based immunopurification of antigens and antibodies

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ABSTRACT

An immunoaffinity purification system using C30N and C1Z Eupergit C beads was developed and optimized. Poly- and monoclonal antibodies were purified using immobilized antigens and antigens were purified using immobilized antibodies. Antigens were used that possess enzymic activities and the efficiency of antigen binding was determined from the enzymic activity of the matrix-bound immunocomplexes. High-performance immunoaffinity purification using Eupergit C beads proved to be highly specific, reproducible, free from protein leakage and possessed a low degree of non-specific adsorption of tissue proteins. These characteristics of the system were illustrated by the isolation of immunoglobulin G from serum and of human decidua proteins from the decidua tissue and from seminal plasma. These proteins were obtained at high purity in a single purification step, as shown by sodium dodecyl sulphate–polyacrylamide gel electrophoresis.

INTRODUCTION

The increasing availability of a variety of monoclonal antibodies and the rapid development of new water-insoluble matrices and covalent binding methods[1] during the last decade have led to a marked increase in the application of immunoaffinity methods to the purification of biologically active proteins. Of the various commercially available matrices, agarose and Sepharose are most widely used for the affinity purification of proteins on the laboratory and industrial scales. However, their application in high-performance immunoaffinity chromatography (HPIAC) is limited by their low mechanical stability[2] and by leakage of antibodies off the matrix, especially when coupled via cyanogen bromide activation[3]. In addition, agarose, as a natural polymer, may be susceptible to biological degradation which may limit the life span of antibody–agarose conjugates. Silica is mostly used as a support for HPIAC[2] and a variety of methods have been developed for covalent coupling of proteins onto this matrix. However, the sensitivity of silica to alkaline pH (unless

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especially capped) may limit its application in immunoaffinity purification processes since buffers of high pH (*e.g.*, ammonia, pH 10) are often used to elute the antigen from the matrix-conjugated antibody.

Recently, a number of synthetic polymeric matrices have been developed that bear active groups for covalent coupling of proteins[4]. We chose to study the applicability of one of these matrices, Eupergit C[5], to the high-performance liquid chromatographic (HPLC)-based immunoaffinity purification of antigens and antibodies. This matrix is a cross-linked copolymer of methylacrylamide, N-methylenebisacrylamide and monomers containing reactive oxirane groups which react with amino, thio and hydroxyl groups of proteins. It is available as beads of 150 μm (standard, porous), 30 μm (C30N, porous) and 1 μm (C1Z, non-porous). The last two are excellent candidates for use as HPIAC supports.

In the process of the development of a carrier for immunoaffinity purification, several parameters have to be determined and optimized. Among these are the capacity of the matrix for protein binding, the activity and specificity of the matrix-bound antibodies, the elution of the antigens from the matrix, the absence of leakage of immobilized antibodies and the reproducibility and the stability of the immunoaffinity purification system. The results of these investigations are presented in this paper.

EXPERIMENTAL

Materials

Carboxypeptidase A (CPA) and horseradish peroxidase (HRP) were purchased from Sigma (St. Louis, MO, U.S.A.). Goat anti-rabbit immunoglobulin G (IgG) was purchased from Bio-Makor (Rehovot, Israel). Eupergit C was obtained from Rohm-Pharma (Darmstadt, F.R.G.).

The preparation of anti-CPA and anti-HRP monoclonal antibodies (mAbs), the determination of enzymic activities of CPA and HRP and the periodate oxidation of antibodies by the multi-step procedure were described previously[6].

Protein determination

Protein was determined according to the method described by Bradford[7] using the Bio-Rad Labs. (Richmond, CA, U.S.A.) protein assay reagent. The reagent was diluted 5-fold in water before use. Samples containing 0.2–8 μg of protein in 10–50 μl of phosphate-buffered saline (pH 7.4) (PBS) were mixed with 200 μl of the diluted reagent. The intensity of the colour developed was measured at 690 nm using an enzyme-linked immunosorbent assay (ELISA) reader (SLT, Grodig, Austria).

Alternatively, protein concentrations were determined spectrophotometrically by the absorbance at 280 nm using molar absorptivities of 1.8, 1.13 and 1.4 for 1 mg/ml solutions of CPA, HRP and IgG, respectively.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed with 10% gels according to the procedure of Laemmli[8].

High-performance immunoaffinity chromatography

The chromatographic system was obtained from Gilson (Villiers-le-Bel, France),

equipped with Model 303 pumps, a Model 111B UV detector and a Model 421 Autoinjector. Unless stated otherwise, all HPIAC separations where carried out with antigens or antibodies conjugated to Eupergit C C30N beads. The beads were packed into the columns in PBS using a high vacuum pump.

Preparation and selection of monoclonal antibodies

Anti-CPA and anti-HRP were prepared as described previously[6,9]. Antibodies which do not affect the activities of these enzymes were selected. The antibodies were assayed by ELISA as described previously[6,9].

Protein iodination

Iodination of proteins was performed by the chloramine T method[10]. Excess of free iodine were removed by gel filtration on a Sephadex G-25 column. ^{125}I-labelled CPA and rabbit IgG possessed specific activities of 4000–6000 cpm/μg.

Immobilization of proteins on Eupergit C

Eupergit C beads were thoroughly washed with PBS until no absorbance at 280 nm was detected in the supernatant and then the beads were washed once with 1 *M* potassium phosphate buffer (pH 7.4). Solutions of proteins to be immobilized (0.025–15 mg, 1–10 mg/ml of the same buffer) were incubated with washed Eupergit C beads (usually at 5–10 mg/g of matrix) for 16 h at 4°C or for 3–4 h at room temperature. The amount of enzyme antigens bound to the matrix was determibed by assays of the matrix-bound enzymic activities as described previously[6]. The amount of antibody bound was determined by adding a ^{125}I-labelled rabbit IgG as a tracer or by a protein assay of the reaction mixture supernatant at the end of the coupling reaction.

Excess oxirane groups on the matrix were blocked by incubation with 0.2 *M* β-mercaptoethanol for 4 h at 4°C. When used in HPIAC the Eupergit C conjugates were packed into stainless-steel HPLC columns (10 × 0.8 cm I.D.) and repeatedly washed with PBS and 0.2 *M* ammonium acetate buffer (pH 10) before application to immunopurification of the respective antibodies or antigens.

Formation of the immunocomplex by Eupergit C-conjugated antigens or antibodies

Antigens or antibodies were bound to the respective Eupergit C-conjugated antibodies or antigens by mixing the beads with a molar excess of the specific protein to be bound in PBS. The mixture was incubated for 3 h at room temperature or 16 h at 4°C. The amount of immunologically bound protein was determined after centrifugation of the reaction mixture at 12 000 *g* by enzymic assays of the matrix-bound CPA or HRP (when the respective antibodies were conjugated to the matrix) or by protein assays of the respective supernatants. In HPLC columns antigens or antibodies were loaded in PBS at a flow-rate of 0.5 ml/min. After the non-bound protein had been washed off the column, elution of the specific protein by washing with 0.2 *M* ammonium acetate buffer (pH 10.0) was monitored by UV adsorption at 280 nm.

RESULTS AND DISCUSSION

Protein-binding capacity of Eupergit C

Protein binding to the different forms of Eupergit C (standard, C30N and C1Z)

was determined by incubation of radioactively labelled IgG with the matrix and direct counting of the matrix-bound radioactive protein. Non-covalently adsorbed protein was excluded by washing the beads with a solution containing 1% SDS in 8 *M* urea. Both the standard Eupergit C and C30N exhibited a high degree of protein binding, which was more than 85% of the protein loaded onto the matrices even at a load of 100 mg protein per gram (dry weight) of matrix. With protein concentrations not exceeding 5 mg per gram of C30N binding was achieved within 15 min. With higher protein loads maximum binding was observed only after 4 h of incubation at room temperature (Fig. 1). Binding onto standard Eupergit C was similar to that onto C30N and is not shown. As might be expected, with the non-porous 1-μm beads C1Z the degree of protein binding was much lower, reaching a maximum at about 7 mg per gram of matrix. For all the bead forms the amount of protein non-covalently adsorbed to the matrix was relatively low (<2% of the input protein).

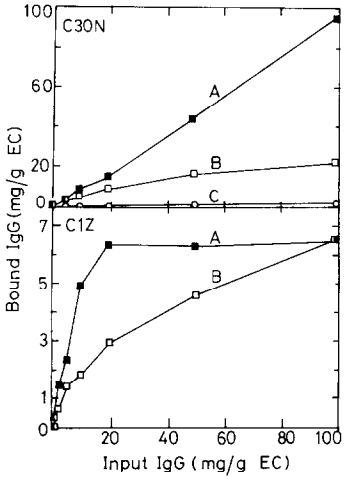


Fig. 1. Protein binding to Eupergit C (EC) C30N and C1Z beads. Aliquots of rabbit IgG (0–1000 μg) were mixed with 5 μg of ^{125}I-labelled rabbit IgG (36 600 cpm) and incubated in 1 *M* potassium phosphate buffer (pH 7.4) with 10 mg portions of Eupergit C C30N or C1Z for (□) 15 min or (■) 4 h at room temperature with slow agitation. At the end of the reaction, the beads were washed three times with PBS, transferred into counter tubes and counted. The amount of radioactive material adsorbed on the beads (○) was determined after washing the beads three times with 1% SDS in 8 *M* urea and counting the radioactivity released to the solution. (A) 4 h; (B) 15 min; (C) adsorption.

Blocking of residual oxirane groups on the matrix

Titration of the oxirane groups with thiosulphate showed that Eupergit C (standard and C30N) contains about 1.6 mmol of oxirane groups per gram of matrix[5,7]. About half of these groups are readily available to reaction with amino or thio groups whereas the other half are much less reactive and require a long time (hours to days) to react. Coupling of a protein usually uses only a tiny amount of these oxirane groups; the remainder have to be blocked to prevent undesirable covalent protein binding at a later stage. We found β-mercaptoethanol to be an efficient blocker. In

contrast to the commonly used ethanolamine, it may by applied to the matrix at almost neutral pH (pH 8.0) and, as it does not form charged groups when bound to the matrix, non-specific ionic adsorption of proteins is eliminated. Our investigations indicated that the immobilized antibodies were not affected by the presence of 0.2 *M* of β-mercaptoethanol used for blocking of the matrix (data not shown).

Immunoaffinity purification of antibodies using immobilized antigens

An efficient immunoaffinity purification is characterized by high selectivity, specificity, capacity and reproducibility. In order to study the applicability of Eupergit C to immunoaffinity purification various antigens (CPA, HRP and rabbit IgG) were coupled to C30N beads, packed into HPLC columns and used to isolate poly- and monoclonal antibodies from the respective antisera and ascites fluids. Purification of poly- and monoclonal anti-CPA antibodies is shown in Fig. 2. The purified antibodies were eluted as sharp peaks, in contrast to about 10-fold broader peaks which eluted using a comparable LC affinity purification system. Reproducibility of the purification system was demonstrated by reapplication of the purified antibodies (peak II), after dialysis against PBS, to the same column and re-elution with ammonium acetate buffer (pH 10). As shown by an ELISA test for anti-CPA antibodies (Fig. 3), most of the antibody loaded onto the column was eluted in peak II whereas peak I did not contain any active antibody. Assay for purity by SDS-PAGE showed that under the above conditions the antibody obtained by the single-step purification was about 90% pure. The impurities have been shown to result from non-specific adsorption of serum proteins by the matrix. Inclusion of polyethylene glycol (PEG) 400 in the loading buffer was found to reduce this type of adsorption, leading to the elution of almost pure antibody[11]. Purification of anti-HRP and anti-IgG mono- and polyclonal antibodies on the respective C30N-conjugated antigens yielded similar results to those described above (data not shown).

Immunoaffinity purification of antigens using immobilized antibodies

The different affinity-purified antibodies were conjugated to standard Eupergit C and their capacity to bind the antigen was tested. As shown in Table I, the specific antigen-binding activity of these antibodies depended markedly on the amount of antibody loaded onto the matrix. At a low antibody load (*ca.* 1 mg per gram of matrix) 0.60–0.80 mol of antigen were bound per mole of antibody whereas at a high antibody load (*ca.* 10 mg per gram of matrix) the specific activity decreased to 0.15–0.50 mol/mol. The polyclonal antibody immobilized on Eupergit C showed comparable binding activity. As shown in Table II, the activity of C30N-conjugated monoclonal antibodies was 10–20% higher than that observed for standard Eupergit C conjugates. C1Z-conjugated antibodies possessed higher activities (30–50% higher than the standard Eupergit C conjugates, but the efficiency decreased markedly at antibody concentrations higher than 2 mg per gram of C1Z.

In order to illustrate the applicability of the conjugated antibodies to HPIAC antigen isolation, C30N-conjugated anti-CPA mAb9 was packed into an HPLC column and CPA was applied, washed with PBS and eluted with ammonium acetate (pH 10) (Fig. 4). From the amount of CPA eluted a specific antigen-binding activity of 0.16 mol/mol was calculated, compared with 0.5 mol/mol observed under batch operation. The different efficiencies of antigen binding by immobilized antibodies in

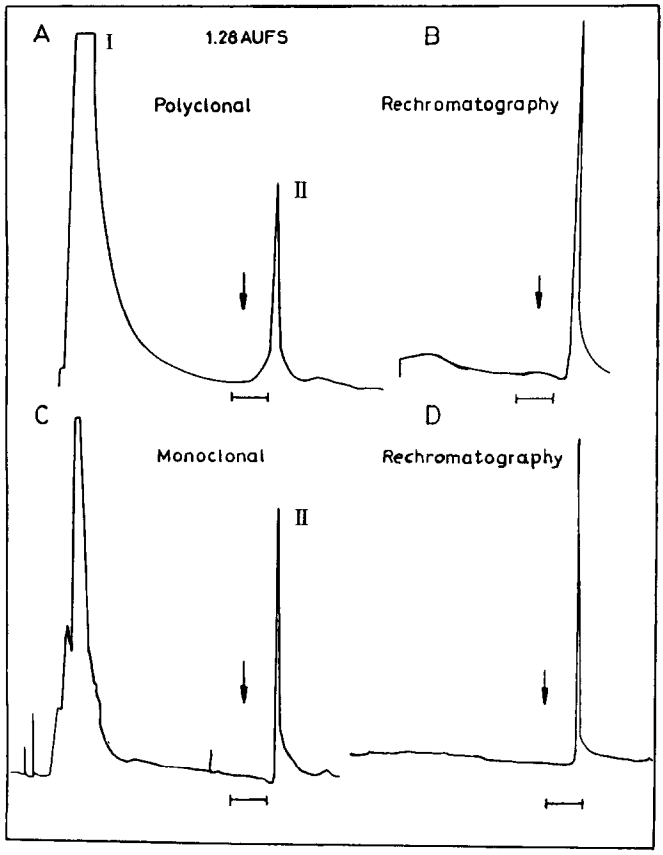


Fig. 2. Immunopurification of anti-CPA antibodies on a Eupergit C C30N–CPA column. (A) Purification of polyclonal antibodies from anti-serum: 0.5 ml of immunized rabbit serum was applied to a column (250 × 4 mm I.D.) containing Eupergit C-bound CPA (10 mg/g of matrix) in PBS at a flow-rate of 0.5 ml/min. Immunologically bound antibodies were eluted from the column with 0.2 *M* ammonium acetate buffer (pH 10.0) at a flow-rate of 1 ml/min. (B) Rechromatography of peak II in A. The material eluted in this peak was collected, dialysed against PBS (18 h, 4°C), concentrated and reapplied to the same column. (C) Purification of monoclonal antibody anti-CPA mAb14 from ascites fluid; 0.5 ml of ascites fluid was applied to the column and chromatographed under the same conditions as described for A. (D) Rechromatography of peak II in C as described for B. Arrows mark the application of ammonium acetate buffer (pH 10). The bars indicate 10 min.

column and batch operations may result from diffusion barriers which occur in the column. This phenomenon is still under investigation. Recently, we have developed binding methods that led to increased antigen-binding activity of the immobilized antibodies. These include coupling of oxidized antibodies (by sodium periodate or enzymatically) to adipic dihydrazide-modified Eupergit C[6,13,14] and reversible protection of the amino groups of the antibodies by dimethylmaleic anhydride prior to coupling to the matrix[15].

By immobilization of properly selected antibodies we were able to purify from decidua tissue two specific proteins named hDP55[16] and hDP71[17]. The last protein

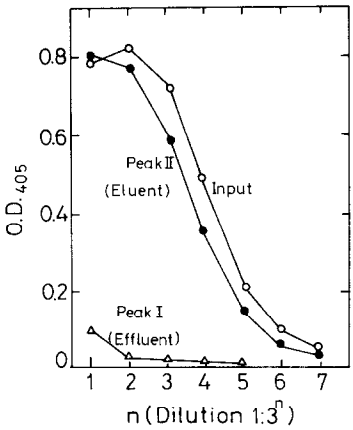


Fig. 3. Ag-Binding activity of antibodies purified by immunoaffinity chromatography. ELISA test for anti-CPA antibodies in the fraction loaded into the column (Input) (○), peak I (△) and peak II (●) (see Fig. 2 A). CPA was adsorbed on the wells of a polystyrene microtitre plate. Serial dilutions of the solutions to be tested were added and the amount of antibody bound was determined by reaction with alkaline phosphatase-labelled goat anti-rabbit IgG.

was found also to be present in large amounts in seminal plasma. Using immunoaffinity purification with C30N-immobilized monoclonal antibody (DEC21) we purified this protein to homogeneity from seminal plasma in one immunopurification step (Fig. 5).

This antigen was also used to test the durability of our HPLC-based immunopurification system. Samples of cell-free seminal plasma (0.01–0.5 ml) were applied to

TABLE I

BINDING OF ANTIBODIES TO STANDARD EUPERGIT C

Antibody[a]	*Anti-CPA*[b]			*CPA*[c]			*[CPA]/[Ig] (mol/mol)*
	Input (mg/g)	*Bound (mg/g)*	*%*	*Input (mg/g)*	*Bound (mg/g)*	*%*	
mAb1	0.7	0.7	100	0.5	0.10	20	0.60
	7.7	5.0	65	2.5	0.15	6	0.15
mAb9	1.5	1.4	93	0.5	0.23	46	0.69
	15.7	8.0	51	2.5	0.80	32	0.50
mAb14	1.4	1.4	100	0.5	0.25	50	0.75
	14.2	9.3	65	2.5	0.45	18	0.24
Polyclonal	5.0	4.5	90	1.0	0.60	60	0.55

[a] Names of antibodies are abbreviated; mAb1 is anti-CPA mAb1, etc.

[b] Antibodies were immobilized onto 100-mg fractions of Eupergit C as described under Experimental. Input and bound protein were determined by the Bradford reaction.

[c] The amount of bound antigen was determined by enzymatic assay of the antigen bound to the matrix-conjugated antibody as described under Experimental.

TABLE II

CHEMICAL AND IMMUNOLOGICAL PROPERTIES OF VARIOUS EUPERGIT C BEADS AND MATRIX-CONJUGATED ANTIBODIES

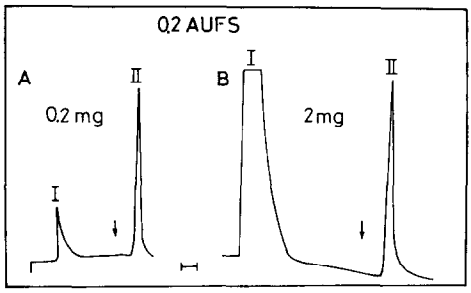
Eupergit C	*Size (μm)*	*Porosity*	*Oxirane content (mmol/g)*	*Protein-binding capacity[a] (mg/g)*	*Antigen-binding capacity[b] (mol/mol)*
Standard	150	Porous	>1000[c]	>100	0.75 (1.4) 0.24 (14.2)
C30N	30	Porous	>1000[c]	>100	0.8 (1.2) 0.4 (11.5)
C1Z	1	Non-porous	20[d]	6.5	0.95 (2.0)

[a] See legend to Fig. 1 for details of analysis of protein binding.

[b] Anti-CPA mAb14 was immobilized onto 100-mg fractions of Eupergit C as described under Experimental. Bound protein (numbers in parentheses, mg/g matrix) was determined by a Bradford assay of the supernatants before and after the coupling reaction. The amount of bound antigen was determined by enzymic assay of CPA as described under Experimental.

[c] Determined by reaction with thiosulphate[12]. See also ref. 8.

[d] Determined by binding of ^{35}S-labelled cysteine.



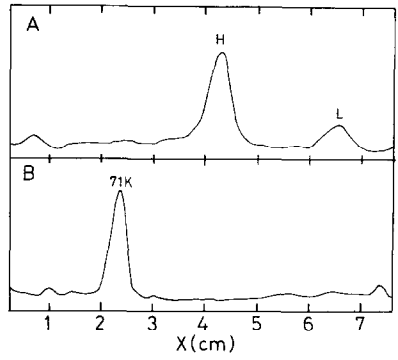


Fig. 4. Purification of CPA by Eupergit C C30N-conjugated anti-CPA mAb9. (A) 0.2 mg of enzyme was applied to a column (60 × 8 mm I.D.) packed with 0.8 g of matrix containing 4 mg of immobilized antibody. Protein eluted in peak I was found to be devoid of enzymatic activity. See text for experimental details. (B) Effect of overloading of the column by 2 mg of CPA. Peak II was used to calculate the column capacity for binding of CPA. Arrows mark the application of ammonium acetate buffer (pH 10). The bar indicates 5 min.

Fig. 5. SDS-PAGE densitometer patterns of IgG and hDP71 purified in the presence of 1% PEG 400. (A) Rabbit IgG (0.1 mg) was mixed with 0.5 ml of horse serum and purified by C30N-conjugated goat anti-rabbit IgG antibodies (0.5 mg Ab bound to 100 mg of matrix). (A) 100-mg amount of matrix, containing 0.5 mg of antibody, was packed into an HPLC column (60 × 6 mm I.D.) onto which 0.5 ml of rabbit IgG-containing horse serum was applied. After washing the column with PBS, IgG was eluted with ammonium acetate buffer (pH 10) as described in the legend to Fig. 2. (B) Human decidua protein (hDP71) was purified from cell-free seminal plasma by C30N-conjugated mAb DEC21. The beads were packed into an HPLC column as described in A. A 0.2-ml volume of seminal plasma was applied to the column. Elution conditions were as in A. H and L denote the peaks corresponding to the heavy and light chains of IgG. 71K denotes the peak corresponding to hDP71.

the column in PBS, hDP71 was eluted with ammonium acetate buffer (pH 10) and the column was re-equilibrated with PBS. This process was automatically repeated and the peak area of the eluted protein was recorded. A high efficiency of hDP71 purification was maintained for over 200 cycles of operation, lasting altogether over 2 months.

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