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Effect of polyethylene glycol on the non-specific adsorption of proteins to Eupergit C and agarose

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ABSTRACT

Non-specific adsorption of serum proteins to Eupergit C (EC) and agarose during the process of immunoaffinity chromatography often leads to contamination of the specifically eluted antigens to be purified. This effect was studied by application of serum samples to a β-mercaptoethanol-blocked EC (EC-βME) column followed by analysis of proteins eluted with various elution buffers. Inclusion of polyethylene glycol (PEG 400 or 1500) in the loading buffer reduced the non-specific adsorption of proteins to EC but had an adverse effect on agarose. Covalent attachment of amino-PEG to EC and to epoxy-activated Sepharose mimicked the effect of PEG in solution with EC and resulted in a marked reduction in non-specific adsorption of serum proteins. Inclusion of PEG in the loading buffer during immunopurification of a serum protein (immunoglobulin G) or seminal plasma protein (human decidua protein hDP71) resulted in a marked improvement in the purity of these proteins eluted from the respective columns by ammonium acetate (pH 10).

INTRODUCTION

During the last decade, affinity chromatography has become a major tool in the purification of biologically active proteins[1]. The interaction between the immobilized ligand and the protein(s) to be purified is usually highly specific, resulting in a high separation power. Thus, protein purification may be achieved by a small number of purification steps. One of the major difficulties which occurs with this method, however, is the contamination of the affinity-purified protein(s) of interest by foreign proteins which are non-specifically adsorbed to the matrix during the loading step and are later eluted during the elution step as contaminants of the product.

Non-specific adsorption of proteins has been shown to increase with the hydrophobicity of the matrix[2]. In addition, different proteins tend to be adsorbed to a given matrix to different extents, depending on their hydrophobicity, size and shape and their concentration in the solution[2]. This type of adsorption is characterized by a hysteretic behaviour[3]. When a protein is purified from a crude solution (*e.g.*, serum

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or ascites fluid), some of the more hydrophobic proteins present in the preparation may be adsorbed non-specifically and reversibly to the matrix and later eluted under the specific elution conditions, contaminating the purified protein(s) of interest.

In an attempt to reduce non-specific adsorption of foreign proteins to Eupergit C and agarose, we have studied the effect of water-soluble polymers, particularly polyethylene glycol (PEG), on such adsorption. PEG is an amphipathic reagent that readily associates with protein surfaces. The interaction of proteins with PEG has been shown to increase the solubility in organic solvents[4], decrease the immunogenicity[5], prolong the lifetime in blood circulation[6] and confer protection against proteolytic degradation[7]. PEG has been also used as a ligand in the hydrophobic chromatography of proteins[8].

PEG 400 and 1500 are not toxic to humans[9] and are even included in skin ointments for human use[10]. Therefore, it is expected that their inclusion in the loading buffer of injectable proteins to be immunopurified will not be hazardous to humans.

In this paper, we describe studies on the reduction of the non-specific adsorption of proteins to the surface of Eupergit C after treatment of the matrix with PEG.

EXPERIMENTAL

Polyethylene glycols 200, 400 and 1500 were purchased from Fluka (Buchs, Switzerland). Ammonium sulphate fractionated goat anti-rabbit immunoglobulin G (IgG) was obtained from Bio-Makor Biochemicals (Rehovot, Israel), agarose (Type I) from Sigma (St. Louis, MO, U.S.A.) and epoxy-activated Sepharose from Pharmacia (Uppsala, Sweden). Eupergit C was obtained from Rohm-Pharma (Darmstadt, F.R.G.). Amino-polyethylene glycol 1500 was kindly provided by Prof. Kula of the Institute for Enzyme Technology Jülich, F.R.G.

Preparation of blocked Eupergit C column

A 2-mg amount of Eupergit C (150-μm beads) were extensively washed with phosphate-buffered saline (PBS) (pH 7.4) and then incubated with 20 ml of 0.2 *M* β-mercaptoethanol (βME) (pH 8.0) for 4 h at room temperature. The beads were then packed into a stainless-steel column (10 × 0.6 cm I.D.) (Knauer, Bad Homburg, F.R.G.). The packed column was connected to a high-performance liquid chromatographic (HPLC) system (Gilson, Villiers-le-Bel, France; Model 303) equipped with a Model 111B UV detector and an HP Model 3390 integrator (Hewlett-Packard, Sunnyvale, CA, U.S.A.). Prior to use the column was extensively washed alternatively with PBS (pH 7.4) and with 0.2 *M* ammonium acetate (pH 10.0).

Agarose column

One gram of agarose was packed into a 3 × 1.2 cm I.D. plastic column which was connected to a peristaltic pump (Pharmacia) and a Gilson Model 111B UV detector equipped with an LC optical cell. Prior to the application of serum samples to the column it was thoroughly washed with PBS (pH 7.4), 0.2 *M* ammonium acetate buffer (pH 10.0) and again with PBS (pH 7.4).

Amino-polyethylene glycol-modified Eupergit C and agarose

Eupergit C beads (2 g) were extensively washed with PBS (pH 7.4) and once with

1 *M* potassium phosphate buffer (pH 7.4). Excess of buffer was decanted and 1.0 g of amino-PEG 1500, dissolved in 5 ml of the same buffer (with gentle heating), was mixed with the beads for 18 h at 4°C. Unbound amino-PEG was washed off with PBS (pH 7.4.).

Similarly, an amino-PEG derivative of agarose was prepared by reaction of amino-PEG (1.0 g) with 2 g of epoxy-activated Sepharose.

The amino-PEG derivatives of Eupergit C and Sepharose were packed into columns and used as described above.

Adsorption of serum proteins

Normal horse serum (NHS) (0–1 ml) was applied to the Eupergit C or the agarose columns. The two columns were washed at a flow-rate of 1.0 or 0.5 ml/min, respectively, with PBS (pH 7.4) or PBS containing 1% PEG (200, 400 or 1500) until all the non-adsorbed proteins were washed off. Elution of adsorbed proteins was achieved by washing the columns with either a solution of 10% sodium dodecyl sulphate (SDS) in 8 *M* urea or 0.2 *M* ammonium acetate buffer (pH 10.0). The eluting material was monitored by following the absorbance at 280 nm. The amount of protein eluted was determined as the area under the protein peaks (in A_{280} units) derived by peak integration. Normal serum contains *ca.* 80 A_{280} units/ml. In some experiments the fraction of proteins adsorbed to the matrix and later eluted with ammonium acetate buffer (pH 10) was collected, dialysed against PBS at 4°C for 16 h and reapplied to the PBS-washed Eupergit C–βME column.

SDS polyacrylamide gel elctrophoresis (PAGE)

SDS-PAGE was performed with 10% gels according to the procedure of Laemmli[11].

Immunopurification of human decidua protein (hDP71)

hDP71 was purified on a Eupergit C-based immunoaffinity column as described previously[12].

Immunopurification of rabbit IgG

Ammonium sulphate fractionated goat anti-rabbit IgG [2 ml of PBS containing 5.2 mg of antibody (Ab) and 20 mg of total protein] was coupled to 2 g of Eupergit C beads as described previously[13,14]. One gram of the beads was incubated with βME for 18 h at 4°C and 1 g was treated with amino-PEG as described above.

IgG of normal rabbit serum (50 ml) was fractionated by ammonium sulphate precipitation (37% saturation). IgG solution (2 mg in 50 μl of PBS) was mixed with 0.5 ml of normal horse serum and loaded onto each of the anti-IgG columns in PBS or in PBS containing 1% PEG 400. Unbound protein was washed off the column with the respective buffer at a flow-rate of 1 ml/min, then the bound IgG was eluted with 0.2 *M* ammonium acetate buffer (pH 10.0). Samples of the eluates were analysed for contaminating proteins by SDS-PAGE.

RESULTS AND DISCUSSION

Adsorption of serum proteins to Eupergit C and to agarose

Eupergit C is a polymeric matrix bearing oxirane groups which are capable of covalently binding proteins via their amino, thio or hydroxy moieties[15]. Owing to the slightly hydrophobic nature of the matrix, non-specific adsorption of proteins may occur in the process of affinity purification, especially when crude biological fluids are loaded onto the column. In order to distinguish between non-specific adsorption and covalent binding of proteins to Eupergit C beads, reactive oxirane groups on the surface of the beads were blocked by reaction with βME, thus eliminating the possibility of covalent binding of proteins. The modified Eupergit C beads were packed into an HPLC column. Aliquots of normal horse serum were applied to the column and washed with PBS until no protein was eluted. Washing of the column with an elution buffer [0.2 *M* ammonium acetate buffer (pH 10.0) or 1% SDS in 8 *M* urea] resulted in the elution of non-specifically adsorbed proteins (Figs. 1b and 2). The mechanism of protein adsorption to the matrix seems complicated; when the non-adsorbed protein (Fig. 1, peak I) was collected and reapplied to the same column, a certain amount (similar to the amount of protein adsorbed to the column at the first run) was adsorbed to the matrix and later eluted with 0.2 *M* ammonium acetate buffer

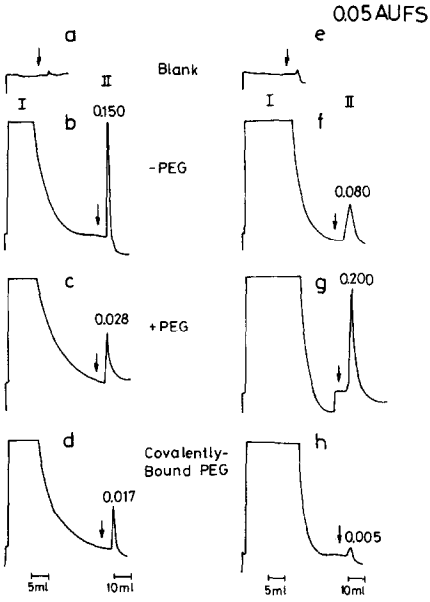

Fig. 1. Adsorption of serum proteins on Eupergit C derivatives (HPLC patterns), onto intact agarose and onto Sepharose 6B-bound PEG (LC patterns). Normal horse serum (0.5 ml) was applied to the columns in PBS (pH 7.4) or PBS containing 1% PEG 400 as described under Experimental. After removal of the non-adsorbed material (peak I), the adsorbed material (peak II) was eluted with 0.2 *M* ammonium acetate buffer (pH 10.0). The absorbance of the eluent was monitored at 280 nm. (a–d) β-Mercaptoethanol-blocked Eupergit C C30N; (e–g) intact agarose; (h) epoxy-activated Sepharose 6B modified with amino-PEG 1500. (a, e) Blank runs, no serum applied; (b, f) control runs in absence of PEG; (c, g) runs in presence of 1% PEG 400; (d, h) runs on covalently bound amino-PEG columns. Numbers on peak II denote peak area in $A_{280\ nm}$ units. Arrows indicate the application of the elution buffer, 0.2 *M* ammoniom acetate (pH 10.0).

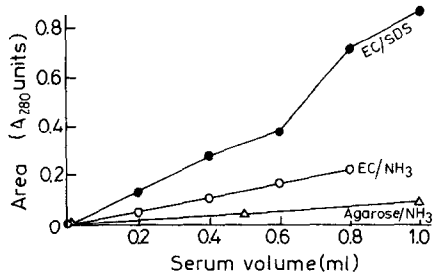

Fig. 2. Dependence of protein adsorption from serum by β-mercaptoethanol-blocked Eupergit C and intact agarose on the amount of serum loaded onto the columns. ●, Protein eluted from Eupergit C with SDS–urea buffer; ○, protein eluted from Eupergit C with 0.2 *M* ammonium acetate buffer (pH 10.0); △, protein eluted from agarose with 0.2 *M* ammoniom acetate buffer (pH 10.0).

(pH 10.0). In contrast, when the protein previously adsorbed to the matrix (peak II) was reapplied to the same column, no protein was re-adsorbed and eluted by the elution buffer.

The amount of serum proteins adsorbed to the matrix and later eluted by the elution buffers was directly proportional to the amount of serum applied to the column (about 0.4% of the protein loaded; Fig. 2). The amount of protein adsorbed on the column increased with increasing NaCl concentration in the loading buffer; in 0.1 *M* sodium phosphate buffer (pH 7.4) the absorption was about 50% of that in PBS, whereas increasing the salt concentration to 1 *M* resulted in the adsorption of about 2% of the protein applied to the column. This observation indicates that the adsorption is of a hydrophobic, rather than ionic, nature.

The adsorption of serum proteins to the agarose beads was followed using a similar approach. Aliquots of NHS were applied to the agarose column in PBS (pH 7.4) and the amount of protein eluted from the column on washing with 0.2 *M* ammonium acetate (pH 10.0) was determined. As shown in Fig. 1, the amount of protein adsorbed to the agarose beads was lower than that of the proteins adsorbed to the Eupergit C beads. Still, the adsorption of proteins was directly proportional to the amount of serum applied to the column (Fig. 2).

Of all the proteins that may be adsorbed to an immunoaffinity column, only those which are eluted under the specific conditions used to elute the protein of interest may lead to contamination of the product. Although those proteins which may be eluted only with SDS–urea treatment may cause other difficulties (*e.g.*, affect column performance), they will not cause product contamination. Therefore, we decided to concentrate our efforts on those proteins which elute under the specific elution conditions, namely with 0.2 *M* ammonium acetate buffer (pH 10.0).

As shown above, when serum is applied to the Eupergit C–βME and the agarose columns, about 0.4% and 0.2% of its proteins, respectively, are adsorbed to the matrices and eluted with 0.2 *M* ammonium acetate buffer (pH 10.0). Only a few of the proteins of the normal serum were adsorbed to Eupergit C–βME beads, as shown by SDS-PAGE of the ammoniom acetate-eluted fraction (data not shown). It is pertinent to note that when bovine serum albumin (20 mg in 0.5 ml of PBS, which is approximately equivalent to the amount of albumin in 0.5 ml of serum) was applied to the same column under similar conditions, no protein was apparently adsorbed to the matrix.

Effect of PEG on protein adsorption to Eupergit C and agarose

In an attempt to reduce the adsorption of serum proteins to Eupergit C, we examined the effect of the addition of organic polymers to the loading buffer (PBS, pH 7.4) on protein adsorption. When poly(vinyl alcohol) (M_r = 25 000, 1%) was included in the loading buffer, a dramatic increase (about 10-fold) in the adsorption of serum proteins to the Eupergit C–βME column was observed (data not shown). In contrast, inclusion of PEG in the loading buffer at a final concentration of 1% reduced the amount of adsorbed material. As shown in Fig. 3, PEG 1500 and 400 were more effective than PEG 200. The protective effect of PEG 1500 was more pronounced at low ionic strength (<0.15 *M* NaCl). In the presence of 1 *M* NaCl almost no effect of PEG on protein adsorption was observed (data not shown).

After the Eupergit C column had been washed once with PBS containing 1% PEG, a "memory effect" was observed; in the following run, even if PEG was omitted from the loading buffer, the column behaved as if PEG was still present and the amount of protein adsorbed was very low. This effect lasted for 3–5 runs before the column returned to "normal" behaviour (Fig. 4).

In contrast to the protective effect of PEG against protein adsorption observed with Eupergit C beads, an adverse effect of PEG on protein adsorption to agarose was observed. Inclusion of PEG in the loading buffer resulted in a 20-fold increase in protein adsorption to the agarose beads. No "memory effect" was observed in this instance. When PEG was omitted from the loading buffer, adsorption was at the "normal" level, regardless of whether or not the column had been washed with PEG in the previous run.

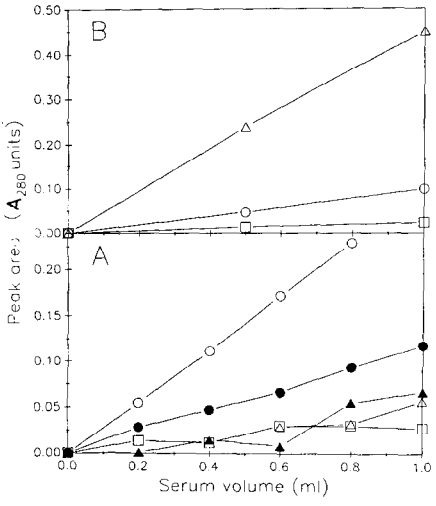

Fig. 3. Effect of polyethylene glycol on the non-specific adsorption of proteins to β-mercaptoethanol-blocked Eupergit C (A) and intact agarose (B). PEG of various molecular weight was included at a concentration of 1% in the loading buffer of different aliquots of normal horse serum. Adsorbed protein was eluted from the columns by 0.2 *M* ammonium acetate buffer (pH 10.0). ○ = No PEG included in the loading buffer; ● = PEG 3; △ = PEG 400; ▲ = PEG 1500; □ = covalently bound amino-PEG, no PEG included in the loading buffer.

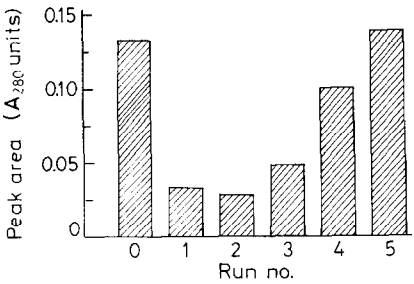

Fig. 4. Memory effect of PEG 400 on protein adsorption to β-mercaptoethanol-blocked Eupergit C in successive runs. Run 1 was carried out in the presence of 1% PEG 400 in the sample loading buffer. Runs 0 and 2–5 were carried out in the absence of PEG.

Immunoaffinity purification in the presence of PEG

As shown above (Figs. 1c and 3), inclusion of PEG in the loading buffer resulted in a decrease in the amount of proteins adsorbed to a Eupergit C–βME column. It was still necessary to show that PEG has a protective against non-specific adsorption of proteins to the matrix in an immunoaffinity purification process. This was demonstrated with the immunopurification of rabbit IgG from serum using Eupergit C-immobilized polyclonal anti-IgG and of the human decidua protein hDP71 from seminal plasma using Eupergit C-immobilized monoclonal antibodies DEC21[12].

Rabbit IgG (ammonium sulphate fraction) was first "contaminated" by mixing it with normal horse serum and then loaded onto the Eupergit C-conjugated anti-IgG column in PBS (pH 7.4), in the absence or presence of 1% PEG 400. As shown in Fig. 5A, the IgG fraction loaded on the matrix in the absence of PEG was eluted

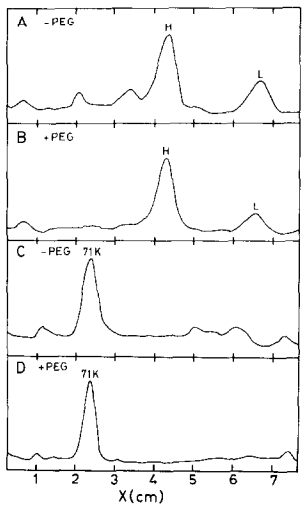

Fig. 5. Immunoaffinity purification of rabbit IgG (A and B) and hDP71 (C and D) using Eupergit C-immobilized goat anti-rabbit IgG and monoclonal anti-hDP71 (DEC21), respectively. Patterns of SDS-PAGE of the materials eluted in peak II of runs carried out in the absence (A and C) or presence of 1% PEG 400 (B and D) in the sample loading buffer are shown.

contaminated with a few other protein bands (containing about 10–20% of the total protein, by peak integration). In contrast, when PEG 400 (1%) was included in the loading buffer the IgG was eluted much purer.

Similar results were obtained with the immunopurification of hDP71 from crude cell-free seminal plasma. As shown in Fig. 5B, in the absence of PEG in the loading buffer the hDP71-containing fraction eluted from the column with 0.2 *M* ammonium acetate buffer (pH 10.0) also contained several minor protein bands, as revealed by SDS-PAGE. When PEG 400 was included in the loading buffer, however, the hDP71 eluted from the column was essentially pure.

Preparation and properties of covalently linked PEG–Eupergit C and -agarose

As described above, in order to reduce non-specific adsorption of proteins onto Eupergit C, PEG 400 or 1500 should be included in the loading buffer. Although PEG is considered to be non-toxic and non-immunogenic, in some instances one may wish to avoid the inclusion of any foreign material in the loading buffer during an immunoaffinity purification process. Therefore, we examined the possibilily of modifying the surfaces of Eupergit C and agarose with covalently bound PEG in an attempt to achieve protection against non-specific adsorption of proteins to the modified matrices.

PEG-modified Eupergit C and agarose were prepared by covalent binding of diamino-PEG 1500 to Eupergit C and to expoxy-activated agarose. As shown in Figs. 1d and h and 3, the reduction of non-specific adsorption of proteins by the modified matrices was similar to, or even slightly better than, the reduction observed when PEG was included in the loading buffer with the non-modified matrices. Interestingly, in contrast to the increase in protein adsorption observed when PEG 400 was included in the loading buffer of the agarose column (see Fig. 1), the covalent linking of PEG 1500 to the agarose resulted in a decrease in protein adsorption. Possibly two different mechanisms are involved. When the serum samples are injected into the agarose column in the presence of PEG, its proteins may react with PEG and their surface properties may be altered. In contrast, when PEG is reacted with the insoluble matrix it may create a modified matrix which possesses different adsorption properties.

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