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DIVINYLSULPHONE-ACTIVATED AGAROSE

FORMATION OF STABLE AND NON-LEAKING AFFINITY MATRICES BY IMMOBILIZATION OF IMMUNOGLOBULINS AND OTHER PROTEINS

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SUMMARY

Divinylsulphone-activated agarose is an attractive alternative to several of the activated supports usually used. Unlike CNBr-activated gels, it does not leak the immobilized protein at high pH. It reacts readily with proteins at near-neutral pH (unlike the epoxy-activated supports). Generally, divinylsulphone-activated agarose reacts with amino, hydroxyl, and sulphhydryl groups, thus allowing immobilization of a wide spectrum of ligands. Moreover, it is available in an aqueous suspension free of organic solvents and neither requires time-consuming swelling nor washing.

INTRODUCTION

Cross-linking and activation of agarose with divinylsulphone (DVS) was described by Porath and co-workers in 1975 [1, 2] but has achieved little recognition since then, even though the method has been applied to the immobilization of carbohydrates [3] and hormones [4].

Our preliminary investigations lead us to believe that this method for the immobilization of ligands for affinity chromatography has more useful applications than has been recognized so far. Thus, it has been our aim to investigate the coupling parameters of DVS-derivatized agarose gel as well as the functional integrity of coupled ligands, with special reference to immunosorption techniques. We conclude that DVS-activated agarose is a useful matrix for the immobilization of biomolecules, comparing favourably with CNBr- and epoxy-activated gels.

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EXPERIMENTAL

Materials

DVS-activated agarose (MINI-LEAK), horseradish peroxidase (HRP), crude goat antiserum against rabbit immunoglobulin G (IgG) and lectins concanavalin A (Con A), wheatgerm agglutinin (WGA), *Lens culinari* agglutinin (LCA), *Pisum sativum* agglutinin (PSA), soya bean agglutinin (SBA) peanut agglutinin (PNA), *Solanum tuberosum* agglutinin (STA) and *Vicia vilosa* agglutinin (VVA) were a gift from Kem-En-Tec Biotechnology Corp. (Hellerup, Denmark). Bovine albumin and bovine γ-globulins were from Sigma (St. Louis, MO, U.S.A.). CNBr-activated agarose was from Pharmacia (Uppsala, Sweden) as was CNBr-activated Sepharose 6B. Rabbit and human immunoglobulins were prepared on immobilized protein A according to standard procedures. Human IgG was labelled with ^{125}I by the chloramine-T method.

Methods

For experimental conditions, see legends to Figs. 1–4 and Table I.

RESULTS

Coupling as a function of ionic strength

Coupling of proteins was found to proceed in near-neutral to weakly alkaline media from pH 7.5 to 9.5, coupling faster the more alkaline the pH. In the following experiments, as well as for routine coupling, we chose to couple at pH 8.6. All coupling procedures were performed at room temperature for 14—20 h.

We found that various proteins couple with different efficiencies depending upon the ionic strength of the coupling medium. Thus, as indicated in Fig. 1,

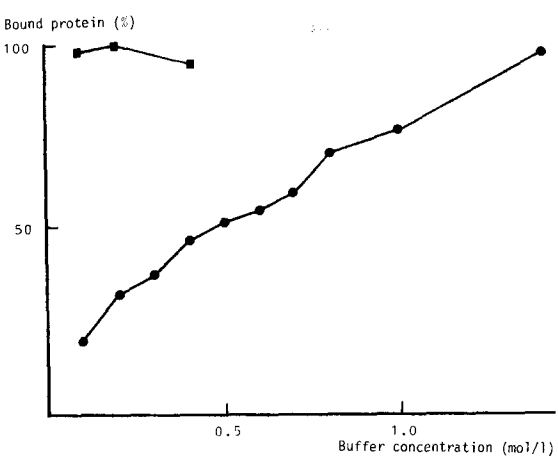

Fig. 1. Coupling of pure proteins to DVS-activated agarose. (▪) Bovine γ-globulins; (•) bovine serum albumin. The coupling was performed at pH 8.6 for 14 h at room temperature at a concentration of 15 mg/ml protein, corresponding to 30 mg of protein per g of wet gel. The coupling is expressed as the percentage of bound protein as a function of the concentration of potassium phosphate buffer, pH 8.6 (0.05–1.4 *M*).

albumin only coupled efficiently to the gel at high ionic strength, while the coupling of γ-globulins showed no dependence on ionic strength.

Coupling capacity and yield

As the coupling capacity and coupling yield are important parameters in practical work with affinity chromatographic procedures, we investigated these parameters as a function of the amount of added protein. We defined the coupling capacity as the maximal amount of protein that can be coupled to 1 ml of wet, sedimented gel, and the coupling yield as the percentage of the total amount of added protein that is coupled. Bovine albumin and bovine γ-globulin were used as model proteins, and for comparison we performed parallel experiments with CNBr-activated Sepharose (Fig. 2). We used optimal conditions for the coupling, e.g. high ionic strength for albumin and moderate ionic strength for γ-globulins when coupling to DVS-activated gel. Moderate ionic strength was used when coupling to CNBr-activated gel, as coupling of albumin to this gel is independent of ionic strength (over a broad range). As seen in Fig. 2, the DVS-activated gel has a higher capacity for albumin at high albumin concentration than the CNBr-activated gel, whereas the two gels show similar capacity for binding of γ-globulins. We were pleased to find that the DVS-activated gel couples with yields better than 80%, up to a load as high as 80 mg of protein per ml of activated gel.

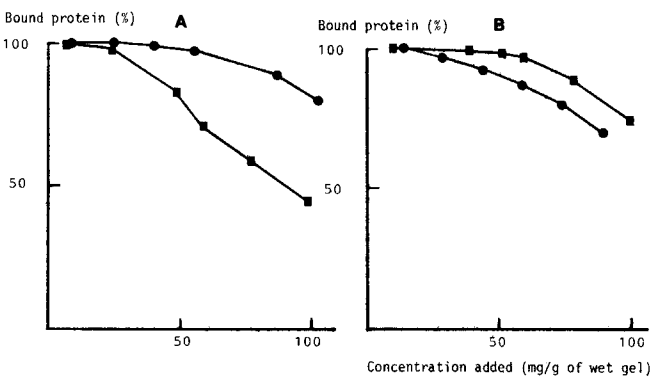

Fig. 2. Coupling of pure proteins to DVS-activated agarose (•) and to CNBr-activated Sepharose 6B (▪). The yield of bound protein (%) is given as a function of the amount of protein added per g of wet activated gel. (A) Coupling of bovine serum albumin: the coupling buffer was 1.4 *M* potassium phosphate (pH 8.6) for DVS-activated agarose and 0.2 *M* potassium phosphate (pH 8.6) for CNBr-activated Sepharose 6B. (B) Coupling yield of bovine γ-globulin: the coupling buffer was 0.2 *M* potassium phosphate (pH 8.6). During coupling, the protein concentration was 20–30 mg/ml of coupling suspension. The coupling was performed for 14 h at room temperature.

Stability of the coupled gel

As high and low pH values are often applied for elution of immunosorption columns and, generally, with deforming buffers in affinity chromatography, we examined the leakage of immobilized protein at pH 2.5—11.5. For this experiment two different proteins, HRP and radioactively labelled human IgG, were immobilized on DVS- and CNBr-activated gels.

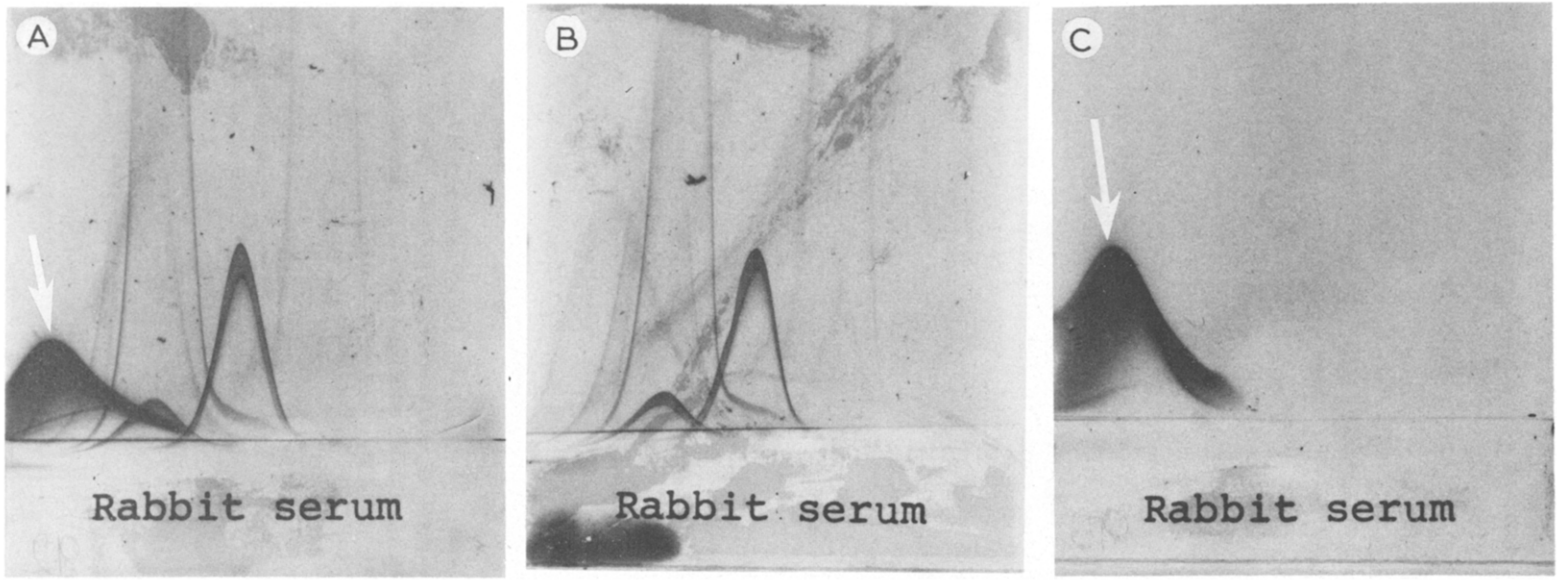

Fig. 3. Preparation of immunosorbed antibodies. (A) Crossed immunoelectrophoresis of 2 μl of rabbit serum with 300 μl of crude goat antiserum against rabbit IgG (arrow indicates IgG). (B) Crossed immunoelectrophoresis of 2 μl of rabbit serum with 300 μl of non-bound fraction (arrow indicates that IgG is not precipitated by this fraction). (C) Crossed immunoelectrophoresis of 2 μl of rabbit serum with 300 μl of eluted antibody fraction. After elution at pH 11.5, the antibody fraction was neutralized and dialysed. The only precipitate is formed by anti-IgG. First-dimension anode is to the right (electrophoresis for 1 h at 10 V/cm); second-dimension anode on top (electrophoresis for 16 h at 1.8 V/cm). Buffer was 1% Tris-veronal (pH 8.6) Litex agarose HSA. Plate dimensions 7 × 10 cm.

TABLE I

PROTEIN LEAKING

HRP: purified HRP was coupled to DVS- and CNBr-activated gels at a concentration of 5 mg/g of wet sedimented gel, washed with high- and low-pH buffers and then incubated for 15 h at pH 11.5 and 2.5. Release of HRP from the gels was determined continously by spectrophotometry at 403 nm and calculated as HRP liberated (ng/min) per mg HRP immobilized. IgG: purified, radioactively labelled human IgG was immobilized on the two gels at a concentration of 10 mg/g wet sedimented gel, washed with high- and low-pH buffers and then incubated for 24 h at pH 11.5, 7.0 and 2.5. Release of radioactive protein was determined and calculated as IgG liberated (ng/min) per mg IgG immobilized.

Protein released	HRP (ng/min mg)		IgG (ng/min mg)		
	pH 11.5	pH 2.5	pH 11.5	pH 7.0	pH 2.5
From DVS-activated gel	0.2	< 0.1	1.5	0.35	1.1
From CNBr-activated gel	11.0	2.8	36.8	0.35	1.4

The leakage from the two gels was calculated from the absorbance at 403 nm owing to released HRP and from radioactivity of the released immunoglobulin. Table I shows that the DVS-activated gel gives coupled products that are at least one order of magnitude more stable at alkaline pH than the products of the CNBr-activated gel.

Applications in affinity chromatography

The high coupling capacity, combined with the high stability of the chemical bonds, makes DVS-activated gel a potentially useful matrix in affinity chromatography. In particular, the stability as expressed by the comparatively low leakage at high pH values makes this gel an attractive alternative for immunosorbant techniques.

Coupling of antigen for immunosorption: immobilized rabbit immunoglobulin for preparation of goat anti-rabbit immunoglobulin. For the preparation of affinity-purified goat anti-rabbit immunoglobulin from crude oligospecific antiserum, we immobilized pure rabbit immunoglobulins to DVS-activated agarose to a final concentration of 15 mg of rabbit immunoglobulins per g of wet gel. The matrix obtained was used in an immunosorbent column, which was loaded with the crude antiserum, washed with saline and eluted with 0.1 *M* phosphate buffer (pH 11.5). Fig. 3A illustrates the oligospecificity of the crude antiserum when used in the second dimension of a crossed immunoelectrophoresis of rabbit serum. The crude antiserum precipitates a number of rabbit proteins in addition to IgG. In Fig. 3B the non-binding fraction, the run-through, is used as antibody in the second dimension. As seen from the disappearance of the IgG precipitate, all anti-IgG has been adsorbed to the column. After desorption at pH 11.5, the desorbed antibody fraction, the no-nonsense goat anti-rabbit IgG, showed only activity against IgG (Fig. 3C).

When loading the column at its maximal capacity, 10 ml of crude antiserum could be completely adsorbed for anti-IgG per ml of gel. On a 2-ml column we could prepare 28 mg of affinity-purified goat anti-rabbit IgG. These data indicate that the immobilized rabbit IgG, to a large extent, is sterically available and active as antigen on the matrix.

Coupling of antibodies for immunosorption: goat anti-rabbit immunoglobulin immobilized for preparation of rabbit immunoglobulin. To investigate the performance of antibodies coupled to DVS-activated agarose, we immobilized the no-nonsense affinity-purified goat anti-rabbit IgG (described above) to a final density of 1 mg of antibody per ml of gel.

A column with 10 ml of this antibody gel was prepared and, after careful washing with high- and low-pH buffers (pH 2.2 and 11.5, respectively), the column was loaded with rabbit serum. After washing with saline, the column was eluted with 0.1 *M* glycine hydrochloride (pH 2.2) and the eluate was immediately neutralized. When loaded to its maximal capacity, the column could yield between 4 and 5 mg of pure rabbit IgG repeatedly, without loss of activity. Fig. 4 shows an analysis of the rabbit serum and the eluted immunoglobulin by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS PAGE). Only IgG was detected in the eluate; other rabbit proteins could not be seen (column 1).

Our data indicate that between 40 and 50% of the DVS-activated agarose-immobilized antibody is exposed on the matrix in a sterically available and active form.

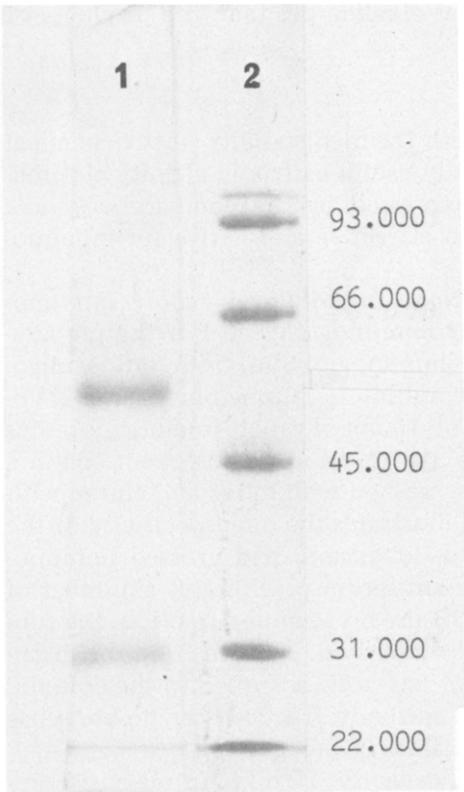

Fig. 4. Preparation of rabbit immunoglobulin. Analysis by SDS-PAGE. Column 1: the eluted immunoglobulin fraction with heavy and light chains; electrophoresis was performed at 30 mA overnight. Column 2: a set of marker proteins with the molecular weights given (Kem-En-Tec).

DISCUSSION

The DVS-activated agarose appears to exhibit an extraordinarily high reactivity when coupling immunoglobulins, as compared to albumin (Fig. 1). In other experiments, we have been able to demonstrate that this selectivity is also expressed during immobilization of complex protein mixtures as crude antiserum, mouse ascites fluid and tissue culture supernatant (in preparation). The selective adsorption of immunoglobulins to the mercaptoethanol derivative of DVS-activated agarose (the so-called T-gel [7]) was presented at this meeting by Porath. This adsorption is dependent upon the ionic strength and may be based upon a structural interaction between a surface area of the immunoglobulin molecule and the C—S—C configuration. This promising absorbent can be prepared easily by incubating DVS agarose with mercaptoethanol at slightly alkaline pH [7].

To assess the activity of another group of binding proteins after coupling to DVS-activated agarose, we chose to immobilize lectins. This was done at a concentration of 5—10 mg of lectin per ml of gel and the immobilized lectins (see *Materials*) were found to be fully active and able to separate human serum proteins into a binding fraction and a non-binding fraction (in preparation).

In contrast to CNBr-activated agarose, the DVS-activated agarose also reacts with hydroxyl groups. We immobilized L-fucose on DVS-activated agarose at pH 11 for 24 h at room temperature [5]. We then used this gel successfully as an affinity adsorbent for purification of the lectin UEA-I from crude extracts of *Ulex europeaeus* seeds. The capacity of this gel was determined to be 4–5 mg of lectin per ml of gel [5], which is more than three times as much as reported earlier [6].

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