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Compared stability of Sepharose-based immunoadsorbents prepared by various activation methods

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ABSTRACT

During the use of chromatographic supports for the purification of proteins or the selective removal of substances by immunoaffinity, leakage of the antibodies immobilized on the matrix is systematically observed. When the cleansing of blood plasma by extracorporeal circulation is concerned, it is of prime importance that the immunoadsorbents exhibit an extensive chemical stability over the whole range of experimental conditions. To study and minimize this leakage, a matrix, Sepharose CL-4B, was activated by various chemical reagents and coupled to goat anti-apolipoprotein B polyclonal antibodies. Immunoadsorbents thus prepared were compared with those obtained earlier by cyanogen bromide activation. It turns out that divinyl sulphone- and tresyl chloride-activated supports lead to similar results in terms of coupling yield and adsorption capacity, but to a significant reduction in released antibodies.

INTRODUCTION

Immunoadsorption is an attractive method used for the specific extraction of proteins or, conversely, the cleansing of biological fluids such as blood plasma. However, in addition to its cost, the leakage of significant amounts of antibodies from the support [1,2] considerably hampers the practical use of this method. In fact, these released antibodies contaminate the purified proteins or detoxified media, and, when clinical use

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is concerned, may lead to side-effects in patients, especially if they are of animal origin. It is therefore of prime importance that immunoadsorbents exhibit a satisfactory stability over the widest range of chemical and biochemical conditions occurring during their clinical handling.

In the present article, we report on the preparation of immunoadsorbents designed for the selective removal of low-density lipoproteins (LDLs) by extracorporeal circulation from the blood plasma of patients affected by familial hypercholesterolaemia [3]. These immunoadsorbents were prepared by covalent coupling of goat anti-apolipoprotein B polyclonal antibodies on Sepharose CL-4B previously activated with various chemical reagents. The resulting immunoadsorbents are compared, in terms of adsorption capacity and stability, with that obtained earlier by activation of Sepharose CL-4B with cyanogen bromide [4].

EXPERIMENTAL

Materials

The matrix used was Sepharose CL-4B from Pharmacia (Uppsala, Sweden). Carbonyldiimidazole (CDI), 1,4-butanediol diglycidyl ether (BDGE), divinyl sulphone (DVS) and tresyl chloride (TC) were purchased from Aldrich (Milwaukee, WI, USA). Goat immune serum was purchased from Sebia (Issy-les-Moulineaux, France). Goat anti-apolipoprotein B polyclonal antibodies (anti-apo B) were obtained by affinity chromatography of goat plasma on Sepharose-immobilized human apolipoproteins B (apo B). Fresh blood plasma from healthy donors was provided by the Centre Régional de Transfusion Sanguine de Nancy (Nancy, France). All other chemical reagents were obtained from Merck (Darmstadt, Germany) and Prolabo (Strasbourg, France) and were of analytical grade.

Carbonyldiimidazole activation

This activation was carried out according to Bethell and co-workers [5,6]. Decanted Sepharose CL-4B (5 ml) was washed on a glass filter successively with water (100 ml), water–dioxane (7:3, 5:5, 3:7, v/v, 100 ml of each) and finally anhydrous dioxane (100 ml). The gel was suspended in 2.5 ml of anhydrous dioxane and CDI (20, 30 or 50 mg/ml of gel) was added. The suspension was rotated at 40°C for 2 h, then washed with 100 ml of anhydrous dioxane, dioxane–water (7:3, 5:5, 3:7, v/v) and distilled water and used immediately.

The amount of imidazole and carbonyl groups was determined according to a procedure previously described [6].

1,4-Butanediol diglycidyl ether activation

This activation was performed according to the procedure described by Sundberg and Porath [7]. A 5-ml volume of decanted Sepharose CL-4B was washed on a glass filter with water (100 ml) and stirred by rotation at room temperature for 8 h with 5 ml (or 10 ml) or BDGE, in the presence of 5 ml (or 10 ml) of 0.6 *M* sodium hydroxide containing 10 mg (or 20 mg) of sodium borohydride. The activation reaction was stopped by washing the gel on a glass filter with 1 l of distilled water.

The amount of oxirane groups immobilized on the support was determined after treatment of the activated matrix with sodium thiosulphate [7].

Divinyl sulphone activation

This activation was effected according to the procedure described by Porath *et al.* [8]. A 5-ml volume of decanted Sepharose CL-4B was washed on a glass filter with 25 ml of water, then with 15 ml of 0.5 *M* sodium carbonate buffer–sodium hydroxide pH 11. The gel was suspended in 5 ml of the same buffer and reacted under stirring with DVS (0.01, 0.10 or 0.15 ml/ml of gel) at room temperature. The resulting matrix was then washed with water (2 l) in order to remove the excess DVS.

The amount of vinyl groups was determined after preliminary treatment of the activated matrix by sodium thiosulphate [8]. The amount of total sulphur was measured according to the procedure described by Wagner [9].

Tresyl chloride activation

This activation was performed according to Nilsson and Mosbach [10]. Typically, a 5-ml volume of decanted Sepharose CL-4B was washed on a glass filter successively with water (500 ml), water–acetone (7:3, 5:5, 3:7, v/v) and finally with dry acetone (100 ml). The gel was suspended in 3 ml of dry acetone containing 20, 40 or 60 μl of pyridine, according to the amount of tresyl chloride (10, 20 or 30 μl/ml of gel) subsequently added dropwise to the suspension. After the mixture was shaken for 15 min, the gel was washed with dry acetone (100 ml), acetone–1 m*M* hydrochloric acid (7:3, 5:5, 3:7, v/v) and finally 1 m*M* hydrochloric acid (100 ml).

The amount of total sulphur was determined according to the procedure described by Wagner [9].

Typical coupling procedure

Each activated support (5 ml) was washed on a glass filter with 0.1 *M* sodium bicarbonate, 0.5 *M* sodium chloride pH 8.5 buffer and mixed under gentle stirring at room temperature to the goat anti-apo B solution (6 mg/ml) in the same buffer, volume to volume. This concentration has previously been determined as the one affording an optimal adsorption capacity [4]. The reaction time was 22 h for the gels activated by CDI or TC, 48 h for the BDGE-activated supports and 24 h for the DVS-activated matrices. Resulting immunoadsorbents were then washed successively with sodium bicarbonate buffer, 1 *M* ethanolamine pH 8.5 for 2 h to block the unreacted active groups, then with 0.3 *M* glycine–hydrochloric acid pH 2.8 in order to disrupt possible antibody–antibody interactions. After final washings in phosphate-buffered saline (PBS) pH 7.3, each immunoadsorbent was stored at 4°C in 25% aqueous ethanol.

Adsorption capacity

Each immunoadsorbent (5 ml) was packed in a column (5.3 cm × 1.1 cm I.D.) and washed successively with PBS pH 7.3, glycine–hydrochloric acid pH 2.8 and again PBS. Blood plasma (60 ml) was pumped into the column containing each immunoadsorbent (30 ml/h). Elution with PBS pH 7.3 was then carried out until 60 ml of plasma thus purified were recovered. Adsorbed apo B were desorbed thereafter with glycine–hydrochloric acid pH 2.8. Each immunoadsorbent was then washed with PBS and stored again in 25% aqueous ethanol. The amount of apo B in the initial plasma, the eluted plasma and the apo B solution was determined by Laurell immunoelectrophoresis rockets [11].

Assay of released antibodies

The amount of released antibodies was measured by an enzyme-linked immunosorbent assay (ELISA), both in the eluted plasma at pH 7.3 and in the apo B solution desorbed at pH 2.8. Microtitre plates (Costar) were coated with 100 μl of rabbit anti-goat IgG antibodies in 50 m*M* sodium carbonate buffer, pH 9.6 (2.5 μg/ml), overnight at 4°C. After washing with PBS–Tween (130 m*M* sodium chloride, 5 m*M* disodium hydrogenphosphate, 1 m*M* potassium dihydrogenphosphate, pH 7.2, containing 0.05% Tween 20), 125 μl of 0.5% cold-water fish-skin gelatin were added to each well, and the plates were incubated at 37°C for 3 h. A 100-μl aliquot of samples diluted in the initial plasma or PBS–Tween was added to the wells, and the plates were incubated at 37°C for 2 h. Goat IgGs were used as a standard. After washing with PBS–Tween, 100 μl of biotinylated rabbit anti-goat IgG antibodies were added to each well. The plates were incubated at 37°C for 2 h and washed with PBS–Tween. A 100-μl volume of peroxidase–streptavidin (Zymed) (1:1000) was then dispensed into each well and the plates were incubated at 37°C for 10 min. Finally, after washing with PBS–Tween, then with 0.14 *M* acetate–citrate buffer pH 6, 0.1 mg/ml 3,3′,5,5′-tetramethylbenzidine and 0.01% hydrogen peroxide in the acetate–citrate buffer were placed in each well. The reaction was stopped by addition of 25 μl of 2 *M* sulphuric acid and the absorbance was measured colorimetrically at 450 nm in a Titertek plus micro ELISA reader (Flow Laboratories, Middlesex, UK).

Adsorption specificity. For each immunoadsor-

bent, the adsorption specificity was tested by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) on a Phast System apparatus, according to the Pharmacia procedure.

RESULTS AND DISCUSSION

The efficient removal (or purification) of apo B from blood plasma requires the preparation of immunoadsorbents exhibiting both a high adsorption capacity and an extensive stability over the widest range of chemical or biochemical conditions to which they are submitted. To optimize this synthesis, the activation of Sepharose CL-4B was carried out with four different reagents, selected from those most often used during the elaboration of affinity chromatography matrices. Various amounts of CDI, BDGE, DVS and TC were reacted with Sepharose CL-4B, and each of the resulting activated matrices was subsequently coupled to goat anti-apo B polyclonal antibodies at different pH values. The conditions for optimal coupling yield are collected in Fig. 1. Obviously, CDI and also BDGE activations afford rather low coupling yields. In contrast, DVS- and TC-activated supports lead to highly substituted immunoadsorbents having an adsorption capacity for apo B similar to that obtained with the previously described cyanogen bromide-activated gel [4] (Fig. 2).

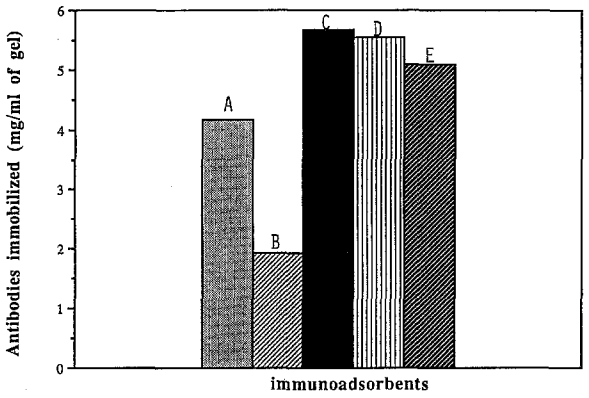

Fig. 1. Optimal coupling yields of the immunoadsorbents prepared by reaction of 5 ml of goat anti-apo B polyclonal antibodies (6 mg/ml) with Sepharose CL-4B (5 ml) previously activated with various chemical reagents: (A) CDI (30 mg/ml of gel); (B) BDGE (2 ml/ml of gel; (C) DVS (0.15 ml/ml of gel); (D) TC (20 μl/ml of gel); (E) cyanogen bromide (100 mg/ml of gel). The coupling was performed at pH 9 for CDI-, BDGE- and DVS-activated supports, pH 7.5 for the TC-activated matrix and pH 8.5 for the cyanogen bromide-activated one.

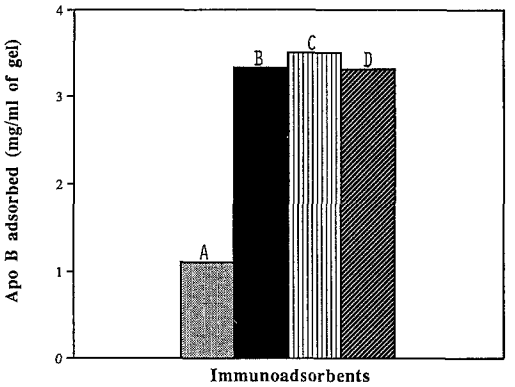

Fig. 2. Optimal adsorption capacities of the various immunoadsorbents (5 ml) obtained by activation of Sepharose CL-4B with (A) CDI (4.20 mg of antibodies per ml of gel), (B) DVS (5.80 mg of antibodies per ml of gel); (C) TC (5.55 mg of antibodies per ml of gel and (D) cyanogen bromide (5.10 mg of antibodies per ml of gel), when loaded with blood plasma (60 ml).

Fig. 3 shows the antibodies released at pH 7.3 in the effluent plasma, according to the nature of the immunoadsorbent employed, and Fig. 4 those released from the various immunoadsorbents during the elution at acidic pH (2.8) of adsorbed apo B.

The most striking finding is that pH has a strong influence on the release. Appreciable

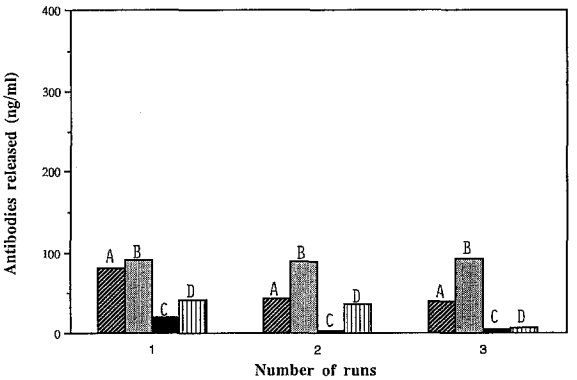

Fig. 3. Stability of the various immunoadsorbents at pH 7.3. Antibodies released in the eluted plasma at pH 7.3 *versus* the number of runs for each immunoadsorbent previously activated by (B) CDI, (C) DVS, (D) TC and (A) cyanogen bromide.

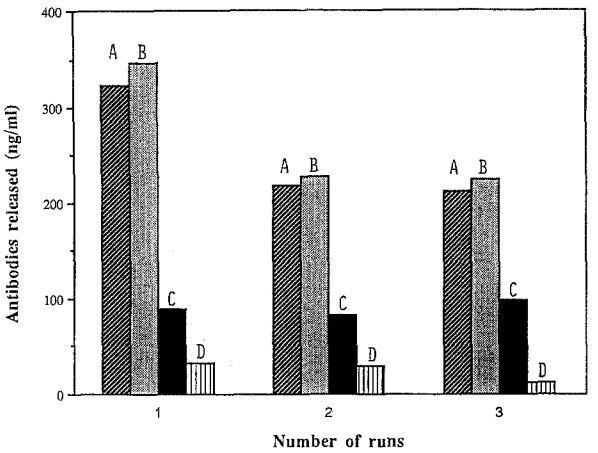

Fig. 4. Stability of the various immunoadsorbents at pH 2.8. Amounts of antibodies released at pH 2.8 in the apo B solution *versus* the number of runs for (B) CDI-, (C) DVS-, (D) TC- and (A) cyanogen bromide-activated immunoadsorbents.

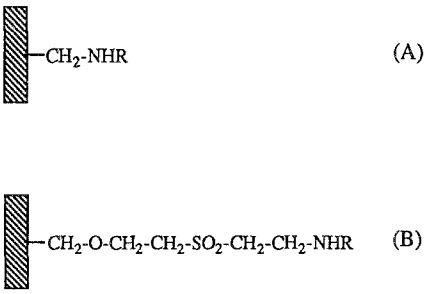

Fig. 5. Structures of immunoadsorbents prepared by activation of Sepharose CL-4B with TC (A) and DVS (B), followed by coupling to goat anti-apo B polyclonal antibodies (R-NH_2).

amounts of antibodies are released at acidic pH and therefore contaminate the resulting apo B solutions, whereas in neutral pH conditions, a limited leakage is observed.

The second observation concerns the stability of the various immunoadsorbents according to the activation procedure employed for their preparation. Clearly, whether in the effluent plasma at pH 7.3 (Fig. 3) or in the eluted apo B solution at pH 2.8 (Fig. 4), the use of DVS- or TC-activated supports brings a significant improvement, relative to that prepared by cyanogen bromide activation. In fact, the amount of antibodies released from the immunoadsorbents is systematically lower than that observed with the cyanogen bromide-activated support, and the decrease may reach, under certain conditions, a factor of 20.

Finally, important information may be obtained from the behaviour of TC- and DVS-activated immunoadsorbents at the two different pH values. Generally speaking, the release of an antibody from a matrix may be caused by various factors, among which, besides the denaturation of the antibody itself, the fragility of the matrix and the breaking of the spacer arm between the support and the antibody are the two effects most likely to occur. Fig. 3 shows that at pH 7.3 only very limited leakage occurs. This is not surprising since around neutrality the nature of the chemical bonds between the matrix and the antibody (Fig. 5) makes them unlikely to be broken. At this pH, the small leakage observed would therefore be essentially the result of some degradation of the Sepharose structure. The fact that the DVS-activated adsorbent is even more stable than the TC-activated one supports this hypothesis. In fact, the bifunctional character of this activation reagent probably results in some additional cross-linking of the matrix and therefore enhanced stability.

On the other hand, the stabilities of TC- and DVS-activated supports at pH 2.8 (Fig. 4) are quite different. Upon decreasing pH from 7.3 to 2.8, the leakage from DVS-activated immunoadsorbents increase significantly (four-fold), whereas that of TC-activated supports is unaffected. Since the matrix is identical for both immunoadsorbents, this difference must be ascribed to the relative stabilities of the two spacer arms. In this respect, it is clear that secondary amino groups of TC-activated supports are rather insensitive to acidic conditions, in contrast to CH_2-SO_2 groups, or ether links involved in the structure of immunoadsorbents prepared by DVS activation.

From these results, it appears that the preparation of immunoadsorbents, highly stable over a wide pH range and therefore taking into account the additive fragilities of the matrix and the spacer arm, would be optimal if carried out with tresyl chloride activation and with highly cross-linked matrices.

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