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Comparison of three activated agaroses for use in affinity chromatography: effects on coupling performance and ligand leakage

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ABSTRACT

Three commercially available activated supports, N-hydroxysuccinimide (NHS)-, tresyl chloride- and hydrazide-activated agarose, were compared with respect to coupling rate and coupling efficiency for the ligand and ligand leakage during both storage and chromatography. A monoclonal antibody against the E_1 protein of the rubella virus was used as a ligand. For each support the monoclonal antibody (mab) was immobilized at three concentrations: 0, and *ca.* 2.5, 5.0 and 10 mg IgG per ml gel. The NHS-activated support showed very fast and complete binding of the ligand. Moreover, using this support the ligand leakage was considerably less both during storage and chromatography as compared with the other two supports. It was also shown that the static binding capacity was comparable for the NHS- and tresyl chloride-derivatized agaroses and it was about a factor of two lower for the hydrazide-derivatized agarose.

INTRODUCTION

Affinity chromatography based on antigen–antibody interactions, called immunosorption is an extremely powerful technique and has been increasingly successful since the advent of monoclonal antibodies (mabs). When there is a suitable monoclonal antibody at hand, immunosorption is an especially attractive technique for protein purification. Purification factors of 2000–20 000-fold are often achievable, and it is sometimes possible to achieve purification to homogeneity in a single step.

The extensive use of antibody-containing affinity columns in the purification of biologically active compounds is severely hampered by the leaching of antibody or portions thereof from the immunoaffinity support during elution of the target antigen. Part of the problem is caused by the combined use of reducing agents (*i.e.*, thiols) and chaotropic agents (*e.g.*, detergents and denaturants) in the elution step, which causes the dissociation of heavy and/or light chains from the immobilized antibody. This part of the leakage problem can be diminished by, amongst other things, intramolecular cross-linking of the antibody chains at their sites of disulphide interlinkage using bifunctional SH-specific reagents [1] or via the lysine groups using glutaraldehyde. A decrease in the problems in this context can also be obtained by the selection of conjugation methods that yield a more stable chemical linkage between the matrix and the spacer and between the spacer and the antibody [2].

Many activated gel matrices ready for the reaction with a ligand are commercially available [3]. They differ in, amongst other things, the reactive group, the extent of activation, introduced spacer length and type, particle size and porosity. In practice, most of these gel matrices are based on beaded agarose.

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In spite of some disadvantages, activation by cyanogen bromide (CNBr) remains a popular method. The main disadvantages of CNBr-activated agarose stem from the isourea linkage [4] between the gel and the amino groups of lysine. The isourea derivative introduces an extra positive charge at neutral pH ($pK \approx 9.5$), causing the gel to act as a weak ion exchanger at low salt concentrations. This does not usually present a problem. More serious is the fact that the isourea bond is reversible and can be cleaved, *e.g.*, by hydrolysis at weakly alkaline pH (>8) and by aminolysis with low-molecular-mass amines. In addition, very slow leakage of the (protein) ligand from the column occurs over a period of months to years [5].

Proteins may also be coupled to agarose which has been derivatized by spacer arms with N-hydroxysuccinimide ester at their ends [6]. Succinimide esters are very susceptible to nucleophilic attack by the ϵ-amino groups of lysine, resulting in the formation of a stable amide bond between the protein and the spacer arm. Limitations of NHS esters in affinity chromatography and protein immobilization were described by Wilchek and Miron [2]. On reaction with ligands containing an amino group, the columns were unstable to alkali and were plagued by constant leakage during use. However, they also described an alternative two-step method for the preparation of NHS esters, based first on the reaction of a carboxyl-containing matrix with a carbodiimide, that yields stable affinity columns. Tresyl chloride-activated gel matrices are suitable for immobilization of amino- and thiol-containing ligands and allow efficient immobilization even at neutral pH. The ligand becomes immobilized to the matrix by stable $-CH_2-S-$ (thioether) or $-CH_2-NH-$ (amine) linkages. The thiol groups are more reactive than amines and also imidazole and tyrosine hydroxyl groups can displace the sulphonate ester [7].

A hydrazide matrix can be used for immobilization of ligands containing aldehyde and ketone groups [8]. The reaction occurs at low pH (*ca.* 5) and the chemical bond that is formed is a stable hydrazone, obviating the need for reduction (although this can be performed if desired) [9]. In the case of antibodies, carbohydrate residues in the oligosaccharide chains, mainly at the Fc-part of the molecule, are oxidized at the vicinal hydroxyls to form aldehydes. This should have the advantage that antibodies are immobilized with optimum orientation [10].

The purpose of this investigation was to evaluate three commercially available products, hydrazide-, tresyl chloride- and N-hydroxysuccinimide (NHS)-activated agarose, with respect to their performance with regard to ligand leakage during storage and immunoaffinity chromatography. Moreover, the coupling speed and coupling efficiency for a mab and the performance of the immunosorbents with respect to static binding capacities at three ligand densities were determined. These factors can be influenced by, amongst other things, temperature, pH, type and concentration of coupling buffer, use and type of spacer arm and nature of the ligand [7,11]. Because investigations according to these factors were beyond the scope of this work, preparation of the immunosorbents was performed according to the manufacturer's protocol.

The purification of the E_1–E_2 glycoprotein complex (M_r 300 000) of the rubella virus from culture fluid was used as a model for this study. A mab directed against the E_1 protein of the antigen complex was immobilized on the three gels at three different concentrations. It has been reported that rubella virus proteins are unstable at low pH [12] and that they can be purified by immunochromatography using 0.5 *M* diethanolamine (pH 11.5) [13]. Also in this investigation basic conditions were used to elute the bound antigen from the column. Because the isourea bond is not stable under these conditions, CNBr-activated agarose was excluded from this evaluation.

EXPERIMENTAL

Materials and reagents

Tresyl-activated Sepharose 4 fast flow (FF) and protein A Sepharose 4 FF were purchased, and NHS-activated Sepharose 4 FF (a prototype gel containing 22 μmol of NHS groups per ml gel stored in 2-propanol) was obtained as a gift from Pharmacia (Woerden, Netherlands). Affi-Gel Hz

was purchased as a complete kit from Bio-Rad (Veenendaal, Netherlands). A Micro BCA protein assay kit was purchased from Pierce (Oud-Beijerland, Netherlands). A glycan detection kit was purchased from Boehringer (Mannheim, Germany). All other chemicals were of analytical-reagent grade.

The liquid chromatographic system was supplied by Pharmacia and consisted of two P-500 piston pumps, an MV-7 valve, a UV-M II monitor, a FRAC-200 fraction collector and a flat-bed recorder. A microwell system (model 510 reader, model 500 incubator and model 500 washer) was obtained from Organon Teknika (Turnhout, Belgium).

Protein purification

Monoclonal antibodies. Mab OT-Ru-5, 27 and 28, all specific for the E_1 protein of the rubella virus, were produced under protein-free conditions in a hollow-fibre dialysis system [14,15] and were purified by means of protein A affinity chromatography [16].

Antigen. Rubella virus was produced in baby hamster kidney (BHK) cells persistently infected with the vaccine strain HPV-77. The protein fraction was extracted with Tween–diethyl ether and concentrated 20-fold by ultra-filtration using an M_r 100 000 filter. Phenylmethylsuphonyl fluoride (PMSF), sodium azide and cinnamaldehyde were added to the antigen solution at concentrations of 2, 3 and 5 m*M*, respectively. The pH was adjusted to 7.3 using 1.0 *M* sodium phosphate–potassium phosphate buffer (pH 6.5). The solution was centrifuged for 15 min at 4000 *g* and the supernatant was filtered through a 0.45-μm pore size filter. It should be noted that the amount of active protein was only 0.02% (w/w) of the total amount of protein in the starting material.

Preparation of immunosorbents

Mab OT-Ru-28 was coupled to the activated supports according to the manufacturer's protocol. A schematic illustration of the bonds formed between the activated supports and the mab is given in Fig. 3.

Hydrazide-activated support. The solvent in which the mab was dissolved was exchanged for coupling buffer (pH 5.5) using an Econo-Pac 10 DG column. The column and buffer were included in the Affi-Gel Hz kit. Oxidation of IgG carbohydrates was performed by adding 10% (v/v) of 0.1 *M* aqueous $NaIO_4$ to the mab solution. This mixture was incubated for 1 h by end-over-end rotation in the dark at ambient temperature. The solvent was exchanged for coupling buffer by means of gel filtration using an Econo-Pac 10 DG column. The final IgG concentration was determined by UV measurement [A_{280} (1 cm, 1 mg/ml) = 1.45].

A portion of 6 ml of gel in 2-propanol was washed twice with 12 ml of coupling buffer. Mab in coupling buffer was added in amounts of 0, 3.4, 7.4 and 14.8 mg to portions of 1.5 ml of gel. The volume of the incubation mixtures was adjusted to *ca.* 6 ml by adding coupling buffer. Incubation was performed by end-over-end rotation for 24 h at ambient temperature. After incubation for 2, 4 and 24 h samples were taken from the supernatant to determine the coupling performance. To remove non-covalently bound antibody [17] the gels were washed on a sintered-glass filter with *ca.* 25 bed volumes of 7 m*M* phosphate–0.1 *M* NaCl (pH 7.3) [phosphate-buffered saline (PBS)], PBS–1 *M* NaCl, cold water, 0.1 *M* Na_2CO_3–0.5 *M* NaCl (pH 11), 0.1 *M* sodium acetate–0.5 *M* NaCl (pH 4), 0.1 *M* Na_2CO_3–0.5 *M* NaCl (pH 11), 0.1 *M* sodium acetate–0.5 *M* NaCl (pH 4) and again PBS. The gels were stored at 4°C in PBS containing 3 m*M* sodium azide.

Before each incubation with antigen solution, the gels were washed with approximately four bed volumes of 10 m*M* Na_2CO_3 (pH 11) and PBS.

Tresyl-activated support. Lyophilized tresyl-activated Sepharose (1.8 g) was swollen for 1 h in 1 m*M* HCl. The swollen gel was washed on a sintered-glass filter successively with 360 ml of 1 m*M* HCl and 150 ml of 9 g/l NaCl. The gel was suspended in an equal volume of 0.1 *M* $NaHCO_3$–0.5 *M* NaCl (pH 8.3) (coupling buffer). To 3-ml portions of this gel suspension, mab in coupling buffer was added in amounts of 0, 4.2, 8.4 and 16.8 mg. The volume of the incubation mixtures was adjusted to *ca.* 6 ml with coupling buffer. Incubation was performed by

end-over-end rotation for 24 h at ambient temperature. During and at the end of incubation samples were taken from the supernatant to determine the coupling performance. After coupling the supernatant was removed, 3 ml of 0.1 *M* Tris–HCl (pH 8.0) were added to the gels and incubation was continued for 4 h at ambient temperature. The gels were washed, stored and equilibrated as described for the hydrazide-activated support.

NHS-activated support. Approximately 12 ml of a 66% (v/v) slurry was washed on a sintered-glass filter with 120 ml of 1 m*M* HCl at 4°C. The gel was suspended with an equal volume of 0.2 *M* $NaHCO_3$–0.5 *M* NaCl (pH 8.2) (coupling buffer). Immediately afterwards, mab in coupling buffer was added in amounts of 0, 4.3, 8.6 and 17.2 mg to 3-ml portions of gel suspension. The volume of the incubation mixtures was adjusted to *ca.* 6 ml with coupling buffer. Incubation was performed by end-over-end rotation for 5 h at ambient temperature. During and at the end of incubation samples were taken from the supernatant to determine the coupling performance. Although not prescribed, the gels were incubated with 3 ml of Tris–HCl (pH 8.0) for 17 h at 4°C. The gels were washed, stored and equilibrated as described for the hydrazide-activated support.

Determination of static binding capacity and binding efficiency

The static binding capacity was measured in batch experiments, for which it was determined that 16–20 h of incubation was ample for equilibrium to be established. Six different amounts of antigen were incubated with 40 μl of a 25% (v/v) gel suspension of each immunosorbent. Incubation was performed by end-over-end rotation for 16–20 h at ambient temperature. After settling of the gel, the amount of antigen in the supernatant was determined by means of an enzyme-linked immunosorbent assay (ELISA).

Determination of ligand leakage

In the supernatants obtained during a period of up to 3 months after preparation of the gels and storage at 4°C and in preparations obtained during purification, the IgG content was determined by means of ELISA.

Chromatography

Purification was performed with the immunosorbents containing 3.0, 2.6 and 2.9 mg of IgG/ml gel based on the hydrazide-, tresyl- and NHS-activated supports, respectively. Portions of 1 ml of a 25% (v/v) gel suspension (duplicates) were incubated with antigen solution (1000 U/ml gel). Incubation was performed batchwise by end-over-end rotation for 16–20 h at ambient temperature. After incubation the gel was transferred to a C10/10 column (Pharmacia) and washed with PBS (75 cm/h) until the original baseline (absorbance at 280 nm) was reached. The adsorbed antigen was eluted with 10 m*M* Na_2CO_3 (pH 11) (15 cm/h). The pH of the eluted antigen fraction was neutralized with 1 *M* 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES) (pH 6.5).

Determination of rubella E1-antigen activity using ELISA

Polystyrene microtitration strip-plates were coated overnight at ambient temperature with a 2.5 μg/ml solution of mab OT-Ru-5. The plates were washed with PBS containing 0.05% (w/v) of Tween 20 (PBST), dried and stored at 4°C. Samples and standards were diluted with PBST and incubated (100 μl per well) for 1 h at 37°C. The wells were washed with PBST (300 μl per well) and incubated with 100 μl of conjugate solution (HRP conjugated to mab OT-Ru-27) in PBST for 1 h at 37°C. The wells were washed again and incubated for 0.5 h at ambient temperature with 100 μl per well of substrate (urea peroxide)–chromogen (tetramethylbenzidine) solution. The reaction was stopped by addition of 1 *M* sulphuric acid (100 μl per well). Absorbances at 450 nm were determined using a microtitration plate reader. The measuring range of the assay was 0.025–0.1 U antigen/ml. Units are obtained by defining an in-house rubella antigen solution arbitrarily as 10 U/ml.

Determination of mouse IgG using ELISA

Polystyrene microtitration strip-plates were coated with sheep anti-mouse IgG at a concen-

tration of 0.7 μg/ml in 50 m*M* sodium carbonate buffer (pH 9.6) at ambient temperature for 24 h. The coated plates were washed, dried and stored at 4°C. A 100-μl volume of diluted sample or standard (mab OT-Ru-28) was pipetted into each well and incubated for 1 h at 37°C. The wells were washed and incubated with sheep anti-mouse IgG conjugated with HRP for 30 min at 37°C. The wells were washed again and incubated for 0.5 h at ambient temperature with 100 μl per well of substrate–chromogen solution. The reaction was stopped by addition of 1 *M* sulphuric acid (100 μl per well). Absorbances at 450 nm were determined using a microplate reader. The measuring range of the assay was 1–10 ng IgG/ml.

Determination of protein content

Samples and standard [bovine serum albumin (BSA)] solutions were diluted in 0.15 *M* NaCl and 100 μl were pipetted into the well of a microtitration strip-plate. A 100-μl volume of Micro BCA working reagent was added per well and the plate was incubated for 1 h at 50°C. Absorbances at 540 nm were determined using a microplate reader. The measuring range of the assay was 1–20 μg/ml.

RESULTS AND DISCUSSION

Determination of coupling performance

The amount of mab OT-Ru-28 IgG in the samples taken during and at the end of the coupling and taken during the wash cycles (see preparation of the immunosorbents) was determined by means of ELISA. It was confirmed that periodate oxidation of the mab had no effect on the reactivity in the ELISA for mouse IgG. The ligand density, defined as mg of IgG coupled per ml of gel, and the coupling efficiency, defined as (amount of IgG coupled/amount of IgG offered) × 100%, were calculated and are given in Table I. The amount of IgG coupled was calculated by subtracting the total amount of IgG found in the supernatant after coupling and in all wash fractions from the amount of IgG offered.

For hydrazide- and tresyl-activated supports, coupling efficiencies based on the ELISA results were confirmed by measurements of the absorbance at 280 nm (A_{280}) of the supernatant (results not shown). Because of the high A_{280} of NHS, which is released during coupling, supernatants obtained after coupling were analysed for IgG content using HPSEC [Zorbax GF-250 column, 20-μl injection volume, 0.2 *M* phosphate buffer (pH 7.0) and detection at 206 nm]. No IgG could be detected, which confirmed the results obtained from the ELISA.

TABLE I

LIGAND DENSITY AND COUPLING EFFICIENCY

Immobilization of mab OT-Ru-28 to hydrazide-, tresyl- and NHS-activated agarose matrices were performed by overnight coupling at ambient temperature, followed by an extensive washing procedure as described in detail under Experimental. The amount of uncoupled mab was determined using ELISA for mouse IgG and used to calculate coupling efficiency and ligand density.

Immunosorbent	Ligand density (mg IgG/ml gel)	Coupling efficiency (%)
Hydrazide	0	0
	1.3	54
	3.0	60
	7.8	79
Tresyl	0	0
	2.6	93
	5.3	96
	8.9	81
NHS	0	0
	2.9	100
	5.4	100
	11.1	100

Washing the gel directly after coupling to remove non-covalently bound IgG resulted with the tresyl- and hydrazide-activated agarose only in a minimal loss of ligand compared with the amount of IgG coupled. No IgG could be detected in the wash fractions of the NHS-activated gels.

The coupling of mab OT-Ru-28 to NHS-activated agarose is completed within 30 min for all three concentrations tested (Fig. 1). This fast and efficient coupling of mab agreed very well with the results obtained by Matson and Little [18]. After incubation for 4–5 h, coupling of mab to the hydrazide- and tresyl chloride-activated supports is almost at its maximum (81–96%);

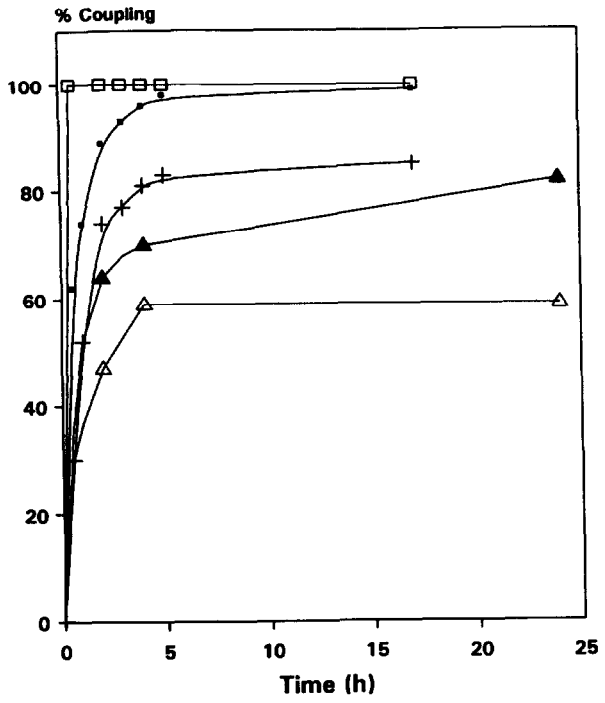

Fig. 1. Determination of coupling rate and coupling efficiency of mab OT-Ru-28 to NHS-activated agarose offering 2.9, 5.4 or 11.1 mg/ml gel [all the same curve (□)], hydrazide-activated agarose offering (△) 2.3 or (▲) 9.9 mg/ml and tresyl-activated agarose offering (■) 2.8 or (+) 11.2 mg/ml.

longer incubation times result only in a slightly higher ligand density. These results were also in good agreement with those reported [7,19].

A relatively low binding efficiency of 54–79% after incubation for 24 h was observed for the hydrazide-activated support. This may be due to the degree and type of glycosylation of the antibody molecule, causing a limiting amount of aldehyde groups on the oxidized antibody, or to sub-optimum coupling conditions for this particular mab.

Determination of ligand leakage during storage

The leakage of ligand from the gel during storage is the strongest for the tresyl-activated gel followed by the hydrazide-activated matrix. Detailed results are given in Table II. With the NHS-activated gel almost no IgG could be detected in the supernatants.

TABLE II

LIGAND LEAKAGE DURING STORAGE

IgG leakage was determined by ELISA for mouse IgG on the supernatants of the immunosorbents after a storage period of 3 months in PBS + 0.02% NaN_3 at 4°C.

Immunosorbent	Ligand density (mg IgG/ml)	Ligand leakage (μg/ml gel)
Hydrazide	1.3	11
	3.0	21
	7.8	31
Tresyl	2.6	15
	5.3	104
	8.9	>120
NHS	2.9	0.008
	5.4	0.028
	11.1	0.291

Static binding capacity

The static binding capacities of the immunosorbents are summarized in Table III, and were determined as described under Experimental. They were read from the binding efficiency curve at the 80% point. This definition gives a practically useful binding capacity and is not the maximum binding capacity of the immuno-

TABLE III

DETERMINATION OF BINDING CAPACITY

Binding capacities of the immunosorbents were determined using batchwise incubation (16–20 h, ambient temperature) with antigen. The amount of unbound antigen was determined by ELISA. The binding capacity was defined as the amount of antigen (U) bound per ml of immunosorbent with an efficiency of 80%.

Gel	Ligand density (mg IgG/ml)	Binding capacity (U/ml gel)
Hydrazide	1.3	325
	3.0	500
	7.8	555
Tresyl	2.6	1130
	5.3	1115
	8.9	1235
NHS	2.9	1060
	5.4	1083
	11.1	1080

sorbent. The binding efficiency curves for the three gels which were formerly used in purification experiments are shown in Fig. 2.

No binding was observed when the antigen was incubated with the gels containing no ligand, which excludes non-specific adsorption.

Static binding capacities for immunosorbents based on the tresyl- and NHS-activated gels were about equal and increasing the ligand density from 2.6 to 11.1 mg IgG/ml did not result in an increase in static binding capacity. Instead of an improved binding capacity as expected by oriented coupling of the mab molecules, immunosorbents based on the hydrazide-activated gels had binding capacities that were only half or less those that of the other two supports.

Orthner *et al.* [20] found that their mabs against human plasma proteins factor IX or protein C also contained carbohydrates in the Fab′ region. Carbohydrate analysis of intact IgG and $F(ab')_2$ fragments of mab OT-Ru-28B under reducing and non-reducing conditions showed that sugars were only present on the Fc part. This excluded coupling via the antigen-binding site.

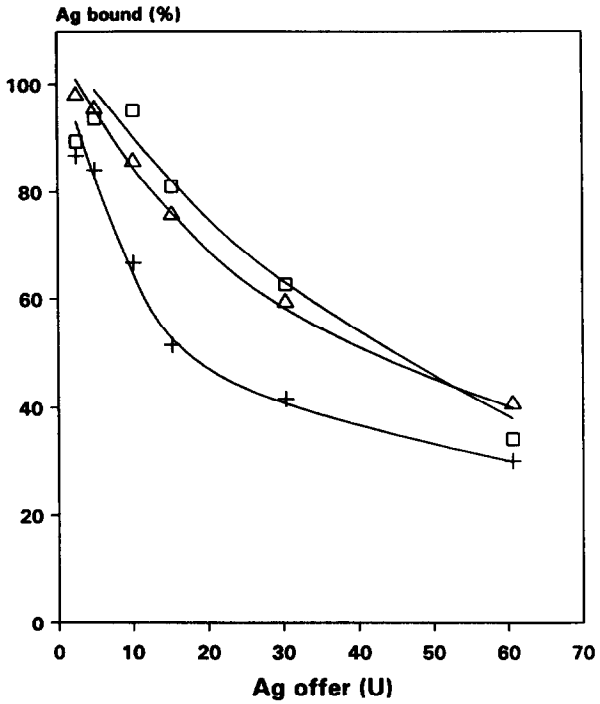

Fig. 2. Determination of the binding efficiency of the immunosorbents based on (□) NHS-activated agarose, (+) hydrazide-activated agarose and (△) tresyl-activated agarose with ligand densities of 2.9, 3.0 and 2.6 mg mab/ml gel, respectively.

Whether the reduced activity is due to the oxidation step was not investigated. Orthner *et al.* [20] found for their mabs no reduced activity caused by oxidation. However, Fleminger *et al.* [21] showed for several mabs a loss of activity up to 26% after incubation with 10 m*M* sodium periodate for 1 h in the dark at 4°C. They also showed that increasing the temperature during the oxidation step to ambient temperature resulted in a rapid inactivation of the more sensitive antibodies. Another reason for the reduced activity may be that sodium periodate oxidizes not only the carbohydrate moieties, but also certain amino acid residues, particularly N-terminal serine, threonine and methionine. Whenever these residues are essential for the antigen-binding activity of an antibody, their oxidation may harm its activity.

Because the immunosorbents were useful for isolation of rubella virus proteins and optimization of the binding capacity, which was outside the scope of this investigation, has been thoroughly discussed in the literature [22,23], no further attention was devoted to this subject.

Determination of ligand leakage during chromatography

Ligand leakage during chromatography using an immunosorbent based on NHS-activated agarose is about a factor of fifteen lower than that using the sorbent prepared from tresyl-activated agarose and more than a factor of twenty lower than that using the sorbent based on hydrazide-activated agarose (see Table IV). This means that instability towards alkali of ligands coupled to an NHS group-containing support, as been noted by Cuatrecasas and Parikh [6] and Wilchek and Miron [2], is almost overcome.

Because the amine linkage formed after reaction of tresyl with amino groups is a stable bond, the higher ligand leakage for the tresyl-activated agarose in comparison with the NHS-activated gel can be explained by the lower stability at high pH of the bonds probably formed by side-reactions with imidazole or

TABLE IV

LIGAND LEAKAGE DURING CHROMATOGRAPHY

The amount of mouse IgG was determined using ELISA and the amount of total protein was determined using the Micro BCA protein assay. For details, see Experimental. Contamination of the purified antigen fraction with anti-rubella mab was determined in duplicate. Contamination was expressed as (amount of mouse IgG/amount of total protein) × 100%.

Immunosorbent	Ligand density (mg IgG/ml gel)	Contamination (%, w/w)	
		Run 1	Run 2
Hydrazide	3.0	0.44	0.50
Tresyl	2.6	0.26	0.33
NHS	2.9	0.02	0.02

tyrosine hydroxyl groups. As IgG has no free thiol groups, the presence of a thioether bond is not likely.

The relatively high contamination of the final product with mouse IgG (expressed as %, w/w) observed for the hydrazide gel can partly be explained by the lower binding capacity of this immunosorbent. Other explanations may be the lower stability of the hydrazone bond at high pH as compared with the amine and amide bonds (see Fig. 3), or that leakage is related to the mode of introduction of the hydrazide group into the support.

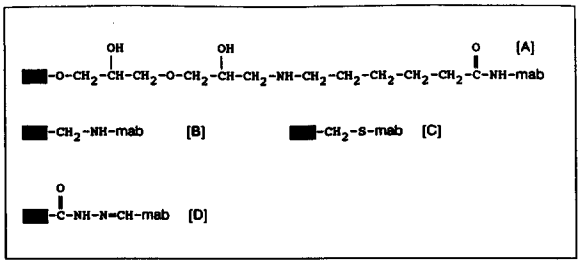

Fig. 3. Immobilization of an amino-containing ligand (mab-NH_2) to the (A) NHS- and (B) tresyl-activated support. Coupling of a thiol-containing ligand (mab-SH) to the (C) tresyl-activated support and coupling of an aldehyde-containing ligand (mab-CHO) to the (D) hydrazide-activated support [D].

CONCLUSIONS

It was concluded that of the three activated supports evaluated, NHS-activated Sepharose 4 fast flow is the most suitable activated matrix with respect to (mab) ligand leakage both during storage at neutral pH and during chromatography using alkaline (pH 11.0) desorption conditions. An improvement in ligand leakage for this gel in comparison with earlier available NHS-activated agaroses was obtained by introducing the spacer arm via epoxy activation to the polysaccharide matrix [24], which results in a stable ether bond, followed by a suitable method for introduction of the NHS ester. For the mab used in this investigation it was shown that the desired ligand density can be exactly achieved because coupling is complete within 30 min of incubation.

Two minor practical disadvantages of this gel are that the succinimide ester gives COOH groups after hydrolysis, which introduce an extra charge, causing the gel to act as an ion exchanger at low salt concentrations, and that the progress and efficiency of coupling of protein cannot be followed by simple ultraviolet absorption measurement because NHS also absorbs strongly.

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