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Affinity purification of monoclonal antibodies, using a bifunctional oligosaccharide hapten

SRINIVAS UPPUGUNDURI[1], JAMAL DAKOUR[1], ELISABETH KALLIN[2], THOMAS NORBERG[2], DAVID ZOPF[3] and ARNE LUNDBLAD[1,2]

[1] *Department of Clinical Chemistry, University Hospital, S-22185 Lund, Sweden*
[2] *BioCarb AB, S-22370, Lund, Sweden*
[3] *BioCarb Inc, PO Box 7409, Gaithersburg, MD 20898, USA*

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A bifunctional hapten was synthesized consisting of a blood group A active tetrasaccharide (A-tetra) and a blood group Le^a active pentasaccharide. lacto-*N*-fucopentaose II (LNF II), linked to each other with a phenylaminothiourea spacer connecting the reducing ends (A-tetra–LNF II). The hapten was demonstrated to retain both blood group A and Le^a activity and could be easily bound to both monoclonal anti-A and anti-Le^a affinity columns. Due to the strong temperature dependence of the two antibodies in their binding to oligosaccharides, the bifunctional hapten could be utilized to achieve easy desorption in the final step of affinity purification of either monoclonal anti-Le^a or anti-A. The system is postulated to have general applicability in affinity purification of any ligate that binds with an avidity too high to achieve non-denaturing desorption.

Keywords: oligosaccharide, monoclonal antibody, affinity chromatography, ELISA

It is now well established that the binding of several monoclonal antibodies to carbohydrate structures is temperature dependent [1–5]. Anti-A/A oligosaccharides [2, 3] or anti-Le^a/Le^a oligosaccharides [4] are examples of such ligate/ligand pairs in which the binding is dependent on temperature. The affinity constants approximately double for each 8°C downward shift in temperature from 37°C to 4°C [6]. Affinity purification of biological molecules (ligates) with high affinity for their immobilized ligands often requires harsh chemical conditions for desorption, leading to loss of biological activity in the purified products. The aim of the present investigation was to utilize the temperature dependence in one ligand/ligate system for easy desorption of any other ligate. A model was created using a bifunctional molecule consisting of a blood group A active tetrasaccharide (A-tetra) bound through its reducing end to the reducing end of Le^a active lacto-*N*-fucopentaose II (LNF II). This bifunctional link (A-tetra–LNF II) could easily be bound to and desorbed from affinity columns that contain immobilized monoclonal anti-A or anti-Le^a antibodies. In the present study we have investigated the potential of this bifunctional link to isolate specifically monoclonal anti-Le^a and anti-A antibodies. The method was compared with the conventional, more harsh, type of affinity chromatography using columns with blood group A or Le^a active oligosaccharides covalently immobilized on an insoluble matrix.

* To whom correspondence should be addressed.

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Materials and methods

Monoclonal antibodies. Monoclonal anti-A antibody (A003, IgM) was obtained from MonoCarb AB (Lund, Sweden), as a cell culture supernatant. The hybridoma cell line producing anti-Le^a (CO 514, IgG3) monoclonal antibodies was purchased from the American Type Culture Collection (Rockville, MD, USA). A cell culture supernatant was produced and harvested as previously described [7].

Oligosaccharides. A-tetra and LNF II were obtained from BioCarb Chemicals (Lund, Sweden).

Affinity chromatography columns. All columns were prepared in 0.3 × 10 cm water-jacketed glass columns with an approximate bed volume of 1 ml. Schematic diagrams of the different columns used are illustrated in Fig. 1.

Column I – Monoclonal anti-Le^a covalently bound to Mini Leak®: Mini Leak® (BioCarb Chemicals, Lund, Sweden) is a divinyl sulphone agarose derivative. Equal volumes of anti-Le^a cell culture supernatant and saturated (100%) ammonium sulphate were mixed together and the anti-Le^a was allowed to precipitate at 4°C for two days. The mixture was centrifuged (5000 × **g**, 20 min) and the pellet dissolved in Tris-HCl buffered saline (TBS, 0.05 M Tris-HCl, 0.14 M

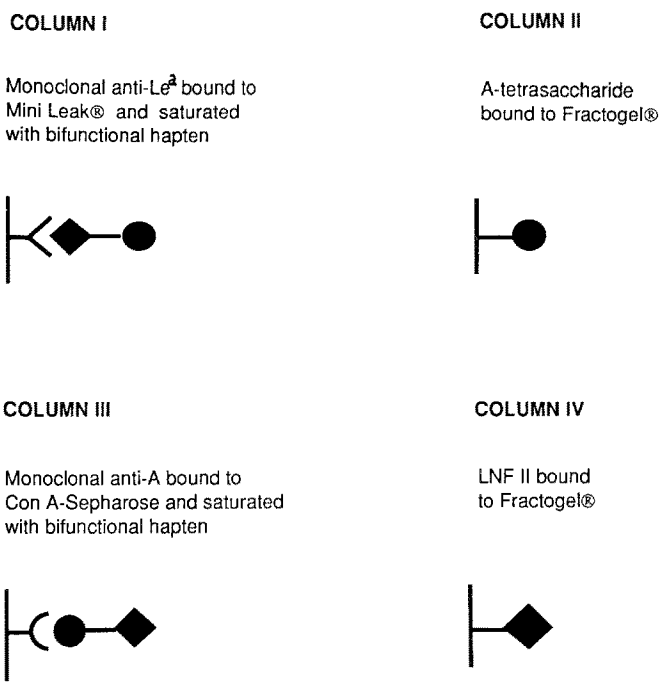

Figure 1. Diagrammatic representation of different columns and the bifunctional hapten A-tetra–LNF II.

NaCl, pH 8.0). Further purification was achieved by adsorption to a Protein G column (1 ml bed volume; Perstorp Biolytica AB, Lund, Sweden), followed by elution with 0.1 M glycine-HCl buffer (pH 2.5). The eluted pool was dialysed against TBS for two days and then coupled to Mini Leak®, as previously described [8, 9]. The total amount of bound anti-Le^a antibody was estimated to be 12.2 mg by measuring the absorption at 280 nm.

Column II – Blood group A active tetrasaccharide immobilized on Fractogel®: A column was prepared by immobilizing A-tetra on Fractogel® TSK HW65 (BioCarb Chemicals) through an aminophenylethyl spacer [10]. The degree of substitution is approximately 5 μmol carbohydrate/g gel.

Column III – Monoclonal anti-A bound to Concanavalin A-Sepharose: Equal volumes of anti-A cell culture supernatant and saturated ammonium sulphate (100%) were mixed together and the antibody was precipitated at 4°C over a period of two days. The mixture was centrifuged (5000 × *g*, 20 min) and the pellet was dissolved in TBS. Approximately 15 mg of A003 was dissolved in 5 ml of TBS and applied to a 1 ml Concanavalin A-Sepharose column (ConA-Seph, Pharmacia, Uppsala, Sweden). The column was washed with at least ten column volumes of TBS before use. The amount of anti-A antibody bound was estimated to be 0.85 mg by eluting the column with 0.5 M α-D-methylmannoside to release the bound antibody and measuring the absorption at 280 nm.

Column IV – LNF II immobilized on Fractogel®: A column was prepared by immobilizing LNF II on Fractogel® TSK HW65 (BioCarb Chemicals) through an aminophenylethyl spacer [10]. The degree of substitution is approximately 5 μmol carbohydrate/g gel.

Synthesis of the bifunctional hapten A-tetra–LNF II. The 1-amino-1-deoxyalditol derivative of LNF II (LNF II–NH_2, Fig. 2A) was prepared in two steps (65% yield) from LNF II as previously described [11, 12]. The *N*-acetyl-*N*-(*p*-trifluoroacetamidophenyl)-1-amino-1-deoxyalditol derivative of A-tetra (A-tetra–TFAC, Fig. 2B) was prepared from A-tetra (74% yield) as described [11, 12]. The bifunctional hapten A-tetra–LNF II (Fig. 2D) was prepared from the above derivatives in the following way: A-tetra–TFAC (25 mg) was dissolved in aqueous NaOH (0.5 M, 2.0 ml) and kept at 4°C overnight. Ethanol (4.0 ml) and glacial acetic acid (0.1 ml) were added. The mixture was stirred at room temperature and monitored by a pH meter while thiophosgene (20 μl) was added. The pH was maintained at 6–7 by addition of aqueous NaOH. After 10 min the mixture was concentrated to a small volume, diluted with water and washed with diethyl ether. The aqueous layer, containing reasonably pure A-tetra isothiocyanate derivative (Fig. 2C) was concentrated to approximately 3 ml and directly added

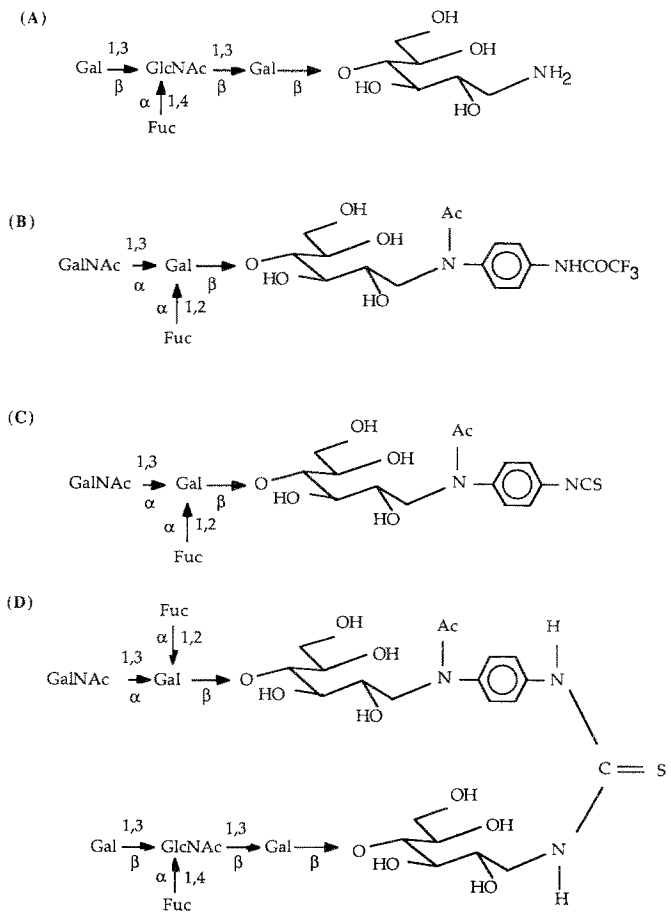

Figure 2. Scheme of synthesis of bifunctional link.

to a solution of LNF II–NH_2 (17 mg) in sodium phosphate buffer (0.1 M, pH 9.5, 3.0 ml). The solution was stirred at room temperature for three hours, during which time the pH was maintained at 9.5 by addition of aqueous NaOH when necessary. The mixture was purified by gel chromatography on a Sephadex G-15 column. Lyophilization of appropriate fractions gave 11 mg of material corresponding to a 32% yield. Positive ion FAB-MS showed the presence of an expected $(M + 1)^+$ ion ($m/z = 1723$). The 500 MHz ^{1}H-NMR spectrum gave the expected signals from A-tetra–LNF II and the aromatic protons. However, for unknown reasons the signal from the aromatic *N*-acetyl group was weak. The molecular weight of the bifunctional compound was calculated to be 1722.4. Tritiated A-tetra–LNF II was prepared by treatment with galactose oxidase followed by reduction with tritiated sodium borohydride as previously described [13].

Determination of column capacity for A-tetra–LNF II. The maximum amount of A-tetra–LNF II that could bind to either the anti-A or the anti-Le^a column was determined by isotope dilution. In successive chromatographic runs increasing amounts of unlabelled A-tetra–LNF II were added to a constant amount of its tritiated derivative and run on the column at 4°C. Following each run, bound ligate was released by increasing the temperature of the column to 40°C. The columns were saturated when the total amount of retarded ligate released was constant in two consecutive runs. The respective column capacities were estimated to be 18 µg for column I (anti-Le^a) and 8 µg for column III (anti-A).

ELISA. Anti-A and anti-Le^a antibody activities in eluted fractions were determined by enzyme linked immuno sorbent assay (ELISA, [12]). The A-tetrasaccharide and LNF II, both linked to human serum albumin (HSA, BioCarb Chemicals AB) were used as antigens coated to microtiter plates (Dynatech Immulon, Plochingen, Germany). Rabbit anti-mouse immunoglobulin, conjugated to alkaline phosphatase (Dakopatts, Copenhagen, Denmark) was used as second antibody and absorbance measured at 405 nm.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS– PAGE). Purity of the eluted antibodies was determined by electrophoresis on vertical polyacrylamide slab gels [15]. The pooled fractions were lyophilized and resuspended in 1–2 ml TBS in order to concentrate the samples. The samples were electrophoresed using the Phast System (Pharmacia, Uppsala, Sweden) and stained with silver nitrate. The samples (7.5 µl) were dissolved in the samples buffer (2.5 µl) and heated at 100°C for 4 min before applying the gel. A 4–15% acrylamide gradient was employed along with a 3% stacking gel.

Protein determinations. Protein content was determined as described earlier [16], using a method based on bicinchoninic acid protein assay reagent (BCA, Pierce, Oud-Beijerland, The Netherlands). The assay was carried out in microtiter plates and absorbance was noted at 540 nm [17].

Results

Affinity purification of monoclonal anti-A antibody on column I

Column I was washed with TBS, saturated with an excess of unlabelled bifunctional link (80 µg), and eluted isocratically with TBS at 4°C at a flow rate of 0.2 ml min^{-1} (Fig. 3). Since the bifunctional hapten absorbs light at a wavelength of 240 nm the eluate was monitored continuously for the presence of the bifunctional link by passage through a UV detector (Uvicord S 2138, LKB, Bromma, Sweden). After approximately 12 column volumes of TBS had passed through the column, when no further hapten could be detected by the UV detector (data not shown in Fig. 3), 750 µg of anti-A antibody was applied onto the column at 4°C. A major fraction of protein was noted initially after the application of antibody which was associated with a minor peak of ELISA activity. The column was washed with TBS and after approximately eight column volumes of TBS, the temperature was raised to 40°C. The increase in temperature resulted in the elution of a protein peak which was associated with a major peak of ELISA activity. The fractions were pooled, as indicated (Fig. 3), and ultrafiltered (PM 10, Amicon Corp., Lexington, MA, USA). The amount of retarded protein was calculated from UV absorption to be 196 µg. The pooled fraction containing the retarded antibody was also run on SDS–PAGE after prior treatment with mercaptoethanol. Electrophoresis showed two pure bands of the light and heavy chains of IgM (see Fig. 7, Lane 2).

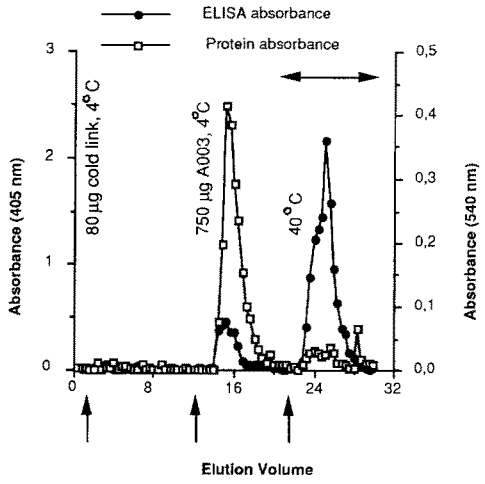

Figure 3. Affinity purification of anti-A antibody on column I (anti-Le^a antibody coupled to Mini Leak®).

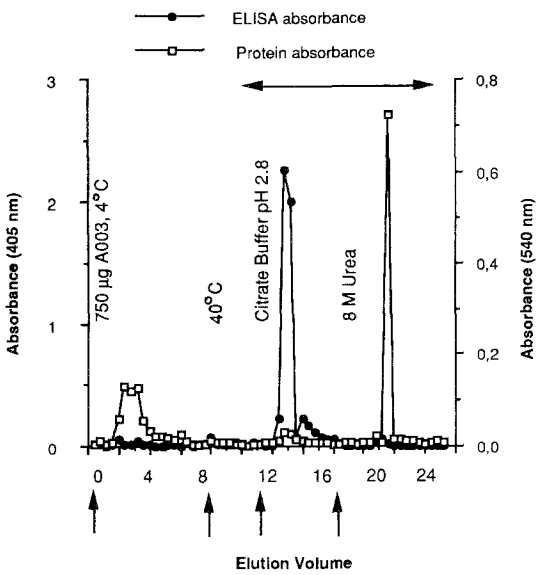

Figure 4. Affinity purification of anti-A antibody on column II (A-tetrasaccharide bound to Fractogel®).

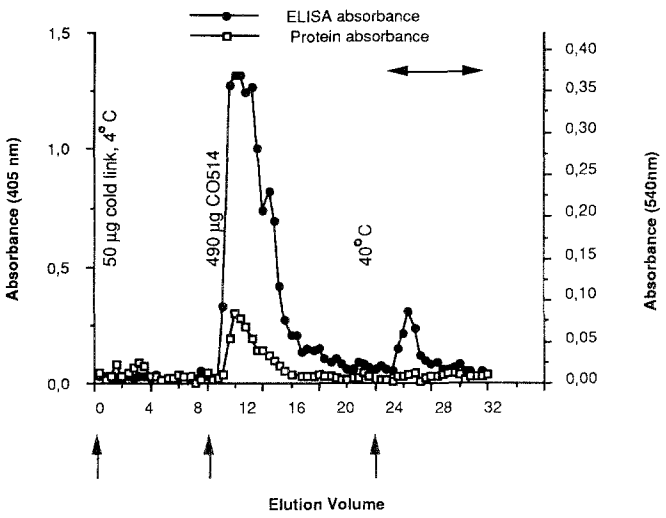

Figure 5. Affinity purification of anti-Le^a antibody on column III (anti-A antibody coupled to Con A-Sepharose).

In a separate experiment using radiolabelled bifunctional hapten (data not shown), we ascertained that almost 96% of the A-tetra–LNF II hapten that was eluted along with the anti-A could easily be removed by ultrafiltration (PM 10, Amicon).

Affinity purification of monoclonal anti-A antibody on column II

Monoclonal anti-A antibody was purified on column II containing the A-tetrasaccharide covalently bound to Fractogel® (Fig. 4). Approximately 750 µg of anti-A antibody was applied to column II at 4°C. The sample was run isocratically in TBS at 4°C at a flow rate of 0.2 ml min^{-1}. Protein determination showed an initial protein peak without any activity on ELISA. After elution with approximately ten column volumes the temperature was increased to 40°C. No detectable protein was released even after approximately six column volumes had passed through the column. The eluting buffer was then changed to 0.1 M sodium citrate pH 2.8 in 0.15M NaCl at 25°C. This resulted in the release of a small amount of retarded protein with major activity on ELISA (Fig. 4). After approximately eight column volumes, stripping the column with 8 M urea, 0.02 M sodium acetate (pH 5.0) did not result in release of appreciable additional protein. The fractions were pooled, as indicated (Fig. 4) and ultrafiltered (PM 10, Amicon). The total amount of antibody eluted in the pooled fraction was calculated from UV absorption to be 205 µg. Electrophoresis of the pooled fractions containing the retarded protein showed two bands of the light and heavy chains of IgM (see Fig. 7, Lane 3).

Affinity purification of monoclonal anti-Le^a antibody on column III

The column was saturated with an excess of the bifunctional oligosaccharide hapten (50 µg) at 4°C, and eluted with TBS at a flow rate of 0.2 ml min^{-1}. The eluate was monitored continuously for the presence of the bifunctional link by passage through an UV detector (Uvicord S 2138). After eight column volumes of TBS, when no further hapten could be detected (data not shown in Fig. 5), 490 µg of anti-Le^a antibody was applied to the column. An initial protein peak observed after the application of protein was coincident with a major peak of ELISA activity. After increasing the column temperature to 40°C, elution with 12 column volumes of TBS gave a protein peak coincident with a minor peak of ELISA activity (Fig. 5).

The fractions were pooled, as indicated (Fig. 6), and ultrafiltered (PM 10, Amicon). The total content of protein in the pooled fractions containing the retarded antibody was calculated by UV absorption to be 53.3 µg. Electrophoresis of the retarded protein showed two faint bands (Fig. 7, Lane 5).

Affinity purification of monoclonal anti-Le^a antibody on column IV

An affinity column containing the LNF II oligosaccharide directly immobilized on Fractogel® was used as an alternative way to purify monoclonal anti-Le^a (Fig. 6). Anti-Le^a antibody (490 µg) was applied to column IV at 4°C. The sample was run isocratically in TBS, at 4°C and a flow rate of 0.2 ml min^{-1}. The void peak was devoid of ELISA activity indicating that the active antibody bound to the column. After washing with approximately four column volumes the temperature was increased to 40°C. No protein

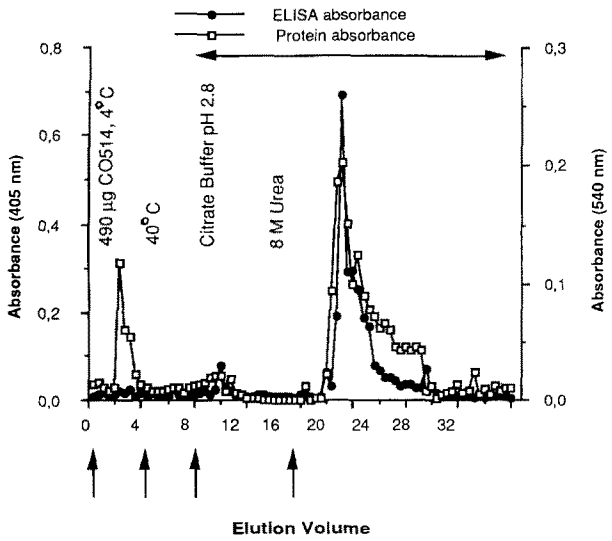

Figure 6. Affinity purification of anti-Le^a antibody on column IV (LNF II immobilized on Fractogel®).

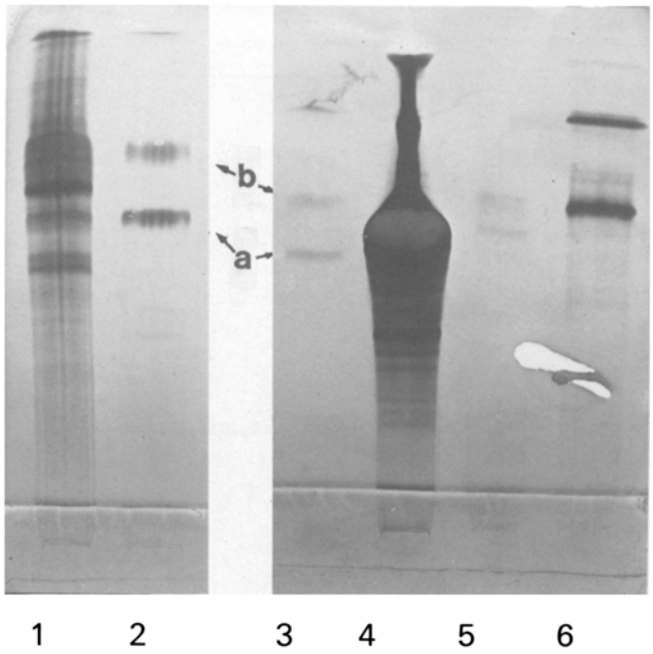

Figure 7. Electrophoresis of the pooled lyophilized samples after treatment with mercaptoethanol. Lane 1: Partially purified anti-A. Lane 2: Anti-A purified using the bifunctional link. Lane 3: Anti-A purified using A-tetra immobilized on Fractogel®. Lane 4: Partially purified anti-Le^a. Lane 5: Anti-Le^a purified using the bifunctional link. Lane 6: Anti-Le^a purified using LNF-II immobilized on Fractogel®. Heavy chains and light chains of the different antibodies are denoted by (a) and (b) respectively.

was released even after washing the column with four bed volumes. The eluting buffer was then changed to 0.1 M sodium citrate pH 2.8 in 0.15 M NaCl at 25°C, resulting in the release of a very small amount of protein which showed ELISA activity. After washing with approximately eight column volumes, the column was eluted with 8 M urea, 0.02 M sodium acetate (pH 5.0), giving a peak containing coincident protein and ELISA activity (Fig. 6).

Fractions containing the eluted antibody were pooled and the total content of retarded protein was estimated to be 409 µg. Electrophoresis of the retarded protein showed two bands corresponding to the light and heavy chains of IgG (Fig. 7, Lane 6).

Determination of biological activity

Elution of antibodies from antigens immobilized on solid matrices often leads to loss of biological activity. To ascertain whether purification of antibodies using the bifunctional link was more beneficial with respect to retention of biological activity we determined the titer of antibodies purified using either the bifunctional link or immobilized antigens. The antibodies eluted from the respective columns (columns I–IV) were pooled as described above, ultrafiltered and then reconstituted to equal volumes with TBS. An ELISA was performed using different dilutions of the antibodies to determine the titer. We then compared the dilution of antibody that was required to produce a 50% reduction in ELISA absorbance (data not shown). Anti-A antibody purified using immobilized antigen had approximately four-fold lower titer as compared to anti-A antibody purified using the bifunctional link. Similarly anti-Le^a antibody purified utilizing immobilized antigens had approximately five-fold lower titer as compared to anti-Le^a antibody purified using the bifunctional hapten.

Discussion

Results presented above demonstrate that the bifunctional hapten A-tetra–LNF II retains both its blood group A and Le^a activity. The anti-A and the anti-Le^a columns exhibited a disparity in the maximum amount of hapten that could be bound to the column. The total amount of anti-A antibody purified using the bifunctional link was 196 µg, whereas the total amount of anti-Le^a antibody purified using the same method was 53 µg.

This difference might be accounted for in part by the larger amount of anti-Le^a antibody coupled covalently to Mini Leak® as compared to a smaller amount of anti-A antibody non-covalently coupled to Con A-Sepharose. Since the columns had different capacities for the bivalent linker, A-tetra-Le^a, they also exhibited a disparity in the amount of antibody that could be purified from the mobile phase. The yields obtained with the different columns are summarized in Table 1, which suggest that an IgG antibody covalently bound to Mini Leak® is the preferred column for this type of affinity chromatography.

Both the A/anti-A and Le^a/anti-Le^a, ligand/ligate pairs used in the model system exhibit temperature dependent binding. An increase in temperature was however not

Table 1. A summary of the type of support used, antibody bound and yields of purified antibodies.

Column, matrix	*Antibody/antigen bound*	*A-tetra–LNF II bound*	*Antibody purified*
I: Mini Leak®	Anti-Le^a, IgG, 81 nmol	10.4 nmol	Anti-A, 0.206 nmol
II: Fractogel®	A-Tetra, 3.33 μmol		Anti-A, 0.215 nmol
III: Con A-Sepharose	Anti-A, IgM, 0.89 nmol	4.6 nmol	Anti-Le^a, 0.35 nmol
IV: Fractogel®	LNF II, 3.33 μmol		Anti-Le^a, 2.7 nmol

sufficient to release the antibodies from their corresponding oligosaccharide antigens covalently linked to an insoluble column matrix. Only a more harsh decrease of pH could effect elution. In the case of LNF II immobilized on Fractogel® (column IV) a major portion of the bound protein was released only on elution with 8 M urea.

Although similar amounts of anti-A antibody were purified utilizing either the bifunctional link or immobilized antigens (196 μg and 205 μg respectively), the anti-A antibody purified using the bifunctional link had a four-time higher titer. Similarly anti-Le^a purified utilizing the bifunctional link exhibited a five-fold higher titer than anti-Le^a antibody eluted with citrate buffer or 8 M urea.

The results presented here suggest an important general scheme for recovering specifically bound ligates from affinity matrices by simply warming the affinity column. For example, at 4°C anti-A covalently bound to an affinity matrix can immobilize a bivalent hapten containing A-tetra coupled to ligand X. Molecules that bind to X might then be specifically adsorbed to the matrix and eluted by warming the column to 40°C to dissociate the binding of anti-A to A-tetra complex. In the experiments presented here the X–anti-X complex (Le^a–anti-Le^a) is itself thermolabile; however, it is anticipated that thermal elution would proceed even if the X–anti-X interaction were of high affinity and virtually insensitive to temperature. Under these circumstances thermal elution might provide a worthwhile alternative to chemical elution methods that often denature protein ligates during elution. Once A-tetra-X–anti-X complex is eluted at elevated temperature, the bivalent hapten could be separated from the anti-X ligate by methods such as dialysis or gel filtration to recover pure anti-X.

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