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NON-BONDED AFFINITY CHROMATOGRAPHY: A BIOSPECIFIC OIL-SOLUBLE COATED SUPPORT FOR CONCANAVALIN A

RODNEY R. WALTERS and RICHARD P. BUCK*
Kenan Laboratories of Chemistry, The University of North Carolina, Chapel Hill, N.C. 27514 (U.S.A.)
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SUMMARY

Affinity chromatographic separations using ligands chemically bonded to supports have been performed for many years; the first non-bonded affinity chromatographic method is reported here. An oil-soluble compound containing glucosamine was synthesized. When dissolved in a grease and coated on a solid support, this compound bound concanavalin A specifically. Concanavalin A could be eluted by a competitively binding sugar added to the mobile phase. The column separated concanavalin A from bovine serum albumin rapidly and efficiently. The solubility of the concanavalin A–ligand complex in the mobile phase and dissociation of the complex during washing of the column caused a slow leakage of bound material from the column, and a slow degradation of the column performance. Non-bonded affinity chromatography offers a flexible and potentially important new method of performing biospecific separations.

INTRODUCTION

Interest has arisen in the concept of immuno-electrodes in which an antigen or antibody is attached to an electrode surface and a potential is generated when the corresponding antibody or antigen from solution binds to the electrode[1–3]. In this laboratory, our approach has been to synthesize a water-insoluble affinity ligand that can be dissolved in a suitable polymeric phase and coated on an electrode. As a way of testing the binding ability of this ligand, we prepared an affinity chromatographic column from the compound dissolved in a hydrophobic grease, Apiezon L, and coated on a readily available support material, Chromosorb W. In addition to aiding the electrochemical study, this chromatographic method is useful in its own right as an alternative to the standard chemically bonded affinity chromatographic methods[4,5]. The first test of this method, which we call non-bonded affinity chromatography, is described here.

The concanavalin A–polysaccharide precipitation reaction has been used as a

* To whom inquiries should be addressed.

model of antibody–antigen reactions[6,7]. Concanavalin A (Con A) was chosen for our study because it is readily available in purified form and because small sugars such as glucose behave like haptens and form soluble complexes[6]. Glucosamine has been coupled to agarose through a six-carbon spacer[8] and used to isolate Con A[9]. Decanol was chosen to provide the hydrophobic portion of the affinity ligand in this study. It was coupled to 6-aminohexanoic acid via epichlorohydrin, and finally to glucosamine using dicyclohexylcarbodiimide.

MATERIALS AND METHODS

Synthesis

Con A (grade IV), α-methyl-D-mannoside (grade III) and bovine serum albumin (fraction V) were obtained from Sigma (St. Louis, Mo., U.S.A.). *n*-Heptane was technical grade, and all other chemicals were reagent grade.

1-Chloro-2-hydroxypropyl decyl ether (I, Fig. 1) was prepared by the method of Zielinska and Gasztych[10], as follows. Decanol (1.75 moles), 1.00 mole of epichlorohydrin and 0.0089 mole of $HClO_4$ (60%) were heated under reflux for 5 h at 90–110°. After cooling, the reaction mixture was washed with water and allowed to equilibrate with 0.01 *M* NaOH overnight; it was then washed with water until the washings were neutral, dried over Na_2SO_4 and filtered. The products were purified by distillation under reduced pressure. Excess decanol was distilled at 41–44° at a pressure of *ca.* 5 mTorr. Compound I was obtained as a colorless liquid at 94–95° in 71% yield. It was identified by infrared (IR)[10,11] and nuclear magnetic resonance (NMR) spectroscopy.

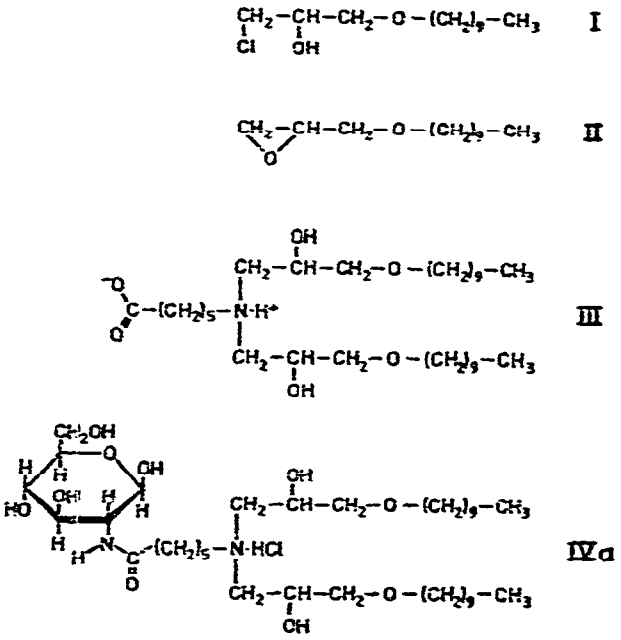

Fig. 1. Structures of the synthesized compounds. III is shown in the neutral zwitterionic form, and IV as the amine salt, IVa.

1,2-Epoxypropyl decyl ether (II, Fig. 1) was prepared by the method of Zielinska and Gasztych[10], as follows. A 0.53-mole portion of I, 52.5 g of 40% NaOH solution and 540 ml of dioxane were heated under reflux, with stirring, for 22 h. The NaCl was filtered off, the small aqueous layer was removed, and distillation was used to remove first the dioxane and then II at 54–64° at a pressure of *ca.* 5 mTorr. Compound II was obtained as a colorless liquid in 52% yield and was identified by IR[10,11] and NMR spectroscopy.

N,N-Bis-[3-decoxy-2-hydroxypropyl]-6-aminohexanoic acid (III, Fig. 1) was prepared by a method similar to that of Ulsperger[12]. A 0.079-mole portion of 6-aminohexanoic acid was dissolved in 60 ml of 5.3% NaOH solution, and while heating at 80–90° and stirring, 0.079 mole of II was added dropwise. Heating and stirring were maintained for several hours until a heavy white gel had formed. The gel was acidified with dilute HCl, and the resulting white precipitate was filtered off, air-dried, recrystallized from acetone and washed with light petroleum (b.p. 30–60°). The product was isolated as the hydrochloric acid salt of III in 42% yield.

This product was insoluble in water but somewhat soluble in ethanol–water mixtures. The equivalent weight was determined by suspending 0.1 to 0.2 g of the acidic form of III in 10 ml of absolute ethanol and 50 ml of water, and titrating the suspension potentiometrically with 0.05 *M* NaOH containing 10% of ethanol until the first sharp end-point was seen near pH 7; 250 ml of absolute ethanol were then added to decrease the pK_a of the protonated tertiary amine, and titration was continued to the weak second end-point.

The zwitterionic form was isolated by titrating the acidic form in water to the first end-point with a calculated amount of NaOH solution. The suspension was stirred overnight to reach equilibrium. Filtration and drying produced a fine white powder (m.p. 84–90°); IR and NMR spectroscopy supported the proposed structure. This form was used in the subsequent synthesis.

2-Deoxy-2-amino-D-glucose was coupled to III by means of an amide bond. The hydrochloric acid salt of this compound, IVa (Fig. 1), was synthesized by a method similar to that of Vafina and Molodtsov[13]. To 0.004 mole of D-glucosamine hydrochloride at 0° in 2.0 ml of water were added 0.004 mole of III suspended in 10 ml of pyridine, followed by 0.006 mole of N,N'-dicyclohexylcarbodiimide in 2 ml of pyridine. III was dissolved by diluting the reaction mixture to 30 ml with pyridine, and the solution was stirred for 18 h at room temperature. Then 80 ml of water were added to the cloudy yellow solution. The solution and precipitate were evaporated to dryness (rotary evaporator) below 45° with care to avoid bumping and foaming. The residue was dissolved in 90 ml of absolute ethanol on a steam bath. On cooling overnight, N,N'-dicyclohexylurea (DCU) crystallized out and was filtered off; the remaining solution was dried over Na_2SO_4 and again evaporated (rotary evaporator). The residue was dissolved in 100 ml light petroleum (b.p. 30–60°), and the remaining DCU was filtered off. The solution was allowed to evaporate nearly to dryness at room temperature. A small amount of IVa precipitated as a white gel and was filtered off after cooling. The white waxy solid was obtained in 10% yield [m.p. = 98–103°; $[\alpha]_D^{22} = 20°$ (0.025 g/ml in absolute ethanol)]. The product slowly turned yellow on exposure to air. The remaining light petroleum solution was evaporated (rotary evaporator) to produce an amber amorphous solid with similar optical activity in 73% yield. Both products were identified as IVa by IR and

NMR methods. By thin-layer chromatography on silica gel, with chloroform–ethanol–water (75:25:1.5) as mobile phase, it was shown that the amber solid was mostly IVa ($R_F = 0.2$), with a small amount of III ($R_F = 0.3$) and traces of yellow compounds (probably decomposition products of IVa or pyridine). Visualization of the chromatogram with *p*-anisidine phthalate[14] showed that IV was a reducing sugar.

Chromatography

The chromatographic flow system (Fig. 2) consisted of a Gilson Minipuls 2 peristaltic pump with 0.030-in. I.D. pump tubing; flow-rates of up to 1.25 ml/min could be obtained. A Hamilton 3-way valve was connected before the pump to allow switching between two solvents. A glass column (15 cm × 7 mm I.D.) was used. Home-made fittings allowed injection of the sample through a septum into the flow stream immediately before the column; 0.030-in. I.D. polyethylene tubing and 20-gauge syringe needles were used for connections. A home-made flow-through cell with a path of diameter 1/16 in. and length 1 cm was used for detection at 280 nm in a Beckman DB-G spectrophotometer with recorder. The system was operated at room temperature.

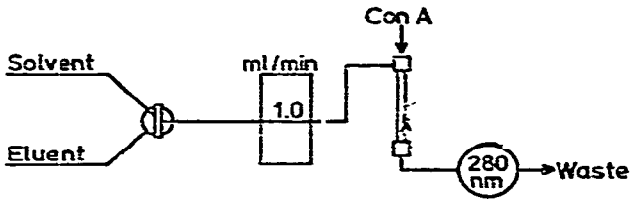

Fig. 2. The affinity chromatographic flow system. Solutions of Con A were injected by means of a syringe into the flowing stream of solvent at the top of the column. The bound Con A was later eluted by switching the 3-way valve so that the eluent passed through the column.

The adsorbent was prepared using the basic form of the affinity compound, IVb. A 0.62-g portion of a solution of IVa in absolute ethanol was stored in a freezer. An aliquot of this was treated with a twofold molar excess of 1 *M* Na_2CO_3 (pH 11), and the solution and precipitate were immediately evaporated (rotary evaporator) to dryness below 32°. Heptane was added, and the solution was dried over Na_2SO_4 and filtered. This heptane solution of IVb was used to prepare the adsorbents.

Apiezon L (typically 0.2 g) was dissolved in heptane in a 100-ml round-bottomed flask. An aliquot of the IVb solution (typically containing 0.06–0.13 g of IVb) was added, followed by 1.5 g of Chromosorb W (100–120 mesh). This mixture was evaporated to dryness below 32°, and during evaporation, the flask was removed from the evaporator several times and the walls were scraped with a spatula to ensure a uniform adsorbent. Then 1 *M* NaCl was added to the dried packing, and the mixture was degassed under a 50-Torr aspirator vacuum for several minutes. The resulting aqueous solution was twice decanted to remove any fines.

The column was packed as follows. The column was filled to one-third of its height with the 1 *M* NaCl solvent, then packing was added with a disposable pipette and allowed to settle; any fines that did not settle rapidly were removed with the

pipette, along with excess of solvent. This was procedure repeated until the column was full. An additional 6-cm length of column was attached with a piece of flexible tubing to the top of the column and more packing was added as before. When all the packing had been added, the column was allowed to settle for 2 min, with an occasional gentle tap with a finger. The upper column fitting was then attached to the extension, and solvent was pumped through at 0.1 ml/min until settling appeared to have stopped. The packing was further settled by pumping solvent at 0.25, 0.50, 0.75, 1.00 and 1.25 ml/min for 2, 3, 2, 3, and 1 min, respectively. Gentle tapping on the column helped to settle the packing and so produce narrow symmetrical peaks. However, too much tapping decreased the permeability of the column and caused asymmetrical or double peaks.

The extension and the extra packing in it were then removed, and a piece of filter paper was placed at the top of the column so that the packing would not be disturbed. The upper column fitting was attached to the column, and solvent was pumped backwards through the system to remove air bubbles from the upper fitting. The column was then ready for use.

A solution (10 mg/ml) of Con A in 1 *M* NaCl was prepared; it also contained 1 m*M* $MnCl_2$ and 1 m*M* $CaCl_2$, as these metals are required for binding[15]; 1 *M* NaCl was chosen, since Con A is soluble only in high salt concentrations[16], and 1 *M* NaCl has been used for most Con A purifications[17]. The eluting solvent was 0.02 *M* α-methyl-D-mannoside in 1 *M* NaCl[18].

The procedure for a typical experiment is as follows. Solvent was pumped through the column at 1.0 ml/min, and 49 μl of the Con A solution (0.49 mg of Con A) were injected. After 5 min, any material that did not bind to the column began to elute (the column void volume is *ca.* 5 ml), as shown in Fig. 3 for a typical run. After 15 min, the solvent flow was switched to the eluting solvent; 5 min later, the Con A was eluted as a sharp peak. When the absorbance had dropped

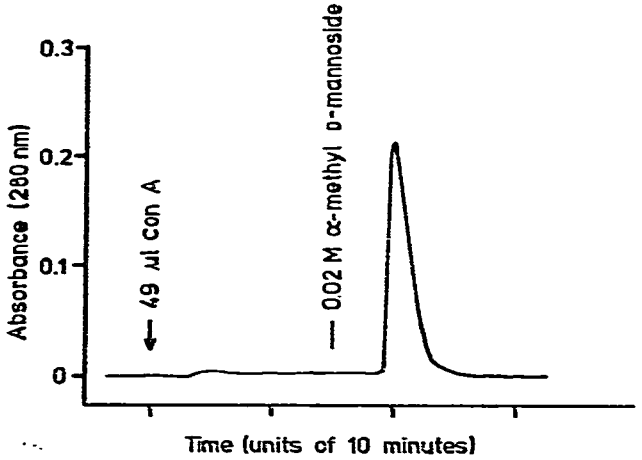

Fig. 3. A typical injection. The Con A solution is injected into 1 *M* NaCl flowing at 1.0 ml/min. Some of the Con A does not bind to the column and begins eluting 5 min after injection; the remaining Con A is eluted as a sharp peak by changing the solvent to a competitively binding sugar in 1 *M* NaCl.

nearly to the base-line value, the solvent was again switched back to 1 *M* NaCl. Another run could be made 30 to 35 min after the previous injection.

Experiments on the specificity of binding and elution, flow-rate, amount of IVb, leakage of bound Con A, pH, ionic strength and the analytical utility of this system were performed. The solutions and procedure were as described above except where otherwise stated.

Peak areas were measured with a planimeter. The percentage of Con A bound and then eluted from the column was calculated by comparison with the peak areas of totallynon-bound Con A injections; these values were obtained by injecting Con A while the eluting solvent was flowing through the column.

RESULTS AND DISCUSSION

Compounds III and IV are new to the literature. The equivalent-weight determinations of III gave values of 591 ± 11 and 555 ± 8 g-equiv. for the first and second end-points, respectively. These compare fairly well with an expected value of 595 and indicate that both amino protons of 6-aminohexanoic acid are substituted by the epoxy group of II. This is in agreement with the results of Ulsperger[12], who obtained disubstituted products in similar reactions. The acidic form of III showed a strong carboxyl stretch at 1710 cm^{-1}, while the zwitterionic and basic forms showed this band at 1580 cm^{-1}.

The structure of IVa was confirmed by the strong amide bands at 1550 cm^{-1} and 1650 cm^{-1} and by the broad hydroxyl stretch at 3300 cm^{-1}. Crystalline IVa, obtained in low yield, showed an almost identical IR spectrum, with the amide bands shifted slightly to 1565 and 1625 cm^{-1}. The non-crystalline product, although not completely pure, was used in all the chromatography experiments. It showed definite surfactant properties when mixed with water, but was soluble in most organic solvents. When converted into the basic form, IVb, it became visibly less soluble in water.

Both forms of IV tended to become yellower and more oily when exposed to air for several days, some batches eventually losing their Con A-binding ability. The petroleum ether purification step greatly decreased this problem. However, storage under nitrogen or in an ethanol solution was often used as a precaution. Columns prepared from IVb retained Con A-binding ability indefinitely when stored in 1 *M* NaCl or dry.

In performing the column chromatographic experiments, it was feared that the very hydrophobic stationary phase, Apiezon L, or the hydrophobic tails of IVb would cause severe non-specific adsorption, such as is seen in hydrophobic affinity chromatography[19], particularly since Con A has been shown to have a weak hydrophobic binding site[20]. Indeed, this effect was seen for a column containing 0.2 g of Apiezon L only. Several injections, for a total of *ca.* 0.7 mg of Con A, were totally or partially adsorbed on the column. None of this material could be eluted with the α-methyl-D-mannoside solvent. However, columns prepared from Apiezon L with IVa or IVb showed no non-specific adsorption. The first injection made into these columns showed the same peak size as later injections. We hypothesize that the hydrophobic tails of IV dissolve in the Apiezon L and cover or deactivate the adsorption sites. The hydrophilic heads of IV tend to extend into the aqueous

mobile phase. As a result, proteins in the mobile phase "see" only sugar molecules, just as they would in a conventional agarose gel column, and non-specific adsorption remains minimal.

The specificity of the binding of Con A to columns containing IVb was tested in several ways. First, it was observed that 0.1 *M* glucose or 0.02 *M* α-methyl-D-mannoside would elute the Con A as a sharp peak (Fig. 3). However, D-mannitol, which does not inhibit the precipitation of Con A with dextran[18], also does not prevent Con A from binding to the column when 0.1 *M* mannitol is present in the solvent (Fig. 4, injection on right). Similarly 0.1 *M* mannitol does not cause Con A to elute from a column to which the latter is already bound (Fig. 4, injection on left). This shows that the elution of Con A from the column is the result of a specific interaction with the eluting sugar and is not caused merely by a change in concentration of the species in the solvent.

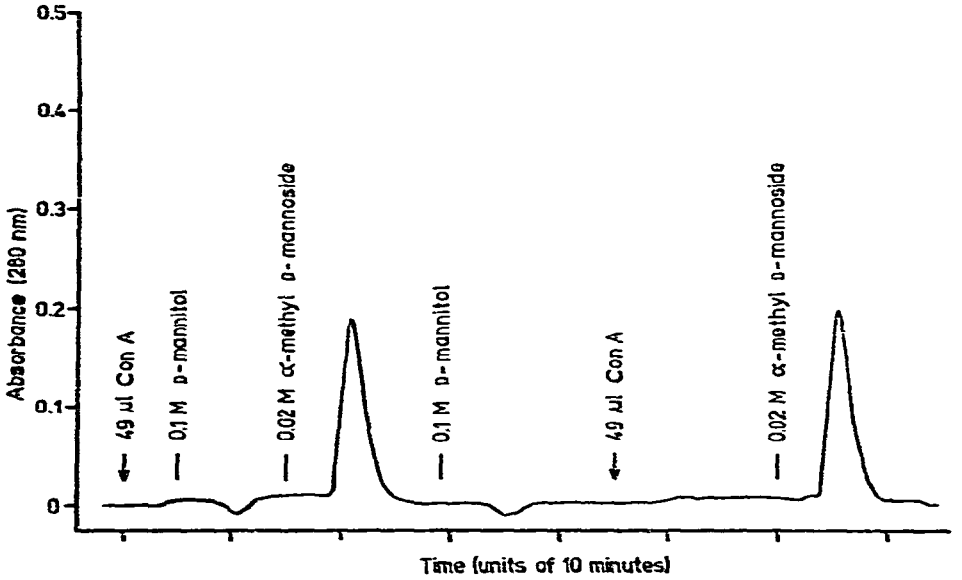

Fig. 4. Specificity of binding and elution. In the first injection, changing the solvent from 1 *M* NaCl to 1 *M* NaCl containing 0.1 *M* D-mannitol does not cause the Con A to elute. In the second injection, the presence of 0.1 *M* D-mannitol in the 1 *M* NaCl solvent does not prevent the Con A from binding to the column. The small negative peaks are caused by mixing of the two solvents of different concentration in the flow cell. The much more dilute 0.02 *M* α-methyl-D-mannoside 1 *M* NaCl solution does not cause a significant change in the baseline.

The analytical utility, as well as the specificity, of the columns was tested by separating a mixture of bovine serum albumin (BSA) and Con A. Portions (300 µl) of 1 *M* NaCl containing 2.5 mg of BSA and 0, 0.25, or 0.50 mg of Con A were injected into a column containing 0.12 g of IVb and 0.2 g of Apiezon L. Fig. 5 shows that this separation can be accomplished quite easily in 20 min, with baseline resolution. The BSA peak is eluted at the void volume of the column, with no tailing; it is slightly contaminated with Con A that has leaked from the

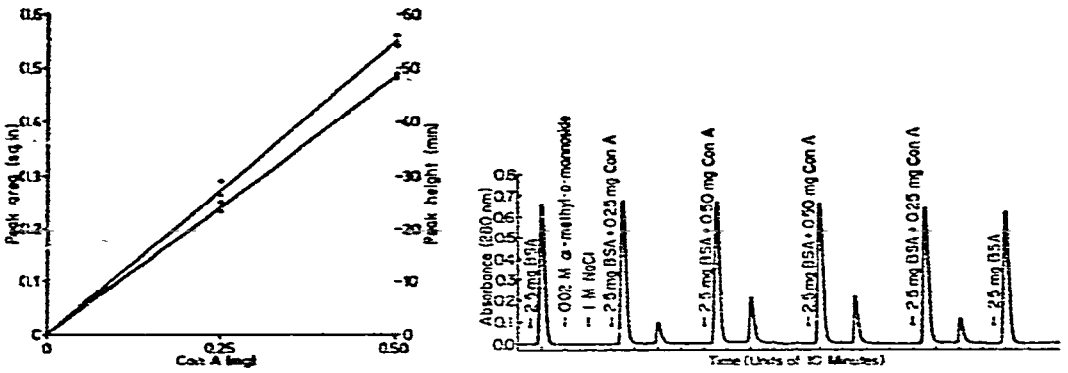

Fig. 5. Separation of BSA from Con A (right) on a column containing 0.12 g of IVb and 0.2 g of Apiezon L. The solvent changes are shown for the first injection only. The left graph shows the linearity of the Con A peak height (+) and peak area (●) calibration curves in the presence of BSA.

column early. Approximately 90% of the injected Con A remains and is eluted after 20 min with α-methyl-D-mannoside. The lack of any adsorption of BSA on the column is significant, as BSA has considerable hydrophobicity[21] and is non-specifically adsorbed on gels with hydrophobic arms[22,23]. This is further evidence that the non-specific adsorption sites of the column have been inactivated. The ease and speed of the separation show that the method is readily applicable to quantitative analysis as well as to purification. The graph in Fig. 5 shows the linear calibration curves obtained for Con A with this small data set.

The quantitative analytical ability of the system was further evaluated on a column containing 0.095 g of IVb; 25 to 200 μl of a solution (10 mg/ml) of Con A were injected, with the results shown in Fig. 6. The peak heights and areas, and the percentage of Con A bound, were evaluated. The peak area calibration curve (Fig. 7) is linear, with a correlation coefficient of 0.998 and an intercept close to zero. The peak height calibration curve (Fig. 8) has a correlation coefficient of 0.999 and an intercept of zero. The percentage of Con A bound was found to be independent of the amount of Con A injected within experimental error.

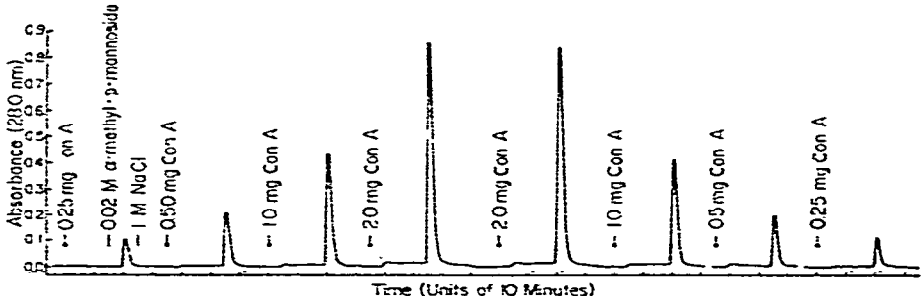

Fig. 6. Peaks obtained from injections of 25 to 200 μl of Con A solution. The column contained 0.095 g of IVb and 0.2 g of Apiezon L.

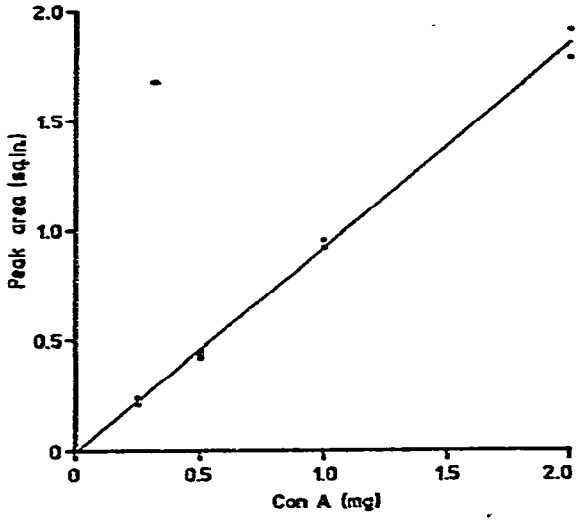

Fig. 7. The peak area calibration curve. The correlation coefficient is 0.998.

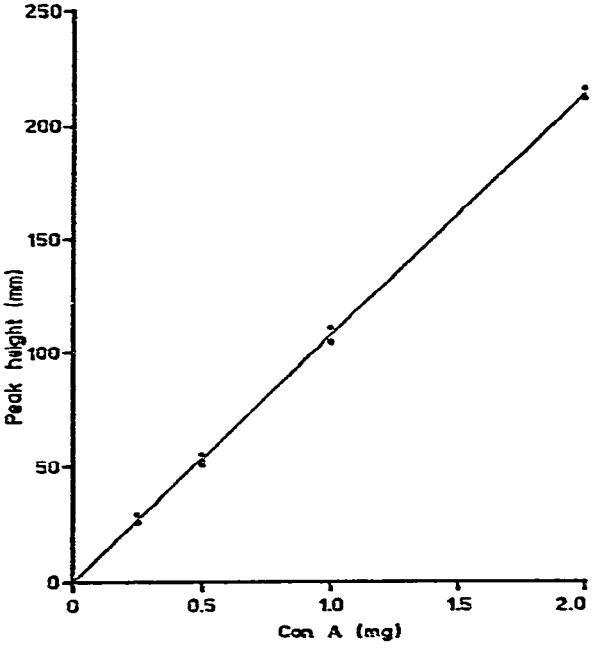

Fig. 8. The peak height calibration curve. The correlation coefficient is 0.999.

The calibration curves changed slightly from day to day as the column slowly degraded in binding ability; the peak height calibration curve also changed as the column settled slightly, producing narrower and taller peaks.

It has been mentioned that some of the Con A injected into the column leaks off. Depending on the initial amount of IVb and the length of use of the column,

typically 60–90% of the Con A remains bound and can be eluted after 20 min. There are several possible reasons for this leakage: (1) at the high flow-rates used, some of the Con A does not have time to reach binding equilibrium in the column; (2) the equilibrium constant for dissociation is too large, and a significant amount of Con A is always free in the mobile phase; and (3) the Con A–IVb complex has a significant solubility in the mobile phase, and thus the complex itself leaks off of the column. These possibilities should be kept in mind as the results of the remaining experiments are discussed.

The effect of flow-rate on Con A binding was studied over the range 0.50 to 1.25 ml/min. The retention volume was fixed at 20 ml for each flow-rate. If the rate of binding of Con A to the column were slow, one would expect decreased binding at high flow-rates. If the rate of dissociation were slow, one would expect decreased peak heights and broadened peak widths at high flow-rates. The data in Table I show that the amount of Con A bound and the peak heights are independent of flow-rate over the range studied. One can conclude from this that the Con A-binding reaction is fairly rapid and readily reversible. This conclusion is in agreement with experiments that have shown that the binding of 4-methylumbelliferyl-α-D-mannopyranoside to Con A in solution requires only 300 msec to reach equilibrium[24].

TABLE I

EFFECT OF FLOW-RATE ON BINDING OF CON A

The column packing contained 0.06 g of IVb and 0.2 g of Apiezon L. The retention volume was held constant at 20 ml.

Flow-rate (ml/min)	*Con A bound (%)*	*Peak height (mm)*
0.50	75	55
0.75	75	54
1.00	75	55
1.25	73	53

The total and relative amounts of IVb and Apiezon L should affect the binding properties of the column. These two parameters were varied, and the percentages of Con A bound to the column were calculated and are shown in Table II. The error range for each point varies from 2 to 8%, with 5% a typical value. Looking across Table II at a constant IVb value of 0.058 g, it can readily be seen that the amount

TABLE II

BINDING OF CON A TO COLUMNS CONTAINING DIFFERENT AMOUNTS OF IVb AND APIEZON L

Values are the percentages of Con A bound. The retention time was 20 min in each instance.

Column content of IVb (g)	*Column content of Apiezon L (g)*			
	0.05	*0.10*	*0.20*	*0.40*
0.017	52	—	—	—
0.033	—	74	68	—
0.058	77	80	79	76
0.095	—	—	89	—

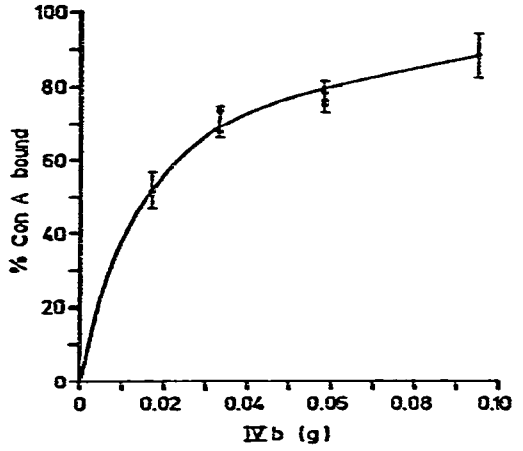

Fig. 9. Effect of amount of IVb on binding of Con A. The amount of Apiezon L varied from 0.05 to 0.40 g.

of Apiezon L is unimportant. In Fig. 9, the percentage of Con A bound is plotted against the amount of IVb without regard for the amount of Apiezon L. The dependence here is dramatic, with more IVb yielding a better column. This same effect is seen experimentally when the equilibrium dissociation constant for the complex, K_L, is too large or the concentration of affinity ligand in the column, L_0, is too small[25]. Graves and Wu[26] have derived equations for the fraction of protein specifically bound to the affinity ligands. A simplified approximate expression is given for f, the fraction bound:

$$f = \frac{L_0}{K_L + L_0} \tag{1}$$

The data in Table II give a value of approximately $4 \cdot 10^{-3}$ M for K_L. This compares with a K_L value of $1.7 \cdot 10^{-3}$ M for free glucose[27] and one of $1.1 \cdot 10^{-4}$ M for an O-α-D-glucosyl polyacrylamide gel[28] and indicates fairly weak binding. We also note that, although the ligand concentration is of the order of 10 to 20 mM, possibly a considerable fraction of this is buried in the Apiezon L or is otherwise unavailable for binding. The variable orientation of the IVb molecules may also be responsible for a wide range of K_L values in the column. The equation assumes that equilibrium is reached, whereas Con A is continually being washed from the column, so dissociation of the complex will continue indefinitely. Hence, the value of K_L may not be accurate, but dissociation of the Con A–IVb complex is certainly a major factor in the less-than-total Con A binding. This problem can be minimized by increasing the concentration of the affinity ligand in the column and by washing the column as little as possible[29].

The leakage problem was further examined by allowing the Con A to remain on the column for longer periods. Thus, 1.0-mg portions of Con A were injected and allowed to remain on the column for 6 to 200 min with 1 M NaCl flowing through the column at 1.0 ml/min. Table III shows the percentage of Con A remaining bound

TABLE III

BINDING OF CON A AS FUNCTION OF PERIOD OF WASHING COLUMN

Values are the percentages of Con A remaining bound as a function of the length of time the column was washed with 1 *M* NaCl before elution. The column contained 0.095 g of IVb and 0.2 g of Apiezon L.

*Retention time (min)**	*Column volumes***	*Con A bound (%)*
10	1.9	92
100	19.2	53
5	1.2	98
200	38.5	33
20	3.8	76
150	28.8	35
50	9.6	46

* Listed in the order run; 1.0 mg of Con A injected in each instance.

** Based on a void time of 5.2 min and a flow-rate of 1.0 ml/min.

to the column and the number of column volumes of solvent that have passed through the column at each time-point. The time-points, listed in the order run, were staggered to minimize any systematic error caused by degradation of the column; the data are plotted in Fig. 10. If the loss of Con A shown in Fig. 10 is a result of a relatively large K_L value, the data should fit an exponential decay. If a column-chromatography experiment can be approximated by a multiple-batch separation, then the concentration of Con A remaining, C, after some number of batches, n, will be a function of the original concentration, C_0:

$$C = C_0 f^n \tag{2}$$

Thus, C will decay exponentially. The data in Fig. 10 were fitted to an exponential curve as shown. The last data point, taken after 50 min, was deleted from the fit. The exponential fit is rather poor because the binding, which drops off rapidly at

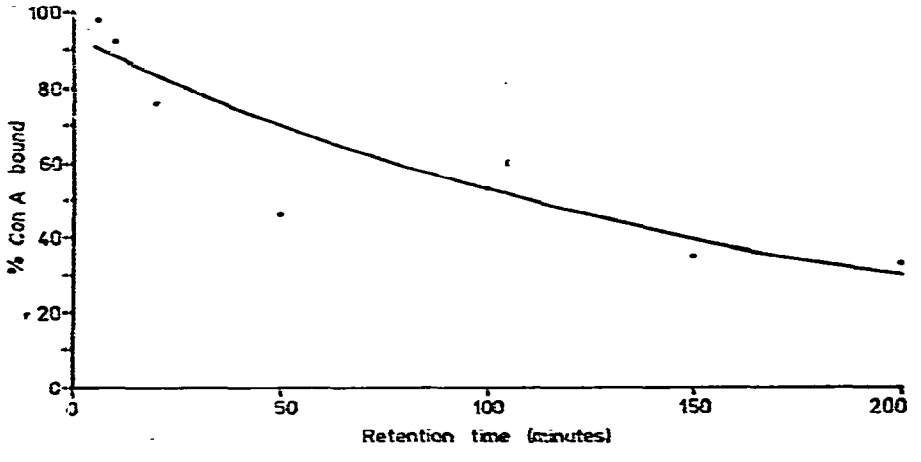

Fig. 10. Effect of Con A retention time on percentage of Con A remaining bound to the column. The data of Table III (except for the 50 min time-point) was fitted to an exponential decay as shown.

first, appears to level off around 30% at long retention times. Also, the last three data points taken all lie below the line. This leads to the conclusion that the column performance deteriorated as the experiment progressed. The column, which contained 0.095 g of IVb, originally bound 89% of the Con A injected. After 21 injections and 4 re-packings, it still bound approximately 80% of the Con A injected. Pumping 1 *M* NaCl through the column for long periods produced no observable column degradation. However, the 7 injections of this experiment decreased the binding ability to less than 60% (based on an injection with the standard 20-min retention time performed immediately after the experiment was completed).

This deterioration of the affinity column cannot be explained by an argument involving dissociation of the complex. Rather, we note that it is the presence of Con A in the column that causes the deterioration. Based on evidence to be presented later in this discussion, we believe that IVb is only marginally insoluble in 1 *M* NaCl. When Con A is bound to IVb, the solubility of the complex is large enough to ensure that the Con A–IVb complex itself is washed from the column. This source of loss of Con A would add a second term to eqn. 2 and thus modify the exponential decay. Some IVb may be more strongly attached to the stationary phase by virtue of adsorption or the extent to which the hydrophobic groups are dissolved in the Apiezon L. Thus, some IVb–Con A complexes may never become detached from the stationary phase, while others are readily lost. Since Con A is tetrameric[30], multiple binding would also cause some complexes to be more stable. As IVb is removed from the column, the fraction of Con A bound (eqn. 1) will also decrease, and the column will appear to deteriorate even more rapidly.

The properties of this affinity-chromatographic system were further elucidated by varying the pH of the mobile phase. Precipitin reactions of Con A with glycogen and dextran are observed over a pH range of 5 to 9 (see refs. 31 and 32). Ultraviolet difference studies[15] show that binding of simple carbohydrate haptens extends down to pH 2. Maximum binding in all these studies is observed for pH values of *ca.* 5 to 8. Con A binds strongly to Sephadex gels[33], and solutions of pH 2.4 will elute the Con A, while at pH 8.5 elution is incomplete[34]. Experiments in our laboratory show that, at pH 5, 7 or 9 in 1 *M* NaCl, Con A will bind to Sephadex G-75-120. At pH 3.5, binding is only partial and the Con A is eluted from the column as a broad tailing peak.

The effect of pH on the dissolved ligand system was studied on a column containing 0.058 g of IVb. Buffers (0.01 *M*) of Na_2CO_3 (pH 11.0), $NaHCO_3$ (pH 9.0), NaH_2PO_4 (pH 7.0) and sodium acetate (pH 5.0) were used. The binding of Con A, shown pictorally in Fig. 11, exhibited a sharp decrease at pH 9.0 from that with 1 *M* NaCl only. A return to 1 *M* NaCl restored most of the binding ability. Binding decreased still further at pH 7.0 and fell to zero at pH 5.0. A return to 1 *M* NaCl restored only a trace of binding. At pH 11.0, no binding was seen, but, when the column was returned to 1 *M* NaCl, considerable binding was restored. Soaking overnight in pH 11.0 buffer followed by 1 *M* NaCl restored much of the original binding ability.

These results follow from the acid–base nature of the affinity ligand. The basic form was used in all the columns so far discussed. When the acidic form, IVa, was used in a column, no Con A was bound. Some retardation of the peak was seen, with the Con A tailing off slowly, as shown in Fig. 12. When this column was

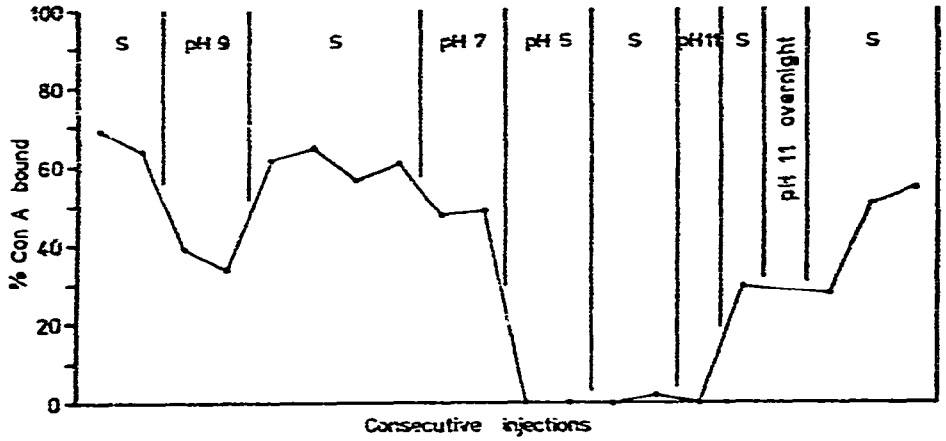

Fig. 11. The effect of pH on binding of Con A. All buffers contained 1 *M* NaCl; the eluent also contained both the buffer and 1 *M* NaCl. S = 1 *M* NaCl with no buffer. The column contained 0.058 g of IVb and 0.2 g of Apiezon L.

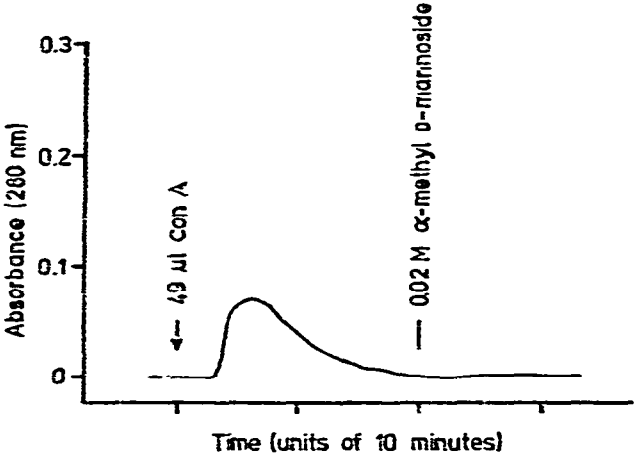

Fig. 12. Effect of using IVa as affinity agent. The column contained 0.05 g of IVa and 0.2 g of Apiezon L.

soaked overnight in 0.1 *M* Na_2CO_3 of pH 11.0 in 1 *M* NaCl and then run in 1 *M* NaCl, it too showed Con A-binding ability of *ca.* 60%.

This information gives a clearer picture of the effect of pH changes. At pH 11, the solution is too basic for Con A to be bound. At pH 9, binding is still somewhat impaired. At pH 7, and more so at pH 5, IVb is converted into the acidic form, with a loss of binding ability. By deprotonating IVa in a buffer of high pH, some of the binding ability is restored. The inability of IVa to retain Con A on a column has two possible explanations: (1) the positive charge increases K_L and thus interferes with the binding of the ligand to Con A; or (2) the charge increases the solubility of

the affinity ligand, and of the complex, in water to the point at which the complex itself is lost from the column.

The charge on IVa is separated from the glucosamine part of the ligand by the six-carbon spacer. An agarose column with glucosamine bound to it through a six-carbon spacer is able to bind Con A[9], so it is likely that, if steric hindrance at the end of the spacer is not a problem, then a charge in that position will also not affect K_L. We do note, however, that it may not take a very large change in K_L to decrease considerably the binding in a column chromatography system[26] and thus we cannot totally rule out this possibility.

An increase in the solubility of the Con A–IVa complex over that of the Con A–IVb complex could also explain the observed results. The earlier discussion noted that the marginal solubility of the Con A–IVb complex would cause the long-term deterioration observed. The even higher solubility of the Con A–IVa complex should cause even greater deterioration in column performance. Since no Con A binding is observed, each injection of Con A would remove a corresponding amount of IVa from the column. The solubility of IVa itself is probably sufficiently low to ensure that most of it remains on the column except when bound to Con A. Hence, a column containing IVa can be made active by a change in pH, even though it has already been thoroughly washed with solvent.

The ionic strength should have a major effect on the solubilities. Con A is more soluble at high ionic strength[16], while one would expect IVb to be salted out of solution at high ionic strength. A column containing 0.12 g of IVb was re-packed and run in 1.0 *M*, 0.5 *M* and 0.1 *M* NaCl (the eluting solvent also contained these salt concentrations). Con A was injected in 1 *M* NaCl in all instances. The results of this experiment are shown in Fig. 13. Decreasing the ionic strength to 0.5 *M* decreased the binding by about 50%. At 0.1 *M*, little binding was observed. When the column was returned to 1 *M* NaCl, only about half of the initial binding ability returned, indicating that some of the IVb had been stripped from the column. A blank column containing only Apiezon L showed no apparent loss of stationary

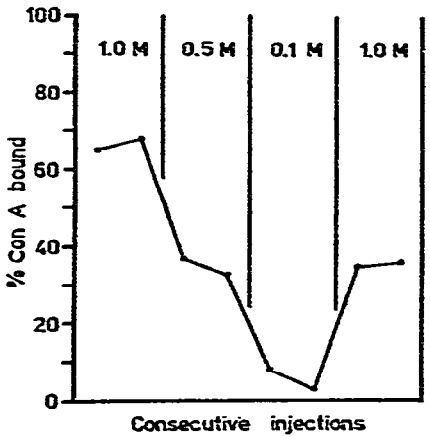

Fig. 13. Effect of ionic strength on binding of Con A. The solvent and eluent contained the indicated concentrations of NaCl. The column was re-packed in each new solvent and contained 0.12 g of IVb and 0.2 g of Apiezon L.

phase when run in 0.1 *M* NaCl, so that we conclude that loss of IVb was not caused by a concomitant loss of Apiezon L, but rather by its own intrinsic solubility.

These experiments have indicated many of the properties of this non-bonded affinity chromatographic system, which are summarized below. The affinity ligand IVb was held in a hydrophobic stationary phase by means of long hydrocarbon tails, the biospecific hydrophilic head projecting into the aqueous solvent. Under conditions of high salt concentration and the absence of buffer, this column was quite stable. The many affinity ligands formed a layer of sugar molecules on the surface of the stationary phase, which rapidly, specifically and reversibly bound Con A, but showed little tendency to adsorb other proteins non-specifically. Some Con A slowly leaked from the column as a result of dissociation of the complex and the solubility of the complex in the mobile phase; the leakage could be minimized by increasing the concentration of IVb. The solubility problem caused long-term deterioration in column performance. Decreasing the ionic strength or buffering the mobile phase to medium or low pH values decreased retention of Con A, either because of changes in the dissociation constant or a change in the solubility of the complex.

CONCLUSIONS

The work presented here has demonstrated a new way of performing affinity-chromatographic separations without using ligands chemically bonded to the stationary phase. The non-bonded coated stationary phase is specific and provides a separation that can be repeated at intervals of 35 min or less. Non-specific adsorption is very low, and the quantitative analytical ability is excellent. We believe that future applications are possible in the areas of enzyme or antibody analysis, with immobilized inhibitors and haptens, respectively. The major problem of the method is the solubility of the ligand–analyte complex in the mobile phase. The ligand synthesis and the chromatographic conditions must be carefully planned and controlled to produce an insoluble complex.

This sytem has several potential advantages over the conventional method. The affinity ligand can be purified from troublesome impurities before use, since it is not attached to a polymeric gel. For example, ionic side products of the synthesis could be removed by ion exchange and thus eliminated as a site for non-specific adsorption. A mixture of affinity ligands can easily be prepared in any concentration simply by mixing the desired compounds together. It may be possible to elute difficult-to-dissociate complexes from the column simply by changing the ionic strength, thereby increasing the solubility of the complex in the mobile phse.

Other improvements may be achieved by using standard liquid-chromatography packing materials to produce narrower peaks. It may even be possible tightly to adsorb ligands like IVb on an octadecyl bonded stationary phase, thus eliminating the need for an Apiezon L coating. Ion-pairing could prove useful for retaining charged ligands on the stationary phase, particularly if the charge is not necessary for binding to the complementary protein.

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