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High-performance affinity chromatography of a chick lectin on an adsorbent based on hydrophilic polymer gel

HIROTAKA KAKITA*, KOJI NAKAMURA and YOSHIO KATO

Central Research Laboratory, Tosoh Corporation, Tonda, Shin-nanyo, Yamaguchi (Japan)

and

YUKO ODA, KIYOHITO SHIMURA and KEN-ICHI KASAI

Faculty of Pharmaceutical Sciences, Teikyo University, Sagamiko, Kanagawa (Japan)

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ABSTRACT

A mouse monoclonal antibody (SIA4-5) which reacts with a chick 14K lectin (C14K) was covalently attached to a new support for high-performance affinity chromatography, TSKgel Tresyl-5PW, which is a preactivated, polymer-based particle. The immobilized antibody (SIA4-5–5PW) thus prepared proved to be useful in measuring not only the molecular properties of C14K but also specific interactions of C14K with SIA4-5 and hapten sugars. The C14K preparation was fractionated according to the oligomeric structure and with slight differences in affinity to SIA4-5 although the former was homogeneous in sodium dodecyl sulphate polyacrylamide gel electrophoresis. Application of the method for quantitative analytical purposes was successful.

INTRODUCTION

The development of suitable supports for affinity chromatography has always been required in order to extend the field of application of the method. Properties required for suitable supports are as follows: hard, small, spherical, homogeneous in size, highly porous with large pores, hydrophilic and devoid of non-specific adsorption. A new preactivated support for high-performance affinity chromatography has become commercially available under the trade-name TSKgel Tresyl-5PW. According to the manufacturer (Tosoh, Yamaguchi, Japan), it is prepared by introducing tresyl groups into TSKgel G5000PW [1], which is hydrophilic resin-based material of large pore size (particle diameter 10 μm, approximate pore size 1000 Å), intended for high-performance gel filtration. The amount of tresyl groups introduced is about 20 μmol per ml of swollen gel. Protein can be easily coupled via its ε-amino or thiol groups [2]. Affinity adsorbents prepared from this support should be useful not only for preparative but also for other purposes such as analysis and diagnosis. We intend to use this new material for investigations on specific interactions of biomolecules.

In the course of studies on vertebrate β-galactoside-binding lectins, we cloned anti-chick 14K lectin mouse monoclonal antibody (SIA4-5). Chick 14K lectin (C14K)

has a monomer molecular weight of 14 970 [3] and an isoelectric point of 7.0 [4]. Both monomeric and dimeric forms were usually found in purified preparation. To investigate the interaction between C14K and SIA4-5, we attached SIA4-5 to TSKgel Tresyl-5PW and studied the chromatographic behaviour of C14K. Useful information such as oligomeric structure and dissociation constants between C14K and its soluble hapten sugars were obtained.

EXPERIMENTAL

The columns and proteins investigated are summarized in Table I.

Materials

Human γ-globulin (HG) and mouse γ-globulin (MG) were purchased from Miles Diagnostics Division (Elkhart, IN, U.S.A.), concanavalin A (carbohydrate-free) from Seikagaku Kogyo (Tokyo, Japan), thiodigalactoside, N-acetyl-D-lactosamine, proteins for molecular weight markers, from Sigma (St. Louis, MO, U.S.A.) and Bradford protein assay dye reagent was purchased from Bio-Rad Labs. (Richmond, CA, U.S.A.). Other reagents and solvents were of analytical-reagent grade. TSKgel Tresyl-5PW (Tosoh) with particle size 10 μm and average pore size 1000 Å was used as a support for affinity adsorbent. C14K and SIA4-5 were purified as described [4,5].

Chromatography

Chromatography was performed at ambient temperature (20–25°C) with an HLC-803D pump equipped with a variable-wavelength Model UV-8000 UV detector and a Model FS-8000 variable-wavelength fluorescence detector (all from Tosoh). Fluorescence of proteins was detected with excitation at 285 nm and emission at 340 nm. The recovery of proteins was determined from the areas of the eluted peaks [6]. As controls, we used areas obtained when the column was replaced with empty 1 mm I.D. stainless-steel tubing of 1 ml total inner volume and elution was performed isocratically at the eluent compositions at which each protein was eluted. Each protein was injected. Peak areas were measured with a Model CP-8000 data processor (Tosoh).

TABLE I

AFFINITY CHROMATOGRAPHIC COLUMNS

Column	Immobilized protein	Column size	Sample	Fig. No.
C14K–5PW	C14K	50 mm × 4.6 mm I.D.	SIA4-5	1A
C14K–5PW	C14K	50 mm × 4.6 mm I.D.	MG	1C
Non-protein–5PW	–	75 mm × 4.6 mm I.D.	SIA4-5	1B
Non-protein–5PW	–	75 mm × 4.6 mm I.D.	C14K	1F
SIA4-5–5PW	SIA4-5	75 mm × 4.6 mm I.D.	C14K	1D, 2A, 2B, 2C, 3B, 3C, 5A, 5B, 6A, 6B
HG–5PW	HG	75 mm × 4.6 mm I.D.	C14K	1E

Coupling of proteins to TSKgel Tresyl-5PW

Protein solution was applied to a Bio-Gel P-6 column that had been equili-brated with buffer [1 *M* sodium potassium phosphate buffer (pH 7.5) or 0.2 *M* NaH-CO₃ containing 0.5 *M* NaCl (pH 8.0)] for buffer change. The protein concentration of SIA4-5 was calculated from the absorbance at 280 nm assuming a molar absorptivity (1% w/v, 1 cm) of 14 [5]. The protein concentration of C14K was determined by the protein assay method of Bradfood [7]. The protein-containing fraction obtained (4 ml) was mixed with 0.5 g (dry weight) of TSKgel Tresyl-5PW and shaken at 4°C overnight. After washing with the same buffer, unreacted tresyl groups were deactiv-ated by resuspending the gel in 12 ml of 0.5 *M* Tris–HCl buffer (pH 8.0) at 4°C overnight. As a control, 4 ml of the buffer without protein were mixed with 0.5 g (dry weight) of TSKgel Tresyl-5PW and the same treatments were carried out. The cou-pling yield of protein was determined by amino acid analysis of pure and immobilized protein. After immobilization of protein, a portion of the gel and pure protein solu-tion were treated with 6 *M* HCl for 20 h at 110°C. Hydrolysis was performed *in vacuo*. This treatment did not destroy the gel. The amino acid composition of supernatant was determined with a Hitachi Model 835 automatic amino acid analyser. As alanine is stable, its value is not easily influenced by contamination and it is a major compo-nent of the proteins investigated in this work, its value was used for determining the coupling yield. The amount of coupled protein was calculated from the protein con-centration of pure solution and the coupling yield. The adsorbent thus prepared was usually stored in 0.05% sodium azide solution at 4°C. It could be used repeatedly for at least 1 year without an appreciable decrease in efficiency.

Miscellaneous methods

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by the method of Laemmli [8] using 3% stacking gel and 12.6% separat-ing gel. Haemagglutination activity was measured according to Nowak *et al.* [9]. In a microtitre plate (Cooke Engineering), 25 μl of serially diluted sample, 25 μl of buffer [20 m*M* sodium phosphate buffer (pH 7.2) containing 150 m*M* NaCl, 2 m*M* EDTA and 4 m*M* 2-mercaptoethanol], 25 μl of 1% bovine serum albumin and 25 μl of a 4% suspension of trypsinized glutaraldehyde-fixed rabbit erythrocytes were mixed. One unit of hemagglutination activity was defined as the reciprocal of the highest dilution of the sample which gives detectable agglutination. Concanavalin A was used as a positive control. Protein concentration was determined by using a dye reagent ac-cording to Bradford [7]. Bovine serum albumin was used as a standard. The molec-ular weight of C14K under non-denaturing conditions was determined by high-per-formance gel filtration chromatography on TSKgel G2000SWxl. Sodium phosphate buffer (0.1 *M*, pH 6.8) containing 0.1 *M* Na₂SO₄ was used.

Analysis of the interaction between C14K and immobilized SIA4-5

Interaction between C14K and immobilized SIA4-5 was analysed quantitative-ly according to the theory developed for frontal affinity chromatography [10,11] and that for zonal chromatography [12]. In frontal affinity chromatography, we use a relatively weak affinity adsorbent so that only retardation instead of adsorption oc-curs. The dissociation constant (K_d) of the complex formed between a soluble protein

(A; in this case, C14K) and its immobilized ligand (B; SIA4-5) can be described as follows:

$$K_d = [A][B]/[AB]$$

$$= \frac{B_t}{V - V_0} - [A]_0 \tag{1}$$

where B_t is total amount of the immobilized ligand, V the elution volume of A, V_0 the elution volume of A under the conditions where specific interaction is completely suppressed and $[A]_0$ the initial concentration of A, which is maintained in the column. Eqn. 1 can be rearranged to

$$V = V_0 + \frac{B_t}{[A]_0 + K_d} \tag{2}$$

If $[A]_0$ is negligibly small in comparison with K_d, V approaches the maximum value V_m, hence

$$V_m = V_0 + (B_t/K_d) \tag{3}$$

Eqn. 3 can be rearranged as follows:

$$K_d = \frac{B_t}{V_m - V_0} \tag{4}$$

This means that we can determine K_d from the elution volume provided that we apply a very dilute solution of A. Eqn. 3 is equivalent to the basic equation derived for partition chromatography (not for frontal chromatography but for zonal chromatography), although in the latter the volume of stationary phase and partition coefficient are used instead of B_t and K_d, respectively. This indicates that when we apply a very small amount of protein to the column, adsorption chromatographic techniques such as affinity chromatography can be treated by the same theory as developed for partition chromatography, because the amount of the occupied immobilized ligand becomes negligible and consequently the ratio of free and bound protein ($[A]/[AB]$) becomes constant. This consideration also indicates that eqn. 3 is applicable not only to frontal chromatography but also to zonal chromatography under these conditions, because frontal chromatography can be considered as a repetition of independent zonal chromatography. By expanding eqn. 3, the interactions between C14K (A) and other soluble molecules (I) that affect the binding of C14K to SIA4-5–5PW can be analysed. When chromatography is carried out in the presence of a competitive ligand I, A is eluted at an elution volume V_i which is smaller than V_m. If the concentration of I ($[I]_0$) is extremely high in comparison with $[A]_0$, we obtain the following equation [10,11]:

$$\frac{1}{1 + \frac{[I]_0}{K_i}} = \frac{V_i - V_0}{V_m - V_0} \tag{5}$$

where K_i is the dissociation constant of the AI complex. Eqn. 5 can be rearranged as follows:

$$V_i = V_0 + \frac{K_i(V_m - V_i)}{[I]_0} \tag{6}$$

When V_i is plotted against $(V_m - V_i)/[I]_0$, a straight line is obtained and K_i and V_0 can be determined from the slope and the intercept, respectively. Dunn and Chaiken [12] also proposed another analytical procedure for affinity chromatography, which was developed to analyse the effect of soluble molecules (I) which inhibit the interaction of the immobilized ligand (B) and its counterpart (A). It was originally developed for zonal chromatography and the K_i value can be obtained from the ratio of intercept and slope of the $1/(V_i - V_0)$ versus $[I]_0$ plot.

RESULTS

Immobilization of SIA4-5 and C14K on TSKgel Tresyl-5PW

SIA4-5–5PW and C14K–5PW were prepared as described under Experimental. The protein concentration of SIA4-5 solution was calculated as 0.9 mg/ml from the absorbance at 280 nm. The coupling yield was determined by amino acid analysis of pure and immobilized SIA4-5, using the value of alanine. A 4-ml volume of pure SIA4-5 solution contained 153.9 μg of alanine and 0.5 g (dry weight) of SIA4-5–5PW contained 144.7 μg of alanine; therefore, the coupling yield was determined as 94%.
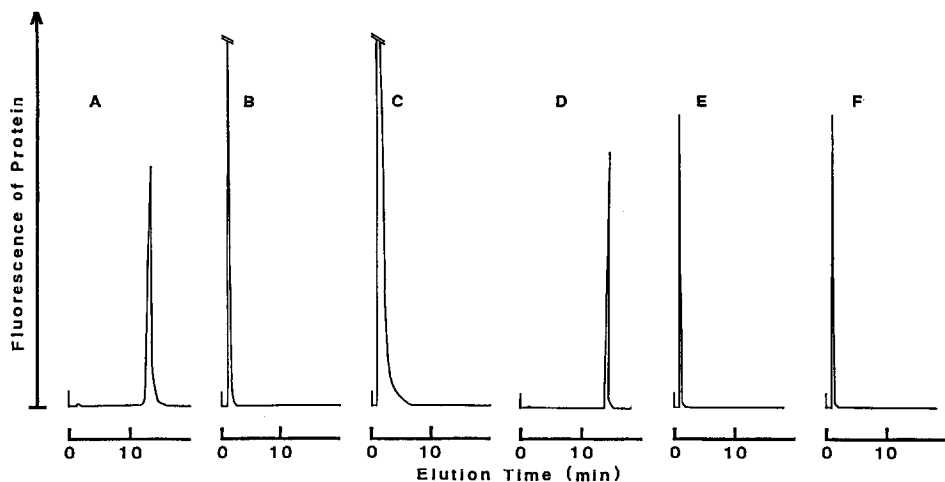

Fig. 1. Properties of several derivatized Tresyl-5PW. Columns, (A, C) C14K–5PW (50 mm × 4.6 mm I.D.), (B, F) non-protein–5PW (75 mm × 4.6 mm I.D.), (D) SIA4-5–5PW (75 mm × 4.6 mm I.D.) and (E) HG–5PW (75 mm × 4.6 mm I.D.) (containing 1.9 mg of HG). Applied samples, (A, B) SIA4-5 (100 μl, 100 μg/ml), (C) commercial MG (100 μl, 2.2 mg/ml) and (D–F) C14K (100 μl, 18 μg/ml). Flow-rate, 1 ml/min. Two elution programmes were used: for A–C, 0–10 min, 0.1 M sodium phosphate buffer (pH 6.8) containing 0.1 M Na$_2$SO$_4$ and 10–20 min, 0.1 M sodium citrate buffer (pH 5.0) containing 0.1 M Na$_2$SO$_4$; for D–F, 0–10 min, 0.1 M sodium phosphate buffer (pH 7.4) and 10–20 min, 0.1 M sodium citrate buffer (pH 5.0). Non-protein–5PW refers to derivatized Tresyl-5PW which coupled Tris base instead of protein.

The coupling yield of C14K was determined as 91% by the same method. Each gel was packed in a stainless-steel column. The column of SIA4-5–5PW (75 mm × 4.6 mm I.D.) contained 2.1 mg of SIA4-5 and that of C14K–5PW (50 mm × 4.6 mm I.D.) contained 70 μg of C14K.

SIA4-5 was adsorbed on a C14K–5PW column and eluted with sodium citrate buffer (0.1 M, pH 5.0) containing 0.1 M Na$_2$SO$_4$ (Fig. 1A). The recovery of SIA4-5 was 96%. Commercial MG was not adsorbed on the C14K–5PW column (Fig. 1C). C14K was strongly adsorbed on SIA4-5–5PW column at pH 7.4 (0.1 M sodium phosphate buffer) and eluted with 0.1 M sodium citrate buffer (pH 5.0). The recovery of C14K was more than 90% (Fig. 1D). On the other hand, C14K was not adsorbed on an HG–5PW column (Fig. 1E). No protein was adsorbed on non-protein–5PW, which coupled Tris base instead of protein (Fig. 1B and F).

Separation of C14K into several components with the SIA4-5–5PW column

Under certain conditions, C14K was separated into several peaks by SIA4-5–5PW chromatography, although the preparation used had given only one band (14K) on SDS-PAGE. Fig. 2A shows that four peaks (1–4) appeared when a descending pH gradient was applied. Apparently peak 1 has the weakest affinity to immobilized SIA4-5 and peak 4 the highest. A more detailed study showed that they can be classified into two groups, weaker (peaks 1 and 2) and stronger (peaks 3 and 4). Under the initial conditions used (pH 6.0), peaks 1 and 2 were not adsorbed on the column, whereas peaks 3 and 4 were adsorbed and eluted with 0.1 M sodium phosphate buffer (pH 5.0).

To characterize each peak, the C14K preparation was size-fractionated by gel filtration on TSKgel G2000SWxl and the resulting protein fractions were applied to
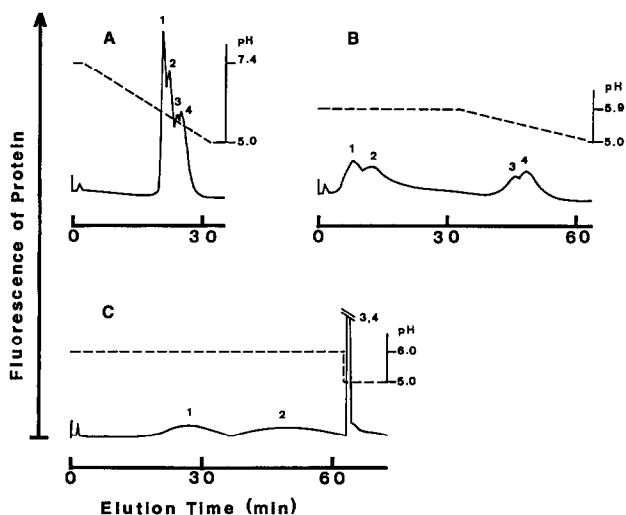

Fig. 2. Chromatograms of C14K (100 μl, 18 μg/ml) on SIA4-5–5PW column with various elution programmes. C14K protein, 1.8 μg in 100 μl, was applied. Elution programmes: (A) linear gradient from 0.1 M sodium phosphate buffer (pH 7.4) to 0.1 M sodium citrate buffer (pH 5.0) for 30 min; (B) isocratic elution with 0.1 M sodium citrate buffer (pH 5.9) for 30 min, then linear gradient from pH 5.9 to 5.0 in 0.1 M sodium citrate buffer for 30 min; (C) isocratic elution with 0.1 M sodium phosphate buffer (pH 6.0).
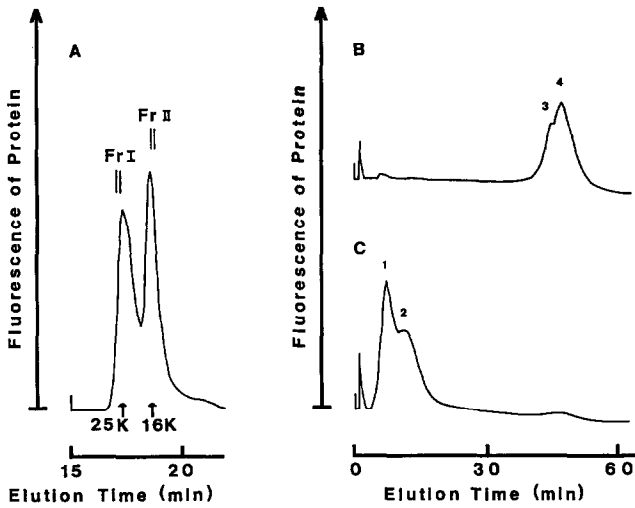
Fig. 3. (A) Gel permeation chromatography of C14K preparation on TSKgel G2000SWxl (30 cm × 7.8 mm I.D.). Mobile phase, 0.1 M sodium phosphate buffer (pH 6.0). Eluate between the two vertical lines of each peak was collected. Flow-rate, 0.5 ml/min. (B) and (C) chromatograms on SIA4-5-5PW of fractions I and II obtained in (A). The elution programme was the same as in Fig. 2B. Flow-rate, 1 ml/min. K = Kilodalton.
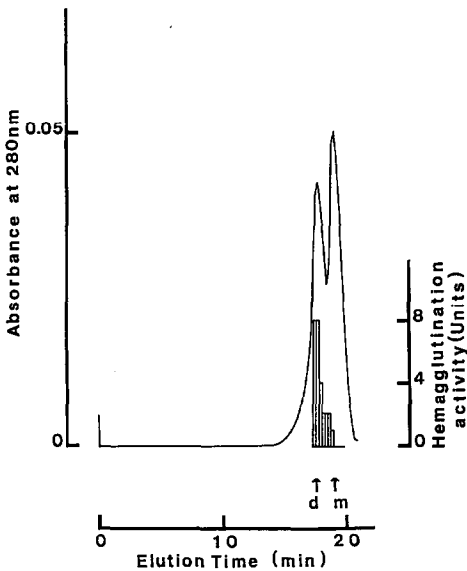

Fig. 4. Gel permeation chromatography of C14K on TSKgel G2000SWxl (30 cm × 7.8 mm I.D.). Mobile phase, 0.1 M sodium phosphate buffer (pH 6.8) containing 0.1 M Na$_2$SO$_4$. Flow-rate, 0.5 ml/min. Portions of 100 μl of column eluent were collected and haemagglutination activity was measured (indicated by histogram). Letters d and m below the chromatogram indicate C14K dimer and monomer, respectively. Elution of protein was detected by absorbance at 280 nm.

an SIA4-5-5PW column. Fig. 3A shows that C14K was separated into two peaks by gel filtration. The molecular weight of the first peak (fraction I) was calculated to be about 25 000 and that of the second peak (fraction II) about 16 000. Fraction I corresponds to C14K dimer and fraction II to C14K monomer. This was further supported because, as shown in Fig. 4, the haemagglutination activity of fraction I was much higher than that of fraction II. Fraction I gave only peaks 3 and 4, and fraction II only peaks 1 and 2 on SIA4-5-5PW chromatography under the same conditions as in Fig. 2B. Thus, peaks 1 and 2 in Fig. 2 correspond to C14K monomer. On the other hand, peaks 3 and 4 correspond to C14K dimer. It is reasonable that the dimer species has a higher affinity than the monomer species. However, the reason why each of them was further separated into two peaks has not been clarified.

Comparison of old and fresh preparations of C14K

As the behavior of C14K described above was attributable to some modification of C14K protein that occurred after purification, a C14K preparation which had been stored for 50 days at 4°C after preparation (old preparation) was applied to the column. This preparation also gave only a 14 000-dalton band on SDS-PAGE. The specific activity of the old preparation was at least eight times lower than that of freshly prepared C14K. Elution conditions that allowed only discrimination between the dimer and monomer were chosen in the experiments shown in Fig. 5. Apparently,
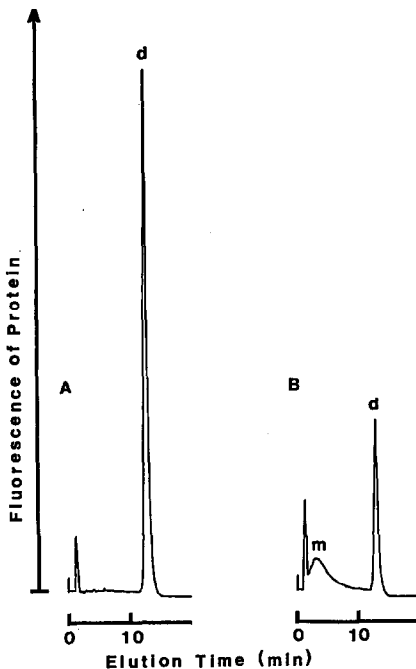

Fig. 5. Chromatograms of (A) fresh and (B) old C14K preparation on SIA4-5-5PW. C14K protein, 3 μg in 30 μl, was applied. Elution programmes were as follows; 0–10 min, 75% buffer A [0.1 *M* sodium phosphate buffer (pH 6.8) containing 0.1 *M* Na₂SO₄, 6.7 m*M* lactose] + 25% buffer B [0.1 *M* sodium citrate buffer (pH 5.0) containing 0.1 *M* Na₂SO₄]; 10–20 min, 100% buffer B. Flow-rate, 1 ml/min. Letters d and m indicate C14K dimer and monomer, respectively.

the fresh C14K preparation contained only dimer (peak d, retention time 12.5 min). On the other hand, the old preparation contained both monomer (peak m, retention time 3 min) and dimer (peak d). This suggests that C14K had partially lost its ability to form dimer during storage.

Quantitative analysis of the interaction between C14K and SIA4-5

The amount of effective immobilized ligands of the SIA4-5–5PW column (B_t) was determined as $5.5 \cdot 10^{-9}$ mol by applying an excess amount of C14K. V_0 was 1.0 ml. Under the conditions as described in Fig. 2C, when C14K monomer ($3.9 \cdot 10^{-11}$ mol, 0.59 μg) was applied to the SIA4-5–5PW column, the retention volume of peak 1 was 28 ml and that of peak 2 was 48 ml. When a smaller amount of C14K ($1.1 \cdot 10^{-12}$ mol) was applied, the same elution volume was observed. As the elution volume was unaffected by the change in the amount of protein applied, these elution volumes can be considered as V_m. Thus, K_d values were calculated to be $2.0 \cdot 10^{-7} M$ for peak 1 and $1.1 \cdot 10^{-7} M$ for peak 2 by using eqn. 4. K_d values for peaks 3 and 4 were not determined because they were adsorbed on the column.

Effect of hapten sugars

It was found that addition of hapten sugars affected the chromatographic pattern. Chromatography of C14K was carried out under conditions where peaks 1 and 2 were not adsorbed but retarded. In the presence of lactose, the elution volumes of both peaks 1 and 2 were diminished whereas sucrose and maltose had no effect (Fig.
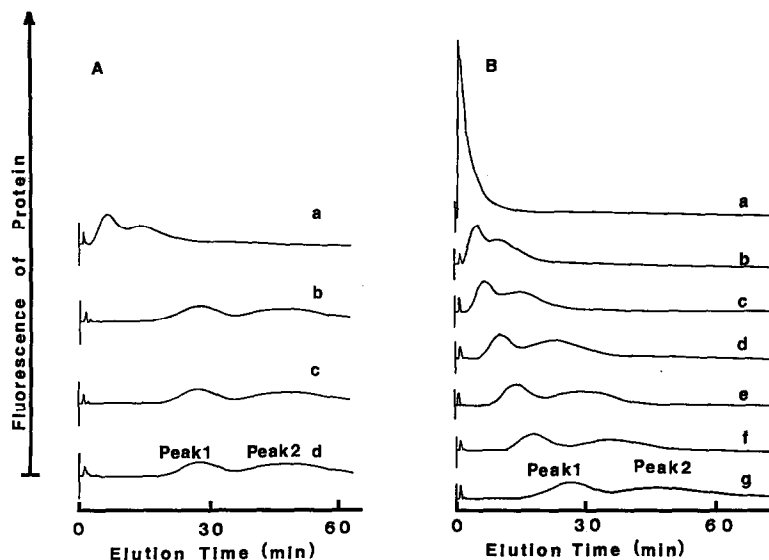

Fig. 6. (A) Effect of sugars on the chromatograms of C14K on SIA4-5–5PW. C14K protein, 0.6 μg in 100 μl was applied. Mobile phase, 0.1 M sodium phosphate buffer (pH 6.0) containing disaccharides: (a) 2 mM lactose; (b) 10 mM maltose; (c) 10 mM sucrose; (d) none. Flow-rate, 1 ml/min. (B) Effect of lactose of various concentration on chromatograms of C14K on SIA4-5–5PW. C14K protein, 0.6 μg in 100 μl, was applied. 0.1 M Sodium phosphate buffer (pH 6.0) was used. Concentration of lactose: (a) 20; (b) 3; (c) 2; (d) 1; (e) 0.6; (f) 0.3; (g) 0 mM. Flow-rate, 1 ml/min.
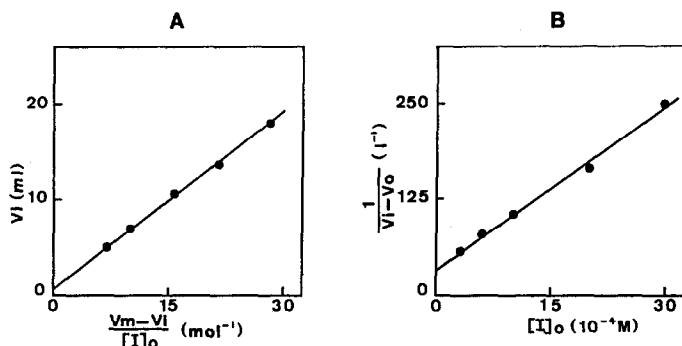
Fig. 7. Analysis of the effect of lactose. SIA4-5-5PW column was equilibrated with sodium phosphate buffer (pH 6.0) containing various concentrations of lactose. C14K protein, 0.6 μg in 100 μl was applied, then the elution volumes of C14K peak 1 were determined. Flow-rate, 1 ml/min. (A) V_i versus $(V_m - V_i)/[I]_0$ plot; (B) $1/(V_i - V_0)$ versus $[I]_0$ plot.

6A). Peaks 3 and 4 were not examined because under the conditions used they were adsorbed on the column. Other sugars containing a β-galactoside moiety, thiodigalactoside and N-acetyl-D-lactosamine, showed a similar effect. As the SIA4-5 used is sensitive to conformational changes of C14K protein, a conformational change of C14K induced by the binding with specific sugars seemed to diminish the interaction with SIA4-5. Chromatograms obtained in the presence of various concentrations of lactose (20–0.3 mM) are shown in Fig. 6B. A plot of V_i versus $(V_m - V_i)/[I]_0$, where $[I]_0$ is the concentration of lactose, according to eqn. 6 is shown in Fig. 7A. The retention volume of C14K peaks in the absence of lactose was used as V_m. Straight lines were obtained for both peaks 1 and 2 [correlation coefficients (r) were 0.999 and 0.997, respectively]. K_i values calculated for lactose were 0.6 mM (peak 1) and 0.88 mM (peak 2). K_i values for thiodigalactoside and N-acetyl-D-lactosamine were also determined and are summarized in Table II. K_i values were also calculated using the equations developed by Dunn and Chaiken [12], and similar values were obtained. Thiodigalactoside bound most strongly and lactose most weakly. These values are

TABLE II

K_i VALUES OF LECTIN PEAKS FOR β-GALACTOSIDE-CONTAINING SACCHARIDES BY QUANTITATIVE AFFINITY CHROMATOGRAPHY

Saccharide	K_i (mM)[a]			
	Peak 1		Peak 2	
	a	b	a	b
Lactose	0.60	0.51	0.88	0.76
Thiodigalactoside	0.063	0.060	0.12	0.12
N-Acetyl-D-lactosamine	0.14	0.15	0.25	0.25

[a] a, K_i values were calculated by plots according to eqn. 6; b, K_i values were calculated by plots according to Dunn and Chaiken [12].

consistent with previous reports [13]. Peak 1 always had a slightly stronger affinity than peak 2 for sugars.

DISCUSSION

The advantages of TSKgel Tresyl-5PW for the preparation of affinity adsorbents have been demonstrated. Proteins can be immobilized under mild conditions either at ambient temperature or in a cold room. The coupling reaction is completed within 1-2 h at 20-25°C. The procedure is simple and convenient, because it does not require aggressive reagents such as cyanogen bromide [14] or reducing agents [15]. The ratios of tyrosine and arginine to alanine particularly decreased after immobilization. The decrease in the ratio of arginine to alanine is undoubtedly due to coupling to the gel. Although tyrosine is unstable during hydrolysis in the presence of the gel, this appreciable decrease in the ratio of tyrosine to alanine may be due to coupling to the gel. Non-specific adsorption of the proteins investigated to TSKgel Tresyl-5PW was absent.

Purified C14K was separated into four peaks by the SIA4-5-5PW column in spite of apparent homogeneity in the molecular weight of the monomer. C14K is known to require a thiol-reducing agent to maintain haemagglutination activity [16,17]. However, it was suggested that no cysteine residue is involved in the sugar-binding site. Modification of cysteine residues seems to affect the dimer-forming ability of C14K, which is supported by the results shown in Fig. 5. Although the fresh C14K preparation was composed exclusively of dimer, the old preparation contained monomer in addition to dimer. The C14K preparation stored for a long period after purification seems to have been chemically modified and to have become unfavourable for forming the dimer. The reason why the monomeric C14K was separated into two peaks (peaks 1 and 2 in Fig. 2) has not been elucidated. Peak 1 seemed to have a slightly stronger affinity to saccharides than peak 2, although the former had a weaker affinity to the SIA4-5-5PW column than the latter. Some modification might have occurred which affected both sugar-binding ability and interaction with SIA4-5.

Employment of the new support for high-performance affinity chromatography proved to be promising, especially for analytical purposes. In this work, we used less than 1 μg C14K protein and each chromatographic run took less than 1 h. This method proved to be very sensitive in detecting slight differences between C14K molecules and also very powerful in determining dissociation constants. This will be also useful in a variety of applications such as diagnosis and quality control.

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