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COMPARATIVE STUDY OF AFFINITY CHROMATOGRAPHY OF COMPONENTS OF THE HYALURONATE–PROTEOGLYCAN COMPLEX TO IMMOBILIZED HYALURONATE

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SUMMARY

Hyaluronate-containing protein was bound by several substrates through its reducing end, carboxyl groups or free protein functional groups. The derivatized matrices were used to purify components of the hyaluronate–proteoglycan complex of cartilage by affinity chromatography.

INTRODUCTION

Hyaluronate is a linear high-molecular-weight polysaccharide found in nearly all types of connective tissue[1]. Proteoglycans isolated from hyaline cartilages have been characterized in some detail[2,3]. These macromolecules contain many chondroitin sulphate and keratan sulphate chains covalently bound to a protein core. They are present in the cartilage matrix primarily as aggregates, in which large number of proteoglycan molecules bind to single strands of hyaluronate through specific, ionic interactions[4–6]. The aggregates are stabilized by proteins of low molecular weight (link proteins)[5–8], which appear to interact with both hyaluronate and the hyaluronate binding region of proteoglycan molecules[6,9,10]. The aggregates are dissociated in 4 *M* guanidinium hydrochloride solution or a buffer of pH 3.1[11].

In early studies the isolation and purification of extracted proteoglycans in aggregated or monomeric form was performed by complicated and expensive methods[11,12]. Recently several techniques have been used to immobilize hyaluronate on gels by covalent linkages, in order to make possible the isolation and purification of the components of the hyaluronate–proteoglycan complex by affinity chromatography[13–15]. Christner *et al.*[13] reported that the amount of hyaluronate incorporated on the affinity adsorbent was small, and proteoglycan monomers did not bind to their affinity gel. Similarly Tengblad[14] found that the bulk of proteoglycans did not bind to their affinity matrix. I have been pursuing similar studies with hyaluronate coupled to cellulose–ε-amino-*n*-caproic acid[16]. In this study it was found that this matrix bound chondroitinase-degraded aggregable proteoglycans, link proteins and also aggregable proteoglycans. These contradictory results may not be surprising in view of the different reaction conditions used in the preparation of the affiity adsorbents.

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In this paper studies are reported on the coupling of hyaluronate on several substrates with various insoluble supports, spacer molecules, groups on the hyaluronate chain used for the coupling reaction and chromatographic properties of the received affinity matrices.

EXPERIMENTAL

Materials

Sepharose 2B, AH-Sepharose 4B and Sephadex G-50 were obtained from Pharmacia, aminoethylcellulose (0.3 mequiv./g) and Dowex 50X8, H^+ (250–400 mesh) from Sigma, cellulose (CF-11) and ECTEOLA-cellulose (ET-11, total capacity 0.29 mequiv./g) from Whatman (U.K.) and aminoethyl-Bio-Gel (100–200 mesh) from Bio-Rad. Papain (E.C. 3.4.22.2) twice crystallized and hyaluronidase, Type VI (E.C. 3.2.1.35) were obtained from Sigma, chondroitinase ABC (E.C. 4.2.2.4) from Miles Labs. Sodium cyanoborohydride was purchased from Aldrich and was crystallized as described by Borch *et al.*[17]. 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) was obtained from Sigma.

Analytical procedures

Uronic acid and protein were determined by the methods of Bitter and Muir[18] and Lowry *et al.*[19], respectively, automated by Heinegård[20]. Total hexosamines were determined according to the method of Antonopoulos *et al.*[21].

Preparation of proteoglycans and protein–keratan sulphate cores

Proteoglycans were isolated from ovine nasal cartilage in the disaggregated form (A_1D_1) as described by Sajdera and Hascall[22], Theocharis and Tsiganos[23] and Oegema *et al.*[24]. Protein–keratan sulphate cores were prepared from A_1D_1 according to Hascall and Heinegård[9].

Preparation of hyaluronate

Hyaluronate from papain digests of rooster comb (white Leghorn) was isolated and purified following a combination of well established purification methods for glycosaminoglycans[16]. Oligosaccharides from hyaluronate were prepared as follows. Hyaluronate (measured as uronic acid) (15 mg) was dissolved in 4 ml of 0.15 *M* sodium chloride, 0.1 *M* sodium acetate, pH 5 and incubated for 1 h at 37°C with 1260 I.U. of hyaluronidase. The digest was dialysed in water for 4 h at 4°C and chromatographed on a column of ECTEOLA-cellulose (10 × 2 cm) at 4°C, eluted sequentially with 100 ml each of 0.05 *M* (a), 0.1 *M* (b) and 0.7 *M* sodium chloride (c). Oligosaccharides greater than decasaccharides were isolated by chromatography of fraction c on a standardized column of Sephadex G-50 according to the method of Hascall and Heinegård[9].

Coupling of hyaluronate to several substrates

Procedure A: coupling of partially digested hyaluronate to aminoethyl-Bio-Gel. This method relies upon the ability of cyanoborohydride anion to reduce selectively the Schiff base formed between the reducing end of hyaluronate and the amino groups of aminoethyl-Bio-Gel.

A 200-mg amount of aminoethyl-Bio-Gel (previously swollen), 440 μg of par-

tially digested hyaluronate (measured as uronic acid) and 100 mg of sodium cyanoborohydride were mixed in 20 ml of 0.2 *M* K_2HPO_4, pH 9. The mixture was agitated by gentle shaking at room temperature for a week. The reacted gel was washed on a sintered glass filter with 500 ml each of water (a), 2 *M* sodium chloride (b) and 4 *M* guanidinium hydrochloride. The remaining unsubstituted amino groups on the gel were blocked by N-acetylation[25].

Procedure B: coupling of partially digested hyaluronate to aminoethylcellulose. This was performed as in procedure A.

Procedure C: coupling of intact hyaluronate to AH-Sepharose 4B. Intact hyaluronate was coupled via its carboxyl groups to the amino groups of AH-Sepharose 4B by the carbodiimide technique[26].

Hyaluronate (measured as uronic acid) (17 mg) and 5 g of AH-Sepharose 4B were mixed in 45 ml of water (pH 4.5). A 200-mg amount of EDC was added to the mixture at room temperature and the pH was held at 4.5–6 for 24 h by addition of 0.1 *M* hydrochloric acid. The excess of amino groups on the gel was removed by performing a further carbodiimide reaction using acetic acid as blocking agent. The washing of the reacted gel was performed as described previously.

Procedure D: coupling of intact hyaluronate to hydrazidocellulose. Intact hyaluronate was coupled via its reducing end to the amino groups of hydrazidocellulose by forming a Schiff base.

Cellulose (2 g) was treated with cyanogen bromide to "activate" the glycan, followed by coupling of the "activated" cellulose to adipic acid dihydrazide by the method described by Wilchek and Miron[27]. The coupling of intact hyaluronate on hydrazidocellulose was performed as described in procedure A.

Procedure E: coupling of partially digested hyaluronate to aminohexylcellulose. Aminohexylcellulose was synthesized by condensing an excess of 1,6-diaminohexane with periodate-oxidized cellulose as described by Junowicz and Charm[28]. Unreacted aldehyde groups on the cellulose were reduced with $NaBH_4$ during the reduction step of the 1,6-diaminohexane coupling. Partially digested hyaluronate was then coupled via its reducing end to ε-amino groups of the spacer molecule as described in procedure A. The excess of amino groups was blocked by N-acetylation.

Procedure F: coupling of intact hyaluronate to cellulose–ε-amino-n-caproic acid. This was performed as previously described[16].

Analysis of bound hyaluronate

Three methods have been devised for quantitation of the amount of hyaluronate covalently bound on the affinity matrices: (a) from the difference between the total amount of hyaluronate added to the coupling mixture and that recovered after exhaustive washing; (b) by determination of uronic acid in the supernatant after digestion of immobilized hyaluronate with hyaluronidase[13] and (c) from the hexosamine content of the substituted matrix.

Affinity chromatography

A 1-g amount of each affinity adsorbent was suspended in 10 ml of 0.05 *M* sodium chloride–0.05 *M* sodium acetate, pH 5.8, containing samples of A_1D_1, protein–keratan sulphate cores or a mixture of A_1D_1 and link proteins. This mixture was incubated overnight at 4°C, with gentle shaking. The slurry was packed into a

column (7.1 × 0.6 cm) and washed with three bed volumes each of 0.05 *M*, 0.5 *M*, 2 *M* sodium chloride and 4 *M* guanidinium hydrochloride all buffered to pH 5.8 with 0.05 *M* sodium acetate. The effluents from the chromatography of proteoglycans were analyzed for uronic acid and those from protein–keratan sulphate cores for protein. The column was operated at 4°C and in some experiments the 4 *M* guanidinium hydrochloride solution was substituted by 0.02 *M* phosphate–citrate buffer, pH 3.1.

Control experiments using unsubstituted affinity adsorbents were made to account for non-specific binding of proteoglycans or protein–keratan sulphate cores.

Gel chromatography

Chromatography of various fractions from the affinity chromatography, alone or after mixing with exogenous hyaluronate, was performed on Sepharose 2B at 4°C on 86 × 0.8 or 147 × 0.8 cm columns, eluted with 0.5 *M* sodium acetate, pH 6.8. The flow-rate was 3.5 ml/h and 1.5-ml fractions were collected and analyzed for uronic acid or protein.

Electrophoresis

Sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis was carried out in 8% polyacrylamide gel according to Laemmli[29].

RESULTS AND DISCUSSION

The effects of the support matrix, spacer arm and molecular size of hyaluronate on the yield of immobilized hyaluronate are summarized in Table I. Apparently, the

TABLE I

EFFECTS OF THE SUPPORT MATRIX, SPACER ARM AND MOLECULAR SIZE OF HYALURONATE ON THE YIELD OF IMMOBILIZED HYALURONATE

The coupling of intact or partially digested HA to several substrates was performed as described in the text. Immobilized HA was measured as uronic acid or total hexosamines per g of dry substituted matrix.

Procedure	*Support matrix*	*Spacer arm*	*Bound ligand*	*Immobilized HA (mg/g)*
A	Bio-Gel	Ethylene-diamine	Partially digested HA*	0.49
B**	Cellulose	Ethylene-diamine	Partially digested HA	0.00
C	Sepharose 4B	1,6-Diamino-hexane	Intact HA	2.58
D	Cellulose	Adipic acid dihydrazide	Partially digested HA	0.17
E	Cellulose	1,6-Diamino-hexane	Partially digested HA	0.64
F	Cellulose	ε-Amino-*n*-caproic acid	Intact HA	1.46

* Oligosaccharides of hyaluronate greater than decasaccharides were prepared as described in the text.

** Aminoethylcellulose was purchased from Sigma and was synthesized by covalent coupling of ethylenediamine to cyanogen bromide-activated cellulose.

affinity matrix with the highest concentration of bound hyaluronate (2.58 mg of uronic acid per g of dry AH-Sepharose 4B or 0.64 mg/ml of moist Sepharose) was obtained by using Sepharose 4B as support matrix and 1,6-diaminohexane as spacer arm. Tengblad[14] coupled hyaluronate on AH-Sepharose 4B by the carbodiimide technique, utilized some of the hyaluronate carboxyl groups and amino groups of AH-Sepharose 4B and obtained comparable results (0.6–1.7 mg of HA per ml of gel). However, this linkage between hyaluronate and gel was not stable during prolonged storage in the presence of 4 *M* guanidinium hydrochloride. It seems that this is due to the instability of the linkage formed between the spacer arm and cyanogen bromide-activated Sepharose[28,30,31]. Besides the continuous decrease in the column capacity, the released hyaluronate from the gel, during the elution of this column by 4 *M* guanidinium hydrochloride, contaminates coeluted proteoglycans or protein–keratan sulphate cores. Moreover several reports have noted that the spacer arm–polysaccharide produced by the cyanogen bromide procedure contains positive charges[31,32]. These charges may lead to spurious results in the sense that will retain anions, such as proteoglycans or protein–keratan sulphate cores, during their affinity chromatography on this matrix, not through coupled hyaluronate but in a non-specific manner. It is likely that non-specific adsorptions observed during the chromatography of proteoglycans on hyaluronate–hydrazidocellulose (procedure D) may be due mostly to such interactions. Christner *et al.*[13] used hydrazido-Sepharose to couple hyaluronate via its terminal aldehyde group. They incorporated about 0.06 mg of uronic acid per ml of gel and reported that proteoglycan monomers were not bound to their affinity gel.

In order to avoid the cyanogen bromide procedure, I attempted to attach hyaluronate to aminoethyl-Bio-Gel (procedure A). Unfortunately the spacer arm–polymer alone interacted very strongly and irreversibly with proteoglycans (recovery 23%). Presaturation of hyaluronate–aminoethyl-Bio-Gel with albumin minimized non-specific adsorptions but it was also accompanied by a reduction in the ability of the matrix to undergo affinity chromatography. Since chondroitin sulphate did not interact with aminoethyl-Bio-Gel, it is possible that these adsorptions occur through the protein core of proteoglycans.

On the other hand, my attempt to bind hyaluronate on aminoethylcellulose, purchased from Sigma and synthesized by covalent coupling of ethylenediamine to cyanogen bromide-activated cellulose, was unsuccessful (procedure B). The most plausible explanations are either that the large molecular size of hyaluronate inhibited its incorporation on aminoethylcellulose (Gray[33] reported that the rate of coupling is highly dependent on the molecular size of the polysaccharide), or that the spacer arm was unsuitable as regards accessibility to the ligand.

These results led to the use of a longer spacer arm, such that its functional groups, critical in the interaction with hyaluronate, were sufficiently distant from the solid matrix support. At first 1,6-diaminohexane was employed as a spacer arm. The oxidation of cellulose with periodate produced aldehyde groups on the fibres, which reacted with one of the amino groups of 1,6-diaminohexane by forming a Schiff base. The bond between cellulose and the spacer arm was reduced with cyanoborohydride, giving a stable derivatized cellulose (procedure E). By titration with sodium 2,4,6-trinitrobenzenesulphonate[34], it was found that each gram of derivatized cellulose contained 1.4 mequiv. of amino groups. When aminohexylcellulose was treated with

TABLE II

AMOUNTS OF A_1D_1 PROTEOGLYCANS AND PROTEIN–KERATAN SULPHATE CORES BOUND TO SEVERAL AFFINITY MATRICES AND ELUTED WITH SOLUTIONS OF DIFFERENT SALT CONCENTRATIONS

Different amounts of proteoglycans (μg of uronic acid) or protein–keratan sulphate cores (μg of protein) interacted with 1 g of affinity matrix as described in the Experimental. The columns were eluted sequentially with 0.05 *M* (a), 0.5 *M* (b), 2 *M* sodium chloride (c) and 4 *M* guanidinium hydrochloride (d) or a buffer, pH 3.1. Link proteins were mixed with proteoglycans in a ratio of 8 μg of protein per 100 μg of uronic acid.

Affinity matrix	*Sample*		*μg of uronic acid or protein per g of affinity matrix eluted by*				*Recovery (%, w/w)*
	A_1D_1 ± link proteins	*Protein cores*	*0.05 M NaCl*	*0.5 M NaCl*	*2 M NaCl*	*4 M Guanidinium hydrochloride or buffer, pH 3.1*	
Hyaluronate-hydrazido-cellulose	450		365	45	0	0	
	900		720	100	0	0	
	1800		1630	98	0	0	92 ± 4
		250	124	63	0	43	
		500	340	70	0	50	
Hyaluronate-aminohexyl-cellulose	200		130	18	13	10	
	400		261	37	25	19	85 ± 5
	800		665	40	24	19	
Cellulose-ε-amino-*n*-caproic acid-hyaluronate	710		560	129	0	14	99 ± 0.5
	1500		1350	130	0	14	
		250	60	30	0	140	
		1500	–	–	–	820	
		3000	–	–	–	810	

hyaluronate, the amino groups of the former were coupled with the terminal aldehyde group of the latter to form a Schiff base. This bond was also reduced with cyanoborohydride. The amount of hyaluronate linked to aminohexylcellulose was 0.64 mg of uronic acid or hexosamine per g of cellulose (Table I). This is lower than the amount linked to cellulose–ε-amino-*n*-caproic acid (about 1.46 mg of uronic acid per g of cellulose)[16]. The difference between these results may be due to the difference between the number of reducing ends in a sample of hyaluronate and the number of its protein amino groups.

Affinity chromatography of proteoglycans and protein–keratan sulphate cores

Hyaluronate-cellulose, prepared by procedures D, E or F, was used to isolate the population of proteoglycans or of their protein–keratan sulphate cores, which in free solution reacts with hyaluronate to form complexes. These complexes are stabilized by low-molecular weight proteins (link proteins), which appear to interact with both hyaluronate and proteoglycan molecules[5–8]. The aggregates are dissociated in 4 *M* guanidinium hydrochloride solution or a buffer of pH 3.1 and can be reformed upon dialysis to 0.4–0.5 *M* guanidinium hydrochloride or to neutral pH[11].

When intact monomeric proteoglycans either alone (A_1D_1) or in the presence of link proteins were mixed with the affinity matrices in 0.05 *M* sodium chloride–0.05 *M* sodium acetate, pH 5.8, the major part of the material did not bind to the matrices (Table II). Very little material was recovered when the columns were eluted with solutions of 0.5 *M* sodium chloride or 2 *M* sodium chloride–0.05 *M* sodium acetate, pH 5.8. No or small amounts were retained and eluted with 4 *M* guanidinium hydrochloride. Some proteoglycans (11–15% measured as uronic acid) were bound very firmly to the hyaluronate-cellulose prepared by procedure D or E. These proteoglycans seemed to be trapped within the matrix by non-specific forces. Tengblad[14] reported that only a small fraction of undigested proteoglycans bind to their affinity adsorbent. It has previously been shown[16] that the results were improved when mixing of proteoglycans with the affinity matrix was performed in the presence of 4 *M* guanidinium hydrochloride and under constant dialysis.

The affinity matrices retained higher amounts of protein–keratan sulphate cores, somewhat increasing with increasing quantity in the reaction mixture, reaching a maximum value of 820 μg of protein per g of cellulose–ε-amino-*n*-caproic acid–hyaluronate or 50 μg of protein per g of hyaluronate-hydrazidocellulose; since the protein–keratan sulphate cores contain 40–50% protein[3], these figures correspond to 1.8 and 0.11 mg of material respectively. It is known that each protein–karatan sulphate core of molecular weight 4.5×10^5 occupies 8–10 disaccharide units along the hyaluronate polysaccharide chain[9]. Thus, the theoretical binding capacity of 1 g of the substituted matrix is manifold greater than that experimentally obtained. These results suggest that the majority of the proteoglycans or the protein–keratan sulphate cores are too bulky to approach the coupled hyaluronate. Alternatively, the stability of this interaction is weaker than that occurring when the components are in free solution.

The proteoglycans eluted from the affinity matrices were further characterized by their ability to bind to exogenous hyaluronate (Fig. 1). The results, summarized in Table II and Fig. 1, led to the following conclusions.

(1) Chromatography of proteoglycans on hyaluronate hydrazidocellulose

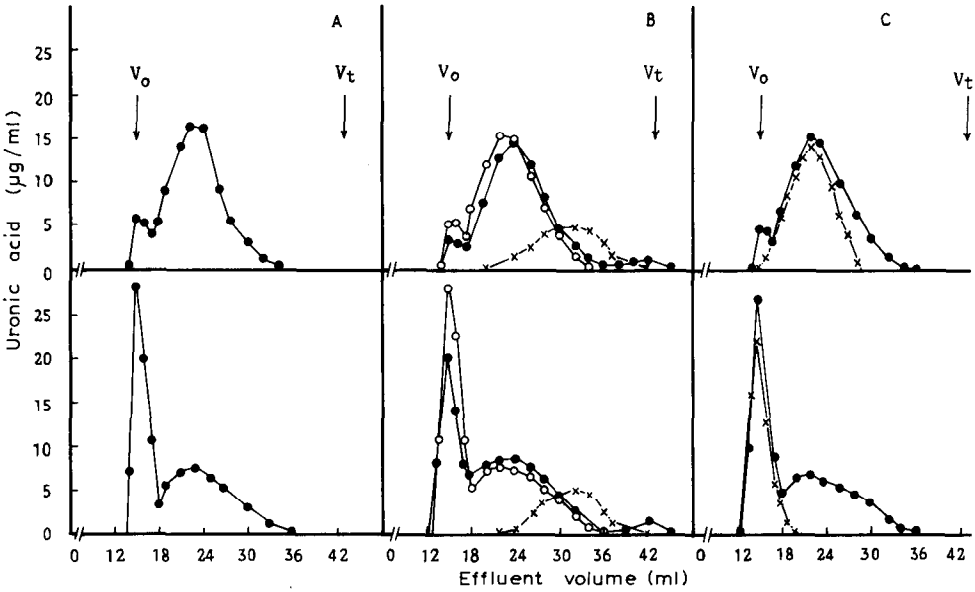

Fig. 1. Gel chromatography on Sepharose 2B of proteoglycans eluted from hyaluronate-hydrazidocellulose (A), hyaluronate-aminohexylcellulose (B) and cellulose-ε-amino-*n*-caproic acid-hyaluronate (C), before and after mixing with exogenous hyaluronate. The column of Sepharose 2B (86 × 0.8 cm) was eluted with 0.5 *M* sodium acetate, pH 6.8. Top: proteoglycans eluted from the affinity matrix with 0.5 *M* and 2 *M* sodium chloride (●—●) or 4 *M* guanidinium hydrochloride (×—×). A_1D_1-proteoglycans were used as control (○—○). Bottom: proteoglycans eluted from the same affinity matrices and mixed with exogenous hyaluronate at a ratio of 100:1 (μg of uronic acid:μg of uronic acid). The solutions were left at room temperature for 1 h, dialysed overnight at 4°C against 0.05 *M* sodium acetate, pH 5.8 and chromatographed on Sepharose 2B.

was not detrimental to their structure or their ability to bind to exogenous hyaluronate. Furthermore, the partial binding of proteoglycans (0.5 *M* sodium chloride fraction) was non-specific and non-preferential with a particular macromolecule of the proteoglycan population.

(2) Proteoglycans eluted from hyaluronate-aminohexylcellulose with 0.5 *M* or 2 *M* sodium chloride consisted of aggregable and non-aggregable molecules, since they were partially excluded (33%) by Sepharose 2B after mixing with exogenous hyaluronate. However the extent of exclusion was lower than that of the whole population of proteoglycans (56%, Fig. 1B). It is emphasized that the material eluted from hyaluronate-aminohexylcellulose with 4 *M* guanidinium hydrochloride consisted of small and non-aggregable proteoglycans similar to those reported by Christner *et al.*[13]. Thus these small proteoglycans were bound to the affinity matrix independently of the specific interaction through hyaluronate. A similar non-specific binding and a decrease in the ability to bind exogenous hyaluronate have been shown for proteoglycans eluted from unsubstituted aminohexylcellulose. Since the excess of amino groups was blocked by N-acetylation, some activation of "sites with high reactivity" occurs during the preparation of the affinity matrix. Because of these serious restrictions, the use of this affinity matrix must be avoided (Fig. 1B).

(3) Proteoglycan chromatographed on cellulose–ε-amino-*n*-caproic acid–hyaluronate maintained its structural and functional properties. Furthermore the pro-

teoglycans eluted from this affinity matrix with 4 *M* guanidinium hydrochloride, when mixed with exogenous hyaluronate, became of larger hydrodynamic volume, since they were excluded by Sepharose 2B (Fig. 1C).

On the other hand, the fraction of protein–keratan sulphate cores that was retained by hyaluronate-hydrazidocellulose or cellulose–ε-amino-*n*-caproic acid–hyaluronate consisted entirely of aggregable molecules (Fig. 2). If it was combined with exogenous hyaluronate and then fractionated on Sepharose 2B, the protein eluted at the void volume of the column.

Whenever link proteins were present in the interaction mixtures, they were eluted from the affinity matrix by 4 *M* guanidinium hydrochloride together with aggregable proteoglycans. This was demonstrated by SDS-polyacrylamide gel electrophoresis of the 4 *M* guanidinium hyaluronate fractions from affinity chromatography of A_1D_1-proteoglycans ± link proteins (not shown). This behaviour of the link proteins has been reported previously[14,16].

The present experiments show that cellulose–ε-amino-*n*-caproic acid–hyaluronate minimizes non-specific adsorptions and steric problems associated with immobilization of the hyaluronate. Most important of all, this matrix is not detrimental to the structure of proteoglycans or protein-keratan sulphate cores which are chromatographed intact. Other substrates also bind hyaluronate through its carboxyl groups or its reducing end. However the percentage of the hyaluronate binding and the chromatographic properties of the products, which were obtained, were significantly inferior to those obtained in the case of cellulose–ε-amino-*n*-caproic acid–hyaluronate.

The affinity chromatography of proteoglycans can be employed for rapid and non-expensive separation of aggregable and non-aggregable proteoglycans in order

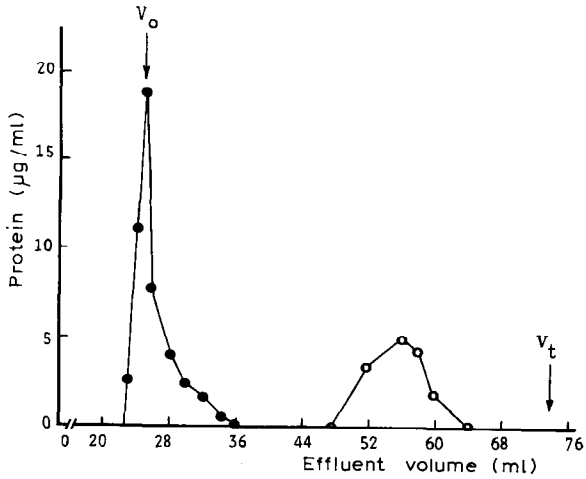

Fig. 2. Gel chromatography on Sepharose 2B of protein–keratan sulphate cores eluted from hyaluronate-hydrazidocellulose or cellulose-ε-amino-*n*-caproic acid-hyaluronate with 4 *M* guanidinium hydrochloride, before (○—○) and after (●—●) mixing with exogenous hyaluronate. The Sepharose 2B column (147 × 0.8 cm) was eluted with 0.5 *M* sodium acetate, pH 6.8. In aggregation experiments, protein cores in 4 *M* guanidinium hydrochloride were mixed with exogenous hyaluronate at a ratio of 80:2.5 (μg of protein:μg of uronic acid), left at room temperature for 1 h, dialysed overnight at 4°C against 0.05 *M* sodium acetate, pH 5.8 and then chromatographed on Sepharose 2B.

to study their structure and mainly the protein core, which exhibits a specific non-covalent interaction with hyaluronate. Furthermore this technique can be used to isolate link proteins from untreated cartilage extracts or enzymes implicated in hyaluronate biosynthesis.

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