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Immunochemical Analysis of Cartilage Proteoglycans

RADIOIMMUNOASSAY OF THE MOLECULES AND THE SUBSTRUCTURES

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Antibodies specifically reacting with the link proteins, the hyaluronic acid-binding region and chondroitin sulphate–peptides were used to design specific radioimmunoassay procedures. The sensitivity of the method used for the link protein was about 20ng/ml, and the other two components could be determined at concentrations of about 2ng/ml. The radioimmunoassay procedures were tested by using proteoglycan subfractions or fragments thereof. The procedures used to quantify link protein and hyaluronic acid-binding region showed no cross-interference. Fragments of trypsin-digested proteoglycan monomers still reacted in the radioimmunoassay for hyaluronic acid-binding region. Subfractions of proteoglycan monomers separated according to size had a gradually higher relative content of the hyaluronic acid-binding region compared with both chondroitin sulphate–peptides and uronic acid, when the molecules were smaller. The proteoglycans therefore may contain a variably large chondroitin sulphate-rich region, which has a constant substitution with polysaccharide side chains.

Cartilage proteoglycans are large molecules with a very complex structure. The proteoglycan monomer contains a central protein core to which a large number of polysaccharide side chains of chondroitin sulphate and keratan sulphate are covalently attached (Muir, 1958; Anderson *et al.*, 1965; Helting & Rodén, 1968). The polysaccharides, however, are not distributed randomly along the core. Substructures containing preferentially one type of polysaccharide have been identified (Heinegård & Axelsson, 1977). At one end of the molecule, the major portion of the chondroitin sulphate side chains are bound to a portion of the protein core, the chondroitin sulphate-rich region (Heinegård & Axelson, 1977). The keratan sulphate-rich region (Heinegård & Axelsson, 1977) is located next to the chondroitin sulphate-rich region and contains a large proportion of the keratan sulphate chains in the proteoglycan, but only few chondroitin sulphate chains (Heinegård & Axelsson, 1977). The protein at the other end of the monomer contains few or no polysaccharide chains (Heinegård & Hascall, 1974). This hyaluronic acid-binding region has a tertiary structure, stabilized by disulphide bonds (Hascall & Heinegård, 1974*a*; Heinegård, 1977), allowing a very specific interaction with hyaluronic acid (for references see Hascall & Heinegård, 1979). In the cartilage tissue several proteoglycan monomers are bound to one hyaluronic acid molecule, forming a proteoglycan aggregate (Hascall & Heinegård, 1974*a*). The non-covalent bond between the hyaluronic acid-binding region of the proteoglycan and hyaluronic acid is stabilized by an additional protein component, the link protein (Hascall & Heinegård, 1974*b*; Keiser *et al.*, 1972), which does not contain glycosaminoglycan side chains. An important additional feature of the proteoglycan monomer is the variable size (Heinegård, 1977; Hascall & Sajdera, 1970; Hardingham *et al.*, 1976). Available data indicate that proteoglycans may vary in size and in composition, although they have the same ability to bind to hyaluronic acid, indicating that they contain at least one constant portion, the hyaluronic acid-binding region (Heinegård, 1977). Chemical analysis indicate that the content of the chondroitin sulphate-rich region of the monomers may vary.

In a previous paper we analysed antibodies specific for the link protein, the hyaluronic acid-binding region and the chondroitin sulphate–peptides liberated from the proteoglycan monomer (or aggregate) by trypsin digestion (Wieslander & Heinegård, 1979). These antibodies are used in radioimmunoassay procedures to develop sensitive and specific procedures for measuring some of the important structural features of the proteoglycans.

Materials and Methods

Proteoglycans and subfractions were prepared as described elsewhere (Wieslander & Heinegård, 1979). Antibodies were raised in rabbits against the

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link protein, the hyaluronic acid-binding region and the glycosaminoglycan–peptides isolated from trypsin-digested proteoglycans (fraction A1-T-A1) as described previously (Wieslander & Heinegård, 1979). The operational nomenclature is that outlined by Heinegård & Axelsson (1977): A1-T-A1 refers to glycosaminoglycan peptides isolated from trypsin-digested proteoglycan aggregates by using CsCl-density-gradient centrifugation; A1, proteoglycan aggregate fraction; A1-D1, proteoglycan monomer fraction.] The immunoglobulin-G fraction was isolated from the antisera as previously described (Wieslander & Heinegård, 1979; Steinbuch & Audran, 1969).

Pig antiserum to rabbit immunoglobin G was a kind gift from Dr. B. G. Johansson, Department of Clinical Chemistry, University Hospital, Lund, Sweden. Antigens to be used for radioimmunoassay were rechromatographed on Sephadex G-200 columns eluted with 4M-guanidinium chloride as described by Wieslander & Heinegård (1979). The antigens were [^{125}I]iodinated by using lactoperoxidase, by the procedure described by Thorell & Johansson (1971). Usually more than 20×10^6 c.p.m. were incorporated per μg of antigen.

In order to remove any contaminating antibodies, the anti-(fraction A1-T-A1) antiserum was passed through an affinity column containing hyaluronic acid-binding region covalently attached to Sepharose 4B by using the CNBr procedure (March *et al.*, 1974). The link proteins were not soluble in the buffers used. Therefore they were maleoylated before analysis essentially as described elsewhere (Wieslander & Heinegård, 1979).

Chondroitin sulphate–peptides were prepared from fraction A1-T-A1 by chondroitinase digestion and Sepharose 6B chromatography as described previously (Heinegård & Axelsson, 1977). This preparation incorporated ^{125}I, but very little of the labelled material could be precipitated with any of the antisera. It is likely that the radioisotope was only incorporated into traces of chondroitinase remaining with the preparation. In contrast, ^{125}I-labelled proteoglycan core preparations prepared from a chondroitinase ABC digest of fraction A1-D1 as described elsewhere (Hascall & Heinegård, 1974*a*) were precipitated with the anti-(fraction A1-T-A1) antibodies. As discussed below, this reaction could be inhibited by addition of chondroitin sulphate–peptides. Therefore the core preparation was used for the radioimmunoassay of chondroitin sulphate–peptides.

The following scheme was utilized for the precipitation of antigens and subsequently antigen–antibody complexes. Portions (50μl) of [^{125}I]iodinated antigen (containing about 10000 c.p.m.) were mixed with 50μl of sample or standard amounts of the antigen. The antigens were dissolved in diluent buffer (0.05M-sodium phosphate, 0.15M-NaCl, 0.02% NaN_3 and 0.5% bovine serum albumin, pH 7.5). Antibody (purified immunoglobulin G) in diluent buffer was then added. Usually 50μl of a dilution of antibody of 1:10000–1:100000 was used. The mixture was incubated for 4 days or more in a cold-room (4°C). The pig anti-(rabbit immunoglobulin G) [50μl of a 1:10–1:100 dilution in 5% (w/v) poly(ethylene glycol) (PEG 6000)] was then added and the samples were left overnight in the cold-room. In order to increase the relative proportion of solvent to precipitate, and thereby minimize the non-specific contamination of the precipitate by unprecipitated antigen, the samples were then diluted by the addition of 1 ml of diluent buffer. The samples were then immediately centrifuged at 2000$g_{av.}$ for 20 min in a swing-out rotor in a refrigerated centrifuge. The supernatants were then decanted and analysed, as were the precipitates, for ^{125}I contents by using a LKB–Wallac gamma counter. The radioactivity ratio, i.e. the amount of bound (precipitated) label over the amount of free (non-precipitated) label, was calculated.

The amount of antibody required for optimal precipitation was determined by using the procedure described above, with a set of dilutions of the antibody solutions (the stock solution of the antibody contained approximately the same amount of immunoglobulin G as the original rabbit serum). From the results the appropriate amount of antibody for the radioimmunoassay could be determined. A dilution of antiserum sufficient to precipitate 30–50% of the iodinated antigen was chosen, and standard curves with added non-labelled antigens to inhibit precipitation were prepared.

In an attempt to test the methods, they were applied in the analysis of chromatograms of complexes of hyaluronic acid-binding region, link protein and hyaluronic acid isolated from aggregates after trypsin digestion (Heinegård & Axelsson, 1977). Such complexes (4 mg) were chromatographed on a Sephadex G-200 column (1.0 cm × 150 cm) eluted with 4M-guanidinium chloride/0.05M-sodium acetate, pH 5.8, and the effluent fractions were analysed for contents of protein by their A_{280}. The link proteins in the fractions were made soluble in weak salt solutions by maleoylation. Samples (1 ml) were diluted with 1M-Na_2CO_3 (100μl) and maleic anhydride (1M; 10μl) was added. The samples were then incubated for 5 min. Samples were then diluted with an appropriate volume of electrophoresis buffer or diluent buffer and analysed for their contents of hyaluronic acid-binding region and link protein by using electroimmunoassay (Laurell, 1966; Wieslander & Heinegård, 1979) or the radioimmunoassay discussed above, respectively.

In another experiment 200mg of bovine nasal-cartilage proteoglycan monomer (A1-D1) was chromatographed on a Sepharose 2B column (2.5cm × 150cm) eluted with 0.5M-sodium acetate, pH 7.0, and pooled to yield eight fractions (indicated in Fig. 6), which were diluted and analysed for content of uronic acid, by using the carbazole procedure (Heinegård, 1973). In addition, contents of hyaluronic acid-binding region and chondroitin sulphate–peptides were determined by using the radioimmunoassay procedure.

A sample (50mg) of bovine nasal-cartilage proteoglycan monomers (A1-D1) was sequentially digested with chondroitinase and trypsin as described elsewhere (Heinegård & Axelsson, 1977; Heinegård, 1977). The digest was chromatographed on Sepharose 6B eluted with 0.5M-sodium acetate, pH 7.0, in order to separate the keratan sulphate–peptide from the chondroitin sulphate–oligosaccharide peptides as described elsewhere (Heinegård & Axelsson, 1977). The effluent fractions were analysed for contents of protein, hexose and uronic acid by using automated procedures (Heinegård, 1973). Samples of the fractions were diluted and analysed for contents of substances reactive in radioimmunoassay for hyaluronic acid-binding region and chondroitin sulphate–peptides.

Results and Discussion

In the present investigation all antigens used for immunoassay were labelled with ^{125}I. No other isotope was tried for labelling, primarily because of the extremely high specific radioactivity of the ^{125}I, allowing highest sensitivity of the assay procedure. In other experiments the hyaluronic acid-binding region has been labelled with [^{3}H]acetate by using acetic anhydride (Heinegård & Hascall, 1979). The maximum amount of radioactivity incorporated was, however, 200–500d.p.m./μg, a value many orders of magnitude lower than the values recorded with ^{125}I and therefore not useful for radioimmunoassay. Some preliminary experiments using enzyme-linked immunosorbent assay (ELISA) for quantifying the link protein indicate, however, that a higher sensitivity can be obtained compared with the radioimmunoassay (results not shown), possibly because of less problems with solubility when tubes are coated with antigens.

The degree of precipitation of the ^{125}I-labelled antigens was tested. Serial dilutions containing progressively decreasing amounts of antibodies were prepared, mixed with ^{125}I-labelled antigen and precipitated by using the procedure described in the Materials and Methods section. It was shown that the maximal amount of antigen that could be precipitated varied from about 85% (Fig. 1*a*) for the ^{125}I-labelled hyaluronic acid-binding region with anti-(hyaluronic acid-binding region) serum to about

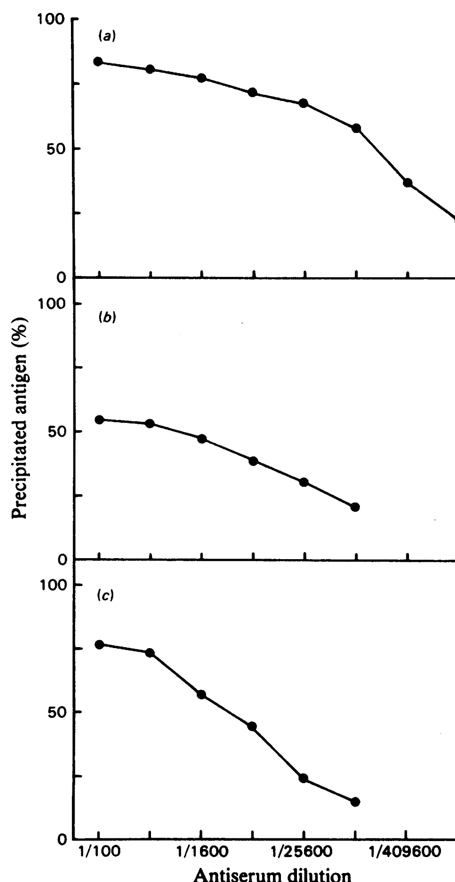

Fig. 1. *Reaction of ^{125}I-labelled antigens with dilutions of specific antibodies*

Bound antigens were isolated by precipitation of the immunoglobulins by using the double-antibody technique, and the proportion of the label recovered in the precipitate is indicated. (*a*) ^{125}I-labelled hyaluronic acid-binding region and dilutions of anti-(hyaluronic acid-binding-region) immunoglobulin G. (*b*) ^{125}I-labelled link protein and dilutions of anti-(link protein) immunoglobulin G. (*c*) ^{125}I-labelled proteoglycan core protein and dilutions of anti-(fraction A1-T-A1) (chondroitin sulphate-peptides) immunoglobulin G.

60% for the ^{125}I-labelled link-protein (Fig. 1*b*) and core-protein (Fig. 1*c*) preparations with anti-(link protein) and anti-(fraction A1-T-A1) chrondroitin sulphate–peptides] sera, respectively. In separate experiments it was shown that more than 60% of the ^{125}I-labelled core-protein preparation could be precipitated with anti-(hyaluronic acid-binding region) immunoglobulins. It is possible that the core-protein preparation contained small amounts of non-precipitable chondroitinase labelled with ^{125}I, explaining

the relatively low degree of precipitation. The relatively inefficient precipitation of the ^{125}I-labelled link protein may be due to blocking of antigenic sites by the maleoylation procedure interfering with the immune precipitation. The precipitation curves were used to calculate the dilution of antiserum that was optimal for radioimmunoassay. A degree of precipitation of about 30–50% was chosen, which is on the slope of the curves. The dilution of antiserum, then, varied from 1:10000 to 1:100000 (Figs. 1*a*–*c*).

Standard curves with added non-labelled antigen to inhibit precipitation of the ^{125}I-labelled material were prepared (Figs. 2, 3 and 4). It appears that both the hyaluronic acid-binding region and the chondroitin sulphate–peptides can be determined in amounts not less than 0.1 ng in the reaction mixture, that is about 2 ng/ml of solution (Figs. 2 and 3). The isolated hyaluronic acid-binding region was digested with trypsin as previously described (Wieslander & Heinegård, 1979), before radioimmunoassay. The peptides in the digest still reacted in the immunoassay, although the degree of inhibition was only about half that of the intact hyaluronic acid-binding region, indicating that some antigenic sites had been destroyed.

As discussed above, trypsin digests of proteoglycans can be used as the antigen for the determination both of the hyaluronic acid-binding region and of the chondroitin sulphate–peptides. Therefore it is feasible to use trypsin digestion to extract proteoglycan fragments from very small pieces of

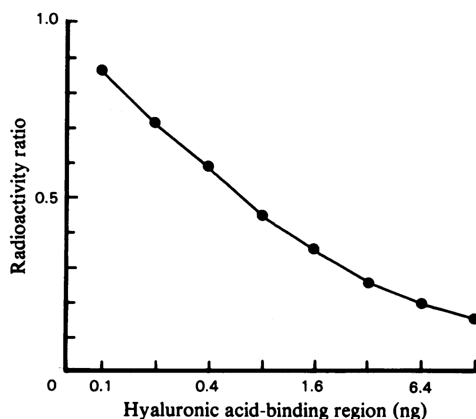

Fig. 2. *Standard curve for the radioimmunoassay of hyaluronic acid-binding region*

^{125}I-labelled hyaluronic acid-binding region was incubated with various amounts of non-labelled hyaluronic acid-binding region and anti-(hyaluronic acid-binding region) immunoglobulin G. Bound antigen was isolated by precipitation of the immunoglobulins by using the double-antibody technique. The degree of precipitation is indicated as the radioactivity ratio, i.e. the ratio of bound ^{125}I-labelled antigen to free ^{125}I-labelled antigen.

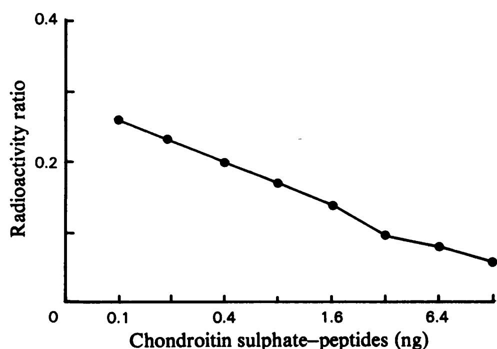

Fig. 3. *Standard curve for the radioimmunoassay of chondroitin sulphate–peptides*

^{125}I-labelled proteoglycan core protein was incubated with various amounts of non-labelled chondroitin sulphate–peptides and anti-(fraction A1-T-A1) immunoglobulin G. Bound antigen was isolated by precipitation of the immunoglobulins by using the double-antibody technique. The degree of precipitation is indicated as the radioactivity ratio, i.e. the ratio of bound ^{125}I-labelled antigen to free ^{125}I-labelled antigen.

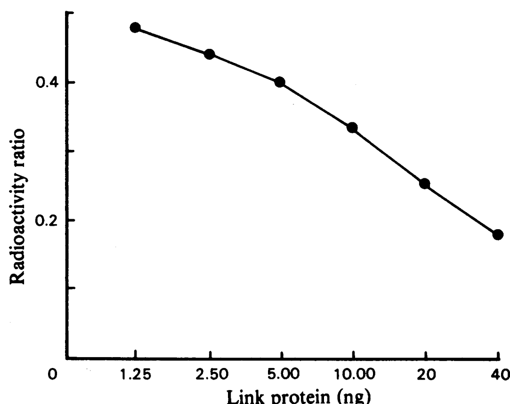

Fig. 4. *Standard curve for the radioimmunoassay of link protein*

^{125}I-labelled link protein was incubated with various amounts of non-labelled link protein and anti-(link protein) immunoglobulin G. Bound antigen was isolated by precipitation of the immunoglobulins by using the double-antibody technique. The degree of precipitation is indicated as the radioactivity ratio, i.e. the ratio of bound ^{125}I-labelled antigen to free ^{125}I-labelled antigen.

cartilage followed by radioimmunoassay to determine the quantity and also some structural features, as revealed by proportion of substructures, of the cartilage proteoglycans. It was also considered important subsequently to use trypsin digests of proteoglycans, since the large size and charge density of the intact molecules may interfere with the immunoprecipitation procedure.

The sensitivity of the assay procedure for the link protein was not as good as for the other two antigens (Fig. 4). Approx. 20ng of link protein/ml was required for quantification. This lower sensitivity may partly be due to the smaller amount of ^{125}I incorporated into the link-protein preparation (about half as much as of the other antigens). It may also be that the maleoylation procedure used to render the link proteins soluble in the buffers may interfere with the subsequent assay.

The radioimmunoassay procedures developed were applied in three sets of experiments. From previously published data it is known that hyaluronic acid, hyaluronic acid-binding region and link protein can be isolated as a very large complex from trypsin digests of proteoglycan aggregates (Heinegård & Axelsson, 1977; Hascall & Heinegård, 1979). The components in this complex can be separated under dissociative conditions on a Sephadex G-200 column, eluted with 4M-guanidinium chloride. Two major u.v.-absorbing peaks are obtained (Fig. 5; see Heinegård & Hascall, 1974), which have been identified as the hyaluronic acid-binding region (the larger component) and the link protein respectively (Heinegård & Hascall, 1974). The effluent fractions from such a chromatogram were therefore analysed for contents of hyaluronic acid-binding region and link protein both by the electroimmunoassay procedure and by the radioimmunoassay (Fig. 5). It appears that both procedures are specific and only react with components in the expected peak, i.e. only the material in the first peak reacted in the assay procedures for the hyaluronic acid-binding region, and only the material in the second peak reacted in the assay procedure for link protein. Specific measurements of either of the two components can therefore be made with no interference from the other. These data corroborate and extend previous results obtained with precipitating antibodies (Wieslander & Heinegård, 1979), indicating that the two components are not antigenically related.

In another experiment a bovine nasal-cartilage proteoglycan monomer (fraction A1-D1) preparation

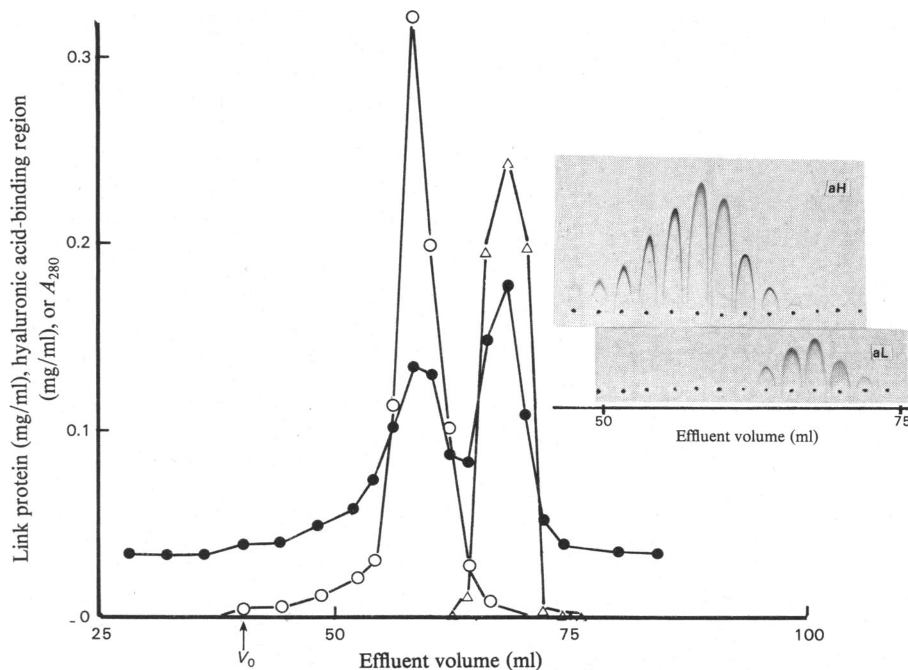

Fig. 5. *Sephadex G-200 chromatography under dissociative conditions (4M-guanidinium chloride) of the complex of hyaluronic acid-binding region, link protein and hyaluronic acid*

The contents of the tubes were assayed for content of hyaluronic acid-binding region by radioimmunoassay (○) and by electroimmunoassay (insert aH) and for contents of link protein by radioimmunoassay (△) and by electroimmunoassay (insert aL). ●, A_{280} (protein); V_0, void volume. For full details see the text.

was chromatographed on Sepharose 2B, essentially as described previously (Heinegård, 1977). The effluent was monitored for content of uronic acid (Fig. 6), and subfractions were pooled as indicated on the horizontal axis in Fig 6(a). The material in the void volume, which may represent monomers bound to small amounts of remaining hyaluronic acid (Hascall & Heinegård, 1974b), was not further analysed. The materials in the included seven fractions were sequentially digested with chondroitinase ABC and trypsin and analysed for contents of uronic acid (chondroitin sulphate) by the carbazole procedure, and for contents of hyaluronic acid-binding region and of chondroitin sulphate–peptides by using the radioimmunoassay procedures developed here. The ratios of the various components are shown in Fig. 6(a). It appears that the more retarded and therefore smaller the proteoglycan monomers, the higher was the relative content of hyaluronic acid-binding region. Measured as ratio to uronic acid or chondroitin sulphate–peptides, this ratio gradually increased with increasing elution volume of the fraction. Interestingly, the ratio of uronic acid (chondroitin sulphate) to chondroitin sulphate–peptides remained constant for the differently sized proteoglycans, maybe indicating that the polysaccharide-attachment portion of the protein core has a relatively constant substitution with chondroitin sulphate chains. On the basis of data from analyses of intact and enzyme-treated fractions from Sepharose 2B chromatography, it has been suggested that proteoglycans differ in size mainly by having a constant hyaluronic acid-binding region and a variably large chondroitin sulphate-rich region (Heinegård, 1977). The direct measurements of these structures, discussed above, give supporting evidence for such a model and also indicate that the radioimmunoassay procedures discussed can be useful in structural analysis of proteoglycans.

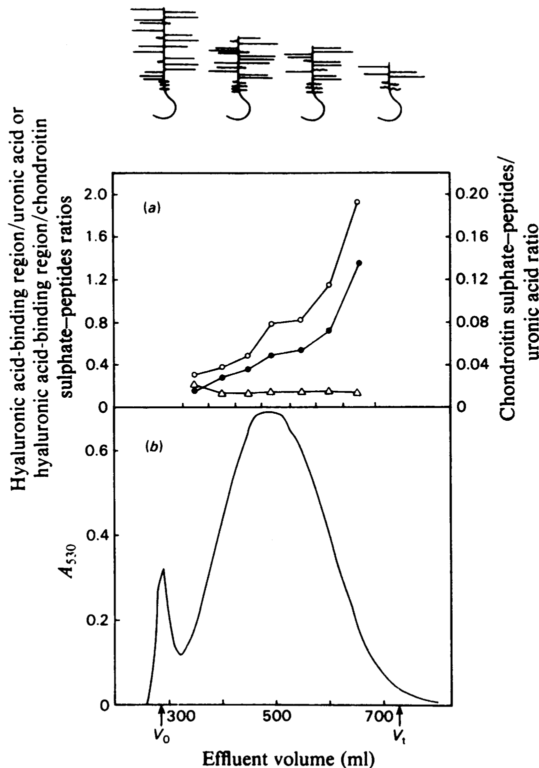

Fig. 6. *Sepharose 2B chromatography of proteoglycan monomers*

For experimental details see the text. (b) ——, A_{530} (carbazole reaction). The fractions were pooled as indicated on the horizontal axis of part (a) of the Figure. The material recovered was analysed for contents of hyaluronic acid-binding region and chondroitin sulphate–peptides by using radioimmunoassay. Uronic acid contents of fractions were determined by using the carbazole reaction. The ratios (w/w) of hyaluronic acid-binding region to uronic acid (○), hyaluronic acid-binding region to chondroitin sulphate–peptides (●) and chondroitin sulphate–peptides to uronic acid (△) are shown in part (a). V_0, void volume; V_t, total volume.

A third set of experiments was performed to determine the size distribution of chondroitin sulphate–peptides and hyaluronic acid-binding-region fragments prepared by proteolysis. A sequential chondroitinase–trypsin digest of proteoglycan monomers (A1-D1) was chromatographed on a Sepharose 6B column (Fig. 7). The distribution of neutral sugar, uronic acid and protein in the effluent fractions reproduced previously obtained patterns (Heinegård & Axelsson, 1977), showing an early-eluted peak (A) containing the keratan sulphate-rich region, an intermediate peak (B) containing the chondroitin sulphate-linkage region oligosaccharide peptides and a total-volume peak containing disaccharides produced from chondroitin sulphate by the action of chondroitinase.

Samples of the effluent fractions were diluted as discussed in the Materials and Methods section and assayed for contents of antigenic fragments from the hyaluronic acid-binding region and the chondroitin sulphate–peptides (Fig. 7). As discussed above, trypsin digestion does not destroy the antigenicity of the hyaluronic acid-binding region. The antigenic fragments were eluted in two peaks, at the positions of the intermediate and the small-size chondroitin sulphate-linkage-region oligosaccharide–peptides, respectively. Previous experiments (Heinegård & Axelsson, 1977) indicate that a major proportion of the peptides formed by trypsin digestion of isolated hyaluronic acid-binding region is eluted at a position indicating sizes corresponding to those observed

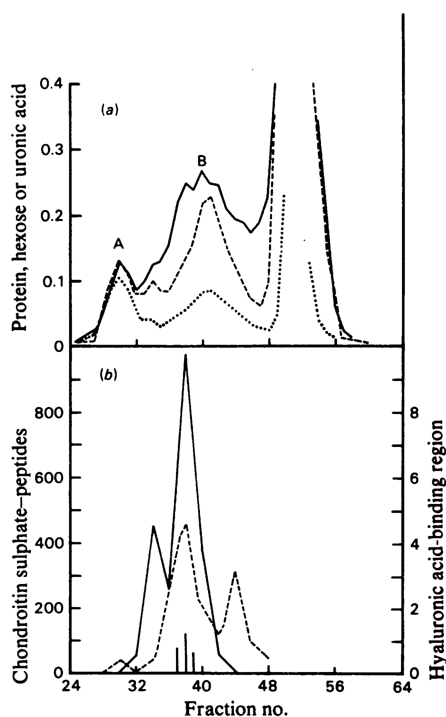

Fig. 7. *Sepharose 6B chromatography of a sequential chondroitinase/trypsin digest of proteoglycan monomers*
For experimental details see the text. (*a*) The effluent fractions were analysed for contents of uronic acid (----), hexose (····) and protein (——). (*b*) Samples of the fractions were diluted and analysed by radioimmunoassay for contents of hyaluronic acid-binding-region peptides (----) and chondroitin sulphate–peptides (——). Bars in part (*b*) indicate heights of 'rockets' obtained on electroimmunoassay for hyaluronic acid-binding region against anti-(hyaluronic acid-binding region) antibodies.

for the antigenic fragments. It is likely, then, that the major fragments produced by trypsin digestion contain antigenic sites. The results also show that when chondroitin sulphate linkage-region oligosaccharides are prepared directly from proteoglycan monomers, the preparations also contain peptides from the hyaluronic acid-binding region. The antigenic fragments from the chondroitin sulphate–peptides were eluted at the position of the corresponding peak identified by chemical methods. The peak, however, is very broad, whereas the antigenic fragments are eluted in one minor early peak and one major peak of intermediate elution position. The latter coincided with the peak position observed on chemical analyses. It is possible that the antigenic fragments represent relatively short sequences of the chondroitin sulphate-rich region. It may be that they are repeating sequences, since larger molecules have a higher relative content of these antigenic fragments as well as of uronic acid, as discussed above.

General discussion

The data presented in this paper deal with the determination of substructures of proteoglycans by very sensitive methods.

In order to understand better the function of normal and diseased cartilage, it is important to develop procedures that can assist in establishing structural variations of the proteoglycans. Preferably the techniques should be sensitive enough to allow analysis of small biopsies and therefore more homogeneous pieces of tissue. Such techniques are particularly valuable in the analysis of cartilage degeneration. By using the radioimmunoassay procedures developed it is quite feasible to use such thin sections, representing microgram quantities of tissue which are normally used for microscopy, also for chemical analysis. It should be stressed, however, as shown in the present and previous papers (Wieslander & Heinegård, 1979), that proteoglycans contain more than one antigenic site and that the relative proportion of the antigenic sites may vary in different preparations, e.g. in fractions differing in the size of the molecules. Therefore it is essential that well-defined antisera only reacting with single substructures are used in radioimmunoassay procedures. If this requirement is not fulfilled similar quantities (by weight) of proteoglycan preparations may give very different results in the immunoassay procedures.

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