Isolation and biochemical characterization of the tryptic fragments of bovine nasal-cartilage proteoglycan monomer of high buoyant density

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Relatively homogeneous fractions of proteoglycan fragments were prepared from tryptic digests of the 4M-guanidinium chloride extract of bovine nasal cartilage. Glycosaminoglycan-containing fragments were separated from non-proteoglycan contaminants by ion-exchange chromatography and fractionated by equilibrium density-gradient centrifugation under dissociative conditions. The fractions of highest buoyant density were chromatographed on a column of Sepharose 4B, digested with chondroitinase ABC and chromatographed on a column of Sepharose 6B, yielding two distinct fractions: fraction B/6B-4 contained fragments from the chondroitin sulphate-bearing region of the proteoglycan monomer, and fraction B/6B-2 fragments from the keratan sulphate-rich region, most probably including a chondroitin sulphate-bearing monomer segment. By dansyl chloride analysis, fraction B/6B-2 had alanine and leucine as sole and fraction B/6B-4 had isoleucine and leucine as greatly predominant N-terminal amino acids, indicative of the relative homogeneity of these preparations of cartilage proteoglycan monomer fragments.

The cartilage proteoglycan monomer appears to contain three chemically and functionally distinct portions: at one end there is a mannose-containing glycoprotein region responsible for the binding to hyaluronic acid, at the other end there is a region of protein core of variable length with chondroitin sulphate side chains, and in between there is a region of protein core to which are attached keratan sulphate and oligosaccharides that lack mannose (Heinegård & Hascall, 1974; Heinegård & Axelsson, 1977; Lohmander *et al.*, 1980).

Homogeneous preparations of cartilage proteoglycan link proteins have been produced by several groups of investigators (Bonnet *et al.*, 1978; Baker & Caterson, 1979; Tang *et al.*, 1979; Treadwell *et al.*, 1980). However, despite considerable effort over many years, no comparably homogeneous preparation of the cartilage proteoglycan monomer, or of a monomer fragment, has been obtained. Indeed, evidence has been presented suggesting that cartilage extracts may contain multiple different proteoglycan monomers with different electrophoretic or functional properties (Roughley & Mason, 1976; Stanescu *et al.*, 1977; Swann *et al.*, 1979; Heinegård & Hascall, 1979; Heinegård *et al.*, 1981).

The studies reported in the present paper represent part of an effort aimed at the systematic

isolation of the fragments of bovine nasal-cartilage proteoglycan monomer produced by digestion with trypsin and at the characterization of the fragments in terms of their structural and immunological properties. We describe here the isolation of two relatively homogeneous fractions of high-buoyant-density fragments of bovine nasal-cartilage proteoglycan that, like the fragments described by Heinegård & Axelsson (1977), appear to be derived from the keratan sulphate-rich and the chondroitin sulphate-bearing regions of the proteoglycan monomer respectively.

Materials and methods

Chemicals and enzymes

Guanidinium chloride was purchased from Heico (Delaware Water Gap, PA, U.S.A.), trypsin (diphenylcarbamoyl chloride-treated; type XI) and pepstatin A were from Sigma Chemical Co. (St. Louis, MO, U.S.A.), chondroitinase ABC and endo- β -galactosidase were from Miles Laboratories (Elkhart, IN, U.S.A.), dansyl (5-dimethylaminonaphthalene-1-sulphonyl) chloride, dansyl-amino acid standards and constant-boiling HCl were from Pierce Chemical Co. (Rockford, IL, U.S.A.), micro-polyamide sheets and DEAE-cellulose (type 20)

were from Schleicher and Schuell (Keene, NH, U.S.A.), Ultrogel AcA-54 was from LKB Instruments (Rockville, MD, U.S.A.) and Spectrapor membrane tubing was from Spectrum Medical Industries (Los Angeles, CA, U.S.A.).

Proteoglycan extract

Bovine nasal cartilage was extracted with 4M-guanidinium chloride in 50mM-sodium acetate buffer, pH 5.6, containing 1mM-EDTA and pepstatin, by the method of Hascall & Sajdera (1969). The extract was filtered, dialysed against distilled water and freeze-dried.

Proteoglycan fractionation (see Scheme 1)

A 300mg portion of crude proteoglycan extract was dissolved in 30ml of 10mM-sodium phosphate buffer, pH 7.0. Diphenylcarbamoyl chloride-treated trypsin (1mg) was added, and the solution was incubated at 37°C for 6h. The trypsin digest was applied to a 60cm × 2.5cm column filled with DEAE-cellulose equilibrated with 10mM-sodium

phosphate buffer, pH 7.0, at room temperature, and was eluted in stepwise fashion with 400ml each of 0.1M-KCl, 0.5M-KCl and 1.0M-KCl in 10mM-sodium phosphate buffer, pH 7.0 (Fig. 1). The 0.5M-KCl eluates ('DEAE-I' fraction) and the 1.0M-KCl eluates ('DEAE-II' fraction) were pooled, dialysed against distilled water and freeze-dried. Typical yields were: DEAE-I fraction, 30mg; DEAE-II fraction, 180mg.

DEAE-II fraction was dissolved at a concentration of 1.5mg/ml in 4M-guanidinium chloride in 50mM-sodium acetate buffer, pH 5.6, containing sufficient CsCl to give a density of 1.50g/ml. The solution was centrifuged in a Beckman model 50Ti rotor at 32000rev./min for 42–68h at 5°C. The density gradient was fractionated by suction from below with a timed peristaltic pump into six fractions of equal volume, which were then dialysed against distilled water and freeze-dried. Typical runs starting with 180mg of DEAE-II fraction yielded approx. 65mg, 25mg, 20mg, 15mg, 10mg and 20mg in successive fractions from the bottom upward.

The bottom fraction of the dissociative density gradient was applied to a 60cm × 2cm column of Sepharose 4B in 0.5M-NaCl/1mM-EDTA/50mM-sodium phosphate buffer, pH 7.3, at room temperature, and the column was eluted with the same buffer (Fig. 2). The indicated eluate pools were dialysed against distilled water and freeze-dried, then dissolved in 0.1M-sodium acetate/0.1M-Tris buffer, pH 7.4, at a concentration of 5mg/ml and digested with chondroitinase ABC (0.025 nominal unit/ml) for 18h at 37°C under toluene. About one-third the amount of material subjected to chondroitinase digestion was recovered after dialysis against distilled water and freeze-drying.

Approx. 50mg of chondroitinase digest was applied to a 60cm × 2.5cm column of Sepharose 6B in 0.5M-NaCl/1mM-EDTA/50mM-sodium phosphate buffer, pH 7.3, at room temperature, and the column was eluted with the same buffer. Eluate fractions from the two major peaks of carbazole reactivity (Fig. 3) were pooled, and after dialysis and freeze-drying the yield of fraction B/6B-2 was about 12mg and that of fraction B/6B-4 was about 8mg.

β-Elimination and keratanase digestion

A 20mg portion of fraction B/6B-2 or B/6B-4 was incubated in 1ml of 1M-NaBH₄ in 50mM-NaOH for 48h at 45°C (Lohmander *et al.*, 1980). After neutralization with acetic acid, the solution was applied to a 60cm × 2.5cm column of Ultrogel AcA-54 in 0.5M-NaCl/1mM-EDTA/50mM-sodium phosphate buffer, pH 7.3, and the column was eluted with the same buffer. The eluate pools indicated in Fig. 4 were dialysed in sacs of 2000-mol.wt.-cut-off dialysis tubing and freeze-dried. After the setting aside of material for sugar analysis, the remainder

Scheme 1. Method of preparation of bovine nasal cartilage proteoglycan tryptic fragments of high buoyant density

For full experimental details see the text.

was dissolved in 1 ml of 0.2 M-sodium acetate buffer, pH 5.8, incubated with 0.01 unit of endo- β -galactosidase (keratanase) for 18 h at 37°C under toluene, and applied to and re-eluted from the same Ultrogel AcA-54 column. Fractions B/6B-2 and B/6B-4 (5 mg/ml each) were digested with keratanase under the same conditions.

Analysis

Carbazole reactivity was determined by the method of Bitter & Muir (1962), and anthrone reactivity by the method of Yemm & Willis (1954). Amino acid analyses were performed on 1 mg samples hydrolysed in 6 M-HCl for 18 h at 120°C, with and without performate oxidation, by using the Beckman 118CL amino acid analyser. For determination of *N*-terminal amino acids, 1 mg samples were dissolved in 0.2 ml of 6 M-urea in 0.1 M-NaHCO₃, and 0.2 ml of dansyl chloride (10 mg/ml) in acetone was added and the pH was adjusted to about 8.5 with 15% (w/v) Na₂CO₃. The solution was incubated for 18 h at 37°C, dialysed against ten changes of 100 vol. of distilled water over 4 days and freeze-dried. Hydrolysis and t.l.c. were performed by standard procedures (Woods & Wang, 1967). Sugar compositions were determined by g.l.c. of the alditol acetate derivatives (Sawardeker *et al.*, 1965). Immunodiffusion and sodium dodecyl sulphate/polyacrylamide-gel electrophoresis utilized antisera and methods described elsewhere (Keiser *et al.*, 1972; Keiser, 1982).

Results

Fractionation of proteoglycan fragments (Scheme 1)

Crude 4 M-guanidinium chloride extract of bovine nasal cartilage was digested with diphenyl-carbamoyl chloride-treated trypsin and separated into three fractions by DEAE-cellulose column chromatography (Fig. 1). Fraction DEAE-0 con-

tained material with absorption at 280 nm, anthrone reactivity but no carbazole reactivity, and no reactivity with anti-proteoglycan sera by immunodiffusion; it was discarded. Fraction DEAE-I showed considerable absorption at 280 nm and minimal carbazole reactivity, and was found on analysis by immunodiffusion and sodium dodecyl sulphate/polyacrylamide-gel electrophoresis to contain an assortment of proteoglycan monomer fragments and a large link-protein fragment. Fraction DEAE-II contained the great bulk of carbazole-reactive material of the original crude cartilage extract. No additional carbazole-reactive material was eluted by KCl solutions of concentrations greater than 1 M.

The DEAE-II fraction of tryptic proteoglycan fragments was subfractionated by CsCl-equilibrium-density-gradient ultracentrifugation, and the subfractions of greatest buoyant density were chromatographed on a Sepharose 4B column. Material recovered from the eluate fraction pools indicated in Fig. 2 was digested with chondroitinase ABC and rechromatographed on Sepharose gel columns. After chondroitinase digestion, the material originally isolated as Sepharose 4B pool 1 was eluted exclusively in the fractions corresponding to Sepharose 4B pools 3 and 4. The results of Sepharose 6B column chromatography of the chondroitinase ABC digest of Sepharose 4B pool 1 are shown in Fig. 3; similar results were obtained with the digests of pools 2–4. Pooled eluate fractions from the column void and total volumes yielded very small amounts of material that did not react on immunodiffusion against anti-proteoglycan sera BN40A and BN41B (Keiser, 1982). The earlier-eluted peak of carbazole reactivity in the included volume (B/6B-2; $K_D = 0.36$) coincided with a peak of 280 nm absorption; little 280 nm absorption was found with the second peak (B/6B-4; $K_D = 0.70$). The elution positions of these two peaks of carbazole reactivity, and the coincidence of the first

Fig. 1. Purification of tryptic fragments of cartilage proteoglycan by DEAE-cellulose column chromatography. Samples of volume 0.1 ml were used in the carbazole determination. —, A_{530} (carbazole reaction); — — —, A_{280} ; ·····, concn. of KCl. For full experimental details see the text.

Fig. 2. *Sepharose 4B* column chromatography of the tryptic fragments of cartilage proteoglycan of highest buoyant density. Samples of volume 0.05 ml were used in the carbazole determination. Eluate fractions were pooled as indicated by the numbered horizontal bar above the carbazole peak. —, A_{530} (carbazole reaction); ----, A_{280} . For full experimental details see the text. In this and subsequent Figures V_0 and V_t indicate the void volume and the total volume respectively.

Fig. 3. *Sepharose 6B* column chromatography of the chondroitinase ABC digest of the high-buoyant-density tryptic fragments of cartilage proteoglycan from *Sepharose 4B* pool 1 (Fig. 2)

Samples of volume 0.2 ml were used for the carbazole determination. Eluate fractions were pooled as indicated by the labelled horizontal lines. —, A_{530} (carbazole reaction); ----, A_{280} . For full experimental details see the text.

with a peak of 280nm absorption, were unchanged on redigestion of the isolated fractions with trypsin or on gel chromatography under dissociating conditions, i.e. *Sepharose* CL-6B in 4M-guanidinium chloride.

Characterization of fractions B/6B-2 and B/6B-4

The *O*-serine-linked carbohydrate of the chondroitinase-treated proteoglycan fragments in fractions B/6B-2 and B/6B-4 was removed by the alkaline borohydride reaction under mild conditions (Kieras, 1975) and chromatographed on a column of *Ultrogel* AcA-54 (Fig. 4). Both fractions yielded coincident high peaks of anthrone reactivity and carbazole reactivity shortly before the column total volume; however, the eluates from fraction B/6B-2

showed, in addition, a broad earlier-eluted peak of coincident anthrone reactivity and carbazole reactivity, with anthrone reactivity predominant. The sugar composition of the material recovered from these eluate peaks (Table 2) suggests that peak 1 of fraction B/6B-2 contained keratan sulphate with its Gal-GluNAcSO₄ repeating unit and terminal GalNAc-*O*-serine (Bray *et al.*, 1967), that the fraction B/6B-4 peak contained the Gal-Gal-Xyl chondroitin sulphate-protein bridge, along with a short stretch of the GluA-GalNAcSO₄ repeating unit of chondroitin sulphate (Rodén & Smith, 1966), and that peak 2 of fraction B/6B-2 contained a mixture of chondroitin sulphate 'stubs' and other oligosaccharides.

Fractions B/6B-2, B/6B-4 and *Ultrogel* AcA-54

Fig. 4. Ultrogel AcA-54 column chromatography of alkaline-borohydride-treated fractions B/6B-2 and B/6B-4. Samples of volume 0.2 ml were used for the carbazole determination, and samples of volume 0.3 ml for the anthrone determination. Eluate fractions were pooled as indicated by the horizontal bars. ·····, A_{625} (anthrone reaction); —, A_{530} (carbazole reaction); ---, A_{280} . For full experimental details see the text.

peak 1 of alkaline-borohydride-treated fraction B/6B-2 were digested with keratanase, the endo- β -galactosidase from *Escherichia freundii* specific for the linkages in keratan sulphate (Fukuda & Matsumura, 1976), and the digests were rechromatographed on the same Sepharose 6B or Ultrogel AcA-54 column. After digestion with this enzyme, the anthrone reactivity and carbazole reactivity of the material initially isolated as fraction B/6B-2/AcA-54 peak 1 (Fig. 4) was found in the eluate fractions corresponding to peak 2. The keratanase digest of fraction B/6B-2 formed a later-eluted peak of 280nm absorption, carbazole reactivity and anthrone reactivity (K_D before keratanase, 0.36; K_D after keratanase, 0.45), as well as a peak of anthrone reactivity in eluate fractions from the column total volume. The elution position of fraction B/6B-4 was unchanged after digestion with keratanase, and there was no peak of anthrone reactivity at the column total volume.

The amino acid composition of fraction B/6B-4 (Table 1) is typical of that described for fragments from the chondroitin sulphate-containing portion of the proteoglycan monomer core protein (Mathews, 1971), and is distinctly different from that of fraction B/6B-2.

When sodium dodecyl sulphate/polyacrylamide-

Table 1. Amino acid composition of fractions B/6B-2 and B/6B-4

	Amino acid composition (residues/1000 residues)	
	Fraction B/6B-2	Fraction B/6B-4
Asp	32	60
Thr	53	39
Ser	85	153
Glu	191	158
Pro	199	94
Gly	78	172
Ala	58	56
CyS	10	4
Val	62	61
Met	0	0
Ile	22	42
Leu	51	95
Tyr	17	4
Phe	59	17
His	29	25
Lys	34	7
Arg	20	13
GlcN/GalN ratio	3.41	0.12

gel electrophoresis was performed with fractions B/6B-2 and B/6B-4, no discrete protein bands were found in gels stained with Coomassie Blue. Caputo

et al. (1980) have reported similar negative results.

Sephacrose 4B pool 1, both before and after digestion with chondroitinase ABC, was found to contain strongly predominant leucine, isoleucine and alanine *N*-terminal amino acids by the dansyl chloride method. Fraction B/6B-2 contained only leucine and alanine *N*-terminals and fraction B/6B-4 a great predominance of *N*-terminal leucine and isoleucine; these results were unchanged after digestion with keratanase or redigestion with trypsin. Early and late eluate pools from the peak comprising fraction B/6B-2 (Fig. 3) yielded spots corresponding to leucine and alanine of similar relative intensity. Sepharose 6B fractions comparable with fractions B/6B-2 and B/6B-4 derived from the gradient fraction of second highest density (Scheme 1) or from Sepharose 4B pools 2–4 (Fig. 2) yielded the same predominant *N*-terminals, but were more likely to be contaminated with additional end groups. Similar results were obtained when density-gradient-purified aggregate (Hascall & Sajdera, 1969) was used instead of crude proteoglycan aggregate.

Discussion

We describe here the preparation of two chemically distinct and relatively homogeneous fractions of bovine nasal-cartilage proteoglycan monomer fragments of high buoyant density. The fractionation scheme employed relies on ion-exchange chromatography rather than the classical density-gradient centrifugation method of Hascall & Sajdera (1969) for the separation of proteoglycan fragments from collagen and other cartilage components not covalently associated with glycosaminoglycans. It has the advantage of allowing more efficient use of the ultracentrifuge and of not systematically excluding proteoglycans of low buoyant density that have relatively short chondroitin sulphate-bearing segments (Rosenberg *et al.*, 1976) or that represent different proteoglycan species (Stanescu & Sweet, 1981; Heinegård *et al.*, 1981).

Trypsin has been shown to degrade cartilage proteoglycan aggregates into three major components: monomer fragments bearing chondroitin sulphate, a monomer fragment enriched in keratan sulphate, and a complex consisting of hyaluronic acid, the hyaluronic acid-binding portion of the monomer and a large fragment of the link proteins (Heinegård & Hascall, 1974; Heinegård & Axelsson, 1977). All three components are present in the DEAE-II fraction (Keiser, 1982). The late elution of keratan sulphate-rich monomer fragments from DEAE-cellulose could be due to the presence of some degree of oversulphation in the keratan sulphate of bovine nasal cartilage, similar to that found in the chondroitin sulphate produced by cultured

rabbit chondrocytes (Srivastava *et al.*, 1974). Alternatively, the keratan sulphate-rich monomer fragment produced by trypsin may also contain one or more chondroitin sulphate side chains. The latter explanation is supported by the finding that all the material in Sepharose 4B pool 1 (Fig. 2), including the keratan sulphate-containing fragments later isolated as fraction B/6B-2, is decreased in apparent hydrodynamic size by digestion with chondroitinase ABC. The presence of xylose in the material from peak 2 of alkaline-borohydride-treated fraction B/6B-2 (Fig. 4 and Table 2) also supports the possibility that the keratan sulphate-rich monomer fragments contain some chondroitin sulphate. We have no explanation for the delayed elution from DEAE-cellulose of the proteoglycan fragments of lower buoyant density ascribed to the hyaluronic acid–monomer fragment–link protein complex (Keiser, 1982).

Our keratan sulphate-rich fragment fraction, B/6B-2, and our chondroitin sulphate-bearing fragment fraction, B/6B-4, resemble in amino acid and sugar composition and in elution position from Sepharose 6B columns the proteoglycan monomer fragment fractions previously described by Heinegård & Axelsson (1977). Heinegård & Axelsson (1977) used two methods to produce their fractions, neither identical with the procedure used in the present work, and, though similar, their fractions appear to be not identical with ours. They recovered 20–30% of total keratan sulphate in their chondroitin sulphate-bearing fragment fraction, whereas we find no keratan sulphate in fraction B/6B-4 and, contrariwise, find evidence suggestive of the presence of a small amount of chondroitin sulphate in fraction B/6B-2. We find that fractions B/6B-2 and B/6B-4 contain cysteine, which was not reported by Heinegård & Axelsson (1977) in their fractions. The absence of a peak of 280nm absorption in association with fraction B/6B-4 (Fig. 3) stands in contrast with the substantial peak of Folin reactivity shown by Heinegård & Axelsson (1977) in asso-

Table 2. Sugar composition of Ultrogel AcA-54 eluates of alkaline borohydride-treated fractions B/6B-2 and B/6B-4

	Sugar composition (μmol)		
	Fraction B/6B-2		Fraction B/6B-4
	Subfraction AcA-54-1	Subfraction AcA-54-2	
Xylose	0.007	0.338	1.119
Mannose	0.050	0.074	0.032
Galactose	1.255	1.726	1.902
Glucose	0.047	0.258	0.246
Glucosamine	1.056	1.055	0.166
Galactosamine	0.151	0.816	1.794

ciation with their chondroitin sulphate-bearing fragment eluates (their Figs. 5 and 13). These differences could, at least in part, be due to differences in the preparative methods used. For example, a cysteine-bearing portion of core protein may be protected during trypsin digestion of proteoglycan aggregate but not of the monomer, as is the case with the hyaluronic acid-binding region (Heinegård & Hascall, 1974; Björnsson & Heinegård, 1981), hence present in our fraction B/6B-2 but not in Heinegård & Axelsson's (1977) fraction A1-D1-CB-T-6B1. The dissociative density-gradient fractionation step that we employ may serve to remove from fraction B/6B-4 the chondroitin sulphate-bearing fragments relatively enriched in aromatic amino acids present in the corresponding fractions obtained by Heinegård & Axelsson (1977). The report by Heinegård & Axelsson (1977) presents insufficient data to allow assessment of the degree of chemical homogeneity of their fractions; fractions that we prepared from purified proteoglycan monomer by their method were more heterogeneous than fractions B/6B-2 and B/6B-4, as judged by the number of amino acid end groups detected. Finally, their immunological evidence for homogeneity (Wieslander & Heinegård, 1979) should be re-evaluated in light of the immunodiffusion studies presented in the following paper (Keiser, 1982).

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