Extraction of Glycosaminoglycan Peptide from Bovine Nasal Cartilage with 0.1 M Sodium Acetate

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Cartilage is a nonedible byproduct with little saleable value, but it is rich in glycosaminoglycan (GAG), which can be converted to a highly profitable product. In this study, GAG was extracted from bovine cartilaginous tissues including nasal cartilage, occipital articular cartilage, and temporomandibular joint disk without using the expensive exogenous proteinase commonly used to extract GAG from tissues. The GAG was extracted with highest yield (≈80% of total tissue uronic acid) by incubating nasal cartilage in 0.1 M sodium acetate, pH 4.5, at 37 °C. Tissue autolysis was suggested to occur under the incubation condition to release GAG-peptide. The GAG-peptide purified by DEAE ion-exchange chromatography contained approximately 7% protein, 89% chondroitin sulfate, and 4% keratan sulfate and had no capability to interact with hyaluronic acid. The results suggest that the extraction method with 0.1 M sodium acetate described in this paper is useful for the preparation of GAG-peptide at a low cost.

Keywords: *Glycosaminoglycan; chondroitin sulfate; cartilage; animal waste; autolysis*

INTRODUCTION

Cartilaginous tissues are nonedible byproducts with little saleable value, but they may contain materials which, if extracted and purified, are useful. Cartilage contains collagen and proteoglycans as its major extracellular matrix constituents. Aggrecan is the predominant proteoglycan in hyalin cartilage. The structure of aggrecan has been extensively studied (Heinegård and Oldberg, 1989; Hardingham et al., 1994). The aggrecan monomer consists of core protein of M_r approximately ≈200 000 to which glycosaminoglycan (GAG) chains including approximately 100 chondroitin sulfate (CS) chains of M_r 10 000−25 000, 30−60 keratan sulfate (KS) chains of M_r 3 000−15 000, and N- and O-linked oligosaccharides are covalently attached. The core protein has three distinct globular domains, G1 and G2 at the amino terminus and G3 at the carboxy terminus. Most of the GAG chains are found between G2 and G3. All of the CS and about half of the KS chains are found in the CS-rich region, accounting for more than half of the core protein. A second region, the KS-rich region, is located between the CS-rich region and the G2 globular domain of core protein. The G1 amino-terminal region can interact noncovalently with GAG hyaluronic acid to form large aggregate with total molecular mass in the hundreds of millions.

GAG chains account for ≈90% of aggrecan mass, in which the majority (>90%) of GAG is CS with small amount of KS. Chondroitin sulfate is an anionic molecule that consists of repeating disaccharide units of *N*-acetylgalactosamine 4- or 6-sulfate and D-glucuronic acid. This GAG has been reported to have a wide range of applications in the pharmaceutical, cosmetic, and food industries. For example, CS has been shown to have chondroprotective (Dean et al., 1991) and antiatherogenic (Matsushima et al., 1987) effects in experimental animals. Chondroitin sulfate−iron complex has been reported as a potent antianemic agent, in which CS contributes to an increased bioavailability of iron (Barone et al., 1988). In eye banks, CS is used to increase storage time of corneas (Keates and Rabin, 1988).

Currently available GAGs from chemical companies are too expensive to prepare GAG products, and thus the development of techniques of low-cost production of GAGs is needed. Chondroitin sulfate can be prepared from intact aggrecan by proteolysis or alkaline treatment (Rodén et al., 1972). More practically, CS is directly liberated from the cartilage by digestion with an exogenous proteinase (e.g. papain) (Rodén et al., 1972). Chondroitin sulfate can also be liberated by activation of endogenous enzymes (autolysis). Jibril (1967) incubated calf scapula cartilage in 0.1 M sodium acetate, ranging in pH from 2 to 9, at three different temperatures (3, 25, and 37 °C) and found that the amount of uronic acid released by tissue autolysis is highest at pH 4.2 and 37 °C. The yield of extractable GAGs may vary between cartilaginous tissues from different anatomical location, but there is limited information available in this regard. There is also not much information available concerning the chemical composition of autolysis products of cartilage, which is important for preparation of GAG products. In the present study, samples of bovine nasal cartilage, occipital articular cartilage, and temporomandibular joint disk (fibrocartilage) were incubated in an acidic sodium acetate buffer at 37 °C, and the yield of extractable GAG was compared among the three tissues. GAG fractions from the nasal cartilage, which had a higher extraction yield than did the others, were further examined using gel chromatography, ion-exchange chromatography, enzymatic digestion, and cellulose acetate electrophoresis.

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EXPERIMENTAL PROCEDURES

Materials. Bovine nasal cartilage, occipital articular cartilage, and fibrocartilaginous disk of the temporomandibular joint from five young adult (2−3-year-old) cattle were obtained fresh at a local abattoir (Alsask Beef Co. Ltd., Edmonton). These tissues were transported to the laboratory on ice, rinsed with cold water, and dissected free of noncartilaginous adherent tissues. They were stored at −20 °C until extracted. The whale CS was obtained from Miles Laboratories. The chicken CS was prepared from tibial articular cartilage of 1-month-old broiler chickens as described previously (Nakano and Sim, 1995). It was the CS precipitated at an ethanol concentration of 40%. Bovine nasal cartilage proteoglycan was prepared by chromatography of 4 M guanidinium chloride extract of tissue on a DEAE-cellulose column (Antonopoulos et al., 1974).

Investigation of Factors Affecting Extractability of GAG. The first experiment was carried out to determine the effects of time, pH, and temperature on the extractability of uronic acid from bovine nasal cartilage. Frozen samples of nasal cartilage were thawed at 4 °C, finely diced (approximately 1 mm^3 in size), and thoroughly mixed. The diced samples (≈1 g wet weight) were incubated in duplicate in 10 volumes of 0.1 M sodium acetate buffer, pH 4.0, at 37 °C. Aliquots of supernatants of incubation mixtures were then collected every hour until 7 h, and after 12 and 24 h, and assayed for uronic acid by the carbazole reaction (see below). Further portions of diced cartilage were then incubated in duplicate in 0.1 M sodium acetate buffer at various pH (3, 4, 5, 6, 7, 8, and 9) at 37 °C for 7 h. The effect of temperature was determined by incubating cartilage samples in 0.1 M sodium acetate buffer, pH 4.0, at various temperatures (4, 21, 37, 50, 60, and 70 °C) for 7 h.

The extractability of uronic acid was then compared among the bovine cartilage tissues. Triplicate samples of each of nasal cartilage, occipital articular cartilage, and temporomandibular joint disk fibrocartilage were incubated in 0.1 M sodium acetate buffer, pH 4.0, at 37 °C for 7 h. Each incubation mixture was then centrifuged to separate the extract from the residual tissue. The latter was digested with papain, and uronic acid contents in both the extract and the residual tissue were determined to estimate the proportion of uronic acid liberated as percent of total uronic acid recovered.

Effects of proteinase inhibitors on the release of uronic acid were examined by incubating the nasal cartilage in triplicate at 37 °C for 7 h in 0.1 M sodium acetate, pH 4.0, with and without 7.3 mM pepstatin (an inhibitor of carboxypeptidase; Aoyagi et al., 1972) or 0.4 M 6-aminohexanoic acid (an inhibitor of plasmin; Steffen and Steffen, 1976). Both inhibitors were obtained from Sigma Chemical Co. After incubation, aliquots of medium collected were assayed for uronic acid by the carbazole reaction.

Extraction of GAGs from Bovine Nasal Cartilage. The nasal cartilage (≈10 g) was incubated in 0.1 M sodium acetate, pH 4.5, at 37 °C for 7 h to overnight. This tissue was chosen because it had the highest extractability of uronic acid among the three types of cartilage examined. The incubation mixture was centrifuged, and the supernatant, which was referred to as fraction I, was collected. Pieces of residual tissues were collected for histochemical examination. They were fixed in 4% buffered formalin, pH 7.3, routinely dehydrated and embedded in paraffin (Drury and Wallington, 1967), and stained for GAG with Safranin-O (Rosenberg, 1971). Safranin-O is a cationic dye commonly used to stain GAGs in the cartilage. The remaining portions of residual tissues were incubated in 0.5 N NaOH at 4 °C overnight and then in 1 N NaOH at 4 °C overnight to obtain fractions II and III, respectively. These fractions were neutralized with 6 N HCl. The residual tissue obtained after the alkaline treatment was then digested with papain (see below). After proteolysis, the digest (fraction IV) was deproteinized by adding cold trichloroacetic acid to a final concentration of 7% (w/v). All fractions (I−IV) obtained were dialyzed in water, freeze-dried, and stored at 4 °C for subsequent analyses.

Column Chromatography. Fractions I−IV and two samples of standard CS from whale and chicken cartilages were examined for molecular size using Sephacryl S-300 (Pharmacia Biotech. Canada Inc.). A portion of each sample was applied to a 1 × 110 cm column equilibrated and eluted with 0.5 M sodium acetate buffer, pH 5.8, containing 0.02% sodium azide. Blue dextran and tritiated water were used to determine void volume (V_0) and total volume (V_t) of the column, respectively. Eluates collected at a flow rate of 7 mL/h were assayed for GAG by the dimethylmethylene blue dye-binding method (Farndale et al., 1982). Fraction I had materials excluded from the Sephacryl S-300 column. These were incubated in 0.5 N NaOH at 4 °C overnight to remove peptide attached to GAG chains. The incubation mixtures were then neutralized with HCl and rechromatographed on the same column. A portion of fraction I was also applied to an ion-exchange column of DEAE-Sephacel (Pharmacia Biotech. Canada Inc.) to separate GAG-peptide from non-GAG materials (including collagen) and hyaluronic acid present in the fraction. The GAG-peptide fraction obtained was then chromatographed on Sepharose CL-2B (Pharmacia Biotech. Canada Inc.).

Analytical Methods. Moisture content in tissue was estimated from the loss of sample weight by heating at 110 °C overnight. The content of hydroxyproline (reflecting that of collagen) was determined according to the method of Stegemann and Stalder (1967) after hydrolysis in 6 N HCl at 110 °C for 20 h. Uronic acid was determined by the carbazole reaction (Kosakai and Yoshizawa, 1979) after proteolysis of samples with twice-crystallized papain (Sigma Chemical Co.) as described (Harab and Mourão, 1989). Sialic acid was determined according to the method of Warren (1959) after hydrolysis of samples in 0.1 N sulfuric acid at 80 °C for 1 h. The chromophore formed was extracted using 1-propanol (Nakano et al., 1994) instead of cyclohexanone as used by Warren (1959). Protein was determined according to the method of Lowry et al. (1951) using bovine serum albumin as a standard. Nitrogen was analyzed by the LECO model F-428 nitrogen analyzer (LECO Corp., St. Joseph, MI). Sulfate was determined using gelatin−barium chloride reagent (Dodgson and Price, 1962) after hydrolysis in 1 N HCl at 105 °C for 5 h. Galactose, glucosamine, and galactosamine were analyzed as alditol acetate derivatives by gas−liquid chromatography (Blakeney et al., 1983). Analysis of amino acid was carried out by HPLC as previously described (Sunwoo et al., 1995). A *t* test was used to determine significant differences between means.

Cellulose Acetate Electrophoresis. Samples of GAG preparations were electrophoresed on strips of cellulose acetate (Sepraphore III, Gelman Sciences) in 0.1 M pyridine/1.2 M acetic acid, pH 3.5 (Habuchi et al., 1973). The strips were stained in 0.1% (w/v) Alcian Blue 8GX in 0.1% (v/v) acetic acid containing 0.02% sodium azide for 3 min, washed in 0.1% acetic acid, cleared in Sepra Clear II (Gelman Sciences), and subjected to densitometric scanning at 600 nm.

Other Methods. Gel electrophoresis was carried out as described previously (Scott et al., 1989) using 5% polyacrylamide gels containing 0.1% sodium dodecyl sulfate in Tris−borate buffer, pH 8.6. Gels were stained with 0.2% Toluidine Blue in 0.1 M acetic acid. Digestion of GAGs with *Streptomyces hyaluronidase*, chondroitinase-ABC, chondroitinase-ACI, and endo-β-galactosidase was carried out as described (Murata, 1985; Nakano and Scott, 1989; Nakano et al., 1993). The proportion of unsaturated disaccharides released after chondroitinase-ABC digestion was determined using thin-layer chromatography (Shimada et al., 1987). For estimation of KS content in the sulfated GAG, a sample of GAG-peptide was digested with papain, and the resultant digest was fractionated with ethanol to obtain five fractions which were precipitated at ethanol concentrations of 18, 25, 40, 50, and 75%, respectively. Portions of the 75% ethanol fraction, which was the only fraction containing KS, were incubated with and without chondroitinase-ABC and electrophoresed on cellulose acetate. The chondroitinase-ABC resistant band, which was susceptible to the KS-degrading enzyme endo-β-galactosidase, was densitometrically scanned, and the content of KS was calculated

after correction for the lower staining intensity of this GAG relative to that of CS (0.73 vs 1; Hata and Nagai, 1973).

RESULTS

Tissue Composition. The moisture contents were similar ($p > 0.05$) among the three cartilaginous tissues (mean ± SD with five replicates = 71.7 ± 0.7% of wet weight). The concentration of hydroxyproline, expressed as milligrams per gram of dry tissue, was lower ($p < 0.05$) in the nasal cartilage (72.6 ± 9.6) than in the articular cartilage (91.5 ± 5.6) and the fibrocartilage (88.5 ± 7.9), with the concentration being similar ($p > 0.05$) between the latter two tissues. In contrast, the concentrations of uronic acid (milligrams per gram of dry tissue) in the nasal cartilage (68.7 ± 6.6) were approximately 4 and 7 times higher than those in the articular cartilage (19.1 ± 0.9) and fibrocartilage (10.1 ± 1.7), respectively. The concentration of sialic acid (milligrams per gram of dry tissue) was highest ($p < 0.05$) in the nasal cartilage (2.1 ± 0.1) and higher ($p < 0.05$) in the articular cartilage (1.6 ± 0.1) than in the fibrocartilage (0.9 ± 0.1).

Effects of Various Factors on the Extractability of GAG. The content of GAG uronic acid liberated from the nasal cartilage into the 0.1 M sodium acetate was dependent on time, pH, and temperature. The content of released uronic acid was found to increase during the 7-h incubation period and reached a plateau thereafter (Figure 1a). The content of released uronic acid was highest at pH 4−5 (Figure 1b) and 37 °C (Figure 1c). The yield of uronic acid released from tissue by incubation in 0.1 M sodium acetate, pH 4.0, at 37 °C (see Materials and Methods) was highest in the nasal cartilage (85 ± 5% of total uronic acid recovered). This value was 5 and 14 times higher ($p < 0.05$) than those from the articular cartilage (18 ± 3%) and the fibrocartilage (6 ± 1%), respectively. The release of uronic acid was reduced by 52% in the presence of pepstatin but not affected by 6-aminohexanoic acid.

Extraction of GAG from Bovine Nasal Cartilage. The GAG uronic acid extracted from the nasal cartilage with 0.1 M sodium acetate buffer, pH 4.5, at 37 °C (fraction I) accounted for ≈80% of total uronic acid recovered (Table 1). This was consistent with the results of histochemical examination of tissues showing a marked loss of GAG staining with Safranin-O in the cartilage after incubation (results not shown). The yield of uronic acid released was apparently lower than that found in the earlier experiment (85%, see above) with 10 times smaller amount of cartilage sample. However, the difference was not significant ($p > 0.05$). Glycosaminoglycans in the residual cartilage were then extracted with alkaline treatment and papain digestion (Table 1). Most uronic acid (70% of total) was recovered in fraction II solubilized with 0.5 N NaOH. Four times smaller proportions of uronic acid were recovered in fractions III and IV, solubilized with 1 N NaOH and papain, respectively. Hydroxyproline concentrations were highest in fraction III, higher in fraction IV than in fractions I and II, and similar between the latter two fractions. Sialic acid concentrations were highest in fraction I and decreased in fractions III, II, and IV in that order. Protein concentrations were lowest in fraction I and increased as fraction number increased.

On Sephacryl S-300 chromatography, the majority (84%) of fraction I GAG eluted in the void volume (Figure 2a). However, when treated with 0.5 N NaOH,

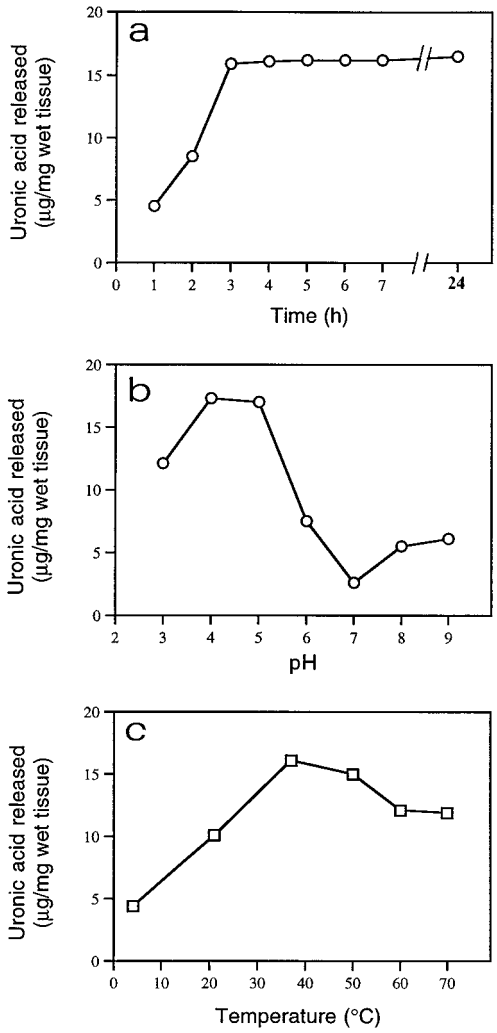

Figure 1. Effect of time, pH, and temperature on the release of uronic acid from bovine nasal cartilage: (a) diced bovine nasal cartilage samples incubated in duplicate in 0.1 M sodium acetate buffer, pH 4.0 at 37 °C (aliquots of supernatant of the incubation mixture were collected at different times); (b) cartilage samples incubated in duplicate in 0.1 M sodium acetate buffers with various pH at 37 °C for 7 h; (c) cartilage samples incubated in duplicate in 0.1 M sodium acetate buffer, pH 4.0, at various temperatures for 7 h. The total uronic acid released was determined in each experiment.

it was retarded in the column (Figure 2b). This suggested that fraction I contained GAG-peptide and that the peptide was separated from polysaccharide by the β-elimination reaction. The partition coefficient (K_{av}) of the retarded peak of fraction I was 0.18, which was less than those of fractions II ($K_{av} = 0.31$; Figure 2c) and III ($K_{av} = 0.31$; Figure 2d). These GAG fractions extracted with NaOH had apparently smaller size than did the standard CS from either whale cartilage ($K_{av} = 0.25$, not shown) or chicken cartilage ($K_{av} = 0.25$, not shown). In contrast, fraction IV (Figure 2e) had a broader peak (with K_{av} of eluates ranging from 0 to near 0.6) than did other fractions. The fraction I was further chromatographed on a column of DEAE-Sephacel to separate collagenous materials from the GAG-peptide. A representative chromatogram is shown in Figure 3. No GAG was detected in fractions that failed to bind to DEAE-Sephacel. Fractions 105−115, accounting for 1.6% of recovered uronic acid, gave two bands on cellulose acetate electrophoresis (Figure 4a). The slow moving band having mobility of hyaluronic acid was

Table 1. Analysis of the Fractions Extracted from Bovine Nasal Cartilage[a]

fraction	incubation medium	% of total uronic acid	hydroxyproline (μg/mg of dry wt)	sialic acid (μg/mg of dry wt)	protein (μg/mg of dry wt)
I	0.1 M sodium acetate	79.6 ± 8.3[a]	21.3 ± 1.0[a]	10.0 ± 1.4[a]	121.0 ± 4.0[a]
II	0.5 N NaOH	14.2 ± 7.8[b]	20.7 ± 4.1[a]	4.9 ± 0.6[b]	222.5 ± 20.3[b]
III	1 N NaOH	3.2 ± 2.9[c]	135.8 ± 7.3[b]	7.0 ± 0.2[c]	365.4 ± 29.7[c]
IV	papain solution	3.0 ± 1.6[c]	87.8 ± 5.5[c]	1.0 ± 0.2[d]	779.8 ± 56.0[d]

[a] Values presented are mean ± SD obtained from four experiments (n = 4). The cartilage samples used in each experiment were derived from different animals. Means in the same column with different superscripts are significantly different (p < 0.05).

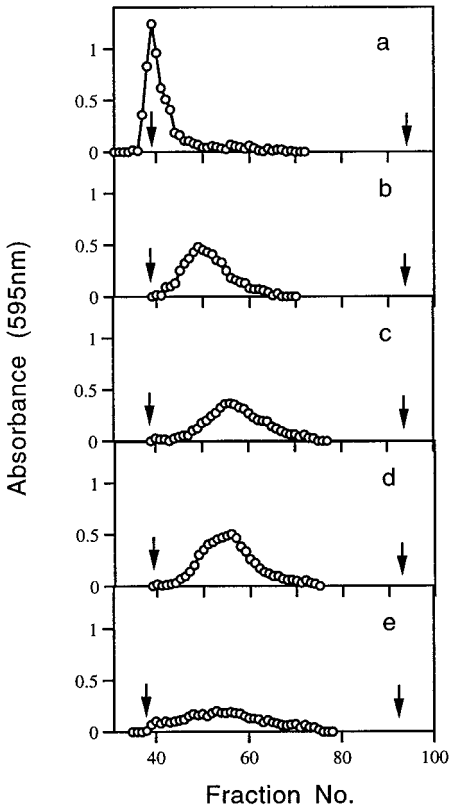

Figure 2. Sephacryl S-300 chromatograms of bovine nasal cartilage extracts (fractions I−IV): (a) fraction I; (b) fraction I treated with 0.5 N NaOH; (c) fraction II; (d) fraction III; (e) fraction IV. The void volume and total column volume in each chromatogram are shown by the left and right arrows, respectively.

highly susceptible to *Streptomyces hyaluronidase*, an enzyme specific to hyaluronic acid (Ohya and Kaneko, 1970) (results not shown). The fast moving band, with its mobility slightly slower than that of standard CS, was highly susceptible to chrondroitinase-ACI (results not shown), indicating that the band consisted of CS.

Approximately 98% of recovered GAG was in fractions 116−180 (Figure 3). Major fractions (116−165) were pooled, dialyzed in water, and freeze-dried to obtain a final preparation of GAG-peptide, which accounted for 18.7% of dry tissue weight. Cellulose acetate electrophoresis of the GAG-peptide showed a band with its mobility slightly slower than that of standard CS (Figure 4b). Identical electrophoretic results were obtained when the sample was treated with 0.5 N NaOH to separate GAG chains from peptide (results not shown). Digestion with chondroitinase-ACI resulted in complete elimination of bands of GAG-peptide treated with and without 0.5 N NaOH (results not shown).

Sepharose CL-2B chromatography showed that the molecular size of GAG-peptide of fraction I (Figure 5a) was apparently smaller than that of bovine nasal cartilage proteoglycan (Figure 5b). Addition of hyaluronic acid did not affect the elution pattern of fraction I (Figure 5a), indicating no interaction of the GAG-peptide with hyaluronic acid. In contrast, the bovine nasal cartilage proteoglycan showed interaction with hyaluronic acid, which was observed as the appearance of a peak excluded from Sepharose CL-2B (Figure 5b). On gel electrophoresis with 5% polyacrylamide, the GAG-peptide remained at the top of the gel as did the standard bovine nasal cartilage proteoglycan (results

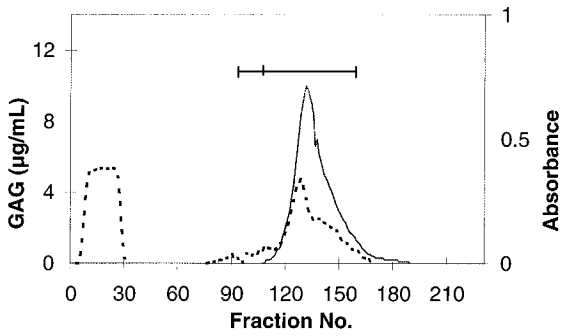

Figure 3. Chromatography of bovine nasal cartilage extract, fraction I, on DEAE-Sephacel. Fraction I containing GAG-peptide was applied to a 2.5 × 14.5 cm column equilibrated with 0.1 M sodium acetate buffer, pH 4.5. The column was then eluted with a gradient formed from 350 mL of the same buffer and 350 mL of 0.1 M sodium acetate, pH 4.5, containing 1 M NaCl, followed by a further 100 mL of 0.1 M sodium acetate, pH 4.5, containing 2 M NaCl. Fractions 60−200 contained eluates with the 0−1 M NaCl gradient. Fractions (5 mL) were collected at a flow rate of 21 mL/h. (- - -) Absorbance at 280 nm; (—) results of dimethylmethylene blue dye-binding assay for GAG. Bars denote fractions pooled (105−115 and 116−165) for further study.

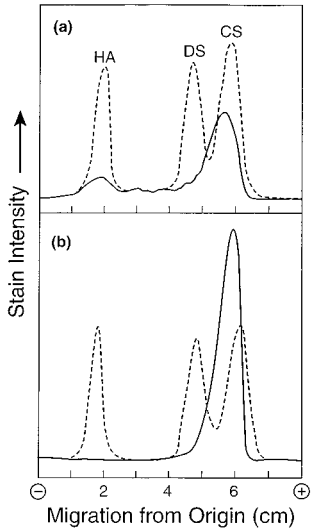

Figure 4. Cellulose acetate electrophoresis of GAGs eluted from the column of DEAE-Sephacel: (a) pooled sample of fractions 105−115 (—) and standard GAGs (- - -); (b) pooled sample of fractions 116−180 (—) and standard GAGs (- - -). HA, hyaluronic acid; DS, dermatan sulfate; CS, chondroitin sulfate.

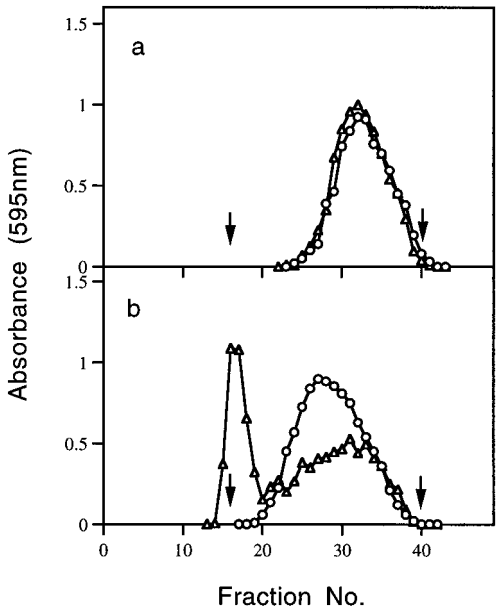

Figure 5. Chromatography on Sepharose CL-2B of bovine nasal cartilage GAG-peptide (a) and proteoglycan (b) with (△) and without (○) prior incubation with hyaluronic acid. Each sample was applied to a 0.7 × 70 cm column equilibrated and eluted with 0.5 M sodium acetate buffer, pH 5.8, containing 0.02% sodium azide, and fractions (0.65 mL) collected at a flow rate of 0.60 mL/h were monitored for GAG by the dimethylmethylene blue dye-binding method. The left and right arrows in each chromatogram show the void volume and total column volume, respectively.

Table 2. Analysis of GAG-Peptide[a]

carbohydrate, sulfate, nitrogen, and protein		amino acid	
uronic acid	288.0	aspartic acid	37
galactose	24.7	glutamic acid	102
galactose:uronic acid	1:12	serine	76
sialic acid	7.9	histidine	9
glucosamine:galactosamine	1:30	glycine	130
CS	95.7	threonine	36
KS	4.3	arginine	22
sulfate	125.0	alanine	48
sulfate:uronic acid	0.95:1	tyrosine	5
ΔDi4S:ΔDi6S	88:12	methionine	ND
nitrogen	33.1	valine	46
protein (Lowry et al. method)	89.3	phenylalanine	25
protein (sum of amino acid residues)	68.4	isoleucine	24
		leucine	57
		lysine	11
		cysteic acid	5
		hydroxyproline	0.6

[a] Concentrations of carbohydrates (with the exception of CS and KS), sulfate, nitrogen, and protein are expressed as μg/mg of dry weight, and those of amino acids as nmol/mg of dry weight. The contents of CS and KS are expressed as percent of total sulfated GAG. The proportion of CS was calculated by subtracting the percentage of KS from 100%, assuming that all sulfated GAGs in the GAG-peptide were derived from proteoglycan (aggrecan) bearing CS and KS. Ratio values are presented as molar ratios. ΔDi4S, unsaturated 4-sulfated disaccharide; ΔDi6S, unsaturated 6-sulfated disaccharide; ND, not detected.

not shown). Digestion of GAG-peptide with chondroitinase-ABC resulted in disappearance of its band.

Chemical composition of GAG-peptide (Table 2) indicated that <10% of the preparation is peptide and most of the remaining portion GAG. Amino acid analysis showed high concentrations of glutamic acid, glycine, and serine residues characteristic of CS proteoglycan of cartilage (Hascall and Sajdera, 1969; Heinegård, 1977). Chondroitin sulfate was the major GAG (≈96% of total GAG) with a small amount of KS (4%). The molar ratio of sulfate to uronic acid was close to 1. Analysis of the chondroitinase-ABC digest of CS showed the ratio of 4-sulfated unsaturated disaccharide to 6-sulfated unsaturated disaccharide close to 9 to 1. The predominance of 4-sulfated unsaturated disaccharide was further confirmed by digesting the GAG-peptide by papain and fractionating the digest according to solubility in ethanol. Most (74%) of total uronic acid was in the 40% ethanol fraction [chondroitin 4-sulfate fraction according to Meyer et al. (1956)], and the remaining 2, 1, 15, and 8% were in the 18, 25, 50, and 75% ethanol fractions, respectively. Small amounts of galactose, sialic acid, and glucosamine were also detected in the GAG-peptide preparation (Table 2).

DISCUSSION

The highest extractability of uronic acid in the nasal cartilage appears to be related in part to its highest concentration of uronic acid and lowest concentration of collagen. It is, however, unknown why the nasal cartilage, which is presumably subjected to less load than the occipital articular cartilage or temporomandibular joint disk fibrocartilage, needs more extractable uronic acid.

It is well-known that the extractability of GAG from cartilage is very low with a low ionic strength buffer having pH >5 and temperature <37 °C. For example, Sajdera and Hascall (1969) incubated bovine nasal cartilage in 0.15 M KCl/0.05 M Tris-HCl, pH 7.5, at 25 °C for 48 h and reported extraction of ≈20% of total tissue uronic acid. This is confirmed by the present study (Figure 1b,c). However, if the pH and temperature are changed to the levels optimal for autolysis, many more GAGs are liberated. The optimal pH (Figure 1b) and temperature (Figure 1c) found in the present study are consistent with those reported previously (Jibril, 1967; Dziewiatkowski et al., 1968). The observed inhibition of release of GAGs by pepstatin, an inhibitor of carboxypeptidase (Aoyagi et al., 1972), is also consistent with the findings of Jibril (1967) and suggests partial contribution of the activity(ies) of endogenous proteinase(s) to degrade the protein core of proteoglycan to release GAG-peptide. Cathepsin D, which is known to be in the lysosomes and active at pH 5, may be one of the enzymes involved (Sapolsky et al., 1973). Jibril (1967) further observed inhibition of release of GAGs from ossifying scapula cartilage with 6-aminohexanoic acid, a plasmin inhibitor (Steffen and Steffen, 1976). This, however, did not occur in the bovine nasal cartilage examined in the present study. This difference may reflect differences in functions between calcifying cartilage of scapula and noncalcifying nasal cartilage.

Development of a simple low-cost method of preparation of useful chemicals from animal byproducts with little saleable value is of economic importance in the livestock industry. Glycosaminoglycans are polysaccharides of potential importance with wide applications (see above). However, not much attention has been given to the ecomomical production of GAGs. With the method described in this paper, we can extract GAG-peptide from the bovine nasal cartilage without using any exogenous proteinase(s) (e.g. papain to liberate GAGs) or chaotropic solvent(s) (e.g. guanidinium chloride to solubilize proteoglycans). This minimizes the

extraction cost and the work to remove chemicals (e.g. trichloroacetic acid used for deproteinization of papain digests) that are harmful to the human body. The toxicity of sodium acetate used in this experiment, if not completely removed from the final product, is unknown. In this regard, we have recently found that sodium acetate can be replaced by NaCl without decreasing the extraction yield of uronic acid (T. Nakano and J. S. Sim, unpublished results), suggesting possible production of GAG-peptide suitable for human consumption. Approximately 80% of bovine nasal cartilage uronic acid is extracted as GAG-peptide by incubating tissues in 0.1 M sodium acetate, pH 4.5, at 37 °C for 7 h. This is higher than the yield of extractable GAG-peptide (65%) calculated from the data of Hoffman et al. (1967), who incubated bovine nasal cartilage in 0.1 M sodium acetate buffer, pH 5, containing 0.9% NaCl at 37 °C for 24 h and reported the extractable GAG-peptide to be 26% of dry weight of cartilage, of which 40% is proteoglycan. The present yield of GAG-peptide is also higher than the previously reported 60% yield of extractable uronic acid from human articular cartilage (Bayliss and Ali, 1978). The extractability may be increased by reducing the thickness of tissues to be incubated (Pottenger et al., 1983). Further treatment of the residual tissue with cold NaOH, which releases peptide-free GAG chains by the β-elimination reaction (Rodén et al., 1972), can increase the yield of extractable uronic acid by >15% (Table 1). Thus, >95% of total uronic acid can be extracted from the bovine nasal cartilage without exogenous proteinase or chaotropic agent.

The GAG-peptide is a degradation product of proteoglycan ignored by many researchers, and thus the properties and chemical composition of the material are not well understood. In the present study, the GAG-peptide obtained after ion-exchange chromatography had no capability to interact with hyaluronic acid (Figure 5a), suggesting loss or degradation of the G1 domain containing hyaluronic acid binding region. No further characterization of the peptide was made in this study. The high proportion of 4-sulfated disaccharide observed in the GAG-peptide (see above) is consistent with the study of Seno et al. (1975), who analyzed nasal cartilages from cattle, sheep, and whale and found that the majority of disaccharides of CS are sulfated at C4. This is, however, in contrast with the disaccharide composition of bovine articular cartilage proteoglycan, in that the 6-sulfated disaccharide is the major disaccharide with the ratio of 4-sulfated disaccharide to 6-sulfated disaccharide ranging from 0.30 to 0.69 (Murata and Bjelle, 1977). These different results may be related to the difference in function between the two tissues (non-weight-bearing nasal cartilage vs weight-bearing articular cartilge).

Keratan sulfate, a minor sulfated GAG found in the present preparation of GAG-peptide (Table 2), can be separated, if necessary, from CS by ethanol precipitation fractionation technique (Meyer et al., 1956) after GAG chains are released from peptides by alkaline treatment (Rodén et al., 1972).

There is little information available concerning the effects of room temperature storage of cartilage and of small amounts of noncartilaginous tissues attaching to cartilage on the yield and quality of GAG. Studies on these questions are important for the development of methods of large scale production of GAG.

In conclusion, the results obtained in the present study with bovine tissues confirm the previous reports of optimal pH and temperature for autolysis of cartilage to release GAGs and further provide previously unreported observations on (1) variations in the extractability of GAGs among the nasal and occipital cartilages and temporomandibular joint disk fibrocartilage and (2) chemical composition of GAG-peptide extracted by tissue autolysis. The GAG-peptide can be extracted with highest yield by incubating nasal cartilage in 0.1 M sodium acetate buffer, pH 4−5, at 37 °C. The yields of GAG-peptide found in this study are much higher than that previously reported for GAG-peptide from the bovine nasal cartilage. The GAG-peptide purified using ion-exchange chromatography contains approximately 89% CS, 4% KS, and 7% peptide and has no capability to interact with hyaluronic acid. The extraction method is useful for the preparation of GAG-peptide at a low cost.

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