An economical method to extract chondroitin sulphate-peptide from bovine nasal cartilage

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Nakano, T., Ikawa, N. and Ozimek, L. 2000. **An economical method to extract chondroitin sulphate-peptide from bovine nasal cartilage**. Can. Agric. Eng. 42:205-208. Utilization of animal by-products by extracting useful material is of economic importance in the livestock industry. This report describes a simple low cost procedure to release chondroitin sulphate (CS) from bovine nasal cartilage without introducing any chemicals except acetic acid used for pH adjustment. More than 70% of total CS in nasal cartilage can be released as CS-peptide by incubation in water at pH 4.5 and 37°C. This is likely the most economical method currently available to extract CS from cartilage. Endogenous proteinases are probably involved in the release of CS. The extract is boiled and dried at 90°C to obtain a crude CS preparation. The purity of CS can be improved approximately 1.4 fold by anion-exchange chromatography. This information may contribute to the development of a low cost method of preparation of CS for commercial purposes.

L'extraction de substances utiles d'origine animale est d'une grande importance économique pour l'industrie de l'élevage. Ce rapport décrit un procédé simple et peu coûteux pour extraire la chondroitine sulfate (CS) du cartilage nasal des bovins sans utiliser de produits chimiques sauf l'acide acétique pour l'ajustement du pH. Plus de 70% de la CS totale présente dans le cartilage nasal peut être libérée sous forme de CS-peptide par incubation dans l'eau à un pH de 4.5 et une température de 37°C. Cette méthode d'extraction de la CS du cartilage est vraisemblablement la plus économique à l'heure actuelle. Les protéinases endogènes sont probablement impliquées dans la libération de la CS. On obtient une préparation brute de CS en faisant bouillir l'extrait, et en le faisant sécher à 90°C. On peut améliorer la pureté de la CS par un facteur de 1.4 en utilisant la chromatographie par échange d'anions. Cette information pourrait contribuer à l'élaboration d'un procédé commercial de préparation de la CS qui soit peu coûteux.

INTRODUCTION

In the livestock industry, meat is the primary product accounting for the majority of the economic value of cattle and hogs. Meat produced by these animals accounts for less than half of their liveweight, the remainder being edible and non-edible by-products. Beneficial ways of utilization of these by-products (e.g. extraction of useful materials from them) are of economical importance in the livestock industry. Cartilage is a relatively low-value by-product but is rich in chondroitin sulphate (CS). Chondroitin sulphate is an anionic polysaccharide comprised of repeating disaccharide units of N-acetylgalactosamine 4- or 6-sulphate and D-glucuronic acid. In cartilaginous tissue, this polysaccharide is covalently attached to protein to form proteoglycan (Muir and Hardingham 1975). Chondroitin sulphate has 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 anti-atherogenic (Matsushima et al. 1987) effects in experimental animals. A chondroitin sulphate-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). Chondroitin sulphate has also been used in eye lotion, cosmetics (as moisturants), and mayonnaise and dressings.

Commercially available CS is, however, too expensive to prepare CS products, and thus the development of a low cost method of preparing this polysaccharide is needed. Chondroitin sulphate can be extracted from cartilage by digestion with an exogenous proteinase (e.g. papain) or alkaline treatment (Rodén et al. 1972). Chondroitin sulphate can also be released from tissue by activation of endogenous proteinases (autolysis). Nakano et al. (1998) incubated bovine nasal cartilage in 0.1 M sodium acetate at pH 4.5 and 37°C, and reported high yield extraction of CS-peptide accounting for approximately 80% of total recovered uronic acid by tissue autolysis. There are, however, few reports of extraction of CS from tissues without the use of exogenous enzyme or chemical which makes the preparation of CS too expensive for commercial purposes. This study was, therefore, undertaken: to extract CS from bovine nasal cartilage with water at pH 4.5 and 37°C without introducing any exogenous materials other than acetic acid used for pH adjustment; and to determine the chemical composition of the CS-peptide purified from the extract by ion-exchange chromatography.

MATERIALS and METHODS

Materials

Samples of bovine nasal cartilage were obtained fresh from young adult (2 – 3 year old) animals at a local abattoir. They were transported to the laboratory on ice, rinsed with cold water, and dissected free of non-cartilaginous adherent tissues. They were stored at −20°C until extracted. Standard CS proteoglycan from bovine nasal cartilage was obtained from ICN Biomedicals Inc., Costa Mesa, CA.

Extraction of CS

Frozen samples of nasal cartilage were thawed at 4°C, finely diced (approximately 1 mm^3) and thoroughly mixed. A portion of the diced sample (approximately 1 g wet mass in six replicates) was incubated in 10 volumes of water at pH 4.5 and 37°C for 7 h. This condition was confirmed to be optimal for the extraction of CS from bovine nasal cartilage (T. Nakano and L. Ozimek unpublished data). Acetic acid was used to adjust the pH of the incubation mixture. The pH was occasionally checked and adjusted to 4.5 if increased. Portions of the sample were also incubated separately at 37°C for 7 h in: 1) 0.1 M sodium acetate (pH 4.5); and 2) deionized water without adjusting the pH. Nine replicates were used in each case. The incubation mixtures were then centrifuged to obtain the supernatant (acidic water soluble fraction) and tissue residue. An aliquot of each acidic water soluble fraction was assayed for uronic acid by the carbazole reaction (Kosakai and Yoshizawa 1979). The amount of uronic acid remaining in the tissue residue was estimated after proteolysis with papain followed by deproteinization with trichloroacetic acid (Nakano et al. 1998). Since most (> 98%) uronic acid is present in bovine nasal cartilage as a sugar moiety of CS (Nakano et al. 1998), the content of uronic acid reflects that of CS. The proportion of uronic acid released from the tissue was calculated as the mean ± standard deviation. One way analysis of variance (Schlotzhauer and Littell 1987) was used to detect differences ($P < 0.05$) between means of the proportion of uronic acid released from the tissue with different incubation media including acidic water, pH 4.5, deionized water, and 0.1 M sodium acetate (see above).

Preparation of crude CS fraction

The acidic water soluble fraction (see above) was concentrated by boiling and then held at 90°C until dry to obtain a crude CS fraction.

Isolation of CS-peptide

A portion of the acidic water soluble fraction or crude CS fraction (see above) was chromatographed on DEAE-Sephacel (Pharmacia Biotech Inc., Baie d'Urefé, QC) to separate CS containing material from non-CS materials including collagen and hyaluronic acid present in the fraction (Nakano et al. 1998).

Other methods

Gel chromatography on Sephacryl S-300 and Sepharose CL-2B was carried out as described previously (Nakano et al. 1998). Eluates collected were determined for the content of CS by the dimethylmethylene blue dye-binding method (Farndale et al. 1982). The partition coefficient (K_{av}) of CS peak was calculated from:

$$K_{av} = \frac{V_e - V_0}{V_t - V_0}$$

where:

V_e = volume of the peak fraction,
V_0 = void volume, and
V_t = total volume.

V_0 and V_t were determined using blue dextran and tritiated water, respectively. Alkali treatment of crude CS was carried out as described (Nakano et al. 1998). Amino acid analysis was performed by reversed phase high performance liquid chromatography using a 4.6 mm × 150 mm column packed with 3 µm Supelcosil LC-18 (Supelco Canada, Mississauga, ON). Samples were derivatized using *o*-phthaldialdehyde, and hydrolyzed in 6 N HCl at 110°C for 24 h (Sedgwick et al. 1991). The contents of cysteine and methionine were estimated by determining cysteic acid and methionine sulfone, respectively after oxidizing CS-peptide with performic acid (AOAC 1998) and subsequent acid hydrolysis. Protein content was determined by the method of Lowry et al. (1951) using bovine serum albumin as standard.

RESULTS and DISCUSSION

The uronic acid released from the nasal cartilage with water at pH 4.5 and 37°C accounted for an average 73.2 ± 3.0% of total recovered uronic acid. This value was approximately eight times higher ($P < 0.05$) than the content of uronic acid (8.8 ± 1.5% of total) released from the tissue with deionized water at near neutral pH. The pH of the incubation mixture with deionized water was 7.5 at the start of incubation and gradually decreased to 6.8 at the end of incubation. When the tissue was incubated in 0.1 M sodium acetate, pH 4.5, the proportion of released uronic acid increased ($P < 0.05$) to 78.8 ± 3.4% a value similar to the previously reported value (79.6 ± 8.3%) obtained under similar experimental conditions (Nakano et al. 1998). These results suggest that pH is the important factor affecting the yield of soluble CS measured as uronic acid, and that the majority of CS present in the nasal cartilage can be liberated from the tissue at pH 4.5 and 37°C without using 0.1 M sodium acetate. Tissue autolysis (Nakano et al. 1998) may be one explanation for the high extractability of CS. Therefore, the cost of extraction of CS can be significantly reduced if liberated with acidic water only. On the other hand, the use of 0.1 M sodium acetate which slightly improves extractability of CS (by approximately 6%, $P<0.05$) does not appear to be advantageous for the purpose of cost reduction. Approximately 1 ml of acetic acid is required to maintain the pH of the mixture of 100 g of bovine nasal cartilage and 1 L of water at 4.5 for 7 h, while approximately 8 mL of acetic acid and 4.1 g of sodium acetate are required to obtain 1 L of 0.1 M sodium acetate, pH 4.5, which has enough buffering capacity during incubation with 100 g of cartilage. Bovine nasal cartilage is an appropriate by-product from which CS-peptide can be prepared with high yield. Articular cartilage with a low content of extractable CS (Nakano et al. 1998) is not recommended for this purpose.

The acidic water soluble fractions from six replicates were pooled, boiled and dried at 90°C to obtain a crude CS fraction, which accounted for 11.5% of the wet weight of the tissue, and which contained 21.6 % uronic acid by dry weight. This heating process is important to obtain a dry sterilized preparation of crude CS. Freeze-drying is a more expensive technique than heating used in this study and is not efficient to destroy contaminating microorganisms. Sephacryl S-300 gel chromatography results (Fig. 1) showed that the majority (75%) of the crude CS fraction eluted in the void volume. However, when treated with 0.5 N NaOH, the fraction was retarded in the column. These results are consistent with a previous report of the 0.1 M sodium acetate soluble fraction from bovine nasal cartilage (Nakano et al. 1998) and suggested that the crude CS

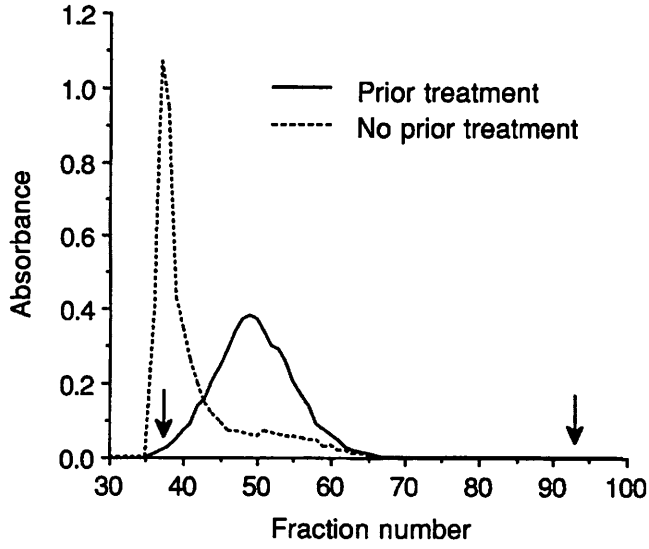

Fig. 1. Gel chromatography of crude CS on a 1 x 110 cm column of Sephacryl S-300. Fractions (1 mL) collected were determined for CS by the dimethylmethylene blue dye reaction (absorbance at 595 nm) (see the Methods). The void volume and total column volume, determined using blue dextran and tritiated water, respectively, are shown by the left and right arrows, respectively.

fraction contained CS-peptide, and that the peptide was separated from CS by the β-elimination reaction (Rodén et al. 1972). The crude preparation was further chromatographed on a column of DEAE-Sephacel to give the final preparation of CS-peptide, which accounted for 7.8% of wet weight of cartilage, and contained 29.8% uronic acid and 6.5% protein by dry weight. Comparison of uronic acid concentrations determined in the crude (see above) and purified preparations of CS-peptide suggests that its purity is improved approximately 1.4 fold by using anion-exchange chromatography.

Amino acid composition of the CS-peptide (Table I) was similar to that of a previous preparation of CS-peptide extracted from bovine nasal cartilage with 0.1 M sodium acetate (Nakano et al. 1998). The CS-peptide contained high contents of glutamic acid (or glutamine), glycine and serine characteristic of a CS proteoglycan (Hascall and Sajdera 1969). On Sepharose CL-2B chromatography (chromatogram not shown), the CS-peptide showed a single peak with its K_{av} (0.65) similar to that (0.67) of the previous preparation of CS-peptide (Nakano et al. 1998), suggesting a similar molecular size between the two preparations of CS-peptide. Sepharose CL-2B chromatography of CS-peptide following incubation with exogenous hyaluronic acid showed an absence of the CS peak that was excluded from the column (chromatogram not shown). This is consistent with a previous report of CS-peptide (Nakano et al. 1998), and suggests that the present preparation of CS-peptide lacks a hyaluronic acid binding region found in CS proteoglycan.

Table I. Amino acid composition of CS-peptide.

Amino acid	Mol %
Aspartic acid and asparagine	8.4
Glutamic acid and glutamine	17.0
Serine	10.1
Histidine	1.6
Glycine	15.5
Threonine	5.4
Arginine	2.9
Alanine	9.4
Methionine	0.3
Tyrosine	0.7
Valine	8.2
Phenylalanine	4.2
Isoleucine	4.6
Leucine	9.4
Lysine	1.8
Cysteine	0.5

CONCLUSIONS

The present procedure which does not require any chemicals except acetic acid used to adjust the pH of the incubation mixture may be the most economical way among published methods to liberate CS from cartilaginous tissues. Using this procedure, more than 70% of total CS can be extracted from bovine nasal cartilage to provide a crude CS-peptide fraction. This preparation may be used as an ingredient for food, cosmetic or pharmaceutical products. The purity of CS-peptide can be improved, if necessary, by anion exchange chromatography. The technique described here may be useful for the development of an industrial scale low cost preparations of CS.

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