ISOLATION AND CHARACTERIZATION OF GLYCOSAMINO- GLYCANS FROM THE BOVINE FOURTH STOMACH

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Five glycosaminoglycans were isolated from tryptic digest of bovine fourth gastric mucosa, and were assayed by determining the carbohydrate content of materials. Separation of these five polymers was achieved by preparative zone electrophoresis in a Pevikon C-870 block and by Dowex 1 × 2 column chromatography. They were identified as hyaluronic acid, heparan sulfate, chondroitin-4-sulfate, dermatan sulfate and chondroitin-6-sulfate, respectively.

INTRODUCTION

Glycosaminoglycans in the several organs of the animals have well been studied by many authors. At present, however, little is known about these complex carbohydrates in the stomachs of animals except those of the pig and dog (Kimura et al., 1965; Häkkinen et al., 1965). A recent study on the glycosaminoglycans from the bovine fourth gastric mucosa reported that four alcian blue positive fractions were observed on cellulose acetate electrophoresis (Masuda et al., 1967). The present paper deals with a demonstration of these substances in the bovine fourth gastric mucosa.

MATERIALS AND METHODS

Materials

The bovine stomachs were obtained from the local abattoir. They were separated into four different parts of the stomach including the first, second, third and fourth stomachs after histological examination. The wall of the fourth stomach was separated into two different layers — mucosal layer and muscular layer including serosa, and then the mucosal layer of the fourth stomach was used in these experiments. It was cut into cherry-sized pieces, put into several changes of acetone for several days and dried over CaCl₂ in a desiccator. It was ground in a Wiley mill sufficiently small to pass through a 60 mesh sieve.

Isolation procedure

Crude glycosaminoglycans were extracted from dry tissue powder by the

method of Schiller et al. (1961). It was digested with 1/50 weights of papain (EC 3.4.4.10) in the presence of EDTA and cysteine (5:9:4, w/w/w) at 65°C for 48 hr, after homogenesis in 5 volumes of 1/15 M potassium phosphate, monobasic-sodium phosphate, dibasic buffer of pH 6.4. The mixture was adjusted to pH 7.8 and then digested with 1/50 weights of trypsin (EC 3.4.4.4) at 37°C for 72 hr with simultaneous dialysis against 1/15 M phosphate buffer of pH 7.8. The digested solution was deproteinized with 5 % trichloroacetic acid in the cold and the supernatant was dialyzed against several changes of cold deionized water for 72 hr. Then 4 volumes of alcohol containing 1 % acetic acid and 1 % potassium acetate were added to precipitate the crude glycosaminoglycans. The precipitate was washed with alcohol and ether, dried in a desiccator over CaCl_2 and then redissolved for the use of fractionation.

The scheme of the further fractionation of the glycosaminoglycans is shown in the flow diagram (Fig. 1).

Fractionation of the glycosaminoglycans

Glycosaminoglycans were fractionated using Pevikon C-870 powder (copolymer of polyvinylchloride and polyvinylacetate: Fabriks, Sweden) as supporting medium by the method of Müller-Eberhard (1960). The dimensions of the block were 45×14×1 cm, and a few ml of concentrated glycosaminoglycan solution were applied to the block. Electrophoresis was run in barbital buffer pH 8.6, $\mu=0.06$ at 1.5 mA per cm width for 15 hr. Then one cm segments were cut off, eluted with 15 ml of deionized water and the eluates were subjected to uronic acid and hexose analyses (Dische, 1962; Winzler, 1955). Isolated fractions (fraction A and B) were dialyzed against deionized water and the contents of the

Fig. 1 Flow diagram of the fractionation of glycosaminoglycans from the bovine fourth gastric mucosa.

dialysis bag were mixed with 4 volumes of alcohol to precipitate the glycosaminoglycans which were used for further fractionation.

Dowex 1×2 (Dow Chemicals, U.S.A., 100-200 mesh, Cl^- form) was recycled twice with 0.5 N NaOH and 0.5 N HCl, equilibrated with 0.25 M NaCl, which was used as the starting solution for the elution system, and packed into the column (2.7×46 cm). Glycosaminoglycans fraction dissolved in 10 ml of 0.25 M NaCl was applied to the column in two portions of 5 ml each, and the column was washed with the same salt solution. Stepwise elution with 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 2.0 and 4.0 M NaCl was performed at a flow rate of 10 ml per hour. Fractions (15 ml) were collected and the uronic acid content of each was determined. The material eluted at each step was pooled, concentrated in a rotary evaporator, dialyzed, and lyophilized. The preparation was dissolved in a small amount of deionized water, and was utilized both for cellulose acetate electrophoresis and for digestion with neuraminidase as well as mucopolysaccharase.

Cellulose acetate electrophoresis

The material was run in a horizontal type apparatus at a constant current of 1.0 mA/cm on an OXOID strip (9×1.2 cm) using barbital buffer, pH 8.6, $\mu=0.06$ for 30 min. Aqueous suspensions of preparations, 5 to 10 μ l of 1.0 mg per ml of preparations, were used in this study. After electrophoresis, the strip was stained with both alcian blue and periodic acid-Schiff reaction (PAS) and then scanned in an Atago densitometer (Tokyo, Japan). Marker samples containing hyaluronic acid (human umbilical cord), heparan sulfate (bovine liver), chondroitin-4-sulfate (whale cartilage), dermatan sulfate (pig skin) and chondroitin-6-sulfate (shark cartilage) were applied alongside each sample; hyaluronic acid, chondroitin-4-sulfate and chondroitin-6-sulfate were all purchased from Seikagaku Kogyo Co., Ltd, (Tokyo, Japan), and heparan sulfate and dermatan sulfate were kindly given by Dr. A. Cifonelli (Chicago University) and Dr. Y. Tanaka (Kurume University), respectively.

Digestion of the materials with neuraminidase and mucopolysaccharase

A 0.3 mg portion of every NaCl fraction from both the fraction A and B on Pevikon block electrophoresis was incubated with 0.1 mg of neuraminidase [EC 3. 2. 1. 18, mucopolysaccharide N-acetylneuraminyldiolase from *Clostridium perfringens* Type V, Sigma Chem. Co., U. S. A.] in 5 ml of 1.0 M acetic acid-sodium acetate buffer of pH 4.8 saturated with toluene at 37°C overnight. The digestion was repeated twice in the same manner. The digest was deproteinized with 5% trichloroacetic acid, and then the solution was dialyzed against several changes of cold deionized water for 48 hr. A 0.3 mg of same

Samples was digested with 3 turbidity-reducing unit (TRU) of hyaluronidase from *Streptomyces hyalurolyticus* (Seikagaku Kogyo, Co., Ltd., Tokyo, Japan) in 1 ml of 1/15 M potassium phosphate, monobasic-sodium phosphate, dibasic buffer of pH 5.0 saturated with toluene at 37°C overnight, and followed by further treatment. A 0.3 mg portion of same materials was incubated with 0.5 units of chondroitinase AC and chondroitinase ABC (Seikagaku Kogyo, Co., Ltd., Tokyo, Japan) according to the procedure of Saito et al. (1968), respectively, and followed by further treatment. After enzymatic digestion the samples were freeze-dried. The dried samples were dissolved in 0.2 ml of deionized water and this solution was again used for cellulose acetate electrophoresis. After enzymatic digestion of both the 1.5 and 2.0 M NaCl fractions from the fraction A and B on Pevikon block electrophoresis with chondroitinase AC, the samples were used for paper chromatography. Paper chromatography was carried out by the method of Suzuki et al. (1968), using 2-acetamido-2-deoxy-3-0-4-0-sulfo-D-galactose (Δ Di-4S) and 2-acetamido-2-deoxy-3-0-6-0-sulfo-D-galactose (Δ Di-6S) as standards. After paper chromatography, compounds containing Δ Di-4S and Δ Di-6S were detected by viewing under ultra violet ray (wave length 2536 Å, Manasulu Chemicals; Tokyo, Japan).

Analytical procedures

The separation and quantitative determination of glucosamine and galactosamine were carried out with a Hitachi KLA-3 Amino Acid Analyser (Tokyo, Japan), using a 20 cm column in citrate buffer of pH 5.28 (Na: 0.35 N), after hydrolysis with 4 N HCl for 6 hr. Sulfate was determined by the method of Dodgson and Price (1962)

after hydrolysis with 4 N HCl for 6 hr ; uronic acid by both the carbazole reaction (Dische, 1962) and the orcinol-hydrochloric acid method (Khym and Deherity, 1952), respectively.

RESULTS AND DISCUSSION

Carbohydrate analysis of block segments revealed the presence of three hexose peaks and two uronic acid peaks. The fractions under the hexose peaks were identified as glycopeptides, as reported previously (Masuda et al, 1977). Consequently materials corresponding to these uronic acid peaks were num-

bered in order of increasing electrophoretic mobility, fraction A and fraction B, and then were selected for further fractionation.

The elution patterns of the Dowex 1×2 column chromatography of both the fraction A and B are depicted in Fig. 2. The stepwise elution of the fraction A on Pevikon block electrophoresis gave six main uronic acid peaks at the molarity of NaCl solution ranging from 0.5 to 2.0 M, while the fraction B on Pevikon block electrophoresis separated into four main uronic acid peaks at the molarity of NaCl solution ranging from 1.0 to 2.0 M. Therefore,

Fig. 2 Dowex 1×2 column chromatography of fractions after zone electrophoresis on a Pevikon C-870 block of the material obtained from bovine fourth stomach.
A, top : fraction A obtained from zone electrophoresis on a Pevikon C-870 block ;
B, bottom : fraction B obtained from zone electrophoresis on a Pevikon C-870 block.
——— : uronic acid.

the fractions corresponding to these peaks were pooled and dialyzed against cold deionized water for three days; the insoluble materials were centrifuged out and the supernatants were freeze-dried. These samples were then studied by cellulose acetate electrophoresis, enzymatic digestion and chemical analyses.

Cellulose acetate electrophoresis of the various fractions was carried out on an OXOID strip in the barbital buf-

fer, pH 8.6, $\mu=0.06$ (Fig. 3). Electrophoresis of all fractions from the fraction A on Pevikon block electrophoresis resulted in two components, while each of the fractions from the fraction B on Pevikon block electrophoresis had a single band. Although the one component which was observed in the material from the fraction A on Pevikon block electrophoresis moved slightly to the anode and was stained with both alcian blue and PAS, the other component

Fig. 3 Cellulose acetate electrophoretic patterns of fractions obtained from Dowex 1×2 column chromatography in barbital buffer.

After electrophoresis, the strip was stained with alcian blue. A, top: fraction A obtained from zone electrophoresis on a Pevikon C-870 block; B, bottom: fraction B obtained from zone electrophoresis on a Pevikon C-870 block. C, crude preparation from bovine fourth stomach; I: 0.5 M NaCl fraction; II: 0.75 M NaCl fraction; III: 1.0 M NaCl fraction; IV: 1.25 M NaCl fraction; V: 1.5 M NaCl fraction; VI: 2.0 M NaCl fraction; HA: hyaluronic acid; HS: heparan sulfate; Ch-4-S: chondroitin-4-sulfate; DS: dermatan sulfate; Ch-6-S: chondroitin-6-sulfate.

migrated rapidly toward the anode with the same mobility of control glycosaminoglycans and was alcian blue positive but failed to stain with PAS. The rapid-moving bands of both 0.5 and 0.75 M NaCl fractions from the fraction A on Pevikon block electrophoresis corresponded in mobility with hyaluronic acid, those of both 1.0 and 1.25 M NaCl fractions corresponded in mobility with heparan sulfate and those of both 1.5 and 2.0 M NaCl fractions corresponded in mobility with chondroitin sulfates, respectively. Both the 1.0 and 1.25 M NaCl fractions from the fraction B on Pevikon block electrophoresis had one component which corresponded in mobility with heparan sulfate and both the 1.5 and 2.0 M NaCl fractions also had one band but it corresponded in mobility with chondroitin sulfates. Therefore, all fractions of the molarity of NaCl solution ranging from 0.5 to 2.0 M from both the fraction A and B on Pevikon block electrophoresis were digested further with neuraminidase, hyaluronidase from streptomyces hyalurolyticus, chon-

droitinase AC and chondroitinase ABC, and then used again for cellulose acetate electrophoresis. After digestion of the concentrates with neuraminidase the slow-moving component faded away in each of all NaCl fractions from the fraction A on Pevikon block electrophoresis, whereas other bands in all fractions from both the fraction A and fraction B remained unchanged. After treatment of the samples with hyaluronidase, the materials with same mobility as hyaluronic acid were resultedly disappeared in both the 0.5 and 0.75 M NaCl fractions from the fraction A, but all other bands from the total fractions remained. After digestion of the concentrates with chondroitinase AC, the materials with mobility of hyaluronic acid disappeared in both the 0.5 and 0.75 M NaCl fractions and the components with mobility of chondroitin sulfates faded in both the 1.5 and 2.0 M NaCl fractions while other bands with mobility of heparan sulfate remained in both the 1.0 and 1.25 M NaCl fractions. After treatment with chondroi-

TABLE 1
*Molar carbohydrate composition of glycosaminoglycans isolated from the mucosa of the bovine fourth stomach**

Fraction		Component				
Pevikon	Dowex 1 × 2	Glucosamine	Galactosamine	Sulfate	Uronic acid	
					Carbazole	c/o ratio**
A	0.5 M NaCl	1.0	trace	—	0.9	1.4
	0.75M NaCl	1.0	0.3	—	0.7	0.7
	1.0 M NaCl	1.0	0.4	0.9	0.8	1.1
	1.25M NaCl	1.0	0.3	0.8	1.2	1.4
	1.5 M NaCl	0.6	1.0	0.7	1.2	1.2
	2.0 M NaCl	0.5	1.0	0.8	0.5	0.7
B	1.0 M NaCl	1.0	trace	1.1	2.5	1.4
	1.25M NaCl	1.0	0.2	1.3	1.7	1.1
	1.5 M NaCl	0.2	1.0	1.1	1.1	0.7
	2.0 M NaCl	trace	1.0	1.4	0.7	0.4

* Expressed on the basis of glucosamine or galactosamine.

** The ratio of carbazole to orcinol.

tinase ABC, the slow-moving components remained as usual in both the 0.5 and 0.75 M NaCl fractions which were stained with both alcian blue and PAS, as well as the materials which found in the 1.0 and 1.25 M NaCl fractions also remained, whereas all other spots from the all fractions disappeared.

The chemical analyses of every fraction are summarized in TABLE 1. The fraction at the molarity of NaCl solution ranging from 0.5 to 1.25 M contained more glucosamine than galactosamine, while both the 1.5 and 2.0 M NaCl fractions contained more galactosamine than glucosamine. Sulfate was detected in the 1.0 to 2.0 M NaCl fractions, whereas it was scarcely found in the 0.5 and 0.75 M NaCl fractions. Both the uronic acid content which was measured by the carbazole reaction and the ratio of carbazole to orcinol reaction for uronic acid were lowest in the 2.0 M NaCl fraction. Both the 1.5 and 2.0 M NaCl fractions from the fraction B on Pevikon block electrophoresis were digested with chondroitinase AC, and the digested solution was studied by the paper chromatography. Paper chromatography of both the 1.5 and 2.0 M NaCl fractions revealed the presence of two components which corresponded in flow rate (Rf) with Δ Di-4S and Δ Di-6S, respectively.

Comparison of the results obtained by the chemical, enzymatic and electrophoretic studies together with paper chromatographical facts shows that there are five glycosaminoglycans in bovine fourth gastric mucosa. They are consisted of hyaluronic acid, heparan sulfate and chondroitin-4-sulfate, dermatan sulfate and chondroitin-6-sulfate. Paper chromatography and electrophoresis after enzymatic digestion of both the 1.5 and 2.0 M NaCl fractions from the fraction B on Pevikon

block electrophoresis with chondroitinase AC and chondroitinase ABC showed the presence of chondroitin-4-sulfate, dermatan sulfate and chondroitin-6-sulfate.

Contents of the carbohydrate components of glycosaminoglycans isolated from the mucosa of the bovine fourth stomach showed more scattered values in each of the fractions from the fraction A on Pevikon block electrophoresis than those in each of the fractions from the fraction B (TABLE 1), and each of the fractions from the fraction A contained the material which was stained with both alcian blue and PAS, but none of the fractions from the fraction B contained this material. Sialic acid-rich glycopeptide, which was stained with both alcian blue and PAS, was present in the mucosa of the bovine fourth stomach (Masuda, et al., 1977), and so this may suggest that such a glycopeptide might possibly be contaminated into each of the fractions from the fraction A on Pevikon block electrophoresis. Further purification of each of the glycosaminoglycans will be necessary and is now in progress.

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