Acid Mucopolysaccharides in the Salivary Glands of Dogs

Tetsuya Sakaki, Tadayoshi Honda, Hiroshi Morita, Hiromasa Akahori, Yoshiji Miyai and Dirk Keun Lee

Summary

Acid mucopolysaccharides were isolated from the salivary glands of dogs and determined qualitatively and quantitatively by chemical analysis, CPC fractionation, electrophoresis on cellulose acetate strips, paper chromatography and enzymic digestion.

The ratio of the yield of whole acid mucopolysaccharides to tissue dry weight was highest in the sublingual gland followed by submaxillary and parotid glands. No difference was found in the components of acid mucopolysaccharides in the salivary glands. Hyaluronic acid, dermatan sulfate and heparan sulfate constituted major components, while chondroitin sulfate A and C constituted minor components. The composite ratios of each acid mucopolysaccharide were different depending upon the kind of salivary glands.

Thus, it may be considered that the components of acid mucopolysaccharides in the dog salivary glands were qualitatively the same as those of the rat, bovine and pig salivary glands, but in quantity, different from those of their salivary glands.

Introduction

It is well known that acid mucopolysaccharides in the salivary glands of the rat1-2, bovine3-4 and pig5-6 are mainly composed of hyaluronic acid, dermatan sulfate and heparan sulfate. The presence of chondroitin sulfate A and C in the salivary glands of these animals is also reported1-4).

Since the functional significance of acid mucopolysaccharides in various organs has not yet been clearly defined, it seemed to be important to examine the presence of acid mucopolysaccharides in the salivary glands of various animals which have a physiological function, such as saliva production and secretion.

In the present work, the isolation and determination of acid mucopolysaccharides from the dog salivary glands were studied.

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Materials and Methods

1. Preparation of materials

The submaxillary, sublingual and parotid glands were obtained from adult male dogs, weighing about 15 kg, rinsed in physiological saline solution, and kept in aceton in a cold room for several days. The pooled salivary glands obtained from five individuals were homogenized with Polytron, defatted and dehydrated with several changes of acetone, with methanol-chloroform (1:1, v/v) and then with ether. The material was dried in a vacuum exsiccator and weighed.

2. Extraction of acid mucopolysaccharides

Defatted dry samples were suspended in ten volumes of water, heated at 100°C for 1 hr. The suspension was digested with pepsin for 72 hr at pH 1.8 and 38°C, and with trypsin for 48 hr at pH 7.5 and 38°C. After being deproteinized with trichloroacetic acid and dialysis, the crude acid mucopolysaccharides in the dialysate were precipitated with three volumes of ethanol containing 1% potassium acetate, allowed to stand for 24 hr at 4°C, and centrifuged. The precipitate was washed with 80% ethanol, redissolved in water, and precipitated by addition of 0.4 M NaCl solution containing 1% of cetylpyridinium chloride (henceforth abbreviated as CPC). The CPC-acid mucopolysaccharide complex collected by centrifugation was washed several times with 0.04 M NaCl solution containing 0.1% CPC. Whole acid mucopolysaccharides were extracted from the precipitate with 30 volumes of 3.0 M NaCl solution containing 0.1% CPC and then precipitated by adding three volumes of ethanol. The precipitate was collected, washed several times with ethanol, redissolved in water, dialyzed against water for 48 hr, and lyophilized.

3. Separation of acid mucopolysaccharides

Acid mucopolysaccharides were separated following the method of Schiller et al. (1961) with a minor modification. The acid mucopolysaccharides were dissolved in 0.04 M NaCl solution, and a 1% solution of CPC was added until precipitation was complete. After the precipitate was incubated at 37°C for 1 hr, celite 535 was added. The precipitate collected by centrifugation was washed with 0.04 M NaCl solution containing 0.1% CPC. Non-sulfated and sulfated mucopolysaccharides were then extracted from the precipitate successively with 0.4 M and 3.0 M NaCl solutions containing 0.1% CPC. They were precipitated from solutions by adding two volumes of ethanol and one-tenth volume of methanol, collected by centrifugation, redissolved in water, dialyzed against water and lyophilized.

4. Analytical method

Uronic acid was determined with a modified carbazole reaction by Bitter et al. (1962), the primary carbazole reaction by Dische (1947) and orcinol reaction. Glucuronic acid (Nakarai Chemicals, Kyoto, Japan) was used as standard.

Glucosamine and galactosamine were determined by a Hitachi liquid chromatograph 034 with a 0.9×20 cm column at 55°C, using 0.35 M citrate buffer (pH 5.28) at a flow rate of 60 ml/hr, after hydrolysis in 4.0 M HCl in stoppered tubes at 100°C for 16 hr. D-Glucosamine HCl and D-galactosamine HCl (Nakarai Chemicals, Kyoto, Japan) were
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used as standard.

Sulfate was determined by Antonopoulos' benzidine method after hydrolysis in 25% formic acid in stoppered tubes at 100°C for 24 hr.

Hexose was determined by an anthrone method\textsuperscript{13} with D-galactose (Nakarai Chemical, Kyoto, Japan) used as standard.

Isomeric chondroitin sulfate was determined following the method of Saito et al. (1968)\textsuperscript{13}, based on conversion of chondroitin sulfate that is inactive in the Morgan-Elson reaction to an active form in the color reaction by chondroitinase and chondrosulfatase digestion. The amount of 2-acetamido-2-deoxy-3-O-(β-D-gluc-4-enepyrano-syluronic acid)-D-galactose (\(\text{Di-OS}\)) formed under the influence of the three multienzyme systems, i.e., chondroitinase ABC plus chondro-6-sulfatase, chondroitinase AC plus chondro-4-sulfatase plus chondro-6-sulfatase, and chondroitinase ABC plus chondro-4-sulfatase plus chondro-6-sulfatase, was measured with a modified Morgan-Elson reaction by Reissig et al. (1955)\textsuperscript{14}. The amount of each isomer was calculated from the yield of \(\text{Di-OS}\) in the three systems.

5. Electrophoresis on cellulose acetate strips

A two-dimensional electrophoresis of the whole acid mucopolysaccharides on a cellulose acetate strips (Separax, Jookoo Sangyo Co., Tokyo, Japan) was performed first in 0.1 M pyridine-0.47 M formic acid (pH 3.0) at a constant current of 1 mA/cm for 1 hr and, after the strip was dried in the air, a second run was performed in 0.1 M barium acetate (pH 8.0) at 1 mA/cm for 3 hr\textsuperscript{10}.

Electrophoresis of the separated acid mucopolysaccharides was performed in 0.1 M pyridine-0.47 M formic acid at 1 mA/cm for 35 min or in 0.2 M calcium acetate at 1 mA/cm for three hr\textsuperscript{19}. The strips were stained with 0.5% alcian blue 8GS in 3% acetic acid for 20 min, rinsed with 1% acetic acid for 10 min and then with water for 10 min, and dried.

Superspecial grade samples of chondroitin sulfate A and C, dermatan sulfate and heparin were purchased from Seikagaku Kogyo Co., Tokyo, Japan, and hyaluronic acid was from Fluka AG, Switzerland. Heparan sulfate from the rat kidney was kindly supplied by Dr. Nagase, the Medical Institute of Sasaki Foundation, Tokyo.

6. Enzymic digestion

The acid mucopolysaccharides in 0.4 M NaCl fraction were digested with streptomyces hyaluronidase (3000 T. R. U./mg protein, purchased from Seikagaku Kogyo Co., Tokyo, Japan) in 0.02 M acetic acid buffer (pH 5.0) at 50°C for 48 hr\textsuperscript{13}. The acid mucopolysaccharides in 3.0 M NaCl fraction were digested with chondroitinase ABC or AC (Seikagaku Kogyo Co., Tokyo, Japan) according to the method of Saito et al. (1968)\textsuperscript{13}.

The unsaturated disaccharides obtained were separated by paper chromatography in n-butyrlic acid-0.5 N ammonia (5: 3, v/v) for 48 hr at room temperature and detected by staining with silver nitrate reagent\textsuperscript{15}.

The effect of enzymic digestion were evaluated on the basis of electrophoretic behaviors of these products on a cellulose acetate strip.

The amount of heparan sulfate in 3.0 M NaCl fraction was calculated from the
yield of uronic acid in the acid mucopolysaccharides which were resistant to chondroitinase ABC or AC.

Results

1. Preparation of whole acid mucopolysaccharides

The yield of the whole acid mucopolysaccharides is shown in Table 1 as the amount of glucuronic acid. That yield in the sublingual, submaxillary and parotid glands of dogs was 0.10, 0.08 and 0.07% of dry tissue, respectively.

The results of chemical analysis are summarized in Table 2. The molar ratio of uronic acid to hexosamine was 1/1.2, and the sulfate content was low in all the salivary glands. In the submaxillary and sublingual glands, the content of glucosamine was over two times that of galactosamine, and the ratio of carbazole to orcinol was lower than 1. While in the parotid gland, the content of glucosamine was less than that of galactosamine, and the ratio of carbazole to orcinol was approximately 0.5.

Table 1. Acid mucopolysaccharide content in salivary glands of five dogs

<table>
<thead>
<tr>
<th></th>
<th>Parotid</th>
<th>Submaxillary</th>
<th>Sublingual</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry weight (g)</td>
<td>2.56</td>
<td>3.00</td>
<td>1.65</td>
</tr>
<tr>
<td>Total amount of acid mucopolysaccharides (µg as glucuronic acid*)</td>
<td>1780</td>
<td>2408</td>
<td>1623</td>
</tr>
<tr>
<td>Acid mucopolysaccharides (µg) / dry weight (g)</td>
<td>687</td>
<td>802</td>
<td>984</td>
</tr>
</tbody>
</table>

* Modified carbazole reaction by Bitter-Muir

Table 2. Analysis of whole acid mucopolysaccharides in dog salivary glands

<table>
<thead>
<tr>
<th></th>
<th>Parotid</th>
<th>Submaxillary</th>
<th>Sublingual</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uronic acid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bitter-Muir* (%)</td>
<td>28.4</td>
<td>23.9</td>
<td>25.9</td>
</tr>
<tr>
<td>Carbazole** (%)</td>
<td>23.2</td>
<td>20.5</td>
<td>21.5</td>
</tr>
<tr>
<td>Orcinol (%)</td>
<td>45.3</td>
<td>25.2</td>
<td>33.9</td>
</tr>
<tr>
<td>C/O***</td>
<td>0.51</td>
<td>0.81</td>
<td>0.63</td>
</tr>
<tr>
<td>Hexosamine (%)</td>
<td>32.5</td>
<td>26.1</td>
<td>28.7</td>
</tr>
<tr>
<td>Glu/Gal****</td>
<td>0.79</td>
<td>2.42</td>
<td>2.04</td>
</tr>
<tr>
<td>Sulfate (%)</td>
<td>7.7</td>
<td>3.9</td>
<td>6.2</td>
</tr>
<tr>
<td>Hexose (%)</td>
<td>12.0</td>
<td>15.3</td>
<td>14.0</td>
</tr>
</tbody>
</table>

*Modified carbazole reaction by Bitter-Muir
**Primary carbazole reaction by Dische
***Ratio of carbazole to orcinol
****Ratio of glucosamine to galactosamine
2. Electrophoretic determination of acid mucopolysaccharides

On a two-dimensional electrophoresis, the whole acid mucopolysaccharides in each salivary gland showed three spots corresponding to hyaluronic acid, heparan sulfate and dermatan sulfate, with a faint spot corresponding to chondroitin sulfate A (and/or C) (Fig. 1).

3. Separation of acid mucopolysaccharides

For further characterization of the acid mucopolysaccharide components, separation based on a differential solubility of mucopolysaccharide-CPC complexes in NaCl solution was carried out. The yield of these fractions is shown in Table 3. In the parotid gland, about 65% of the acid mucopolysaccharide content was extracted with 3.0 M NaCl, and nearly 35% was found in the 0.4 M NaCl fraction. However, in the sublingual gland, about 53% of the acid mucopolysaccharide content was found in the 0.4 M NaCl fraction.

### Fig. 1. Two-dimensional electrophoretic patterns of whole acid mucopolysaccharides.

Electrophoretic system:
0.1 M pyridine-0.47 M formic acid (pH 3) at 1 mA/cm for 1 hr in the first dimension, and 0.1 M barium acetate (pH 8) at 1 mA/cm for 3 hr in the second.

Standard samples:
1. Chondroitin sulfate A
2. Dermatan sulfate
3. Hyaluronic acid
4. Heparan sulfate
Table 3. Fractionation of acid mucopolysaccharides by CPC method

<table>
<thead>
<tr>
<th>NaCl fraction</th>
<th>Uronic acid Amount (μg)*%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parotid</td>
<td></td>
</tr>
<tr>
<td>0.4 M</td>
<td>251.5</td>
</tr>
<tr>
<td>3.0 M</td>
<td>469.6</td>
</tr>
<tr>
<td>Submaxillary</td>
<td></td>
</tr>
<tr>
<td>0.4 M</td>
<td>970.9</td>
</tr>
<tr>
<td>3.0 M</td>
<td>967.1</td>
</tr>
<tr>
<td>Sublingual</td>
<td></td>
</tr>
<tr>
<td>0.4 M</td>
<td>350.2</td>
</tr>
<tr>
<td>3.0 M</td>
<td>313.0</td>
</tr>
</tbody>
</table>

*Modified carbazole reaction by Bitter-Muir

fraction, and remaining 47% or so was found in the 3.0 M NaCl fraction. In the submaxillary gland, about the same content was observed in the 0.4 M and 3.0 M NaCl fractions.

4. Characterization of separated mucopolysaccharides
   a. The 0.4 M NaCl fraction

   Hexosamine of this fraction was found to consist of nearly 100% of glucosamine and a trace amount of galactosamine. On a cellulose acetate electrophoresis, this fraction from each salivary gland showed a single spot corresponding to standard hyaluronic acid (Fig. 2), and after digestion with streptomyces hyaluronidase, no spot was found (Fig. 3). From these results, this fraction seems to contain only hyaluronic acid.

   b. The 3.0 M NaCl fraction

   This fraction from each salivary gland afforded three spots (Fig. 2). A faint spot with mobility equal to that of standard heparan sulfate was not affected when digested with chondroitinase ABC or chondroitinase AC (Fig. 3). A faint spot with mobility the same as that of standard hyaluronic acid (and/or C) was affected following chondroitinase AC or chondroitinase ABC digestion (Fig. 3). A spot corresponding to standard dermatan sulfate was affected when subjected to chondroitinase ABC digestion, but unchanged when digested with chondroitinase AC (Fig. 3).

   In order to confirm the type of chondroitin sulfate, paper chromatography of products of this fraction resulting from digestion with chondroitinase ABC or chondroitinase AC was also performed. The paper chromatogram afforded two spots corresponding to Di-4S and Di-6S in each salivary gland. Moreover, a spot of Di-4S produced by chondroitinase ABC digestion was slightly stronger than that by chondroitinase AC digestion (Fig. 4).

   From the above results, this fraction seems to contain dermatan sulfate as a major component and chondroitin sulfate A and C and heparan sulfate as minor components.

5. Amount of heparan sulfate and chondroitin sulfate isomers

   The amount of heparan sulfate in the 3.0 M NaCl fraction was calculated from the yield of uronic acid in the acid mucopolysaccharides which were resistant to chondroitinase ABC or AC (Table 4). The amount of heparan sulfate in the parotid, submaxillary and sublingual glands accounted for 11.6, 17.2 and 10.3% of the total amount of acid mucopolysaccharides, respectively.
Fig. 2. Electrophoretic pattern of each fraction obtained by CPC method.
Electrophoretic system:
0.2 M calcium acetate at 1 mA/cm for 3 hr
Standard samples:
1. Heparin and heparan sulfate
2. Chondroitin sulfate C, A, dermatan sulfate and hyaluronic acid
Abbreviation used:
P. Parotid gland
M. Submaxillary gland
L. Sublingual gland

Table 4. Chondroitinase ABC sensitivity of acid mucopolysaccharides in 3.0 M NaCl fraction

<table>
<thead>
<tr>
<th>Chondroitinase ABC</th>
<th>Sensitive (%)</th>
<th>Resistant (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parotid</td>
<td>82.23</td>
<td>17.77</td>
</tr>
<tr>
<td>Submaxillary</td>
<td>65.61</td>
<td>34.39</td>
</tr>
<tr>
<td>Sublingual</td>
<td>78.10</td>
<td>21.90</td>
</tr>
</tbody>
</table>

Quantities of isomeric chondroitin sulfate in the 3.0 M NaCl fraction, measured following the method of Saito et al. (1968), are presented in Table 5 against total chondroitin sulfates and total acid mucopolysaccharides. In all the salivary glands, dermatan sulfate amounted to over 25% of total acid mucopolysaccharides, while the amount of chondroitin sulfate C was as slight as below 5%. The amount of chondroitin sulfate A in the parotid gland was about 18% of total acid mucopolysaccharides, while those in the other glands were below 4%.
Fig. 3. Electrophoretic patterns of each fraction digested with hyaluronidase or chondroitinase.

Electrophoretic system:
0.2 M calcium acetate at 1 mA/cm for 3 hr

Standard samples:
1. Heparin and heparan sulfate
2. Chondroitin sulfate C, A, dermatan sulfate and hyaluronic acid

Abbreviation used:
P. Parotid gland
M. Submaxillary gland
L. Sublingual gland
SH. Streptomyces hyaluronidase
CHase. Chondroitinase

Table 5. Distribution of chondroitin sulfate isomer in dog salivary glands

<table>
<thead>
<tr>
<th></th>
<th>3.0 M NaCl fraction</th>
<th>Compositive ratio to whole acid mucopolysaccharides</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ChS-A (%)</td>
<td>DS (%)</td>
</tr>
<tr>
<td>Parotid</td>
<td>15.7</td>
<td>81.3</td>
</tr>
<tr>
<td>Submaxillary</td>
<td>8.0</td>
<td>76.3</td>
</tr>
<tr>
<td>Sublingual</td>
<td>11.0</td>
<td>79.9</td>
</tr>
</tbody>
</table>
6. Composition of acid mucopolysaccharides

From the above results, the acid mucopolysaccharides in the dog salivary glands proved to be hyaluronic acid, chondroitin sulfate A and C, dermatan sulfate and heparan sulfate. The major component in the submaxillary and sublingual glands proved to be hyaluronic acid, corresponding to 50% of total acid mucopolysaccharides, while in the parotid gland, dermatan sulfate was the major component accounting for 44% of total acid mucopolysaccharides. The amount of dermatan sulfate in the submaxillary and

**Fig. 4.** Paper chromatogram of acid mucopolysaccharides digested with chondroitinase.

Developer: n-Butyric acid—0.5 N ammonia (5: 3, v/v)
Paper: Toyo Roshi filter paper No. 51A

**Fig. 5.** Composition of acid mucopolysaccharides in dog salivary glands
sublingual glands was approximately 35%, respectively, less than that in the parotid gland. The amount of the other acid mucopolysaccharides was different depending upon the kind of salivary glands (Fig. 5).

Discussion

In the present study, the concentration of acid mucopolysaccharides from the adult dog salivary glands was highest in the sublingual gland, intermediate in the submaxillary gland, and lowest in the parotid gland on the basis of tissue dry weight. The findings are different from that for the adult rat2). In addition, in the previous studies in authors' laboratory, differences in acid mucopolysaccharide content were recognized between bovine3,4) and pig5,6) salivary glands, both of which were young adult. From these findings, it may be considered that such differences in the acid mucopolysaccharide content in the salivary glands depend upon the difference of the kind of animals.

However, no difference in the constituent components of acid mucopolysaccharides was found in any salivary glands of dogs. The acid mucopolysaccharides contained hyaluronic acid, dermatan sulfate and heparan sulfate as major components with chondroitin sulfate A and C as minor components. While, the compositive ratios of each acid mucopolysaccharide were different depending upon the kind of salivary glands. From the above findings, it was assumed that the components of acid mucopolysaccharides in the dog salivary glands were almost the same qualitatively, but different quantitatively when compared with those of the rat2), bovine3,4) and pig5,6) salivary glands.

It has generally been considered that the production and secretion of acid mucopolysaccharides is one of the properties characteristic of animal connective tissue cells, though, more recently, there have been several reports19-23) indicating the production of acid mucopolysaccharides by cultured epithelial cells. The acid mucopolysaccharides in the dog salivary glands may thus originate from mesenchymal tissue which is generally rich in the salivary glands.

References

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