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Regional Distribution of Mucopolysaccharides in the Pig Kidney

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Summary

Mucopolysaccharides were isolated from the cortex and medulla of pig kidney, and characterized by chemical analysis, electrophoresis and enzymic digestion. The mucopolysaccharides in the cortex were found to be mainly heparan sulfate and dermatan sulfate, with trace amounts of hyaluronic acid and chondroitin sulfate A. In the medulla, heparan sulfate was also the major mucopolysaccharide, with dermatan sulfate and hyaluronic acid, and a trace of chondroitin sulfate A. This mucopolysaccharide pattern of the medulla was quite different from those of other animals.

Three heparan sulfate fractions obtained from both the cortex and medulla by Dowex 1 chromatography showed different contents of sulfate, but there was practically no difference in the amounts of *N*-sulfated hexosamine. After alkaline reduction, it was proved that the heparan sulfates are linked to peptides through *O*-glycosidic bonds between xylose and serine, as in the chondroitin sulfates.

Introduction

In relation to the physiological functions of the kidney, such as transport of water and electrolytes, much interest has been concentrated on the mucopolysaccharides of this organ. However, although several studies have been reported (1–7), no distinct evidence has been obtained to establish a relationship between any of the functions and the mucopolysaccharide components, because of the different procedures of extraction and identification used in the previous investigations. Therefore, it is necessary to re-study quantitatively the details of the constituents of kidney mucopolysaccharides.

The present paper describes the isolation and characterization of mucopolysaccharides from the cortex and medulla of pig kidney, in order to compare the constituents of the mucopolysaccharide in these two tissues. The heterogeneity of the heparan sulfates isolated is also described.

Methods

Chemical analysis — Chemical analysis of uronic acid, hexosamine, sulfate, *N*-sulfated hexosamine and *N*-acetyl were performed as described previously (8).

Amino acid analysis — Amino acids were analyzed by means of a JEOL-JLC-5AH amino acid analyzer, after hydrolysis in 6 *M* HCl at 110°C for 22 h in a vacuum-sealed tube.

Analysis of neutral sugar and sugar alcohol — For analysis of neutral sugar and alditol after alkaline reduction, the sample was hydrolyzed in 0.5 *M* H_2SO_4 at 100°C for 4 h and neutralyzed; arabinitol was added as an internal standard and the reaction mixture was deionized as described previously (9). The sample was acetylated (10) and analyzed by gas liquid chromatography, using a Shimadzu GC–4BM equipped with a glass column packed with 3% ECNSS-M on Gas Chrom Q(100–120 mesh), with nitrogen as carrier gas at a column temperature of 190°C.

Electrophoresis on cellulose acetate strips — Electrophoresis of mucopolysaccharides on cellulose acetate strips (Separax) was carried out in the following three systems: (1) 1 *M* acetic acid-pyridine (pH 3.5) at 0.5 mA/cm for 20 min; (2) 0.1 *M* barium acetate at 1 mA/cm for 2.5 h; and (3) 0.1 *M* HCl at 1 mA/cm for 2.5 h. The spots of mucopolysaccharides were detected by staining with 0.5% alcian blue or 0.5% toluidine blue (11).

Enzymic digestion — Mucopolysaccharide (200 μg/20 μl) dissolved in 0.25 *M* Tris-HCl buffer (pH 8.0) was incubated overnight with chondroitinase-ABC (0.2 units/20 μl) or chondroitinase-AC (0.2 units/20 μl) at 37°C. The reaction mixture was used for paper chromatography. Sample (1.0 *M* NaCl, 1.5 *M* NaCl fractions, 50 mg/ml) dissolved in 0.1*M* acetate buffer (pH 4.5) containing 0.15*M* NaCl were digested with testicular hyaluronidase (5 mg/ml) at 37°C for 16 h.

Paper chromatography — For unsaturated disaccharides obtained by digestion with chondroitinases, descending paper chromatography was performed according to the method of Saito *et al.* (12).

Preparation of mucopolysaccharides from pig cortex and medulla — After removing the capsule and vessels from fresh kidneys, they were dissected into cortical and medullary portions and cut into small pieces. The wet tissue was homogenized with acetone in a Waring blendor and dehydrated and defatted with three changes of acetone followed by two changes of chloroform-methanol (2:1, v/v), and dried. Extraction and fractionation of mucopolysaccharides from the defatted cortex and medulla were carried out essentially according to the procedure of Seno *et al.*, including Dowex 1 chromatography with stepwise elution of NaCl (0.5 *M*, 0.7 *M*, 1.0 *M*, 1.25 *M*, 1.5 *M*, 2.0 *M* and 3.0 *M*) (6).

Isolation of heparan sulfate fractions — In order to isolate heparan sulfate fractions, two fractions (1.0 *M* NaCl Fr. and 1.5 *M* NaCl Fr.) obtained by Dowex 1 fractionation were digested with testicular hyaluronidase and chondroitinase-ABC and applied to a column of Sephadex G–50 equilibrated with 1 *M* NaCl–10%

EtOH as described previously (8). The undigested polymer fraction was desalted by gel filtration on Sephadex G–25 in 10% EtOH and lyophilized.

Alkaline-reduction — For ß-elimination-reduction of heparan sulfate-peptides, the heparan sulfate fraction (3–4.5 mg/ml) was mixed with 0.04 *M* $PdCl_2$-$2H_2O$ (0.5 ml) and 1.2 *M* $NaBH_4$ in 1.6 *M* NaOH (0.5 ml) at room temperature for 24 h under nitrogen (9).

In order to analyze the position of the glycosidic linkage and the position substituted by sulfate, alkaline treatment of the heparan sulfate fractions was carried out according to the method of Sampson and Meyer (13): heparan sulfate was treated at a concentration of 10 mg/ml in 1.0*M* NaOH containing 20 mg of $NaBH_4$ at 80°C for 7 h. The polymer fraction re-isolated by gel filtration on Sephadex G–25 in 10% EtOH was hydrolyzed in 4 *M* HCl at 100°C for 1 h. The hydrolysate was analyzed by the amino acid analyzer, using a short column in citrate buffer (pH 5.28).

Results

Characterization of mucopolysaccharide components

The yields of mucopolysaccharides extracted from pig cortex and medulla were 0.15% and 0.29% of the dry tissues, respectively. The yield and chemical analysis of each fraction, after fractionation of the mucopolysaccharides on a Dowex 1 column, were summarized in Table I. The major mucopolysaccharides of the cortex and medulla were eluted with 1.25 *M* NaCl and 1.0 *M* NaCl, respectively.

In the 0.5 *M* NaCl fraction, the sulfate content was very low; glucosamine was found as the only hexosamine present. A single band corresponding to hyaluronic acid was detected by electrophoresis. These results showed that this fraction is mainly hyaluronic acid.

In the 1.0 *M* NaCl fraction, the only hexosamine found was glucosamine; a small amount of sulfate and *N*-sulfated hexosamine were detected. On electrophoresis, this fraction gave a major band corresponding to heparan sulfate and a trace of hyaluronic acid.

In the 1.25 *M* NaCl fraction, the only hexosamine found was glucosamine. On electrophoresis, this fraction gave a single band corresponding to heparan sulfate, and contained *N*-sulfated hexosamine; its total sulfate content was higher than that of the 1.0 *M* NaCl fraction. These results showed that this fraction includes only heparan sulfate. It was designated as "1.25 *M* heparan sulfate".

The 1.5 *M* NaCl fraction contained both glucosamine and galactosamine. Three bands corresponding to heparan sulfate, dermatan sulfate and chondroitin sulfate A were found by electrophoresis.

In the 2.0 *M* NaCl fraction, the ratio of carbazole to orcinol was low. This fraction gave ⊿Di-4S and ⊿Di-6S on digestion with chondroitinase-ABC but no

Table 1. Analysis of each fraction obtained by Dowex 1 chromatography

	0.5 *M* Fr.		1.0 *M* Fr.		1.25 *M* Fr.		1.5 *M* Fr.		2.0 *M* Fr.	
	Cortex	Medulla	Cortex	Medulla	Cortex	Medulla	Cortex	Medulla	Cortex	Medulla
Yield (%)	5.6	4.3	18.6	23.2	30.4	20.4	21.3	21.1	16.5	19.3
Uronic acid										
Carbazole (%)	29.6	23.4	43.1	43.0	39.8	40.0	24.2	32.8	13.6	15.5
Orcinol (%)	25.0	18.1	23.0	22.4	18.3	22.5	12.6	29.8	22.0	22.2
C/O	1.18	1.29	1.87	1.92	2.18	1.80	1.92	1.10	0.61	0.69
Hexosamine										
Elson-Morgan (%)	23.6	19.2	26.9	26.1	23.6	24.4	20.3	28.4	22.5	23.0
GlcN/GalN	GlcN	GlcN	GlcN	GlcN	GlcN	GlcN	2.6/1.0	1.0/2.0	1.0/3.7	1.0/5.2
N-Sulfated hexosamine (%)	—	—	6.5	8.5	9.3	8.5	2.0	2.7	—	—
Sulfate (%)	0.5	0.4	4.4	4.0	8.4	9.0	9.5	15.0	14.5	16.2
Neutral sugar as Gal (%)	4.8	2.2	5.2	3.3	4.7	2.9	1.3	2.2	1.9	2.6
Lowry protein (%)	6.2	5.2	3.7	3.5	3.9	4.4	3.0	2.8	1.5	2.1

C/O: ratio of carbazole to orcinol; GlcN: glucosamine; GalN: galactosamine.

Table II. Comparison of heparan sulfate fractions

	1.0 *M* HS				1.25 *M* HS				1.5 *M* HS			
	Cortex		Medulla		Cortex		Medulla		Cortex		Medulla	
	%	Molar ratio	%	Molar ratio	%	Molar ratio	%	Molar ratio	%	Molar ratio	%	Molar ratio
Glucosamine	26.9	1.0	26.1	1.0	23.6	1.0	24.4	1.0	24.0	1.0	27.4	1.0
Uronic acid												
Carbazole	43.1	1.5	43.0	1.5	39.8	1.6	40.4	1.6	38.8	1.5	35.2	1.2
Orcinol	23.0		22.4		18.3		22.5		18.2		21.6	
C/O	1.87		1.92		2.18		1.80		2.10		1.63	
Sulfate	4.4	0.3	4.0	0.3	8.4	0.7	9.0	0.7	9.7	0.7	13.0	0.9
N-Sulfated hexosamine	9.0	0.3	6.7	0.3	9.3	0.4	8.5	0.3	9.7	0.4	5.3	0.2
N-Acetyl	6.0	0.67			5.9	0.74			5.7	0.70		
$[\alpha]_D$	+42.5° (c 1.9, H_2O)				+64.5° (c 1.5, H_2O)				+38.5° (c 0.8, H_2O)			

HS: heparan sulfate.

unsaturated disaccharides with chondroitinase-AC.

Variation of heparan sulfate in pig kidney

Isolation and characterization of three heparan sulfate fractions — After enzymic digestion followed by gel filtration of the 1.0 *M* and 1.5 *M* NaCl fractions, the polymer fractions remaining gave a single band corresponding to heparan sulfate, on electrophoresis (in barium acetate). These purified heparan sulfates were obtained in a yield of 46% and 35% of each fraction, and were designated as "1.0 *M* and 1.5 *M* heparan sulfate", respectively. The chemical analyses of these heparan sulfates and 1.25 *M* heparan sulfate are listed in Table II. Glucosamine was found as the only hexosamine present; the ratio of carbazole to orcinol was about 2. Total sulfate and the electrophoretic mobilities in 0.1 *M* HCl increased gradually in the order of 1.0 *M*, 1.25 *M* and 1.5 *M* heparan sulfate. The content of *N*-sulfated hexosamine of these heparan sulfate fractions ranged between 0.33 and 0.40. All cortex heparan sulfate fractions were eluted at the void volume of a Sephadex G–50 column, and they were retained as a relatively broad peak on a Sephadex G–75 column.

When 1.25 *M* heparan sulfate and 1.5 *M* heparan sulfate were treated with alkali in the presence of $NaBH_4$ at 80°C for 7 h and analyzed by an amino acid analyzer, a new peak at the relative retention time of 1.55 to glucosamine was detected, as reported previously (13).

Linkage region of heparan sulfate-peptides — The results of amino acid and neutral sugar analyses of the cortical heparan sulfate fractions are shown in Table III. The content of amino acids in 1.0 *M* heparan sulfate was higher than those

Table III. Effect of alkaline reduction on amino acids and neutral sugars of heparan sulfate fractions

	1.0 *M* HS		1.25 *M* HS		1.5 *M* HS	
	Before	After	Before	After	Before	After
	(μmole/g)		(μmole/g)		(μmole/g)	
Aspartic acid	21.4	21.8	12.5	12.7	11.9	12.2
Threonine	6.4	3.3	4.5	1.9	5.1	3.1
Serine	22.8	9.3	13.1	5.2	12.1	6.5
Glutamic acid	13.2	12.9	9.5	9.4	10.2	10.4
Proline	2.3	2.3	—	—	—	—
Glycine	83.1	82.0	39.0	39.4	21.2	19.4
Alanine	11.4	23.6	7.4	14.5	8.7	14.4
Valine	—	—	6.8	6.6	8.0	10.8
Isoleucine	1.4	—	1.4	0.8	2.3	2.1
Leucine	3.6	—	3.9	3.2	4.5	4.8
Total amino acids	165.6		98.1		84.0	
Fucose		8.9		1.2		
Xylose		10.4		2.5		
Xylitol		12.8		9.9		
Galactose		50.0		27.5		
Glucose		17.9		5.6		
Mannose		20.3		10.0		

in 1.25 *M* and 1.5 *M* heparan sulfate. The predominant amino acids were Gly, Ser, Asp, Glu and Ala in all the fractions. Alkaline reduction of these heparan sulfates caused the destruction of 59%, 60% and 46% of the amounts of Ser originally present, respectively, and Ala and xylitol were produced in amounts equivalent to the amount of serine decreased. The neutral sugars in 1.0 *M* heparan sulfate and 1.25 *M* heparan sulfate were found to be mainly Gal and Xyl, with Glc, Man and Fuc, and the molar ratio of Gal to (Xyl+Xylitol) was about 2.

Discussion

From the results of the elution pattern on Dowex 1 and the electrophoretogram on cellulose acetate strips of each fraction, the mucopolysaccharide compositions were listed as shown in Table IV. The major mucopolysaccharide was heparan sulfate in both tissues. These results differ from the previous reports: rabbit and dog medulla were found to contain hyaluronic acid as the major component (50% and 60% of the total mucopolysaccharides, respectively (3, 2). In order to check the loss of hyaluronic acid during pretreatment of the tissues with acetone, fresh kidney was separated into cortex and medulla and each tissue was immediately lyophilized. The mucopolysaccharides obtained from them by the same procedure still mainly consisted of heparan sulfate (Table IV). In the medulla, the percent of hyaluronic acid was slightly greater and dermatan sulfate was smaller, whereas in the cortex, the dermatan sulfate content was slightly varied. Although it is possible that some of the hyaluronic acid was lost during acetone treatment of the tissue, there is no doubt that the major mucopolysaccharide of pig medulla is heparan sulfate.

Each sample of heparan sulfate obtained at the same NaCl concentration from cortex and medulla was similar in its chemical analysis to the others. On the basis of gel filtration results, the molecular weights of these heparan sulfates

Table IV. Relative amounts of mucopolysaccharides obtained from cortex and medulla

		% of whole mucopolysaccharides				
		HS	DS	HA	ChS-A	ChS-C
Cortex	Acetone treated	57	24	8	11	—
	Freeze dried	79	5	7	7	2
Medulla	Acetone treated	49	25	15	10	—
	Freeze dried	52	3	30	15	—

HA: hyaluronic acid; DS: dermatan sulfate; ChS-A: chondroitin sulfate A; ChS-C: chondroitin sulfate C.

were considered to be about $1\text{–}2\times10^4$, similar to those of hog mucosa (14), and beef lung (14, 15).

Chemical analysis of 1.0 *M* heparan sulfate showed that its sulfate consisted of *N*-sulfate, with no *O*-sulfate. The low-sulfated heparan sulfate reported in beef lung heparan sulfate (16) consisted of *O*-sulfate, and the ratio of *O*-sulfate to *N*-sulfate was over 1 in amyloid liver (15) and about 1 in rat brain (17). Comparison with these results suggests that the 1.0 *M* heparan sulfate of pig kidney may be a new type of heparan sulfate.

In 1.25 *M* heparan sulfate and 1.5 *M* heparan sulfate, *O*-sulfate was present in addition to *N*-sulfate, and the position of the ester sulfate may be C–2 or C–3 of uronic acid, based on the absorption at 800 cm^{-1} in infrared spectra (18). The formation of 3,6-anhydroglucosamine on alkaline treatment suggested that the ester sulfate links at C–3 or C–6 of glucosamine, although further study may be required to elucidate the position of the sulfate.

The mode of linkage was proved to be an *O*-glycosyl linkage between Ser and Xyl by quantitative ß-elimination-reduction. It is of interest in connection with the biosynthesis that the linkage regions of heparan sulfate-peptides were similar to those of other mucopolysaccharides such as chondroitin sulfate A, dermatan sulfate and heparin.

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