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Short Communication

Study of the measurement of chondroitin sulphates in rabbit plasma and serum

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ABSTRACT

A highly sensitive high-performance liquid chromatography method, which was established by us for the determination of chondroitin sulphates in biological substances as their unsaturated disaccharides, was applied to elucidate the qualitative and quantitative differences in chondroitin sulphates in rabbit plasma and serum samples. In this work, it was found that rabbit plasma contains low-sulphated chondroitin 4-sulphate (approximately 40% sulphation at the 4-position of N-acetyl galactosamine), while serum contains the low-sulphated chondroitin 4-sulphate and fully sulphated chondroitin 4-sulphate (approximately 96% sulphation). The latter was released from platelets during coagulation of blood.

INTRODUCTION

Concentrations of glycosaminoglycans (GAGs) in mammalian serum are reported to be higher than those in plasma when GAGs are determined as uronic acids [1]. However, the species and an origin of increased GAGs in serum are not clear. For physiological and clinical studies on chondroitin sulphates (CSs), it is very important to measure the concentrations in plasma or serum and to determine the origin of CSs.

Recently, we developed a highly sensitive fluorimetric high-performance liquid chromatography (HPLC) method for the determination of unsaturated disaccharides obtained from CSs [2]. In the present work, our method was applied to the qualitative and quantitative comparison of serum and plasma CSs in rabbits.

EXPERIMENTAL

Reagents and materials

Standard unsaturated disaccharides, 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-D-galactose (ΔDi-0S), 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-4-O-sulpho-D-galactose (ΔDi-4S) and 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-6-O-sulpho-D-galactose (Δ-Di-6S), and chondroitinase ABC (EC 4.2.2.4) were obtained from Seikagaku Kogyo (Tokyo, Japan). 2-Cyanoacetamide was purchased from Kanto Chemicals (Tokyo, Japan), and pronase from Kaken Pharmaceutical (Tokyo, Japan). All other chemicals were of reagent grade. TSKgel NH_2-60 (particle size 5 μm) was purchased from Tosoh (Tokyo, Japan).

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Apparatus and chromatographic conditions

Unsaturated disaccharides were measured by a sensitive and specific HPLC method which was established by us [2]. The chromatographic conditions were as follows: column, TSKgel NH_2-60 (150 mm × 4.6 mm I.D.); eluent, 20 m*M* ammonium formate buffer (pH 5.0) containing 10 m*M* sodium sulphate in 4% acetonitrile at a flow-rate of 0.5 ml/min. The fluorimetric detection was based on the reaction of unsaturated disaccharides with 2-cyanoacetamide in alkaline solution.

Collection of rabbit plasma, serum, platelet-rich plasma and platelets

Collection of plasma, serum and platelet-rich plasma were carried out according to conventional methods. Blood was collected from the marginal ear vein of adult rabbits. Plasma was prepared as follows; one volume of 3.8% sodium citrate solution was added to nine volumes of blood and then the mixture was centrifuged at 1200 *g* for 15 min. Serum was prepared as follows; blood was incubated at 37°C for 30 min, and then stood overnight in an ice-water bath. After centrifugation at 1200 *g* for 15 min, the serum was transferred to a tube. Platelet-rich plasma and platelets were prepared as follows; one volume of 3.8% sodium citrate solution was added to nine volumes of blood and the mixture was centrifuged at 200 *g* for 15 min. A portion of the supernatant was transferred to a tube (platelet-rich plasma). The other portion of the supernatant was further centrifuged at 750 *g* for 15 min. The pellet was suspended with 12.3 m*M* Tris–HCl buffer containing 0.139 *M* sodium chloride and 1.54 m*M* EDTA (pH 7.4) and then centrifuged at 450 *g* for 15 min. The pellet was resuspended with phosphate-buffered saline solution (platelets).

Preparation of GAGs

GAGs were separated by a modification of the method of Emura and Mukuda [3] as follows: to a 100-μl portion of sample solution, 40 μl of 0.05 *M* Tris-HCl buffer (pH 8.0) containing 1% pronase were added, and the mixture was incubated at 45°C for 3 h. To the solution, 340 μl of sodium chloride solution and 60 μl of 0.1 *M* acetic acid solution were added. Then the mixture was heated in a boiling-water bath for 5 min. After being cooled in a water bath, the solution was centrifuged at 2300 *g* for 15 min. To 360 μl of the supernatant, 40 μl of 0.1 *M* sodium hydroxide solution and 1.6 ml of cooled ethanol saturated with sodium acetate were added. The mixture was left to stand overnight at 0°C and centrifuged at 2300 *g* for 15 min at 5°C. To the precipitate was added 0.6 ml of 0.015% cetylpyridinium chloride containing 0.03 *M* sodium chloride warmed at 37°C before use. The mixture was left to stand overnight at 0°C. The solution was centrifuged at 2300 *g* for 15 min at 5°C. The precipitate was washed twice with 0.6 ml of ethanol saturated with sodium chloride, and then lyophilized.

Enzymatic digestion

Digestion of GAGs with chondroitinase ABC was carried out according to the method of Kodama *et al.* [4].

Extraction of GAGs from cellulose acetate strips

Extraction of GAGs from cellulose acetate strips was achieved as follows; cellulose acetate strips corresponding to each band were incubated with water at 20°C for 20 min. The extraction efficiency of GAGs from a cellulose acetate strip was about 80% [5].

RESULTS AND DISCUSSION

Analysis of CSs in rabbit plasma, serum, and platelet-rich plasma

CSs were prepared from rabbit plasma, serum and platelet-rich plasma according to the procedure in the text and were digested with chondroitinase ABC. The analytical results of unsaturated disaccharides obtained from the samples are shown in Table I.

The concentrations of both ΔDi-0S and ΔDi-6S in plasma are almost the same as in serum, but serum contains a higher concentration of ΔDi-4S. These data suggest that the serum CS released from blood cells is fully sulphated chondroitin 4-sulphate. The CS concentrations in platelet-rich plasma were then measured and compared with those in plasma and serum. This result shows that the increase in CS in serum during coagulation of blood was approximately

TABLE I

ANALYSIS OF UNSATURATED DISACCHARIDES PRODUCED FROM CHONDROITIN SULPHATES IN NORMAL RABBIT PLASMA, SERUM AND PLATELET-RICH PLASMA AFTER DIGESTION WITH CHONDROITINASE ABC

Each value represents the mean ± S.D. of six analyses. The values in parentheses represent the ratio (%) of disaccharide to the total amount of unsaturated disaccharides.

Sample	Concentration of unsaturated disaccharide (μM)			
	ΔDi-0S	ΔDi-6S	ΔDi-4S	Total
Plasma	13.1 ± 1.7 (51.1)	1.0 ± 0.3 (3.8)	11.5 ± 2.1 (45.1)	25.6 ± 3.7
Serum	12.4 ± 3.1 (35.6)	1.2 ± 0.5 (3.5)	21.3 ± 2.9 (60.9)	34.9 ± 4.0
Platelet-rich plasma	14.6 ± 1.6 (40.0)	1.1 ± 0.4 (3.0)	20.8 ± 2.5 (57.0)	36.5 ± 4.0

equal to the amount of CS released from platelets in platelet-rich plasma (Table I).

Cellulose acetate membrane electrophoresis of GAGs

GAGs in rabbit plasma, serum, platelet-rich plasma and platelets were analysed by cellulose acetate membrane electrophoresis (Fig. 1).

Rabbit plasma GAGs consist of a major fast-migrating component (band B) and a minor slow-migrating component (band C). However, in the case of serum GAGs, a band of GAGs (band A) also appeared at the same location as chondroitin 4-sulphate (Ch-4S), and GAGs from rabbit platelets also migrated to the same location. From each band on the cellulose acetate strips, GAGs were extracted and digested with chondroitinase ABC. The chromatograms of unsaturated disaccharides obtained from serum GAGs corresponded to bands A, B and C and are shown in Fig. 2.

The CS at band B was low-sulphated Ch-4S, and its sulphation ratio was approximately 40%

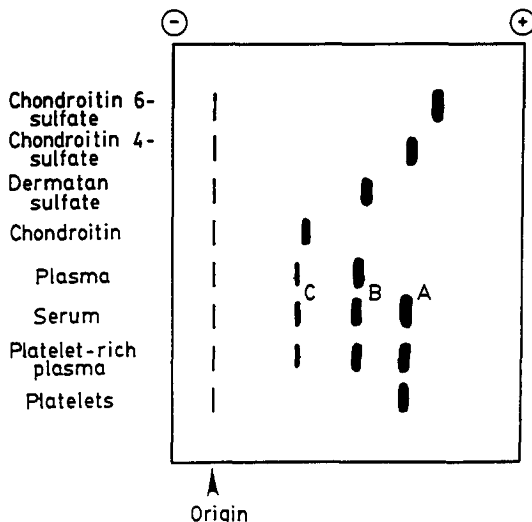

Fig. 1. Electrophoretic patterns of glycosaminoglycans from rabbit plasma, serum, platelet-rich plasma and platelets. Electrophoresis was carried out in 0.3 M calcium acetate at 1.0 mA/cm for 3 h on a cellulose acetate strip. A strip was stained with 0.1% alcian blue in 0.1% acetic acid.

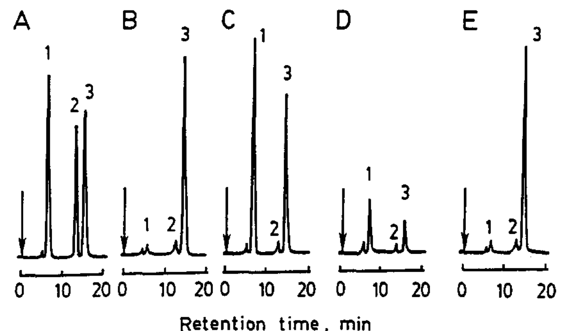

Fig. 2. Chromatograms of unsaturated disaccharides produced enzymatically from chondroitin sulphates which were extracted from a cellulose acetate strip after carrying out electrophoresis. Sample size: 10 μl. (A) Standard mixture of ΔDi-0S, ΔDi-6S and ΔDi-4S (the concentration of each unsaturated disaccharide was 5 μg/ml); (B) serum band A; (C) serum band B; (D) serum band C; (E) platelet band A. Peaks: 1 = ΔDi-0S; 2 = ΔDi-6S; 3 = ΔDi-4S.

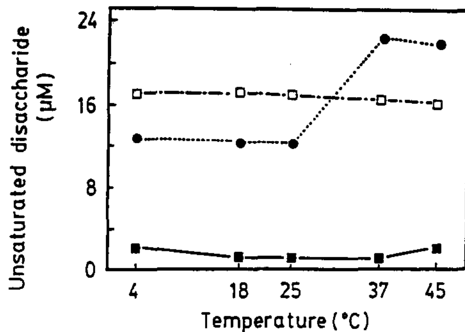

Fig. 3. Effects of incubation temperature on changes in serum chondroitin sulphate levels. Blood was collected from normal rabbits, and then incubated for 30 min at each temperature. After centrifugation at 1200 *g* for 15 min, CSs were prepared from the supernatant. CSs were digested with chondroitinase ABC and submitted to unsaturated disaccharide analysis. ● = ΔDi-4S; ■ = ΔDi-6S; □ = ΔDi-0S.

(Fig. 2C). On the other hand, the serum CS at band A was fully sulphated Ch-4S and its sulphation was approximately 96% (Fig. 2B). This component was identical to platelet CS (Fig. 2E).

Analysis of increased CS in serum during blood coagulation

It is reported that GAGs in serum increase during blood coagulation [6]. We therefore examined the increase in CS in serum during blood coagulation. Fig. 3 shows the effect of incubation temperature on the liberation of CS from rabbit cells. The concentration of Ch-4S in serum was maximum when rabbit blood was incubated at 37°C for 30 min. However, the concentration of chondroitin and chondroitin 6-sulphate did not change during the coagulation of blood.

CONCLUSION

The analytical results described above indicate that one origin of the increased Ch-4S in serum during coagulation of blood is platelets, which have proteo-chondroitin sulphate in their surface coats and in their granules. It may be possible to establish a simple assay method to determine Ch-4S released from platelets by measuring the difference between serum and plasma ΔDi-4S concentrations.

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