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Urinary crystal surface binding substances on calcium oxalate crystals

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Summary. In order to study the effect of urinary crystal surface binding substances (CSBS), we extracted the naturally existing CSBS from urine from healthy individuals by conducting homogeneous crystallization of calcium oxalate. CSBS proved not to be promoters but rather strong inhibitors of calcium oxalate crystal growth and aggregation. It is suggested that CSBS exhibited their inhibitory effect by masking the growing sites and aggregating sites on the crystal surface. As for the characteristics of CSBS, we found around 10 peaks of molecular weight, and all of them contained both peptides and saccharides. The findings suggest that CSBS are composed of various kinds of glycoproteins and proteoglycans.

Key words: Calcium oxalate – Crystallization – Crystal surface – Adsorption – Inhibitors – Glycoprotein

Various kinds of macromolecules, such as glycosaminoglycans [3, 21, 25, 26], acid peptides [11, 18], and naturally existing urinary macromolecules [12, 26] as well as various other substances have been reported to be strong inhibitors of crystal growth and/or aggregation. In fact, there is no doubt that urinary macromolecules play a more important role in preventing crystal growth and/or aggregation than do small molecular inhibitors [6, 8, 12, 22].

It is not clear, however, how urinary macromolecules achieve their effect. One possibility is that they bind onto the crystal surface and mask the growth and/or aggregation sites of crystals. The other possibility is that they change the ambient environment surrounding crystallization by adsorbing stone-forming ions onto themselves or in some other manner.

In our preliminary experiment, we confirmed that the urinary macromolecules as well as various kinds of sulfated acid glycosaminoglycans were adsorbed onto calcium oxalate crystals by measuring the alucian blue-positive product in the substances incorporated into the calcium oxalate crystals. This led us the speculate that the naturally existing urinary macromolecules would achieve their effect by binding onto the crystal surface.

In this paper, we extracted naturally existing urinary macromolecules which bind to the calcium oxalate crystal surface (we call these crystal surface binding substances, CSBS, in this paper) and studied the effect of CSBS on calcium oxalate crystallization and attempted to clarify the characteristics of these substances.

Materials and methods

Preparation of crystal surface binding substances

We collected a large volume of urine from eight healthy male subjects between 24 and 33 years of age. The urine was collected in sterile glass containers with 0.02% sodium azide as an antibacterial agent and stored at 4°C until enough urine samples were obtained. After reheating at 37°C with shaking, the urine was pre-filtered through a Whatman no. 1 paper filter and filtered through a 0.22-μm Millipore filter. We conducted the spontaneous crystallization of calcium oxalate in a mixture using the pooled urine (1 *M* $CaCl_2$:0.1 *M* sodium oxalate = 1,000:32:320) and incubated it for 6 h in a 37°C water bath with shaking. After incubation the samples were centrifuged at 2,000 rpm for 10 min. After decanting the supernatant, the crystal pellet produced during centrifugation was washed thoroughly with a sufficient volume a saturated solution of calcium oxalate to prevent contamination from the remaining urinary constituents. Infrared spectrophotometry confirmed that the crystal composition consisted of calcium oxalate. The crystals were then suspended in a 10% EDTA 4 Na solution (pH 8), and they dissolved within 30 min. Next, ultrafiltration was employed to remove EDTA 4 Na and a Labomodule (Asahi Kasei Co. Ltd., Japan) to concentrate this solution with a cut-off for molecules at 6,000 dalton. Finally, the concentrated and desalinated CSBS solutions was freeze-dried.

Inhibition assay

The inhibitory activity of CSBS on calcium oxalate crystallization was examined as follows: One milliliter each of 0.1 *M* $CaCl_2$ and 0.1 *M* sodium oxalate solution was added simulutaneously to a mixture of 90 ml of the ultrafiltered urine (cut-off MW = 6,000 dalton) and 10 ml of CSBS solution.

The final concentration was adjusted approximately so that the alucian blue-positive product reaction was equal to that of the original urine. Doubly distilled water instead of CSBS was used as a control. After 4 h of incubation at 37°C in a water bath with shaking, the particle distribution assay was performed with a Coulter Counter, Multisizer. Macroscopic and microscopic observation of the crystals formed was also performed. The resulting particle distribution patterns represent the mean of sextuple measurements.

Promotion assay

Using the method reported previously by us [13], the promotive effect of CSBS in a whole urine system was studied. One milliliter each of 0.1 *M* $CaCl_2$ and 0.1 *M* sodium oxalate solution was added simulutaneously to the mixture of 90 ml of the whole pooled urine and 10 ml of CSBS solution. The final concentration was adjusted to that of the inhibition assay. Doubly distilled water was used instead of CSBS in the control experiment. After 4 h of incubation at 37°C in a water bath with shaking, the particle distribution assay was carried out with a Coulter Counter, Multisizer. The resulting particle distribution patterns represent the mean of sextuple measurements.

Gel chromatography

CSBS was fractionated with a TSK-gel G3000SW (Toyo Soda, Japan) column (600×7.5 mm) preequilibrated with 0.1 *M* sodium phosphate buffer (pH 7.0). Fractions were eluted at a flow rate of 1.0 ml/min and monitored by absorbance at 220 nm to detect the peptide peak. Each peak fraction was subjected to a phenol-sulfuric acid reaction to find saccharides. The molecular weight of each peak was determined by marker proteins.

Reversed-phase liquid chromatography

CSBS were also fractionated with a Bio-Rad RP-304 (Bio-Rad, USA) column (250×4.6 mm) preequilibrated with 0.1% (v/v) trifluoroacetic acid. Fractions were eluted with the acetonitrile gradient from 0% to 50% in 50 min at a flow rate of 1.0 ml/min and monitored with the absorbance at 220 nm. Each peak fraction was subjected to a phenol-sulfuric acid reaction to detect saccharides and to the following other experiments.

Gas chromatography mass spectrometer analysis

Fractions from reversed-phase chromatography were hydrolyzed with 2 *N* HCl at 100°C for 2 h and applied to a gas chromatography mass spectrometer (GC-MS; Shimazu QP-1000, Japan) analysis after trimethylsilyl derivatization. Trimethylsilyl-derivatized hydrolyzates were fractionated on a capillary column (Durabond DB-5, J&W Co., USA, 60×0.32 mm) and introduced into the mass spectrometer. Column temperature was started at 80°C and increased to 100°C in 1 min and to a final temperature of 250°C in the following 15 min.

Amino acid analysis

Fractions from reversed-phase chromatography were hydrolyzed with 6 *N* HCl at 110°C for 24 h. Amino acid and amino sugar (glucosamine and galactosamine) were analyzed with a JEOL-JLC amino acid analyzer (Nihondenshi, Japan).

Amino acid sequence analysis

The N-terminal amino acid sequence of carbohydrate-rich fractions from reversed-phase chromatography was determined with a Gas Phase Protein Sequencer 470A (Applied Biosystem, USA). PTH-amino acid from the sequencer was identified with a PTH-Amino Acid Analyzer SP8700XR (Spectra Physics, USA)

Cellulose acetate membrane electrophoresis

Cellulose acetate membrane electrophoresis was carried out employing the method reported by Kondo et al. [14]. Commercially available chondroitin sulfate A, B, C, heparan sulfate, keratan sulfate, and hyaluronic acid (Sigma, USA) were used as marker glycosaminoglycans.

Thin layer gel chromatography

Thin layer gel chromatography was done with the method reported by Marzullo and Lash [16]. The commercially available glycosaminoglycans described above were used as markers.

Results

Inhibition assay

Despite the concentration of alucian blue-positive product being similar to that of the original urine, CSBS exhibited very strong inhibitory activity on both crystal growth and aggregation. This was confirmed by the particle distribution assay with the Coulter Counter, Multisizer, but it was also observed macroscopically and microscopically. The postincubated, ultrafiltered urine with CSBS contained small calcium oxalate dihydrate crystals which did not adhere to the wall of the glass container, while that without CSBS formed large aggregated crystals, and the wall of the glass container became opaque because of the adherence of many crystals. Figure 1 shows the comparison of particle size distribution between the experiments with and without CSBS. Crystal size distribution clearly was smaller in the experiment with CSBS than in that without CSBS.

Promotion assay

In contrast to the results of the inhibition assay, there was no difference in the particle distribution patterns between the experiments with and without CSBS (Fig. 2).

Gel chromatography and reversed-phase liquid chromatography

We obtained 9 peaks of macromolecules in gel chromatography by using TSK gel, G3000SW. These peaks were detected by ultraviolet absorbance at 220 nm and a further 11 peaks, by reversed-phase liquid chromatography (Fig. 3). The estimated molecular weight of each sub-

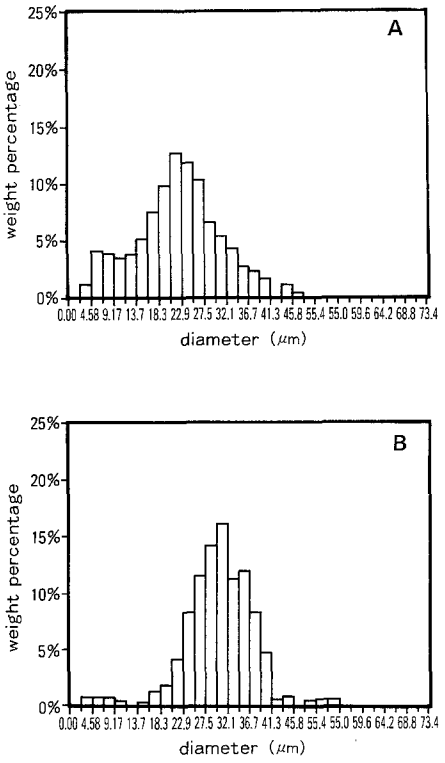

Fig. 1A, B. Particle distribution patterns of the inhibition assay. Experiment with (**A**) and without (**B**) crystal surface binding substances

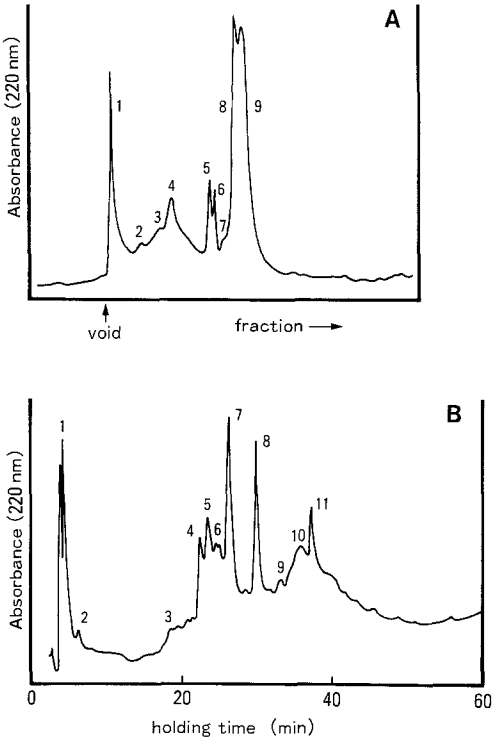

Fig. 3A, B. The results of gel chromatogryphy (**A**) and reversed-phase liquid chromatography (**B**)

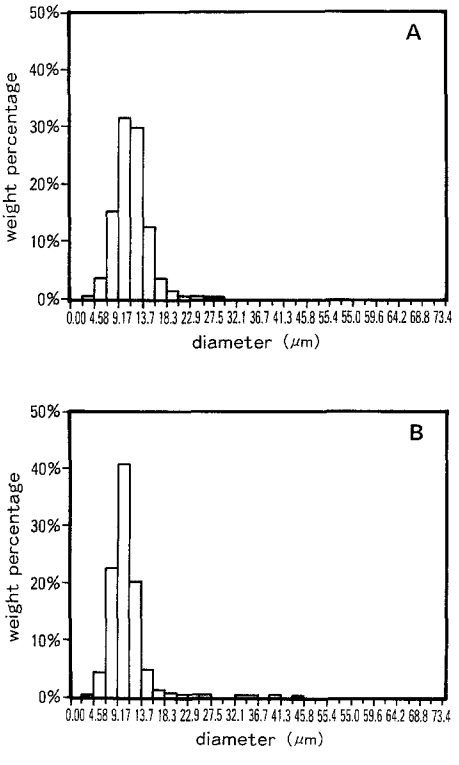

Fig. 2A, B. Particle distribution patterns of the promotion assay. Experiment with (**A**) and without (**B**) crystal surface binding substances

stance obtained by gel filtration was more than 300,000 dalton at peak 1, 200,000 at peak 2, 80,000 at peak 4, 55,000 at peak 5, 18,000 at peak 6, and 5,800 at peak 8. During the phenol-sulfuric acid reaction, we examined the saccharide content macroscopically. Peak 6 in gel filtration and peak 4 in reversed-phase liquid chromatography revealed a strong reaction while the other peaks responded to the phenol-sulfuric acid reaction to some extent in comparison with control.

Gas chromatography mass spectrometer analysis

Saccharide analysis by GC-MS was performed on the peaks obtained by reversed-phase liquid chromatography. Although each peak showed various amounts of saccharide contents, peak 4 revealed an especially high saccharide content including galactose, mannose, xylose, and glucose. Peak 4 also contained several unknown saccharides (Fig. 4).

Amino acid contents and amino acid sequence

The amino acid contents of each of the 11 fractions obtained in reversed-phase liquid chromatography are listed in Table 1. During the amino acid sequencing of crude CSBS, no amino acid in particular was observed, even in peak 4.

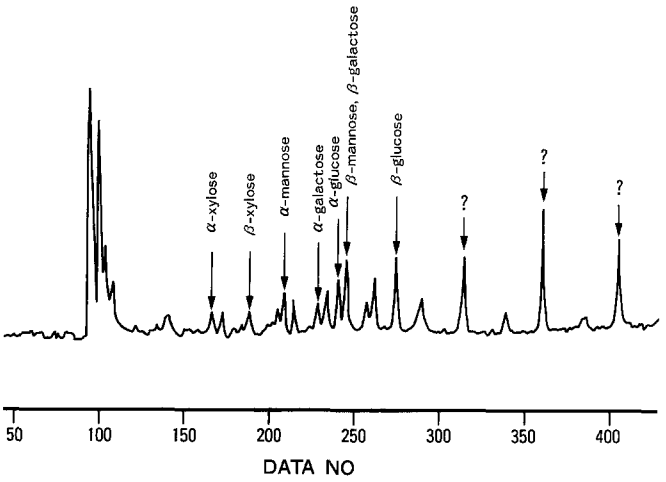

Fig. 4A, B. Results of gas chromatography mass spectrometry analysis of peak 4 obtained from the reversed-phase liquid chromatography; indicates unidentified saccharide peaks

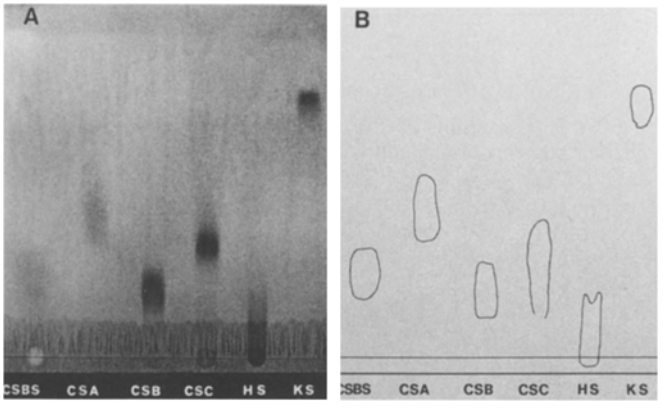

Fig. 5A, B. Results of thin layer gel chromatography. Chondroitin sulfate (*CS*) A, B and C forms, heparan sulfate (*HS*), keratan sulfate (*KS*=, and crystal surface binding substances (*CSBS*)

Cellulose acetate membrane electrophoresis

With cellulose acetate membrane electrophoresis, CSBS showed some obscure bands near the chondroitin sulfates A, B, and C, but no particular glycosaminoglycans could be isolated.

Thin layer gel chromatography

CSBS moved to the area close to chondroitin sulfates B and C, and no staining occurred at the positions of heparan sulfate or keratan sulfate (Fig. 5). Furthermore, since hyaluronic acid does not move under these conditions [16], CSBS seem to be different from hyaluronic acid as well.

Table 1. Net amino acid content of each peak obtained from reversed-phase liquid chromatography

Fraction no.	Amino acid content (nmole/0.5 ml sample)
1	5.05
2	14.78
3	10.14
4	29.57
5	35.95
6	38.17
7	70.31
8	26.91
10	19.26
11	55.41

Discussion

It has long been speculated that some macromolecules bind to the crystal surface and have some effect on crystallization [1, 3, 7, 15, 17]. However, as to the urinary macromolecules which interact with the crystal surface, there are still many aspects which must be clarified.

The first problem is whether or not naturally existing urinary macromolecules really interact with the crystal surface. The second is whether or not urinary macromolecules have a promotive effect on crystallization, although there is little doubt that they have an inhibitory effect. The third is what kind of substances influence crystallization, and the last problem is how do the macromolecules affect crystallization.

As to the first question, Morse and Resnick [17] recently proved an interaction between the crystal surface of calcium oxalate and naturally existing macromolecules. In this paper, we also confirmed beyond doubt that some part of the urinary macromolecules is adsorbed onto the calcium oxalate crystal surface.

The second problem, the promotive effect of urinary macromolecules, has been controversial. Several investigators reported that some of the urinary macromolecules or part of the stone matrix might stimulate the formation of abnormally large crystals of calcium oxalate [4, 5, 10], and they considered these particular macromolecules to be promoters of crystallization. On the other hand, Gjaldbaek and Robertson [9] stressed that urinary macromolecules do not have any promotive effect on crystallization and, on the contrary, act as inhibitors. In our study limited to the urinary macromolecules adsorbable onto calcium oxalate crystals, these macromolecules had no promotive effect on either crystal growth or aggregation, but were very strong inhibitors.

Concerning the third problem, what kind of substances influence crystallization, varied opinions have been expressed by numerous investigators. Glycosaminoglycans [3, 21, 25, 26], acid peptides [11, 18], RNA-like substances [11, 27], acid glycoproteins [19], glycoproteins or proteoglycans [12], Tamm–Horsfall glycoprotein [23, 28], and others have been nominated as possible inhibitors of

crystallization, while mucoproteins including Tamm–Horsfall glycoprotein [24] or a part of glycosaminoglycans [20] have been hypothesized as promoters of crystallization. This confusion is due to the difficulties inherent in the analytical chemistry of macromolecules and the variety of methods for investigating the crystallization processes. In the analysis of CSBS in this study, we were also not able to clarify completely the characteristics of CSBS. However, our study enabled us to obtain certain evidence that CSBS have a wide range of molecular weights, supported by Azoury et al. [2], and that they are composed of both peptides and saccharides, suggesting that they constitute a complex of various glycoproteins. Furthermore, it seems that CSBS contain glycosaminoglycans other than heparan sulfate, keratan sulfate, and hyaluronic acid. These findings are based on the results from thin layer gel chromatography, although the glycosaminoglycans contained in CSBS did not completely correspond to the commercially available glycosaminoglycans. We speculate that their multidimensional structure must have a strong influence on the effect of CSBS, but so far we have not been able to clarify the importance of this influence. For example, we cannot assert that a single chain of naturally existing urinary glycosaminoglycans has a strong inhibitory activity.

As to the last problem, how macromolecules affect crystallization, it was strongly suggested that a major proportion of the urinary macromolecular inhibitors implements the effect by masking the crystal growth and aggregation sites. Urinary macromolecules adsorbed onto the crystal surface did not exhibit any promotive effect on the crystallization of calcium oxalate. In other words, CSBS did not act as a cementing substance for crystallization but as direct inhibitors of crystal growth an aggregation. In this respect we agree with the opinion stated by Angell and Resnick [1]. However, it does not seem to be correct that the substances with a stronger affinity to the crystal surface are better inhibitors of crystallization in urine, because we speculate that their multi dimensional structure might have a greater influence on their inhibitory activity.

In conclusion, CSBS seem to constitute a major part of the urinary macromolecular inhibitors we reported on previously [12] and to consist of various substances, probably glycoproteins and/or proteoglycans. More chemical analysis, including enzyme analysis in combination with inhibition assays, will be needed to clarify the relationship between the inhibitory activity of CSBS and their structure.

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