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Heparan sulfate in the stone matrix and its inhibitory effect on calcium oxalate crystallization

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Summary. The nature of the soluble stone matrix and its possible role in urinary stone formation was studied. For this purpose we performed two-dimensional cellulose acetate membrane electrophoresis of the glycosaminoglycans (GAGs) which were contained in the soluble stone matrix, substances adsorbed onto calcium oxalate crystals in vitro (crystal surface binding substances, CSBS) and urinary macromolecules (UMMs). The main GAG in the soluble stone matrix and CSBS was found to be heparan sulfate, whereas the UMMs contained various GAGs usually seen in urine. An inhibition assay showed the soluble stone matrix to have the strongest inhibitory activity among these macromolecular substances when inhibitory activity was expressed in terms of uronic acid concentration. It is suggested that the main GAG in the soluble stone matrix consists of heparan sulfate, which has a strong inhibitory activity on calcium oxalate crystal growth and aggregation and constitutes part of the CSBS.

Key words: Crystal surface binding substances – Haparan sulfate – Inhibitory activity – Stone matrix – Urinary macromolecules

Various macromolecular organic substances such as glycosaminoglycans (GAGs), Tamm-Horsfall glycoprotein (THGP), other glycoproteins and urinary proteins, may play important roles in calcium oxalate stone formation. The role of the organic stone matrix, however, has not been clarified.

Some investigators have reported that the organic matrix is selectively incorporated into urinary calculi as a substance promoting urinary crystal growth and aggregation [3, 15, 17]. Recently, Resnick et al. [12, 16] reported that some particular proteins which were adsorbed onto crystals might promote calcium oxalate crystallization. Others, however, have suggested that stone matrix formation is the result of nonspecific adsorption of urinary macromolecules (UMMs) onto urinary crystals and that the stone matrix does not act as a promoter [7, 13, 21]. Moreover, still other researchers have shown that the soluble stone matrix has an inhibitory effect on crystal growth and aggregation in vitro [20].

On the basis of studies at our institutes, we have already reported that UMMs have a strong inhibitory activity [10] and that some UMMs are adsorbed as inhibitors onto the surface of calcium oxalate crystals (crystal surface binding substances, CSBS) [11]. The aim of this study was to determine the characteristics of GAGs in the soluble stone matrix as well as in the CSBS and UMMs, and to clarify the role of these GAGs in calcium oxalate stone formation.

Materials and methods

Preparation of pooled urine

We collected a large volume of urine from 10 healthy males between 24 and 37 years old. The urine was collected in sterile glass containers with 0.02% sodium azide as an antibacterial agent and stored at 4°C. After warming the urine to 37°C while it was being shaken, it was filtered trough a Whatman no. 1 paper filter and a 0.22-μm Millipore filter prior to the experiments.

Preparation of urinary macromolecules (UMMs)

After filtration the pooled urine was ultrafiltered with a Labomodule (Asahi Kasei, Japan; cut-off molecular weight 3,000 Da). The concentrated and desalinated urinary macromolecular solution was lyophilized and stored at −20°C until use.

Preparation of crystal surface binding substances (CSBS)

We obtained CSBS by the following procedures, also reported previously [11]. Spontaneous crystallization of calcium oxalate was induced by adding $CaCl_2$ and $Na_2C_2O_4$ to the pooled urine (urine :

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1 M $CaCl_2$: 0.1 M sodium oxalate = 1,000 : 32 : 320) and incubating for 6 h in a water bath at 37°C while being shaken. The crystals were determined to be calcium oxalate monohydrate by infrared spectrophotometry. After incubation, the samples were centrifuged at 2,000 rpm for 10 min. The crystal pellets precipitated during centrifugation were washed thoroughly with a sufficient volume of saturated calcium oxalate solution to prevent contamination from the remaining urinary constituents and then suspended in a 10% EDTA·4Na solution (pH 8); they dissolved within 30 min.

Ultrafiltration was performed with a Labomodule (Asahi Kasei, Japan) to remove EDTA·4Na and to concentrate this solution with a cut-off for molecules at 3,000 Da. Finally, the concentrated and desalinated CSBS solution were lyophilized and stored at −20°C until use.

Preparation of seed-crystal surface binding substances (seed-CSBS)

The organic substances adsorbed onto commercially available calcium oxalate monohydrate crystals were obtained by the following procedure. Fifty grams of calcium oxalate monohydrate crystal (Nakalai Tesque, Japan) were added to 3 L pooled urine and incubated for 6 h in a water bath at 37°C while being shaken. After incubation, the solution was centrifuged at 2,000 rpm for 10 min. The rest of the procedure was the same as for the preparation of CSBS [11]; the resultant substances are referred to here as seed-CSBS.

Preparation of soluble stone matrix

Thirty-two calcium oxalate stones obtained from 26 male patients by open surgery between 1985 and 1990, and which had been stored at room temperature, were used in this experiment. All the stones were sterile. The composition of the stones was redetermined by infrared spectrophotometry before the experiments and was shown to be predominantly calcium oxalate monohydrate. The stones were crushed into a powder with a mortar and pestle, placed in a dialysis sac (Spectra/Por 3 membrane; cut-off molecular weight 3,500 Da and dialyzed against an abundant volume of 5% EDTA·4Na solution (pH 7.8) for 2 weeks. The completely decalcified material was dialyzed against distilled water for 72 h to remove EDTA, the distilled water being changed every 6 h. The dialyzed solution was centrifuged at 3,000 rpm for 10 min. The supernatant, which contained the soluble stone matrix, was lyophilized and stored at −20°C until use.

Preparation of glycosaminoglycans

UMMs, seed-CSBS, CSBS and the soluble stone matrix were treated with 0.5 N NaOH overnight at 4°C and then neutralized with 1 N HCl. Proteolysis was then performed using the method reported by Nishio et al. [15]. Two milliliters pronase E (4 mg/ml) were added and the mixture incubated for 24 h while being constantly shaken at 50°C. An additional 2 ml pronase E were added and proteolysis continued for a further 24 h. The suspension was cooled to 4°C and 60% trichloroacetic acid added to a final concentration of 10%. After having been left standing for 2 h at 4°C, the suspension was centrifuged at 15,000 rpm for 15 min at 4°C. The supernatant was then dialyzed for 48 h against distilled water in a Spectra/Por 3 membrane tube. The dialyzed material was lyophilized and stored to measure the amount of GAGs.

Uronic acid was measured with the method of Bitter and Muir [2]. D-Glucuronic acid (Sigma, St. Luis, Mo.) was used as the standard.

The protein concentrations of UMMs, seed-CSBS, CSBS and the soluble stone matrix was also measured with the Bradford protein assay method [5] before proteolysis.

Two-dimensional cellulose acetate membrane electrophoresis of glycosaminoglycans

Two-dimensional electrophoresis was done using the method of Hata and Nagai [9] on a Sepraphore III instrument (Gelman Sciences, Germany) using 0.1 M pyridine and 0.47 M formic acid (pH 3.0) as a solvent in the first dimension for 60 min, and 0.1 M barium acetate (pH 8.0) in the second dimension for 4.5 h. UMMs, seed-CSBS and the soluble stone matrix were applied after the proteolysis. Commercially available GAGs, chondroitin-6-sulfate, dermatan sulfate, hyaluronic acid (Sigma, USA) and heparin (Wako, Japan) were applied as marker GAGs simultaneously. Staining was done with 0.1% alcian blue in 0.1% acetic acid for 10 min. The Sepraphore III instrument was washed with 300 ml 0.1% acetic acid for 20 min.

Inhibition assay

The inhibitory activity of UMMs, CSBS and the soluble stone matrix on calcium oxalate crystal growth and aggregation was measured with the modified method of Robertson et al. [18]. A 2.5 ml aliquot of calcium oxalate crystal suspension (1 g/l) was added to 100 ml 10 mM sodium cacodylate buffer solution (pH 6.0) which initially contained 1 mM $CaCl_2$, 0.2 mM $Na_2C_2O_4$ and 0.15 M NaCl. The mixture was incubated while being constantly stirred for 4 h at 37°C. Crystal distribution was measured with the Coulter Multisizer (Coulter, UK). Percentage inhibition was calculated on particles over 9 μm diameter before and after 4 h of incubation.

The fraction of particles greater than 9 μm in diameter at $t = 0$ and $t = 4$ h in the control flask (with no inhibitor) was taken as C_0 and C_4, and in the flask containing the test compound as T_0 and T_4, respectively. The increase in the fraction of particles greater than 9 μm after 4 h is $(C_4 - C_0)$ in the control flask and $(T_4 - T_0$ in the flask containing the test compound. The degree of inhibition (I) was expressed as: $I = [(C_4 - C_0) - (T_4 - T_0)]/(C_4 - C_0) \times 100\%$.

The effect on the growth and aggregation of calcium oxalate crystals of adding various concentrations of UMMs, CSBS and the soluble stone matrix was studied. The percentage inhibitory activity was expressed per unit of uronic acid content and protein content.

Results

Two-dimensional cellulose acetate membrane electrophoresis

Two-dimensional electrophoresis revealed that UMMs contained various GAGs usually seen in normal human urine. These included chondroitin-6-sulfate and chondroitin-4-sulfate, dermatan sulfate, keratan sulfate, hyaluronic acid and heparan sulfate (Fig. 1 a). Seed-CSBS, the substances adsorbed onto the seeded calcium oxalate monohydrate crystals, showed a similar GAG pattern to UMMs but the heparan sulfate spot was stained fairly strongly (Fig. 1 b). As for CSBS, the substances adsorbed onto calcium oxalate crystals during crystallization in whole urine, and the GAGs in the CSBS, consisted of heparan sulfate and included a small amount of dermatan sulfate (Fig. 1 C). Furthermore, the only GAG in the soluble stone matrix was heparan sulfate; other GAGs could not be found (Fig. 1 d). We have to modify our previous report [11] in which we stated on the basis of that CSBS contained a chondroitin-sulfate-like substance thin layer chromatography and one-dimensional electrophoresis: after proteolysis, two-dimensional electrophoresis

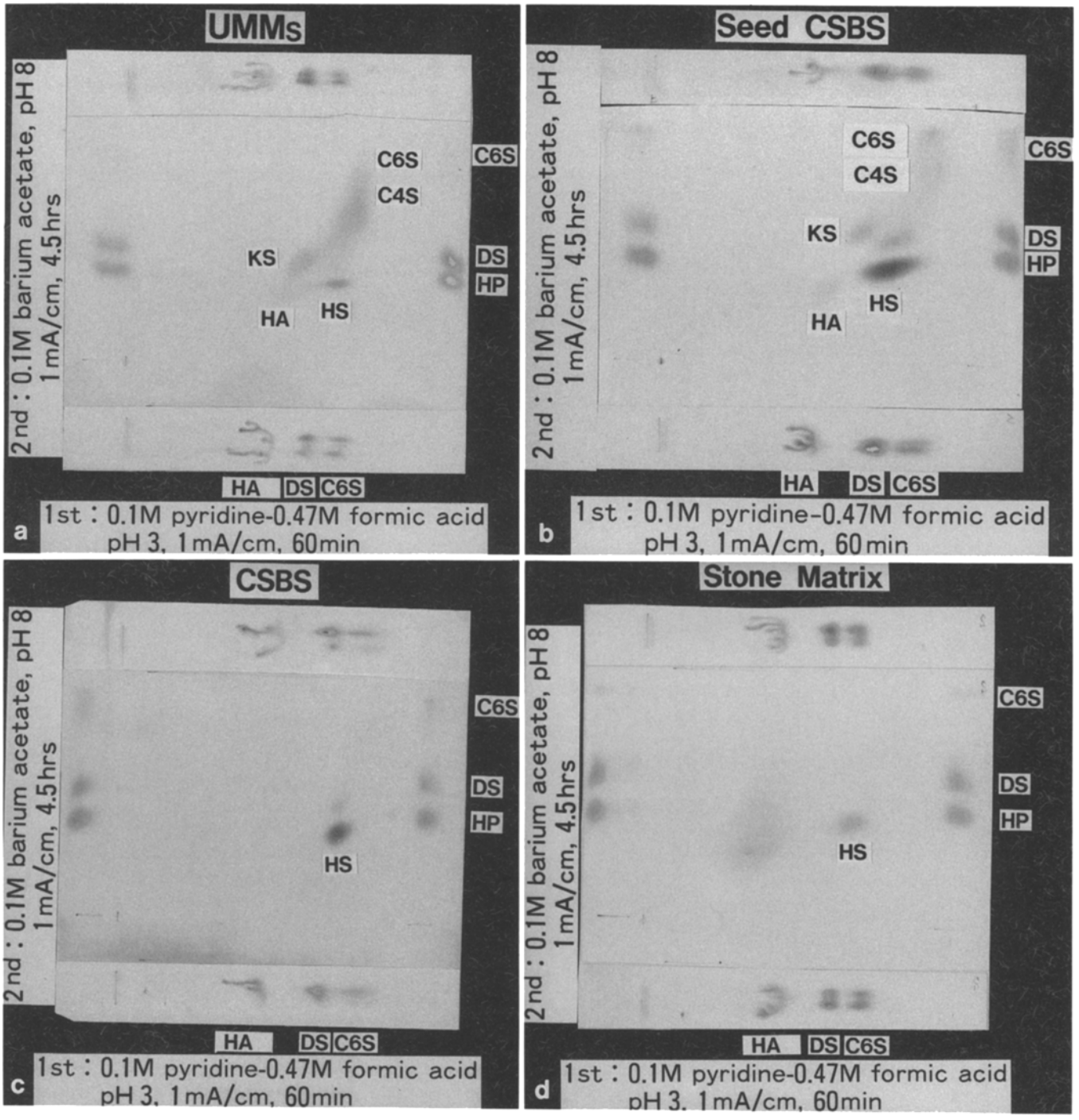
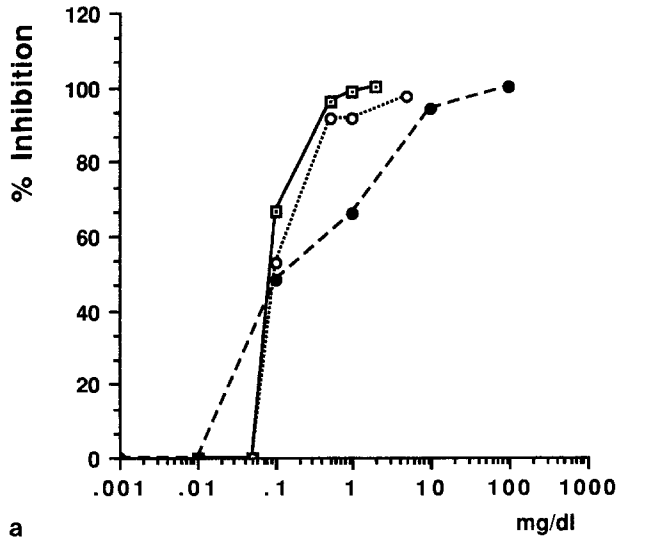


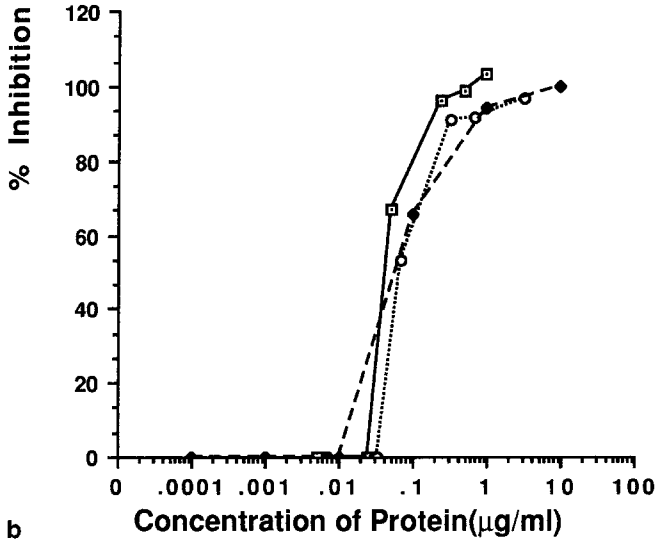
Fig. 1a–d. Two-dimensional electrophoresis shows four GAG patterns. Urinary macromolecules (UMMs) contain all of the known glycosaminoglycans (GAGs) in urine: chondroitin-6-sulfate (*C6S*), chondroitin-4-sulfate (*C4S*), keratan sulfate (*KS*), dermatan sulfate (*DS*), heparan sulfate (*HS*) and hyaluronic acid (*HA*). Seed-crystal surface binding substances (seed-CSBS) have a GAG pattern similar to UMMs, although the proportion of heparan sulfate is increased. Heparan sulfate is predominantly identified in the CSBS, while a small amount of dermatan sulfate is recognized in the other GAGs. Only heparan sulfate is identified in the soluble stone matrix. *HP*, heparin

revealed that the main GAG in CSBS was not chondroitin sulfate but heparan sulfate.

Inhibitory activity of UMMs, CSBS and the soluble stone matrix

A comparison of the inhibitory activity of UMMs, CSBS and the soluble stone matrix on calcium oxalate crystal growth and aggregation is shown in Fig. 2. Although the inhibitory activities of the soluble stone matrix, CSBS and UMMs did not differ greatly from each other when expressed as dose-responce curves and in terms of protein





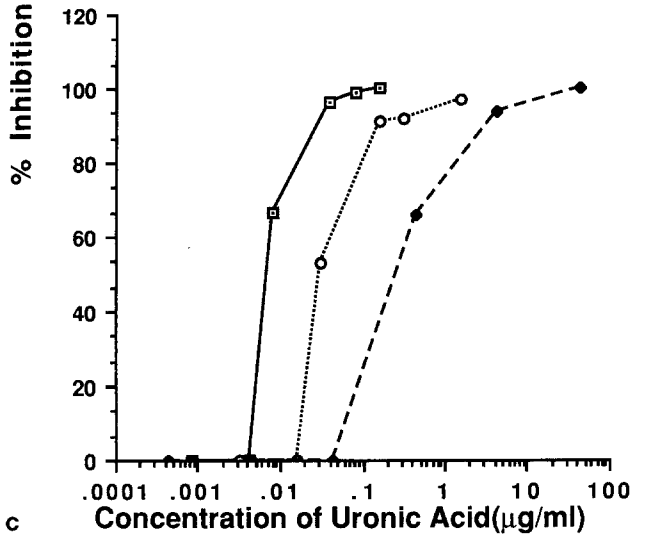
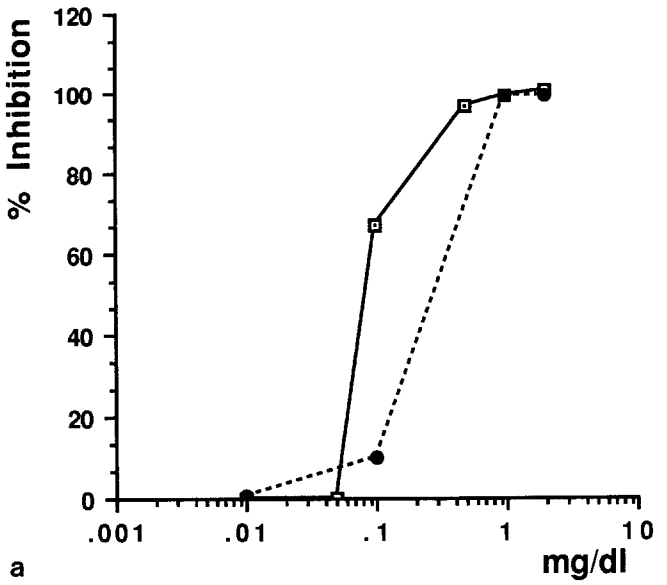


Fig. 2a–c. Inhibitory activity of UMMs, CSBS and soluble stone matrix (*SM*). **a** Expressed in terms of protein concentration; **b** expressed as a dose-reponse curve; **c** expressed in terms of uronic acid concentration. -⊡-, SM; ····○····, CSBS; – –◆– –, UMMs



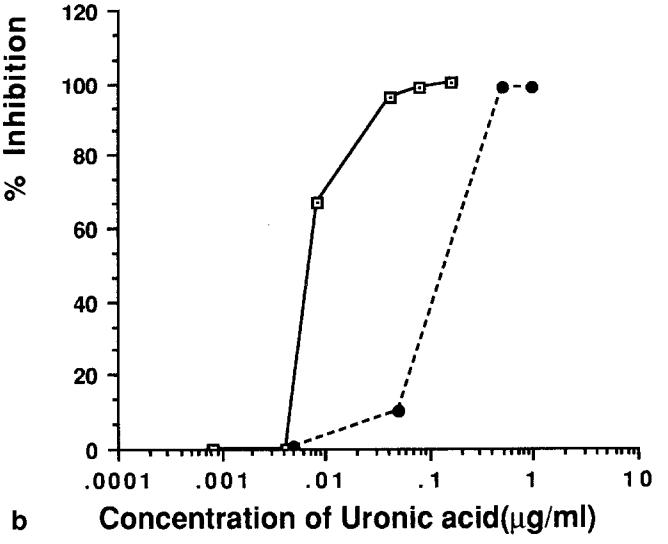


Fig. 3a,b. Inhibitory activity of the soluble stone matrix before (-⊡-) and after (···●···) proteolysis. **a** Expressed as a dose-responce curve; **b** expressed in terms of uronic acid concentration

concentration (Fig. 2a,b), there was a major difference when they were expressed in terms of uronic acid concentration (Fig. 2c). Percentage inhibition in terms of uronic acid was strongest in the soluble stone matrix and CSBS exhibited fairly strong inhibition (Fig. 2c). The inhibitory activity of the soluble stone matrix was not changed by proteolysis when expressed as dose-response curves. The strong inhibitory activity of the soluble stone matrix, however, was weakened by proteolysis when expressed in terms of uronic acid concentration (Fig. 3).

Discussion

The organic matrix of calcium oxalate stones has been shown to account for 2–3% of the total stone weight [3].

The role of this stone matrix in the formation of urinary calculi is not well understood.

Boyce and associates [3] proposed that the stone matrix (matrix substance A) is incorporated into stones in a highly selective manner and acts to promote urinary stone formation. Resnick et al. [12, 16] reported that some particular proteins are selectively adsorbed onto crystals in urine, and then speculated that these proteins play a promotive role in the stone-forming state.

On the other hand, Vermeulen and others [7, 13, 21] have stated that matrix formation occurs by random adsorption of urinary macromolecules onto urinary crystals. Gjaldbaek and Robertson [8] showed that "matrix substance A" is not a promoter of calcium oxalate crystal growth but an inhibitor, and that this inhibitory effect is associated with the concentration of GAGs [8]. This is consistent with the hypothesis that GAGs are powerful inhibitors of crystal growth and aggregation of calcium oxalate [4, 19]. Scurr et al. [20] suggested that the soluble fraction, which should contain "matrix substance A", is a potent inhibitor of both crystal growth and combined crystal growth and aggregation.

Regarding the GAGs in the stone matrix, Nishio [15] showed that heparan sulfate is the sole GAG in the calcium oxalate monohydrate stone matrix and speculated that it is a promoter rather than an inhibitor of urinary stone formation and enhances stone growth by affecting crystal growth rate and crystal aggregation. In this study, however, we proved that the soluble stone matrix shows a very strong inhibitory activity on calcium oxalate crystal growth and aggregation, although the composition of GAGs in the stone matrix is still consistent with the findings of Nishio et al. [15].

In a previous report we showed that CSBS, the substance adsorbed onto crystals, was not a promoter but a strong inhibitor of crystal growth and aggregation [11]. Moreover, Doyle et al. [6], in a recent study, demonstrated that the crystal matrix protein exhibits a marked affinity for calcium oxalate crystals and is a potent inhibitor of crystal aggregation. The exact nature of the relationship between the substances adsorbed onto crystals and the stone matrix has not, as far as we are aware, been researched in detail. When we examined the composition of GAGs we found that the proportion of heparan sulfate was the highest in the stone matrix, the second highest in the CSBS, the third highest in the seed-CSBS, and the lowest in the UMMs. These results indicate that with regard to GAGs, the seed-CSBS appears to be a part of the UMMs, the CSBS a part of the seed-CSBS, and the soluble stone matrix a part of the CSBS.

The difference between CSBS and seed-CSBS is that the CSBS was formed under conditions of more rapid crystallization than the seed-CSBS. It is our hypothesis that, in the rapid crystallizing state, stronger inhibitors are adsorbed more actively onto crystals than weaker inhibitors. This agrees with the finding that weaker inhibitors bind with less affinity than do stronger inhibitors [1].

It follows from this hypothesis that heparan sulfate might have the strongest affinity to the crystals and have its strongest inhibitory activity on crystal growth and aggregation. GAGs other than heparan sulfate might not contribute significantly to this inhibitory activity. Therefore, the stone matrix, which is predominantly composed of heparan sulfate, exhibits the strongest inhibitory activity.

It must also be stressed that the inhibitory strength of the soluble stone matrix was markedly reduced by protein digestion. There are two possible explanations. One is that the heparan sulfate in the stone matrix usually occurs in the form of protein-heparan sulfate complex, so that its strongest inhibitory activity appears in the form of proteoglycans; consequently, this inhibitory power is weakened by proteolysis. The other possible explanation is that some matrix proteins, such as nephrocalcin [14], exhibit a strong inhibitory effect, which is reduced by proteolysis. Since CSBS contain various substances, as reported previously [11], the latter possibility cannot be ignored in relation to the soluble stone matrix. We want to stress, however, that a strong heparan sulfate spot could not be obtained during two-dimensional electrophoresis without proteolysis. This finding indicates that heparan sulfate is incorporated into the calcium oxalate stone in the form of proteoglycan. Gjaldbaek et al. [8] have reported that a strong inhibitory activity in the soluble stone matrix was recognized in the uronic acid-rich fraction, suggesting that GAG components play an important role in inhibitory activities even if they occur in the form of proteoglycans. Therefore, it is suggested that heparan sulfate occurs in the form of a proteoglycan, and exhibits its stronger inhibitory activity as a form of protein-heparan sulfate complex.

In conclusion, it was found that the soluble stone matrix is composed of part of the CSBS, at least with regard to GAGs, and has a very high affinity to the crystals. The GAGs in the soluble stone matrix are mainly composed of heparan sulfate, which may exist as a form of protein-heparan sulfate complex, and exhibit a strong inhibitory activity on calcium oxalate crystal growth and aggregation. The insoluble fraction of the stone matrix should be investigated further to determine whether the stone matrix itself has any stone-promoting function.

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