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Isolation and purification of a new glycoprotein from human urine inhibiting calcium oxalate crystallization

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Summary. A calcium oxalate crystal growth inhibitor was isolated from human urine using DEAE-Sephacel gel followed by Sephacryl S-300 chromatography and FPLC column. The isolated inhibitor was a uronic-acid-rich protein (UAP). It was found to be a glycoprotein with a molecular weight of 35000 Da as determined by SDS-polyacrylamide gel electrophoresis. Inhibitory activity was demonstrated using a calcium oxalate crystallization system. In addition UAP, nephrocalcin (NC) or nephrocalcin-like (NC-like) activity was an effective inhibitor in this system. However, the inhibitory activity of UAP appeared to be higher than that of NC or NC-like activity. This finding suggests that NC or NC-like activity is not only urinary protein with strong inhibitory activity. UAP and probably other proteins also play a role in the control of urinary crystal growth.

Key words: Calcium oxalate – Nephrocalcin – Chromatography – Crystallization – Uronic-acid-rich protein

The urine of healthy subjects is supersaturated with calcium oxalate, the most common component of renal calculi in Western countries [25, 29, 39]. However, Robertson et al. [28] considered that supersaturation alone is not responsible for the difference in the size of crystals between normal subjects and recurrent stone-formers. In previous studies it has been shown that normal urine contains substances strongly inhibiting crystal growth and aggregation. The urine from recurrent stone-formers appears to contain less of this material and inhibitory activity is lower than in normal urine [4, 5, 19, 27, 30].

The effect of several low molecular weight substances has been tested in different crystal growth systems. Several workers have suggested that citrate, isocitrate, magnesium, phosphate, pyrophosphate and zinc were acting as inhibitors [9, 11, 16, 19, 35]. However, others consider that these low molecular weight substances account for only a small proportion of crystal growth inhibition in urine [22, 36]. The urinary macromolecular substances of molecular weight more than 10000 Da show a marked inhibitory activity [1, 7, 8, 15]. Isolation, purification and determination of the nature of this material is still controversial. The role of Tamm-Horsfall glycoprotein (THP) in the formation of renal calculi has not yet been definitively clarified. Some reports have suggested that THP is an inhibitor [10, 13]. Others propose that it is a promoter [12, 31] or an inactive substance regarding crystal growth [33, 40]. Nakagawa et al. have isolated from human urine [22], from human kidney tissue culture medium [21], and from a rat kidney and urine [23] a glycoprotein with a molecular weight of approximately 14000 Da, named nephrocalcin. The authors claim that this glycoprotein accounts for 90% of the inhibitory activity against calcium oxalate monohydrate growth. Recently, Sorensen et al. [34] have isolated a protein which was identified as inter-α-trypsin inhibitor by determination of the first 16 residues at the amino end. Their study seems to indicate that α-trypsin inhibitor is the only urinary protein which might have an inhibitory activity on crystal growth.

The presence of glycosaminoglycans (GAGs) in urine is thought to be important for the prevention of calcium oxalate stone formation [24, 32, 42]. Bowyer et al. [3] have isolated GAGs from normal urine that showed a strong inhibitory activity towards calcium oxalate growth. However, Koide et al. [15] considered that GAGs account for only a small amount of inhibitory activity, and that 70%–90% of the inhibition was lost after digestion with pronase.

Pak et al. [14, 43], using an ultrafiltration technique for separation of the urinary substances, have demonstrated that inhibition rises progressively with decreasing size of organic material. This material contains proteins and uronic acid, a finding that suggests it may be glycoproteins. The material is in the 12000–42000 Da molecular weight range, as determined by SDS-polyacrylamide gels.

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The daily excretion of the small glycoproteins was lower in stone-formers and exhibited a weak inhibitory activity [14, 43].

In this study, we were interested in the glycoprotein material. The aim was to purify this material from urine and to determine which glycoproteins could be involved in crystal growth inhibitory activity.

Materials and methods

Materials

Twenty-four hour urine samples were collected from one healthy man who had no previous history of kidney stone disease. They were stored in a polypropylene bottle containing sodium azide as a preservative. During the collection the urine was kept at 4°C. DEAE-Sephacel and Sephacryl S-300 gels were purchased from Pharmacia and recycled according to the manufacturer's procedure. Dialysis tubing with a molecular weight cut-off of 8000 Da was purchased from Spectrapor. Columns and electrophoresis apparatus were obtained from LKB. ^{45}Ca was obtained from Amersham. All other reagents were purchased from Sigma.

Inhibition assay

The measurement was performed using a calcium oxalate crystallization system. The assay solution contained 1 ml of 2 mmol/l $CaCl_2$ in 0.05 mol/l TRIS-HCl, 0.15 mol/l NaCl, pH 7.3 with a trace of ^{45}Ca (0.067 μCi/ml). The $CaCl_2$ solution was distributed in tubes containing 5–10 μg of proteins dissolved in 50 μl 0.15 mol/l NaCl. Tubes with no added proteins were used as controls. The assay was initiated by adding 1 ml of 2 mmol/l ammonium oxalate in 0.05 mol/l TRIS-HCl, 0.15 mol/l NaCl, pH 7.3 for 1 h with stirring at room temperature. At the end of the assay tubes were centrifuged at 4500 rpm for 5 min and 500 μl aliquots of the supernatant assayed for ^{45}Ca radioactivity in a liquid scintillation counter. The inhibition (I) of calcium oxalate crystallization was calculated according to the formula:

$$I(\%) = 100 \times \frac{(C_i - C_{fo}) - (C_i - C_{fI})}{(C_i - C_{fI})}$$

where C_i represents the concentration of calcium at initial time T_0, C_{fo} the concentration of calcium at the final time T in the absence of inhibitor, and C_{fI} the concentration of calcium at time T in the presence of inhibitor. The coefficient of variation was 5% at low inhibition percentages ($n=5$) and 1.5% at high inhibition percentages ($n=5$). In our assay we examined the effects of TRIS, NaCl, THP and different GAGs. TRIS and NaCl were used as eluent in different steps of chromatography. THP was chosen as a high molecular weight protein which is abundant in urine. GAGs are considered potent inhibitors of calcium oxalate crystallization [24].

Purification procedure

The urine samples was dialyzed against 10 l of deionized water for 24 h at 4°C with one change. The dialysate was adjusted to pH 7.3 and 0.1 mol/l NaCl with the addition of NaOH 1 mol/l and solid NaCl, respectively. Then it was mixed with DEAE-Sephacel gel preequilibrated with 0.05 mol/l TRIS-HCl, 0.1 mol/l NaCl, pH 7.3. After 30 min of stirring the mixture was filtered and the gel washed with 2×500 ml of equilibration buffer. The proteins were eluted from the gel by washing successively with 300 and 200 ml of 0.05 mol/l TRIS-HCl, 0.5 mol/l NaCl, pH 7.3. The two eluents were mixed together and dialyzed overnight against 10 l of deionized water at 4°C. The sample was adjusted to pH 7.3 and 0.1 mol/l NaCl then added to the DEAE-Sephacel column (0.8×16 cm). The column was rinsed and eluted with buffer using a linear sodium chloride gradient from 0.1 to 0.5 mol/l with a flow rate of 20 ml/h. Each 3 ml was collected and the concentration of proteins, uronic acid, sodium and inhibitory activity measured in each fifth tube. The B fraction, which is rich in proteins and uronic acid, was dialyzed and incubated with EDTA 0.05 mol/l pH 8 for 4 days at 4°C. The fraction was dialyzed and lyophilized to give a volume of approximately 4–5 ml. The sample was then submitted to chromatography on a Sephacryl S-300 column (2.6×90 cm) with TRIS-HCl 0.05 mol/l, NaCl 0.1 mol/l, pH 7.3 as eluent (flow rate of 25 ml/h). In addition, each 3 ml was collected and the concentration of proteins, uronic acid and inhibitory activity measured in each fifth tube. This step was followed by a separation of proteins with an FPLC column (Mono Q) using a linear gradient of NaCl from 0.1 to 0.5 mol/l in TRIS-HCl 0.05 mol/l, pH 7.3.

Protein determination

Protein concentrations were determined by Lowry's method [18] using bovine albumin as standard. In all chromatographic procedures proteins were detected by their absorbance at 280 nm.

Uronic acid determination

The uronic acid content was determined by the carbazole reaction according to the Bitter and Muir method [2] with glucuronic acid as standard.

Gel electrophoresis

Electrophoresis was performed with a linear polyacrylamide gradient from 8% to 20% according to the method of Weber [38]. The proteins and standards were electrophoresed in the presence of SDS (0.1%) and β-mercaptoethanol (0.1%). The protein bands were revealed by staining with Coomassie blue R or using the silver staining procedure [20].

Carbohydrate analysis

The presence of carbohydrate was detected using periodic acid-Schiff staining on polyacrylamide gels after electrophoresis [41].

Qualitative analysis of GAGs

GAGs were precipitated every tenth tube fractionated from DEAE-Sephacel according to Diferrante's method [6]. Samples were applied to cellulose acetate electrophoresis using the procedure outlined by Renzo [26].

Enzyme assay

The inhibitor might be a GAG, so it was submitted to enzymatic digestion using the protocol described by Sorensen et al. [34]. We used chondroitinase AC (Sigma, C 2262), hyaluronidase (Sigma, H 1136) and $NaNO_2$. In addition, it was also submitted to protease (Calbiochem, 537088, 100 PUK/mg).

Table 1. Effect of various substances on the calcium oxalate crystallization system

Substances tested	% inhibition
Tris, 0.05–0.25 mol/l	No effect
NaCl, 0.05–0.8 mol/l	No effect
THP, 1.25–10 μg/ml	18% to 8%

THP, Tamm-Horsfall protein

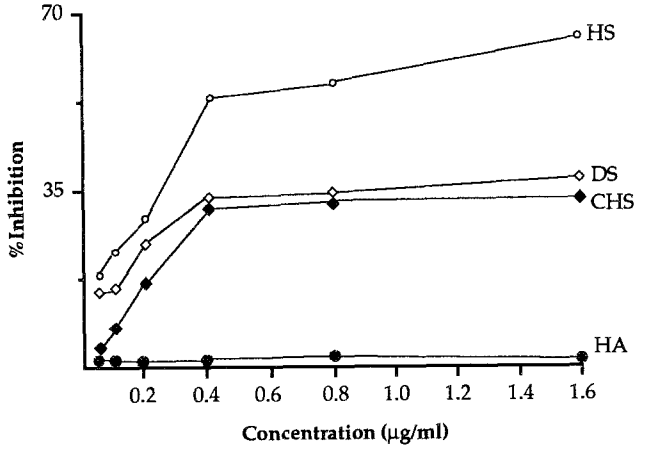

Fig. 1. Effect of various glycosaminoglycans on the calcium oxalate crystallization test. HS, heparan sulfate, DS, dermatan sulfate; CHS, chondroitin sulfate; HA, hyaluronic acid

Immunological technique

Immunoelectrophoresis was carried out as described by Towbin et al. [37] using a polyclonal anti-THP antibody.

Results

Inhibition assay

In order to investigate the degree of inhibition of urinary proteins we developed a calcium oxalate crystallization system. First, we tested the effect of TRIS and NaCl used as eluent in different steps of chromatography and which were found in excess after lyophilization. The results, summarized in Table 1, indicate that TRIS and NaCl had no influence on the calcium oxalate crystallization system in our assay. Second, we tested several substances considered to be potent urinary inhibitors such as THP and different GAGs. As shown in Table 1, THP had only a minor effect on calcium oxalate crystallization at low concentration and no effect at high concentration. The effects of GAGs were examined for a wide range of concentrations between 0.1 and 1.6 μg/ml. Chondroitin sulfate and dermatan sulfate exhibited a moderate inhibition not exceeding 35%. However, hyaluronic acid had no effect and heparan sulfate exerted a strong inhibitory activity (Fig. 1).

Purification procedure

The purification procedure included three chromatography steps. The first was the fractionation of urinary proteins by a DEAE-Sephacel column using a linear gradient of NaCl, from 0.1 to 0.5 mol/l. The B fraction, which is rich in proteins and uronic acid, was eluted between 0.2 and 0.3 mol/l NaCl corresponding to 20%–75% inhibition. A typical chromatographic pattern is shown in Fig. 2. The inhibition was still marked in the latest fractions. The analysis of GAGs performed by electrophoresis in cellulose acetate showed that these fractions contained chondroitin sulfate and dermatan sulfate.

It has been shown that calcium and other divalent ions contribute to protein polymerization [21, 40]. The B fraction was therefore fractionated according to molecular size in a Sephacryl S-300 column after if had been treated with EDTA 0.05 mol/l for 4 days to chelate calcium. The elution pattern is shown in Fig. 3. It can be seen that the first peak, corresponding to THP, showed only a minor effect on our test and that the major inhibitory activity was observed in tubes 100 to 140. This fraction was rich in proteins and uronic acid.

Electrophoresis was performed to find out which proteins were in this fraction. As shown in Fig. 4, exclu-

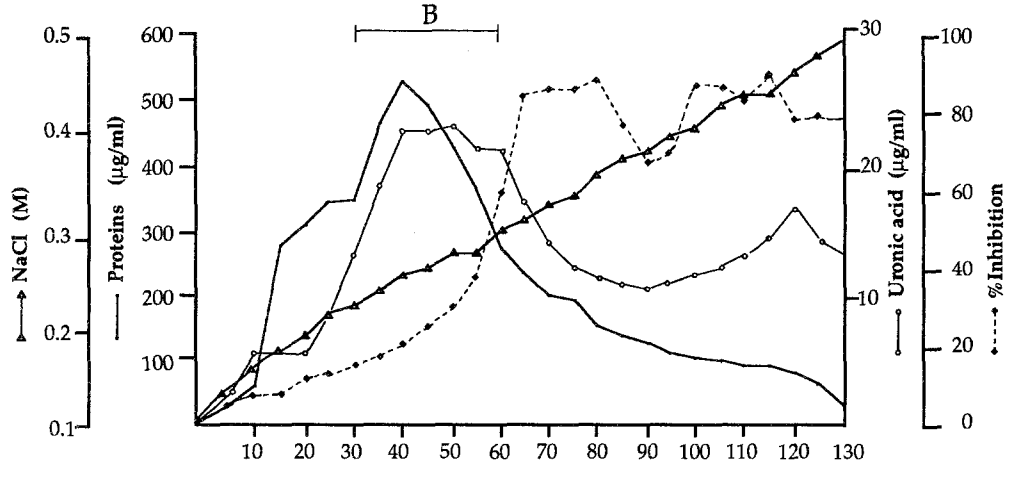

Fig. 2. DEAE-Sephacel ion-exchange chromatography of urinary proteins. The column was eluted with a linear gradient of NaCl from 0.1 to 0.5 mol/l in 0.05 mol/l TRIS-HCl, pH 7.3. Fractions of 3 ml were collected. Protein concentration was determined by Lowry's method and uronic acid by the carbazole reaction. Salt concentration was measured by flame spectrophotometry and inhibition was assayed using a calcium oxalate crystallization system. Fraction B was taken for further purification

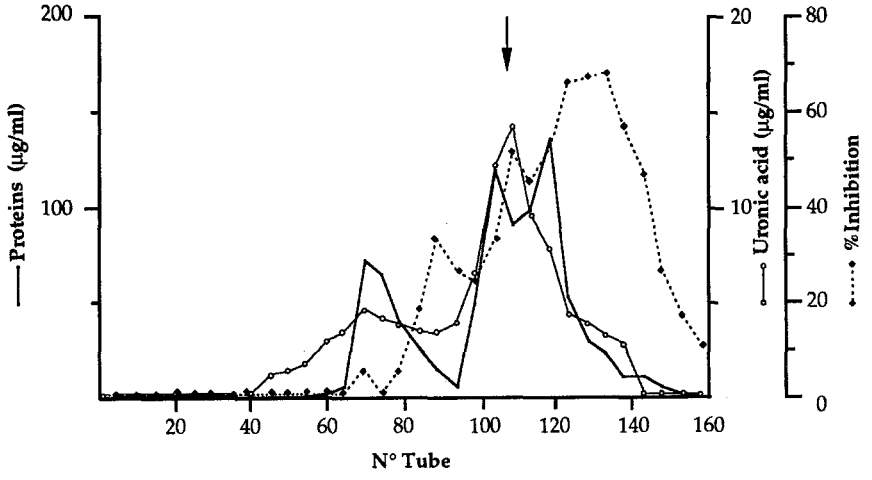

Fig. 3. Sephacryl S-300 column chromatography. Fraction B from the DEAE-Sephacel column was fractionated in a Sephacryl S-300 colunm using TRIS-HCl 0.05 mol/l, NaCl 0.1 mol/l, pH 7.3 as eluent. The *arrow* indicates the elution position of uronic-acid-rich protein (UAP)

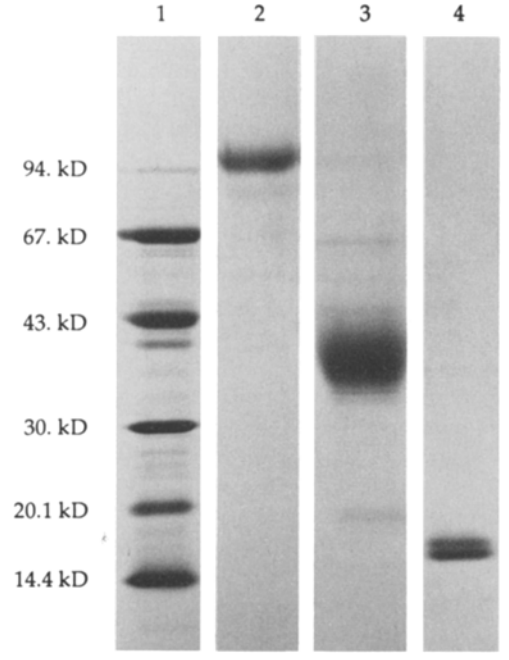

Fig. 4. SDS-polyacrylamide gel electrophoresis: *Lane 1,* molecular weight markers: phosphorylase b, 94. kD; Albumin, 67. kD; Ovalbumin, 43. kD; Carbonic anhydrase, 30. kD; Trypsin inhibitor, 20.1 kD; and α-Lactalbumin 14.4 kD; *Lane 2,* THP; *Lane 3,* UAP; *Lane 4,* NC

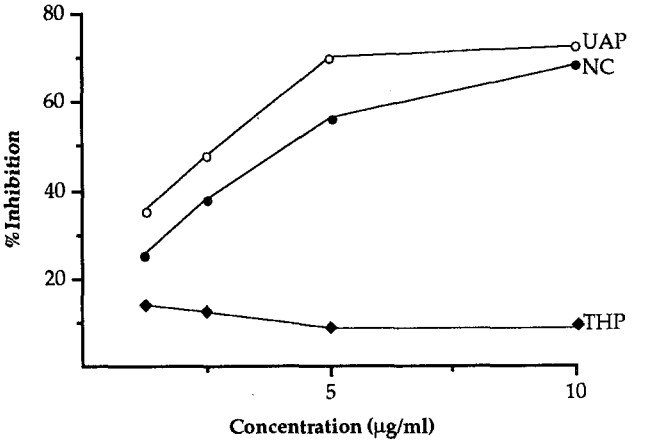

Fig. 5. Effect of nephrocalcin (NC) or NC-like activity, uronic-acid-rich protein (UAP) and Tamm-Horsfall protein (THP) on the calcium oxalate crystallization system after purification by an FPLC column using a linear gradient of NaCl from 0.1 to 0.5 mol/l TRIS-HCl 0.05 mol/l, pH 7.3

sively the 45000 Da band, three bands (45000, 35000 and 16000 Da) were detected. The second band corresponded to uronic-acid-rich protein (UAP) and the third presumably to nephrocalcin (NC). In fact several properties of nephrocalcin, as described by Nakagawa et al. [21, 40], were found: The protein eluted between 0.2 and 0.3 mol/l of NaCl in the DEAE-Sephacel column, it was a glycoprotein able to bind to THP, and it could be dissociated with EDTA. Moreover, this protein showed a strong inhibitory activity towards calcium oxalate crystal growth.

Purification was followed by separation with an FPLC column using an NaCl gradient from 0.1 to 0.5 mol/l. We separated the three proteins and tested them in our assay.

We also tested the effect of purified THP by the same procedure. The results showed only a small effect of THP and the 45000 Da protein, but UAP and NC or NC-like activity showed a remarkable inhibitory activity which was proportional to the concentration used. The inhibitory activity of UAP appeared to be higher than that of NC or NC-like activity (Fig. 5).

There was no immunological identity between THP and UAP when immunoelectrophoresis was performed with anti-THP antibody and UAP inhibitor. We could not test the immunological reactivity between UAP and NC antibodies because NC antibodies were not available. However, the amino acid composition of UAP is different from that of NC (data not shown).

As shown in Fig. 6, the inhibitory activity was not influenced by chondroitinase or hyaluronidase, but the protease did degrade UAP and the inhibitory activity was lost. However, the mixture of $NaNO_2$ and acetic acid itself strongly affected the test activity. We therefore cannot draw a conclusion at this time about the effect of $NaNo_2$ on UAP.

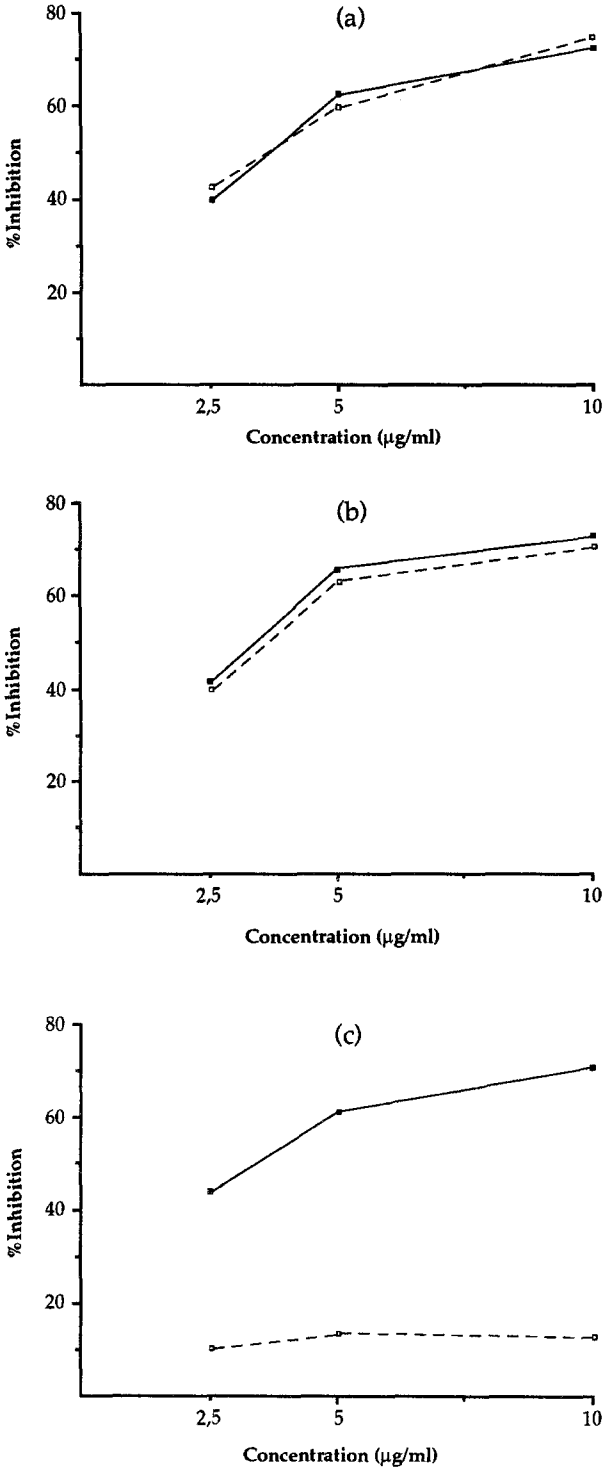

Fig. 6a–c. Effect of chondroitinase AC (a), hyaluronidase (b), and protease (c) on inhibitory activity of UAP. Incubation without enzyme *(continuous line)* and with enzyme *(dashed line)* respectively

Discussion

Human urine is usually supersaturated with various salts and often contains crystals, which can grow and aggregate to form calculi. Nevertheless stones generally do not form, suggesting that the urine of healthy subject contains inhibitory substances. Robertson et al. [28] demonstrated that supersaturation alone is not responsible for crystal formation. Indeed, there is a large body of experimental evidence showing that normal urine contains highly effective inhibitors of crystal formation and aggregation [1, 3, 7, 8, 14, 15, 22, 34]. According to Nakagawa et al. [21–23] nephrocalcin accounts for approximately 90% of total urinary inhibition. These authors have suggested that low molecular weight substances account for only a small part of crystal growth inhibition in urine, and they totally ignored the effect of GAGs. Pak et al. [14, 43] have found that inhibition is partly supported by a urinary macromolecular material, with a significant augmentation of inhibitory activity occurring in the presence of increasing calcium concentrations. This material was rich in uronic acid, suggesting that it was composed of glycoproteins. This finding led the authors to hypothesize that this material might contain nephrocalcin [43]. We attempted to clarify this point in our study.

On the one hand, in agreement with other studies [24, 32, 42], we also found that urinary GAGs were effective macromolecular inhibitors. The fractions obtained from DEAE-Sephacel column, which were rich in chondroitin sulfate and dermatan sulfate, showed a high level of inhibition of calcium oxalate crystal formation. On the other hand, our results showed that the macromolecular material which we isolated from urine effectively contained a protein with a molecular weight of about 16000, presumably nephrocalcin. Moreover, we isolated another glycoprotein with a molecular weight of about 35000 (UAP) and showed that it also had a potent inhibitory effect on crystal growth. UAP, in contrast to α-trypsin inhibitor [34], was damaged by protease, suggesting that these two proteins are different. Further studies on UAP are in progress.

These findings suggest that NC or NC-like activity alone does not account for the total inhibitory activity in urine. The mechanisms of the inhibitory activity have been addressed in numerous studies. It has been proposed that inhibitors bind onto specific sites of crystal growth so that inhibition is achieved with a small quantity of macromolecular substances. Another proposed mechanism for inhibition is that the inhibitors bind onto the surface of crystals and lead to a change in the zeta potential of the surface, thereby modifying attractive or repulsive forces between crystals. Several groups have demonstrated that the inhibitory activity phenomenon is not additive [17, 36]. Our results confirm these findings, demonstrating that urinary GAGs are effective inhibitors and that urine contains another glycoprotein, different from NC, which is a potent inhibitor of calcium oxalate crystal growth. Finally, we believe that urine is a complex system where inhibitors interact between themselves and with promoters to give an unstable and changing equilibrium in which one inhibitor alone cannot be responsible for the total urinary inhibitory activity.

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