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An unidentified macromolecular inhibitory constituent of calcium oxalate crystal growth in human urine

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Summary. We have detected and isolated a macromolecular constituent in normal human urine possessing calcium crystal growth inhibitory activity. The purification procedure consisted of two anion exchange chromatographies and one affinity chromatography. The crystal growth inhibitor was found to be heterogeneous in net charge as well as in size. It has not been identified. It is not an uronic acid-containing glycosaminoglycan, hitherto presumed to be responsible for the inhibitory activity. Whether an urinary fragment of inter-α-trypsin inhibitor is responsible has yet to be resolved.

Key words: Calcium oxalate – Glycosaminoglycans – Growth inhibitors – Proteins – Urine – Urine calculi

Abnormal urine composition seems to be involved in the formation of urinary calculi, but the aetiology is still unknown. Four major theories have been put forward to explain the mechanisms behind the formation of the stones [3]: (i) supersaturation of urine with a substance that can crystallize at a given pH; (ii) presence of a matrix that may promote crystallization and crystal aggregation; (iii) absence of inhibitors that can prevent, or at least limit, crystal growth and aggregation in normal urine; (iv) overgrowth of one crystal upon the surface of another (epitaxy) owing to a resemblance in the lattice between two different types of crystals.

In recent years much attention has been directed towards urinary constituents that are able to reduce crystal nucleation and inhibit the growth and aggregation of already formed crystals, particularly those consisting of calcium oxalate, the most common constituent of urinary calculi. Substances with a high molecular weight seem to be responsible for most of this inhibitory activity in urine [2, 7, 8, 20, 23, 26] whereas those with a low molecular weight, such as pyrophosphate and citrate, seem to contribute little [17].

Several urinary macromolecules have been reported to be inhibitors including glycosaminoglycans [2, 14, 22, 24, 29], a highly acidic glycoprotein (M_r 14,000) [20], acidic peptides [11], non-polymerised Tamm-Horsfall mucoprotein (uromucoid) [23] and RNA [11, 23], but extensive purification and identification of these constituents have only been reported in a very few studies.

Isolation of naturally occurring urinary inhibitors of calcium oxalate may have important implications for the pathogenesis and treatment of urinary stone because, if crystal growth and aggregation of calcium oxalate crystals could be prevented or controlled, the small crystalline nuclei might pass harmlessly into the urine. Here we report our attempts to isolate and identify a calcium oxalate crystal growth inhibitor from normal human urine.

Materials and methods

Materials

[^{14}C] Oxalic acid (60–80 mCi/mmol) and $^{45}CaCl_2$ (10–40 mCi/mg Ca) were purchased from Amersham International, Amersham, UK; diethylaminoethyl (DEAE)-Sephacel, Mono Q HR 5/5 column, Superose 12 HR 10/30 and molecular weight standards were from Pharmacia Fine Chemicals, Uppsala, Sweden; TSK G3000SW from LKB, Bromma, Sweden, and agarose (type HSA) from Litex, Glostrup, Denmark.

Rabbit anti-human normal urine antiserum (code no. A 211) was obtained from Dakopatts, Glostrup, Denmark; Chelex 100 (dry mesh 200–400) acrylamide and *N, N'*methylene-bis-acrylamide from Bio-Rad, Richmond, CA, USA. Vinylsulfone agarose (Mini Leak) was delivered by Kem-en-tec, Copenhagen, Denmark; calcium oxalate monohydrate crystals (cat. no. 27609) were purchased from BDH Chemicals, Poole, UK; protease (Pronase, cat. no. 537088, 110 PUK/mg, nuclease free <0.0005 U/mg) was from Calbiochem, San Diego, USA; and hyaluronic acid (no. H 1504), chondroitinase AC (no. C 2262) and hyaluronidase (no. H 1136) were from Sigma Chemical, St. Louis, USA. Heparin was obtained from Leo Pharmaceutical Products, Ballerup, Denmark (173 IU/mg heparin). Instagel II was from Packard Instrument, Downers Grove, IL, USA.

Polystyrene tubes (100 × 15 mm; cat. no. 342919) and polypropylene tubes (72 × 12.5 mm; cat. no. 366524) were obtained from Teknunc, Roskilde, Denmark; dialysis tubing (Servapor) from Serva, Heidelberg, FRG; and Cuprophan-dialysis foil (type 150 PM) from Enka AG, Wuppertal, FRG.

Urine concentration and dialysis

Urine from one or more healthy laboratory workers was collected over 120 h (≈ 6–8 l). Preservative was not added, but all samples were kept refrigerated until the entire amount had been colleted. No care was taken to prevent precipitates forming during collection. The urine was concentrated and dialysed against deionized water using a disposable dialyser and a peristaltic pump [28]. We used a parallel flow dialyser (Lundia 10, 3N) or a hollow fibre dialyser (GF 180 M) from Gambro, Lund, Sweden (M_r cut-off about 1,000–3,000). A volume of 6–8 l urine was concentrated to about 400–500 ml in a few hours. After use the dialyser was rinsed in 0.075% (v/v) sodium hypochlorite and reused not more than five times. The urine was then dialysed twice against 0.05 *M* TRIS-HCl buffer pH 7.5 with 15 m*M* NaN_3 at 4°C using a 10,000 MW cut-off dialysing tubing. After centrifugation at 2,000 *g* for 10 min, the clear supernatant was removed from a minor precipitate.

Anion exchange chromatography

The dialysed urine was applied to a 26 × 100 mm DEAE-Sephacel column equilibrated with 0.05 *M* TRIS-HCl buffer, pH 7.5 (flow/rate 15 ml/h), and eluted with a linear NaCl gradient from 0 to 800 m*M* in 0.05 *M* TRIS-HCl buffer, pH 7.5. The flow rate was 12–15 ml/h, and 4–5 ml was collected in each fraction.

Uronic acid determination

The uronic acid content in the fractions of the anion exchange chromatography (DEAE-Sephacel) and size-exclusion chromatography (Superose 12 HR 10/30) was measured as described in [1].

Protease treatment

A volume of 150 µl (<200 µg protein) of the fractions of anion exchange chromatography (DEAE-Sephacel) was incubated with 10 µl (100 µg) protease. After 3 h at 37°C the crystal growth inhibitory activity was measured, and fused rocket immunoelectrophoresis was performed on the fractions (both methods are described below).

Ca^{2+} binding activity

Ca^{2+} binding activity in eluted fractions from anion exchange chromatography (DEAE-Sephacel) was assayed by the Chelex $^{45}Ca^{2+}$ assay [27]. In polypropylene tubes (72 × 12.5 mm), 200 µl of 50% (v/v) Chelex 100 in 0.05 *M* TRIS-HCl buffer (pH 7.5), 500 µl of sample and 100 µl (1 µCi) of $^{45}CaCl_2$ were incubated by rotating for 15 min at room temperature. After centrifugation at 2,000 *g* for 10 min, 200 µl supernatant was mixed with 5 ml of scintillation fluid (Instagel II), and radioactivity was measured in a β-counter (Isocap/300, Nuclear Chicago Division).

Fused rocket immunoelectrophoresis

Fused rocket immunoelectrophoresis was performed as described in [9]. An appropriate volume (2–10 µl) of every or every other fraction from chromatography was transferred consecutively to the sample wells in the antibody-free agarose gel in the lower third of a 10 × 10 cm glass plate. The samples were allowed to diffuse for 60 min before the electrophoresis was carried out (18–20 h at 2.5 V/cm) with 10% (v/v) rabbit anti-human normal urine antiserum in the upper two-thirds of the agarose plate. A TRIS-barbital buffer, I=0.02, pH 8.6, was used. After electrophoresis the plate was pressed, then washed overnight in 155 m*M* NaCl, dried and stained in Coomassie Blue R-250, and dried.

Inhibition of calcium oxalate crystal growth

The inhibition of the crystal growth rate in urine and in fractions of various chromatograpies of urine was measured by the amount of [^{14}C] oxalic acid remaining dissolved in a metastable solution of calcium chloride and sodium oxalate after addition of seed crystals of calcium oxalate. This is basically the method described by Ligabue et al. [14], except that we reduced the incubation volume to a fourth. In each polystyrene tube (100 × 15 mm), 3.75 ml of 2 m*M* $CaCl_2 \cdot 2H_2O$, 50–250 µl of sample, 200–0 µl of 150 m*M* NaCl, 3.75 ml of 0.4 m*M* sodium oxalate in 10 m*M* sodium cacodylate buffer, pH 6.0, and 25 µl (12 nCi) of [^{14}C] oxalic acid were mixed. The reaction was initiated by adding 300 µl of a calcium oxalate crystal suspension (1 g/l). The ion strength was maintained in all solutions with NaCl in a concentration of 150 m*M*, and they were passed through a 0.45-µm filter before use, except for the sodium chloride solution for the calcium oxalate suspension, which was filtered beforehand. The tubes were closed with tight-fitting polyethylene caps and incubated horizontally in a shaking apparatus at 37°C for 2 h. After centrifugation (2,000 *g*, 10 min) 500 µl supernatant was withdrawn, and 10 ml scintillation fluid (Instagel II) was added, and samples were counted for 20 min in a liquid scintillation counter (Isocap/300, Nuclear Chicago Division).

Anion exchange chromatography

Fractions from the first anion exchange chromatography (DEAE-Sephacel) with inhibitory activity were pooled and dialysed twice against 0.05 *M* TRIS-HCl buffer, pH 7.5. The pool was then placed on a Mono Q HR 5/5, flow rate 1 ml/min, and a linear NaCl gradient from 0 to 800 m*M* TRIS-HCl buffer was applied by means of FPLC equipment (Pharmacia, Sweden). Fractions of 0.5 ml were collected. Measurement of crystal growth inhibitory activity and fused rocket immunoelectrophoresis were performed on every fraction.

Negative affinity chromatography

An immunoadsorbent column was established by coupling 25 ml rabbit anti-human normal urine antiserum (protein concentration 40 g/l) to 25 ml vinylsulfone agarose according to the manufacturer's directions. The coupling was done in 0.2 *M* potassium phosphate buffer, pH 8.6, with 1 *M* NaCl. The remaining active groups were blocked with 0.1 *M* ethanolamine at pH 8.6 (3 h). Positive inhibitor fractions obtained from anion exchange chromatography were combined and further purified by negative affinity chromatography on a 25 × 50 mm column with the immunoadsorbent at a flow rate of 50 ml/h followed once by the bed volume of 0.05 *M* TRIS-HCl buffer, pH 7.5. Fractions of 1.0 ml were collected. The immunoadsorbent was regenerated with 0.1 *M* glycine, pH 2.5. Break-through fractions were dialysed against distilled water, lyophilized and redissolved in distilled water.

Size-exclusion chromatography

Some 100 µl (≈ 0.6 l urine) of the purified product was fractionated on a 0.75 × 60 cm column of TSK G3000 SW with 0.05 *M* TRIS-HCl buffer, pH 7.5, and 100 m*M* NaCl as the mobile phase. The flow was 0.1 ml/min and the volume of each fraction, 0.5 ml. The crystal growth inhibitory activity was measured on every fraction.

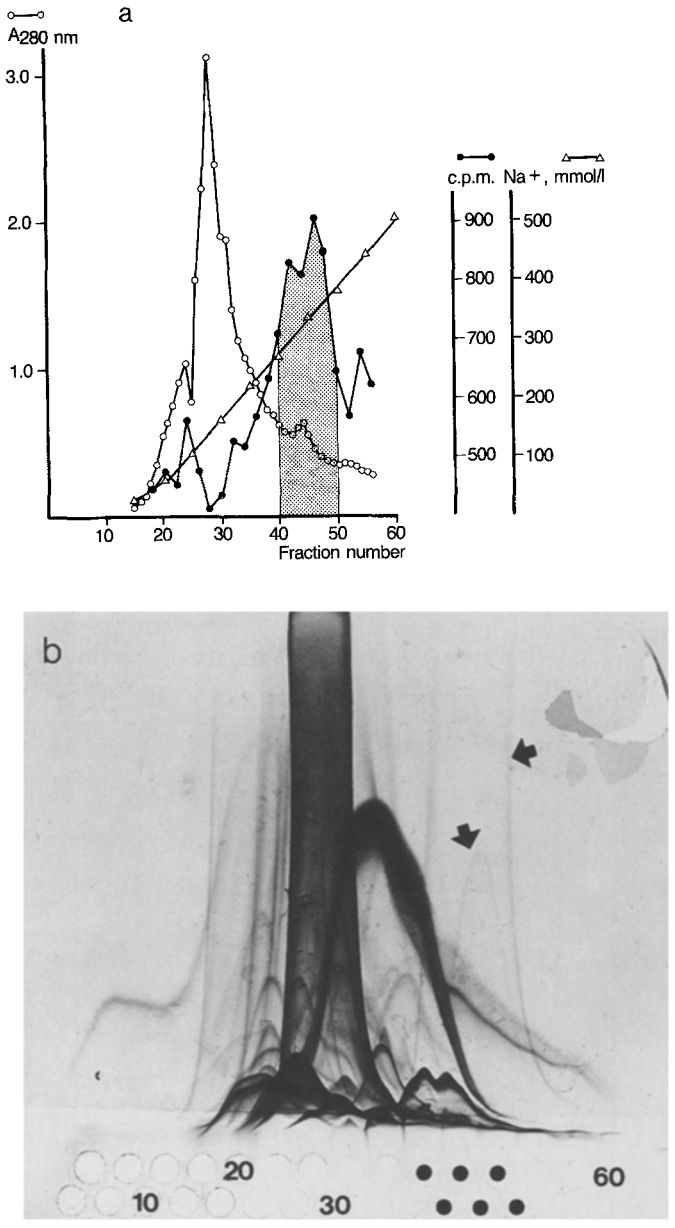


Fig. 1. **a** Anion exchange chromatography (DEAE-Sephacel, 26 × 100 mm) of a 120-h human urine specimen concentrated and dialysed against 0.05 *M* TRIS-HCl buffer, pH 7.5. Fractions with calcium oxalate crystal growth inhibitory activity were combined (*hatched area*). **b** Fused rocket immunoelectrophoresis of 10 µl of every other fraction of the anion exchange chromatography in **a**. Electrophoresis was carried out against 10% (v/v) anti-human normal urine antiserum in 1% (w/v) agarose gel. Two immunoprecipitates could be identified in the fractions with crystal growth inhibitory activity (*arrows*)

Sodium dodecyl sulphate polyacrylamide gel electrophoresis

A sample of 25 µl of every fraction with crystal growth inhibitory activity by size-exclusion chromatography (TSK G3000 SW) and 5 µl (11.6 µg) of molecular weight standards were denatured and reduced in 2% (w/v) sodium dodecyl sulphate (SDS) and 5% (v/v) 2-mercaptoethanol (2 min at 100°C). Stacking gel consisted of 6% (w/v) acrylamide, 0.25% (w/v) bisacrylamide and 0.1% (w/v) SDS in 0.125 *M* TRIS-HCl buffer, pH 6.8. In the separation gel there were 15% (w/v) acrylamide, 0.15 (w/v) bisacrylamide and 0.1% (w/v) SDS in 0.75 *M* TRIS-HCl buffer, pH 8.8. The electrophoresis was carried out at 8 mA overnight in 0.125 *M* TRIS-HCl, 0.1 *M* glycine and 0.1% (w/v) SDS. The gel was then stained using a silver staining technique [18] and dried between two layers of cellophane [13].

Glycosaminoglycan degradation

Since the inhibitor might be a glycosaminoglycan or galactosaminoglycan, 100 µl of purified inhibitor (≈ 2.6 l urine) having a uronic acid content of 82 µg was incubated with: (a) 60 µl (≈ 0.15 unit) of chondroitinase AC and 40 µl 0.25 *M* TRIS and 0.29 *M* sodium acetate with 0.25 *M* NaCl, pH 8.0 [25], (b) 10 µl (≈ 10 units) of hyaluronidase, 40 µl of 0.1 *M* sodium acetate, pH 5.0, and 50 µl of distilled water [12] or (c) 50 µl of 3.0 *M* $NaNO_2$ and 50 µl of glacial acetic acid [10] to degrade heparin and haparan sulphate. After incubation for 16–24 h at 37°C the preparation was fractioned on Superose 12 HR 10/30 with 0.1 *M* sodium acetate, pH 5.0, and 0.15 *M* NaCl as the mobile phase. The flow rate was 0.5 ml/min, and the volume of each fraction was 1.0 ml. The crystal growth inhibitory activity and uronic acid concentration were measured on the fractions. In a control experiment the purified inhibitor was replaced by 50 µg heparin or 50 µg hyaluronic acid.

Inhibitory activity of the purified preparation

The purified inhibitor (1 ml ≈ 6 l urine) was diluted with urine [1:100, v/v (%)] and 150 m*M* NaCl [1:100, v/v (%)], respectively, and investigated for inhibitory activity as described above. Incubation periods of 20, 40, 60 and 120 min were carried out.

Results

Isolation of calcium oxalate crystal growth inhibitor

A strong crystal growth inhibitory activity was found in fractions eluted late in the first anion exchange chromatography (DEAE-Sephacel), from about 250 to 400 m*M* Na^+ (Fig. 1a). By fused rocket immunoelectrophoresis carried out against anti-human normal urine antiserum on the same fractions, only two immunoprecipitates had a peak in the same fractions (Fig. 1b). Fractions from the anion exchange chromatography were investigated for uronic acid content, influence of protease on crystal growth inhibitory activity and Ca^{2+} binding activity. No uronic acid peaks coincided with the crystal growth inhibitory peak (Fig. 2), and treatment of the crystal growth inhibitor with proteinase did not significantly change its inhibitory activity (data not shown), even though this treatment had a strongly denaturing influence on the proteins (data not shown). The proteinase itself had no inhibitory properties. Ca^{2+} binding activity was not found in the fractions with crystal growth inhibitory activity, only in the fractions just after the A_{280}nm maximum but before the inhibitory activity peak (data not shown).

Fractions having a crystal growth inhibitory activity were subjected to one more anion exchange chromatography (Mono Q). The crystal growth inhibitor was still eluted late and in many fractions with a Na^+ concentra-

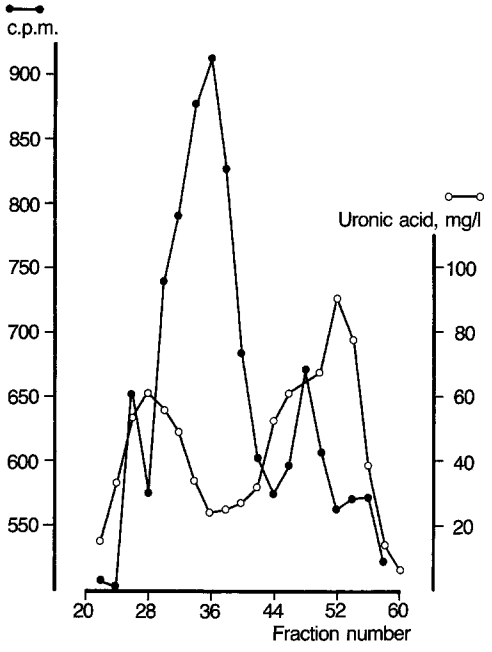
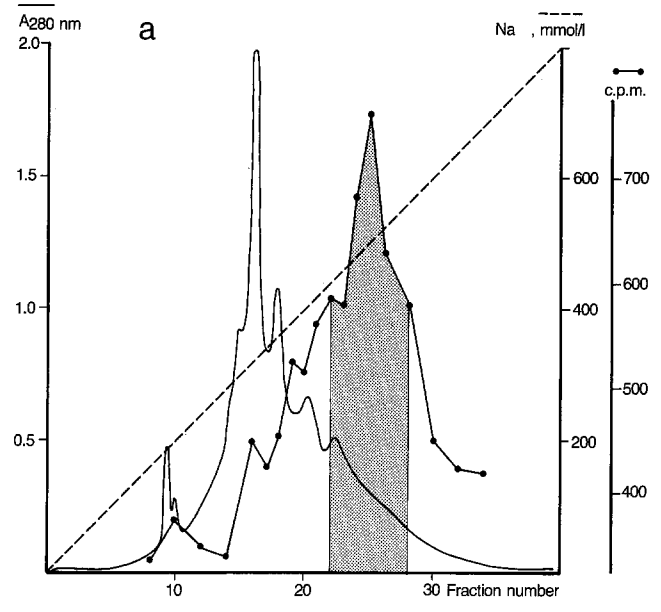
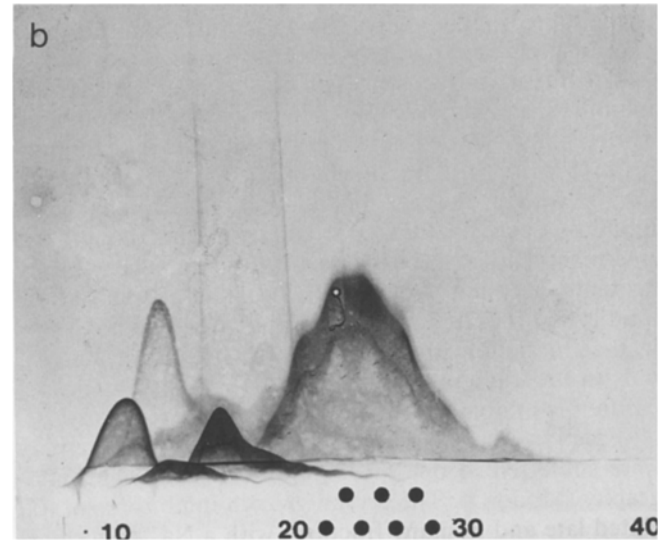


Fig. 2. Calcium oxalate crystal growth inhibitory activity and uronic acid concentration in the fractions of anion exchange chromatography (DEAE-Sephacel) of 120-h human urine (similar to that in Fig. 1a but not identical)

tion from about 350 to 600 m*M* (Fig. 3a). Fused rocket immunoelectrophoresis of the same fractions against anti-human normal urine antiserum is shown in Fig. 3b. Only one precipitate coincided with the crystal growth inhibitory activity, but this was different from the two that coincided with the inhibitory activity in the first anion exchange chromatography (Fig. 1b). This arouses a suspicion that no precipitating antibodies against the crystal growth inhibitor are present in rabbit anti-normal human urine antiserum.

Negative affinity chromatography was then used to try to remove the remaining normal urine proteins. The crystal growth inhibitor appeared in the break-through fractions whereas mostly proteins were adsorbed, not the inhibitor, provided that this was not inactivated by acid treatment (Fig. 4). The fractions containing the crystal growth inhibitor were dialysed against distilled water and lyophilized.





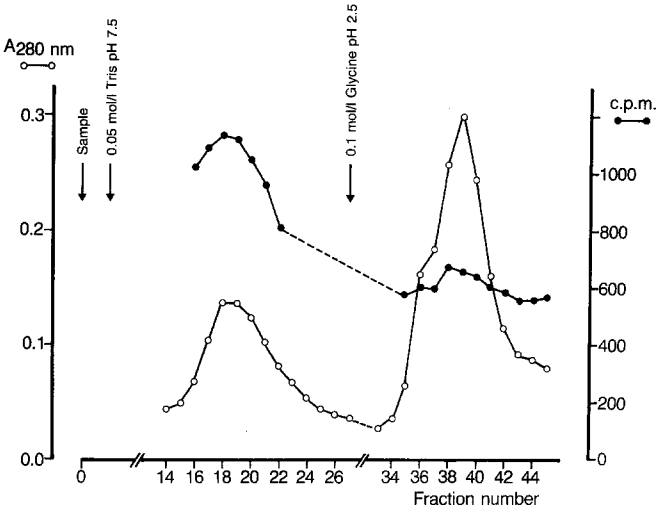


Fig. 4. Negative affinity chromatography of pooled fractions with crystal growth inhibitory activity in Fig. 3a. They passed over a 25 × 50 mm column of immunoadsorbent coupled with rabbit anti-human normal urine antiserum at a flow rate of 50 ml/h. Fractions of about 1 ml were collected. Sample volume for measuring the inhibitory activity was 150 µl. Glycine-containing fractions were dialysed against 0.05 *M* TRIS-HCl, pH 7.5, before the inhibitory activity was measured

Fig. 3. a Anion exchange chromatography (Mono Q, 5 × 50 mm) of the combined fractions with crystal growth inhibitory activity in Fig. 1a. Fractions of 0.5 ml with crystal growth inhibitory activity were pooled (*hatched area*). **b** Fused rocket immunoelectrophoresis of 2 µl of every fraction of anion exchange chromatography in **a**. The experimental conditions were the same as described under Fig. 1b. Only one precipitate coincides with the crystal growth inhibitory activity (●), but it is not identical with one of the two precipitates coinciding with the inhibitory activity in Fig. 1b

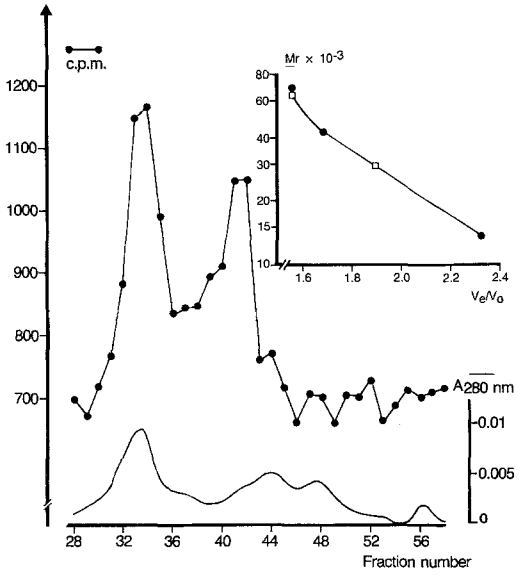


Fig. 5. Size-exclusion chromatography of 100 µl (≈ 0.6 l urine) of the purified crystal growth inhibitor on TSK G3000 SW (0.75 × 60 cm). Volume of each fraction was 0.5 ml. Crystal growth inhibitor was eluted in two peaks, the second inconstantly. *Insert,* Estimation of the relative molecular weight of the inhibitor (□)

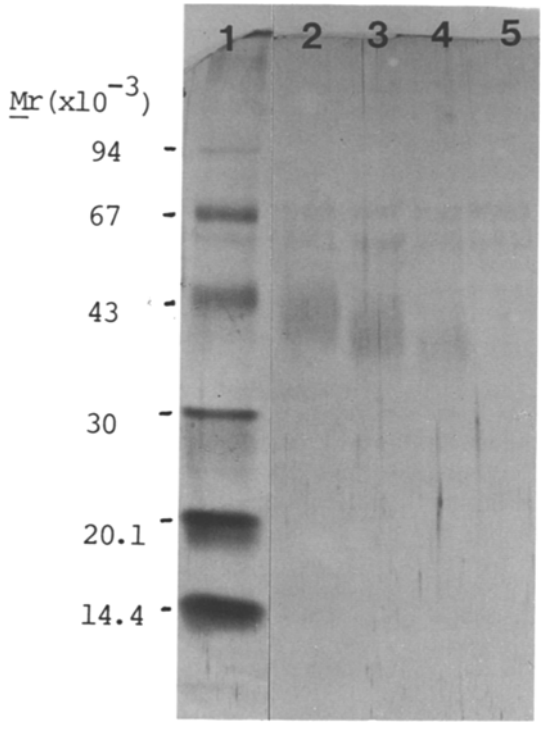


Fig. 6. Sodium dodecyl sulphate polyacrylamide gel electrophoresis of 5 µl (11.6 µg) of molecular weight standards (*lane 1*) and 25 µl of fractions 33–35 from size-exclusion chromatography of purified crystal growth inhibitor in Fig. 5 (*lane 2–4*). *Lane 5* is buffer. Samples were denatured and reduced (see text). Gel was stained by a silver staining technique

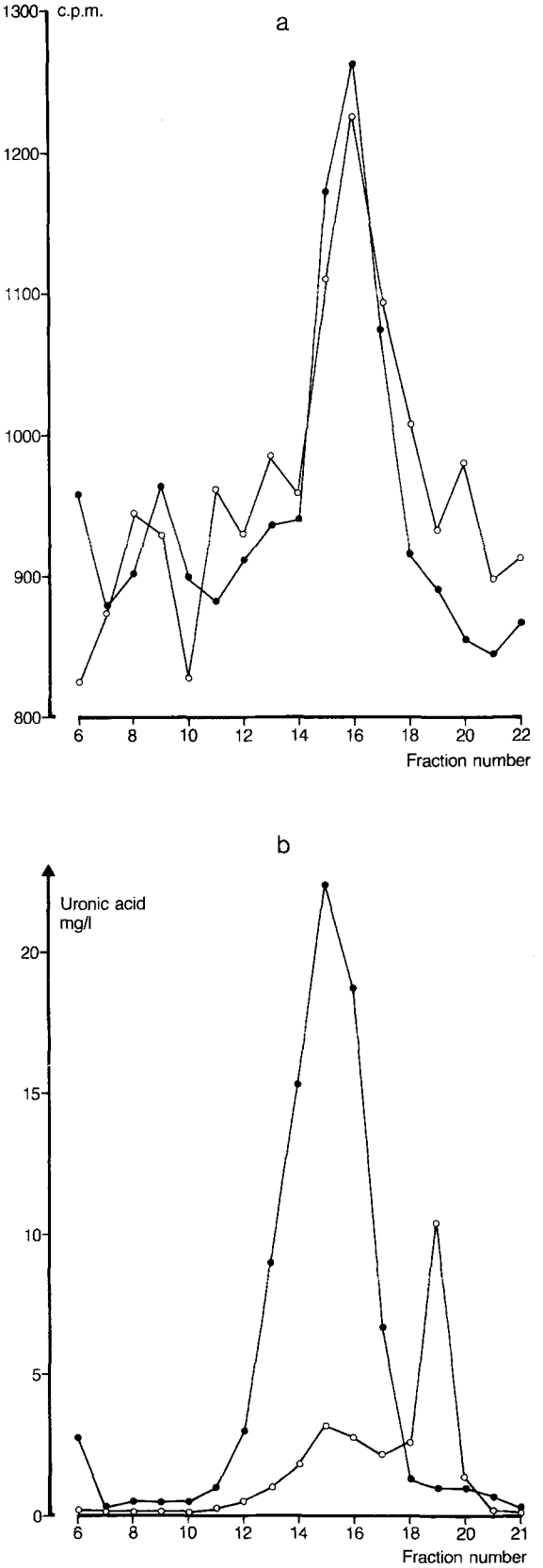


Fig. 7a, b. Size-exclusion chromatography (Superose 12 HR 10/30) of purified crystal growth inhibitor incubated overnight at 37°C with chondroitinase AC (○—○) and without enzyme (●—●). The volume of each fraction was 1.0 ml. The crystal growth inhibitory activity was not influenced by the enzyme (**a**), whereas the uronic acid disappeared (**b**)

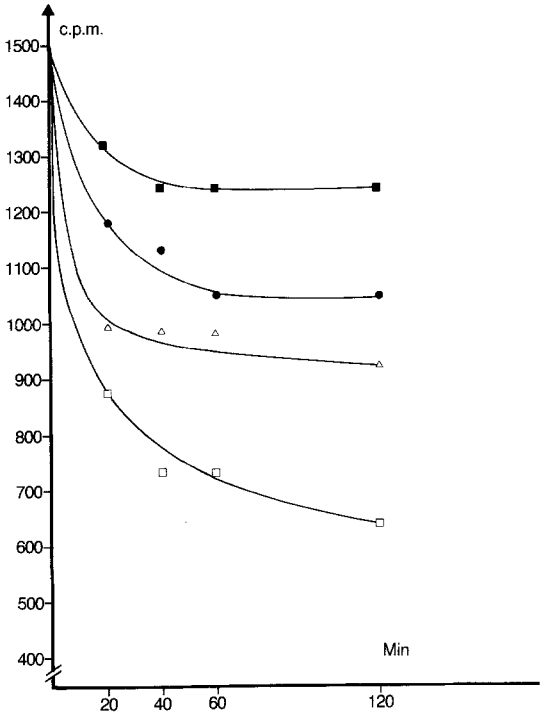


Fig. 8. Crystal growth inhibitory activity of purified inhibitor preparation (1 ml ≈ 6 l urine) mixed 1:100 [v/v (%)] with urine (■—■) or 150 m*M* NaCl (△—△). Sample volume was 150 µl and incubation periods, 20, 40, 60 and 120 min. Control experiments: urine (●—●) and 150 m*M* NaCl (□—□)

By size-exclusion chromatography of the purified product the inhibitory activity appeared in two peaks (Fig. 5) with a relative molecular weight (M_r) of about 67,000 and 30,000, respectively (Fig. 5, insert). SDS-PAGE of fractions in the first peak showed a band with M_r about 40,000 (Fig. 6). No band was detected corresponding to the second inhibitory peak (not shown). The protein in the first peak was identified as inter-α-trypsin inhibitor by determination of the first sixteen residues of the amino end (details not given). The crystal growth inhibitor was not degraded by chondroitinase AC (Fig. 7a), but uronic acid disappeared from the fractions with inhibitory activity (Fig. 7b) due to chondroitin sulphate in the purified preparation. The inhibitor was not susceptible to digestion by hyaluronidase and $NaNO_2$ (not shown).

Purified crystal growth inhibitor added to urine strongly increased the inhibitory activity (Fig. 8).

Discussion

There is increasing agreement that one or more macromolecular constituents of urine possess major crystal growth inhibitory activity. This inhibitory activity has been particularly attributed to the glycosaminoglycans (GAG) [2, 14, 22, 24, 29]. However, although some GAG might be lost by dialysis, the lack of coelution of uronic acids (glycosaminoglycans) with the crystal growth inhibitor activity by anion exchange chromatography observed in this study rules out the possibility that one of the major components of GAG in urine (dermatan sulphate, chondroitin sulphate or heparan sulphate [21]) possesses such activity. This is further supported by the fact that the purified inhibitor was unsusceptible to digestion by chondroitinase AC and $NaNO_2$.

The appearance of inter-α-trypsin inhibitor as a single band in SDS-PAGE of the fractions from size-exclusion chromatography (Fig. 6) with crystal growth inhibitory activity might indicate that this protein or a fragment is responsible for the urinary crystal growth inhibitory activity. The anti-proteinase effect of the protein might explain why protease treatment did not influence the crystal growth inhibitory activity. One might suppose that a commercial antiserum against normal human urine contains antibodies against inter-α-trypsin inhibitor and thereby the crystal growth inhibitor. But antibodies could not be demonstrated by immunoelectrophoretic techniques against the inhibitor. Perhaps the inhibitor is removed to a certain extend by affinity chromatography. However, an estimate of loss of crystal growth inhibitory activity by the purification procedure could not be established, since the measurement of the crystal growth inhibitory activity is not really a quantitative method. Our study seems to indicatee that trypsin inhibitor is the only urinary protein which might have a crystal growth inhibitory activity but further studies are necessary.

In other studies acidic peptides [11] as well as a highly acid glycoprotein [20] have been described as having crystal growth inhibitory activity. The inhibitor described by Nakagawa et al. was heterogeneous in net charge as well as in size, in agreement with our findings estimated by anion exchange chromatography and gel filtration chromatography. However, they found that their inhibitor was a glycoprotein bound to Tamm-Horsfall protein (uromocoid) [15], which was not observed in our study. Other investigators have also shown the crystal inhibitor to be molecularly heterogeneous [5, 7, 26], with the major part larger than a M_r of 10,000, since the inhibitory activity was retained after dialysis with tubing with a M_r cut-off of 10,000.

The highly acid glycoprotein described by Nakagawa et al. [20] contained carboxyglutamic acid (Gla) which may have calcium-binding properties, thus inhibiting crystal precipitation and decreasing the rate of crystal growth in vivo, but we found no coelution of calcium-binding constituents and crystal growth inhibitor on anion exchange chromatography. The inhibitor is produced both by a culture medium of human kidney tissue [19] and by bladder mucosa [4, 16]. The mechanism of action is uncertain, but the inhibitory effect appears to be related to the binding of the inhibitor onto the crystal surface where it inhibits induction of new crystal growth and aggregation [6], perhaps by forming a complex with exposed ions on the crystal surface through carboxyl and phosphate moieties [20].

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