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Identification of a macromolecular crystal growth inhibitor in human urine as osteopontin

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Abstract Macromolecules occurring in human urine inhibit the growth and/or aggregation of calcium oxalate crystals and may prevent the formation of kidney stones. Attention has focused particularly on proteins, as these seem to be most responsible for the inhibitory activity; three proteins, nephrocalcin, an unidentified protein rich in uronic acid, and uropontin have all been described as possessing such activity. We have recently isolated an unknown inhibitor of calcium oxalate crystal growth that co-eluted with trypsin inhibitor in several separation steps, which suggested its identity. The aim of the present study was to outline a simple procedure for isolating and identifying this inhibitor. Purification was done as follows: precipitation of the major proteins (albumin and uromucoid) with trichloroacetic acid, followed by anion exchange chromatography, hydroxyapatite chromatography, anion exchange chromatography, negative affinity chromatography, and twice reversed phase chromatographies of the supernatant. By this procedure, the inhibitor was identified as being a fragment of osteopontin; urinary trypsin inhibitor and nucleic acids were excluded as being responsible for inhibitory action.

Key words Calcium oxalate urine · Growth inhibitors urine · Growth inhibitors, isolation and purification · Osteopontin · Precipitation · Trichloroacetic acid

Precipitation of crystals in urine oversaturated with calcium oxalate, i.e. urine where the ion product in excess of the level at which spontaneous precipitation occurs, may be the initial event of stone formation. However, urine contains inhibitor(s) against growth and/or aggregation of calcium oxalate crystals and most of the activity can be ascribed to macromolecules [4, 7]. These may thus be potent inhibitors of urinary stone formation. A great deal of effort has been expended on identifying these constituents. Whether the inhibitory effect is due to one or more specific macromolecules is not clear. Glycosaminoglycans were long believed to be potent inhibitors, but recent studies have been unable to confirm these findings [19, 26]. In recent years, more proteins have been regarded as the most likely candidates responsible for the inhibitory activity in urine. Particular attention has focused on nephrocalcin [19], an unidentified protein rich in uronic acid [1], and uropontin [24]. We have recently isolated an unknown crystal growth inhibitor that co-eluted with an α_1-trypsin inhibitor in several separation steps which suggested a possible identity [26]. We now report the isolation of the crystal growth inhibitor by extending conventional chromatographic techniques [19, 26] to include hydroxyapatite chromatography, a technique that may be useful for isolating substances that bind to the surface of calcium oxalate crystals [15, 18]. The crystal growth inhibitor was identified as a fragment of osteopontin; the study rules out urinary trypsin inhibitor and nucleic acids as inhibitors of the growth of calcium oxalate crystals.

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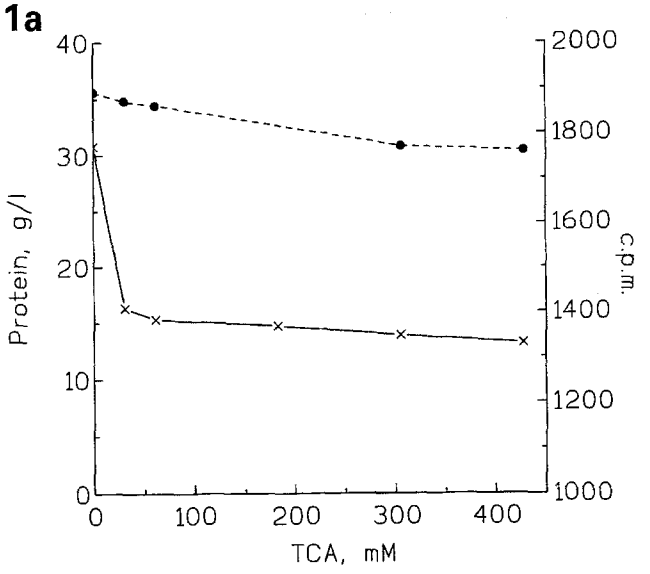
Materials and methods

Urine concentration

A volume of 25 l urine was collected, stored without preservative at 4 °C for a few days and concentrated approximately 50 fold to about 0.5 l by ultrafiltration [26]. The relative molar mass cut off was about 1000–3000. The urine was centrifuged to remove cellular debris.



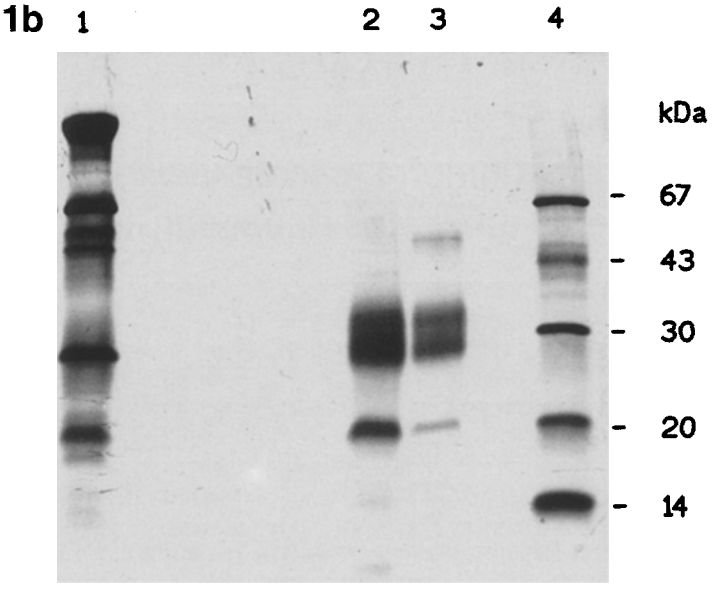


Fig. 1 **a** Trichloroacetic acid (*TCA*) precipitation of urinary proteins. Only minimal amounts of crystal growth inhibitor were eliminated by TCA precipitation, at least up to 428 mM (~7.5%). ×—× protein, ●– –● crystal growth inhibitor activity (c.p.m.). **b** SDS-PAGE (15%) of urine concentrated by ultrafiltration (M_r cutoff 1000–3000) and subsequently treated with different concentrations of TCA. The supernatants were dialysed against distilled water, lyophilized, and resuspended in distilled water (see text). A sample volume was denatured in 2% (w/v) SDS [1 + 1 (v/v)]. *Lane 1* 10 µl (308 µg protein) untreated urine, *lane 2* 20 µl (326 µg protein) supernatant after TCA treatment (incubation concentration 30.6 mmol/l), *lane 3* 20 µl (278 µg protein) supernatant after TCA treatment (incubation concentration 306 mmol/l), *lane 4* 20 µl (46 µg protein), low molecular weight standard

Protein precipitation

Most urinary proteins are precipitated by trichloroacetic acid (TCA), leaving the crystal growth inhibitor in the supernatant. The optimal TCA concentration was established by bringing 15 ml concentrated urine to 0, 30.6, 61, 183, 306, and 428 mM TCA in a final volume of 25 ml by using a 2.45 M TCA. The temperature of all fluids before mixing was 4 °C. After 15 min at 4 °C and subsequent centrifugation (8000 *g*, 30 min), the supernatant was neutralized with 0.1 M HEPES buffer/1.33 M KOH (approximately the TCA volume) and adjusted to pH 7.5, dialysed against distilled water, lyophilized, and resuspended in 1.5 ml distilled water. Crystal growth inhibition and the protein concentration [17] were measured in each of the TCA concentrations. Inhibition 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 [26]; results are given as c.p.m. Resuspended material after treatment with, respectively, 0, 30.6, and 306 mM TCA was examined by means of SDS-PAGE [26]. Precipitation with TCA reduced the protein concentration, but had no substantial influence on the crystal growth inhibitory activity (Fig. 1a). When concentrated urine was treated with 306 mM TCA, the major proteins, albumin and uromucoid, were eliminated, leaving low molecular mass proteins in the solution (Fig. 1b). This TCA concentration was used for batch production of the crystal growth inhibitor, but with a slight modification of the method: To 510 ml of the concentrated urine, 340 ml 765 mM TCA was added and incubated for 15 min (final incubation 306 mM). After centrifugation (24 000 *g*, 30 min) the supernatant was neutralized with NaOH to pH 6.0, dialysed several times against distilled water, and lyophilized.

Preparative chromatography

The lyophilized material was resuspended in 50 ml 0.05 M TRIS-HCl buffer, pH 7.5, and applied to a DEAE-Sephacel column. The adsorbed material was eluted with a linear gradient of NaCl from 0 to 800 mM in 0.05 M TRIS-HCl buffer, pH 7.5, as described elsewhere [26]. The fractions were monitored for inhibitory activity on crystal growth and the content of uronic acid [26]. Those exhibiting the greatest activity were pooled, dialysed against distilled water, and lyophilized.

After anion exchange chromatography, the lyophilized material containing the crystal growth inhibitor was resuspended in 1.5 ml 10 mM K_2HPO_4, pH 6.8, and fractionated on a 10 × 45-mm hydroxyapatite column (cat. no. 44225, BDH Chemicals, Poole, UK). Components bound to the matrix were eluted with a K_2HPO_4 linear gradient from 10 to 1000 mM. The flow rate was 0.5 ml/min and the volume of each fraction was 3 ml. From each fraction, 50 µl was assayed for crystal growth inhibition.

Enriched fractions of the inhibitor from hydroxyapatite chromatography were dialysed against distilled water, lyophilized, resuspended in 0.5 ml 0.05 M TRIS-HCl, pH 7.5, and injected onto a Mono Q column. A linear gradient of NaCl was used for the elution [26]. The flow rate was 0.5 ml/min and each fraction contained 3.0 ml. The sample volume for determination of the inhibitory activity was 25 µl.

We tried to remove the remaining urinary proteins, which reacted to the rabbit antihuman normal urine antiserum, with negative affinity chromatography [26]. The fractions from Mono Q containing crystal growth inhibitor were applied to an affinity column coupled with antihuman normal urine antiserum (16 × 130 mm) and eluted with 0.05 M TRIS-HCl, pH 7.5. The flow rate was 0.33 ml/min, and the volume of each fraction was 2 ml. To assay crystal growth inhibition 250 µl sample was used. The column was regenerated with 0.1 M glycine, pH 2.5. The fractions containing the crystal growth inhibitor were dialysed against distilled water and lyophilized.

Lyophilized material from negative affinity chromatography was resuspended in 1 ml 5% acetic acid and 500 µl was injected onto a 7.5 × 75-mm C18 column (Hema-S 1000, Tessek, Praha, Czech Republic). The crystal growth inhibitor was eluted with a flow rate of 0.5 ml/min and a gradient from 5% to 90% acetic acid. The fractions of 1 ml were dried in a vacuum centrifuge and redissolved in 1 ml

distilled water. The inhibitory activity was measured on 25 µl of sample.

The fractions with the highest inhibitory activity were further separated on a C4 column (2.1 × 150 mm, Vydac, Hesperia, Calif., USA). The gradient was from 5% to 30% acetonitrile in 0.1% trifluoroacetic acid for 50 min at a flow of 0.2 ml/min. The fractions were evaporated in a vacuum centrifuge and reconstituted in 200 µl distilled water. Inhibition assay was performed on 50 µl on every second fraction. Several fractions were analysed on a protein sequencer (475 A, Applied Biosystems, Foster City, Calif., USA). The phenylthiohydantoin derivatives were identified by on-line reversed phase HPLC.

Separate experiments

Fused rocket immunoelectrophoresis

Fused rocket immunoelectrophoresis was performed on the fractions obtained from the hydroxyapatite chromatography against 10% (v/v) rabbit anti-human normal urine antibodies (Dako, Glostrup, Denmark) in the agarose gel [26].

Absorption curve

The absorption of the partially purified crystal growth inhibitor (after negative affinity chromatography) was measured from 220 nm to 350 nm in order to detect distinct absorption peaks for identification purposes. For comparison, we measured absorption of 1 g/l bovine albumin (no. A-4503, Sigma, Mo., USA) and 50 mg/l calf thymus DNA single-stranded (No. D-8889, Sigma).

DNA/RNAase treatment

To exclude the possibility that small amounts of DNA/RNA may be responsible for the crystal growth inhibitory activity, the partially purified inhibitor after negative affinity chromatography (~6 l urine) was reconstituted in 250 µl 50 mM TRIS-HCl, pH 7.5, 1 mM $MgCl_2$, and 0.1 g/l bovine albumin and incubated with 1 µl (25 units) nuclease (Benzonase, cat. no. 1694, E. Merck, Darmstadt, Germany). The mixture was then fractionated on Superose 12 HR 10/30 (Pharmacia LKB, Uppsala, Sweden) with 0.05 M TRIS-HCl, pH 7.5. The flow rate was 0.5 ml/min and the volume of each fraction was 1.0 ml. A sample volume of 250 µl was used to assess the crystal growth inhibition. The control experiment was done without enzyme added. The DNA/RNAase activity of the nuclease was confirmed by replacing the crystal growth inhibitor with 12.5 mg DNA.

Results

Anion exchange chromatography (DEAE-Sephacel) separated the crystal growth inhibitory activity from the major protein peaks (Fig. 2); the uronic acid containing glycosaminoglycans almost co-eluted with the third protein peak (not shown). The crystal growth inhibitor bound to hydroxyapatite and was eluted from 50 to 150 mmol/l K_2HPO_4 (Fig. 3a). The uronic acid with the glycosaminoglycans remaining after anion exchange chromatography did not bind to the matrix and passed through the column together with almost all remaining proteins (Fig. 3a, b) including α_1-trypsin

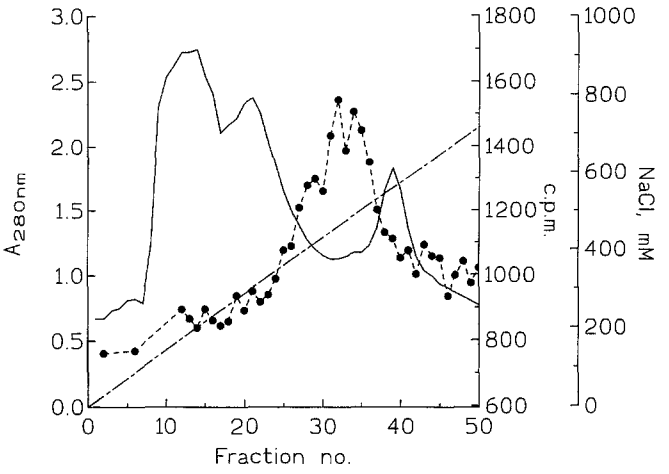
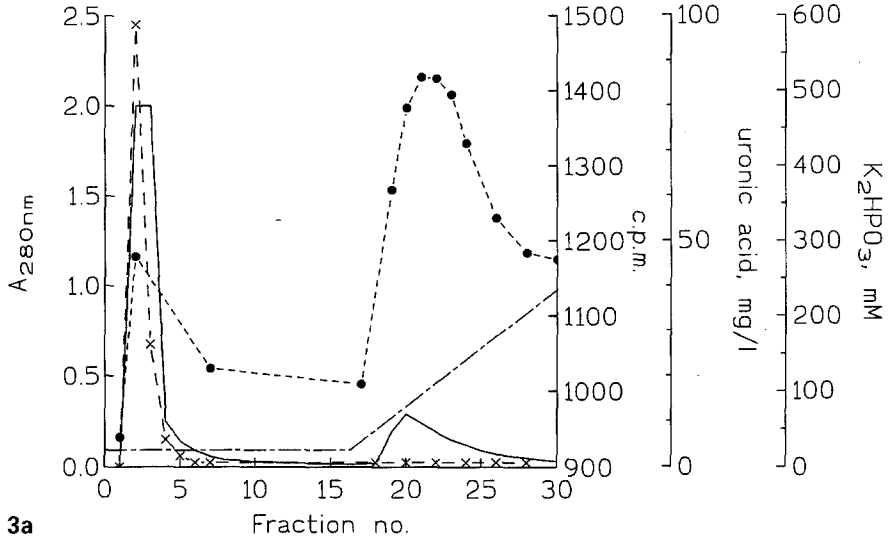


Fig. 2 Anion exchange chromatography (DEAE-Sephacel) of non-TCA-precipitated material. A volume of concentrated urine of 0.5 l was treated with TCA (originally 25 l before ultrafiltration). The supernatant equilibrated against 0.05 M TRIS-HCl 7.5 was loaded on the column and eluted by a linear NaCl gradient. The fraction Nos. 27–37 with the highest crystal growth inhibitory activity were pooled, dialysed against distilled water, and lyophilized for hydroxylapatite chromatography. — $A_{280\,nm}$, ●– –● crystal growth inhibitor activity (c.p.m.), — – NaCl gradient

inhibitor. Further purification was achieved with anion exchange chromatography on Mono Q (Fig. 4). The inhibitor was eluted between 450 and 550 mmol/l NaCl without a distinct absorption peak appearing at 280 nm. The fractions containing the crystal growth inhibitor were further purified by negative affinity chromatography (Fig. 5). Absorbance at 280 nm and the crystal growth inhibitory activity did not coincide. This could be due to a combination of impurities and low absorbance of the inhibitor at 280 nm. The crystal growth inhibitor was eluted in two peaks at 220 nm by reversed phase chromatography (Fig. 6). Fraction No. 13 and fraction Nos. 15–16 were further chromatographed on a narrow bore C4 reversed phase column, giving rise to broad inhibitory peaks (Fig. 7a, b). Several fractions were analysed by sequence analysis. The N-terminal sequence of fraction No. 27 (Fig. 7a) could unequivocally be determined as Phe-Lys-Gln-Glu-Thr-Leu-Pro-Ser-Lys-Ser-Asn-Glu-Ser-His-Asp-His-Met-Xxx-Xxx-Met. About 20% contamination consisting of two to three other sequences was also seen. A search in the PIR data bank revealed the identification of the major sequence as part of osteopontin. During the sequencing of these fractions, a high background to Asp was seen which obscured identification of Asp. Accordingly, the two unidentified residues in the sequence given above corresponded to Asp in the cDNA-deduced osteopontin sequence. The osteopontin sequence also constituted a significant part of the sequences obtained from the fractions analysed of the second peak (Fig. 7b): No. 51 (~50%), No. 57 (~70%), No. 63 (~70%), and No. 67 (~60%). The absolute amount of osteopontin in the four fractions determined



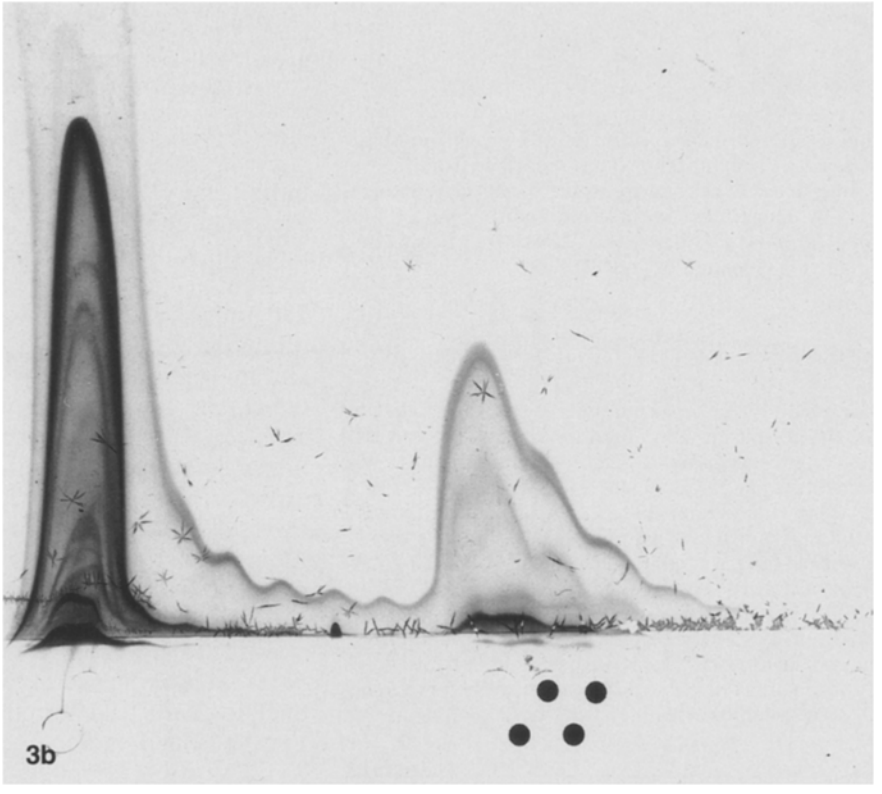


Fig. 3 a Hydroxyapatite chromatography. Enriched fractions of crystal growth inhibitor in anion exchange chromatography were further purified. Lyophilized material was resuspended in 10 mM phosphate buffer and applied on a 10 × 45-mm hydroxyapatite column. A linear phosphate gradient was used to elute the inhibitor. Fractions 19–24 were pooled for anion exchange chromatography (Mono Q). — $A_{280\,nm}$, ●– –● crystal growth inhibitor activity (c.p.m.), × — — × uronic acid, — – phosphate gradient. **b** Fused rocket immunoelectrophoresis. A volume of 10 µl each fraction from the hydroxyapatite chromatography (Fig. 3a) was applied in the wells. The gel contained 10% (v/v) rabbit anti-human normal urine antibodies. No precipitates coincided with the inhibitory peak in Fig. 3a. The fractions containing inhibitory activity are indicated by ●

from the sequencing yields parallelled the crystal growth inhibitor activity. The amino acid sequence of the crystal growth inhibitor is given in Fig. 8, together with that given by other authors.

The absorption spectrum of the partially purified crystal growth inhibitor showed a broad indistinct peak in the range 260–270 nm and no peak at 278 nm (Fig. 9). For comparison, albumin and DNA have absorption maxima at, respectively, 278 nm and 260 nm. By size exclusion chromatography, the partially purified crystal growth inhibitor after negative affinity chromatography appeared as a broad double peak, the first of which did not have absorption at 280 nm (Fig. 10). Treatment of the purified inhibitor with DNA/RNAase had no effect on the elution pattern of the inhibitor (not shown).

Discussion

Urine contains macromolecules that inhibit the aggregation and growth of calcium oxalate crystals, by

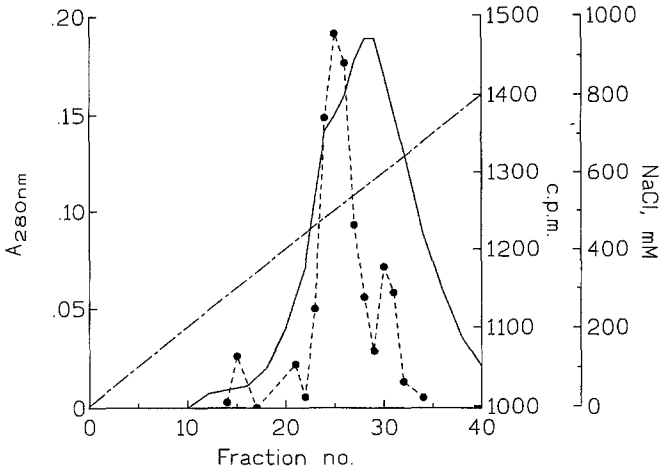


Fig. 4 Anion exchange chromatography (Mono Q). Crystal growth inhibitor containing fractions in Fig. 3a were dialysed against distilled water, freeze dried, resuspended in 0.5 ml 0.05 M TRIS-HCl, and applied on the column. — $A_{280\,nm}$, ●– –● crystal growth inhibitor activity (c.p.m.), — – NaCl gradient

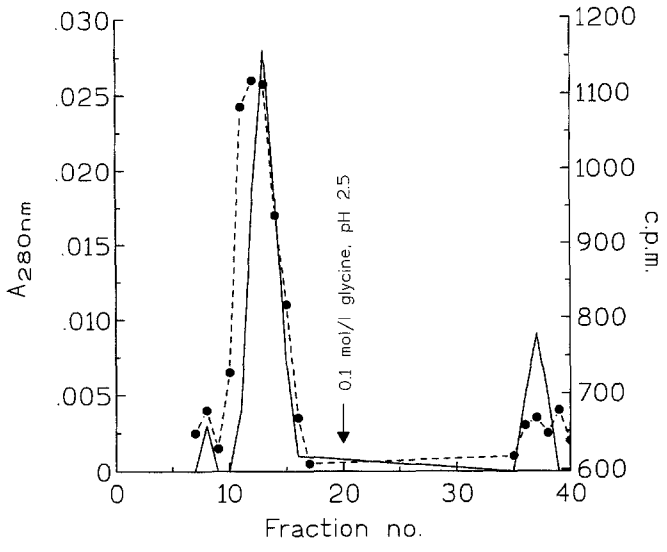


Fig. 5 Negative affinity chromatography. Crystal growth inhibitory containing fraction Nos. 24–27 in Fig. 4 were pooled and loaded on a 16 × 130-mm column of immunoadsorbent coupled with rabbit anti-human normal urine antiserum to remove small amounts of urine proteins. The crystal growth inhibitory activity appeared earlier than the absorbance peak at 280 nm, which indicates either impurities in the preparation or non-absorbance of some of the inhibitor at 280 nm. — $A_{280\,nm}$, ●– –● crystal growth inhibitor activity (c.p.m.)

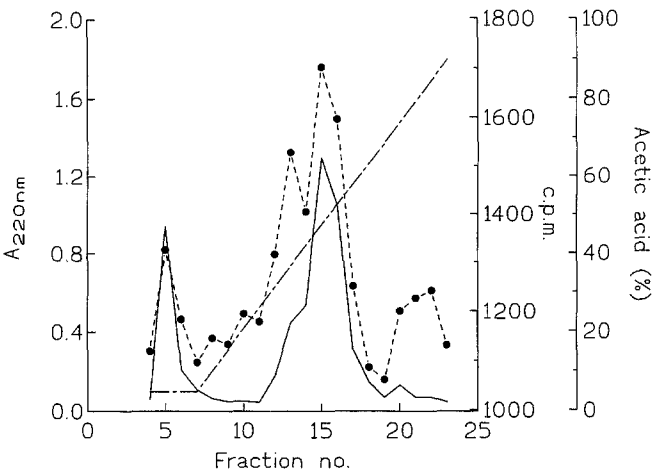
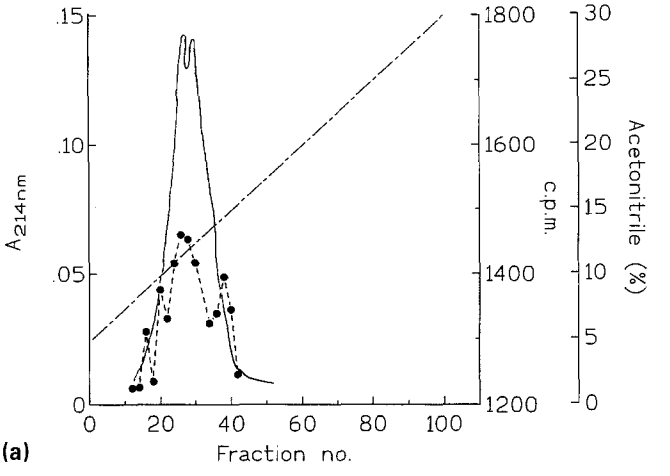


Fig. 6 Reversed phase chromatography (C18). The fraction Nos. 10–15 from negative affinity chromatography were dialysed against distilled water and lyophilized, reconstituted in 5% acetic acid and fractionated on a C18 column. An acetic acid gradient from 5% to 90% was used. Flow rate was 0.5 ml/min and each fraction 1 ml. — $A_{220\,nm}$, ●– –● crystal growth inhibitor activity (c.p.m.), — – acetic acid gradient



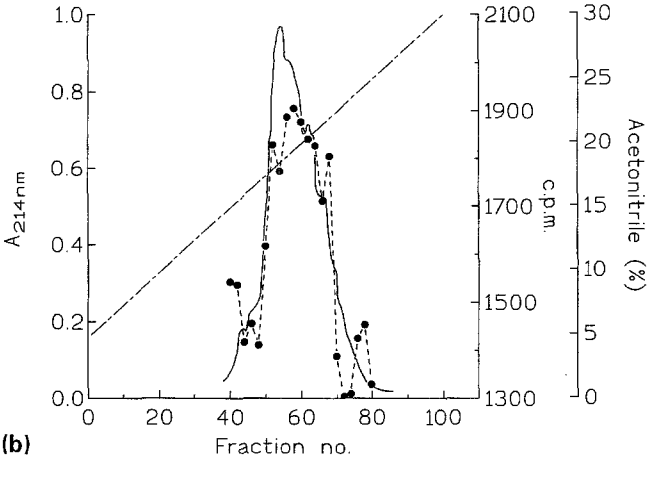
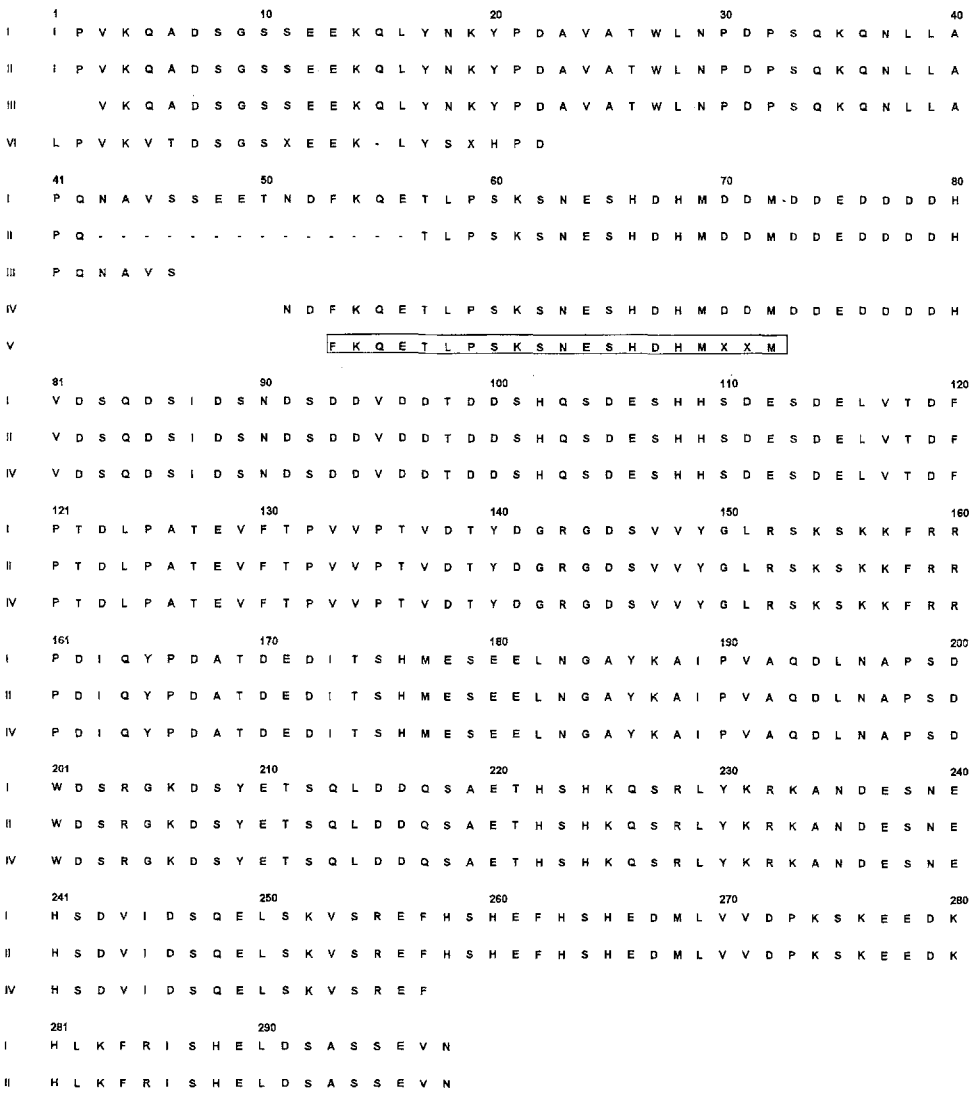


Fig. 7a, b Reversed phase chromatography (C4). Fraction No. 13 and fraction Nos. 15 + 16 in Fig. 6 were dried down, redissolved in distilled water, and injected on a C4 column (**a**, **b**, respectively). The gradient was 5–30% acetonitrile in 0.1% trifluoroacetic acid. The flow rate was 0.2 ml/min and each fraction 200 µl. — $A_{214\,nm}$, ●– –● crystal growth inhibitor activity (c.p.m.), — – acetonitrile gradient

Fig. 8 Comparison of osteopontin sequences and the crystal growth inhibitor. *I* human osteopontin in osteosarcoma deduced from cDNA. A signal peptide containing 16 amino acids has been omitted [12], *II* human osteopontin in bone cells deduced from cDNA [28], *III* human uropontin from urine [24], *IV* urinary stone protein in human kidney cells deduced from cDNA [13], *V* human crystal growth inhibitor from urine (this study), *VI* mouse crystal growth inhibitor from mouse kidney cells [27], *X* no assigned amino acid, - deletion of one amino acid



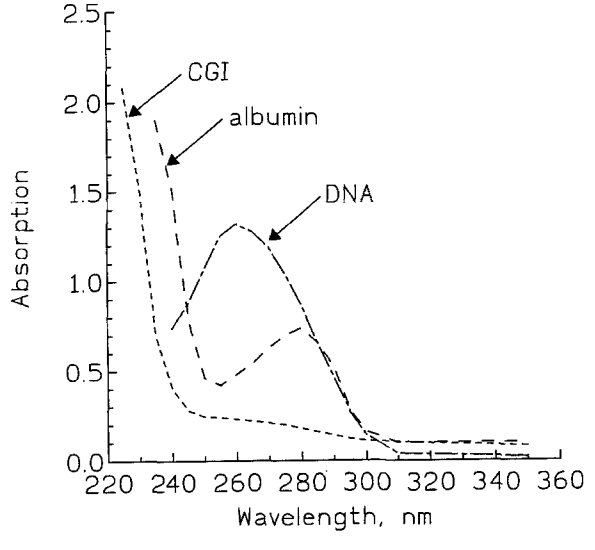


Fig. 9 Absorption spectrum. Partially purified crystal growth inhibitor (after negative affinity chromatography) compared with albumin (1 g/l) and DNA (50 mg/l)

reducing the size of the crystal clusters and the deposition of calcium oxalate material [4]. Crystal growth inhibitors have been purified and (partly) characterized in only a few studies, and are believed to be proteins [1, 19, 24, 26]. The first purification step in this study, TCA precipitation of the majority of urinary proteins including albumin and uromucoid, leaves α_1-trypsin inhibitor and other lower molecular mass proteins in the supernatant. This is consistent with the findings of Lim et al. [16], who also reported that TCA readily precipitated high molecular mass proteins, whereas precipitation of low molecular mass proteins was absent or incomplete. Thus, this step significantly reduced the amount of protein material that had no inhibitory activity on crystal growth. The hydroxyapatite chromatographic step was an excellent affinity chromatographic method for the further removal of those proteins and especially the remaining glycosaminoglycans that do not possess crystal growth inhibitory properties.

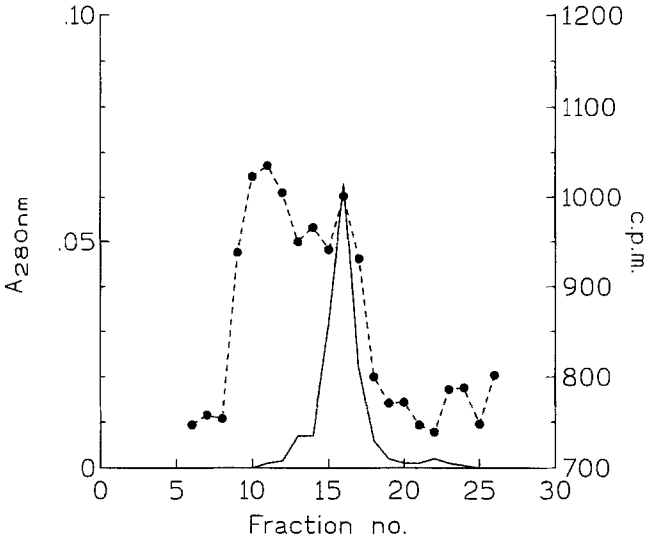


Fig. 10 Size exclusion chromatography (Superose 12 HR). Partially purified crystal growth inhibitor (after negative affinity chromatography) was loaded on a Superose 12 HR column. The flow rate was 0.5 ml/min and each fraction 1.0 ml. The inhibitor appeared as a double peak. The first peak apparently had only minimal absorbance at 280 nm. — A_{280nm}, ●– –● crystal growth inhibitor activity (c.p.m.)

By high-resolution reversed phase chromatography the crystal growth inhibitor eluted as two broad peaks, thus indicating a heterogeneous composition or post-translational modification. The N-terminal sequence determination of the crystal growth inhibitor revealed identity with residues 69–78 of human preosteopontin [12]. Shiraga et al. have also found a fragment of osteopontin (uropontin) that inhibited the calcium oxalate crystal growth [24]; their protein was isolated from urine by monoclonal antibody immuno-affinity chromatography and the N-terminal sequence from residue 1 to residue 44 was identical with residue 3–46 in osteopontin. The shorter fragment described in our study might be caused by acid-catalysed cleavage of the native protein in the first separation step without reduction of the crystal growth inhibitory activity, or it might be that the osteopontin in urine is originally heterogeneous in size as a result of alternatively spliced mRNA, post-translational processing and/or different tissue origin.

Osteopontin is a non-collageneous protein originally isolated from bone matrix [5, 6, 21]. However, this protein is also expressed on many luminal epithelial surfaces, in particular the gall bladder, mammary tissue, urinary tract (distal tubules and collecting ducts), and endometrium in the secretory phase [3]. It has a molecular mass of about 34 kDa as predicted from the cDNA sequence [12], whereas it is determined as a 45-kDa protein on 15% SDS-PAGE and 75 kDa on 5–15% SDS-PAGE [21]. Almost half of its amino acids are serine, glutamic acid/glutamine, and aspartic acid/asparagine residues [5]; the protein is post-translationally sialated [21], threonine and serine phosphorylated [21], and it contains an Arg-Gly-Asp cell-binding sequence [20] (amino acid Nos. 143–145, in Fig. 8), which is responsible for binding to cell surface receptors.

Whereas crystal nucleation in urine is a common event, probably initiated by cellular degradation products, crystal aggregation is generally limited to stone formers [22]. The matrix in the stone centre is highly disorganized, whereas in the peripheral area it is organized in concentric laminations [11]. Whether it plays an active or a passive role in crystal aggregation (and stone formation) is at present unclear. The paradox that osteopontin possesses crystal growth inhibitory activity in vitro and also occurs as a urinary stone protein [13] remains to be explored. Osteopontin mRNA and protein were sporadically present in the renal distal tubular cells of healthy normal rats and were substantially increased in rats with urinary stones [14]. It has been proposed that differences in post-translational processing modulate the physiological activity of osteopontin [25]. Thus, osteopontin was identified in healthy rat kidney cells in two forms: a phosphorylated form and a non-phosphorylated form. The phosphorylated protein interacted with the cell surface and the non-phosphorylated protein formed complexes with soluble fibronectin. Hence, several structurally different forms of osteopontin exist in vivo and may have different physiological functions. Osteopontin probably exerts its inhibitory effect on the growth and aggregation of crystals by interacting with calcium ions on the crystal surfaces through carboxyl and phosphate moieties [2]. Dephosphorylation and chemical modification studies indicate that phosphate groups are quantitatively more important than carboxylate groups [9].

Uronic acid containing glycosaminoglycans [23] and Tamm-Horsfall's glycoprotein (identical with uromucoid) [8] have been proposed as inhibitors of crystal growth and aggregation. However, neither of these macromolecules was found to possess inhibitory potential in fractionated urine in our previous study [26]. Nor do nucleic acid polymers seem to be responsible, as the purified inhibitor was unaffected by nuclease and had no absorption at 254 nm. This conflicts with the study by Ito and Coe [10], who found that RNA-like substances possessed inhibitory properties. The partially purified crystal growth inhibitor did not have a distinct absorption at 280 nm, in keeping with a low number of 8 (2.7%) of tyrosine and 2 (0.7%) of tryptophan out of 298 amino acids in intact osteopontin; therefore, the inhibitor is probably better monitored at, for example, 214 or 220 nm.

In summary, this report describes the isolation of a macromolecular calcium oxalate crystal growth inhibitor in human urine by classical protein-chemical techniques, and its identification as osteopontin or as

a large fragment of it. Whether this protein is implicated in the inhibition of stone formation in vivo remains to be elucidated.

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