Effects of inter-α-inhibitor and several of its derivatives on calcium oxalate crystallization in vitro

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ABSTRACT

The bikunin peptide chain of the protease inhibitor inter-α-inhibitor (IαI) has been reported to be an inhibitor of calcium oxalate (CaOx) crystallization, and hence has been proposed as having a role in CaOx kidney stone formation. However, further experimental evidence is required to assess if fragments of IαI other than bikunin may play a role in the regulation of crystallization events in stone formation. The aim of the present study was to assess the effects of IαI and several of its derivatives on CaOx crystallization in a seeded inorganic system and to compare these effects with those of a known inhibitor of crystallization, prothrombin. IαI was purified from a preparation of human plasma and fragmented by alkaline hydrolysis, and two of its peptide chains, bikunin and heavy chain 1 (H1), were purified further by HPLC. Their purity was confirmed by SDS/PAGE. Using Coulter counter and [14C]oxalate analysis and scanning electron microscopy, IαI, its H1 chain and bikunin from urine and from plasma were shown to be relatively weak inhibitors of CaOx crystallization in vitro at expected physiological concentrations. It was concluded that members of the IαI family may not be as important in kidney stone formation as has been generally proposed, although further studies are required before a possible role for IαI and its fragments in stone formation can be unambiguously discounted.

INTRODUCTION

Inter-α-inhibitor (IαI) is a complex molecule with a remarkable structure, in that three distinct protein chains with separate genetic origins form the mature protein. The IαI native/intact molecule has two heavy chains, H1 and H2, with respective molecular masses in the ranges 65–101 and 70–106 kDa respectively. These are linked covalently via a chondroitin sulphate moiety to a glycosylated light chain known as bikunin, which has a molecular mass of approx. 30 kDa [1]. The unusual covalent chondroitin sulphate inter-chain link between these three peptides has been termed a protein–glycosaminoglycan–protein cross-link [2].

Even more remarkable is the fact that the physiological role of IαI remains unclear. The portion of the protein responsible for the protease-inhibitory activity is located at the N-terminus of the parent molecule and is assumed to be the bikunin fragment [3]. Although IαI has been reported to inhibit trypsin [4], more recently it has been reported ([5]; cited in [6]) that IαI in human serum accounts for less than 5% of the total trypsin-inhibitory capacity. Despite the demonstration that IαI is a protease inhibitor, the direct action of the protein upon proteases...
**in vivo** has yet to be unequivocally demonstrated. Weak interactions observed between IαI and several human proteases **in vitro** were suggested by Potempa et al. [7] to be irrelevant under physiological conditions. Despite the lack of evidence that IαI is a strong protease inhibitor **in vivo**, the molecule may have a role in stabilizing extracellular matrix after ovulation is stimulated [8]. In fact, the heavy chains of IαI have only recently been ascribed a possible function in extracellular-matrix stabilization and integrity [9].

IαI and its derivatives have been linked to various pathological states, such as Alzheimer’s disease [10–12] and severe inflammatory processes such as septic shock [13,14], adult respiratory distress syndrome [15] and cancer [16–18]. Bikunin has been used therapeutically in preliminary studies to reduce tissue damage associated with surgical complications such as shock [19] and hyperamylasaemia [20].

Urolithiasis is another disease that has been linked with IαI. Results of several studies have suggested that IαI derivatives may be associated with urinary stone formation by virtue of their ability to inhibit the deposition of calcium oxalate (CaOx) from inorganic solutions [21–25]. In addition, a protein resembling bikunin [26] was extracted from the urine of stone formers and shown to be a weaker inhibitor of crystallization than that from normal individuals [24].

Bikunin has long been regarded as the only portion of IαI that is present in urine and hence has been the sole derivative tested for its effects upon CaOx crystallization. However, the detection of H1 and H2 in normal human urine, calcium stones and CaOx crystals [27,28], and the presence of intact IαI in several normal urines [29], suggest that bikunin might not be the only IαI derivative with a possible involvement in stone formation.

The aim of the present work was to test independently the effects of several IαI derivatives on CaOx crystal growth and aggregation in a seeded crystallization system **in vitro**. The derivatives tested that were isolated from human plasma were bikunin (bikunin-p), H1, intact IαI and prothrombin; we also tested bikunin isolated from urine (bikunin-u). Prothrombin was included as a control in the crystallization experiments because its inhibitory effects on CaOx crystallization are well documented in our laboratory [30,31].

**METHODS**

**Chemicals**

All reagents used were of analytical grade and all solutions were prepared with high-purity water (Permutit Australia, Brookvale, NSW, Australia). Unless stated otherwise, biochemicals were supplied by Sigma Chemical Co. (St. Louis, MO, U.S.A.). Other reagents were obtained from the following sources: acrylamide, N,N’-methylene-bis-acrylamide, β-mercaptoethanol, N,N,N’,N’-tetramethylthelenediamine (Bio-Rad Laboratories, Richmond, CA, U.S.A.); CaCl₂, glycerol, hydrogen peroxide (BDH Chemicals Australia, Kilsyth, Victoria, Australia); CaOx, glycine, sodium acetate, sodium thiosulphate, methanol, KH₂PO₄ (Ajax Chemicals, Auburn, NSW, Australia); Bromophenol Blue, glutaraldehyde (Merck, Darmstadt, Germany), K₂HPO₄ (Fisons Scientific Equipment, Loughborough, U.K.); [¹⁴C]oxalate (NEN* Products; Dupont, Wilmington, MA, U.S.A.); Ready Safe scintillation fluid (Beckman Instruments Inc.).

**Purification of IαI, bikunin and H1 from Prothrombinex*-HT by HPLC**

IαI was purified from Prothrombinex*-HT, a concentrate of human blood coagulation factors manufactured from human plasma by CSL Ltd. (Victoria, Australia), by gel permeation on a Superose 6 (6 cm x 90 cm) column (Pharmacia, Uppsala, Sweden) with a citric acid/phosphate buffer (pH 7.4) as described elsewhere [27]. All protein fractions were monitored at 280 nm and were analysed by SDS/PAGE and Western blotting (see below), and those fractions containing IαI were subjected to alkaline hydrolysis as detailed in [27], which dissociated IαI into various fragments.

Using the method of Malki et al. [32], dissociated IαI was dialysed overnight against 0.02 mol/l Tris/ HCl, pH 7.6, and loaded on to an ion-exchange Mono Q HR 5/5 column (Pharmacia). The Mono Q HR 5/5 column was equilibrated with 0.02 mol/l Tris/HCl, pH 7.6, at a flow rate of 1 ml/min and proteins were eluted using a linear gradient of NaCl (0–1 mol/l). All fractions were analysed by SDS/PAGE and Western blotting.

Material in one fraction containing two protein bands with molecular masses of approx. 77 and 25 kDa was purified further using gel permeation on a Waters Protein Pak 125 column (7.8 mm x 300 mm; Waters Corp., Milford, MA, U.S.A.) in the same citric acid/phosphate buffer (pH 7.4) as used above. Two proteins collected from the Waters Protein Pak 125 column were also analysed by SDS/PAGE and Western blotting before assessing their effects on CaOx crystallization **in vitro**. These two proteins were also sent for N-terminus sequencing at the Peptide Biology Laboratory, Baker Medical Research Institute, Melbourne, Victoria, Australia.

**SDS/PAGE and Western blotting of IαI and derivatives**

SDS/PAGE and Western blotting were carried out as previously described [27], except that the percentage of acrylamide varied according to the range of molecular masses of proteins to be analysed. Detection of IαI and
related fragments on blots was performed using a rabbit anti-(human IxI) (RazI) antibody (1:100 dilution; code A301; Dako Corp., Carpinteria, CA, U.S.A.) followed by the addition of horseradish peroxidase (HRP)-conjugated goat anti-(rabbit IgG) (GzR–HRP; catalogue no. 170–6515; Bio–Rad) at a dilution of 1:2000. In order to confirm that the GzR–HRP did not bind non-specifically to the blot, GzR–HRP was added to a blotted alkaline-hydrolysed IxI in the absence of the RazI antibody and stained using diaminobenzidine as usual. GzR–HRP did not bind non-specifically to the blot (results not shown). Rabbit anti-bikunin antibody was a gift from Dr. J. J. Enghild (Pathology Department, Duke University Medical Center, Durham, NC, U.S.A.) and was detected using a 1:2000 dilution of GzR–HRP.

Preparation of proteins for inhibitory assays
IxI, bikunin-p and H1, isolated as described above from Prothrombinex*-HT, were dialysed separately against phosphate buffer (pH 7.3) containing 0.145 mol/l NaCl, 6.26 mmol/l K2HPO4·3H2O and 1.84 mmol/l KH2PO4 at 4 °C for approx. 24 h. Phosphate buffer was used as it was found not to affect the crystallization of CaOx in the 1 % seeded system (results not shown).

Prothrombin, which was purified from Prothrombinex*-HT as described by Grover et al. [33], was also dialysed in the phosphate buffer (pH 7.3). Prothrombin was used as a control in the experiments rather than urinary prothrombin fragment 1, which is a more powerful inhibitor, because supplies of the former were more readily available in our laboratory.

Bikunin-u was isolated by Mr Boda Zhang (Biochemical Factory of Nanjing University, China) and kindly provided by Dr D. Ollis (Research School of Chemistry, Australian National University, Canberra, Australia). Lyophilized bikunin-u was dissolved in distilled water. It was not known at the time whether the bikunin-u supplied was in a buffer, but because of the small volume of the sample ( < 100 μl) it was decided not to dialyse the protein against the phosphate buffer. In any event, the volume of bikunin-u sample added to the incubation solution in the inhibition assay comprised only 0.015 % of the total volume of solution. Hence it was assumed that any direct influence of an unidentified buffer upon CaOx crystallization would be negligible.

Protein concentrations in all protein samples were determined using the Bio–Rad Laboratories protein assay (catalogue no. 500-006).

Determination of inhibitory activity
The effects of bikunin-p, bikunin-u, H1, IxI and prothrombin on CaOx crystallization were determined using the method of Ryall et al. [34], as modified by Grover and Ryall [31]. CaOx monohydrate seed crystals were prepared by grinding with a mortar and pestle, suspended in distilled water at a concentration of 1 g/l, and stirred by a Teflon-coated magnetic bar at room temperature for at least 2 weeks to equilibrate. An incubation solution containing 1 mmol/l CaCl2, 0.15 mol/l NaCl and 10 mmol/l Mes at pH 6.0 was prepared and filtered (0.22 μm pore size).

Bikunin-p (molecular mass 25 kDa), bikunin-u (36 kDa), H1 (77 kDa), IxI (230 kDa) and prothrombin (72.5 kDa) were added at a final concentration of 16 nmol/l to separate flasks containing the solution just described; the samples were incubated in a shaking water bath at 37 °C for the duration of the experiment. Stock CaOx seeds were added to achieve a final concentration of 0.02 mg/ml. The volume–size distributions of the seed crystals at zero time were determined using a Coulter counter (model TAI1; Coulter Electronics) fitted with a Population Count Accessory and a 70 μm orifice. CaOx crystallization was induced by dropwise addition of sodium oxalate (10 mmol/l) to achieve a final oxalate concentration of 0.2 mmol/l. Readings were taken using the Coulter counter at 30 min intervals for a total of 120 min. Amounts of bikunin-p, bikunin-u, H1 and prothrombin were very limited; hence the effects of each protein are reported as the means of duplicates. A Bonferroni statistical test using SPSS 7.5 for Windows was used to compare each pair of means for crystal size at 120 min for each of the proteins.

Scanning electron microscopy
At the end of the crystallization experiment, 2 ml aliquots of each sample were filtered (0.2 μm pore size; #GSWP 01300; Millipore, Bedford, MA, U.S.A.) and the filtration membranes were dried overnight at 37 °C. The filters were then mounted on aluminium stubs and sputtered with gold for 180 s (Autocoating Unit E5200; Polaron Equipment Ltd, Watford, U.K.). Representative fields were photographed on stubs examined by an ETEC Auto Scan Electron Microscope (Siemens AG, Karlsruhe, Germany) at an operating voltage of 20 kV.

Measurement of CaOx deposition by [14C]oxalate analysis
Incubations similar to those described above using the Coulter counter were carried out in parallel, except that [14C]oxalate was added to each flask at a final concentration of 0.1333 μCi/ml. Before the induction of CaOx crystallization, 1 ml aliquots of each sample were filtered into 100 μl of conc. HCl using disposable syringes fitted with 0.22 μm filters (Minisart®; Sartorius AG, Göttingen, Germany) to provide zero-time values. After addition of the sodium oxalate, further 1 ml aliquots were removed every 30 min for 120 min. Duplicate 0.3 ml aliquots of these solutions were then added to 10 ml of
Ready Safe scintillation fluid. The samples were then counted for radioactivity for 5 min in a liquid scintillation counter (Beckman LS 3801 Liquid Scintillation System). Duplicate samples containing bikunin-p, bikunin-u and IαI were used. However, the amounts of H1 and prothrombin were not sufficient to be used in duplicate, and only one estimate was therefore obtained for these proteins. Where possible, results are expressed as mean values. Inhibition of crystallization was calculated as the percentage of $[^{14}C]$oxalate remaining in solution at each time point compared with the amount of $[^{14}C]$oxalate that was in solution before the initiation of crystallization (zero time). One-way analyses of variance were carried out to compare differences between proteins and between duplicates using SPSS 7.5 for Windows.

RESULTS

Purification and analysis of IαI derivatives from Prothrombinex®-HT by HPLC

IαI and its derivatives were purified from the blood concentrate Prothrombinex®-HT. The predominant component of IαI (Figure 1, lane 4) ran with a molecular mass of approx. 230 kDa. Minor contaminants, which are very likely to be various combinations of bikunin and the heavy chains [35] with approximate molecular masses of 60–230 kDa, were also present in this preparation, along with a band at 16 kDa.

IαI was then dissociated by alkaline hydrolysis and fractionated by ion-exchange chromatography followed by gel-permeation chromatography. One protein isolated (Figure 1, lane 1) was heavily overloaded on the SDS/PAGE gel in order to visualize the single band with a molecular mass of 25 kDa. This protein was immunoreactive with both the RαI antibody and the more specific rabbit anti-bikunin antibody (results not shown), confirming that the 25 kDa protein was bikunin. Furthermore, sequence analysis of the 25 kDa protein yielded the sequence Ala-Val-Leu-Pro-Gln-Glu, which corresponds to the bikunin portion of IαI [36,37], and this protein was thus designated bikunin-p.

The other protein isolated from the alkaline-hydrolysed IαI ran as a single band with a molecular mass of 77 kDa (Figure 1, lane 3). This 77 kDa protein was immunoreactive with the RαI antibody (results not shown) and is presumably H1, as it appears to be the smaller of the two heavy chains. Sequencing data for this protein were not obtained because of N-terminal blocking. Purification of the H2 protein was unsuccessful.

Bikunin-u (Figure 1, lane 2) was not seen as a discrete band, but rather as a smear with a molecular mass ranging from approx. 21 to 43 kDa. Some minor contaminants were visible in the prothrombin preparation (Figure 1, lane 5), particularly the F1 + 2 fragment of prothrombin with a molecular mass of approx. 48 kDa, but the main band was prothrombin at 72 kDa. Where possible, the protein samples were overloaded on the gel to ensure visualization of the complete sample.

Crystallization inhibition experiments were therefore performed using IαI, H1, bikunin-p, bikunin-u and prothrombin.

Effects of bikunin-p, bikunin-u, H1, IαI and prothrombin on crystal size

The crystal sizes at the end of the 2 h incubation period in the control incubation (containing no added protein) and in the presence of bikunin-u, H1, IαI and prothrombin are depicted in Figure 2. Size is expressed as the mode of the crystal size–volume distribution curve. The data for
bikunin-p are presented separately in Figure 3, as the inhibitory activity of this protein was measured in a separate experiment.

The overall particle size was only slightly decreased in the presence of bikunin-u and H1, with the mode of the crystal volume distribution curve reduced to 11.5 μm in the presence of either protein, compared with 12.7 μm in the control. In contrast, the effects of IαI and prothrombin were more pronounced, reducing the modal particle size to 7.3 and 8 μm respectively. Bonferroni statistical analysis of these data revealed three homogeneous subsets of treatments: control, bikunin-u and H1, and IαI and prothrombin were revealed to be significantly different. Bikunin-p had no discernible effect on crystal size, with the mode of the volume distribution curve being identical to that of the control (Figure 3). These data were not tested statistically, as the lack of any difference was self evident.

These results were confirmed by scanning electron microscopy (Figure 4). In the absence of proteins, the CaOx monohydrate seed crystals were highly aggregated, with large clumps of crystals being observed in the control sample. Crystals derived from samples containing bikunin-p, bikunin-u and H1 were also clustered and of similar size to those observed in the control. However, in the presence of IαI or prothrombin the crystals tended to be clustered into smaller, loose clumps, indicating that these two proteins had markedly inhibited crystal aggregation. It is also noteworthy that individual crystals

![Figure 3](image1.png)

**Figure 3** Effect of bikunin-p on crystal size

The particle size distribution of CaOx crystals is shown, after the 2 h incubation period, in the control (no added protein) and in the presence of bikunin-p at a final concentration of 16 nmol/l.

![Figure 4](image2.png)

**Figure 4** Scanning electron micrographs at low power of CaOx crystalline material deposited in samples after a 2 h incubation

Crystal aggregates were photographed in the control sample (no protein) and in solutions containing bikunin-p, bikunin-u, H1, IαI or prothrombin (PT). The bar represents 10 μm.
The amount of $[^{14}\text{C}]$oxalate remaining in solution over a 2 h incubation period in a metastable solution of CaOx is shown for the control (no added protein) and in the presence of bikunin-u, H1, IzaI and prothrombin (PT), each at a final concentration of 16 nmol/l.

Figure 5: Effects of bikunin-u, H1, IzaI and prothrombin on CaOx deposition

The amount of $[^{14}\text{C}]$oxalate remaining in solution over a 2 h incubation period in a metastable solution of CaOx is shown for the control (no added protein) and in the presence of bikunin-u, H1, IzaI and prothrombin (PT), each at a final concentration of 16 nmol/l.

Figure 6: Effect of bikunin-p on CaOx deposition

The amount of $[^{14}\text{C}]$oxalate remaining in solution over a 2 h incubation period in a metastable solution of CaOx is shown for the control (no added protein) and in the presence of bikunin-p at a final concentration of 16 nmol/l.

Measurement of CaOx deposition by $[^{14}\text{C}]$oxalate disappearance

The rates of disappearance of $[^{14}\text{C}]$oxalate from solution during the 2 h incubation period in the control sample, and in the presence of bikunin-u, H1, IzaI and prothrombin, are shown in Figure 5, expressed as the percentage of $[^{14}\text{C}]$oxalate remaining in solution. Once again, the data for bikunin-p are presented on a separate graph (Figure 6). To normalize the data, the percentage inhibition of crystallization produced by each protein was calculated as the percentage of $[^{14}\text{C}]$oxalate remaining in solution at each time point in relation to that at zero time. A value less than that in the control indicates promotion of $[^{14}\text{C}]$oxalate deposition; a value identical to the control indicates no inhibition, and a higher value reflects inhibition of deposition.

It can be seen from Figure 5 that all the proteins tested inhibited $[^{14}\text{C}]$oxalate deposition. However, Figure 6 shows that the rate of disappearance of $[^{14}\text{C}]$oxalate in the presence of bikunin-p was similar to that of the control sample.

At the end of the experiment, the amount of $[^{14}\text{C}]$oxalate detected in the control sample was 39.5% less than that at zero time (Figure 6), while those in the presence of bikunin-u, H1, IzaI and prothrombin were 41.5, 43.5, 48 and 58% respectively. In the separate experiment testing the effect of bikunin-p (Figure 6), the mean percentage decrease in $[^{14}\text{C}]$oxalate was only marginally greater (41.4%) than in the control (40.7%), and consequently no statistical analysis was performed. Thus, in relation to the control sample, bikunin-p, bikunin-u, H1, IzaI and prothrombin inhibited $[^{14}\text{C}]$oxalate deposition by 1.1, 3.1, 9.9, 22.5 and 31.4% respectively. One-way analyses of variance demonstrated that all pairs of means for each protein differed significantly at each time point ($P < 0.05$). It is noteworthy that, although IzaI inhibited aggregation more than prothrombin, their relative effects on deposition were reversed.

**DISCUSSION**

It has been shown recently [27,28] that the heavy chains H1 and H2 of IzaI are present in calcium stones, human urine and CaOx crystals precipitated from urine, suggesting that bikunin may not be the only IzaI derivative with a potential role in stone formation. The aim of the present study was to measure the inhibitory effects of IzaI and its derivatives on CaOx crystallization, which necessitated purification of each protein. In addition to bikunin-u, proteins IzaI, H1 and bikunin-p were isolated from a plasma concentrate, as was the unrelated prothrombin, which was included to provide a control, since it is known to significantly inhibit CaOx crystallization in vitro [30,31]. Purified H1 and bikunin-p were shown to be single bands on silver-stained SDS/PAGE gels. However, IzaI was not as pure, as it contained several other bands, which probably reflects the complex nature of the molecule and its inherent instability. Unfortunately, a pure sample of the H2 derivative of IzaI was not obtained by the isolation procedures used.

It is noteworthy that the two bikunin preparations from urine and plasma exhibited different staining patterns and molecular masses. On SDS/PAGE, bikunin-p ran as a single sharp band at 25 kDa, whereas remaining in solution at each time point in relation to that at zero time. A value less than that in the control indicates promotion of $[^{14}\text{C}]$oxalate deposition; a value identical to the control indicates no inhibition, and a higher value reflects inhibition of deposition.

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Prothrombin inhibited $[^{14}]$C]oxalate disappearance more potently (31%) than did IxI (22%), which was in contrast with their relative effects on overall crystal size. The shift to higher diameters in the crystal volume distribution curve which occurred in the presence of prothrombin, relative to IxI, may possibly have resulted from occlusion of prothrombin into the crystal structure. This phenomenon has been proposed to explain increases in crystal volume in the absence of any accompanying enhancement of $[^{14}]$C]oxalate deposition [45]. However, this does not appear likely from the scanning electron micrographs, as individual crystals actually appeared smaller in the presence of prothrombin, and the aggregates larger, than those with IxI, confirming that prothrombin is a more powerful inhibitor of CaOx deposition than IxI, but a weaker inhibitor of aggregation. Although these observations are subjective, they support both the Coulter counter and the $[^{14}]$C]oxalate data. IxI may in fact reduce aggregation by binding to the crystal surface, or by simply blocking crystal–crystal interactions in suspension. IxI is a large protein and its activity may be similar to that of Tamm–Horsfall glycoprotein, which does not bind irreversibly to the CaOx crystal surface and therefore has a negligible effect on growth. It is, however, an efficient inhibitor of aggregation, an effect it appears to achieve by stereospecific hindrance [46].

The effects on CaOx crystallization of bikunin itself, or several of its urinary forms, have been tested using different methods [21–23,25,47]. Nephrocalcin, which may be part of bikunin [25], inhibited mineral deposition by approx. 19% at a concentration of 240 nmol/l [48]. Atmani et al. [22,24] showed that uronic-acid-rich protein, which is now recognized as a form of bikunin [41], inhibited CaOx mineral deposition by 72% at a concentration of 286 nmol/l [22], while 60% inhibition of crystallization was reported at a concentration of 10000 nmol/l [47]. The concentrations of protein used in those studies were far greater than those used here, where bikunin-u and bikunin-p inhibited CaOx deposition by only 1.1 and 3.1% respectively at the same final concentration of 16 nmol/l. Although the marked differences in inhibitory potencies of the bikunins between the published studies may reflect variations in the different crystallization systems used, it is important to consider these differences in relation to the reported physiological urinary concentration of the protein.

There are several published estimates of the concentration of bikunin in human urine: 500 nmol/l [47]; 10.13 ± 1.13 µg/ml (280.5 nmol/l) and 6.72 ± 0.93 µg/ml (186 nmol/l) in normal men and women respectively [49]; 5.01 ± 0.91 µg/ml (208 nmol/l) [50]; and 4.82 ± 2.46 mg/day (138 nmol/l) and 3.86 ± 1.35 mg/day (110 nmol/l) in normal men and women respectively [51]. The lowest estimate of urinary bikunin to date is 0.25 ± 0.11 µg/mg of creatinine [52]. By using a reference level of 8–23 mmol/day for creatinine excretion [53] and
assuming that a person excretes approx. 1 litre of urine per day, the approximate average daily excretion of bikunin can be calculated to be between 0.225 μg/ml (6.42 nmol/l) and 0.650 μg/ml (18.6 nmol/l).

The value for urinary bikunin concentration of 500 nmol/l reported by Kobayashi et al. [47] is almost certainly an overestimate, since it translates to an approximate equivalent of 17.5 mg/day, which is an unlikely amount considering that human serum albumin, the second-most abundant urinary protein, is excreted at a concentration of less than 20 mg/l [53]. Furthermore, our SDS/PAGE data reproducibly demonstrate that bikunin is a relatively minor component of urine, whereas human serum albumin is a major protein band. In the absence of any methodology or data, doubt must be cast on the accuracy of the value cited by Kobayashi et al. [47].

All studies measuring urinary bikunin [49–52] used various forms of immunoassays with antibodies which were presumed to cross-react, were demonstrated to do so, or are known to cross-react, with IgX derivatives containing bikunin. None were specific to bikunin. Therefore values obtained are probably overestimates of the urinary bikunin concentration. Furthermore, these values by no means constitute definitive normal ranges, since only two studies [50,52] related bikunin excretion to urinary output. Trefz et al. [52] used a monoclonal antibody generated against bikunin, as opposed to a polyclonal commercial antibody generated against the entire IgX molecule [50]. Presumably the antibody used by Trefz et al. [52] has a higher affinity for bikunin. We conclude, therefore, that the true physiological range for bikunin is likely to be in the region of 0.225–0.650 μg/ml (6.42–18.6 nmol/l).

Thus, at approximate urinary physiological concentrations, the urinary and plasma forms of bikunin do not greatly influence CaOx crystallization in vitro, despite the fact that the inorganic crystallization system used is generally acknowledged to be highly sensitive. Furthermore, it is widely recognized that urine is typically diluted 100-fold in order to construct a dose–response curve to produce measurable effects on CaOx deposition and aggregation in inorganic, seeded reaction systems. So even if the physiological concentration of bikunin in urine were of the order of 208 nmol/l [50], a bikunin concentration of 2.08 nmol/l would be appropriate for use in inorganic systems, compared with those of 1.25–10 μg/ml (35–280 nmol/l) [22,24] and 10000 nmol/l [47] used by others. Thus even the concentrations (16 nmol/l) of bikunin-u and bikunin-p used in the present investigation (0.56 and 0.403 μg/ml, based on molecular masses of 25 and 35 kDa respectively) exceed the 100-fold dilution of the biologically relevant concentration range of bikunin. Given the high sensitivity of the inorganic crystallization system, it is highly probable, therefore, that biologically relevant concentrations of bikunin would have a negligible effect, or none at all, on CaOx crystallization in undiluted urine. These results therefore suggest that bikunin may not be as influential in stone formation as has been generally assumed.

The same is also probably true for H1. H1 inhibited CaOx crystallization slightly, although Kobayashi et al. [47], using much higher protein concentrations (0–100 000 nmol/l), reported that the heavy chains of IgX had no effect. Unfortunately, purification of H2 was unsuccessful using the procedures described here. Future experiments investigating its effect on CaOx crystallization might prove worthwhile, as this peptide chain reportedly contains two γ-carboxyglutamic acid residues [54], which should enhance its calcium-binding properties. In fact, both heavy chains of IgX contain calcium-binding sites (Asp-Asn-Asn-Asp) [55], which should have facilitated their binding to the CaOx seed crystals used in the present study. It is difficult to assess, therefore, what contribution, if any, the heavy chains of IgX make to the inhibitory effect of urine on CaOx crystallization. The presence of the heavy chains of IgX in human urine has only recently been reported [28], and at the present time there are no published reference ranges for the urinary concentrations of either H1 or H2. In any event, the heavy chains are almost certainly only minor protein constituents of urine, as they are not as obvious as, say, human serum albumin or Tamm–Horsfall glycoprotein on SDS/PAGE gels or Western blots. Moreover, prothrombin was 3–4 times more inhibitory than H1, and its activation fragment, F1, is an even stronger inhibitor of crystal growth and aggregation in the physiological concentration range [30]. Because the concentration of H1 used in the present study is almost certainly in the physiological range, or perhaps even higher, and its effects in a sensitive crystallization system were minimal, it would appear that H1 is unlikely to play a significant role in crystal or stone formation in the urinary tract.

Inorganic seeded crystallization systems like those used here provide valuable information about the individual inhibitory effects of urinary proteins on CaOx crystallization, as they permit evaluation of a protein’s effects on crystallization without interference by other substances in urine, thus enabling direct comparisons of relative potencies. Nonetheless, these results cannot be extrapolated directly to predict effects expected in urine in vivo. For this reason, it would have been advantageous to assess the relative inhibitory activities of the bikunins, H1 and prothrombin in undiluted urine. However, the use of undiluted urine would have required quantities of each protein far greater than those required for the inorganic system, and supplies of the proteins were very limited.

Nonetheless, in view of the low activity exhibited by bikunin and H1, it is doubtful whether any useful information would be gained by testing their effects in undiluted urine, since their effects in that medium would...
be expected to be even less. They are therefore unlikely to play a direct role in crystallization processes in stone pathogenesis. Nonetheless, Izl and its derivatives may indirectly influence stone formation by preventing the degradation of other proteins, such as urinary prothrombin fragment 1. Another possible, although also indirect, function for the Izl family of proteins could be in the regulation of calcium handling and excretion in the kidney. Kanayama et al. [56] hypothesized that bikunin may be involved in the regulation of intracellular calcium, as it reduced the influx of calcium ions into neutrophils and into smooth muscle cells [57]. Further evidence to support the notion that a physiological function of the Izl family of proteins may be to control calcium levels is that calcidulin, a protein isolated from snake venom and structurally homologous to members of the superfamily of Kunitz-type protease inhibitors, is a potent blocker of calcium channels [58]. Recently [29], increased amounts of intact Izl were detected in the urine of stone-forming individuals when compared with normal individuals. However, these data were only semi-quantitative, and it is reasonable to postulate that raised levels in stone formers could simply be the result of an inflammatory response arising from stone formation itself.

In summary, therefore, the available evidence does not support a significant inhibitory role for Izl or its derivatives in CaOx stone pathogenesis. A definitive function for these proteins must therefore remain in doubt until such time as they are shown to inhibit CaOx crystal formation, growth or aggregation in undiluted human urine at physiologically relevant concentrations.

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