Urol Res (1999) 27:69–75

ORIGINAL PAPER

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Inhibitory effect of bikunin on calcium oxalate crystallization in vitro and urinary bikunin decrease in renal stone formers

Received: 16 December 1997 / Accepted: 23 June 1998

Abstract Two proteins of 17 and 24 kDa, respectively, which were immunologically related to bikunin, were purified from urine of healthy men, using in the last step a trypsin CNBr-sepharose affinity column. These proteins strongly inhibited calcium oxalate (CaOx) crystallization in two in vitro models. In the first model, the presence of 8 μg/ml protein in a medium containing 0.76 mM $CaCl_2$ (with ^{45}Ca) and 0.76 mM ammonium oxalate inhibited the crystallization process by 80%, as estimated by supernatant radioactivity after 60 min of incubation. A similar inhibition was observed in the second turbidimetric model, where the CaOx crystallization kinetics were followed for 10 min at 620 nm in a medium containing 4 mM $CaCl_2$ and 0.5 mM Na_2Ox. These proteins were used as standard protein for the development of an enzyme-linked immunosorbent assay (ELISA) in urine. Mean (± SEM) urinary bikunin concentration in 18 healthy subjects was 5.01 ± 0.91 μg/ml. This was a concentration range of strong inhibitory activity in vitro. Bikunin values were nearly 50% lower (2.54 ± 0.42 μg/ml, $P = 0.007$) in 31 CaOx renal stone formers (having weddelite crystals in their first morning urine) than in the healthy volunteers. A correlation was found between urinary bikunin and alpha-1 microglobulin concentrations in the control group ($y = 0.73x + 1.09$, $r^2 = 0.8$) while no such correlation existed in the lithiasis group. In conclusion, bikunin exerts a strong inhibitory action of CaOx crystallization in vitro. Its involvement in urinary CaOx crystallization of stone formers is highly probable, based on the significant decrease in its urinary concentration in the majority of stone formers studied.

Key words Bikunin · Calcium oxalate · Crystallization · Stone formers · Urine and ELISA

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Introduction

During the past few decades, the prevalence of calcium oxalate (CaOx) calculi has increased, especially in industrialized countries [23, 30]. However, CaOx crystals are not present in large amounts in the urine of healthy subjects despite considerable supersaturation, partly because of the presence of inhibitors of nucleation, crystal growth and aggregation [8]. To date, considerable interest has been devoted to urinary macromolecules, especially glycoproteins that are shown to have an inhibitory effect on CaOx crystallization in vitro [20, 31]. Although urinary glycoproteins have been reported to be responsible for 70%–90% of the total inhibitory activity in urine [18, 26], it is difficult to compare the relative contribution of urinary glycoproteins to total urinary inhibitory activity which is not equal to the sum of individual inhibitors. Among these proteins bikunin, also referred to as UTI (urinary trypsin inhibitor), HI 30 (human inhibitor of 30 kDa), ASPI (acid stable proteinase inhibitor), urinastatin and ITI (inter-α-trypsin inhibitor) light chain, has been described as a potent inhibitor of calcium oxalate crystallization in vitro, under the name of uronic acid rich protein (UAP) [1, 4]. The purification procedure used involved nonspecific chromatographic steps, i.e., ion exchange chromatography and gel filtration which did not completely avoid the problem of slight contamination with other proteins, in particular with alpha 1 microglobulin (a1m) [2]. On the other hand, bikunin has been purified by other methods, especially immunoaffinity chromatography [33] using anti-ITI antibodies, since bikunin is the light chain of ITI.

Bikunin is synthesized in the liver, in the form of a precursor also containing a1 m [7, 17, 22, 37]. After cleavage in Golgi apparatus, it is secreted into blood in free form and mainly combined to other protein chains essentially to form ITI, pre-alpha-trypsin inhibitor (PI) and other molecules which are catabolized in the bloodstream [32, 34]. Bikunin is eliminated in free form in urine, mainly as a low molecular weight protein, and is filtered intact in urine. No heavy chains (H1, H2) have been detected in urine (33) and consequently polyclonal anti-ITI antibodies can be used for quantification of bikunin in urine.

Hochstrasser et al. [12] first described a physiological urinary trypsin inhibitor having an apparent molecular mass of 34 kDa. The anti-trypsin properties of bikunin have been successfully used to purify bikunin in urine, by means of trypsin coupled to a CNBr sepharose column [36, 41], since it has been reported that bikunin was the only protein responsible for all anti-trypsin activity in normal urine (24).

The aim of the present work was (1) to obtain pure bikunin from a trypsin adsorbent column and to verify that it was active in inhibiting CaOx crystallization, as shown with previous purification procedures, and (2) to compare bikunin concentration in the urine of healthy men and of CaOx stone formers, using an enzyme-linked immunosorbent assay (ELISA) technique.

Materials and methods

Subjects and materials

Urine collections. Randomly collected first morning urine specimens were obtained from apparently healthy men in the laboratory (for bikunin purification and for the control group of healthy subjects of the in vivo study, $n = 18$). These subjects were all asymptomatic, with no history of personal renal stone disease and receiving no medication.

Thirty-one hypercalciuric patients regularly followed in the Nephrology Division at Necker Hospital, Paris constituted the lithiasis group. Urine examination by polarizing microscopy revealed no crystalluria in the control group but the presence of calcium oxalate dihydrate crystals in all stone formers (mean ± SEM weddellite crystal number, 12.43 ± 4.64/mm^3).

The age of control and lithiasis subjects was comparable (mean ± SEM = 39 ± 3.9 years vs 47 ± 2.8, respectively, P = NS).

Serum C reactive protein level was measured in all subjects to exclude individuals with inflammatory conditions and was found to be normal in all.

Primary rabbit monospecific anti-human ITI antibodies and primary rabbit monospecific anti-human a1m antibodies were purchased from Dako, Denmark (reference A 0301 and A 256, respectively). Primary rabbit anti-human albumin (HSA) antibodies and anti-rabbit IgG peroxidase conjugate, developed in goat, were purchased from Sigma, USA (reference A 0433 and A 9169). Polystyrene microtitration plates were from Nunc Immuno, Denmark, ^{45}Ca (specific activity, 37 mBeq/ml) from Amersham, UK, CNBr-activated sepharose 4B and Sephadex G25 from Pharmacia, Sweden and peroxidase, glutaraldehyde, 4-chloro-1-naphtol, O-phenylenediamine dihydrochloride (OPD) and all usual chemical products from Sigma.

ITI (purity exceeding 99%, determined by 2D electrophoresis and SDS PAGE) was a gift of Hoechst-Behring, Rueil-Malmaison, France.

Methods

Bikunin purification procedure. Two liters of pooled first morning urine from six healthy subjects were dialyzed for 24 h at 4°C against distilled water in the presence of sodium azide (0.1% w/v) and phenylmethylsulfonyl fluoride (5 μM). Then urinary volume was concentrated approximatively 10-fold across a dialysis membrane of 8000 Da cut-off, in the presence of polyethylene glycol 17 500 Da. The addition of ammonium sulfate (20% saturation) eliminated some urinary proteins, in particular the Tamm-Horsfall protein (THP) as estimated by SDS PAGE. The supernatant was dialyzed and lyophilized. After solubilization of residual proteins in 10 mM EDTA, 0.05 M sodium citrate buffer pH 6.5 in order to solubilize the proteins, 30 mg of protein extract were applied on a CNBr-activated sepharose column coupled with trypsin, according to the recommendations of the manufacturer: 14 ml of gel coupled with 70 mg of trypsin were packed in a 20 ml column (1 cm × 20 cm). After washing the column with seven volumes of a 50-mM sodium acetate buffer, pH 4.0, the proteins linked to trypsin were eluted with a 250 mM glycine buffer, pH 2.5, at a constant rate of 40 ml/h. The collected fractions were immediately neutralized with concentrated TRIS buffer.

The protein purity was checked by SDS PAGE and revelation was done by silver staining or immunoblotting using various antibodies, especially anti-ITI, anti-a1m and anti-HSA.

Electrophoresis and Western blotting. SDS PAGE, in the presence of 0.1% SDS and 0.5% dithiothreitol, was carried out according to Laëmmli [19] with a concentrating gel of 4% acrylamide and a separating gel of 12% acrylamide on a mini protean II electrophoresis cell (Bio Rad USA). The proteins were stained with a silver technique (kit from Biorad). Immunoblotting was carried out on 0.2-μm nitrocellulose membranes. The primary antibodies (anti-ITI, anti-a1 m and anti-HSA) were diluted 200-fold. Immunoblot revelations were made with 4-chloro-1-naphtol (0.05% in PBS 50 mM, pH 7.4, added to 30 μl/100 ml of 30% hydrogen peroxide).

Peroxidase-labeled anti-human ITI antibodies. Anti-ITI was labelled with peroxidase using a two-step procedure [5]. Peroxidase was firstly coupled to glutaraldehyde (10 mg dissolved in 0.2 ml of 0.1 M PBS buffer, pH 6.8, containing 1.25% glutaraldehyde). The mixture was allowed to stand for 18 h at room temperature and then filtered through a Sephadex G25 column (60 × 0.9 cm), equilibrated with 0.15 M NaCl. The brown-colored fractions were pooled, concentrated to 1 ml and coupled, in a second step, to 1 ml of anti-ITI solution containing 5 mg of antibodies and 0.2 ml of 1 M carbonate-bicarbonate buffer, pH 9.5. After 18–24 h at 4°C, 0.2 ml of 1 M glycine dissolved in 0.1 M PBS buffer were added and the mixture was left to stand for 2 h. Then it was dialyzed extensively with PBS buffer, pH 7.4. The conjuguates were stored with 0.01% thimerosal at 4°C.

ELISA procedure for bikunin quantification in urine. The polystyrene plates were coated overnight at 4°C with 100 μl anti-ITI antibodies, 300-fold diluted in PBS buffer, pH 7.4. After washing with PBS-Tween (Tween 0.2% v/v), the wells were saturated with PBS containing 2% bovine serum albumin (PBS-BSA) (w/v). Then, after washing with PBS-Tween, 100 μl of urine samples diluted in PBS-BSA (with a protein concentration ranging from 20 to 1000 ng/100 μl) were added and incubated at room temperature for 2 h. After washing with PBS-Tween, 100 μl of peroxidase anti-ITI conjugate were added to the wells and the plates were held overnight at 4°C. After washing with PBS-Tween, 200 μl of OPD solution (Sigma fast OPD kit) were added and the coloration was stopped by adding 50 μl of 3 N HCl before reading optical density at 490 nm. Each urine sample was assayed in octuplicate (at least four different dilutions in duplicate).

In vitro inhibition tests of CaOx crystallization

Model using ^{45}Ca as indicator of remaining soluble calcium. Bikunin was assayed in the CaOx crystallization model developed by Atmani et al. [1]. In a test tube, up to 10 µg of proteins solubilized in 0.3 ml of 0.15 M NaCl were mixed extemporaneously with 0.5 ml of 2 mM ammonium oxalate, 50 mM TRIS HCl buffer pH 7.4. The assay was initiated by adding 0.5 ml 2 mM $CaCl_2$ (containing a trace of ^{45}Ca), 50 mM TRIS HCl buffer pH 7.4. After 1 h of stirring at room temperature, the tubes were centrifuged at 10 000 *g* for 5 min and the radioactivity of the supernatant counted. Each point of protein concentration has been measured in duplicate or triplicate.

Model based on a turbidimetric method. Bikunin and ITI were assayed in the CaOx crystallization system developed by Hennequin et al. [10]. In each test cuvette, the CaOx precipitation kinetics were followed for 3 to 10 min by reading the turbidity at 620 nm. The presence of proteins (0 to 50 µg/ml) in a medium containing 4 mM $CaCl_2$ and 0.5 mM Na_2Ox (TRIS HCl, pH 6.8, NaCl 0.15 M) inhibited CaOx crystallization as estimated by slope modification of the turbidimetric curve. The time of induction (ti) corresponded to the time between the addition of oxalate and the moment at which the growth was experimentally measurable (i.e., 2.5% of the maximal OD value) [11]. Each point of protein concentration was measured in duplicate or triplicate.

Urinary biochemical determinations

Urinary samples were analyzed for Ca (orthocresolphtaleine method, Boehringer kit) and creatinine (Jaffe method, Boehringer kit) on a multiparametric Hitachi 717 analyzer (Boehringer, Mannheim). Oxalate concentration was determined by ionic chromatography.

The protein concentration was determined with a modified Bradford method (Bio Rad protein microassay). Urinary a1m concentration was measured by an ELISA technique similar to the procedure used for bikunin in the present study. Urinary crystals were analyzed by polarizing microscopy according to the recommendations of Bader et al. [6].

Expression of results and statistical analysis

The results of the in vivo study have been expressed as means ± SEM. Analysis of variance (ANOVA) was used to compare the results between stone formers and healthy subjects.

Results

Urinary bikunin purification

The addition of ammonium sulfate (20% saturation) to a urine sample, which had been previously dialyzed and concentrated 10-fold, caused marked depletion of THP protein as shown on SDS PAGE and by silver staining (Fig. 1, compare trace A and B for elimination of the 94-kDa protein). Then the protein extract was applied on trypsin CNBr sepharose column. The proteins eluted from the column with 0.25 M glycine buffer, pH 2.5 showed, after SDS PAGE and silver staining, two protein bands of 24 and 17 kDa apparent molecular weight (Fig. 1, trace C). The same sample, after transfer on a nitrocellulose membrane, showed by immunorevelation against anti-ITI antibodies two corresponding bands of similar molecular weight (Fig. 1, trace D). No revelation occurred by contact with anti-human serum albumin and anti-human a1m.

In vitro inhibition of calcium oxalate crystallization by bikunin and ITI

Bikunin purified by trypsin affinity was assayed in two in vitro models of CaOx crystallization (see Methods). In the model based on turbidimetry as well as in the one based on radioactive calcium remaining in solution, bikunin strongly inhibited CaOx crystallization in a concentration-dependent manner, with an inhibitory effect reaching approximately 80% for a bikunin concentration of 8 µg/ml (Fig. 2 and Table 1).

The results presented in Table 1 show that, in the turbidimetric model, increasing bikunin concentrations proportionally increased induction time (ti), decreased turbidimetric slope and inhibited CaOx crystallization. Under the same experimental conditions, ITI moderately inhibited CaOx crystallization with no significant effect at 5 µg/l but a 16.4% inhibition at 50 µg/ml. ITI did not modify induction time.

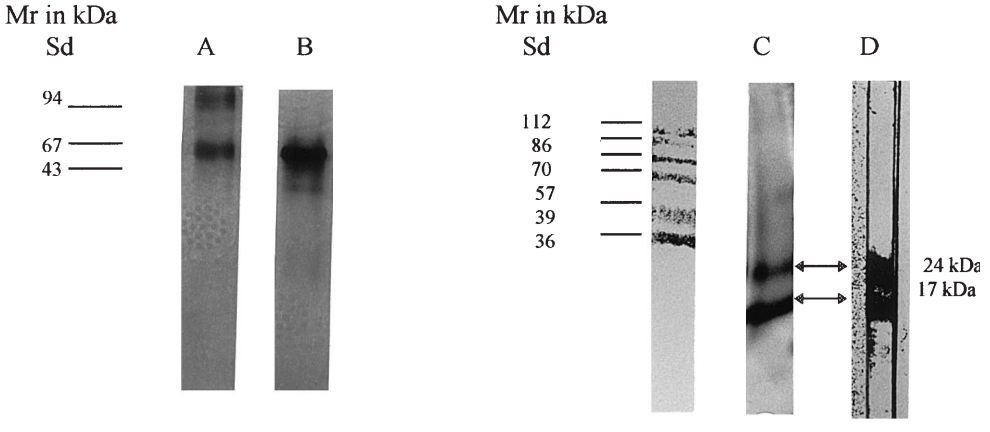

Fig. 1 Silver staining (A,B,C) and Western blotting (D) of urinary proteins on SDS PAGE analysis after purification on trypsin CNBr sepharose chromatography. *A* dialyzed urine, *B* urinary extract after ammonium sulfate addition (20% saturation), *C* purified bikunin after trypsin CNBr sepharose chromatography, *D* Western blot of sample C. Immunostaining against anti-ITI antibodies

Human study

Comparison of bikunin excretion between healthy subjects and CaOx stone formers: relation with urinary oxalate and calcium excretion

Urinary bikunin concentration, determined by ELISA, was compared between healthy subjects without crystalluria and stone formers with CaOx crystalluria. Mean values were significantly different between normal subjects and stone formers whether they were expressed in mg/l (5.01 ± 0.91 and 2.54 ± 0.42, respectively) or in mg/mmol creatinine (0.47 ± 0.11 and 0.25 ± 0.05, respectively). Urinary total protein (65.81 ± 7.54 vs 58.38 ± 3.72 mg/l) and creatinine (13.74 ± 1.65 vs 11.93 ± 1.11 mmol/l) were similar in both groups (Table 2).

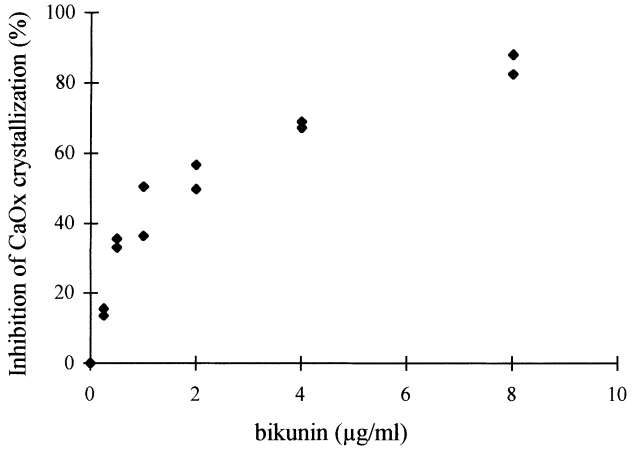

Fig. 2 Inhibitory activity of bikunin on CaOx crystallization in vitro using the model based on radioactive calcium remaining in solution after 1 h of incubation

Calciuria and the calcium oxalate product (CaOx) expressed in $(mmol/l)^2$ were significantly higher in urine of renal stone formers than in urine of healthy subjects ($P < 0.0001$ and $P = 0.022$, respectively). Oxaluria was not statistically different between the two groups (Table 3).

Correlations between urinary bikunin and a1m excretion in healthy subjects and CaOx stone formers

The urinary concentration of bikunin was positively correlated with that of a1 m in healthy subjects ($y = 0.73x + 1.09$, $r^2 = 0.8$, $P < 0.0001$) whereas no such correlation was observed in the stone formers ($y = 0.17x + 2.029$, $r^2 = 0.015$, $P =$ NS) (Fig. 3).

Discussion

The results of the present study show that two proteins related to bikunin have been purified from urine of healthy subjects. Both proteins exhibited immunoreactivity against polyclonal anti-ITI antibodies and strongly inhibited CaOx crystallization in vitro.

The proteins, bound to the trypsin-CNBr sepharose column and eluted with a low pH buffer, showed two bands of, respectively, 17 and 24 kDa apparent molecular weight after SDS PAGE and protein staining, and which were also revealed by immunoreaction with anti-ITI antiserum. To test preparation purity, we have verified that there was no cross-reaction between the proteins eluted specifically from the column and antibodies of proteins susceptible to contaminate them, i.e., a1 m as previously observed in the study by Atmani et al. [2], and albumin as a major constituent of urinary proteins.

Table 1 Inhibitory effect of-bikunin and inter-α-trysin inhibitor (ITI) on CaOx crystallization in vitro using the turbidimetric test

Protein	Concentration (µg/ml)	Induction time (min)	Turbidimetric slope	Inhibition of CaOx crystallization (%)
0	0	0.8	0.113	0
Bikunin	0.5	1.2	0.0927	17.9
	1	1.1	0.0761	32.6
	2	1.6	0.0637	43.6
	4	1.8	0.0441	60.9
	8	2	0.0201	82.2
ITI	5	1	0.1088	3.7
	50	0.8	0.0944	16.4

Table 2 Comparison of bikunin concentration in urine of healthy subjects and in calcium oxalate (CaOx) stone formers

	Healthy subjects (n = 18)	CaOx stone formers (n = 31)	Significance
Total protein (mg/l)	65.81 ± 7.53[a]	58.38 ± 3.72	NS (P = 0.328)
Creatinine (mmol/l)	13.74 ± 1.65	11.93 ± 1.11	NS (P = 0.352)
Bikunin (mg/l)	5.01 ± 0.91	2.54 ± 0.42	S (P = 0.007)
Bikunin (mg/mmol of creatinine)	0.47 ± 0.11	0.25 ± 0.05	S (P = 0.036)

[a] Values are mean + SEM

Table 3 Comparison of Ca, Oxalate and CaOx product in urine of healthy subjects and in CaOx stone formers

	Healthy subjects (n = 14)	CaOx stone formers (n = 15)	Significance
Ca (mmol/l)	2.90 ± 0.34[a]	6.65 ± 0.50	S (P < 0.0001)
Oxalate (mmol/l)	0.46 ± 0.07	0.41 ± 0.04	NS (P = 0.537)
CaOx (mmol/l)^2	1.42 ± 0.33	2.78 ± 0.31	S (P = 0.0067)

[a] Values are mean + SEM

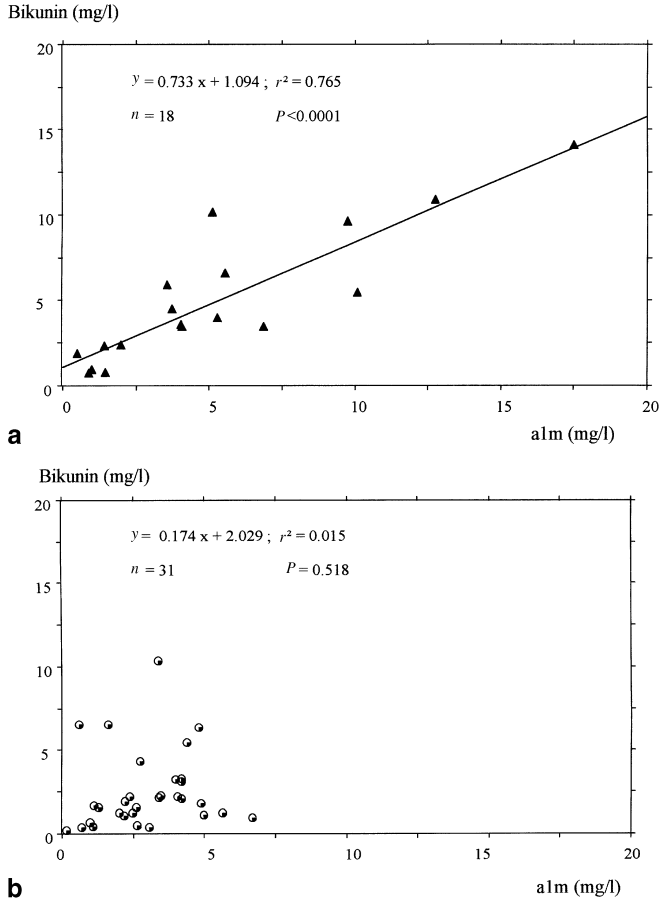

Fig. 3 Comparison between the urinary alpha 1 microglobulin (a1m) and bikunin concentrations in healthy subjects (n = 18) and in CaOx stone formers (n = 31)

In the literature, varying results have been reported concerning the molecular weight of bikunin in urine, which differed according to the techniques used. By gel filtration, an apparent molecular weight of 60–70 kDa has been found [15, 27, 29]. Using SDS PAGE, bikunin appeared in urine as a single band of either 68 kDa [28], 40–43 kDa or 38 kDa [15, 38] or 35 kDa [1, 27, 39]. Finally, the whole protein was composed by a 16-kDa polypeptide chain [9, 38], an 8-kDa chondroitin sulfate chain and a N-linked oligosaccharide of about 1 kDa [38]. The nucleotide and protein sequence of bikunin corresponded to 145 amino acids in rat [22], and to 147 amino acids in both human [22] and bovine species [21]. The molecular weight of the two molecular species of bikunin purified in the present study probably corresponded to digestion products of the native protein by trypsin on the affinity column. In accord with this, it has been demonstrated that bikunin is sensitive to trypsin digestion even though a relatively stable domain of 17 kDa, corresponding to the antitrypsin site, may be resistant to proteolysis [12]. With the purification procedure used by Nakagawa et al. [26] to isolate nephrocalcin, without affinity chromatography steps, Tang et al. [39] have isolated a protein of 21 kDa (SDS PAGE), which strongly inhibited CaOx crystal growth. After gel digestion, this last protein yielded two peptides of which both sequences showed homology with the internal sequence of bikunin and with the longer one matching the N-terminus of HI-14. It is possible that the 24 kDa protein isolated in our work, and which is immunologically related to bikunin, corresponds to the protein of 21 kDa isolated by Tang et al. [39].

The in vitro inhibitory activity of bikunin has been demonstrated in the present study with two different tests: a kinetic test essentially taking into account the nucleation and the crystal growth phases, and an end-point test essentially taking into account crystal growth. Thus the inhibitory effect of bikunin on crystal growth has been confirmed by two models. Furthermore, a slight inhibitory activity was observed with ITI, which contains bikunin within its stucture.

The inhibitory activity of the bikunin fragments isolated in the present work was strong. It reached approximately 80% at a protein concentration of 8 μg/ml (Fig. 2). In the same test, Atmani et al. [1] found approximately 70% inhibitory activity with the whole molecule (intact molecule) of 35 kDa at a protein concentration of 10 μg/ml, indicating that the break provoked by trypsin did not notably modify the inhibitory action on CaOx crystallization. The inhibitory activity of bikunin is probably supported by the polypeptide chain since it was not lost after chondroitinase AC treatment [4]. Among the urinary inhibitory proteins, bikunin is a potent inhibitor since, when tested in the same model and with identical salt concentration, it exerted an inhibitory activity on crystal growth similar to nephrocalcin and had a much higher level of activity than THP [1].

Mean bikunin concentration in healthy subjects in the present study was 5.01 ± 0.91 mg/l in urine samples collected at the first morning urination. This result is in agreement with normal urinary trypsin inhibitor (UTI) excretion (8.17 ± 1.18 mg/24 h), using radioimmunological quantification [16], and urinary UTI concentration (8.95 ± 1.65 mg/l for men aged 40–49 years) determined by enzyme immunoassay [27]. However, another report indicated higher values (17 ± 0.9 mg/l) for acid-stable trypsin inhibitor, previously called UTI [25], in normal human urine samples [42]. Whatever the exact physiological bikunin concentration in urine of healthy men it was in a concentration range shown to exert a strong inhibitory effect on CaOx crystallization in vitro. Therefore, in addition to its already known anti-inflammatory properties, it is of interest to investi-

gate the possible involvement of bikunin in the development of urinary crystalluria.

The results of the present study showed that mean urinary bikunin concentrations differed significantly between healthy subjects and CaOx stone formers. A similar decrease has been observed for urinary a1m concentration in CaOx stone formers (data not shown).

In the present study, a positive correlation has been observed between the urinary excretion of bikunin and that of a1 m in healthy subjects ($y = 0.73x + 1.09$, $r^2 = 0.8$, $P < 0.0001$), while no such correlation was found in CaOx stone formers. Since the two proteins are synthesized from a common precursor, it is not surprising to observe a correlation between their rates of elimination. A similar correlation was previously reported by others [35] not only for healthy subjects, but also for patients with various urological diseases. In contrast to the present finding, the patients of the latter report had been selected according to various urological diseases (bladder carcinoma, urethral polyp, and chronic renal failure). This may explain the discrepancy between their results and ours. To date, the significance of the absence of a correlation between a1m and bikunin urinary concentration in stone formers is unknown. However, in other pathologies, for instance in human mood disorders and in dementia of Alzheimer's type, the slopes of regression plots also differed from that of healthy control subjects [13, 14].

Bikunin could be involved in CaOx crystallization in stone former urine through a decrease of its concentration. This, however, does not exclude a role of structural modifications of bikunin. Thus urinaryUAP, which more recently has been associated with bikunin [4], has been suggested to play a role as a CaOx inhibitor, as demonstrated by its in vitro inhibitory activity in association with its lower degree of action on crystallization in stone formers [3]. On the other hand, urinary antitrypsin activity was not found to be statistically significantly different between healthy subjects and renal stone formers [40].

To date, no functional link has been shown to exist between a1m and bikunin although both are derived from a common precursor. We demonstrate here for the first time a common inhibitory activity against CaOx crystallization in vitro for these proteins. One can suppose that, even if the diminished concentration of one inhibitory protein alone may not be sufficient to modify CaOx crystalluria, the additional qualitative or quantitative defect of other proteins such as bikunin, a1m and others, may favor the crystallization of CaOx in urine. Their possible role in urinary CaOx lithogenesis warrants further studies.

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