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Characterization of uronic-acid-rich inhibitor of calcium oxalate crystallization isolated from rat urine

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Abstract Human urine contains several macromolecules which inhibit calcium oxalate crystallization. Uronic-acid-rich protein (UAP), a glycoprotein with a molecular weight of approximately 35 kDa, is one such inhibitor. Here we report the characterization of UAP extracted from rat urine using three chromatographic steps including diethylaminoethanol (DEAE)-Sephacel, Sephacryl S-300 and Mono Q column and compare it with human UAP. The molecular weight of rat UAP (UAP_r) is similar to that of human UAP (UAP_h), being approximately 35 kDa as estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Their amino acid compositions are identical, they contain a high percentage of aspartic and glutamic acids and they react positively in the carbazole reaction, suggesting that they contain uronic acid. The inhibitory activities of UAP_h and UAP_r were assayed on a calcium oxalate crystallization system in vitro using [^{45}Ca]calcium chloride. Both exert a strong inhibition, suggesting that UAP_r, like UAP_h, plays an important role in preventing and reducing calcium oxalate crystallization in the urine. On Western blot analysis, both UAP_h and UAP_r immunoreact with inter-α-trypsin inhibitor (ITI) antibody. Nevertheless, using the Ouchterlony immunodiffusion technique, there was no precipitation line between ITI antibody and UAP. Therefore, we hypothesize that UAP is related to ITI and that they may have the same epitope but are not completely identical. We conclude that UAP belongs to the ITI superfamily of macromolecules which contribute to the regulation of the calcium oxalate crystallization process.

Key words Nephrolithiasis · Calcium oxalate · Chromatography · Uronic-acid-rich protein · Inter-α-trypsin inhibitor

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Urine is supersaturated with respect to calcium oxalate, the most common component of kidney stones. For many years attention has been focused on urinary macromolecules which can inhibit or reduce calcium oxalate crystal formation. These macromolecules may bind to the crystal's surface, preventing growth and aggregation of small crystals into calculi which could be retained in the urinary tract. Recently various urinary macromolecules have been isolated and characterized including glycosaminoglycans (GAGs) [5], Tamm-Horsfall protein (THP) [8], nephrocalcin (NC) [13], uropontin [19], inter-α-trypsin inhibitor (ITI) [20] and more recently uronic-acid-rich protein (UAP) [1]. This latter macromolecule may play an important role in preventing crystal growth because it is a potent inhibitor of calcium oxalate crystallization and its inhibitory activity is reduced in the urine of stone formers [3]. It is a glycoprotein with a molecular weight of approximately 35 kDa as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The carbohydrate content averages 8.5% of the total molecular weight [2]. Its partial structure shows a homology with the inter-α-trypsin inhibitor [2], a serum protein implicated in the inflammation process, cancer and renal failure [6, 9, 14, 21]. Inte-α-trypsin inhibitor encompasses several proteins consisting of bikunin referred to as HI-30 chain and one or two heavy chains designated H1, H2 and H3 [18]. ITI is produced mainly in the liver. Nevertheless, ITI immunoreactivity is also present in kidneys [24]. The most intense staining was observed in proximal tubules, suggesting that ITI or other macromolecules related to this protein could be synthesized by these cells [23]. It is also reported that human endothelial cell growth factor isolated from cultured human hepatoma cells were similar to ITI and HI-30 [11]. The molecular weight of ITI remains a matter of much controversy [6]. One of the most important characteristics of ITI is its inhibition of the action of various enzymes such as trypsin and chymotrypsin [7, 16]. HI-30, a macromolecule with a molecular weight of about 30 kDa, is considered to be the inhibitory active part of ITI [17].

In this paper, we attempted to purify UAP from rat urine in the same way as human UAP and to test its inhibitory

activity against calcium oxalate crystallization in vitro. We also characterized it and compared it to the UAP isolated from normal human urine.

Materials and methods

Materials

Twenty four hour urine was collected from one healthy man who had no previous complications related to kidney stone disease. The urine sample was kept in a polypropylene bottle containing sodium azide at 4°C. Pooled rat urine was collected using metabolic cages from male Sprague Dawley rats (Harlan Sprague Dawley Inc. In., USA) fed a normal diet. Urine samples were kept in plastic receptacles containing one or two drops of 20% sodium azide. During the collection, vials were surrounded by dry ice.

Diethylaminoethyl (DEAE)-Sephacel, Sephacryl S-300 and the Mono Q HR 5/5 column were purchased from Pharmacia (Pharmacia Biotech, N.J., USA). Before each use, gels or columns were recycled according to the manufacturer's recommendations. The salt concentration was measured using a conductivity meter (Fisher Scientific. Pa., USA). Dialysis tubing with a molecular weight cutoff of 6–8 kDa was obtained from Spectrapor (Spectrum Medical Industries, Inc. Calif., USA). Protease (Code 537088, 110 PUK/mg) and normal human serum inter-α-trypsin inhibitor polyclonal antibody (Code AXL 424) were purchased from Calbiochem (Calif., USA) and Accurate (Chemical and Scientific, N.Y., USA), respectively. [^{45}Ca]Calcium chloride was purchased from Amersham (Ill., USA). Electrophoresis, transfer blot apparatus, prestained low molecular weight markers and reagents for immunostaining were obtained from Bio-Rad (Calif., USA). Polyvinyldene fluoride (PVDF) membrane was from Millipore (Bedford, USA) and the second antibody was from Hyclone Laboratories (Utah, USA). All pure reagents were purchased from Fisher Scientific (Pa., USA).

Methods

Protein isolation

The isolation procedure for purification of UAP from human and rat urine was as described previously [1]. In both cases, the urine sample was dialyzed against deionized water for 1 day at 4°C. The pH and the ionic strength of the dialysate were adjusted to 7.3 and 0.1 M NaCl by adding NaOH and solid NaCl, respectively. It was then incubated with DEAE-Sephacel gel preequilibrated with 0.05 M TRIS, 0.1 M NaCl, pH 7.3, for 30 min with stirring. The suspension was filtered, washed with equilibrated buffer and eluted successively with 300 and 200 ml 0.05 M TRIS, 0.5 M NaCl, pH 7.3. Eluents were collected and dialyzed overnight against deionized water. The sample was then adjusted to pH 7.3 and 0.1 M NaCl and injected into the DEAE-Sephacel column (0.8×16 cm). Protein was eluted with a linear gradient from 0.1 to 0.5 M NaCl in TRIS 0.05 M, pH 7.3. The main protein and uronic acid peak contained UAP and named fraction B (for human) or B' (for rat). These fractions were dialyzed, lyophilized and injected into the Sephacryl S-300 column (2.6×90 cm) after treatment with 0.05 M ethylenediamine-tetraacetic acid (EDTA), pH 8, for 1 day at 4°C. To complete the purification, UAP was then subjected to fast protein liquid chromatography (FPLC) on a Mono Q HR 5/5 column using a linear gradient of NaCl from 0.1 to 0.5 M NaCl buffered with TRIS 0.05 M, pH 7.3.

Protein and uronic acid assay

After each chromatographic step, protein and uronic acid concentrations were determined in every fifth tube using Lowry's method [10] and the carbazole reaction [4], respectively. Depending on the method, albumin and glucuronic acid were used as standards.

Inhibition assay

To determine the inhibitory activity of isolated urinary macromolecules, the calcium oxalate crystallization system was used as described in a previous paper with a minor modification [1]. One milliliter of 2 mM calcium chloride in TRIS 0.05 M, NaCl 0.15 M, pH 7.3, with a trace of ^{45}Ca was pipetted into tubes containing macromolecules lyophilized from 100–200 µl sample obtained from different chromatographic steps despite the use of a constant concentration of proteins. The assay was started by adding 1 ml of 2 mM ammonium oxalate in TRIS 0.05 M, NaCl 0.15 M, pH 7.3, for 1 h with stirring. Tubes were then centrifuged at 2000 *g* for 5 min and 0.5 ml supernatant was withdrawn for radioactivity determination in an LS 7500 liquid scintillation counter (Beckman Instruments. Calif., USA).

Gel electrophoresis

SDS-PAGE was used to check the degree of purity of protein and to estimate their relative molecular weights. Proteins were migrated into 1-mm-thick 10% polyacrylamide gel after they were subjected to reduction using β-mercaptoethanol. Gels were stained with Coomassie blue R-250 or by silver staining procedure [12].

Amino acid analysis and sequencing

Amino acid composition was determined by using a Beckman 6300 amino acid analyzer after hydrolysis in 6 N HCl for 24 h. Amino acid microsequence analysis was carried out by an automated Edman degradation on a protein sequencer. In both analyses, UAP was first electrophoresed by SDS-PAGE and transferred onto a PVDF membrane.

Western blotting and immunodiffusion technique

After electrophoresis, proteins were transferred onto nitrocellulose membrane for immunological identification. Proteins were transferred at 100 V for 1 h in transfer buffer (25 mmol/l of TRIS, 192 mmol/l of glycine and 200 ml/l methanol) at 4°C. The membrane was stained with inter-α-trypsin inhibitor antibody by the method described by Towbin et al. [22]. A second antibody conjugated to alkaline phosphatase was used for immunostaining. The immunodiffusion technique was performed on 1% agarose in phosphate buffer salin (PBS) (136.9 mmol/l NaCl, 2.68 mmol/l KCl, 5.37 mmol/l Na_2HPO4, 1.76 mmol/l KH_2PO_4, pH 7.4) according to the Ouchterlony technique [15] using the same antibody. Human serum and urine and rat serum and urine were used as controls. The diffusion was allowed to run for 48 h at room temperature.

Protease treatment

UAP was submitted to protease digestion as described by Sorensen et al. [20], whereby the inhibitory activity was assayed on the calcium oxalate crystallization system with and without protease treatment after 3 h at 37°C.

Results

Urine samples obtained from human and rat were subjected to a DEAE-Sephacel bath and column chromatography using a linear gradient of NaCl from 0.1 to 0.5 M in TRIS 0.05 M, pH 7.3, as described in "Methods" above. The elution patterns are shown in Figs. 1 and 2, respectively. Two hundred microliters of every fifth fraction was lyophilized

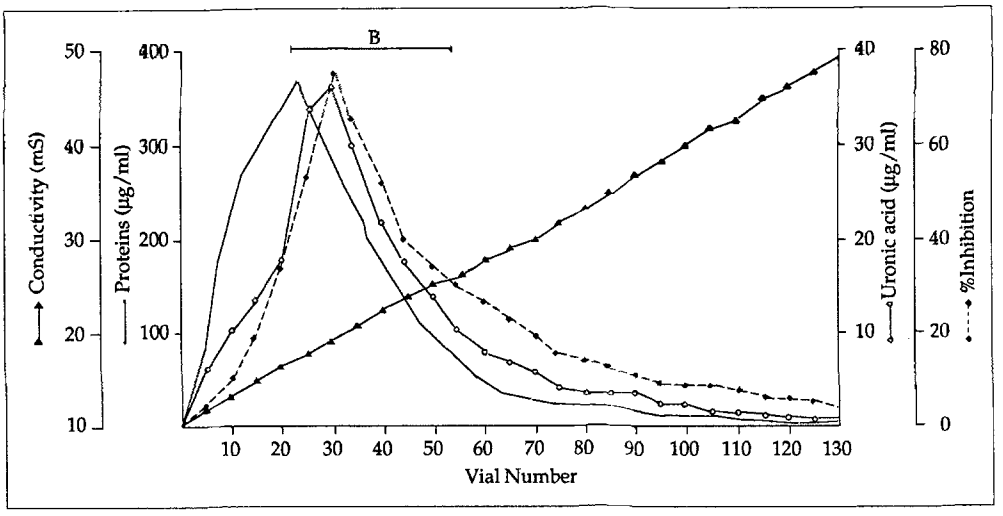

Fig. 1 Elution of human urinary protein from DEAE-Sephacel ion exchange chromatography using a linear gradient of NaCl from 0.1 to 0.5 M in TRIS 0.05 M, pH 7.3. Protein and uronic acid concentration were determined according to Lowry's method and the carbazole reaction respectively. Salt concentration was measured by a conductivity meter. Inhibition of fractions was assayed on a calcium oxalate crystallization system. Fraction B, which is rich in UAP, was collected for further chromatographic steps

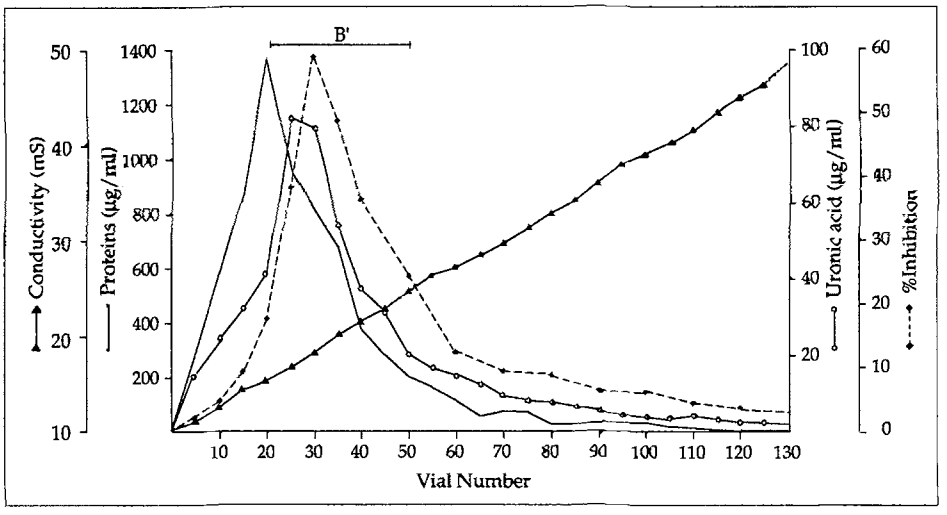

Fig. 2 Isolation of urinary rat UAP using DEAE-Sephacel column chromatography in same manner as described in Fig 1. B' was used for subsequent purification

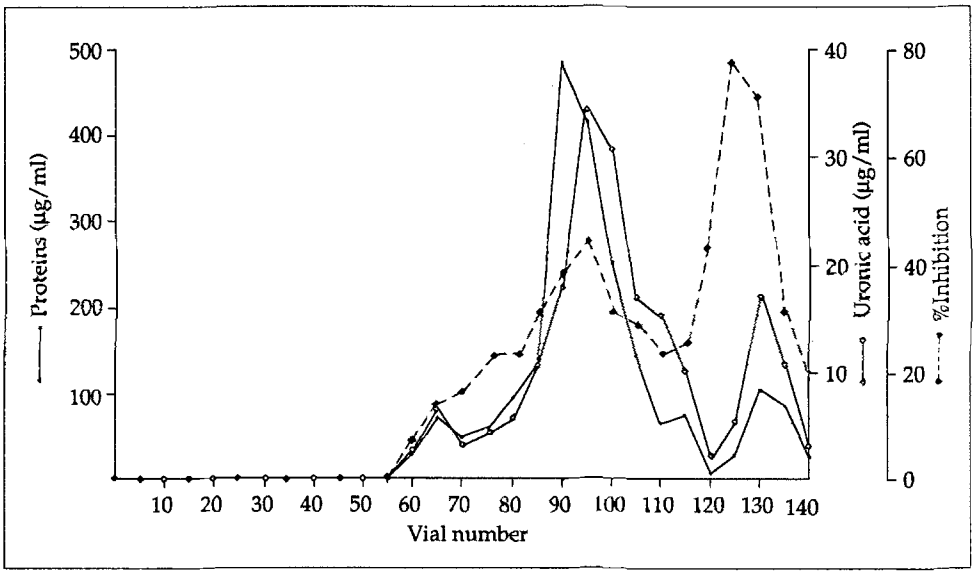

Fig. 3 Fraction B obtained in Fig. 1 was chromatographed on a Sephacryl S-300 column using TRIS 0.05 M, NaCl 0.1 M, pH 7.3, as eluent. Fractions of 3 ml were collected. Protein concentration, uronic acid content and inhibitory activity in every fifth tube were determined as described in "Methods"

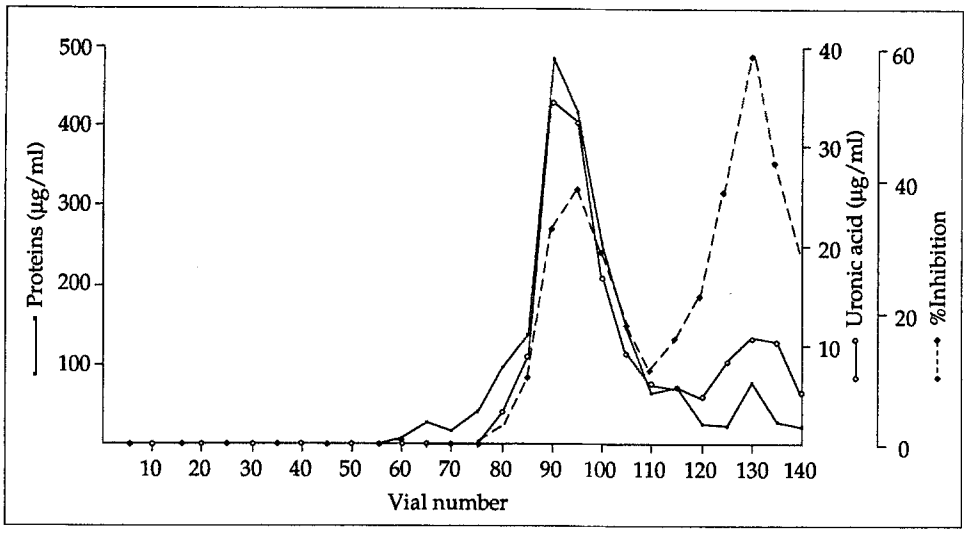

Fig. 4 Size exclusion chromatography on Sephacryl S-300 column of urinary rat UAP. Same details as for Fig. 3

Table 1 Amino acid composition of UAP isolated from human (UAP_h) and rat (UAP_r) urine. Values are given as the number of amino acid residues/100 residues

Amino acid	UAP_h		UAP_r	
	Number of residues	Nearest integer	Number of residues	Nearest integer
Asp	10.94	11	9.93	10
Thr	5.59	6	6.68	7
Ser	7.48	7	7.18	7
Glu	13.25	13	13.89	14
Pro	4.24	4	4.51	5
Gly	15.11	15	10.75	11
Ala	6.04	6	5.69	6
Val	5.46	5	5.45	5
Met	1.23	1	1.68	2
Ile	2.36	2	4.23	4
Leu	6.59	7	7.61	8
Tyr	3.84	4	5.14	5
Phe	4.54	5	4.93	5
His	1.87	2	1.44	1
Lys	5.15	5	5.47	5
Arg	6.31	6	5.43	5

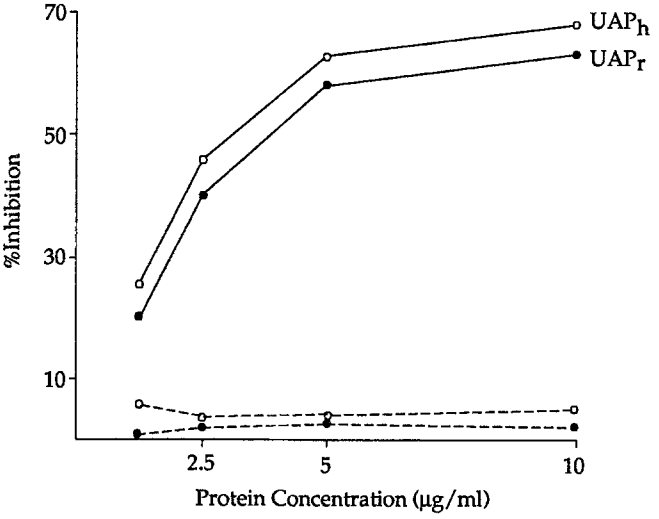

Fig. 5 Inhibitory activity of UAP isolated from human (UAP_h) and rat (UAP_r) urine after incubation without enzyme (*continuous line*) and with enzyme (*dashed line*), respectively

and tested in our calcium oxalate crystallization system. The inhibitory activity coincided with peak protein and uronic acid and the greatest inhibition was observed in fraction B (for human urine) and B' (for rat urine) containing UAP. The second chromatography step was to submit B and B' fractions to analysis with the Sephacryl S-300 column. Typical patterns are illustrated in Figs. 3 and 4, respectively. In this step only 100 µl of every fifth fraction was lyophilized and tested for the inhibition of calcium oxalate crystallization. Both figures show two principal inhibitory activity peaks. By using SDS-PAGE, we found that the first peak corresponds to 35 kDa UAP and associated proteins while the second peak corresponds to a protein with a molecular weight of approximately 16 kDa. As can be seen in Figs. 3 and 4, UAP reacts positively in the carbazole reaction, suggesting it contains uronic acid. The 16 kDa protein demonstrated a weak response in the carbazole reaction. At this stage of the chromatography, human and rat UAP remain contaminated by other proteins. To achieve the purification, UAP_h and UAP_r were completely purified using the FPLC system on a Mono Q column. They were assayed in our calcium oxalate crystallization system using different concentrations of protein. As shown in Fig. 5, both human and rat UAP exert a strong inhibitory activity. UAP_r showed less inhibitory activity than UAP_h at each protein concentration used.

The molecular weights of human and rat UAP are identical, being about 35 kDa as determined by SDS-PAGE (Fig. 6). The amino acid compositions of UAP_h and UAP_r are similar. Results are shown in Table 1. In both inhibitors, aspartic and glutamic acids represent 24% of the total amino acid residues, while basic and aromatic amino

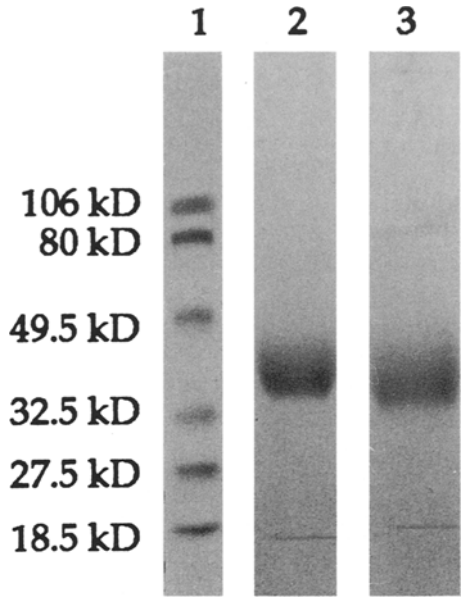

Fig. 6 SDS-PAGE: *lane 1*, prestained molecular weight markers; *lane 2*, human UAP; *lane 3*, rat UAP

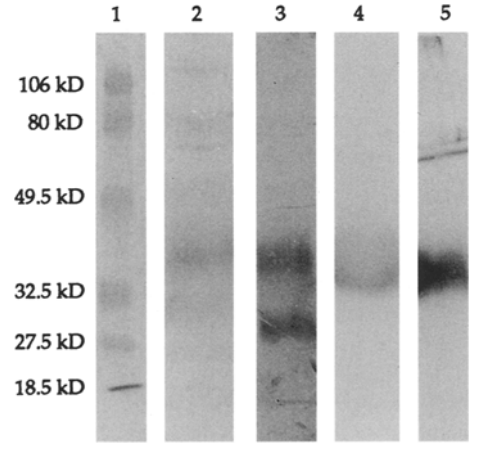

Fig. 7 Immunoblotting analysis after SDS-PAGE: *lane 1*, prestained molecular weight markers; *lane 2*, human urine; *lane 3*, rat urine; *lane 4*, human UAP; *lane 5*, rat UAP

Table 2 Determination of the 18 amino acids from the N-terminal of human and rat UAP by Edman degradation. The sequence has been identified in homologous proteins

	1 5 10 15
	AVLPQEEEGTGGTQLVXE
Proteins	**Sequence**
	1 5 10 15
Inter-α-trypsin inhibitor	AVLPQEEEGSGGGQLVTE
Inter-α-trypsin inhibitor chain HI-30	AVLPQEEEGSGGGQLVTE
Endothelial cell growth factor 2b	AVLPQEEEGXGGGQLVTE

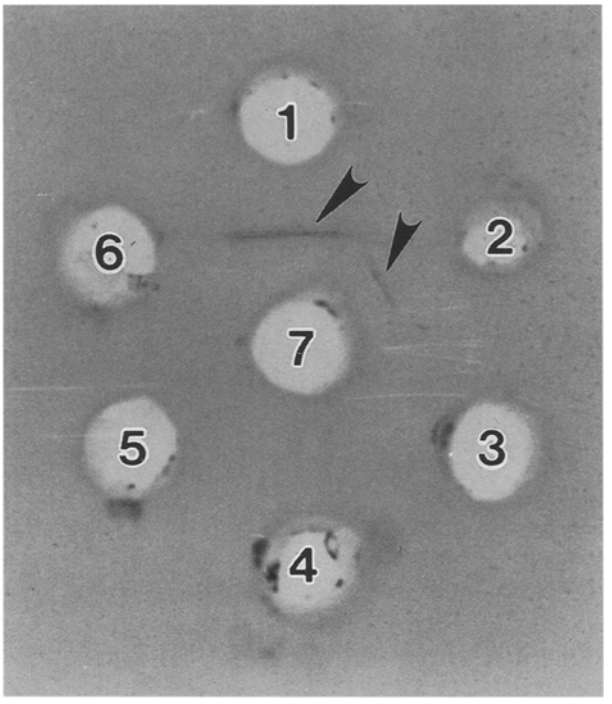

Fig. 8 Immunodiffusion technique on agarose gel: *1*, human serum; *2*, human urine; *3*, rat serum; *4*, rat urine; *5*, human UAP; *6*, rat UAP; *7*, ITI antibody

acids represent 10% and 13% of the total amino acid residues, respectively.

The microsequence of 18 amino acid residues from the N-terminal of human and rat UAP was determined and is shown in Table 2. This sequence exhibits a structural homology with inter-α-trypsin inhibitor (ITI), HI-30 fragment or bikunin, and with an endothelial cell growth factor 2b. After electrophoresis and transfer blot, both human and rat UAP were immunostained using polyclonal ITI antibody. Human and rat urine showed two bands, one at 35 kDa and the other at 30 kDa (Fig. 7). Using the Ouchterlony technique, we obtained only a continuous precipitation line between ITI and human serum and urine. The immunodiffusion between ITI and human and rat UAP on one side, and between ITI and rat serum and urine on other side, were negative (Fig. 8).

UAP isolated from human and rat urine was treated with protease and assayed on a calcium oxalate crystallization system at different concentrations. In both cases, inhibitory activity of UAP was completely destroyed (Fig. 5).

Discussion

It is now widely known that normal human urine contains several macromolecules inhibiting calcium oxalate formation in vitro [1, 5, 8, 13, 19, 20]. However, the mechanism of how these macromolecules bind onto the crystal surface

preventing and reducing calcium oxalate growth and aggregation has not been definitely clarified. Purification and characterization of urinary macromolecules constitutes an important means of better understanding urolithiasis. In respect of this idea, we have recently found in normal human urine a glycoprotein named uronic-acid-rich protein (UAP) [1]. The essence of our finding is that UAP may play an important role in the inhibition of calcium oxalate precipitation and that one inhibitor alone cannot be responsible for the total urinary inhibitory activity. UAP isolated from the urine of stone formers showed less inhibitory activity toward calcium oxalate formation in vitro [3]. Detailed biochemical characterization of this glycoprotein is in progress.

Here we reported the characterization of UAP isolated from rat urine and compared with human UAP using the same purification procedure. In the first purification step on the DEAE-Sephacel column, the inhibitory activity agreed with peak protein and uronic acid after we made a minor modification in our inhibition assay (see "Methods" above). Fractions B and B' exhibiting the highest inhibitory activity contained UAP.

In the second chromatographic step, we observed two inhibitory activity peaks with the highest peak corresponding to the 16 kDa protein. Our earlier work has shown that this protein may be nephrocalcin (NC) or an NC-like macromolecule based on several characteristics of NC or an macromolecule NC-like [1, 13]. In both human and rat urine, the latter macromolecule is virtually pure. However, UAP remains largely contaminated by albumin and other macromolecules. The degree of purity of proteins was followed using the SDS-PAGE technique. After a complete purification by using FPLC on a Mono Q column, human and rat UAP exhibited a strong inhibitory activity toward calcium oxalate crystallization. Moreover, we have already demonstrated that the inhibitory activity of human UAP is higher than that of NC or NC-like macromolecules [1]. This reconfirms our finding that UAP is one of many macromolecules which contributes to the total urinary inhibitory activity.

Their apparent molecular weight is identical, being about 35 kDa as determined by SDS-PAGE. The amino acid composition of human and rat UAP is similar, both containing 24% acidic amino acids. Basic and aromatic amino acids represent 10% and 13% of total amino acids, respectively.

UAP_h and UAP_r react positively in the carbazole reaction, suggesting they contain uronic acid. This finding allowed us to postulate that UAP could be a glycosaminoglycan (GAG). Nevertheless, we have demonstrated that chondroitinase AC had no effect on the inhibitory activity of UAP [2], but that it affects its molecular weight by splitting it into two peptide chains. It appears that chondroitin sulfate ensures the junction between the two peptides.

The first 18-amino-acid microsequence from the N-terminal of human and rat UAP was determined and showed a structural homology with ITI, HI-30 or bikunin, and endothelial cell growth factor 2b. Also, the immunoreaction between UAP and human serum ITI polyclonal antibody was positive, but the immunodiffusion between UAP and ITI antibody was negative. These results allowed us to hypothesize that UAP and ITI are not completely identical but may have the same epitope localized in the N-terminal of the peptide chain. In another study, investigators [20] have isolated an unidentified calcium oxalate inhibitor from human urine. This inhibitor has a molecular weight of approximately 40 kDa and the first 16-amino-acid sequence matches with the ITI sequence. Moreover, its inhibitory activity was not destroyed after treatment with proteinase. This finding is in agreement with several studies demonstrating that ITI and other macromolecules related to this protein are capable of inhibiting the action of various enzymes such as trypsin and chymotrypsin [7, 16]. In order to investigate the effect of different enzymes, the inhibitory activity of UAP was assayed in our calcium oxalate crystallization system with and without proteinase treatment using the same enzymes and Sorensen's procedure [20]. The inhibitory activity of UAP was completely destroyed after treatment with protease. We speculate that UAP and Sorensen's protein are different. This discrepancy could be explained by the fact that we have used a different purification procedure from that used by Sorensen et al. In addition, UAP could be a fragment of other proteins. Nevertheless, the latter hypothesis could be discarded because normal urine in both cases, human and rat, showed two bands when stained with ITI antibody. One band at 35 kDa may correspond to UAP and another band at 30 kDa which may be the HI-30 chain, also named bikunin. So far, no analyses have been performed on the latter macromolecule. Accordingly, UAP is a physiological component of normal urine and not an artifact due to the purification procedure.

It is known that some of macromolecules related to ITI are present, not only in the liver, but in other organs including the kidneys [23, 24]. Indeed, an acid-stable trypsin inhibitor (ASTI) immunoreactivity was observed in proximal tubules, suggesting that ASTI could be secreted into the urine or synthesized by these cells. We have also observed a positive immunoreaction in rat kidney using ITI antibody located in the junction between the cortex and medulla (result not shown). Unfortunately, the relationship between UAP and ITI remains unclear and it appears that the characterization of both proteins isolated from the same urine sample using the same purification procedure is necessary to clarify this relationship between these macromolecules. This constitutes our objective in the next step of our investigation.

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Note added in proof. Another important urinary macromolecule involved in calcium oxalate crystallization, named calcium matrix protein (CMP), has been identified as urinary prothrombin fragment I (personal communication and Stapleton AMF, Simpson RJ, Ryall RL; Biochem Biophys Res Commun 195:1199)