Urol Res (1999) 27:57–61

ORIGINAL PAPER

Fouad Atmani · Saeed R. Khan

Role of inter-α-inhibitor and its related proteins in urolithiasis. Purification of an inter-α-inhibitor related protein from the bovine kidney

Received: 18 February 1998 / Accepted: 9 July 1998

Abstract Urine contains several macromolecules that inhibit calcium oxalate (CaOx) crystallization. Among them is bikunin, the light chain of most of the inter-α-inhibitor (IαI) family of glycoproteins. This study aimed to verify whether bikunin and other members of the IαI family are synthesized in the kidneys or derived exclusively from the plasma. Proteins extracted from homogenized bovine kidney were applied successively to three chromatographic steps on DEAE-Sephacel, Sephacryl S-300, and Mono Q column. The inhibitory activity was assayed using a CaOx crystallization system. The presence of IαI-related proteins was determined by electrophoresis and Western blotting. The results showed that kidney extract contained a 125-kDa protein that cross-reacted with anti-IαI antibodies. This protein inhibited CaOx crystallization efficiently. According to its molecular weight and immunoreaction with anti-IαI antibody, the 125-kDa protein could be pre-α-inhibitor. The latter is known to encompass a heavy chain and bikunin, which may explain its inhibitory activity against CaOx crystallization. Consequently, we hypothesize that kidneys may produce some IαI-related proteins that are involved in the inhibition of stone formation.

Key words Nephrolithiasis · Bovine kidney · Calcium oxalate crystallization · Inter-α-inhibitor · Bikunin · Chromatography

F. Atmani
Université Mohamed 1er, Faculté des Sciences,
Département de Biologie, Oujda-Morocco

S. R. Khan
University of Florida, College of Medicine,
Department of Pathology, Gainesville, Florida, USA

F. Atmani (✉)
Université Mohamed 1er, Faculté des Sciences,
Département de Biologie,
60000 Oujda-Morocco
e-mail: atmani@sciences.univ-oujda.ac.ma

Introduction

Urine is normally metastable with respect to calcium oxalate (CaOx), a common constituent of most human kidney stones. Thus under appropriate conditions CaOx may readily precipitate in the urine and lead to stone formation in the urinary tract. Fortunately, the presence of efficient inhibitory macromolecules, reduces the likelihood of crystallization in the urine [8, 11, 13, 16, 17, 24, 27]. The past 15 years have seen a tremendous increase in research in this area and a substantial amount of information is currently available about purification, characterization, and identification of inhibitory macromolecules [1, 2, 10, 12, 19, 27]. We identified one such molecule, uronic acid-rich protein (UAP), a glycoprotein with a molecular weight of 35 kDa in human [1, 3] and rat urine [3]. In these studies, we have shown that UAP is a potent inhibitor of CaOx crystallization. Interestingly, both human and rat UAP exhibited structural similarity to bikunin, a subunit of inter-α-inhibitor (IαI) [3,5]. Later, we confirmed that UAP is bikunin [4]. Moreover, we have demonstrated that urine contains two bikunins, one of which is a potent inhibitory of CaOx crystallization. The differences between the two urinary bikunins may be due to their origin. We hypothesized that one form of bikunin may derive from plasma and the other from the kidneys. The aim of this study was to purify inter-α-inhibitor-related proteins from bovine kidney and test their inhibitory activity on CaOx crystallization.

Materials and Methods

Materials

Bovine kidneys were obtained from the Animal Facility of the Veterinary College at the University of Florida (Gainesville, FL). During transportation to the laboratory, kidneys were kept on ice and then processed immediately on arrival as described in the Method section.

Diethylaminoethyl (DEAE)-Sephacel, Sephacryl S-300, and Mono Q HR 5/5 column were purchased from Pharmacia Biotechnology, N.J. Before each use, gels or columns were recycled according to the manufacturer's recommendations. Salt concentration was measured by conductivity meter (Fisher Scientific, Pa). Dialysis tubing with a molecular weight cut off of 6000–8000 Da was obtained from Spectrapor (Spectrum Medical Industries Calif.). Normal human serum inter-α-inhibitor polyclonal antibody (Code AXL 424) was purchased from Accurate Chemical and Scientific, N.Y. [^{45}Ca] Calcium Chloride was purchased from Amersham Corporation, IL Electrophoresis, transfer blot apparatus, low pre-stained molecular weight markers, and reagents for immunostaining were obtained from Bio-Rad Laboratories, Calif. Nitrocellulose membranes were purchased from Millipore Bedford, USA and the second antibody was from Hyclone Laboratories, Utah. All reagent grade chemicals were acquired from Fisher Scientific.

Methods

Kidney tissue preparation

Part of bovine kidney (256 g) was sliced into small pieces after removing fatty tissue and washed several times with TRIS buffer (TRIS 0.05 M, NaCl 0.1 M, pH 7.3) to remove any blood contaminant. Then, the tissue was homogenized in a blender with 500 ml of TRIS buffer and centrifuged at 8000 rpm for 20 min. The pellet obtained was resuspended in 300 ml of TRIS buffer, homogenized again, and centrifuged. This procedure was repeated three times. All supernatants were combined and heated to 65°C in a boiling-water bath with stirring. After the solution reached 65°C, it was immediately cooled on ice and centrifuged at 8000 rpm for 20 min. The supernatant was dialyzed overnight against demineralized water at 4°C. The dialysate obtained was adjusted to pH 7.3 and NaCl 0.1 M by adding NaOH and solid NaCl, respectively and then processed for protein purification as described below.

Protein isolation

The procedure used to purify proteins from bovine kidney extract was similar to that used for human urine as previously described [1]. Briefly, the sample was incubated with DEAE-Sephacel gel pre-equilibrated with TRIS 0.05 M, NaCl 0.1 M, pH 7.3 for 30 min with stirring. The suspension was filtered, washed with equilibrated buffer, and eluted successively with 300 and 200 ml of TRIS buffer. The combined eluents were dialyzed overnight against deionzed water. Then, the sample was adjusted to pH 7.3 and NaCl 0.1 M and injected to the DEAE-Sephacel column. Proteins were eluted with a liner gradient of NaCl solution from 0.1 to 0.5 M in TRIS buffer. The fractions containing IαI-related proteins were dialyzed, lyophilized, and injected into the Sephacryl S-300 column. The final purification step is the use of fast protein liquid chromatography (FPLC) chromatography on Mono Q HR 5/5 column using a linear gradient of NaCl solution from 0.1 to 0.5 M 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 each fifth tube using Lowry's method [26] and carbazole reaction [9], respectively. According to the method used, albumin and glucuronic acid were used as standards.

Inhibition assay

To determine the inhibitory activity in the fractions, a calcium oxalate crystallization system was used as described in a previous paper [3]. The inhibition assay involves using a mixture of 1 ml solution of calcium chloride (2 mM in TRIS 0.05 M, NaCl 0.15 M, pH 7.3) in the presence of [^{45}Ca] calcium chloride and 1 ml ammonium oxalate (2 mM in TRIS 0.05 M, NaCl 0.15 M, pH 7.3). The tubes containing proteins, lyophilized from 100-µl samples, were compared with those without proteins. At the end of the assay, tubes were centrifuged at 2000 *g* for 5 min and 0.5 ml of supernatant was withdrawn for radioactivity determination in an LS 7500 liquid scintillation counter (Beckman Instruments, Calif.).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting

Electrophoresis was performed on 1 mm thick 10% polyacrylamide gel by using Bio Rad Mini Proteinase II apparatus. Proteins and standards were electrophoresed after they were subjected to the reduction in the presence of SDS and β-mercaptoethanol. The protein bands were revealed by staining with Coomassie blue R-250 or transferred onto nitrocellulose membrane for immunological identification.

Gels and nitrocellulose membranes were pre-equilibrated with transfer buffer (TRIS 25 mM, glycine 192 mM, methanol 200 ml). Proteins were transferred by using the same buffer at 100 volts for 1 h at 4°C. The immunochemical staining steps were performed at room temperature by using polyclonal IαI antisera at the dilution of 1:1000 as primary antibody according to the procedure described by Towbin et al. [34]. The second antibody conjugated to alkaline phosphatase was used at the dilution of 1:5000. The staining was developed by using an alkaline phosphatase kit.

Results and discussion

It is now widely recognized that urine contains several macromolecules that prevent, reduce or modulate crystallization [1, 2, 10, 12, 19, 27]. Among them, bikunin, the light chain of the IαI-related family of proteins, with a molecular weight of 35 kDa, is a potent inhibitor of CaOx crystallization [2–5]. Based on our observations that urine contains two bikunins with slightly different crystallization inhibitory activity and molecular weights, we hypothesized that kidneys may produce glycoproteins related to IαI [4]. Since then we have demonstrated that bikunin as well as the two heavy chains of IαI, namely H1 and H2, are found in the matrices of human CaOx kidney stones and are also included in CaOx crystals experimentally induced in the human urine [6]. Recent results from our laboratory have shown additionally that rat kidneys as well as LLC-PK$_1$ and MDCK cells in culture, both express bikunin m-RNA [7, 20]. It is interesting then to purify these proteins from the kidneys and test their inhibitory activity against CaOx crystal formation.

The first step in the preparation of protein extract from bovine kidney is the homogenization of the tissue. In order to avoid blood contaminants, the kidney was sliced in tiny pieces and washed several times with TRIS buffer. The protein extract obtained as described in the Materials and Methods section was processed for protein purification procedure using three chromatographic steps. The sample was subjected to a DEAE-Sephacel bath and column chromatography using a linear gradient of NaCl solution from 0.1 to 0.5 M in TRIS 0.05 M, pH 7.3. The elution profile is shown in the Fig. 1. The

first half of the fractions (1 to 60) are rich in proteins. The CaOx crystallization inhibitory activity increases rapidly in the first 10 fractions and becomes stable between 70% and 80% in the remaining fractions. The electrophoresis followed by Western blot analysis showed that fractions 30 to 70 (fraction A) contain a protein with a molecular weight of about 125 kDa which cross-reacted with anti-IαI antibodies (Fig. 2). This evidence, by itself, cannot substantiate the real presence of IαI-related proteins in the kidney. Contamination from plasma cannot be ruled out even though the precautions and controls adopted during the preparation procedure seem to exclude this event. The same conclusion has been drawn by other researchers [14].

The second chromatography step was to submit the fraction "A" to the Sephacryl S-300 column. A typical elution profile is illustrated in Fig. 3. The electrophoresis followed by Western blot analysis showed that fractions 80 to 110 contain a 125-kDa protein which immunoreacted with anti-IαI antibodies (Fig. 4). Fractions containing this protein (fraction B) were subjected to the FPLC chromatography on Mono Q column for a final purification. After elution, samples from the fractions were subjected to gel electrophoresis to evaluate their protein content. According to the result of this analysis, the fractions were pooled into three fractions named "a", "b" and "c". These latter were then assayed on our calcium oxalate crystallization system using different concentrations from 2.5 to 20 µg/ml. The results are summarized in Table 1. As it can be seen, fraction "c" exhibits a strong inhibitory activity compared with "a" and "b" fractions. Electrophoresis and Western bolt analysis confirmed the presence of the 125-kDa protein in fraction "c" which cross-reacted with anti-IαI antibodies (not shown), According to its molecular weight and its positive immunoreaction with IαI antibodies, the 125-kDa protein could be the pre-α-inhibitor (PαI). PαI contains two chains: heavy chain H3 and bikunin [15, 28]. According to a new nomoclature proposed by Salier et al. [32], PαI is expressed by IαIH3,B. However, presence of the H3 chain has to be proven before the 125-kDa IαI-related protein obtained from bovine kidneys can be confirmed as PαI.

Figs. 1 and 2 illustrate the elution profiles, protein contents, uronic acid contents, and inhibitory activity of various fractions during chromatographic separation of the protein. It is clear that the inhibitory activity does not fully coincide with either the proteins or the uronic

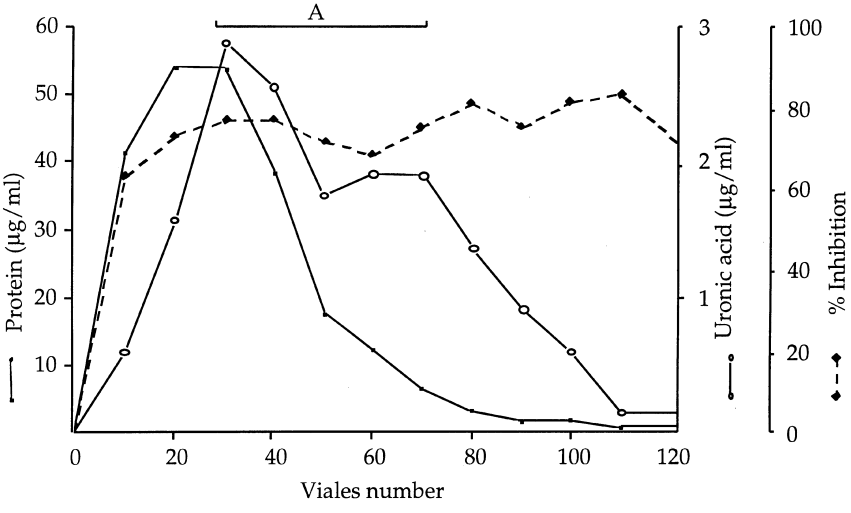

Fig. 1 DEAE-Sephacel ion exchange chromatography of proteins extracted from homogenized bovine kidney. The column was eluted by a linear gradient of NaCl solution from 0.1 to 0.5 M in TRIS 0.05 M, pH 7.3. Protein and uronic acid concentration were determined according to Lowry's method and the carbazole reaction respectively. Salt concentration was measured by a conductivity meter. Inhibition assay of fractions was assayed on a calcium oxalate crystallization system. Fraction "A" was collected for further chromatographic purification

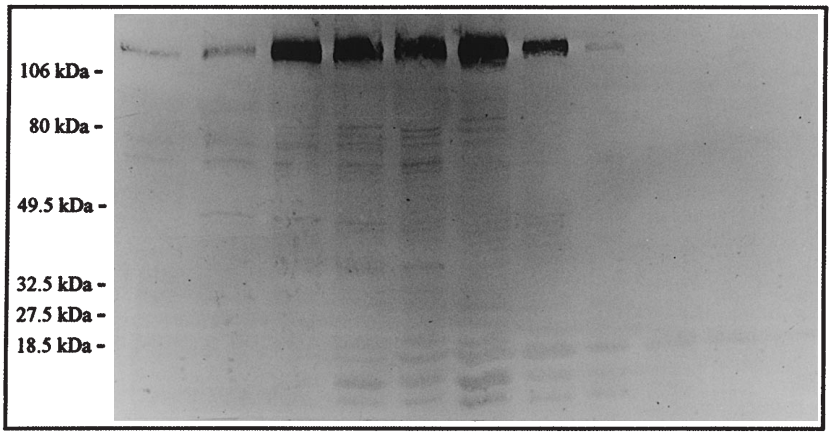

Fig. 2 Western bolt analysis using inter-α-inhibitor antibody of each tenth fraction obtained from the DEAE-Sephacel column. Molecular weight markers are shown on the left

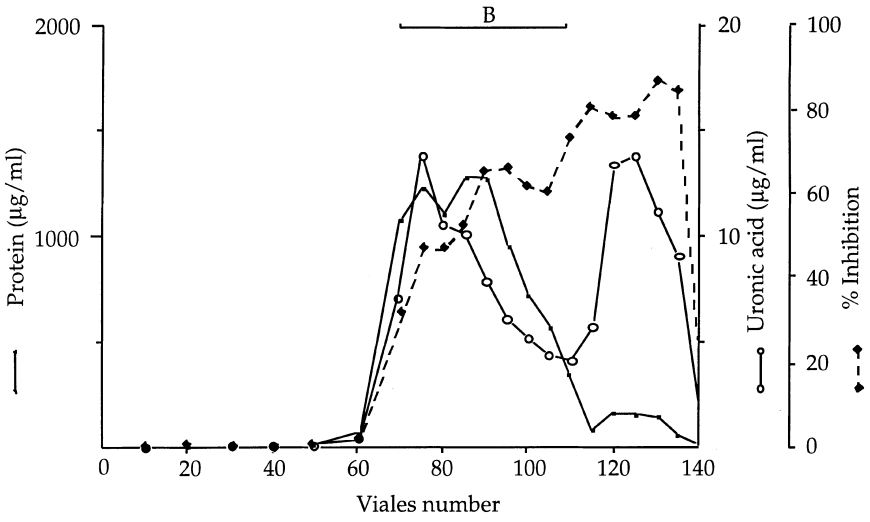

Fig. 3 Fraction "A" obtained from DEAE-Sephacel chromatography was eluted on a Sephacryl S-300 column using TRIS 0.05 M, NaCl 0.1 M, pH 7.3 as eluent. Protein concentration, uronic acid content, and inhibition activity were determined as in Fig. 1. Fraction "B" was taken for a further chromatographic step

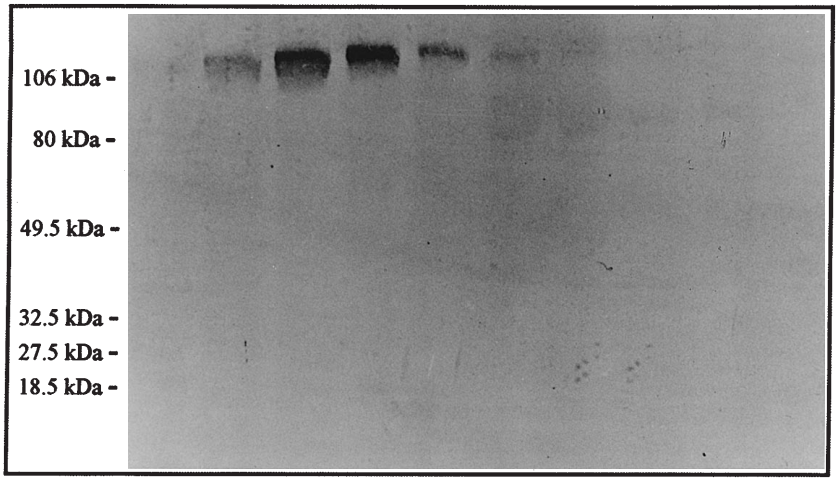

Fig. 4 Western bolt analysis by using inter-α-inhibitor antibody of each tenth fraction (from 70 to 140) obtained from Sephacryl S-300 column. Molecular weight markers are shown on the left

Table 1 Calcium oxalate crystal growth inhibitory activity of fractions obtained from the Mono Q column. The results are expressed as the percentage of calcium oxalate growth inhibited

Fraction	Concentration (µg/ml)			
	2.5	5	10	20
a	4.0	15.5	20.0	27.6
b	7.3	26.7	52.4	70.1
c	20.9	46.6	62.4	74.2

acid contents, indicating that substances other than proteins detectable by Lowry's method or macromolecules containing uronic acid may participate in inhibition of crystallization. It is also possible that these macromolecules are active at very low concentrations and no change in the activity is noticed after a certain level is reached. It is clear that fractions A and B contained the 125-kDa IαI-related protein which is a potent inhibitor of CaOx crystallization. The 125-kDa protein itself can be a modulator of CaOx crystallization or its light chain, bikunin may be responsible for the inhibitory activity against CaOx crystallization since we have demonstrated in several studies that bikunin is a potent inhibitor of CaOx crystal formation [1–4].

It is generally recognized that IαI-related proteins are synthesized in the liver [29, 31]. In addition Kastern et al. [21] have shown that α1-microglobulin mRNA levels, studied by an RNA dot blot assay, were high in the liver and kidney of rats. This finding leads to the hypothesis that bikunin is also produced by the kidney since α1-microglobulin and bikunin are synthesized by a common mRNA precursor [22, 25]. Moreover, in some pathological conditions such as inflammation, an increase in bikunin synthesis occurs in the absence of proteolytically induced breakdown of IαI complexes [18, 30]. Recent results from our laboratory have shown an expression of bikunin mRNA in rat kidneys as well as LLC-PK_1 and MDCK cells in culture [7, 20]. Presence of the 125-kDa protein described here and most other IαI-related proteins, in the kidneys and urine, indicate that these proteins are also produced by the kidneys, since under normal conditions they would be unable to pass through the glomerular sieves. Results of our recent studies de-

scribed here and elsewhere suggest that various IαI-related proteins can be produced by renal cells and some of them may be involved in modulation of crystallization within the nephron.

Acknowledgment We thank Dr. Abderahime Bouali for reading and revising this manuscript. This work was done at the Department of Pathology, University of Florida, Gainesville, USA and was supported by grants PO1-DK20586 and RO1-DK41434 of the National Institutes of Health, Bethesda, Maryland, USA.

References

1. Atmani F, Lacour B, Drüeke T, Daudon M (1993) Isolation and purification of a new glycoprotein from human urine inhibiting calcium oxalate crystallization. Urol Res 21:61
2. Atmani F, Lacour B, Streker G, Parvy P, Drüeke T, Daudon M (1993) Molecular characteristics of uronic acid-rich protein, a strong inhibitor of calcium oxalate crystallization in vitro. Biochem Biophys Res Comm 191:1158
3. Atmani F, Khan SR (1995) Characterization of uronic acid-rich inhibitor of calcium oxalate crystallization isolated from rat urine. Urol Res 23:95
4. Atmani F, Mizon J, Khan SR (1996) Identification of uronic acid-rich protein as urinary bikunin, the light chain of inter-α-inhibitor. Eur J Biochem 236:984
5. Atmani F, Mizon J, Khan SR (1996) Inter-α-inhibitor: a protein family involved in the inhibition of calcium oxalate crystallization. Scanning Microsc 10:425
6. Atmani F, Khan SR (1996) Inter-α-inhibitor, another serum protein with potential involvement in calcium oxalate nephrolithiasis. J Am Soc Nephrol 7:1798
7. Atmani F, Khan SR (1998) Role of inter-α-inhibitor and its related proteins in experimentally induced calcium oxalate urolithiasis. Localization of proteins and expression of bikunin gene in the rat kidney. Urol Res. In press
8. Azoury R, Goldwasser B, Wax Y, Perlberg S, Garti N, Sarig S (1985) Evaluation of the relative inhibitory potential of fractionated urinary macromolecules. Urol Res 13:199
9. Bitter T, Muir HM (1962) A modified uronic acid carbazole reaction. Anal Biochem 4:330
10. Bowyer RC, Brockis JG, McCulloch RK (1979) Glycosaminoglycans as inhibitors of calcium oxalate crystal growth and aggregation. Clin Chim Acta 95:23
11. Dent CE, Sutor DJ (1971) Presence or absence of inhibitor of calcium oxalate crystal growth in urine of normals and stone-formers. Lancet II:775
12. Doyle IR, Marshall VR, Dawson CJ, Ryall RL (1995) Calcium oxalate crystal matrix extract: the most potent macromolecular inhibitor of crystal growth and aggregation yet tested in human undiluted human urine in vitro. Urol Res 23:53
13. Edyvane KA, Hibberd CM, Harnett RM, Marshall VR, Ryall RL (1987) Macromolecules inhibit calcium oxalate crystal growth and aggregation in whole human urine. Clin Chim Acta 167:329.
14. Eleuteri AM, Angeletti M, Fioretti E (1994) Proteinase inhibitors of the Kunitz family in fallow deer organs: a comparative study. Comp Biochem Physiol 107B:539
15. Enghild JJ, Thøgersen IB, Pizzo SV, Salvesen G (1989) Analysis of inter-α-trypsin inhibitor and a novel trypsin inhibitor, Pre-α-trypsin inhibitor, from human plasma. Polypeptide chain stoichiometry and assembly by glycan. J Biol Chem 264:15975
16. Fleisch H (1978) Inhibitor and promoters of stone formation. Kidney Int 13:361
17. Felix R, Monod A, Broge L, Hansen NM, Fleisch H (1977) Aggregation of calcium oxalate crystals: effect of urine and various inhibitors. Urol Res 5:21
18. Gebhard W, Hochstrasser K (1986) Inter-α-trypsin inhibitor and its close relatives. In: Proteinase inhibitors. Amsterdam, Elsevier, pp 389–401
19. Hess B (1994) Tamm-Horsfall glycoprotein and calcium nephrolithiasis. Miner Electrolyte Metab 20:393
20. Iida S, Johnson-Tardieu J, Glenton PA, Byer K, Peck AB. and Khan SR (1997) Molecular detection of bikunin in normal and oxalate exposed kidneys and renal epithelial cells. J Am Soc Nephrol 8:563A
21. Kastern W, Björck L, Åkerström B (1986) Developmental and tissue-specific expression of α1-microglobulin mRNA in the rat. J Biol Chem 261:15070
22. Kaumeyer JF, Polazzi JO, Kotick MP (1986) The mRNA for a proteinase inhibitor related to the HI-30 domain of inter-α-trypsin inhibitor also encodes α1-microglobulin (protein HC). Nucleic Acids Res 14:7839
23. Khan SR (1997) Intercation between stone-forming calcific crystals and macromolecules. Urol Int 59:59
24. Lanzalaco AC, Singh RP, Smesko SA, Nancollas GH, Sufrin G, Binette M, Binette JP (1988) The influence of urinary macromolecules on calcium oxalate monohydrate crystal growth. J Urol 139:190
25. Lindqvist A, Bratt T, Altieri M, Kastern W, Åkerström B (1992) Rat α1-microglobulin: co-expression in liver with the light chain of inter-α-trypsin inhibitor. Biochim Biophys Acta 1130:63
26. Lowry OH, Rosebrouch NR, Farr AL, Randall RJ (1951) Protein measurement with the folin phenol reagent. J Biol Chem 193:265
27. Nakagawa Y, Abram V, Kezdy FJ, Kaiser ET, Coe FL (1983) Purification and characterization of the principal inhibitor of calcium oxalate monohydrate crystal growth in human urine. J Biol Chem 258:12594
28. Rouet P, Daveau M, Salier JP (1992) Electrophoretic pattern of the inter-α-trypsin inhibitor family proteins in human serum, characterized by chain-specific antibodies. Biol Chem Hoppe-Seyler 373:1019
29. Salier JP, Diarra-Mehrpour M, Sesboüé R, Bourguignon J, Benarous R, Ohkubo I, Kurachi S, Kurachi K, Martin JP (1987) Isolation and characterization of cDNAs encoding the heavy chain of human inter-α-inhibitor (IaTI): unambiguous evidence for multipolypeptide chain structure of IaTI. Proc Natl Acad Sci USA 84:8272
30. Salier JP (1990) Inter-α-trypsin inhibitor: emergence of a family within the Kunitz-type protease inhibitor superfamily. TIBS 15:435
31. Salier JP, Chan P, Raguenez G, Zwingman T, Erickson RP (1993) Developmentally regulated transcription of the four liver-specific genes for inter-α-trypsin inhibitor family in mouse. Biochem J 296:85
32. Salier JP, Rouet P, Raguenez G, Daveau M (1996) The inter-α-inhibitor family: from structure to regulation. Biochem J 315:1
33. Shiraga H, Min W, Vandusen WJ, Clayman MD, Miner D, Terrell CH, Sherbotie JR, Foreman JW, Przysiecki C, Neilson EG, Hoyer JR (1992) Inhibition of calcium oxalate crystal growth in vitro by uropontin: another member of the aspartic acid-rich protein superfamily. Proc Natl Acad Sci USA 89:426
34. Towbin H, Stachelin T, Gordon J (1979) Electrophoretic transfer of protein from polyacrylamide gels to nitro-cellulose sheets: procedures and some applications. Proc Natl Acad Sci USA 76:4350