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Concentration of a Potent Calcium Oxalate Monohydrate Crystal Growth Inhibitor in the Urine of Normal Persons and Kidney Stone Patients by ELISA-Based Assay System Employing Monoclonal Antibodies

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Abstract Standardized calcium oxalate monohydrate (COM) crystal growth assay system was employed to study the ability of various test samples to influence growth rates of COM crystals. The inhibitory activity (IA) of various samples was expressed in terms of inhibitory units. Urine samples obtained from normal persons and kidney stone patients were found to have IA of 3.18 ± 0.62 and 1.02 ± 0.08, respectively. A potent inhibitor having molecular weight between 14.2 and 16.2 kDa was found to be primarily responsible for the differences observed in the urinary IAs between normal persons and kidney stone patients. The potent inhibitor was found to be tightly associated with a chromophore resembling Urobilirubin. An ELISA based assay system, using monoclonal antibodies against the above most potent inhibitor confirmed the difference observed in the urinary IA between the normal persons and kidney stone patients. This assay system has the potential to be routinely used to screen human beings for potential stone formers. J. Cell. Biochem. 90: 1261–1275, 2003.

Key words: calcium oxalate; stones; inhibitors; urine; monoclonal antibody

Urolithiasis, the formation of calculi in urinary tract, is a widespread disease afflicting mankind and continues to pose a universal health problem even today. It is a complex, multifactorial disease resulting from an interaction between environmental and genetic factors. Although calculi can be lodged in any part of the urinary tract, e.g., urethra, bladder, ureters, and kidneys, yet during the last century interestingly the incidence of its occurrence has shifted from the lower (bladder and ureter) to the upper (kidneys) urinary tract.

Calcium oxalate has been shown to be the main component of about two thirds of all urinary calculi. Calcium oxalate monohydrate (COM) has been found to initiate mineralization followed by the deposition of calcium oxalate dihydrate (COD) on it [Cao et al., 1993; Verkoelen et al., 1995]. In addition to calcium oxalate, urinary stones have also been found to contain phosphates, uric acid, and magnesium ammonium phosphates with apatite and struvites predominating.

In vertebrates, the physicochemical studies have shown that various body fluids are supersaturated with respect to various mineral phase forming constituents. The question that naturally arises is why there are so few cases of pathological mineralization and why all our body tissues do not get mineralized under physiological conditions to turn us into pillars of stones. To explain this paradox, scientists

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have postulated that either the body fluids are undersaturated with respect to various obligatory precursors of the final forms of the mineral phase or the body fluids may contain certain biomolecules which can control the mineralization process by acting as inhibitors or promoters of mineralization. Studies by various workers have shown that body fluids contain both low molecular weight (e.g., Mg^{2+}, citrate, pyrophosphate, amino acids, nucleotides, etc.) and high molecular weight substances (e.g., polypeptides, proteins, glycoproteins, etc.), which by acting as inhibitors of mineral phase formation may play an important role in the etiology of urolithiasis [Howard and Thomas, 1958; Grover et al., 1994; Tandon et al., 1997, 1998; Aggarwal et al., 2000].

The role of inhibitors in controlling biological mineralization may be of evolutionary importance. Crystallization in specific organs from supersaturated body fluids may impair their functions. Slavkin et al. [1992] and Goodman et al. [1995] observed that biomineralization of certain specific tissues under specific pathological conditions could be due to a metabolic defect leading to either the synthesis of specific promoters or inactivation of potent inhibitory bioregulatory molecules. Whatever may be the nature of this effect at the molecular level, it is logical to presume that this endogenous disorder must occur well in advance of the occurrence of the stone problem. Hence, an early detection of this abnormality could go a long way in the clinical management of the urolithiasis problem.

Review of literature revealed that no available analytical method has the potential to be routinely used in a clinical laboratory to determine the levels of inhibitors/promoters in body fluids/samples.

The present study was thus undertaken with the following objectives.

i. To investigate the effect of urine samples from normal persons and kidney stone patients on in vitro mineralization by the assay system involving the precipitation of calcium and oxalate as mineral phase.
ii. To isolate, purify, and characterize the potent biomolecule(s) from human urine which are responsible for its inhibitory potency.
iii. To raise monoclonal antibodies against the most potent human urinary inhibitor for developing an ELISA based method to estimate the concentration of potent inhibitor in urine of normal persons and kidney stone patients.

MATERIALS AND METHODS

Materials

DEAE–cellulose, (Sigma, St. Louis, MO); Biogel-10, Sephadex G-150, Acrylamide, *N*,*N*-methylene bis-acrylamide, ammonium persulphate, *N*,*N*,*N*,*N* tetramethylenediamine (TEMED), SDS, molecular weight markers (Bio-Rad, Hercules, CA).

Assay Systems

Ability of urine samples to influence COM crystal growth was determined by the method of Nakagawa et al. [1981]. Aliquots of 24 urine samples were filtered to remove any suspended material. Urine sample containing 1.0 μg creatinine was added to 10.0 ml of 5×10^{-2} M sodium acetate buffer (pH 5.7) containing 9.6×10^{-2} M NaCl, 1.0×10^{-3} M calcium chloride, and 2.0×10^{-4} M sodium oxalate with traces of ^{14}C-oxalate. The reaction was started by addition of 1.0 ml of seed crystal slurry (1.5 mg/ml). Rate of decrease of ^{14}C-oxalate from the solution at different time intervals was determined in case of a time course study of COM crystal growth. To measure the inhibitory activity (IA) units of a given inhibitor, 1.0 ml aliquot was withdrawn after 40 min of incubation at 37°C and filtered through 0.22 μm pore size Millipore filter. The radioactivity in the filtrate was counted using a liquid Scintillation Counter (Packard 1900 CA). The IA was expressed as inhibitory units. A control flask containing 1.0 ml of buffer instead of urine was processed identically. The IA was thus calculated using the following equation:

$$\text{Inhibitory activity} = \frac{C_t - C_w}{C_i - C_t} \times \frac{C_i - C_\infty}{C_w - C_\infty}$$

$C_i = {}^{14}$C-cpm at zero time.

$C_t = {}^{14}$C-cpm in the presence of an inhibitor at 40 min.

$C_w = {}^{14}$C-cpm in absence of an inhibitor at 40 min.

$C_\infty = {}^{14}$C-cpm at 24 h.

SELECTION OF SUBJECTS

Twenty four-hour urine samples were collected form normal male and female volunteers

and kidney stone patients between 20 and 55 years of age. Kidney stone patients were those who were admitted to the Urology Department of PGIMER, Chandigarh for surgical removal of their stones. The stones were detected by X-ray of the abdomen and confirmed by intravenous pyelography. Only those male and female patients who were suffering from no other abnormality and whose stones were of non-recurrent and non-infectious nature were selected for the present study. All patients and normal subjects were advised to void their bladder at 8 O'clock in the morning (which was discarded and thereafter, all the urine was collected in plastic bottles (containing a few crystals of thymol as preservative) till 8 O'clock the next day morning. The samples were chemically analyzed and preserved at 4°C to conduct other investigations.

Isolation, Purification, and Characterization of Potent Biomolecules From Urine Samples

Urine samples from normal persons and kidney stone patients were filtered through Whatman No. 1 filter paper and dialyzed against double distilled water with constant stirring using 12.5 kDa cut off molecular weight dialysis bags. Both dialysate and dialysant were concentrated to a known volume by lyophilization using Eyela Japanese lyophiliser and assayed for their ability to influence COM crystal growth. Further, the dialysant (>12.5 kDa) samples were used to isolate, purify, and characterize potent inhibitory biomolecules from normal persons. The activated DEAE–cellulose was pre-equilibrated with 5×10^{-2} M Tris-HCl buffer (pH 7.3) for batch separation of the potent biomolecules from the dialysant. Three hundred eighty milliliters of the gel were mixed with dialysant and stirred magnetically for 30 min at room temperature. The slurry was filtered through a sintered glass funnel (G-2) under vacuum in two batches. Each batch was washed with 5 L of equilibrating buffer. The adsorbed biomolecules were desorbed from the ion-exchanger by resuspending the gel in 800 ml of 5.0×10^{-1} M NaCl in 5×10^{-2} M. Tris-HCl buffer (pH 7.3). The slurry was filtered and the filtrate obtained was dialyzed against 20 L of glass distilled water for 36 h at 4°C by changing water after 12 h.

The pH of the known volume of dialysant was adjusted to 7.3. For further purification of potent biomolecules, the sample having 5×10^{-2} M NaCl was subjected to DEAE–cellulose column chromatography. A 20 cm long column (X K26) having an internal diameter of 2.6 cm obtained from Pharmacia (LKB, Sweden) was used. Pre-swollen DEAE–cellulose gel was mixed with starting buffer (5.0×10^{-2} M Tris HCl containing 5×10^{-2} M sodium chloride). The sample was eluted by a linear sodium chloride gradient (5×10^{-2} to 5.0×10^{-1}) in 5.0×10^{-2} M Tris-HCl buffer (pH 7.3) using a gradient mixer and the flow rate of the column was maintained at 8 ml/cm^2/h with the help of a peristaltic pump. Gilson's fraction collector was used to collect fractions of 4 ml each in test tubes containing two drops of 1% NaN_3. Concentration of protein in the fractions was determined spectrophotometrically by monitoring the absorption at 280 nm. Salt concentration of various fractions was determined by the conductivity meter and every fifth fraction was assayed for its ability to influence COM crystal growth.

Biogel-P10 Gel Filtration Chromatography

The main inhibitory fractions from DEAE–cellulose column were combined and dialyzed against distilled water. After dialysis, the color of dialysant was still found to be brownish, the color could be due to association of some pigment with the inhibitory biomolecule(s) as reported by Nakagawa et al. [1983]. The chromophore was found to absorb at 625 nm. The chromophore was dissociated from the main inhibitor by treatment of the pooled fractions with 50% aqueous formamide solution. The main inhibitory biomolecule(s) were isolated by gel-filtration using Biogel-P10 column. A 50 cm long column (X K26 column from Pharmacia) having an internal diameter of 2.6 cm was used for chromatography. The column was packed and equilibrated with eluant (50% aqueous formamide solution). Two milliliters fractions were collected using a Gipson's fraction collector by maintaining a flow rate of 5 ml/cm^2/h. Each fraction was assayed for its IA and protein concentration.

Sephadex G-150 Gel Filtration Chromatography

Further purification of the potent biomolecules from Biogel-P10 fractions influencing COM crystal growth was done by Sephadex G-150 gel filtration chromatography. Sephadex G-150 (super fine grade, dry bead diameter

10–40 μm) was allowed to swell over night in distilled water. A 110 cm long column having internal diameter of 2.6 cm with X K26 column adoption was used. The swollen gel was mixed with 5×10^{-2} M Tris-HCl buffer (pH 7.3) containing 2.0×10^{-1} M NaCl and 2.0×10^{-2}% NaN_3. The fractions were collected at a flow rate of 2.9 or 1.95 ml/cm^2/h. Protein concentration was determined spectrophotometrically by measuring absorption at 280 nm. Each fraction was also assayed for its ability to influence COM crystal growth.

Molecular Weight Determination of Purified Inhibitor(s)

Method of Whitaker [1963] was followed to determined the molecular weights of the potent biomolecules by standardizing the G-150 column with the standard proteins obtained from Sigma. Standard proteins (kDa) were: apo protein (6.5), cytochrome c (12.4), carbonic anhydrase (29.0), and albumin (66.0). Void volume was determined using dextran blue (2,000). The molecular weight of the potent inhibitory biomolecule(s) purified by Sephadex G-150 column was also estimated by SDS–PAGE as described by Laemmli [1970]. Resolving (12.5%) and stacking (5%) gels were prepared. After electrophoresis, protein bands were silver stained (Bio-Rad10). Standard proteins for calibration (kDa) were: phosphorylase b (97.4), serum albumin (66.2), ovalbumin (45), carbonic anhydrose (31), trypsin inhibitor (21.5), and lysozymc (14.4).

Quantitative Chemical Analysis

The concentration of calcium, phosphorous, oxalate, creatinine, citrate, total carbohydrates, and protein in the samples were determined by the methods of Trinder [1960], Gomori [1941], Hodgkinson and Wiliams [1972], Bonsenes and Taussky [1945], White and Davies [1963], Roe [1995], Lowry et al. [1951].

Establishment of Hybridoma Clones

Six to eight weeks old inbred BALB/c mice were given three subcutaneous injections of purified inhibitor (50 μg) at intervals of 15 days. Before injections, the inhibitor was emulsified in Freund's complete adjuvant for first injection while boosters were given in incomplete adjuvant. The mice were bled through the cardiac puncture and sacrificed by cervical dislocation. Spleen cells were fused with PAI-O mouse myeloma cells in a ratio of 10:1 (spleen cells to myeloma cells) by using PEG 4000 (Sigma) following a procedure described by Galfre and Milstein [1981]. Hybridoma clones were screened by testing the supernatant in an ELISA as described below and eventually subcloned by limiting dilution technique. Monoclonal antibody sub-types were determined by ELISA using sub-type specific antisera (Sigma).

Purification of Monoclonal Antibodies

Immunoglobulin was purified from ascites fluid by affinity chromatography on protein A-Sepharose (Pharmacia) according to standard procedure of Eye et al. [1978].

ELISA

ELISA plates (M/s Costar Corp., Cambridge, MA) were coated with 100 μl/well purified antigen (1 μg/μl) in 5.0×10^{-2} M carbonate buffer (pH 9.6) and incubated overnight at 9°C. Antigen solution was flicked from the plate and the plate was washed six times with phosphate-buffered saline (PBS) (pH 7.4) containing Tween-20. To saturate the wells, 150 μl of 5% skimmed milk powder in PBS (pH 7.4) was added to each well before incubating at 37°C for 2 h. The plate was washed six times with PBST and 100 μl of test supernatants were added to each well. The plate was again incubated at 4°C overnight. The plate was then washed six times with PBST and 100 μl of (1:5,000 dilution) of goat anti mouse horse radish peroxidase (HRP) conjugate was added to each well and the plate was re-incubated for 2 h at 37°C. It was washed six times with PBST and developed with 5.0×10^{-1}% 2,2′-azinobis-(3-ethyl benz-thiazoline-6-sulfonic acid) (ABTS) obtained from Sigma in 1.8×10^{-1} M citrate phosphate buffer, pH 4.0 containing 2.5×10^{-2}% H_2O_2. The plate was then incubated at room temperature by the addition of 10 μl of 10% SDS optical density was measured by reading absorption at 405 nm on ELISA reader.

Immunoblotting

The purified inhibitor band on polyacrylamide gel was transferred on the nitrocellulose paper (NCP) in transblot cell (Bio-Rad) containing transfer buffer (2.0×10^{-2} M Tris-base, 1.5×10^{-1} M glycine, and 20% methanol) and subjected to 100 mA current for 1 h. The transfer efficiency of the inhibitory protein was assessed by staining one of the NCP strip with Ponceau

red. The remaining NCP strips containing the inhibitor were saturated with 5% skimmed milk powder in PBS (pH 7.4) at 4°C over night. The strips after washing five times with PBS containing 5.0×10^{-2}% Tween-20 were incubated for 2 h at room temperature with purified monoclonal antibodies in PBS containing 1.0×10^{-1}% skimmed milk. The strips were washed four times with PBST, and then incubated with 1:1000 diluted goat anti mouse HRP conjugate for 2 h at room temperature. The color was developed by using diaminobenzidine (DAB) with freshly added 15 μl of 30% H_2O_2. The reaction was stopped by washing the strips with distilled water.

RESULTS

Chemical Composition of Urine Samples From Normal Persons and Kidney Stone Patients

On an average kidney stone patients were found to excrete significantly higher amounts of lithogenic substances (calcium and oxalate) and lower amounts of known inhibitors of mineralization (citrate and magnesium) in the urine samples per day as compared to the normal persons (Table I). In contrast to 24 urinary excretions, kidney stone patients were found to excrete lower concentrations of not only the two important known inhibitors of mineralization but also the lithogenic substances as compared to the normal persons.

Ability of Urine Samples From Normal Persons and Kidney Stone Patients to Influence COM Crystal Growth Rate

The data presented in Figure 1 revealed that urine samples from both normal persons and kidney stone patients contain biomolecules which act as potent inhibitors of the rate of COM crystal growth. When the inhibitory potency was expressed in terms of the IA, urine samples obtained from kidney stone patients were found to be significantly less potent as compared to normal persons. The IAs of the urine samples obtained from kidney stone patients and normal persons were found to be 1.02 ± 0.08 and 3.18 ± 0.62, respectively.

It is further apparent from Figure 1 that the urinary IAs both in the case of normal persons and kidney stone patients are directly proportional to the urine volumes used in the assay systems. At identical volumes, the urine samples obtained from normal persons were found to be much more potent to inhibit the growth rate of COM crystals as compared to the kidney stone patients.

Isolation, Purification, and Characterization of the Potent Inhibitory Biomolecules From the Urine of Normal Persons

It is apparent from the data presented in Table II that the significant differences, in the inhibitory potencies, observed between the whole urine samples obtained from normal

TABLE I. Analysis of Urine Samples From Normal Persons and Kidney Stone Patients

Parameter studied	Units	Normal persons (17)	Kidney stone patients (19)
Volume	ml/day	$1{,}012 \pm 122$	$1{,}881 \pm 135$***
pH	—	5.40 ± 0.095	5.75 ± 0.106^{NS}
Calcium	mmoles/day	4.12 ± 0.114	4.98 ± 0.242*
	μmoles/ml	3.68 ± 0.101	2.65 ± 0.161**
Phosphate	mmoles/day	23.37 ± 0.448	17.11 ± 0.554**
	μmoles/ml	23.09 ± 0.421	10.01 ± 0.298***
Oxalate	mmoles/day	0.34 ± 0.010	0.53 ± 0.022**
	μmoles/ml	0.33 ± 0.009	0.28 ± 0.013*
Citrate	mmoles/day	2.01 ± 0.100	1.39 ± 0.109*
	μmoles/ml	1.99 ± 0.092	0.74 ± 0.72***
Magnesium	mmoles/day	10.17 ± 0.280	6.22 ± 0.199**
	μmoles/ml	10.01 ± 0.254	3.31 ± 0.112***
Creatinine	mmoles/day	10.09 ± 0.475	9.80 ± 0.492^{NS}
	μmoles/mly	9.97 ± 0.457	5.21 ± 0.271**
Carbohydrates	g/day	0.011 ± 0.002	0.012 ± 0.005^{NS}
	mg/ml	0.0108 ± 0.002	0.006 ± 0.003**
Peptidal nitrogen	g/day	0.201 ± 0.108	0.281 ± 0.38**
	mg/ml	0.191 ± 0.015	0.149 ± 0.020*

All values are mean ± SE.
NS, not significant; *$P < 0.05$; **$P < 0.1$; ***$P < 0.001$ as compared to normal persons.

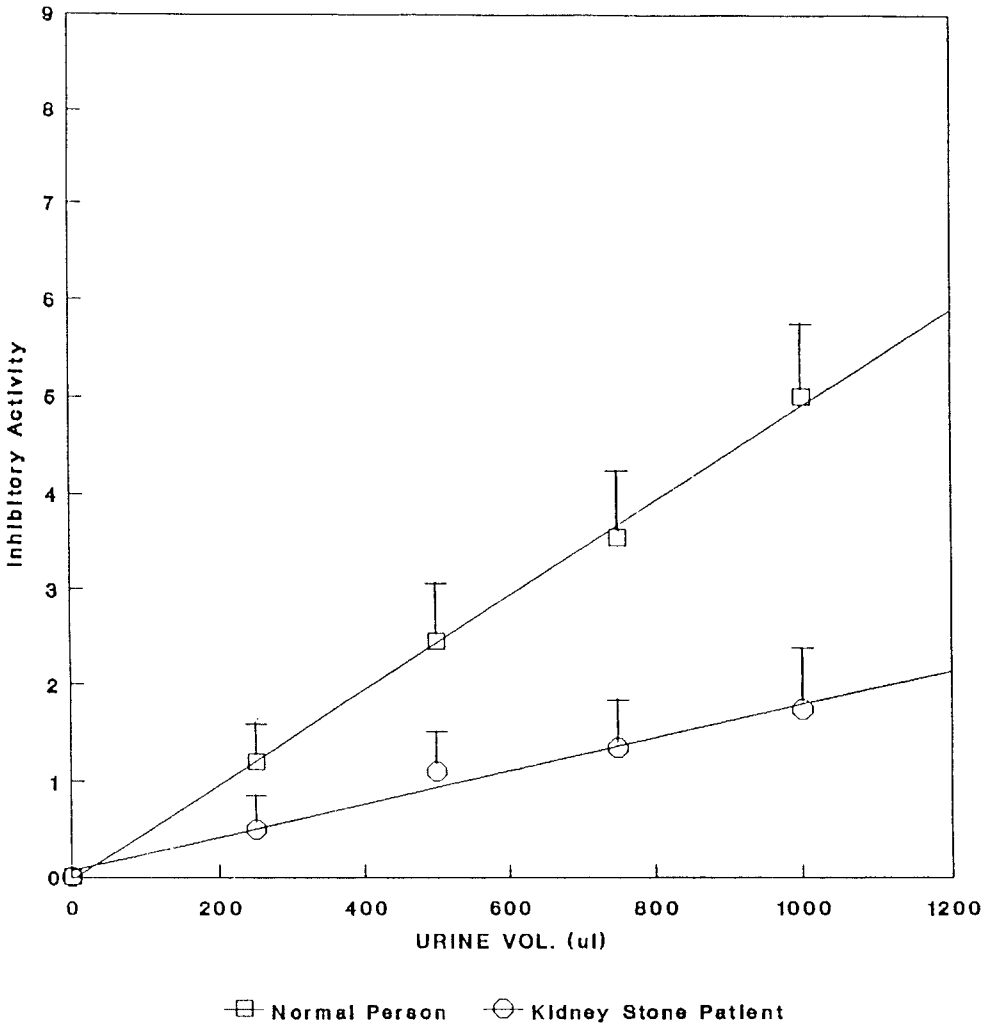

Fig. 1. Effect of urine concentration on the inhibitory activities (IAs) of urine samples from NP and KSP (2 μmoles creatinine/ml).

TABLE II. Effect of Filtration and Dialysis on the Inhibitory Activity (IA) of Urine Samples Obtained From Normal Persons and Kidney Stone Patients

		IA[a]	
S. no.	Samples used	Normal persons	Kidney stone patients
1.	Whole urine	3.18 ± 0.62	1.02 ± 0.08 (0.061)[e]
2.	Filtrate	1.25 ± 0.19[b**]	0.91 ± 0.11[b,NS] (0.065)[e]
3.	Dialysant	2.22 ± 0.42[c*,d**]	0.54 ± 0.09[c**,d**] (−0.122)[e]

NP, normal persons; KSP, kidney stone patients.
For dialysis Sigma's dialysis sacs (cut off molecular weight 12.5 kDa) were used.
One milliliter of sample used had 1 μmole of creatinine.
*$P < 0.01$, **$P < 0.05$; NS, not significant.
[a]Mean ± SE.
[b]Filtrate vs. whole urine (NP and KSP).
[c]Dialysant vs. whole urine (NP and KSP).
[d]Dialysant (NP vs. KSP).
[e]One kidney stone patient, whose urine sample after dialysis was found to stimulate COM crystal growth.

persons and kidney stone patients were primarily due to the biomolecules having molecular weight >12.5 kDa. Following the dialysis of the whole urine samples, the potent inhibitory biomolecules present in dialysant were concentrated by using DEAE–cellulose. DEAE–cellulose was washed with 5 L of equilibrating buffer to remove the adventitiously bound biomolecules. The inhibitory biomolecules bound to DEAE–cellulose were desorbed by washing the gel with 5.0×10^{-1} M NaCl in 5×10^{-2} Tris HCl buffer (pH 7.3). The sample thus obtained was dialyzed against distilled water using dialysis sacs having cut off molecular weights of 8 kDa to remove the salts.

Purification of Potent Inhibitory Biomolecules by DEAE–Cellulose (Anion-Exchange) Chromatography

DEAE–cellulose column was eluted by a linear sodium chloride gradient (5.0×10^{-2} M to 5.0×10^{-1} M) in 5.0×10^{-2} M Tris-HCl buffer (pH 7.3). It is apparent from the studies presented in Figure 2 that the inhibitory biomolecules got eluted out mainly under one major inhibitory peak (fractions 135–170) and two minor inhibitory peaks (fractions 91–112 and 115–134) at conductivity of 28, 23, and 25, respectively. All the fractions responsible for the three inhibitory peaks (i.e., fractions 90–170) were pooled together and dialyzed against glass distilled water to remove the salts. The dialysant was concentrated to a known volume by lyophilization and it was found to be brownish in color. The color was due to the presence of a chromophore showing absorption at 625 nm.

Separation of Chromophore From the Pooled Concentrated DEAE–Cellulose Inhibitory Fractions by Using Biogel-P10 Gel-Filtration Chromatography

Method of Nakagawa et al. [1983] was employed to separate the chromophore associated with the urinary biomolecules from the concentrated pooled DEAE–cellulose inhibitory fractions. The concentrated sample was mixed with 50% aqueous formamide solution. It is apparent from Figure 3 that the majority of the IA got eluted out in the void volume (fractions 30–36). The fractions responsible for this peak were found to be colorless. The rest of the IA got eluted out in a second peak corresponding to fractions 37–46. Both the inhibitory peaks were found to correspond to the protein peaks as determined by absorbance at 280 nm. The third peak (fractions 45–55) as measured by absorbance at 280 nm was found to have negligible IA and also showed absorbance at 625 nm.

It is further apparent from the present studies that the chromophore is not an essential part of the urinary inhibitor as no loss of IA was observed upon the dissociation of the chromophore form the main inhibitor.

Sephadex G-150 Gel Filtration Chromatography of the Potent Colorless Inhibitor of COM Crystal Growth Obtained From Biogel-P10 Chromatography

Fractions 30–36 responsible for the IA of colorless main inhibitory fractions of Biogel-P10 column were pooled, dialyzed, and concentrated before being subject to G-150 gel filtration chromatography. It is apparent from Figure 4 that three inhibitory peaks I_1, I_2, and I_3 corresponding to the apparent molecular weight of 68, 29, and 14.2 kDa were obtained. The above elution pattern strongly suggested that either the three inhibitory peaks were due to the presence of three distinct inhibitor biomolecules or these might be due to the presence of different aggregation states of the same inhibitory biomolecule. To differentiate between the above two possibilities, the various fractions responsible for the inhibitory potencies of I_1, I_2, and I_3 were pooled together and incubated with 1.0×10^{-2} M EDTA for 4 days. At the end, EDTA was removed by dialysis against distilled water. The dialyzed fractions were concentrated to a known volume. The concentrated sample was again subjected to G-150 column chromatography and eluted with Tris-HCl buffer (pH 7.3) containing 2.0×10^{-2} M NaCl. Only one inhibitory peak corresponding to the molecular weight of 14.2 kDa was obtained (Fig. 5). This peak corresponds to the peak I_3 observed in Figure 4. When this inhibitor was subjected to SDS–PAGE it gave a value of 16.2 kDa (Fig. 6).

Effect of Increasing Concentration of the Purified Inhibitor on the Rate of COM Crystal Growth

Different concentrations (7.5–17.5 μg/ml) of purified inhibitor were added to the assay system to study their effect on the rate of COM crystal growth under metastable concentrations. The purified inhibitor was found to be very potent inhibitor of the growth of COM

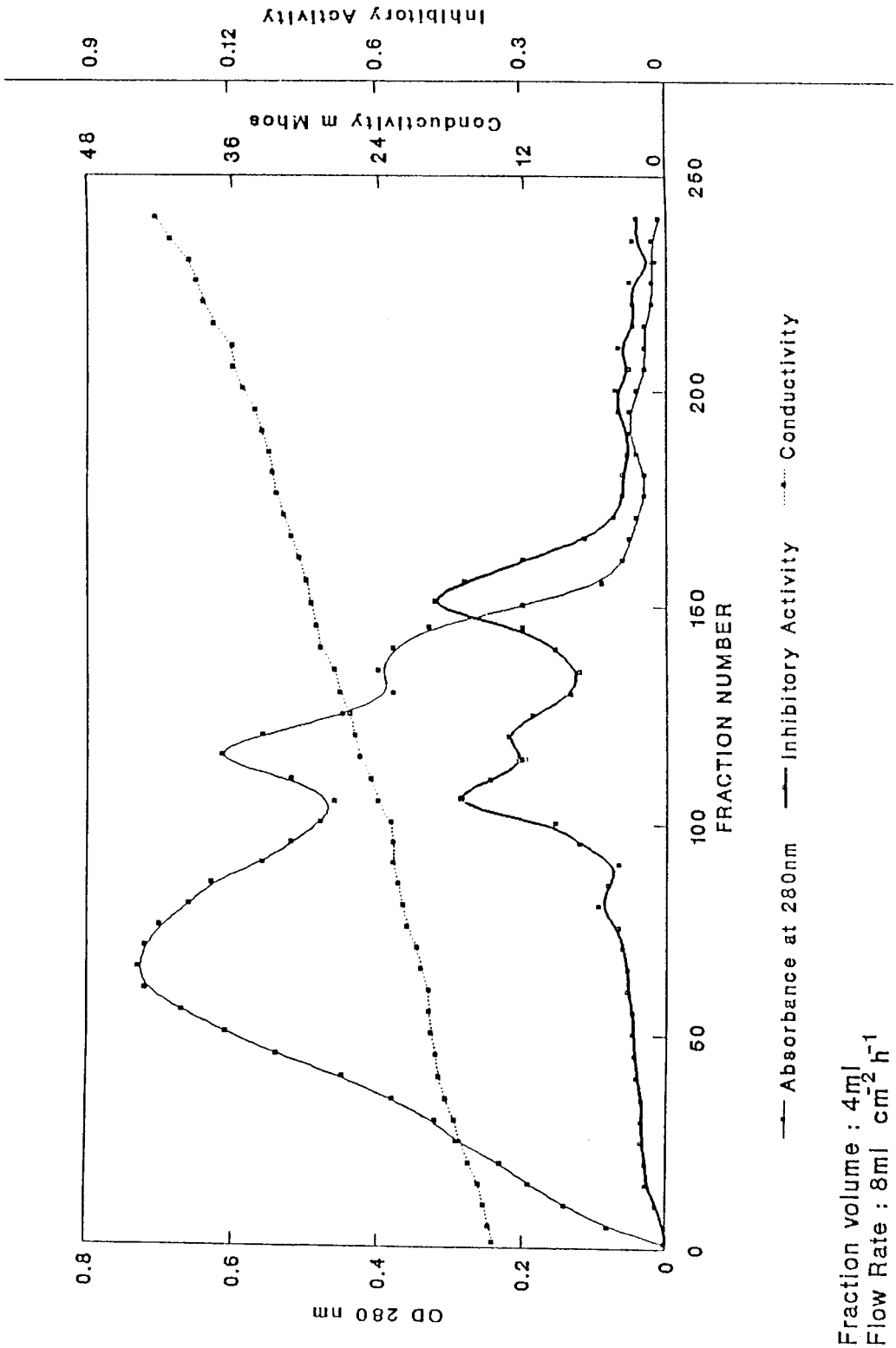

Fig. 2. DEAE–cellulose column chromatography of >8 kDa fraction from human urine.

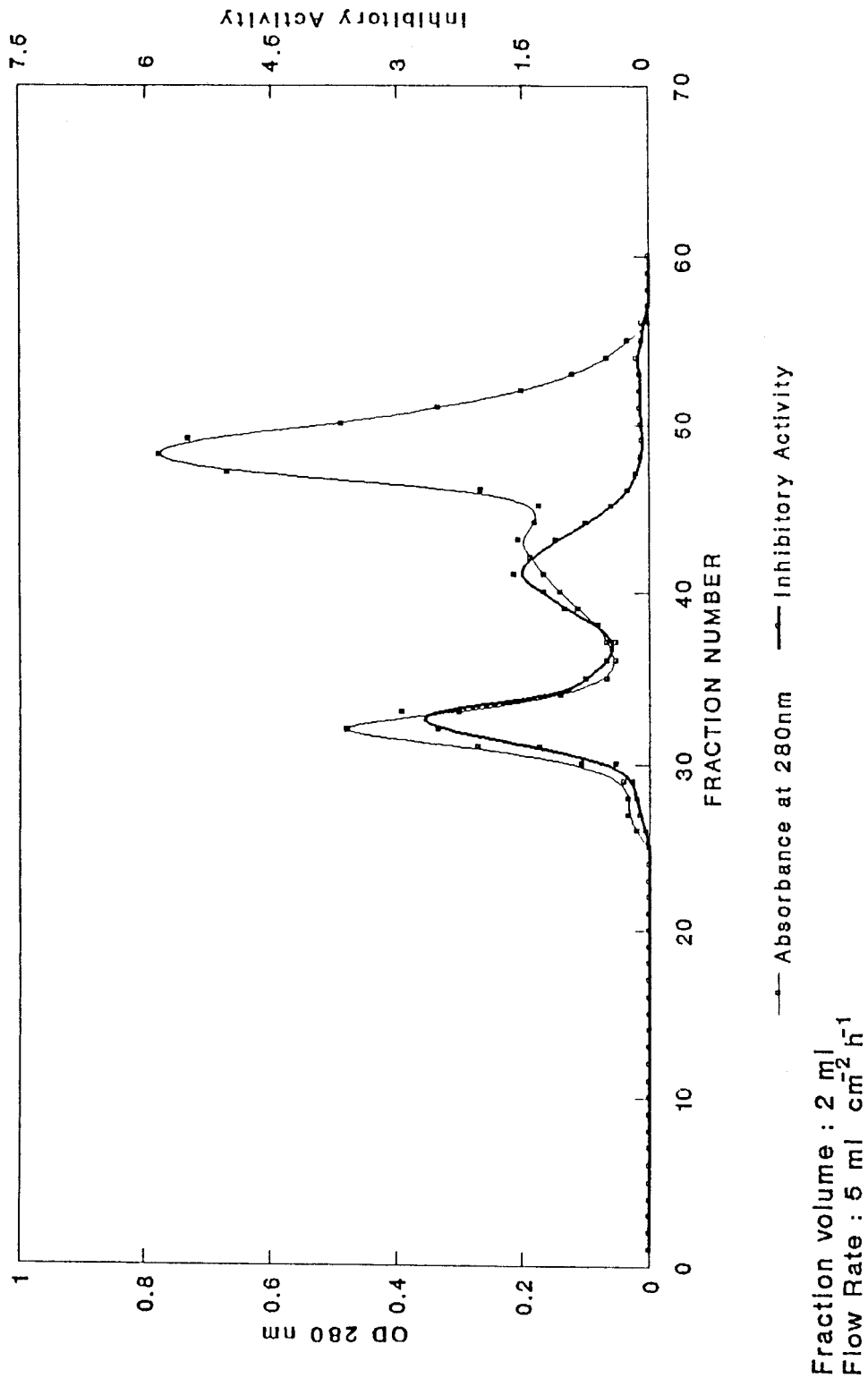

Fig. 3. Biogel-P10-chromatography of DEAE–cellulose pooled inhibitory fractions.

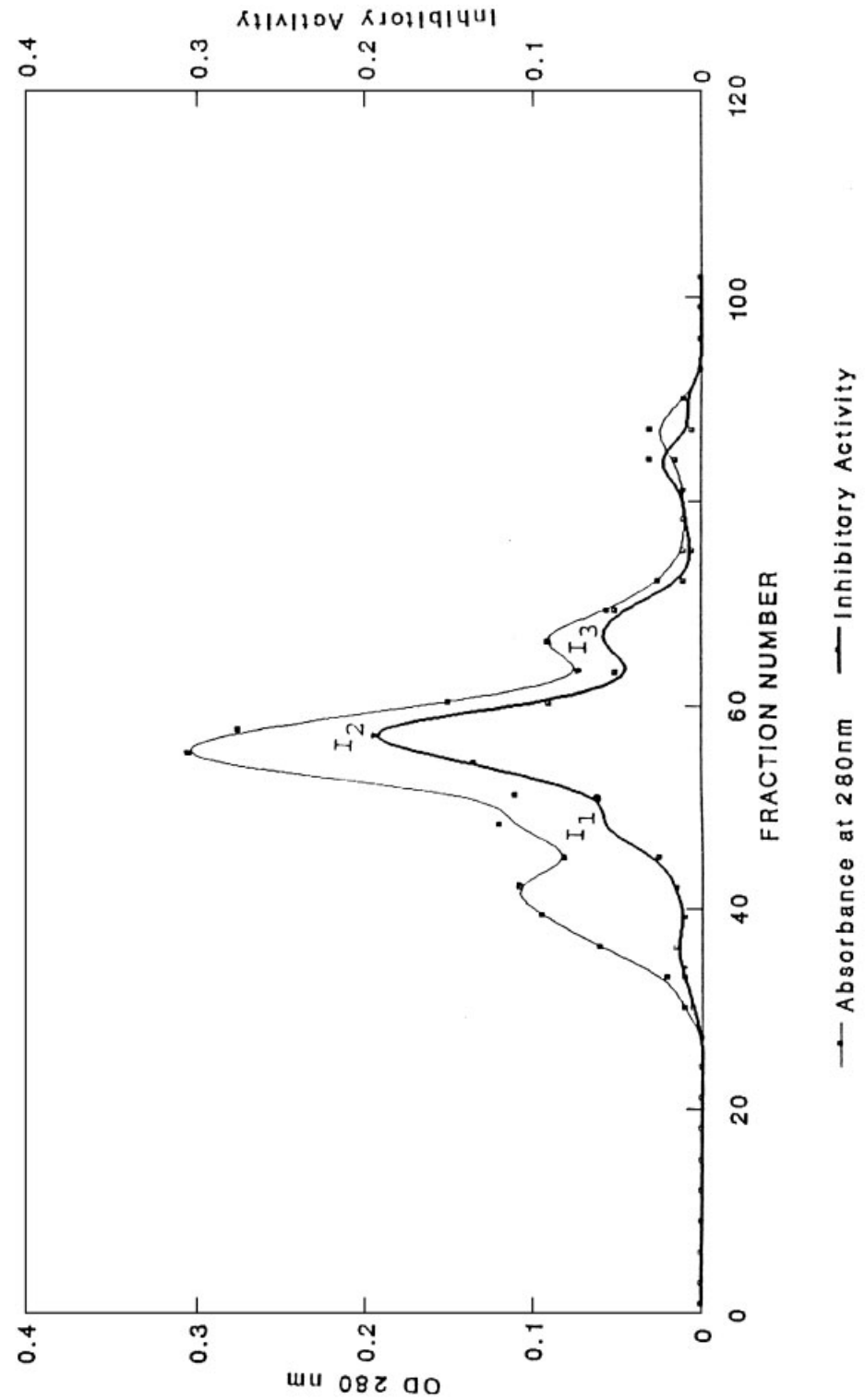

Fig. 4. Sephadex G-150 chromatography of potent inhibitors obtained from Biogel-10.

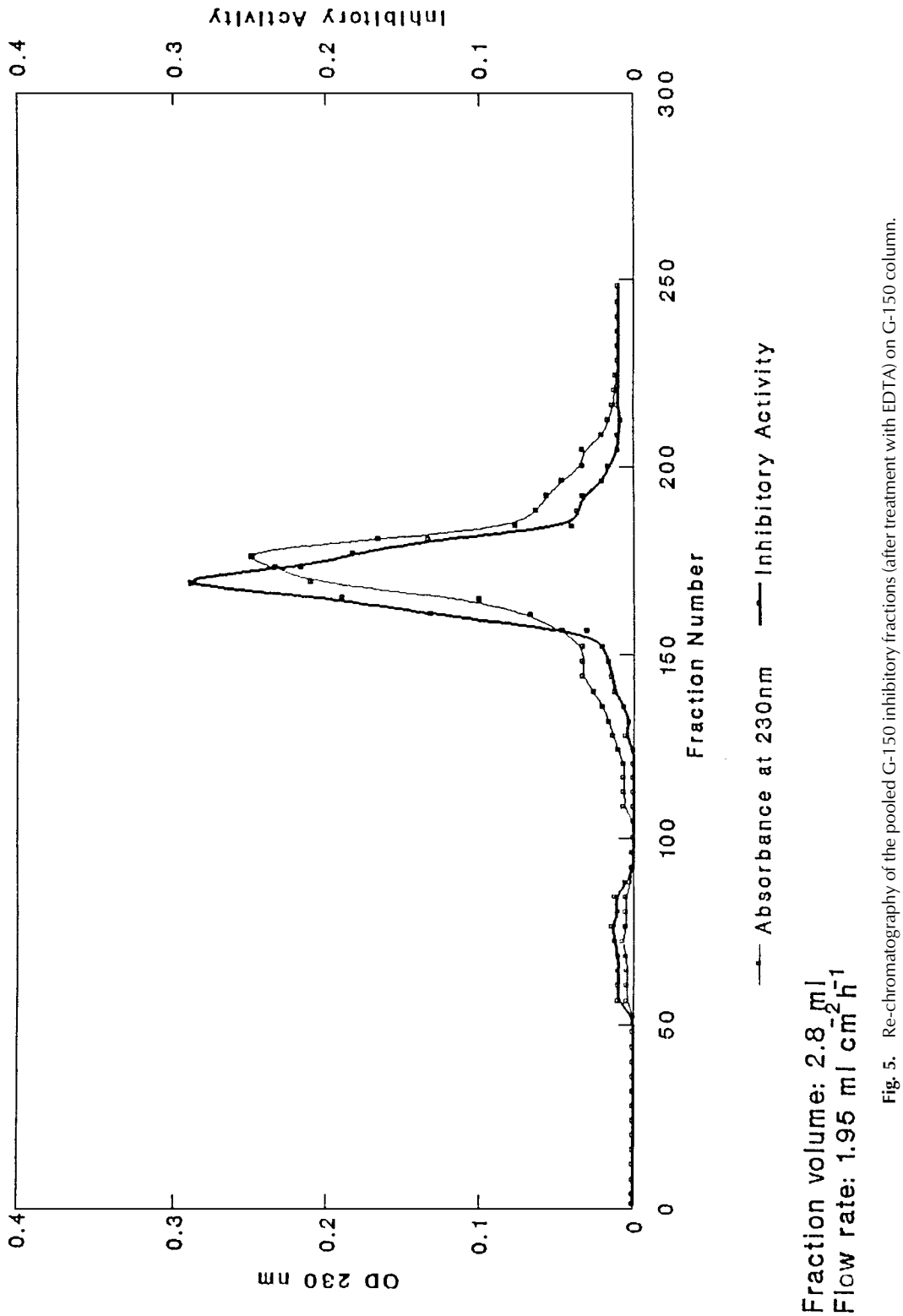

Fig. 5. Re-chromatography of the pooled G-150 inhibitory fractions (after treatment with EDTA) on G-150 column.

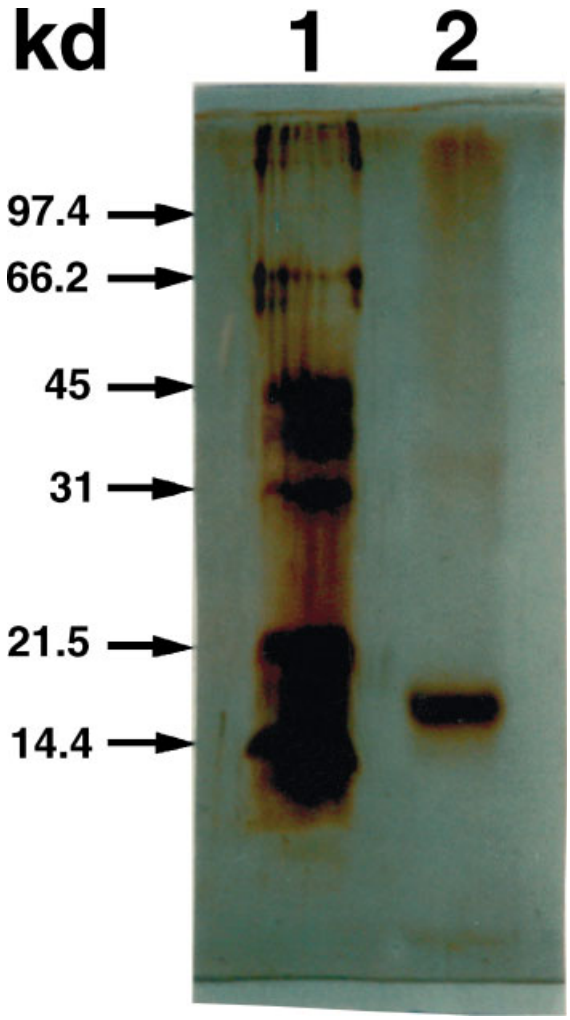

Fig. 6. SDS–polyacrylamide gel electrophoresis of purified inhibitory protein. **Lane 1**: Molecular weight markers. **Lane 2**: Purified inhibitory protein. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

crystal. At concentrations of inhibitor equivalent to 7.5, 12.5, 15.0, and 17.5 μg of protein, the IA units were found to be 1.8, 2.4, 3.2, and 3.9, respectively (Table III). The IA was found to be directly related to the inhibitor concentration expressed in terms of protein.

TABLE III. Effect of Different Concentrations of the Purified Urinary Inhibitor on the IA Units

S. no.	Amount of inhibitor added (μl)	Concentration of inhibitor (μg protein)	IA units
1.	90	7.5	1.8
2.	150	12.5	2.4
3.	180	15.0	3.2
4.	210	17.5	3.9

TABLE IV. Fusion Efficiency of Immune Spleen Cells With PAI-O Myeloma Cells

S. no.	Description of wells	No. of wells
1.	Total no. of wells with fused myeloma and splenocytes	72
2.	Wells with growing hybrids	29 (40.2%)
3.	Secretory hybrids as assessed by ELISA	11 (15.2%)

Production of Monoclonal Antibody Against Purified Inhibitor

BALB/c mice were immunized against the potent inhibitor and the immune splenocytes were obtained from the mice. Hybridization of PAI-O mouse myeloma cells with immune splenocytes resulted in successful fusion forming growing hybrids in 40.2% of the wells (Table IV). Assessment of the growing hybrids for the production of anti-inhibitor antibodies by ELISA, revealed that 15.2% of the hybrids were secretory. One hybrid from these wells was selected for further cloning and subcloning by limiting dilution method. Finally, based upon their anti-inhibitory antibody secreting abilities, one clone (10F9) was selected for the production of ascites in BALB/c mice. Isotyping of monoclonal antibody produced by 10F9 revealed that the antibodies produced by this clone were of IgG2a subclass.

Anti-inhibitor antibodies from ascetic fluid were purified by affinity chromatography using protein A-Sepharose column. The concentration of antibodies was determined by measuring the absorbance at 280 nm.

The Western–immunoblotting of the affinity chromatography purified monoclonal antibodies showed the presence of two inhibitory proteins with apparent molecular weights of 16 kDa (major band) and 29 kDa (minor band). The latter band perhaps represented the dimer form of the major inhibitor (Fig. 7). Normal mouse serum and an irrelevant monoclonal antibody were also tested against the main inhibitor or its aggregates. These antibodies were found not to react either with the inhibitory protein or with any of its aggregates.

Assay of the Potent Inhibitor of COM Crystal Growth in Urine Samples of Normal Persons and Kidney Stone Patients by ELISA Using Monoclonal Antibody

Table V presents the levels of the potent inhibitor in the urine sample of normal persons

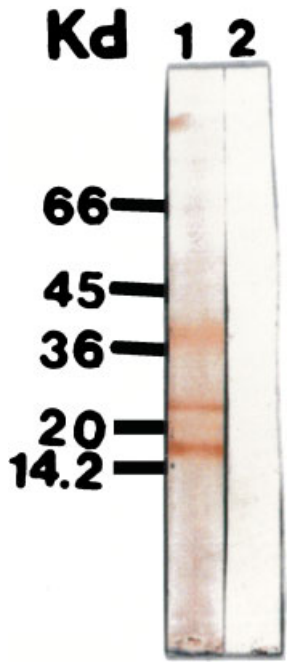

Fig. 7. Specificity of monoclonal antibody 10F9. Western–immunoblotting of inhibitory protein with MOAb 10F9 (**lane 1**), with serum from normal mouse (**lane 2**), with an irrelevant MOAb (**lane 3**). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

and kidney stone patients. The concentration was measured by ELISA method as per the procedure given in the methods. One hundred microliters/well of the urine samples (1:10 dilution) were used for coating ELISA plates. While 100 ml of affinity purified monoclonal anti-inhibitor antibodies (5 μg/ml) was used for the detection. It is apparent that females both in the case of normal persons and kidney stone patients excreted lower levels of the potent inhibitor in their urine as compared to their male counterparts. The studies further revealed that both sexes of kidney stone patients were found to excrete lower levels of the potent inhibitor in the urine as compared to those obtained in case of normal persons.

TABLE V. Average Levels of the Potent Urinary Inhibitor as Determined by ELISA Method Using Monoclonal Antibodies

	Normal persons (μg/ml)	Kidney stone patients (μg/ml)
Males (6)	$6.38 \pm 1.69^{a*,c**}$	$4.46 \pm 0.41^{b*,c**}$
Females (6)	$4.91 \pm 1.02^{a*,d**}$	$3.72 \pm 0.56^{b*,d**}$
Total subjects (12)	$5.65 \pm 1.65^{e**}$	$4.18 \pm 0.72^{e**}$

All values are mean ± SD.
*$P < 0.05$ and **$P < 0.01$.
NP, normal person; KSP, kidney stone patient.
[a]NP males vs. KSP females.
[b]KSP males vs. KSP females.
[c]NP males vs. KSP males.
[d]NP females vs. KSP females.
[e]NP vs. KSP.

DISCUSSION

The per day urinary excretions of lithogenic substances were found to be significantly higher and that of inhibitors of mineralization lower in kidney stone patients as compared to normal persons. However, when the urinary excretions of lithogenic substances or the inhibitors of mineralization were expressed on per unit volume basis, the urinary excretion of both inhibitors of mineralization and lithogenic substances were found to be lower in kidney stone patients as compared to normal persons. This is due to the fact that on an average kidney stone patients were found to excrete higher volumes of urine/day as compared to the normal persons. Both normal persons and kidney stone patients excreted in their urine potent inhibitors of COM crystal growth. As compared to normal persons the lower inhibitory potency of urine samples, obtained from kidney stone patients observed during the present studies are in agreement with the findings of other workers [Kumar and Jethi, 1975; Singla et al., 1978; Schwille et al., 1979; Leskovar, 1982; Thomas, 1982; Jarrar et al., 1984; Abraham and Smith, 1987; Zimmerhackl et al., 1996; Yasui et al., 1999; Suzuki et al., 2000]. We observed that approximately 35% of the total IA of urine samples from normal persons was found to be due to biomolecules, having molecular weights <12.5 kDa. As reported by various workers, cations (Mg^{2+}, Cu^{2+}, Zn^{2+}, Sn^{2+}, Al^{3+}, etc.), anions (F^-, PPi, citrate, phosphocitrate, NTP, small molecular weight acid polypeptides, etc.) may be responsible for the IA of <12.5 kDa fraction. Biomolecules having molecular weights >12 kDa were found to almost quantitatively account for the differences observed in the urinary IAs between normal persons and kidney stone patients. Determination of the molecular weight of the purified inhibitor by Sephadex G-150 and SDS–PAGE gave values of 14.2 and 16.2 kDa, respectively. It is thus apparent that the potent inhibitory biomolecule primarily responsible for the IA of >12 kDa fraction has a molecular weight between 14.2 and 16.2 kDa. The potent inhibitor was found to

have a tendency for aggregation and it was found to exist in at least three multiple molecular weight forms under the present experimental conditions. The aggregated forms of the inhibitor were found to dissociate upon treatment with EDTA, thus strongly suggesting that divalent cations may be involved in this aggregation process. A possible role for calcium in the aggregation has been postulated because it was the only cation present in appreciable amounts under the present experimental conditions.

The studies further revealed that the potent inhibitors in the urine were strongly associated with a chromophore showing absorbance at 625 nm. Nakagawa et al. [1981] isolated α_1-microglobulin from human urine and similarly observed that it has a chromophore associated with the protein. Although no attempt was made to characterize the chromophore but spectral studies tend to suggest that it could be urobilirubin. It is further apparent from the present studies that the chromophore is not an essential part of the urinary inhibitor as no loss of IA was observed upon the dissociation of the chromophore from the main inhibitor. The purified inhibitor was found to be very potent inhibitor of the growth of COM crystals. The IA was found to be directly related to the inhibitor concentration expressed in terms of protein. The inhibitor was found to significantly inhibit COM crystal growth even at protein concentrations between 7.5 and 17.5 μg. Nakagawa et al. [1981] observed that the factor responsible for 90% of the crystal growth IA of human urine is a non-dialyzable glycoprotein having a molecular weight of 1.4×10^4 daltons (14 kDa), having 10.3% carbohydrates, rich in acidic amino acids including γ-carboxy glutamic acid.

Although the inhibitory biomolecule isolated from human urine has not been fully characterized yet the observations made during the present studies have physiological and clinical relevance. It is further apparent that based upon molecular weight, elution profile, and behavior on SDS–PAGE, the potent inhibitory biomolecule is identical to the one isolated and purified from human embryonic kidney tissue culture medium by Nakagawa et al. [1981] but quite different from other known inhibitors/promoters, e.g., 220–240 kDa inter-alpha-inhibitor (IalphaI); 40 kDa (25 kDa when deglycosylated) bikunin; 44 kDa highly acidic phosphorylated glycoprotein; 35 kDa uronic acid-rich glycoprotein (UAP), and 95 kDa Tamm–Horsfall protein (THP) [Atmani and Khan, 1995, 1999; Zimmerhackl et al., 1996; Yasui et al., 1999; Suzuki et al., 2001] postulated from time to time to play an important role in urolithiasis.

During the present studies monoclonal anti-inhibitor antibodies of IgG2a subclass have been used for developing an ELISA method using polystyrene microplates coated with inhibitory protein for determining the concentration of the potent inhibitor in the urine of the normal persons and kidney stone patients. Using such an assay system, the concentrations of potent inhibitor in the urine samples of kidney stone patients were found to be significantly less ($P < 0.05$) than those of normal persons which is in conformity with the results obtained by direct non-immunological biochemical assay system (Table I). Since, it is logical to presume that whatever may be the nature of the endogenous defect(s) at the molecular level resulting in the lower concentrations of the potent inhibitor in the urine of kidney stone patients as compared to normal persons, these endogenous defects must occur well in advance of occurrence of urinary calculosis. It is hoped that the assay system developed during the present studies may help us to screen human population for potential stone formers. Needless to say that the above findings studies could prove to be of immense practical importance in the management of urinary calculosis disease. The assay system developed here can further be used to detect the site responsible for the biosynthesis of the potent inhibitor. Further biochemical and immunological studies are being planned to characterize the potent inhibitor obtained from the urine of normal persons and kidney stone patients so as to have a better understanding of its role in the etiopathogenesis of urinary stone disease.

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