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Proteins in renal stones and urine of stone formers

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Abstract Knowledge of the essential characteristics of macromolecules constituting the organic matrix of the nidus of urinary stones is required to understand the mechanism of urolithogenesis. The aim of this study was to isolate and characterise those stone nidus proteins. Using an extraction buffer containing SDS and β-mercaptoethanol, we were able to overcome known problems of protein isolation from urinary stone matrix. These proteins were characterised by a strong tendency to aggregate under reducing and denaturing conditions. On SDS-PAGE, their molecular weights range from ≤12 to 66 kDa. Antisera raised against stone matrix proteins showed a cross-reactivity between proteins isolated from different stones irrespective of their origin or mineral composition. Moreover, urinary proteins from stone formers also cross-reacted with these whereas there was no reaction with urinary proteins of non-stone formers. Western blotting confirmed these findings. Given the above summarised properties, it can be safely concluded that these proteins are prevalent in urines of stone formers, that they are selectively incorporated into renal stones of all aetiologies, and that they most likely have a role in nidus and, therefore, early stone formation.

Key words Urolithiasis · Urinary proteins · Stone matrix proteins · Low molecular weight proteins · SDS-PAGE

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Introduction

Renal calculi are composed of an outer crystalline phase which mostly contains urate, calcium (Ca), oxalate (Ox), phosphates (PO_4), as well as, to a lesser extent, magnesium (Mg). The inner core of the stone contains the organic stone matrix. Electron microscopy imaging shows that the arrangement of proteins and crystals in the matrix is distinctively different from that in the crystalline phase [2]. A better understanding of the components of stone matrix and their interaction on a molecular level seems therefore mandatory in order to gain insight into urolithogenesis in its early stages of stone nidus formation.

Although proteins derived from urinary calculi, crystals and urines constitute the group of macromolecules that has been studied most extensively by researchers all over the world, their exact role in urolithogenesis remains unclear [7, 9]. Various proteins have been suggested as promoters and inhibitors in urolithiasis [7]. However, so far only a few proteins extracted from stone matrices have been fully characterised, which is mainly due to technical difficulties in isolating them in an intact form. Although various methods for the extraction of proteins from organic stone matrix have been tried [2, 30], all these methods could not completely isolate all the proteins. Consequently, a complete profile of renal stone proteins is not available to date.

The aim of this study was, therefore, to find a suitable method of extraction for these proteins in order to extract a maximum amount of biological material without causing a significant degradation. Moreover, we aimed at characterising some of the physico-chemical and antigenic properties of these stone matrix proteins in order to further elucidate their possible role in crystal–protein interaction, and, therefore, their potential role in urolithogenesis.

Materials and methods

Urinary proteins

Twenty-four h urines were collected from eight normal subjects without any evidence or history of stone formation, and from 25 known stone formers who, however, were stone free at the time of the urine collection and had not been subjected to stone treatment for at least 6 weeks prior to collection. Urine samples were frozen at −70°C until further use.

Renal stones

For this study, 35 human renal stones of various sizes and composition were analysed. All stones had been removed by means of open surgery from patients of the Urology Section of the Aga Khan University Hospital (AKUH) in Karachi, Pakistan. Chemical stone analysis of all 35 stones was performed by the clinical laboratory of the AKUH by means of a Merckognost kit (Merck, Darmstadt, Germany). The stone mineral composition is shown in Table 1.

The unbroken stones were washed with 0.1 M nitric acid (HNO_3) and, consecutively, with a 2% sodium dodecyl sulphate (SDS) solution in order to remove any contaminating mucus [28].

Stones were then mechanically broken in halves. In some cases, the structure of the nidus was macroscopically well distinguishable from the outer stone phase. In other cases, this demarcation line was determined microscopically. Consecutively, the stone nidus was mechanically separated from the rest of the stone with the help of a scalpel, and powdered.

Protein extraction buffer

In order to optimise the extraction of the stone proteins, we compared three different extraction buffers (EB1–3):

- EB1: 0.06 M TRIS HCl (pH 6.8), 10% glycerol, 5% β-mercaptoethanol, 2% SDS
- EB2: 0.06 M TRIS HCl (pH 6.8), 10% glycerol, 5% β-mercaptoethanol, 1% Triton X 100
- EB3: 0.06 M TRIS HCl (pH 6.8), 10% glycerol, 5% β-mercaptoethanol, 1 mM EDTA, 8 M urea

The powdered stone nidus material was repeatedly suspended in each buffer solution (material/buffer 1:4). Consecutively, each sample was heated in a boiling water bath for 30 min and centrifuged in a DPR 6000 centrifuge (Damon/IEC Division, Mass.) at 3000 rpm for 15 min. The supernatant was collected, and centrifugation was repeated twice in order to obtain a maximum yield of protein. All extracts from identical samples, extracted with the same buffer, were then pooled and centrifuged again at 2500 rpm for 15 min in order to remove any debris. Samples were stored at −20°C until further use.

Dialysis and concentration of samples

For de-salting, the resulting supernatants were dialysed against 2 l de-ionised water over a period of 24 h with five changes of water.

Table 1 Stone mineral composition ($n = 35$)

Mineral composition	No. of stones
$CaOxPO_4$	10
CaOx	5
$CaOxPO_4$ Mg	8
CaOx-urate	4
Struvite	8

The dialysed extracts were then concentrated to small volumes by means of a Savant Concentrator (Savant Instruments, Farmingdale, N.Y.).

Electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

The concentrated stone extracts were subjected to SDS-PAGE according to the method described by Görg et al. [14]. Polyacrylamide gels with a gradient of 7.5%–22% were used. The electrophoresis buffer contained 0.06 M TRIS-glycine (pH 8.3) with 0.1% SDS. Gels were stained with Coomassie brilliant blue R-250 (Sigma, St Louis, Mo.). Small portions of whole, undiluted and unconcentrated urines from non-stone formers and stone formers were similarly loaded onto SDS-PAGE and processed accordingly.

Non-denaturing and non-reducing gel electrophoresis

To determine whether the extracted stone matrix proteins were either subunits of larger and more complex macromolecules or genuine low molecular weight proteins, the extracted proteins were again subjected to electrophoresis under identical conditions. However, this time reducing and denaturing agents, such as SDS and β-mercaptoethanol, were omitted in order to allow the renaturation of the proteins.

Gel filtration chromatography

The protein extracts were then fractionated by gel filtration with a Sephadex G-75 gel system (Sigma). A column 2 cm in diameter and 90 cm long was equilibrated with a 0.01 M PO_4 buffer (pH 7.5). After the determination of the void volume, the column was calibrated with proteins of known molecular weight. The renal stone protein extracts were then applied to the column, and fractions of 2 ml each were collected. The individual protein content of each fraction was assessed by the BCA method (Pierce, Rockford, Ill.). Fractions of identical peaks were pooled separately, and concentrated to a small volume as described above (see Section on protein extraction buffer.). Each of the fractions was then subjected to SDS-PAGE.

Removal of SDS

For later immunological procedures, the protein extracts had to be rendered SDS-free again in order to get renatured and to regain their original antigenic specificity. This was achieved by passing them through ion retardation resins AGIIA8 (BioRad, Richmond, Calif.). A column 2.5 cm in diameter and 12 cm long was filled with resins in de-ionised water. Proteins were loaded and then eluted from the column using de-ionised water. The removal of SDS from the sample was confirmed by the method of Arand et al. [1]. Protein recovery was estimated at 96%.

Immunisation

The so-obtained SDS-free protein extracts were mixed with Freund's adjuvant and injected in the foot pads of rabbits in a concentration of 2 mg/ml. This was followed by two booster doses given at weekly intervals. An immune response was first detected after the second booster dose by an indirect enzyme-linked immunosorbent assay (ELISA).

ELISA

In this study, we used an antibody capture assay (indirect ELISA). A 96-well microtitre plate was coated with both purified and crude

extracts of stone proteins (9 μg/ml), and incubated overnight at 4°C to allow for maximum binding. On the following day, the wells were washed twice with PBS tween solution. Remaining binding sites were blocked by the application of 3% BSA in PBS for 2 h. Consecutively, the wells were incubated for 2 h with rabbit antisera previously raised against the stone proteins. After washing off the excess antibodies, the wells were incubated for another 2 h with anti-rabbit IgG conjugated to horseradish peroxidase. Colouring was achieved by the use of orthophenylenediamine (6 mg/100 ml) in 0.05 M PO_4 citrate buffer. The reaction was stopped after 15–30 min with 2 M H_2SO_4. With an identical procedure, the cross-reactivity of urinary proteins in whole, undiluted and unconcentrated urines of normal individuals and stone formers was determined.

Western blotting

Stone matrix proteins and urinary proteins of non-stone formers and stone formers were run on SDS-PAGE. The gels were subjected to an immunoreaction with the same antibodies raised against stone matrix proteins. The presence of reactive proteins was confirmed and visualised by Western blot technique. For comparative purposes, a commercially available urine concentrate was also included in the tests (Sigma).

Results

We compared three different buffers for the extraction of proteins from renal stones. The buffer containing both SDS and β-mercaptoethanol (EB1), appeared to be the most suitable buffer for our study as it gave a maximum yield of proteins. Protein yields were quantified by the prominence of their bands in identical electrophoresis gels (SDS-PAGE).

As a continuous finding, the electrophoretic profiles of the extracted stone nidus proteins showed bands of low molecular weight proteins in a range from ≤12 to 66 kDa, with the most prominent band consistently being at around 12 kDa (Fig. 1). Similarly, proteins derived from urines of stone formers also were to a large proportion in the same low molecular weight range (Fig. 2). When SDS and β-mercaptoethanol were removed from the extracts, these proteins tended to form large aggregates. These aggregated proteins tended to remain stationary at the loading point on the SDS-gels.

The gel elution profile of the extracted proteins subjected to gel-permeation chromatography is shown in Fig. 3. Most of the proteins were eluted in the void volume (I), indicating their molecular weight being greater than 75 kDa. Matching with our SDS-PAGE findings, however, a large proportion of proteins were also present in the 30–50 kDa (II) and 10–15 kDa range (III). A smaller fourth peak is consistently present in all

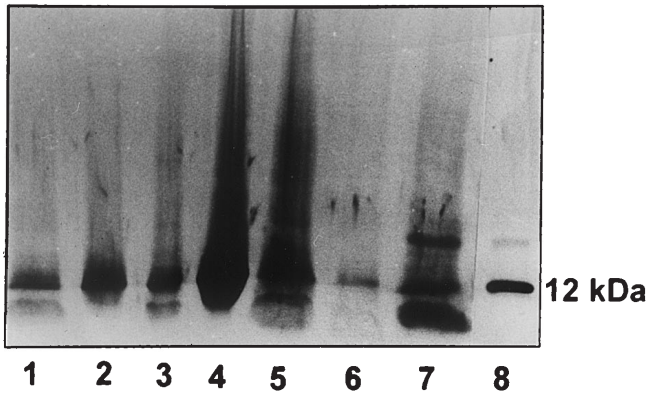

Fig. 1 SDS-PAGE of stone matrix protein extracts from different stones (Lanes 1–7). Lane 8 contains low molecular weight markers. Note the consistent prominent protein band at 12 kDa in all samples

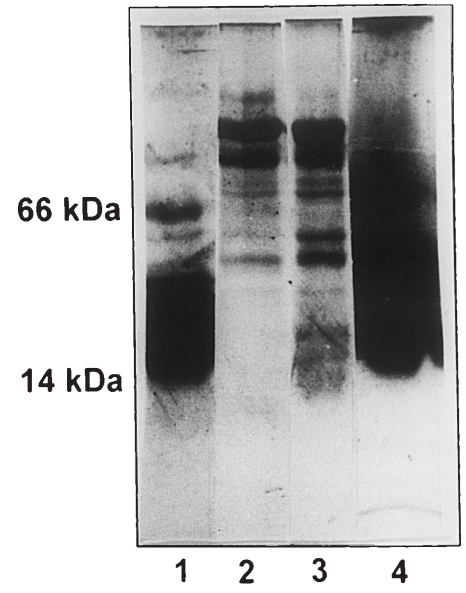

Fig. 2 SDS-PAGE comparing urinary proteins of two stone formers (Lanes 2, 3) and stone matrix protein extract (Lane 4). Lane 1 contains low molecular weight markers. In spite of a limited quality of the image, the presence of low molecular weight proteins down to a weight of <14kDa is clearly visible

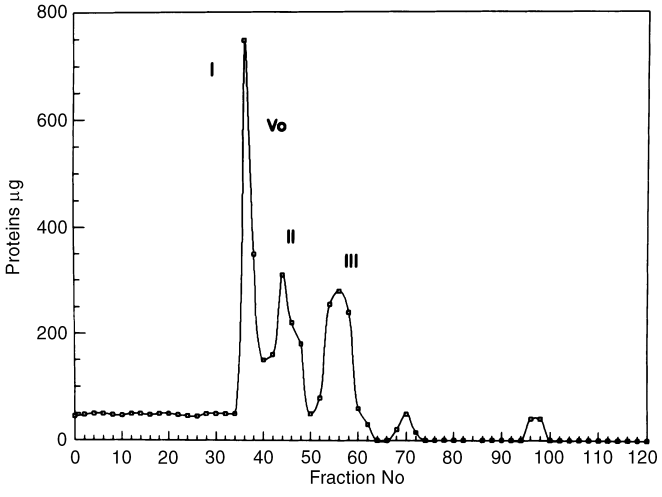

Fig. 3 Gel filtration profile of stone matrix protein extracts. The three main peaks correspond to proteins with a molecular weight >75kDa (I), 30–50 kDa (II), and 10–15 kDa (III)

stone types and comprises polypeptide species that may constitute degradation products of larger proteins.

Individual fractions were then subjected to SDS-PAGE. There was a content of low molecular weight proteins in all fractions. However, fraction III clearly shows the prominent 12 kDa band (Fig. 4). Again, these low molecular weight proteins in all fractions showed the aggregation tendency after renaturation as described above. Electrophoretic analysis of fraction I under denaturing and reducing conditions revealed that it consists of such reaggregated low molecular weight proteins.

Antibodies were raised against both crude and purified stone protein fractions to allow a comparison between the immunological properties of renal stone proteins from different individuals and of various mineral composition ($CaOxPO_4$ and CaOx-urate), as well as with urinary proteins. Antibodies raised against $CaOxPO_4$ stone proteins showed a cross-reactivity with proteins from CaOx-urate stones and pure CaOx stones. The reaction of the same antibodies with stone proteins was compared with their reaction with urinary proteins derived from both urines of normal individuals and stone formers. They indeed cross-reacted with urinary proteins derived from the urines of stone formers. However, there was no reaction with urinary proteins derived from the urines of normal individuals. Western blots of gels containing stone matrix proteins and urinary proteins confirmed these findings in showing low molecular weight proteins, and, in particular, ≤12 kDa proteins in all stones, in urines of stone formers but not, or only to a very minor extent, in normal concentrated urine (Fig. 5).

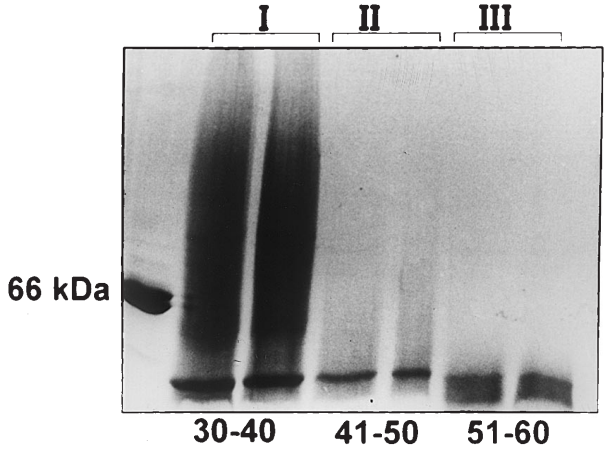

Fig. 4 SDS-PAGE of the gel filtration fractions. The lanes marked with roman numbers represent protein peaks (see Fig. 3). Arabic numerals below each lane represent fraction of gel filtration column. The left lane shows bovine serum albumin (BSA) which was used as a molecular weight marker (66 kDa). Low molecular weight proteins are present in all fractions; however, the prominent band at 12 kDa is prevalent in fraction III

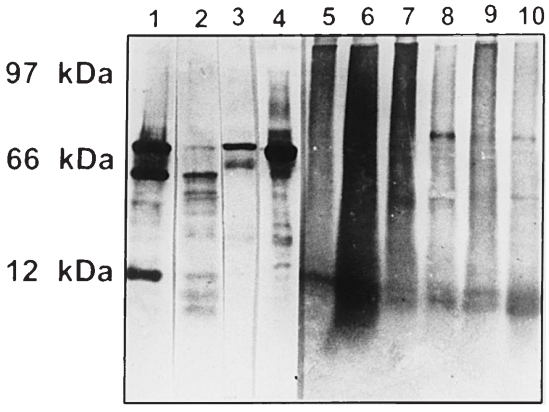

Fig. 5 Western blot immunodetection of proteins ≤12 kDa with an antibody raised against stone matrix proteins. The first lane (1) shows low molecular weight markers as indicated left of it. There are no proteins in the urine of a normal individual (3), and only very few in a commercially available urine concentrate (4). In contrast, the urine of a stone former shows an abundance of the proteins (2), as do stone matrix protein extracts from different renal stones (5–10)

Discussion

Proteins are found in both the outer crystalline phase and the inner organic matrix of urinary stones, suggesting a close protein–crystal interaction and, therefore, a potential role in stone formation. It is known that proteins influence the morphology of crystals, their aggregating properties, and their ability to adhere to renal epithelial cells [8, 27]. A layer of proteinaceous material can indeed cover every single CaOx crystal up to 75% of its surface. Consequently, this protein–crystal complex then binds to other CaOx crystals [24].

The bulk of the proteins in urinary stones is located within the inner organic stone matrix. Proteinaceous material, such as mucoproteins, glycoproteins and various antigenic components, constitute about 64% of the matrix [5, 13, 19–22]. The characterisation of these proteins is a crucial step towards the elucidation of the role of organic matrix in urolithogenesis, and, in particular, in its early stages of nidus formation. It is obvious that the inner core represents the former nidus around which the outer crystalline shell of the stone forms in concentric layers. However, the highly mineralised and, widely insoluble nature of the stone matrix has been a major impediment in achieving a complete profile of its component macromolecules in the past. Only 25% of the lyophilised matrix material is readily soluble [4].

A number of proteins have been isolated from urinary stones with different methods. Amongst these are matrix substance A, Tamm-Horsefall protein (THP), albumin, transferrin, and uromucoid [3, 11, 12, 15]. The last has also been attributed to the stone matrix as a minor component [3]. It is immunologically similar to subunits of THP which are secreted by the renal tubules [5]. Other proteins found in urinary stones are alpha-1

and alpha-2 globulins [3], urinary prothrombin fragment 1 (UPTF1) [17, 29], and various glycosaminoglycans (GAG) in different stone types which form complexes with proteins and may form insoluble calcium salts in urines of stone formers [8, 26].

To date, controversy persists with regard to the role of all the various stone proteins in the formation of urinary stones [6–8, 23]. Whereas some authors attribute a crucial role either as promoters or inhibitors of crystallisation to some of them [6–8, 16, 23], others suggest that proteinaceous material is merely incorporated by the crystalline growth front [30], or represents a co-precipitating species of proteins [10, 31]. Moreover, in spite of the fact that a number of proteins have been identified in urinary stones, there is no profile of proteins present in the nidus of stones available to date. These nidus proteins, as mentioned, represent players in the initial stages of stone formation, and, therefore, in one of the crucial acts of that process. In this study, we aimed, therefore, at characterising some of the physico-chemical and antigenic properties of proteins present in stone matrix in order to elucidate further their possible role in crystal–protein interaction and stone formation.

As mentioned earlier, the highly insoluble nature of the protein–crystalline complexed matrix rendered it very difficult in the past to extract proteins from stone matrix in a sufficient amount without destroying their structure and identifiable properties. Methods for the extraction of proteins from urinary stones have been mildly aggressive in order to avoid excessive protein degradation. Low-strength EDTA buffers, electroelution, and suspension in 10% acetic acid have been used with limited success [2, 18, 25]. The yield of protein varies considerably with these methods. Therefore, in an attempt to select the best method for extracting a maximum amount of stone proteins, we compared three different extraction buffers. The SDS and β-mercaptoethanol-containing buffer resulted in the best protein yield and was consequently used in all following experiments.

The major reason for using this combination of a denaturing (SDS) and a reducing agent (β-mercaptoethanol) was the strong tendency of the extracted stone proteins to aggregate. Whenever these agents were removed, the proteins aggregated and the isolation procedure inevitably failed. This strong tendency towards aggregation was a continuous finding and has been reported previously for stone proteins [2]. It represents an ubiquitous feature of all the low molecular weight stone proteins isolated and analysed in this study, independent of their origin and mineral composition. However, all stones contained CaOx as a common component, combined with either PO_4 or urate, and occasionally NH_4 and Mg. This may suggest that these proteins are somehow associated with and have a preferential affinity to CaOx crystals, and, moreover, that the incorporation of proteins into urinary stones is a selective process involving specific proteins.

Consequently, these aggregating proteins isolated from stone nidus were subjected to SDS-PAGE and gel filtration. In both methods, proteins were characterised by a relatively small molecular weight between 12 and 66 kDa with the bulk being present at a molecular weight of ≤12 kDa.

Given their low molecular weight and their aggregation tendency, it seems possible that these aggregating proteins represent in fact subunits of larger peptides and, therefore, after renaturing, resume their natural affinities and re-form the larger mother peptide.

In a further step, we attempted to shed light on the immunological features of this group of stone matrix proteins. Therefore, we raised antisera to the renal stone proteins. With these antibodies, we tested stone protein extracts from different individuals and of different mineral compositions, as well as urinary proteins derived from urines of normal subjects and stone formers. A positive cross-reaction was found with proteins of all stones, as well as with urinary proteins from stone formers. However, there was no reaction with the urinary proteins of non-stone formers. This confirms the presence of the same proteins in all urinary stone nidus irrespective of their origin or their mineral composition. Furthermore, it shows that these proteins are present in the urines of stone formers, but not in those of non-stone formers. Since we used undiluted, unconcentrated, whole urine samples, the concentration of these proteins in the urines of stone formers, and, in particular of their 12-kDa component, can be assumed to be very high. The more sensitive method of Western blotting confirmed these findings but showed, however, the presence of these low molecular proteins in a commercially available urine concentrate, which indicates that they might be, after all, present in urines of normal individuals but in a much lesser concentration than in stone formers. These findings, again, strongly suggest a selective protein incorporation into the stones in the early phases of stone and nidus formation, and, moreover, underline their role in the process. In conclusion, we recommend a buffer containing SDS and β-mercaptoethanol as the most effective method available to date to extract proteins from urinary stone material.

A distinct group of strongly aggregating proteins with a low molecular weight of ≤12 – 66 kDa constitutes a major component of renal stone matrix. Most of these proteins have a molecular weight of around ≤12 kDa. The same proteins occur in all renal stones irrespective of their origin or their mineral composition. This suggests their potential role in stone and nidus formation in general, and in stones of all aetiologies. However, all stones contained CaOx as a common component. It therefore seems likely that these proteins are associated with CaOx crystallisation.

Moreover, the same proteins are present in urines of stone formers in a high concentration, but not or only to a very minor extent in urines of non-stone formers. This further strengthens the assumption of their role in stone formation, but also underlines the incorporation of these

proteins into stones as being a selective process. Their low molecular weight in association with their strong aggregation tendency may indicate that this group of proteins is in fact a group of subunits of larger peptides which, once being renatured, redevelop their original properties to aggregate into the form of the mother molecule.

Although we have characterised this group of proteins with regard to some of their important physicochemical and antigenic features, to date we have not yet fully identified them. Efforts in this regard are currently being made in our laboratory. However, given their properties summarised above, it can be safely concluded that these proteins are prevalent in urines of stone formers, that they are selectively incorporated into renal stones, and that they most likely have a role in nidus and, therefore, early stone formation.

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