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Selective protein enrichment in calcium oxalate stone matrix: a window to pathogenesis?

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Abstract

Urine proteins are thought to control calcium oxalate stone formation, but over 1000 proteins have been reported in stone matrix obscuring their relative importance. Proteins critical to stone formation should be present at increased relative abundance in stone matrix compared to urine, so quantitative protein distribution data were obtained for stone matrix compared to prior urine proteome data. Matrix proteins were isolated from eight stones (> 90% calcium oxalate content)

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Human studies Four CaOx kidney stones in this study were obtained from de-identified, pathological waste specimens previously characterized at the Mandel International Stone and Molecular Analysis Center (MIS.MAC, Milwaukee, WI, USA) or the National VA Crystal Identification Center (Milwaukee, WI, USA) and were used without obtaining IRB approval. The use of these samples for publication was reviewed with the VA IRB. While the VA IRB could not grant retrospective approval for studying these samples, they did agree that the data could be published with appropriate acknowledgment of their origin and lack of IRB approval. An additional four stones were obtained from newly recruited patients following their presentation for stone removal surgery at Froedtert Hospital under IRB approval (protocol number PRO21952), and these samples were identified as S1 through S4. All procedures performed in these studies were in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki Declaration and its later amendments.

by crystal dissolution and further purified by ultrafiltration (> 10 kDa membrane). Proteomic analyses were performed using label-free spectral counting tandem mass spectrometry, followed by stringent filtering. The average matrix proteome was compared to the average urine proteome observed in random urine samples from 25 calcium oxalate stone formers reported previously. Five proteins were prominently enriched in matrix, accounting for a mass fraction of > 30% of matrix protein, but only 3% of urine protein. Many highly abundant urinary proteins, like albumin and uromodulin, were present in matrix at reduced relative abundance compared to urine, likely indicating non-selective inclusion in matrix. Furthermore, grouping proteins by isoelectric point demonstrated that the stone matrix proteome was highly enriched in both strongly anionic (i.e., osteopontin) and strongly cationic (i.e., histone) proteins, most of which are normally found in intracellular or nuclear compartments. The fact that highly anionic and highly cationic proteins aggregate at low concentrations and these aggregates can induce crystal aggregation suggests that protein aggregation may facilitate calcium oxalate stone formation, while cell injury processes are implicated by the presence of many intracellular proteins.

Keywords

Nephrolithiasis; Urine proteome; Calcium oxalate; Kidney calculi

Introduction

Despite many decades of research, the pathogenesis of stone formation remains uncertain beyond the obvious association with urine supersaturation, particularly for calcium oxalate (CaOx) stones, the most common type. Considerable research effort has been focused on anionic urine macromolecules, specifically proteins, because crystallization processes are strongly influenced by these macromolecules [1, 2]. Stone matrix, which comprises 2–3% of CaOx stone weight, is predominantly protein [3]. In recent years, mass spectrometry-based studies have identified over 1000 proteins in stone matrix, most of which have been found in urine [4, 5]. The complexity of the protein mixtures in urine and in stone matrix obscures the identity of those proteins critical to the stone formation process; however, the physical properties of these macromolecules (isoelectric point and hydrophobicity) and their relative abundance are likely to be controlling features in stone formation [2]. As knowledge pertaining to these proteins in stone disease is limited, further studies are necessary.

Proteins rich in anionic side chains are known to strongly influence CaOx crystallization processes, and they demonstrate strong crystal surface interactions in surface science studies [1, 2, 6]. Most proteins contain some of these same anionic side chains. Since proteins differ mainly in the relative quantities and ordering of the same amino acids in their backbones, differences in their interactions with CaOx crystals are likely to be quantitative, rather than qualitative. While strongly anionic proteins generally inhibit all crystallization processes in vitro (nucleation, growth, aggregation and cell surface attachment) [7–10], many of these same proteins are found in stone matrix suggesting that they may also induce stone formation under some circumstances [4, 7, 11, 12].

In vitro studies on protein mixtures have demonstrated that protein aggregate formation, induced by either mixing of strong polycations with strong polyanions [2, 13] or by placing weakly anionic proteins in higher salt concentrations [2, 14], can induce CaOx crystal aggregation, as well as nucleation [2, 14]. These observations suggest that protein aggregation could be a triggering event in stone formation. Forming protein aggregates by either pathway in the presence of urine proteins results in many unrelated urine proteins joining the aggregation processes [2, 14]. Consequently, we anticipate that the formation of CaOx stones may involve contributions from a large number of proteins (urine or cell surface), differing more in magnitude than in the nature of their interactions, as this solid phase is formed in urine. We also anticipate that strong polyanions might be principally involved in CaOx crystal surface interactions, while other protein features (hydro-phobicity or opposite charge attractions) could contribute to the buildup of other proteins in the matrix through protein–protein interactions. The proteins critical to stone formation should be enriched in stone matrix compared to those proteins included through non-selective interactions. A careful examination of the relative abundance of stone matrix proteins compared to their urinary abundance should identify the most relevant proteins. In particular, examining the isoelectric point distribution weighted by relative protein abundance of the CaOx stone matrix proteome may demonstrate a patterned enrichment collectively of polyanions and polycations, if polyanion–polycation aggregates are important to stone formation.

In this study, the protein matrix was isolated from 8 CaOx kidney stones and characterized using label-free spectral counting tandem mass spectrometry (MS/MS) methods, followed by bioinformatics analysis to identify urine proteins enriched in stone matrix. The relative abundances of stone matrix proteins were compared to the average urine protein abundances observed in a previous study of 25 CaOx stone formers (SFU) [15], finding that only a handful of urine proteins were both highly enriched and present at high abundance in stone matrix. On the other hand, most highly abundant urine proteins were found in matrix, but at lower relative abundance than in urine, suggesting that their inclusion was by nonspecific mechanisms. Insight into the collective actions of the many proteins found in stone matrix was obtained by comparing the isoelectric point distributions between stone matrix proteins and urine proteins, revealing that both highly anionic proteins and highly cationic proteins were selectively enriched in stone matrix. This finding is a previously unrecognized characteristic of the CaOx matrix proteome with mechanistic implications that are explored in more detail below.

Methods

Stone analysis

Four CaOx kidney stones in this study were obtained from de-identified, pathological waste specimens previously characterized at the Mandel International Stone and Molecular Analysis Center (MIS.MAC, Milwaukee, WI, USA) or the National VA Crystal Identification Center (Milwaukee, WI, USA) and were used without obtaining IRB approval (identified as M1 through M4). No additional patient data beyond age and gender were available for these stones. An additional four stones were obtained from newly recruited

patients following their presentation for stone removal surgery at Froedtert Hospital under IRB approval (protocol number PRO21952), and these samples were identified as S1 through S4. None of these four new patients had underlying conditions predisposing them to CaOx stones, and they did not have a history of urinary tract infections at the time of surgery. All stone samples were characterized using FTIR and determined to be 90% or greater calcium oxalate (CaOx) by mass.

Stone matrix isolation

The stones were individually pulverized using a Wig-L-Bug grinder/mixer with vial adapter (Dentsply International, Elgin, IL) and solubilized with three sequential washes of 0.4 M EDTA with 2% SDS (50 °C water bath, 1 h), followed by centrifugation for 1 min at 14,000g. The final supernatant was ultrafiltered (10 kDa cutoff) against 10 mM Tris-HCl, pH 8 until SDS no longer interfered with protein assays. Samples were stored at – 80 °C until analyzed.

Urine sample collection and initial characterization

The freshly voided random, midday urine samples were collected and analyzed following the procedure described earlier [15]. Protease inhibitors were added (PMSF, leupeptin and sodium azide), and samples were centrifuged briefly to remove cellular debris (1000g × 5 min). Urine dipsticks were used as a preliminary urine diagnostic tool (Multistix 10SG, Siemens, Malvern, PA USA). While all urine samples in the earlier study were negative for blood by dipstick, many newly acquired samples were positive for blood. Data from these samples are discussed, due to the lack of samples negative for blood by dipstick in patients S1 through S4, but these data were not utilized in the matrix proteome comparison, because of the excessive blood present. Urine anions, cations and creatinine were determined using ion chromatography (Dionex; Thermo-Fisher Scientific, Waltham, MA). Samples were ultrafiltered (10 kDa) against 10 mM NaCl with the same protease inhibitors and washed three times with 10 mM NaCl, and the recovered retentate was stored at – 80 °C.

All protein assays were performed in triplicate, using bovine serum albumin as a standard. Initial urine protein concentrations for protein-creatinine normalization and abundance calculations were determined using a pyrogallol red-based protein assay (Sigma-Aldrich, Milwaukee, WI) matching our clinical laboratory method. Isolated protein samples were assayed using a commercially available protein dye reagent (Bio-Rad, Hercules, CA).

Tandem mass spectrometry-based proteomics (MS/MS)

Proteomic studies were performed at the MCW Mass Spectrometry Facility, Milwaukee, WI, as described previously [15]. Equivalent protein samples (20 µg) were lyophilized and resuspended in 100 µl of 2% SDS, 310 mM DTT, 62.5 mM Tris pH 6.8, followed by addition of 100 µl of 30%T 2.7%C acrylamide/bis-acrylamide, 10 µl 10% APS, and 5 µl TEMED. Subsequently, in-gel reduction (30 min at 37 °C with 50 mM DTT in 50 mM ammonium bicarbonate) and alkylation with iodoacetamide (45 min at 37 °C) were performed, followed by in-gel trypsin digestion overnight at 37 °C, as described previously [15]. Tryptic peptide digests were extracted with trifluoroacetic acid and acetonitrile solutions, and the combined extracts were dried by vacuum centrifugation lyophilization

and desalted with C18 zip tips according to the manufacturers' protocol (EMD Millipore). Peptide samples were then lyophilized and resuspended in 80 μ l of 95% water/5% acetonitrile/0.1% formic acid buffer. Resuspended peptide samples were then separated by HPLC using capillary columns (10 cm $\times$ 75 μ m) packed with 3 μ m Magic C18AQ particles (Michrom-Bruker, Auburn, CA). The effluent was loaded onto a ThermoFinnigan LTQ Mass Spectrometer (MSⁿ capability) with a Thermo nanoelectro-spray ionization source coupled to an Eksigent chromatography system [16]. Two technical replicates were run for each biological replicate.

Protein spectral peaks were extracted from the RAW files using MSConvert via in-house workflow [17] and searched using the Sequest search algorithm against the UniProtKB human database (16,890 proteins, 9,512,957 amino acids). Database searches included modifications for oxidation of methionine (+ 16-Da), alkylation of cysteine (+ 57-Da), a peptide tolerance of 2.5-Da, and up to 3 missed cleavages (K, R) were allowed. Protein peaks were determined using established criteria and the data exported as.EZ files to be further analyzed using proteomic Visualize software [17, 18]. Filters were applied to the datasets, including exclusion of common contaminants and redundant proteins. Peptides with a Visualize software probability score ≥ 0.85 (5% FDR) and a peptide count ≥ 2 were also excluded to ensure the appropriate stringency in filtering the data. The total spectral counts (SC) in the filtered dataset must be ≥ 1000 for sample inclusion. Relative protein abundance was then defined as percent total spectral counts (%SC) for each given sample [16].

Bioinformatics analysis

Proteins identified were entered into the bioinformatics analysis data mining tool to derive protein locations reported in the literature (<http://www.ingenuity.com/IPA>). Protein parameters data tool was used to confirm calculated protein isoelectric point (pI) and GRAVY (Grand Average of Hydropathicity) scores based on the amino acid backbone of the proteins (<http://www.uniprot.org>).

Statistical analysis

All data are reported as averages plus or minus the standard deviation (SD). The unpaired Welch's *t* test was used to report statistical significance ($p < 0.05$).

Results

The stone composition and patient demographics are summarized in Table 1 for the eight CaOx stone samples analyzed. The patients included seven men and one woman in this study, compared to 10 men and 15 women in the earlier study [15]. The average age for these patients was 63 ± 9 years, somewhat older than the SFU patients (age = 46 ± 13 years) used to define the urine proteome [15]. All stones were predominantly CaOx monohydrate (COM; mean $81 \pm 14\%$; range 60–100%), with $15 \pm 11\%$ CaOx dihydrate (COD; range 0–30%). A small fraction (10%) of basic calcium phosphate (BCP) was detected in 3 samples (mean $4 \pm 5\%$). Thus, these stones were effectively pure CaOx and predominantly COM.

The observed protein mass fraction ($0.8\% \pm 0.4\%$) was consistent with previously published proteomic analyses [3, 4, 19, 20].

Mass spectrometry analyses from these stone matrix samples identified a total of 366 unique proteins, and all proteins are listed in Supplementary Data (SD1). As shown in Fig. 1, 88 proteins (68% of stone matrix protein SC) were overlapping with the reported urine proteome (73% of urine protein SC, which included 298 proteins) [15]. Reasoning that the proteins critical to stone formation will be both highly abundant and present in nearly all COM stones, we selected 60 proteins (see Table 2) as being dominant in CaOx stone matrix, based on either high abundance (average $> 0.5\%$ SC) or high prevalence (observed in 6 of 8 samples), for more detailed examination. Most proteins in this list met both criteria, but 13 proteins were less prevalent (found in either 4 or 5 stones, but meeting abundance criteria) and 13 other proteins were less abundant (found in 6 or more stones, but at $< 0.5\%$ SC) than these cutoffs. The remaining 306 proteins met neither criterion. All were low abundance, and most were only found in 1 or 2 stones (of 8 total), suggesting that these 306 proteins had no significant role in stone formation.

Average abundance data for the 60 dominant stone matrix proteins are included in Table 2, along with the abbreviations, accession numbers, *pI* and GRAVY (Grand Average of Hydrophobicity) scores. The first 32 proteins in Table 2 were reported previously in stone former urine studies [15], and their average urinary abundances from that study are also included in Table 2. Proteins are shown in order of decreasing urinary abundance for proteins common to both sets and alphabetically for the remainder. Standard deviations for most stone matrix protein abundances were comparable in magnitude to their mean values and even exceeded the mean values for lower abundance proteins, representing large sample to sample variations. The relative abundance variations between urine samples were also substantial, though generally smaller than observed in matrix samples.

Most of these dominant matrix proteins have been reported by others in earlier stone matrix proteomic studies [4, 19–25]. Albumin (ALBU) and Tamm–Horsfall protein (uromodulin, UROM), the most highly abundant urine proteins (20% and 13%, respectively) [15], were both found in all stone matrix samples, but at lower relative abundance (6% and 3%, respectively). This finding argues against an alternate model we have proposed [2, 14], where incompletely sialylated Tamm–Horsfall Protein self aggregates and triggers crystal aggregate (stone) formation. The same was true for most other moderate to high abundance urine proteins, suggesting that their inclusion in matrix was through non-selective processes. Most likely they were detectable in matrix only because their rich abundance in urine increased their probability of non-selective inclusion. Notable by their absence from Table 2 and the complete list of matrix proteins (SD1) were some moderately abundant urine proteins ($> 1.5\%$ SC), specifically prostaglandin-H2D-isomerase, galactin-3-binding protein, Ig alpha-2 chain C, and zinc- α -2-glycoprotein, while bikunin (AMBP), kininogen-1, Ig alpha-1 chain C, Ig lambda chain C, transferrin, and α -1-antitrypsin were present in stone matrix, but only at low abundance and low prevalence (see SD1). Almost all proteins in Table 2 demonstrated statistically different relative abundances in stone matrix than in urine, but the highly enriched proteins are more easily identified graphically.

In Fig. 2, the relative abundances of these 60 dominant matrix proteins (connected dots) are compared to the reported urinary abundances (bars), plotted from left to right in the same order as listed in Table 2. Five proteins were both highly abundant and highly enriched in stone matrix compared to their urinary abundance implying both selective inclusion in stone matrix and a dominant role in stone formation, including osteopontin (OSTP), mannan-binding lectin serine protease 2 (MASP2), vitamin K-dependent protein Z (PROZ), prothrombin (THRB), and hemoglobin β chain (HBB). Each of these proteins accounted for $> 4\%$ of the stone matrix proteome, and in aggregate these proteins accounted for $30 \pm 20\%$ of matrix protein mass (compared to only $3 \pm 2\%$ in urine). Many other proteins were highly enriched compared to their urinary abundances, implying a selective inclusion in matrix, but individually these proteins contributed only $< 2\%$ to the stone matrix proteome, suggesting a minor role in stone formation. While a pattern of selective protein enrichment appears to be characteristic of stone formation, the list of candidate proteins remains too large and diverse to infer possible mechanisms of action by identity alone.

The proteome distribution was examined for changes characteristic of a cationic shift in protein distribution as noted in the stone former urine proteome [15] by grouping the proteins in 0.5 pI/unit intervals spanning the pI range from 4 to 13. The total relative abundances of proteins in each group are plotted vs pI for both CaOx stone matrix (shown as connected dots) and stone former urine (shown as bars) in Fig. 3, and strikingly different distributions were observed. Strong enrichment of both highly anionic proteins ($pI < 5$) and highly cationic proteins ($pI > 9$) in stone matrix compared to the urine proteome distribution was obvious. Conversely, many intermediate pI range proteins (5–9) clearly exhibited reduced abundance in matrix. These same general features were evident in each individual stone matrix protein distribution (see Supplementary Data SD2).

Relatively few proteins possess these extreme isoelectric points (either < 5 or > 9) and account for this proteomic shift (see Table 3). Generally, these proteins were highly enriched ($5\times$ to $> 100\times$) in stone matrix, though the enrichment factors were only approximated for proteins not detected in urine, by assuming a urinary abundance of 0.01% SC. Among the intermediate isoelectric point proteins, only eight were prominently enriched and highly abundant. Hemoglobins are grouped separately in Table 3, but also showed prominent enrichment. The significance of hemoglobin enrichment remains uncertain, given the association of stones with urinary tract bleeding. Data indicating tissue locations for these proteins from network analysis (ingenuity.com) and the Uniprot database (uniprot.com) are included in Table 3, as well specifically highlighting extracellular, nuclear, and cytoplasmic locations. Most of the proteins observed at extreme isoelectric points ($pI < 5$ or > 9) are found in either nuclear or cytoplasmic locations, while most intermediate isoelectric point proteins are found in extracellular locations.

Discussion

Ideally, the comparison of matrix with urine protein relative abundances should be made with a stone and urine from the same patient, since generally repeat urine proteomes from a single patient demonstrated smaller variances than was observed between patients in the earlier study [15]. No urine samples could be obtained from patients M1–4, so the

previously published urine proteome data were required for comparison with these samples. Unfortunately, nearly half of urine samples from patients S1–4 (7 of 17) were positive for blood by dipstick (See Supplementary Data tables, SD3), which had been used as part of exclusion criteria in the previous study. Also, all samples from one patient (S2) contained abnormally large amounts of protein. While in all other respects the urine samples from patients S1–4 demonstrated similar chemistries and proteome distributions to those reported earlier, the presence of blood and excess protein in many samples from this cohort made additional proteome comparisons between the newly acquired urine samples and the SFU cohort problematic. Consequently, the previously published urine proteome data (SFU) have been used throughout the remaining discussion for consistency in comparison with the observed COM stone matrix proteome.

Our observed CaOx stone matrix proteins are consistent with many prior publications, demonstrating that many urinary proteins are found in stone matrix [4, 19–24, 26, 27]. Most of these studies have only enumerated the proteins present, so only the degree of overlap between proteomic sets can be compared. A few studies included quantitative information on proteins observed making them directly comparable to this study, and they reported generally similar protein distributions [4, 20]. Most studies do report some unique protein identifications, but typically only for lower abundance proteins. We must anticipate that proteins at low or trace abundance will be inconsistently detected using this method, reducing the reliability of calling their inclusion “unique”. These observations could indicate biological diversity, but they may simply reflect protein extraction differences or the statistical vagaries of analyzing complex mixtures using a method with finite sensitivity.

Data from this study must be characteristic of COM stone matrix, since the average stone composition was more than 80% COM. Our samples were derived from taking a major fraction (10–50%) of the powdered stone sample for protein extraction. Any selective distribution of additional components (COD or BCP) between surface and interior or nidus will have been similarly averaged in both the stone mineral composition and proteome distribution measurements by using the powdered stone material. Furthermore, published data have demonstrated a large and diverse set of proteins in each stone type described to date, with only limited data indicating qualitative differences in proteomes between different stone types (identifying individual protein differences without quantitative, relative abundance information) [19, 22, 27]. These same studies also highlight similar levels of proteome variation when comparing individual samples of the same stone type [19, 20, 22, 27], similar to data from this study, where substantial variations in individual protein contents were observed between stone samples. Consequently, the influence of either COD (minor component with average content < 15%) or BCP (trace component with average content < 5%) on the observed proteome is likely undetectable in the face of this large variance, especially since truly comparable relative abundance data are not available for these other stone mineral compositions.

Many have argued that stone matrix plays a critical role in stone formation, but the complexity of this mixture of organic components has defied mechanistic interpretation to date. While most earlier studies have focused on individual protein differences, we have taken the approach that the stone aggregate is fundamentally an organic–inorganic

composite material whose formation is dictated by the physical–chemical interactions between the organic and inorganic phases. The organic components have long been thought to be a “glue” holding the aggregate together, and therefore the organic components must demonstrate affinity (adhesiveness) to the CaOx crystal surfaces, as well as internal attraction, to act in this manner. Since proteins are all composed of the same amino acids, they contain the same functional groups, which contribute to these fundamental processes. Different proteins are defined by differences in the relative quantities and arrangement along the protein backbone of specific amino acids. Hence, differences in their contributions to a physical chemical process like stone formation are more likely to be quantitative rather than qualitative, and the relative importance of these interactions should manifest as differences in the relative abundance of specific proteins in stone matrix.

Viewed from the perspective of the physical chemical properties, we are not surprised by enrichment of strong polyanions in stone matrix ($pI \leq 5$), since anionic side chains are well known for their strong interactions with CaOx surfaces [2], presumably through binding to calcium ions on the crystal surface. Strongly cationic proteins ($pI \geq 9$) were not expected to be important in stone matrix based on their lack of influence on CaOx crystallization in earlier in vitro work [2], yet they are prominently present in all eight stone matrix samples, as well as in earlier studies [4, 19, 21, 24, 27], suggesting a critical role for polycations in stone formation. Polycations demonstrated strong affinity for strongly anionic proteins, and when present together at the crystal surface, the mixture differed greatly from either component alone in its influence of crystal nucleation, growth, and aggregation [2, 13]. Furthermore, the prominence of individual proteins characterized by these extreme isoelectric points also highlights the importance of cytoplasmic and nuclear proteins in stone formation, implying a significant role for cell injury in the process.

The mechanism of stone formation cannot be proven from these data, since these data cannot distinguish COM crystals first forming and collecting the observed protein mixture from polyanion–polycation aggregates first forming and inducing COM crystal nucleation, growth and aggregation. Likewise, the enrichment of proteins in matrix that are either low abundance or not routinely seen in urine cannot be taken as proof of cell injury, since in most patients, stones grow slowly over many months or years. Consequently, the observed distribution of proteins could be achieved by selective adsorption over a long period of time, rather than by acute bursts of activity, even for proteins normally found at very low concentrations in the urine. We favor the polyanion–polycation aggregate interpretation as the mechanism for stone formation, because the polyanions are strong inhibitors of these same crystallization processes, when polycations are absent. We also note that a competing mechanism that we have explored in the laboratory based on aggregation of Tamm–Horsfall Protein with low sialic acid content [2, 14] is not consistent with the observed COM stone matrix proteome based on the relative low abundance of Tamm–Horsfall Protein in matrix compared to urine.

Regardless, under this physical chemical model of stone formation, most abundant urine proteins should be expected to be present because they contain the same anionic amino acids that interact strongly with the CaOx surfaces, with their lower abundance resulting from their lower affinity for the CaOx crystal surfaces compared to strongly anionic proteins.

On the other hand, these lower abundance matrix proteins might have joined a polymer aggregate, based on their limited solubility in water; driven by hydrophobic forces (entropic forces), which are otherwise independent of the stone forming process. Regardless of the relative importance of these two pathways to stone formation, the presence of many different proteins is to be expected in a process driven by physical–chemical interactions. Evidence for the affinity of many unrelated urine proteins with protein aggregate formation has been seen in both UROM aggregate formation and with polycation–polyanion aggregates in earlier studies [2, 14]. Therefore, in this physical–chemical model of stone formation, the many low abundance matrix proteins likely have little to do with stone formation, but rather join stone matrix in preference to being dissolved in water.

Taking an alternative approach to analyzing these data based on a biological perspective, we have also performed an over-enrichment analysis using WebGestalt on gene identifiers for the proteins that were found in at least half of the eight stone matrix samples (103 total proteins; found in 4 samples). Using these methods, we examined the [Gene Ontology (GO)] biological process (see Supplementary Data SD4), [GO] molecular function (see Supplementary Data SD5), and Kyoto Encyclopedia of Genes and Genomes [KEGG] pathways that were significantly enriched compared to those expected in a randomized sample. The pathway enrichment analysis only demonstrated significant enrichment in the ‘Complement and Coagulation Cascade (hsa04610)’ (14 of 79 identified; 0.54 expected by random chance; enrichment ratio = 25.77; $p < 0.05$; FDR < 5%), consistent with a role for cell injury in stone formation. Additionally, the main enrichment for [GO] biological processes was ‘Chaperone-mediated protein complex assembly (GO:0051131)’ (4 of 20 identified; 0.081 expected by random chance; enrichment ratio = 49.36; $p < 0.05$; FDR < 5%). In examining the same list for molecular function, the main enrichment was related to the ‘Binding’ gene ontology category (22 of 32 enriched terms were related), suggesting the protein list identified in the stone matrix had a high binding capacity. These enrichment data suggest that the protein composition was not likely from a coordinated pathway and that there was an enrichment of high binding proteins involved, consistent with the physical–chemical model interpretation above.

Since these data and subsequent analyses were largely observational and hypothesis generating, the main limitations of this study should not be judged on the basis of proving or disproving a mechanistic model, but rather must be judged on the basis of the validity of the observations. Admittedly, the COM stone matrix pool did not match the SFU patient pool demographics very closely, but with only eight stone samples in this study, subgroup analysis based on gender or age was not appropriate. Thus, no statistical adjustment or evaluation was possible for the stone matrix proteome coming from a pool of older and predominantly male patients, compared to the urine proteome cohort. We do note, however, that no gender difference was found in the urine proteomes from the earlier larger pool [15], suggesting that the preponderance of men in this study was likely not an important factor in the outcome. In addition, we note that the differences in proteome isoelectric point distributions between stone matrix and urine proteins were profound, and evident in all eight stone matrix samples, individually. Likewise, the urine proteome isoelectric point distributions were generally similar, including the urine samples from the newly recruited patients. These observations support the hypothesis that the observed stone matrix proteome

distribution is characteristic of the stone forming process, and not related to other underlying disease processes (known to not be present in newly recruited patients, but unknown in the pathological specimens, M1–M4). Finally, newer mass spectrometers, which provide greater depth of penetration, would generally increase the number of low abundance proteins detected, but the highly abundant proteins would still be expected to account for most of the protein detected and dominate the isoelectric point distribution without changing the overall abundance weighted distribution of proteins.

Conclusions

The observed COM stone matrix proteome demonstrated a diverse array of mainly urinary proteins, clouding the interpretation of stone pathophysiology. Five proteins, highly enriched compared to their urinary abundance, accounted for > 30% of the protein mass, though this observation provided no specific insight into stone formation processes. However, the COM stone matrix proteome prominently displayed enrichment of strongly polyanionic proteins and strongly polycationic proteins, a combination of proteins that would form aggregates that could induce COM growth and aggregation, and potentially induce stone formation. Most of these highly charged proteins are normally found in intracellular or nuclear locations, suggesting a significant role for cell injury in stone formation. Most abundant urinary proteins were present in matrix, but at lower relative abundance than in urine, suggesting that their inclusion was through non-selective mechanisms. Future studies should include a larger pool of patients with both stones and urine samples to address limitations noted above. New studies will need to be developed to differentiate the potential mechanistic pathways and provide greater insight into stone pathogenesis to guide development of more effective stone prevention therapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.
Venn diagram of proteome overlap between SFU and CaOx stone matrix

Fig. 2.

SFU and CaOx stone matrix protein abundances. The dominant stone matrix proteins ($n = 8$; black line dots) defined by being highly abundant ($> 0.5\%$ SC average) and/or highly incident (detected in ≥ 6 stones) that are outlined in Table 2 are compared to the relative urinary abundance of the same proteins (gray bars) reported earlier [15]. The proteins were sorted based on the urinary concentrations (high to low) and alphabetically for those not detected in the SFU cohort. Abbreviations used on the x -axis are defined in Table 3 and given in the same order. The 60 matrix proteins illustrated here account for approximately 80% of the stone matrix proteome and roughly 50% of the SF urine proteome

Fig. 3.

pI histograms of SFU vs. CaOx stone matrix proteomes. Relative protein abundance sorted by 0.5 *pI* units is shown for SFU (gray bars; $n = 25$) and the average stone matrix proteome (black dot line; $n = 8$). Four *pI* regions show significant enrichment in the matrix samples ($p < 0.05$). Increases for *pI* 4–4.5 were due primarily to OSTP and SET, increases for *pI* 6.5–8.5 were principally due to HBB, HRG, HBA, and HBD, increases for 9.5–10 were mainly due to PERE and ECP, and increases for *pI* 11–12.5 were due primarily to H4 and SRSFs. Decreased inclusion of proteins in the range *pI* 5–5.5 was mainly due to UROM. Many proteins contributed in the range *pI* 7–7.5. Significant differences are indicated with an asterisk

Table 1

Patient demographics and stone composition

ID	M/F	Age, years	Stone compositions, % COM–COD–BCP
M1	M	65	70–20–10
M2	M	69	80–20–0
M3	M	46	100–0–0
M4	M	69	80–20–0
S1	M	60	100–0–0
S2	F	58	60–30–10
S3	M	66	80–10–10
S4	M	73	80–20–0

Stone compositions are reported for eight calcium oxalate stones, along with gender and age data. Samples M1–4 were archival stones from Dr. Mandel's commercial stone analysis operation, while samples S1–4 were obtained by surgical extraction from patients at Froedtert Hospital, specifically recruited for this study. *COM* calcium oxalate monohydrate, *COD* calcium oxalate dihydrate, *BCP* basic calcium phosphate

Table 2

Dominant stone matrix proteins

Abbr	Accession no.	Protein	pI	GRAVY	Matrix, %SC	SFU, %SC
ALBU	Q9P1I7	Albumin	5.97	-0.35	6 ± 7	20 ± 9
UROM	P07911	Uromodulin (THP)	5.07	-0.11	3 ± 3	13 ± 7
APOD	P05090	Apolipoprotein D	5.07	-0.05	0.5 ± 0.5	3.1 ± 1.6
OSTP	P10451	Osteopontin	4.39	-1.10	11 ± 6	2.4 ± 1.0
EGF	P01133	Epidermal growth factor	5.61	-0.30	0.8 ± 0.6	2.0 ± 1.1
IGHG1	P01857	Ig gamma-1 chain C	7.95	-0.43	0.4 ± 0.4	1.9 ± 1.2
IGKC	P01834	Ig kappa chain C	5.63	-0.50	0.9 ± 1.1	1.8 ± 1.2
CLUS	P10909	Clusterin	5.95	-0.66	0.9 ± 0.6	0.6 ± 0.5
IPSP	P05154	Plasma serine protease inhibitor	9.08	-0.12	0.2 ± 0.2	0.6 ± 0.5
MASP2	Q9UBP3	Mannan-binding lectin serine protease 2	5.52	-0.27	5 ± 3	0.5 ± 0.6
HBB	Q9BX96	Hemoglobin beta	6.80	0.01	6 ± 3	0.3 ± 0.6
LMAN2	Q12907	Lectin, mannose-binding 2	6.53	-0.36	0.6 ± 0.8	0.3 ± 0.4
VMO1	Q7Z5L0	Vitellogen membrane outer layer protein 1	4.92	-0.26	0.7 ± 0.7	0.3 ± 0.4
S10A8	P05109	S100-A8 (Calgranulin-A)	6.57	-0.40	1.2 ± 1.8	0.2 ± 0.3
CO3	P01024	Complement C3	6.08	-0.32	0.8 ± 0.7	0.1 ± 0.4
APOA1	P02647	Apolipoprotein A-I	5.59	-0.72	0.5 ± 0.7	0.1 ± 0.2
FIBA	P02671	Fibrinogen alpha	5.75	-0.82	0.5 ± 0.5	0.1 ± 0.2
PROZ	P22891	Vitamin K-dependent protein Z	5.69	-0.27	4 ± 4	0.1 ± 0.1
THRB	P00734	Prothrombin	5.67	-0.54	8 ± 6	0.08 ± 0.11
SODE	P08294	Superoxide dismutase [Cu-Zn]	6.21	-0.37	0.8 ± 1.0	0.1 ± 0.2
APOA4	P06727	Apolipoprotein A-IV	5.30	-0.80	0.5 ± 0.5	0.02 ± 0.08
FIBG	P02679	Fibrinogen gamma	5.40	-0.59	0.4 ± 0.3	0.02 ± 0.07
HBA	P69905	Hemoglobin alpha	8.41	0.05	1.6 ± 0.6	0.02 ± 0.05
VTNC	P04004	Vitronectin	5.59	-0.72	0.8 ± 0.6	0.02 ± 0.07
HRG	P04196	Histidine-rich glycoprotein	7.11	-0.96	0.8 ± 0.7	0.01 ± 0.04
HSPB1	P04792	Heat-shock protein beta-1	6.06	-0.57	0.8 ± 0.8	0.01 ± 0.02
MA1A1	P33908	Alpha-1,2-mannosidase IA	6.11	-0.27	0.7 ± 0.5	0.01 ± 0.03
APOE	P02649	Apolipoprotein E	5.65	-0.60	0.7 ± 0.9	0.00 ± 0.02

Abbr	Accession no.	Protein	pI	GRAVY	Matrix, %SC	SFU, %SC
CFAB	P00751	Complement factor B	6.68	-0.50	0.8 ± 0.7	0.00 ± 0.02
GGH	Q92820	Gamma-glutamyl hydrolase	6.68	-0.09	0.3 ± 0.3	0.00 ± 0.02
H4	P62805	Histone H4	11.76	-0.52	1.8 ± 0.9	0.00 ± 0.01
PRG2	P13727	Bone-marrow proteoglycan	6.28	-0.40	0.5 ± 0.8	0.01 ± 0.02
ASSY	P00966	Argininosuccinate synthase	7.69	-0.37	0.3 ± 0.3	nd
ATPB	P06576	ATP synthase subunit beta	5.28	0.02	0.14 ± 0.13	nd
CATG	P08311	Cathepsin G	11.64	-0.43	0.6 ± 0.9	nd
CO9	P02748	Complement C9	5.45	-0.45	0.2 ± 0.2	nd
ECP	P12724	Eosinophil cationic protein	9.88	-0.28	1.3 ± 1.9	nd
GAS6	Q14393	Growth-arrest-specific protein 6	5.90	-0.30	1.3 ± 1.1	nd
HBD	P02042	Hemoglobin delta	7.59	-0.05	0.8 ± 0.9	nd
HEP2	P05546	Heparin cofactor 2	6.49	-0.24	0.8 ± 0.7	nd
HMGB1	P09429	High mobility group protein B1	5.62	-1.61	0.2 ± 0.2	nd
HS90A	P07900	Heat-shock protein HSP 90-alpha	4.95	-0.75	1.1 ± 1.0	nd
HS90B	P08238	Heat-shock protein HSP 90-beta	4.97	-0.68	1.2 ± 1.2	nd
ITIH1	P19827	Inter-alpha-trypsin inhibitor 1	6.39	-0.28	0.2 ± 0.3	nd
MYH9	P35579	Myosin-9	5.52	-0.85	0.8 ± 0.5	nd
NPM	Q96EA5	Nucleophosmin	4.65	-0.97	0.8 ± 0.6	nd
NUCL	P19338	Nucleolin	4.60	-1.13	0.9 ± 0.9	nd
PERE	P11678	Eosinophil peroxidase	10.17	-0.31	1.3 ± 1.9	nd
PERM	P05164	Myeloperoxidase	8.75	-0.27	0.7 ± 1.5	nd
PGDH	P15428	Prostaglandin dehydrogenase	5.60	-0.01	1.1 ± 1.8	nd
PON1	P27169	Serum paraoxonase	5.11	-0.10	0.7 ± 0.9	nd
PROS	P07225	Vitamin K-dependent protein S	5.51	-0.29	0.6 ± 0.5	nd
SET	Q01105	Phosphatase 2A inhibitor I2PP2A	4.23	-1.33	0.6 ± 0.3	nd
SRSF2	Q01130	Ser/Arg splicing factor 2	12.35	-1.62	1.0 ± 0.7	nd
SRSF3	P84103	Ser/Arg splicing factor 3	12.13	-1.52	0.4 ± 0.2	nd
SRSF7	Q16629	Ser/Arg splicing factor 7	12.32	-1.40	0.2 ± 0.2	nd
TBB2C	P68371	Tubulin beta-2C	4.80	-0.36	1.1 ± 1.8	nd
TBB5	P07437	Tubulin beta	4.79	-0.35	1.5 ± 1.5	nd
TRAP1	Q12931	Tumor necrosis factor type 1 receptor-associated protein	7.92	-0.34	0.3 ± 0.3	nd

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Abbr	Accession no.	Protein	pI	GRAVY	Matrix, %SC	SFU, %SC
UBIQ	Q91888	Ubiquitin	6.60	−0.49	0.15 ± 0.12	nd

The dominant matrix proteins are defined as highly frequent matrix proteins (> 6 of 8 stones) or highly abundant (average > 0.5% SC), and they are compared to the previously reported urinary abundances of the same proteins. These 60 proteins account for 82 ± 9% of the matrix sample total spectral scans. Eleven proteins were found in all stone matrix samples (OSTP, THRB, ALBU, HBB, UROM, H4, HBA, GAS6, SRSF2, SET, and SRSF3), and another 11 proteins are found in 7 of 8 stone matrix samples (MASP2, PROZ, CLUS, NUCL, NPM, EGF, MYH9, CO3, VTNC, MA1A1, and IGHG1). The proteins are sorted based on the urinary concentrations (high to low) for proteins found in both urine and stone matrix, and alphabetically for the remainder. Protein abbreviations, accession numbers, isoelectric point (pI), and GRAVY (Grand Average of Hydropathicity) scores were extracted directly from the Uniprot database (<http://www.uniprot.org>). A complete listing of all 366 stone matrix proteins is given as supplemental data (SD1)

Table 3

Dominant enriched matrix proteins grouped by isoelectric point

Gene name	Protein abbr.	Description	Tissue location	Incidence (<i>n</i> = 8)	Fold enrichment MX vs. urine
Anionic proteins: <i>p</i> / < 5 (10–29% of matrix in individual samples; list mean = 13 ± 8%)					
SPP1	OSTP	Osteopontin	EC	8	5
SET	SET	Phosphatase 2A inhibitor (I2PP2A)	NUC	8	50
NCL	NUCL	Nucleolin	NUC	7	50
NPM1	NPM	Nucleophosmin	NUC	7	100
Intermediate proteins: <i>p</i> / 5–9 (55–77% of matrix in individual samples; list mean = 22 ± 16%)					
F2	THRB	Prothrombin	EC	8	100
GAS6	GAS6	Growth arrest protein 6	EC	8	125
MYH9	MYH9	Myosin 9	CYT	7	80
MAN1A1	MA1A1	Alpha-1,2-Mannosidase IA	CYT	7	70
PROZ	PROZ	Vitamin K-dependent protein Z	EC	7	50
VTN	VTNC	Vitronectin	EC	7	40
MASP2	MASP2	Mannan-binding serine protease 2	EC	7	10
C3	CO3	Complement factor 3	other	7	6
Cationic proteins: <i>p</i> / > 9 (4–16% of matrix in individual samples; list mean = 7 ± 8%)					
SRSF ^a	SRSF	Serine-arginine splicing factors	NUC	8	230
HIST1H4	H4	Histone H4	NUC	8	180
EPX	PERE	Eosinophil peroxidase	CYT	5	130
RNASE3	ECP	Eosinophil cationic protein	EC	5	130
CTSG	CATG	Cathepsin G	CYT	5	60
Hemoglobin (5–17% of matrix in individual samples; list mean = 8 ± 5%)					
	HBB	Hemoglobin, beta chain		8	
	HBA	Hemoglobin, alpha chain		8	
	HBD	Hemoglobin, delta chain		5	

Abundant and enriched proteins are reported as either anionic, intermediate, or cationic, with hemoglobins listed separately. Ranges of weight percentage values for each category are given on individual sample basis. Average total weight percentage and standard deviation data for only the proteins listed in the table are also given. Enrichment factors were determined by comparing matrix relative abundances to reported stone former urine abundances. A minimum urine value of 0.01%SC was used to estimate matrix enrichment when the urinary abundance was not determined. OSTP, SET, NCL, and NPM account for almost all the anionic protein group spectral scans. THRB, GAS6, MYH9, MA1A1, PROZ, VTNC, MASP2, and CO3 account for almost half of the intermediate p/protein spectral scans, while abundant but not enriched intermediate proteins account for roughly 15% of the matrix (ALBU, UROM, CLUS, EGF, and immunoglobulins). Histone 4 and serine-arginine splicing factors account

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for 90% of the cationic protein group. (SRSFs include serine—arginine splicing factors 1, 2, 3, 5, 6, 7 and TRA2B.) Network analysis (ingenuity.com) and Uniprot database (uniprot.org) provided protein notes and tissue locations: extracellular (EC), nuclear (NUC), and cytoplasmic (CYT)