Mass Spectroscopic Characteristics of Low Molecular Weight Proteins Extracted From Calcium Oxalate Stones: Preliminary Study

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It is believed that boundary compositions of matrix proteins might play a role in stone formation; however, few proteomic studies concerning matrix proteins in urinary stones have been conducted. In this study, we extracted low molecular weight proteins from calcium oxalate stones and measured their characteristic patterns by mass spectroscopy. A total of 10 stones were surgically removed from patients with urolithiasis. Proteins were extracted from the stones and identified by one-dimensional electrophoresis (sodium dodecyl sulfate buffer [SDS]–polyacrylamide gel electrophoresis [SDS-PAGE]). After in-gel digest, samples were analyzed by the surface-enhanced laser desorption ionization-time of flight (SELDI-TOF) technique. The peptide sequences were analyzed from the data of mass spectroscopy. Proteins were identified from Database Search (SwissProt Protein Database; Swiss Institute of Bioinformatics; <http://www.expasy.org/sprot>) on a MASCOT server (Matrix Science Ltd.; <http://www.matrixscience.com>). A total of

three bands of proteins (27, 18, and 14 kDa) were identified from SDS-PAGE in each stone sample. A database search (SwissProt) on a MASCOT server revealed that the most frequently seen proteins from band 1 (27 kDa) were leukocyte elastase precursor, cathepsin G precursor, azurocidin precursor, and myeloblastin precursor (EC 3.4.21.76) (leukocyte proteinase 3); band 2 (18 kDa) comprised calgranulin B, eosinophil cationic protein precursor, and lysozyme C precursor; band 3 (14 kDa) showed neutrophil defensin 3 precursor, calgranulin A, calgranulin C, and histone H4. The modifications and deamidations found from the mass pattern of these proteins may provide information for the study of matrix proteins. Various lower molecular weight proteins can be extracted from calcium oxalate stones. The characteristic patterns and their functions of those proteins should be further tested to investigate their roles in stone formation. *J. Clin. Lab. Anal.* 22:77–85, 2008. © 2008 Wiley-Liss, Inc.

Key words: calcium oxalate; matrix protein; matrix-assisted laser desorption-time of flight mass spectrometry (MALDI-TOF-MS); surface-enhanced laser desorption ionization-time of flight mass spectrometry (SELDI-TOF-MS); leukocyte elastase precursor

INTRODUCTION

Urinary stones are formed by abnormal biomineralization (1). It has been reported that proteins are involved in the formation of calcium oxalate depositions in plants (2). Therefore, proteins that present in the stones or urine can be considered to modify crystal formation. Many proteins found in urine have been reported to be promoters or inhibitors of human

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urolithiasis, such as inter- α -trypsin inhibitor, prothrombin families, osteopontin, albumin, Tamm-Horsfall protein, nephrocalcin, and α 1-microglobulin (3–5). However, it is unclear which of the matrix proteins found in the stones are promoters or inhibitors of urolithiasis.

Several studies of the function of matrix proteins have indicated that certain matrix proteins may act as inhibitors and that certain proteins act as promoters in the formation of urinary stones. Boyce (6) reported that the matrix of stone was the primary substance that promotes crystal formation. However, Vermeulen and Lyon (7) questioned whether the matrix was a promoter; they found evidence that the matrix was incorporated into stone by nonspecific adsorption. Doyle et al. (8) used undiluted urine to form stone *in vitro*; they found that certain stone matrix proteins act as inhibitors of calcium oxalate crystal growth and aggregation but do not inhibit nucleation. Urinary prothrombin fragment 1 (UPTF1) was postulated to be an inhibitor of stone formation (9). Khan et al. (10) showed that osteopontin is a significant constituent of the matrix of calcium oxalate stones and is a strong inhibitor of crystal nucleation, growth, and aggregation. However, the reason why inhibitors are present in stones is unknown. In another series of studies, Govindaraj and Selvam (11) extracted matrix proteins from human stones and found that peroxidized protein promoted crystal formation.

There are many types of proteins in stone matrix. Different stones may contain their unique proteins. Matrix protein material could certainly be vastly different in calcium phosphate and struvite stone former, particularly calgranulin and other calcium binding proteins (12). However, previous studies of matrix proteins are individualized. Mass spectroscopy is a powerful analytical tool to analyze a group of proteins in a sample when combined with matrix-assisted laser desorption–time of flight mass spectrometry (MALDI-TOF-MS) and surface-enhanced laser desorption ionization–time of flight mass spectrometry (SELDI-TOF-MS), which analyze peptide mass sequences in solid and liquid states, respectively. Mass spectroscopy is widely used in proteomic studies in urology (13). The boundary proteins in urine can be studied from their peptide sequences after enzyme digestion. Therefore, mass spectroscopy can be a good tool for the studying of variable amounts of matrix proteins. Calcium oxalate stones are the most frequently seen in human urinary stones. Therefore, we extracted proteins from calcium oxalate stones and measured their characteristic patterns by mass spectroscopy in order to provide more information for the study of matrix proteins.

MATERIALS AND METHODS

Extraction of the Protein

Because stone specimen can be obtained enough from surgery and recurrent stone formers may have more derangement than single episode, we chose calcium oxalate stones as study materials. We used sodium dodecyl sulfate (SDS) as an extracted agent for stone matrix proteins. A total of 10 stones were surgically removed from 10 patients with recurrent calcium oxalate stones. They did not have relevant metabolic disorder, genetic inheritance concerning stone disease, or urinary tract infection (seven men and three women, age range from 40 to 60 years, average age 45.2 years). Each stone was washed by 0.1 M phosphate buffer solution (PBS) and 0.15% sodium azide for 15 min. Before fragmenting the stone, it was stored at -70°C . Then the stone was washed in 2% SDS solution with gentle stirring at 4°C to remove any contaminating mucus. A small fragment was analyzed by infrared spectroscopy to confirm the content of calcium oxalate. The stone was weighed after being lyophilized and ground in a mortar. The powder was suspended in 5-mL cold extraction buffer (0.06 M Tris-HCl, pH 6.8, 10% glycerol, 5% β -mercaptoethanol, and 2% SDS). Protease inhibitors used in the buffer included 0.05 M ethylene diamine tetraacetic acid (EDTA), pH 8, 1 mM phenylmethanesulphonylfluoride (PMSF), 1 M, 1% thioglycol, and 1 mM sodium azide. The suspension was bathed in 37°C water for 30 min and then centrifuged for 15 min at 10,000 rpm (Kubota 5800; Kubota, Tokyo, Japan). The resultant supernatant was then centrifuged for 15 min at 10,000 rpm (14). Supernatants were pooled and stored at -20°C . The supernatants were dialyzed against double-distilled (dd) water for 24 hr with five changes at 4°C by in a 6–8-kDa cutoff dialysis membrane. Protein was extracted from the dialyzate by centrifuging with Amicon Ultra (MWCO 5000; Millipore, Beverly, MA) at 3,200 rpm for 30 min at 4°C . This study has been reviewed by the Institutional Review Board (DMR-94-IRB-67).

Protein Assay

The protein concentration of the supernatant was measured by the Bradford method (BioRad, Richmond, CA). Before electrophoresis, samples were diluted with solubilization buffer to achieve uniform protein concentrations of $150\text{ }\mu\text{g}$ per $130\text{ }\mu\text{L}$ of final sample volume (ratio of protein/buffer = 2:1). Samples were heated to 95°C for 5 min and then loaded in a 12% SDS gel. One-dimensional electrophoresis (SDS–polyacrylamide gel electrophoresis [SDS-PAGE]) was performed at a current of 50 V for stacking gel and 120 V for separating gel. The gel was then stained by Coomassie blue stain.

The band shown on the gel after staining was cut and digested.

In-Gel Protein Digestion

In-gel digestion of proteins from Coomassie blue-stained gels for later mass spectroscopic analysis was performed as follows (15,16). Each spot of interest in the Coomassie blue-stained gel was sliced into 1-mm cubes and washed three times with 50% vol/vol acetonitrile (ACN) in 25 mM ammonium bicarbonate buffer (pH 8.0) for 15 min at room temperature. Then, the stained spots were washed with 100 mM ammonium bicarbonate and ACN, reduced with dithiothreitol (DTT) at 60°C for 40 min, and then alkylated by iodoacetamide (IAA) in a dark place for 30 min. The gel pieces were soaked in 100% ACN for 5 min, dried in a lyophilizer for 20–30 min and rehydrated in 25 mM ammonium bicarbonate buffer (pH 8.0) containing 35 μ L of 10 μ g/mL porcine trypsin (Promega, Madison, WI) until the gel pieces were fully immersed. After incubating at 37°C overnight, the remaining trypsin solution was transferred into a new microtube for nano-liquid chromatography (LC)/mass spectroscopy (MS)/MS analysis.

Nanoelectrospray Mass Spectrometry

Nanoscale capillary LC-MS/MS analysis was performed using an Ultimate capillary LC system (LC Packings, Amsterdam, The Netherlands) coupled to a QSTAR_{XL} quadrupole-time of flight (Q-TOF) mass spectrometer (Applied Biosystems/MDS Sciex, Foster City, CA). The nanoscale capillary LC separation was performed on a RP C18 column (15 cm $\times$ 75 μ m inner diameter [i.d.]) with a flow rate of 200 nL/min and a 70-min linear gradient of 5–50% buffer B. Buffer A contained 0.1% formic acid in 2% aqueous ACN; buffer B contained 0.1% formic acid in 98% aqueous ACN. The nano-LC tip for on-line LC-MS used was a PicoTip (FS360-20-10-D-20; New Objective, Cambridge, MA). Data acquisition was performed by an automatic Information Dependent Acquisition system (IDA; Applied Biosystem/MDS Sciex). The IDA automatically finds the most intense ions in a TOF-MS spectrum, and then performs an optimized MS/MS analysis on the selected ions. The product ion spectra generated by nano-LC-MS/MS were searched against the National Center for Biotechnology Information (NCBI) databases (<http://www.ncbi.nlm.nih.gov>) for exact matches using the ProID program (Applied Biosystem/MDS Sciex) and the MASCOT search program (17). A *Homo sapiens* taxonomy restriction was used and the mass tolerance of both precursor ion and fragment ions was set to ± 0.3 Da. Carbamidomethyl cysteine was set as a fixed modification, while serine, threonine, and tyrosine

phosphorylation were set as variable modifications. All phosphopeptides identified were confirmed by manual interpretation of the spectra.

RESULTS

The stones ranged in weight from 0.76 to 0.11 g (average 0.296 ± 0.187 g). The amount of protein extracted from stones ranged from 0.01 to 5.16 μ g (average 0.70 ± 1.57 μ g) in weight. The extracted proteins comprised 0.07–0.68% of the total weight (average $0.14 \pm 0.18\%$). To identify the peptide sequence, we performed Q-TOF after in-gel digestion of extracted stone matrix proteins. This approach allowed us to rapidly identify several peaks in each sample although the amount of proteins in each sample was low. Coomassie blue stain revealed three bands (molecular weight markers 27, 14, and 10 kDa; Fig. 1). The bands were individually digested and analyzed by mass spectroscopy. By comparing each sample, we identified proteins which frequently appeared in each sample (Table 1). A total of 11 proteins were extracted in this study. Leukocyte elastase precursor, cathepsin G precursor, azurocidin precursor, and myeloblastin precursor (leukocyte proteinase 3) were identified from band 1 by Database Search (SwissProt Protein Database; Swiss Institute of Bioinformatics; <http://www.expasy.org/sprot>) on a MASCOT server (Matrix Science Ltd.; <http://www.matrixscience.com>) (Fig. 2a). Calgranulin B, eosinophil cationic protein precursor, and lysozyme C precursor were identified from band 2 (Fig. 2b).

Fig. 1. One-dimensional SDS-PAGE of proteins extracted from stones. M = molecular markers (kDa).

TABLE 1. Identification of the digestive proteins bands by database search (SwissProt) on MASCOT server

Sequence ^a	Residues	Mass ^b		Mass accuracy (Delta)	MS/MS ions score ^c	Modification
		M _r found	M _r calc			
A. Band 1						
Leukocyte elastase precursor (EC 3.4.21.37) (neutrophil elastase) (Mowse score: 285) ^c , (M _r : 28500, pI: 9.71) ^d , (sequence coverage: 36%)						
GGHFC*GATLIAPNFVMSAAHC	51–78	2985.40 (+4)	2985.40	−0.00	12	Deamidation (N63, N74), methylation (R78)
*VANVNVR						
VVLGAHNLSR	82–91	1064.59 (+2)	1064.61	−0.02	52	
QVFAVQR	97–103	846.44 (+2)	846.47	−0.03	45	
		1800.85 (+2)	1800.91	−0.06	40	
		1816.86 (+3)	1816.91	−0.05	29	Oxidation (M154)
		1817.86 (+3)	1817.89	−0.03	48	Deamidation (Q150), oxidation (M154)
SNVC*TLVR	184–191	947.46 (+2)	947.49	−0.03	50	
		2817.34 (+3)	2817.37	−0.03	25	
		2818.29 (+4)	2818.35	−0.06	74	Deamidation (N209)
		2898.25 (+3)	2898.32	−0.07	64	Deamidation (N209), phosphorylation (S202)
Cathepsin G precursor (EC 3.4.21.20) (CG) (Mowse score: 136) ^c , (M _r : 28819, pI: 11.19) ^d , (sequence coverage: 40%)						
C*GGFLVR	49–55	807.39 (+2)	807.41	−0.02	46	
		1543.81 (+3)	1543.84	−0.03	52	
		1544.76 (+2)	1544.82	−0.06	49	Deamidation (N107)
		1560.77 (+2)	1560.82	−0.05	45	Deamidation (N107), oxidation (M110)
NVNPVALPR	123–131	978.53 (+2)	978.56	−0.03	29	
AQEGLRPGTLC*TVAGWGR	132–149	1927.94 (+3)	1927.97	−0.03	27	
IFGSYDPR	175–182	953.45 (+2)	953.46	−0.02	28	
QIC*VGDR	184–190	846.38 (+2)	846.40	−0.02	36	
GDSGGPLLC*NNVAHGIVSYGK	199–219	2113.97 (+3)	2114.02	−0.05	30	
SSGVPPEVFTR	220–230	1174.57 (+2)	1174.60	−0.03	47	
VSSFLPWIR	231–239	1103.59 (+2)	1103.61	−0.03	34	
Azurocidin precursor (cationic antimicrobial protein CAP37) (Mowse score: 74) ^c , (M _r : 26869, pI: 9.75) ^d , (sequence coverage: 48%)						
QFPFLASIQNQGR	37–49	1504.74 (+3)	1504.78	−0.04	20	
HFC*GGALIHAR	50–60	1237.59 (+3)	1237.61	−0.02	12	
FVMTAASC*FQSQNPQGVSTVVLGAYDLR	61–87	2933.33 (+3)	2933.41	−0.08	36	Oxidation (M63)
QTFSSMSSENGYDPQ	95–123	3388.49 (+3)	3388.56	−0.06	38	Oxidation (M102, M117)
QNLNDLMLLQLDR						
C*QVAGWGSQR	149–158	1147.53 (+2)	1147.52	0.01	26	
RGGIC*NGDGGTPLVC*	193–224	3228.44 (+4)	3228.49	−0.05	17	Deamidation (N198)
EGLAHGVASFSLGPC*GR						
Myeloblastin precursor (EC 3.4.21.76) (leukocyte proteinase 3) (Mowse score: 42) ^c , (M _r : 27789, pI: 8.72) ^d , (sequence coverage: 7%)						
LVNVVLGAHNVR	80–91	1289.74 (+3)	1289.76	−0.02	37	
LFPDFFTR	228–235	1041.50 (+2)	1041.53	−0.03	10	
B. Band 2						
Calgranulin B (migration inhibitory factor-related protein 14) (S100 calcium-binding protein) (Mowse score: 919) ^c , (M _r : 13234, pI: 5.71) ^d , (sequence coverage: 81%)						
		1805.82 (+3)	1805.93	−0.11	54	
		1806.82 (+2)	1806.92	−0.10	34	Deamidation (N17)
LGHPTLNQGEFK	26–38	1454.63 (+2)	1454.72	−0.09	54	
		876.41 (+2)	876.47	−0.06	14	
		877.41 (+2)	877.45	−0.05	16	Deamidation (N47)
		1741.72 (+2)	1741.82	−0.10	48	
		1757.72 (+2)	1757.81	−0.09	53	Oxidation (M63)
		1773.71 (+3)	1773.81	−0.1	32	Oxidation (H61,M63)
		1613.69 (+2)	1613.79	−0.11	79	
		1629.66 (+2)	1629.79	−0.13	40	Oxidation (M81)
		1645.67 (+2)	1645.78	−0.11	31	Oxidation (M81,M83)
		970.44 (+2)	970.49	−0.04	34	

TABLE 1. Continued

Sequence ^a	Residues	Mass ^b		Mass accuracy (Delta)	MS/MS ions score ^c	Modification
		M _r found	M _r calc			
B. Band 2						
		1002.44 (+2)	1002.48	−0.04	19	Oxidation (W88,H91)
MHEGDEGPGHHHKPGLGEGTP	94–114	2190.85 (+3)	2190.95	−0.10	9	Oxidation (M94)
Eosinophil cationic protein precursor (EC 3.1.27.–) (ECP) (ribonuclease 3) (Mowse score: 124) ^c , (M _r : 18428, pI:10.31) ^d , (sequence coverage: 41%)						
AQWFAIQHISLNPPR	35–49	1776.81 (+2)	1776.94	−0.13	50	
NQNTFLR	66–72	891.41 (+2)	891.46	−0.05	20	
		1778.77 (+2)	1778.87	−0.10	47	
		1779.77 (+3)	1779.86	−0.09	34	Deamidation (N84)
FYVVAC*DNR	133–141	1142.46 (+3)	1142.52	−0.06	13	
YPVVPVHLDTTI	149–160	1352.64 (+2)	1352.73	−0.09	34	
Lysozyme C precursor (EC 3.2.1.17) (1,4-beta-N-acetylmuramidase C) (Mowse score: 62) ^c , (Mr: 16526, pI: 9.38) ^d , (sequence coverage: 55%)						
LGMDGYR	33–39	826.32 (+2)	826.36	−0.04	21	Oxidation (M35)
GISLANWMCLAK	40–51	1362.60 (+2)	1362.68	−0.08	18	
WESGYNTR	52–59	1011.39 (+2)	1011.44	−0.05	16	
STDYGIFQINSR	69–80	1399.59 (+2)	1399.67	−0.08	51	
YWC*NDGK	81–87	941.32 (+2)	941.37	−0.05	20	
TPGAVNAC*HLSC*	88–115	2926.19 (+3)	2926.37	−0.18	41	
SALLQDNIADAVAC*AK						
QYVQGCGV	141–148	909.35 (+2)	909.40	−0.05	14	
C. Band 3						
Neutrophil defensin 3 precursor (HNP-3) (HP-3) (HP3) (defensin, alpha 3) (Mowse score: 817) ^c , (M _r : 10238, pI: 5.71) ^d , (sequence coverage: 26%)						
IPAC*IAGER	70–78	985.43 (+2)	985.50	−0.07	61	
YGTC*IYQGR	80–88	1116.43 (+2)	1116.50	−0.08	62	
LWAFCC*	89–94	855.27 (+2)	855.34	−0.07	30	
Calgranulin A (migration inhibitory factor-related protein 8) (MRP-8) (Mowse score: 340) ^c , (M _r : 10828, pI: 6.51) ^d , (sequence coverage: 76%)						
		862.37 (+2)	862.45	−0.07	14	
		878.38 (+2)	878.44	−0.06	36	Oxidation (M1)
		1271.60 (+3)	1271.69	−0.09	40	
		1272.57 (+3)	1272.67	−0.10	31	Deamidation (N10)
GNFHAVYR	24–31	962.40 (+2)	962.47	−0.07	36	
		1420.57 (+2)	1420.70	−0.13	44	
		1421.56 (+2)	1421.69	−0.12	59	Deamidation (Q44)
GADVWFK	50–56	821.34 (+2)	821.41	−0.07	35	
		2390.07 (+3)	2390.27	−0.20	47	
		2391.10 (+3)	2391.26	−0.16	27	Deamidation (N61)
Calgranulin C (CAGC) (CGRP) (neutrophil S100 protein) (Mowse score: 109) ^c , (M _r : 10437, pI:5.82) ^d , (sequence coverage: 79%)						
LEEHLLEGIVNIFHQYSVR	3–20	2181.95 (+3)	2182.12	−0.17	40	
GHFDTLSK	22–29	903.38 (+2)	903.44	−0.07	28	
AVIDEIFQGLDANQD	51–82	3577.50 (+3)	3577.82	−0.32	72	
EQVDFQEFISLVIAIALK						
Histone H4 (Mowse score: 57) ^c , (M _r : 11229, pI: 11.36) ^d , (sequence coverage: 21%)						
ISGLIYEETR	46–55	1179.52 (+2)	1179.61	−0.09	60	
		1309.60 (+2)	1309.70	−0.10	14	
		1325.59 (+2)	1325.69	−0.10	31	

[†]All mass values are listed as monoisotopic mass. Calculated masses were obtained from the NCBI database.

^aAmino acid sequence of peptides identified from tryptic digests on basis of their low-energy CID spectrum. C* denotes carbamidomethyl cysteine.

^bAll mass values are listed as monoisotopic mass. Calculated masses (M_r calc) were obtained from the NCBI database.

^cMowse and MS/MS ions scores were obtained from the MASCOT.

Neutrophil defensin 3 precursor, calgranulin A, calgranulin C, and histone H4 were identified from band 3 (Fig. 2c).

The mass pattern of leukocyte elastase precursor revealed the following amino acid modifications: oxidation, deamidation, methylation, and phosphorylation.

Fig. 2. Nano-LC-MS analysis of tryptic proteins from band 1 (a), band 2 (b), and band 3 (c). The peptides, which were identified from CID spectra in a separate LC-MS/MS analysis, are labeled and listed in Table 1.

Methylation was found at amino acid sequence of R78, deamidation was found at amino acids N63, N74, Q150, N209, and N209, oxidation at M154, and phosphorylation at S202. Deamidation at N107 and oxidation at M110 of the cathepsin G precursor were found from the mass pattern. Modifications were also seen on the azurocidin precursor at M63, M102, and M117 (oxidation) and at N198 (deamidation). Modifications such as deamidation and oxidation were seen for calgranulin A and B but not C. There was no modification on histone H4.

DISCUSSION

The aim of this study was to find the characteristic mass pattern of frequently seen proteins within calcium oxalate stones. Lower molecular weight proteins were more easily seen on SDS-PAGE bands, which enabled us to study them. Many proteins were found on three bands after SDS-PAGE. Each of them was repeatedly seen in the 10 stones, indicating that they may be common in calcium oxalate stones. However, no reports have been published concerning their role in the formation of stones. This is our preliminary study on matrix proteins present in calcium oxalate stones by means of mass spectroscopy. Further investigation, including functional and immunohistochemical studies should be performed to investigate their role. More stones should be collected to confirm the data.

Traditionally, stone matrix proteins are analyzed biochemically using antibodies to identify special proteins. However, this method can only be used on one protein at a time. Total protein contents cannot be predicted from antibody-related methods, such as Western blotting and enzyme-linked immunosorbent assay (ELISA). It is difficult to identify the functions of each protein from a mass of proteins obtained from biological tissue or materials. In addition, not all amino acids in a mass of proteins exert a specialized function in biological fields. Furthermore, acidic amino acids were reported to be found within stones and it was hypothesized that they are involved in the formation of stones in specialized plant cells (2). Understanding the characteristics of amino acids in peptide or proteins might help in the understanding of how proteins are related to stone formation.

A detailed and systemic isolation, identification, and characterization of urine proteins has been achieved through proteomics. Many biomarkers for bladder cancer, renal cancer, and prostate cancer have been detected using that method. Therefore, proteomics has the potential to be used in the diagnosis and management of urologic diseases. However, few studies have used proteomics in the investigation of stone disease.

Recently, Cadieux et al. (18) reported isolating urinary proteins that are involved in the formation of stone disease. They found a relatively large amount of urinary proteins and a high concentration of albumin in patients with stone disease. Two major bands of proteins (67-kDa and 42-kDa) were found from electrophoresis but they have yet to be characterized. By combining conventional and proteomic methods, Chutipongtanate et al. (19) found a new stone inhibitor in urine, Trefoil factor 1. However, in both of those studies proteins were identified from urine. Our study is the first to identify proteins directly from stones by proteomic method, providing an alternative direction of survey for stone disease.

The most common calcium-related protein isolated in our study was leukocyte elastase, a serine protease isolated from human bone marrow cell mitochondria (20). Weinrauch et al. (21) concluded that their findings established neutrophil elastase as the first neutrophil factor that targets bacterial virulence proteins. Cathepsin G is a member of the peptidase S1 protein family and may participate in the killing and digestion of engulfed pathogens, and in connective tissue remodeling at sites of inflammation (22). Azurocidin is an inflammatory protein secreted from granulocytes that is homologous to neutrophil elastase, myeloblastin, and cathepsin G (23). All of the above-mentioned proteins are involved in the inflammatory process. Whether they induced stone formation or were simply engulfed into the stone by means of inflammation caused by the stone is still unknown. Calgranulin is a member of the S100 family of proteins and is involved in the regulation of a number of cellular processes, such as cell cycle progression and differentiation (24). This protein can bind with calcium and is a tumor marker for endometrial cancer (25). Moreover, Bergsland et al. (26) used Western blotting to investigate urine inhibitors from patients with stones and patients without stones and found that calgranulin B was different between these two groups. They concluded that changes in form or amount of calgranulin B may be associated with stone formation. The binding activity of these proteins with calcium may indicate an indirect relationship. More studies are needed.

It is unclear whether the proteins found in urinary stones are associated with the formation of urolithiasis. Tawada et al. (27) reported that osteopontin and calprotectin are present in the matrix of urinary calcium stones. Siddiqui et al. (14) isolated proteins (molecular weights ranged from ≤ 12 kDa to 66 kDa) from urinary stone matrix and found that these proteins were also prevalent in the urine of patients with stones. These proteins were selectively incorporated into renal stones and most likely play a role in nidus. They concluded that

these proteins promote the formation of calcium oxalate stone. However, a study from stone former urine by Kleinman et al. (28) found that osteopontin has endogenous defense against crystal formation. Osteopontin acts in vivo primarily to inhibit the retention of crystal rather than to retard crystal nucleation, growth, or aggregation from a preliminary data of Kleinman et al. (28). Osteopontin found in stone maybe cause by its adsorption on crystal surface to prevent further crystal adhesion thereby as an inhibitor. Therefore, its existence in stone matrix may not indicate osteopontin is a promoter. Further study should be done to determine its role in stone formation.

The modifications of amino acids can alter the function of proteins. Selvam and Kalaiselvi (29) found several oxalate-specific binding proteins in human and rat kidney. The purified oxalate binding proteins comprised mitochondrial protein (62 kDa), histone H_{1B} (27.5 kDa), nuclear membrane protein (68 kDa), nuclear pore complex protein (205 kDa), and 45-kDa protein. The enhancement of oxalate binding activity was correlated positively with increased lipid peroxidation and correlated negatively with decreased thiol status. In the calcium oxalate crystal growth system, the oxalate binding proteins behaved either as promoters or inhibitors of the nucleation and aggregation of crystals. Following the peroxidation of the proteins, the degree of the effect of the promoter protein was further stimulated while the degree of inhibition caused by the inhibitor protein further declined. The oxalate binding proteins reported by Selvam and Kalaiselvi (29) were thought to modulate the crystallization process and as modulators depending their modifications. From their study, the modifications of the proteins found in our study may have some implications in stone formation.

There were some unknown proteins in urine that could not be detected by mass spectroscopy. Some unknown peptide sequences and proteins have only been identified by their amino acid compositions, making identification from mass spectroscopic data impossible. Proteins that have previously been found in urine, such as Tamm-Horsfall protein, nephrocalcin, and osteopontin, were not detected in our study; this could have been due to the small number of stones analyzed. However, it could also be caused by problems of methodology, such as composition of stones, protein destroyed during preparation, or the exclusion of high molecular weight proteins by electrophoresis such as Tamm-Horsfall protein. It may be helpful to search other biochemical methods for the protein study to solve this problem. Therefore, more stones should be collected to further investigate the proteins. Furthermore, immunochemical staining should be applied to

the surface of stones to prove the existence of our reported proteins.

In conclusion, our study provides a proteomic study of matrix proteins in urinary stones. There were abundant proteins in the SDS-PAGE bands 27 kDa, 14 kDa, and 10 kDa, which showed modifications on mass spectroscopy. The role these proteins play in the formation of calcium oxalate stones needs to be investigated.

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