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Analysis of urinary calculi obtained from a patient with idiopathic hypouricemia using micro area x-ray diffractometry and LC-MS

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Abstract Urolithiasis is a common complication in patients with hypouricemia. Using a microarea x-ray diffractometer and nanoflow liquid chromatography-mass spectrometry (LC-MS) following SDS-polyacrylamide gel electrophoresis (PAGE), recurrent urinary calculi complicating a hypouricemic patient were analyzed. Analysis with the microarea x-ray diffractometer showed that one of the calculi was composed of calcium oxalate monohydrate and hydroxyapatite. The other was found to be formed from calcium oxalate dihydrate. After determination with LC-MS, both were found to contain uromodulin, albumin, osteopontin, protein Z, and defensins. Lysozyme and calgranulin A were also identified in these calculi. Defensins, which were antimicrobial peptides, and lysozyme, a mucopeptide glycohydrolase, were identified as new organic components of urinary stones. The role of these proteins in the process of urolithiasis is of particular interest.

Keywords Urinary calculi · LC-MS · Microarea x-ray diffractometer · Calcium oxalate · Defensins · Lysozyme

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Introduction

Hypouricemia due to an isolated tubular defect of uric acid transport is a relatively rare disease. Patients with idiopathic renal hypouricemia show a high uric acid clearance because of abnormal tubular transport of uric acid in the kidney. It is known that urinary calculi are often complications in patients with hypouricemia [1]. It is important to carefully analyze each patient's individual urinary calculus in order to investigate the mechanisms of calculi recurrence.

Several proteins, collectively dubbed "the matrix", have been reported as organic components of renal stones [2, 3]. Albumin, Tamm-Horsfall protein, α-globulins and γ-globulins have been reported to be present within the calculi as matrix proteins. Osteopontin and prothrombin, which are calcium-binding proteins, have been reported in the matrix of calcium oxalate stones [4–7]. Calprotectin was also found both in struvite stones and calcium oxalate stones [8–10]. Furthermore, we identified protein Z, which is reported to be a vitamin K-dependent calcium-binding protein [11–16], in a urinary calculus composed of calcium oxalate monohydrate [17]. This protein has been reported to be a factor acting in the coagulation process [18, 19].

In other studies, after polyacrylamide gel electrophoresis (PAGE) was performed, liquid chromatography-mass spectrometry (LC-MS) was applied for the analysis of various proteins [20–23]. LC-MS is of importance because of its high sensitivity.

In this study, we employed LC-MS equipped with a nanoelectrospray interface, and an ion-trap detector, in order to analyze the matrix protein in recurrent calculi from a hypouricemic patient. An algorithm with tandem mass spectrum database matching tools was also employed for identifying proteins [24]. Furthermore, in order to determine the inorganic components of the calculus, a microarea x-ray diffractometer [25, 26] was used, enabling the fine inorganic components of the calculus to be analyzed.

Materials and methods

Urinary calculi

Urinary calculi were obtained from a male patient with idiopathic hypouricemia [27]. He had suffered from recurrent urolithiasis and came to the Department of Internal Medicine at Teikyo University Hospital for treatment. Calculus 1 (2×3 mm) was discharged in 1994, and calculus 2 (1.5×3 mm) in 1997. The patient brought the calculi to the hospital for further clinical examination. Routine IR analysis showed that they were mainly composed of calcium oxalate (more than 95%). The patient was informed that his urinary calculi would be used in further analysis.

Microarea x-ray diffractometry

The urinary calculi were analyzed with a microbeam of x-rays at several spots. After the outer surface of the unbroken calculus was determined, it was divided into two or three pieces and was analyzed at several points on its inner face. For this study, a microarea x-ray diffractometer (JEOL JDX-8030, DX-MAP2, Tokyo, Japan) with a microscope was used according to previously reported methods [25]. The analytical conditions were as follows: target: Cu; filter: Ni; voltage: 40 kV; current: 40 mA; diameter of the collimator: 100 μm. The diffraction pattern obtained was compared with the data registered in the Joint Committee on Powder Diffraction Standards (JCPDS) database.

Protein extraction from urinary calculi

Extraction was performed mainly according to previously reported methods [17]. Calculus 1 was powdered and extracted with 4 M guanidine hydrochloride in 50 mM Tris/HCl buffer (pH 7.4) containing 1 mM phenyl methyl sulfonyl fluoride (PMSF), and 0.5 M EDTA in 50 mM Tris/HCl buffer (pH 7.4). Calculus 2 was powdered in the same way, and then extracted separately with 0.6 M KCl, 10% formic acid, 4 M guanidine hydrochloride in 50 mM Tris/HCl buffer (pH 7.4) containing 1 mM phenyl methyl sulfonyl fluoride (PMSF), and 0.5 M EDTA in 50 mM Tris/HCl buffer (pH 7.4). After every extraction, the sediment was washed three times and the supernatant dialyzed and concentrated by lyophilization. The dialyzed samples were kept at −80°C until electrophoresis.

SDS-PAGE

Proteins were analyzed by sodium dodecyl sulfated-polyacrylamide gel electrophoresis (SDS-PAGE) or two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) [20]. The sample was dissolved in 125 μl of buffer containing 8 M urea, 2% CHAPS, IPG buffer and bromophenol blue. The gel was stained with Coomassie brilliant blue (CBB).

In gel digestion with trypsin

Protein spots were excised from the gel and digested with trypsin according to published procedures [28].

Liquid chromatography-mass spectrometry

A portion of the tryptic peptide solution was analyzed by LC-MS. The HPLC system used was the Magic 2002 from Michrom BioResources (USA). The analytical conditions were as follows: column: Magic C_{18}, 0.2 mm ID×50 mm; solvent: 0.1 M formic acid with CH_3CN gradient (5–65% in 20 min); flow rate: 2 μl/min.

The mass spectrometer employed was a ThermoQuest LCQ_{DACA} (USA) equipped with an ion trap, and a nano ESI interface from Michrom BioResources. The electrospray voltage was 1.5 kV and the temperature of the heated capillary was 170°C. During the chromatographic separation, the LCQ_{DACA} performed the "dynamic exclusion" experiment. The experiment produced a full-scan MS spectrum and full-scan MS/MS spectrum based on a maximum intensity threshold. Using ion-trap detection, the mass fragmentation from the selected ions was easily collected. The MS/MS spectra were subjected to a database search using SEQUEST (X calibur 1.3, Bioworks 3.0) [29]. SEQUEST correlates uninterpreted tandem mass spectra on peptides with amino acid sequences in a protein and nucleotide database.

Chemicals

All chemicals were of the highest commercial quality available. Chemicals were purchased from Wako Pure Chemicals (Japan) or Sigma (USA).

Results

Analysis using a microarea x-ray diffractometer

Figure 1 indicates the x-ray diffraction pattern of a spot on the outer surface of the unbroken calculus 1. The brown portion on the surface of this calculus was composed mainly of calcium oxalate monohydrate (whewellite). The intensity of the diffracted x-rays was strong, and the diffraction angles agreed well with those of calcium oxalate monohydrate as listed in the JCPDS database. Calculus 1 also contained a white colored internal area. When this portion was determined, the intensity of the diffracted x-ray was not very strong

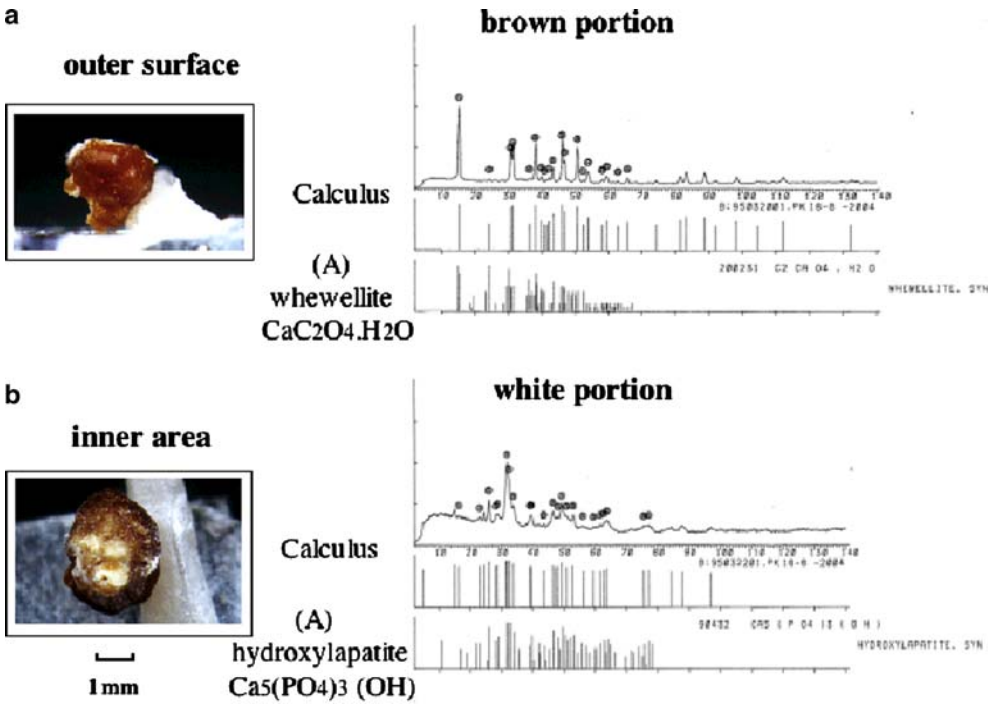


Fig. 1 X-ray diffraction pattern of: **a** a spot on the outer surface and **b** a spot in the inner area of calculus 1. Several points of calculus 1 were analyzed using microbeam x-rays. The x-ray diffraction pattern was recorded with an x-ray goniometer and is represented in intensity as a function of twice the diffraction angle (2θ) curve. The *vertical line* shows the intensity of x-ray diffraction. The retrieved standard substance in the JCPDS database is indicated by (A) at the bottom

when compared with the outer surface. However, the data indicated a high degree of similarity to hydroxyapatite as listed in the JCPDS database (Fig. 1). Therefore, we determined this portion to be hydroxyapatite, an inorganic component.

Figure 2 shows the x-ray diffraction pattern of calculus 2. Both the brown-colored outer surface and the white-colored inner portion were considered to be calcium oxalate dihydrate (weddellite).

SDS-PAGE of urinary stones

In Fig. 3a, the polyacrylamide gel electrophoresis of guanidine and EDTA extracted proteins from calculus 1 is shown. From calculus 1, several protein bands were stained with CBB. Most of the proteins migrated to 40–75 kDa and < 25 kDa. Figure 3b shows SDS-PAGE of extracted proteins from calculus 2 with four different solvents: 0.6 M KCl, 10% formic acid, 4 M guanidine

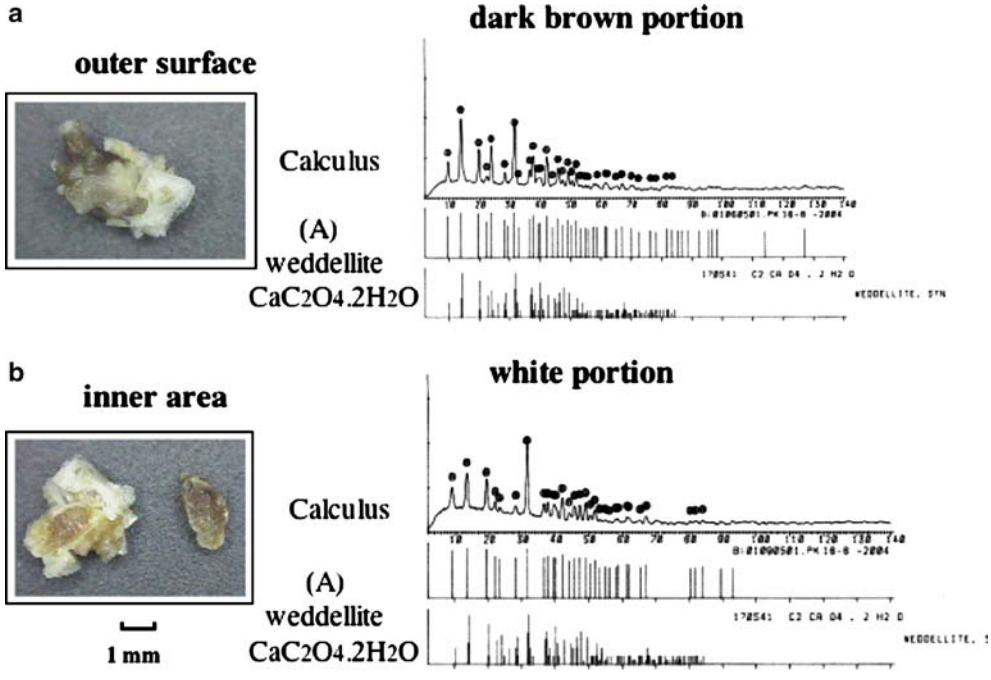


Fig. 2 X-ray diffraction pattern of: **a** a spot on the outer surface and **b** a spot in the inner area of calculus 2. Refer to the legend in Fig. 1 for details

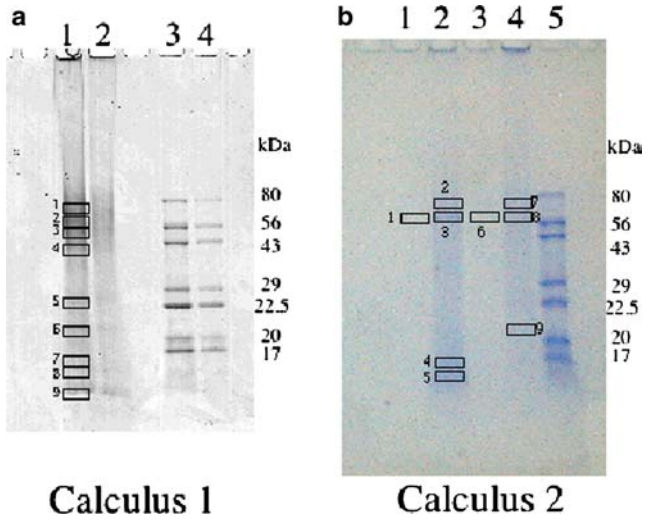


Fig. 3 SDS-polyacrylamide gel electrophoresis (PAGE) of calculus 1 (**a**) and calculus 2 (**b**). **a** Extracted protein, together with guanidine-HCl and EDTA, from calculus 1 were subjected to SDS-PAGE. Proteins were stained with Coomassie brilliant blue (CBB). *Lane 1*: extract 10 µl; *lane 2*: extract 5 µl; *lane 3*: marker 10 µl; *lane 4*: marker 5 µL. **b** Proteins in calculus 2 were extracted in sequence with 0.6 M KCl, 10% formic acid, 4 M guanidine-HCl, and 0.5 M EDTA. Proteins were subjected to SDS-PAGE and stained with CBB. *Lane 1*: extract with 0.6 M KCl; *lane 2*: extract with 10% formic acid; *lane 3*: extract with 4 M guanidine-HCl; *lane 4*: extract with 0.5 M EDTA; *lane 5*: marker

hydrochloride, and 0.5 M EDTA. Stained bands were seen in both the formic acid and EDTA fractions.

Though protein bands were not well isolated as individual bands, these spots were excised, and digested with trypsin in gel, then applied to LC-MS.

Protein detection using LC-MS

Analysis of proteins from each excised spot was conducted, and multiple MS/MS spectra obtained. The computerized protein identification program, SEQUEST, a tandem mass spectrum database-matching tool, was applied.

Proteins identified in these urinary calculi are summarized in Table 1. Protein Z, which is a vitamin K-dependent protein that we previously identified in a renal stone [17], was detected in bands 1, 2, 3 and 4 of the SDS-PAGE of calculus 1, as shown in Fig. 3a. The molecular weight of protein Z is reported to be 62 kDa, but in calculus 1, protein Z was detected in four bands with molecular weights of 40–75 kDa. After LC-MS analysis, four peptides of protein Z were detected in calculus 1. The positions of the detected peptides were 204–215, 232–240, 320-327 and 413–418 amino acids, respectively. The protein coverage rate was 8.3%. The spectrum obtained from calculus 1 was similar to the theoretical fragmentation of the corresponding peptide.

Lysozyme, a protease with a molecular weight of 17 kDa, was detected in band 8 of calculus 1. Six peptides were detected and the protein coverage rate was 46.6% (Fig. 4a). The MS/MS spectrum of the peptide, positions 69–80, is indicated in Fig. 4b.

Neutrophil defensins, which have a molecular weight of 10 kDa, were identified in band 9. Three peptides were detected by LC-MS analysis and the protein coverage rate was 26.6% (Fig. 5a). The MS/MS spectrum of the peptide, positions 70–78, are shown in Fig. 5b.

From calculus 1, three other proteins, uromodulin, albumin and osteopontin, were determined in several areas. Uromodulin was identified in bands 1, 3, 4, and 6. Albumin was detected in bands 2 and 4, and osteopontin was identified in bands 1, 2, and 3.

In calculus 2, the protein extraction procedure from the calculi was changed and the elusion pattern of the proteins studied. Few proteins were stained with CBB in both 0.6 M KCl and 4 M guanidine hydrochloride extracts, as indicated in Fig. 3b. Protein candidates could not be detected in these extracts (Table 1). On the other hand, several bands were stained with CBB in 10% formic acid extract and in the 0.5 M EDTA extract. In

Table 1 Proteins identified in calculus 1 and calculus 2

		Identified protein	Bands
Calculus 1	SDS-PAGE	Uromodulin	Band 1, 3, 4, 6
		Osteopontin	Band 1, 2, 3
		Protein Z	Band 1, 2, 3, 4
		Albumin	Band 2, 4
		Lysozyme	Band 8
		Defensins	Band 9
	2D-PAGE	Uromodulin	
		Protein Z	
Calculus 2	SDS-PAGE		
	0.6 M KCl	Not detected	
	10% Formic acid	Calgranulin A	Band 2, 3, 4, 5
		Uromodulin	Band 2
		Albumin	Band 3
		Defensins	Band 5
	4 M Guanidine-HCl	Not detected	
	0.5M EDTA	Osteopontin	Band 8
		Protein Z	Band 8

database=C:\Xcalibur\database\human.fasta, accession=gi|126615|sp|P00695|LYC_HUMAN **a**
peptide(s)=YWCNDGK WESGYNTR QYVQGCGV AWVAWR STDYGIFQINSR
TPGAVNACHLSCSALLQDNIADAVACAK

Analyzing ...

>gi|126615|sp|P00695|LYC_HUMAN LYSOZYME C PRECURSOR (1,4-BETA-N-ACETYLMURAMIDASE C)□gi|2144473|pir||LZHU lysozyme (EC 3.2.1.17) c precursor - human□gi|307140 (J03801) lysozyme precursor (EC 3.2.1.17) [Homo sapiens]□gi|307142 (M19045) lysozyme precursor (EC 3.2.1.17) [Homo sapiens]□gi|1790941 (U76912) lysozyme c precursor [Pan troglodytes]□gi|1790961 (U76933) lysozyme c precursor [Pan paniscus]
[MASS=16537]
MKALIVLGLV LLSVTVQGKV FERCELARTL KRLGMDGYRG ISLANWMCLA KWESGYNTRA
TNYNAGDRST DYGIFQINSR YWCNDGKTPG AVNACHLSCS ALLQDNIADA VACAKRVVRD
PQGIRAWVAW RNRCQNRDVR QYVQGCGV
>average mass = **16519**

position	sequence (**NCBI BLAST** link)
81- 87	YWCNDGK
52- 59	WESGYNTR
141- 148	QYVQGCGV
126- 131	AWVAWR
69- 80	STDYGIFQINSR
88- 115	TPGAVNACHLSCSALLQDNIADAVACAK

Protein Coverage: 69/148 = **46.6%** by amino acid count, 7587/16519 = **45.9%** by mass

Search SWISS-PROT with gi|126615|sp|P00695|LYC_HUMAN via accession, descr./ID, or full text field.

Done.

Lysozyme C precursor peptide, position 69-80 (STDYGIFQINSR) **b**

Seq #		b	y	(+1)
S	1	88.1	1401.5	12
T	2	189.2	1314.4	11
D	3	**304.3**	**1213.3**	10
Y	4	**467.4**	**1098.2**	9
G	5	**524.5**	**935.0**	8
I	6	**637.7**	**878.0**	7
F	7	**784.8**	**764.8**	6
Q	8	**913.0**	**617.7**	5
I	9	**1026.1**	**489.5**	4
N	10	**1140.2**	**376.4**	3
S	11	1227.3	**262.3**	2
R	12	1383.5	175.2	1

Seq #		b	y	(+2)
S	1	44.5	701.3	12
T	2	95.1	657.7	11
D	3	152.6	**607.2**	10
Y	4	234.2	**549.6**	9
G	5	**262.8**	**468.0**	8
I	6	319.3	**439.5**	7
F	7	**392.9**	382.9	6
Q	8	457.0	309.3	5
I	9	513.6	245.3	4
N	10	570.6	188.7	3
S	11	614.2	131.6	2
R	12	**692.3**	88.1	1

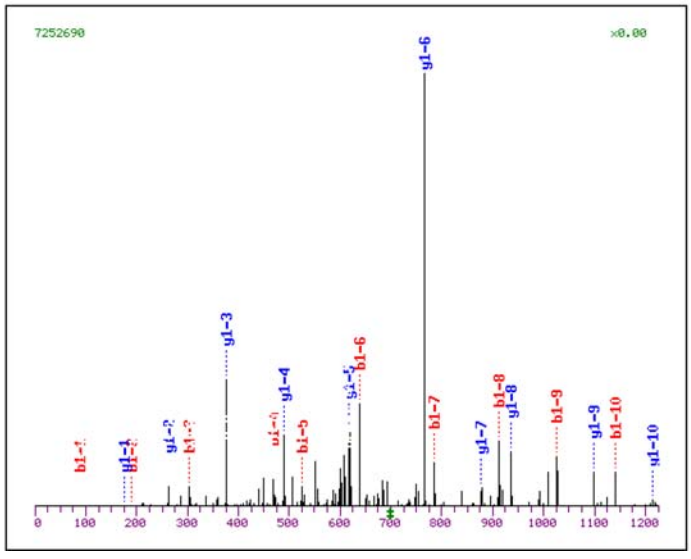


Fig. 4 Lysozyme identified by LC-MS of calculus 1. With the SEQUEST search program from MS/MS spectra obtained from the calculus 1 analysis, six peptides in protein Z were detected. The results of the protein search are shown in **a**, and the protein coverage rate was 46.6%. MS/MS spectrum of a peptide, positions 69–80 (STDYGIFQINSR), is indicated in **b**. The theoretical mass numbers of MS/MS fragmentation are shown on the *left*. The MS/MS spectrum actually obtained from the extract of the calculus is shown in the *box* to the right. The theoretical mass numbers, which were found to be identical to the actual mass numbers observed in calculus, are indicated in *bold* or *colored print* in the theoretical data table and are marked accordingly on the spectrum. Most of the spectra agreed with the theoretical fragmentation mass numbers in the calculus

the extract with 10% formic acid, calgranulin A, uromodulin, and albumin were identified. In the EDTA extract, osteopontin and protein Z, which are both calcium-binding proteins, were detected.

Discussion

Urinary calculi obtained from a hypouricemic patient were analyzed in detail. Microarea x-ray diffractometry was first carried out with unbroken stones in order to determine the inorganic components. For the analysis of matrix proteins, extraction, SDS-PAGE and LC-MS were performed.

Using SDS-PAGE, proteins in calculi could not be clearly isolated as individual protein bands because of a strong tendency to aggregate, as reported previously [3]. Proteins in the calculus seem to interact and associate with each other, and are not easily isolated.

Calculus 1 was composed of calcium oxalate monohydrate and hydroxyapatite as crystal components. When this calculus was extracted with guanidine-HCl and EDTA, it contained uromodulin, osteopontin, albumin, protein Z, defensins, and lysozyme as the matrix proteins.

Calculus 2 was mainly composed of calcium oxalate dihydrate as an inorganic component. When we used 10% formic acid, we were able to obtain calgranulin A,

a

database=C:\Xcalibur\database\human.fasta, accession=gi|118434|sp|P11479|DEFN_HUMAN
peptide(s)=IPACIAGERR RYGTCIYQGR IPACIAGER YGTCIYQGR LWAFCC

Analyzing ...

>gi|118434|sp|P11479|DEFN_HUMAN NEUTROPHIL DEFENSINS 1, 2 AND 3 PRECURSOR (HNP) (DEFENSIN, ALPHA 1)□gi|107410|pir||A40499 defensin HNP-1 precursor - human□gi|32402 (X52053) HP-1 (AA 1-94) [Homo sapiens]□gi|181527 (M21130) neutrophil peptide 1 precursor [Homo sapiens]□gi|181529 (M26602) defensin 1 [Homo sapiens]□gi|292363 (L12690) neutrophil peptide-1 [Homo sapiens]□gi|446635|prf||1912193A defensin [Oryctolagus cuniculus]□gi|1098031|prf||2115200A neutrophil peptide [Homo sapiens] [MASS=10201]
MRTLAILAAI LLVALQAQAE PLQARADEVA AAPEQIAADI PEVVVSLAWD ESLAPKHPGS
RKNMACYCRI **PACIAGERRY GTCIYQGRLW AFCC**
>average mass = **10182**

position	sequence (**NCBI BLAST** link)
70- 79	IPACIAGERR
79- 88	RYGTCIYQGR
70- 78	IPACIAGER
80- 88	YGTCIYQGR
89- 94	LWAFCC

Protein Coverage: 25/94 = **26.6%** by amino acid count, 2833/10182 = **27.8%** by mass

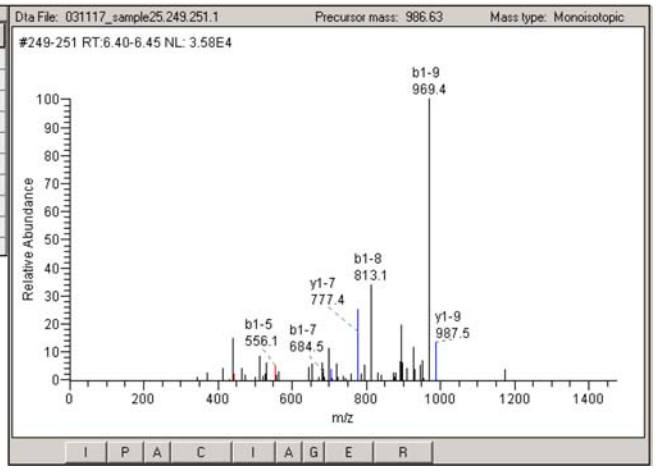
Search SWISS-PROT with gi|118434|sp|P11479|DEFN_HUMAN via accession, descr./ID, or full text field.

Done.

b

Defensins precursor peptide, position 70-78 (IPACIAGER)

	AA	A Ions	B Ions	Y Ions	
1	I		114.09	-	9
2	P		211.14	874.39	8
3	A		282.18	777.34	7
4	C		443.20	706.30	6
5	I		556.28	545.29	5
6	A		627.32	432.20	4
7	G		684.34	361.17	3
8	E		813.38	304.14	2
9	R		-	175.10	1



uromodulin, albumin, and defensins. Furthermore, osteopontin and protein Z were detected with 0.5 M EDTA.

The common proteins in these two calculi were uromodulin, osteopontin, albumin, protein Z and defensins. The three former proteins are widely known to exist in urinary calculi [2–5, 7, 10]. We previously reported the presence of protein Z, a vitamin K-dependent calcium-binding protein, in a calculus [17]. Although the physiological role of protein Z is little understood, it is believed to act as a regulator in the coagulation process [11–16, 18, 19]. Osteopontin and protein Z are considered to be relevant to the formation of calcium oxalate stones.

Defensins are reported to be antimicrobial peptides in vertebrates [30, 31]. The present study is the first to detect defensins in urinary calculi. Defensins contain a relatively large number of basic amino acids, and therefore we were able to extract them with formic acid in calculus 2. The presence of defensins in urinary calculi is thought to be the result of a preventive reaction to some type of infection.

In addition to these proteins, calculus 1 contained lysozyme and calculus 2 calgranulin A. Calgranulin A has previously been reported in urinary calculi [8–10]. Because it is a calcium binding protein, it probably plays this role in the matrix of calcium stones. Lysozyme has not previously been reported as a matrix protein in urinary calculi. Because lysozyme is a comparatively small enzyme, it has been used as a marker for renal dysfunction and abnormal urinalysis [32, 33]. The patient had idiopathic renal hypouricemia with a defect in post-secretary reabsorption of uric acid [27]. The presence of lysozyme in his calculus is probably related to this renal abnormality.

The differences in protein between the two calculi can be derived from their crystal components. Calculus 1 contained calcium oxalate monohydrate and hydroxyapatite and calculus 2 was composed of calcium dihydrate. There have been few reports in which different calculi from the same individual have been analyzed minutely using sensitive methods. It is considered important to carefully analyze each calculus from a patient with recurrent urolithiasis and to compare the data obtained.

In this study, urinary calculi obtained from a hypouricemic patient with recurrent urolithiasis were

Fig. 5 Defensins identified by LC-MS of calculus 1. **a** Shows the results of the protein search. Three peptides were detected by LC-MS and the protein coverage rate was 26.6%. **b** Indicates the MS/MS spectrum of peptide, positions 70–78 (IPACIAGER). Most of the spectra in the calculus agreed with the theoretical fragmentation mass numbers

analyzed minutely using a microarea x-ray diffractometer and LC-MS. Novel matrix proteins, defensins and lysozyme, were detected in urinary calculi mainly composed of calcium oxalate. In recurrent urolithiasis, these organic substances may well play a significant role in the formation of the calculus. Further investigation is considered to be important.

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