KURENAI 紅

Kyoto University Research Information Repository

KYOTO UNIVERSITY

Title	Detection of osteopontin as matrix protein in calcium-containing urinary stones
Author(s)	YAMATE, Takanori; UMEKAWA, Tohru; IGUCHI, Masanori; KURITA, Takashi; KOHRI, Kenjiro
Citation	(1997), 43(9): 623-627
Issue Date	1997-09
URL	http://hdl.handle.net/2433/116036
Right	
Type	Departmental Bulletin Paper
Textversion	publisher

Kyoto University

DETECTION OF OSTEOPONTIN AS MATRIX PROTEIN IN CALCIUM-CONTAINING URINARY STONES

Takanori Yamate, Tohru Umekawa, Masanori Iguchi
and Takashi Kurita

From the Department of Urology, Kinki University School of Medicine

Kenjiro Kohri

From the Department of Urology, Medical School, Nagoya City University

Osteopontin (OPN) has been identified as a matrix protein of calcium oxalate urinary stones by sequencing c-DNA for urinary stone protein. OPN, a phosphoprotein, is susceptible to thrombin digestion and specifically stainable with Stains-All. We examined the matrix in 4 kinds of urinary stones, calcium oxalate dihydrate, calcium phosphate, magnesium ammonium phosphate and uric acid, for the presence of OPN by staining thrombin-digestion and undigested matrix with Stains-All. Matrix was extracted with a 0.1 M ethylenediamine tetraacetic acid (EDTA) solution. Furthermore, the amino acid sequence was determined for the NH2-terminal 20 amino acid residues. OPN was identified in calcium oxalate dihydrate and calcium phosphate stones, but was absent in magnesium ammonium phosphate and uric acid stones. Our findings suggest that OPN, which binds tightly to hydroxyapatite and is related to bone formation as bone matrix, also participates in the formation of calcium-containing usinary stones.

(Acta Urol. Jpn. **43** : 623–627, 1997)

Key words : Calcium-containing stone, Matrix protein, Osteopontin, Stains-All

INTRODUCTION

Although many reports have suggested the importance of the matrix in urinary stone formation, its role has remained unidentified. Boyce et al.[1] and King et al.[2] reported that the matrix was composed of 64% protein, 5% proteoglycans and 10% bound water. Umekawa et al. reported the presence of calprotectin[3] and alpha 1-antitripsin[4] in the matrix of calcium oxalate urinary stones. According to the amino acid analysis conducted by Spector et al.[5] acidic amino acids such as aspartic acid and glutamic acid are the major constituent amino acids of the matrix. Lian et al.[6] reported the presence of gamma carboxyglutamic acid, and Melick et al.[7] identified sialic acid. Two dimensional polyacrylamide gel electrophoresis by Jones et al.[8] revealed that most of the proteins in the matrix of the urinary stones are low molecular weight proteins.

Our recent study revealed that the c-DNA sequence of osteopontin (OPN) encodes the urinary calcium oxalate stone protein, suggesting that OPN is involved in stone formation as the stone matrix[9] The expression of OPN mRNA and protein was observed in stone forming kidneys[10]. OPN was isolated from the mineralized matrix of bovine bone. This protein, a secreted phosphoprotein-1, binds tightly to hydroxyapatite[11] Since previous studies on the stone matrix protein primarily used Coomassie brilliant blue stain, highly phosphorylated OPN

protein may not have been identified. In this study, we demonstrated the presence of OPN in the stone proteins by using Stains-All staining, a new molecular biological technique that can stain highly phosphorylated protein. The purpose of this study is to examine the existence of OPN in urinary stone components as a matrix.

MATERIALS AND METHODS

Extraction procedures of osteopontin protein

The urinary stones obtained after extracorporeal shock wave lithotripsy (ESWL) were analyzed by infrared spectroscopy. Four stones with over 96% of any of the 4 kinds of stone components of calcium oxalate dihydrate, calcium phosphate, magnesium ammonium phosphate, and uric acid were used as specimens. Stones were destroyed by extracorporeal shock wave lithotripsy, allowed to spontaneously discharge, washed with water, dried, and stored. One hundred fifty mg of each powdered stone was put into a 1.5 ml Safe-Lock (Eppendorf) microfuge tube and extracted with 1 ml of 0.5 M ethylenediamine tetraacetic acid (EDTA), prepared in 50 mM Tris/ hydrochloric acid (HCl) buffer, pH 7.4, containing 1 mM phenylmethyl sulfonyl fluoride (PMSF). The extraction was performed for 24 hours at 4°C with gentle shaking. The specimen was washed three times in phosphate buffered solution (PBS) containing PMSF for 30 minutes each, between extractions. The suspension was centrifuged at 8,000

rpm on an Eppendorf microfuge tube for 10 minutes, and the supernatant was dialyzed against three changes of 10 mM ammonium bicarbonate containing 0.05% Bolyxyethleme Lauryl ether (BRIJ 35) lyophilized and kept at −80°C.

Thrombin digestion

Digestion was performed at 37°C for 30 minutes in 10 μl of 10 mM Tris/HCl buffer pH 8.0 containing 10 mM calcium chloride ($CaCl_2$) and 1 unit of thrombin (Sigma Chem. Co., St Louis). Digestion was terminated by the addition of 0.25 volume of a 4-fold concentrated sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer containing 150 μg dithiothreitol (DTT) and heating to 56°C for 25 minutes.

Poylacrylamide gel electrophoresis

Proteins were analyzed by SDS-PAGE electrophoresis using the discontinuous Tris/glycine buffer system with 10% cross-linked linear gels as described by Goldberg et al.[12] and Nagata et al.[13]. Specimens were dissolved in 10 μl of sample buffer containing 1% SDS, 2M urea, and bromophenol blue marker. For analysis of proteins under reducing conditions, 150 μg DTT was induced. The specimens were heated to 56°C for 30 minutes and cooled immediately before application to individual wells. A 10% gel plate was put into an electrophoresis tank filled with electrolyte buffer (15.1 g/l, Tris, 72 g/l

glycine). Each lane was applied with thrombin undigested sample, thrombin digested sample, and prestained SDS-PAGE standard markers. Electrophoresis was carried out for 1 hour at 150 V Following separation, the gels were stained in the dark for at least 48 hours with 0.025% Stains-All (3,

3′-diethyl-9-methyl-4, 5, 4′, 5′-dibenzothiacarbo-cyanine bromide) (Nakalai Tesque Co., Kyoto), 25% isopropyl alcohol, 7.5% formamide and 30 mM Tris base, pH 8.8.

Amino acid sequence analysis

NH2-terminal analysis of 20 amino acids using 400–600 pmol samples of protein was carried out by a gas phase protein sequencer 477A (Applied Biosystems).

RESULTS

Figures 1–3 and 4 show the electrophoretic patterns for the matrix protein extracted from 4 kinds of stone components and stained with Stains-All. The calcium oxalate dihydrate stones showed 3 bands of 49.5, 32.5, and 25 kDa (Fig. 1, lane C). The 49.5 kDa band disappeared after thrombin digestion, and the 32.5 band was intensified (Fig. 1, lane T). The calcium phosphate stones showed 3 bands of 45, 32.5, and 25 kDa (Fig. 2, lane C). After thrombin digestion, the 45 kDa band disappeared and the 32.5 kDa band was intensified (Fig. 2, lane T). Magne-

C   Thrombin undigestion
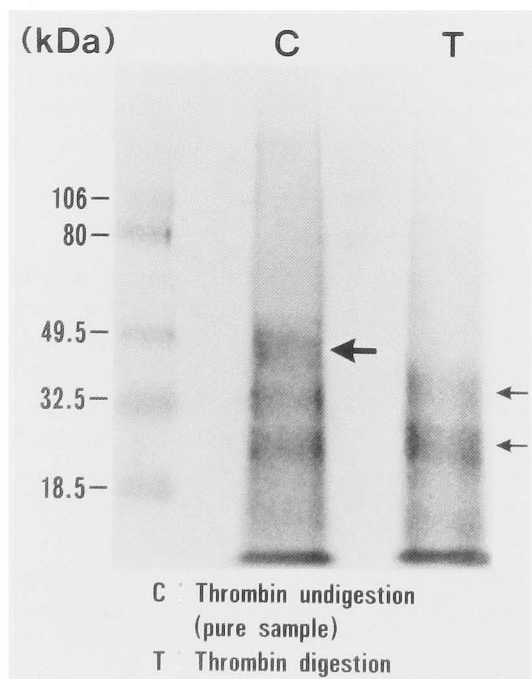   (pure sample)
T   Thrombin digestion

Fig. 1. Electrophoretic patterns by Stains-All staining of matrix protein extracted from calcium oxalate dihydrate (weddelite) The 3 bands of 49.5, 32.5 and 25 kDa appeared in the thrombin undigested lane (lani C). The 49.5 kDa band disappeared after thrombin digestion, and the 32.5 kDa band was intensified (lane T).

C   Thrombin undigestion
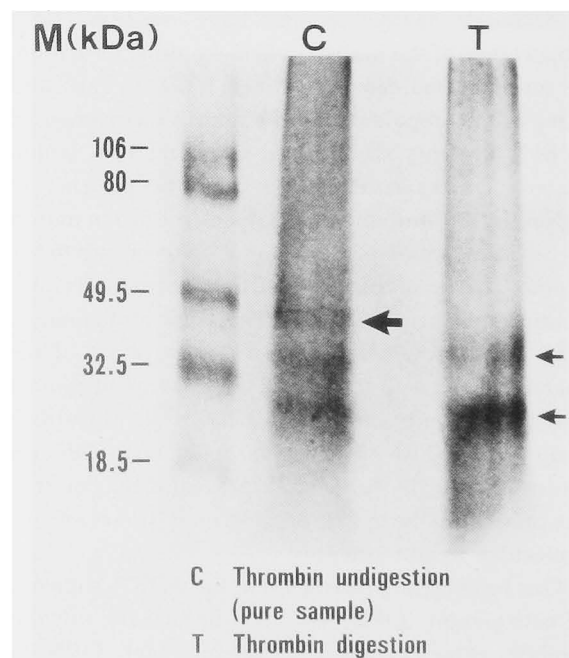   (pure sample)
T   Thrombin digestion

Fig. 2. Electrophoretic patterns by Stains-All staining of matrix protein extracted from calcium phosphate (brushite) The 3 bands of 45, 32.5 and 25 KDa appeared in the thrombin undigested lane (lane T) The 45 kDa band disappeared after thrombin digestion, and the 32.5 kDa band was intensified (lane T).
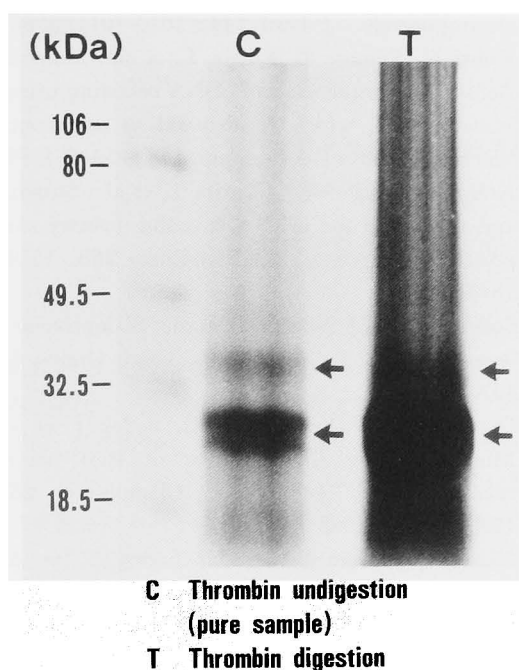
Fig. 3. Electrophoretic patterns by Stains-All staining of matrix protein extracted from magnesium ammonium phosphate (struvite) The 2 bands of 35 and 25 kDa appeared in the thrombin undigested lane (lane C). They were unchanged after thrombin digestion (lane T).
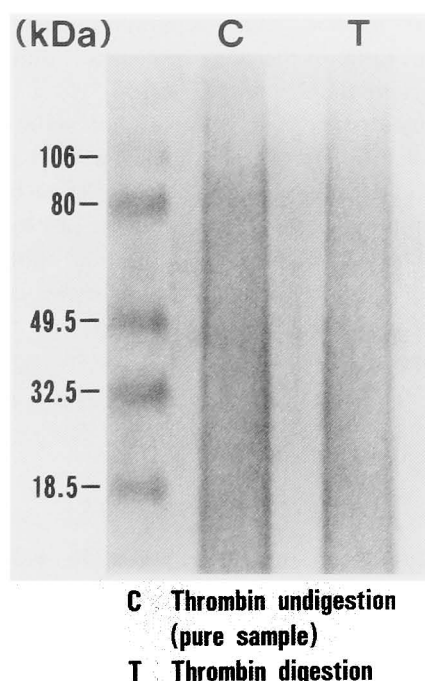

Fig. 4. Electrophoretic patterns by Stains-All staining of matrix protein extracted from uric acid. No bands appeared in the thrombin undigested lane (lane C) and after thrombin digestion (lane T).

sium ammonium phosphate stones showed 2 bands of 35 and 25 kDa (Fig. 3, lane C). The bands were unchanged after thrombin digestion (Fig. 3, lane T). No bands appeared after Stains-All staining for the uric acid stones (Fig. 4, lane C, lane T). The NH2-terminal 20 amino acids in the band were the same as those of OPN[9].

DISCUSSION

Although many reports have suggested the importance of the matrix in urinary stone formation, little is known about the composition of the stone matrix[1-8]. Our previous molecular study showed that the c-DNA sequence of OPN encoded the calcium oxalate urinary stone protein[9]. The present study using Stains-All showed that the calcium containing urinary stones (calcium oxalate, and calcium phosphate) contained OPN protein.

OPN is a major component of mineralizing connective tissues such as bone and dentine[14]. Nagata et al.[15] reported that OPN could be extracted from bone and identified with Stains-All staining. Stains-All is a cationic carbocyanine dye and is suitable for staining sialoglycoproteins and phosphoproteins[16]. OPN is a sialated phosphorylated glycoprotein. Since OPN has a thrombin-susceptible site (Arg-Gly-Asp tripeptides) in the middle of the molecule, thrombin digestion produces 23 kDa and 33 kDa bands from a 52 kDa band[17] The 49.5 and 45 kDa bands (in lane C of Fig. 1 and 2) from

the calcium oxalate and calcium phosphate stones, respectively were attributed to OPN. The 2 low molecular bands of 32.5 and 25 kDa were obtained from both the calcium oxalate and calcium phosphate stones after thrombin digestion. Only 2 low molecular bands of 35 and 25 kDa were observed in the proteins extracted from the struvite stones (Fig. 3). The absence of OPN in struvite stones may be because calcium ions are not involved in the formation of struvite stones as with uric acid stones, and therefore, OPN with affinity for calcium ions was not necessary. Another possibility is degradation of OPN in the formation process of struvite stones. Further studies are necessary. On the other hand, the lack of a definite band in Fig. 4 suggested that the uric acid stones did not contain OPN. This study revealed that OPN was extracted from only calcium-containing stones among 4 kinds of stones. OPN binds Ca^{2+}, and has a negative charge, in addition to having a high affinity for hydroxyapatite. It has a potential to act as both a nucleator of hydroxyapatite crystal formation as well as a regulator of crystal growth and dissolution, these activities being dependent upon the physical state of the protein[18] Shiraga et al.[19] discovered in human urine uropontin, which is a protein with homology at 40 sites of amino acids for rat, mouse and porcine OPN. They reported that this uropontin inhibited the crystal growth of calcium oxalate in vivo, although they had expected it to promote the crystal growth in vivo. We previously identified OPN by a molecular

biological method. The separation and identification of OPN in the stone protein was difficult by the conventional method. OPN is a highly phosphorylated protein and was not stained with Coomassie brilliant blue. All conventional staining methods of the stone protein used Coomassie brilliant blue. In this study, OPN in the stone protein could be stained with Stains-All, but further studies are needed on the role, of OPN in stones and its involvement in *in vitro* crystallization.

The finding obtained in the present study and the strong binding properties of OPN to Ca^{2+} suggest that OPN is related to stone formation in the calcium-containg stones.

REFERENCES

1) Boyce WH and Garvey FK: The amount and nature of the organic matrix in urinary calculi. J Urol 76: 213–227, 1956
2) King JS and Boyce WH: Amino acid and carbohydrate_ composition of the mucoprotein matrix in various calculi. Proc Soc Exp Biol Med 95: 183–187, 1957
3) Umekawa T and Kurita T: Calprotectin-like protein is related to soluble organic matrix in calcium oxalate urinary stone. Biochem Molec Bio Int 34: 309–313, 1994
4) Umekawa T, Yamate T, Kurita T, et al.: Sequencing of a urinary stone proteins, identical to alpha-one antitripsin, which lacks 22 amino acids. Biochem Biophys Res Commun 193: 1049–1053, 1993
5) Specter AR, Gray A and Prien WL Jr: Kidney stone matrix. Differences in acidic protein composition. Invest Urol 13: 387–389, 1976
6) Lian JB, Prien Jr, Glimcher EL, et al.: The presence of protein-bound γ-carboxyglutamic acid in calcium-containing renal calculi. Clin Sci 59: 1151–1157, 1997
7) Melic RA, Quelch KJ and Rhodes M: The demonstration of sialic acid in kidney stone matrix. Clin Soi 59: 401–404, 1980
8) Jones WT and Resnick MI: The characterization of soluble matrix proteins in selected human renal calculi using two-dimensional polyacrylamide gel electrophoresis. J Urol 144: 1010–1014, 1990
9) Kohri K, Yamate T, Kurita T, et al.: Molecular cloning and sequencing of cDNA encoding urinary stone protein, which is identical to osteopontin. Biochem Biophys Res Commun 184: 859–864, 1992
10) Kohri K, Umekawa T, Kurita T, et al.: Structure and express of the mRNA encoding urinary stone protein (osteopontin). J Biol Chem 268: 15180–15184, 1993
11) Sodek J, Chen J, Nagata T, et al.: Sialoproteins in bone remodeling. Bio Mecha Tooth Cranio 38: 127–136, 1992
12) Goldberg HA, Domenicucci C, Sodek J, et al.: Mineral-binding proteoglycans of fetal porcine calvarial bone. J Biolumin Chemilumin 263: 12092–12101, 1988
13) Nagata T, Domenicucci C, Sodek J, et al.: Biosynthesis of bone proteins by fetal porcine calvariae in vitro. Rapid association of sulfated sialoproteins and chondroitin sulfate proteoglycan with bone mineral. Matrix 11: 86–100, 1991
14) Mark MP, Butler WT, Ruch JV, et al.: Developmental expression of 44-kDa bone phosphoprotein (osteopontin) and bone γ-carboxyglutamic acid-containing protein (osteocalcin) in calcifying tissues of rat. Differentiation 37: 123–136, 1988
15) Nagata T, Todescan R, Goldberg HA, et al.: Sulphation of secreted phosphoprotein I (SPPI, osteopontin) is associated with mineralized tissue formation. Biochem Biophys Res Commun 165: 234–240, 1989
16) Campbell KP, MacLennan DH and Jorgensen AO: Staining of the Ca^{2+}-binding proteins, Calsequestrin, Calmodulin, Troponin C, and S-100, with the cationic carbocyanine dye 'Stains-All'. J Biolumin Chemilumin 18: 11267–11273, 1983
17) Nagata T, Kasugai S, Sodek J, et al.: Biosynthesis of bone proteins (secretad phosphoprotein-I, osteopontin), BSP (bone sialoprotein) and SPARC (osteonectin) in association with mineralized-tissue formation by fetal-rat carvarial cells in culture. Biochem J 274: 513–520, 1991
18) Linde A and Lussi A: Mineral induction by polyanionic dentin and bone proteins at physiological ionic conditions. Connect Tissue Res 21: 197–202, 1989
19) Shiraga H, Min W, Hoyer JR, et al.: Inhibition of calcium oxalate crystal growth in vitro by uropontin: another member of aspartic acid-rich protein superfamily. Proc Natl Acad Sci USA 82: 426–430, 1992

和文抄録

カルシウム含有結石マトリックス内蛋白におけるオステオポンチンの同定

近畿大学医学部泌尿器科学教室（主任：栗田　孝教授）

山手　貴詔，梅川　　徹，井口　正典，栗田　　孝

名古屋市立大学医学部泌尿器科学教室（主任：郡健二郎教授）

郡　健二郎

　尿路結石蛋白の c-DNA sequence にて，蓚酸 Ca 結石内のマトリックス蛋白としてオステオポンチン（OPN）が見つかった．OPN はリン酸化蛋白であり，トロンビンにより分割をうけ，Stains-All 染色に特異的に染色される特徴を持っている．私達は蓚酸カルシウム，リン酸カルシウム，尿酸，リン酸マグネシウムアンモニウムの4種類の結石内マトリックスを，Stains-All 染色によりトロンビン分割の有無で OPN の存在を確認した．マトリックス成分は 0.1 M EDTA 溶液で抽出した．そのうえ N 末端より20残基のアミノ酸分析を施行した．蓚酸カルシウム，リン酸カルシウムのみ OPN が存在し尿酸，リン酸マグネシウムアンモニウム結石内には認めなかった．今回の結果は骨のマトリックスとして骨形成に関わり，強固にヒドロキシアパタイトと結合する OPN が，カルシウム含有結石形成過程においても重要であることを示唆した．

（泌尿紀要　**43**：623-627，1997）