

<Original Article>

Development of protein extraction method from urinary calculus and identification of protein in calculus extract

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Summary Correct extraction of proteins contained within the calculi of urolithiasis patients is important to correctly identify these diverse proteins. Based on protein extraction solutions previously reported, we developed an efficient method to extract protein from the urinary calculus and confirmed the presence of the following proteins in the extract: albumin (Alb), Tamm-Horsfall protein (THP), and IgG, which are all major proteins in urine, and α_1 -antitrypsin, which is a marker of inflammation. Using this extraction method, we further reveal for the first time that cystatin C, a marker of tubular injury, is also present in the urinary calculus. We observed an abundance of Alb, THP, and IgG proteins in the calculus; in addition, each of these proteins exhibited a variety of molecular weights by western blot, possibly indicating protein polymerization within the calculus material. Overall, these improved methods can contribute to determining urinary stone formation mechanisms.

Key words: Urolithiasis, Calcium oxalate calculi, Extraction method, SDS-polyacrylamide gel electrophoresis (SDS-PAGE), Cystatin C

1. Introduction

The prevalence and morbidity rate of urolithiasis is steadily increasing in Japan as well as in other advanced countries. A nationwide survey conducted by the Japanese Society on Urolithiasis Research in 2005 revealed that the morbidity rate was 15.1% in males and 6.8% in females. Urolithiasis symptoms range from asymptomatic to severe. Even with treatment, urolithiasis is liable to recur. Among the

several types of calculi, calcium-containing calculus accounts for approximately 80% of all cases.

Besides the known calculi constituents, urine contains various inorganic and organic substances that can influence the mechanism of stone formation; some reports argue that urinary protein components are an important part of the calculus formation mechanism^{1,2,3)}. To address whether and what protein components were present in calculus, we compared the known urinary proteins to the protein components

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Received for Publication December 26, 2011

Accepted for Publication January 4, 2012

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we analyzed in calculus extract. Based on extraction solutions for calculus already reported^{4,5,6}, this study aimed to find the most optimal protein extraction solution and identify the main proteins in calculus extract by SDS - polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis.

2. Materials and Methods

2.1. Subjects

Urinary calculi were obtained from 5 urolithiasis patients (4 males and 1 female, aged 35-63) being treated with extracorporeal shock wave lithotripsy (ESWL) at the Department of Urology, Yokosuka Kyosai Hospital. The Yokosuka Kyosai Hospital ethical committee approved this study; all patients provided informed consent. All 5 calculi were of the calcium oxalate subset and were stored at room temperature (RT).

2.2. Pretreatment of urinary calculi

Each urinary calculus was cleaned with saline plus the protease inhibitor 1 mM phenyl methane sulfonyl fluoride (PMSF) for 30 min and allowed to dry naturally. The 5 calculi were pulverized and mixed together using an agate mortar. 0.05 g of mixed calculi was aliquoted into 1.5 mL tubes.

2.3. Protein Extraction

Five solutions were prepared for protein extraction from urinary calculus: distilled water (Formula 1), saline (Formula 2), 50 mM Tris-HCl (pH 7.4) plus 0.5 mM EDTA⁴ (Formula 3), 60 mM Tris-HCl (pH 6.8) plus 10% glycerol and 2% SDS⁶ (Formula 4), and 50 mM Tris-HCl (pH 7.4) plus 4 M guanidine hydrochloride and 5 mM EDTA⁵ (Formula 5). Each of the extracting solutions were added to the 0.05 g aliquots and shaken at RT. The extracted solution was centrifuged at $10,000 \times g$ for 5 min.

2.4. Protein yield measurement

Semiquantitative analysis of relative protein concentration in the extracted solutions was performed by silver dot blot assay⁷.

2.5. Removal of contaminants

2.5-1. Acetone precipitation

0.2 mL of extracted solution was mixed with $4 \times$ volume of cold acetone, incubated for 1 h at -80°C , and centrifuged at $10,000 \times g$ for 15 min. This was repeated $3 \times$.

2.5-2. Desalination

Protein extract was desalinated by PAGEprep Advance Clean-UP Kit (Takara Bio Inc., Shiga, Japan) according to manufacturer's instructions.

2.6. Protein identification and analysis

2.6-1. SDS-PAGE

SDS-PAGE was performed using a 7.5% gel, following the Laemmli method⁸. After electrophoresis, gels were stained using a silver staining kit (Silver Stain Kit Wako) (Wako Pure Chemical Industries Ltd., Osaka, Japan). Molecular mass (kDa) of each protein band was calculated using the LMW Calibration Kit (GE Healthcare UK Ltd., England). Electrophoretic patterns were analyzed by densitometry using Image J software (NIH, USA).

2.6-2. Western blot analysis

Proteins were transferred from SDS-PAGE gels to polyvinylidene fluoride (PVDF) membranes (Immobilon-P, Millipore, Billerica, MA, USA) for western blotting. Each membrane was incubated overnight with rabbit antibodies against human albumin (Alb) (polyclonal), IgG, α_1 -antitrypsin (α_1 -AT), cystatin C (Dako Cytomation, Glostrup, Denmark), and Tamm-Horsfall protein (THP), followed by 1 h incubation with peroxidase-conjugated swine anti-rabbit IgG antibody (Dako Cytomation). Subsequently, the membranes were treated with DAB solution (0.02% 3,3-diaminobenzidine, tetrahydrochloride salt-containing 0.02% H_2O_2) to detect peroxidase.

3. Results

3.1. Formula 5 yielded the most protein at the 1 h and 24 h extraction time points

To determine the optimal protein extraction

Table 1 Protein concentration by different extracting solutions and extraction time

		Types of extracting solutions				
		Formula 1	Formula 2	Formula 3	Formula 4	Formula 5
Extraction time	1 hour	40 mg/L	85 mg/L	94 mg/L	328 mg/L	664 mg/L
	24 hours	67 mg/L	81 mg/L	153 mg/L	339 mg/L	814 mg/L

Formula 1: Distilled water

Formula 2: Saline

Formula 3: 50 mM Tris-HCl (pH 7.4) plus 0.5 mM EDTA

Formula 4: 60 mM Tris-HCl (pH 6.8) plus 10 % glycerol and 2 % SDS

Formula 5: 50 mM Tris-HCl (pH 7.4) plus 4M guanidinium hydrochloride and 5 mM EDTA

Table 2 The method developed for the extraction of proteins from the urinary calculus

Step	Extraction procedure
1. Pretreatment	Urinary calculi were cleaned using saline containing 1 mM phenyl methane sulfonyl fluoride [PMSF] (volume, 10 times that of the sample) 30 min.
2. Drying	The samples were dried naturally.
3. Pulverization	Pulverization and mixing were performed using an agate mortar.
4. Extraction	The mixed calculus (0.05 g) was divided into 1.5 ml tubes. Formula5 (Tris-HCl (50 mM, pH 7.4) including guanidinium hydrochloride (4 M) and EDTA (5 mM) containing 1 mM PMSF) was added to the 1.5 ml tube containing 0.05 g of mixed calculus. The tube was shaken at room temperature. The extraction solution was centrifuged at 10,000_g for 5 min.
5. Desalination	Guanidine hydrochloride was removed using PAGEprep Advance Protein Clean-Up Kit (Takara Bio Inc., Shiga, Japan).

solutions for obtaining the highest protein yield, extraction from urinary calculus was performed using 5 types of extraction solutions. Comparing among the 5 solutions, Formula 5 produced the highest protein concentration at both the 1 h (664 mg/L) and 24 h (814 mg/L) extraction time points (Table 1). Therefore, Formula 5 was optimal, as it demonstrated a protein extraction efficiency several times higher than the 4 other solutions.

3.2. Desalination by PAGEprep Advance Clean-UP Kit improves band resolution

When the Formula 5 extract was run on an SDS-PAGE gel to identify the extracted proteins, the electrophoretic pattern background was too densely stained to resolve the bands. We determined that deposition of insoluble salts masked the identification

of the true protein bands (data not shown). The PAGE prep Advance Clean-UP Kit further optimized the Formula 5 extraction protocol by introducing a desalination step that resulted a clear electrophoretic pattern and better band resolution for calculi proteins.

3.3. Thirty minutes of extraction time is sufficient for high yield and band density

To find the shortest extraction time that generates maximum protein yield, we performed a time course to determine optimal extraction efficiency. Using Formula 5, we compared the band densities after 30 min, 1 h, 6 h, and 24 h extraction times. Comparing protein bands at 30 min and 24 h, no difference was detected in number or contrast density (Fig. 1). Thus, the 30 min extraction was the shortest time point sufficient to generate high protein band density.

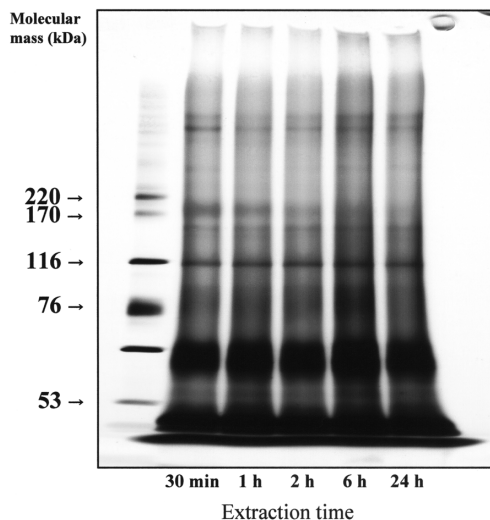


Fig. 1 Electrophoretic patterns of protein of extraction calculus obtained during an extraction time course using Formula 5 (50 mM Tris-HCl [pH 7.4] plus 4M guanidine hydrochloride and 5 mM EDTA).

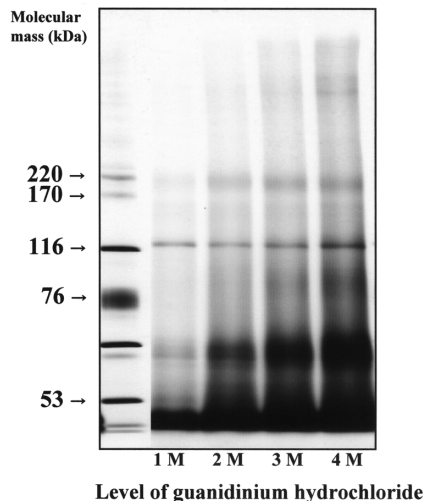


Fig. 2 Electrophoretic patterns of protein of extraction calculus obtained using a dose curve of guanidine hydrochloride concentration in Formula 5 (guanidine hydrochloride when 50 mM Tris-HCl (pH 7.4) plus 4M guanidine hydrochloride and 5 mM EDTA).

3.4. 4 M guanidine hydrochloride concentration optimally detected protein band number and intensity

We optimized band resolution in 30 min extracts by comparing 1, 2, 3, and 4 M guanidine hydrochloride concentrations in Formula 5. The number and intensity of protein bands detected on the gel increased with higher guanidine hydrochloride concentration (Fig. 2). Thus, we used 4 M of guanidine hydrochloride in Formula 5 to identify the proteins contained in the mixed calculi.

3.5 Overall scheme of the optimized protein extraction method

A schematic detailing the optimal protein extraction method we developed to identify proteins in urinary calculus is outlined in Table 2.

3.6. Analysis of extracted protein identified Alb, THP, α_1 -AT, IgG, and cystatin C proteins in urinary calculus

Using our optimized protein extraction protocol, 19 protein bands were detected within a range of 54-

262 kDa by SDS-PAGE (Fig. 3). A 66 kDa protein band (Alb) accounted for an estimated 20% of the total band density by densitometric quantification. Western blot analysis identified Alb, THP, α_1 -AT, IgG, and cystatin C proteins in calculi extract. In addition, 6 additional bands of Alb, 4 bands of THP, 5 bands of α_1 -AT, 5 bands of IgG, and 2 bands of cystatin C were observed in protein ranges greater than the expected bands detected by the specific antibodies. Within a single 240 kDa band, Alb and THP were both detected; the 251 kDa band contained Alb, THP, and IgG; and the 227 kDa band contained all 5 proteins. Collectively, these results indicated that multiple forms of each protein existed within the calculus material.

4. Discussion

We aimed in this study to optimize and analyze the protein extract from calcium-containing calculus by examining the efficiencies of 5 types of extraction solutions, including distilled water and saline, by using solutions determined previously^{4, 5, 6}. In our

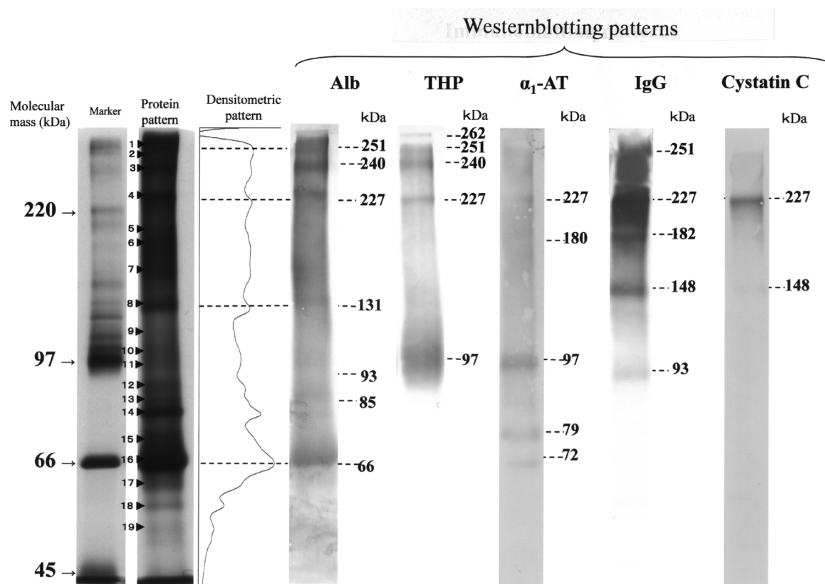


Fig. 3 Electrophoretic and western blotting patterns of the urinary calculus proteins Alb, THP, α_1 -AT, IgG, and cystatin C.

study, we optimized the extraction protocol in order to obtain high protein yield, optimally resolve the proteins on SDS-PAGE gel, and determine the specific identity of the bands by western blot analysis. We show here that extracting urinary calculus protein for 30 min in buffer containing 50 mM Tris-HCl (pH 7.4) plus 4 M guanidine hydrochloride and 5 mM EDTA (Formula 5) followed by treatment with a desalinating agent produces the highest protein yield and best resolves the protein bands with low background staining.

Yokomizo et al. harvested the calculus protein by soaking calculus powder in saline and extracting the protein by shaking the powder in saline overnight at RT. They reported an extracted protein yield ranging from 1.5×10^{-3} to 18.8×10^{-3} mg harvested from 0.1 g of calculus starting material. From this extraction, 18 bands were separated ranging from 26.8-143 kDa⁹. Using our method, we obtained 6.6×10^{-3} mg of protein from 0.1 g of starting mixed calculi material, which is approximately a 5-fold increase over the earlier study. Additionally, we found more bands than the earlier study, as 19 separated bands were observed ranging from 45 to 262 kDa.

The SDS-PAGE banding patterns obtained in the 3 previously published extraction methods were not clearly resolved^{4,5,6}. Kaneko et al. explained that the smear and poorly resolved bands were attributed to the interaction and cohesion of proteins within calculus^{5,10}. In our study, high protein yield was obtained from calcium-containing calculus in a short time, and the SDS-PAGE pattern was clearly resolved. Therefore, our extraction solution improved upon existing extraction efficiencies.

In terms of probing for specific proteins, we focused on Alb, THP, and IgG because they are major proteins in urine, α_1 -AT because it is marker of inflammation, and cystatin C because it is a marker of tubular injury. Our study confirmed that α_1 -AT was present in calculus^{4,11}, as well as Alb, THP, and IgG. Interestingly, western blot analysis detected multiple molecular weights for each of these proteins. These results confirmed our previous report showing that multiple molecular varieties of Alb, THP, and IgG proteins were present in urine of patients with ureterolithiasis and nephrolithiasis¹². This molecular heterogeneity may be related to calculus formation in some way, although this remains to be determined.

We reveal for the first time that cystatin C is also present. Biologically, plasma cystatin C is freely filtered from tubular and glomerular basement membranes, and more than 99% of total amount is normally reabsorbed in the proximal tubule. However, cystatin C excretion can increase due to abnormal function of these basement membranes¹³. We speculate that the existence of cystatin C in the calculus confirms tubular disorder of urolithiasis patients.

Our interesting observation that single bands from calculi extracts contain multiple proteins may indicate that proteins within the calculus can polymerize together. In the future, we may examine the protein variations in individual calculi and determine why these proteins polymerize in the calculus material.

Although it not currently known whether all of these proteins are present in each of the individual calculus samples we tested, our results using the mixed calculi sample strongly indicate that protein in urine may be incorporated during calculus stone formation. In the future, it would be interesting to analyze the variations of these proteins in individual calculi. We think this would be very interesting to evaluate and the results would be clinically useful. Overall, we developed an efficient method to extract protein from urinary calculus. Importantly, this method revealed the presence of cystatin C in urinary calculus. In the future, analysis of urine and calculus obtained from urolithiasis patients using these improved methods would contribute to determining urinary stone formation mechanisms.

Acknowledgments

The authors would like to thank the staff of the the Clinical Medicine Laboratory of Bunkyo Gakuin University.

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