

Cellulose Acetate Membrane Electrophoresis Based Urinary Proteomics for the Identification of Characteristic Proteins

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Background: Analysis of urinary proteins using cellulose acetate membrane electrophoresis (CAME) is a useful and challenging method for the recognition of damaged sites in the kidney. However, protein content of each CAME fraction is still not completely understood. **Methods:** In this study, an effective method of protein extraction from each band fractionated by CAME was established, which enabled us to examine the extracted proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and mass spectrometry. **Results:**

Key words: cellulose acetate membrane electrophoresis; kidney; proteome; SDS-PAGE; urinary protein

Proteins were extracted from the gel and analyzed by mass spectrometry. In all, 31 proteins were identified, including 20 urinary proteins that were newly identified in the CAME-based analysis. **Conclusion:** This methodology was useful for identifying the proteins responsible for creating unique bands on CAME in a urine sample of a patient with drug-induced interstitial nephritis. These findings provide in-depth characterization of urinary protein contents in each CAME fraction. *J. Clin. Lab. Anal.* **30:** 359–367, 2016. © 2015 Wiley Periodicals, Inc.

INTRODUCTION

Currently, high-throughput mass spectrometric analysis of urinary proteins is being widely used in renal studies (1–3). Since urine sampling is noninvasive and relatively easy compared with serum samples, urine is attractive for biomarker discovery. A urinary protein panel, which is a renal disease assessment involving several novel protein markers (3), increases the diagnosis accuracy in diabetes mellitus, lupus nephritis (4), and recurrent bladder cancer (5). Therefore, a multiplex urinary protein assay is valuable for monitoring renal diseases.

Cellulose acetate membrane electrophoresis (CAME) is one of the most classic approaches of protein panel analysis. In clinical practice, urinary protein electrophoresis has been mainly used for confirming the Bence Jones protein in multiple myeloma (6), but it has not been

widely used. In the previous study, we analyzed the potential of this easy and inexpensive method to predict renal dysfunction. First, we developed a highly sensitive colloidal silver stain for CAM instead of conventional Ponceau 3R or other dyes (7). The silver staining technique is sensitive enough to visualize urinary protein patterns even in urine samples of healthy subjects (8). Second, we

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established the classification of urinary protein patterns using the relative mobility (RM) of each band on CAME by using urine samples of patients with several kidney diseases (9). The urinary protein patterns of patients with persistent proteinuria, including IgA nephropathy, tubulointerstitial nephritis, and other nephropathies, were clearly classified into three groups according to the damaged location within the nephron, as evidenced by the characteristic protein bands on CAME: glomerular, tubular, and mixed patterns. The representative proteins in the glomerular pattern are albumin (Alb) at the Alb fraction and transferrin (Tf) at the β fraction. In the tubular pattern, retinol-binding protein (RBP) appears at the anodic side of the β (β_a) fraction and β_2 -microglobulin (β_{2m}) at the cathodic side of the β (β_c) fraction. RBP and/or β_{2m} with Alb and Tf appeared in the mixed pattern. Since the protein profiles classified with this technique exhibited good agreement with the histological findings of the subsequent renal biopsy (9), the urinary protein profile based on CAME is expected to become a useful method to predict the regional site and provide supportive data for histopathological diagnosis of renal diseases.

Different profiles are often seen in the CAME of urinary proteins from patients with renal diseases (10, 11). To identify the proteins on CAME, immune fixation and Western blot have been employed using specific antibodies (12–14); however, the protein contents in each fraction have not been fully identified. Identification of urinary proteins in each fraction is expected to be more efficient, effective, and accurate if it is coupled with proteomic approaches such as mass spectrometry once the proteins are effectively extracted from CAM after electrophoresis. In the present study, we established a method for extracting proteins from each fraction of CAM. By employing the proteomic approach, characteristic proteins were identified from the unique fractions of a sample of a patient with drug-induced interstitial nephritis.

MATERIALS AND METHODS

Patients

The analytical samples of two patients with tubulointerstitial nephritis at the Niigata university hospital were collected before renal biopsy (4). A urine sample was also collected from a patient with IgA nephropathy, which showed five fractions of glomerular-type protein pattern, to establish the protein extraction method. Informed consent was obtained from all patients. Freshly voided urine was centrifuged ($1,500 \times g$, 5 min, 4°C), and the supernatant was stocked at -80°C until analysis.

Methods

Measurement of total protein (TP) and Alb concentrations in urine samples

The urinary TP concentration was determined by the pyrogallol red method using the Micro TP-Test Wako (Wako, Osaka, Japan). The urinary Alb concentration was determined by the immunoturbidimetric method using the N-Assay TIA Micro Alb (Nittobo, Tokyo, Japan). Both proteins were analyzed using an autoanalyzer (Accute, Toshiba, Tokyo, Japan).

CAME following colloidal silver staining

Urine samples ($0.8 \mu\text{l}$) were loaded on CAM (SELECA-VSP, Toyo Roshi Kaisha, Tokyo, Japan) using a sample applicator (Jokoh, Tokyo, Japan). Urinary proteins were electrophoresed at a constant current of 0.7 mA/cm for 40 min using 0.6 mol/l Veronal buffer at pH 8.6 (Jokoh). After electrophoresis, the proteins were stained with the colloidal silver staining kit (Jokoh) as previously described (9).

Protein extraction after CAME

To investigate the protein content of each fraction on CAM, we established the protein extraction method. First, a urine sample was loaded into ten lanes on CAM, and electrophoresed using the conditions listed above (Fig. 1a). Then, both ends of CAM were cut and stained with colloidal silver (Fig. 1b). Each fraction in the unstained part of the CAM from lane 2 to 9 was cut out following the stained area (Fig. 1c), and each fraction strip was further cut into 5 mm^2 segments (Fig. 1d). The proteins in each fraction on the CAM segments were extracted with $40 \mu\text{l}$ of each protein extraction buffer, and $10 \mu\text{l}$ of the extracted proteins (approximately $1 \mu\text{g/well}$) was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (15). After electrophoresis, the gel was stained with Silver Stain KANTO III (Kanto Kagaku, Tokyo, Japan).

To establish the basic conditions for protein extraction from CAM, we carried out the following validations:

- (1) Spread of protein fraction after electrophoresis: While staining both ends of CAM after electrophoresis, the unstained part was kept moist in a chamber. Proteins might diffuse into the adjacent fractions, leading to misidentification of proteins. To investigate the influence of protein diffusion on CAM, the membrane was kept in a moisture chamber for 0, 15, 30, 60, and 120 min after electrophoresis, and each protein pattern was compared.

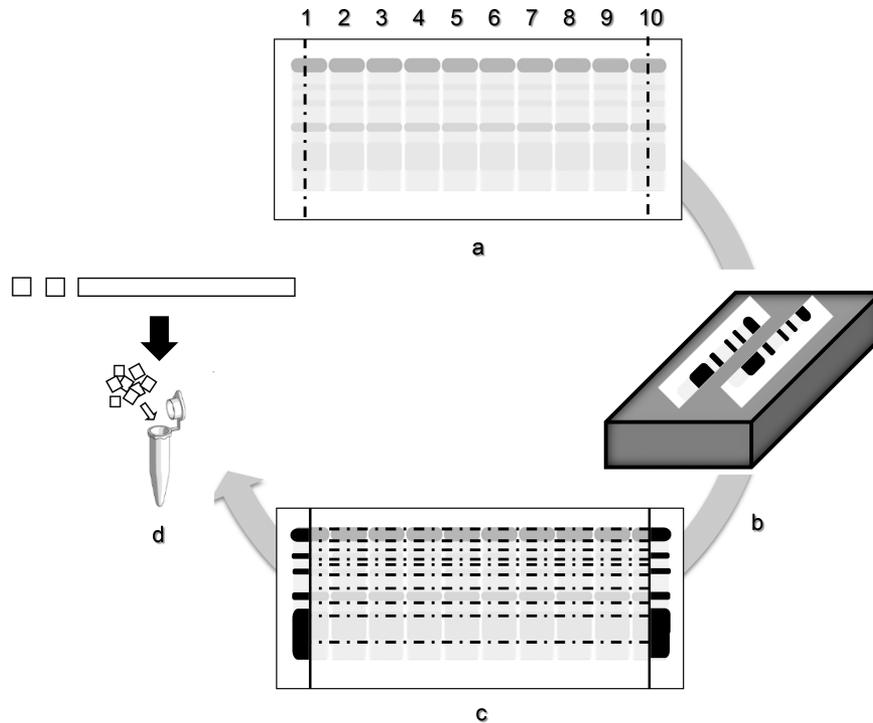


Fig. 1. Schematic representation of the protocol for protein extraction from CAM. Loading of ten lanes with urine sample on CAM (a), silver staining of both ends after separation (b), cutting each fraction of the unstained membrane following the stained area (c), and fragmenting each fraction strip for protein extraction (d).

- (2) Optimal concentration of protein application: The urine sample from the patient with IgA nephropathy was diluted to a final TP concentration of 100–1600 mg/l, and each protein pattern was compared to assess the conditions for obtaining well-separated protein bands on CAM with maximum protein load.
- (3) Selection of protein extraction buffer: Buffers at four different pH values were tested for protein extraction, and the protein profile under the different conditions was compared: 0.2 mol/l glycine-HCl buffer (Gly-HCl, pH 2.5), phosphate-buffered saline (PBS, pH 7.4), 96 mmol/l carbonate-bicarbonate buffer (Carb-bicarb, pH 9.6), and Laemmli sample buffer (LSB; 14 mmol/l SDS, 0.5 mol/l Tris amino methane, 0.7 μ mol/l bromophenol blue, and 14 mol/l glycerol, pH 6.8), which is the buffer solution used for SDS-PAGE sample preparation.
- (4) Protein extraction time: The protein profiles obtained after 1, 2, 4, and 8 h were extracted, and each protein pattern was compared.

In-gel digestion

The protein bands were excised manually from the SDS-PAGE gel, cut into pieces, and destained using the

Silver Stain KANTO Gel Washing Solution for MS (Kanto Kagaku). Gel pieces were washed with 50% v/v acetonitrile (ACN) in 25 mmol/l ammonium bicarbonate. The gel pieces were reduced with 40 μ l of 10 mmol/l dithiothreitol in 100 mmol/l ammonium bicarbonate at 56°C for 1 h. After removal of dithiothreitol solution, 40 μ l of 55 mmol/l iodoacetamide in 100 mmol/l ammonium bicarbonate was added, and the gel pieces were incubated for 30 min at room temperature in the dark. Following reduction and alkylation, the gel pieces were washed with 30 μ l of 100 mmol/l ammonium bicarbonate and 40 μ l of ACN and placed in a centrifugal concentrator (VC-36N, Titec, Saitama, Japan) until dry. Then, sufficient volume (20–40 μ l) of modified porcine trypsin (Sequencing Grade Modified Trypsin; Promega, Madison, WI) at a final concentration of 25 ng/ μ l in 50 mmol/l ammonium bicarbonate was added to the gel pieces and allowed to rehydrate for 30 min on ice. Then, 10 μ l of 50 mmol/l ammonium bicarbonate was added, and tryptic digestion was performed overnight at 37°C. The resulting peptides were extracted from the gel pieces with 10 μ l of 25 mmol/l ammonium bicarbonate for 10 min, and the extracted peptide solution was removed and saved. Then, 20 μ l of ACN was added and dehydrated for 10 min. The extracted solution was also saved in the same tube. The gel pieces were then incubated with 10 μ l of 5% formic acid in 50% ACN for 10 min, and the solution was collected.

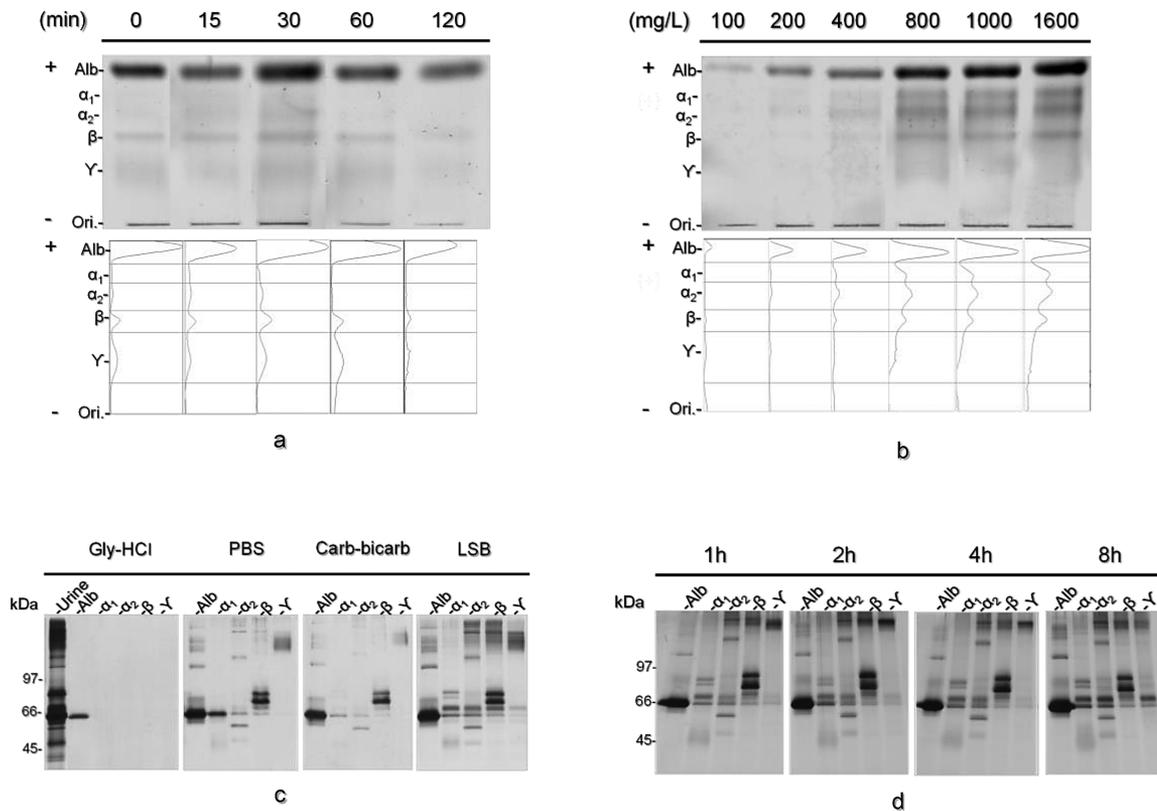


Fig. 2. Validation of the protein extraction procedure. CAME pattern of the urine sample kept in a chamber for 0, 15, 30, 60, and 120 min after separation and the corresponding densitometric profiles (a). CAME profile of the urine sample at different loading amounts (b). Result of the SDS-PAGE of the urine sample using four different extraction buffers (c). Result of the SDS-PAGE of the urine sample with Laemmli sample buffer at different extraction times (d).

This procedure was repeated three times. The eluates were combined and concentrated in a centrifugal concentrator and stored at -20°C until analysis.

Mass spectrometry analysis

Digested peptides were reconstituted with $20\ \mu\text{l}$ of 0.1% formic acid in water and analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS, LC: Advance-nano UHPLC, AMR, Tokyo, Japan, MS: amazon ETD, Bruker Daltonics, Billerica, MA) using a C_{18} reverse-phase capillary column (L-column 2 ODS, $0.1 \times 50\ \text{mm}$, $0.3\ \mu\text{m}$ i.d., Chemicals Evaluation Research Institute, Tokyo, Japan) at a flow rate of 500 nl/min using the following mobile phases: A, 0.1% formic acid in water; B, 0.1% formic acid/ACN. The gradient step was from 5% to 45% B in 20 min.

Subsequently, all acquired MS/MS spectra were searched against the Swiss-Prot database R51.5 human taxonomy using the MASCOT search engine (v. 2.1, Matrix Science, London, UK) using the following parameters: missed cleavage = 1, peptide mass tolerance = 0.5, MS/MS mass tolerance = 0.5 Da. Carbamidomethyla-

tion was used for fixed modification and methionine oxidation for variable modification.

RESULTS

Extraction Conditions of Protein From CAM

Spread of protein fraction after electrophoresis

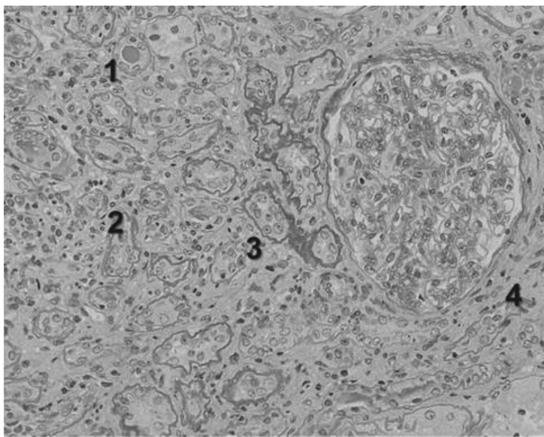
After 15 and 30 min, which is the colloidal silver-staining incubation time, the proteins of each fraction did not diffuse compared with the control (Fig. 2a). Remarkable protein diffusion was observed after 120 min especially in the fraction peaks of α_1 -G, α_2 -G, and γ -G, which became flat, making it difficult to distinguish the borders of each fraction.

Optimal concentration of protein application

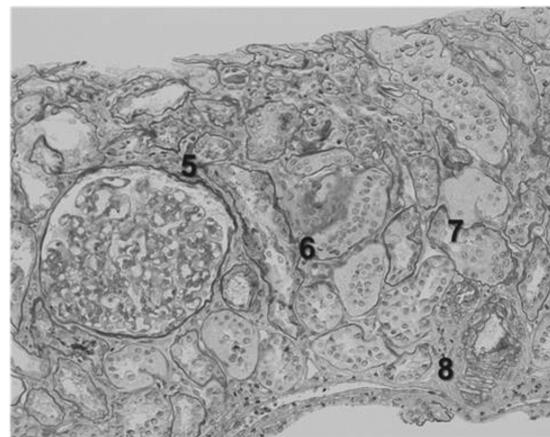
At the concentration $<800\ \text{mg/l}$, all protein fractions were detected. However, each fraction became turbid when $>1000\ \text{mg/l}$ protein was loaded (Fig. 2b).

	Patient A	Patient B
sex	Male	Female
Age (years)	57	46
Urinary protein	83.0 mg/L	1313 mg/L
Urinary albumin	12.6 mg/L	160 mg/L
Urinary creatinine	331 mg/L	330 mg/L
Clinical diagnosis	renal insufficiency, sarcoidosis, pneumoconiosis, tubulointerstitial nephritis	HIV (+), drug-induced tubulointerstitial nephritis
Diagnosis by renal biopsy	tubulointerstitial nephritis, minor glomerular abnormalities, granuloma	tubulointerstitial nephritis, minor glomerular abnormalities,
Medication	—	LPV/RTV, TDF, EFV
Number of protein fractions on CAME	6	9

a



b



c

Fig. 3. Clinical characteristics and histological features of kidney biopsies from two patients with tubulointerstitial nephritis. Basic information of the two patients with tubulointerstitial nephritis (a). The renal tissue of patient A (b) shows tubular atrophy, granuloma formation, inflammatory cell infiltrate, and cast formation (1–4). In addition, tubular atrophy, interstitial fibrosis, tubular dropout, and interstitial edema (5–8) were observed in the tissue of patient B (c).

Selection of protein extraction buffer

The SDS-PAGE results of the urine sample before extraction were compared in several extraction buffers. Only one band was observed in Gly-HCl buffer. A couple of proteins were extracted by PBS or Carb-bicarb buffer. The best results were obtained in LSB. Therefore, we employed LSB for protein extraction in the following experiments (Fig. 2c).

Time required for protein extraction

There were no major differences in the protein patterns between 1 and 8 h, with the exception of the high molecular mass area of the Alb and Y-G fractions (Fig. 2d). Therefore, in the following experiments, protein extraction was performed for 8 h.

Patient Profile and Pathological Characteristics

The diagnosis and medications of the two patients with tubulointerstitial nephritis are summarized in Figure 3a. In patient A, biopsy revealed tubular atrophy, granuloma formation, inflammatory cell infiltrate, and cast formation (Fig. 3b, 1–4). In patient B, tubular atrophy, interstitial fibrosis, tubular dropout, and interstitial edema were observed (Fig. 3c, 5–8).

CAME Profile of Urinary Proteins

The urinary protein patterns on CAME of the patients with tubulointerstitial nephritis are shown in Figure 4a. The RM and area percentage of each fraction were calculated by densitometric analysis (Fig. 4b). Since patient A showed the typical tubular pattern on CAME, the abundance of the Alb fraction was less than 50%, while the

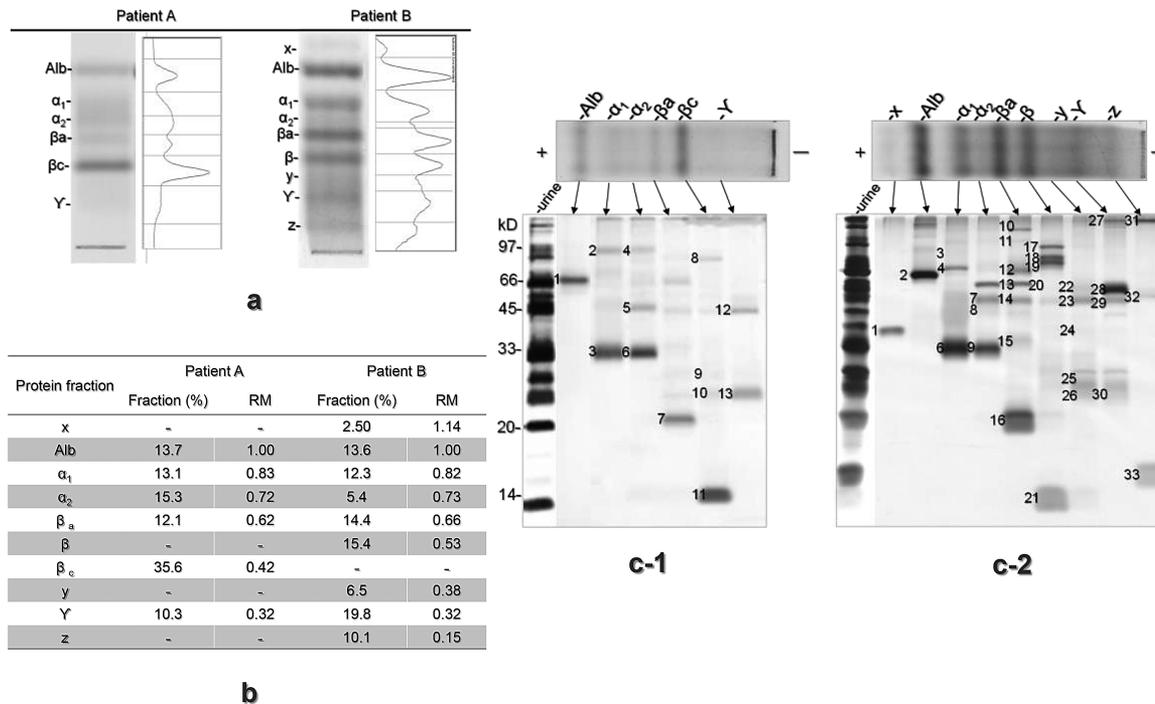


Fig. 4. CAME and SDS-PAGE patterns of the proteins extracted from the urine samples of the patients with tubulointerstitial nephritis and RM of each fraction. Silver-stained CAM gel and densitometric profiles of the urine samples from two patients with tubulointerstitial nephritis (a). RM of each fraction from each patient (b). SDS-PAGE gels of the fractions extracted from the urine samples of patient A (c-1) and patient B (c-2).

bands of β_a and β_c represent RBP at RM of 0.62 (reference 0.60–0.65) and β_{2m} at RM of 0.42 (reference 0.40–0.45), respectively. In patient B, on the other hand, urinary proteins were separated into nine characteristic fractions. Although Alb (<50% abundance) and β_a fractions indicated tubular type, the β fraction represented a glomerular protein of Tf at the RM of 0.53 (0.48–0.57). Furthermore, this pattern was similar to the mixed pattern, even though it lacked the β_c fraction. Three unknown bands were detected at RM of 1.14, 0.38, and 0.15 (x, y, and z). The relatively sharp band of the γ -G fraction was also a characteristic feature of the band shape.

Comparison of the Proteins Extracted From Each CAM Fraction

Figure 4c shows the SDS-PAGE results of the extracted proteins in each pattern. In total, 31 proteins from 46 bands in the SDS-PAGE gels were identified with this method from the samples of the two patients with tubulointerstitial nephritis (Table 1). The characteristic fraction x of patient B was consistent with transthyretin. The fraction y showed similar protein content of the γ -G fraction, except for the lower amount of beta-2-glycoprotein. Fraction z was mainly consistent with lysozyme.

Among the proteins identified, 11 have been previously identified by immunofixation (6), and the

remaining 20 proteins (i.e., zinc-alpha-2-glycoprotein, prostaglandin-H2 D-isomerase, Ig kappa chain C region, Ig gamma-1 chain C region, alpha-1B-glycoprotein, angiotensinogen, vitamin D binding protein, apolipoprotein A-IV, plasma protease C1 inhibitor, complement C1s subcomponent, antithrombin-III, spermatogenesis-associated protein 7, complement C3 fragment, pigment epithelium-derived factor, beta-2-glycoprotein 1/apolipoprotein H, complement factor H related protein 1, apolipoprotein A-I, complement factor D, immunoglobulin light chain, Ig gamma-3 chain C region) were newly detected in the present CAM-based analysis.

DISCUSSION

Urinary protein profile using CAME coupled with the highly sensitive colloidal silver staining enables the separation of urinary proteins without concentration and provides the entire scope of the urinary protein abnormalities. Urinary protein profiles on CAME also allow to identify damaged portions of the kidney. To increase the information that CAME could provide, identification of proteins in each fraction was necessary.

In the present study, our protein extraction method was validated. As expected, the LSB buffer was the most effective and appropriate solution to extract the proteins fractionated on CAM. The subsequent SDS-PAGE

TABLE 1. Protein Bands Identified by LC-MS/MS

Band no.	Fraction	Protein name	Gene name	AC	Nominal mass	pI value	Score	Peptide no.	Sequence coverage (%)
Patient A									
1	Alb	Serum Alb	ALBU.HUMAN	P02768	71,317	5.92	104	11	26
2	α_1	Uromodulin	UROM	P07911	72,451	5.05	24	2	3
3		Alpha-1-microglobulin	AMBP.HUMAN	P02760	39,886	5.95	136	4	15
4	α_2	Uromodulin	UROM	P07911	72,451	5.05	65	4	5
5		Zinc-alpha-2-glycoprotein	ZA2G.HUMAN	P25311	34,465	5.71	86	6	23
6		Alpha-1-microglobulin	AMBP.HUMAN	P02760	39,886	5.95	70	8	19
7	β_a	RBP 4	RET4.HUMAN	P02753	23,337	5.76	68	4	30
8	β_c	Serotransferrin	TRFE.HUMAN	P02787	79,294	6.81	22	3	7
9		Prostaglandin-H2 D-isomerase	PTGDS.HUMAN	P41222	21,243	7.66	88	2	8
10		Ig kappa chain C region	IGKC.HUMAN	P01834	11,773	5.58	31	2	32
11		Lysozyme C	LYZ.HUMAN	P61626	16,982	9.38	79	3	19
11		Cystatin-C	CYTC.HUMAN	P01034	16,017	9.00	176	3	19
12	γ	Ig gamma-1 chain C region	IGHG1.HUMAN	P01857	36,596	8.46	81	3	50
13		Ig kappa chain C region	IGKC.HUMAN	P01834	11,773	5.58	123	3	48
Patient B									
1	X	Transthyretin	TTHY.HUMAN	P02766	15,991	5.52	79	3	17
2	Alb	Serum Alb	ALBU.HUMAN	P02768	71,317	5.92	254	12	48
3	α_1	Alpha-1-antitrypsin	A1AT.HUMAN	P01009	46,878	5.37	166	4	10
4		Alpha-1B-glycoprotein	A1BG.HUMAN	P04217	54,790	5.56	66	9	24
5		Alpha-1-microglobulin	AMBP.HUMAN	P02760	39,886	5.95	76	10	21
6	α_2	Angiotensinogen	ANGT.HUMAN	P01019	53,406	5.87	100	3	8
7		Vitamin D binding protein	VTDB.HUMAN	P02774	54,526	5.40	77	10	32
8		Apolipoprotein A-IV	APOA4.HUMAN	P06727	45,371	5.28	21	3	13
9		Zinc-alpha-2-glycoprotein	ZA2G.HUMAN	P25311	34,465	5.71	108	4	10
10		Alpha-1-microglobulin	AMBP.HUMAN	P02760	39,886	5.95	130	10	31
11	β_a	Plasma protease C1 inhibitor	IC1.HUMAN	Q96FE0	55,347	6.09	89	7	19
12		Complement C1s subcomponent	C1S.HUMAN	Q9UCV3	78,174	4.86	73	9	19
13		Antithrombin-III	ANT3.HUMAN	Q9UC78	53,025	6.32	60	7	21
14		Spermatogenesis-associated protein 7	SPAT7.HUMAN	Q9P0W8	68,190	5.90	35	3	7
15		Vitamin D binding protein	VTDB.HUMAN	P02774	54,526	5.40	103	7	28
16		Apolipoprotein A-IV	APOA4.HUMAN	P06727	45,371	5.28	28	3	21
16		Complement C3 fragment	CO3.HUMAN	P01024	188,569	6.02	67	9	10
17		RBP 4	RET4.HUMAN	P02753	23,337	5.76	81	7	45
18	β	Apolipoprotein A-IV	APOA4.HUMAN	P06727	45,371	5.28	169	8	21
19		Serotransferrin	TRFE.HUMAN	P02787	79,294	6.81	148	6	10
20		Hemopexin	HEMO.HUMAN	P02790	52,385	6.55	56	5	16
21		β_2m	B2MG.HUMAN	P61769	13,715	5.30	34	3	14
22	γ	Pigment epithelium derived factor	PDEF.HUMAN	P36955	46,454	5.97	220	8	20
23		Beta-2-glycoprotein 1/Apolipoprotein H	APOH.HUMAN	P02749	39,548	8.34	55	4	17
24		Complement factor H related protein 1	PHR1.HUMAN	Q03591	38,766	7.38	37	6	18
25		Apolipoprotein A-I	APOA1.HUMAN	P02647	30,759	5.56	80	9	32
25		Complement factor D	CFAD.HUMAN	P00746	27,529	7.99	79	7	27
26		Ig kappa chain C region	IGKC.HUMAN	P01834	11,773	5.58	85	3	18
27	γ	Ig kappa chain C region	IGKC.HUMAN	P01834	11,773	5.58	97	2	18
28		Beta-2-glycoprotein 1/Apolipoprotein H	APOH.HUMAN	P02749	39,548	8.34	63	6	28
29		Beta-2-glycoprotein 1/Apolipoprotein H	APOH.HUMAN	P02749	39,548	8.34	337	15	54
29		Apolipoprotein A-IV	APOA4.HUMAN	P06727	45,371	5.28	62	12	29
30		Immunoglobulin light chain	gi218783334	BAH03697	23,438	8.31	55	5	39
31	Z	Ig gamma-1 chain C region	IGHG1.HUMAN	P01857	36,596	8.46	63	2	9
31		Ig kappa chain C region	IGKC.HUMAN	P01834	11,773	5.58	116	4	51
31		Ig gamma-3 chain C region	IGHG3.HUMAN	P01860	42,287	8.23	57	3	9
32		Apolipoprotein A-IV	APOA4.HUMAN	P06727	45,371	5.28	29	6	18
33		Lysozyme C	LYZ.HUMAN	P61626	16,982	9.38	95	6	37
33		Cystatin-C	CYTC.HUMAN	P01034	16,017	9.00	62	5	45

The protein band number used in Figure 4c, protein identity, gene name, AC, normal mass, pI value, score, number of peptide matches (peptide no.), and sequence coverage.

AC, accession code.

analysis enabled the estimation of the approximate abundance of the extracted proteins. Furthermore, this method could discriminate both the well-separated proteins on CAM, such as transthyretin, Alb, RBP, β_2m , and lysozyme C, as well as overlapped-proteins such as uromodulin, alpha-1-microglobulin, vitamin D binding protein, and apolipoprotein A-IV. These detailed results confirm the feasibility of CAME in urinary protein analysis. In our preliminary study, better results were obtained when 15% acrylamide gel was used than when 10% and 12.5% acrylamide gel were employed (data not shown). However, the protein identification may be limited by the polyacrylamide concentration, and wide molecular mass range might not be fully covered. Shotgun proteomics of the extracted proteins could also be an effective method for protein identification if the sample preparation method is successfully confirmed.

Our developed CAME-based proteomic approach was useful to identify the proteins contained in each fraction. Most of these proteins have already been reported as renal dysfunction related markers. For example, zinc-alpha-2-glycoprotein was reported as the candidate marker for nonalbuminuric diabetic nephropathy (16) and active lupus nephritis (17). In lupus nephritis, urinary prostaglandin-H2 D-isomerase was also reported as a novel marker (17). Alpha-1B-glycoprotein expression was increased in the urine of patients with interstitial cystitis (18). Urinary angiotensinogen level was found to be elevated in children with obstructive nephropathy (19). Vitamin D binding protein is one of the typical megalin-mediated proteins (20), and it has been reported as the biomarker of several kidney diseases including tubular dysfunction and IgA nephropathy (21,22). Apolipoprotein A-IV is also taken up by megalin expressed in proximal tubular cells, and many studies have demonstrated that the decreased clearance of apolipoprotein A-IV was due to renal dysfunction (23,24). Vivekanandan-Giri et al. have reported that the levels of several N-linked glycoproteins such as zinc-alpha-2-glycoprotein, antithrombin-III, pigment epithelium derived factor, and complement factor H related 1 were elevated in urine samples of patients with chronic kidney diseases (25). Our patient B showed a relatively sharp band in the Y-G fraction, which usually presents as a broad diffuse band because of a slightly different pI state of immunoglobulins. This fraction was mainly consistent with beta-2-glycoprotein, which is a highly glycosylated plasma protein of approximately 40 kDa, and because of its high isophoretic point (8.34), it is thought to be filtered in the glomerulus more easily than other anionic proteins (16). Beta-2-glycoprotein is also metabolized through megalin-mediated endocytosis like other low-molecular mass proteins, and is thought to be an important marker of renal tubular diseases (17). In a previous analysis using agarose gel electrophoresis,

beta-2-glycoprotein also produced a prominent band in the β to Y-G region in urine of patients with renal tubular diseases, including low syndrome, Fanconi syndrome, and Dent's disease (26). Therefore, the characteristic band in the Y-G fraction may be used as a useful indicator of deterioration of tubular reabsorption.

Low amounts of proteins may not highly contribute to the alteration of the mobility or formation of a protein fraction; however, a band on CAM is an assembly of proteins that have a similar charge. Pathophysiological changes can differentiate the composition of proteins from a damaged kidney in individual patients, and this may cause slight mobility shifts or create new fractions. The two patients enrolled in this study presented tubulointerstitial nephritis; however, patient B exhibited exacerbation of tubular proteinuria, while patient A did not. To clarify whether their different protein profiles on CAME were caused by a different disease stage, pharmacological treatment, or other reasons, further studies conducted on a larger number of patients are needed.

Renal biopsy is the most accurate method to diagnose renal diseases, not only for differentiating the damaged site of nephrons (e.g., glomerular or tubular) but also for evaluating the treatment results; however, since it is invasive, expensive, and risky, it cannot be performed frequently. Therefore, a noninvasive urine analysis is needed to predict and diagnose renal diseases. The coexistence of characteristic urinary protein fractions on CAME may lead to quick and inexpensive laboratory tests for renal injury. To broaden the clinical application of urinary protein profiles obtained via CAME, such as screening renal diseases or providing data that support the pathological findings of renal biopsy, the relationship between each protein profile and tissue damage needs to be clarified. In conclusion, the present study provides a useful procedure for protein identification from characteristic fractions on CAME.

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