Improved Recovery of Human Urinary Protein for Electrophoresis

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Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is frequently used to analyze urinary proteins, but appropriate urine sample preparation is essential, because urinary proteins are present in small concentrations, and urine contains high concentrations of salts and metabolic wastes. The aim of this study was to determine the optimum method for urine sample preparation. The effects on urinary protein recovery of pretreating the ultrafiltration apparatus with various agents (Triton X-100, Tween20, PEG compound, and SDS) were studied; Triton X-100 was found to be the most effective agent. Ultrafiltration and acetone precipitation were compared as sample preparation methods for SDS-PAGE. Recovery of urinary protein using ultrafiltration (89.5 ± 8.1%) was better than that achieved by using acetone precipitation (75.1 ± 12.2%) ($p < 0.01$). Integrated densitometric values for five protein bands (62, 52, 39, 31, and 6 kDa) were higher for ultrafiltration samples than for acetone samples (42350 ± 2568 *vs.* 37010 ± 725, 34665 ± 1519 *vs.* 32355 ± 2278, 10755 ± 1683 *vs.* 8870 ± 941, 65920 ± 3354 *vs.* 60680 ± 3651, and 1355 ± 160 *vs.* 1130 ± 211, respectively) ($p < 0.05$), although differences for the other nine bands were not significant. In conclusion, ultrafiltration is a suitable method for preparing urine samples for SDS-PAGE.

Key words —— ultrafiltration, human urinary protein, sample preparation, sodium dodecyl sulfate polyacrylamide gel electrophoresis

INTRODUCTION

Urine contains various components, both physiological and pathophysiological, which, given correct analysis, can be very useful for understanding disease.[1–3] Excretion of urinary proteins sensitively reflects renal disorders,[1,4] and both quantitative and qualitative changes can occur in urinary protein excretion, depending on the disease. Therefore, measuring only total urinary protein concentration may be insufficient for understanding a pathophysiological condition.[5] Qualitative analysis of protein is frequently performed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and the technique is also used for separation of urinary proteins. However, two problems exist with respect to using SDS-PAGE for protein detection in urine: first, urine contains abundant salts and metabolic wastes, and second, protein concentrations in urine are typically very low.[6,7] Therefore, appropriate sample preparation is vital for achieving good urinary protein separation by SDS-PAGE.

Both chemical and physical separation have been used for such preparative purposes. Acetone is one of the most commonly used agents to effect chemical separation; it functions via precipitation, and can be used in combination with various organic solvents or acids.[6,8–11] Ultrafiltration is one method of physical separation.[6,12] Both preparation methods have a common problem; that is, protein loss occurs during the procedure. Improvement of protein recovery by ultrafiltration has been reported recently for bovine liver lysate;[13] however, the effectiveness of ultrafiltration has not yet been evaluated for human urine. Furthermore, pretreatment of the ultrafiltration unit with various passivation agents is known to decrease nonspecific binding of compounds to the

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unit itself, thus minimizing protein loss.[14] However, the effectiveness of pretreatment has not been investigated for human urine. The aim of the present study was to determine the optimum method of preparing human urine samples for SDS-PAGE.

MATERIALS AND METHODS

Materials —— Acetone, diethylether, ethanol, methanol, Tween20, and glycerol were purchased from Kanto Chemical Co. (Tokyo, Japan). SDS was purchased from ACROS Organics (New Jersey, U.S.A.). Coomassie brilliant blue (CBB) G-250, Bradford reagent, EZ Blue Gel Staining Reagent, and polyethyleneglycol (PEG) compound (MW 15000–20000, Cat. No. P2263) were purchased from Sigma (St. Louis, MO, U.S.A.). Acrylamide, methylene bis-acrylamide, N,N,N′,N′-Tetramethylethylenediamine (TEMED), and ammonium persulfate (APS) were purchased from GE Healthcare Bio-Sciences Corp. (Piscataway, NJ, U.S.A.). Polyoxyethylene (10) octylphenyl ether (Triton X-100) was purchased from Wako Pure Chemical Industries (Tokyo, Japan). 2-Mercaptoethanol was purchased from Merck Schuchardt (Hohenbrunn, Germany). Tris and Tricine were purchased from MP Biomedicals, LLC (Aurora, OH, U.S.A.). The ultrafiltration devices used were Vivaspin-500 centrifugal filter concentrator units from Vivascience (Hannover, Germany) with 5-kDa polyethersulfone filters. Bovine serum albumin solution (2 mg/ml) was purchased from Pierce (Rockford, IL, U.S.A.). A broad-range protein molecular weight marker was purchased from BioRad (Hercules, CA, U.S.A.).

Urine Samples —— Urine samples from 21 patients with essential hypertension were used in this study. The urine was stored at –80°C until use. The ethics committee of our institution approved the experimental protocol.

Pretreatment Conditions for Ultrafiltration —— Five-hundred microliter aliquots of each of four passivation agents (5% v/v Triton X-100, 5% w/v PEG compound, 5% v/v Tween-20, and 5% w/v SDS) were loaded onto centrifugal ultrafiltration units, respectively. The agents were left in the units overnight, then the units were washed five times with pure water. Urine samples (500 μl) were then added to the pretreated units, such that urine from each patient was added to units pretreated with each of the four passivation agents, respectively. The units were spun in a centrifuge at 7500 g at 4°C for 60–90 min, until the sample volume decreased to about one-twentieth of the initial volume. The volume of the concentrated urine was measured by weight in accordance with instructions provided by the manufacturer of the ultrafiltration device. Total urinary protein in both native and concentrated urine was measured using the CBB protein dye-binding assay as described elsewhere, with modifications.[15,16] Bovine serum albumin was used as the protein standard. Briefly, 0.125–3.5 μg of protein in a sample volume of 50 μl was mixed with 150 μl of Bradford reagent. The increase in absorbance at 590 nm was measured using a Spectrafluor Plus microplate reader (Tecan, Mannedorf/Zurich, Switzerland) after 10 min. Protein recovery values were calculated for the concentrated and the native urine samples, respectively.

Comparison between Ultrafiltration and Acetone Precipitation —— Urine from one patient was used for both ultrafiltration and acetone precipitation. Different urine samples from those used in the former experiment were used in this experiment. For acetone precipitation, urine was added to four times the volume of cold acetone as described elsewhere, with modifications.[6,8] The mixture was stored for 3 hr at –30°C, after which it was centrifuged at 10000 g at 4°C for 15 min. The pellet thus obtained was washed with a cold diethylether : ethanol (6 : 1) mixture and centrifuged at 10000 g at 4°C for 5 min. After the organic solvent was removed, the pellet was dried in air. For ultrafiltration, pretreatment with Triton X-100 and subsequent ultrafiltration were carried out as described above. The total amounts of protein concentrated by both methods were measured.

The urine samples concentrated by ultrafiltration and acetone precipitation, respectively, were also compared by SDS-PAGE using a 10% polyacrylamide slab gel and a Tris/Tricine system.[17] The acetone-precipitated pellets were mixed with sample buffer (50 mM Tris-HCl (pH 6.8), 4% w/v SDS, 12% w/v glycerol, 2% v/v 2-mercaptoethanol, and 0.01% w/v CBB-G250). The urine concentrated by ultrafiltration was mixed with four times the volume of concentrated sample buffer. Then, samples were heated at 95°C for 5 min. A broad-range molecular weight marker was used as a reference. The SDS-PAGE gel was run using miniVE (GE Healthcare Bio-Sciences Corp.) at 30 V for 1.5 hr and then at 100 V for 2.5 hr. The gel was then washed in water and fixed in a solution containing 50% methanol and

Table 1. Protein Recovery Using Ultrafiltration with Various Pretreatments

Pretreatment agent	Protein recovery (%)	*p* value				
		Triton X-100	PEG compound	Tween-20	SDS	No pretreatment
Triton X-100 ($n = 12$)	86.6 ± 8.1	—				
PEG compound ($n = 12$)	80.3 ± 8.9	N.S.	—			
Tween-20 ($n = 12$)	80.1 ± 8.3	N.S.	N.S.	—		
SDS ($n = 12$)	75.3 ± 9.9	< 0.05	N.S.	N.S.	—	
No pretreatment ($n = 12$)	76.2 ± 8.2	< 0.05	N.S.	N.S.	N.S.	—

N.S., not significant.

10% acetic acid. After the gel was washed in water, protein bands in the gel were visualized by CBB-G 250 staining using the EZ Blue Gel Staining Reagent. The gel was destained in water until the background of the gel was clear. Images of gels were captured by using the Printgraph system (ATTO, Tokyo, Japan), and integrated densitometric values (IDV) for each protein band were measured with lane spot analyzer software (ATTO) as described elsewhere.[18]

Statistical Analysis —— Statistical analyses were performed using the paired *t*-test and one-way analysis of variance (ANOVA). All data are presented as mean ± standard deviation (S.D.). A value of $p < 0.05$ was considered significant.

RESULTS AND DISCUSSION

There are various methods that can be used to concentrate diluted protein samples for electrophoresis, including ultrafiltration,[6,12] and precipitation with acetone,[6,8] trichloroacetic acid (TCA),[8,11] and TCA/acetone.[6,9,10] These methods can also be used for urine. However, with each method there is potential for urinary protein loss. For instance, some urinary proteins, for example β2-microgloblin, are unstable at low pH.[19] Thus, because TCA makes urine acidic, it is not appropriate for urine concentration. Acetone precipitation has the problem of solubility into organic solvents.[20] In ultrafiltration, adhesion of material to the equipment and leakage of proteins into the filtrate must be considered. Recently, ultrafiltration has been reported to improve protein recovery in bovine liver lysate;[13] however, the effectiveness of ultrafiltration has not been evaluated for human urine before now.

Pretreatment Conditions for Ultrafiltration

There are many ultrafiltration systems available, which have various MW cut-off points and use various membrane materials. The smallest pore size currently available is 5 kDa. Devices with low-MW cut-offs hold low-MW urinary proteins in the retentate and prevent leakage of low-MW proteins through the membrane into the filtrate during centrifugation. Polyethersulfone membranes have a much higher flux than regenerated cellulose, so use of the former permits a shorter centrifugation time than the latter. In the present study, we used a 5-kDa ultrafiltration system with a polyethersulfone membrane.

With respect to optimization of ultrafiltration, pretreatment of ultrafiltration units with various passivation agents is known to reduce nonspecific binding of compounds to the unit itself.[14] However, the effects of pretreatment have not been studied for human urine. Table 1 shows the effects of the various pretreatments used in the present study on urinary protein recovery. Pretreatment with Triton X-100 enabled significantly higher protein recovery than no pretreatment ($p < 0.05$) and SDS pretreatment ($p < 0.05$). However, protein recovery with Triton X-100 pretreatment was not significantly different from that when PEG compound or Tween-20 were used, and protein recovery after pretreatment with PEG compound, Tween-20, and SDS did not differ from that when no pretreatment was used. Our results show that Triton X-100 is the most suitable agent for ultrafiltration pretreatment for human urine because it allows recovery of the greatest amount of protein. It is quite possible that Triton X-100 might have prevented the adhesion of urinary proteins to the ultrafiltration unit in this case.

Comparison between Ultrafiltration and Acetone Precipitation

In previous reports, most urinary proteins detected have been less than 200 kDa in size.[21] The electrophoretic conditions used in the present study were thus set for analyses in this range. The electrophoretic patterns were analyzed in order to compare

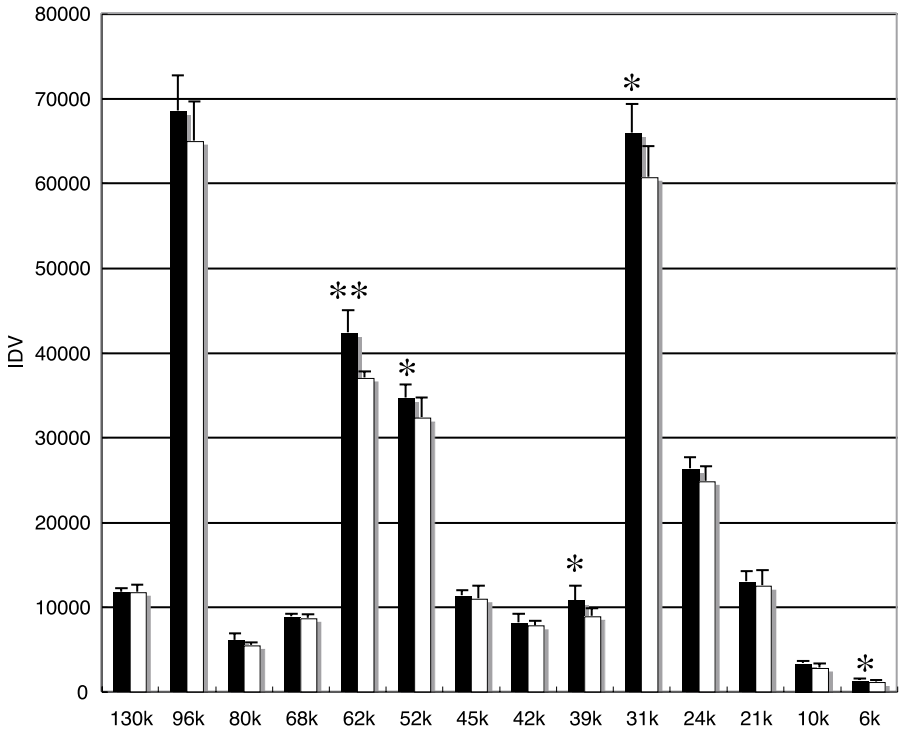

Fig. 1. IDVs for Each SDS-PAGE Band from Urine Samples Prepared by Ultrafiltration (Black Columns) and by Acetone Precipitation (White Columns)
Data are mean ± S.D.; *$p < 0.05$, **$p < 0.01$.

urinary proteins obtained by using two different methods: acetone precipitation and ultrafiltration. Protein recovery is a key factor when selecting a preparation method for SDS-PAGE. Protein recovery from urine using ultrafiltration (89.5 ± 8.1%) was significantly greater than that achieved by acetone precipitation (75.1 ± 12.2%) ($p < 0.01$). Furthermore, the CV% for ultrafiltration (9.1%) was smaller than that for acetone precipitation (16.3%). Our results showed that the recovery rate using ultrafiltration was superior to that using acetone. Consistency in protein recovery is also important, and CV% for recovery using ultrafiltration was smaller than that for recovery using acetone precipitation. Furthermore, SDS-PAGE gels of urine concentrated using the two different methods had the same numbers of bands. A quantitative comparison for each band on the SDS-PAGE gels is shown in Fig. 1. The IDVs of five bands (64, 52, 39, 31, and 6 kDa) when obtained via ultrafiltration were significantly higher than corresponding IDVs for the same bands obtained via acetone precipitation ($p < 0.05$). However, there were no differences in IDVs for the other nine bands (130, 96, 80, 68, 45, 42, 24, 21, and 10 kDa). The electropherogram for both preparation methods is shown in Fig. 2.

There are three reasons why protein recovery using ultrafiltration might be superior. First, hydrophobic proteins can remain solubilized in the liquid fraction during ultrafiltration, whereas acetone can easily precipitate hydrophilic proteins. Second, considerable quantities of urinary salts and metabolic wastes are also precipitated during the acetone-precipitation procedure; these substances could interfere with redissolution of the urinary proteins. It is possible that insoluble protein complexes or protein agglomerate bands of more than 200 kDa in size might appear in gels of the acetone-precipitated samples. Third, ultrafiltration is better able to recover low-MW proteins. Leakage of low-MW proteins into the filtrate through a 5-kDa MW cut-off membrane is a potential shortcoming of ultrafiltration, but in the present study, in which a 6 kDa band was the smallest protein identified, the IDV of that band in the ultrafiltration sample was significantly higher than the IDV of the band in the acetone sample. This means that the ultrafiltration retentate is better able to retain even low-MW urinary proteins in comparison with acetone.

In conclusions, ultrafiltration with a 5-kDa cut-

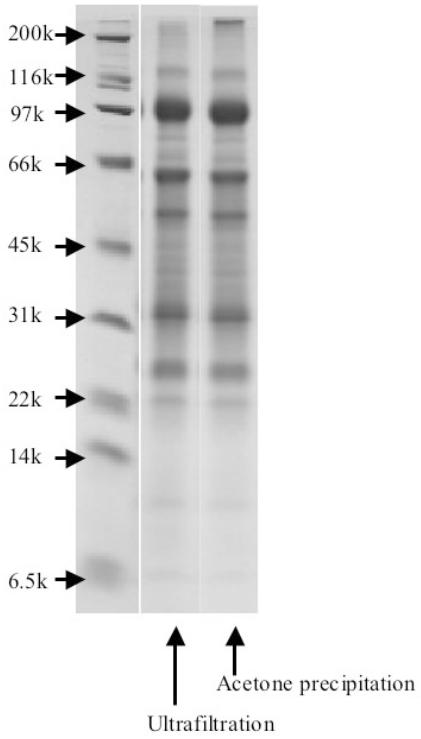

Fig. 2. SDS-PAGE Electropherogram for Urine Samples Prepared by Ultrafiltration (Center Lane) and Acetone Precipitation (Right Lane)
Left lane is the protein MW marker.

off, using Triton X-100 pretreatment, is a suitable sample preparation method for SDS-PAGE analysis of human urinary proteins. Ultrafiltration is superior to the acetone precipitation method for comparing the quantitative and qualitative differences in urinary proteins between healthy and unhealthy persons.

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