[Technical Note]

Comparison of urinary exosomal protein solubilization methods
for two-dimensional gel electrophoresisAsami Kosaka¹, Aki Nakayama², Ikue Yamaguchi², Takeshi Kasama³,
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SUMMARY

Background: Urinary exosomal proteins have recently emerged as important candidates for elucidating the mechanisms underlying physiological events and disease-related metabolism in the kidney. Here, we evaluated standard sample preparation methods for two-dimensional gel electrophoresis (2DE) to determine which one yielded the maximum protein recovery from urinary exosomes for protein identification.

Materials and Methods: Urinary exosomes were purified from a healthy subject by using ultracentrifugation. The final pellets were dissolved with PBS or RIPA buffer. After being desalted, these exosomal protein solutions were each treated with 1 of 4 rehydration buffers (Rbs) containing detergents in the following formulations: CHAPS (Rb1), CHAPS and Triton X-100 (Rb2), dodecyl maltoside (Rb3), and ASB-14 (Rb4).

Results: For all Rbs, a much greater number of protein spots was detected in the samples isolated with RIPA than with PBS. Only minor differences were observed in the number of protein spots for Rb1–3. The largest protein spots were detected using the combination of RIPA buffer and Rb4; however, the background on the 2DE gel was high in the region of >66 kD and at the lower pH values. For all combinations, the co-precipitation of the urinary Tamm-Horsfall protein masked the protein spots in the 66–100 kD region.

Conclusion: For extracting a large number of proteins with a relatively clear background on silver-stained 2DE gels, the optimal exosomal protein-dissolving buffer is RIPA buffer. All of the evaluated Rbs, except for the one containing ASB-14 as a detergent, is suitable for solubilizing exosomal proteins on 2DE.

Key words: two-dimensional gel electrophoresis, urinary exosome, protein recovery

INTRODUCTION

Urinary exosomes are membrane vesicles, 40–100 nm in diameter, secreted by renal epithelial cells such as podocytes and proximal tubular cells, and are expected to become a new biomarker source^{1–4}. This potential as a new biomarker is based on exosome genesis, which initially involves the fusion of endosomes to multivesicular bodies (MVBs) and the subsequent inward budding of the MVBs. Through fusion of the outer MVB membranes with the apical membrane, exosomes are released into the urine^{1–6}. Urinary exosomes are composed of plasma membrane from the renal epithelial cells and contain cytoplasmic components. Hence, it is important to obtain the protein profile of urinary exosomes from healthy subjects in order to under-

stand the in vivo physiological events in renal epithelial cells, so that basic data can be acquired for the discovery of biomarkers related to different forms of kidney disease.

In standard sample preparation protocols, urinary exosomes are often isolated through ultracentrifugation at 2 different speeds (i.e., 17,000×g and 200,000×g)⁷. Recently, rapid methods using nanomembrane ultracentrifugation have been established^{8–10}. These proactive efforts at reducing the processing time and the number of steps have further enabled the use of urinary exosomal proteins for clinical laboratory analysis.

To date, 1160 proteins have been identified in urinary exosomes¹¹ through LC-MS/MS analysis coupled with one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)¹¹. The typical protein profile

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of urinary exosomes in a one-dimensional SDS-PAGE shows a significant amount of Tamm-Horsfall protein (THP) at 100 kD and densely detected proteins between ~30–70 kD¹⁾. Hence, as an effective approach for protein identification, two-dimensional gel electrophoresis (2DE) is preferable as the first step. However, we have not found any studies reporting the optimal method of sample preparation for 2DE in the proteome analysis of the urinary exosome.

In the present study, we tested the optimal combination of dissolving buffer for the precipitation of urinary exosomes following ultracentrifugation and the rehydration buffer for 2DE to maximize the number of proteins solubilized.

MATERIALS AND METHODS

Exosome purification

The preliminary research showed that few differences in of the number of protein bands and their patterns on SDS PAGE between 26 healthy subjects. In the present study, therefore, we analyzed a urine sample that was selected at random from the population (female, 57 years old) after obtaining her written consent. Protease inhibitor and antibiotics (2.5 mL of 11.5 mmol/L 4-[2-aminoethyl] benzene-sulfonyl fluoride, 50 µL of 1 mol/L leupeptin, and 1.67 mL of 100 mmol/L NaN₃) were added to 50 mL of the freshly obtained urine sample²⁾. The urine sample was centrifuged at 17,000×g using an MLA-80 rotor in an Optima-Max ultracentrifuge (Beckman Coulter, Fullerton, CA, USA), and the pellets were incubated with 200 mg/mL dithiothreitol (DTT) for 10 min at 37°C and recentrifuged at 17,000×g. Next, the supernatants from the first and second rounds of centrifugation were mixed and ultracentrifuged at 200,000×g. The final pellet was suspended in phosphate-buffered saline (PBS) or commercial RIPA lysis buffer (Pierce, Rockford, IL, USA) containing 25 mmol/L tris (hydroxymethyl) aminomethane, 150 mmol/L NaCl, 1% NP-40, 1% sodium deoxycholate, and 0.1% SDS (pH 7.6)^{12, 13)}, and then incubated for 1 hour at room temperature. The total protein concentrations of the final pellets containing the exosomes were measured using the dot blot assay with colloidal silver staining¹⁴⁾.

One-dimensional gel electrophoresis and silver staining

Urinary exosomal proteins extracted by PBS or RIPA buffer (0.5 µg) were mixed with 6× Laemmli sample buffer (1 mol/L tris (hydroxymethyl) aminomethane, 10% SDS, 1 mol/L DTT, and 1% bromophenol blue (pH 6.8)). The samples were heated for 5 minutes at 95°C and analyzed by one-dimensional SDS-PAGE using a 15% polyacrylamide gel. After electrophoresis, the protein bands were visualized by silver staining (Silver Stain II kit Wako, Wako Pure Chemical Industries, Ltd., Osaka, Japan)¹⁵⁾.

Two-dimensional gel electrophoresis

Five micrograms of the urinary exosomes extracted by PBS or RIPA buffer were treated using a 2-D Clean-Up Kit (GE Healthcare, Piscataway, NJ, USA) to remove detergent, salts, and other interfering substances. The protein pellets were solubilized by 4 rehydration buffers (Rbs) consisting of different formulations (summarized in Table 1)^{16–18)} and incubated for 1 hour at room temperature.

Two-dimensional gel electrophoresis was performed by using the ZOOM IPGRunner System (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s instructions. In brief, exosomal samples treated with the different Rbs were separated by isoelectric focusing using a pH 3–10 immobilized gel strip for 20 min at 200 V, 15 min at 450 V, 15 min at 750 V, and 40 min at 2000 V. The gel strip was incubated with DTT and iodoacetamide each for 15 min, and then electrophoresed in a second dimension using a 15% polyacrylamide gel. The proteins were detected by the silver staining mentioned above. Gels were made in triplicate for each condition and compared for the number of protein spots.

RESULTS

One-dimensional gel electrophoresis pattern of the urinary exosomes

The one-dimensional gel electrophoresis pattern of the urinary exosomes was characterized by a broad band of urinary THP (97 kD) above a number of dark protein bands. Although the number of proteins between the bottom of the THP band and the 42 kD band was visually confirmed, a very high background was present (Fig. 1). No difference in the number and the type of the exosomal protein bands (38

Table 1. Components of the 4 rehydration buffers.

	Rehydration buffers (Rbs)			
	1	2	3	4
Chatropes	7 mol/L urea 2 mol/L thiourea	6 mol/L urea 2 mol/L thiourea	7 mol/L urea 2 mol/L thiourea	7 mol/L urea 2 mol/L thiourea
Detergents	4% CHAPS	3% CHAPS 1% Triton X-100	2% dodecyl maltoside	2% ASB-14
Carrier amphorites	0.5% pH 3–10	0.5% pH 3–10	0.5% pH 3–10	0.4% pH 3–10
Reducing agents	20 mmol/L DTT	20 mmol/L DTT	20 mmol/L DTT	20 mmol/L DTT
References (no.)	Welton <i>et al.</i> (16)	Suehara <i>et al.</i> (17)	Rabilloud <i>et al.</i> (18)	Rabilloud <i>et al.</i> (18)

Fig. 1. One-dimensional gel electrophoresis with silver staining (1: PBS, 2: RIPA) showing the difficulty of identifying urinary exosomal protein bands, especially those in the range of 42–66 kD.

protein bands) was found between the samples prepared with PBS and RIPA buffer, showing the strong ability of SDS to solubilize the proteins regardless of the buffer. We employed a polyacrylamide concentration of 15% for the running gel because the highest number of protein bands was detected in this concentration when compared to concentrations of 7.5% and 10% (data not shown). Using these different concentrations of polyacrylamide gels, exosomal proteins ranging in size from approximately 10 to 300 kD were detected.

The two-dimensional gel electrophoresis pattern of urinary exosomes

For the 2DE (Fig. 2), the protein pattern was characterized by a major spot ($pI=3$) and a widespread THP band at 97 kD (the white dotted line in Fig. 2); and the protein separation between the bottom of the THP and the 42 kD bands was dramatically improved. Moreover, there was less streaking of the THP spot for the samples prepared with RIPA than with PBS. Further, the total number of proteins was greater for the samples dissolved in the RIPA buffer than that dissolved in PBS. The average number of spots from the triplicate 2DE gels slightly varied according to the combination of the 2 buffers and the 4 Rbs (Table 2). The combinations of RIPA-Rb3, RIPA-Rb2, and PBS-Rb2 yielded in the X, Y, and Z regions respectively, the highest number of detectable protein spots with a relatively clear background.

The protein patterns from the sample in the Rb4, which contains the zwitterionic detergent ASB-14, were obscured by a black shadow that covered the running gel for the samples prepared with the PBS and RIPA buffers (Fig. 2g and h).

DISCUSSION

In the present study, we obtained a urinary exosomal protein map using 2DE. The visualization of the 42–97 kD range, which showed a dense banding pattern and high

background in the one-dimensional SDS-PAGE, was clearly improved in the 2DE. For the 2DE, the number of proteins dissolved with the RIPA buffer was greater than that with PBS, as expected. Based on the results shown in Table 2, the combination of RIPA-Rb4 resulted in a better protein resolution relative to other combinations. However, the compatibility of this detergent with some urinary exosomal proteins, such as THP seemed to be poor. Therefore, ASB-14 containing Rb may not be suitable for solubilizing exosomal proteins on 2DE. These results suggest that the best choice of detergent for obtaining a clear protein profile will depend on the sample properties.

The commonly used CHAPS-containing Rbs, such as the Rb1 and 2, showed better protein recovery with a clear background in the present study, whereas Wubbolts *et al.* have mentioned the influence of a detergent-resistant membrane (DRM)-like property for CHAPS-containing Rbs in the analysis of human B-cell-derived exosomal proteins¹⁸. In this study, some of the exosomal proteins showed resistance to solubilization by CHAPS. When the DRM lipid components (e.g., cholesterol) were removed, the solubility of the proteins increased¹⁸. These results suggest that the protein solubility of the exosomes purified from biological fluids will require further research in order to improve protein recovery.

The polymer structure of THP is one of the major problems in the proteome analysis of urinary exosomes, especially in healthy subjects⁹. Various factors are known to cause THP aggregation, including the temperature of sample storage, pH, and ionic strength^{19, 20}. In an electron microscopic study, urinary exosomes were trapped in the fiber-like structure of THP⁷. The exosomes that aggregated with THP were depleted during the purification procedure, and fewer proteins derived from the exosomes were detected². For overcoming the co-precipitation of THP with exosomes, a new protocol has been reported that uses DTT to depolymerize the THP structure at the step involving the 17,000×g centrifugation⁷. In the present study, we employed the improved method, and the THP co-precipitation declined. Nonetheless, the THP still affected the analysis of the 2DE electrophoresis. Further studies are needed to remove urinary THP effectively to increase the amount of protein detected from the urinary exosomes.

Similar problems with co-precipitation have been reported to result from other major proteins. The significant amount of glomerular-filtered serum proteins, including albumin and α_1 -antitrypsin, results in major problems with co-precipitation with exosomal samples obtained from nephritic patients, such as those with membranous nephropathy¹⁰. In these cases, the use of size-exclusion chromatography following ultracentrifugation resulted in better coverage of the exosomal proteins. The 2DE techniques may be also be expected to result in a better separation of urinary exosomes from such abundant proteins.

Fig. 2. Two-dimensional gel electrophoresis of urinary exosomal proteins using 4 rehydration buffers coupled with 2 different dissolving buffers. The white dotted line indicates the main spot representing urinary THP.

These results indicate that optimizing the sample preparation or the protein solubilization of urinary exosomes according to the properties of the urine sample is needed to achieve a greater resolution of protein spots in urinary exosome proteomics.

In conclusion, the present study showed the profile of healthy urinary exosomal proteins on 2DE with the optimal sample solubilization method. These results provide basic information for biomarker discovery in urinary exosomes.

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Table 2. Different number of protein detection in the couple with the 2 dissolving buffers and the 4 rehydration buffers.

Rehydration buffers	Dissolving buffers	The number of proteins			
		Total area	X region	Y region	Z region
1–4	PBS	98±21	26±8	27±7	45±10
	RIPA	127±14	36±8	40±7	50± 7
1	PBS	87±12	25±2	20±1	42± 9
	RIPA	121±19	24±5	43±7	52±10
2	PBS	92±27	34±5	34±3	57± 5
	RIPA	127±11	36±3	46±1	46± 8
3	PBS	92±27	22±9	29±7	41±11
	RIPA	124±14	42±9	30±1	52± 4
4	PBS	89± 5	22±7	25±5	42± 7
	RIPA	135± 1	44±1	43±1	49± 1

ABBREVIATIONS

2DE, 2-dimensional electrophoresis; MVBs, multi-vesicular bodies; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; THP, Tamm-Horsfall protein; Rb, rehydration buffer

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