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RESEARCH ARTICLE

A qualitative proteome investigation of the sediment portion of human urine: Implications in the biomarker discovery process

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Inherent to the biomarker discovery process is a comparative analysis of physiological states. It is therefore critical that the proteome detection protocol does not bias the analysis. With urine, the sediment portion, obtained upon thawing frozen urine, is routinely discarded prior to proteome analysis. However, our results demonstrate that such a practice inadvertently induces bias, having significant implications in the biomarker discovery process. We present the first proteome investigation of human urinary sediments, identifying 60 proteins in this phase by MS. Many sediment proteins were also detected in the urinary supernatant, indicating that several proteins partition between the two phases. This partitioning is dependant on the pH of the sample, as well as the degree of sample agitation. As a consequence of discarding the sediment portion of urine, the concentration of potential candidate biomarkers in the supernatant phase will be altered or, in other instances, may be completely removed from the sample. To minimize this, the pH of all samples should first be normalized, and the samples vigorously vortexed prior to discarding the sediments. For more comprehensive biomarker investigations, we suggest that urinary sediments be analyzed along with the supernatant proteins.

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1 Introduction

Among the human body fluids, urine has gained widespread popularity as a protein source for disease biomarker discovery [1]. Not only is sample collection noninvasive, but urine also contains an abundance of proteins; recent investigations suggest the presence of well over 1500 proteins in

urine [2]. Given the proximity to the kidneys, bladder, and urinary tract, urine is particularly suited as a source of candidate biomarkers for genitourinary disorders, including inflammations [3], obstructions [4], and carcinomas of the bladder [5]. Moreover, as a product of blood filtration, urine provides an opportunity to survey proteins derived from essentially all organs of the body. Candidate protein biomarkers for lung cancer [6] as well as obstructive sleep apnea in children [7] have also been discovered through proteomic examinations of urine.

A variety of proteome detection protocols have been applied to urinalysis. Despite the analytical diversity, aspects of these protocols are universally accepted. For example, to preserve the proteome over extended periods, urine is frozen shortly after collection [8, 9]. Upon thawing, sediments from

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Abbreviation: SCX, strong cation exchange

the sample are typically removed by filtration or centrifugation, a necessary step in proteome visualization by gel electrophoresis [10]. This practice continues with modern gel and gel-free proteome analysis techniques, as it is assumed that the sediments may interfere with subsequent analysis.

Despite widespread acceptance, it was reported as early as 1986 that a thawed urine sample should be thoroughly vortexed but *not* centrifuged prior to SDS-PAGE, as this practice would lead to protein loss [11]. The identities of the so-called *lost* proteins were not discussed in this report. Furthermore, Zhou *et al.* [9] determined that the sediment phase of urine accounts for approximately 50% of the total protein content by mass and is primarily comprised of uromodulin. Thus, as a simple prefractionation technique, removal of the sediment phase can be considered beneficial for the analysis of low-abundant protein components in urine supernatants [12]. However, Zhou *et al.* clearly demonstrated (through SDS-PAGE images) that several other proteins contribute to the sediment phase of urine; a comprehensive study to identify these proteins has yet to be conducted. Most recently, it has been shown that vortexing a urine sample aids in the solubilization of proteins from urinary exosomes [9]. Interestingly, although urinary sediments are routinely analyzed through microscopy as a means of diagnosing physiological conditions [13], current proteome investigations focus only on the solubilized portion of the sample. Nonetheless, discarding the sediment phase undoubtedly removes other potentially important protein components from the sample.

It is also realized that any variation imposed by the analytical procedure will result in a superfluous bias upon detection or quantitation of the proteome. A bias induced by a sample handling protocol may have significant implications in the biomarker discovery process. For example, seemingly differentially expressed proteins may simply correlate to relative changes in protein solubility, induced by minor differences in the sample (*e.g.*, pH, protein concentration, extent of sample agitation). We believe a comprehensive analysis of the urinary proteome, including urinary sediments, would lend the greatest probability of biomarker discovery and validation.

In this report we perform the first proteome analysis of human urinary sediments. We explore the effects of sample vortexing on the partitioning of proteins between the supernatant and sediments of urine. We also examine the effect of sample pH on the relative solubility of proteins in urine. The implications of our results toward the biomarker discovery process are discussed.

2 Materials and methods

2.1 Materials

Reagents for SDS-PAGE were purchased from BioRad (Mississauga, Canada). Solvents were of HPLC grade and

were obtained from Fisher (Ottawa, Canada). Protease inhibitor cocktail (cat. P2714-1BTL) was from Sigma (Oakville, Canada), along with trypsin, TPCK treated (cat. YSC2), and all other chemicals used in these experiments.

2.2 Urine collection

Urine samples were collected from healthy male and female donors with approval of the Dalhousie University Ethics Committee (ethics approval #2007-1520). Individuals were determined to be healthy from previous blood tests and had no indications of kidney abnormalities. Immediately following collection, protease inhibitors were added to the sample (10:1 v/v urine/cocktail) and frozen at -20°C .

2.3 Sample manipulations for qualitative analysis by MS

Four samples (50 mL/sample) from individual healthy donors (two male, two female) were thawed undisturbed at room temperature and centrifuged at $3716 \times g$ to pellet the sediments. The supernatants were decanted and the sediments washed with ~ 5 mL of ice-cold water. Each pellet was suspended in 0.5 mL of solubilizing buffer (50 mM DTT, 2 M thiourea, 8 M urea, 1% CHAPS). Of this, 50 μL was reserved for SDS-PAGE. The remaining ~ 450 μL from each of the sediment samples were pooled and brought to a total volume of 15.0 mL with solubilizing buffer, and subject to sIEF (Section 2.8).

The supernatant obtained from these samples were desalted and concentrated by SPE using a C18 cartridge (Varian, Palo Alto, CA). Briefly, ~ 50 mL of the supernatant was loaded on a SPE column, washed with 20% ACN/water + 0.1% formic acid, and eluted with 250 μL of 30% ACN, followed by 250 μL of 50% ACN, each in 0.1% formic acid/water. All samples were then pooled, brought to a total volume of 8 mL with solubilizing buffer and subjected to sIEF (Section 2.8).

2.4 Effect of freezing on amount of sediment proteins

Immediately upon collection, four 1 mL urine samples, obtained from four donors, were centrifuged ($3716 \times g$, 6 min) to obtain the sediments present prior to freezing. The supernatant was removed and both the sediments and supernatants were frozen overnight at -20°C . Samples were thawed at room temperature and the sediments which accumulated in the solution fraction during the freeze-thaw cycle were again collected by centrifugation. Sediments obtained before and after freezing were individually solubilized in 60 μL of $1 \times$ gel solubilization buffer (Section 2.7), heated for 5 min at 95°C and 30 μL of each sample was subject to SDS-PAGE (Section 2.7).

2.5 Effect of vortexing on protein distribution between phases

Samples of urine, obtained from four donors (three male, one female), were thawed and aliquoted into 10 mL volumes, with gentle agitation to suspend the sediments between transfers. Samples were then either vortexed for 2 min at full power on a VWR mini vortexer (Equipland, Fremont, CA), or left undisturbed. The samples were then centrifuged at $3716 \times g$ for 5 min, the supernatant decanted, and the proteins in this phase were acetone precipitated at 4°C using a 3:1 ratio of acetone/urine. All pellets (supernatant and sediment) were then solubilized in 5 mL of PBS, and subjected to SDS-PAGE (Section 2.7).

2.6 Effect of pH on the partitioning of proteins between phases

Two urine samples (~50 mL/sample) from separate donors were thawed, vigorously vortexed for 5 min, pooled, and divided into ten equivalent 1 mL aliquots. Five of these aliquots were used as controls (no pH adjustment) to assess the reproducibility of the visualization protocol. For the remaining five samples, the pH was adjusted over the range 0.5–8.0 through addition of 500 μ L HCl or NaOH (prepared at different concentrations). All samples were agitated on a vortex mixer, allowed to equilibrate for 15 min, and then centrifuged to pellet the insoluble material. The solution phase was carefully removed from the precipitate, and the proteins in these solutions were precipitated with cold acetone as described previously. Along with the original sediment fraction, the acetone-pelleted proteins were suspended in 10 μ L of water and 20 μ L of $5 \times$ gel solubilization buffer (Section 2.7) for visualization by SDS-PAGE.

For quantitative analysis through iTRAQ, a urine sample obtained from a single donor was divided into 4×50 mL portions immediately following collection. The pH was adjusted to 8.8 for one sample, pH 3.9 for another, and unaltered at pH 5.9 for the remaining two, through the addition of 2 mL of 1 M HCl, 1 M NaOH, or water, respectively. Samples were aliquoted into 10 mL fractions and frozen at -20°C . One fraction of each sample type was allowed to thaw at room temperature, and centrifuged at $3716 \times g$ in order to collect the sediments. Sediments were suspended in 1 mL of 1 M urea with gentle heating at 30°C to encourage solubilization. Proteins were digested with trypsin as described in Section 2.8. Digestion was stopped with 100 μ L of 10% formic acid. Samples were then centrifuged to pellet any insoluble material, the soluble portion was evaporated to dryness, and subject to iTRAQ labeling, as described in Section 2.10.

2.7 Protein visualization by SDS-PAGE

If not already suspended in gel solubilization buffer, 40 μ L of a processed urine protein sample was combined

with 10 μ L of $5 \times$ gel solubilization buffer (0.5 M Tris-HCl, pH 6.8, 10% w/v SDS, 0.5% w/v bromophenol blue, 26% glycerol). Following 5 min heating at 95°C , 20 μ L were loaded onto individual lanes of a 15% acrylamide gel, cast according to standard protocols [14]. The proteins were separated by electrophoresis and visualized with silver staining [15].

2.8 Solution IEF and protein digestion

Solution IEF was performed using a custom designed eight-chamber solution IEF device. Details on the apparatus are provided elsewhere [16]. Prior to loading, 200 μ L of carrier ampholytes (pI 3–10) were added *per* 4 mL of sample. Four hundred microliters were loaded into each of the eight chambers, and the device was subject to 2 W constant power for 1.5 h to effect protein focusing. The eight resulting fractions were subject to protein precipitation with cold acetone (overnight, 4°C). These fractions were then centrifuged at $3716 \times g$ for 10 min to obtain the protein pellets. Pellets were dissolved in 100 μ L of water, adjusted to pH 8 with 1 M ammonium bicarbonate and digested with trypsin. Briefly, the proteins were reduced with 200 mM DTT, alkylated with 200 mM iodoacetamide, and digested at 37°C overnight at an approximate 10:1 ratio of protein/ trypsin.

2.9 Qualitative LC-MS/MS and data analysis

Digested proteins were separated with a 1200 HPLC system (Agilent Technologies, Mississauga, Canada) using a Bio-basic-C18 column (Thermo Scientific, San Jose, CA). Solvent B consisted of 0.1% formic acid in ACN, while solvent A was 0.1% formic acid in water. Following a 5 min initial hold at 2% B, the gradient consisted of a ramp from 2 to 40% B over 40 min, then to 80% B in 1 min. The injection volume was 1 μ L with a constant flow rate of 2 μ L/min. Nanospray MS was performed on an LTQ linear IT mass spectrometer (Thermo Scientific, San Jose, CA). Each fraction was analyzed twice (*i.e.*, 2×1 μ L injections with LC-MS/MS), the first run being performed with dynamic exclusion disabled, and in the second, with dynamic exclusion set to 1.5 min. Data were obtained in data-dependant mode (MS scan followed by MS/MS of top five ions). MS/MS data were searched using the Bioworks Browser software package (Thermo Scientific) against human Uniprot database (14 804 entries). Criteria for positive protein identification were as follows: for all peptides, ΔCn had to be at least 0.1 and R_{sp} had to be less than or equal to 4. For triply charged peptides, X_{corr} had to be at least 3.75, doubly charged at least 2.2, and singly charged at least 1.9. Peptides also had to have a peptide probability not greater than 0.05. Reversed database searches were performed on each of the runs using identical search criteria to obtain an assessment of false positive scores.

2.10 iTRAQ analysis of effect of pH on partitioning of proteins

The digested sediment proteins were first subject to RP column cleanup on a Betasil C18 Dash HTS column ($20 \times 2.1 \text{ mm}^2$, $5 \mu\text{m}$ particle size, Thermo Scientific). The fractions containing peptides were pooled, evaporated, and subject to iTRAQ labeling according to the manufacturer's instructions. Label 114 represented the acidified sample (pH 3.9), label 115 and 116 were the pH 5.9 samples, and 117 was the pH 8.8 sample. Following labeling, all samples were combined and again subject to RP cleanup as described above. Fractions were dissolved in $100 \mu\text{L}$ of strong cation exchange (SCX) mobile phase A (10 mM ammonium formate, 25% ACN, pH 3) and separated using a polysulfoethyl A $100 \times 2.1 \text{ mm}^2$ column with $5 \mu\text{m}$ particle size (PolyLC, Columbia, MD). The gradient was selected as follows: hold at 0% B for 5 min (600 mM ammonium formate, 25% ACN, pH 3), to 10% B at 20 min , 20% B at 30 min , 60% B at 40 min , hold to 45 min . The flow rate was 0.2 mL/min and fractions were collected in 2 min intervals from 0 – 45 min . SCX fractions 4–8 were selected for LC/MS/MS analysis, being the only fractions showing peaks in the UV chromatogram.

The SCX fractions were dried down and resuspended in $30 \mu\text{L}$ 5% ACN/ 0.2% formic acid and subjected to RPLC-MS/MS using a nanoAcquity UPLC system (Waters, Milford, MA) with a Q-TOF premier mass spectrometer (Waters) equipped with a nanospray source. The column used was a $1.7 \mu\text{m}$ BEH130 C18 $75 \mu\text{m} \times 100 \text{ mm}$ column (Waters, Milford, MA). The injection volume was $6 \mu\text{L}$ and the gradient was as follows: 5% B (ACN/ 0.1% formic acid) to 40% B over 30 min followed by a ramp to 95% B over 2 min . Solvent A was water/ 0.1% formic acid. MS parameters were set to scan from m/z 450 to 1600 for the MS scan followed by MS/MS of the top three ions at a mass range of 100 – 1500 . Data analysis was performed using the MASCOT search engine with Swiss-Prot-human database [17]. Peak lists were exported using ProteinLynx version 2.3 (Waters). Exported lists were then combined and searched using the MASCOT search engine. Search criteria was set using iTRAQ modifications for four labels, a peptide mass tolerance of 0.8 Da , fragment mass tolerance of 0.6 Da , and one missed cleavage. Quantitation of iTRAQ reporter ions was also performed using MASCOT. False positive rates were obtained by searching a reversed database using the exact same search criteria.

3 Results and discussion

3.1 Urinary sediments are variable and contain several proteins

With a high risk of proteolysis, urine is inevitably frozen upon collection to help preserve the sample. Although necessary, this manipulation step undesirably alters the native

state of the sample. Indeed, Fig. 1A demonstrates that a simple freeze–thaw cycle significantly increases the content of the sediment portion of urine (up to 50-fold increase by mass, depending on the sample). The sediment portion of urine collected before freezing, along with those which result from a freeze–thaw cycle were subject to SDS-PAGE for protein visualization. It can be seen from Fig. 1B that many proteins are present in the sediment phase of urine prior to freezing the sample. Perhaps more significantly, however, is that additional proteins are observed in the sediment portion of urine following a freeze–thaw cycle, despite having removed the sediments obtained immediately following collection of the sample. Such proteins represent those which are not intrinsic to the sediment portion of urine, but are rather induced by the freeze–thaw cycle. Moreover, it can be seen from the gel image that the sediment proteins vary greatly according to the individual, both before and after a freeze–thaw cycle. As we demonstrate here, the sediment portion of urine contains a significant number of urinary proteins, which are easily detected following proper solubilization.

Urinary sediments correspond to the solid portion of urine that can be isolated from the urine supernatant *via* low-speed centrifugation or through filtration. The sediments comprise numerous species including epithelial cells, salt crystals, leukocytes, and protein casts primarily composed of Tamm–Horsfall protein (uromodulin) and serum albumin [9, 13]. Figure 1C displays a sample photomicrograph ($25 \times$ magnification, crystal violet stained) of the urinary sediments isolated from frozen urine *via* low-speed centrifugation. As expected, the photomicrograph provides evidence of sloughed epithelial cells, calcium oxalate crystals, uric acid crystals, mucus, and many other clumps, which are speculated to contain protein. Compared to the suspected protein in the precipitate, epithelial cells contribute very little to the total mass of proteins in the sediment phase. This observation is consistent with Fig. 1A, as well as the newly precipitated proteins observed in the gel image of Fig. 1B. Since much of the precipitated material was obtained as a result of a freeze–thaw cycle, we attempted to resolubilize the material, using PBS at a volume equal to the original volume of the urine sample. The majority of the sediments dissolved, and upon microscopic analysis of the sediment that remained, essentially only salt crystals were observed (Fig. 1D).

Urinary sediments from four individual donors were subject to SDS-PAGE to provide a qualitative look at the protein content present in these samples. The sediments obtained following a freeze–thaw cycle from $\sim 50 \text{ mL}$ of urine varied from as little as 3 mg to as much as 120 mg . Consistent with Fig. 1B, the gel image shown in Fig. 2 shows extremely variable protein profiles from the urinary sediments of the four donors. Here, samples were normalized by volume, with the most concentrated protein lanes corresponding to the sample with a larger portion of sediments by mass. It is apparent that the differences in these gel lanes

Figure 1. (A) Photograph showing human urine before being frozen at -20°C (left tube) and after being frozen and thawed (right tube). (B) 1-D gel image shows sediment proteins from four donors (I–IV) before *versus* after a freeze–thaw cycle. Lanes 1, 3, 5, and 7 are sediments obtained before freezing, while lanes 2, 4, 6, and 8 are sediments exclusively induced from the freeze–thaw cycle. (C) Also shown is a photomicrograph at $25\times$ magnification of the sediments isolated from human urine prior to solubilization and (D) after solubilization in PBS.

cannot be accounted for by simple variations in sample loading. Individual protein bands have significant variability in intensity, indicating that the sediment portion of urine, much like its soluble counterpart, varies according to the donor. We have also observed considerable variation in the protein content of urinary sediment obtained from the same individual donor, collected at different times (results not shown). Although the gel image of Fig. 2 provides only a qualitative view of the urinary sediments proteome, it firmly establishes that the sediment portion of urine is highly variable. This suggests that discarding it will bias the urinary proteome prior to analysis.

3.2 Analysis of the sediment proteome has implications for biomarker discovery

MS analysis was conducted on urinary proteins recovered from the sediment and supernatant of a urine sample, pooled from four donors. Table 1 provides a comprehensive listing of all proteins identified in the sediment portion of the pooled urine sample. Further details on the number of peptides identified and associated probability scores are provided as Table S1 of Supporting Information. A similar MS investigation was conducted using the supernatant portion of urine (sediments discarded), and the complete results are also provided as Table S2 of Supporting Information and summarized in Fig. 3. Briefly, 60 individual proteins were identified from urine sediments, of which 44 of these were common to the supernatant. As a point of reference, we

Figure 2. 1-D gel image showing sediment proteins isolated from a constant volume of urine from four healthy donors (I–IV).

identified 160 proteins from the urinary supernatant. Reversed database searches revealed a false positive rate of 5% for peptides of the sediments and 9% for peptides of the supernatant.

The fact that a large percentage of sediment proteins (73%) were also observed in the supernatant phase is not surprising. As we have shown, the freeze–thaw cycle causes

Table 1. Proteins identified by LC-MS/MS from the sediment phase of a pooled urine sample, with references indicating potential bio-marker significance of the proteins

Access. no.	Protein name	Num. peptide	Unique to sediment?	Tissue specificity	Biomarker significance	Refs.
P02763	α -1 Acid glycoprotein precursor	3	No	–	Metastatic melanoma, Hodgkin's disease	[18, 19]
P02768	Serum albumin	9	No	–	Hodgkin's disease, idiopathic normal pressure hydrocephalus	[19, 27]
P04264	Keratin type 2 cytoskeletal	31	No	–	–	–
P01042	Kininogen	4	No	–	Hodgkin's disease	[19, 22]
P13645	Keratin type 1 cytoskeletal	14	No	–	–	–
P35908	Keratin type 2 cytoskeletal	13	No	–	–	–
P10451	Oteopontin	1	No	–	Focal segmental glomerulosclerosis, inflammatory liver disease, mesothelioma	[28–30]
P19652	α -1-Acid glycoprotein	2	No	–	Neuroblastoma, spontaneous preterm birth	[31, 32]
P41222	Prostaglandin-H ₂ -D isomerase	4	No	–	Proliferative diabetic retinopathy	[20]
P04259	Keratin type 2 cytoskeletal	5	Yes	Epithelia	–	–
P07911	Uromodulin	25	No	–	Fanconi syndrome	[33]
P02533	Keratin type 1 cytoskeletal	4	no	–	–	–
P02533	Keratin type 1 cytoskeletal	1	No	–	–	–
P13647	Keratin type 2 cytoskeletal	6	No	–	–	–
P02760	α -1 -Microglobulin	3	No	–	Hodgkin's, diabetic nephropathy, IgA nephropathy, ossification of posterior longitudinal ligament, renal damage, urinary tract infections, idiopathic normal pressure hydrocephalus	[19, 27, 34–38]
P13646	Keratin type 1 cytoskeletal 13	5	No	–	–	–
P28799	Granulins	1	No	–	–	–
P08779	Keratin type 1 cytoskeletal	7	Yes	Squamous epithelia	–	–
P01857	Immunoglobulin γ 1 chain	1	No	–	–	–
O75594	Peptidoglycan recognition protein	1	No	–	–	–
P05109	Protein S100-A8	1	Yes	Urinary stone protein	–	–
P13987	CD59 glycoprotein	1	No	–	–	–
P05090	Apolipoprotein D	1	No	–	Idiopathic normal pressure hydrocephalus	[27]
P01834	Immunoglobulin κ chain	1	No	–	–	–
P06870	Kallikrein-1	1	No	–	–	–
P07477	Trypsin-1 -precursor	2	No	–	–	–
P62380	TATA box binding protein	1	Yes	Ubiquitous	–	–
Q14624	Inter- α trypsin inhibitor	1	No	–	Hodgkin's disease, pancreatic cancer	[19, 39]
P01842	Immunoglobulin λ chain	1	No	–	Autoimmune disease	[40]
Q9GZM5	Protein YIPF3	1	No	–	–	–
Q9UI46	Dynein intermediate chain 1	1	Yes	Testis/sperm	–	–
Q9BZK7	F box like/WVD repeat containing protein	1	Yes	Ubiquitous	–	–
O15297	Protein phosphatase 1-D	1	Yes	Skin	–	–
P11137	Microtubule associated protein	1	Yes	Epithelium	–	–
Q53H12	Acylglycerol kinase	1	No	–	Prostate cancer	[41]
P51512	Matrix metalloproteinase	1	No	–	Type 1 diabetes	[42]
Q5XUX0	F-box only protein	1	Yes	Testis	–	–
P01833	Polymeric immunoglobulin receptor	1	No	–	–	–
P25311	Zinc- α -2 glycoprotein	4	No	–	Liver fibrosis, bladder cancer, prostate cancer	[21, 23, 43]
Q14508	WAP four disulfide core domain protein	2	No	–	–	–
P10153	Nonsecretory ribonuclease	1	No	–	–	–
Q7Z5L0	Vitellin membrane outer layer protein	1	No	–	–	–
Q43653	Prostate stem cell antigen	1	No	–	Urogenital cancer	[44]
Q8WZ75	Roundabout homolog	1	No	–	–	–
P55290	Cadherin 13	2	No	–	–	–
Q12907	Vesicular integral membrane protein	1	No	–	–	–

Table 1. Continued

Access. no.	Protein name	Num. peptide	Unique to sediment?	Tissue specificity	Biomarker significance	Refs.
P02765	α -2-HS-glycoprotein	1	No	–	Proliferative diabetic retinopathy	[20]
Q8IWU5	Extracellular sulfatase	2	No	–	–	–
P16070	CD44 antigen	2	No	–	Gynecologic malignancies	[45]
P07602	Proactivator polypeptide	1	No	–	–	–
P05451	Lithostathine 1- α	1	No	–	Transposed intestinal segments	[46]
O95998	Interleukin-18 binding protein	2	Yes	Leukocyte	HIV-1	[47]
Q6ISS4	Leukocyte-associated immunoglobulin	1	Yes	Leukocyte associated	–	–
Q96GW7	Brevican core protein	1	Yes	Brain	–	–
P61221	ATP binding cassette sub familiy	1	Yes	Duodenum/uterus	–	–
Q14184	Double C2-like domain containing protein	1	Yes	High levels in kidney	–	–
Q8IWG1	WD repeat containing protein	1	Yes	Testis/brain	–	–
P67809	Nuclease sensitive element binding protein	1	Yes	High levels in kidney	–	–
Q9BZJ0	Crooked neck-like protein	1	No	–	–	–
P35527	Keratin type 1 cytoskeletal	10	No	–	–	–

Figure 3. A Venn diagram summarizing the MS results of proteins identified in the supernatant and sediments of a pooled sample from four healthy donors. The diagram also illustrates the proteins that were common to both phases of the sample.

proteins to partition between the solution and sediment phases. Some proteins undoubtedly precipitate to a larger extent than others. The proteins which were exclusively observed in the sediment portion of the sample do not provide direct evidence that they completely partitioned to this phase, but may be taken as an indication that their relative concentration is perhaps highest in this phase. This illustrates the importance of analyzing both portions of the sample for a more comprehensive investigation of the urinary proteome as well as for reliable biomarker discovery and validation.

Upon closer examination of the unique sediment proteins (Table 1), it can be seen that several of these originate from squamous epithelia, leukocytes, or aggregate on urinary stones. This is consistent with the type of proteins one would

expect to find in the sediments. In addition, the larger portion of keratins in the sediment phase is consistent with sloughed tissues. Given the type of proteins found in this sample, sediment proteins should be considered part of the urinary proteome. These proteins may prove important for the survey of various disorders involving the entire body, or in particular, the urinary tract, which could ultimately be used as an additional source of candidate protein biomarkers.

Many of the proteins identified in the sediments have been reported in the literature as potential biomarkers for various physiological disorders. For example, α -1-acid glycoprotein, α -1-microglobulin, prostaglandin-H₂-D-isomerase, inter- α trypsin inhibitor, kinninogen and zinc- α -2-glycoprotein were all identified in the sediments, and have been reported in the literature as biomarkers for various diseases [18–23]. These diseases range from liver fibrosis, bladder cancer, prostate cancer, and Hodgkin's disease to urinary tract infections and more. A detailed list of the potential biomarker capabilities of the sediment proteins identified is listed in Table 1.

Urinary sediments contain potential biomarkers and, therefore, should be combined with the supernatant or analyzed in parallel with the supernatant. This is particularly important when doing comparative biomarker studies since one sample may have a larger portion of sediments than another, resulting in apparent differences in protein concentration that result from variations in the degree of precipitation induced by a freeze–thaw cycle rather than a pathogenic process. It is realized however, that the consequence of discarding the sediments may vary depending on the analytical protocol. For example, using the ligand library bead method, as described by Castagna *et al.* [24], less

emphasis is placed on protein concentration, and thus discarding the sediments may not significantly alter the resulting protein profile. However, discarding the sediments while using this method may still impose consequences with respect to unique sediment proteins since these would ultimately be discarded.

3.3 Vortexing a urine sample prior to analysis resolubilizes some sediment proteins

In some instances of the proteome sample workup, it may be impractical to analyze the urinary sediments. In such a case, it is critical to minimize any sample bias induced by discarding the sediment portion of urine. Figure 4 displays a silver stained 1-D gel image of proteins from the urine of four different healthy donors (denoted as I–IV) isolated from the (A) sediment and (B) supernatant phases when the sample was either vortexed or not vortexed prior to separation of the two phases. As would be expected, the vortexed samples from each donor show a decrease in protein intensity from the sediment portion and a corresponding increase in intensity from the supernatant. This implies that some of the sediment proteins were solubilized upon vortexing. The extent of sample agitation imposed as urine is initially processed upon thawing is difficult to control. Here we see that simple agitation of the sample is sufficient to redissolve a portion of the precipitate. It is therefore recommended that as part of all proteome analysis protocols, the urine samples be vigorously vortexed for at least 2 min prior to separating the sediment portion of the sample. This simple step affords a greater opportunity of minimizing protein loss due to discarding the sediments.

3.4 The pH of a sample influences the protein profile of urinary sediments

The relative solubility of urinary proteins is dependant on several variables, such as protein concentration, salt content, temperature, and solution pH [25] and these conditions vary from one physiological state to the next. The pH in particular

could have a drastic effect on the partitioning of proteins in urine, since protein solubility is greatly reduced as sample pH approaches the *pI* of the protein. It has been shown that the pH of urine in a healthy individual varies over time from 4.4 to 8 [26], and this variability increases when considering an unhealthy individual.

Upon examining the partitioning of urinary proteins between supernatant and sediment phases by 1-DE, (Fig. 5), it is clear that changing the pH of the solution will greatly affect the solubility of certain proteins in the sample. The control lanes (A) serve as illustration that our sample analysis protocol is highly reproducible when considering identical samples of both the soluble and precipitated portions. However, clear differences are observed in the gels as the pH of the urine is adjusted from its original value of 6.5 (B). The distribution of certain proteins between the sediment and supernatant of urine is significantly altered. Again, since the sediment portion is typically discarded during sample preparation, it is critical that such a practice results in discarding an equivalent portion of the proteome from all samples being compared. Thus, when sampling only a portion of the urine (*i.e.*, the supernatant) it is critical that all variables which may influence the partitioning of proteins to the sediment is controlled, including sample pH and degree of agitation.

In addition to the qualitative visualization of the effect of pH, iTRAQ labeling was conducted in order to quantify some of the differences in protein distribution which result from altering the pH of the sample. These results are summarized in Table 2. Reversed searches revealed a false positive identification rate of 4% and additional information for the peptides identified from each protein can be seen in Table S3 of Supporting Information. As expected, differences in protein concentrations in the sediments were observed at different pH values. As would be predicted, the proteins detected in the samples at identical pH values (5.9) all had ratios close to 1. This was not the case for the samples whose pH was modified. In particular, the acidic sample revealed that clusterin precursor protein was downregulated by a factor of almost three compared to the control, while kallekrein-

Figure 4. 1-D gels of (A) sediment and (B) supernatant proteins from four healthy donors (I–IV), isolated when the samples were not vortexed or were vortexed.

Figure 5. Supernatant (left) and sediment proteins (right) from a pooled sample in which the pH has been adjusted to the values indicated (B). Controls (A), having no pH adjustment, show excellent reproducibility thus differences in gel lanes represent changes induced by manipulation of pH.

Table 2. Proteins from the sediment phase of a urine sample, adjusted to different pH values, and quantified by MS/MS with iTRAQ labeling

Protein name	Expression ratio of sediment proteins from urine		
	Control 2/control 1	Acid/control 1	Base/control 1
Prostaglandin H ₂ -D isomerase precursor	0.73	0.71	0.68
α-1-Microglobulin	0.92	0.60	0.87
Serum albumin precursor	0.96	0.88	0.65
Deoxyribonuclease-1-precursor	0.96	0.58	0.58
Clusterin precursor	1.03	0.27	1.40
Pro-epidermal growth factor precursor	1.03	0.85	1.11
Kallikrein-1-precursor	1.31	6.29	1.16
Uromodulin	1.34	1.37	1.19

Control 1: pH 5.9; Control 2: pH 5.9; Acid: pH 3.9; Base: pH 8.8.

1-precursor protein was upregulated by over a factor of six compared to the control. These variations in protein concentration are observed despite the fact that they originate from a single urine sample. It is noted that the pH ranges employed are within normal biological variation for urine sample; the pH of urine from an unhealthy donor may have even larger variation. These results are consistent with the gel image in Fig. 5 and serve to demonstrate that a change in sample pH can have a significant influence on the partitioning of proteins between sediments and the soluble portion of urine. It is thus critical in a biomarker discovery assay to ensure that all variations between samples be minimized.

4 Concluding remarks

Human urinary sediments contain a significant number of proteins whose relative distribution between sediment and supernatant is highly variable from sample to sample, and is influenced within a sample by the degree of agitation (vortexing/nonvortexing), and pH. No attempts were made to profile and compare proteomes extracted from different individuals, as it is not the objective of this work to explore this variability. Here, we establish that the sediment portion of urine is rich in protein, and discuss its implications. It is likely that other variables (concentration, salt content, temperature) will also change the relative distribution of pro-

teins, and further work is presently being conducted to quantify these effects and will be reported in the near future. From the present work, it is clear that variations in a sample, as well as minor differences in the analytical protocol will affect the detection or quantification of proteins in urine. In order to achieve an unbiased analysis, urinary sediments should be analyzed along with the supernatant of the sample. As an alternate strategy, where it is desired that the urinary sediments be discarded, we suggest that the sample pH is normalized and that urine be thoroughly vortexed prior to centrifugation or filtration, in order to resolubilize the largest portion of proteins possible from the sediment phase. This step will ensure that any sample bias introduced by discarding the sediment portion of urine is, at best, minimized.

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