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Profiling of urinary proteins in *Karan Fries* cows reveals more than 1550 proteins☆

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ABSTRACT

Urine is a non-invasive source of biological fluid, which reflects the physiological status of the mammals. We have profiled the cow urinary proteome and analyzed its functional significance. The urine collected from three healthy cows was concentrated by diafiltration (DF) followed by protein extraction using three methods, namely methanol, acetone, and ammonium sulphate (AS) precipitation and Proteo Spin urine concentration kit (PS). The quality of the protein was assessed by two-dimensional gel electrophoresis (2DE). In-gel digestion method revealed more proteins (1191) in comparison to in-solution digestion method (541). Collectively, 938, 606 and 444 proteins were identified in LC-MS/MS after in-gel and in-solution tryptic digestion of proteins prepared by AS, PS and DF methods, respectively resulting in identification of a total of 1564 proteins. Gene ontology (GO) using Panther7.0 grouped the majority of the proteins into cytoplasmic (location), catalytic activity (function), and metabolism (biological processes), while Cytoscape grouped proteins into complement and coagulation cascades; protease inhibitor activity and wound healing. Functional significance of few selected proteins seems to play important role in their physiology. Comparative analysis with human urine revealed 315 overlapping proteins. This study reports for the first time evidence of more than 1550 proteins in urine of healthy cow donors. This article is part of a Special Issue entitled: Proteomics in India.

1. Introduction

Urine contains numerous biomolecules including proteins, peptides and metabolites, which are the byproducts of the physiological events taking place in the organism. Urine is formed via glomerular filtration of plasma in the kidneys, which act as a filter to retain most of the plasma proteins [1]. However, many low molecular weight proteins and peptides pass through the glomerular membrane, are catabolized in the proximal tubule and then secreted in the urine. In addition, abundant serum proteins such as albumin, immunoglobulin light chain, transferrin, myoglobin, and receptor-associated protein, after passing through the glomeruli, are reabsorbed by endocytic receptors in the proximal renal tubules [2–6]. Overall, the protein concentration in normal urine is usually very low [7]. Moreover, there is interest in using urine for diagnostic applications due to its simple and non-invasive collection compared to other biological fluids. In particular, many systemic diseases may cause a change in the composition of the urinary constituents, which might be useful for diagnosis and disease monitoring [7]. In humans, biomarkers in urine have been used for the diagnosis of kidney and urinary tract diseases [8]. However, very few studies have been reported on the bovine urinary proteome as a potential source for biomarker discovery in disease and pregnancy [9–11].

The human urinary proteome has been extensively studied by a variety of methods including two-dimensional gel electrophoresis (2DE) followed by its characterization using matrix assisted laser desorption ionization (MALDI) and liquid chromatography coupled tandem mass spectrometry (LC-MS/MS) [12]. In one study, Sun and colleagues [13] identified 226 unique human proteins in urine using one-dimensional gel electrophoresis (1D-GE) and multidimensional LC-MS/MS. However, to date, no information is available on the bovine urinary proteome. The aim of the present study was to profile the urinary proteome of *Karan Fries* cows, a crossbred cattle of Holstein Friesian male (*Bos taurus*) × Tharparker (*Bos indicus*) female. The *Karan Fries* breed has adapted well to tropical climate and plays important role in milk

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production in India [14,15]. In this proteomic study, a combination of extraction procedures was used to isolate urinary proteins from *Karan Fries* cows and characterized by LC-MS/MS leading to the identification of a total of 1564 proteins. This is the first report of urinary proteome of cow and will provide a reference database for future studies in biomarker discovery linked to diseases and associated physiological events.

2. Materials and methods

2.1. Animal selection and sampling

Karan Fries (KF) heifers from the dairy herd of National Dairy Research Institute, Karnal, India were maintained under expert veterinary supervision. For this study, urine was collected from three healthy KF heifers after screening for the absence of pus cells under microscope to rule out any infection. One liter each of urine sample was then collected aseptically in urine bags from three healthy cows. Immediately after urine collection, phenylmethylsulfonyl fluoride (PMSF, 0.01%) was added to prevent proteolytic degradation.

2.2. Sample preparation

The freshly collected pooled urine was then centrifuged at 6000 rpm for 30 min to remove insoluble materials. Subsequently, the urine was concentrated by centrifugation using 3 kDa hollow fiber membrane cartridge in Marlow Benchtop System (GE Healthcare, USA). Next, the urine was diafiltered in PBS, pH 7.4 (133 mM NaCl, 2.7 mM NaCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4) [7,16]. By this approach, one liter of urine was concentrated to 100 ml and divided into 2 aliquots of 50 ml each to which additional protease inhibitor cocktail (Sigma, USA) was added to prevent proteolysis. Subsequently, samples were stored at −80 °C until further use.

2.3. Precipitation of primary proteins

The concentrated protein samples as described above were extracted from 50 ml of diafiltered urine with four different protein extraction methods namely; methanol (Merck, Germany) precipitation, acetone (Merck, Germany) precipitation, ammonium sulphate (AS) (Sigma, USA) precipitation and Proteo Spin urine protein concentration (Norgen Biotek, Canada). Details of the precipitation protocols are described below.

2.4. Methanol precipitation

Methanol was used to precipitate protein from urine as reported earlier [17]. Briefly, 10 ml diafiltered urine and methanol were mixed in a ratio of 1:9 (v/v) and incubated at −20 °C for 14–16 h followed by precipitating protein by centrifuged at 14,000 ×*g* at 4 °C for 30 min. The supernatant was discarded and the pellet was washed twice with methanol by centrifuging it at 14,000 ×*g* for 10 min. The pellet was dried at room temperature to remove residual methanol and re-suspended in PBS (pH 7.4, 133 mM NaCl, 2.7 mM NaCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4). Subsequently, the sample was dried in vacuum and stored at −80 °C.

2.5. Acetone precipitation

Ten milliliters of diafiltered urine and chilled acetone (−20 °C) was mixed in a ratio of (1:1 v/v) and centrifuged at 12,000 ×*g* for 30 min. Supernatant was discarded and the pellet was dried and dissolved in solubilizing buffer (7 M Urea, 2 M Thiourea, 4% CHAPS, 30 mM Tris HCL, pH 8.8) and stored at −80 °C until further analysis [1].

2.6. Ammonium sulphate precipitation (AS)

Ammonium sulphate (AS) was used to precipitate proteins as described by Sudha et al. [18]. In brief, AS was added to 50 ml of diafiltered urine to obtain 90% saturation. The mixture was stirred at 4 °C for 90 min until complete dissolution of AS and centrifuged at 12,000 ×*g* for 10 min in Oak Ridge tube at 4 °C. The pellet was re-suspended in PBS and dialyzed against the same buffer for 16–18 h. Subsequently, the sample was centrifuged and stored at −80 °C until further analysis.

2.7. Protein concentration using Proteo Spin™ Urine Maxi Kit (PS)

Protein precipitation from urine by Proteo Spin™ Maxi Kit (Norgen Bioteck, USA) was performed as per the manufacturer's instructions. Briefly, the pH of the urine sample was adjusted to 3.5 by adding binding buffer. The Proteo Spin column was activated by adding 5 ml of the column activation and wash buffer and centrifuged for 3 min at 1000 ×*g*. The flow through was discarded and the same step was repeated twice. 20 ml of the pH adjusted urine was loaded onto the column and centrifuged for 5 min at 1000 ×*g*. The column was again washed by applying column activation and wash buffer and centrifuged for 3 min at 1000 ×*g*. Protein was eluted with elution buffer (10 mM Na_2HPO_4, pH 12.5) in a fresh collection tube containing the neutralizer. The eluted proteins were concentrated and preserved at −80 °C until further use.

2.8. Clean up and protein estimation

Interfering substances such as detergents, salts, lipids and nucleic acids were removed from the precipitated urinary protein preparations using 2D-Clean Up kit (GE Healthcare, USA). The pellet was rehydrated in the same 2D-DIGE lysis buffer and total protein concentration was estimated using 2D-quant kit (GE Healthcare, USA) as per the manufacturer's instruction.

3. Bottom up approach

3.1. Two dimensional gel electrophoresis (2DE)

2DE was carried out as per the published protocols [12,19]. Briefly, 320 μg protein sample was dissolved in 125 μl of rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris) and rehydrated overnight on IPG strips (7 cm; pH range: 4–7) along with ampholytes. Isoelectric focusing (IEF) was performed in Ettan IPGphor III (GE Health, USA) at 150 V for 70 min, 1000 V for 27 min, 5000 V for 90 min, and 5000 V for 24 min. The IPG strips were equilibrate with 1% w/v DTT in 2.5 ml of equilibration buffer (6 M urea, 50 mm Tris–HCl, pH 8.8, 30% w/v glycerol and 2% w/v SDS) to reduce disulfide bonds followed by 2.5% w/v iodoacetamide in the same buffer to alkylate cysteine residues. The strips were then loaded on top of 12% separating gel and electrophoresis was carried out in standardized conditions of temperature and voltage. For visualization, gels were stained with Coomassie brilliant blue (R-350) stain.

3.2. One dimensional gel electrophoresis (1D-GE) and in-gel tryptic digestion

Ten micrograms of protein (pooled from 3 animals) from DF, AS and PS methods, respectively was subjected to 12% SDS-PAGE (10 × 10.5 cm) in a Mini VE complete gel electrophoresis system (GE Healthcare, USA). The gel was stained with colloidal Coomassie brilliant blue (R-350) followed by destaining. Subsequently, each lane of the gel was cut into 6 equal pieces, further destained using 40% ACN and 40 mM NH_4HCO_3 at a ratio of 1:1 (v/v) and in-gel digestion of protein bands was performed as reported previously [20,21]. In brief, destained bands were reduced with 5 mM dithiothreitol (DTT) in 40 mM NH_4HCO_3 followed by alkylation with 20 mM iodoacetamide in 40 mM NH_4HCO_3. Digestion was carried out overnight using 12.5 ng/μl trypsin (modified sequencing grade; Promega, USA) at 37 °C. Subsequently, peptides were extracted from the gel, lyophilized and desalted using zip-tip (Millipore, Germany) following manufacturer's instruction and stored at −80 °C until MS analysis.