

IPD Project Details

Project ID: IPD2682

Project Title: Profiling of cow urinary proteins using various extraction methods reveals more than 1550 proteins

Description: Urine can help in diagnosis of different diseases including cancer and other patho-physiological conditions. Urine proteome studies have been mainly human-centric. No information is available on urinary proteome from bovine till date. In the present study, we have used 3 protein extraction methods such as ammonium sulphate precipitation, ProteoSpin column and diafiltration method from bovine urine for identification of urinary proteome. The tryptic peptides generated after In-gel and In-solution method were identified using LC/MS/MS (ESI-qTOF) which resulted in identification of 1582 proteins. In-gel trypsin digestion method revealed more protein (1191) in comparison to in-solution digestion method (541). Maximum proteins were identified in ammonium sulphate precipitation method (938) followed by ProteoSpin (606) and diafiltration (444) methods respectively. The profile of the identified proteins were compared with human urinary proteome of which 311 bovine urinary proteins matched with human. An exclusive list of 38 bovine urinary proteins with high protein scores were listed which are absent in human urine. All identified proteins were analyzed according to Gene Ontology which were classified according to cellular component, biological processes and molecular functions. This study reports for the first time an exclusive evidence of more than 1550 proteins in urine of healthy cow donors.

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Sample Preparation: Freshly collected urine samples were centrifuged at 6000 rpm for 30 minute to remove insoluble materials. After centrifugation urine samples were concentrated using 3 kDa hollow fiber membrane cartridge in Marlow Benchtop System (GE Healthcare, USA) and dia-filtered in PBS pH 7.4 (133 Mm NaCl, 2.7 mM NaCl, 10mM Na₂HPO₄, 2mM KH₂PO₄) [7, 14]. One liter of urine was concentrated to 100 mL and divided into 2 aliquots of 50 ml each. Protease cocktail (Sigma, USA) was added to prevent proteolysis. Subsequently, samples were stored at -80oC until further analysis. Precipitation of urinary proteins The concentrated protein samples as described above samples were precipitated with four different protein precipitation agents viz., methanol

(Merck, Germany), acetone (Merck, Germany), ammonium sulphate (Sigma, USA) and ProteoSpin urine protein concentration kit (NorgenBiotek, Canada). Details of the precipitation protocols. Clean up and protein estimation The extracted proteins prepared by different procedures was precipitated from all the fractions using 2D-Clean Up kit (GE Healthcare, USA) to remove interfering substances like detergents, salts, lipids and nucleic acid. The pellet was rehydrated in the same 2D-DIGE lysis buffer and the total protein concentrations were estimated using 2D-quant kit (GE Healthcare, USA) as per the manufacturer's instructions. Bottom Up approach Two Dimensional Gel Electrophoresis (2D-GE) 2D-GE was carried out by following the published protocols [12 &17]. Briefly, 320 µg protein sample was dissolved in 125 µl of rehydration buffer (7M Urea, 2M Thiourea, 4% CHAPS, 30 mM Tris) using IPG strips (7cm) in the pH range 4-7. IEF was performed at standardized conditions of 150V for 70 min., 1000V for 27 min, 5000V for 90 min., 5000 V for 24 min. The IPG Strips were subjected to equilibration with 1% w/v DTT in 2.5 ml of equilibration buffer (6 M urea, 50mM Tris –HCl, pH 8.8 30% w/v glycerol and 2% w/v SDS) to reduce disulfide bonds followed by 2.5 % w/v IAA in same buffer to alkylate cysteine residues. The strips were then loaded on top of 12% separating gel and electrophoresis was carried out at standardized conditions of temperature and voltage. For visualization, gels were stained with Coomassie brilliant blue (R-350) stain.

Peptide Separation: One dimensional gel electrophoresis (1D-GE) and In-gel trypsin digestion Equal amount of proteins for each extraction method were pooled from each animals separately. 10 µg protein of pooled samples from different extraction methods (Ammonium precipitation, ProteoSpin column, diafiltered) were resolved individually by 12% SDS-PAGE (10x10.5 cm.) using the gel electrophoresis system (GE Healthcare, USA). The gel was stained with colloidal Coomassie Brilliant blue (R-350) followed by destaining. Each lane of gel was cut into 6 equal parts of approximately 1mm² pieces which were further destained using 40% ACN and 40 mM ammonium bicarbonate at a ratio of 1:1 (v/v). In- gel digestion of protein bands was carried following the earlier reported protocol [16]. In brief, the destained bands were reduced with 5mM dithiothreitol (DTT) in 40mM ammonium bicarbonate followed by alkylation with 20 mM iodoacetamide in 40 mM ammonium bicarbonate. Digestion was carried out using 12.5 ng/µl trypsin (modified sequencing grade; Promega, USA) at 37°C for overnight. The peptides were extracted from the gel, lyophilized and desalted using ziptip (Millipore, Germany) following manufacturer's instruction and stored at -80°C until MS analysis. In-solution Digestion of urine proteins: For in-solution digestion, 10 µg of protein individually extracted from ammonium acetate precipitation, Proteospin column and diafiltered fraction was processed separately. In-solution digestion method was performed following the reported protocol with slight modification [7]. In brief, 45mM DTT in 50mM ammonium bicarbonate was used to reduce disulfide bonds followed by alkylation of cysteine residues using 10 mM Idoacetamide in 50mM ammonium bicarbonate. Digestion were carried out using trypsin 1:20 at 37°C for overnight. The reaction was then stopped with 10% TFA. The peptides were vacuum dried, desalted by zip tip and stored at -80°C.

Protein Characterization: Data Analysis Peak lists were generated by Otof control (version 24.8) using the Hystar post processing program to automatically baseline subtract, smooth and centroid the data before submission to Proteinscape software (Bruker, Germany) which uses the database search program Mascot (2.4.1 Matrix Science, UK) with proteins identified by correlation of mass spectra to entries in the NCBI database (January 2015). Mascot MS/MS ion search criteria were as follows: taxonomy—other mammalia, trypsin digestion allowing up to one missed cleavage, variable modification—oxidation of methionine, fixed modification, cysteine as carboxyamidomethylation or propionamide, peptide tolerance of 50 ppm, and MS/MS tolerance of 0.05 Da. The “ion score cutoff” was manually set to 15 thereby eliminating the lowest quality matches. To eliminate false positives, 1% FDR was applied at both protein and peptide level.

Experiment Type: Shotgun proteomics, Gel-based experiment

Species: Data in species_details No Data

Tissue: Data in tissue_details No Data

Cell Type: Data in cell_details No Data

Disease: Unknown No Data

Instrument Details: Data in instrument_details Data in instrument_details

Protein Modifications: monohydroxylated residue, iodoacetamide derivatized residue

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