Analytical Biochemistry 515 (2016) 9–13

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

Use of focused ultrasonication in activity-based profiling of deubiquitinating enzymes in tissue

Bindu Nanduri [a], Leslie A. Shack [a], Aswathy N. Rai [a], William B. Epperson [b], Wes Baumgartner [b], Ty B. Schmidt [c], Mariola J. Edelmann [d, *]

[a] College of Veterinary Medicine, Department of Basic Sciences, Mississippi State University, MS, USA
[b] College of Veterinary Medicine, Department of Pathobiology and Population Medicine, Mississippi State University, MS, USA
[c] Animal Science Department, Institute of Agriculture and Natural Resources, University of Nebraska - Lincoln, USA
[d] Department of Microbiology and Cell Science, University of Florida, Gainesville, FL, USA

ARTICLE INFO

Article history:
Received 2 June 2016
Received in revised form
31 August 2016
Accepted 19 September 2016
Available online 20 September 2016

Keywords:
Deubiquitinases
Focused-ultrasonication
Activity-based profiling
Chemical proteomics
Tissue lysis

ABSTRACT

To develop a reproducible tissue lysis method that retains enzyme function for activity-based protein profiling, we compared four different methods to obtain protein extracts from bovine lung tissue: focused ultrasonication, standard sonication, mortar & pestle method, and homogenization combined with standard sonication. Focused ultrasonication and mortar & pestle methods were sufficiently effective for activity-based profiling of deubiquitinases in tissue, and focused ultrasonication also had the fastest processing time. We used focused-ultrasonicator for subsequent activity-based proteomic analysis of deubiquitinases to test the compatibility of this method in sample preparation for activity-based chemical proteomics.

1. Introduction

Ubiquitination is a post-translational modification (PTM) critical for regulating the function, activity and stability of a vast majority of proteins [1–3]. It is removed from proteins by deubiquitinases (deubiquitinating enzymes, DUBs) [4–6]. As such, DUBs are critical in determining the turnover rate, activation, and localization of proteins, and are implicated in several diseases [7]. Unlike conventional proteomics that analyzes changes in protein abundance, activity-based protein profiling (ABPP) permits identification of enzyme function using activity-based probes [8]. ABPP could be used to systematically identify and quantify deubiquitinases in animal tissues. To date over 98 DUBs [7] are known in humans, but in most agriculturally relevant animals, such as cattle, majority of DUBs remain either unidentified, or their activity has not yet been shown. UCH37 and USP14 are the only bovine DUBs, which were characterized at a protein level from isolated bovine red blood cell 26S proteasome, and shown to possess catalytic activity [9]. UCH-L1 and UCH-L3 are present in bovine oocytes, but their DUB activity has not been demonstrated [10,11]. In this report, we used chemical proteomics for identification of novel DUBs in bovine tissue based on their reactivity with a ubiquitin-based active site directed probe.

2. Materials and methods

2.1. Animal procedures

Calves were transported to and raised conventionally in individual hutches as established in IACUC (Institutional Animal Care and Use Committee) project 10–024 (Calf Rearing Experience). Holstein bull calves were procured at ~7 days of age, raised on site and were found negative for persistent viral infections. Calves underwent physical examination daily (temperature, pulse, respiration, navel size, fecal score, attitude, and feeding behavior) and received routine viral respiratory vaccination (but were not vaccinated for *Mannheimia haemolytica*). Animals were approximately 6

Abbreviations used: ABPP, activity-based protein profiling; BRD, bovine respiratory disease; DUBs, deubiquitinating enzymes; PTM, post-translational modification; Ub, ubiquitin; Ub-VS, ubiquitin vinyl sulfone.

* Corresponding author. Department of Microbiology and Cell Science, University of Florida, Bldg 981, 1355 Museum Rd., Gainesville, FL 32611, USA.
E-mail address: medelmann@ufl.edu (M.J. Edelmann).

http://dx.doi.org/10.1016/j.ab.2016.09.016
0003-2697/

months old when challenged.

2.2. M. haemolytica challenge

Six animals (three challenged and three sham) were used in this study. Six-month old dairy breed calves were either challenged with *Mannheimia haemolytica* serotype A1 (NADC D153) or sterile saline solution (sham). Challenge dose of 4×10^{10} organisms was administered by intra-tracheal instillation. Clinical observations including depression, nasal/ocular discharge, respiratory rate and intensity etc. were recorded three times a day for seven days. Based on these observations, a clinical score was calculated and calves were considered to show clinical signs of bovine respiratory disease (BRD) when the clinical score was 6 [1]. The following animals were included int he study: two animals from the challenge group with a clinical score of 6 and two control animals, which were not challenged. The challenged calves exhibited mild to moderate pneumonia with peak clinical scores of 6, 6, and 4 (with a 12 point maximum). Seven days post-challenge control and challenged cows were euthanized and lung tissue was collected, snap frozen and stored at −80 °C.

2.3. Apparatus

The following equipment were used in this study: LTQ Velos Orbitrap mass spectrometer (a next generation hybrid Fourier Transform mass spectrometer, released in May 2009, Thermo Scientific, USA). Covaris S220 Focused-ultrasonicator instrument was used for tissue disruption/lysis (Covaris, USA). Microson XL-2000 Ultrasonic Cell Disruptor (Misonix, USA).

2.4. Tissue sample preparation

We used three different lung tissue sections (~40 mg) from one sham treated cow with two technical replicates from each section, making it a total of six replicates. The protein extracts were prepared as follows: (A) *Mortar and pestle*: sections were ground in a mortar in liquid nitrogen (N_2) and were solubilized in cold NP-40 lysis buffer (0.5% NP-40, 0.15 M NaCl, 0.02 M $CaCl_2 \cdot 2H_2O$, 0.05 M Tris, pH 7.4) with 1 mM phenylmethanesulfonyl fluoride (PMSF). (B) *Sonication*: Sections were ground in a mortar in liquid N_2 on dry ice, solubilized in cold 0.5% NP-40 lysis buffer with 1 mM PMSF. The lysates were sonicated on ice (5×5 s, setting 3). (C) *Homogenization and sonication*: Cold 0.5% NP-40 lysis buffer containing 1 mM PMSF was added to the tissue sample, which was homogenized with a tube homogenizer and sonicated on ice as above. (D) *Focused ultrasonicator*: Each tissue section was pulverized and solubilized in cold 0.5% NP-40 lysis buffer containing 1 mM PMSF following manufacturer's instructions. The pulverized sections were processed at 200 W, 20%, 500 cycle/burst by using Covaris S220 focused ultrasonicator (Covaris, USA). All lysates were clarified by centrifugation (3×13000 rpm, 15 min, 4 °C), filtered with centrifugal filter units (Ultrafree-MC 0.1 μm units; Millipore, USA) and quantified by the DC Protein Assay Kit according to the manufacturer's instructions (Bio-Rad, USA). Protein yield was determined using a cuvette spectrophotometer (Evolution 160 UV–Vis, Thermo Scientific, Rockford, IL) at 750 nm.

2.5. Active-site probe labeling of deubiquitinases

The reaction mixture for the HA-tagged ubiquitin vinyl sulfone probe (HA-Ub-VS, Boston Biochem, USA) labeling included protein extracts, a sucrose-based buffer (0.15 M NaCl, 0.005 M $CaCl_2$, 0.25 M sucrose, 0.05 M Tris-HCl, pH 7.4), 1 mM DTT and HA-Ub-VS probe (0.5 μg probe). The input reaction (without the probe) included protein (100 μg) and sucrose-based buffer. For Ub-VS probe reactions, samples were incubated for 1 h at 37 °C. All samples (input and VS probe) were boiled in reducing SDS sample buffer at 95 °C.

Protein samples from the input and HA-Ub-VS probe reactions (~20 μg each) were separated on 4–12% gels (Criterion Cell, Bio-Rad, USA), transferred to a PVDF membrane and probed with anti-HA (Sigma-Aldrich, USA), anti-USP15 (Proteintech, USA) and beta-actin (Sigma-Aldrich, USA) antibodies. Proteins were detected by HRP- conjugated anti-mouse antibody (Dako, USA) and visualized by ECL Plus (GE Healthcare). ImageJ (v.1.46r) was used to compare the band intensities between replicates, to measure the six different HA-reactive bands per sample, representing deubiquitinases that reacted with the HA-tagged Ub-VS probe. The bands were selected for analysis if they were sufficiently resolved from other bands. The intensity for each DUB corresponding to the same molecular weight in each replicate was measured. Statistical analysis and calculation of standard deviation were performed by GraphPad Prims software (Fisher's LSD test).

2.6. Immunoprecipitation

Frozen sections (200 mg) were collected from bovine lung tissue (2 each from control and 2 from BRD-affected animals) for the immunoprecipitation. Proteins obtained from lung tissue (16 mg) were incubated with ~8.1 μg HA-Ub-VS probe (as described above). The samples were diluted to a concentration of 5 mg/mL with lysis buffer and incubated with agarose-A beads for 30 min at 4 °C on a rotator, after which the supernatant was used for immunoprecipitation by using anti-HA-agarose (Sigma-Aldrich) for ~14 h at 4 °C. The HA-agarose beads were washed three times with NP-40 lysis buffer and eluted with 100 mM glycine, pH 2.5 for 30 min, at 4 °C. This eluate was then subjected to in-solution tryptic digestion and label-free proteomic quantification as described in the section below.

2.7. Mass spectrometry

Protein samples from the eluates were precipitated with methanol/chloroform, digested with trypsin and purified by C18 columns as we described earlier [2]. The purified peptides were resuspended in 20 μL of 2% acetonitrile, 0.1% formic acid, and 5 μL was used for mass spectrometric analysis. The peptides were analyzed by Ultimate 3000 HPLC and LTQ Velos ion trap mass spectrometer. To separate peptides, 75-μm i.d. x 15 cm C18 column was used, controlled by an Ultimate 3000 nanoflow HPLC (Dionex, Thermo Scientific, USA). Peptides were eluted using a 60 min gradient from 2% to 55% solvent B (99.9% acetonitrile, 0.1% formic acid) at a flow rate of 0.3 μl/min, and further introduced into a mass spectrometer (Thermo Fisher). Full scan MS spectra (300–2000 amu) were analyzed in a profile mode by the LTQ analyzer, and the ions were selected for collision-induced fragmentation (CID) at normalized collision energy of 35% and activation time of 40 ms. Tandem mass spectra were extracted, charge state deconvoluted and deisotoped by Proteome Discoverer (Thermo Fisher Scientific). MS/MS samples were analyzed using Sequest (version 1.4.0.288) to search bovine proteins from NCBI database (44,138 entries). Fragment ion mass tolerance was 1 Da and a parent ion tolerance was 1.8 Da. Carbamidomethylation (cysteine) was set as fixed modification and Oxidation (methionine) was specified as a variable modification. Scaffold (version 4.4.1, Proteome Software Inc., USA) was used to validate the identifications and perform quantification. Peptide identifications were at FDR $\leq$2.0%, as determined by Scaffold FDR algorithm. Protein identifications were accepted at >99.0% probability with at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm [3]. Proteins which