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

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Abstract

To develop a reproducible tissue-lysis method that retains enzyme function for activity-based protein profiling, we compared four different tissue lysis methods of 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 had also 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.

Graphical Abstract

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Keywords

deubiquitinases; focused-ultrasonication; activity-based profiling; chemical proteomics; tissue lysis

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 reported [10, 11]. In this report, we used an activity-based method for identification of DUBs in bovine tissues to identify novel active DUBs.

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 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]. Two animals from the challenge group with a clinical score of 6 were included in this study 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 sections lung tissue (~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 and were solubilized in cold NP-40 lysis buffer (0.5% NP-40, 0.15 M NaCl, 0.02 M $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 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 sec, 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 hour 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 μ g 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 minutes at 4°C on a rotator, after which the supernatant was used for immunoprecipitation by using anti-HA-agarose (Sigma-Aldrich) for ~14 hours 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 μ L of 2% acetonitrile, 0.1% formic acid, and 5 μ L 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- μ m i.d. $\times$ 15 cm C18 column was used, controlled by an Ultimate 3000 nanoflow HPLC (Dionex, Thermo Scientific, USA). Peptides were eluted using a 60-minute gradient from 2%–55% solvent B (99.9% acetonitrile, 0.1% formic acid) at a flow rate of 0.3 μ L/min, and further introduced into an mass spectrometer (Thermo Fisher). Full scan MS spectra (300–2,000 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 2.0%, as determined by Scaffold FDR algorithm. Proteins 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 could not be differentiated based on MS/MS analysis alone were grouped into clusters. For quantitation, normalization has been performed, fudge factor of 0.2 was used, and the quantitation was done based on the weighted spectra. The Fisher's exact test ($p < 0.05$) was performed.

3. Results and Discussion

To identify active DUBs in tissues, there is a need to design and optimize a tissue lysis method, which is compatible with activity-based quantitative proteomics. Tissue lysate is often contaminated with blood, lipids and other components, which interferes with the proteomic analysis [11]. Here we used bovine lung tissue to compare different tissue lysis methods to obtain protein extracts that retains DUB function for activity-based profiling: focused-ultrasonicator, standard sonicator, mortar & pestle method, and homogenization in combination with standard sonication. In these comparisons, two main criteria were used: the ability to retain the catalytic activity of enzymes studied and reproducibility. Lung tissue sections were derived from a healthy calves, and total of six replicates for each method were used. The obtained protein extracts by each method were incubated with the HA-tagged ubiquitin-specific active-site probe (HA-Ub-VS) to label enzymatically active DUBs. The reaction samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), followed by anti-HA western blotting to detect labeled DUBs. The intensities of six different bands for each replicate were measured to determine the sample variability. The intensities of bands representing specific DUBs were comparable for each sample with equal loading of material (Figure 1; Ponceau Red staining not shown due to space limitations). The statistical analysis revealed that the standard deviation was the lowest for focused-ultrasonicator and mortar & pestle methods. Homogenization combined with sonication had the highest and sonication had the second highest standard deviation. In terms of reproducibility in activity-based profiling of enzymes, focused-ultrasonicator-based sample preparation method is similar to the mortar & pestle method. It is apparent from the western blotting analysis that one of the samples subjected to homogenization combined with sonication was not reproducible (sample 5, Figure 1) and it had a very different profile than the remaining samples, which was not due to sample loading or protein amounts as repetition of this western blot gave similar results (data not shown). The intensity of these bands was still measured by ImageJ.

Since focused-ultrasonicator (Covaris) is a relatively unexplored method for protein extraction, and it has a fast processing time, we used this method for proteomic identification of novel bovine DUBs in lung tissues. We also determined whether DUBs were differentially regulated in BRD disease, thereby demonstrating the use of this method in quantitative studies. BRD is a multifactorial disease in cattle, most frequently characterized by the clinical onset of bronchopneumonia associated with *Mannheimia haemolytica* [12]. BRD is the leading cause of morbidity in feedlot cattle, causing significant economic loss [13]. We lysed lung tissue from two control animals and two animals with clinical symptoms of BRD (*IACUC project 10 – 024*), labeled DUBs by using HA-Ub-VS probe, and analyzed the samples by anti-HA western blotting (Figure 2A), which showed regulation of several DUBs in BRD-affected animals. Please note that addition of a HA-Ub-VS probe (MW: 8.6 kDa) increases the molecular weight of a protein. We then isolated the probe-bound DUBs, and subjected them to proteomic identification. Five bovine DUBs were identified (Figure 2B): Ubiquitin carboxyl-terminal hydrolase 15 (USP15), predicted ubiquitin carboxyl-terminal hydrolase 19 (bUSP19), predicted ubiquitin specific peptidase 5 isoform 1 (bUSP5), ubiquitin carboxyl-terminal hydrolase isozyme L3 (bUCH-L3), and

ubiquitin carboxyl-terminal hydrolase isozyme L1 (bUCH-L1). Two of these five enzymes are predicted proteins in National Center for Biotechnology Information (NCBI) protein database, and none of them have been previously shown to possess DUB activity. The alignment of bovine DUBs with human and mouse orthologs demonstrated that the amino acid sequence was highly homologous and the catalytic site was within conserved domains (Figure 2C, Supplementary Figure 1–5). Moreover, bUSP15 expression was found to be significantly down-regulated in BRD cow tissue, which was validated by western blotting (Figure 2A). The host immune responses to BRD have been relatively well characterized, although less is known on the specific host responses in the lungs at the protein level. Our data shows that bovine USP15 is down-regulated in lungs obtained from the BRD-affected cows, which could indicate that USP15 might be involved in the host responses to BRD. Identifying novel targets, whose function is disrupted in BRD could help develop biomarkers of BRD. Human USP15 is involved in regulation of TGF- β [14], APC tumor suppressor [15], NF- κ B pathway [16], caspase-3 [17], and MAP kinase [18]. Human USP15 is also involved in immune responses by regulating deubiquitination of TRIM25, which promotes the RIG-I-dependent antiviral response [19]. The identified down-regulation of this enzyme in BRD cow tissue could suggest possible functions of this protein in host responses to a pathogen. Amongst the other DUBs identified in bovine lung tissue, bUSP19 and bUSP5 have not been previously identified at protein level, and none of these enzymes have been previously shown to be active deubiquitinase by using DUB activity assays. Future studies focused on the DUBs identified in bovine lung tissue from animals with BRD will help elucidate the role of this class of enzymes in lung pathophysiology.

Focused-ultrasonicator instrument has been successfully used in genomic approaches, such as DNA shearing [20], small molecule or metabolite extraction from tissues [21], and for High Performance ELISA assays [22]. The use of focused-ultrasonicator for proteomics has not yet been established. The fast processing time (<5 minutes) was a positive feature of this method, especially for samples where proteolysis, labile PTMs or time-sensitive enzymatic activity is of concern. Protein extraction from animal tissues by homogenization using mortar and pestle is commonly used [23]. Similarly, sonication can also provide some disruption of biological material, but compared to conventional sonicators operating at 20 kHz and using longer wavelengths (~10cm), Covaris' focused-ultrasonicator works at 500 kHz with a wavelength of ~1 mm, which applies the acoustic energy to the samples in an isothermal mode, thereby minimizing protein degradation. Since no probe is used, it eliminates contamination between the samples. Our results indicating that sample preparation by focused-ultrasonicator are similar to the mortar/pestle method in terms of reproducibility and are consistent with previous findings. In comparison with mortar/pestle method or vortex/sonication, use of focused-ultrasonicator helped to obtain an improved protein pattern on two-dimensional electrophoresis, although the protein separation was quite comparable for all methods [23]. Moreover, focused-ultrasonicator has been shown to preserve the enzymatic activity of some proteins [24], but no direct comparison was made with other methods in this regard.

4. Conclusions

In summary, we identified focused-ultrasonicator-based tissue lysis method as compatible with activity-based protein assays and proteomics and concluded that it is as reproducible as mortar & pestle method for sample preparation. We experimentally confirmed annotation of five novel bovine enzymes by functional proteomics approach and we detected up-regulation of USP15 deubiquitinase in lungs from BRD-diseased cows.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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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

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Figure 1. Comparison of different methods for protein extraction from tissue for active site labeling with chemical probes

(A). Four methods were tested to extract proteins from bovine tissue: focused-ultrasonication (Covaris), standard sonication, mortar/pestle and homogenizer used in combination with standard sonication. The protein extracts were subjected to labeling with ubiquitin-specific active-site probe (HA-tagged Ub-VS) to label active deubiquitinases in lung tissue, followed by anti-HA immunoblotting (B). The visualized bands were quantified in ImageJ to calculate standard variation between the replicates and determine the most reproducible method. The data was analyzed by GraphPad software. The confidence intervals are also shown as calculated by Fisher's LSD test (C).

Figure 2. Identification of active deubiquitinases in bovine lung tissue

(A). Lung tissue from control and BRD-affected cows was subjected to lysis by focused-ultrasonicator, followed by reaction with HA-Ub-VS probe to label active deubiquitinases. The anti-HA western blotting was done to visualize the deubiquitinases, anti-beta actin blot was done as a loading control. Anti-USP15 western blot was also performed. (B). Bovine deubiquitinases from (A) were subjected to immunoprecipitation and proteomic analysis. Relative fold change for one significantly down-regulated protein ($p \leq 0.05$) is shown. (C). Partial sequence alignment generated in Uniprot is shown for human (UBP15_HUMAN), mouse (UBP15_MOUSE) and bovine (UBP15_BOVIN) USP15 protein detected in (B). The catalytic site is shown in dark grey; the Uniprot-mapped conserved domain is highlighted in light grey.