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Assessing the impact of protein extraction methods for human gut metaproteomics

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ABSTRACT

Metaproteomics is a promising methodology for the functional characterizations of the gut microbiome. However, the performance of metaproteomic analysis is affected by protein extraction protocols in terms of the amount of protein recovered and the relative abundance of different bacteria observed in microbiome. Currently, there is a lack of consistency on protein extraction methods in published metaproteomics studies. Here we evaluated the effects of different protein extraction methods on human fecal metaproteome characterizations. We found that sodium dodecyl sulfate (SDS)-based lysis buffer obtained higher protein yields and peptide/protein group identifications compared to urea and the non-ionic detergent-based B-Per buffer. The addition of bead beating to any of the extraction buffers increased both protein yields and protein identifications. As well, bead beating led to a significant increase of the relative abundances of Firmicutes and Actinobacteria. We also demonstrated that ultrasonication, another commonly used mechanical disruption approach, performed even better than bead beating for gut microbial protein extractions. Importantly, proteins of the basic metabolic pathways showed significantly higher relative abundances when using ultrasonication. Overall, these results demonstrate that protein extraction protocols markedly impact the metaproteomic results and recommend a protein extraction protocol with both SDS and ultrasonication for metaproteomic studies.

Biological significance: The gut microbiome is emerging as an important factor influencing human health. Metaproteomics is promising for advancing the understanding of the functional roles of the microbiome in disease. However, metaproteomics suffers from a lack of consistent sample preparation procedures. In the present study, protein extraction protocols for fecal microbiome samples were evaluated for their effects on protein yields, peptide identifications, protein group identifications, taxonomic compositions and functional category distributions. While different protocols favor different microbial taxa and protein functions, our results suggest that a protein extraction protocol using sodium dodecyl sulfate (SDS) and ultrasonication provides the best performance for general shotgun metaproteomics studies.

1. Introduction

The human gut microbiome is an important factor influencing human health and has been linked with many diseases including inflammatory bowel diseases (IBD), obesity, type 2 diabetes and cardiovascular diseases [1]. Our understanding of the microbiome has been primarily driven by next-generation sequencing [2,3]. Recent studies have emphasized the importance of understanding microbiome functions using integrated multi-omics approaches, including metagenomics, metatranscriptomics, metaproteomics, and meta-metabolomics [4,5]. The metaproteomic approach, which directly measures the expressed proteins of a microbiome, has been applied to the fields of human microbiome [6] as well as other microbial communities such as ocean [7], soil [8] and food [9]. However, currently its application is far less common than metagenomics or metatranscriptomics. The limitations and challenges of metaproteomics include the inability of mass spectrometric platforms to measure low-abundance proteins from complex microbial communities, the lack of efficient bioinformatic tools, and the lack of consistent protocols for metaproteomic sample preparation [10–13].

Some of the sample preparation protocols typically used in proteomics, such as protease digestion and desalting, are compatible with microbial proteins [14]. However, extracting proteins from complex gut microbial community is more challenging than that from cells or tissues, largely due to the significant differences of the bacterial cell wall structures between different microorganisms [13]. Therefore, cell lysis procedures typically used in proteomics need to be adapted for gut metaproteomics studies. In particular, the Gram-positive bacteria such as Firmicutes and Actinobacteria, two major microbial phyla in the human gut, have thick peptidoglycan layer in their cell

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walls, which provides great resistance to cell lysis [15,16]. Some metaproteomics studies have added physical or mechanical cell disruption methods to facilitate the protein extractions, such as heating, bead beating or ultrasonication [6,13,17–20]. For example, Tanca et al. have reported that the additional bead beating and freeze-thawing procedures dramatically increased the protein extraction yields from individual species of yeast and Gram-positive bacteria, while there was no detrimental effects on Gram-negative bacteria, and suggested a protocol combining SDS, bead beating, heating and freeze-thawing for gut metaproteomics studies [13]. Kolmeder et al. have reported the sole use of bead beating for protein extraction from human stools for metaproteome characterizations relating [18,19]. Otherwise, McNulty et al. have reported the combination of ultrasonication, 4% SDS (w/v) and heating (95 °C) for protein extraction from mouse cecal microbiome for metaproteomics study [20]. We have also reported the utilization of ultrasonication in combination with SDS/Urea-based lysis buffer for protein extraction from both human and mouse gut microbiota, and identified up to 30,000 gut microbial protein groups [6]. However, as indicated above, the protein extraction protocols lack consistency between different studies, and more importantly, there has been no systematic evaluation of the effects of different protein extraction methods on the gut metaproteomics analyses.

Given that the protein extraction methods may dramatically bias the metaproteomics results, in this study, we evaluated the effects of different lysis buffer and mechanical disruption methods on the metaproteome characterizations of gut microbial communities. We demonstrated that SDS achieved better performance than other studied lysis buffers and mechanical disruption methods are important for gut microbial protein extraction. Furthermore, we reported that ultrasonication is a good alternative to bead beating with higher protein yields and peptide/protein group identifications. Our multi-layer metaproteomic analysis also demonstrated obvious taxonomic and pathway differences using different protein extraction protocols, highlighting the importance of the optimization of protein extraction protocols for metaproteomics studies.

2. Materials and methods

2.1. Stool sample collection and preprocessing

A fresh stool sample was collected from a healthy adult volunteer at the University of Ottawa, Ottawa, Ontario, Canada, with protocol (Protocol # 20160585-01H) approval by the Ottawa Health Science Network Research Ethics Board at the Ottawa Hospital. The fresh stool sample was immediately put on ice, transferred to the laboratory and subjected to differential centrifugation for microbial cell harvesting as described previously [6]. Briefly, ~2 g of the stool was re-suspended in 10 ml cold phosphate buffer (PBS) through vortexing with ten 2.5-mm glass beads. The fecal slurry was centrifuged at 300 g, 4 °C for 5 min to collect supernatant. The pellets were then subjected to two more rounds of resuspension in fresh PBS followed by low-speed centrifugation as described above. All collected supernatants (~30 ml) were then combined and subjected to another three centrifugations at 300g, 4 °C for 5 min to remove remaining debris or large particles. The resulting supernatant was transferred to a new tube for high speed centrifugation at 14,000g, 4 °C for 20 min to pellet the microbial cells followed by three washes with PBS. The washing step was performed by resuspension in cold PBS followed by high speed centrifugation (14,000 g, 4 °C for 20 min). The resulting washed microbial cells were equally aliquoted (~30 mg wet weight per aliquot) for protein extraction using different protocols as described below.

2.2. Protein extraction methods

2.2.1. Protein extractions with different lysis buffers with or without bead beating

Sodium dodecyl sulfate (SDS)-based protein lysis buffer were freshly prepared containing 4% SDS (w/v) in 50 mM Tris-HCl buffer (pH 8.0). Bacterial Protein extraction reagent (B-Per) was purchased from Thermo Fisher Scientific Inc. (catalog number, 78248). Urea-based lysis buffer was freshly prepared containing 8 M urea in 50 mM Tris-HCl buffer (pH 8.0). For all three lysis buffers, protease inhibitor cocktails were added before use, including one Roche cOmplete™ mini tablet and one Roche PhosSTOP™ tablet for every 10 ml lysis buffer according to the manufacturer's instructions.

Protein extractions without bead beating were conducted through re-suspending the microbial cells in 500 μl lysis buffer with pipetting up and down until there was no visible particles in the lysate. Remaining cell debris was removed through centrifugation at 16,000g, 4 °C for 10 min. Three technical replicates were conducted for each protein extraction method.

For bead beating, the lysates were transferred to a 2-ml screw-cap tube containing 0.3 g zirconia/silica beads (0.1 mm, BioSpec Products Inc). Bead beating were carried out using a FastPrep-24 machine (MP Biomedicals Inc., USA) at a speed of 6.5 ms^{-1} for 225 s (45 s each with 1 min interval on ice). Beads or cell debris were removed through centrifugation at 16,000g, 4 °C for 10 min. The supernatant was carefully transferred into a new tube for protein precipitation and quantification as detailed below.

2.2.2. Protein extraction protocols with bead beating or ultrasonication

Protocol 1 was modified from [13,17]. Briefly, the microbial cells were re-suspended in 500 μl 4% SDS (w/v) lysis buffer followed by incubation at 95 °C for 10 min with agitation in an Eppendorf Thermomixer. After cooling, the lysates were transferred to a 2-ml screw-cap tube containing 0.3 g zirconia/silica beads. Bead beating was carried out and beads/cell debris were removed as described above.

Protocol 2 was modified from [20]. Briefly, the microbial cells were re-suspended in 500 μl 4% SDS (w/v) lysis buffer followed by incubation at 95 °C for 10 min with agitation in an Eppendorf Thermomixer. After cooling, the lysates were subjected to three ultrasonications (30 s each with 1 min interval on ice) using Q125 Sonicator (Qsonica, LLC) with an amplitude of 25%. Remaining cell debris was removed through high-speed centrifugation at 16,000g, 4 °C for 10 min.

Protocol 3 was performed according to Protocol 1 but without 95 °C incubation. In addition, 8 M urea was added to the 4% SDS (w/v) lysis buffer for microbial cell lysis at room temperature with bead beating.

Protocol 4 was performed according to Protocol 2 but without 95 °C incubation. In addition, 8 M urea was added to the 4% SDS (w/v) lysis buffer for microbial cell lysis at room temperature with ultrasonication.

2.3. Bradford assay for protein quantitation

To avoid the influence of different lysis buffers on the protein assay, protein lysates from different protein extraction protocols were precipitated using 5-fold volume acidified acetone/ethanol buffer at −20 °C overnight. Proteins were spun down with centrifugation at 16,000g for 20 min at 4 °C, and washed with ice-cold acetone for three times. The protein pellets were then re-suspended in 6 M urea (in 50 mM ammonium bicarbonate buffer) for protein quantitation using the Bradford Protein Assay Kit (Bio-Rad Laboratories, Inc.) according to the manufacturer's instructions.

2.4. Trypsin digestion, desalting and LC-MSMS analysis

In-solution trypsin digestion was conducted as described previously [6]. Briefly, 50 μg proteins were reduced and alkylated with 10 mM dithiothreitol (DTT) and 20 mM iodoacetamide (IAA), respectively. One

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