Evaluation of a High-Intensity Focused Ultrasound-Immobilized Trypsin Digestion and O-18-Labeling Method for Quantitative Proteomics

ARTICLE *in* ANALYTICAL CHEMISTRY · JUNE 2009

Impact Factor: 5.64 · DOI: 10.1021/ac802540s · Source: PubMed

6 AUTHORS, INCLUDING:

Daniel Lopez-Ferrer
Caprion
33 PUBLICATIONS **1,371** CITATIONS
SEE PROFILE

Kim Hixson
Pacific Northwest National Laboratory
41 PUBLICATIONS **2,027** CITATIONS
SEE PROFILE

Heather S Smallwood
The University of Tennessee Health Science …
20 PUBLICATIONS **446** CITATIONS
SEE PROFILE

Richard D Smith
Pacific Northwest National Laboratory
1,131 PUBLICATIONS **46,015** CITATIONS
SEE PROFILE

Anal. Chem. **2009,** *81,* 6272–6277

Evaluation of a High-Intensity Focused Ultrasound-Immobilized Trypsin Digestion and ^{18}O-Labeling Method for Quantitative Proteomics

Daniel López-Ferrer,[†] Kim K. Hixson,[‡] Heather Smallwood,[†] Thomas C. Squier,[†] Konstantinos Petritis,[†] and Richard D. Smith*,[†]

Biological Sciences Division and Environmental Molecular Sciences Laboratory, Pacific Northwest National Laboratory, Richland, Washington 99352

A new method that uses immobilized trypsin concomitant with ultrasonic irradiation results in ultrarapid digestion and more thorough ^{18}O labeling for quantitative protein comparisons. The method was reproducible and provided effective digestions within <1 min with lower amounts of enzyme, compared to traditional methods. This method was demonstrated for digestion of both simple and complex protein mixtures, including bovine serum albumin, a global proteome extract from the bacteria *Shewanella oneidensis,* and mouse plasma, as well as ^{18}O labeling of complex protein mixtures, validating this method for differential proteomic measurements. This approach is simple, reproducible, cost-effective, rapid, and well suited for automation.

Recent advances in high-resolution liquid chromatography and mass spectrometry have enabled high-throughput and sensitive global proteome analyses. As a result, mass-spectrometry-based proteomics has become a preferred analytical tool for obtaining quantitative protein comparisons. In typical proteomic workflows, the largest time bottlenecks result from tedious and/or time-consuming sample preparation protocols. Digestions alone can require several hours to complete, and this time can be doubled if performing an additional isotopic labeling step (e.g., ICAT,[1] SILAC,[2] and ^{18}O[3,4]) for quantitative proteomics. Of all the current isotopic labeling methods, ^{18}O labeling is arguably one of the most affordable, facile, and versatile. Qian et al. recently demonstrated ^{18}O labeling on a universal pooled reference sample, which was added to 18 plasma samples from severe burn patients.[5] Using this strategy, label-free quantitation was attainable simultaneously with precise peptide abundance ratios on large numbers of samples. The label-free and labeled ratios together provided more precision and a greater coverage of protein abundance changes than either method alone, demonstrating the utility of the approach.

In a typical ^{18}O-labeling protocol, proteins are first digested with trypsin over several hours, dried, and then labeled overnight by incubation with $H_2{}^{18}O$ in the presence of trypsin. After quenching the trypsin with formic acid or thermal denaturation to stop the reaction, the two samples are combined prior to liquid chromatography−mass spectrometry (LC-MS) analysis. In addition to long digestion times, traditional ^{18}O-labeling processes are challenged by trypsin autodigestion, back-exchange, and sample cleanup steps that can result in significant sample losses. In response to these challenges, we have investigated the use of immobilized trypsin in conjunction with high-intensity focused ultrasound (HIFU) to produce a more robust digestion, as well as a quick and easy ^{18}O-labeling procedure. Immobilized trypsin is advantageous in that it is less susceptible to autolytic digestion, which leads to a higher stability than that for free trypsin,[6] and it can readily be removed after the reaction is complete, thereby helping prevent back-exchange.[7] The catalytic activity of trypsin is improved by immobilization, because of an increased enzyme concentration. Because the enzyme concentration is directly proportional to the reaction rate (according to the Michaelis−Menten equation), a higher trypsin concentration results in shorter incubation periods. HIFU also increases the reaction kinetics by producing microcavitation events, i.e., quick and centralized bursts of increased temperature and pressure.[8] In addition, the

* To whom correspondence should be addressed. Contact information: Richard D. Smith, Pacific Northwest National Laboratory, P.O. Box 999, MSIN: K8-98, Richland, WA 99352. E-mail: rds@pnl.gov.

[†] Biological Sciences Division.

[‡] Environmental Molecular Sciences Laboratory.

(1) Gygi, S. P.; Rist, B.; Gerber, S. A.; Turecek, F.; Gelb, M. H.; Aebersold, R. *Nat. Biotechnol.* **1999**, *17*, 994–999.

(2) Ong, S. E.; Blagoev, B.; Kratchmarova, I.; Kristensen, D. B.; Steen, H.; Pandey, A.; Mann, M. *Mol. Cell. Proteomics* **2002**, *1*, 376–386.

(3) Yao, X.; Freas, A.; Ramirez, J.; Demirev, P. A.; Fenselau, C. *Anal. Chem.* **2001**, *73*, 2836–2842.

(4) Lopez-Ferrer, D.; Ramos-Fernandez, A.; Martinez-Bartolome, S.; Garcia-Ruiz, P.; Vazquez, J. *Proteomics* **2006**, *6* (Suppl. 1), S4–S11.

(5) Qian, W. J.; Liu, T.; Petyuk, V. A.; Gritsenko, M. A.; Petritis, B. O.; Polpitiya, A. D.; Kaushal, A.; Xiao, W.; Finnerty, C. C.; Jeschke, M. G.; Jaitly, N.; Monroe, M. E.; Moore, R. J.; Moldawer, L. L.; Davis, R. W.; Tompkins, R. G.; Herndon, D. N.; Camp, D. G.; Smith, R. D. *J. Proteome Res.* **2009**, *8*, 290–299.

(6) Freije, J. R.; Mulder, P. P.; Werkman, W.; Rieux, L.; Niederlander, H. A.; Verpoorte, E.; Bischoff, R. *J. Proteome Res.* **2005**, *4*, 1805–1813.

(7) Sevinsky, J. R.; Brown, K. J.; Cargile, B. J.; Bundy, J. L.; Stephenson, J. L., Jr. *Anal. Chem.* **2007**, *79*, 2158–2162.

(8) Mason, T. J.; Lormer, J. P. *Sonochemistry, Theory, Applications and Uses of Ultrasound in Chemistry*, 1st ed.; Ellis Horwood Limited: Chichester, England, 1988.

10.1021/ac802540s CCC: $40.75
Published on Web 06/25/2009

use of ultrasonic energy aids in mixing and solubilizing proteins, which enhances enzymatic digestions.[9−11]

Herein, we report the evaluation of a HIFU-immobilized trypsin method for quantitative proteomic applications. The method was evaluated by digesting a diverse array of protein mixtures that ranged in protein complexity (i.e., BSA, *Shewanella onedensis* proteome extract, and mouse plasma), followed by ^{18}O labeling of some samples to demonstrate the rapid HIFU-immobilized trypsin technique for determination of changes in protein abundances between samples. The entire process, including denaturation, digestion, and labeling, was reduced to <5 min.

EXPERIMENTAL PROCEDURES

Materials and Reagents. Immobilized trypsin was purchased from Applied Biosystems (Foster City, CA), Tris[2-carboxyethyl]phosphine (TCEP), and a BCA protein assay kit was purchased from Pierce (Rockford, IL). Bovine serum albumin (BSA), urea, iodoacetamide (IAA), ammonium bicarbonate, formic acid, ^{18}O water, mouse plasma, and high-performance liquid chromatography (HPLC)-grade solvents were purchased from Sigma−Aldrich (St. Louis, MO).

Digestion Procedures. BSA was used to evaluate the method under several different conditions. First, equal aliquots of 100 μg of BSA were denatured in 8 M urea, reduced, and then alkylated simultaneously with 5 mM TCEP and 50 mM iodoacetamide in 25 mM ammonium bicarbonate (pH 8.25) for 3 min under ultrasonic irradiation. The reduced and alkylated samples were diluted 4-fold with 25 mM ammonium bicarbonate to decrease urea, TCEP, and IAA concentrations. A range of 1−10 μL of immobilized trypsin slurry (as supplied by the manufacturer) was added to a final volume of 100 μL, and the solutions were sonicated for 15, 30, 60, or 120 s at 2% of the maximum ultrasound amplitude, which yielded 1−3 W of power at the end of the tip. All of the experiments used a Sonicator 3000 instrument and a 3.2-mm microtip, which were obtained from Mixonix (Farmingdale, NY). The slurry was washed three times with 25 mM ammonium bicarbonate, to eliminate nonvolatile preservatives before adding it to the reaction tube. Finally, the enzymatic digests were filtered to remove the trypsin beads using 0.45-μm cutoff spin filters from Beckman Coulter, Inc. (Fullerton, CA) and the filtered peptide mixtures were transferred to new centrifuge tubes, acidified, and frozen with liquid nitrogen to stop the reaction. The samples were then dried by centrifugal evaporation and stored at −20 °C.

The *Shewanella oneidensis* proteome was prepared as described previously,[12] and the soluble portion of the proteome was aliquoted as 50 μg samples and dried down. Dried proteins were resuspended in 8 M urea in 25 mM ammonium bicarbonate, 5 mM TCEP, and 50 mM IAA and then were subjected to 3 min of ultrasonic irradiation. The samples were diluted and 5 μL of previously washed immobilized trypsin slurry was added after which the samples were sonicated for 30−90 s. A control was digested in parallel for 4 h in a thermomixer at 37 °C with shaking at 1500 rpm. Two replicate digestions were performed for each time point. After digestion, samples were acidified and then immobilized trypsin was filtered out, again using a 0.45 μm cutoff. Finally, digestion products were snap-frozen in liquid nitrogen and dried by centrifugal evaporation.

Mouse plasma was diluted 5-fold, using 8 M urea in 25 mM ammonium bicarbonate, and then reduced, alkylated, and digested in the same way as described previously. In this case, no technical replicates were performed.

Post-Digestion ^{18}O Labeling. Plasma samples were prepared as previously described with minor modifications.[13] Briefly, dried peptides were cleaned by loading them onto a 1-mL SPE C18 Discovery solid-phase extraction (SPE) column (Supelco, Bellefonte, PA). Each sample was washed with 4 mL of 0.1% trifluoroacetic acid (TFA)/5% acetonitrile (ACN). Peptides were eluted from the SPE column with 1 mL of 0.1% TFA/80% ACN and then lyophilized. One-hundred-microgram aliquots of the desalted peptides were dried by centrifugal evaporation in a 0.6-mL centrifuge tube and the peptides were redissolved in 50 μL of 100 mM ammonium acetate (pH 6.75) with 5 μL of immobilized trypsin. After drying again by centrifugal evaporation, the samples were dissolved in 100 μL of either $H_2{}^{16}O$ or $H_2{}^{18}O$ (95%, Sigma−Aldrich) and sonicated for 30−90 s. Labeling was stopped by adding formic acid to 1% and the immobilized trypsin was filtered out. Samples were then mixed in a 1:1 ratio and prepared for LC-MS analysis.

As a control experiment to evaluate the complete proteomic workflow, two identical aliquots of a *Shewanella oniedensis* protein extract were subjected to reduction and alkylation, digestion, and labeling (either ^{18}O or ^{16}O). To avoid sample cleanup for post-digestion labeling, we incorporated a trifluoroethanol (TFE)-assisted digestion, previously developed in our laboratory.[14] Dried extracts were solubilized in a 50:50 TFE:50 mM ammonium bicarbonate solution and reduced and alkylated by adding TCEP and IAA to a final concentration of 5 mM and 50 mM, respectively, followed by a 3-min sonication. Samples were subsequently diluted 10 times and 5 μL of immobilized trypsin was added. The samples were again sonicated at 2% intensity for 30 s to perform the digestion. The enzymatic activity was stopped by acidification of the samples and physical removal of the immobilized trypsin by filtration. Filtrates were dried down by centrifugal evaporation. Using TFE as an organic cosolvent acting as a chaotrope, there was no need for a separate sample cleanup. Post-digestion enzymatic labeling was performed as explained previously. After the labeling process was finished, samples were combined in a 1:1 ratio and submitted for LC-MS analysis.

LC-MS(/MS) Analysis. For the BSA analyses, 500 fmol of the protein digest were analyzed by LC-MS/MS using an Agilent HPLC-Chip system coupled to a MSD XCT Ultra ion trap (Agilent Technologies, Santa Clara, CA). Separations were performed on a chip that contained a 40-nL enrichment column and a 43 mm × 75

(9) López-Ferrer, D.; Cañas, B.; Vázquez, J.; Lodeiro, C.; Rial-Otero, R.; Moura, I.; Capelo, J. L. *Trends Anal. Chem.* **2006**, *25*, 996–1005.

(10) Lopez-Ferrer, D.; Capelo, J. L.; Vazquez, J. *J. Proteome Res.* **2005**, *4*, 1569–1574.

(11) Capelo, J. L.; Ximenez-Embun, P.; Madrid-Albarran, Y.; Camara, C. *Anal. Chem.* **2004**, *76*, 233–237.

(12) Shen, Y.; Zhao, R.; Belov, M. E.; Conrads, T. P.; Anderson, G. A.; Tang, K.; Pasa-Tolic, L.; Veenstra, T. D.; Lipton, M. S.; Smith, R. D. *Anal. Chem.* **2001**, *73*, 1766–1775.

(13) Liu, T.; Qian, W. J.; Mottaz, H. M.; Gritsenko, M. A.; Norbeck, A. D.; Moore, R. J.; Purvine, S. O.; Camp, D. G., 2nd; Smith, R. D. *Mol. Cell. Proteomics* **2006**, *5*, 2167–2174.

(14) Wang, H.; Qian, W. J.; Chin, M. H.; Petyuk, V. A.; Barry, R. C.; Liu, T.; Gritsenko, M. A.; Mottaz, H. M.; Moore, R. J.; Camp Ii, D. G.; Khan, A. H.; Smith, D. J.; Smith, R. D. *J. Proteome Res.* **2006**, *5*, 361–369.

μm analytical column, both packed with 5 μm ZORBAX 300SB C18 particles. A flow rate of 1 μL/min was used for loading and enriching the sample. Peptides were eluted at 600 nL/min, using either (i) a 5-min gradient from 5% B to 90% B (solvent B: 0.5% formic acid in water:acetonitrile, 10:90; solvent A: 0.5% formic acid in water: acetonitrile, 97:3) with a separation window of ~2 min and total analysis time of 12 min, or (ii) a 55-min gradient from 5% B to 90% B.

For the *S. oneidensis* proteome and mouse plasma analyses, 2 μg of the digested peptides were analyzed using an in-house-developed capillary liquid chromatography (LC) system coupled online to a linear ion-trap mass spectrometer (Thermo-Fisher, San Jose, CA) with an in-house developed ESI source.[12] Reversed-phase capillary columns were prepared by slurry packing 5-μm Jupiter C18-bonded particles (Phenomenex, Torrence, CA) into a 150 μm × 65 cm fused silica capillary (Polymicro Technologies, Phoenix, AZ) that utilized a 2-μm stainless steel retaining screen within a stainless steel union (Valco Instruments Co., Houston, TX). The mobile phase consisted of 0.2% acetic acid and 0.05% TFA in water (A) and 0.1% TFA in 90% acetonitrile/10% water (B). Mobile phases were degassed online using a vacuum degasser (Jones Chromatography Inc., Lakewood, CO), and the HPLC system was equilibrated at 10 000 psi with 100% mobile phase A for the initial starting conditions. After loading 2.5 μg of peptides onto the column, the mobile phase was held at 100% A for 20 min. Exponential gradient elution was performed by increasing the mobile-phase composition from 0 to 77% B over ~100 min, using a stainless steel mixing chamber, followed by column washing at 100% B for 10 min. Data were collected in a data-dependent mode (m/z 400−2000), where the ten most-abundant ions were selected for tandem mass spectroscopy (MS) after a full scan. A dynamic exclusion time of 1 min and a normalized collision energy of 35% were used for the entire run. A potential of 2.2 kV was applied to the union to form the electrospray. Quantitative analyses were performed using the same chromatography system but, in this case, were coupled online to a hybrid LTQ-Orbitrap mass spectrometer. Data were collected in a data-dependent mode, where the six most-abundant ions were selected for tandem MS in the ion trap after a full, high-resolution survey scan.

Protein Identification and Validation. Peptide identification was performed using SEQUEST[15] to search MS/MS spectra against an IPI *Mus musculus* or *Shewanella oneidensis* databases downloaded from NCBI. Database search parameters included a dynamic modification for Met oxidation and for carbamidomethylation of Cys. Peptide identification statistics that included estimating random match probabilities and false discovery rates (FDRs) were performed using the two-variable Gaussian method[16] with some modifications, as described elsewhere.[17] For application of the accurate mass and time (AMT) tag strategy, the high-resolution LC-MS spectra were processed using Decon-2LS software (publicly available at http://omics.pnl.gov/software/) to obtain peak lists that contained the monoisotopic mass and observed charge. A reference AMT tag database was generated from peptides previously identified by LC-MS/MS, using MTDB Creator software (publicly available at http://omics.pnl.gov/software/). Once generated, high-resolution MS datasets were matched to the reference database, as described below.

Quantitative Proteomic Strategy. LC-MS datasets were analyzed using VIPER software (publicly available at http://omics.pnl.gov/software/). Details of the quantitation strategy are described elsewhere.[17] Briefly, data processing ratios were calculated across the chromatographic peak, taking into account the labeling efficiency,[18] which corrects the ratio as a function of the remaining unlabeled peptide from the labeled sample. The ratio calculated for each pair then is averaged from several ratios calculated across the chromatographic peak, and an error in terms of the standard deviation is used to discriminate between poorly matched peaks and real pairs. Pairs were then identified by comparing the mass and the elution time of the pairs with the mass and the normalized elution time (NET) of the peptides in the AMT tag database[19] (match tolerance of ±1.4 ppm and ±0.02 LC NET). Finally, a statistical analysis was performed in the same way as that described previously,[4] i.e., fitting a histogram of the ratios to a Gaussian distribution and using the average and standard deviations to discriminate between pairs that represented a real differential expression event.

RESULTS AND DISCUSSION

Evaluation of HIFU and Immobilized Trypsin Digestion Performance. Protein digestion was initially evaluated using BSA as a standard protein. HIFU time varied from 15 s to 2 min for a sample volume of 100 μL that contained 100 μg of total protein and 10 μL of washed immobilized trypsin slurry. Following digestion, the samples were acidified and the trypsin-coated beads were removed by filtering. The tryptic digests were then analyzed by LC-Chip-MS/MS in an automated fashion, using a 5-min gradient. Optimal digestion occurred within 15 s, and no significant differences in terms of coverage or number of peptides were observed for sonication times in the range of 15−120 s (see Figure 1a). In addition, no traces of undigested protein were detected. Figure 1b shows the chromatograms of all the experiments, in addition to a control BSA digestion under unfavorable conditions, where undigested or partially digested protein was observed. Protein coverage was typically >60% using the aforementioned fast 5-min gradient (Figure 1a). This coverage is equivalent to that of the control experiments performed with a typical overnight digestion (data not shown). Furthermore, this rapid digestion technique increased protein coverage by 2.3-fold, compared to a similar technique that utilized a combination of microwave technology and magnetic silica microspheres, which only obtained 26% coverage.[20] The minimal amount of immobilized trypsin slurry necessary for maximal protein coverage was 2 μL (see figure given in the Supporting Information), which suggests that costs can effectively be minimized to less than $1 for immobilized trypsin per 100 μg of sample digested. While the price of 2 μg of free trypsin is similar, activity decreases more rapidly with free trypsin (especially when used in conjunction with ultrasound techniques), which may lead to incomplete digestions under certain circum-

(15) Eng, J. K.; McCormack, A. L.; Yates, I. I. I. J. R. *J. Am. Soc. Mass Spectrom.* **1994**, *5*, 976–989.

(16) Lopez-Ferrer, D.; Martinez-Bartolome, S.; Villar, M.; Campillos, M.; Martin-Maroto, F.; Vazquez, J. *Anal. Chem.* **2004**, *76*, 6853–6860.

(17) López-Ferrer, D.; Heibeck, T. H.; Petritis, K.; Hixson, K. K.; Qian, W.; Monroe, M. E.; Mayampurath, A.; Moore, R. J.; Belov, M. E.; Camp, D. G.; Smith, R. D. *J. Proteome Res.* **2008**, *7* (9), 3860–3867.

(18) Ramos-Fernandez, A.; Lopez-Ferrer, D.; Vazquez, J. *Mol. Cell. Proteomics* **2007**, *6*, 1274–1286.

(19) Jaitly, N.; Monroe, M. E.; Petyuk, V. A.; Clauss, T. R.; Adkins, J. N.; Smith, R. D. *Anal. Chem.* **2006**, *78*, 7397–7409.

(20) Lin, S.; Yao, G.; Qi, D.; Li, Y.; Deng, C.; Yang, P.; Zhang, X. *Anal. Chem.* **2008**, *80* (10), 3655–3665.

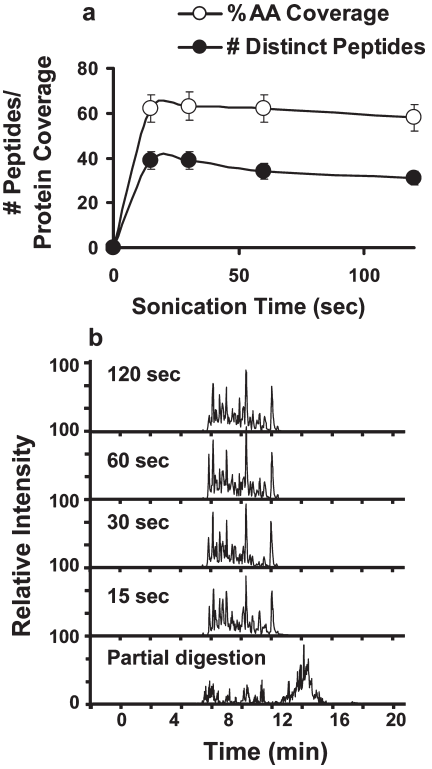

Figure 1. (a) Comparison of the number of uniquely identified peptides and BSA amino acid coverage at different irradiation times of 15, 30, 60, and 120 s. Empty circles correspond to protein coverage, whereas filled circles correspond to number of identified peptides. (b) Reprentative LC-MS/MS chromatograms for each of 15, 30, 60, and 120 s digestion experiments, as well as for a control BSA "incomplete digestion".

stances. Moreover, free trypsin cannot be removed quickly and thoroughly from a protein mixture, which is important for ^{18}O labeling strategies, to avoid back-exchange.

A *S. oneidensis* (an anaerobic soil bacteria) cell lysate was digested to evaluate the utility of the immobilized trypsin combined with ultrasound on a complex proteome sample. *S. oneidensis* cell lysates were digested in duplicate using HIFU and immobilized trypsin for 30 s, 60 s, or a standard 4-h digestion (i.e., control) at 37 °C with mixing at 1500 rpm (where rpm denotes revolutions per minute). Representative chromatograms of each digestion and the amount of information generated in terms of unique peptide and protein identification was similar in all cases (see Figures 2a and 2b) and the reproducibility of peptide identification among duplicates was >80% (see Figure 2d). In addition to being comparable in terms of digestion efficiency and reproducibility to standard digestions, the combined use of immobilized trypsin with HIFU provides significant advantages over the traditional protocol in that the digestion procedure is complete within a few minutes rather than a few hours and the trypsin can be removed quickly. Furthermore, by rolling up the identified peptides to the protein level, we show, in Figure 2c, that more than 60% of the identified proteins are identified in all three of the three experiments, which represents an extraordinary achievement, in terms of reproducibility in a proteomic experiment.

Having demonstrated that the method worked well on prokaryotic samples, the strategy was next evaluated using a more-complex proteome, represented by a nondepleted mouse plasma sample. The plasma was diluted, reduced, and then alkylated during a 3-min ultrasonic irradiation. Trypsin beads were added, and the plasma-bead mixture was sonicated for 30 s. For comparison, we used a standard 4-h digestion, using a thermomixer set at 37 °C with shaking at 1500 rpm. The histogram in Figure 3a shows the number of unique peptides identified with an FDR of <1%, along with the corresponding number of proteins identified. A 76% overlap of shared peptides was identified between the 4-h thermomix digestion and the 30-s HIFU digestion, relative to all peptides identified in the 4-h thermomix digestion and a 55% overlap of shared peptides was identified in the 30-s HIFU digestion, relative to all peptides identified in the 30 s HIFU digestion (see Figure 3b). Overall, the HIFU digestion identified 470 more peptides (~40%) than the 4-h thermomix digestion. The overlap between the two digestion methods was greater than the

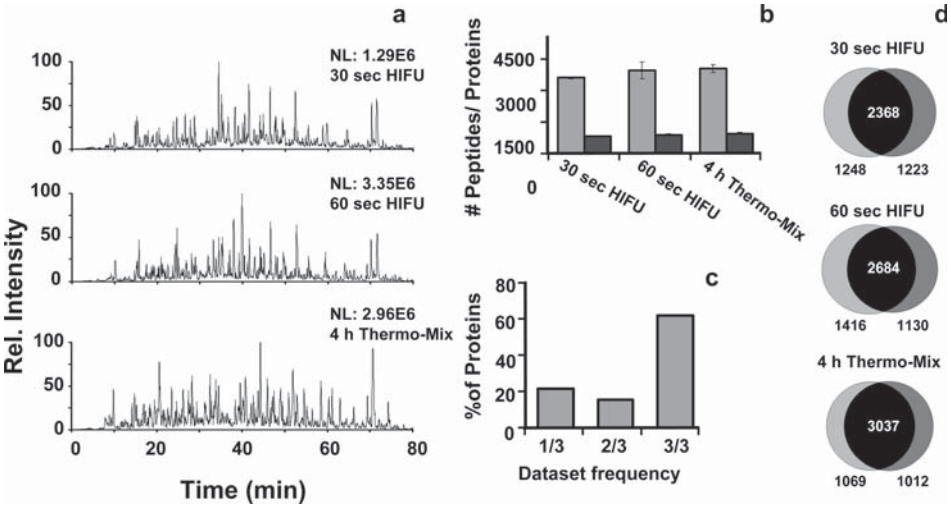

Figure 2. Evaluation of the digestion process combining ultrasonic energy and immobilized trypsin using a *Shewanella oneidensis* cell extract. (a) The representative chromatograms corresponding to different digestion times show a great similarity, which is further demonstrated by (b) the histograms obtained for each sample. Gray bars indicate unique peptide identifications, and dark bars indicate the number of identified proteins. (c) Histograms obtained by analyzing the percentage of proteins common to the different datasets. (d) Venn diagrams showing the peptide overlap between technical duplicates.

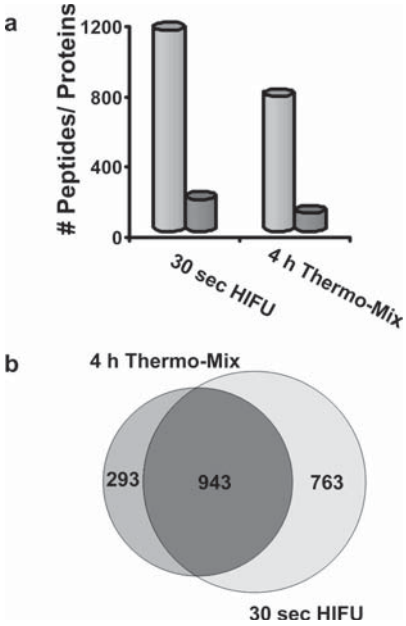

Figure 3. (a) Histogram showing the number of identified peptides (light-gray bars) and proteins (dark-gray bars), comparing a 30-s HIFU digestion versus a 4-h digestion using the thermomixer. (b) Venn diagram showing the overlap between the samples in terms of peptide identifications.

levels expected, even between technical replicates of the same sample in a typical shotgun proteomics experiment.[21]

Evaluation of HIFU and Immobilized Trypsin for the Digestion and ^{18}O Labeling of Complex Protein Mixtures. ^{18}O labeling strategies are useful for quantitative protein comparisons among samples.[4,22] However, C-terminal oxygens can back-exchange over time in the presence of residual active trypsin and nonlabeled aqueous solutions (i.e., when reconstituted in solution for LC-MS analysis),[4,21] and this can lead to inaccurate quantitation. HIFU coupled with immobilized trypsin beads can reduce such back-exchange by enabling fast removal of the trypsin-coated beads. To determine whether this method allowed for complete and reproducible labeling, we applied the labeling method to a mouse serum proteome for 30, 60, and 90 s. Afterward, the samples were quickly acidified to quench any remaining trypsin activity, the trypsin was removed by filtering the beads, and the ^{16}O- and ^{18}O-labeled samples were mixed in a 1:1 ratio and dried.

Statistical analysis shows that all quantified peptides followed a normal distribution with statistically significant changes (i.e., those with an FDR of <10%) occurring in <1% of the identified peptides (see Figure 4). This level of false identifications remained consistent through multiple replicate analyses (data not shown), which highlights the reproducibility and accuracy of the method for quantitative experiments. As a control experiment to evaluate the complete proteomic workflow, two identical aliquots of *Shewanella oniedensis* proteins were subjected to reduction and alkylation, digestion, and labeling, using immobilized enzymes and HIFU. As shown in Figure 5, more than 90% of the quantified pairs had a labeling efficiency of >90%, which allowed for accurate peptide quantitation, even with an irradiation time of only 30 s.

The work presented here demonstrates that the use of immobilized trypsin in the presence of ultrasonic energy produces a faster and more-complete digestion and/or ^{18}O exchange compared to classic thermomixing techniques. Several components in this method contribute to the acceleration of the process. First, in the immobilized form, trypsin maintains an active conformation in the presence of ultrasonic energy and other harsh conditions, such as the presence of organic solvents and elevated temperatures. Second, the increased load capacity of immobilized trypsin increases the reaction rate, because of the higher enzyme-to-protein ratio. Third, with our method, we create microstreaming events in the solution, which are propagated by the immobilized trypsin beads. Microstreaming is a phenomenon whereby the acoustic waves facilitate

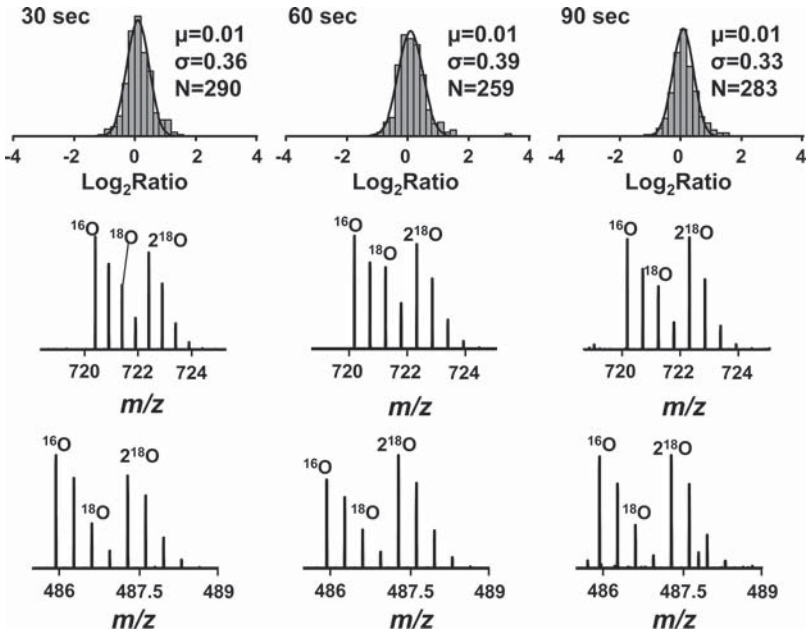

Figure 4. Results obtained in a negative control experiment using HIFU and immobilized trypsin for post-digestion labeling. Upper panel shows the results obtained by fitting the histogram of the $\log_2(^{16}O/^{18}O)$ ratios to a Gaussian function for different irradiation times. The bottom panel shows representative MS spectra for two peptides from the experiment: VVIEDGVGDAVLTR and PPTVTITSR.

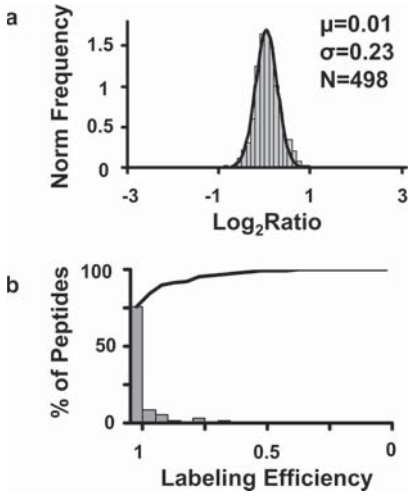

Figure 5. Summary of the results obtained following HIFU assisted digestion and labeling with immobilized trypsin applied to the *Shewanella oneidensis* proteome. (a) Histogram of the $\log_2(^{16}O/^{18}O)$ ratios fitted to a Gaussian function. (b) Histogram of the labeling efficiency calculated from the quantified peptides. The solid line represents the cumulative frequency of the labeling efficiency.

mixing because of the presence of microparticles or air bubbles,[22−24] and has been applied to accelerate DNA hybridization reactions in biochips..[25,26] This phenomenon is often incorporated into ultrasound processes to avoid a potential drawback of sonication, which is that acoustic waves in the liquid can lose energy due to resistance as they propagate through the solution. These loses are considerable and especially prevalent when the waves encounter the plastic microfuge tube walls that absorb most of the energy.[22]

CONCLUSIONS

We successfully demonstrated a method for coupling HIFU with immobilized trypsin to perform ultrarapid digestion and/or ^{18}O labeling for proteome applications. The method proved successful for rapidly digesting the globular protein bovine serum albumin and the complex proteome mixtures of *S. oneidensis* lysates and mouse plasma. Although the denaturing effects of ultrasonic irradiation enhanced the digestion of plasma proteins, no appreciable differences were observed between this and traditional protocols, although the digestion time was reduced from 4 h to less than a few minutes. When the technique was applied to post-digestion ^{18}O-labeling experiments, more than 90% of the quantified pairs had a labeling efficiency of >90%, which allowed for accurate peptide quantitation, even with an irradiation time of only 30 s. In addition, the digestions proved to be robust and reproducible, as illustrated by the extremely tight Gaussian distributions.

We anticipate the ultrarapid labeling method presented here will become the mainstay for quantitative proteome analysis, not only for two-sample comparisons but also for large-scale multiple sample analyses.[5] The combined use of highly stable enzymes and ultrasound provides a means of dramatically reducing both sample preparation times and digestion and labeling costs. The method is compatible with many downstream workflows in proteome analysis such as HPLC fractionation and it is amenable to automation, making faster, more reproducible high-throughput proteomics achievable. The combination of highly stable enzymes and HIFU cavitation microstreaming in microfluidic devices holds the potential for making the ultimate lab-on-a-chip a reality for proteomic experiments.

Abbreviations

ACN	acetonitrile
AMT	accurate mass and time
BSA	bovine serum albumin
ESI	electrospray ionization
FDR	false discovery rate
HIFU	high-intensity focused ultrasound
HPLC	high-pressure liquid chromotography
IAA	iodoacetamide
ICAT	isotope-coded affinity tags
LC-MS/MS	liquid chromotography tandem mass spectrometry
PCR	polymerase chain reaction
SPE	solid-phase extraction
TCEP	Tris[2-carboxyethyl]phosphine
TFA	trifluoroacetic acid

ACKNOWLEDGMENT

The authors thank Dr. Tyler Heibeck, Dr. Weijun Qian, Dr. Matthew Monroe, Angela Norbeck, and Penny Colton for their helpful assistance and suggestions. Moreover, the EMSL High-Throughput Proteomics group for technical assistance in the mass spectrometry portion of this work. Portions of this work were supported by the NIH National Center for Research Resources (RR018522), NIH National Cancer Institute (R21 CA12619-01), and the Pacific Northwest National Laboratory's (PNNL) Laboratory Directed Research and Development Program. PNNL is operated for the U.S. Department of Energy by Battelle, under Contract No. DE-AC05-76RLO1830.

SUPPORTING INFORMATION AVAILABLE

This material is available free of charge via the Internet at http://pubs.acs.org.

(21) Resing, K. A.; Meyer-Arendt, K.; Mendoza, A. M.; Aveline-Wolf, L. D.; Jonscher, K. R.; Pierce, K. G.; Old, W. M.; Cheung, H. T.; Russell, S.; Wattawa, J. L.; Goehle, G. R.; Knight, R. D.; Ahn, N. G. *Anal. Chem.* **2004**, *76*, 3556–3568.
(22) Shoh, A. *IEEE Trans. Sonics Ultrason.* **1975**, *Su22*, 60–71.
(23) Liu, R. H.; Lenigk, R.; Druyor-Sanchez, R. L.; Yang, J.; Grodzinski, P. *Anal. Chem.* **2003**, *75*, 1911–1917.
(24) Liu, R. H.; Yang, J.; Pindera, M. Z.; Athavale, M.; Grodzinski, P. *Lab Chip* **2002**, *2*, 151–157.
(25) Liu, R. H.; Yang, J.; Lenigk, R.; Bonanno, J.; Grodzinski, P. *Anal. Chem.* **2004**, *76*, 1824–1831.
(26) Paxton, W. F.; O'Hara, M. J.; Peper, S. M.; Petersen, S. L.; Grate, J. W. *Anal. Chem.* **2008**, *80* (11), 4070–4077.

Received for review December 1, 2008. Accepted May 24, 2009.

AC802540S