Impact of AFA-energetics™ on Enzymatic Hydrolysis of Proteins

Abstract: A novel fully automatable approach to improve trypsin digestion of protein samples is presented. Proof of concept was achieved through the quantification of model substrate BSA Proteinase K- and Trypsin-hydrolysis efficiency via MALDI-TOF. Proteinase K-hydrolysis was performed in 96 deep well plates and Trypsin digests in 384-well plates through the application of Adaptive Focused Acoustics® (AFA®) and compared to standard heat block-mediated incubations. MALDI-TOF traces of small peptides resulting from heat block controls and AFA treatments revealed a significant increase hydrolysis efficiency, observed as early accumulation of low molecular weight peptides and intermediate products from the AFA-treated samples. The presented assay in 384-well plates is readily transferable to other plate or strip tube formats and for any enzyme. Compatible Covaris instrumentation includes the LE220-plus/LE220R and LE220Rsc Series (96-well and 384-well plates), and the ML230 (8-strip tubes).

Introduction

Enzymatic digestion is a crucial step of sample preparation before mass spectrometry (MS), if not the most important protein processing step in proteomics. Trypsin is a serine protease that specifically cleaves proteins at the C-terminus of lysine or arginine residues, creating peptides in the preferred mass range for MS analysis. It is by far the most used enzyme, and numerous protocols have been established [1]. With the emergence of proteomics in clinical use, it became important to set up recommendations for proper peptide generation, as introduced by the Clinical Proteomic Tumor Analysis Consortium (CPTAC) of the National Cancer Institute [2]. These recommendations would also be in favor of more harmonization between methods.

In addition, proteins represent a very heterogenous population by nature, differing for instance in their sequences and posttranslational modifications, which can affect digestion efficiency and speed. Clinical practice requires a rapid delivery of reproducible results in a suitable throughput. Covaris AFA can make digestion faster, more reproducible, and at the required scale, which naturally suits clinical needs while also benefiting any other mass spectrometry lab.

It has already been published that AFA can be used to accelerate the enzymatic hydrolysis process [3,4]. Additionally, recent internal data has shown a high degree of temperature control as well as an increased rate of BSA intermediate Proteinase K digestion with AFA. In this document, we present new data which elevates trypsin digestion to the next level, by 1) reaching efficient and reproducible digestion in under 30 minutes, and 2) for up to 384 samples at a time. The suggested protocol can be utilized with enzymes other than trypsin and can be adapted to any Covaris instrument to achieve similar precision, efficiency, speed, and reproducibility. This method will be of utmost complementarity with existing single pot protocols already involving AFA for cell lysis and sample homogenization [5,6].

Materials

Trypsin Digestion

Equipment

- LE220Rsc Focused-ultrasonicator (PN 500652)

Consumables

- 384 AFA-TUBE 20 PP Plate (PN 520303)
- Foil Seal (PN 520073)

Reagents

- Pierce™ Bovine Serum Albumin (Thermo Fisher, 23209)
- Pierce™ Trypsin Protease (Thermo Fisher, 90057)
- DTT (Sigma, 646563)
- Pierce™ Iodoacetamide (Thermo Fisher, A39271)
- Ammonium bicarbonate (Sigma, A6141)
- Acetic acid (Sigma, 320099)
- Urea (Sigma, U5378)
- α-cyano-4-hydroxycinnamic acid (Sigma, C2020)

Instrument	LE220Rsc
PIP (W)	300
DF (%)	30
CPB	50
Water Bath (C)	30
Scan Speed (mm/s)	10
Iteration	N is based on desired incubation time

Table 1. Trypsin digestion AFA settings for LE220Rsc with the 384 AFA-TUBE 20 PP Plate. AFA treatment iteration number is dependent on the desired digestion time and can be varied to suit user needs.

Methods

Prior to AFA-based trypsin digestion, BSA was reduced and alkylated. A 20 µg sample of BSA was diluted in a buffer containing 8 M urea, 20 mM DTT, 50 mM ammonium bicarbonate (ABC), pH 7.7 to a final volume of 100 µL. The sample was reduced for 1 hour at 37 °C. Following the incubation, iodoacetamide was added to a final concentration of 40 mM and the sample was alkylated at room temperature for 30 minutes in the dark. The reaction was quenched by adding DTT to a final concentration of 10 mM. To facilitate trypsin digestion, the sample was diluted with 50 mM ammonium bicarbonate to reduce urea concentration below 1 M.

Reduced/alkylated BSA (300 ng/10 µl per well) was pipetted into a 384 AFA-TUBE 20 PP Plate. Immediately before AFA treatment, 1 µL of diluted trypsin was added to all samples for a final Trypsin : BSA mass ratio of 1:20. Plates were covered with foil seals, inserted into the Focused-ultrasonicator and treated as outlined in *Table 1*. To achieve an incubation time of 10 or 30 minutes, 26 and 78 iterations of AFA scanning were utilized, respectively. Control samples were prepared in 1.5 mL Eppendorf tubes and incubated in a heat block for either 10 or 30 minutes. Following incubation, all samples were frozen at -80 °C to halt the reaction.

Hydrolysates obtained via heat block and AFA-enhanced incubations were analyzed by MALDI-TOF Mass Spectrometry. Samples were desalted with C18 pipette tips (Thermo Scientific, 87782) according to manufacturer's protocol, and eluted in 70% acetonitrile/0.1% TFA. Eluates were mixed with an equal volume of a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile/1% TFA. A 2 µL aliquot was pipetted onto a MALDI target plate (Bruker, 8280800) before drying in a desiccator over silica gel. MALDI analysis was performed with a Bruker Microflex LT system in positive ion linear mode with a nitrogen laser (337 nm) set to 20% power, with a 0.50 GS/s sample rate, and a detector voltage of 2990, firing 10,000 shots per sample in a randomized pattern.

Proteinase K Digestion

Equipment
- LE220-plus Focused-ultrasonicator (500569)
- Electrophoresis cell (Bio-Rad Criterion Cell, 1656001 or equivalent)
- Power Supply (Bio-Rad PowerPac HC High-Current Power Supply, 1645052 or equivalent)
- Gel Imager (Bio-Rad Gel Doc EZ System, 1708270EDU or equivalent)

Consumables
- 96 Well 0.8mL Polypropylene Deepwell Storage Plate (Abgene AB0765)
- Foil Seal (PN 520073)
- Protein Gel (Bio-Rad 4–20% Criterion TGX Stain-Free Protein Gel 5678093)

Reagents
- Pierce™ Bovine Serum Albumin (Thermo Fisher, 23209)
- Proteinase K (Roche, 03654672103)
- Low EDTA TE (Thermo Scientific, J75793-AP)
- Laemmli Sample Buffer (Bio-Rad, 1610737)
- Tris/Glycine/SDS Running Buffer (Bio-Rad, 1610732)
- 2-Mercaptoethanol (Sigma, 63689)

Instrument	LE220Rsc	
PIP (W)	500	390
DF (%)	50	20
CPB	1000	1000
Water Bath (C)	20	20
Scan Speed (mm/s)	8	3 on / 3 off
Iteration	1	N is based on desired incubation time

Table 2. Proteinase K digestion AFA settings for LE220-plus with 96 Well 0.8 mL Polypropylene Deepwell Storage Plate. AFA treatment iteration number is dependent on the desired digestion time and can be varied to suit user needs.

A40 µg BSA in 190 µL of Low-EDTA TE was pipetted into the wells of a 0.8 mL Polypropylene Deepwell Storage Plate. Plates were covered with foil seals, inserted into the Focused-ultrasonicator and treated as outlined in *Table 2*. Immediately after the initial 8 second treatment, 10 µL Proteinase K was added to sample wells with a multichannel pipette. Samples were treated with AFA for 10 minutes as outlined in *Table 2* using 100 iterations of AFA pulses. Control samples were prepared in 1.5 mL Eppendorf tubes and incubated in a heat block for 2 minutes at 56 °C prior to addition of Proteinase K and further incubation at 56 °C for 10 minutes.

Reactions were stopped by addition of Laemmli sample buffer containing 2-mercaptoethanol and boiling for 5 minutes. Control and AFA-energetics treated samples were analyzed via SDS-PAGE

(4 to 20% Criterion TGX Stain-Free Protein Gel; electrophoresis for 40 min at 200 V). Gels were imaged in a Gel Doc EZ imager utilizing a 5 minute gel activation treatment. Gel densitometry was performed with Image Lab Version 6.

Results

Proteinase K Digestion

In contrast to conventional heat-block incubation, AFA-energetics can be used to incubate samples at a target temperature of 52 to 54 °C. The instrument water bath temperature was set to 20 °C. During AFA-settings optimization, the internal temperature of a 200 µL sample in a 96-well 0.8 mL Polypropylene Deepwell Storage Plate was monitored for 5 minutes. The established settings are shown in *Table 2*. An initial 8 second burst is used to raise the temperature to >52 °C, and is followed by a pulsed treatment to maintain a constant temperature. Temperature profiles oscillate slightly as theresult of the pulsing feature (*Figure 1*). The average temperature was calculated to be 52.6 °C (±1.0 °C).
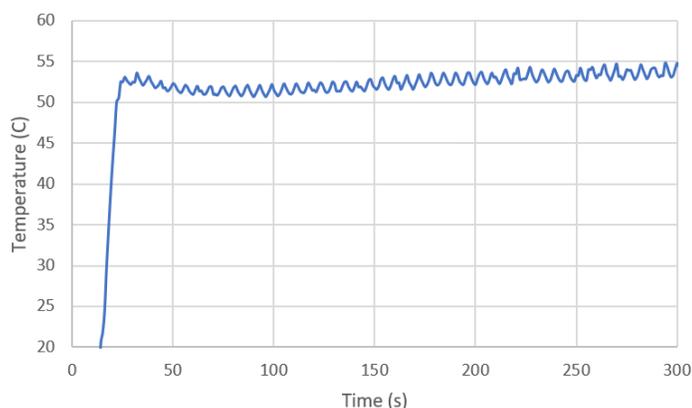
Figure 1. Temperature profile of a 200 µL sample inside the wells of a 96-well 0.8 mL Polypropylene Deepwell Storage Plate treated with AFA on the LE220-plus.

BSA hydrolysis was analyzed by SDS-PAGE (disappearance of the main undigested BSA band at 60 kDa; *Figure 2A*) as described in Methods. Note, that AFA-treated BSA solutions in absence of protease shows no detectable degradation/fragmentation of BSA (not shown). Intermediates of the hydrolysis were also analyzed via gel densitometry. Generation and subsequent extinction of a 50 kDa intermediate is shown in *Figure 2B*. While a similar rate of BSA digestion is observed when comparing AFA-energetics and heat block-treated samples, the rate of intermediate generation and subsequent further hydrolysis suggests a significantly higher turnover rate of digest intermediates.
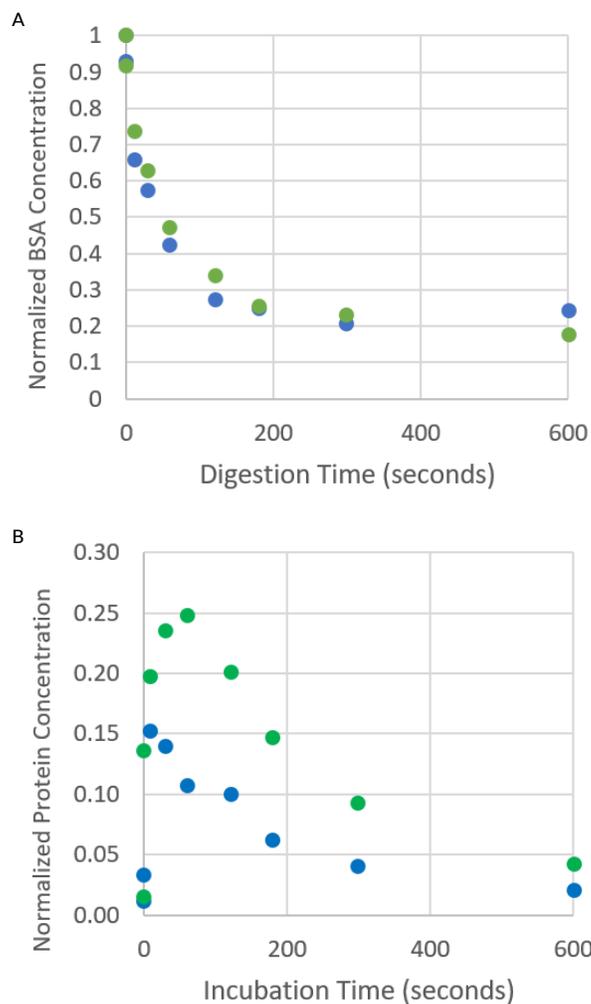
Figure 2. (A) Gel densitometry analysis of BSA during a Proteinase K digestion time course (AFA - blue; Heat Block - green). (B) Gel densitometry analysis of 50 kDa BSA intermediate during a PK digestion time course (AFA - blue; Heat Block - green).

Trypsin Digestion

In contrast to incubation with a simple heat block, AFA-energetics was used to heat samples to a target temperature of 37 °C. The instrument water bath temperature was set to 30 °C. During optimization, the internal temperature of a 10 µL sample in a 384 AFA-TUBE 20 PP Plate was monitored for 20 minutes. The established settings are shown in *Table 1*. Temperature profiles oscillate slightly due to the scanning feature, exposing samples for 0.44 seconds per column as the plate moves over the Focused-ultrasonic transducer (*Figure 3*). The average temperature was calculated to be 35.8 °C (±1.4 °C).

Trypsinized BSA was analyzed by MALDI-TOF mass spectrometry as described in **Methods**. AFA-treated and heat block sample mass spectra were analyzed to compare each peptide by calculating the difference between their relative peak intensities. Results obtained after 10 minute (**Figure 4**) and 30 minute (**Figure 5**) trypsinizations are shown. Data was plotted such that greater positive values indicate a higher concentration of peptide for AFA-treated samples. Conversely, more negative values indicate a higher concentration of peptide for heat block samples. The results in **Figure 4A** reveal that trypsinization with AFA yields more digested peptide fragments than with a heat block after 10 minutes. The results in **Figure 5A** reveal that trypsinization with AFA yields a higher concentration of low molecular weight fragments, while standard heat block digestion produces more high molecular weight peptides.
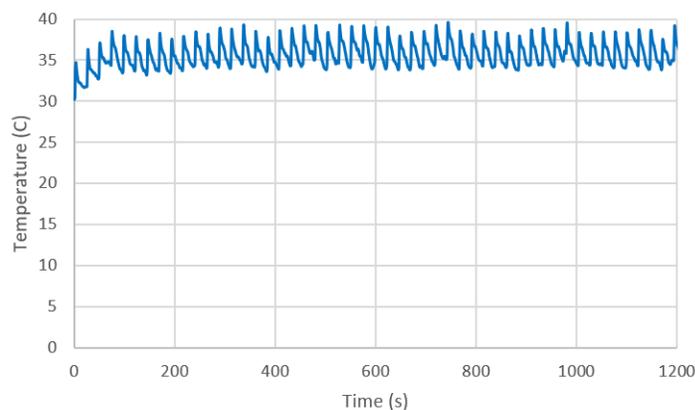

Figure 3. Temperature profile of a 10 μL sample inside the wells of a 384 AFA-TUBE 20 PP Plate treated with AFA on the LE220Rsc.
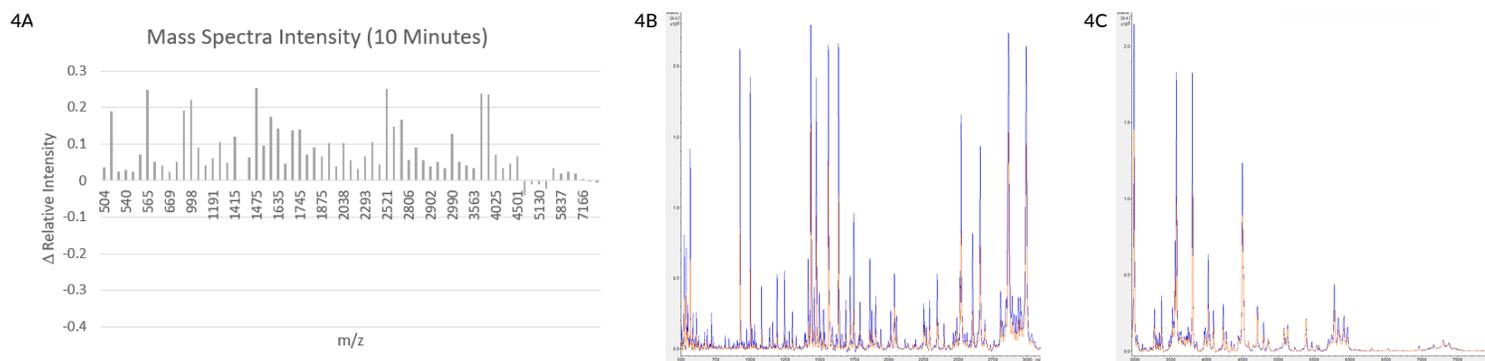

Figure 4. Difference between the relative mass spectrometry intensity data of BSA trypsinized using AFA or heat block for 10 minutes (A). Overlay of MALDI-TOF mass spectra (AFA - blue; Heat Block - orange) ranging 500 to 3000 m/z (B) and 3000 to 7500 m/z (C). The bar graphs were generated by subtracting the peak intensity of the heat block samples from peak intensities of the AFA treated samples.
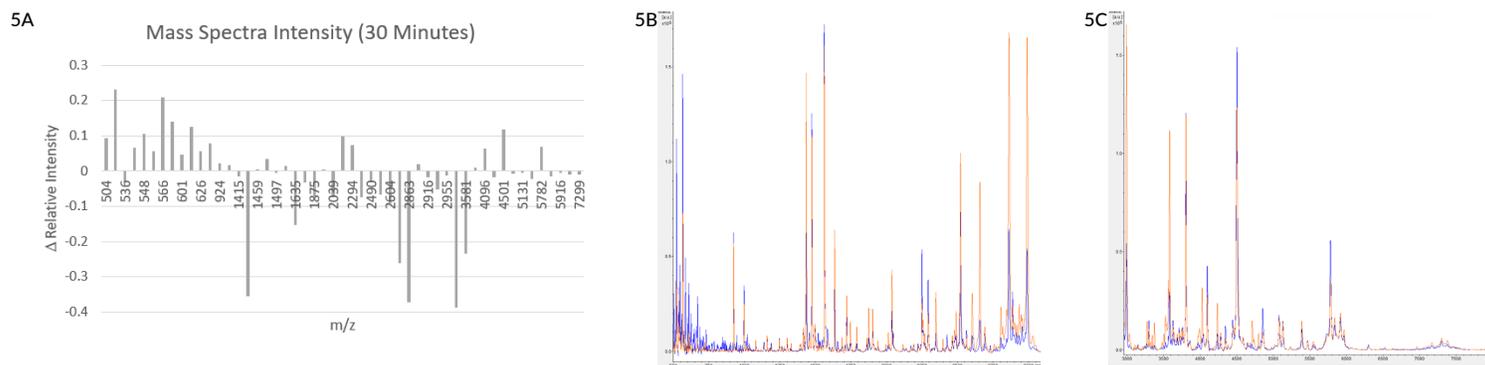

Figure 5. Difference between the relative mass spectrometry intensity data of BSA trypsinized using AFA or heat block for 30 minutes (A). Overlay of MALDI-TOF mass spectra (AFA - blue; Heat Block - orange) ranging 500 to 3000 m/z (B) and 3000 to 7500 m/z (C). The bar graphs were generated by subtracting the peak intensity of the heat block samples from peak intensities of the AFA treated samples.

Conclusion

AFA-energetics is a contact-free method that can provide energy in the form of heat when required. More importantly however, the energy can be utilized to cause fluctuations in matrix pressure. This contributes to micro-mixing and to increased molecular movement of peptide and protein chains/residues. As demonstrated in the Proteinase K digestion experiment performed under non-denaturing conditions (Low-EDTA TE), these factors may facilitate faster presentation/binding to the protease and accelerate disassociation of cleaved fragments from the initial substrate for faster turnover of intermediates.

Data from our trypsin digestion experiments report the rapid accumulation of peptide fragments from BSA trypsinized with AFA for 10 minutes, suggesting an increased rate of protein digestion as compared to trypsinization with a heat block. The majority of peptides produced with heat block incubation are unlikely to be fully digested and are therefore beyond the investigated MALDI-TOF mass range. Increasing treatment time to 30 minutes drastically changes the mass spectra. AFA samples continue to digest, resulting in an accumulation of low molecular weight fragments and a loss of high molecular weight fragments. The partially digested peptides of heat block samples are further trypsinized, yielding a higher concentration of peptides in the 1500 to 3500 m/z range. These results suggest that the rate of trypsin digestion of BSA is higher when heating with AFA than with a standard heat block.

We have shown robust data demonstrating the acceleration of BSA proteolysis with AFA as compared to standard heat block incubation. These protocols utilize either 96 deep well plates (large volume Proteinase K digests) or 384 AFA-TUBE 20 PP Plates and the Covaris LE220-plus or LE220Rsc Focused-ultrasonicator to perform trypsin digestion of protein samples. Reaction volumes and treatment times can be adjusted to meet the needs of the end user; they can be adapted for other Covaris instruments in 8-strip or 96-well formats, and for other enzymes. Ultimately, protocols outlined in this technical note will be utilized by researchers aiming to accelerate their digestion steps or looking for an optimized single pot protocol involving cell lysis.

References

1. Gundry RL, White MY, Murray CI, et al. Preparation of proteins and peptides for mass spectrometry analysis in a bottom-up proteomics workflow. In: Ausubel FM, Brent R, Kingston RE, et al., eds. Current Protocols in Molecular Biology. John Wiley & Sons, Inc.; 2009:mb1025s88. DOI: 10.1002/0471142727.mb1025s88

2. Hoofnagle AN, Whiteaker JR, Carr SA, et al. Recommendations for the generation, quantification, storage, and handling of peptides used for mass spectrometry–based assays. Clinical Chemistry. 2016;62(1):48-69. DOI: 10.1373/clinchem.2015.250563

3. Acoustic technology-assisted rapid proteolysis for high-throughput proteome analysis, Kim et al, ANALYTICAL SCIENCE & TECHNOLOGY, Vol. 24, No. 6, 510-518, 2011 DOI: 10.5806/AST.2011.24.6.510

4. Lee B, Kim BC, Chang MS, et al. Efficient protein digestion using highly-stable and reproducible trypsin coatings on magnetic nanofibers. Chemical Engineering Journal. 2016;288:770-777. DOI: 10.1016/j.cej.2015.12.003

5. Müller T, Kalxdorf M, Longuespée R, Kazdal DN, Stenzinger A, Krijgsveld J. Automated sample preparation with SP 3 for low-input clinical proteomics. Mol Syst Biol. 2020;16(1). DOI: 10.15252/msb.20199111

6. AFA-sonication Followed by Modified Protein Aggregation Capture (APAC) Enables Direct, Reproducible and Non-toxic Sample Preparation of FFPE Tissue for Mass Spectrometry-based Proteomics. Schweitzer et al., Covaris Application Note, May 2020 - covaris.com/wp/wp-content/uploads/2020/06/M020141.pdf