Analysis of Microwave-Assisted Enzymatic Digests of Hemoglobin by Mass Spectrometry

Luchuan Mi, Hubert W. Vesper, Maria Ospina, Gary L. Myers

Protein Biomarker Laboratory
Division of Laboratory Sciences
National Center for Environmental Health
Centers for Disease Control and Prevention
Atlanta, Georgia
Background

- Increasing numbers of applications to quantify peptides and proteins in clinical and environmental chemistry created a demand for rapid identification and quantification of proteins and protein modifications. Most of these applications focus on modifications of the N-terminal valine of hemoglobin.

- The standard procedure for obtaining information about these hemoglobin modifications is digesting the protein with endopeptidases and analyzing resulting peptides by mass spectrometry.

- The digestion time depends on the nature of the proteins and enzyme, and varies from hours to days. Thus, digestion time has become a limiting factor in the speed of the protein identification and quantification processes.

- Previous research using other proteins indicated microwave energy is a viable technology in accelerating protein digestion and increasing sample throughput. However, its applicability to protein quantification still remains unknown.
Objective

- Fully-controlled, programmed, and focused microwave energy may greatly accelerate enzymatic digestions. This study assesses this technology for its applicability to protein quantification study.

- The main objective is to reduce digestion time of hemoglobin using this new technology while maintaining the digestion efficiency and reproducibility of conventional digestion.
Methods

- Enzymatic digestion of hemoglobin was carried out with trypsin.
- Microwave digestion was performed with a CEM® Discover™.
- Reaction products were analyzed on a Finnigan LCQ™ DECA ion trap mass spectrometer.
- Hexapeptide (VHLTPE, N-terminal hexapeptide of the β-chain of hemoglobin) was used as the internal standard (IS) since it is not affected by trypsin.
- The same amount of IS and protein were used in all experiments, so the digestion efficiency can be estimated as the area ratio between the N-terminal octapeptide of the β-chain of hemoglobin and the IS. The doubly-charged ions for both peptides were selected to assess the extent of the digestion due to their better S/N ratio.
Different Digestion Methods

Microwave-Assisted Digestion

Conventional Digestion
LC/MS Details

- Samples were injected onto a 2-mm i.d., C-12, reverse phase column (Phenomenex Jupiter 4µ Proteo 90 Å).
- Gradient: from 100%A (A = 0.025% TFA in water and B = 0.025% TFA in acetonitrile) to 40%A over 100 min., flow rate: 200 µL/min.
- Full MS scan was selected for all experiments.
  - Mass range: 150-2000 m/z
  - Spray voltage of ESI source: 5.5 kV
  - Sheath gas: 80 (arbitrary unit)
  - Auxiliary gas: 20 (arbitrary unit)
  - Inlet capillary temperature: 175 °C
  - Inlet capillary voltage: 25 V
Microwave Instrument

Key Features:

- Single-mode cavity design
- Temperature and pressure feedback control
- Vessel flexibility
- Reaction quenching

Microwaves couple directly with the molecules present in the reaction mixture, leading to a rapid rise in temperature and increasing the reaction rate.
Example Chromatogram

Total ion chromatogram of trypsin digest of Hb-A0 (20 minutes, 50 °C, 100 mM ammonium bicarbonate buffer)
Experiment Details

- Hemoglobin-A0 was treated with trypsin at a protease-to-protein ratio (w/w) of 1:25, 1:50, 1:100, and 1:200.

- Ammonium bicarbonate buffer concentrations were 25, 50, and 100 mM (pH: 8.5).

- Microwave irradiation was performed at controlled temperatures of 40, 45, 50, 55, and 60 °C.

- Microwave irradiation was performed for a duration of 5 to 45 minutes, in steps of 5 minutes.

- Conventional digestion was performed at 37 °C for 18 hours.
Efficiency vs. Digestion Time

- Microwave-Assisted Digestion
- Conventional Digestion

Digestion Time (min.)

Efficiency
Effect of Temperature & Buffer Conc. (20-min. digestion time)
Results

- Digestion efficiency of hemoglobin-A0 reached plateau after 20 minutes.
- The highest degree of digestion occurred at 50 °C.
- Increasing buffer concentration from 25 mM to 50 mM resulted in a significant efficiency gain (50% at 50 °C), but increasing it further to 100 mM resulted only in a minor increase in efficiency (6%).
- Increasing the protease-to-protein ratio resulted only in a small increase in digestion efficiency (13% gain from 1:200 to 1:25 ratio).
- The observed digestion efficiency at optimal conditions was higher than that of conventional digestion (2.0 vs. 1.6).
- The reproducibility at optimal conditions was 5% CV.
Discussion

- Microwave-assisted protein digestion is described in literature mainly for peptide-mapping and qualitative protein analysis. The described optimal digestion conditions are similar to those found in this study (60 vs. 50 °C, 10 vs. 20 min.). Differences may mainly be due to the use of different proteins.

- The reproducibility was found to be comparable to conventional methods.

- The digestion time is profoundly reduced with this technology (20 min. vs. 18 hours) at increased efficiency, making this technology an interesting alternative to conventional digestion.

- This study focused on N-terminal octapeptide of the β-chain of hemoglobin-A0 only. The digestion efficiency with regard to other peptides remains to be assessed.
Conclusion

Microwave-assisted digestion of hemoglobin A0 with trypsin is an alternative to conventional digestion technology. The main advantages seem to be:

- Shorter digestion time (20 minutes vs. 18 hours)
- Increased efficiency (2 vs. 1.6)
- Good reproducibility (5% CV)
References


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Questions? Comments?
- Dr. Hubert W. Vesper: hvesper@cdc.gov
- Dr. Luchuan Mi: lmi@cdc.gov