A Comparison of Methods for Efficient Digestion of Protein Therapeutics

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ABSTRACT

Accomplishment of enzymatic proteolysis via conventional overnight digestion of a therapeutic monoclonal antibody with endoprotease Lys-C was compared to digestion using microwave assisted and pressure cycling technologies. Efficient digestion of proteins using both technologies has been reported in the literature; however there has been limited application to protein therapeutics.

The degree of digestion (number of missed cleavages), and time to achieve complete digestion were investigated for each technique, with conventional digestion used as the control for all experiments performed. Pressure cycling was shown to be most effective at achieving complete digestion in a short time, without unintended perturbation of the molecule. Microwave digestion did not achieve complete digestion and induced oxidation of methionine residues.

INTRODUCTION

Peptide mapping is a critical tool for identification and characterization of protein therapeutics, including monoclonal antibodies. Indeed, this method serves as a fundamental technique for analysis of a protein's primary structure and degradation products. Conventional proteolysis, however, has limitations associated with the long duration required to achieve acceptable levels of digestion. By exploring new techniques such as microwave assisted or pressure cycling digestion, new, more efficient protocols for achieving protein digestion could potentially improve sample preparation times thereby creating cost savings and improving throughput without compromise of data quality.

Monitoring of digestion efficiency may be accomplished in several ways, such as SDS-PAGE or SE-HPLC to assess the presence of intact protein. However, in the presented work, the ratio of the L1-L2 miscleave and cleavage products were monitored as a surrogate for the entire protein. This peptide contains a Lys-Pro cleavage site as well as a disulfide bond, conferring resistance to proteolysis that has been well characterized through prior method development. The utility of a non-reduced digest rather than preliminary reduction and alkylation of the protein also presented a more challenging digest target.

METHODS

A recombinant monoclonal antibody of the IgG1 subclass was denatured with a chaotropic agent (8 M guanidine HCl) for 30 min at 37°C, then digested with endoprotease Lys-C (10:1 substrate:enzyme). A matrix experimental design was employed to compare efficient approaches to digestion to an overnight control, as shown in Table 1.

Peptide mapping was performed via a rapid resolution reverse-phase separation employing two 50 x 2.1 mm C18 columns with two different particle sizes (3.5 µm and 1.8 µm) in series. Peptides were eluted using a linear gradient of 0.1% TFA in acetonitrile at 1%/min with a flow rate of 0.5 mL/min. Detection was accomplished by absorbance at 215 nm and mass spectrometry (Thermo LTQ XL).

REFERENCES

1. **"Microwave-assisted proteomics", Lill JR, et al, Mass Spectrom, 2007**

2. **"Application of pressurized solvents for ultrafast trypsin hydrolysis in proteomics: proteomics on the fly", Lopez-Ferrer, et al, J Proteome Res, 2008**

Table 1 – Experimental Design for Efficient Digestion

Pressure Cycling Instrument - Barocycler NEP2320 (Pressure Biosciences Inc.)	Microwave Assistance Instrument - Discover (CEM Corp.)
Experiment 1 – 25 kpsi to 35 kpsi (20 cycles, 90 sec/cycle constant)	Experiment 1 – 1, 2, 3 hr (10 W, 45 C constant)
Experiment 2 – 10, 20, 30 cycles (30 kpsi, 90 sec/cycle constant)	Experiment 2 – 1, 2, 3 hr (25 W, 45 C constant)
Experiment 3 – 60, 99 sec/cycle (30 kpsi, 20 cycles constant)	Experiment 3 – 1, 2, 3 hr (50 W, 45 C constant)
	Experiment 4 – 25, 37, 55 C (25 W, 1 hr constant)

Figure 1. Comparison of IgG1 peptide maps under non-reducing conditions

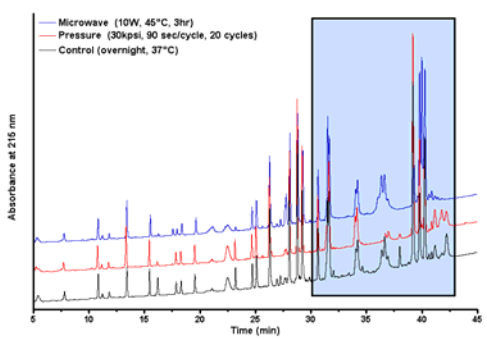


Figure 2. Zoomed region indicating markers for incomplete digestion

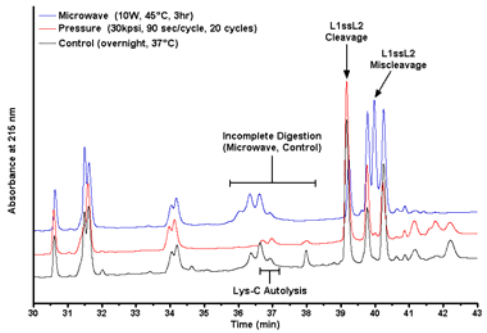


Figure 3. Digestion efficiency (cleavage:miscleave ratio of L1ssL2)

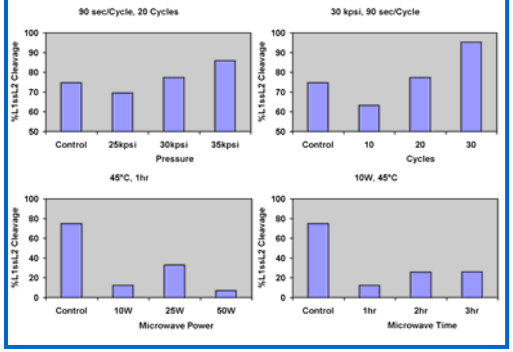


Figure 4. Digestion and degradation trends with varying microwave power

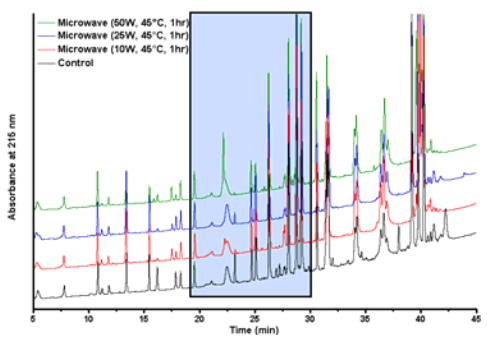


Figure 5. Zoomed region indicating methionine oxidation

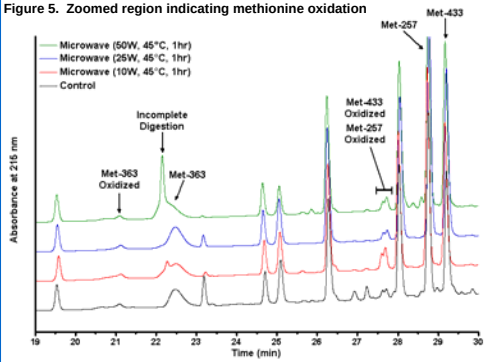
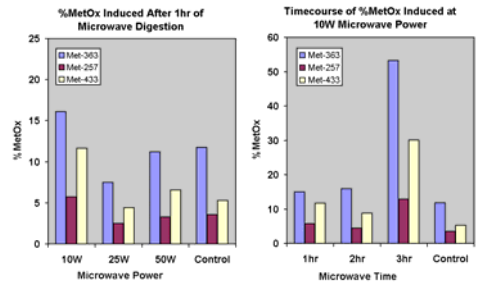


Figure 6. Induced methionine oxidation from microwave digestion

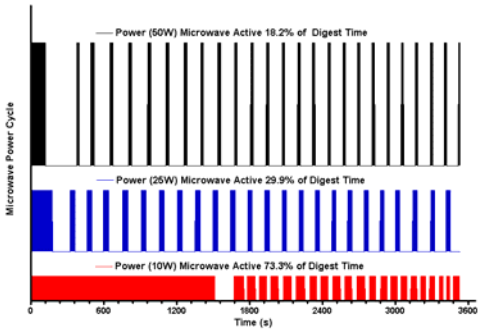


RESULTS

All three approaches demonstrated the ability to generate generally comparable peptide maps. The digestion times for control, microwave, and pressure cycling methods were overnight, 3 hr, and 30 min, respectively, clearly indicating a processing time and throughput advantage to the pressure cycling technology. Closer examination of the peptide maps indicate that two measures of cleavage efficiency (i.e. peaks corresponding to incompletely undigested material, and the ratio of signals for L1ssL2 cleavage and miscleave products) further support the pressure cycling method as yielding more complete digestion, even in a shorter time. The L1ssL2 miscleave product (eluting at ~40.0min) was the dominant species relative to the L1ssL2 cleavage product (eluting at ~39.5min) in all microwave assisted peptide maps; therefore the ability of the microwave method to drive digestion to completion was limited for challenging cleavage sites. In comparison, although in Figure 1 and 2 a small L1ssL2 miscleave peak is evident in the pressure cycled map, this was reduced further by either increasing the pressure above 30 kpsi, or increasing the number of 90 sec cycles from 20 to 30. In any event, the total digest time of the pressure cycling approach to yield essentially complete digestion was still less than 1hr, representing an increase in throughput and efficiency of at least 20-fold over the control conditions.

Figure 3 also illustrated that the microwave technique did not achieve more than 50% digestion of L1ssL2 for any of the time points investigated. Furthermore, as highlighted in Figures 4 and 5, the peptide maps derived from microwave digestion resulted in the presence of additional new peaks. Mass spectrometric analysis concluded that these were related to induced methionine oxidation. The degree of oxidation appears to be independent of the microwave power applied (Figure 6, left panel), but more likely correlated to the proportion of time that the microwave is active (Figure 6, right panel). This correlates with the fact that the microwave cycles are more frequent at higher power due to thermostatted shut-off. Hence, as shown in Figure 7, the overall net power applied is not directly correlated with the instrument setting.

Figure 7. Microwave power cycling and net power delivery



CONCLUSIONS

This study demonstrated that pressure cycling provided the most effective method for digesting monoclonal antibodies. Complete digestion can be obtained in a short period of time without inducing modifications such as methionine oxidation. While the microwave technique has established applicability in a proteomics setting, the more stringent requirements of the biopharmaceutical arena suggest limitations of the technique with respect for characterization of protein primary structure