Side Effect-Free Biotherapeutic Protein Sample Preparation for Peptide Mapping

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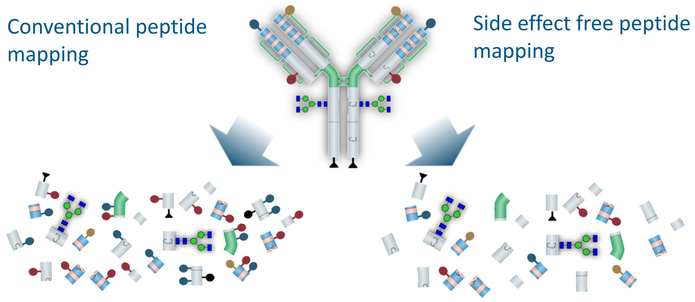
1. Introduction

Conventional peptide mapping



Side effect free peptide mapping

Artificial protein modifications are induced.

Artificial protein modifications are suppressed. Only authentic modifications are retained.

Non-enzymatic modifications such as deamidation, disulfide bond scrambling and oxidation can affect both the stability and efficacy of biotherapeutic proteins. Peptide mapping is the primary analytical method used to monitor these modifications. Unfortunately, steps involved in peptide mapping sample preparation are also a source of these modifications. We address this problem by performing the entire sample preparation at mildly acidic conditions which suppress deamidation and disulfide bond scrambling. Artificial oxidation is suppressed with a reactive oxygen scavenger. Procedural steps of the method are optimized to match the efficiency of the steps in conventional procedure.

The procedure has been adapted for use in common peptide mapping sample preparation procedures based on protein denaturation with GuHCl or Urea and size exclusion clean-up. It is readily amenable to automation.

2. Development of the low pH, side effect-free peptide mapping sample preparation method

Reduction and alkylation at low pH

Reduce with TCEP at low pH

Add IAM, incubate for 30 min and initiate digestion (at low pH)

Alkylation is allowed to proceed over the digestion period to compensate for decreased IAM activity at low pH.

Alkylation efficiency at low pH in a model IgG (mass spec analysis)

Alkylated peptides	Peptide peak intensity	
	Alkylated	Non-alkylated
CCVECPPCPAPPVAGPSVFLFPPKPK	1.69E+10	2.05E+07
LSSVTAADTAIYYCVR	6.4E+10	8.66E+07
NQVSLTCLVK	1.66E+11	1.75E+06
SGTASVVCLLNNFYPR	1.86E+11	6.93E+06

Cysteines are efficiently reduced and alkylated at low pH.

Trypsin protein digestion at low pH

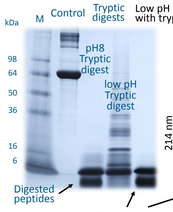
Missed cleavages (undigested cleavage sites) in yeast protein tryptic digest at low pH

Trypsin cleavage specificity

Trypsin activity at low pH is restored upon supplementation with a special, low pH resistant Lys-C protease.

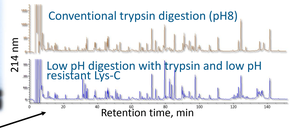
BSA digests with trypsin at low pH

SDS-PAGE



IgG digestion at low pH

RP HPLC



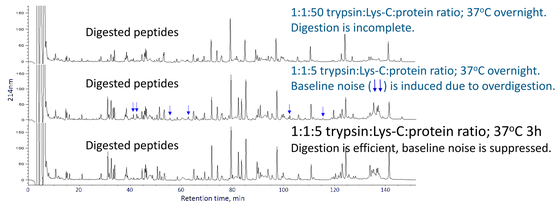
Efficient protein digestion at low pH with trypsin and low pH resistant Lys-C

3. Method optimization

Optimization of protein digestion at low pH

A protein was denatured with GuHCl and the reaction was diluted prior to digestion. The entire sample prep procedure was performed at low pH. Alternative sample prep procedures were also optimized for use with the low pH method (Chapter 4).

RP HPLC analysis of digests of a model IgG (Panitumumab)



3h digestion at 1:5 protease:protein ratio provides efficient digestion with suppressed baseline noise in the method.

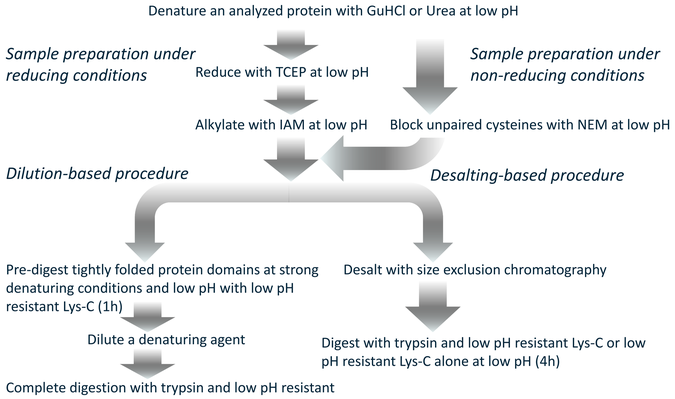
LC/MS analysis of low pH IgG digest

A model IgG (Panitumumab) was denatured, reduced, alkylated and digested at pH8 or low pH and analyzed with LC/MS.

	Conventional digestion (pH8)	Low pH digestion
Total spectra	1197	1289
Sequence coverage	97%	98%

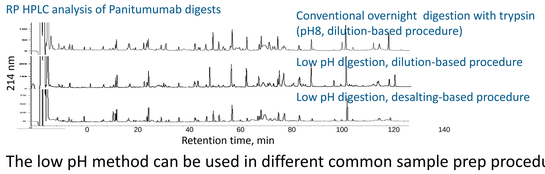
No deterioration in key performance parameters was observed in the low pH method.

4. Adaptation of the method for common sample preparation procedures



Performance of the method in different sample prep procedures

RP HPLC analysis of Panitumumab digests



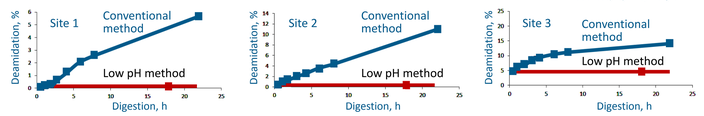
The low pH method can be used in different common sample prep procedures with high efficiency. It can also be readily automated (data not shown).

5. Case studies

Suppression of artificial deamidation induced in digestion step

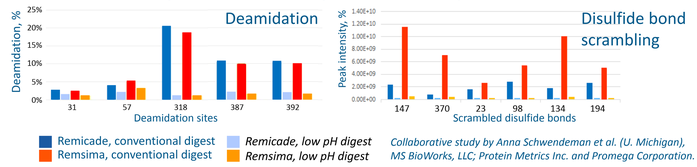
Model IgG was digested according to conventional and low pH methods and analyzed with LC/MS.

Courtesy of RIC, Belgium



Artificial deamidation is induced over the course of conventional protein digestion reaction. In the low pH method, artificial deamidation is suppressed. Authentic deamidation remains intact.

Accurate non-enzymatic PTM analysis in the low pH method



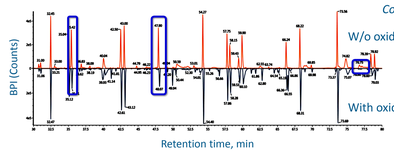
Remicade, conventional digest
Remsima, conventional digest
Remicade, low pH digest
Remsima, low pH digest

Collaborative study by Anna Schwendeman et al. (U. Michigan), MS BioWorks, LLC; Protein Metrics Inc. and Promega Corporation.

Artificial deamidation and disulfide bond scrambling are suppressed in the low pH method. This allows for accurate analysis of authentic non-enzymatic modifications.

Suppression of protein oxidation with the oxidation suppressant

Courtesy of Quality Assistance, Belgium



A model IgG (Adalimumab) was digested according to the method in the presence of an oxidation agent (hydrogen peroxide) with or without the oxidation suppressant, which we selected. The graph shows methionine oxidation.

6. Summary

- We have developed a peptide mapping sample preparation method, in which artificial deamidation, disulfide bond scrambling and oxidation commonly induced during sample preparation are suppressed.
- The entire sample preparation procedure is performed at low pH. All steps of the method including trypsin digestion are optimized to match the efficiency of conventional sample preparation.
- A compound with prominent oxygen scavenging capability is added to a sample to suppress artificial oxidation.
- The method assures high reproducibility and minimal baseline noise. The sample preparation is complete within 5h.
- The method can be readily incorporated into common sample preparation procedures and automated.

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