NIRMA UNIVERSITY

INSTITUTE OF PHARMACY

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PEPTIDE MAPPING: CUTTING EDGE TECHNIQUE- RECENT ADVANCEMENTS

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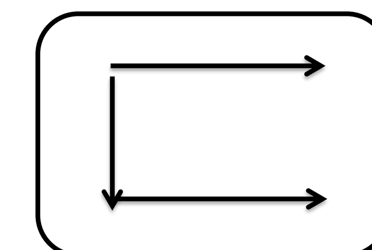


ABSTRACT:

Peptide mapping is a very important tool in biopharmaceuticals. It is a powerful technique most widely used to identify the sequence of amino acids in proteins. In this technique the digestion of protein is done by various protease enzymes like trypsin for degrading protein into amino acid and further into peptides. Peptides are like the biomatrix of the protein and by studying them we can study the whole protein formation and its structure. All proteins have different sequences of amino acids and peptide chain and to study the sequence of peptide chain in proteins, peptide mapping technique is useful. This technique becomes highly useful while manufacturing biosimilars or similar biologics to the reference biologic originator product because in the case of biosimilar to get exact quality, safety and efficacy as of the reference biologic the amino acid sequence should be exactly similar and thus to see whether the amino acid sequence is similar to the originator or not peptide mapping is used. Peptide mapping is usually carried out on HPLC or UPLC to separate and resolve the peptides and then as detector mostly mass spectroscopy is used thus combining LC-MS/MS for accurate identification and sequence analysis.

1. Introduction

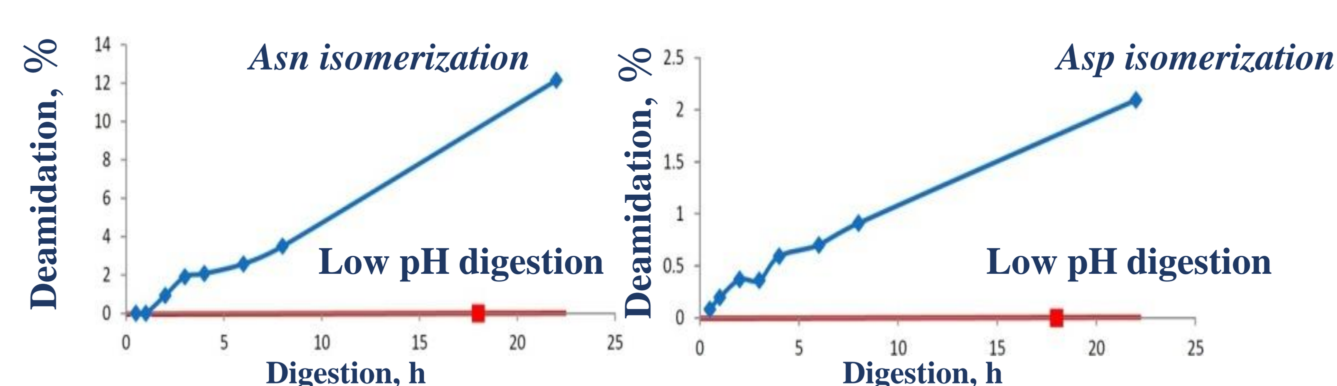
Non-enzymatic chemical modifications such as deamidation, disulfide bond scrambling and oxidation can affect the stability and efficacy of biotherapeutic proteins. Peptide mapping, the method of choice for monitoring these modifications, has a significant drawback which is that peptide mapping sample preparation induces the same modifications.

A new sample preparation method is developed which prevented the above side effects. In this method, all sample preparation steps including reduction, alkylation and digestion are performed at low pH (mildly acidic conditions). Efficient reduction and alkylation at low pH utilized both specialized reagents and procedural modifications. The proteolytic step represented the major bottleneck since trypsin, the most commonly used protease in peptide mapping, is inhibited at acidic pH. This problem was overcome by supplementing trypsin with a low pH resistant Lys-C protease. A reagent with oxygen scavenging activity was also selected, which was successfully applied to suppress protein oxidation during sample preparation.

The method was optimized for use with common sample preparation procedures based on protein denaturation followed by dilution or size exclusion clean-up. Necessary optimizations were introduced to ensure efficient digestion of proteolytically- resistant protein domains, minimal baseline noise in UV HPLC and LC/MS and high analytical reproducibility.

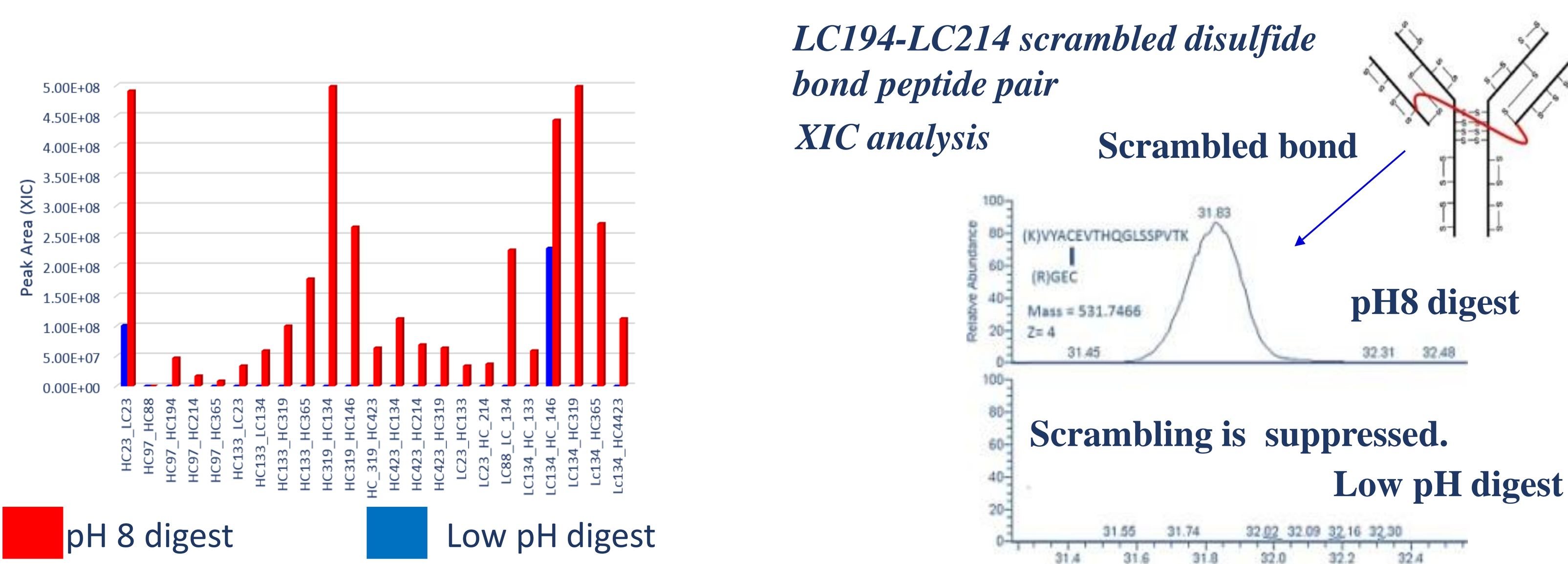
2. Artificial non-enzymatic PTMs induced during sample preparation and low pH solution

Asn deamidation and Asp isomerization induced during protein digestion



mAb was digested at 37ºC at conventional (pH8) or low pH (mildly acidic) conditions. Aliquots of the digests were collected over the course of digestion reaction and analyzed with LC/MS.

Disulfide bond scrambling induced during sample preparation



Panitumumab was digested at 37ºC at conventional (pH8) or low pH (mildly acidic) conditions and disulfide bond scrambling was analyzed with LC/MS.

Alkaline conditions favored by conventional sample preparation induce non-enzymatic PTMs. Suppression of these PTMs requires use of acidic conditions (low pH).

3. Establishing efficient trypsin digestion at low pH

Step 1. Determining the reaction pH for suppression of artificial PTMs

Monitoring of deamidation during protein digestion at increasing reaction pH

Selected pH range for low pH sample preparation

Deamidation, %

PH5 pH5.5 pH5.7 pH5.8 pH6.0 pH6.4 pH8.0

Panitumumab was digested overnight at 37ºC at indicated pH and analyzed with LC/MS. The graph shows analysis of SGTASVVCLLNNFVPR tryptic peptide used as a deamidation reporter. **Deamidation was induced at above pH6. We selected pH5.7-5.8 as an optimal reaction pH to suppress artificial PTMs**

Step 2. Establishing efficient tryptic digestion at low pH

Tryptic cleavages in low pH protein digest

Cleaved Arginine Sites

Cleaved Lysine Sites

Digestion efficiency, %

Trypsin cleavage specificity

NNNNNR/KNNNN

R – arginine

K - lysine

Yeast protein extract was digested overnight at 37ºC at pH5.7 and analyzed with LC/MS. The data show that trypsin inhibition primarily occurs at lysine cleavage sites at low pH. This suggested that trypsin activity at low pH can be restored by supplementing it with low pH resistant Lys-C protease.

Efficient trypsin digestion at low pH after supplementing with low pH resistant Lys-C

BSA digestion, SDS-PAGE

Panitumumab digestion, RP HPLC

Efficient digestion at low pH

We restored efficient trypsin digestion at low pH by supplementing it with a special, low pH resistant Lys-C protease.

4. Establishing efficient reduction and alkylation at low pH

Conventional reagents used to reduce and alkylate disulfide bonds favor alkaline conditions. To achieve efficient reduction and alkylation at low pH following steps were taken:

- Tris(2-carboxyethyl)phosphine) (TCEP), a reducing reagent retaining high activity over a broad pH range including mildly acidic conditions, was selected for the method.
- Iodoacetamide (IAM) was selected as an alkylating agent. To compensate for its decreased activity at acidic pH, we allowed alkylation to proceed over digestion period.

Procedure for protein reduction and alkylation at low pH

Reduction with TCEP, *37ºC 30 min*

↓

Alkylation with IAM, *37ºC 30 min* (initial alkylation step)

↓

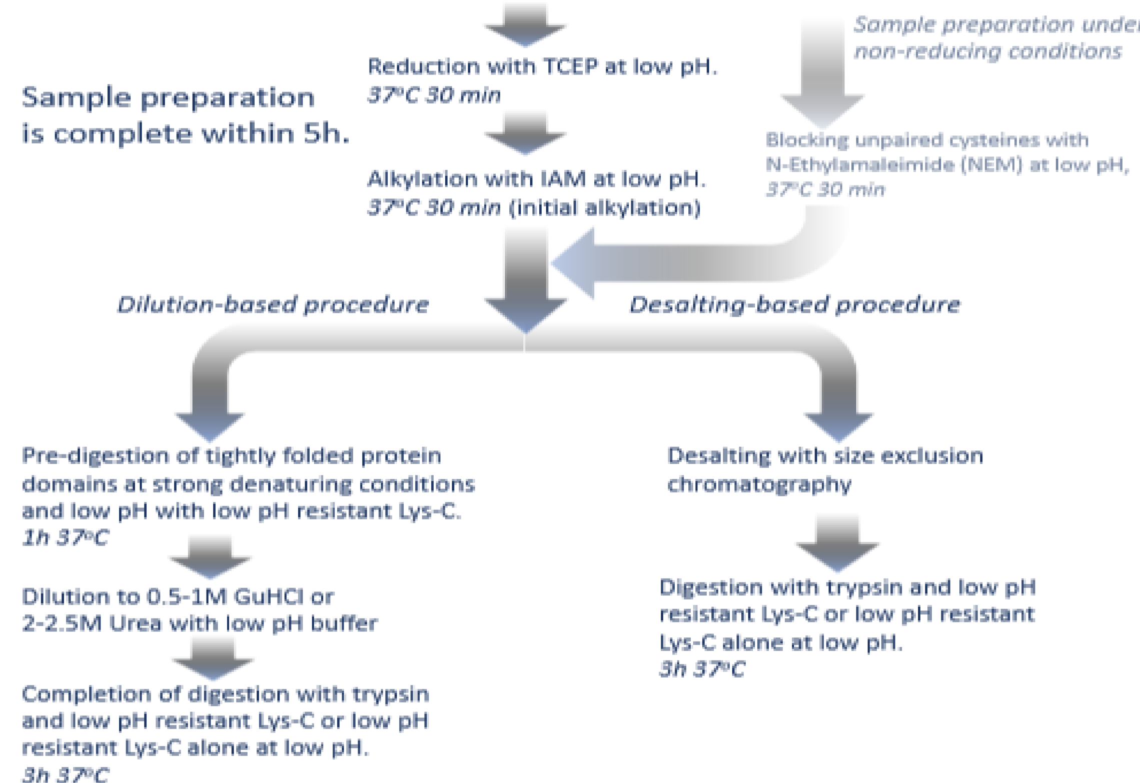
Digestion (alkylation is allowed to proceed over digestion period)

Panitumumab was reduced with TCEP, alkylated with IAM, digested and analyzed with LC/MS (Q Exactive, Thermo). All steps were performed at low pH as shown on the left.

Note: Iodoacetic acid (IAA) supported similar efficient alkylation in our low pH method.

Efficient reduction and alkylation was achieved at low pH requiring minimal procedural modifications.

6. Adaptation of the method for common sample preparation procedures



7. Conclusion

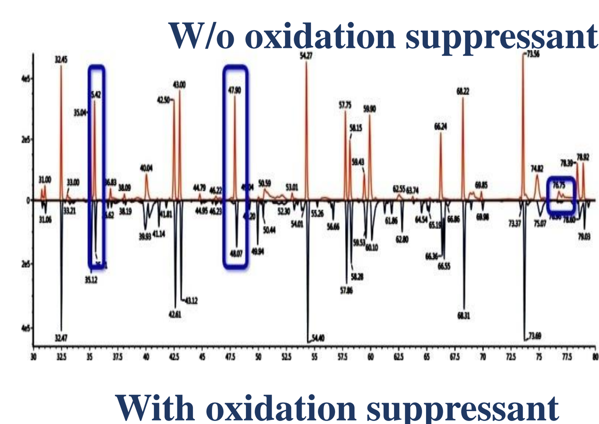
- A new method for peptide mapping is developed in which all sample preparation steps are performed at low pH (mildly acidic conditions).
- A robust tryptic protein digestion at low pH by supplementing trypsin with a specialized, low pH resistant Lys-C was developed.
- The method was optimized for efficient reduction, alkylation and digestion efficiency at low pH, as well as minimal baseline noise and high reproducibility. It was successfully incorporated into common sample preparation procedures based on use of denaturing agents followed by dilution or size exclusion clean-up prior to digestion.
- Sample preparation is complete within 5h in our method.
- A reagent with high oxygen scavenging activity was selected for optional use as a protein oxidation suppressant during sample preparation.
- The method offers an efficient means for preventing artificial non-enzymatic PTMs commonly induced during peptide mapping sample preparation.

5. Suppression of protein oxidation with an oxygen scavenger reagent

Various excipients and impurities can cause protein oxidation during sample preparation.To protect proteins from oxidation we selected a reagent with high oxygen scavenging activity ('oxidation suppressant').

A model IgG (Adalimumab) was denatured, reduced, alkylated and digested according to the low pH sample preparation procedure in the presence of an oxidation agent (hydrogen peroxide) with or without the oxidation suppressant. The digests were analyzed with LC/MS. The data indicate suppression of oxidation.

W/o oxidation suppressant



With oxidation suppressant

Suppression of H_2O_2-induced methionine oxidation during IgG digestion (oxidized methionine residues, %)

HC:M34 HC:M83 HC:M256 HC:M432 LC:M4

The oxidation suppressant suppressed protein oxidation during sample preparation in a model experiment.

8. References

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