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APPLICATION NOTE 21834

Automated chymotrypsin peptide mapping of proteins by LC-MS

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Keywords

Peptide mapping, Magnetic SMART Digest, KingFisher, automated chymotrypsin digestion, biotherapeutics, bottom-up, biopharmaceutical, protein digestion

Application benefits

- An easily automated proteolytic digestion using chymotrypsin
- Orthogonal digestion to trypsin
- High reproducibility from a robust protocol and automation

Goal

To demonstrate an easy-to-use, robust, high-precision, automated approach to a chymotrypsin peptide mapping characterization workflow that provides alternative digestion selectivity to trypsin digestion. Additionally, to show confirmation of a peptide map with high sequence coverage using high-resolution, accurate-mass mass spectrometry. To further display that it is possible to develop a highly reproducible, automated digestion procedure for use in high level characterization of biotherapeutic proteins.

Introduction

The biopharmaceutical industry continues to develop protein-based biotherapeutics in increasing numbers. Due to their complexity and biotechnological production, there are many attributes that need to be analyzed to guarantee their safety and efficacy.

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Peptide mapping is used to measure several critical quality attributes (CQAs) required for the characterization of any biotherapeutic protein. The analysis is used to confirm that the correct sequence has been expressed for the protein and to check for post-translational and chemical modifications (PTMs). Mass spectrometry (MS) is coupled to liquid chromatography (LC) for peak identification and sequence confirmation.

Trypsin is the most commonly used proteolytic digestion enzyme due to its high specificity for cleavage at arginine and lysine residues. However, there are proteins that do not digest well with trypsin due to too many or too few of the specific trypsin cleavage sites in the sequence. There are also cases where the cleavage sites can be too close together, producing very short hydrophilic tryptic peptides that do not retain on the reversed-phase HPLC columns and are therefore difficult to detect.

Chymotrypsin is a protease that has alternative cleavage specificity to trypsin. Chymotrypsin will cleave primarily at the hydrophobic aromatic amino acid residues of tryptophan, tyrosine, and phenylalanine. However, chymotrypsin is also known to additionally cleave at other sites such as leucine, histidine, and methionine, but with a lower level of specific activity. The consequence of chymotrypsin's selectivity is that the aromatic amino acids are among the first peptide bonds to be broken with other sites being cleaved at a slower rate.

Unlike trypsin, which has a high specificity for only two amino acid residues, the chymotrypsin digestion pattern alters as the digestion time increases due to a slower rate of activity at its alternative digestion sites. Unless the time of digestion is carefully controlled, errors can occur that will compromise the ability to reproducibly characterize a protein to the required standard. This is especially true where workflows only employ UV detection without peptide confirmation by MS. The digestion must be reproducible and chromatography must be extremely stable to allow unambiguous peptide identification based on chromatographic retention time.

This work details the automation of a chymotrypsin digestion of recombinant somatotropin as a model protein. The applicability and reproducibility of an automated chymotrypsin digestion protocol and subsequent analysis was investigated. In addition, this work also shows the effects of digestion time on chymotrypsin activity.

Magnetic beads are a proven support used for many purification and sample preparation approaches in life science research and biotechnology. The Thermo Scientific™ KingFisher™ Duo purification system enables robotic handling and easy automation of any magnetic-bead-based application resulting in superior performance and reproducibility. The combination of Thermo Scientific™ Magnetic SMART Digest™ beads and the KingFisher Duo purification system was used to automate the digestion process to produce high quality, reproducible peptide mapping data using an alternative protease.

The Thermo Scientific™ Vanquish™ Horizon UHPLC system was subsequently used to analyze the samples and coupled to a Thermo Scientific™ Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ mass spectrometer for MS confirmation of the peptide sequence.

Experimental

Consumables

- Deionized water, 18.2 MΩ·cm resistivity
- Fisher Scientific™ HPLC grade water (P/N 10449380)
- Fisher Scientific™ LCMS grade acetonitrile (P/N 10489553)
- Fisher Chemical™ Optima™ LC/MS grade water with 0.1% formic acid (v/v) (P/N 10429474)
- Fisher Chemical™ Optima™ LC/MS grade acetonitrile with 0.1% formic acid (v/v) (P/N 10468704)
- Fisher Chemical™ Optima™ LC/MS trifluoroacetic acid (P/N 10125637)
- SMART Digest Chymotrypsin Kit, Magnetic Bulk Resin option (P/N 60109-104-MB)
- Thermo Scientific™ KingFisher™ Deepwell, 96 well plate (P/N 95040450)
- Thermo Scientific™ KingFisher™ Duo 12-tip comb (P/N 97003500)
- Thermo Scientific™ Acclaim™ VANQUISH™ C18 UHPLC column, 2.1 × 250 mm (P/N 074812-V)

Equipment

- KingFisher Duo Prime purification system (P/N 5400110)
- Thermo Scientific™ Hypersep™ 96 well positive pressure system (P/N 60103-357)
- Vanquish Horizon UHPLC system, including:
  - Binary Pump H (P/N VH-P10-A)
  - Column Compartment H with (P/N VH-C10-A)
    - Active Pre-heater VH-C1 (P/N 6732.0110)
    - Post-column Cooler 1 µL VH-C1 (P/N 6732.0510)
  - Split Sampler HT (P/N VH-A10-A)
  - Diode Array Detector HL (P/N VH-D10-A)
- MS Connection Kit Vanquish (P/N 6720.0405)
- Q Exactive Plus hybrid quadrupole-Orbitrap mass spectrometer (P/N IQLAAEGAAPFALGMBDK)

Sample preparation

Lyophilized powder of recombinant somatotropin was dissolved in deionized water and adjusted to a final concentration of 10 mg/mL.

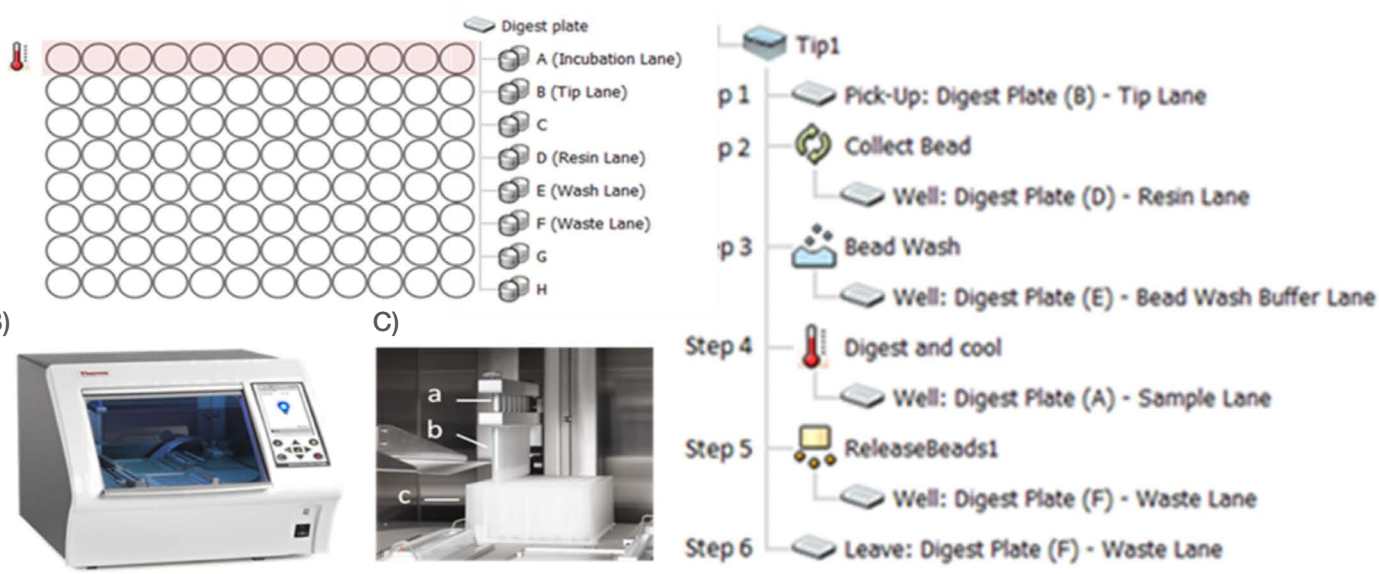
Magnetic SMART Digest, automated digestion protocol

- Magnetic SMART Digest resin slurry was diluted and uniformly suspended in SMART Digest buffer to create a suspension of 15 µL original resin into 100 µL of buffer in each well of the dedicated “resin lane” of a KingFisher Deepwell 96 well plate (96 DW plate).
- 200 µL of 1:4 diluted SMART Digest buffer was prepared in each well of a separate row of the plate as the optional wash buffer.
- 50 µL of the sample solution was diluted into 150 µL of SMART Digest buffer in the dedicated “incubation lane” that allows for heating and cooling (row A).
- Thermo Scientific™ BindIt™ software (version 4.0) was used to control the KingFisher Duo Prime with the program outlined in Figure 1.
- The digestion step was completed at 70 °C.
- Sedimentation of beads was prevented by repeated insertion of the magnetic comb using the mixing speed setting “medium”.
- Incubation times for somatotropin are shown in the text; 15 minutes was used as the optimal time to ensure complete digestion of the protein in the shortest time period.
- Immediately after incubation the magnetic beads were collected and removed from the reaction, and the digest solution was actively cooled to 15 °C.

Figure 1. Automated SMART Digest configuration using the KingFisher Duo Prime purification system

A) Schematic of plate layout and digestion program.
B) The KingFisher Duo Prime Purification System.
C) Robotic handling compartment: 12 magnetic rods (a), disposable comb tip (b), 96 DW plate (c)
D) Plate layout showing the volumes and solutions in each well
E) Protocol step details

A)



B)

C)

D) Plate Layout:

Lane	Content	Volume (µL)
A	Buffer	150
	Sample	50
B	Tip Comb	
D	Beads	15
	Bead Buffer	100
E	Bead Wash Buffer	200
F	Waste Lane	250

E) Protocol Step Details:

Step	Release Bead	Mixing	Collect Beads	Temp	Lane
Collect Bead	–	10 s Bottom Mix	3 count, 1 s	–	D
Bead Wash	Yes	1 min Medium Mix	3 count, 1 s	–	E
Digest and Cool	Yes	8 min, 30 s Medium Mix	3 count, 15 s	70 °C heating while mixing 5 °C post temperature	A
Release Beads	Yes, Fast	–	–	–	F

UHPLC-MS separation conditions

Column:	Thermo Scientific™ Acclaim™ VANQUISH™ C18, 2.2 µm, 2.1 × 250 mm
Mobile phase:	A: Water + 0.1% formic acid B: Acetonitrile + 0.1% formic acid
Flow rate:	0.3 mL/min
Column temperature:	70 °C (still air mode)
Post column cooler:	50 °C
Injection volume:	5 µL
UV wavelength:	214 nm
Gradient	Table 2

Table 2. Mobile phase gradient.

Time (min)	%A	%B	Flow (mL/min)	Curve
0.0	96	4	0.3	5
30	25	75	0.3	5
30	0	100	0.3	5
35	0	100	0.3	5
35	96	4	0.3	5
45	96	4	0.3	5

MS conditions

The Q Exactive Plus mass spectrometer equipped with a HESI-II probe was used for mass spectrometric detection using a full MS / dd-MS2 (Top5) experiment.

Ionization:	HESI Positive ion
Scan range:	140 to 2000 *m/z*
Source temperature:	350 °C
Sheath gas pressure:	45 psi
Auxiliary gas flow:	10 Arb
Spray voltage:	3.4 kV
Capillary temperature:	320 °C
Resolution [fullMS/MS2]:	70,000/17,500
Top-N MS2:	5
S-lens RF level:	60
Max inject time:	100 mS
Collision energy:	27 eV

Data processing and software

MS data acquisition	Thermo Scientific™ Xcalibur™ software v 2.2 SP1.48
Analysis	Thermo Scientific™ BioPharma Finder™ 2.0 software

Results and discussion

The applicability of the automated protein chymotrypsin digestion with the KingFisher Duo Prime purification system was tested with somatotropin. Three replicate digests were conducted for 15 minutes and the generated peptides were separated and analyzed by UHPLC-MS. The corresponding peptide map is shown in Figure 2 with the sequence coverage at 100%.

Somatotropin was readily digested using the automated SMART Digest protocol with chymotrypsin, resulting in complete digestion of the protein. The magnetic SMART chymotrypsin cleaves efficiently at the aromatic amino acids, tyrosine, phenylalanine, and tryptophan, with no missed cleavages observed with somatotropin; however, there are also a significant number of cleavages after some leucine residues.

Extra cleavages increase with longer incubation times to eventually include all the leucine residues, some methionine, histidine, and even an arginine residue (Figure 3). This produces an increasingly complex pattern with more peptides than required for full coverage. Care must be taken when optimizing the length of digestion time to avoid this. This can easily be achieved however because of the reproducibility of the automated SMART digestion process, which allows for precise timing and therefore reproducible digestion. This is more difficult to achieve with traditional in-solution digests.

The increasing number of peptides released during a digestion time course is depicted in Figure 3 over a 30 minute time period for the C-terminus of somatotropin. Initial cleavage is restricted to sites following aromatic amino acid residues. However, at 15 minutes digestion time, cleavage at lysine residues starts to occur, with less specific cleavage sites appearing at 30 minutes digestion. Automation with the KingFisher Duo Prime purification system allows optimization with a very specific time of digestion, in this case 15 minutes. The number of cleavage sites produced during the digestion can therefore be controlled to suit the protein of interest.

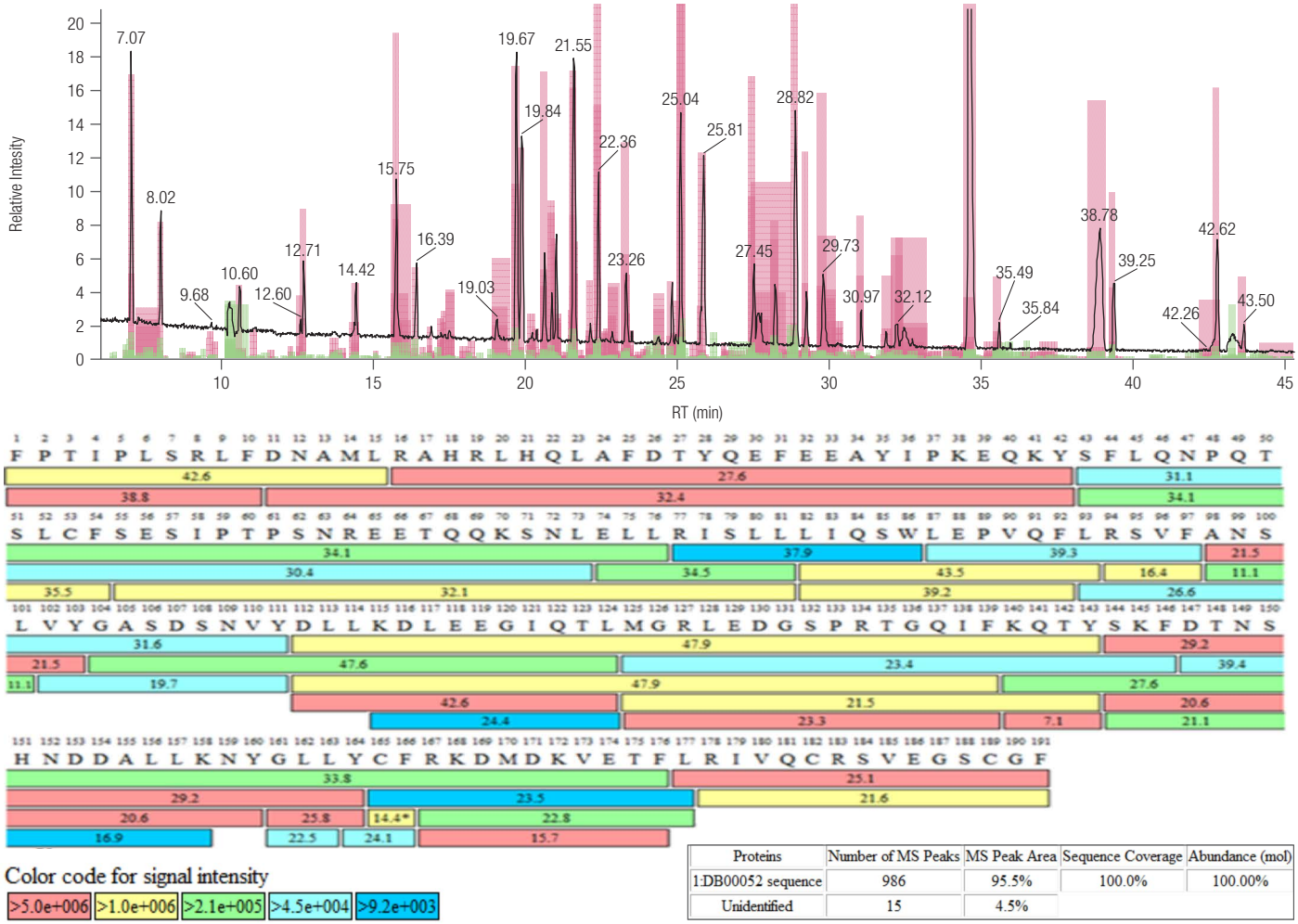
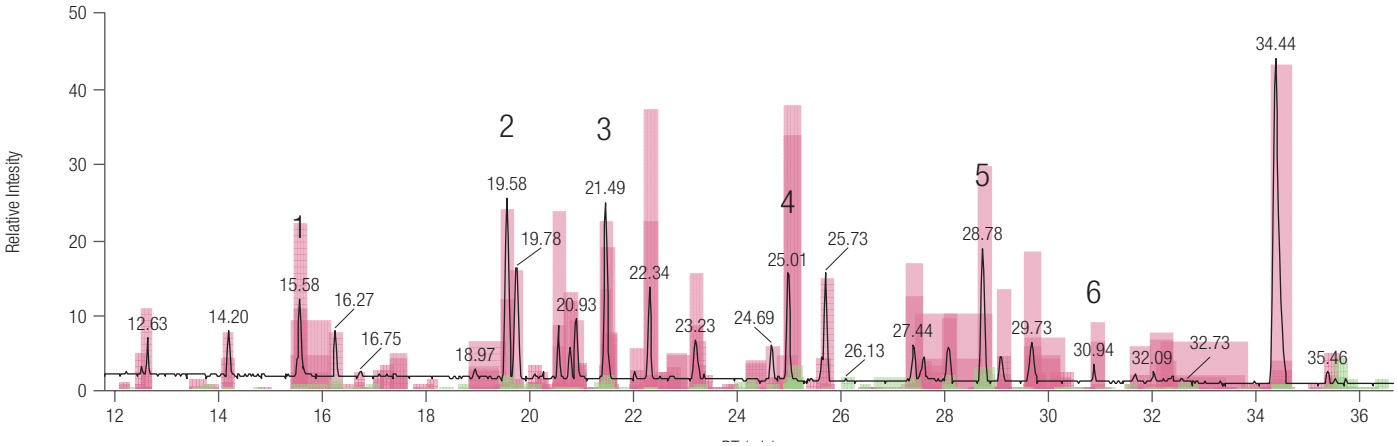


Figure 2. Sequence coverage of somatotropin with magnetic chymotrypsin digestion. The upper trace shows the base peak chromatogram (BPC) with the sequence coverage underneath. The colored bars show the identified peptides, with the number in the bars reflecting the retention time (min) and the intensity of the peptide in the MS1 scan: red = high abundant > yellow > green > light blue > cyan = low abundant.

Figure 3. The controlled appearance of sites of cleavage from SMART chymotrypsin over the C-terminal sequence of somatotropin. A time course of 5, 10, 15, 20, and 30 minutes is shown with increasing numbers of cleavages over time.

The part of the sequence shown in Figure 3 has two cleavage sites very close together at Y 164 and F 166 producing a short dipeptide (highlighted with a red circle). If a short dipeptide is produced from a trypsin digest, one of the amino acids would be charged [R or K] almost certainly, making the peptide very hydrophilic and not retained on the reversed-phase separation column. The elution in the column void would make the peptide very difficult, if not impossible, to detect. As chymotrypsin cleaves after hydrophobic aromatic amino acids, the peptides are usually retained and detectable with the higher UV absorbance of the aromatic amino acid. The short $C^{165}F^{166}$ peptide can be seen to elute in 14.4 minutes in the peptide gradient shown in Figure 2. This is another advantage with chymotrypsin digestions.

In addition to peptide coverage, reproducibility was investigated over three replicates of the 15 minute digestion. Measurements were made using six selected peptide peaks from the chromatogram. An average percentage relative standard deviation (% RSD) of 3.56% was archived for the peaks annotated in Figure 4. Three of these peak areas had % RSD values of 2.2% and below. This level of reproducibility is enhanced by the consistency of the Vanquish Horizon UHPLC system gradient generation and injection accuracy allowing near identical chromatography, which makes integration and peak interpretation easier.



Peak	1	2	3	4	5	6
RSD	6.1%	1.2%	6.6%	3.4%	1.9%	2.2%

Figure 4. Overlaid base peak chromatograms (BPCs) of three replicates showing reproducibility of automated chymotrypsin digestion using magnetic beads. Overlay of n = 3 different digestions.

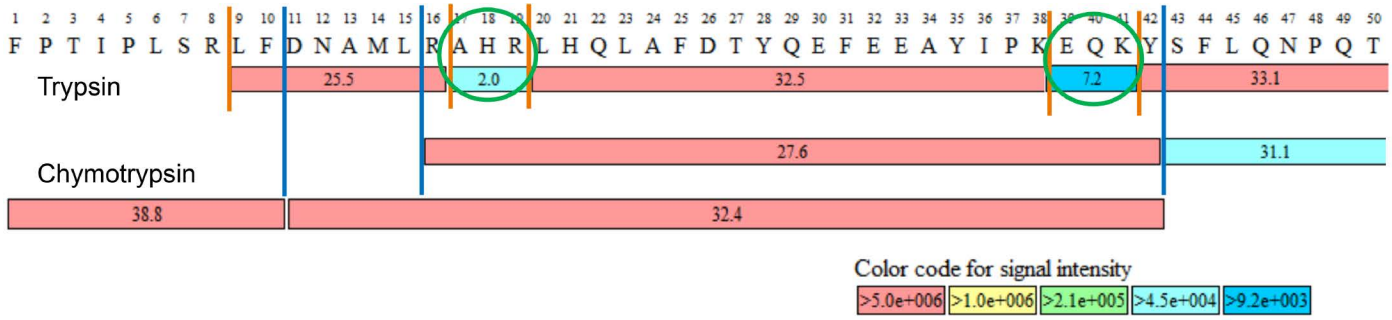


Figure 5. Comparisons of magnetic SMART trypsin and chymotrypsin cleavage over part of the somatotropin sequence. Trypsin cleavage sites are shown with a red line and chymotrypsin cleavage sites are shown with a blue line. The colored bars show the identified peptides, with the number in the bars reflecting the retention time (min) and the intensity of the peptide in the MS1 scan: red = high abundant > yellow > green > light blue > cyan = low abundant.

The complimentary cleavage patterns for trypsin and chymotrypsin allow overlapping sequence analysis, which is useful to confirm parts of the sequence that could be missed through the presence of cleavage sites too close together or too far apart to give good sequence coverage with one enzyme alone. It can also be used to find the correct order of peptides for an unknown protein sequence. Figure 5 shows part of the somatotropin sequence that produces two short peptides (marked with a green circle) following a trypsin digestion that are difficult to detect. In comparison, chymotrypsin digestion produces two longer, high abundant peptides that contain the sequence for both of these short trypsin cleavage peptides, allowing unambiguous sequence coverage with the two digestion protocols.

The results show that an automated chymotrypsin digestion can be easily reproduced with careful optimization of the digestion time. There are instances where the application of an alternative protease activity to the commonly used trypsin can be extremely useful.

Conclusions

- Automated magnetic SMART chymotrypsin digestion can be reproducibly undertaken with ease.
- Optimization of the time the digestion is allowed to proceed is important.
- Reproducibility is further improved with chymotrypsin when automated.
- Chymotrypsin is a viable alternative digestion protocol to trypsin.
- There are useful applications for this alternative digestion enzyme.

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