Comprehensive protein sequence coverage using MagReSyn® Trypsin & MagReSyn® Chymotrypsin

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Abstract

The application note provides a rapid protocol and adaptable guideline for in-depth characterization of protein sequence. Digestions were performed on a four-protein mix using magnetic microparticles with immobilized proteolytic enzymes, MagReSyn® Trypsin and MagReSyn® Chymotrypsin. The orthogonal cleavage sites of Trypsin and Chymotrypsin provided complementary sets of peptides that resulted in increased sequence coverage of cumulative peptide maps. Furthermore, the digestions were rapid (1 hr, Trypsin; 0.5 hr Chymotrypsin), highly efficient, and reproducible. The method was also adapted for use with the Thermo Kingfisher™ Duo magnetic microparticle handling station, in order to fully automate the sample preparation protocol for increased reproducibility in routine operation.

Introduction

Protein digestion is a critical part of any bottom-up mass spectrometry (MS) workflow, where proteins have to be cleaved into their surrogate peptides prior to analysis. The most widely used enzyme to digest proteins is Trypsin, which cleaves polypeptides with high specificity at the C-terminal ends of the amino acids Lysine (K) and Arginine (R). However, other proteases are gaining in popularity primarily due to different cleavage specificities, or the ability to work under denaturing conditions. Their distinctive specificities can allow for the generation of peptide sets complementary to those obtained by Trypsin cleavage, resulting in improved sequence coverage and consequently more confident protein identifications. This information is particularly valuable when studying closely related species such as isoforms, monitoring of point-mutations (e.g. requisite during the manufacture of protein-based biologics or biosimilars) as well as in post-translational modification (PTM) studies. Chymotrypsin in particular can digest orthogonally to Trypsin due to its preference for hydrophobic residues including Phenylalanine (F), Tyrosine (Y), Leucine (L), Tryptophan (W) and Methionine (M). This specificity toward non-polar amino acids makes Chymotrypsin especially effective for digestion of membrane proteins, which constitute approximately one third of the total proteome. Furthermore, membrane proteins are critical to all physiological processes and represent 60% of approved drug targets, making them an interesting class of proteins in proteomics studies. Here we describe the application of covalently immobilized sequencing-grade Trypsin and Chymotrypsin for rapid and efficient protein digestion, and demonstrate that improved sequence coverage can be achieved when combining datasets from enzymatic digestion of protein samples using Trypsin and Chymotrypsin. Further, the high content immobilization of Trypsin and Chymotrypsin on magnetic microparticles allows for the development of rapid automated digestion workflows using bead handling stations.

Materials

- *Denaturation Buffer*: 50 mM Tris pH 8.0, 8 M urea
- *Reducing Reagent*: 1 M Dithiothreitol (DTT) in 50 mM Tris pH 8.0, 8 M urea
- *Alkylating Reagent*: 1 M Iodoacetamide (IAA) in 50 mM Tris pH 8.0, 8 M urea
- *Digestion Buffer*: 50 mM Tris pH 8.0
- *0.5 ml and 2 ml Eppendorf® LoBind microcentrifuge tubes (recommend for sample storage and digestion due to low non-specific binding, low leaching of MS-interfering components).*

A magnetic separator is required to isolate magnetic microparticles between each step of the protocol when performing the manual digestion procedure.

Methods

Protein Sample Preparation – Reduction and Alkylation

A standard protein mix containing Thyroglobulin, Ovalbumin, IgG (Light Chain) and Myoglobin was prepared in *Denaturation Buffer* at a concentration of 16 mg.ml^{-1}. Reduction was initiated by the addition of *Reducing Reagent* (DTT) to a final concentration of 50 mM from the 1 M stock solution. Samples were mixed by vortexing for 30 sec and incubated for 45 min at 23°C. Alkylation was initiated by addition of *Alkylation Reagent* (IAA) to a final concentration of 100 mM from the 1 M stock solution. Samples were mixed by vortexing for 30 sec and incubated for 45 min at 23°C in the dark. The reduced and alkylated protein mix was diluted to 2 mg.ml^{-1} using *Digestion Buffer*.

Protein Digestion - Manual

***Note**: The quantity of microparticles may be scaled to match the quantity of protein to be digested. Generally, 20 µl of MagReSyn® Trypsin or Chymotrypsin is sufficient to digest 50 µg of total protein.* Microparticle solutions were thoroughly resuspended by vortex mixing for 3 sec and 20 µl transferred to a microcentrifuge tube. The shipping solution was removed by aspiration with a pipette and discarded after magnetic isolation of the microparticles. The microparticles were subsequently equilibrated in 2 x 200 µl *Digestion Buffer* (30 sec mixing times per incubation) with magnetic recovery and aspiration of the *Digestion Buffer* after each magnetic recovery step. 25 µl (50 µg) reduced and alkylated protein mix was added to the equilibrated MagReSyn® Trypsin or Chymotrypsin microparticles. 25 µl *Digestion Buffer* was added for a total volume of 50 µl. Samples were incubated for 30 min (Chymotrypsin) or 60 min (Trypsin) at 37°C on a vortex or alternate suitable microcentrifuge tube mixer, to ensure the microparticles remain in suspension during digestion. ***Note**: Mixing by inversion is not recommended for low sample volumes as the sample will not mix efficiently.* After digestion, microparticles were recovered using a magnetic separator and the supernatant (digested peptides) transferred to a 0.5 ml microcentrifuge tube. Samples were acidified using formic acid, added to a final concentration of 0.5%, and samples subsequently stored at -20°C until LC-MS/MS analysis. ***Note**: An optional microparticle wash step (post digestion) can be performed with Digestion Buffer when working with low sample quantities. This can potentially improve peptide recovery. When using low quantities of starting protein we recommend reducing the volume of the digested proteins pre-MS by lyophilization or vacuum drying. Alternatively the acidified samples can be desalted using C18 Stage Tips, ZipTip®, or other suitable clean-up methods.*

Protein Digestion - Automated

Automated sample clean-up and digestion was performed on a KingFisher™ Duo magnetic handling station (Thermo Scientific, USA), using 96 deep-well microtiter plates, suitable for processing up to 12 samples in parallel. The system has a 12-pin robotic magnet head with disposable plastic comb. This prevents sample cross-contamination during binding, mixing and transfer steps between wells (**Figure 1)**. The KingFisher™ Duo Peltier block (row A) with a temperature range from 4-75°C, was used for protein digestion at 37°C. The protocol file is freely available upon request (info@resynbio.com).

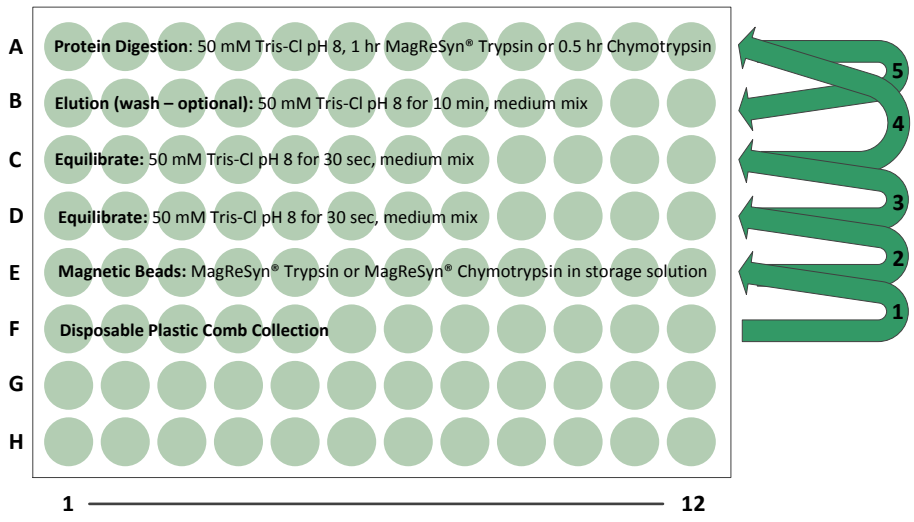

Figure 1: A KingFisher™ Duo magnetic handling station was configured for protein digestion. Immobilized Trypsin or Chymotrypsin microparticles are placed in row E and protein samples in row A. The microparticles are collected from row E, transferred to row D, then row C for equilibration in Digestion Buffer. Thereafter the microparticles are transferred to row A containing the protein mix and incubated at 37°C. Digestion proceeds for 0.5 hr with Chymotrypsin or 1 hr if using Trypsin. Microparticles are then removed from the digested protein mixture and may be transferred to a wash solution (secondary elution in Digestion Buffer: row B) or back to the storage position in row E.

LC-MSMS Analysis

LC-MSMS analysis was performed using an AB Sciex TripleTOF® 6600 coupled to a Dionex™ 3500 nanoRSLC fitted with a capillary flow selector (1-10 µl.min^{-1}). A volume of 5 µl (5 µg total protein) from the MagReSyn® Trypsin or Chymotrypsin digested samples was loaded on an Acclaim® PepMap C18 trap column (100 µm × 2 cm) and desalted for 2.5 min at 15 µl.min^{-1} using 2% acetonitrile (ACN), 0.2% FA. Peptides were separated on an Acclaim® PepMap C18 column (300 µm × 15 cm, 3 µm particle size) coupled to the TripleTOF® 6600 MS (AB SCIEX) using a 25 µm electrospray probe. Peptide elution was achieved using a flow-rate of 8 µl.min^{-1} with a gradient of 4-35% solution **B** over 40 min (**A**: 0.1% FA; **B**: 80% ACN with 0.1% FA. Data acquisition: One survey scan (360–1500 m/z) with 250 ms accumulation time and 50 product ion scans (100-1500 m/z) each with 50 ms accumulation time. Product ions scans were triggered automatically when multiply charged ions (2^{+} to 5^{+}) mass 360-1500 m/z and intensity ≥200 counts were detected. An exclusion window of 30 sec was applied. Rolling collision energy with a collision energy spread (CES) of 10 eV was used.

Data Processing

LC-MSMS spectral data was analysed using PEAKS Studio 6 software (Bioinformatics Solutions Inc., Canada). Spectral data was searched against a database containing sequences of bovine thyroglobulin, bovine IgG, chicken ovalbumin and equine myoglobin (the standard protein mix) as well as a list of common protein contaminants. The following search parameters were applied: parent mass tolerance error 20 ppm; fragment mass tolerance error 0.1 Da; up to four missed cleavages per peptide; non-specific cleavages allowed at one end; cysteine carbamidomethylation (fixed modification); methionine oxidation and N-terminal acetylation (variable modification). A false discovery rate of 0.1% was used at the peptide level and at least one unique peptide was required for protein identification.

Results & Discussion

The standard mix used for digestion was made up of four proteins: thyroglobulin (165 kDa), ovalbumin (43 kDa), IgG light chain (24 kDa) and myoglobin (17 kDa). Rapid digestion was performed using MagReSyn® Trypsin (1 hr digest time) and MagReSyn® Chymotrypsin (0.5 hr digest time) resulting in highly reproducible protein cleavage as indicated by the Total Ion Chromatograph (TIC) overlays below (**Figure 2**).

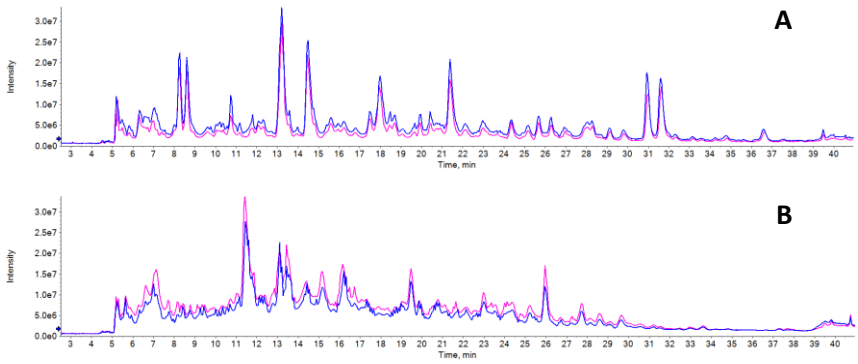

Figure 2. Precursor TICs acquired in the analysis of 5 µg protein digests generated using (A) MagReSyn® Trypsin and (B) MagReSyn® Chymotrypsin. Each digest was performed in duplicate and resulting TICs overlaid as represented by the blue and pink traces.

Protein digestion was efficient with both MagReSyn® Trypsin and MagReSyn® Chymotrypsin resulting in high sequence coverage for all four standard proteins (refer **Table 1**).

The peptides generated by the MagReSyn® Trypsin digest were complementary to those obtained from the MagReSyn® Chymotrypsin digest (**Figure 3).** Between digests, only six overlapping peptides (0.3%) were detected, indicating that the two immobilized enzymes are performing at high specificity with very low Chymotryptic activity in the case of MagReSyn® Trypsin and very low Tryptic activity in the case of MagReSyn® Chymotrypsin.

Table 1: Unique peptide identifications and sequence coverage

	MagReSyn® Trypsin		MagReSyn® Chymotrypsin	
Protein Standard	Unique peptides	Sequence Coverage (%)	Unique peptides	Sequence Coverage (%)
Thyroglobulin	182	60	479	79
Ovalbumin	68	88	131	97
IgG (Light Chain)	36	77	64	75
Myoglobin	54	100	63	99

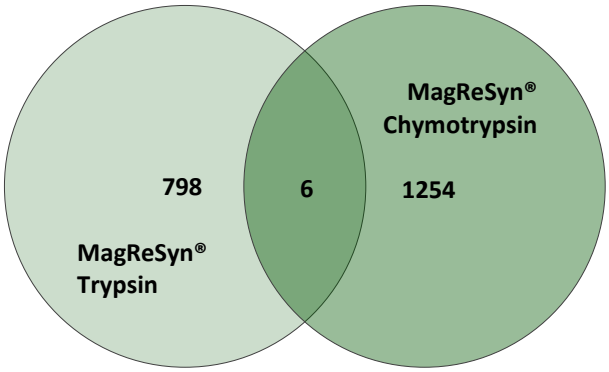

Figure 3: Venn diagram indicating the quantity of peptides identified, as well as the level of total peptide overlap between proteins enzymatically digested with MagReSyn® Trypsin and MagReSyn® Chymotrypsin.

The complementary cleavage sites resulted in an increase in cumulative sequence coverage when datasets from MagReSyn® Trypsin and MagReSyn® Chymotrypsin digests were combined; thereby allowing for a more comprehensive characterization of the primary sequence of the proteins (**Figure 4**).

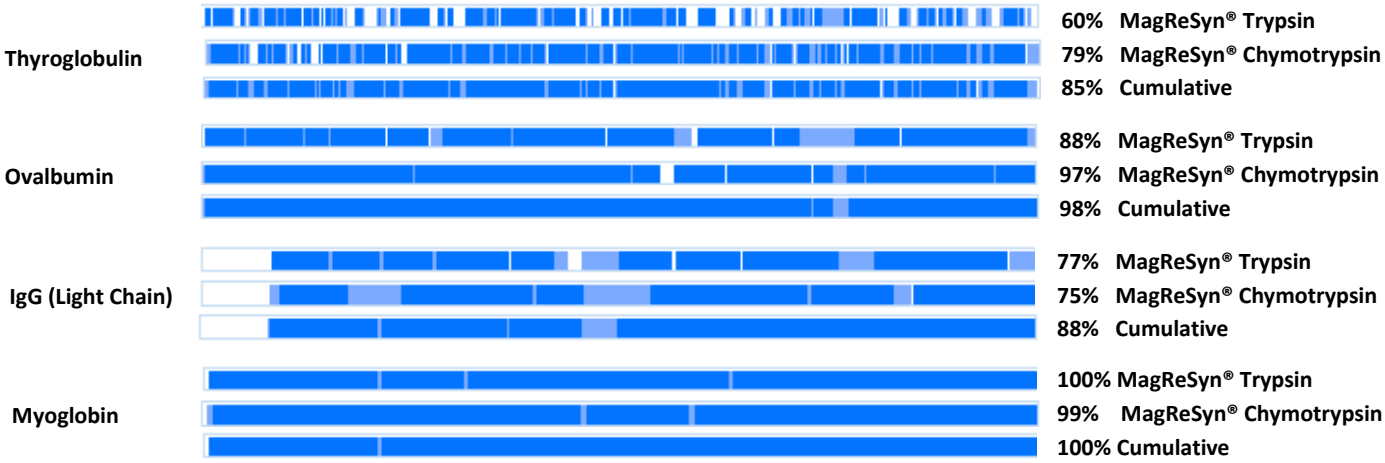

Figure 4: Graphical representation of sequence coverage for each of the proteins present in the standard protein mix digested with MagReSyn® Trypsin or MagReSyn® Chymotrypsin. The cumulative sequence coverage was calculated from the combined pool of peptides generated from digestions from either MagReSyn® Trypsin or MagReSyn® Chymotrypsin. Blue filled portions are indicative of coverage by LC-MS/MS analysis of samples. The percentage sequence coverage for each protein is indicated on the right of each graphic.

Conclusions

The application note provides an adaptable protocol for protein sequence coverage by LCMSMS analysis of protein samples. Efficient and rapid protein digestion was achieved using MagReSyn® Trypsin and MagReSyn® Chymotrypsin, while combining the LC-MS/MS datasets from the digestions proved complimentary and provided improved sequence coverage for all of the proteins analysed. The digestion protocol was adapted for use with a magnetic handling station (KingFisher™ Duo), illustrating the compatibility of the magnetic microspheres with robotic handling stations for automation of workflows and protocols.

Ordering Information

Description	*Product Code*
MagReSyn® Trypsin 2 ml	**MR-TRP002**
MagReSyn® Trypsin 5 ml	**MR-TRP005**
MagReSyn® Trypsin 10 ml	**MR-TRP010**
MagReSyn® Chymotrypsin 2 ml	**MR-CHY002**
MagReSyn® Chymotrypsin 5 ml	**MR-CHY005**
MagReSyn® Chymotrypsin 10 ml	**MR-CHY010**

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