Improved stability of MagReSyn® Trypsin in the presence of denaturants: SDS and Urea

Stoyan Stoychev & Justin Jordaan, ReSyn Biosciences (Pty) Ltd, Johannesburg, South Africa

Previn Naicker, CSIR Biosciences, Pretoria, South Africa

Abstract

This application note illustrates the efficient digestion of a standard protein mix in denaturants urea and SDS. MagReSyn® Trypsin consists of high content immobilized TPCK treated Trypsin on ferromagnetic polymer particles. Digestions were performed in the presence of urea and SDS where MagReSyn® Trypsin retained activity in concentrations of up to 5 M urea and 0.5% SDS, with complete protein cleavage within 2 hrs under these conditions. Increased microsphere loading can be used to increase the digestion efficiency. LC-MSMS analysis of proteins digested in the presence of 5 M urea showed improved sequence coverage, without reduction in the specificity of the enzymatic cleavage.

Introduction

Bottom-up mass spectrometry (MS) workflows often require the use of proteolytic enzymes for the cleavage of proteins into peptides prior to analysis. Efficient protein cleavage, predominantly performed using the enzyme Trypsin, is highly dependent on the accessibility of the proteolytic cleavage sites. Digestion is often ineffective in the case of proteins that are tightly folded or for example membrane proteins that are not soluble in aqueous solutions. This in turn results in poor sequence coverage or even lack of detection. Protein solubilization using detergents such as sodium dodecyl sulfate (SDS), or denaturation using chaotropes such as urea and guanidine hydrochloride are common practice to improve sequence coverage using proteolytic digestion. SDS is a frequently used detergent due to its high efficiency in denaturing proteins as well as disassociation of protein-protein interactions. However, most enzymes, including Trypsin, are sensitive to high concentrations of denaturants and detergents since these agents may in turn destabilize and denature the enzyme performing the digestion. Therefore, prior to digestion dilution of the denaturant or detergent is required to ensure proteolytic enzyme activity. In the case of sequencing grade Trypsin the recommended urea and SDS concentrations are $\leq$ 1M and $\leq$ 0.1 % respectively. The covalent immobilization of Trypsin on MagReSyn® ultra-capacity magnetic microparticles stabilizes the enzyme for use in the presence of increased concentrations of these common denaturants. The increased stability is enabled through multi-point covalent attachment of the enzyme to the polymer microspheres. Here we describe the application of magnetically immobilized, high content, sequencing-grade Trypsin for rapid and efficient protein digestion under denaturing conditions. Enzyme activity is retained in the presence of 5 M Urea and up to 0.5% SDS. LC-MSMS digest analysis demonstrated that significant improvements in sequence coverage are achieved when digestion was performed in the presence of 5 M urea. The immobilization of Trypsin on magnetic microparticles also allows for the automation of the digestion workflows using magnetic handling stations.

Materials

- *Resuspension Buffer*: 50 mM Ammonium bicarbonate pH 8.0, 8 M urea
- *Reducing Reagent*: 1 M Dithiothreitol (DTT) in 50 mM Ammonium bicarbonate pH 8.0, 8 M urea
- *Alkylating Reagent*: 1 M Iodoacetamide (IAA) in 50 mM Ammonium bicarbonate pH 8.0, 8 M urea
- *Native Digestion Buffer*: 50 mM Ammonium bicarbonate pH 8.0
- *Denaturing Digestion Buffer:* 50 mM Ammonium bicarbonate pH 8.0, 5 M urea
- *Detergent Digestion Buffer:* 50 mM Ammonium bicarbonate pH 8.0, 0.01 – 1 % SDS
- *0.5 ml and 2 ml Eppendorf® LoBind microcentrifuge tubes (recommend use for sample storage and digestion due to low non-specific binding, low leaching of MS-interfering components)*

A magnetic separator will be 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, IgG, Ovalbumin, Bovalbumin and Myoglobin was prepared in *Resuspension buffer* (50 mM Ammonium bicarbonate pH 8.0, 8 M Urea) at a concentration of 16 $mg.ml^{-1}$. Reduction was initiated by the addition of *Reducing Reagent* 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 *Alkylating Reagent* to a final concentration of 100 mM from the 1 M stock. 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 subsequently diluted to 2 $mg.ml^{-1}$ using *Native Digestion Buffer, Denaturing Digestion Buffer* or *Detergent Digestion Buffer* (containing a range of SDS from 0.01% – 1% SDS).

Protein digestion

***Note**: The quantity of microparticles may be scaled to match the quantity of protein to be digested. Generally, 20 µl of MagReSyn® Trypsin is sufficient to digest 50 µg total protein under native conditions. For digestion under denaturing conditions we recommend a minimum of 50 µl MagReSyn® Trypsin.* MagReSyn® Trypsin microparticles were thoroughly resuspended by vortex mixing for 3 sec. A volume of 20 µl microparticle suspension was aspirated from the commercial preparation and used for digestion under native conditions, while 50 µl was used for digestion in the presence of Urea or SDS. The microparticles were recovered using a magnetic separator, and the shipping solution was removed by pipette aspiration and discarded. The microparticles were subsequently equilibrated in 2 volumes of 200 µl *Native Digestion Buffer* (30 sec mixing times per incubation) with magnetic recovery and aspiration of the *Native Digestion Buffer* after each magnetic recovery step. equilibrated MagReSyn® Trypsin microparticles. The microparticles were resuspended in 25 µl of *Native Digestion Buffer, Denaturing Digestion Buffer* or *Detergent Digestion Buffer* (range from 0.01 to 1%), and the reaction initiated by the addition of 25 µl (50 µg) reduced and alkylated protein mix (diluted in the various buffers as above) for a total reaction volume of 50 µl. Samples were incubated for 60 - 120 min at 37°C on a vortex (or suitable alternate tube mixer), to ensure the microparticles remain in suspension during digestion. ***Note**: Mixing by inversion is not recommended due to low sample volumes.* After digestion, microparticles were recovered using a magnetic separator and the supernatant (digested peptides) transferred to a 0.5 ml microcentrifuge tube. Formic acid (FA) was added to the digest to a final concentration of 0.5 % and samples were stored at -20°C until LC-MSMS analysis. ***Note**: An optional microparticle wash step, post digestion, can be performed with Digestion Buffer when working with low sample amounts. This can 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. For SDS removal, post digestion, we recommend DS^- precipitation using potassium chloride (KCl) as per the method described by Zhou et al. (2012), followed by C18 StageTips or ZipTip® desalting step for removal of the KCl prior to LC-MSMS analysis.*

Gel Electrophoresis

Digestion efficiency was assessed by gel electrophoresis using NUPAGE® 4-12% Bis-Tris precast gels (Life Technologies) according to the manufacturer's instructions. Protein bands were visualized in the gel by staining proteins for 16 h with Colloidal Coomassie Blue stain (Neuhoff *et al,* 1990). The gels were destained in ultrapure water. Images of the PAGE gels were captured on a Syngene G:BOX gel documentation system (automatic exposure to avoid saturation) and analysed for protein purity using densitometry (GeneSnap).

LC-MSMS analysis

LC-MSMS analysis was performed using a Dionex Ultimate 3000 RSLC system coupled to a QSTAR ELITE mass spectrometer. Peptides were first de-salted on an Acclaim PepMap C18 trap column (75 µm × 2 cm) for 8 min at 5 $µl.min^{-1}$ using 2% acetonitrile in 0.2% formic acid, than separated on Acclain PepMap C18 RSLC column (75 µm × 15 cm, 2 µm particle size). Peptide elution was achieved using a flow-rate of 500 $nl.min^{-1}$ with a gradient: 4-35 % B in 40 min (A: 0.1% formic acid; B: 80 % acetonitrile in 0.1% formic acid). Nano-spray was achieved using a MicroIonSpray (Applied Biosystems) assembled with a New Objective PicoTip emitter. An electrospray voltage of 2.2-2.8 kV was applied to the emitter. The QSTAR ELITE mass spectrometer was operated in Information Dependant Acquisition mode using an Exit Factor of 7.0 and Maximum Accumulation Time of 2.5 sec. Precursor scans were acquired from *m/z* 400-1500 and the three most intense ions were automatically fragmented in Q2 collision cells using nitrogen as the collision gas. Collision energies were chosen automatically as function of *m/z* and charge.

Data processing

LC-MSMS spectral data were analysed using AB SS Protein pilot v4.0.8085. Spectral data was searched against a database containing sequences of bovine thyroglobulin, bovine albumin, chicken ovalbumin and equine myoglobin (The Standard Protein mix) as well as a list of common protein contaminants. Proteins with threshold above ≥ 99.9% confidence were reported.

Results & Discussion

The digestion activity of MagReSyn® Trypsin under denaturing conditions (urea and SDS) was initially evaluated by gel electrophoresis. Under standard conditions (digestion without urea) complete digestion of 50 µg Protein mix was obtained with 20 µl MagReSyn® Trypsin within 1 hr (Figure 1, lane 2). MagReSyn® Trypsin retained sufficient activity for proteolytic digestion in the presence of 5 M urea (Figure 1, lanes 3-5). As a result, when using the standard volume of MagReSyn® Trypsin (20 µl per 50 µg protein) there was substantial amount of undigested protein even after 4 hr incubation (Figure 1, lanes 3-5). Increasing the volume of MagReSyn® Trypsin microparticles from 20 to 50 µl resulted in significant improvement in digestion efficiency (Figure 1, lanes 6-8). MagReSyn® Trypsin retained substantial activity in presence of SDS up to a concentration of 0.5 % with digestion of 50 µg protein mix achieved using 50 µl microparticles within 2 hrs (Figure 2).

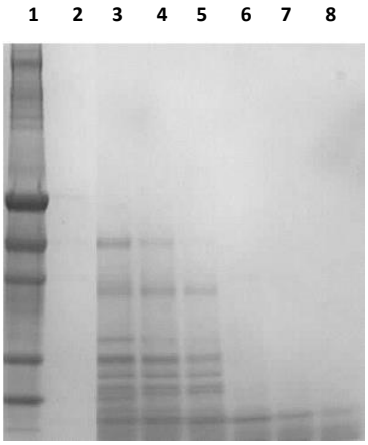

Figure 1: Digestion efficiency of MagReSyn® Trypsin in the presence of 5 M urea. *Lane 1* – undigested protein mix; *Lane 2* – Protein mix digested under native conditions for 1 hr using 20 µl MagReSyn® Trypsin; *Lanes 3-5* – Protein mix digested in the presence of 5 M urea using 20 µl MagReSyn® Trypsin for 1, 2, and 4 hrs respectively; *Lanes 6-8* - Protein mix digested in the presence of 5 M urea using 50 µl MagReSyn® Trypsin for 1, 2, and 4 hr respectively.

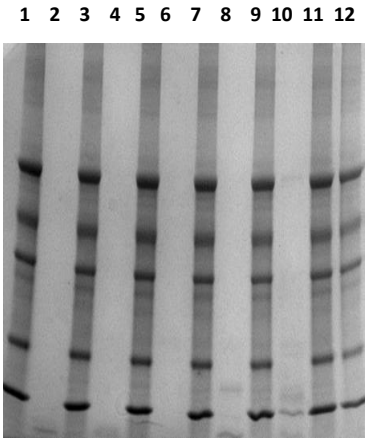

Figure 2: Digestion efficiency of MagReSyn® Trypsin in presence of increasing concentrations of SDS concentrations. *Lanes 1, 3, 5, 7, 9, 11* are undigested protein mix prepared in the various concentrations of SDS; *Lanes 2, 4, 6, 8, 10, 12* are Protein mix digested using 50 µl MagReSyn® Trypsin for 2 hrs in the presence of 0.01, 0.02, 0.1, 0.5 and 1 % SDS respectively illustrating residual protein.

Peptide pools generated from digests performed under native and denaturing (5M urea) conditions were analysed using LC-MSMS. Digestion the presence of 5 M urea using 50 µl MagReSyn® Trypsin resulted in improved sequence coverage for all analysed proteins for both 2 hr and 4 hr incubations (Figure 3). There was a small increase in miss-cleavages in the presence of 5 M urea in comparison to digestion under native conditions (Table 1). This is most likely due to decreased Tryptic activity at such high denaturant concentrations. By increasing the digestion time from 2 to 4 hr miss-cleavage were reduced. The presence of urea did not affect the enzyme specificity with over 90 % of peptides being correctly cleaved at C-terminal of Lysine (K) or Arginine (R) residues (Table 1).

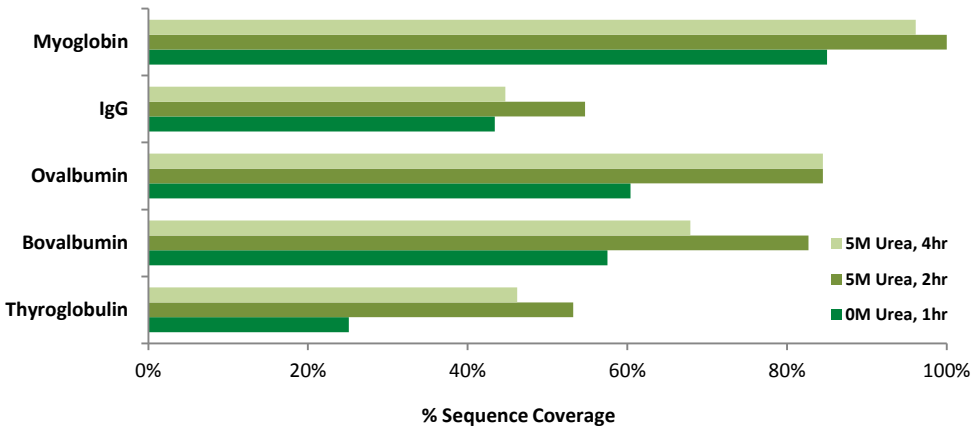

Figure 3: Protein sequence coverage as determined by LC-MSMS analysis. The protein mix was digested under native (with 20 µl microparticles) or denaturing conditions (50 µl microparticles in 5 M urea). Digestion was performed for 1hr under native, and 2 or 4 hrs under denaturing (5M urea) conditions. The sequence coverage for each protein was calculated using LC-MSMS identified peptides having a confidence interval of ≥95%.

Table 1: Specificity and missed-cleavages of proteins digested under experimental digestions conditions.

Digestion Condition	Native	5M - 2Hr	5M - 4Hr
Correct Cleavage	90%	93%	94%
Non Specific (Chymotryptic)	5%	5%	4%
Non Specific (Other)	5%	5%	4%
Increase in miss-cleavage	NA	16%	10%

Conclusions

The application note provides an adaptable protocol for protein sequence analysis under denaturing conditions and enabled through enhanced stability provided by immobilization. PAGE analysis of digests illustrate that MagReSyn® Trypsin retained activity in the presence of high concentrations in the presence of urea and SDS. LC-MSMS peptide sequencing showed that digestion in the presence of urea provided improved sequence coverage without compromising on specificity. The digestion protocol was adapted for use with a magnetic handling station, Thermo Scientific KingFisher™ Duo, illustrating the compatibility of the magnetic microspheres for automation (refer to application note *"Comprehensive protein sequence coverage using MagReSyn® Trypsin & MagReSyn® Chymotrypsin"* available online at www.resynbio.com).

References

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- **Neuhoff** V, Stamm R, Pardowitz I, Arold N, Ehrhardt W, Taube D. Essential problems in quantification of proteins following colloidal staining with Coomassie brilliant blue dyes in polyacrylamide gels, and their solution. Electrophoresis. 2010. 11:101-7

Ordering Information

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

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