Improve Protein Analysis with the New, Mass Spectrometry-Compatible ProteasMAX™ Surfactant

ABSTRACT Incomplete solubilization and digestion and poor peptide recovery are frequent limitations in protein sample preparation for mass spectrometry (MS) analysis. Additives such as SDS or urea can improve protein solubilization and denaturation, but they tend to have negative effects on digestion and MS analysis. Here we present a novel acid- and thermo-labile surfactant, ProteasMAX™ Surfactant, that improves solubilization, digestion and peptide recovery while avoiding negative effects observed with common solubilizing agents. The surfactant is designed to degrade over the course of a typical sample preparation protocol, generating zwitterionic and neutral species that do not interfere with MS.

The improved solubilization properties of the surfactant allowed us to solubilize hydrophobic membrane proteins at room temperature within 1 hour. 2D LC-MS/MS analysis of mouse membrane proteome demonstrated a 70% increase in proteome coverage when the surfactant was added to the sample preparation protocol as a complementary solubilizing agent to urea. We observed up to a 30-fold increase in the rate of proteolysis in trypsin digestion of myoglobin in the presence of the surfactant. In-gel digestion showed up to 1.8-fold increase in protein coverage and up to 6.9-fold increase in MASCOT score. Long peptides in the range of 2,500–4,000 Da, which typically are poorly recovered following in-gel digestion, were present in quantities sufficient for MS/MS analysis. Experiments with model peptides and protein showed that the surfactant also minimizes the loss of peptides and proteins due to adsorption onto laboratory plasticware.

Sergei Saveliev[1], Daniel Simpson[1], William Daily[2], Carolyn Woodroofe[2], Dieter Klaubert[2], Grzegorz Sabat[3], Robert Bulliet[1], and Keith V. Wood[1], [1]Promega Corporation, [2]Promega Biosciences, [3]Mass Spectrometry Facility, Biotechnology Center, University of Wisconsin-Madison.

Surfactant: SURFace ACTive AgeNT. Common examples of surfactants are soap-like detergents including SDS and Triton® X-100.

INTRODUCTION

Advances in mass spectrometry (MS) instrumentation and computational data analysis have driven a transformation in protein analysis, expanding the use of MS into applications such as protein:protein interaction, proteomic profiling, biomarker discovery and analysis of post-translational modification. While MS instrumentation has become increasingly powerful, the limitations on sample protein preparation for analysis have remained relatively unchanged. These limitations include incompatibility of common protein solubilizers with mass spectrometry, inefficient protein digestion, and incomplete peptide recovery. Poor peptide recovery is particularly problematic for in-gel protein digestion.

Most protein analyses done today rely on protease digestion (i.e., trypsin, chymotrypsin, Lys-C, etc.) to reduce the molecular size of the protein and produce a fingerprint of peptides. Efficient digestion requires that the protein(s) of interest be solubilized and at least partially denatured to allow a protease access to proteolytic sites. To achieve this, additives such as urea, guanidine or SDS are often added prior to digestion. While these adjuncts enhance solubility and/or denaturation, they tend to have a negative impact on MS analysis.

Recovery of digested peptides from gel slices represents another problem. Peptide extraction from gel slices relies on diffusion. Because longer peptides diffuse poorly, only relatively short peptides are typically recovered in quantities sufficient for tandem mass spectrometry analysis (MS/MS), decreasing confidence in protein identification (1).

Here we present a new reagent, ProteasMAX™ Surfactant, Trypsin Enhancer[(a)] that helps overcome limitations in protein sample preparation and improve MS results. It can be used to enhance protein solubility, accelerate protease digestion and improve recovery of peptides from polyacrylamide gel slices. ProteasMAX™ Surfactant is a hydrophobic anionic sulfonate (sodium 3-((1-(furan-2-yl)undecyloxy) carbonylamino)propane-1-sulfonate) with a molecular weight of 425.5 Da — effectively an SDS-like molecule, but designed to degrade over the course of a digestion reaction. Degradation of the surfactant produces a hydrophilic zwitterionic species (M.W. = 139.2 Da) and a neutral hydrophobic species (M.W. = 238.4 Da), which do not interfere with MS analysis when the surfactant is used in recommended amounts.

IN-GEL PROTEIN DIGESTION

Figure 1 illustrates the advantages of using ProteasMAX™ Surfactant for in-gel digestions, including significant time savings and improved extraction of long peptides.

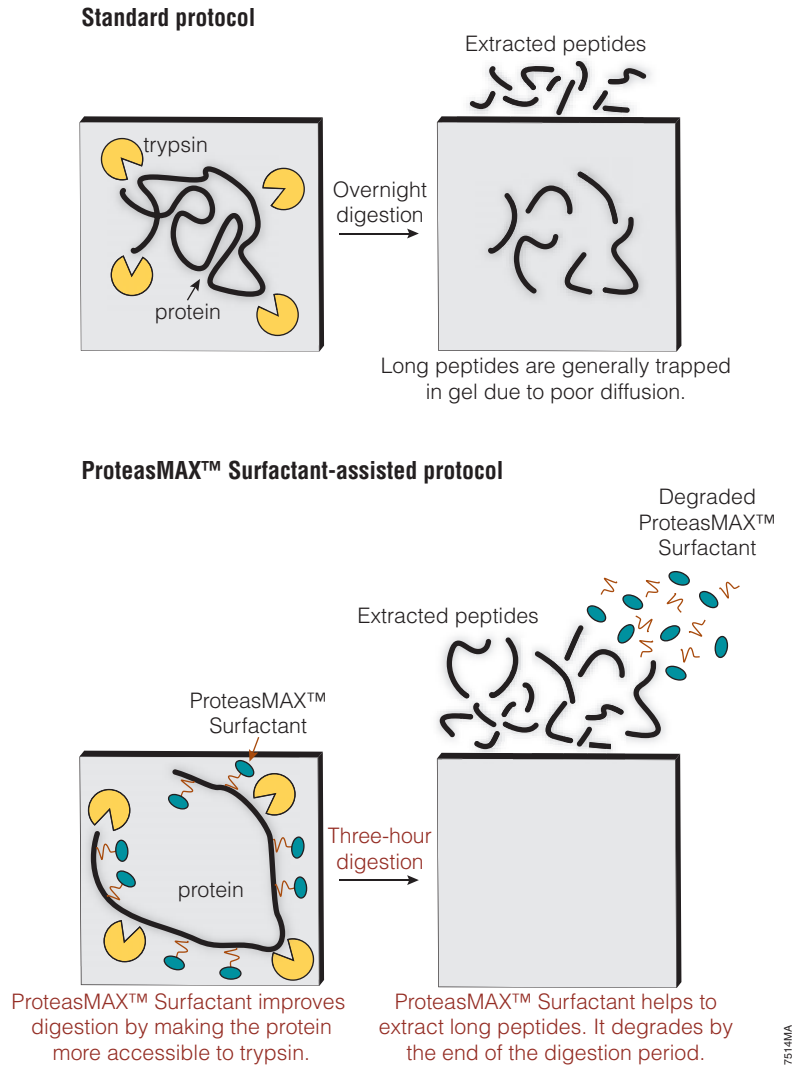


Figure 1. Advantages of in-gel protein digestion with ProteasMAX™ Surfactant, Trypsin Enhancer: significant time savings and improved extraction of long peptides.

Figure 2 presents data from an experiment in which we resolved 20 µg of the membrane protein extract from mouse heart (Sigma) in a 1-D SDS polyacrylamide gel. The gel was stained with Coomassie® Brilliant Blue R-250. Five bands containing proteins with apparent molecular masses ranging from 52 to 114 kDa were excised from the gel and digested with trypsin using either a standard in-gel protocol (2) with overnight digestion, or a ProteasMAX™ Surfactant-assisted protocol with a 3-hour digestion. For the ProteasMAX™ Surfactant-assisted protocol, the digestion was performed in the presence of 0.025% ProteasMAX™ Surfactant. Acetonitrile was omitted from the peptide extraction step (3). We found no decrease in peptide yield using ProteasMAX™ Surfactant in place of acetonitrile. The modified extraction step saves 1–2 hours.

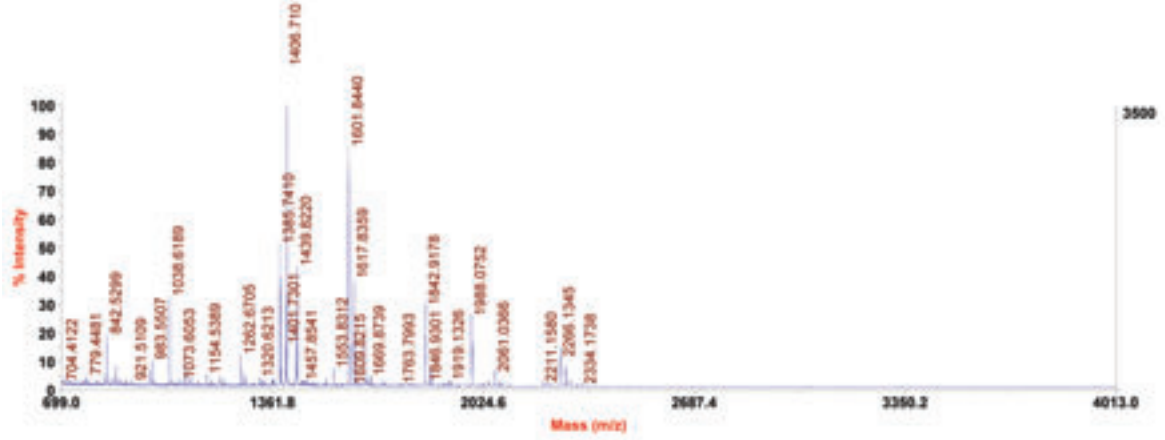
Figure 2 shows the mass spectra of the digested proteins from the isolated protein band having an apparent mass of 55 kDa. A significant improvement in the number and magnitude of peptides is evident in the sample prepared with ProteasMAX™ Surfactant compared to the standard method (compare Figure 2, Panels A and B). The most prominent difference between the standard protocol and the ProteasMAX™ Surfactant-assisted protocol is observed in the peptide range of 2,500–4,000 Da. While this mass range is peptide-free in the sample prepared with the standard protocol (Figure 2, Panel A), it contains prominent peptide peaks in the sample prepared with ProteasMAX™ Surfactant (Figure 2, Panel B). The insert in Panel B shows that these long peptides were extracted in quantities sufficient for MS/MS analysis. The quality of MS/MS analysis was high enough to provide identification of 50% of the amino acid residues in the analyzed peptide (Figure 2, Panel C).

We used MASCOT, a powerful search engine that uses MS data to identify proteins from a primary sequence database, and identified the major 55 kDa protein band as a beta subunit of ATP synthase from H^+ transporting mitochondrial F1 complex (MASCOT search results are not shown; 4). Sequence coverage of the protein increased 31% (from 45% to 76%), and MASCOT score increased 526 points in the sample prepared with ProteasMAX™ Surfactant. Similar increases in protein coverage and MASCOT score were observed for proteins identified from the other four protein bands (data not shown). Improvement in protein coverage and MASCOT score varied for different proteins in this and additional experiments with maximum increase of up to 1.8- and 6.9-fold for protein coverage and MASCOT score, respectively.

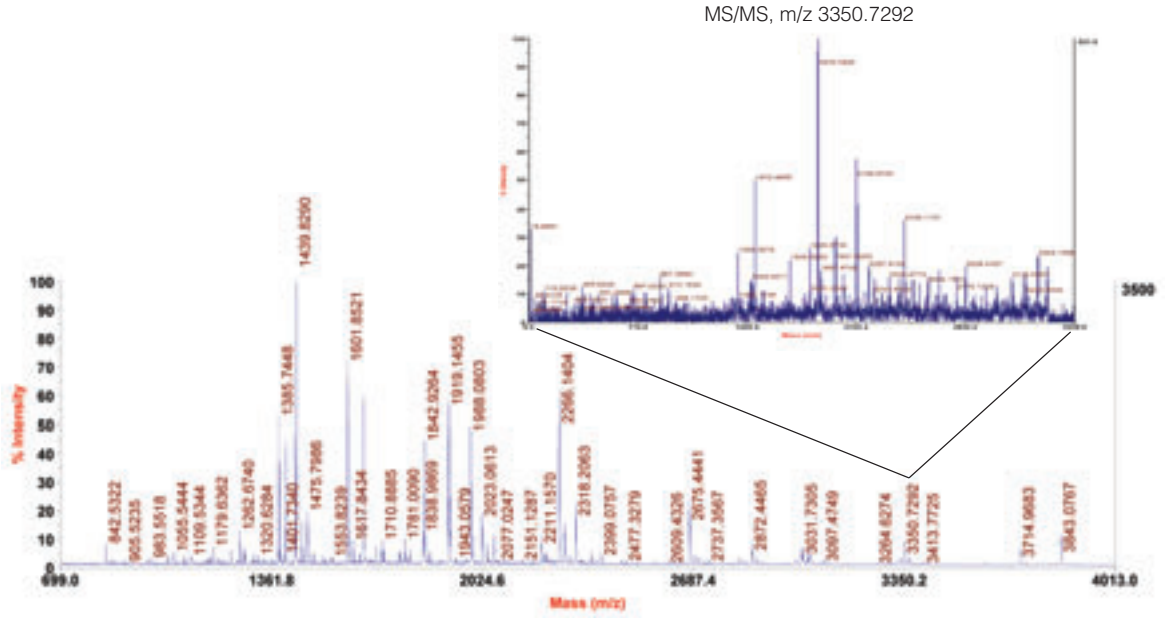
ProteasMAX™ Surfactant has been used for in-gel digestion of 34 different cytoplasmic and membrane proteins. Approximately 75% of these proteins yielded 2,500–4,000 Da peptides in amounts sufficient for MS/MS analysis. By expanding MS/MS analysis into the 2,500–4,000 Da peptide range, ProteasMAX™ Surfactant can improve significantly the sequence coverage and confidence of protein identification.

Benefits of ProteasMAX™ Surfactant for in-gel digestion are not limited to improved peptide recovery. The surfactant also appears to solubilize and unfold proteins that have precipitated in the gel during fixation (Figure 1). As a result we were able to achieve efficient protein digestion within 3 hours. This saves a substantial amount of time compared to standard overnight incubation. Another likely mechanism by which ProteasMAX™ Surfactant enhances digestion is trypsin stabilization. We found that trypsin can be substantially adsorbed by the plasticware typically used during in-gel digestion reactions. Loss of trypsin to adsorption reached 50% or higher in our tests (data not shown). ProteasMAX™ Surfactant minimizes trypsin adsorption to plastic surfaces.

A.



B.



C.

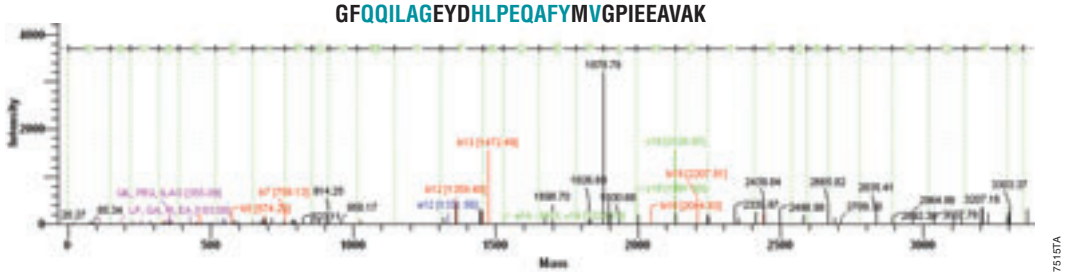


Figure 2. Improved in-gel protein digestion with ProteasMAX™ Surfactant. Membrane protein extract from mouse heart was resolved in 4–20% SDS-PAGE. The gel was stained with Coomassie® Blue R-250. A protein band with an apparent mass of 55 kDa was excised from the gel, and the protein was digested using a standard protocol without ProteasMAX™ Surfactant overnight (**Panel A**) or with ProteasMAX™ Surfactant for 3 hours (**Panel B**). Digestion was performed at 37 °C. The peptides were cleaned up with standard 0.6 µl C18 ZipTip® pipette tips (Millipore) and analyzed with 4800 MALDI-TOF/TOF mass spectrometer (Applied Biosystems). Note the substantial increase in number of peptides and peptide signal in the ProteasMAX™ Surfactant-assisted protocol. This led to a substantial increase in sequence coverage and MASCOT score for the identified protein (see the text for more detail). The major improvement was seen with the extraction of long peptides (compare 2,500–4,000 Da range in Panels A and B). Long peptides were extracted in amounts sufficient for MS/MS analysis. The insert shows MS/MS spectrum of one of those long peptides identified only in the surfactant-enhanced sample. **Panel C** illustrates the annotated sequence for that peptide with its corresponding ions for y and b series highlighted by green dotted lines and red lines, respectively. MS/MS analysis accounted for 50% sequence coverage for this 30 amino acid peptide (the identified residues are highlighted in blue).

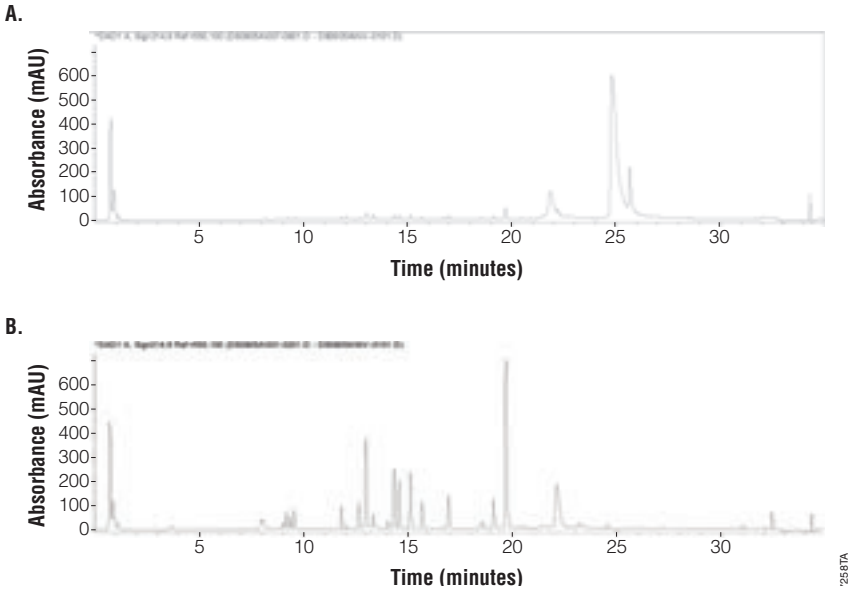


Figure 3. ProteasMAX™ Surfactant reduces the time required for protein digestion. HPLC chromatograms of myoglobin digests. Myoglobin from horse heart was digested with trypsin (50:1) for 30 minutes at 37 °C and resolved on a C18 HPLC column. **Panel A.** Myoglobin was digested under standard conditions (without ProteasMAX™ Surfactant). The large peak at the right is intact myoglobin. **Panel B.** Myoglobin was digested in the presence of 0.025% ProteasMAX™ Surfactant. The peaks on the bottom panel indicate the digested peptides. The data show that the protein is digested in the presence of ProteasMAX™ Surfactant within 30 minutes, whereas it remains largely intact under standard conditions.

ProteasMAX™ Surfactant enhances three major steps in protein MS sample preparation: solubilization, digestion and peptide recovery.

Adsorption is also a known cause of peptide loss. Our experiments involving different peptides showed that ProteasMAX™ Surfactant minimizes peptide adsorption to plastic surfaces. We believe preventing peptide adsorption contributes to the higher peptide recovery with ProteasMAX™ Surfactant.

IN-SOLUTION PROTEIN DIGESTION

ProteasMAX™ Surfactant offers two major benefits for digesting proteins in solution. First, ProteasMAX™ Surfactant efficiently solubilizes proteins. In addition, ProteasMAX™ Surfactant does not interfere with MS analysis when used in the recommended amount. We were able to reproducibly solubilize mouse membrane proteins with ProteasMAX™ Surfactant at room temperature in less than 1 hour.

The second advantage of ProteasMAX™ Surfactant for in-solution protein digestion is an enhanced rate of proteolysis. ProteasMAX™ Surfactant can partially or fully denature proteins in an SDS-like manner, providing the protease easier access to cleavage sites. Figure 3 shows that myoglobin from horse heart was fully digested with trypsin in the presence of as little as 0.01% ProteasMAX™ Surfactant in less than 30 minutes. Myoglobin is moderately resistant to proteolysis and, in the absence of denaturing conditions, requires overnight incubation for full digestion.

Even complex protein mixtures were efficiently digested in solution in the presence of ProteasMAX™ Surfactant. In our model experiment, 500 µg aliquots of the membrane protein from mouse heart were solubilized under three different conditions: 1) with 0.2% of ProteasMAX™ Surfactant, 2) with 8 M urea, 3) with a mix of 0.2% ProteasMAX™ Surfactant and 8 M urea. After solubilization, reaction volumes were adjusted for digestion. Reactions 1 and 3 were digested with trypsin for 3 hours, and reaction 2 was digested overnight. Each digestion was analyzed separately by offline 2D LC-MS/MS. By using ProteasMAX™ Surfactant we increased the number of total MASCOT protein hits from 477 in urea-assisted digest to 812 hits in all the reactions combined. This is an important achievement because membrane proteins are particularly difficult for MS analysis. We note that ProteasMAX™ Surfactant appears to be the most beneficial as a complementary agent or an additive to urea rather than replacement for it. From 812 proteins, 453 were found in the digestion reaction prepared with ProteasMAX™ Surfactant. The other proteins were found in the reactions prepared with urea or urea/ProteasMAX™ mix.

Certain precautions should be taken when using ProteasMAX™ Surfactant for in-solution digestion. We routinely use a 0.1% solution of ProteasMAX™ Surfactant to solubilize cytoplasmic proteins. To solubilize membrane proteins, we increase the concentration to 0.2%. These higher concentrations of ProteasMAX™ Surfactant are not inhibitory for trypsin, but they can be problematic for peptide recovery as well as for mass spectral acquisition. Therefore, before initiating the protein digestion we recommend diluting ProteasMAX™ Surfactant to 0.03 or 0.05%.

The ProteasMAX™ Surfactant degradation product generally does not interfere with mass spectral acquisition, however, to improve signal at very low protein or peptide concentrations it may be desirable to remove or reduce the level of degraded surfactant or any other potential contaminant. Solid phase extraction can be used to capture peptides and reduce the amount of degradation product present; however, it is important to avoid micro-C18 (0.2 µl) tips for this purpose and use only the recommended tip sizes for the amount of surfactant used (0.6 µl ZipTip® or 10 µl OMIX® tips for 50 µl of digest using 0.03% or less ProteasMAX™ Surfactant and 100 µl OMIX® tips for more than 50 µl of digest using 0.03% ProteasMAX™ Surfactant). The use of micro-c18 tips will result in a significant decrease in peptide recovery due to competition with degraded surfactant. For more information regarding this issue see reference 3.

SUMMARY

ProteasMAX™ Surfactant enhances three major steps in protein MS sample preparation: solubilization, digestion and peptide recovery. By increasing the yield of long peptides to quantities sufficient for MS/MS analysis, ProteasMAX™ Surfactant substantially expands the capacity of in-gel protein analysis. ProteasMAX™ Surfactant also enhances digestion of complex protein mixtures in solution, particularly if used concurrently with urea. ProteasMAX™ Surfactant significantly improves protein coverage and overall confidence in protein identification for in-gel and in-solution protein digestion protocols.

REFERENCES

1. Wada, Y. *et al.* (2003) *J. Mass. Spec.* **38**, 117–18.
2. Shevchenko, A. *et al.* (1996) *Proc. Natl. Acad. Sci. USA* **93**, 14440–5.
3. *ProteasMAX™ Surfactant, Trypsin Enhancer Technical Bulletin* #TB373 Promega Corporation.
4. Perkins, D.N. *et al.* (1999) *Electrophoresis* **20**, 3551–67.

PROTOCOL

- *ProteasMAX™ Surfactant, Trypsin Enhancer Technical Bulletin*, #TB373, Promega Corporation
  www.promega.com/tbs/tb373/tb373.html

ORDERING INFORMATION

Product	Size	Cat.#
ProteasMAX™ Surfactant, Trypsin Enhancer	1 mg	V2071
	5 × 1 mg	V2072

[a]Patent Pending.