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Mass Spectrometry Compatible Surfactant for Optimized In-Gel Protein Digestion

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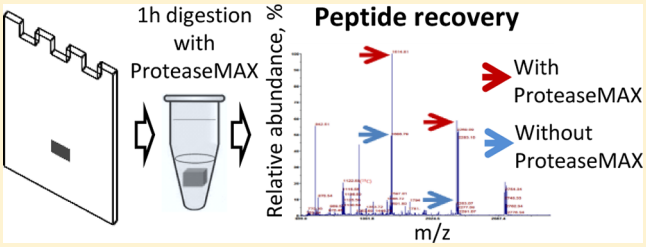
Supporting Information

ABSTRACT: Identification of proteins resolved by SDS-PAGE depends on robust in-gel protein digestion and efficient peptide extraction, requirements that are often difficult to achieve. A lengthy and laborious procedure is an additional challenge of protein identification in gel. We show here that with the use of the mass spectrometry compatible surfactant sodium 3-((1-(furan-2-yl)undecyloxy)carbonylamino)-propane-1-sulfonate, the challenges of in-gel protein digestion are effectively addressed. Peptide quantitation based on stable isotope labeling showed that the surfactant induced 1.5−2 fold increase in peptide recovery. Consequently, protein sequence coverage was increased by 20−30%, on average, and the number of identified proteins saw a substantial boost. The surfactant also accelerated the digestion process. Maximal in-gel digestion was achieved in as little as one hour, depending on incubation temperature, and peptides were readily recovered from gel eliminating the need for postdigestion extraction. This study shows that the surfactant provides an efficient means of improving protein identification in gel and streamlining the in-gel digestion procedure requiring no extra handling steps or special equipment.



Mass spectrometry (MS) has become the leading analytical tool in proteomics because of its sensitivity, speed, and high-throughput capability. However, the analytical capability of mass spectrometry is just one of the requirements for efficient protein identification. Adequate sample preparation is also a necessity. Biological samples typically consist of thousands of proteins; thus, to reduce the biological sample complexity to a level amenable for MS analysis, different methods of protein fractionation are applied. In the first method, peptides generated by proteolysis are separated with liquid chromatography (LC) before being subjected to MS. LC-MS is widely accepted because of its high sensitivity, peptide resolution capacity, and minimal hands-on time. An alternative fractionation method relies on gel electrophoresis. Proteins are first resolved in gel, and then species of interest are excised and digested; generated peptides are extracted from the gel and analyzed with MS. Two-dimensional gel electrophoresis is favored by many researchers because of its high resolving power, whereas one-dimensional gel with lower resolving power allows for much higher throughput. One-dimensional gel electrophoresis is also a popular means of removing impurities such as salts, buffer or detergents from a protein sample prior to MS analysis. Gel electrophoresis is often used in combination with LC-MS in a hybrid method referred to as gel-LC-MS/MS. In gel-LC-MS/MS, proteins are first fractionated according to their electrophoretic mobility. Each fraction is then digested and peptides are resolved chromatographically prior to MS.

There are several shortcomings of protein sample preparation involving gel fractionation. Efficient peptide extraction from gel is often difficult to achieve due to limited peptide diffusion from the gel. Indeed, large and hydrophobic peptides tend to remain in the gel even after rigorous peptide extraction.[1,2] Adsorption to plastic ware (i.e., to tube walls) leads to a further decrease in peptide recovery. Adsorption can cause a loss of up to 50% of the peptides.[3] As a result, in-gel protein digestion requires relatively large protein amounts for reliable protein identification. Furthermore, this procedure is laborious and time-consuming; it includes many manual steps and typically requires overnight digestion. Various approaches have been utilized to improve in-gel protein digestion and streamline the process. For example, ultrasound and infrared radiation have been shown to reduce digestion time and improve peptide recovery from the gel.[4,5] Unfortunately, the requirement for special equipment limits the utility of these approaches.

Detergents provide a viable alternative to address the needs of in-gel protein digestion. By solubilizing and denaturing proteins, detergents enhance the efficiency of protein digestion. The caveat is that detergents are generally not compatible with

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mass spectrometry.[6] For example, the commonly used ionic detergent SDS causes high noise and contributes to signal suppression interfering with peptide identification.[7] To address this limitation, acid-degradable mass spectrometry compatible surfactants ("surfactant" stands for surface acting agent) have been developed. These surfactants begin as ionic, mass spectrometry-incompatible compounds but degrade into species that are innocuous for mass spectrometry analysis after acid hydrolysis. Acid-degradable sodium 3-[(2-methyl-2-undecyl-1,3-dioxolan-4-yl)methoxyl]-1-propanesulfonate, which is known as *RapiGest*, was successfully utilized to improve in-gel protein digestion while avoiding interference with mass spectrometry analysis.[8]

To date, no systematic study of surfactant effect on in-gel protein digestion has been conducted limiting the opportunity to optimize surfactant use in this procedure. Here we analyze the impact of the novel mass spectrometry-compatible surfactant, sodium 3-((1-(furan-2-yl)undecyloxy) carbonylamino) propane-1-sulfonate on key steps of in-gel protein digestion. In the presence of the surfactant the digestion was rapid, with maximal peptide recovery achieved in only a few hours or at elevated temperature, after just one hour of incubation. The most efficient digestion required a narrow range of low surfactant concentrations and actually declined at higher concentrations. Peptides were readily recovered from gel eliminating the need for postdigestion extraction. On the basis of these findings, we established an optimized protocol, in which protein digestion and peptide extraction steps were combined in a single one hour step. Our quantitative assay, based on the use of stable isotope labeling, indicated that peptide recovery from the gel increased 1.5−2 fold, on average, in the optimized surfactant-assisted protocol as compared to a conventional in-gel digestion. The surfactant also dramatically minimized postdigestion peptide loss arising from limited peptide solubility or adsorption to plasticware. The combination of positive effects led to improvement in protein sequence coverage and increased confidence of protein identification.

Simplicity of use is a distinct advantage of the surfactant. The surfactant-assisted in-gel protein digestion does not require special equipment or extra handling steps. The surfactant degrades over the course of a typical digestion reaction eliminating the need for postdigestion degradation. The digested sample is then processed with use of typical sample handling procedures, such as centrifugation or solid phase extraction, and it can be readily analyzed with LC-MS or MALDI.

EXPERIMENTAL SECTION

Materials. Dithiothreitol (DTT), bacteriorhodopsin, myoglobin, ovalbumin (albumin from chicken egg white, grade VII), chymotrypsin, α-cyano-4-hydroxycinnamic acid, acetonitrile (ACN), and ammonium bicarbonate were purchased from Sigma-Aldrich (St. Louis, MO). Trifluoretic acids (TFA), bovine serum albumin (BSA), and iodoacetamide (IAA) were from Thermo Fisher Scientific (Waltham, MA). Trypsin Gold, mass spectrometry grade, was from Promega (Madison, WI). Nanopure water was prepared with use of Milli-Q water purification system (Millipore, Billerica, MA).

Preparation for In-Gel Protein Digestion. Proteins were separated using precast 4−20% Tris-Glycine SDS-PAGE gels (1.0 mm thick) (Life Technologies, Carlsbad, CA). The protein gels were stained with Coomassie Blue or Simply Blue SafeStain (Life Technologies, Carlsbad, CA) and protein bands of interest were cut out of gel. Typically, the dimension of gel slices was 10−12 mm^3. The slices were cut into 1 mm^3 pieces and transferred into 1.5 mL polypropylene tubes (Thermo Fisher Scientific, Waltham, CA). The gel pieces were washed with 200 μL of Nanopure water for 30 s and destained twice with 200 μL of 50% Methanol/50 mM Ammonium bicarbonate mixture for 1 min with intermittent mixing. The pieces were dehydrated with 200 μL of 50% ACN/50 mM Ammonium bicarbonate mixture for 5 min using intermittent mixing, and then with 200 μL of 100% ACN for 30 s. ACN was discarded. Traces of remaining ACN were removed by vacuum drying in SpeedVac for 5 min. The gel pieces were rehydrated in 100 μL of freshly prepared 25 mM DTT in 50 mM Ammonium bicarbonate and incubated at 56 °C for 20 min. The solution was discarded and the pieces were incubated in 100 μL of freshly prepared 55 mM IAA in 50 mM Ammonium bicarbonate at room temperature in the dark for 20 min. The pieces were washed in 400 μL of nanopure water for 1 min with intermittent mixing and, then, were dehydrated, first in 200 μL of 50% ACN/50 mM ammonium bicarbonate mixture for 5 min with intermittent mixing and then with 200 μL of 100% ACN for 30 s. ACN was discarded and traces of remaining ACN were removed by vacuum drying in SpeedVac for 5 min.

Conventional In-Gel Protein Digestion. The dried gel pieces were rehydrated in 20 μL of 12 ng/μL Trypsin Gold/50 mM ammonium bicarbonate for 10 min at room temperature and, then, overlaid with 10−15 μL of 50 mM ammonium bicarbonate to cover gel pieces and incubated at 37 °C overnight. After the digestion was complete, condensed evaporated water was collected from tube walls by 5 s centrifugation using benchtop microcentrifuge (Eppendorf, Hauppauge, NY). The gel pieces and digestion reaction were mixed with 50 μL 2.5% TFA and rigorously mixed for 15 min. The solution with extracted peptides was transferred into a fresh tube. The remaining peptides were extracted with 80 μL 70% ACN/5% TFA mixture using rigorous mixing for 15 min. The extracts were pooled and dried to completion (1.5−2 h) in SpeedVac. The dried peptides were reconstituted in 30 μL 0.1% TFA by mixing for 5 min and stored in ice or at −20 °C prior to analysis.

Streamlined Surfactant-Assisted In-Gel Protein Digestion. The dried gel pieces were rehydrated in 20 μL 12 ng/μL Trypsin Gold/0.01% ProteaseMAX surfactant/50 mM ammonium bicarbonate mixture for 10 min at room temperature, overlaid with 30 μL 0.01% ProteaseMAX surfactant/50 mM ammonium bicarbonate and incubated at 50 °C for 1 h. After the digestion was complete, condensed evaporated water was collected from tube walls by 5 s centrifugation. The digestion reaction (containing extracted peptides) was transferred to a fresh tube, mixed with TFA (0.5% final TFA concentration) to inactivate trypsin and stored in ice or at −20 °C prior to analysis.

MALDI TOF/TOF Analysis. Peptides were solid phase extracted using $ZipTip_{C18}$ (Millipore, Billerica, MA) and eluted with 0.5 μL of acetonitrile/H_2O/TFA (60%:40%:0.05%), deposited onto the Opti-TOF 384 Well plate (Applied Biosystems, Foster City, CA) and crystallized with 0.5 μL of matrix (10 mg/mL α-cyano-4-hydroxycinnamic acid in acetonitrile/H_2O/TFA (60%:40%:0.05%)). Peptide map fingerprint result-dependent MS/MS analysis was performed on a 4800 MALDI TOF-TOF mass spectrometer (Applied Biosystems, Foster City, CA). In short, peptide fingerprint was

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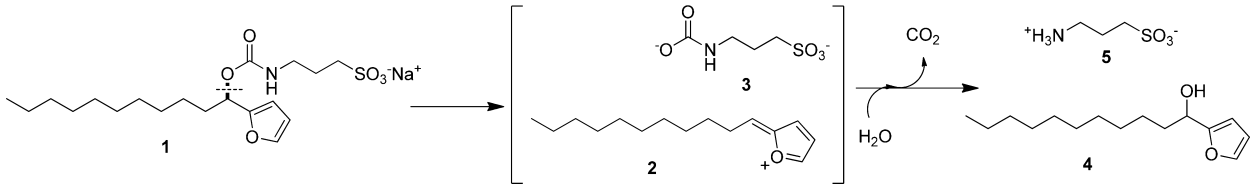

Figure 1. Sulfonate(sodium 3-((1-(furan-2-yl)undecyloxy)carbonylamino)propane-1-sulfonate surfactant and its decomposition pathway.

generated scanning 700−4000 Da mass range using 1000 shots acquired from 20 randomized regions of the sample spot at 3600 intensity of OptiBeam on-axis Nd:YAG laser with 200 Hz firing rate and 3−7 ns pulse width in positive reflectron mode. Fifteen most abundant precursors, excluding trypsin autoproteolytic peptides and sodium/potassium adducts, were selected for subsequent tandem MS analysis where 1200 total shots were taken with 4200 laser intensity and 2 kV collision induced activation (CID) using air. Postsource decay (PSD) fragments from the precursors of interest were isolated by timed-ion selection and reaccelerated into the reflectron to generate the MS/MS spectrum. Raw data was deconvoluted using GPS Explorer software and submitted for peptide mapping and MS/MS ion search analysis against user defined database with an in-house licensed Mascot search engine (Matrix Science, London, U.K.) with cysteine carbamidomethylation and methionine oxidation as variable modifications.

LC-MS/MS Analysis. Samples were centrifuged at 14000−16000 × *g* for 10 min to remove particulate material. Five microliters of each sample were injected into a self-packed fused silica nano C18 reversed phase column (12 cm in length with an inner diameter of 150 μm (Peeke Scientific, Redwood City, CA)). The LC system was an Eksigent nano2D LC run at 0.45 μL/minute. Mobile phase A was 99.9% water/0.1% formic acid, mobile phase B was 99.9% acetonitrile/0.1% formic acid. A linear gradient from 2% mobile phase B to 45% mobile phase B over sixty minutes was used. The ions were infused into the mass spectrometer using an Advion Nanomate source. The mass spectrometer was an LCQ Deca XP+ ion trap set in data dependent acquisition mode to perform a MS/MS experiment on the top 3 most intense precursor ions. Database searching was done using a Sorcerer (SageN) processor, Sequest was used and the database was the ipi Rat v.35. For highest protein stringency and to mitigate the assignment of false positives, 2 peptides with a protein FDR of 0.2% and a peptide FDR of 5% were used for protein assignments.

RESULTS AND DISCUSSION

Novel Acid- and Thermolabile Mass Spectrometry Compatible Surfactant. An ideal surfactant for mass spectrometry protein sample preparation should have enhanced protein solubilizing and denaturing properties while avoiding interference with trypsin and mass spectrometry. In regards to the compatibility with mass spectrometry, incorporation of an acid labile bond into a surfactant offers a viable solution under the caveat that treatment with acid degrades the surfactant into mass spectrometry innocuous species.[9,10]

We screened a wide variety of structurally diverse surfactant-like compounds using acid lability, efficient protein solubilization and digestion as selection criteria. With regard to fulfilling these requirements, sodium 3-((1-(furan-2-yl)undecyloxy)-carbonylamino)propane-1-sulfonate[11] (trade name ProteaseMAX) was selected as the best candidate (Figure 1, compound **1**). This surfactant is structurally similar to SDS, with a sulfonate anion as the hydrophilic head and a long alkyl chain as the hydrophobic tail. A furanyl carbamate group separating the hydrophilic head and hydrophobic tail plays a critical role in acid lability of the surfactant. Upon acid hydrolysis (Figure 1), the furanyl carbamate group stabilizes intermediate **2**, allowing the indicated bond in the surfactant to break easily. Intermediate **3** then spontaneously loses carbon dioxide, providing a strong driving force for irreversible degradation of the surfactant, while intermediate **2** undergoes the addition of water. The degradation is complete within 5 min under acidic conditions at ambient temperature (Supporting Information, Figure S-1) and efficiently transforms an anionic surfactant into one neutral compound **4** and one small zwitterionic species **5** eliminating the surfactant interference with mass spectrometry.

The furanyl group destabilizes the labile bond sufficiently that surfactant degradation occurs not only at low pH but even under neutral conditions. The rate of degradation at neutral pH is dependent on concentration and temperature. The surfactant is stable at higher concentration and low temperature, yet rapidly hydrolyzes at low concentration and increased temperature (Supporting Information, Figure S-2). This leads to an important procedural advantage, since effective surfactant concentrations used in protein digestion reaction are sufficiently low to allow degradation over the course of a typical protein digestion. Thus, unlike other acid-degradable surfactants, this surfactant does not require postdigestion acid degradation.

We made an interesting observation regarding the role of the degradation products in postdigestion period of the in-gel digestion process. It is known that peptides adsorb to the sides of a plastic reaction tube reducing the efficiency of protein identification.[3] We found that the surfactant mitigated peptide loss even after it has been degraded. To demonstrate this we incubated various peptides in a plastic tube in the presence of the predegraded surfactant. Four out of six tested peptides showed significant improvement in recovery (2−17 fold) if the degraded surfactant was present in solution (Supporting Information, Figure S-3). We contend that the improvement in peptide recovery is likely due to the degradation product **4**. This compound retains the hydrophobic tail of the original surfactant, while the hydroxyl and furyl group lend uncharged yet polar character to the other end of the molecule. This combination of properties is consistent with the structure of a nonionic surfactant.

The surfactant demonstrated strong protein solubilizing and proteolysis enhancing capacities. The membrane protein bacteriorhodopsin, insoluble in aqueous solution, was rapidly solubilized and then proteolyzed in the presence of the surfactant (Supporting Information, Figure S-4). Bacteriorhodopsin was solubilized at room rather than high temperature indicating enhanced solubilizing efficiency of the surfactant. This finding is in line with the results of the other group, which showed efficient extraction of membrane proteins from rat brain with the surfactant.[12] This group demonstrated superior

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Table 1. Optimization of the Surfactant-Assisted In-Gel Protein Digestion

optimization of time and temperature (each reaction was performed in duplicates)	protein coverage (%)	MASCOT score
BSA (50 ng (0.75 pmol))		
conventional overnight digestion w/o surfactant, 37 °C	23 ± 5	120.5 ± 4.5
overnight digestion with surfactant, 37 °C	56.5 ± 2.5	257 ± 10
4 h digestion with surfactant, 37 °C	60 ± 5	281 ± 30
2 h digestion with surfactant, 37 °C	68 ± 2	318.5 ± 4.5
1 h digestion with surfactant, 50 °C	63 ± 5	275.5 ± 94.5
ovalbumin (50 ng (1.13 pmol))		
conventional overnight digestion w/o surfactant, 37 °C	30.5 ± 1.5	117.5 ± 7.5
overnight digestion with surfactant, 37 °C	52.5 ± 2.5	198.5 ± 30.5
4 h digestion with surfactant, 37 °C	52.5 ± 2.5	124.5 ± 25.5
2 h digestion with surfactant, 37 °C	52.5 ± 5.5	135.5 ± 34.5
1 h digestion with surfactant, 50 °C	54.5 ± 1.5	223.5 ± 68.5
optimization of the surfactant concentration	**protein coverage (%)**	**MASCOT score**
ovalbumin (50 ng (1.13 pmol))		
0.01% surfactant concentration, 1 h digestion, 50 °C	49 ± 1	602 ± 37
0.025% surfactant concentration, 1 h digestion, 50 °C	50 ± 2.5	469.5 ± 40.5
0.1% surfactant concentration, 1 h digestion, 50 °C	40 ± 4	131 ± 6
mouse heart ATP synthase, b subunit (250 ng (4.5 pmol))		
0.01% surfactant concentration, 1 h digestion, 50 °C	63.5 ± 1.5	841 ± 51
0.005% surfactant concentration, 1 h digestion, 50 °C	51.5 ± 0.5	662 ± 131

effect of postdigestion peptide extraction with ACN	protein coverage (%)	MASCOT score
experiment 1, BSA (0.75 pmol)		
surfactant-assisted in-gel digestion (1 h incubation at 50 °C) without postdigestion peptide extraction step	56 ± 3	106 ± 38
surfactant-assisted in-gel digestion (1 h incubation at 50 °C) followed by peptide extraction with ACN	18 ± 3	35.5 ± 1.5
experiment 2, mouse ATP synthase, b subunit (4 pmol)		
surfactant-assisted in-gel digestion (1 h incubation at 50 °C) without postdigestion peptide extraction step	53.5 ± 0.5	614.5 ± 104.5
surfactant-assisted in-gel digestion (1 h incubation at 50 °C) followed by peptide extraction with ACN, in which drying the extracted peptides was avoided	51.5 ± 2.5	586.5 ± 64.5

performance of the surfactant versus Invitrosol (Life Technologies), the other mass spectrometry compatible surfactant, as judged by the number of identified proteins. In another model experiment, the surfactant induced rapid digestion of a proteolytically resistant protein myoglobin (Supporting Information, Figure S-5). We hypothesize that the surfactant denatured myoglobin allowing for ready protease access to earlier inaccessible internal protein cleavage sites. Importantly, the surfactant did not inhibit trypsin. On the contrary, trypsin activity was higher in the presence of the surfactant (Supporting Information, Figure S-6), which may have been due to prevention of trypsin adsorption to the reaction tube walls.

Overall, the surfactant demonstrated a strong positive impact on the key steps of protein sample preparation for mass spectrometry—protein solubilization, digestion, and peptide recovery. Degradation during the course of protein digestion added a unique advantage by eliminating the need for postdigestion surfactant degradation.

Optimization of the Surfactant-Assisted In-Gel Protein Digestion. We began analysis of the surfactant-assisted in-gel protein digestion by applying conditions similar to those used in a conventional procedure. Overnight incubation at 37 °C is a typical requirement of in-gel protein digestion used to ensure efficient proteolysis. Studies involving other surfactants showed that incorporation of a surfactant into a conventional procedure improved in-gel protein digestion,[8,13,14] and we expected that our surfactant would show similar effect.

In agreement with our expectations, incorporation of the surfactant into conventional in-gel digestion protocol resulted in a strong increase in protein sequence coverage and significant improvement in the probability of identification for both tested model proteins (Table 1, optimization of time and temperature). In the experiment, the digestion was performed in duplicates and the digested samples were analyzed with 4800 MALDI-TOF/TOF. Close analysis of the mass spectra showed that the increase in protein sequence coverage was largely because of improved recovery of larger peptides (Supporting Information, Figure S-7), a phenomenon observed earlier with another surfactant.[13] These peptides typically remain in gel due to poor diffusion.[1,2]

Reducing digestion time was our first optimization to the surfactant-assisted in-gel digestion procedure. We logically expected that, by denaturing proteins, the surfactant would enable ready protease access to internal protein cleavage sites, leading to rapid protein digestion. To test our theory, we examined the time course of the surfactant-assisted digestion. We found that a few hours of digestion in the presence of the surfactant were sufficient to achieve maximal protein sequence coverage and probability of identification (Table 1, optimization of time and temperature). The apparent shortest period at which the surfactant-assisted in-gel protein digestion still retained its maximum efficiency was 2 h (the results of the digestion shorter than 2 h are not shown).

In the study, we used chemically modified trypsin by Promega Corporation (Madison, WI). It has been found earlier that this trypsin maintains high proteolytic activity even at 50 °C.[15] We, therefore, had an option to perform in-gel digestion at 50 °C instead of 37 °C potentially completing digestion within a even shorter time period due to stimulation of enzymatic reaction at an elevated temperature. Indeed, at 50

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°C, the maximal level of protein sequence coverage and probability of identification was achieved after just one hour of incubation (Table 1, optimization of time and temperature). Conventional digestion performed under the same conditions (i.e., at 50 °C for 1 h) showed substantial drop of protein sequence coverage and probability of protein identification indicating that combination of high temperature and the surfactant rather than high temperature alone was required for efficient 1 h in-gel digestion (Supporting Information Table S-1). On the basis of the results, 2−4 h incubation at 37 °C or 1 h incubation at 50 °C were selected as optimal digestion conditions.

We next examined the effect of the surfactant concentration. The data showed that the maximal levels of sequence coverage and probability of identification were observed within a narrow range of low surfactant concentrations (0.01−0.025%) (Table 1, optimization of surfactant concentration). A surfactant concentration of 0.005% afforded approximately 20% less protein sequence coverage and decreased probability of protein identification when compared to 0.01% surfactant solution. Similarly, 0.1% surfactant concentration had a negative effect on in-gel protein digestion. In regards to the last observation, we suspect that reduced solubility of the degraded surfactant was the cause of declining in protein sequence coverage and probability of protein identification. In fact, the degraded surfactant was found to partially precipitate at 0.1% and higher concentrations (data not shown). We hypothesize that peptides coprecipitated with the degraded surfactant at 0.1% surfactant concentration causing the observed negative effect.

Acetonitrile (ACN), typically in combination with trifluoroacetic or formic acid, is often used to improve extraction of peptides from gel.[16] We, therefore, tested if ACN extraction improved peptide recovery in the surfactant-assisted in-gel digestion. Surprisingly, ACN extraction procedure had a detrimental effect. Protein sequence coverage and probability of identification decreased 3-fold after incorporation of ACN extraction into the procedure (Table 1, effect of postdigestion peptide extraction with ACN, experiment 1). To understand the cause of this effect we looked closely at the ACN extraction procedure. In the procedure, peptides were extracted with a relatively large volume of ACN. The extraction volume was then minimized by drying. The dried peptides were reconstituted in a small volume of aqueous solution. Drying also removed ACN, which would otherwise interfered with peptide fractionation using reverse phase LC. We hypothesized that drying was the major cause of the observed drop in sequence coverage and probability of protein identification. During drying the degraded surfactant and peptides coprecipitated forming a pellet. As we noted above, in contrast to the intact surfactant, the degraded surfactant has a limited solubility and, evidently, it did not efficiently resolubilize upon reconstitution of the dried pellet in an aqueous solution. This negatively affected mass spectrometry because peptides remaining in the pellet of the insolubilized degraded surfactant were not available for the analysis. To test this hypothesis, we avoided drying the degraded surfactant. We achieved this by transferring the digestion reaction, which contained most of the surfactant, in a separate tube prior to treatment of gel pieces with ACN. Gel pieces were then treated with ACN and ACN solution with extracted peptides was dried to completion. ACN-extracted peptides were combined with peptides from the digestion reaction by using the digestion reaction as a reconstitution solution. The data showed that, using the modified extraction protocol, we prevented the drop in protein sequence coverage and probability of protein identification (Table 1, effect of postdigestion peptide extraction with ACN, experiment 2). However, the coverage and probability were not improved either, indicating that most if not all peptides were extracted during the digestion. We concluded that the surfactant acted as an efficient peptide solubilizing and extracting agent eliminating the need for postdigestion peptide extraction with ACN.

Quantification of Peptide Recovery. To determine the surfactant-induced increase in peptide recovery, we developed a peptide quantification assay based on differential stable isotope labeling. For the assay, light and heavy isotope labeled forms of the same protein were synthesized. TNT SP6 high-yield wheat germ protein expression system minus amino acids (Promega Corporation, Madison, WI) supplemented with ^{12}C- or ^{13}C-labeled arginine was used to make the proteins. ^{13}C-labeled arginine was incorporated into a protein with 97% efficiency (data not shown). Membrane protein HTR1A (NCBI reference sequence NP_000515.2) was selected for the experiment in order to challenge the in-gel digestion as membrane proteins are particularly resistant to proteolysis. The proteins were purified from the extract using HaloTag technology[17] and equal quantities of each light and heavy isotope labeled protein were then resolved in gel. The light-isotope-labeled protein was digested using a conventional in-gel digestion procedure, and the heavy-isotope-labeled protein, using the surfactant-assisted procedure. After the digestion was complete, equal volumes of peptide extracts from each reaction were mixed and analyzed with MALDI-TOF. Three peptides were selected as quantitation references (Figure 2). Light and heavy isotope labeled peptides were easily differentiated in mass spectra due to 6 Da mass difference. The surfactant-induced increase in peptide recovery was measured on the basis of increased signal intensity of heavy versus light isotope labeled peptide. Reciprocal experiments, in which the light isotope labeled protein was digested using the surfactant-assisted procedure and the heavy isotope labeled protein was digested using the conventional procedure, were also carried out to adjust for possible differences in the loaded amounts of light and heavy isotope labeled proteins.

In the first experiment, the conventional and surfactant-assisted digestions were performed under conditions, which were optimal for the surfactant-assisted digestion −1 h at 50 °C. Under those conditions the surfactant improved peptide recovery 1.38−6.5-fold (Figure 2 and Table 2, experiment 1). In the second experiment, the conventional digestion was performed under most favorable conditions, overnight incubation at 37 °C, followed by peptide extraction with ACN, whereas the surfactant-assisted digestion was still performed in a single 1 h step at 50 °C. The surfactant-assisted procedure again significantly outperformed the conventional procedure, providing 1.45−2-fold higher peptide recovery (Table 2, experiment 2).

Validation of the Surfactant-Assisted In-Gel Digestion Procedure. The experiments above showed that the streamlined surfactant-assisted in-gel protein digestion, in which protein digestion and peptide extraction steps were reduced to a single step, 1 h incubation at 50 °C, provided better results than a conventional procedure. However, just a few model proteins were used in the experiments raising the possibility that the protocol could not work equally well with other proteins. To address this concern, we analyzed a panel of

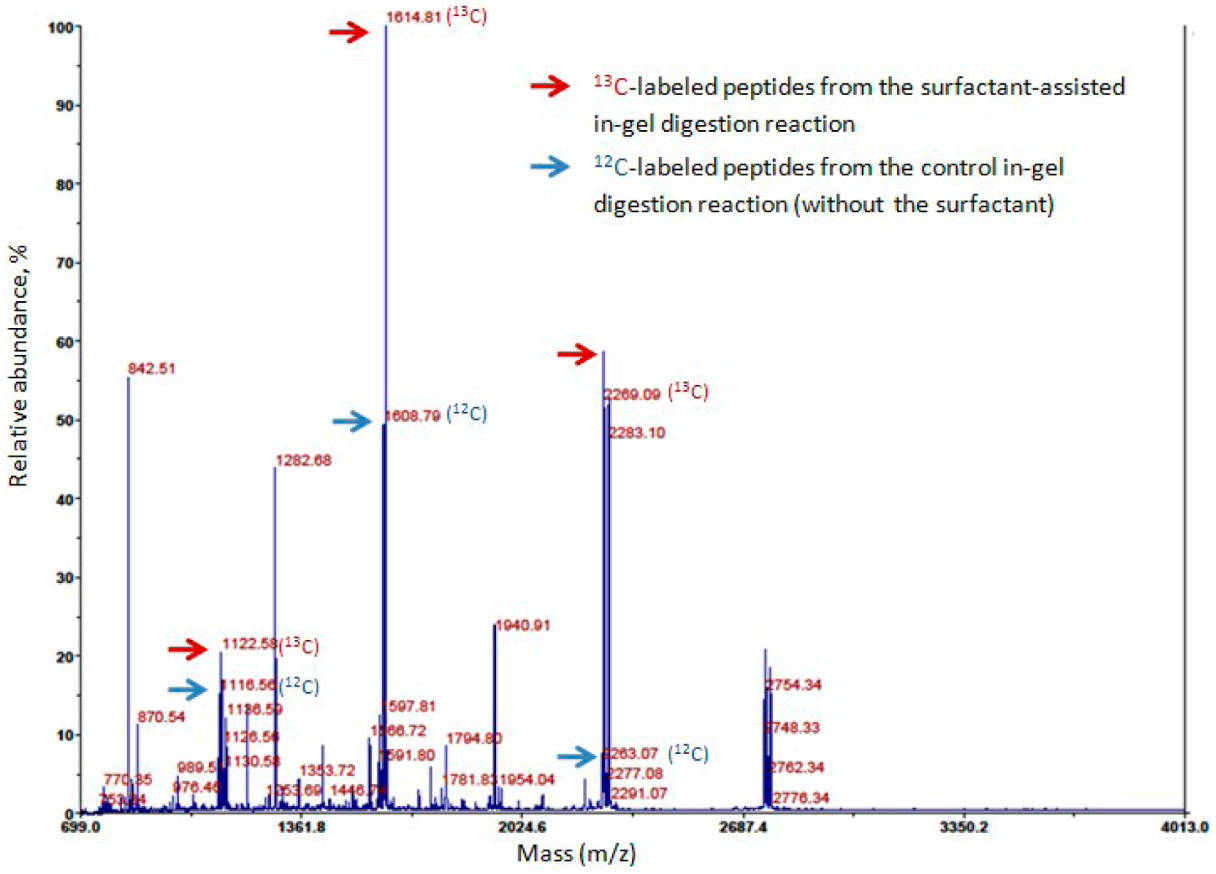

Figure 2. Improved peptide recovery from gel in the presence of the surfactant. ^{13}C-labeled HTR1A protein was in-gel digested for 1 h at 50 °C in the presence of 0.01% surfactant. The same amount of ^{12}C-labeled HTR1A protein was in-gel digested at identical conditions without the surfactant. After digestion reactions were complete equal aliquots from each reaction were mixed, cleaned up with C18 SPE tips, and analyzed with MALDI-TOF MS.

Table 2. Improved Peptide Recovery from Gel in the Presence of the Surfactant

peptides			surfactant-induced increase in peptide recovery, fold change	
	peptide mass			
peptide sequence	light isotope (^{12}C) version (Da)	heavy isotope (^{13}C) version (Da)	experiment 1 (the experiment was performed in four replicates)	experiment 2 (the experiment was performed in four replicates)
AGGALCANGAVR	1116.6	1122.6	1.38 ± 0.09	1.45 ± 0.28
QGDDGAALEVIEVHR	1608.8	1614.8	1.53 ± 0.25	2.06 ± 0.75
EHLPLPSEAGPTPCAPASFER	2263.1	2269.1	6.50 ± 1.51	1.80 ± 0.34

various proteins. Each protein was digested side-by-side using the surfactant-assisted and conventional procedures. Protein sequence coverage was chosen as a performance criterion. The data confirmed the advantage of the surfactant-assisted digestion. Using the surfactant-assisted procedure we improved protein sequence coverage for 26 out of 31 analyzed proteins (Figure 3). The coverage was increased by 30%, on average. A paired t test confirmed a statistically significant surfactant-induced increase in protein coverage ($p < 0.0001$, Supporting Information Figure S-8a and b).

Next, we tested a complex protein mixture using mouse membrane protein extract as a model system. The extract was analyzed with a gel-LC-MS/MS method. In short, the extract was first fractionated with SDS-PAGE. After the gel electrophoresis was complete, each gel lane was cut into nine bands with each band representing a separate protein fraction. The bands were divided onto two equal parts. One part was digested using the surfactant-assisted procedure and other, conventional procedure. Digested peptides were next analyzed via a nanoLC-MS/MS workflow. We found that the surfactant induced a 33% increase in the number of assigned peptide MS/MS spectra (from 3178 to 4218). This led to improved protein sequence coverage. In fact, 25% of the identified proteins saw an increase in sequence coverage with the average increase equaling to 21% (Supporting Information, Table S-2). Use of the surfactant also resulted in the identification of additional proteins. The latter proteins were primarily membrane and vesicle associated proteins (Supporting Information, Table S-3), identification of which failed in a conventional digestion.

We found that the surfactant also improved probability of identification of proteins present in gel at low nanogram levels. Identification of such proteins in gel is often compromised. The surfactant-assisted digestion procedure required certain adjustment for optimal performance in regards to low abundant

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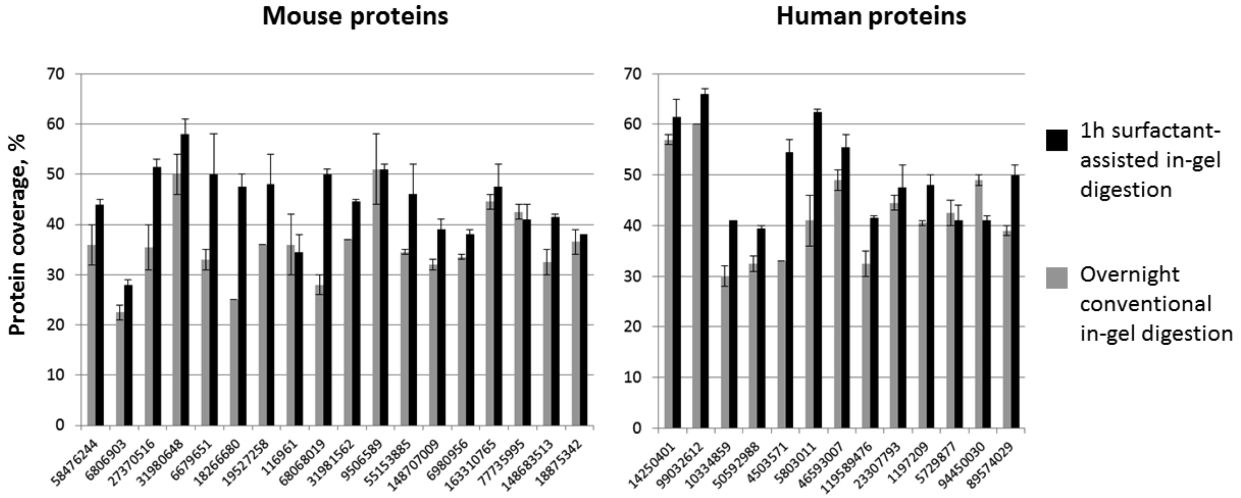

Figure 3. Validation of the surfactant-assisted in-gel protein digestion. Mouse and human proteins were resolved in SDS-PAGE and each protein was side-by-side digested according to 1 h surfactant-assisted and overnight conventional procedures. Peptides were cleaned up with C18 SPE tips and analyzed with MALDI-TOF MS. The graphs show average sequence coverage obtained in two independent in-gel digestion reactions.

proteins. Our data showed that at low nanogram quantities of an analyzed protein tryptic autoproteolytic peptides, which inevitably accumulate during in-gel digestion, dominate mass spectra (Supporting Information, Figure S-9a). To address this problem, we decreased trypsin amount per digestion reaction from 250 ng (typical trypsin load per digestion reaction) to 40 ng. In agreement with our expectation, autoproteolytic tryptic peptide peaks were minimized upon decreasing trypsin amount. The decrease in trypsin amount had no noticeable effect on the surfactant-assisted in-gel protein digestion (Supporting Information, Figure S-9b). We note that trypsin amount could be safely decreased only if the surfactant was added into the reaction. In the absence of the surfactant, trypsin was completely adsorbed by tube walls when present at amounts of 80 ng or less (Supporting Information, Figure S-10). Efficiency of the surfactant-assisted in-gel digestion adjusted as described above for analysis of low abundant proteins was demonstrated using a panel of 27 proteins, each of which was present in gel at low nanogram quantities. We found that the number of identified proteins increased from 10 in the conventional digestion to 18 in the surfactant-assisted digestion, or nearly two times (Supporting Information, Table S-4).

Compatibility with LC-ESI and MALDI. The potential for negative impacts of surfactants on reverse-phase liquid chromatography, electrospray (ESI), or matrix-assisted laser desorption/ionization (MALDI) is a common concern of mass spectrometry users.[6] Therefore, we carefully examined the effect of our surfactant on liquid chromatography and mass spectrometry.

Sample handling prior to mass spectrometry analysis typically involves centrifugation or solid phase extraction. These procedures remove gel pieces, buffer, salts, and other nonpeptide compounds from in-gel digestion reaction. These procedures also remove 90–95% of the degraded surfactant (the data not shown). The remaining degradation products enter liquid chromatography; however, the hydrophobic degradation product **4** elutes very late in the LC gradient (Supporting Information, Figure S-11) without affecting the column binding capacity or peptide elution time, and the zwitterionic hydrophilic product **5** is not retained on the column. A blank run with an acetonitrile gradient, routinely used to prevent sample carry-over between LC-MS runs, also prevents carry-over of the hydrophobic degradation product (Supporting Information, Figure S-12).

Neither peptide signal intensity nor signal-to-noise ratio were affected in the surfactant-containing samples. No deterioration in chromatographic profiles or decrease in ion signal intensity were observed after over one hundred runs with the surfactant-containing samples on nanoLC ESI Ion Trap (Agilent Technologies, Santa Clara, CA) showing no evidence of long-term negative effect of the surfactant in LC-MS. Similarly, we did not observe any interference of the surfactant with MALDI-MS analysis. The samples were cleaned up with solid-phase extraction (C18 tips) prior to MALDI-MS analysis.

Therefore, the surfactant is safe for use in liquid chromatography, ESI, or MALDI under the caveat that recommended handling steps are applied prior to a sample analysis.

CONCLUSIONS

The novel mass spectrometry compatible surfactant sulfonate-(sodium 3-((1-(furan-2-yl)undecyloxy) carbonylamino)-propane-1-sulfonate improves identification of proteins in gel through enhanced protein digestion, increased peptide extraction, and minimized postdigestion peptide loss. Increase in protein sequence coverage is the common outcome of the surfactant introduction to in-gel digestion reaction. For a complex protein mixture, the surfactant also increases number of identified proteins. The surfactant-induced improvement in probability of protein identification is the major benefit for in-gel digestion of low abundant proteins.

Besides improvement in protein identification, the surfactant offers significant procedural improvements for in-gel protein digestion. In the surfactant-assisted procedure, maximal protein digestion is achieved after only 2 h or even 1 h incubation depending on incubation temperature. The surfactant facilitates efficient peptide extraction from gel eliminating the need for acetonitrile extraction step. As a result, in-gel protein digestion and peptide extraction are complete in a single one hour step. This is a significant procedural advantage in comparison with the conventional in-gel digestion, which typically requires overnight incubation and a laborious peptide extraction step.

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The surfactant degrades over the course of in-gel digestion reaction eliminating the need for postdigestion degradation and, thereby, offering additional procedural advantage. In our validation experiments using a panel of various individual proteins and a complex protein mixture, the streamlined surfactant-assisted in-gel digestion procedure consistently demonstrated superior performance as compared to a conventional procedure for both MALDI and ESI MS platforms.

Certain precaution should be taken into account while using the surfactant. Surprisingly, the maximal performance of the in-gel digestion procedure was observed at low rather than high surfactant concentrations. Our data suggest that high surfactant concentrations could be detrimental to in-gel digestion process due to the surfactant degradation products that begin interfering with the process if their concentration exceeds certain optimal level. Other than that the surfactant is simple to use. It degrades over the course of in-gel digestion reaction, thereby eliminating the need for a postdigestion degradation step. The degradation products are largely removed during sample handling steps such as centrifugation or solid phase extraction, which are routinely used prior to analysis of the in-gel digested proteins. Small quantities of the degradation products persisting through sample handling steps are innocuous for liquid chromatography and mass spectrometry. We found that the surfactant did not affect peptide retention, signal intensity, or signal-to-noise ratio even after over one hundred runs with the surfactant-containing samples. Similarly, we did not observe any interference of the surfactant with MALDI-MS analysis.

Use of the surfactant for protein digestion in solution and compatibility with postdigestion peptide treatment such as iTRAQ are the important applications for the surfactant. The examples of such use and proof of the surfactant compatibility with iTRAQ labeling have been demonstrated.[18−21] It should also be noted that this surfactant was independently compared to another commercially available surfactant and showed favorable performance.[12]

Overall, incorporation of the surfactant in in-gel protein digestion significantly improves protein identification and simplifies the digestion procedure imposing no negative effects on liquid chromatography and mass spectrometry.

■ ASSOCIATED CONTENT

Supporting Information

Additional material as described in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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