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Enzyme-Friendly, Mass Spectrometry-Compatible Surfactant for In-Solution Enzymatic Digestion of Proteins

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Improved in-solution tryptic digestion of proteins in terms of speed and peptide coverage was achieved with the aid of a novel acid-labile anionic surfactant (ALS). Unlike SDS, ALS solubilizes proteins without inhibiting trypsin or other common endopeptidases activity. Trypsin activity was evaluated in the presence of various denaturants; little or no decrease in proteolytic activity was observed in 0.1−1% ALS solutions (w/v). Sample preparation prior to mass spectrometry and liquid chromatography analysis consists of sample acidification. ALS degrades rapidly at low-pH conditions, which eliminates surfactant-caused interference with analysis. Described methodology combines the advantages of protein solubilization, rapid digestion, high peptide coverages, and easy sample preparation for mass spectrometry and liquid chromatography analyses.

With the proliferation of mass spectrometry instrumentation into pharmaceutical and biotechnology laboratories, the characterization of proteins by peptide mass fingerprinting has become a widely used technique.[1] Both matrix-assisted laser desorption/ionization time-of-flight (MALDI TOF) and tandem liquid chromatography−mass spectrometry (LC−MS) methods find broad application in analysis, characterization, and quality control of therapeutic and diagnostic proteins.[2−4] In addition, proteomics research utilizing protein separation methods and mass spectrometry analysis has attracted considerable attention in recent years.[5−10]

In a typical experiment, proteins are first subjected to proteolytic cleavage followed by MS analysis of the resulting peptides. An efficient protein digestion is desirable to generate a representative peptide mixture covering a high percentage of protein amino acid sequence. However, it has been shown that enzymatic fragmentation of insoluble and proteolytically resistant proteins is difficult and generates limited amounts of peptides.[11−13] The confidence of the protein identification is compromised by inefficient proteolytic digestion and low peptide coverage. High peptide coverage is especially desirable when posttranslational or other protein modifications are studied. The efficiency of proteolytic digestion also has an impact on analysis of complicated protein mixtures (proteomic studies).[5−7,10,14] It has been found that hydrophobic/proteolytically resistant proteins do not yield a sufficient amount of peptides and may be underrepresented in the mixture submitted for analysis.[11,15]

Current in-solution protein enzymatic digestions are typically carried out for several hours or overnight in order to generate a sufficient amount of peptides for analysis. Despite the extensive time, an inadequate digestion compromises the characterization of hydrophobic and proteolytically resistant proteins and contributes to false protein identification via peptide mass fingerprinting.

To improve the protein digestion, additives such as surfactants, organic solvents, and urea are commonly used to improve the proteins' solubility and hence facilitate a more complete peptide map. The drawback of this approach is that denaturants often reduce the proteolytic activity of enzymes and interfere with mass spectrometry and liquid chromatography (LC).[16−19] Therefore, sample cleanup is often necessary prior to LC, MS, or LC−MS analysis. Various sample preparation methods were developed including dialysis, solid-phase extraction sample cleanup using reversed-phase (RP), hydrophilic interaction chromatography, or

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surfactant precipitation. These methods are time-consuming, only partially efficient, and often lead to reduction in sample recovery.[20,21]

This work describes the use of a novel acid-labile surfactant (ALS), sodium 3-[(2-methyl-2-undecyl-1,3-dioxolan-4-yl)methoxyl]-1-propanesulfonate, for in-solution protein digestion. The impact of ALS on trypsin activity and the speed of protein in-solution digestion were investigated. The degree of trypsin inhibition was compared to other commonly used protein solubilizers/denaturants such as SDS, urea, and organic solvents. Acid-labile surfactant was observed to enhance the rate and extent of in-solution protein digestion. The acid-labile property of ALS enables fast and simple sample cleanup prior to MALDI MS or LC−MS analysis of protein digests without compromising the quality of the analysis.

EXPERIMENTAL SECTION

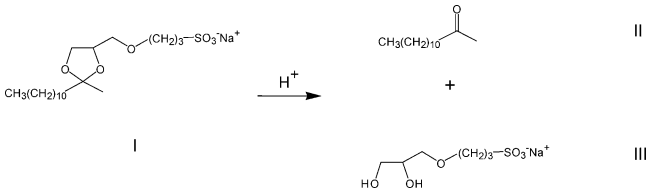
Reagents. Proteins (ubiquitin, myoglobin, lysozyme, ovalbumin, bacteriorhodopsin), MALDI matrix (α-cyano-4-hydroxycinnamic acid, CHCA) and ammonium bicarbonate were purchased from Sigma (St. Louis, MO). Sequencing grade trypsin was purchased from Promega (Madison, WI); sequencing grade chymotrypsin was purchased from Roche Diagnostics. Lyophilized sodium 3-[(2-methyl-2-undecyl-1,3-dioxolan-4-yl)methoxyl]-1-propanesulfonate (**I**) was obtained from Waters Corp. (Milford, MA) under the trade name of *Rapi*Gest SF.

Trypsin Activity Assay. The method for trypsin activity measurement was described elsewhere.[22] Briefly, the activity is measured by monitoring the hydrolysis of trypsin substrate, *N*-α-benzoyl-L-arginine ethyl ester (BAEE), to *N*-α-benzoyl-L-arginine (BA) using a UV spectrophotometer[22] (Ultrospec 2100, Amersham). The BAEE hydrolysis follows zero-order kinetics; an increase in absorbance $A_{253\ nm}$ in time is linear. BAEE (0.2 mM) was prepared in 50 mM ammonium bicarbonate buffer (pH ∼7.8) and spiked with 0.5 μg of trypsin. UV absorbance was recorded in 1-min intervals; the first 5 min of reaction was used to calculate the slope. The slope of $\Delta A_{253\ nm}$ versus time defines the trypsin activity. Change in trypsin activity caused by the addition of SDS, ALS, urea, or organic solvent (MeOH), respectively, were determined by normalizing the BAEE hydrolysis rate against that of a reference sample (no additives).

Enzymatic Digestion. Ubiquitin, lysozyme, myoglobin, and ovalbumin were dissolved in 50 mM ammonium bicarbonate (no denaturant added) at a concentration of 5 pmol/μL each and digested as described below. Alternatively, proteins were solubilized with 0.1% (w/v) ALS solution, buffered with 25 or 50 mM ammonium bicarbonate, and digested with selected endoprotease. The enzyme-to-protein ratio used was 1:100−1:50. Enzymatic digestions were carried out at 37 °C.

Membrane protein, bacteriorhodopsin (BO), was suspended in 0.1% (w/v) ALS, boiled at 100 °C for 5 min, and cooled to room temperature prior to digestion with chymotrypsin overnight. In a second experiment, BO was solubilized in 8 M urea. The solution was diluted to 2 M urea prior to the chymotrypsin digestion to minimize the impact of urea on chymotrysin activity.

Scheme 1[a]



[a] ALS (**I**) rapidly decomposed to two products, tridecan-2-one (**II**) and sodium 3-(2, 3-dihydroxypropoxy)propanesulfonate (**III**) under low-pH conditions.

Surfactant Degradation. ALS breaks down into two products under acidic conditions (Scheme 1). Tridecan-2-one (**II**) is water immiscible and hence can be removed by centrifugation. The other product, sodium 3-(2,3-dihydroxy)propoxypropanesulfonate (**III**), is a water-soluble ionic compound that does not interfere with RP-LC or MS analysis. The half-life ($t_{1/2}$) of ALS hydrolysis at pH 2 is ∼7.8 min at room temperature; less than 5% of the surfactant remains intact after 30 min at pH 2. Strong acids such as hydrochloric acid (30−50 mM total concentration) or trifluroacetic acid (0.5%, v/v) were used to degrade the surfactant and also served the purpose of quenching enzymatic reactions.

MALDI-TOF Analysis. The MALDI matrix was CHCA (10 mM) in 50% acetonitrile/water containing 0.1% TFA. One microliter of sample was mixed with the same volume of matrix on the MALDI target and allowed to evaporate at ambient temperature. The matrix/sample crystals were washed with deionized water to remove salts by spotting a drop of water on the target and aspirating it quickly with a Pasteur pipet connected to a vacuum manifold.

Mass spectra were acquired by a Micromass M@LDI *R* TOF instrument (Waters) equipped with a pulsed N_2 laser (337 nm) and a 2.3-m flight path and controlled by MassLynx 3.5 NT (Waters). This instrument was operated in reflectron mode with delayed extraction and calibrated externally with horse myoglobin tryptic digests or internally with ACTH. The spectra were acquired in mass range 700−3000 amu summing 100 laser shots for each sample. The peptide masses were matched against SwissProt database via ProteinLynx software.

Reversed-Phase HPLC−MS. Protein digests were separated on a capillary HPLC system (CapLC, Waters). The mobile phase A was 0.02% TFA in water; mobile phase B consisted of 0.016% TFA in acetonitrile. Peptides were separated on a 1.0 × 50 mm Symmetry C18 column (Waters) using a linear gradient from 0 to 60% B (2% acetonitrile per minute) with a flow rate of 35 μL/min and column temperature of 40 °C. The LC system was interfaced with an orthogonal acceleration TOF mass spectrometer (Micromass LCT, Waters) via an electrospray ionization source. This instrument was controlled by Micromass MassLynx 3.5 NT software (Waters). The TOF instrument sample cone voltage was 30 V; extraction cone and MCP detector voltages were 2 and 2700 V, respectively. The desolvation temperature and the source temperature were set at 250 and 100 °C, respectively, and desolvation gas flow was 300−400 L/h. The instrument was calibrated by infusion of horse myoglobin in 50% ACN with 0.1% formic acid.

RESULTS AND DISCUSSION

Protein Solubilization. A basic requirement for successful enzymatic proteolysis is to disrupt protein aggregation and bring

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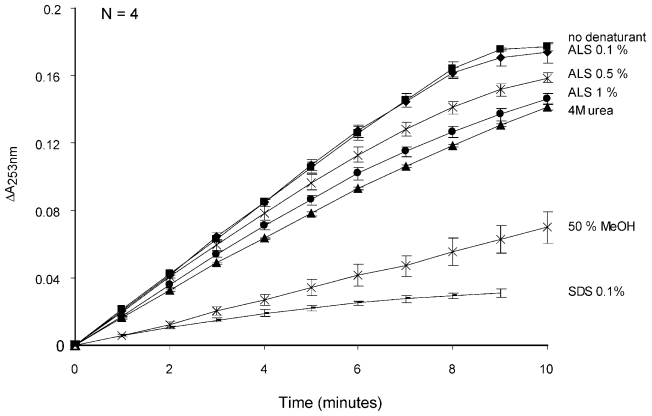

Figure 1. Trypsin activity measured in the presence of various denaturants and normalized against the control experiment (no denaturant). BAEE substrate was hydrolyzed by trypsin to UV-absorbing product BA. Trypsin activity was measured as a slope of the UV absorbance change at $A_{253\ nm}$. Data were averaged from four measurements. Calculated values of relative trypsin activity are given in Table 1.

them into solution. This seemingly trivial requirement is rarely fulfilled, especially when working with more complex mixtures or analyzing hydrophobic and membrane proteins. Surfactants are known to be efficient protein-solubilizing agents, and it is believed that they bind to the proteins and unfold them, exposing the specific sites to enzymatic cleavage.[23] The ALS denaturant employed in this work is an anionic surfactant, which structurally resembles SDS, given that it is composed of an ionic moiety (sulfonate) and hydrophobic alkyl chain (undecyl). It was observed that, like SDS, 0.1% (w/v) ALS solutions were efficient in solubilizing globular proteins, e.g., myoglobin and hydrophobic membrane proteins. A complete solubilization was observed also for transmembrane protein bacteriorhodopsin; 0.1% (w/v) ALS solutions remained clear without noticeable protein precipitation over an extended period of time.

Effect of Denaturants on Trypsin Activity. Trypsin activity was measured as the rate of BAEE substrate hydrolysis into a UV-active product BA.[22] Since the reaction follows zero-order kinetics (enzyme saturation), the absorbance increases in linear fashion until all BAEE substrate is converted into BA product (Figure 1). We evaluated trypsin activity in the presence of ALS, SDS, urea, and methanol. Various degrees of trypsin inhibition were observed, most notably in the SDS solution (Table 1). Little inhibition was found for ALS-containing solutions; the rate of hydrolysis for the control experiment (no denaturant) and 0.1% ALS solution was essentially identical (Figure 1). Enzyme activity was measured as a slope of $\Delta A_{253\ nm}$ versus time (first 5 min of reaction); however, the reaction in the presence of 0.1% SDS shows nonlinear behavior (Figure 1) and did not progress to completion even at an extended time period (data not shown). This is likely due to denaturation of trypsin leading to loss of activity. It appears that in the presence of 0.1% SDS trypsin completely loses activity within 15−30 min. Abrupt and complete loss of trypsin activity was observed at 0.5% SDS solution (Table 1). In contrast, only moderate reduction in trypsin activity was observed for 0.5−1.0% ALS concentrations. The trend of gradual enzyme inactivation is noticeable (Figure 1), but it is substantially slower when compared to the 0.1% SDS. Apparently, ALS is a milder denaturant than SDS, and at low concentrations (≤0.1%), it does not impair the trypsin activity (Table 1).

Table 1. Summary of Trypsin Activity Reduction Caused by the Presence of Different Denaturants Relative to the Control (No Denaturat)

	control (no additive)	ALS			SDS		urea		MeOH
		0.1%	0.5%	1.0%	0.1%	0.5%	2 M	4 M	(50%)
trypsin activity (%)	100	99	87	79	20	0.8	85	71	31

ALS Impact on Protein Digestion. ALS (0.1% solution) was found to substantially accelerate tryptic digestions of proteolytically resistant proteins, compared to control reaction (no denaturant added). An example of digestion of soluble, but moderately proteolytically resistant horse myoglobin[11,13] is shown in Figure 2. Complete protein digestion was observed within 15 min in ALS solution, while more than 50% of horse myoglobin remained undigested after 9 h for the control reaction (without surfactant). In our experience, it is challenging to achieve a complete protein digestion even over an extended period of time (15−20 h).[11,13] Interestingly, an incomplete digestion was observed for all proteins used in this study, including the ones that are susceptible to enzymatic proteolysis.

Peptide coverage of the ALS-aided tryptic digestion of myoglobin (Figure 2, 15-min reaction time) was 83% based on MALDI-

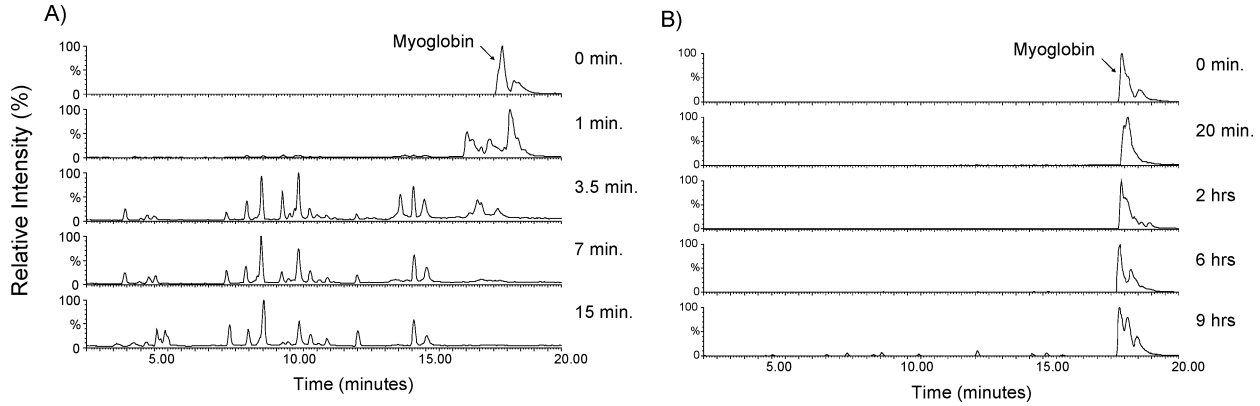

Figure 2. LC−MS total ion chromatogram of tryptic digest of myoglobin (A) solubilized with 0.1% ALS and (B) control reaction (no denaturant). Myoglobin treated with 0.1% ALS was observed to undergo complete tryptic digestion within 15 min, while myoglobin in the control reaction remains mostly undigested after 9 h.

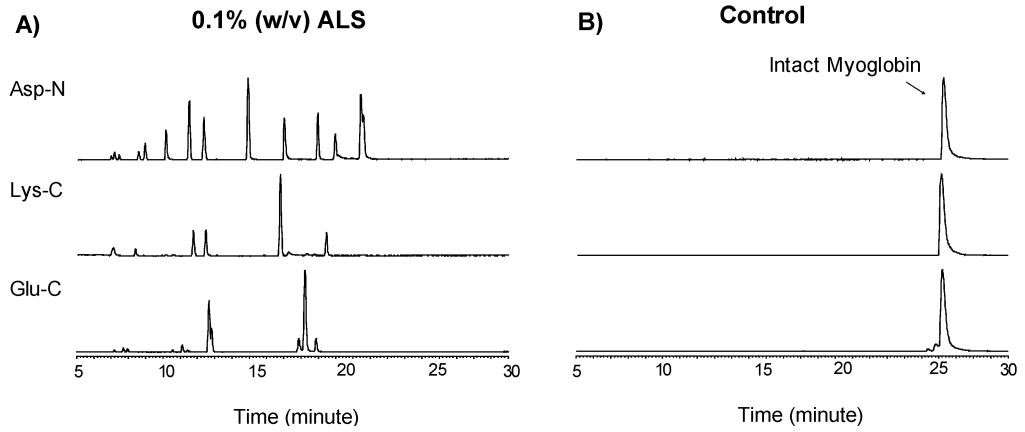

Figure 3. Horse myoglobin (50 pmol/μL) digestion with Asp-N, Lys-C, and Glu-C with or without 0.1% (w/v) ALS. (A) After 1-h incubation at 37 °C with 0.1% ALS, no intact protein was left undigested. (B) Control experiment (no surfactant) showed that majority of the myoglobin remained undigested.

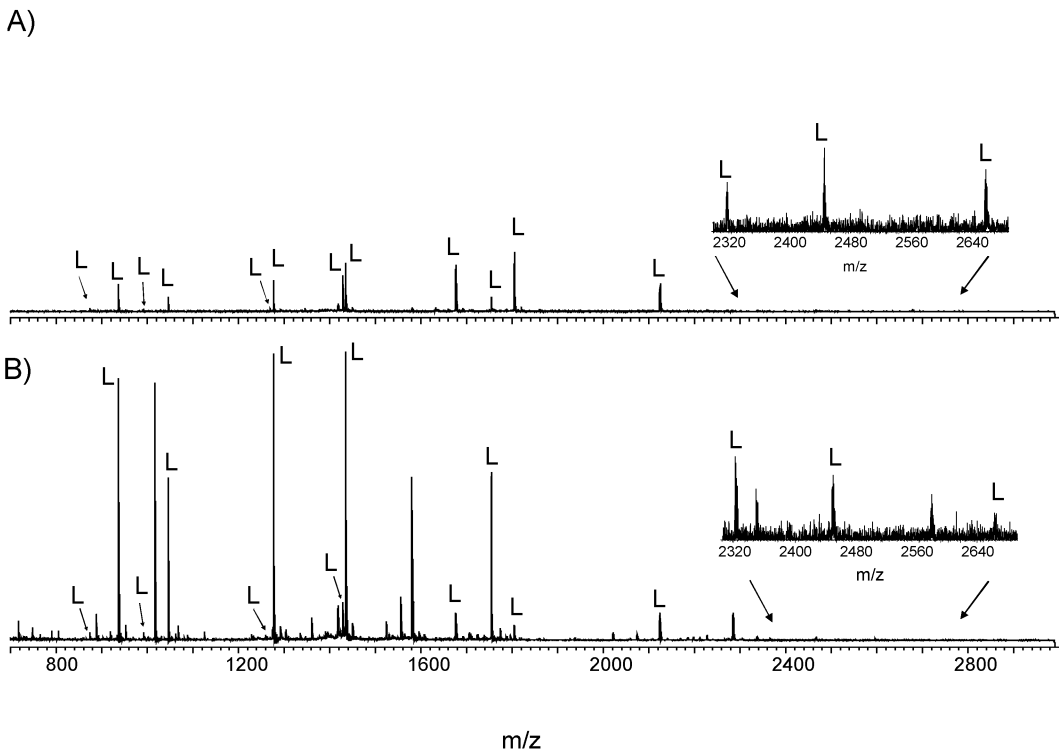

Figure 4. Normalized MALDI-TOF mass spectra of trypsin-digested protein mixture (ubiqutin, horse myoglobin, lysozyme, chicken ovalbumin). (A) Control experiment, no surfactant; (B) 0.1% ALS. Label L indicates only lysozyme peptides.

TOF MS analysis. The coverage is comparable to that of the overnight digestion of myoglobin without denaturation (68%). Since trypsin activity is unaffected by the surfactant (Table 1), the mechanism by which ALS improves digestion efficiency is presumably both protein solubilization and unfolding that exposes the cleavage sites and makes them amenable to proteolysis. LC−MS analysis confirms that proteolysis is nearly complete, and only trace amounts of intact proteins are detected after 30 min of digestion in ALS-containing solutions.

ALS was also found to be compatible with other common proteolytic enzymes, such as Lsy-C, Glu-C, Asp-N, and chymotrypsin. The rate and extent of myoglogin proteolysis is enhanced in the presence of 0.1% ALS (Figure 3).

To mimic a problem often encountered when working with complex protein mixtures, the proteins with different susceptibility toward enzymatic cleavage were studied. The mixture of ubiqutin, lysozyme, myoglobin, and ovalbumin was prepared and digested with trypsin in the presence and absence of ALS for 1 h. Myoglobin and ubiqutin, are resistant to enzymatic digestion, ovalbumin is moderately resistant, and lysozyme is amenable to trypsin cleavage. MALDI-TOF MS analysis of digested samples is shown in Figure 4. A greater number of expected peptides and higher abundance of signal was detected for the ALS-assisted digestion (Figure 4b) compared to the control experiment (Figure 4A). Ion signals observed in the control experiments were mainly peptides from lysozyme. In contrast, peptides generated from ubiqutin, myoglobin, and ovalbumin cleavage were clearly represented in the surfactant-assisted digestion (Figure 4B, Table 2). The results suggest that ALS-mediated proteolysis improvements are more apparent for hydrophobic or enzyme-resistant proteins.

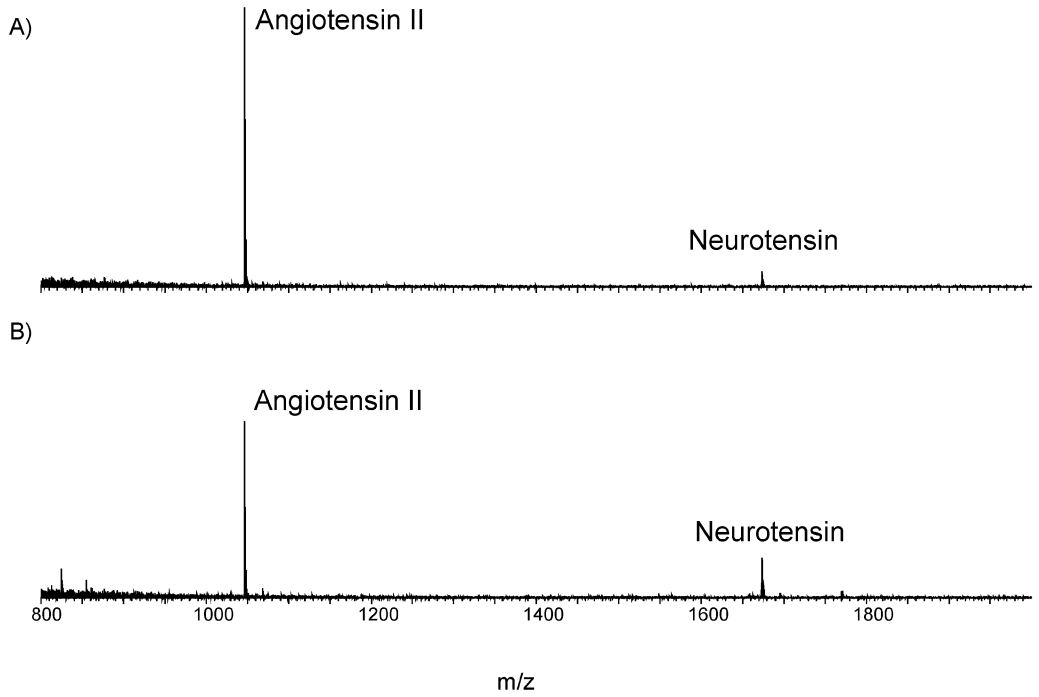

Figure 5. Normalized MALDI-TOF spectrum of Angiotensin II (50 fmol) and neurotensin (30 fmol). (A) Peptide mixtures were solubilized in high-purity water and mixed with the matrix (1:1) on the target. (B) Peptide mixtures were suspended in 0.1% ALS solution before mixing with the matrix. No suppression of signal intensity was observed in the presence of surfactant.

Table 2. Trypsin-Disgested Proteins in the Presence and Absence of ALS[a]

	no. of tryptic peptides observed	
proteins (5 pmol/μL)	control	with 0.1% (w/v) ALS
ubiqutin	1	5
horse myoglobin	2	7
lysozyme	15	15
chicken ovalbumin	1	7

[a] Mixture of four proteins was digested with trypsin in the presence of 0.1% ALS or under control conditions (no ALS), After 1-h digestion, peptides were identified via the MALDI-TOF MS for both experiments; the number of peptides found is listed.

MS Compatibility. After the enzymatic digestion, the enzyme activity is typically quenched by addition of strong acid, e.g., HCl or TFA. Because ALS degrades rapidly under acidic condition to **II** and **III** (Scheme 1), the majority of the surfactant is degraded prior to MS analysis. To achieve complete removal of surfactant, 0.5 M HCl was added to the samples to a final concentration of 30−50 mM. After incubation for 30−45 min at 37 °C (half-life of ALS is 7.8 min at pH 2), the samples were centrifuged to remove insoluble ALS degradant. No signal suppression was observed from degradation products or remaining traces of intact ALS. Interestingly, it was found that skipping the sample preparation step (acid degradation of ALS) has little impact on the MALDI MS signal. To illustrate the compatibility of the intact ALS with the MALDI ionization process, two standard peptide mixtures, angiotensin II (50 fmol) and neurotensin (30 fmol) were mixed with CHCA (1:1) with and without surfactant and directly spotted onto a MALDI target. Peak intensity and resolution of both spectra are similar (Figure 5). This finding is rather surprising, since surfactants such as SDS are known to suppress MS ion signals of analytes and give a high MS background even at trace concentrations.[24] It is possible that acidic MALDI matrix at least partially hydrolyzes ALS during crystallization on MALDI target. Because of the compatibility of ALS with MALDI MS analysis, the sample preparation prior to analysis is optional.

Removal of surfactant to LC−MS was necessary[25,26] in order to maintain RP-HPLC separation efficiency and peak shape. It is important to degrade ALS with HCl or with TFA as described above for a period of at least 30 min. If the remains of the nondegraded surfactant are introduced onto a RP-HPLC column, they act as a strong ion-pairing agent, effectively shifting retention time of peptides and causing poor HPLC reproducibility.[26] If degradation is complete, the degradation products do not interfere with LC-ESI-MS analysis. While **II** is removed by centrifugation, the other degradation product **III** is not retained on a RP LC column, elutes at the void volume, and hence does not interfere with the LC-ESI-MS process of later eluting peptides.

A potential drawback of ALS degradation is that some hydrophobic peptides may coprecipitate with insoluble degradation product **II**. We observed partial loss of hydrophobic peptides (highly retained by RP-HPLC) after ALS degradation and removal of **II**. Experimental results suggest that 0.025−0.05% (w/v) ALS concentrations mediate similar protein digestion with the same efficiency as the 0.1% ALS. At lower ALS concentrations, a minimal loss of hydrophobic peptides was observed.

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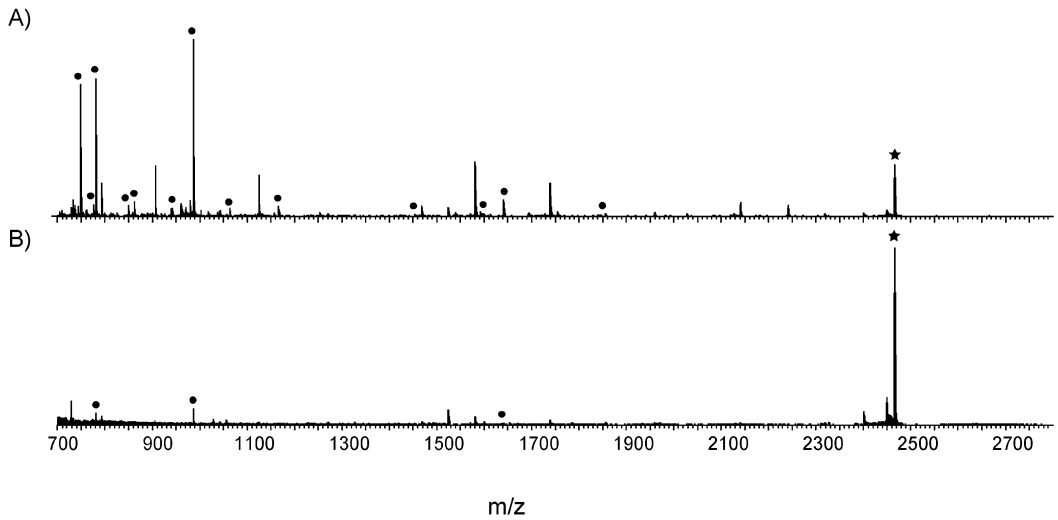

Figure 6. Normalized MALDI-TOF mass spectra of the overnight chymotrypsin digest of bacteriorhodopsin. (A) Bacteriorhodopsin was solubilized with 0.1% ALS; (B) bacteriorhodopsin was solubilzed in 8 M urea and diluted to 2 M urea prior to digestion. Approximately 10 pmol of digest was spotted on the MALDI target. An internal mass calibrant, ACTH (*m*/*z* 2465.199), is labeled with an asterisk. Ions labeled with circles are bacteriorhodopsin peptides.

Hydrophobic/Membrane Proteins. Analysis of hydrophobic proteins such as integral or transmembrane proteins has always been a challenge since a high concentration of strong denaturants is required to solubilize the proteins. Reduction in enzyme activity and ion suppression during MS analysis caused by the presence of denaturants are difficult to overcome. Typically, a high concentration of urea (8 M) is used to solubilize hydrophobic proteins; however, dilution of the sample prior to the digestion is necessary in order to minimize the inhibition of proteolytic enzymes. Removal of urea (most often by solid-phase extraction) is required for MALDI analysis.[18] Using a high concentration of urea also causes carbamylation of peptides, which further complicates protein identification by introducing peptide mass heterogeneity.[17]

ALS was used to solubilize an integral membrane protein, bacteriorhodopsin, prior to digestion and the MALDI-TOF MS analysis of digested peptides. We observed that ALS solubilizes BO completely while 8 M urea only gave partial solubilization. Chymotrypsyn was the proteolytic enzyme of the choice. Although it is a less specific enzyme, it effectively cleaves the peptide bonds consisting of amino acids with aromatic or large hydrophobic side chains. Therefore, more peptide fragments from hydrophobic proteins such as membrane proteins can be generated with chymotrypsin compared to trypsin.

The BO samples dissolved in 0.1% ALS or in 8 M urea (control) were digested overnight (control sample was diluted to 2 M urea prior to digestion). Both samples were further diluted 1:15 (v:v), mixed 1:1 with CHCA matrix, and directly spotted onto a MALDI target. The purpose of the postdigestion dilution was to reduce the urea concentration to a level tolerable for MALDI MS analysis, so that the signal intensity for both experiments can be compared more realistically. Experimental results show that more peptides with greater signal abundance (MALDI TOF) were generated from the ALS-denatured BO compared to the urea-denatured BO (Figure 6). The control experiment gave very few chymotryptic peptides, and the signals were weak (Figure 6B). Shortening the reaction time to 2 h, while keeping other conditions the same, yielded a result similar to that of the overnight digestion (data not show).

CONCLUSIONS

A novel acid-labile ionic surfactant was successfully employed as an aid for fast and complete protein digestions. ALS acts as a mild denaturant that effectively solubilizes proteins and makes them more amenable to proteolysis. This surfactant was found to be compatible with trypsin and other proteolytic enzymes.

The activity of trypsin was unaffected at moderate to high concentrations of ALS surfactant, which allows rapid in-solution digestion of hydrophobic and proteolytically resistant proteins. Experimental results suggest that fast and more efficient digestion for many proteins was achieved. Drastic improvements for enzymatic digestion of proteolytically resistant and hydrophobic proteins were realized. Due to the acid-labile character of the surfactant, sample preparation prior to MALDI MS and LC−MS analysis was reduced to simple acid treatment followed by centrifugation. The surfactant quickly degrades at low pH into mass spectrometry-friendly products, allowing for routine and efficient sample cleanup.

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