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Detection and Quantitation of Succinimide in Intact Protein via Hydrazine Trapping and Chemical Derivatization

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Abstract

Formation of aspartyl succinimide (Asu) is a common post-translational modification (PTM) of protein pharmaceuticals under acidic conditions. We present a method to detect and quantitate succinimide in intact protein via hydrazine trapping and chemical derivatization. Succinimide, which is labile under typical analytical conditions, is first trapped with hydrazine to form stable hydrazide and can be directly analyzed by mass spectrometry. The resulting aspartyl hydrazide can be selectively derivatized by various tags, such as fluorescent rhodamine sulfonyl chloride that absorbs strongly in the visible region (570 nm). Our tagging strategy allows the labeled protein to be analyzed by orthogonal methods, including HPLC-UV, LC-MS, and SDS-PAGE coupled with fluorescence imaging. A unique advantage of our method is that variants containing succinimide, after derivatization, can be readily resolved via either affinity enrichment or chromatographic separation. This allows further investigation of individual factors in a complex protein mixture that affect succinimide formation. Some additional advantages imparted by fluorescence labeling include, the facile detection of the intact protein without proteolytic digestion to peptides; and high sensitivity, e.g. without optimization 0.41% succinimide was readily detected. As such, our method should be useful for rapid screening, optimization of formulation conditions and related processes relevant to protein pharmaceuticals.

Keywords

chemical stability; deamidation; formulation; isoaspartic acid; mass spectrometry; protein; posttranslational modification; succinimide

INTRODUCTION

A common post-translational modification (PTM) in therapeutic proteins and peptides, amino-aspartyl succinimide (Asu) can be generated via two non-enzymatic spontaneous processes: deamidation of asparagine (Asn) or cyclization of aspartic acid (Asp), as illustrated in Scheme 1.¹⁻¹¹ Deamidation of asparagine involves an initial nucleophilic attack of the backbone amide nitrogen to the carbonyl carbon of the side-chain amide and the

subsequent loss of ammonia.¹²⁻¹⁹ Similarly, cyclization of aspartic acid involves an initial attack of the side-chain carboxylic acid and the subsequent loss of water. The resulting aspartyl succinimide typically undergoes further hydrolysis to form aspartic acid and isoaspartic acid (isoAsp or isoD).^{20,21}

The rates of Asu formation and accumulation depend on multiple factors such as the primary sequence and higher-order structures of the proteins, as well as pH and composition of excipients.²²⁻²⁹ Under neutral to basic conditions, hydrolysis of Asu is generally much faster than its formation, hence with little accumulation of Asu.^{30,31} On the other hand, under mildly acidic conditions (e.g., pH 4 to 5, common in protein formulations), conversion from Asp or isoAsp to Asu is faster and hydrolysis of Asu is slower, thus significant amounts (i.e., up to 40%) of Asu may accumulate when its formation and hydrolysis reach a dynamic equilibrium.^{6,7,32-34}

Many formulations are aimed at reducing Asn deamidation which is typically faster at higher pH; however, by exposing proteins to mildly acidic conditions, they may then be more susceptible to aspartic acid isomerization and accumulation of succinimide.^{35,36} As a result, succinimide is commonly observed in a myriad of therapeutic proteins including monoclonal antibodies^{12,36-42}, growth hormones^{43,44}, crystallins⁴⁵, lysozyme^{7,40,46-48} and others⁴⁹⁻⁵². Moreover, once a protein is introduced to a human subject, i.e., to the blood at pH 7.4, succinimide will rapidly hydrolyze into Asp and isoAsp.^{40,53} Succinimide formation, together with Asn deamidation and Asp isomerization, introduces heterogeneity into proteins and may lead to a number of issues including alterations in structure^{9,54}, intermolecular crosslinking⁵⁵⁻⁵⁹, aggregation⁶⁰, loss of activity^{48,61,62}, and even immunogenicity^{63,64}, thereby affecting both efficacy and toxicity.^{36,42,65}

Detection methods for succinimide are limited both in number and practicality, mainly due to its intrinsic instability, i.e., it is rapidly hydrolyzed during tryptic digestion typically carried out at or above neutral pH.⁶⁶⁻⁶⁹ To address the stability issue, Huang et al.⁷⁰ used RapiGest-assisted tryptic digestion carried at pH 6.0 to preserve and detect the labile Asu in a recombinant human monoclonal antibody IgG2 for LC-MS/MS analysis. Given that multiple factors affect the stability of Asu, this method will require considerable optimization for each protein and each site of modification and false negatives are a concern.¹⁸ O-Labeling is a method based on the concept of complete hydrolysis of Asu⁷¹, and detection of the resulting ¹⁸O-labeled Asp and isoAsp peptides by mass spectrometry.⁷²⁻⁷⁶ This method has the advantage of being quantitative. However, isotopic peaks from the relatively small 2 Da mass increase overlap with the natural isotopic distribution of the native peptide and may hide small percentages of succinimide.⁷⁷ This limitation also prevents direct analysis of large intact proteins, making proteolysis to relatively small peptides obligatory.⁷⁶

Following the concept for ¹⁸O-labeling, i.e. taking advantage of the high reactivity of succinimide, but avoiding the limitation of the small (2 Da) mass increase and isotopic peak overlapping, we envision a chemical method for the detection of succinimide in intact proteins by trapping succinimide with hydrazine to generate aspartyl hydrazide. Our chemical labeling strategy offers several advantages. First, the resulting hydrazide is stable

and can be readily detected and quantified by mass spectrometry due to the mass increase of 14 Da (hydrazide vs. aspartic acid). Moreover, highly nucleophilic and orthogonal to other functional groups in proteins, hydrazide can be further derivatized—even in intact proteins—to assist separation, detection, and quantitation. For instance, without proteolytic digestion, UV detection of 0.41% succinimide was achieved after derivatization with a commercially available rhodamine sulfonyl chloride. As such, our method is amenable to parallel high-throughput analysis, such as SDS-PAGE coupled with fluorescence detection, and thus should be useful for rapid screening, optimization of formulation conditions and related processes.

MATERIALS AND METHODS

Reagents

All aqueous solutions were prepared with ultrapure water from a Milli-Q system (Millipore, Bedford, MA). All reagents were ACS grade from Sigma Aldrich (St. Louis, MO) unless otherwise specified. All protein and peptide solutions were stored at -20°C unless otherwise specified. Hydrazine was prepared from the dichloride salt and the pH was adjusted with 10 M NaOH. Sodium acetate and glacial acetic acid were mixed to prepare a buffered 100 mM pH 4 solution. The pH of all protein and hydrazine solutions was determined using EMD Colorphast pH strips (Gibbstown, NJ) with the appropriate range of either 0-6 or 5-10, both with 0.5 pH unit accuracy. Hen egg white lysozyme (EC 3.2.1.14) was from Sigma Aldrich. Lysozyme concentrations were determined from the absorbance at 280 nm measured on a NanoDrop 1000 UV-Vis (Thermo Scientific, Wilmington, DE) and an estimated extinction coefficient of $37,970\text{ M}^{-1}\text{cm}^{-1}$, based on the amino acid composition. Sequencing-grade-modified trypsin was from Promega (Madison, WI). Lissamine rhodamine B sulfonyl chloride was from Life Technologies (Grand Island, NY). Rhodamine aldehyde was synthesized from the commercial product as previously described.⁷⁸

Generation of Succinimide in Lysozyme

Succinimide was generated based on a reported procedure.⁴⁷ Briefly, hen egg white lysozyme was dissolved in 100 mM sodium acetate at pH 4 to a final concentrations of 3.20 mM. The protein solution was incubated for 2 weeks at 37°C (denoted as “aged”), and then stored at -20°C until further analysis.

Quantitation of Succinimide in Protein by LC-MS

After a 2-week incubation, lysozyme was analyzed for succinimide content by LC-MS using an Agilent 1100 HPLC system coupled to an LCQ-Deca XP (Thermo Fisher Scientific, San Jose, CA). The MS was calibrated monthly with ESI positive ion calibration solution (Thermo Fisher Scientific). The flow rate was set to $300\text{ }\mu\text{L}/\text{min}$ and was split pre-column to deliver $300\text{ nL}/\text{min}$ to the analytical column with mobile phase A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile). Aged protein solutions were diluted in 0.1% formic acid in water immediately prior to analysis, maintaining pH ~ 3 -4 to prevent hydrolysis of succinimide. The samples were then loaded onto a self-packed reversed-phase column ($75\text{ }\mu\text{m i.d.} \times 15\text{ cm}$, Magic C4 resin, $5\text{ }\mu\text{m}$ particle size, $300\text{ }\text{\AA}$ pore size, Michrom

BioResources, Auburn, CA). The gradient was as follows: 1-40% B in 40 min, 40-60% B in 5 min, 60-1% B in 5 min, and holding at 1% B for 2 minutes. Data processing was done using XCalibur 2.0 software (Thermo Fisher Scientific, Waltham, MA) and relative amounts of native and succinimide protein were approximated by deconvoluted peak areas.

Hydrazinolysis

Aged lysozyme (1 mL of 3.20 mM) was mixed with hydrazine (2 mL, 2 M, pH 7.5) and incubated at room temperature for 3 hrs. Then excess hydrazine was removed by dialysis in Slide-A-Lyzer mini dialysis units (7,000 MWCO, Pierce, Rockford, IL) against 50 mM ammonium bicarbonate (pH 8, 1 L twice for 2 hours at room temperature). **CAUTION:** Highly concentrated and anhydrous hydrazine is corrosive and flammable and should be handled with care or avoided. We did not have any issues with hydrazine in aqueous solutions up to 2 M.

Tryptic Digestion of Proteins

Lysozyme was reduced with 2 mM tris(2-carboxyethyl) phosphine (TCEP) in 50 mM ammonium bicarbonate (pH 8) containing 10% acetonitrile at room temperature.^{79,80}

Trypsin (Promega, Madison, WI) was added to 100 μ L of lysozyme solution at a ratio of 1:40 mol/mol and incubated overnight at 25 °C. Proteolytic digestion was quenched with 5% v/v phosphoric acid and the mixture stored at -20 °C.

Identification of the Site of Hydrazide by MS

The tryptic digest of lysozyme containing aspartyl hydrazide was fractionated by RP-HPLC (conditions described below) and analyzed using a 4800 MALDI-TOF/TOF mass spectrometer (Applied Biosystems, Framingham, MA). The instrument was calibrated externally daily with a calibration mixture from the Sequazyme peptide mass standard kit (P2-3143-00, Applied Biosystems, Framingham, MA). Digested samples were dried by speed-vac then re-dissolved in 0.05% trifluoroacetic acid (TFA)/50% acetonitrile in water. An aliquot of 0.6 μ L was spotted on the plate and co-crystallized with an equal volume of a saturated solution of α -cyano-4-hydroxycinnamic acid (CHCA) dissolved in the same solvent. For tandem mass spectrometry experiments, the precursor mass chosen was 1818.02 m/z (1817.92 m/z calculated) corresponding to hydrazine-modified tryptic fragment 97-112 (KIVSZGNGMNAWVAWR, Z denoting hydrazide); in the native protein, the corresponding aspartyl peptide 97-112 (1803.89 m/z observed, 1803.90 m/z calculated) was isolated for fragmentation. The 97-112 peptide, with an extra N-terminal lysine due to a missed cleavage, exhibited better fragmentation and was more readily detected than the 98-112 peptide, IVSZGNGMNAWVAWR (1689.97 m/z observed; 1689.80 calculated).

Derivatization of Hydrazide in Intact Protein by Rhodamine Sulfonyl Chloride and Succinimide Quantitation

Native lysozyme was freshly prepared to 2.39 mM in 100 mM sodium acetate (pH 4), then diluted to 300 μ M in the same buffer. This protein solution was kept on ice to minimize the formation of succinimide. Aged lysozyme (2 weeks, pH 4, 3.20 mM) containing 27% succinimide was diluted to 300 μ M in the same buffer. A serial dilution was done to prepare

the following concentration range of succinimide relative to total protein: 27, 13, 6.6, 3.3, 1.6, 0.83, 0.41, 0.21, 0.10, and 0%. Hydrazinolysis was performed as described in the section above; after three hours of incubation the reaction was quenched with 5% v/v phosphoric acid (pH~1.5). Excess hydrazine was removed by dialysis against 200 mM phosphoric acid (pH 2, 1 L, 2 hours at room temperature). To label hydrazide, rhodamine sulfonyl chloride was freshly prepared in acetonitrile to a concentration of 5 mM and shielded from light. The freshly prepared rhodamine sulfonyl chloride was diluted 1:5 v/v into the protein aspartyl hydrazide solution. The final concentration of rhodamine sulfonyl chloride was 1 mM and acetonitrile was 20%. This solution was incubated for 3 hours at 25 °C, shielded from light, then frozen at –80 °C.

Effect of pH on Hydrazinolysis Specificity

Aliquots of 500 µL, 2 M hydrazine (pH 6.0, 7.0, 7.5, 8.0, or 9.0) were added to separate aliquots of succinimidyl lysozyme (250 µL of 282 µM). The resulting mixtures were incubated at room temperature and aliquots of 100 µL were removed at 10 min, 30 min, 1 hr, 2 hr, 3 hr and 24 hr; reactions were quenched with phosphoric acid as described above. Samples were dialyzed against phosphoric acid as described, then aliquots of 20 µL of 1 mM rhodamine aldehyde (synthesized in our laboratory as reported ⁷⁸) in 50% ethanol were added to 75 µL of each dialyzed protein solution and incubated at room temperature for 3 hours. The rhodamine aldehyde was used only to facilitate hydrazide detection of intact protein using SDS-PAGE and fluorescence imaging.

For SDS-PAGE, aliquots of 15 µL of reaction mixture, containing 4 µg of protein, were mixed with 5 µL of 4X SDS loading buffer (90% glycerol, 10% SDS in water) and heated in a 90 °C water bath for 5 minutes. The heat-treated samples were loaded into an 18% Tris-HCl Ready Gel (Bio-Rad, Hercules, CA) to separate protein from excess rhodamine. Voltage was maintained at a constant 200 V using a Bio-Rad Powerpac HV power supply (Bio-Rad, Hercules, CA) and stopped after the excess dye ran off the gel in approximately 50 minutes. After electrophoresis, the gel was analyzed using a Molecular Dynamics Storm 840 imaging system (GE Healthcare, Piscataway, NJ) and Storm Scanner Control v. 5.03. The excitation wavelength was 450 nm and the emission filter was set to 520 nm long pass. Data were analyzed utilizing Imagequant TL 7.0 (GE Healthcare).

Peptide Mapping of Lysozyme Modified by Rhodamine Sulfonyl Chloride

After rhodamine sulfonyl chloride labeling, samples were exchanged into 50 µL of 50 mM ammonium bicarbonate at pH 8 using 5K Vivaspin columns (5,000 MWCO, Sartorius Stedim, Bohemia, NY) using the manufacturer's instructions. The concentrated protein solution was prepared to 100 µL total volume in 50 mM ammonium bicarbonate at pH 8 with 10% acetonitrile and 2 mM TCEP. The protein was digested with trypsin as described above.

Digested samples were analyzed using an Agilent 1100 HPLC (Palo Alto, CA) with a diode array detector monitoring at 214 and 570 nm. Tryptic peptides were separated on a C18 reversed-phase column (4.6 × 50 mm, 3 µm beads, 300 Å pore size, Vydac, Hesperia, CA) with the following mobile phases: A (0.1% TFA in water) and B (0.1% TFA in acetonitrile)

at a flow rate of 1 mL/min. The gradient was as follows: 1-40% B in 40 min, 40-60% B in 5 min, 60-1% B in 5 min, and holding at 1% B for 2 minutes. An aliquot of 5 μ L of 86 μ M protein digest (430 pmol) was injected on the column for each sample for initial quantitation. To collect fractions for peptide sequencing, 50 μ L of 86 μ M protein equaling 4.3 nmol total protein was injected. Fractions were manually collected for each peak observed at 280 and 570 nm, dried by speed-vac, and reconstituted in 5 μ L of 0.05% trifluoroacetic acid (TFA)/50% acetonitrile in water, then analyzed by MALDI-TOF/TOF mass spectrometry as previously described.

RESULTS AND DISCUSSION

Formation of Succinimide

Succinimide was generated and accumulated in lysozyme by incubation at pH 4 at 37 °C for 14 days, based on a published procedure.⁴⁷ Succinimide variant was not resolved from native lysozyme under our RP-HPLC conditions, again highlighting the challenge in resolving closely related protein variants. On the other hand, the deconvoluted MS spectrum (See Figure 1) clearly shows the succinimide species (14287 m/z observed, 14287 calculated), which constituted 27% of the total protein. These findings were similar to those reported by Tomizawa et al., i.e., 30% Asu at residue 101 after reaching equilibrium at pH 4.⁴⁷

As previously mentioned and shown in Scheme 1, succinimide formation from aspartic acid results in the loss of a water molecule (H₂O, 18 Da).^{20,21} When using mass spectrometry, this relatively small mass change is readily detectable in peptides or small proteins (e.g., lysozyme, 14.3 kDa). However, as the mass of the protein increases in larger proteins such as antibodies, which have masses of 150 kDa or more, the -18 Da difference in m/z of native and modified protein may not be distinguishable due to broadening of the isotopic envelope, even with a high-resolution mass spectrometer. Quantitation may also be compromised as the isotopic envelope of the native protein overlaps with the envelope of the modified protein, and significantly, small amounts of modification cannot be detected with high confidence due to narrow dynamic range and intrinsic noise of mass spectra.⁸¹

Trapping with Hydrazine and Specificity

As previously discussed, aspartyl succinimide is labile and rapidly hydrolyzes when exposed to neutral or basic solutions and thus poses challenges for its analysis.⁶⁶⁻⁶⁹ On the other hand, taking advantage of its intrinsic high reactivity, we trap the labile succinimide with hydrazine to form stable hydrazide. The formation of aspartyl hydrazide results in a mass increase of 14 Da from aspartic acid.^{82,83}

The aspartyl hydrazide protein (Asu lysozyme after hydrazinolysis) was digested with trypsin. As seen in Figure 2, the resulting aspartyl hydrazide was stable to proteolytic digestion, as reported.⁸² Sequencing by MALDI-TOF/TOF determined the modification site to be at Asp101. Succinimide was previously established at this site by X-ray crystallography.⁴⁷ The 14 Da mass increase imparted by derivatization with hydrazine places the mass of the hydrazide protein outside the isotopic envelope of the native protein

to facilitate detection of low levels of modification, i.e., less than 1 % succinimide protein variant.

As we demonstrated,⁷⁸ the SDS-PAGE and fluorescence detection platform offers high-throughput detection and quantitation. To facilitate the testing of numerous conditions for hydrazinolysis, it was desirable to label aspartyl hydrazide lysozyme with the rhodamine to impart a fluorescence signal. Therefore, we utilized an aldehyde-functionalized rhodamine tag that we have previously described.⁷⁸ Under the acidic conditions employed (pH 2), the aldehyde tag only reacted with hydrazide to form hydrazine but not other amino acid residues, as we have shown in similar applications.^{78,82,83}

No modification by hydrazine of other sites was observed, indicating that hydrazine is specific towards succinimide under our conditions. This specificity was accomplished by controlling the reaction at pH 7.5. By keeping the reaction pH slightly below the pKa of hydrazine, which is 7.8, the equilibrium was shifted to favor protonated hydrazine, reducing its intrinsic reactivity and minimizing any side reactions. Lowering the pH to 6.0-7.0, little to no detectable background labeling of native lysozyme was observed, but the yield for labeling of succinimide was also lower, due to significant protonation of hydrazine. Conversely, raising the pH to 8.0-9.0, hydrazine reaction with native lysozyme was more pronounced (more background reaction), mostly via deamidation at the NG “hot spot” in lysozyme (i.e., Asn103-Gly104). Fundamentally, its high intrinsic reactivity offers the high selectivity towards succinimide vs. asparagine residues. Reaction time and temperature had less pronounced effects on selectivity but optimal conditions were maintained at a constant 37 °C for 3 hours.

Derivatization of Aspartyl Hydrazide and Specificity

After trapping succinimide with hydrazine to form the stable aspartyl hydrazide, protein aspartyl hydrazide was labeled with rhodamine sulfonyl chloride as shown in Scheme 2. The adduct serves as a telltale sign of succinimide as it can be detected using the visible or fluorescence signal imparted by the rhodamine tag. As we previously reported for the analysis of isoAsp, derivatization of protein hydrazide by sulfonyl chloride could be highly specific under acidic conditions, i.e., no detectable modifications of the amino and other functional groups in proteins.^{78,82,83} By maintaining a reaction pH at or slightly below the pKa of hydrazide (pKa $\approx$ 3), hydrazide will be slightly reactive (partially deprotonated) while protein amines (pKa $\approx$ 9) will be protonated and unreactive. Indeed we found that by reducing the reaction to pH 2, nonspecific labeling was eliminated (not detectable).

The same strategy, albeit with different chemistry, was employed for reacting protein aspartyl hydrazide with rhodamine aldehyde for the purpose of detecting hydrazinolysis. In this case, hydrazide reacts with aldehyde to form hydrazone as shown in Scheme 2. Selectivity for this reaction was greatly enhanced by reducing the reaction pH even further, to pH 1 or 2, most likely due to the reversible and dynamic nature of the Schiff base (imine, formed between amines and aldehydes) under our conditions, as the vast majority of amines are protonated.^{78,82,83} Moreover, hydrazones are intrinsically more stable than Schiff base.⁸⁴ Indeed, Figure 5 shows the high selectivity of rhodamine aldehyde labeling at pH 2 with a

detectable fluorescence band only observed in aspartyl hydrazide protein but not in aspartyl or aspartyl succinimide (aged) protein.

Specificity of Sulfonyl Chloride Derivatization

Specificity of our labeling chemistry was verified in several ways. First, intact aged lysozyme was treated with hydrazine and labeled with rhodamine sulfonyl chloride. The intact protein was directly analyzed by RP-HPLC-UV-vis using a C4 stationary phase for separation and monitoring at 280 and 570 nm, for protein and rhodamine tag, respectively. A single rhodamine-labeled peak was resolved from the native protein (see Figure 6). Native lysozyme was analyzed in the same way and is overlaid for comparison. Background labeling observed in the native protein was minimal and may be explained by deamidation at the NG “hotspot” directly C-terminal adjacent to our targeted DG tandem (although not observed by MS/MS sequencing), or could be some artifactual succinimide formation while in acidic buffer. These samples were also analyzed by LC-MS. The deconvoluted mass spectrum of labeled aged lysozyme showed a single peak with mass increase of 540 Da (data not shown) corresponding to a single rhodamine tag.

Next we digested the rhodamine-labeled lysozyme with trypsin to generate a peptide map, which was resolved by HPLC on a C18 analytical column with UV-vis detection, monitoring 214 and 570 nm. Native lysozyme was treated in the same manner to serve as a control. In the labeled succinimide variant, a single peak was observed at 570 nm (see Figure 4). This peak was also detected in the 214 nm trace, and baseline resolved from the other peptide peaks. This suggests a single labeled peptide with relatively high hydrophobicity, due to the late elution time, imparted by hydrophobic rhodamine.

Finally, the tryptic peaks observed by HPLC-UV-vis were manually collected and identified by MALDI-TOF analysis; the peak eluting at 30.2 min corresponded to the rhodamine-labeled peptide 98-112, see Figure 4. As it is very clearly illustrated in the 570 nm trace, only the target peptide containing the DG tandem was labeled. Then, by tandem mass spectrometry (TOF/TOF), it was confirmed that Asp101 was the only residue labeled with the rhodamine tag. See Figure 3.

Quantitation of Succinimide via Rhodamine Tag in Intact Protein by HPLC-UV-Vis

A key advantage of our method is the ability to detect and quantify succinimide in intact proteins, as illustrated in Figure 6 showing an HPLC trace of intact lysozyme monitoring at both 280 and 570 nm for protein and rhodamine dye, respectively. In the case of lysozyme, a relatively small 14.3 kDa and hydrophilic protein, the addition of a single molecule of rhodamine altered the hydrophobicity of the protein enough to enable baseline separation by RP-HPLC. In the case of a much larger protein, separation of these two species, modified and unmodified, may not be achieved. Even so, because most proteins will not have any interference at 570 nm, only the labeled protein will be detected in this spectral channel.

Our method of detection and quantitation of rhodamine sulfonyl hydrazide is based on the absorbance at 570 nm rather than 280 nm alone and offers several advantages. For instance, no separation of the labeled and unlabeled proteins is required, such as SDS-PAGE and fluorescence imaging. Rather, only excess rhodamine labeling reagent must be separated

from the protein or peptide, which was achieved during standard SDS-PAGE or via dialysis. Detection limits are improved because of the higher extinction coefficient of rhodamine ($88,000 \text{ M}^{-1}\text{cm}^{-1}$) compared to 280 nm ($37,970 \text{ M}^{-1}\text{cm}^{-1}$ in lysozyme). Therefore, the sensitivity of our method can be improved upon simply by substituting a stronger chromophore, or by using fluorescence detection.

To determine the linear dynamic range and limit of detection, Asu-containing lysozyme was spiked into native protein (without succinimide) at various ratios (up to 27% succinimide relative to total protein). These mixtures were treated with hydrazine, then labeled with rhodamine sulfonyl chloride as shown in Scheme 2. A linear correlation was observed between the percentage of Asu vs. the ratio of peak area at 280 vs. peak area at 570 as shown in Figure 7.

Our limit of detection (3 standard deviations above the background) was 0.41% relative succinimide, which in our experiments was the equivalent of $0.16 \mu\text{M}$ succinimide in $40 \mu\text{M}$ of total lysozyme protein. Certainly, relative and absolute sensitivities will likely differ with different proteins. For example, the structures and microenvironments of antibodies and proteins are known to affect reactivities.²⁶⁻²⁹ Optimization of the specific conditions of each reaction may further improve the LOD and LOQ, particularly in the hydrazine-trapping step. Additionally, more sensitive tags can be substituted for UV and fluorescent detection, all considerations that will vary depending on the specific protein to be tested. It is also conceivable that our method can be used in conjunction with ^{18}O -labeling to take advantage of the benefits each method offers.

Separation of Intact Protein Variants

It is critically important to understand the individual factors in a complex protein mixture that affect succinimide formation, hence separation of intact protein variants is highly desirable. While the addition of a single molecule of rhodamine to a single molecule of lysozyme enabled baseline separation by RPHPLC, this may not be the case in larger proteins such as monoclonal antibodies. To markedly reduce sample complexity, affinity enrichment is the most common and powerful approach, and the case for our chemical derivatization method. For instance, aspartyl hydrazides can be affinity-enriched with aldehyde resins, and alternatively, can be enriched and detected with aldehyde-containing affinity tags (e.g., biotin) as others and we have demonstrated.^{78,82,83}

CONCLUSION

Formation of succinimide is a major concern, particularly among protein pharmaceuticals that use common mildly acidic formulations. Detection methods for succinimide, however, are limited both in number and practicality, mainly due to its intrinsic instability and the small mass change. The chemical derivatization method presented here enables detection and quantitation of succinimide in intact proteins. The two-step approach also offers the flexibility that a range of detection platforms can be employed either individually or in combination, such as mass spectrometry and fluorescence imaging. Certainly, a single method for the detection of such a labile protein modification may not be reasonable or adequate, but instead a combination of methods will likely be the answer. For instance, our

method could be used as a first step to discover (screen) succinimide in intact proteins. Subsequently, ^{18}O -labeling can then be used for absolute quantitation. Last but not least, affinity enrichment of the hydrazide or its derivatives should allow the succinimide species to be isolated as intact proteins, so factors that affect succinimide formation can be pinpointed. This is particularly valuable, as protein pharmaceuticals typically contain multiple variants that may behave quite differently.

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Scheme 1.

Formation of aspartyl succinimide (Asu) and isoaspartic acid (isoAsp) via aspartic acid (Asp) isomerization and asparagine (Asn) deamidation. The peptide backbone is traced in bold to illustrate the perturbation upon methylene insertion due to formation of isoaspartic acid. Trapping of the labile Asu to form stable hydrazide is illustrated. Note that, while only α -aspartyl hydrazide is shown, β -aspartyl hydrazide can also be formed.

Scheme 2.

A. Hydrazide can be further derivatized by reaction with sulfonyl chloride to incorporate a UV or fluorescence or affinity (e.g., biotin) tag. B. Hydrazide can be reversibly bound to aldehydefunctionalized resin for affinity enrichment by formation of hydrazone.

Figure 1. Deconvoluted LC-MS spectrum of native lysozyme (top; theoretical average mass 14,305 Da; observed average mass 14,306 Da) compared to aged lysozyme containing 27% succinimide variant (bottom; theoretical average mass 14,287 Da; observed average mass 14,287 Da). The mass difference of 18 Da corresponds to the loss of a water (H_2O) molecule when aspartic acid is converted into the succinimide.

Figure 2. MALDI-TOF mass spectra of tryptic peptide 97-112 (1818.07 m/z, KIVSD*GNMNAWVAWR) showing the +14 Da mass increase in hydrazine-treated lysozyme (bottom, hydrazide) as compared to native lysozyme (1804.01 m/z, no hydrazine treatment, top, carboxylic acid).

Figure 3.

MALDI-TOF/TOF mass spectrum of the precursor ion (2231.89 m/z observed, 2231.97 m/z calculated) for tryptic peptide 98-112, confirming Asp101 is the site of derivatization by rhodamine sulfonyl chloride. D* denotes rhodamine-modified aspartyl hydrazide.

Figure 4. HPLC-UV-vis chromatograms of tryptic digests of lysozyme (native Asp and Asu variants), both treated with hydrazine and rhodamine sulfonyl chloride. The top panel shows all peptides by monitoring at 214 nm (detecting all peptides). The bottom panel shows a single significant peak by monitoring at 570 nm, the wavelength at which only rhodamine absorbs.

Figure 5.

Fluorescence images of SDS-PAGE gels showing the specificity of rhodamine-aldehyde labeling for hydrazide protein when the reaction was carried out at pH 2. The sample labeled “Z” contains hydrazide and after treatment with rhodamine-aldehyde the band displays a fluorescence signal. In comparison, the native lysozyme (Asp) and aged lysozyme containing succinimide (Asu), which were also treated with rhodamine-aldehyde showed no detectable fluorescence signal, indicating no measurable background reaction under these conditions.

Figure 6. HPLC-UV-vis chromatograms of intact native and aged lysozyme (with 0.41% succinimide) that were treated with hydrazine and rhodamine sulfonyl chloride in the same manner. The top trace at 280 nm shows that the rhodamine-modified species and native lysozyme were resolved. The bottom trace shows higher sensitivity achieved by monitoring at 570 nm. Native lysozyme (without spiked succinimide) showed a small peak, which may originate from background reaction or a small amount of succinimide already present in the native lysozyme.

Figure 7.

Intact protein linear correlation of the percentage of succinimide (intact lysozyme) with the UV-vis absorbance (peak area ratio of 570 nm to 280 nm). The insert shows the lower percentage region, which remains linear even below our reported LOD of 0.41%. The native protein (no Asu, control) is included in the plot.