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

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ABSTRACT: The formation of aspartyl succinimide is a common post-translational modification 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-Vis, liquid chromatography mass spectrometry (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 are imparted by fluorescence labeling including the facile detection of the intact protein without proteolytic digestion to peptides; and high sensitivity, for example, 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. © 2014 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 103:3033–3042, 2014

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INTRODUCTION

A common post-translational modification in therapeutic proteins and peptides, amino-aspartyl succinimide (Asu), can be generated via two nonenzymatic spontaneous processes: deamidation of asparagine (Asn) or cyclization of aspartic acid (Asp), as illustrated in Scheme 1.[1–11] Deamidation of Asn 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 Asp 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 Asp 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 contrary, 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]

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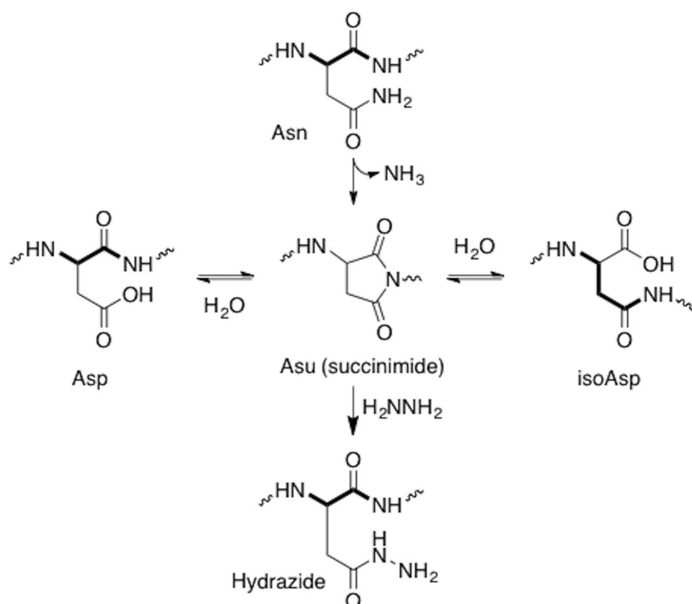
Many formulations are aimed at reducing Asn deamidation that is typically faster at higher pH; however, by exposing proteins to mildly acidic conditions, they may then be more susceptible to Asp isomerization and accumulation of succinimide.[35,36] As a result, succinimide is commonly observed in a myriad of therapeutic proteins including monoclonal antibodies,[12,36–42] growth hormones,[43,44] crystallins,[45] lysozyme,[7,40,46–48] and others.[49–52] Moreover, once a protein is introduced to a human subject, that is, 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 cross-linking,[55–59] aggregation,[60] 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 because of its intrinsic instability, that is, 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 out at pH 6.0 to preserve and detect the labile Asu in a recombinant human monoclonal antibody IgG2 for analysis by liquid chromatography–tandem mass spectrometry (LC–MS/MS). 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 Asu,[71] and the detection of the resulting ^{18}O-labeled Asp and isoAsp peptides by mass spectrometry.[72–76]



Scheme 1. Formation of Asu and isoAsp via Asp isomerization and Asn deamidation. The peptide backbone is traced in bold to illustrate the perturbation upon methylene insertion because of the formation of isoaspartic acid. Trapping of the labile Asu to form stable hydrazide is illustrated. Note that although only α-aspartyl hydrazide is shown, β-aspartyl hydrazide can also be formed.

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 ^{18}O-labeling, that is, 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 because of the mass increase of 14 Da (hydrazide vs. Asp). 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, Massachusetts). All reagents were ACS grade from Sigma–Aldrich (St. Louis, Missouri) 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, New Jersey) 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 obtained from Sigma–Aldrich. Lysozyme concentrations were determined from the absorbance at 280 nm measured on a NanoDrop 1000 UV–Vis (Thermo Scientific, Wilmington, Delaware) and an estimated extinction coefficient of 37,970 M^{-1} cm^{-1}, based on the amino acid composition. Sequencing-grade-modified trypsin was obtained from Promega (Madison, Wisconsin). Lissamine rhodamine B sulfonyl chloride was from Life Technologies (Grand Island, New York). 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, California). The MS was calibrated monthly with ESI positive ion calibration solution (Thermo Fisher Scientific). The flow rate was set to 300 μL/min and was split precolumn to deliver 300 nL/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 approximately 3–4 to prevent hydrolysis of succinimide. The samples were then loaded onto a self-packed reversed-phase column (75 μm i.d. × 15 cm, Magic C4 resin, 5 μm particle size, 300 Å pore size; Michrom BioResources, Auburn, California). 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 min. Data processing was performed using XCalibur 2.0 software (Thermo Fisher Scientific, Waltham, Massachusetts), and relative amounts of native and succinimidyl 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 h. Then, excess hydrazine was removed by dialysis in Slide-A-Lyzer mini dialysis units (7000 MWCO; Pierce, Rockford, Illinois) against 50 mM ammonium bicarbonate (pH 8, 1 L twice for 2 h 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.*

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