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Age estimation of museum wool textiles from *Ovis aries* using deamidation rates utilizing matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

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Cultural heritage contains a large number of precious proteinaceous specimens, such as wool and silk textiles, leather objects, paper, paint, coatings, binders (and associated adhesives), etc. To minimize the degradation of and to preserve these artifacts, it is desirable to understand the fundamental factors that cause their degradation, to identify the deterioration markers that determine their degradation stage and their age, and to use technologies that can provide this information rapidly while consuming a minimal amount of sample. There are several forces that cause protein degradation, including amino acid racemization, protein deamidation, and protein truncation. The purpose of this paper is to study protein deamidation using matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) for high-throughput dating of museums wool specimens. For proof of concept, several well-dated sheep's wool textiles from museum collections were analyzed. For wool samples aged from the present to ~400 years ago, the deamidation of two asparagine-containing peptides obtained from the tryptic digest of sheep wool were found to behave linearly in time, indicating that they could act as a potential biomarker of aging for wool samples.

Non-enzymatic deamidation of the amino acid residues glutamine (Gln, E) and asparagine (Asn, N) in peptides and proteins represents one of the most common posttranslational modifications found in proteins.[1] With average frequencies of 4.1% and 4.3% glutamine and asparagine residues in proteins, respectively, deamidation of these residues is considered an important degradation reaction, occurring *in vitro* in the course of isolation, or storage and *in vivo* during development and/or aging, therefore acting as a biological clock.[1–8] The deamidation reaction of Asn starts with the formation of a succinimide (five-membered cyclic imide) intermediate. The succinimide intermediate is rapidly hydrolyzed and produces L-Asp and L-isoAsp, with a little racemization to D-Asp and D-isoAsp.[9] The L-Asp and L-isoAsp are produced approximately in a mixture of 1:3. This step between the intermediate and L-Asp/isoAsp is reversible in an aqueous solution. In non-enzymatic deamidation, Gln can also participate in the same series of reaction sequences as Asn, but it forms a six-membered glutarimide instead of a five-membered succimide. Formation of the glutarimide is, however, less favorable, so imide deamidation of Gln is slower than Asn by about two orders of magnitude. Though the role of deamidation is not yet clear, deamidation is assumed to be a signal of protein half-life, and therefore is considered to act as a 'molecular timer'.[1] Deamidation from Asn and Gln causes a mass shift of +0.9840 Da. While this shift is distinguishable by mass spectrometry, if the peptide and its deamidated form are not separated by a separation technique or by high-resolution mass spectrometry, their isotopic patterns will overlap.[4,5] Using a conventional mass spectrometer and without using a separation technique, the degree of deamidation can be measured by enzymatic digestion of proteins, identification of the peptide containing Gln and Asn, and quantitation of the deamidated/non-deamidated forms by the deconvolution of the overlapping isotopic pattern. This procedure, however, only works: (1) for samples with a similar chemical/environmental history. This is because deamidation is a chemical reaction, and, as such, the rate of deamidation is affected by chemical and environmental factors such as temperature, humidity, chemical form, pH, etc.[8] Museum textiles with a known history usually satisfy the first requirement, since these textiles are usually stored under extraordinary care in religious or royal treasuries, and later in museums, where many environmental factors are maintained fairly constant.[10,11] (2) When samples of the same species are compared. This requirement is needed because the deamidation rate is also affected by the primary, secondary, tertiary, and the quaternary structure of proteins.[12–17] For example, the half-life of deamidation has been found to depend on the nearest neighboring residues on the peptide, causing significant differences in observed half-lives. Measuring the deamidation of wool samples from the same species (*Ovis aries*) satisfies this condition, since they will have the same protein sequence/structure, rendering the deamidation measurement more reliable. Recently, deamidation was used to molecularly investigate deterioration/aging

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signature of proteinaceous specimens.[18,19] In this article, the deamidation rates for three dated wool textiles from museum collections are compared. One unique peptide (*m/z* 2664) specific to sheep wool keratin type I and one non-specific wool peptide (*m/z* 2144) were analyzed utilizing matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and their deamidation rates were estimated from isotopic distribution of the peptides and their comparison with the theoretical isotopic distribution. Also, the influence of tryptic digestion on the rate of deamidation was assessed using ^{18}O-labeled water.[20]

EXPERIMENTAL

Materials

Urea was purchased from Mallinckrodt Baker (Phillipsburg, NJ, USA). Dithiothreitol (DTT), acetonitrile, and trifluoroacetic acid (TFA) were obtained from Thermo Fisher Scientific (Fair Lawn, NJ, USA). Trypsin was purchased from Promega (Madison, WI, USA). ZipTip C_{18} 10 µL pipette tips were supplied by Millipore Corporation (Billerica, MA, USA). All other chemicals, and $H_2^{18}O$ (97% ^{18}O), were purchased from Sigma-Aldrich (St. Louis, MO, USA). An anchor chip and Autoflex II MALDI-TOF/TOF mass spectrometer were purchased from Bruker Daltonics (Billerica, MA, USA). In MS mode, the resolution of the mass spectrometer was approximately 5000 (FWHH). Wool samples (Table 1) were obtained from Mary Ballard, Smithsonian Museum Conservation Institute. The ages of specimens were obtained using the midpoint of the reported age range, i.e., 1700 was used for a sample dated late 17th century/early 18th century. The modern wool sample was obtained from a commercial yarn.

Protein extraction

The wool samples (5 mg) were washed with water twice and sonicated twice for 30 s in 95% ethanol. The cleaned samples were dried and cut in small pieces (<2 mm) with clean scissors. The samples were extracted in 200 µL of a solution containing 8 M urea, 100 mM ammonium bicarbonate, and 50 mM DTT at pH 9.0 under constant shaking at room temperature for 48 h. The sample solutions were centrifuged at 90 *g* for 5 min, and the insoluble fractions were removed.

Reduction and alkylation

Proteins were reduced and alkylated before analysis. The samples (100 µL) in the extraction solution mentioned above were reduced with 10% V_i of 100 mM DTT for >1 h at 60 °C in a heating block. The samples were then alkylated with 10% V_i of 500 mM iodoacetamide for 1 h in the dark, and 100% V_i of 100 mM DTT was added to quench the reaction. The samples were separated into two tubes and completely dried by Speedvac.

Table 1. Description of the wool samples

Number	Year/Description
1	Modern wool
2	~1800 s-plied warp
3	~1700 s-plied warp
4	~1650 tapestry shied warp

Digestion

The dried samples were dissolved into 40 µL of 100 mM ammonium bicarbonate (pH 8.3) containing 4 µg of trypsin. Then, 20% V_{tot} of 100% acetonitrile was added and the solution was digested overnight at 370 °C. Because this solution contained urea and DTT (with approx. twice the concentration of the above section since the volume of the solution was approx. half the original volume), after digestion an aliquot of the sample was desalted using a ZipTip (C18) prior to MALDI-MS analysis. The following procedure was used for ZipTipping the samples: (1) the ZipTips were wetted in wetting solution (water/acetonitrile; 20/80, v/v) containing 0.1% TFA; (2) the ZipTips were equilibrated in water/acetonitrile solution (95/5, v/v) containing 0.1% TFA by repeatedly aspirating and dispensing this solution; (3) peptides were bound to a ZipTip by repeatedly aspirating and dispensing the peptide solution (~50 µL); (4) peptides were washed on the ZipTip using the solution used in step 2; and (5) peptides were eluted using 5 µL of the solution used in step 1. Half of this solution (2.5 µL) was used for MALDI-MS analysis.

^{18}O-Labeled digestion

To assess potential artificial deamidation during the digestion process, $H_2^{18}O$ was used. The ^{18}O labeling was done by using the same procedures described in the digestion and mass spectrometry analysis sections, except that $H_2^{18}O$ was used in all solution preparations.

Mass spectrometry analysis

For the matrix preparation, 10 mg/mL α-cyano-4-hydroxycinnamic acid was dissolved in 50% acetonitrile and 0.1% TFA. The MALDI spots on an anchor chip were prepared by mixing 2 µL of samples with 2 µL of the matrix. All peptides were analyzed by using the Autoflex in MALDI-TOF MS mode, and the mass spectra were smoothed. The isotopic distribution of a mixture of non-deamidated and deamidated peptides was interpreted using the software *Isotopica*.[21]

RESULTS AND DISCUSSION

Proteomics and MALDI-MS analyses of tryptic digests from several animal wools (sheep, goat, dog, etc.) had revealed the existence of several Asn-containing peptides in sheep wool.[22–25] In this study, keratin type I microfibrillar (47.6 kDa) in *Ovis aries* (UniProt P25690) was mostly extracted from the wool samples, and analyzed by MALDI-MS (Fig. 1). The insets in Fig. 1 show the expanded mass spectra of two of the Asn-containing tryptic peptides, ^{92}SQQQEPLVCP**N**YQSYFR$_{108}$ and ^{307}YSCQL**N**QVQSLIVSVESQLAEIR$_{329}$. The *m/z* values of the monoisotopic masses of the protonated peptides after alkylation by iodoacetamide are 2144.0 and 2664.4,

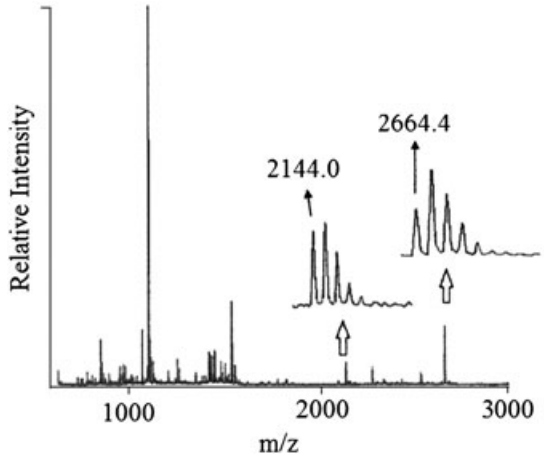

Figure 1. MALDI-TOF MS spectrum of modern sheep wool after extraction, enzymatic digestion, reduction by DTT, alkylation by iodoacetamide, and sample clean-up using a C18 ZipTip. The insets show the expanded areas for *m/z* 2144.0 (SQQQEPLVCPNYQSYFR) and *m/z* 2664.4 (YSCQLNQVQSLIVSVESQLAEIR).

respectively. These two peptides were chosen because *m/z* 2664.4 is considered a biomarker for keratin type I microfibrillar from *Ovis aries*, and, therefore, its deamidation rate is specific to sheep wool protein and is not affected by sequence or structural differences from other species. On the other hand, *m/z* 2144 was chosen as a non-specific peptide because it is present in the hair of several other mammals such as cashmere goat, mohair goat, camel, etc., which have different protein sequences and structures.[22] Moreover, as shown in Fig. 1, both of these *m/z* values exhibit strong peaks in the MALDI MS spectrum of the wool extract, indicating keratin type I microfibrillar from *Ovis aries* was a major protein in our wool extract. In addition, as shown above, the peptide sequence of each tryptic peptide has only one asparagine deamidation site that is the major contributor to the deamidation. However, it is important to note that, since we are considering samples as old as 400 years, it is likely that deamidation also occurred at Gln within the same peptide sequence. Figure 2 shows the experimental isotopic distribution of these two peptides for four wool samples aged from modern to the mid-17th century. As shown, the isotopic pattern almost monotonically shifts to higher masses due to deamidation (the conversion of NH_2 into OH upon aging) and by ~350 years most of the asparagines are deamidated (deamidation half-life = ~200 years). To quantitate this change, the isotopic patterns of 2144.0 and 2664.4 *m/z* were deconvoluted using the on-line version of *Isotopica*. For these calculations, the resolution was set to 5000 in order to match the theoretical resolution to the MALDI-MS experimental resolution. Parameters for the *Isotopica* calculation included: (1) the charge was set to + H; (2) peptide sequence; (3) modifications were added by (a) adding [H]-1[CH_2CONH_2] after the sequences, indicating protonation and alkylation, respectively, and (b) in the modifications box, both the non-deamidation [NH_2]-1 [NH_2] and deamidation [NH_2]-1[OH] were entered; (4) the relative abundances of the isotopic patterns for each sample were entered by averaging the isotopic pattern from three

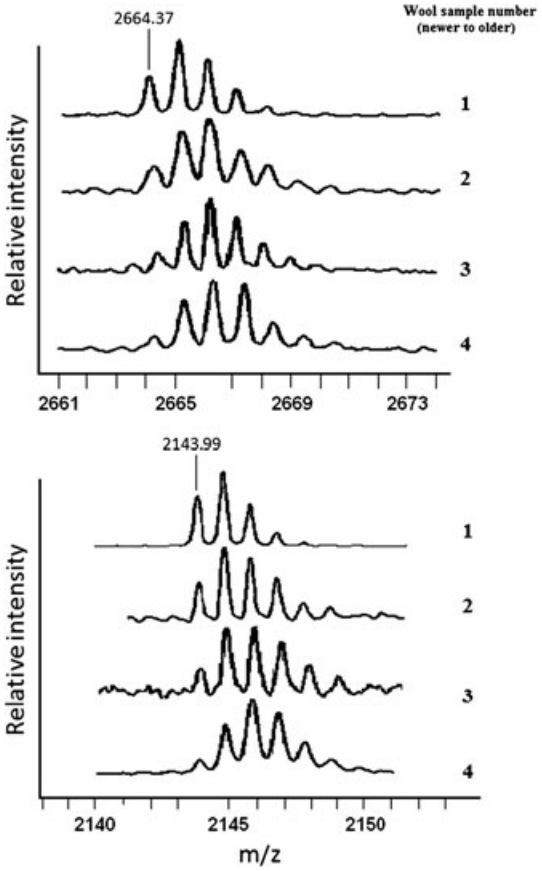

Figure 2. Isotopic patterns for protonated YSCQLNQVQSLIVSVESQLAEIR (*m/z* 2664.4 – top panel) and SQQQEPLVCPNYQSYFR (*m/z* 2143.9866 – bottom panel) after reduction and alkylation for samples 1–4 of Table 1.

MALDI-MS runs. The relative percentages of the deamidated forms were then calculated by *Isotopica*. The results for the peptides with *m/z* 2664.4 and 2144.0 are summarized in the top and bottom panels in Fig. 3, respectively. As shown in Fig. 3, although the two peptides have different sequences, their deamidation rates are very similar. Moreover, in about 400 years, one deamidation site is almost completely deamidated. This rate, however, is much slower than the deamidation of asparagine in the alpha-A crystalline of the human lens during aging, in which 45% of asparagine-101 is deamidated within 30 years.[26] One of the factors affecting faster deamidation in the human lens is its existence under physiological conditions (pH 7.4, 37 °C) compared with storage under room temperature (~20 °C) of these museum textiles. This experiment clearly shows that for wool samples under museum conditions, deamidation is a useful short-term (~400 years) biological clock.

The influence of the trypsin digestion process on deamidation rates

Parallel digestion with trypsin in $H_2^{18}O$ to investigate artificial deamidation during trypsin digestion has been comprehensively described by Li and co-workers.[20] To investigate if any artificial deamidation happened during the digestion

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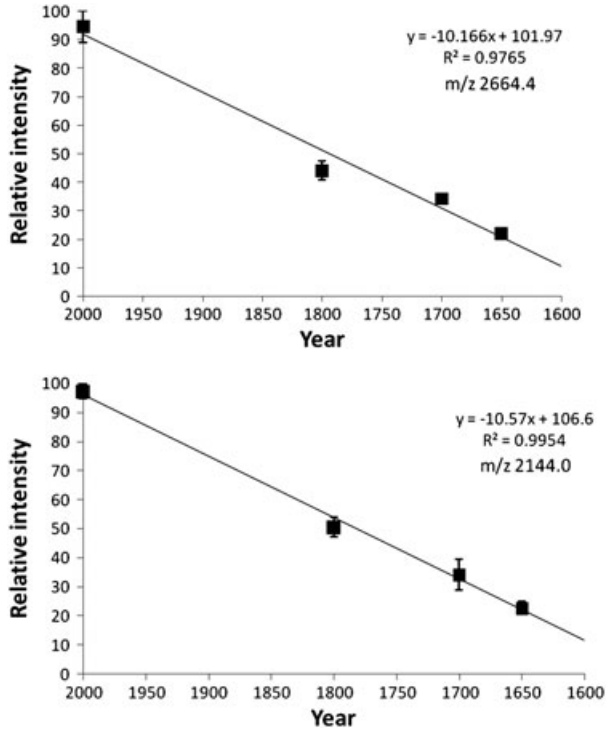

Figure 3. Percent of deamidation vs. time for YSCQLNQVQSLIVSVESQLAEIR (*m/z* 2664.4 – top panel) and SQQQEPLVCPNYQSYFR (*m/z* 2143.9866 – bottom panel) after reduction and alkylation for samples 1–4 of Table 1.

process under our experimental conditions, the enzymatic digestion was also carried out using ^{18}O water ($H_2^{18}O$). By using $H_2^{18}O$, a +2 Da mass shift occurs for each ^{18}O incorporation through peptide bond hydrolysis during the digestion. However, if deamidation occurs during the trypsin digestion, an ^{18}O atom will also be introduced on the newly formed carboxylate moiety (total of +4 Da shift). Comparison of the mass spectra of the two peptides digested in $H_2^{16}O$ and $H_2^{18}O$ waters (Fig. 4), however, showed only +2 Da shift, indicating no significant artificial deamidation during the overnight digestion process used in this study.

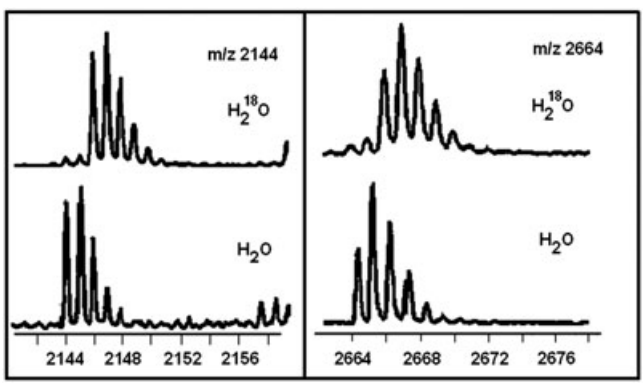

Figure 4. Parallel digestion of a modern wool sample with trypsin in H_2O and $H_2^{18}O$ to investigate artificial deamidation during trypsin digestion.

CONCLUSIONS

Age estimation of recent wool samples (past ~400 years) can be achieved via deamidation studies for museum specimens when sample environmental history is known. Because the deamidation rate depends on environmental factors as much as it depends on protein sequence and structure, a calibration curve must be first established using well-dated wool samples from the same species in order to determine the age of an unknown wool sample. For proof of concept, a calibration curve was established using sheep wool textile samples from museums. In ~400 years, one deamidation site of the two asparagine-containing peptides of the tryptic digest of wool keratins were found to be completely deamidated. While it is definitely true that deamidation at the Asn site is much faster, when considering sample as old as 400 years, it is likely that deamidation is also occurring at Gln within the same peptide sequence. The results obtained from a MALDI-MS study of the three wool samples imply that deamidation of sheep wool keratin can be used as a short-term (<400 years) biological clock. Future works include analyzing more well-dated wool samples from sheep and other species to obtain a more robust calibration curve for sheep, as well as calibration curves for other related species.

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