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Proteomics and spectroscopic analyses for the molecular characterization of collagen-based animal glues

Georgia Ntasi[1], Brunella Cipolletta[1], Carmen Aprea[1], Laura Dello Ioio[2], Celia Duce[3], Emanuele Crisci[3], Emilia Bramanti[4], Alessandro Vergara[1,5], Ilaria Bonaduce[3], Leila Birolo[1,5]

[1] Department Chemical Sciences, University Federico II, Complesso Universitario di Monte Sant'Angelo, Via Cintia, 21, 80126 –Naples, Italy
georgia.ntasi@unina.it, brunella.cipolletta@unina.it, carmen.aprea@studenti.unina.it, birolo@unina.it, avergara@unina.it
[2] Dello Ioio Restauri, Vico Equense, 80069 Naples, Italy
[3] Department of Chemistry and Industrial Chemistry, Via Risorgimento 35, University of Pisa, 56126 Pisa, Italy
[4] Institute of Chemistry of Organo Metallic Compounds, CNR Via Moruzzi 1, 56124 Pisa, Italy
[5] Task Force "Metodologie Analitiche per la Salvaguardia dei Beni Culturali", University Federico II, Complesso Universitario di Monte Sant'Angelo, Via Cintia, 21, 80126 – Naples, Italy

***Abstract* – Animal glues are widely used in restoration, as adhesives, binders and consolidants for organic and inorganic materials. Their variable performances are intrinsically linked to the adhesive characteristics of collagen, which determines the chemical, physical and mechanical properties of the glue. In this work, a multi-analytical approach based on proteomics and spectroscopic analyses is used to characterize a set of animal glues from different sources provided by restoration workshops. Shotgun proteomic analysis provided animal origin, even when blended, and allowed to distinguish between hide and bone glue on the basis of the presence of collagen type III. Proteomics and analytical pyrolysis coupled to GC-MS have been used to analyse chemical modifications in collagen, demonstrating their variability among different glues and showing that, on average, bone glues are less deamidated than hide glues, but more fragmented, and mixed-collagen glues are overall less deamidated than pure glues. Spectroscopic analyses have also been exploited to gain insights in structural changes occurring upon glue preparation from natural materials.**

I. INTRODUCTION

Animal glues have been used for centuries as adhesives in a variety of applications. The earliest uses date back to ancient Egypt, who used animal glues to fix wood inlays with ebony and ivory, to anchor faience to pyramid walls, or as a binder in pigments [1].

The term animal glue usually refers to an adhesive prepared from vertebrate connective tissues, namely bones, skin/hide or sinew. Upon treatment with acids or alkalis in hot water, the otherwise insoluble collagen, the main constituent protein of all these tissues, becomes soluble. Collagen in its natural state is three left-handed helical protein filaments that twist to form a right-handed helix [2] and has the distinctive Gly-X-Y repetitive sequence and a unique high content of Pro and Hyp, making it easily recognizable in the protein universe. It is naturally insoluble in water, and it must be processed into soluble gelatin to be used as animal glue.

The performance of the glue depends on the original source of collagen but is also strongly influenced by the extraction and preparation procedures. Indeed, both the specific sequence and the processing of the starting material affect the resistance of the polypeptide chains to hydrolysis and the (partial) denaturation of the triple helix that occurs upon heating in the gelatinization process [3].

Molecular weight distribution, stabilization of the protein matrix by hydrogen bonding and charge distribution, all have an impact on the performances of the glue and are determined by amino acid composition, but also and most importantly by chemical modifications occurring during preparation procedures.

These factors have a significant influence on glue properties, in terms of viscosity, strength, and overall mechanical behaviour [1].

Conservators' choice of commercial glue to use is primarily based on their empirical experience and often lacks a scientific characterization that could help them in making a properly informed decision.

Although much attention has been devoted to physical properties of collagen-based animal glues [1, 4-6], only recently a systematic characterization of molecular properties of glues started [7] but is far from being complete.

II. RESULTS AND DISCUSSION

A multi-analytical approach, based on proteomics and spectroscopic analyses has been adopted to characterize a set of 19 animal glue samples provided by restoration workshops [7].

A. Identification of collagen chains

Proteins in the samples were identified by a shotgun proteomics approach by LC-MS/MS. This analysis allowed to both establish the glue source and distinguish between hide and bone glues [7].

The proteomic analysis allowed to classify the animal glues on the basis of the specific origin of the identified collagen chains [7]. The discrimination between animal species in the case of collagen is challenging as it relies only on the detection of a very few unique peptides. To simplify species assignment, the search space was reduced to sequences of collagen type I and III of the common domesticates generally used for animal glues [7]. In particular, the detection of collagen alpha 1(III) was used to distinguish between hide and bone glues, as this molecule is abundant in soft connective tissues and skin, while it is poorly synthetized in bones. Instead, collagen alpha-1 and -2(I) are ubiquitous in all the collagen-bearing tissues [8].

Following the identifications, the animal glue samples were clustered based on the proteins content as Bone glues (B) and Hide glues (H) and subdivided in Pure (P) and Mixed (M), as reported in Table 1.

B. Analysis of backbone cleavage and deamidation of collagen

Proteomics and analytical pyrolysis coupled to GC-MS were used to analyse chemical modifications in collagen.

First, backbone cleavage of the polypeptide chain was considered as an expected degradation feature in proteins from animal glues [9]. Collagen is insoluble in cold water and is transformed into soluble gelatin by denaturation and partial hydrolysis, which is achieved by hot water extraction (hydrolytic breakdown) [1]. Such damage at the backbone can be evaluated in LC-MS/MS analyses as semitryptic peptides that will be generated upon trypsin hydrolysis, with a trypsin cleavage site only at one end [7]. The occurrence of semitryptic peptides was semi-quantitatively evaluated by counting peptide to spectrum matches (PSMs) and dividing the PSMs of semitryptic peptides with the total PSMs of identified peptides, including both tryptic and semitryptic peptides [7].

To further investigate the degree of backbone cleavage, analytical pyrolysis coupled with GC-MS analyses were carried out [7]. The most characteristic pyrolysis products of proteins are cyclic dipeptides, 2,5-diketopiperazines (DKPs), whose formation is hypothesized to be a depolymerization involving the cyclization of neighbouring amino acids in a polypeptide chain. A relatively high yield of DKPs might be ascribed to a high degree of protein hydrolysis [10]. As reported in Figure 1, data obtained reveal that generally bone glues are more fragmented than hide ones [7].

Table 1. Collagen chains identified in the animal glue samples by LC-MS/MS. Table from [7]. [a]Protein identification was used to classify glue samples as Bone glues (B) and Hide glues (H) and further subdivide them in Pure (P) and Mixed (M).

Sample	Label on the basis of protein content[a]	Taxonomy	Collagen a1(I)	Collagen a2(I)	Collagen a1(III)
Rabbit glue SOB1	HP1	*Oryctolagus cuniculus*	✓	✓	✓
Rabbit Glue SOB2	HP2	*Bos taurus*	✓	✓	✓
Rabbit glue SOB3	HP3	*Bos taurus*	✓	✓	✓
Rabbit glue SOB4	HP4	*Bos taurus*	✓	✓	✓
Rabbit glue SOB5	HM1	*Bos taurus*	✓	✓	✓
		Sus scrofa	✓	✓	✓
Rabbit glue 10	HP8	*Bos taurus*	✓	✓	✓
Rabbit glue 2	HM2	*Oryctolagus cuniculus*	✓	✓	
		Ovis aries		✓	✓
		Capra hircus	✓		
Rabbit glue 3	HM3	*Bos taurus*	✓	✓	✓
		Oryctolagus cuniculus	✓	✓	✓
		Ovis aries		✓	
Rabbit glue 7	HM5	*Oryctolagus cuniculus*	✓		
		Sus scrofa	✓	✓	✓
Rabbit glue 6	HM4	*Bos taurus*	✓	✓	✓
		Oryctolagus cuniculus	✓	✓	
Fish glue SOB6	HP5	*Sus scrofa*	✓	✓	✓
Fish glue 4	HP6	*Bos taurus*	✓	✓	✓
Fish glue 5	HP7	*Bos taurus*	✓	✓	✓
Sturgeon fish glue SOB7	FM	*Scyliorhinus canicula*	✓	✓	
		Sus scrofa	✓	✓	
Strong glue SOB8	BM1	*Bos taurus*	✓	✓	
		Sus scrofa	✓	✓	
Strong glue SOB9	BM2	*Bos taurus*	✓	✓	
		Sus scrofa	✓	✓	
		Equus asinus	✓	✓	
Strong glue 8	BM4	*Bos taurus*	✓	✓	
		Sus scrofa	✓	✓	
		Equus asinus		✓	
Strong glue 9	BM5	*Bos taurus*	✓	✓	
		Sus scrofa	✓		
		Ovis aries	✓	✓	
		Equus asinus	✓	✓	

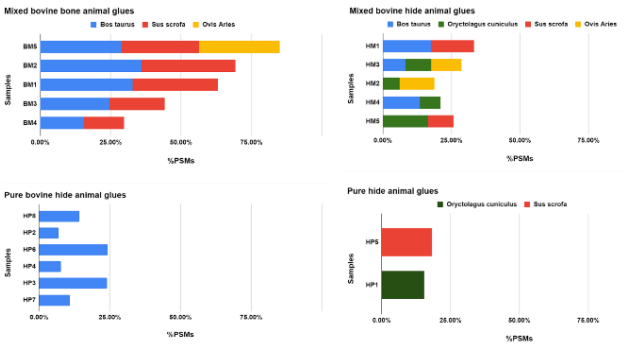

Fig. 1. Occurrence of backbone cleavage in animal glue samples. Figure from [7].

MS/MS data were also used to investigate chemical modifications in the side chains of animal glue collagen. We evaluated deamidation occurrence in the set of samples keeping in mind that deamidation is routinely searched for in aged proteins [11-12] and viewed as a global indicator of sample preservational quality [13], as rates and levels of deamidation are affected by several chemical and environmental factors. This modification is expected to strongly influence the rheological properties of the adhesive material since it changes collagen pI. During glue processing, the acidic or alkaline treatments favour hydrolysis of the amide groups in collagen to a greater or lesser extent by deamidating the lateral chain of Glutamine (Q) and Asparagine (N). Therefore, harsher conditions of collagen extraction in the preparation of animal glues might have been imprinted also in the profile and level of collagen deamidation [7].

Deamidation was evaluated from raw LC-MS/MS data by MaxQuant software with an in-house script based on PSMs intensities for semiquantitative evaluation [7]. Data collected are reported in Figure 2 and show that on average bone glues are less deamidated than hide glues, while mixed-collagen glues are overall less deamidated than pure glues [7].

III. CONCLUSIONS AND NEXT STEPS

Although much attention has been devoted to physical properties of collagen-based animal glues, a systematic characterization of molecular properties of animal glues is still lacking. This work strives to address this issue by providing a molecular characterization of a set of 19 animal glues produced for restoration purposes [7].

Overall proteomic data suggest that, on average, bone glues are less deamidated than hide glues, but more fragmented, and mixed-collagen glues are overall less deamidated than pure glues [7]. There results confirm the heterogeneity of collagen-based animal glues at the molecular level. This heterogeneity is strongly increased by preparation and manufacturing procedures that affect the properties of the glue, possibly more than the collagen origin itself.

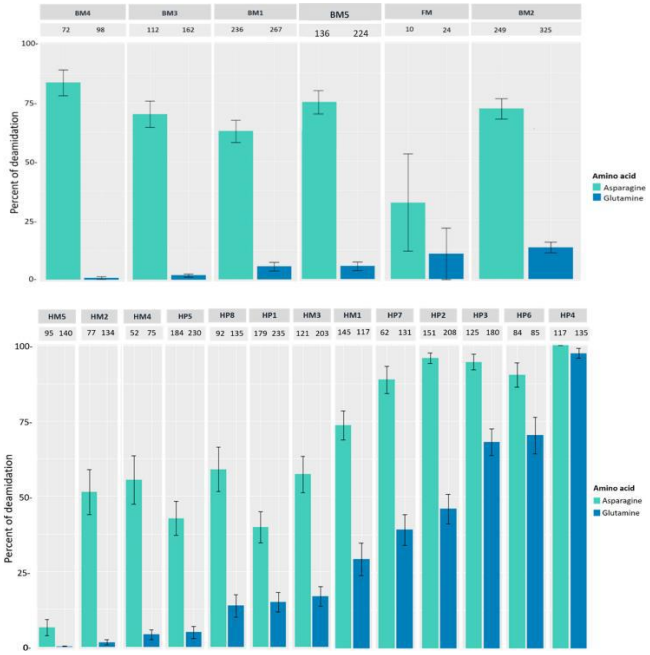

Fig. 2. Overall percentage of deamidation for asparagines (N) and glutamines (Q) residues for the collagen chains identified in the bone (upper panel) and hide (lower panel) glue samples. Error bars represent standard deviation and numbers above each bar represent the number of peptides the data is based on. Figure from [7].

How molecular details are reflected in the three-dimensional structures and in the rheological properties of the animal glues is currently under investigation by a combination of thermalgravimetric analysis (TGA), differential scanning calorimetry (DSC) and spectroscopic techniques.

Data collected so far suggest that animal glues samples deriving from hide tissues are characterized by less degraded collagen, which maintains a high percentage of triple helix structure, thus producing more structured networks. On the contrary, animal glues samples deriving from bone tissues are constituted by more degraded collagen molecules, with very low or absent triple helix structures, which do not produce highly ordered structures.

These preliminary data pave the way to a correlation between molecular modifications and material performances in animal glues.

IV. EXPERIMENTAL SECTION

A. Animal glue samples

A set of 19 animal glues provided by the restoration workshop of the University Suor Orsola Benincasa in Naples, and by Museo Nacional del Prado in Madrid, have been analysed and characterized.

B. Protein Extraction and Digestion

Samples were prepared as reported in ref. [14]. Briefly, 1−2 mg of each pellet was resuspended in 10 μL of 6 M urea. Samples were incubated for 20 min at room temperature and then for 30 min in the sonicator. Samples were then 6-fold diluted with 10 mM ammonium bicarbonate at pH 7.5, and enzymatic digestion was carried out by the addition of 1 μg of trypsin at 37 °C for 16 h. The supernatants were then recovered by centrifugation and filtered on 0.22 μm PVDF membrane (Millipore), and peptides were desalted and concentrated on in-house made C18 extraction stage tips as described by Cappellini et al. [11]. Peptides were eluted with 20 μL of 50% acetonitrile and 0.1% formic acid in Milli-Q water and analysed by LC-MS/MS.

C. LC-MS/MS Analysis

Samples were analysed on a 6520 Accurate-Mass Q-Tof LC/MS System (Agilent Technologies, Palo Alto, CA, U.S.A.) equipped with a 1200 HPLC System and a chip cube (Agilent Technologies) as reported in ref. [14]. Samples were fractionated on a C18 reverse-phase capillary column (Agilent Technologies) at a flow rate of 400 nL/min, with a linear gradient of eluent B (0.1% formic acid in 95% acetonitrile) in A (0.1% formic acid in 2% aceto-nitrile) from 3% to 80% in 50 min.

Peptide analysis was performed using data-dependent acquisition (DDA) of one MS scan (mass range from 300 to 2000 m/z) followed by MS/MS scans of the three most abundant ions in each MS scan. MS/MS spectra were measured automatically when the MS signal surpassed the thresh-old of 50 000 counts. Double and triple charged ions were preferably isolated and fragmented.

Alternatively, peptide fractionation was performed on LTQ Orbitrap XL Hybrid Ion Trap-Orbitrap MS System (Thermo Scientific, Bremen, Germany) on a C18 capillary reverse-phase column (200 mm, 75 μm, 5 μm) at 250 nL/min flow rate, using a step gradient of eluent B (0.2% formic acid, 95% acetonitrile LC-MS grade) in eluent A (0.2% formic acid in 2% acetonitrile) from 5 to 50% over 80 min and to 80% over 5 min.

Mass spectrometric analyses were performed using data-dependent acquisition (DDA) mode over the 400 to 1800 m/z range, at a resolution of 60 000, and the automatic gain control (AGC) target was set to 1 × 106, followed by acquisition in MS/MS of the five most abundant ions. For the MS/MS scans, the resolution was set to 15 000, the AGC target was set to 1×105, the precursor isolation width was 2 Da, and the maximum injection time was set to 500 ms. The CID normalized collision energy was 35%; AGC target was set to 1×105. Data were acquired by Xcalibur software (Thermo Fisher Scientific).

D. Protein identification and semiquantitative evaluation of chemical modifications

MS/MS spectra were transformed in Mascot Generic files (.mgf) format and routinely used to query the SwissProt database. A licensed version of Mascot software (www.matrixscience.com) version 2.4.0. was used. Standard parameters in the searches were trypsin as the enzyme; 3, as allowed number of missed cleavages; 10 ppm MS tolerance and 0.6 Da MS/MS tolerance; peptide charge from +2 to +3. In all the database searches, no fixed chemical modification was inserted but possible oxidation of methionines, deamidation at asparagines and glutamines, and hydroxylation on lysine and proline were considered as variable modifications.

To simplify species assignment and recover more focused results, ultimate searches were carried out using a homemade database, which we named COLLE (60 sequences; 88 859 residues), that collects the sequences of collagen type I and III for all the common domesticates generally used for animal glues). Mass spectrometry data and the COLLE database have been deposited to Mendeley Data (https://data.mendeley.com/datasets/hbmc8yhf7y/2).

Semiquantitative evaluation of deamidation was carried out by MaxQuant software as reported in ref. [7]. Parameters common among all runs are as follows: tryptic search with up to two missed cleavages; minimum peptide length was set to 7; and no fixed modification was set, while oxidation of methionine, hydroxylation of proline and hydroxylation of lysine were set as variable modifications, with up to a maximum of 5 modifications per peptide. Protein identifications were supported by a false discovery rate (FDR) of 0.01 applied (same FDR for dependent peptides when applied) and manually filtered by at least 2 different non-overlapping peptides above the 40-ion score threshold.

After each run, the evidence file.txt of each animal glue was first cross validated for its peptides and proteins according to the protein identification that had already been performed with the use of Mascot. Afterward, the "evidence" files were used for the evaluation of deamidation (N, Q) level both in the total sample (global deamidation) and for the single polypeptide chain identified in each animal glue, with the public available software (https://github.com/dblyon/deamidation).

Backbone cleavage evaluation was carried out by setting the same parameters as for standard protein identification as described above but "semitrypsin" as enzyme and an ion score cut off $\geq$ 25 for unmodified and modified peptides. The assessment of the occurrence of backbone cleavage was carried out by counting the PSMs (Peptide Spectrum Matches) in the single samples, focusing on Type I and Type III collagen chains only.

A site-specific evaluation of the deamidation (N, Q) occurrence along the amino acidic sequence was performed by manually inspection of MS/MS data as reported in ref. [7].

E. Analytical Pyrolysis Coupled to GC-MS (Py-GC-MS)

Samples were subjected to flash pyrolysis at 550 °C for 0.2 min, and the interface temperature was 280 °C. The split/splitless injector was used at a 1:50 split ratio. The Pyrolyser was a Multi-Shot Pyrolyzer EGA/Py-3030D (Frontier Lab) coupled to a gas chromatograph 6890 coupled with a 5973 Mass Selective Detector Agilent Technologies (U.S.A.). To assess the simultaneous presence of other organic components, analytical pyrolysis was also carried out with in situ silylation. Samples were admixed to 2 μL of hexamethyldisilazane in the cup before the pyrolysis, carried out at 550 °C, Py-GC interface set at 280 °C, pyrolysis time of 0.2 min.

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