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A novel LC–MS method using collagen marker peptides for species identification of glue applicable to samples with multiple animal origins

Yuki Kumazawa[1], Yuki Taga[2*], Miho Takashima[3] and Shunji Hattori[1,2]

Abstract

Animal glues derived from collagen-rich tissue are widely used as adhesives and binders in artworks. Identification of the animal source of glue is important not only to understand the artistic technique and historical background, but also to conduct appropriate treatment for restoration and conservation of artworks. Recently, mass spectrometric approaches have been used for species identification of glue. However, it appears that the existing methods were not applied to samples containing multiple animal sources, and the range of target species has been limited because of incomplete sequence information of collagen in public databases. In the present study, we established a novel method for discrimination of glues from eight animals applicable to samples with multiple animal origins. Trypsin-digested glue samples were analyzed by LC–MS in multiple reaction monitoring mode, and the animal source was determined based on the detection patterns of 12 type I collagen-derived marker peptides. Using the database-independent method, we successfully identified the animal source of commercial products and found the declared species for some glues to be incorrect. In addition, some products were identified to contain two different animal origins. Relative abundance of the animal origins in several impure glues was estimated using the marker peptides, which helped to speculate the reason for the detection of multiple species. We analyzed a painting (*The Harvest* by Camille Pissarro, 1882) and clarified that glues used in the ground and size layers of the canvas were derived from cattle (~ 65%) and sheep (~ 35%).

Keywords: Glue, Collagen, LC–MS, Species identification, Marker peptide

Introduction

Skin and bone of mammalians and fish are mainly composed of type I collagen, consisting of Gly–Xaa–Yaa repeats, where Xaa and Yaa are frequently Pro and 4-hydroxyproline (4-Hyp), respectively. Through heating in boiling water, collagen is extracted from these tissues as a partially hydrolyzed form, referred to as gelatin or glue. Animal glue was used by Egyptians more than 4000 years ago and has been commercially manufactured since the 1700s [1]. In the preparation of artworks, artists and craftsmen use animal glue for various purposes; for example, as adhesives for wooden products and paper, as binding media for pigments in paint and ground layers, and as sizings for paper, panel, and canvas. Animal glue exhibits varied physical, chemical, and mechanical properties depending on its biological origin and the method used to prepare it [2]. Therefore, in addition to determining the use of glue, identification of its animal source provides an understanding of artistic material/technique and context of the era. Such information is also helpful in identifying appropriate treatments for restoration and conservation of artworks.

Detection of collagen-based glue in artworks with differentiation from other types of proteinaceous binders, such as milk casein and egg, can be performed based

*Correspondence: y-taga@nippi-inc.co.jp
[2] Nippi Research Institute of Biomatrix, 520-11 Kuwabara, Toride, Ibaraki 302-0017, Japan
Full list of author information is available at the end of the article

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on the high content of Gly and Hyp estimated by HPLC, GC–MS, or capillary zone electrophoresis after acid hydrolysis [3, 4]. However, these methods are unsuitable for identification of the animal source of glue because of the loss of species specificity in the amino acid sequence. Some studies have reported an immunology-based approach using ELISA and a DNA-based approach using PCR for species identification of glue in artworks [5, 6]. ELISA can selectively and sensitively detect the use of glue [5], but cross-reaction of antibodies is a concern because of similarities in the amino acid sequences of collagen among mammals. In contrast, PCR amplification of mitochondrial DNA has high taxonomic specificity for tracing the animal origin of glue [6], but DNA molecules are relatively unstable and can be degraded during glue manufacture and aging after use.

Recently, mass spectrometric approaches have been increasingly used for species identification for various kinds of samples, including bone [7, 8], meat [9, 10], milk [11, 12], and gelatin [13, 14]. Target proteins are enzymatically digested into peptide fragments, typically using trypsin. MS can simultaneously detect the resulting peptides with high sensitivity, which allows discrimination of the animal species based on interspecies differences in the amino acid sequence. As for glue used in artworks, Kuckova et al. successfully identified the animal origin using peptide mass fingerprinting with MALDI–TOF–MS detection [15]. While this analysis can be straightforward using the detected peak pattern in a single mass spectrum, unambiguous species determination is difficult, especially for complex samples. Proteomic approaches with database search of LC–MS/MS spectra also have been reported for species identification of glue [16–19]. This strategy enables more reliable determination of the animal species because of the exact sequence information of the identified peptides determined based on their MS/MS fragmentation patterns. Dallongeville et al. used species-specific peptides identified for type I and type III collagens to discriminate between bovine, rabbit, and sturgeon glues [17]. Using this method, they identified bovine glue in an 18th century gilt sample and found that bovine-derived glue was supplied as rabbit glue for some products. However, identification of species-specific peptides becomes challenging when extending the target species, and all the above existing methods were assumed not to be applied to samples containing multiple glues derived from different animals. It is known that some suppliers provide rabbit glue as a blend with bovine glue to alter its properties [2]. In addition, contamination with other glues is possible during and after the manufacturing process. Furthermore, blending techniques or multiple use of glues derived from different origins may be used in artworks. Therefore, it is important to unambiguously determine the animal source of glue even when multiple-species glues are used.

Collagen-based materials, such as leather, gelatin, and glue, are prepared from various animals. However, collagen sequence information of these domestic animals is scarce in public databases, which makes species identification difficult [16, 20]. For example, only partial sequences of rabbit type I collagen (53 and 526 amino acids for α1(I) and α2(I) chains, respectively) are registered in the UniProt database as reviewed sequence data, and deer α1(I) and α2(I) chains are completely unknown. We recently established a novel approach for species identification of leather using LC–MS [21]. Using authentic leathers, we first performed MS/MS identification of type I collagen-derived peptides using currently available sequence information after trypsin digestion. Among many identified peptides, six tryptic marker peptides were selected with no regard for the animal species of type I collagen to give different and species-specific detection patterns between cattle, horse, pig, sheep, goat, and deer. Detection of the marker peptides by LC–MS in multiple reaction monitoring (MRM) mode enabled simple certification of the animal source of leather. MRM analysis based on MS/MS fragmentation can highly selectively detect target analytes by using a predefined precursor ion and its fragments [22]. Of note, this database-independent methodology can be applied to various animals regardless of whether their collagen sequences are publicly available. In the present study, we applied this approach to species identification of glues from eight animals, including cattle, horse, pig, sheep, goat, deer, rabbit, and sturgeon. In contrast to leather, there is the potential for a mixture of multiple sources for glue. Thus, we further designed the method to give different detection patterns even between samples containing two species. Commercial animal glues and *The Harvest* painted by Camille Pissarro were analyzed using the newly established method.

Experimental

Standard samples

Cattle, horse, and pig collagens were purified from the skin of respective animals by pepsin digestion and salt precipitation as described previously [23]. Sheep collagen and collagen from rabbit skin were purchased from Sigma-Aldrich (St. Louis, MO). Deer glue prepared from deer skin (*Cervus nippon*) was obtained from Amanosan Cultural Heritages Research Institute (Osaka, Japan), and sturgeon glue made from bladder was kindly gifted from Prof. Izuru Seki (Tokyo University of the Arts). A certified chrome-tanned goat leather was used as a standard sample after dechroming as described previously [21].

Glue samples

Commercial animal glues were purchased from several suppliers. The glue samples were dissolved in distilled water at 60 °C for 4 h and stored at 4 °C until analysis.

Trypsin digestion

Collagen or glue samples (protein amount of 50 μg) were heated at 60 °C for 30 min and digested with 2.5 μg of trypsin (Sigma-Aldrich) at 37 °C for 16 h in 100 μL of 100 mM Tris–HCl/1 mM $CaCl_2$ (pH 7.6). The dechromed leather was similarly digested with trypsin as reported previously [21]. The tryptic digests were acidified with formic acid (final 1%) and subjected to LC–MS/MS or MRM analysis.

Analysis of *The Harvest*

Samples from two separate locations (approximately 1 mg each) were taken from the tacking margin of the canvas of *The Harvest* (Camille Pissarro, 1882, 70.3 × 126 cm, glue tempera on canvas), which is part of the collection of The National Museum of Western Art (Tokyo, Japan; inventory number P. 1984-0003). The samples included ground layers, size layers, and fibers of the canvas. The samples were digested with 2.5 μg of trypsin as described above after heating at 60 °C for 30 min in 100 μL of 100 mM Tris–HCl/1 mM $CaCl_2$ (pH 7.6). After acidification of the reactant with formic acid, the supernatants were subjected to MRM analysis.

LC–MS/MS analysis

Identification of collagen marker peptides were performed by LC–MS/MS using a 3200 QTRAP mass spectrometer (AB Sciex, Foster City, CA) coupled to an Agilent 1200 Series HPLC system (Agilent Technologies, Palo Alto, CA). Tryptic digests of standard samples were loaded onto an Ascentis Express C18 HPLC column (2.7 μm particle size, L × I.D. 150 mm × 2.1 mm; Supelco, Bellefonte, PA) at a flow rate of 200 μL/min with a binary gradient as follows: 98% solvent A (0.1% formic acid in water) for 5 min, linear gradient of 2–50% solvent B (100% acetonitrile) for 15 min, 90% solvent B for 5 min, and 98% solvent A for 5 min. Three injections of 10 μL were performed for each sample. The MS scan and MS/MS acquisition were performed over *m/z* ranges of 400–1300 and 100–1700, respectively. The collision energy was automatically determined based on the mass and charge state of the precursor ions using rolling collision energy. Acquired MS/MS spectra were searched against a local type I collagen database, which included type I collagen sequences of various animals [8], using ProteinPilot software 4.0 (AB Sciex) with the Paragon algorithm [24]. The confidence threshold of the identified peptides was defined to be 95%. We have deposited the MS datasets to the ProteomeXchange consortium via the jPOST partner repository with the dataset identifier PXD009059 [25, 26].

MRM analysis

Type I collagen-derived marker peptides were monitored by LC–MS in MRM mode. The tryptic peptide solutions were separated using an Ascentis Express C18 HPLC column (5 μm particle size, L × I.D. 150 mm × 2.1 mm; Supelco) at a flow rate of 500 μL/min with a binary gradient as follows: 98% solvent A (0.1% formic acid in water) for 2 min, linear gradient of 2–60% solvent B (100% acetonitrile) for 4 min, 90% solvent B for 2 min, and 98% solvent A for 2 min. The MRM transitions and collision energy settings for the marker peptides are shown in Additional file 1: Table S1. The detection threshold for marker peptides was set to a peak intensity of 100 with a signal-to-noise ratio of 5.

The relative abundance of the animal origins in glue samples or *The Harvest* was estimated using peak areas of MRM chromatograms of marker peptides detected for either but not both of identified two animal species (P11 for cattle, P9 for rabbit, and P5 for sheep). Type I collagen concentrations in standard samples of cattle, rabbit, and sheep were predetermined using stable isotope-labeled collagen (SI-collagen) as reported previously [27]. Using the standard samples, external calibration curves of cattle, rabbit, and sheep type I collagen were prepared by MRM analysis of the marker peptides (P11, P9, and P5) following trypsin digestion for quantification of the respective animal's type I collagen concentrations in the samples.

Results and discussion

Selection of collagen marker peptides

To establish an LC–MS method for animal source identification of glue, we first selected marker peptides using authentic standards of cattle, horse, pig, sheep, and rabbit (collagen), deer and sturgeon (glue), and goat (leather). The authentic standards were digested with trypsin, and the generated peptides were subjected to LC–MS/MS analysis. Many type I collagen-derived peptides from various animal species were identified for the respective standard samples by searching the acquired MS/MS spectra against an in-house type I collagen database (data have been deposited to the ProteomeXchange consortium with the identifier PXD009059). For example, although the sequence of deer type I collagen is not in public databases, analysis of deer glue identified a total of 396 peptides for type I collagen α1 and α2 chains from various animals, including 55 peptides for bovine (*Bos taurus*) and 37 peptides for sheep (*Ovis aries*). This

is because of the high sequence homology of collagen among mammals. In contrast, almost all peptides identified for sturgeon glue were from fish species.

From the identified peptide list, we ruled out peptides in which not all Pro residues at the Yaa position were hydroxylated to 4-Hyp and a missed cleavage except at the Arg- or Lys-Hyp bond was observed. MRM transitions were then set for the remaining marker peptide candidates to monitor the presence or absence of the peptides for each standard sample (data not shown). From the results, collagen marker peptides were selected to give at least two differences in the patterns of the presence or absence of marker peptides between each animal, as previously reported for leather [21]. Furthermore, we added a rule that there is at least one difference in the detection patterns of marker peptides when glue samples have two animal origins. To satisfy these requirements, we selected seven marker peptides (P1–P7) from α1(I) chain and five marker peptides (P8–P12) from α2(I) chain (Table 1). Two MRM transitions were set for the respective marker peptides to definitively judge the presence of the peptides (Additional file 1: Table S1). From the detection patterns of the MRM peaks, we were able to clearly discriminate the eight animals as shown in Fig. 1 (representing single intense transition for each of the marker peptides). In addition, use of the 12 marker peptides enabled determination of the animal species even when two different origins are present in one sample (Additional file 2: Table S2). For example, marker peptides P1, P4, and P11 are detected for cattle, and P1, P4, and P9 are detected for rabbit, which means P1, P4, P9, and P11 should be detected for a glue sample with cattle and rabbit origins.

Identification of the animal source of commercial glues

We next investigated commercial animal glues using the established method (Table 2). Among 22 glue samples, the animal source was unambiguously determined for 18 samples, including glues for which the biological origin was not provided. In addition, several samples (C, K, and S) were identified to have two different animal origins. In sample S identified as sheep and goat, two marker peptides, P4 and P5, sharing same sequence position (733–756 and 741–756) were detected, which strongly supports the co-existence of different origins in the one product. We could not determine the animal species for some samples (B, F, L, and P) because the detection patterns of the marker peptides did not match any of those established for glue containing one or two animal origins. We assumed that these glue samples contained more than three species or were derived from unexpected animals. In seven samples identified as cattle (A, D, E, G, and H) or sturgeon (Q and R), the identification results matched the animal source given on the label. However, some commercial glues showed detection patterns that were different from those predicted for the declared animal species. For example, the animal source of sample I was declared as deer, but was identified as cattle based on the detection of P1, P4, and P11. Surprisingly, almost all of the glue products supplied as rabbit were identified to be completely derived from a different animal source (pig for sample J and cattle for samples M, N, and O). In addition, sample K was shown to contain cattle as an additional animal source other than rabbit. There was no pure rabbit glue among the commercial products we analyzed. This is consistent with a previous observation that some

Table 1 List of marker peptides for animal species identification of glue

Chain	Position[a]	Marker peptide	Sequence[b]	Cattle[c]	Horse[c]	Pig[c]	Sheep[c]	Goat[c]	Deer[c]	Rabbit[c]	Sturgeon[c]
α1(I)	316–327	P1	GFOGADGVAGPK	+	+	+	−	−	+	+	−
	316–327	P2	GFOGSDGVAGPK	−	−	−	+	+	−	−	−
	688–704	P3	GAAGPOGATGFOGAAGR	−	−	−	−	−	−	−	+
	733–756	P4	GETGPAGROGEVGPOGPOGPAGEK	+	−	−	−	+	+	+	−
	741–756	P5	AGEVGPOGPOGPAGEK	−	−	−	+	−	−	−	−
	889–906	P6	GEAGPAGPAGPIGPVGAR	−	+	−	−	−	−	−	−
	889–906	P7	GETGPAGPAGPAGPAGAR	−	−	−	−	−	−	−	+
α2(I)	238–252	P8	GIOGPAGAAGATGAR	−	+	+	−	−	−	−	−
	253–264	P9	GLVGEOGPAGTK	−	−	−	−	−	−	+	−
	361–374	P10	GFOGSOGNVGPAGK	−	−	+	−	−	−	−	−
	978–990	P11	IGQOGAVGPAGIR	+	−	−	−	−	−	−	−
	978–990	P12	TGQOGAVGPAGIR	−	−	+	+	+	+	−	−

[a] The numbering of residues begins with the triple-helical region of the chains

[b] O indicates 4-Hyp

[c] The presence and absence of marker peptides are denoted by + and −, respectively

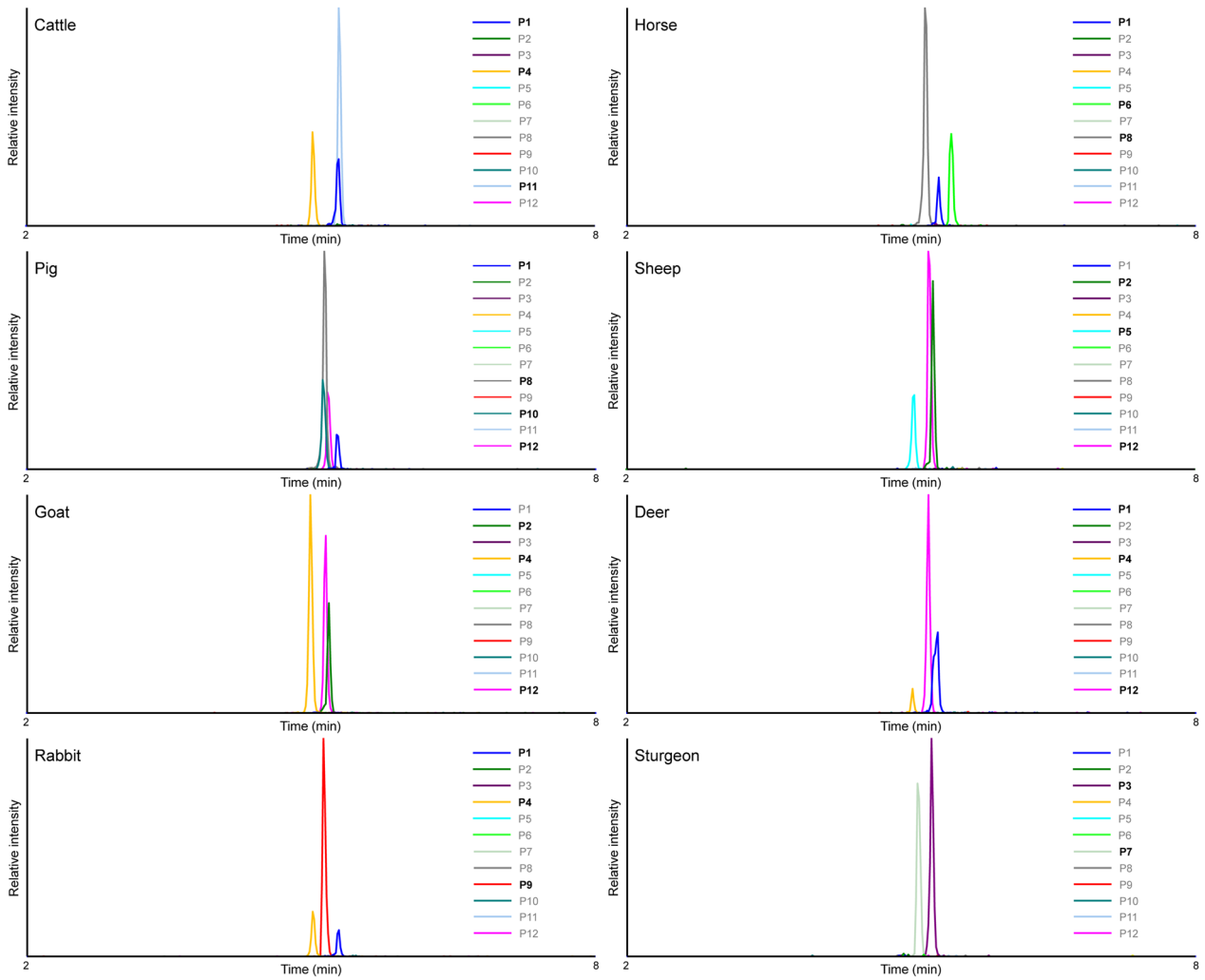

Fig. 1 MRM chromatograms of type I collagen-derived marker peptides for eight animal species. Single intense peaks out of two MRM transitions are depicted as representatives for respective marker peptides. Detected marker peptides for each authentic animal standard are indicated in boldface

modern skin glues provided as rabbit were in fact cattle glues [17].

We also estimated the relative abundance of the animal origin for the glue samples revealed to have both cattle and rabbit origins (samples C and K) (Table 3). Peak areas of MRM chromatograms of marker peptides detected for either but not both animals (P11 for cattle and P9 for rabbit) were used for quantification of respective origins. Concentrations of these animal origins were calculated by external calibration curves prepared using authentic standards of cattle and rabbit for which type I collagen concentrations were predetermined using a previously developed internal standard, named SI-collagen [27]. The proportion of the undeclared origin in sample C was estimated to be low (9.9% for rabbit). From this result, we speculate that cattle glue was accidentally contaminated with rabbit glue, such as when these glues are prepared through the same processes. In contrast, sample K declared as rabbit was composed of nearly equal parts of the two animal origins (55.0% cattle and 45.0% rabbit), suggesting that cattle glue was purposefully blended into rabbit glue.

Identification of the animal source of glue used for *The Harvest*

We applied our method to investigate the glue used in the ground and size layers of *The Harvest* painted in 1882 by Camille Pissarro (Fig. 2a). Samples were taken from two separate locations (#1 and #2) of the tacking margin of the canvas (Fig. 2b). The samples were

Table 2 Species identified for commercial glue products

Sample	Species declared	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	Identified species
A	Cattle	+	−	−	+	−	−	−	−	−	−	+	−	Cattle
B	Cattle	+	+	−	+	−	−	−	+	−	+	+	+	N.D.
C	Cattle	+	−	−	+	−	−	−	−	+	−	+	−	Cattle, rabbit
D	Cattle	+	−	−	+	−	−	−	−	−	−	+	−	Cattle
E	Cattle	+	−	−	+	−	−	−	−	−	−	+	−	Cattle
F	Cattle	+	−	−	+	−	+	−	+	−	+	+	+	N.D.
G	Cattle	+	−	−	+	−	−	−	−	−	−	+	−	Cattle
H	Cattle	+	−	−	+	−	−	−	−	−	−	+	−	Cattle
I	Deer	+	−	−	+	−	−	−	−	−	−	+	−	Cattle
J	Rabbit	+	−	−	−	−	−	−	+	−	+	−	+	Pig
K	Rabbit	+	−	−	+	−	−	−	−	+	−	+	−	Cattle, rabbit
L	Rabbit	+	+	−	+	−	−	−	−	−	−	+	−	N.D.
M	Rabbit	+	−	−	+	−	−	−	−	−	−	+	−	Cattle
N	Rabbit	+	−	−	+	−	−	−	−	−	−	+	−	Cattle
O	Rabbit	+	−	−	+	−	−	−	−	−	−	+	−	Cattle
P	Rabbit	+	−	−	+	−	−	−	+	+	−	+	−	N.D.
Q	Sturgeon	−	−	+	−	−	−	+	−	−	−	−	−	Sturgeon
R	Sturgeon	−	−	+	−	−	−	+	−	−	−	−	−	Sturgeon
S	None	−	+	−	+	+	−	−	−	−	−	−	+	Sheep, goat
T	None	+	−	−	−	−	−	−	+	−	+	−	+	Pig
U	None	+	−	−	+	−	−	−	−	−	−	+	−	Cattle
V	None	+	−	−	+	−	−	−	−	−	−	+	−	Cattle

N.D. not determined

Table 3 Relative abundance of the animal origin in commercial glues containing cattle and rabbit origins

Sample	Species declared	Identified species	Content (%)
C	Cattle	Cattle	90.1 ± 1.4
		Rabbit	9.9 ± 1.4
K	Rabbit	Cattle	55.0 ± 2.5
		Rabbit	45.0 ± 2.5

The data represent the mean ± SD of three separate measurements

directly subjected to trypsin digestion for species identification by MRM analysis. As shown in Fig. 3, collagen marker peptides were clearly detected for location #1. Based on the detection pattern of the marker peptides (P1, P2, P4, P5, P11, and P12), the glue was determined to be derived from two animal species, cattle and sheep (Table 4). Location #2 also showed the same detection pattern (Additional file 3: Figure S1). By estimating the relative abundance of cattle and sheep origins using specific marker peptides P5 for sheep and P11 for cattle, both samples from the two different locations showed similar results; the glues were composed of approximately 65% cattle and 35% sheep (Table 4). Scanning electron microscopy equipped with an energy dispersive X-ray spectroscope revealed that the ground layer of *The Harvest* had a two-layer structure (Additional file 4: Figure S2). Our results indicate that cattle and sheep glues were used for these two layers and also for the size layer(s) located under the ground layers.

Cleavage of peptide bonds of the collagen molecule may occur during the heat extraction process of glue from animal tissues [2]. In addition, during storage, glue present in artworks possibly undergoes various chemical reactions, such as peptide bond cleavage and deamidation of asparagine and glutamine residues [28, 29]. Modified marker peptides cannot be detected by MRM analysis because of the mass shifts caused by these changes. Despite the low sample amount taken from *The Harvest* (approximately 1 mg including fibers of the canvas), identification of the animal source of glue was successfully achieved using collagen-derived marker peptides. This indicates that although protein damage might have occurred during aging, the remaining intact peptides were sufficient to allow species identification of glue for the historical artwork painted more than 100 years ago. Although we cannot rule out the possibility that partial degradation of glue affects the accuracy of quantification of the relative abundance of the animal origin, the similar quantitative values for the different two locations

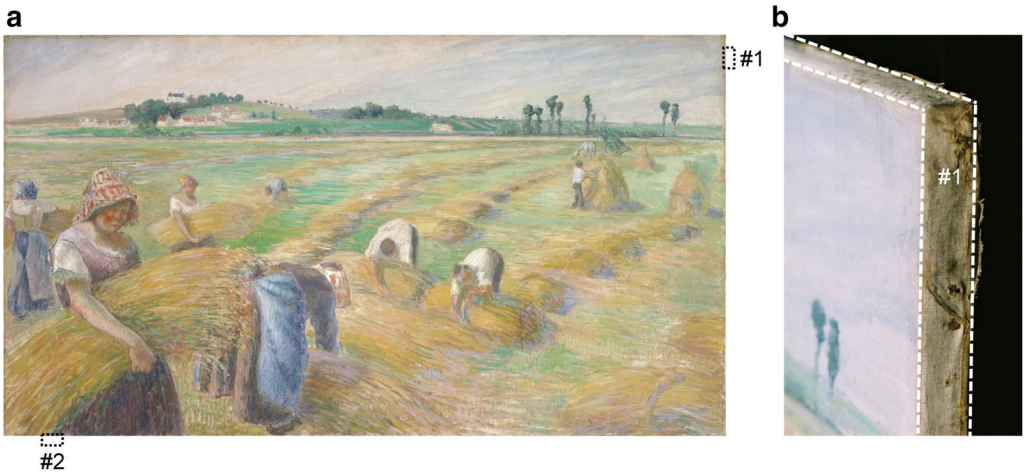

Fig. 2 *The Harvest*. **a** Small samples from the tacking margin of the canvas were analyzed for species identification of glue. The sampling locations are indicated by dotted boxes (#1 and #2). **b** The tacking margin of the canvas is enclosed by white dotted lines

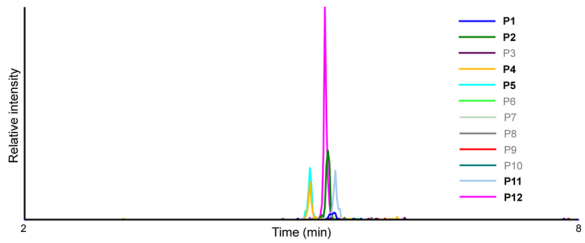

Fig. 3 MRM chromatograms of marker peptides detected for location #1 of *The Harvest*. Detected marker peptides are indicated in boldface

from the painting enhanced the reliability of the analysis (Table 4).

Conclusion

In the present study, we established a novel LC–MS method for animal source identification of glue using detection patterns of 12 type I collagen-derived marker peptides. This database-independent approach enables discrimination between eight animals regardless of whether their type I collagen sequences are known. In addition, our method is applicable not only to simple glue samples but also to samples containing two animal origins. We demonstrated its application in glue certification by detecting undeclared animal species and blends or contamination of different animal origins in commercial glue products. Furthermore, we clarified that both cattle- and sheep-originated animal glues were used in the ground and size layers of *The Harvest*. Many studies have investigated glues present in artworks and identified their animal origins using MS [15–19]. However, only one glue species was determined for each sample in almost all of the investigations, except for the simultaneous detection of bovine- and fish-specific peptides in a polychromy sample [18]. Our novel method may uncover hidden facts about glue used for artworks, which will be helpful to understand the use of artist's materials in historic objects. We could not determine the animal source of four commercial products. These were assumed to be derived from more than three animals or other unexpected animals. Species identification under these situations will be an area of future work. We consider that reselection of marker peptides in response to the target animals should lead to successful determination of the animal source for such complicated glue samples.

Table 4 Glue species identification and determination of relative abundance of the animal origin in *The Harvest*

	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	Identified species	Content (%)
#1	+	+	−	+	+	−	−	−	−	−	+	+	Cattle	65.3
													Sheep	34.7
#2	+	+	−	+	+	−	−	−	−	−	+	+	Cattle	62.4
													Sheep	37.6

Additional files

Additional file 1: Table S1. MRM transitions of marker peptides.

Additional file 2: Table S2. Detection patterns of marker peptides for glue samples with two different animal origins.

Additional file 3: Figure S1. MRM chromatograms of marker peptides detected for location #2 of *The Harvest*. Detected marker peptides are indicated in boldface.

Additional file 4: Figure S2. Cross-section of *The Harvest*. (a) The scanning electron microscopy (SEM) image shows the ground layer consisting of two layers under a paint layer. The upper ground layer contained lead white and calcium carbonate, and the bottom ground layer contained calcium sulfate only, or calcium sulfate and calcium carbonate. The sample was examined by SEM (JSM-5800LV; JEOL, Tokyo, Japan) equipped with an energy dispersive X-ray spectroscope (DX4; EDAX, Mahwah, NJ). (b) Schematic illustration of the cross-section.

Abbreviations

Hyp: hydroxyproline; MRM: multiple reaction monitoring; SI-collagen: stable isotope-labeled collagen.

Authors' contributions

YK and YT designed and developed the method. YK performed the experiments. MT provided the glue/painting samples and historical background. YK, YT, and MT wrote the manuscript. All authors analyzed the data. All authors read and approved the final manuscript.

Author details

[1] Japan Institute of Leather Research, 520-11 Kuwabara, Toride, Ibaraki 302-0017, Japan. [2] Nippi Research Institute of Biomatrix, 520-11 Kuwabara, Toride, Ibaraki 302-0017, Japan. [3] The National Museum of Western Art, 7-7 Ueno-Koen, Taito-ku, Tokyo 110-0007, Japan.

Competing interests

The authors declare that they have no competing interests.

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