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Never boring: Non-invasive palaeoproteomics of mummified human skin

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ABSTRACT

The scientific analysis of mummified individuals can reveal important details on the way people lived and died in the past. Palaeoproteomic approaches are theoretically suitable for obtaining information on the extent of tissue preservation, on the use of protein-based substances for embalming and/or restoration, as well as for characterising the microbiota from both the individual and the environment. However, these analyses usually require the destruction of a sample of tissue, a practice which is (obviously) discouraged by most museums. Unfortunately, this means that in-depth studies, for example by taking multiple samples from each individual, are seldom feasible. Here we show that a non-invasive sampling technique, based on mixed-bed chromatographic media embedded on ethylene vinyl acetate membranes (EVA), which had previously been used exclusively on historical material, was successful in extracting ancient proteins from the surface of Egyptian mummies. We tested the method on a decontextualised fragment of skin and assessed the endogeneity of its metaproteome by comparison with a procedural blank. Furthermore, we retrieved and authenticated sequences of human collagen and keratin, as well as potential bacterial/fungal biodeteriogens, from the mummy of a young woman (Supp. 16747 of the Museum of Anthropology and Ethnography of the University of Turin) who lived and died during the Old Kingdom of Egypt.

1. Introduction

Molecular anthropology began with the cloning of DNA from the most iconic of immortal bodies: Egyptian mummies (Pääbo, 1985). Indeed, the possibility of being able to routinely access and retrieve genetic information encoded and preserved in mummified tissues is of tremendous scientific significance. Thirty-five years later, these great expectations have had to be downsized, due to both poor preservation and contamination issues (Gitschier, 2008; Schuenemann et al., 2017). Ancient proteins, with their overall better preservation potential (e.g. Demarchi et al., 2016), are an ideal alternative to DNA, and could in fact yield information on the extent of molecular preservation, for example aiding to focus palaeogenomic investigations; reveal the presence of protein-based substances (e.g. glues) potentially related to restoration or funerary practices; give precious hints on the cause of death or on potential diseases affecting the individual.

A few studies have described the recovery of protein sequences from mummified tissues. Among these, four used shotgun proteomics by mass

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spectrometry (an approach currently referred to as "palaeoproteomics"): Corthals et al. (2012) detected proteins from the immune response in the proteome extracted from buccal swabs of one of the Llullaillaco children, sacrificed by Inca people 500 years ago; Maixner et al. (2013) characterised the brain proteome of Ötzi (the "ice mummy") and found, among others, sequences specific to brain tissue and wound healing; Jones et al. (2016) similarly found evidence of inflammation and immune response, this time from a much older set of individuals, dating to the First Intermediate Period of Egyptian history, around 4000 years ago. Their work is especially interesting in that these specimens had obviously undergone severe degradation in a hot environment. A fourth study recovered protein sequences from mummified skins of different ages and from different sites, focusing on evaluating the molecular damage (Cappellini et al., 2012). These studies exemplify the main research areas which would greatly benefit from the routine application of palaeoproteomics to mummies: the recovery of information on the lifeways and deathways of ancient people, including ancient diseases and the health of the individual (similarly to studies of ancient oral microbiomes, e.g. Warinner et al., 2014), and the assessment of the preservation of the mummified body. The latter includes a range of conservation-related issues, from controlling the potential growth of microbial deteriogens to reconstructing the history of restoration interventions.

The number of palaeoproteomic publications on mummified bodies is in contrast with these promising beginnings and with the relative abundance of mummies in museums worldwide. The main reason for this being that destructive sampling is strictly regulated by museums and/or national legislation. In Italy, for example, the 2004 Code of Cultural Heritage and Landscape defines "conservation" as "any activity which aims to maintain the *integrity*, identity and function" of a cultural heritage object (including human remains). The number of individuals sampled is therefore low, and multiple samples cannot usually be taken from each body.

Here we report the successful application of a non-invasive protein extraction technique, tagged under the acronym EVA (ethylene vinyl acetate studded with strong cation and anion exchangers as well as with C8 and C18 resins), for the extraction of the surface proteome from 4000-year-old mummified skin. The EVA method was used, in its first application, to harvest and identify morphine in the pages of the famous novel *Maestro y Margarita* by Bulgakov (Zilberstein et al., 2016). In subsequent investigations, though, it was used to extract and identify proteins on the surface of paintings (Barberis et al., 2019), as well as on original manuscripts of famous authors and on the clothes worn by them. This was the case for the detection of the renal pathology of Bulgakov (Zilberstein et al., 2017), of *Yersinia pestis* and other infectious agents in bouts of plague (D'Amato et al., 2018a), of Chekhov's tuberculosis (D'Amato et al., 2018b), and of the gonorrhoea bacillus in Casanova memoirs (Zilberstein et al., 2019). In two papers dealing with the physico-chemical properties of the EVA films, it has been shown that this methodology is non-invasive and does not damage the surface of the artifacts (Barberis et al., 2018; Manfredi et al., 2017). Although the results discussed above appear to be promising, the extraction technique has been applied mostly to items a few centuries old.

There are several instances in which such a non-invasive sampling technique could be useful for the study of mummified specimens: to perform protein-based taxonomic identification of mummies of unknown species; to compare the extent of preservation between different areas of the same organism/body; to discover whether proteinaceous substances had been applied as a surface treatment; to characterise the skin microbiota for studying ancient diseases as well as the action of potential biodeteriogens. For example, Samadelli et al. (2019) recently carried out an extensive non-invasive sampling campaign on a range of organic materials (including mummies) held at the Egyptian Museum of Turin; they used contact agar plates followed by DNA barcoding on the mycelia in order to characterise the fungal communities. We stress that a single EVA sampling could similarly gather information on the presence of bacteria, fungi and other microbes (provided that the sequences are present in the main repositories, e.g. NCBI), and, at the same time, yield protein data from the organism itself. The sequences recovered could also be used to distinguish endogenous proteins (typically those constituting the tissues of the human or animal mummy) from other proteinaceous substances applied to the surface, for example glues applied as part of historical restoration interventions, as these should yield a different collagen signature, provided the most informative parts of the sequence can be recovered. The same approach could also, theoretically, be used to detect the presence of proteinaceous media applied in antiquity; to our knowledge this has never been studied systematically. Overall, EVA sampling would enable comparative studies on multiple samples and individuals, which is important for both conservation and archaeology, and could also be used to guide targeted micro-sampling in order to address specific questions.

One disadvantage of such a "superficial" sampling approach is the trace abundance of "exogenous" proteins relative to the constituents of the bulk sample. However, high-sensitivity liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) techniques, typically used for palaeoproteomics, are able to detect low-abundance proteins and peptides, as demonstrated by the growing number of applications to challenging (very degraded) samples millions of years old (Cappellini et al., 2019; Demarchi et al., 2016; Welker et al., 2019). Furthermore, the taxonomic complexity of the signal recovered from the surface, and the extent of protein sequence degradation (as the molecules are not protected by e.g. a mineral matrix), require extra caution in assessing, authenticating and interpreting the datasets obtained. For example, bioinformatic analyses, by which tandem mass spectra are matched to putative peptide sequences contained in a reference database, should include all possible diagenesis-induced modifications (e.g. deamidation, oxidation, dehydration: see Hendy et al., 2018; Mackie et al., 2018; Mikšík et al., 2016).

The aim of the present work was to assess the suitability of the EVA extraction method for recovering surface proteomes from several-thousand-year-old mummified Ancient Egyptians. We expected to find one or more of the following: (i) keratin, collagen, elastin, i.e. the main skin constituents; (ii) any possible protein related to pathogens or immune response to infection; (iii) any protein-based substance related to either embalming or restoration; (iv) the environmental signal (including biodeteriogens). In order to evaluate the sequences recovered, that is to distinguish between ancient proteins and modern contaminants, we used well-established authentication approaches (i.e. calculation of the extent of glutamine and asparagine deamidation: Geiger and Clarke, 1987; Robinson and Robinson, 2001; Wilson et al., 2012).

2. Materials and methods

2.1. Samples

Three samples were analysed for this pilot study: one procedural blank (EVA_BK) and two fragments of mummified tissue "EVA_C" and "EVA_M16747".

First we tested the efficacy of the method on a decontextualised Egyptian mummified skin (scalp; sample "EVA_C″), part of a teaching reference collection housed at the Department of Life Sciences and Systems Biology (University of Turin), which had already shown the presence of collagen fibres by histological analyses conducted in the 1990s (Boano et al., 1999). This fragment was used to test the suitability of the method for recovering protein sequences and to assess whether the contact between the EVA membrane caused visible damage to the skin. Additionally, we performed a blank extraction of the EVA membrane alongside this sample ("EVA_BK").

Having satisfied these requirements, we proceeded with the sampling of individual Supp. 16747, a mummified young woman from Gebelein (Egypt), dated to 2407-2199 cal BC and currently held at the

Museum of Anthropology and Ethnography (MAET), University of Turin (Fig. 1). This mummy became part of the collections of the MAET in 1926 and its location changed at least three times in the subsequent twenty years: in 1936 it was moved from Palazzo Carignano to the building of the former San Giovanni Hospital (still in Turin), relocated to Fossano (a small town near Turin) during World War II, and brought back to the city in 1945. It is likely that during these movements the mummy had been contained in the original wooden case from the Italian Archaeological Mission in Egypt. In 1975 it was transferred to a glass and metal case, flushed with nitrogen and shown to the public for the first time. Between 1984 and 2017 the mummy remained in this case but was transferred to the storage rooms of the Museum (controlled environmental parameters: ~17 °C, 50% relative humidity). In 2017, the mummy was moved to the Conservation Centre "La Venaria Reale" (Venaria, Italy) in order to begin its restoration. Sampling for this study took place in 2018; a fragment of the scalp (Fig. 1), which was already detached, was sampled with the authorization of the *Soprintendenza*. The scalp was selected because this area was partially covered by bandages and we therefore hoped to minimise environmental contamination. In this pilot study we were fortunate in that we had access to detached skin samples, on which we could test the EVA approach, performing the extraction in the laboratory; however, we stress that the EVA membrane could have been applied directly to the skin surface *in situ*.

Fig. 1. Supp. 16747, "The young woman with the pleated dress". Top: view from the back of the skull, showing the hair arranged in a small pleat. Bottom, detail of the skull, scalp and sampling point.

2.2. Extraction

Small squares of EVA membrane (0.5 by 0.5 cm approximately) were cut, rinsed and then rehydrated in HPLC-grade water for a minimum of 1 h. Two squares were put in contact with skin fragments EVA_C and EVA_M16747 and left for 45 min. Contact was ensured by positioning a small weight on top of the membrane-sample pairs, which had been wrapped in clean aluminium foil. It is however possible to apply the membrane directly to the surface of the body and exert a small amount of pressure in order to avoid dislocation of the EVA square. After contact with the membrane, the skin fragments were left to air-dry in a laminar flow cabinet. Once dry, they did not show any alteration of the surface (assessed by macroscopic observation coupled to visualisation using a stereomicroscope). One square, the procedural blank EVA_BK, was transferred directly to a clean Lo-Bind eppendorf tube after rehydration.

Each EVA sample, i.e. EVA_C, EVA_M16747 and EVA_BK, was placed in clean Lo-Bind eppendorfs and the extracted proteins sequentially eluted using ammonium hydroxide (1M aqueous solution, Sigma), 1% aqueous solution of formic acid (Sigma) and 100% acetonitrile (Sigma) as per published protocols (Manfredi et al., 2017). The solutions were dried in an evaporator or air-dried in a laminar flow cabinet, and the extracts reconstituted in 50 mM ammonium bicarbonate. Alkylation and reduction steps were performed using DL-Dithiothreitol (Sigma) and iodoacetamide (Sigma) and were followed by trypsin digestion overnight and solid-phase extraction (C18 zip-tip) according to usual palaeoproteomic protocols (Hendy et al., 2018).

2.3. Liquid chromatography-tandem mass spectrometry

LC-MS/MS analyses were performed at the Open Access Mass Spectrometry Lab of the University of Turin. Dried peptides were resuspended in 0.1% formic acid and analysed using an Ultimate 3000 Dionex nanoHPLC instrument coupled with an Orbitrap Fusion (Thermo Scientific, Milan, Italy). Separation was achieved using a PepMap RSLC C18, 2 µm, 100 Å, 75 µm × 50 cm column (Thermo Scientific) and an Acclaim PepMap100 C18, 5 µm, 100 Å, 300 µm i.d. × 5 mm, preconcentration column (Thermo Scientific). The eluent used for the preconcentration step was 0.05% trifluoroacetic acid in water/acetonitrile 98/2 and the flow rate was 5 µL/min. The eluents used for chromatographic separation were 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile/water 8/2 (solvent B) in a program which was initially isocratic at 95:5 (A:B %) for 5 min, increased to 75:25 in 55 min, run up to 60:40 in 6 min, and to 10:90 in 5 min. Recondition time was 20 min. The injection volume was 1 µL and the flow rate 300 nL min^{-1}. The nanocolumn was provided with the ESI source. The mass spectrometry parameters were: positive spray voltage 2300 (V), sweep gas 1 (Arb) and ion transfer tube temperature 275 °C. Full scan spectra were acquired in the range of m/z 375–1500 (resolution 120,000 at m/z 200). MS^n spectra in data-dependent analysis mode were acquired in the range between the ion trap cut-off and precursor ion m/z values. HCD collision energy was fixed at 28%, orbitrap resolution 50,000 and the isolation window was 1.6 m/z units. Water (wash) blanks were interspersed between the analyses of the samples.

2.4. Data analysis

Raw tandem mass spectrometry data were analysed using the software program PEAKS Studio v. 8.5 (Ma et al., 2003). Tandem mass spectra were searched against the Uniprot_SwissProt database (downloaded 19/07/2019) including in the search all common contaminants (cRAP). Mass tolerance was set to ±15 ppm on the precursor and 0.05 Da on the fragments. The thresholds for peptide and protein identification were set as follows: protein false discovery rate (FDR) = 0.5%, protein score −10lgP ≥ 20, unique peptides ≥ 2, de novo sequences scores (ALC %) ≥ 80. All the mass spectrometry proteomic data have been deposited in the ProteomeXchange Consortium (http://proteomecentral.proteo

mexchange.org) via the PRIDE partner repository (Perez-Riverol et al., 2019) with the data set identifier PXD015572.

Metaproteomics using the online platform Unipept (Mesuere et al., 2015) was conducted on all peptide sequences assigned to a protein (excluding proteases), as well as all unassigned and de novo peptides. The extent of glutamine and asparagine deamidation was assessed using a VBA macro customised for this study in order to calculate site-specific deamidation.

3. Results

3.1. Procedural blank

In order to evaluate the typical environmental, preparation- and analysis-induced contamination, we carried out a blank extraction of the EVA membrane and performed LC-MS/MS. The proteins identified (excluding trypsin) are reported in Table 1: keratins and other skin-specific proteins (e.g. dermcidin, collagen), common laboratory contaminants (albumin), as well as conserved sequences of enzymes (lactate dehydrogenase, acrosin) and glycoproteins (fibrinogen) are present.

Metaproteomic analysis on the Unipept platform (Fig. 2) yielded a match for 173 of the 284 unique peptide sequences (~60%). Each peptide sequence that could be matched to a non-Metazoan (n = 9; Supplementary Table S1) was checked individually for its occurrence in the deamidated form, as Asn/Gln deamidation is the typical indicator for evaluating the extent of degradation (dependent mainly on age and burial/storage temperature) and therefore the authenticity of a peptide. As expected, none of the sequences were found to be significantly deamidated. The peptide sequences mostly matched with soil bacteria (Table S1 and references therein), generally compatible with environmental contamination due to the aerosolisation of microorganisms (Mirskaya and Agranovski, 2018).

3.2. EVA_C

This sample had originally been selected because of its documented collagen preservation, and indeed collagen and keratins were the only proteins identified using the thresholds applied in this study. Table 2 shows that collagen alpha-2(I) chain (CO1A2) was the top protein, followed by the alpha-1(I) chain (CO1A1), both from *Homo sapiens*. Table 2 also reports a range of collagen sequences from non-human organisms, which had been identified by bioinformatic searches against Uniprot. We include these sequences because we wish to highlight the importance of critically assessing palaeoproteomic data: in fact, the identification of collagen from non-human organisms could be taken to indicate the application of glue-like substances during funerary rituals or as part of conservation practices. However, the organisms identified (except cattle) are unlikely: *Toxodon* (extinct South American ungulate), *Orycteropus* (aardvark), *Mylodon* (extinct South American ground sloth), *Cynops* (Japanese fire belly newt). More importantly, an alignment of the collagen sequences showed that the peptides identified by the PEAKS software programme as uniquely belonging to these organisms were in fact the same as those identified for human collagen, except single residue differences, which were often the isobaric I→ L (isoleucine → leucine) or were weakly supported by the fragmentation spectra. Therefore, we attribute this to bioinformatics bias and will focus the discussion on human type I collagen sequences.

Other types of collagen were also identified: type III and type V (both human, typically found associated with type I collagen and generally localised in hollow organs and skin) and type II (typically found in cartilage). The potential bovine origin of collagen type II is interesting as it could be due to the use of cartilage-based glues, but in fact all putative unique peptides are highly conserved (NCBI BLASTp searches yielded multiple matches with 100% identity). The attribution to *Bos* can therefore also be considered an artefact of the bioinformatic analysis.

While establishing the taxonomic origin of the collagen sequences can be challenging, especially for low-coverage samples, their authenticity in this study was strongly supported by the extent of deamidation of both Q (Gln) and N (Asn) residues, calculated as 75% (Q) and 79% (N) for CO1A1 and 85% (Q) and 87% (N) for CO1A2 (Supplementary Information 1, "Deamidation_EVA_C" sheet). On the contrary, the other group of protein detected, keratins, showed no deamidation, as would be expected from a modern contaminant.

The metaproteomics analysis, conducted on 1195 peptides, was successful for ~26% of the sequences. The overall composition is dominated by Chordata, but the bacterial component is more varied than that observed for the blank sample (Fig. 3). We manually checked all non-Metazoan sequences in order to verify their modern or potentially ancient origin. In all cases in which the peptides contained an Asn or Gln residue, they were invariably undeamidated (Supplementary Table S2). Therefore, the bacterial and fungal component of the metaproteome is most likely simply due to handling and airborne contamination. In particular, some sequences were attributed to fungi and bacteria known for their degradative functions on organic materials (e.g. (hemi-)cellulolytic, proteolytic; Table S2 and references therein), accounting for the modern presence of a biodeteriogenic community on the sample.

3.3. EVA_M16747: the young woman with the pleated dress

The main proteins detected were, also in this instance, collagen and keratin (Table 3). Both protein types were supported by high numbers (>100) of high-scoring peptides and therefore the protein recovery can be considered good. The wash blank analysed prior to this sample contained <10 collagen peptides.

Collagen types I, III and V were recovered, and, in the case of type I,

Table 1
Proteins identified in a blank extract. Proteins highlighted with an asterisk preceding the identifier were also detected in the wash blank analysed prior to this analysis.

Accession	−10lgP	Coverage (%)	#Peptides	#Unique	#Spectra	Description
K2C1_HUMAN|	160.64	52	40	6	583	Keratin, sp|K2C1_HUMAN|
K1C9_HUMAN|	159.8	68	39	38	515	Keratin, sp|K1C9_HUMAN|
K22E_HUMAN|	141.89	55	30	19	307	Keratin, sp|K22E_HUMAN|
K1C10_HUMAN|	138.85	49	37	3	419	Keratin, sp|K1C10_HUMAN|
P02538|K2C6A_HUMAN	101.38	41	22	2	112	Keratin type II cytoskeletal 6A OS=*Homo sapiens* GN = KRT6A PE = 1 SV = 3
P02533|K1C14_HUMAN	85.73	31	14	5	113	Keratin type I cytoskeletal 14 OS=*Homo sapiens* GN = KRT14 PE = 1 SV = 4
P13647|K2C5_HUMAN	78.8	29	17	3	105	Keratin type II cytoskeletal 5 OS=*Homo sapiens* GN = KRT5 PE = 1 SV = 3
*ALBU_HUMAN|	46.05	6	4	4	15	Albumin, sp|ALBU_HUMAN|
*P02671|FIBA_HUMAN	40.28	2	3	3	16	Fibrinogen alpha chain OS=*Homo sapiens* GN = FGA PE = 1 SV = 2
Q9GL10|ACRO_SHEEP	40.12	4	3	2	5	Acrosin (Fragment) OS=*Ovis aries* GN = ACR PE = 1 SV = 1
*Q92055|LDHA_FUNHE	30	5	2	2	7	L-lactate dehydrogenase A chain OS=*Fundulus heteroclitus* GN = ldha PE = 2 SV = 3
P08123|CO1A2_HUMAN	25.06	2	2	2	7	Collagen alpha-2(I) chain OS=*Homo sapiens* GN = COL1A2 PE = 1 SV = 7
P81605|DCD_HUMAN	24.52	13	2	2	9	Dermcidin OS=*Homo sapiens* GN = DCD PE = 1 SV = 2

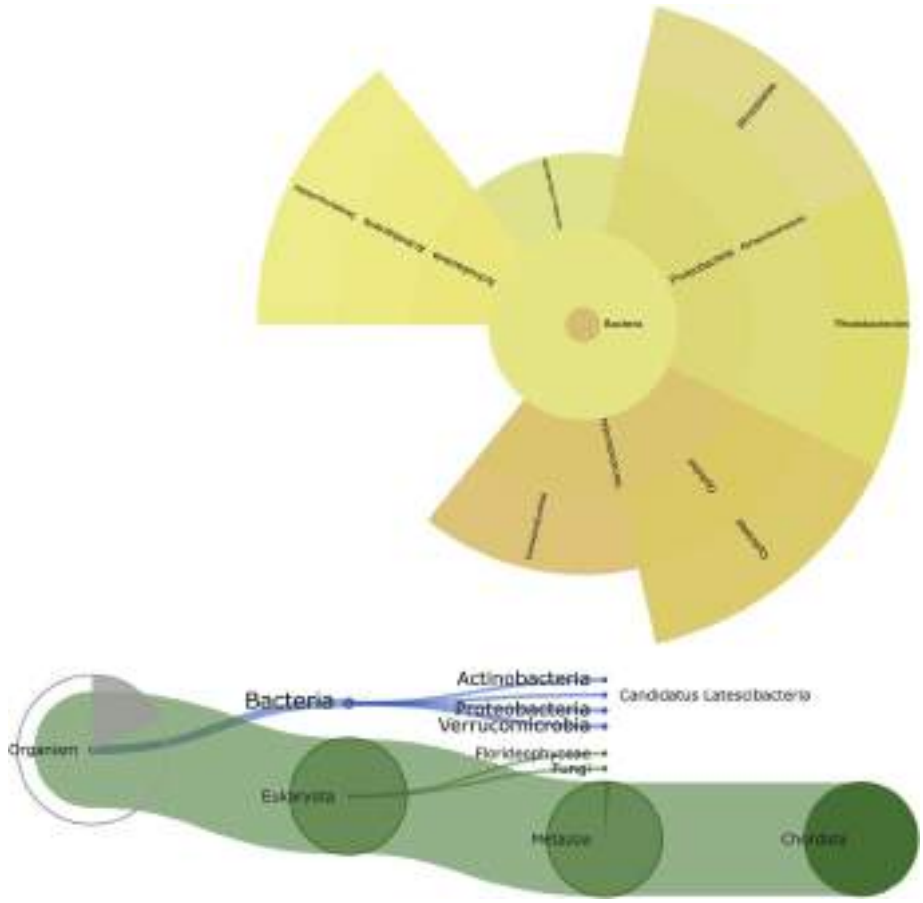

Fig. 2. The metaproteome of a blank extract. Note the dominance of Metazoa (i.e. skin proteins, Table 1) and the putative presence of a range of bacterial sequences.

Table 2
Proteins identified in the mummified human skin sample EVA_C. None of these were detected in the wash blank analysed prior to the sample.

Accession	−10lgP	Coverage (%)	#Peptides	#Unique	#Spectra	Description
P08123|CO1A2_HUMAN	184.47	51	257	72	1899	Collagen alpha-2(I) chain OS=*Homo sapiens* GN = COL1A2 PE = 1 SV = 7
P02452|CO1A1_HUMAN	164.52	50	233	19	1660	Collagen alpha-1(I) chain OS=*Homo sapiens* GN = COL1A1 PE = 1 SV = 5
P02453|CO1A1_BOVIN	158.59	46	189	2	1425	Collagen alpha-1(I) chain OS=*Bos taurus* GN = COL1A1 PE = 1 SV = 3
C0HJP7|CO1A1_TOXSP	153.53	55	162	5	1273	Collagen alpha-1(I) chain (Fragments) OS = *Toxodon* sp. GN = COL1A1 PE = 1 SV = 1
C0HJN3|CO1A1_ORYAF	146.25	53	141	5	996	Collagen alpha-1(I) chain (Fragments) OS=*Orycteropus afer* GN = COL1A1 PE = 1 SV = 1
C0HJP3|CO1A1_MYLDA	142.09	57	129	12	1194	Collagen alpha-1(I) chain (Fragments) OS = *Mylodon darwinii* GN = COL1A1 PE = 1 SV = 1
K2C1_HUMAN|	114.59	40	26	4	218	Keratin, sp|K2C1_HUMAN|
P02461|CO3A1_HUMAN	114.29	22	69	65	381	Collagen alpha-1(III) chain OS=*Homo sapiens* GN = COL3A1 PE = 1 SV = 4
K1C9_HUMAN|	111.08	60	28	27	129	Keratin, sp|K1C9_HUMAN|
K22E_HUMAN|	88.06	35	19	12	101	Keratin, sp|K22E_HUMAN|
Q9YIB4|CO1A1_CYNPY	74.7	11	29	6	186	Collagen alpha-1(I) chain OS=*Cynops pyrrhogaster* GN = COL1A1 PE = 2 SV = 1
P02459|CO2A1_BOVIN	73.53	7	25	6	243	Collagen alpha-1(II) chain OS=*Bos taurus* GN = COL2A1 PE = 1 SV = 4
P02533|K1C14_HUMAN	53.34	21	9	2	36	Keratin type I cytoskeletal 14 OS=*Homo sapiens* GN = KRT14 PE = 1 SV = 4
P13647|K2C5_HUMAN	49.73	17	10	3	22	Keratin type II cytoskeletal 5 OS=*Homo sapiens* GN = KRT5 PE = 1 SV = 3
P05997|CO5A2_HUMAN	40.49	2	4	4	14	Collagen alpha-2(V) chain OS=*Homo sapiens* GN = COL5A2 PE = 1 SV = 3
P20908|CO5A1_HUMAN	26.05	3	5	4	16	Collagen alpha-1(V) chain OS=*Homo sapiens* GN = COL5A1 PE = 1 SV = 3

both alpha-1 and alpha-2 chains could be identified. The taxonomy of COL1A1 was again not entirely straightforward, but non-human collagen sequences were assigned to "unlikely" organisms, as seen in sample EVA_C.

Unipept metaproteomic searches conducted on the peptidome (4345 sequences, excluding those pertaining to proteases) of the sample of

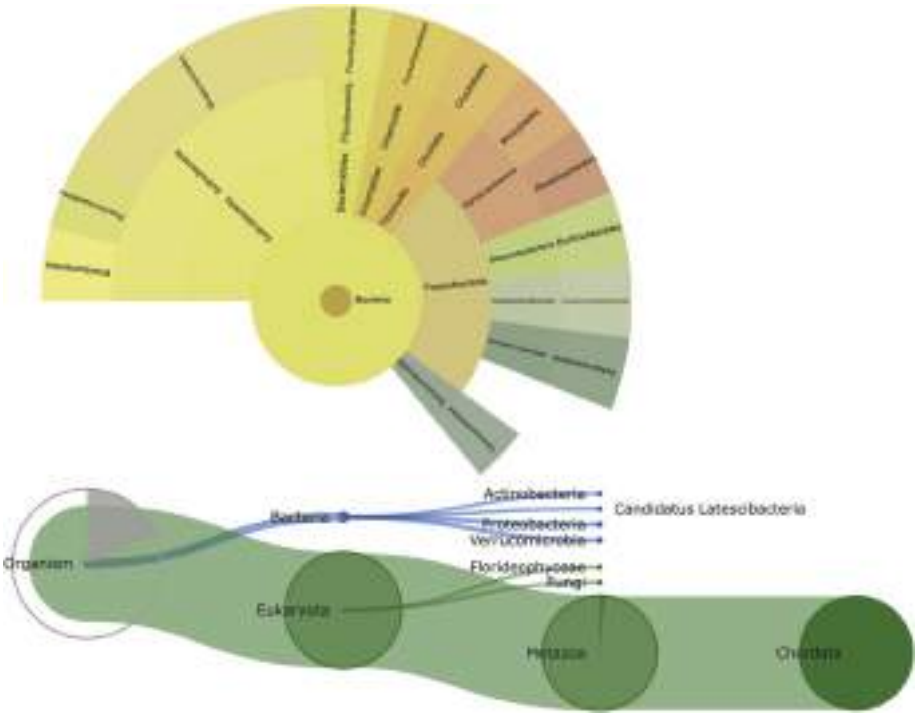

Fig. 3. The metaproteome of mummified human skin sample EVA_C.

Table 3
Proteins identified in the mummified human skin sample of the young woman with the pleated dress, EVA_M16747.

Accession	−10lgP	Coverage (%)	#Peptides	#Unique	#Spectra	Description
P02452|CO1A1_HUMAN	170.02	57	310	23	2206	Collagen alpha-1(I) chain OS=*Homo sapiens* GN = COL1A1 PE = 1 SV = 5
P08123|CO1A2_HUMAN	161.4	58	271	98	1871	Collagen alpha-2(I) chain OS=*Homo sapiens* GN = COL1A2 PE = 1 SV = 7
C0HJP7|CO1A1_TOXSP	156.39	68	226	5	1570	Collagen alpha-1(I) chain (Fragments) OS = *Toxodon* sp. GN = COL1A1 PE = 1 SV = 1
C0HJP3|CO1A1_MYLDA	147.11	57	144	7	1263	Collagen alpha-1(I) chain (Fragments) OS = *Mylodon darwinii* GN = COL1A1 PE = 1 SV = 1
P02454|CO1A1_RAT	133.42	44	193	2	1343	Collagen alpha-1(I) chain OS=*Rattus norvegicus* GN = Col1a1 PE = 1 SV = 5
KRHB6_HUMAN|	96.75	60	48	3	229	Keratin, sp|KRHB6_HUMAN|
KRHB1_HUMAN|	89.98	54	47	4	218	Keratin, sp|KRHB1_HUMAN|
P02461|CO3A1_HUMAN	88.23	25	52	25	245	Collagen alpha-1(III) chain OS=*Homo sapiens* GN = COL3A1 PE = 1 SV = 4
K1H1_HUMAN|	74.9	51	32	21	159	Keratin, sp|K1H1_HUMAN|
P04264|K2C1_HUMAN	58.56	17	9	9	32	Keratin type II cytoskeletal 1 OS=*Homo sapiens* GN = KRT1 PE = 1 SV = 6
Q9BYP9|KRA99_HUMAN	48.86	28	9	9	22	Keratin-associated protein 9-9 OS=*Homo sapiens* GN = KRTAP9-9 PE = 2 SV = 1
Q9BYR7|KRA32_HUMAN	33.83	24	2	2	14	Keratin-associated protein 3-2 OS=*Homo sapiens* GN = KRTAP3-2 PE = 1 SV = 1
K1C10_HUMAN|	31.85	12	10	9	39	Keratin, sp|K1C10_HUMAN|
Q9BYQ5|KRA46_HUMAN	27.93	23	9	7	21	Keratin-associated protein 4-6 OS=*Homo sapiens* GN = KRTAP4-6 PE = 2 SV = 4
Q62168|K1H2_MOUSE	27.31	6	5	3	28	Keratin type I cuticular Ha2 OS = *Mus musculus* GN = Krt32 PE = 1 SV = 2
Q9BYR4|KRA43_HUMAN	26.27	9	3	2	6	Keratin-associated protein 4-3 OS=*Homo sapiens* GN = KRTAP4-3 PE = 2 SV = 2
P20908|CO5A1_HUMAN	23.33	2	3	2	5	Collagen alpha-1(V) chain OS=*Homo sapiens* GN = COL5A1 PE = 1 SV = 3

scalp from the Egyptian mummy Supp. 16747 yielded a match for 431 out of 1888 unique sequences (~23%; Fig. 4). Of these, 61 unique sequences were assigned to non-Metazoan organisms (Supplementary Table S3). Each of these was checked manually in order to verify the frequency of its occurrence, as well as the evidence for deamidation. Most sequences could not be assessed on this basis as they did not contain Asn or Gln residues. Two sequences, QGPSGA and GEQGPA, were found to consistently occur in the deamidated form: this suggests a more ancient origin than that of the rest of the non-Metazoan sequences. These peptides were assigned by Unipept to a member of family Sphingomonadaceae (some members of this group of bacteria are able to degrade aromatic compounds; e.g. Kertesz and Kawasaki, 2010) and to *Escherichia coli*. The presence of bacteria able to degrade aromatics may be perfectly compatible with the presence of resin-like embalming substances, including some potentially used to arrange the hair of the girl (Fig. 1). Indeed, chemical investigations (by gas chromatography –

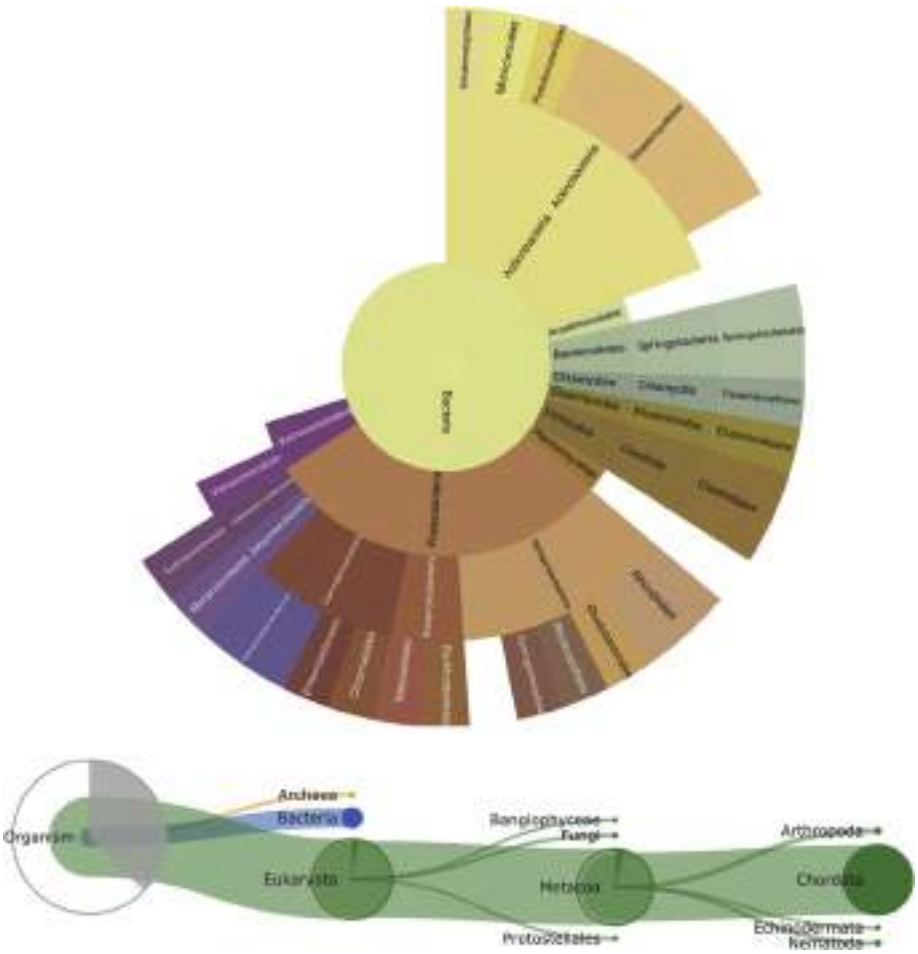

Fig. 4. The metaproteome of mummified human skin sample EVA_M16747, the young woman with the pleated dress.

mass spectrometry, see Supplementary Information 2) revealed the presence of a plant resin from *Pinaceae* on two detached fragments, respectively of skin (from a toe) and bandages (from the hand). *E. coli* is a common human pathogen and its occurrence on the scalp has been described in modern medical cases (Summers et al., 1965). However, both of these sequences (QGPSGA and GEQGPA) are in fact short and glycine- and proline-rich, which strongly suggests that these are simply fragments of collagen. A BLASTp search of the two short sequences yields hundreds of hits, mainly of non-Metazoans but also some Metazoans. Therefore, the taxonomy of these peptides cannot at present be unequivocally determined.

This analysis revealed the presence of a number of fungi and bacteria for which an enzymatic degradation activity towards (hemi-)cellulose -constituents of bandages- or proteins is reported (Table S3 and references therein). The presence of partially deamidated peptides assigned to *Streptomyces*, a genus of Actinobacteria frequently reported on organic cultural heritage materials (Sterflinger and Piñar, 2013), also isolated from mural paintings from ancient Egypt (Abdel-Haliem et al., 2013) and, more recently, from mummies (e.g. Kraková et al., 2018; Pinar et al., 2013), is especially interesting, as it could be ascribed to past microbial biodeterioration. Other sequences, either undeamidated or non characterised in terms of deamidation (N/A), were potentially associated with more recent biodeterioration, including those of bacteria which are usually symbiotically associated to deteriogenic animals, such as termites (*Elusimicrobium minutum*; Geissinger et al., 2009) and mice (*Acetatifavtor muris*; Lagkouvardos et al., 2016).

4. Discussion

The results of this pilot study demonstrate that it is possible to obtain metaproteomes from the surface of a mummified body in a simple, rapid and non-invasive way. We recovered authentic human collagen and keratin sequences, as well as information on fungi and bacteria, deriving from sample handling and environmental sources (e.g. bioaereosol). This is interesting as it helps us to reconstruct the conservation state and to identify potential target materials for biodeteriogens. We also showed that it is of the utmost importance to carry out a full assessment of the authenticity of each of the sequences recovered, especially for samples that have been heavily handled (like in our EVA_C sample, used for teaching), transferred between different locations and/or stored for a long time, which is what happened to the young woman with the pleated dress M16747 (see details in the "Materials and Methods" section).

Contamination introduced in the laboratory during sample preparation should be minimal; for example, all samples in this study were prepared in a dedicated palaeoproteomics laboratory, following the precautions listed by Hendy et al. (2018). However, LC-MS/MS analysis are often carried out in laboratories that routinely process and analyse a range of modern samples, including human tissues (blood, urine, etc), and this is also the case of the Turin Open Access Mass Spectrometry Lab. Therefore, it is important that analytical water blanks (washes) are analysed before and after each sample. In our study we found a limited amount of carryover and environmental contamination (all LC-MS/MS data available at proteomecentral.proteomexchange.org); while this is not problematic in the present case, as the number of peptide hits from the samples was high, it could prevent protein authentication if the samples are particularly protein-poor.

We assessed the authenticity of the sequences by estimating the extent of deamidation. This is a rather crude but usually effective measure of the extent of overall degradation - not necessarily of age (Schroeter and Cleland, 2016; Simpson et al., 2019, 2016; Welker et al., 2016). In our case, we obtained results that were internally consistent: in EVA_C (heavily handled) all collagens appeared to be "ancient" and all keratins "modern", while in EVA_M16747 (not heavily handled but with a century-long post-excavation history), all collagens and all keratins appeared "ancient". Crucially, the extent of deamidation of the potential bacterial/fungal sequences was generally low for all samples, indicating that the surface metaproteome is mostly of recent origin.

5. Conclusions

Overall, we showed that the analysis of protein sequences from mummified individuals can be carried out without impacting on the integrity of the bodies. This opens up the possibility of targeting a higher number of specimens and of conducting intensive sampling of each mummified body. As a consequence, we would be able to obtain valuable data for studying protein diagenesis and survival in extreme environments and as a result of different mummification practices; monitor the preservation of mummies at the molecular level, guiding targeted in-depth destructive studies (e.g. palaeogenomics); recover interesting biomolecular markers of disease as well as potential traces of embalming and/or restoration practices; characterise the biodeteriogens and their effect on the body, informing conservation strategies.

The EVA approach could be used to guide further micro-invasive sampling in order to answer specific questions, for example detection of vascular or metabolic proteins; however, the removal and analysis of bulk samples may not always guarantee the detection of low-abundance sequences, given that the ratio between these and structural proteins (collagen, keratin) would still be skewed in favour of the latter. Therefore, specific pre-treatments, such as filtering of high molecular weight proteins or using collagen-specific enzymes (Wadsworth and Buckley, 2014) would be necessary.

As always when dealing with ancient molecules, but especially so for specimens that are so close to us (i.e. compositionally similar) and with such a complex post-depositional history, it is necessary to be extremely cautious when assessing the authenticity and thus the significance of the data. Furthermore, the taxonomic assignment of peptides is not always straightforward; this is because some sequences are highly conserved (e.g. collagen), but also due to the relative paucity of non-Metazoan reference datasets in public sequence databases. The implementation of specific strategies for sequence validation in human mummies and for potentially discriminating between recent and "ancient" components of the metaproteome (using a wider range of diagenesis-induced modifications) will undoubtedly further increase the impact of palaeoproteomic studies on mummified tissues.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary Tables.

Table S1
Non-Metazoan peptides found by Unipept analysis in sample EVA_BK and, where available, their extent of deamidation.

Peptide	Organism	Taxonomy (https://www.ncbi.nlm.nih.gov/taxonomy)	*Potential deteriogenic activity/#Remarkable ecology	Deamidation?
TATTQAVR	Bacteria			0%
VVTVDLPR	*Candidatus* Latescibacteria	Bacteria	#* bacteria from terrestrial and water habitats, on the basis of metabolic reconstructions with marked capacities for the degradation of proteins, lipids, and polysaccharides (Farag et al., 2017)	N/A
QLSVSLPR	*Chondrus crispus*	Rhodophyta		0%
GSSPATEK	*Fusarium langsethiae*	Fungi, Ascomycota, Nectriaceae	plant pathogen (Thrane et al., 2004)	N/A
AVELSELNR	*Mesorhizobium*	Bacteria, Alphaproteobacteria	soil symbiotic bacteria (Kwon et al., 2005)	0%
LSNPATLR	Opitutaceae	Verrucomicrobia		50%
ESPTEPR	*Pseudovibrio denitrificans*	Bacteria, Alphaproteobacteria	bacterium capable of fermentation, e.g. from loamy soils and water (Gazdag et al., 2019)	N/A
LGELSELNR	*Streptomyces griseochromogenes*	Bacteria, Actonobacteria	#soil bacterium, also inhabiting pathogenic fungi (Wu et al., 2017) [See comments on the genus *Streptomyces* in Tables S2-S3]	0%
QVTVSLPR	Verrucomicrobia	Bacteria		0%

Table S2
Non-Metazoan peptides found by Unipept analysis in sample EVA_C and, where available, their extent of deamidation. Note the presence of glycine- and proline-rich peptides.

Peptide sequence	Organism	Taxonomy (https://www.ncbi.nlm.nih.gov/taxonomy)	*Potential deteriogenic activity/#Remarkable ecology	Deamidation?
VALGLEQR	*Aureimonas jatrophae*	Bacteria, Alphaproteobacteria	plant associated bacteria (Madhaiyan et al., 2013)	0%
APGLPGPR	Bacteria			N/A
AVAGAPGLR	Bacteria			N/A

(*continued on next page*)

Table S2 (*continued*)

Peptide sequence	Organism	Taxonomy (https://www.ncbi.nlm.nih.gov/taxonomy)	*Potential deteriogenic activity/#Remarkable ecology	Deamidation?
AVPGADGR	Bacteria			N/A
DQPWQR	Bacteria			0%
GHAGLAQR	Bacteria			0%
GPAGPER	Bacteria			N/A
LNAGLTQR	Bacteria			0%
TATTQAVR	Bacteria			0%
VVGLPGER	Bacteria			N/A
GPLGPAGAR	*Bifidobacterium thermacidophilum* subsp. *thermacidophilum*	Bacteria, Actinobacteria	# the genus includes bacteria isolated from human and animal faeces (Dong et al., 2000)	N/A
LATVSLPR	*Candidatus* Protochlamydia naegleriophila	Bacteria, Chlamidiae	#potential human pathogen (Casson et al., 2008)	N/A
LVSFVHNAL	*Clostridium*	Bacteria, Firmicutes	#potential human pathogen, contaminating skin (Bobulsky et al., 2008)	0%
QAGLPGPK	*Colletotrichum higginsianum*	Fungi, Ascomycota, Glomerellaceae	*plant pathogen producing pectinolytic, cellulolytic, emicellulolytic enzymes (O'Connell et al., 2012)	0%
GFASFLDK	Crocinitomicaceae	Bacteria, Bacteroidetes		N/A
QPGFSGPR	*Cystobacter ferrugineus*	Bacteria, Deltaproteobacteria	#already reported from historic documents contaminated with body fluids and putrefaction (Jurado et al., 2010)	0%
NSEGPER	Eukaryota			0%
LATVLSPR	Gammaproteobacteria			N/A
AVPGPAGPR	*Lentzea albidocapillata*	Bacteria, Actinobacteria	#human-associated bacteria (Yassin et al., 1995), also retrieved from the Iceman's untreated skin (Rollo et al., 2000)	N/A
LAATVSLPR	*Penicillium subrubescens*	Fungi, Ascomycota, Aspergillaceae	*(hemi-)cellulolytic fungus degrading lignocellulose (Mäkelä et al., 2016)	N/A
VVGLPANR	*Phycomyces blakesleeanus*	Fungi, Mucoromycota, Phycomycetaceae	#on heterogeneous substrates, including faeces of animals, such as mice (e.g. Camino et al., 2015)	0%
VVGLPGQR	Proteobacteria	Bacteria		0%
AFAGATQR	*Burkholderia pseudomallei* group	Bacteria, Betaproteobacteria	#potential human pathogens (Howe et al., 1971) [transmission also by skin contact; Abbink et al., 2001]	0%
GAPGPAQR	*Rhodovulum sulfidophilum*	Bacteria, Alphaproteobacteria	*e.g. proteolytic bacteria (Seangtumnor et al., 2018)	0%
GLAGPPQR	*Streptomyces*	Bacteria, Actinobacteria	*biodeteriogens of organic cultural heritage materials (e.g. proteolytic effect on parchment), also reported from wall-paintings of ancient Egypt (Abdel-Haliem et al., 2013) and isolated from mummies (e.g. Kraková et al., 2018)	0%
QPGPEGPR	*Streptomyces*	Bacteria, Actinobacteria	*see "*Streptomyces*" above	0%
VGLPGLDGR	*Streptomyces*	Bacteria, Actinobacteria	*see "*Streptomyces*" above	N/A
LGELSELNR	*Streptomyces griseochromogenes*	Bacteria, Actinobacteria	#soil bacterium, also inhabiting pathogenic fungi (Wu et al., 2017) [see also the above comments on *Streptomyces*]	0%
QVTVSLPR	Verrucomicrobiae	Bacteria		0%

Table S3

Non-Metazoan peptides found by Unipept analysis in sample EVA_M16747 and, where available, their extent of deamidation. Note the presence of glycine- and proline-rich peptides.

Peptide sequence	Organism	Taxonomy (https://www.ncbi.nlm.nih.gov/taxonomy)	*Potential deteriogenic activity/#Remarkable ecology	Deamidation?
GTLEELQEK	*Acetatifactor muris*	Bacteria, Firmicutes	#gut environment of rodents (e.g. Lagkouvardos et al., 2016)	Undeamidated, 1 peptide only
VGLGPEGR	Actinobacteria			N/A
GPPGAA	Alphaproteobacteria			When present in N/Q-containing sequences, these are deamidated
HSGLMGPR	Archaeoglobales	Archaea, Archaeoglobi		N/A
LGAADDFR	Armatimonadetes	Bacteria, Terrabacteria group		N/A
ADGVAGPK	Bacteria			N/A
GPSGE	Bacteria			When present in N/Q-containing sequences, these are deamidated
VAGPPGPR	Bacteria			N/A
VVGLPGER	Bacteria			When present in N/Q-containing sequences, these are undeamidated
AGYLETLR	Bacteria			N/A
LYEEEL	Bacteria			N/A
APGLPGPR	Bacteria			When present in N/Q-containing sequences, these are undeamidated
GVGPEQR	Bacteria			Undeamidated
WEPTAGR	Bacteria			N/A
APGEPVAR	Bacteria			N/A
TEPLSFR	Bacteria			N/A, 1 peptide only
GPAGPER	Bacteria			N/A
GVGLPEGR	Bacteria			N/A
HSGLPGPR	Bacteria			N/A

(*continued on next page*)

Table S3 (*continued*)

Peptide sequence	Organism	Taxonomy (https://www.ncbi.nlm.nih.gov/taxonomy)	*Potential deteriogenic activity/#Remarkable ecology	Deamidation?
LGYEEELR	*Campylobacter concisus*	Bacteria, Epsilonproteobacteria	#potential human pathogen (Newell, 2005)	N/A
LATVSLPR	*Candidatus* Protochlamydia naegleriophila	Bacteria, Chlamidiae	#potential human pathogen (Casson et al., 2008)	N/A
QAGLPGPK	*Colletotrichum higginsianum*	Fungi, Ascomycota, Glomerellaceae	*plant pathogen producing pectinolytic, cellulolytic, hemicellulolytic enzymes (O'Connell et al., 2012)	When present in N/Q-containing sequences, these are undeamidated
GPAQVGPR	*Cyanidioschyzon merolae*	Rhodophyta	#thermoacidophiles (Kuroiwa et al., 2018)	When present in N/Q-containing sequences, these are undeamidated
YEDEVALR	*Elusimicrobium minutum*	Bacteria, Elusimicrobia	*endosymbiont of termite gut flagellates (Geissinger et al., 2009)	N/A
QPGNAGR	*Enhydrobacter aerosaccus*	Bacteria, Alphaproteobacteria	#human-associated bacteria (hand surface: Park et al., 2017)	N/A
GEQGPA	*Escherichia coli*		#potential human pathogen (Petkovšek et al., 2009)	When present in N/Q-containing sequences, these are deamidated
VGGLPEGR	*Eubacterium*	Bacteria, Clostridia	#e.g. already retrieved from mummies (Ubaldi et al., 1998)	N/A
AEAESWYR	Eukaryota	–	–	N/A
LEGELDTYR	Eukaryota	–	–	N/A
LGGPPGPR	Eukaryota			N/A
NSEGPER	Eukaryota			Undeamidated
QPGLPSK	Eukaryota			Undeamidated
NPLGPPR	Eukaryota			Undeamidated, 1 peptide only
LATVLSPR	Gammaproteobacteria			N/A
NAAGLQAR	Gammaproteobacteria	Bacteria		Undeamidated, 1 peptide only
NPYGPMR	Halobacteriales	Archaea, Euriarchaeota		Undeamidated, 1 peptide only
AVPGPAGPR	*Lentzea albidocapillata*	Bacteria, Actinobacteria	#human-associated bacteria (Yassin et al., 1995), also retrieved from the Iceman's untreated skin (Rollo et al., 2000)	N/A
SVGPAGPR	*Mariprofundus aestuarium*	Bacteria, Zetaproteobacteria	*e.g. iron-oxidizing bacteria (Makita, 2018)	When present in N/Q-containing sequences, these are undeamidated
GLLEWEER	Micrococcales	Bacteria, Actinobacteria		N/A
LPGPAGPR	*Modestobacter*	Bacteria, Actinobacteria, Geodermatophilaceae	#rock dwelling bacteria of arid environments (also reported for Sahara desert stones and monuments; Essoussi et al., 2010)	N/A
NHAGLAR	*Neisseria meningitidis*	Bacteria, Betaproteobacteria	#potential human pathogen (Kahler, 2017)	Undeamidated, 1 peptide only
YGETELSLR	*Pedobacter*	Bacteria, Bacteroidetes	*the genus includes species with cellulolytic activity, inhabiting harsh environments (Soares et al., 2012)	N/A
LAATVSLPR	*Penicillium subrubescens*	Fungi, Ascomycota, Aspergillaceae	*(hemi-)cellulolytic fungus degrading lignocellulose (Mäkelä et al., 2016)	N/A
VVTVSLPR	*Phellinus noxius*	Fungi, Basidiomycota, Hymenochaetaceae	*plant pathogen degrading wood (brown root rot; Zhou et al., 2018)	N/A
AGEFALTK	Planctomycetes	Bacteria		N/A
GLLEGEEER	*Planoprotostelium fungivorum*	Amoebozoa		N/A
LGPGLDGR	*Pseudacidovorax intermedius*	Bacteria, Betaproteobacteria	soil bacterium	N/A, 1 peptide only
FSGLDGAK	Rhizobiaceae	Bacteria, Alphaproteobacteria		When present in N/Q-containing sequences, these are undeamidated
GPPGLA	Rhizobiales	Bacteria, Alphaproteobacteria		When present in N/Q-containing sequences, these are deamidated
GAPGPAQR	*Rhodovulum sulfidophilum*	Bacteria, Alphaproteobacteria	*e.g. proteolytic bacteria (Seangtumnor et al., 2018)	When present in N/Q-containing sequences, these are deamidated
GLASYLEK	*Sphingobacterium*	Bacteria, Bacteroidetes	*e.g. chitinolytic bacteria (Zhou et al., 2016)	N/A
QGPSGA	Sphingomonadaceae	Bacteria, Alphaproteobacteria		When present in N/Q-containing sequences, these are deamidated
QDYEALVER	*Spiribacter salinus*	Bacteria, Gammaproteobacteria	# halophytic bacteria, reported from desert environments (León et al., 2014)	Undeamidated, 1 peptide only
VGLPGLDGR	*Streptomyces*	Bacteria, Actinobacteria	*biodeteriogens of organic cultural heritage materials (e.g. proteolytic effect on parchment), also reported from wall-paintings of ancient Egypt (Abdel-Haliem et al., 2013) and isolated from mummies (Kraková et al., 2018; Pinar et al., 2013)	When present in N/Q-containing sequences, these are undeamidated
VAASDDFR	*Streptomyces*	Bacteria, Actinobacteria	*see "*Streptomyces*" above	N/A, 1 peptide only
GPPGSAG	*Streptomyces chartreusis*	Bacteria, Actinobacteria	*e.g. hemicellulolytic bacteria; (Zhu et al., 2012)	When present in N/Q-containing sequences, these are deamidated
LGELSELNR	*Streptomyces griseochromogenes*	Bacteria, Actinobacteria	#soil bacterium, also inhabiting pathogenic fungi (Wu et al., 2017) [see also the above comments on *Streptomyces*]	Undeamidated, 1 peptide only

(*continued on next page*)

Table S3 (*continued*)

Peptide sequence	Organism	Taxonomy (https://www.ncbi.nlm.nih.gov/taxonomy)	*Potential deteriogenic activity/#Remarkable ecology	Deamidation?
VGLPGEGR	*Streptomyces toyocaensis*	Bacteria, Actinobacteria	*see "*Streptomyces*" above	When present in N/Q-containing sequences, these are undeamidated
QVTVSLPR	Verrucomicrobia	Bacteria		Undeamidated, 1 peptide only
RPGAPGPR	Verrucomicrobia	Bacteria		N/A

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jas.2020.105145.

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