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Technical Note

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Deglycosylation Step to Improve the Identification of Egg Proteins in Art Samples

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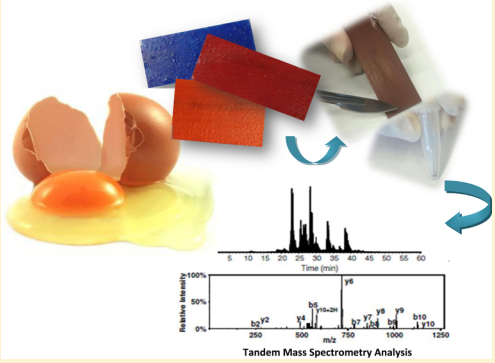
Supporting Information

ABSTRACT: A deglycosylation step using Peptide-N-Glycosidase F (PNGaseF) has been introduced in a standard proteomic protocol to more confidently identify egg based binders. The ingenuity of introducing a PNGaseF digestion was aimed at removing the molecular hindrance, made up by the heavily glycosylated egg proteins, before the protease(s) hydrolysis. This novelty in the protocol resulted in obtaining a significant increase of proteolytic egg peptides thus improving the quality and reliability of egg identification in artwork samples. The protocol has been set up on paint replicas and successfully tested on two historical samples of different origin.



Proteinaceous materials, such as animal glue, egg (both yolk and albumen), and milk have long been used in paintings as binders, coatings, and adhesives. Nowadays the identification of the materials used by artists is of greatest significance in revealing working practices, defining conservation protocols, and occasionally for authenticating and dating the artworks.[1]

Following the seminal papers by Hynek et al.[2] and by Tokarsky et al.,[3] several proteomic strategies, aimed at the unequivocal identification of proteins present in art objects or in archeological remains, have been designed by extensively exploiting modern mass spectrometry. The "bottom-up" approach that has been generally used so far is based on the direct enzymatic digestion of the sample followed by either MALDI-TOF or LC−MS/MS mass spectrometric analysis of peptides released from the object under study.[4−12] The very critical step in this approach is related to the efficiency of either protein extraction from solid matrixes or proteolytic digestion of substrates incorporated within the matrix itself. In our experience[4] the detection of egg proteins in paint samples remains often rather unsatisfactory and is certainly less confident than other most common binders, i.e., milk and animal glue.

It is well-known that the most abundant proteins in hen egg, either in the albumen (i.e., ovalbumin, ovotransferrin, ovomucoid, ovoglobulins, and beta-ovomucin) and in the yolk (i.e., vitellogenin) are heavily glycosylated.[13,14] We reasoned that the extensive glycosylation of egg proteins might create a significant molecular hindrance which hampers proteases to efficiently interact with the proteinaceous substrates, thus greatly decreasing proteolytic efficiency. This, in turn, would result in inefficient and poor production of peptides, impairing a confident protein identification. A possible approach to avoid this molecular hindrance would be to trim out the glycosidic decoration by preceding the proteolytic digestion with a deglycosylating step.[15] This working hypothesis was investigated and results are herein reported. The study demonstrated that the ingenuity of introducing a N-Glycosidase F digestion before the protease(s) treatment greatly increases the release of digested peptides, substantially improving the quality and reliability of egg identification in samples from artworks.

EXPERIMENTAL SECTION

Reagents. Ammonium hydrogen carbonate (AMBIC), ethylenediaminetetraacetic acid (EDTA); tri(hydroxymethyl)-aminomethane (TRIS), TPCK-treated trypsin were from Sigma; recombinant Peptide N-Glycosidase F (PNGaseF) was from Roche. Formic acid and acetonitrile (ACN) were purchased from Baker, respectively. Deionized water was obtained from the Millipore cartridge equipment.

Painting Samples. Paint reconstructions were prepared using egg white, milk, and animal glue as binders and azurite

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($Cu_3(CO_3)_2(OH)_2$), red ochre (Fe_2O_3), minium (Pb_3O_4) and vermilion (HgS) and were applied on glass slides. Paint replicas were prepared in 2010, left to dry at room temperature for 1 month, then artificially aged for 720 h at 25 °C, 50% relative humidity in indoor light conditions, and stored since then in the darkness at room temperature. Details are reported elsewhere.[16,17] Sample DSFL6 was collected from the gilded aureole of the angel in "Holey Conversation", mural painting by Amico Aspertini, 1506−1510, San Frediano Church, Lucca, Italy. Sample Purple 12M080 was collected from a mural painting of the urban district of Cuma archeological site (first century AD)

Protein Deglycosylation. Treatment with PNGaseF was carried out by adding to microsamples (~300−800 μg) 50 μL of AMBIC 50 mM containing 60m U/μL of PNGaseF solution and incubating at 37 °C for 2 h. The reaction was stopped by incubation of the sample in boiling water for 2 min.

Protein Digestion and LC−MS/MS Analysis. Protein samples were enzymatically digested on the basis of the minimally invasive proteomic analytical procedure described by Leo et al.[4] Briefly, trypsin was added to a final concentration of 10 ng/μL both to the samples from PNGaseF pretreatment and, in the case of trypsin alone protocol, to microsamples (~300−800 μg) directly suspended in 50 μL of AMBIC 50 mM. After incubation at 37 °C for 16 h, the supernatants were recovered by centrifugation, and the peptide mixture was filtered on 0.22 μm PVDF membrane (Millipore), concentrated, and purified using a reverse-phase C18 Zip Tip pipet tip (Millipore). Peptides were eluted with 20 μL of a solution made of 50% acetonitrile, 50% formic acid 0.1% in Milli-Q water and analyzed by LC−MS/MS. LC−MS/MS analyses were carried out on a 6520 Accurate-Mass Q-TOF LC/MS System (Agilent Technologies, Palo Alto, CA) equipped with a 1200 HPLC system and a chip cube (Agilent Technologies). After loading, the peptide mixture was first concentrated and washed on a 40 nL enrichment column (Agilent Technologies chip), with 0.1% formic acid in 2% acetonitrile as eluent. The sample was then fractionated on a C18 reverse-phase capillary column (Agilent Technologies chip) 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% acetonitrile) from 3% to 80% in 50 min.

Peptide analysis was performed using data-dependent acquisition 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 threshold of 50 000 counts. Each LC−MS/MS analysis was preceded and followed by blank runs to avoid carryover contamination. Double and triple charged ions were preferably isolated and fragmented. The acquired MS/MS spectra were transformed in Mascot Generic files (.mgf) format and used to query the SwissProt database 2015_04 (548 208 sequences; 195 282 524 residues), with Chordata as the taxonomy restriction for protein identification. A licensed version of MASCOT software (www.matrixscience.com) version 2.4.0. was used with trypsin as the enzyme; 3, as allowed number of missed cleavage; 10 ppm MS tolerance and 0.6 Da MS/MS tolerance; peptide charge from 2+ to 3+. No fixed chemical modification was inserted but possible oxidation of methionine residues, formation of pyroglutamic acid from glutamine residues at the N-terminal position of peptides, and deamidation at asparagines and glutamines were considered as variable modifications.[6] When collagen proteins were identified, a further identification run was carried out, with the insertion of hydroxylation on lysine and proline as variable modifications, since more confident identifications are commonly obtained for these proteins by taking into consideration their extensive post-translational modifications.[4] Only proteins presenting two or more peptides were considered as positively identified. Individual ion score threshold provided by MASCOT software to evaluate the quality of matches in MS/MS data was generally 33. Spectra with a MASCOT score of <10, having low quality were rejected.

RESULTS AND DISCUSSION

The experimental observations that egg proteins are seldom identified in paint samples and, even when analyzing model samples, their identification is based on a small number of peptides, thus impairing a highly confident identification, prompted us to develop a novel analytical protocol to investigate and to circumvent this problem. A deglycosylation step was introduced before trypsin hydrolysis in the hypothesis that the high content of glycosylated proteins of egg might constitute a molecular barrier hampering proteolytic digestion. Several enzymes have been successfully applied to the release of N-linked glycans, such as peptide-N-glycosidase F (PNGaseF), endoglycosidase F and H.[19,20] Among these, PNGase F has emerged as a widely used glycoamidase.[21,22] As already shown in other circumstances,[23] this enzyme is able to remove the glycosidic moieties leaving the protein substrate more amenable to tryptic digestion. A simple protocol was thus set up with a deglycosylating step before the tryptic digestion as described in the Experimental Section.

Paint reconstructions containing red ochre as pigment and alternatively albumen or yolk or whole egg were used. Aliquots of each sample were analyzed using both the classical proteomic approach, based on simple treatment with trypsin (herein indicated as "Trypsin alone") and the protocol with PNGaseF pretreatment (herein indicated as "PNGaseF + Trypsin"). It is worth noting that both steps of PNGaseF and trypsin digestions were carried out in the heterogeneous phase, that means direct enzymatic digestion on solid samples without protein extraction.[4] This can be extremely useful when dealing with paint samples in which proteins are aged and bonded with pigments and fillers[16,17] and are thus difficult to solubilize. After digestion with trypsin, samples were analyzed by LC−MS/MS and proteins were identified by database search with the MS/MS ion search mode within a licensed version of MASCOT, with Chordata as the taxonomic restriction in the SwissProt protein database.

Table 1 reports a comparison of the results obtained on a replica made of albumen and red ochre. It is evident that pretreating the sample with PNGaseF results in a much higher number of peptides obtained, greatly improving the reliability of the identification. In the aliquot treated with trypsin alone, only ovalbumin and ovotransferrin were identified, with a few detected peptides.

PNGaseF pretreatment led to a definitively more confident and reliable identification of eight proteins from chicken egg white, showing also a much larger number of detected peptides for ovalbumin and ovotransferrin. Details on protein identifications are reported in the Supporting Information, Table S1, where the N-glycosylation signature of deamidation in the Asn-X-Ser consensus sequence can be easily spotted in some of the observed peptides demonstrating the efficacy of the

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Table 1. Proteins Identified in the Paint Replica Containing Red Ochre and Albumen by LC−MS/MS[a]

identified protein. (accession number)	protocol					
	Trypsin		PNGaseF + Trypsin		Trypsin + PNGaseF	
	score[b]	no. of peptides	score[b]	no. of peptides	score[b]	no. of peptides
Ovalbumin (P01012)	159	4	960	23	299	11
Ovotransferrin (P02789)	90	2	2147	63	196	7
Ovostatin (P20740)			137	5		
Ovalbumin releted protein Y (P01014)			452	11	85	3
Mucin 5B (Q98UI9)			161	2		
Lysozyme C (P00698)			340	7		
Ovalbumin related protein X (P01013)			82	3		
Protein Tenp (O42273)			82	2		

[a]Aliquots of paint replica were treated in heterogeneous phase with trypsin with or without treatment with PNGaseF (before and after tryptic digestion) and analysed by LC−MS/MS. Details of the identification are given in Table S-1 [b]Protein scores are derived from ions scores as a nonprobabilistic basis for ranking protein hits (http://www.matrixscience.com/help/interpretation_help.html). Ions score is −10 Log(P), where P is the probability that the observed peptide match is a random event.

deglycosylation procedure. As control, an aliquot of sample was digested with trypsin and then with PNGaseF, in order to check whether the complexity of the sample mixture rather than the steric hindrance created by glycosylated peptides was responsible for the lack of results in PNGaseF untreated samples.

Very similar results were obtained on paint replicas containing either whole egg or egg yolk as binder (Table S-2). In the absence of PNGaseF pretreatment, 7 proteins were identified in the whole egg samples while following PNGaseF incubation a total of 14 proteins could be confidently identified, mainly ovalbumin and related protein and vitellogenins ones. Similar results were obtained in the analysis of the egg yolk paint replica, where mainly vitellogenins were identified, demonstrating that the usefulness of PNGaseF treatment is not limited to sample containing proteins from albumen only, but it is extended to any sample where egg proteins are present (Table S-2). Moreover, in all cases a higher number of peptides were detected compared to the trypsin alone protocol (from 2 to 19 times more).

In order to investigate if the PNGaseF treatment affects the identification of milk and animal glue, the other two common proteinaceous paint binders we analyzed with the newly developed protocol paint replicas containing the same pigment, red ochre, mixed with milk and animal glue, as well as different combinations of binders. Table S-3 shows that PNGaseF treatment had no substantial effects on the results when the sample does not contain egg. This result is not surprising, since milk proteins and collagen, the most abundant protein in animal glue, are much less glycosylated than egg proteins and are thus good substrates for tryptic hydrolysis even in the absence of the deglycosylation step. On the contrary, whenever egg is present in mixture with other binders, PNGaseF pretreatment improved identifications.

As in paint samples, inorganic materials are simultaneously present, mostly as pigments and dryers, and it is necessary to investigate whether they can inhibit the activity of PNGaseF. To this aim, paint replicas containing a range of metals commonly occurring in paintings were selected. In particular the replicas contained albumen and cinnabar (HgS), minium (2 $PbO{\cdot}PbO_2$), and azurite ($Cu_3(CO_3)_2(OH)_2$), alongside the previously tested red ochre (Fe_2O_3). Experiments were carried out as above on aliquots of each sample using the trypsin alone and the PNGaseF + trypsin protocols in parallel. Data clearly indicate that PNGaseF treatment always improved the egg identifications, regardless of the pigment used (Table S-4), by increasing the number of peptides that are released from the proteins after tryptic digestion. These results indicate that PNGaseF is effective also in the presence of some widespread metal containing pigments, including copper. Moreover the data clearly show that the newly developed protocol is fundamental in order to maximize the possibility of identifying egg in an aged paint sample. It is well-known, in fact, that pigments can affect the protein identification by amino acid analysis if suitable purification steps are not adopted.[24,25] To fully understand the influence of pigments in the protein identification through proteomics procedures, the effect of pigments still needs be systematically investigated. Despite this, the data presented here clearly indicate that pigments do have an influence in the number of proteins that can be identified by proteomics analysis in a paint sample, as a consequence of the strong interactions taking place between pigments and proteins,[17,18] and the developed protocol is a suitable analytical tool to help us to improve our success rate.

Historical Paint Samples. Finally, our improved protocol was tested on two samples collected from historical objects: sample Purple 12M080 from a mural painting from the urban district of Cuma archeological site and DSFL6 from a mural painting of San Frediano Church in Lucca, Italy (16th century). It is worth noting that sample DSFL6 was previously analyzed by GC/MS and the presence of egg was accordingly inferred.[26] Both samples provided no result when analyzed by the Trypsin alone protocol.

Aliquots of the two historical samples were analyzed following the newly developed procedure including PNGaseF pretreatment before trypsin digestion and LC−MS/MS analysis of the resulting peptide mixtures.

Table 2 reports the identifications of the proteins obtained in the two samples with details of the identifications reported in Table S-5. A number of egg proteins were identified in both samples confirming the presence of an egg-containing binder and showing very clearly that deglycosylation of the samples before tryptic digestion led to the identification of egg proteins that could not be detected before. Moreover, it should be underlined that no proteins from albumen were identified in the sample from Cuma thus allowing us to confidently assess that only yolk was used as paint binder.

■ CONCLUSIONS

Diagnostic methods represent a crucial aspect of the scientific investigation of artworks and proteomics procedures are increasingly applied for the identification of protein binders in samples from archeological objects and works of art. Proteomics strategies applied to artworks has begun to be

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Table 2. Proteins Identified in Historical Paint Samples by LC−MS/MS[a]

sample	identified protein (accession number)	PNGaseF + Trypsin	
		score	no. of peptides
Purple 12M080	Vitellogenin-2 (P02845)	1163	35
	Vitellogenin-1 (P87498)	626	20
	Apolipoprotein B (P11682)	107	4
	Apovitellenin-1 (P02659)	90	2
DSFL6	Ovalbumin (P01012)	20	2

[a]The mural painting fragment "Purple 12M080" from the urban district of Cuma archeological site and a fragment of the mural painting "Sacra Conversazione" (DSFL6) by Amico Aspertini 1506−1510 from the S. Frediano Basilica (Lucca) were subjected to trypsin digestion after PNGaseF pretreatment and the resulting peptide mixtures analyzed by LC−MS/MS. Details of the identification are given in Table S-5

accepted as the gold standard analytical technique when proteins have to be identified and characterized because of the sensitivity, the capability of identifying species-specific proteins, and detecting degradation processes. However, methodological adaptations to specific analytical problems of samples from cultural heritage still need to be developed to tailor appropriate approaches. The physical state of the samples, enormously different from the natural environment of proteins, and the degradation processes undergone during aging provide unusual problems that require to be counteracted by specific adaptations of the classically adopted protocols used in the analysis of biological samples. A typical example of these situations is reported in this paper. Egg proteins can be easily identified when occurring in solution with glycosylation not hampering the procedure. However, when egg is used as binder in paintings, in a solid state mixed with pigments, the oligosaccharide moieties seem to create a molecular hindrance that prevent the accessibility of proteases, greatly impairing the identification process. The implementation of a deglycosylation step in the analysis of paint samples prior to the tryptic digestion has proven to significantly improve the number of identified peptides from egg proteins in several different paint reconstructions as well as on two historical samples of completely different origin. Moreover it was shown that the same glycosylation step does not affect the capability of correctly identifying other proteinaceous paint binders, such as milk and animal glue.

The protocol to identify egg based binders described in this work, based on a sample pretreatment with PNGaseF, has revealed to be useful, reliable, cost-effective, and sensitive enough to cope with the small amounts of degraded proteins that can be found in samples from artworks.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.5b02423.

Additional tables reporting details of protein identifications in paint replicas and historical samples of Table 1 and 2 and the proteins identificated in several combinations of pigment and protein binders (PDF)

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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