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Towards proteomic analysis of milk proteins in historical building materials

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ABSTRACT

The addition of proteinaceous binders to mortars and plasters has a long tradition. The protein additions were identified in many sacral and secular historical buildings. For this method of peptide mass mapping, three model mortar samples with protein additives were prepared. These samples were analysed fresh (1–2 weeks old) and after 9 months of natural ageing. The optimal duration of tryptic cleavage (2 h) and the lowest amount of material needed for relevant analysis of fresh and weathered samples were found; the sufficient amounts of weathered and fresh mortars were set to 0.05 and 0.005 g.

The list of main tryptic peptides coming from milk additives (bovine milk, curd, and whey), their relative intensities and theoretical amino acid sequences assignment is presented. Several sequences have been "de novo" confirmed by mass spectrometry.

1. Introduction

The analysis of historical mortars and plasters is necessary for revealing old technological approaches, for understanding their unusual properties [1] and subsequently, for the selection of the correct technological process for restoration. The identification of inorganic components in the mortars is well mastered, but the identification of organic ones is unjustly marginalized. Although the organic, namely the protein additives, remarkably change the mortar properties [2,3], no analytical methods are available for their routine identification. The analyses are difficult and time consuming because of the extremely low amount of protein additives, aggressive processes during hardening of fresh mortar, and unpredictable changes of the materials caused by their ageing. In several cases pyrolysis [4–6], gas [7–9] and liquid [10–12] chromatography were used for analyses of painted frescoes and baroque artificial marble decoration [13,14], but these methods are not suitable for samples containing lower amounts of proteins, namely because of the losses of the amino acids during the hydrolysis step and the following derivatisation [8,15–21]. Capillary zone electrophoresis has similarly disadvantageous sample preparation [22,23]. The analytical techniques that can be considered non-destructive are infrared (FTIR) and Raman spectroscopy [24–27]. Both methods do not allow the identification of the individual proteinaceous binders.

Currently, the methods that are routinely used by biochemists are also applied to art works analysis. The methods used for the identification of the proteins are the immunological and immunofluorescence methods [28], nanoLC/nanoESI/Q-q-TOF MS/MS [29] and matrix-assisted laser desorption/ionisation time of flight mass spectrometry (MALDI-TOF MS) [30–33].

The method called peptide mass mapping (PMM) has been developed in the last decade for the identification of proteins by biochemists. It is based on specific enzyme cleavage of the proteins and the subsequent analysis of the peptide mixture by mass spectrometry. The obtained mass spectrum enables reliable identification by comparison of the spectrum of building mortar samples with those of reference samples [30]. In our laboratory, the PMM method was applied to the identification of proteinaceous binders in the colour layers of easel paintings [30–33]. Proteinaceous additives are also a significant component of historical building materials [34–36]. Since ancient times milk, curd and whey have been used mainly in murals, plaster and mortars, but not very frequently in paintings because of their inappropriate properties. Milk is a complex emulsion containing many proteins as well as low-molecular weight compounds. Bovine milk contains six predominant proteins (κ-, α_{S1}-, α_{S2}- and β-caseins, α-lactalbumin and β-lactalbumin) and other proteins like globular glycoproteins, macroglobulin and serum albumin. The technology that allows separation of the milk into two components – curd and whey – has been known since time immemorial. All types of caseins are acidic phosphoproteins

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existing in milk in the form of calcium salts; they form the major protein component of milk and curd, while whey contains mainly α- and β-lactalbumin, α- and β-lactoglobulin, globular glycoproteins, macroglobulin and serum albumin [2]. In the presented study the PMM was implemented for identification of protein additives in historical mortars using model samples.

2. Experimental

2.1. Materials

Trypsin (TPCK) from Promega Corporation, trifluoracetic acid and 2,5-dihydroxybenzoic acid both from Sigma, acetonitrile (p.a.) and ammonium hydrogen carbonate from Lachema Brno were used. The commercially available reverse phase ZipTip comes from Millipore Corporation, Bedford, MA, USA. The bovine milk (protein content 3.3%) and curd (protein content 9.6%) comes from Delvita production, whey (protein content 12.8%) from Magador s.r.o., Zlín, Czech Republic.

The three model mortar samples with the protein additives and a blank were prepared according to the recipes shown in Table 1. The protein additives were added to the basic mixture that was prepared from sand, lime and water in ratio of 4:1:1. The samples were shaped on plates into blocks sized 20 × 10 × (2–3) cm where they were left to dry in outdoor conditions (Prague, Czech Republic).

2.2. Method

The pulverized model samples were digested by sequencing grade trypsin (TPCK). Approximately, 50 mg of the solid material were digested in 100 μl of 50 mM of ammonium hydrogen carbonate containing approximately 10 μg/ml of trypsin at room temperature for 1, 2, 4, 8, 16, and 24 h. After the trypsin digestion, the samples were purified on reverse phase ZipTip.

The mass spectra were acquired by Bruker-Daltonics Biflex IV MALDI-TOF and Autoflex II MALDI TOF-TOF mass spectrometer equipped with standard nitrogen laser (337 nm) in reflector mode. The peptide mixture (2 μl) was mixed with 4 μl of 2,5-dihydroxybenzoic acid (DHB) solution (15 mg of DHB in 1 ml of mixture of acetonitrile/0.1% trifluoracetic acid (1/2, v/v)); 1.5 μl of the resulting mixture was spotted on the stainless steel MALDI target and dried in air. At least 200 laser shots were collected for each spectrum and analysed using the XMASS software (Bruker) and our database of proteinaceous binders [30].

Chosen peptides were "de novo sequenced" using MALDI TOF-TOF (method LIFT). Fragmentation spectra were processed by Biotools 3.0 software and peptides were identified using MASCOT database (www.matrixscience.com).

The mass spectra of dried (at room temperature) curd, whey and bovine milk were measured 10 times. The peak occurrence, which is stated in the following tables in Section 3, indicates how often the peak appears in the spectra.

Table 1
Comparison of model mortar samples.

Sample	Proteinaceous binder	Binders weight [g]	Protein additive amount [%]	Basic mixture of mortar [g]
M1	Whey[a]	50	2.50	2000
M3	Curd	40	2.00	2000
M4	Milk	75	3.75	2000
M8	Blank	–	–	2000

[a] The whey solution was prepared by stirring of 40 g of whey powder in 500 ml water.

Table 2
Analysis of the fresh and aged model samples.

Sample	Our results and achieved scores (%)[a]	
	Fresh	Aged
M1	Milk (33%) Whey (21%)	Milk (28%)
M3	Curd (64%) Milk (61%)	Curd (32%) Milk (46%)
M4	Milk (97%)	Milk (44%)

[a] The scores (%) are the ratios of proteinaceous binder to all relevant peaks.

3. Results and discussion

The peptide mass mapping method was tested on model samples of mortars that were prepared according to the recipes in Table 1. The protein additive content denotes the ratio between, for example, the weight of the milk and the overall weight of the mixture. The real content of proteins is much lower; in the case of bovine milk it is approximately 0.12%. The value was calculated with the following procedure: 0.0375 (from Table 1) × 0.033 (the content of the proteins in the milk that was given on the milk's packaging). The original historical recipes are not known, because the art of making quality mortar was handed down in the bricklayers' families and after the coming of modern materials, the knowledge has not been preserved. A suitable duration of trypsin digestion was sought; the mortar samples were digested for 1, 2, 4, 8, 16, and 24 h. The maximum number of mass peaks in the interval 900–2000 m/z was obtained after 2 h.

All model samples were analysed by the peptide mass mapping method (Table 2). The m/z values of the peaks of each model sample were compared with those of the given proteinaceous additive in the database of reference proteinaceous binders [30]. The so called "score" was calculated; this number represents the ratio of the number of peaks for which the corresponding m/z value was found in our database [30] to the number of all peaks detected in the sample mass spectrum. From the table, it can be seen that the scores vary from 20% to almost 100%. It is also clear that very often it is not possible to distinguish between individual components of the material (whey or milk vs. curd).

A decrease of peak numbers was observed in the spectra of the same amounts of fresh (1–2 weeks old) and weathered (9 months old) mortars (Fig. 1). The sufficient amounts of weathered samples for getting quality spectra were set to 0.05 g; this amount was about 10 times lower for the fresh mortar.

The m/z values of the highest peaks from the mass spectra of the protein additives were transcribed (from the Table in Supplements) to Table 3. The furthest highest peaks were found in spectra of curd and milk, but these peaks do not occur frequently. From the table it can be seen that the most intensive peaks are not fully characteristic for the individual additives. The most frequent peaks are shown in Table 4. The most repeated peaks were found in bovine milk and then in the spectra of whey. Although these peaks are often not very intensive, they are more characteristic for the additives than the peaks from Table 3. The characteristic peaks of the milk are mainly: 1098.5, 1137.5, 1299.5, 1608.6, 1660.5, 1744.9, 1867.6, 1871.8, 1915.8, and 1951.8 m/z. The whey contains the highest number of unambiguous peaks such as: 903.4, 925.4, 927.4, 933.4, 955.5, 1090.8, 1097.4, 1157.8, 1193.8, 1222.7, 1237.8, 1301.8, 1311.8, 1337.6, 1439.8, 1473.6, 1479.9, 1567.8, 1627.9, 1635.4, and 1658.9 m/z. The curd has only one most often repeated peak at 1759.7 m/z. The fact that only one characteristic peak occurred in the mass spectra of dried curd can be explained by the presence of a great number of proteins that are preferably cleaved by trypsin. Thus the spectra

Table 3
The highest peaks occurring in mass spectra of curd, whey and milk.

m/z	Curd		Whey		Bovine milk	
	Intensity	Abundance	Intensity	Abundance	Intensity	Abundance
903.4	+++	1	+++	9	+	5
933.4	++	2	+++	9	+	3
977.7	+++	2	+	3	++	2
1005.7	+++	2			++	3
1008.6	+	2			+++	1
1092.8	+++	2			+++	3
1094.9	+++	2			+++	3
1195.8	++	9	+++	7	+	9
1200.5	+	2	+++	9	+	8
1204.0	+++	2			++	1
1249.5			+	1	+++	1
1251.6	++	10	+	5	+++	10
1254.9	++	2			+++	2
1267.5	+++	9	+++	7	+++	9
1269.7					+++	3
1300.9	++	1	+++	2	+++	2
1340.9	+++	2	+	3	++	3
1383.6	+	10	+	10	+++	9
1384.9	++	1	+++	2	+	1
1625.3	+++	2	++	3	+++	3
1658.9			+++	9	+	1
1691.3	+++	2			+	3
1701.3	+++	2	+	1		
1718.4	+++	2			++	2
1759.7	+	8	++	3	+++	5
1881.5	+++	2	+	1	+	1
1994.5	+++	2			+	2

Table 4
The most often occurring mass peaks in mass spectra of the curd, whey and milk.

m/z	Curd		Whey		Bovine milk	
	Intensity	Abundance	Intensity	Abundance	Intensity	Abundance
903.4	+++	1	+++	9	+	5
925.4			+	8	+	2
927.4			+	9	+	1
933.4	++	2	+++	9	+	3
955.5			+	9	+	2
1090.8	++	2	+	9	+	2
1097.4			+	7		
1098.5			+	2	+	7
1137.5	+	5	+	1	+	8
1157.8	+	3	+	8		
1193.8	+	1	++	9	++	6
1195.8	++	9	+++	7	+	9
1200.5	+	2	+++	9	+	8
1222.7	+	1	+	7	+	1
1237.8	+	1	++	10		
1251.6	++	10	+	5	+++	10
1267.5	+++	9	+++	7	+++	9
1289.5	+	7	+	5	+	10
1299.5	+	2			+	8
1301.8	++	1	++	8		2
1311.8			++	7	+	6
1337.6	++	4	+	7	++	6
1383.6	+	10	+	10	+++	9
1406.6	+	2			+	8
1439.8	+	2	+	8	+	3
1473.6			+	9	+	7
1479.9			++	8	+	2
1567.8			++	7	+	1
1608.6					+	8
1627.9			++	8		
1635.4			++	10		
1658.9			+++	9	+	1
1660.5					+	7
1744.9					+	8
1759.7	+	8	++	3	+++	5
1867.6	+	5			+	8
1871.8					+	7
1915.8	+	6	+	1	++	9
1951.8	+	4			++	7

Table 5
The founded amino acid sequences of the diary proteins and their "de novo" confirmation.

m/z	Range	Sequence	Modification	Protein	De novo
903.57	(92–99)	TKIPAVFK		β-Lactoglobulin	+
954.41	(40–47)	NMAINPSK	1x phosphoserin 46	α_{S2}-Casein	
955.53	(38–45)	FFSDKIAK		κ-Casein	
977.52	(166–173)	TKLTEEEK		α_{S2}-Casein	
979.56	(189–196)	FALPQYLK		α_{S2}-Casein	
1022.60	(215–222)	VIPYVRYL		α_{S2}-Casein	
1193.54	(108–118)	SCQAQPTTMAR		κ-Casein	
1193.68	(108–117)	VLVLDTDYKK		β-Lactoglobulin	
1195.65	130–140	NAVPITPTLNR		α_{S2}-Casein	+
1200.65	(118–127)	VGINYWLAHK		α-Lactalbumin	
1251.71	(46–55)	YIPIQYVLSR		κ-Casein	
1251.75	(213–222)	TKVIPYVRYL		α_{S2}-Casein	
1267.71	(106–115)	YLGYLEQLLR		α_{S1}-Casein	+
1337.68	(95–105)	HIQKEDVPSER		α_{S1}-Casein	
1351.78	(129–140)	RNAVPITPTLNR		α_{S2}-Casein	
1355.70	(35–45)	DERFFSDKIAK		κ-Casein	
1367.70	(96–106)	ALNEINQFYQK		α_{S2}-Casein	
1384.73	(38–49)	FFVAPFPEVFGK		α_{S1}-Casein	
1556.89	(3–16)	FFIFTCLLAVALAK		α_{S2}-Casein	
1658.78	(165–178)	LSFNPTQLEEQCHI		β-Lactoglobulin	+
1669.94	(114–127)	ILDKVGINYWLAHK		α-Lactalbumin	
1759.95	(23–37)	HQGLPQEVLNENLLR		α_{S1}-Casein	+
1979.06	(1–16)	EQLTKCEVFRELKDLK		α-Lactalbumin	
1979.84	(40–56)	NMAINPSKENLCSTFCK	1x phosphoserin 46	α_{S2}-Casein	

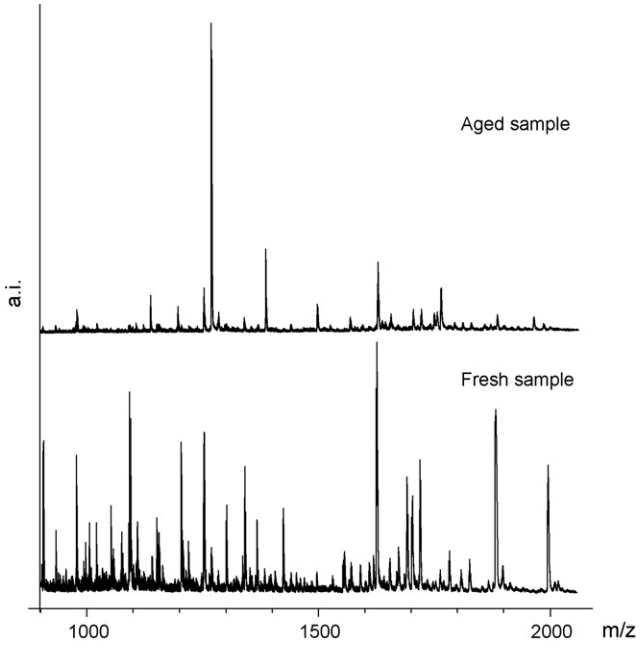

Fig. 1. The decrease of peaks in the mass spectrum of the weathered model mortar sample with curd addition.

contained fewer, yet more intensive peaks, that also occurred in the other diary products.

The sequences of the proteins contained in the bovine milk were found in the publicly available database Expasy and the proteins were subsequently simulated cleaved using the program mMass. The amino acid compositions of some peptides are shown in Table 5. The sequences of the most intensive and most frequently occurring peaks were confirmed "de novo".

4. Conclusions

The peptide mass mapping was successfully implemented for the identification of proteinaceous additives that have been used in historical mortars of different ages. The additives are presented in the mortars in very low concentrations—less than hundredths or even thousandths of percents. For the purpose of this study, three model mortar samples with the protein additives were prepared. The fresh (1–2 weeks old) and the three naturally weathered samples (9 months) were analysed.

Higher scores were obtained within the identification of protein additives in the fresh model samples, although discriminating between whey and curd was also problematic; almost all of the milk proteins were found in both cases.

The amino acid sequences were assigned to 17% of individual peptides coming from the milk proteins. The most intensive mass peaks (peptides) were successfully "de novo" sequenced and some of the theoretical amino acid sequences were confirmed using the publicly available proteomic database Expasy and the program mMass; the sequencing verified the accuracy of the assumption that the model cleavage corresponds to the real process and protein digestion. The relatively low success rate of the assignment can be explained by the small number of proteins that were sequenced and by the large number of posttranslational modifications of milk proteins.

In this study we have started with the sequencing of dairy proteins. In future, we plan to develop a public database containing the amino acid sequences of all tryptic peptide fragments producing mass peaks occurring in used dairy additives. Using this database for the identification of proteinaceous additives should provide unambiguous results [36].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijms.2009.01.011.

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