IDENTIFICATION OF PROTEIN MARKERS FOR THE OCCURRENCE OF DEFROSTED MATERIAL IN MILK THROUGH A MALDI-TOF-MS PROFILING APPROACH

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Abstract – A progressive diffusion of Mozzarella cheese products realized with frozen curd or frozen milk have been observed in the last years. In order to reveal adulterations in raw milk used for cheese manufacturing, we have developed a robust and rapid MALDI-TOF-MS profiling procedure of fresh and frozen buffalo milks. We finally identified 28 polypeptide markers of milk freezing storage. Abundance changes of polipeptide species following freezing treatment were confirmed by an independent approach. We observed the progressive formation in frozen buffalo milk of GLYCAM1-derived phosphopeptides. In addition to these species, β-casein-derived phosphopeptides (1-68), β-casein-derived γ2-, γ3- and γ4-fragments, α-lactalbumin and β-lactoglobulin showed the highest significant abundance changes in frozen milk in comparison with fresh milk.

Keywords: milk adulteration, defrosted milk, MALDI-TOF profiling

1. INTRODUCTION

"Mozzarella di Bufala Campana" is an Italian cheese made from fresh, raw water buffalo (*Bubalus bubalis*) milk, which obtained the Protected Designation of Origin (PDO) registration in the EU product quality legislation (Commision Regulation EC n. 1107/1996). Dedicated analytical methods were previously developed to detect the occurrence of fraudulent procedures in Mozzarella making processes, such as the use of defrosted curd [1]. In this context, in order to evaluate the effect of the freezing storage on raw buffalo milk samples and eventually identify polypeptide markers of the occurrence of frozen material in raw fluid used for mozzarella production, a MALDI-TOF-MS polypeptide profiling approach was developed.

Fresh and frozen buffalo milks, stored at -20°C for different times, were analysed and a robust statistical evaluation was performed on acquired data in order to distinguish milk samples

2. EXPERIMENTAL PROCEDURES

2.1. Sample Preparation

Fresh water buffalo raw milk samples from 30 animals were collected from one local farm after morning milking, kept at 4 °C until delivered in the laboratory and then immediately analyzed (T0) or aliquoted and frozen at -20 °C. Defrosted milk samples at 15 (T15), 30 (T30), 60 (T60), 90 (T90), and 240 (T240) days after freezing were defatted by centrifugation at 3,000 rpm, for 30 min, at 4 °C, diluted with water (1:100) before MS analysis and then quickly processed.

2.2. MALDI-TOF-MS Measurements

For MALDI-TOF-MS polypeptide profiling measurements, performed using an UltraflexExtreme mass spectrometer (Bruker Daltonics), each defatted milk sample was mixed 1:1 with a solution of sinapinic acid (10 mg/ml) (Bruker Daltonics) in 0.1% v/v trifluoroacetic acid (TFA), 30% v/v acetonitrile (ACN), and 1ul was placed onto a Ground Steel Target and dried at room temperature. After optimization of instrumental parameters mass spectra were recorded in the positive linear mode, and externally calibrated.

2.3. Bioinformatics and Statistics

Mass spectral data were analyzed by FlexAnalysis 3.4 and ClinProt Tools 2.2 software packages (Bruker Daltonics). Polypeptides showing a statistically significant difference in signal intensity or mass value were determined by means of specific tests (PWKW, PAD, and PTTA). Principal components analysis (PCA) of MALDI-TOF-MS spectra, which was carried out by an external MATLAB software tool integrated into ClinProTools software, was performed.

2.4. LC quantification of putative markers

Aliquots of defatted buffalo milk samples were resolved by reversed-phase HPLC on a C18 analytical column (Grace Vydac) and then eluted with a linear gradient (5-60% over 60 min) of ACN/0.1% TFA (solvent B) in 0.1% TFA (solvent A), flow rate 1 ml/min. Molecular masses of polypeptide fractions were determined by MALDI-TOF-MS analysis, both in linear and reflector mode. Elaboration of chromatographic profiles and calculation of corresponding peak areas for polypeptide markers for freezing storage times (T0, T30, T60, T90, T240) was performed using the Chromatographic Data system software UniChrom™ (New Analytical System Ltd., Minsk, Belarus).

2.5. Polypeptide structural characterization

Several peptide fractions from chromatographic runs were also subjected to structural characterization. At first, they were subjected to phosphatase treatment in order to ascertain the presence of phosphorylated residues [2]. Lyophilized polypeptide aliquots were digested with trypsin (Roche), at 37 °C for 4 h. Peptide digests were analyzed by nanoLC-ESI-linear ion trap (LIT)-MS/MS procedures, using a LTQ XL mass spectrometer (ThermoFisher) equipped with Easy-nanoLC and nanospray source (Proxeon). Peptide mixtures were separated on a nano column Easy C18 column (Proxeon). Spectra were acquired using data-dependent product ion scanning procedure, over the three most abundant ions, enabling dynamic exclusion. Peptide identification was achieved by using MASCOT (Matrix Science) and SEQUEST.

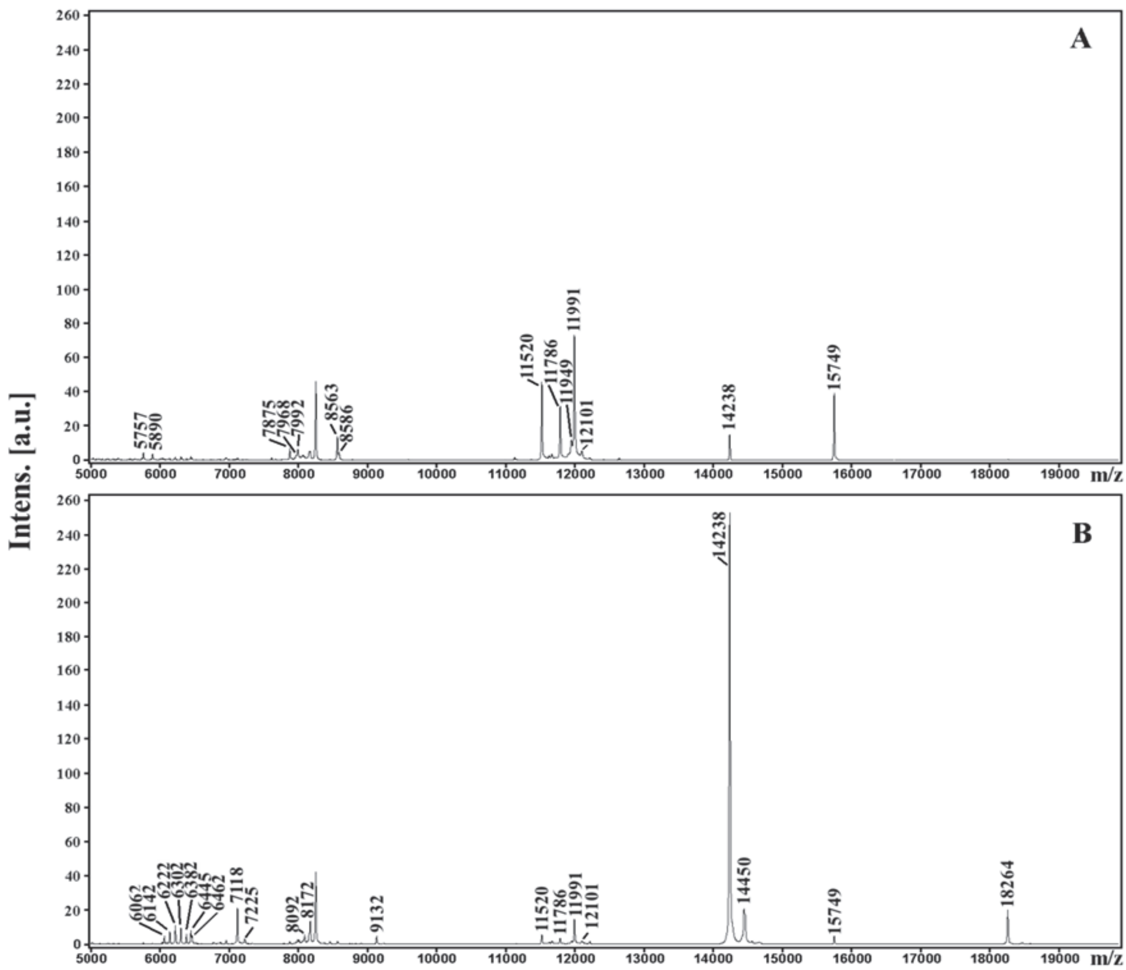

Figura 1. MALDI-TOF-MS profiling (linear mode): average spectra for fresh T0 (panel A) and frozen T240 (panel B) buffalo milk. Only m/z values corresponding to marker signals are reported.

3. RESULTS

We developed a MALDI-TOF-MS peptide/protein profiling protocols for the analysis of diluted skimmed milk. Overall, a data set of 900 MALDI-TOF mass spectra was recorded. In order to estimate technical and biological variability, statistical data analysis was performed considering 20 protein peaks (signal to noise threshold SNR>5) for each condition. Measured intra-experiment coefficient of variation (CV) ranged from 24-28%. Representative examples of MALDI-TOF mass spectra are reported in Figure 1.

Data from PWKW, PAD, and PTTA analyses defined the recognition capability of each specific assay (Table 1). On the basis of acquired MALDI-TOD data, the discrimination of frozen milk samples by MALDI-TOF-MS analysis (100% recognition capability) was guaranteed in most cases of frozen milk (T30, T60, T90 and T240) versus fresh milk (T0), but not between the different freezing times.

Table 1. ClinProt model generation report for the recognition of frozen raw buffalo milk samples.

Model Name	Cross Validation	Recognition Capability
0-15	89.7	98.15
0-30	88.78	100
0-60	96.48	100
0-90	83.94	100
0-240	98.98	100
0-15-30-60-90-240	51.31	87.08

Statistical analysis of MALDI-TOF mass spectra of buffalo milk samples at different freezing times allowed the identification of discriminant peaks based on signal intensity. Thirteen peaks were found decreased and fifteen were found increased in frozen milk samples, in comparison with fresh ones. PCA analysis was performed on MALDI-TOF mass spectral data. A clear separation between fresh and frozen milk was evident even at 30 days of storage in the freezer (data not shown).

Nineteen discriminant marker signals for frozen buffalo milk were identified on the basis of literature data [1,3] and mass value calculations.

In order to achieve peak assignments and, more importantly, confirm the quantitative trend of the polypeptide markers deduced by MALDI-TOF-MS profiling of buffalo milk, defatted T0, T30, T60, T90 and T240 samples were also resolved by reversed-phase LC. Fig. 2 shows the chromatographic profile of a frozen milk sample (T60).

Polypeptide fractions were manually collected, analyzed by MALDI-TOF-MS, and further characterized by nanoLC-ESI-LIT-MS/MS analysis. Assignments are reported in Table 2

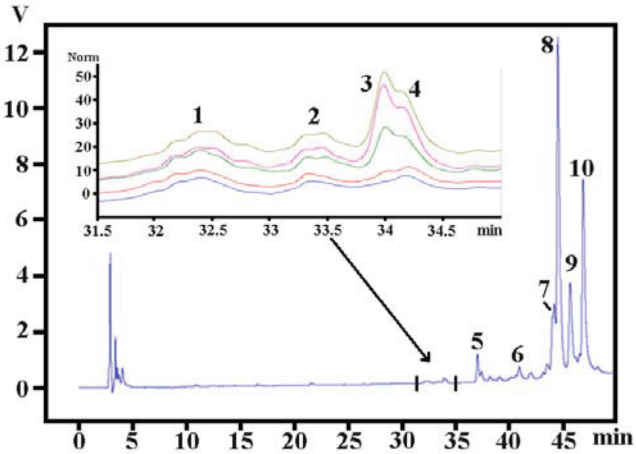

Fig. 2. LC chromatographic profile of a frozen buffalo milk sample (T60). Inset. Comparison of the chromatographic traces for peaks 1, 2, 3 and 4 from T0 (blue), T30 (red), T60 (green), T90 (magenta) and T240 (yellow) samples.

Among protein/polypeptide marker signals identified by MALDI-TOF-MS profiling, worth mentioning are those associated with α-lactalbumin, β-lactoglobulin, and beta-casein, together with fragments related to Glycosylation-dependent cell adhesion molecule 1 (GLYCAM-1) and β-casein, possibly originated by the activity of proteolytic enzymes in buffalo. The GLYCAM1 phosphopeptides (1-53) modification pattern was consistent with a post-translational modification profile of the buffalo protein similar to the bovine counterpart [4]. A quantitative elaboration of the normalized chromatographic trace of the three sample replicates for each freezing time, demonstrated that integrated areas of peaks 1, 2, 3 and 4 in Fig. 2, corresponding to GLYCAM-1 phosphopeptides (1-53), showed a progressive increase as result of the freezing time.

Finally, HPLC fractions 5-10 in Fig. 2 were proved to contain β-casein fragments (1-68), and (1-105), lactosylated α-lactalbumin, N-linked glycated α-lactalbumin, α-lactalbumin, γ2-, γ3- and γ4-caseins, and β-lactoglobulin respectively (Table 2).

Peak areas of γ2- γ3- and γ4-caseins showed an opposite trend to that observed for GLYCAM-1 phosphopeptides. Their intensity decreased as storage freezing time increased. Finally, peak areas of α-lactalbumin derivatives and β-lactoglobulin slightly increased moving from fresh to prolonged-stored frozen milk. Compatibly with the well-known technical limitations of MALDI measurements, quantitative LC results were thus in good agreement with that deduced MALDI-TOF-MS polypeptide profiling, demonstrating the robustness of the latter approach when applied to buffalo milk samples.

classification of this cheese as European Union PDO product, being a parameter highly affecting its organoleptic characteristics. Nowadays, milk freshness has raised increasing importance for other high-quality cheeses, due to the resulting consequences on product properties. This study is the first one in which a dedicated MS approach was developed to face this issue, allowing the proposal of a rapid certification protocol for the starting material.

*Table 2. MALDI-TOF-MS analysis of selected chromatographic fractions of frozen buffalo milk. α--Lactalbumin derivative mass values were calculated on the basis of the primary structure of its B variant [5], reported modifications [6] and major glycans for bovine counterpart [7]. P, phosphate; f, fragment; *glycosylation is N-lynked glycosylation (triantennary -fucosylated monosialylated)*

Fraction	(NCBI/UniProt Code)	Polypeptide fragment	Modification
1	GLYCAM-1 (gi 594080212)	f(1-53)	2P-3P-4P-5P
2			1P-2P
3			0P-1P-2P
4			0P-1P
5	β-Casein (Q9TSI0)	f(1-68)	5P
6	β-Casein (Q9TSI0)	f(1-68)	1P
	β-Casein (Q9TSI0)	f(1-105)	2P-3P-4P-5P
7	α-Lactalbumin variant B	Intact	Lactosylation
		Intact	*glycosylation
8	α-Lactalbumin variant B	Intact	-
9	β-Casein (Q9TSI0)	γ2-Casein/ f(106–209)	-
		γ3-Casein/ f(108–209)	-
		γ4-Casein/ f(69–209)	-
10	β-Lactoglobulin (P02755)	Intact	-

3. CONCLUSION

This study was realized to meet the claimed demand of the producers and consumers for the certification of the raw milk used for manufacturing of Mozzarella di Bufala Campana PDO. In this context, milk freshness is a pre-requisite for the

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