

REGULAR ARTICLE

Carbonylation of milk powder proteins as a consequence of processing conditions

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During industrial treatments, milk proteins could be oxidatively modified, thus leading to the formation of modified/oxidised amino acid residues. The apparition of such modified residues may contribute to the formation of new immunologically reactive structures. Some of these adducts could, in an advanced stage, lead to cross-linked protein species whose proteolytic susceptibility would be drastically decreased. Such protein species, that are resistant to digestion, could also constitute major food allergens. Therefore, these oxidative protein modifications tend to increase the natural allergenicity of milk proteins. For these reasons, monitoring milk protein oxidative modifications could be very useful regarding both product quality and allergenicity issues. In the present paper, we highlight, using different analytical approaches, the preferential carbonylation of β -lactoglobulin (β -Lg) during industrial treatments of milk. This result is particularly interesting since native β -Lg represents one of the major milk allergens.

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1 Introduction

From a general point of view, thermal treatment improves food safety, organoleptic qualities, and storage time of dairy products. However, heat treatment, light exposure and oxygen may adversely cause a decrease of product quality, safety and nutritional value, by means of oxidative damage to food lipids and proteins. Several chemical markers are commonly used for assessing these modifications. For instance, monitoring of lipid oxidation, a major cause of quality deterioration during the processing and storage of milk-based prod-

ucts, is commonly performed by monitoring secondary lipid oxidation products such as malondialdehyde [1] or saturated volatile aldehydes like hexanal [2].

Another important target of modification in milk products is the protein entity. In particular, the well-known Maillard reaction, occurring during food manufacture and storage, is responsible for decreasing protein digestibility and nutritional supply [3]. Reactive dicarbonyl compounds or α,β -unsaturated aldehydes originating from Maillard reaction and/or from lipid oxidation can lead to the formation of protein-bound carbonyls [4]. In addition, free radicals produced by metal-catalysed oxidation in infant formulae [5] can also yield carbonylated amino acid [6]. Therefore, levels of protein carbonyl (PRC) content could be potentially considered as useful markers of protein oxidation in dairy products. For instance, Scaloni *et al.* [7] have recently reported significant differences between heat-treated milk samples, in terms of PRC. The apparition of such modified residues may contribute to the formation of new immunologically reactive structures [8]. Moreover, some of these adducts could, in an advanced stage, lead to cross-linked protein species whose

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Abbreviations: DNPH, 2,4-dinitrophenylhydrazine; HNE, 4-hydroxy-2-nonenal; β -Lg, β -lactoglobulin; MBP, myelin basic protein; PRC, protein carbonyl; UHT, ultra high temperature

proteolytic susceptibility would be drastically decreased [9, 10]. Such protein species, that are resistant to digestion, could also constitute major food allergens [11]. Therefore, these protein modifications could influence the allergenicity of milk proteins, such as β -lactoglobulin (β -Lg) [12]. For all these reasons, monitoring milk protein oxidative modifications could be very useful regarding both product quality and allergenicity issues.

In the present study, we focus on the identification and characterisation of carbonylated proteins during milk processing. Results from measurements of carbonylated proteins by UV spectroscopy and Western blot show that protein carbonylation significantly occur during milk processing. Moreover, the results reported here highlight the preferential oxidation of β -Lg. These data were confirmed and further completed by structural information obtained by means of a targeted proteomic approach involving the analysis of the tryptic hydrolysates from gel-separated proteins by MALDI-TOF MS using 2,4-dinitrophenylhydrazine (DNPH) as matrix [13].

2 Materials and methods

2.1 Materials

Pasteurised, ultra high temperature (UHT), and milk powder samples were purchased from local supermarkets. [Ala96] Myelin basic protein (87–99, MBP, mass 1529.1 Da) and 4-hydroxy-2-nonenal (HNE) were from Neosystem (Strasbourg, France) and Alexis (Laufelfingen, Switzerland) respectively. HNE-modified MBP peptide was synthesised as previously described [14]. All other chemical reagents were of analytical or HPLC grade.

2.2 Measurement of protein carbonyls by UV spectroscopy

Methodology was adapted from Levine *et al.* [15]. Briefly, an aliquot of aqueous milk solution (corresponding to *ca.* 2 mg proteins) was incubated with 10 mM DNPH in 2 N HCl (final concentrations, 0.5 mL final volume) for 30 min at room temperature. Milk proteins were then precipitated with 10% TCA (final concentration), the resulting precipitates recovered by centrifugation. Protein pellets were then washed three times with 1 mL of ethanol/ethyl acetate (50:50) to remove unreacted free DNPH reagent. Milk proteins were redissolved in 0.6 mL of 6 M urea, and solution absorbance measured at 370 nm on a Lambda Bio 20 spectrophotometer (Perkin-Elmer, Rotkreuz, Switzerland). The carbonyl content was calculated using a molar absorption coefficient of 22 000/M $\times$ cm [15], and the protein concentration determined using the Protein Assay kit from Bio-Rad (Munich, Germany). Results were expressed as nmol DNP/mg protein.

2.3 Gel electrophoresis with CBB staining

Gel electrophoresis was performed under denaturing and reducing conditions with 7 μ g of milk proteins loaded on 4–12% gels, as previously described [16].

2.4 Western blot immunoassays

2.4.1 Detection of carbonylated proteins

DNPH derivatisation was performed by incubating for 30 min at room temperature an aliquot of aqueous milk solution (corresponding to *ca.* 70 μ g proteins) in 6% SDS/5 mM DNPH / 5% TFA (final concentrations, 60 μ L final volume). Solution was then neutralised by adding 1/3 vol of reduction buffer (2 M Tris base containing 30% glycerol and 15% β -mercaptoethanol) [17], and 15 μ g of proteins were loaded on 4–12% gels as described in Section 2.3. Then proteins were electroblotted to NC membranes (Novex, Frankfurt, Germany) during 1 h 30 min at 25 V. Blots were blocked with 2% BSA in TBS Tween-20 (TBST) for 1 h at room temperature, and incubated overnight at 4°C with monoclonal antiDNP antibody (mouse IgE, 1:100 dilution; Sigma, Buchs, Switzerland) in 0.5% BSA in TBST, and then for 1 h at room temperature with horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody (1:15 000 dilution; Dako, Zug, Switzerland) in TBST. Bands containing DNP proteins were visualised by chemiluminescence using an ECL Western blotting detection kit (Amersham Biosciences, Buckinghamshire, England).

2.4.2 Detection of HNE-modified proteins

Sample preparation was performed as previously described [16] and 15 μ g of proteins were loaded on gel. Migration, transfer and blocking conditions were as described in Section 2.4.1 for oxidised proteins. Blots were incubated overnight at 4°C with polyclonal antiHNE antibody (rabbit, 1:5000 dilution; Alpha Diagnostic, San Antonio, USA) in 0.5% BSA in TBST, and then for 1 h at room temperature with HRP-conjugated goat antirabbit antibody (1:15 000 dilution; Dako) in TBST. Detection was also performed by chemiluminescence.

2.5 In-gel digestion procedure

This protocol was adapted from Rabilloud *et al.* [18]. In brief, each gel slice was cut into small pieces with a scalpel, washed with 100 μ L of 50 mM NH_4HCO_3 and agitated with a vortex mixer during 10 min. After removal of the supernatant, gel pieces were dehydrated with 100 μ L of ACN, and agitated for another 10 min. The supernatant was then removed. This operation was repeated twice. Gel pieces were then completely dried using a SpeedVac (*ca.* 15 min; Savant, Farmingdale, USA) before reduction/alkylation. Reduction was performed by covering gel pieces with 100 μ L of 10 mM DTT in 50 mM NH_4HCO_3 and the reaction allowed to proceed for

1 h at 57°C. After supernatant removal, 100 µL of 55 mM iodoacetamide in 50 mM NH_4HCO_3 was added and alkylation was performed in the dark at room temperature for 1 h. The supernatant was removed and the washing procedure with 100 µL of NH_4HCO_3 and ACN was repeated three times. Gel pieces were completely dried using a SpeedVac and swollen with 50 µL of TPCK-treated trypsin (12.5 ng/µL in 50 mM NH_4HCO_3). Digestion was performed at 35°C overnight. The gel pieces were centrifuged and 5 µL of H_2O /ACN/formic acid (25:70:5, v/v/v) was added. The mixture was sonicated for 5 min and centrifuged. The supernatant was recovered and the operation repeated twice with 50 µL of the same mixture. Pooled fractions were evaporated to dryness and resuspended in 10 µL of 0.1% TFA. Resulting digests were then concentrated/desalted using C_{18} ZipTip (Millipore, Bedford, MA) according to the manufacturer's instructions, except that peptide elution was performed with 10 µL of 60% ACN containing either 0.1% TFA for MALDI-TOF MS analysis or 0.1% formic acid for nanoESI-MS/MS analysis.

2.6 MS

2.6.1 MALDI-TOF MS analysis

MALDI-TOF MS experiments were carried out on an Autoflex instrument (Bruker Daltonik, Bremen, Germany). For PMF experiments, 1 µL of the peptides mixture was mixed with 1 µL of a saturated solution of 4-hydroxy- α -cyanocinnamic acid (CHCA) in 70% ACN containing 0.1% TFA, and 1 µL of the resulting mixture was deposited on the target and air-dried at room temperature. For identification of oxidised peptides, 1 µL of the peptides mixture was mixed with 1 µL of MBP-HNE peptide solution (gives *ca.* 80 fmol of MBP-HNE on MALDI plate) and 2 µL of DNPH solution (2 mg/mL in 50% ACN containing 0.1% TFA). After a 30 min stay at room temperature (off-line derivatisation required to improve experimental conditions), 1 µL of the mixture was deposited on the target and allowed to dry at room temperature as previously described [13].

2.6.2 NanoESI-MS/MS experiments

Two microlitres of the peptide solutions were loaded into a nanoESI capillary (Protana, Odense, Denmark). NanoESI-MS/MS experiments were performed on a TSQ 7000 API2 mass spectrometer (ThermoElectron, San Jose, CA) equipped with a nanoelectrospray source. Full-scan mass spectra were recorded by scanning an m/z range of 300–1500 with a scan time of 2 s and a spray voltage set between 800 and 1000 V. For CID experiments, the collision gas (argon) pressure was set at 2.7 mTorr (1 Torr = 133.3 Pa), and the scale of collision energy (E_{lab}) ranged from 15 to 35 eV.

3 Results and discussion

3.1 Formation of carbonylated proteins upon heat treatment

In order to evaluate the effect of thermal treatment on milk proteins, the PRC content of differently processed samples was measured using UV spectroscopy. Table 1 summarises the results obtained for pasteurised, UHT and powder milk samples. As expected, a positive correlation was found between PRC content and thermal treatment. For instance, the average PRC content is *ca.* two-fold higher in UHT samples compared to the pasteurised one. This increase is in good agreement with the results previously reported by Scaloni *et al.* [7]. Indeed, the authors reported no significant difference between pasteurised and raw milk samples [7], thus implying that pasteurised samples can be considered as low-oxidised reference samples. Compared to the pasteurised sample, milk powder samples exhibit a ten-fold higher median PRC content. It should be noticed that PRC content of milk powders exhibit a wide range of dispersion (from 8.6 to 60.9 nmol DNP/mg protein) compared to other milk samples, thus reflecting heterogeneous sample compositions and/or processing conditions. However, the lower PRC content of powder samples still remains two-fold higher than those measured on our UHT samples (Table 1). Therefore, PRC content could be used to assess oxidative damages of milk proteins occurring during processing/storage, as already noted for cheese manufacturing [19].

Table 1. PRC contents of processed milk measured by UV spectroscopy

	Pasteurised milk	UHT milk	Milk powders
<i>N</i>	1	3	8
Average, nmol/mg protein	1.9	4.1	25.7
Median, nmol/mg protein	1.9	3.9	19.4
Min., nmol/mg protein	–	3.8	8.6
Max., nmol/mg protein	–	4.5	60.9

3.2 Identification of carbonylated protein(s) in milk samples

To determine whether certain milk proteins were specifically carbonylated, the different samples were analysed by Western blot (Fig. 1). The results were in good agreement with those previously obtained by UV measurement. Only slight differences could be observed between UHT (lanes 1–3) and pasteurised (lane 4) samples. From the protein stain of these samples, which are not adapted samples as can be infant formulae, it could be seen that 80% of bovine milk consists of caseins (variants α_{S1} , α_{S2} , β and κ) and the remaining 20% is whey proteins (mainly α -lactalbumin and β -Lg).

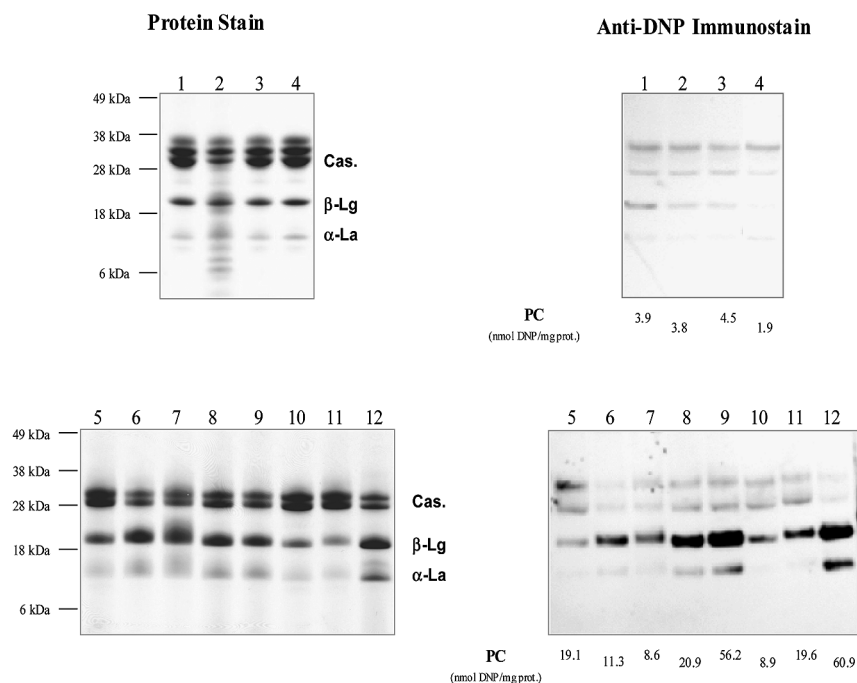


Figure 1. Detection of carbonylated proteins by Western blot immunoassay. Lanes 1–3: UHT samples, lane 4: pasteurised sample and lanes 5–12: milk powder samples. Molecular weights are shown on left, and PRC contents obtained by UV measurement are shown on the lower sides of immunoblots. Cas: caseins, β-Lg: β-lactoglobulin and α-La: α-lactalbumin.

According to these results, it seems that whey proteins, and more especially β-Lg, constitute preferential targets of oxidation in milk powders (lanes 5–12), whereas Scaloni *et al.* [7] showed that caseins constitute the most oxidised proteins in sterilised milk samples. Nevertheless, the susceptibility of β-Lg toward modification in milk powder samples is not unprecedented. For instance, Marvin *et al.* [20] have already demonstrated that β-Lg was among the most lactosylated proteins in heated milk powders, despite the lower quantities originally present in cow milk. In the same way, Galvani *et al.* [21] observed similar lactosylation patterns for whey proteins and caseins, and Guyomarç'h *et al.* [22] identified whey proteins as being the most sensitive indicators of the Maillard reaction in milk. Therefore, oxidative modifications of whey proteins seem to occur preferentially during manufacturing of milk powders. This could be potentially indebted to the high hydrophilicity of whey proteins.

PRC can be generated by several different pathways, *i.e.* by direct oxidation of amino acid side chains or *via* indirect reactions with lipid oxidation products or glycation/glycoxidation products [4], although neither Amadori nor Heyns products contribute significantly to the DNPH reaction [23].

Among the different major lipid oxidation products, HNE can react with histidine, lysine and cysteine residues preferentially *via* a Michael addition, to form derivatives that possess a free carbonyl function [24]. HNE adducts constitute antigenic groups that can be detected by immunochemical techniques, such as Western blot [25]. In an aim of establishing the contribution of lipid oxidation products to PRC formation in milk samples, the most oxidised milk samples, *i.e.* milk powders, were analysed by this technique.

3.3 Contribution of HNE adducts to PRC in milk samples

Figure 2 represents the antiHNE immunoblot of the milk powder samples. These results highlight the existence of HNE adducts on milk proteins and confirm the previous results regarding the specific carbonylation of β-Lg obtained by Western blot. However, the amounts of HNE adducts do not seem to be strictly correlated with those of carbonylated β-Lg. This discrepancy could be easily explained by considering the variations of compositions and processing conditions existing between the various samples. Moreover, it should be noted that no significant

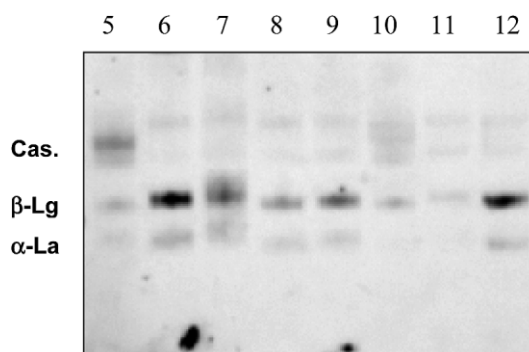


Figure 2. Detection of HNE-modified proteins by Western blot immunoassay. Lanes 1–3: UHT samples, lane 4: pasteurised sample and lanes 5–12: milk powder samples. Molecular weights are shown on left, and PRC contents obtained by UV measurement are shown on the lower sides of immunoblots. Cas: caseins, β-Lg: β-lactoglobulin and α-La: α-lactalbumin.

formation of HNE adducts was observed in liquid samples (data not shown). Therefore, generation of HNE adducts seems to be favoured by 'hard manufacturing conditions'.

The presence of HNE adducts in infant milk powder samples could be explained by considering that vegetal oils, containing polyunsaturated fatty acids such as arachidonic acid or linoleic acid (*w*-6 fatty acids are precursors of HNE), are often supplemented into infant formulae for nutritional purposes [26]. For instance, quite high levels of HNE (up to *ca.* 1 mg/kg) have been found in food systems such as oils or other lipid-containing foodstuffs [27]. This is corroborated by the formation of malondialdehyde, another product of polyunsaturated fatty acid oxidation, which is well-known to occur in milk-based samples [1]. Nevertheless, to further confirm the presence of HNE adduct(s), we have used an alternative method involving a MALDI-TOF MS strategy with DNPH as MALDI matrix [13].

3.4 Identification of HNE binding sites

Recent proteomic technologies have been developed and applied for the identification of carbonylated proteins. For instance, oxidised plasma proteins in Alzheimer's disease have been identified by immunological (antiDNP) 2-DE and MALDI-TOF MS analysis [28], and carbonylated proteins in aged mice brain by LC-MS/MS analysis coupled with hydra-

zide biotin-streptavidin methodology [29]. Although several oxidised proteins have been successfully identified and proved to be in good agreement with previously reported data, no identification/confirmation of the sites of carbonylation has been achieved. This task is rendered difficult both by the numerous carbonylated amino acid residues that could be formed, and by the low stoichiometry of the reactions involved. Therefore, regarding specific carbonylations such as HNE modifications, several specific strategies involving precursor ion scanning [30] or affinity enrichment methodologies [14, 31], followed by MS/MS characterisation have been developed. Recently, we developed a MALDI-TOF MS strategy using DNPH as MALDI matrix. This method converts HNE-modified peptides into the corresponding hydrazones, and the resulting products can be detected with a high sensitivity [13]. In the framework of the present study, spots corresponding to β -Lg from samples 5 and 12 (low and high content of HNE adducts respectively) were excised from gel, in-gel digested and analysed by MALDI-TOF MS using successively CHCA and DNPH as MALDI matrices.

Regarding both samples 5 and 12, the identity of β -Lg could be easily confirmed in both cases by PMF, with CHCA matrix (Fig. 3, CHCA). When DNPH was used as matrix, drastic differences could be noted between MALDI mass spectra of the two milk samples (Fig. 3, DNPH). Only the internal standard (MBP-HNE), artificially modified with

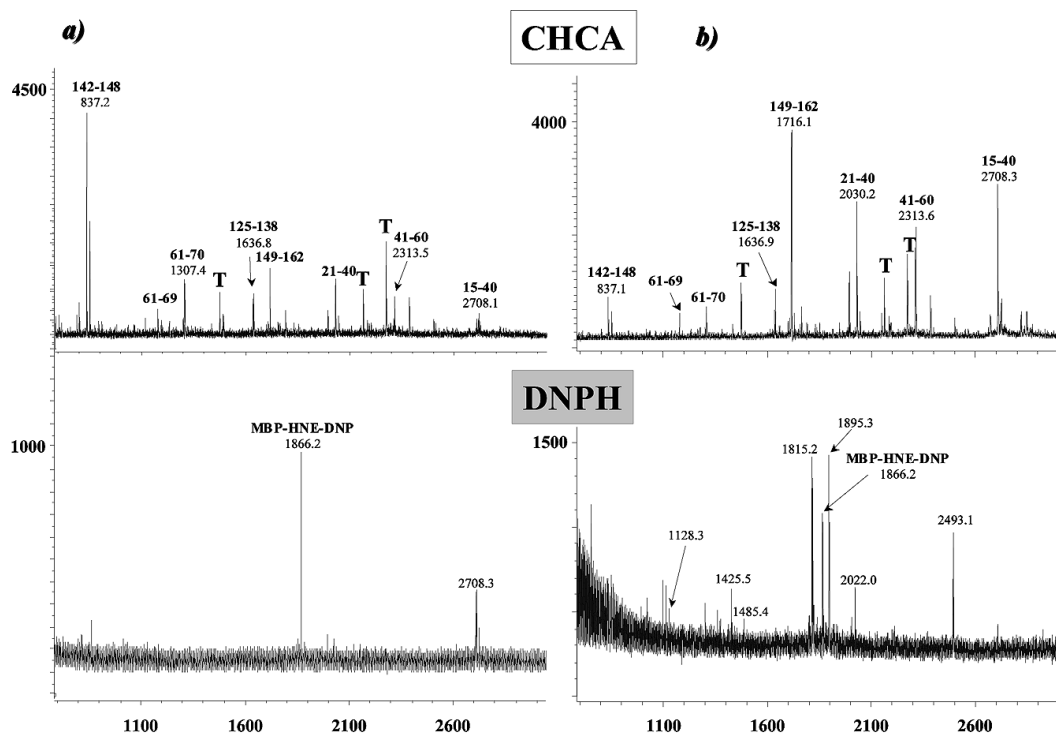


Figure 3. MALDI mass spectra (reflectron mode) using CHCA and DNPH as matrices of the tryptic digests of β -Lg from milk powders with low (a) and high oxidation degree (b), *i.e.* samples 5 and 12 respectively. An equal quantity of HNE-modified MBP peptide (MBP-HNE) was added to each sample to enable quantitative comparisons of the extent of peptide carbonylation. T represents ions originating from trypsin autolysis.

HNE and added to the protein digest before addition of DNPH, is evident at m/z 1866 in the analysis of the MALDI/DNPH spectrum corresponding to the weakly oxidised sample 5. Indeed, a single ion at m/z 2708, corresponding to a native β -Lg tryptic peptide (15–40), could be observed in this mass spectrum. The visualisation of such native peptides can sometimes occur in MALDI/DNPH spectra [13]. This is especially true for peptides with a C-terminal arginine, which show higher MALDI responses compared to Lys-terminated peptides [32, 33]. Thus, it is of prime importance to check the presence of 180 U differences (corresponding to the dinitrophenylhydrazone moiety) between CHCA and DNPH MALDI mass spectra, to confirm the carbonylated nature of the incriminated peptides.

In the MALDI/DNPH spectrum of the highly oxidised sample 12, many other ions are visible and show comparable intensities to that derived from the internal standard. These data confirm those previously obtained by Western blot and UV measurement. The carbonylated nature of the corresponding peptides could be hypothesised since 180 U differences could be observed between CHCA and DNPH MALDI mass spectra [13]. Among these ions, by comparison with theoretical tryptic peptide masses of β -Lg, the ion at m/z 1485 can correspond to the dinitrophenylhydrazone of HNE-modified peptide (139–148: expected masses of 1149 and 1305 Da for the native and HNE-modified tryptic peptides respectively). We have used nanoESI-MS/MS to further confirm the identity of this peptide. Thus, the CID experiment was performed on the doubly charged species of the corresponding peptide without the DNP moiety (*i.e.* m/z 653.3) (Fig. 4). The different fragment ions are consistent with the sequence ALKAL PMH*IR (139–148), where H* is an HNE-modified histidine residue. For instance, the ions at m/z 139 and 266 are characteristic of a protonated dehydrated HNE moiety and an HNE-modified histidine immonium ion respectively [34, 35]. Therefore, His₁₄₆ could be confirmed as

being HNE modified. His residues are well-known to constitute targets of HNE modification, and the two His residues of β -Lg (His₁₄₆ and His₁₆₁) are modified upon incubation with HNE [36]. Besides this His residue, no other amino acid residues were found to be HNE modified, even using selective strategies [14, 30] (data not shown).

From these data, it is difficult to conclude on a strict regiospecificity of HNE modification, since during milk heating the dimeric form of β -Lg dissociates into monomers and formation of aggregates can occur [37]. Therefore, considerable changes in β -Lg conformation occur during milk powder processing, thus potentially exposing amino acids that are normally buried in the native structure and further increasing their original reactivity.

In the MALDI/DNPH spectrum of sample 12 (Fig. 3), many ions could be observed and potentially attributed to carbonylated peptides. Indeed, several examples from the literature tend to demonstrate that using DNPH as MALDI matrix constitutes an efficient way of detecting carbonylated peptides within unfractionated digests [13, 38–40]. The structures of corresponding modified peptides have not been yet fully elucidated. Nevertheless, comparison of these ions with those from native peptides leads to the observation of potential 178, 179 and 195 U differences. For instance, 178 and 179 U differences could potentially correspond to the dinitrophenylhydrazones of oxidised Thr/Tyr and Lys respectively [4, 41, 42]. This is not surprising since, for instance, Lys residues are among the most readily oxidised amino acid residues upon metal-catalysed oxidation [43], which can occur in infant formulae fortified with metals and vitamins [5, 44]. Moreover, it should be noted that Lys residues from β -Lg, representing the main targets of the Maillard reaction [45], could also be oxidatively deaminated *via* this particular modification pathway [46]. For instance, ions at m/z 1815 (125–138) and m/z 2493 (41–60) can potentially correspond to such Thr₁₂₅- and Lys₄₇-modified species

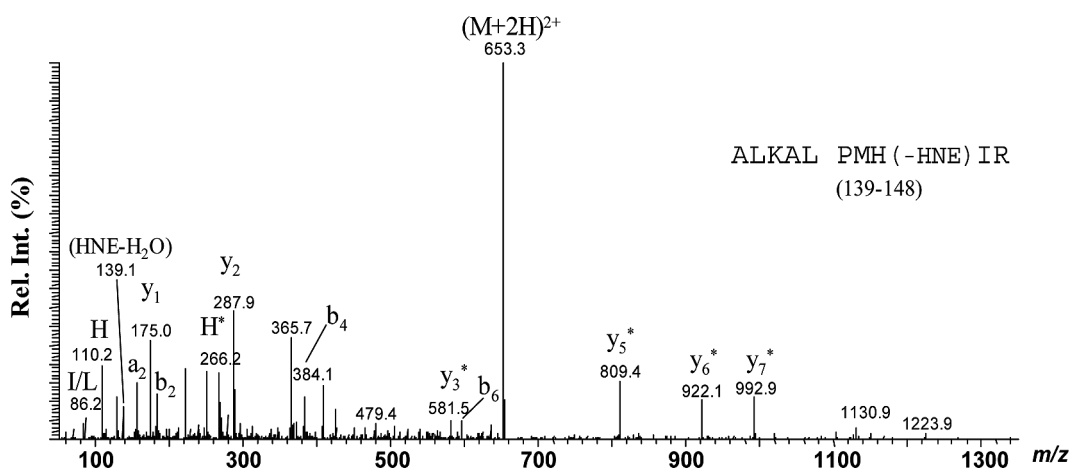


Figure 4. NanoESI CID mass spectrum of m/z 653.3 ($[M + 2H]^{2+}$) corresponding to the peptide fragment (139–148) from β -Lg containing an HNE-modified histidine residue. Fragment ions are labelled according to the nomenclature of Biemann [48].

respectively. To facilitate peptide assignment and interpretation of Fig. 3, the primary sequence of β -Lg (variant B) is given in Fig. 5.

Moreover, a 195 U difference has been shown to correspond to the dinitrophenylhydrazine of the *N*-terminal peptide whose both the *N*-terminal and the Met residues were oxidised upon HOCl treatment [47]. In the case of β -Lg, the dinitrophenylhydrazine of such doubly oxidised *N*-terminal peptide LIVTQ TMK (1–8, M_r 932) would lead to an ion at m/z 1128, as observed in the MALDI/DNPH spectrum of sample 12 (Fig. 3). The Met₇ was confirmed as being oxidised by nanoESI-MS/MS (data not shown), but the *N*-terminal oxidation has not yet been clearly evidenced, this is in progress. Nevertheless, it seems that such targeted proteomic approach using MALDI/DNPH could be an efficient mean to monitor carbonylated proteins after gel electrophoresis separation.

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1  LIVTQ TMKGL DIQKV AGTWY SLAMA ASDIS 30
31  LLDAQ SAPLR VYVEE LKPTP EGDLE ILLQK 60
61  WENGE CAQKK IIAEK TKIPA VFKID ALNEN 90
91  KVLVL DTDYK KYLLF CMENS AEPEQ SLACQ 120
121 CLVRT PEVDD EALEK FDKAL KALPM HIRLS 150
151 FNPTQ LEEQC HI

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Figure 5. Amino acid sequence of β -Lg (variant B).

4 Concluding remarks

In conclusion, we have shown that modifications of milk proteins (especially of β -Lg) during heat treatment involve formation of modified amino acid residues (e.g. carbonylated or HNE-modified residues). The apparition of such modified residues may contribute to the formation of new immunologically reactive and/or biologically active structures. Therefore, monitoring milk protein oxidative modifications could be very useful regarding both product quality and allergenicity issues.

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5 References

- [1] Cesa, S., *J. Agric. Food Chem.* 2004, 52, 2119–2122.
- [2] Ulberth, F., Roubicek, D., *Int. Dairy J.* 1995, 5, 523–531.
- [3] Finot, P. A., Deutsch, R., Bujard, E., *Prog. Food Nutr. Sci.* 1981, 5, 345–355.
- [4] Stadtman, E. R., *Science* 1992, 257, 1220–1224.
- [5] Almaas, R., Rootwelt, T., Oyasaeter, S., Saugstad, O. D., *Eur. J. Pediatr.* 1997, 156, 488–492.
- [6] Stadtman, E. R., Levine, R. L., *Amino Acids* 2003, 25, 207–218.
- [7] Scaloni, A., Perillo, V., Franco, P., Fedele, E., Froio, R., Ferrara, L., Bergamo, P., *Biochim. Biophys. Acta* 2002, 1598, 30–39.
- [8] Wal, J. M., *Allergy* 2003, 58, 727–729.
- [9] Burcham, P. C., Kuhan, Y. T., *Arch. Biochem. Biophys.* 1997, 340, 331–337.
- [10] Friguet, B., Stadtman, E. R., Szveda, L. I., *J. Biol. Chem.* 1994, 269, 21639–21643.
- [11] Astwood, J. D., Leach, J. N., Fuchs, R. L., *Nat. Biotechnol.* 1996, 14, 1269–1273.
- [12] Wal, J. M., *Allergy* 2001, 56 (Suppl. 67), 35–38.
- [13] Fenaille, F., Tabet, J. C., Guy, P. A., *Anal. Chem.* 2004, 76, 867–873.
- [14] Fenaille, F., Tabet, J. C., Guy, P. A., *Anal. Chem.* 2002, 74, 6298–6304.
- [15] Levine, R. L., Garland, D., Oliver, C. N., Amici, A., Climent, I., Lenz, A. G., Ahn, B. W., Shaltiel, S., Stadtman, E. R., *Methods Enzymol.* 1990, 186, 464–478.
- [16] Fenaille, F., Morgan, F., Parisod, V., Tabet, J. C., Guy, P. A., *Rapid Commun. Mass Spectrom.* 2003, 17, 1483–1492.
- [17] Shacter, E., *Methods Enzymol.* 2000, 319, 428–436.
- [18] Rabilloud, T., Strub, J. M., Lucie, S., Van Dorsselaer, A., Lunardi, J., *Proteomics* 2001, 1, 699–704.
- [19] Fedele, E., Bergamo, P., *J. Food Sci.* 2001, 66, 932–935.
- [20] Marvin, L. F., Parisod, V., Fay, L. B., Guy, P. A., *Electrophoresis* 2002, 23, 2505–2512.
- [21] Galvani, M., Hamdan, M., Righetti, P. G., *Rapid Commun. Mass Spectrom.* 2000, 14, 1889–1897.
- [22] Guyomarç'h, F., Warin, F., Muir, D. D., Leaver, J., *Int. Dairy J.* 2000, 10, 863–872.
- [23] Liggins, J., Furth, A. J., *Biochim. Biophys. Acta* 1997, 1361, 123–130.
- [24] Esterbauer, H., Schaur, R. J., Zollner, H., *Free Radic. Biol. Med.* 1991, 11, 81–128.
- [25] Uchida, K., Szveda, L. I., Chae, H., Stadtman, E. R., *Proc. Natl. Acad. Sci. USA* 1993, 90, 8742–8746.
- [26] Carver, J. D., *Am. J. Clin. Nutr.* 2003, 77, 1550S–1554S.
- [27] Lang, J., Celotto, C., Esterbauer, H., *Anal. Biochem.* 1985, 150, 369–378.
- [28] Choi, J., Malakowsky, C. A., Talent, J. M., Conrad, C. C., Gracy, R. W., *Biochem. Biophys. Res. Commun.* 2002, 293, 1566–1570.
- [29] Soreghan, B. A., Yang, F., Thomas, S. N., Hsu, J., Yang, A. J., *Pharm. Res.* 2003, 20, 1713–1720.
- [30] Sangvanich, P., Mackness, B., Gaskell, S. J., Durrington, P., Mackness, M., *Biochem. Biophys. Res. Commun.* 2003, 300, 501–506.
- [31] Ho, J. T. C., Gaskell, S. J., Proceedings of the 51st ASMS Conference on Mass Spectrometry and Allied Topics 2003.
- [32] Krause, E., Wenschuh, H., Jungblut, P. R., *Anal. Chem.* 1999, 71, 4160–4165.
- [33] Fenaille, F., Morgan, F., Parisod, V., Tabet, J. C., Guy, P. A., *J. Mass Spectrom.* 2004, 39, 16–28.
- [34] Bolgar, M. S., Gaskell, S. J., *Anal. Chem.* 1996, 68, 2325–2330.

- [35] Fenaille, F., Guy, P. A., Tabet, J. C., *J. Am. Soc. Mass Spectrom.* 2003, **14**, 215–226.
- [36] Bruenner, B. A., Jones, A. D., German, J. B., *Chem. Res. Toxicol.* 1995, **8**, 552–559.
- [37] Croguennec, T., Bouhallab, S., Molle, D., O’Kennedy, B. T., Mehra, R., *Biochem. Biophys. Res. Commun.* 2003, **301**, 465–471.
- [38] Peng, J., Schmidt, B., von Figura, K., Dierks, T., *J. Mass Spectrom.* 2003, **38**, 80–86.
- [39] Fang, Q., Peng, J., Dierks, T., *J. Biol. Chem.* 2004, **279**, 14570–14578.
- [40] Marquardt, C., Fang, Q., Will, E., Peng, J., von Figura, K., Dierks, T., *J. Biol. Chem.* 2003, **278**, 2212–2218.
- [41] Yang, C. Y., Gu, Z. W., Yang, H. X., Yang, M., Wiseman, W. S., Rogers, L. K., Welty, S. E., Katta, V., Rohde, M. F., Smith, C. V., *Free Radic. Biol. Med.* 1997, **22**, 1235–1240.
- [42] Handelman, G. J., Nightingale, Z. D., Dolnikowski, G. G., Blumberg, J. B., *Anal. Biochem.* 1998, **258**, 339–348.
- [43] Requena, J. R., Chao, C. C., Levine, R. L., Stadtman, E. R., *Proc. Natl. Acad. Sci. USA* 2001, **98**, 69–74.
- [44] Champagne, E. T., Hinojosa, O., Clemetson, C. A. B., *J. Food Sci.* 1990, **55**, 1133–1136.
- [45] Morgan, F., Leonil, J., Molle, D., Bouhallab, S., *Biochem. Biophys. Res. Commun.* 1997, **236**, 413–417.
- [46] Akagawa, M., Sasaki, T., Suyama, K., *Eur. J. Biochem.* 2002, **269**, 5451–5458.
- [47] Yang, C., Wang, J., Krutchinsky, A. N., Chait, B. T., Morrisett, J. D., Smith, C. V., *J. Lipid Res.* 2001, **42**, 1891–1896.
- [48] Biemann, K., *Biomed. Environ. Mass Spectrom.* 1988, **16**, 99–111.