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Journal of Chromatography A, 926 (2001) 105–112

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Characterisation of modified whey protein in milk ingredients by liquid chromatography coupled to electrospray ionisation mass spectrometry

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Abstract

Whey proteins are an important ingredient in the food industry. We have investigated the protein composition of commercial whey samples by liquid chromatography coupled to electrospray ionisation mass spectrometry on a time-of-flight instrument. We found that industrial whey protein contains a multitude of different modifications, ranging from almost native proteins through different degrees of glycosylation and oxidation up to almost completely oxidised forms. The information obtained allows characterisation of the influence of industrial processing on protein modifications and classification of whey protein-based ingredients or milk powders from different suppliers.

Keywords: Milk; Food analysis; Whey proteins; Proteins

1. Introduction

Whey proteins are used in the food industry both for their nutritional and functional properties. They serve as skim milk replacers in ice cream, as fat replacers, whole egg replacers, in dairy and non-dairy dessert products and in enteral nutrition, infant nutrition and sport nutrition [1]. Another important field of application is foamed products based on milk, such as milk shakes, fromage frais desserts, yoghurt foams and cappuccino.

Although it is known that the nutritional and functional properties of whey are linked to the native/denatured state of proteins [1,2], the detailed relationship between structure and function is far from understood. Similar to this, different industrial processing techniques will result in different protein modifications, which in turn will influence the physical properties of the protein mixtures. As a consequence, the food industry is facing the problem that nominally identical products from different suppliers can exhibit very different behaviour regarding foam formation and foam stability.

To understand the detailed composition of various samples from different origin, we have developed an analytical method using separation by reversed-phase high-performance liquid chromatography (HPLC) coupled to detection by electrospray ionisation mass spectrometry (ESI–MS). This combination has previously proven to be valuable for the analysis of milk proteins [3] and milk peptides [4]. The results are reported here.

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0021-9673/01/$ – see front matter
PII: S0021-9673(01)00710-5

2. Experimental

2.1. Chemicals

Peptide standards were obtained from Sigma (Buchs, Switzerland). All solvents were of analytical or HPLC grade and were purchased from Merck (Dietikon, Switzerland). Water was either purified in the laboratory using a Millipore Milli-Q water purification system (Volketswil, Switzerland) or was HPLC-grade (Merck).

We have applied the method described below on a number of samples. A representative selection includes the following commercial products: Globulal 80 (Meggle, Wasserburg, Germany), described by the manufacturer as an acid whey protein concentrate. Protarmor 907 NK (Armor proteines, St. Brice en Coglès, France), an enriched β-lactoglobulin protein isolate obtained by ion-exchange chromatography with low denaturation. Nollibel Z 142 (New Zealand Dairy Board, Wellington, New Zealand), non-commercial blends for ice cream produced on a pilot scale. Domovictus 300 HP (Domo Food Ingredients, Beilen, Netherlands), a whey protein concentrate from partially delactosylated and demineralised whey. Lacprodan DI-9224 (MD Foods, Viby J., Denmark), a heat-stable whey protein isolate.

2.2. Sample preparation

The samples were used ''as received'' and dissolved in distilled water at a concentration of 5 mg whey protein/ml, then acidified to pH 4.6 with 32% hydrochloric acid. After 30 min, samples were centrifuged for 30 min at 4000 *g* and the insoluble material was discarded. The supernatant was filtered through 0.45 μm filters (Gelman Sciences, MI, USA) and 5 μl were injected onto the HPLC system (see below) from polyethylene vials.

2.3. Liquid chromatography

Separations were performed by reversed-phase HPLC on a PLRP-S column (150×4.6 mm; Polymer Labs. 1512.3801; via Ercatech, Bern, Switzerland) using a Waters (Rupperswil, Switzerland) HPLC system, consisting of a type 757 autosampler, a 600-MS pump with system controller and column oven and a type 486-MS UV detector. Solvent A was 5% formic acid in water, solvent B consisted of 800 ml acetonitrile, 200 ml water and 50 ml formic acid. Elution was performed at a column temperature of 50°C and a flow-rate of 1.0 ml/min with a linear gradient from 43 to 63% B in 26 min, 63 to 100% B in 2 min with a 5-min hold and back to initial conditions in 2 min. The column was held at initial conditions for further 5 min before sample injection. The total run time was 40 min.

UV absorption at 280 nm was recorded using an analogue input to the mass spectrometer's data system. Start/stop signals were provided by relay contacts on the HPLC instrument, which were closed during separation; the relay signal was fed to the mass spectrometer's ''I/O port'' and was used to determine analysis duration and to automate batch analyses. The eluent of the HPLC was split at a ratio of 1:50 using an AcuRate flow splitter (LC Packings, via Omnilab, Mettmenstetten, Switzerland) so that approximately 20 μl/min entered the electrospray ion source of the mass spectrometer.

2.4. Mass spectrometry

The mass spectrometer used here was a PerSeptive Biosystems MARINER time-of-flight instrument equipped with an electrospray ion source. The electrospray voltage was set to 3.25 kV, the nozzle voltage to 90 V and the nozzle temperature to 150°C. Data acquisition and data evaluation were performed on a generic, pentium-class personal computer using the instrument manufacturer's software, ver. 2.1.0.0. Mass spectra were acquired in profile mode without threshold and over the mass range from m/z 301 to m/z 2000 in 1.5 s. The instrument's mass resolution was about 5000 (50% valley definition).

The spectra of multiply charged ions were deconvoluted using the same software. Calibration of the mass scale was established using a mixture of horse heart myoglobin [5] and the tetrapeptide MRFA. Furthermore, the instrument calibration was verified regularly using the peaks of α-lactalbumin and β-lactoglobulin B as internal standards.

Protein sequence data were obtained from the SwissProt database [6]. Calculation of the protein masses was performed on a DECstation 5100 running under Ultrix 4.4 (Digital Equipment) using the

Finnigan (San Jose, CA, USA) BioWorks software Ver. 8.2 which is part of Finnigan's ICIS2 package.

3. Results and discussion

3.1. Separation and detection

Traditionally, peptide separations are performed by reversed-phase HPLC with about 0.1% trifluoroacetic acid (TFA) as ion pairing reagent in the mobile phase. However, depending on the construction of the mass spectrometer's electrospray interface, the use of TFA is disadvantageous as it may lead to decreased sensitivity [7,8]. This observation was confirmed with the present MS apparatus, where the use of TFA in the mobile phase led to almost complete loss of sensitivity (data not shown). Replacing TFA by 5% formic acid resulted both in greatly enhanced MS sensitivity and in improved chromatographic separation compared to earlier runs of the same samples using TFA.

Electrospray ionisation yields spectra of multiply charged ions, which can be transformed to a zero-charge scale [9]. While conventional quadrupole mass analysers allow the acquisition of raw spectra with approximately unit resolution, a time-of-flight instrument as employed in this study usually provides a working resolution of 5000 (50% valley definition) or higher. The use of a mass spectrometer with increased resolution is especially beneficial for protein analyses, as the improved ''granularity'' of the raw data enables finding of finer structures in the spectra. As an example, Fig. 1a shows the raw and Fig. 1b the deconvoluted spectrum for a protein mixture, obtained by averaging over both β-lactoglobulin peaks from the chromatogram shown in Fig. 2.

The error in mass assignment of the acquired raw data was generally ± 0.03 m/z or better, and accuracy after deconvolution was better than 0.01% of the value calculated from the sequence, that is, better that ± 2 u at M_r 18 000. This is superior to any other technique used for the determination of molecular mass of proteins.

3.2. Unmodified proteins

The principal proteins in these mixtures are α-lactalbumin, β-lactoglobulin A (β-LgA) and β-lactoglobulin B (β-LgB). Under the chromatographic conditions given above, unmodified α-lactalbumin

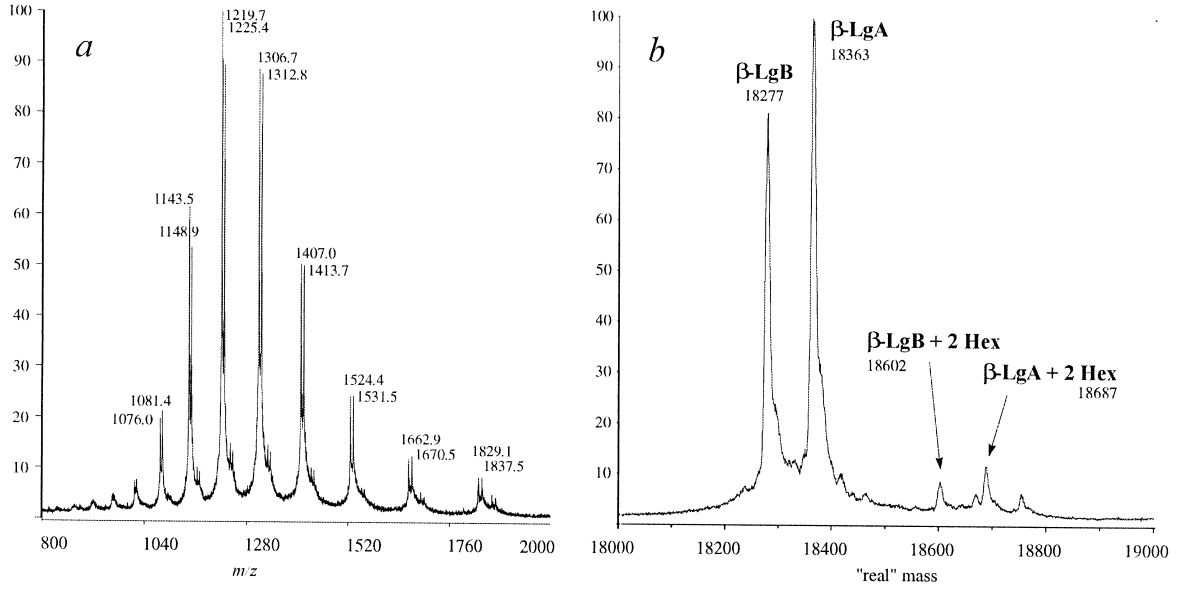

Fig. 1. Mass spectra obtained from the Protarmor 907 NK sample, (a) raw data and (b) deconvoluted spectrum. Data were averaged over both β-lactoglobulin peaks from Fig. 2. The proteins are almost pure and show only minor glycosylation.

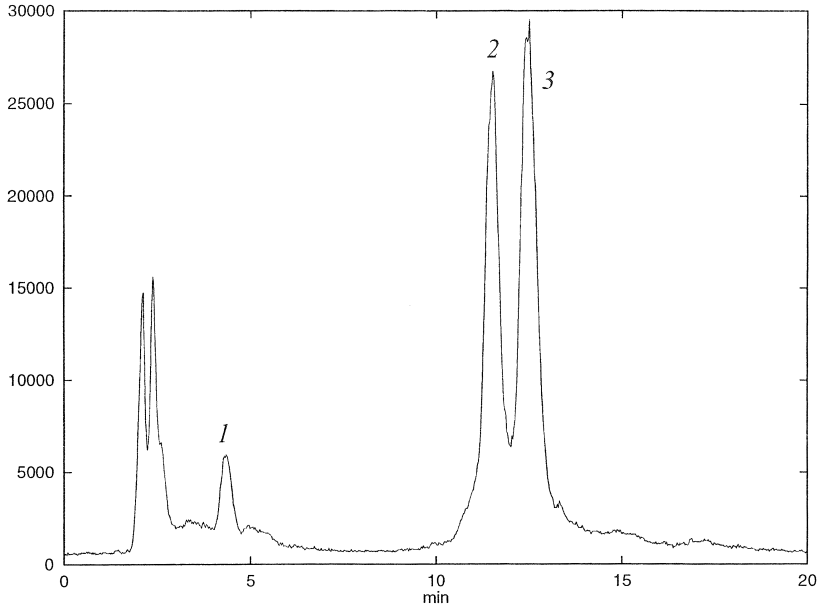

Fig. 2. Total ion chromatogram (TIC) obtained from Protarmor 907 NK. The peaks are: 1, α-lactalbumin; 2, β-lactoglobulin B; 3, β-lactoglobulin A. See text for discussion.

elutes at about 4.3 min. The two genetic variants of β-lactoglobulin are fully separated, as β-lactoglobulin B elutes at about 11.5 min and β-lactoglobulin A 1 min later. An example is shown in Fig. 2 using Protarmor 907NK, which is an almost pure protein mixture with minor traces of glycosylation and no visible oxidation. The retention times of the individual compounds were verified using pure standards and were stable within a time window of ±0.1 min throughout this study.

In addition to the three proteins above, we observed that almost all of the commercial samples contained proteins which had undergone a variety of modifications. The examples discussed below are representative for the diversity of compounds on the market.

3.3. Glycosylation products

The prevailing modification encountered here was the addition of hexose units, commonly referred to as glycosylation or glycation (although not only glucose but also lactose and other sugars may be involved). The natural presence of large amounts of lactose in milk suggests that the most abundant glycosylation can be expected to be a lactosylation. This is in agreement with both literature [10] and with our experimental data, as we found a mass difference of

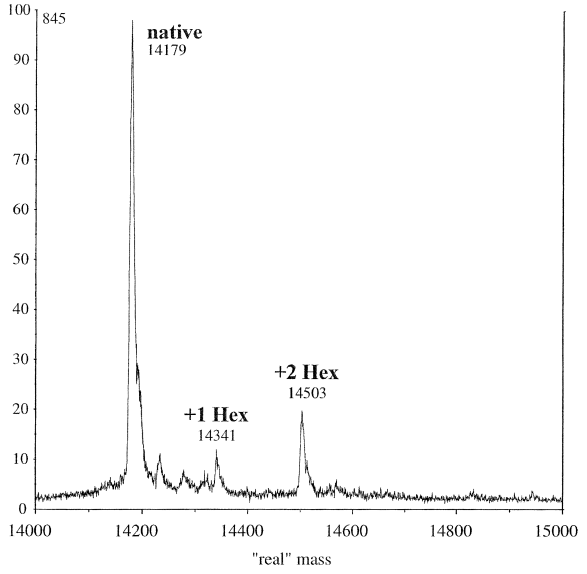

Fig. 3. Deconvoluted mass spectrum obtained over the α-lactalbumin peak from Nolibel Z141. The spectrum shows multiple glycosylation. See text for discussion.

$n\times324$ mass units ($0<n<4$) for almost all samples. An example is given in Fig. 1: in addition to the unmodified protein at M_r 18 277 and 18 363, peaks at M_r 18 602 and 18 687 are observed, corresponding to two modified sites. The same observation was made for α-lactalbumin (Fig. 3).

The presence of large amounts of lactose in milk favours the formation of a lactosyl-amino compound that undergoes Amadori rearrangement to form a Maillard product [11]. As ''suitable'' modification sites, Burr et al. [10] suggested lysine neighbouring either to another lysine or glutamic acid, i.e. Lys69–Lys70, Glu74–Lys75, Lys100–Lys101 and Glu134–Lys135, which fits the observation of up to four modified sites. Léonil et al. described different reaction sites [12], but it should be noted that these experiments were performed under conditions that are different from industrial processing. Still, we can state that the ''additional'' compounds in Fig. 1 represent the formation of one lactulosyllysine adduct each.

The total ion current traces obtained for Nollibel Z142 (Fig. 4a) and Globulal 80 (Fig. 4b) are typical for samples with moderate and with strong lactosylation, respectively. The glycosylated proteins elute slightly earlier than their corresponding non-modified counterparts, and their retention times decrease with increasing degree of glycosylation. These peaks generally overlap with the native proteins, so they can not be differentiated by conventional HPLC–UV.

It should be noted that the MS data alone do not allow for a quantification of the individual com-

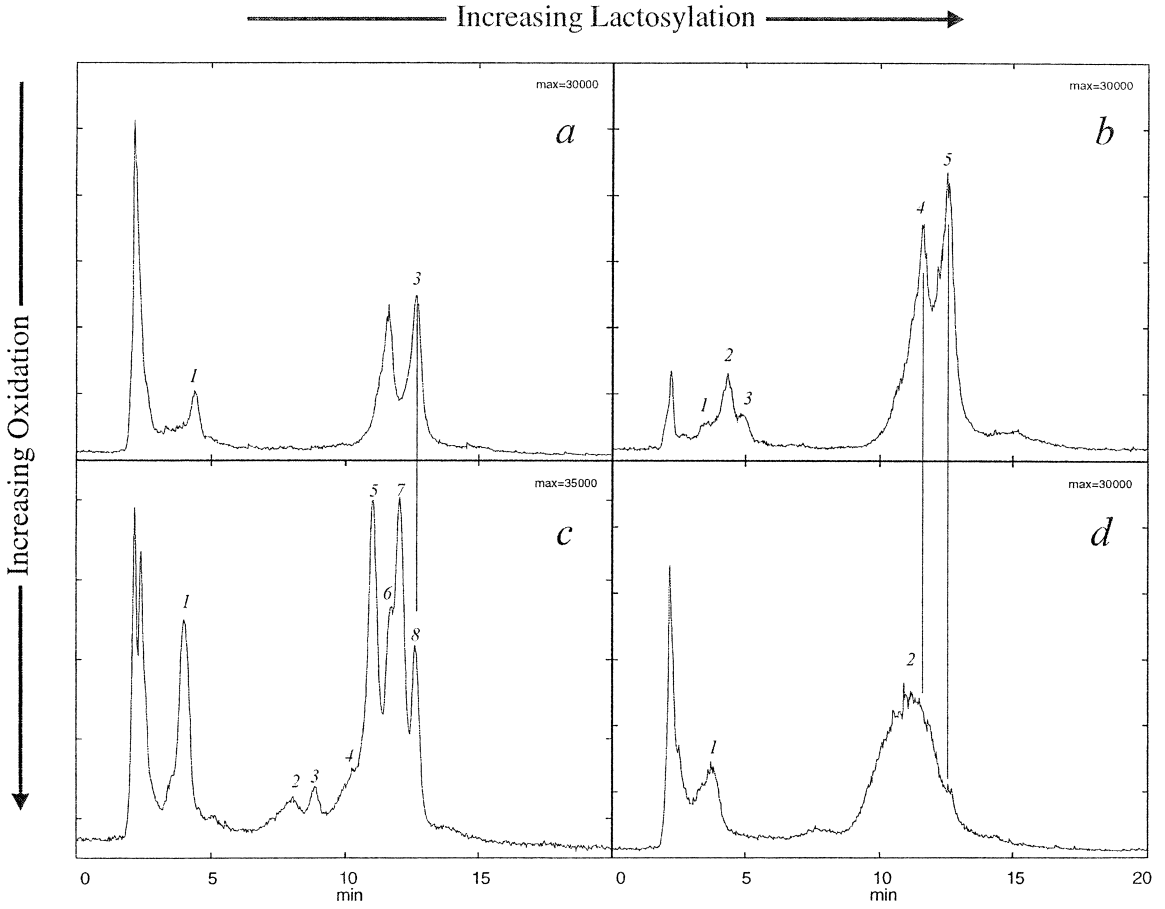

Fig. 4. Total ion chromatogram (TIC) obtained from (a) Nollibel Z142, (b) Globulal 80, (c) Lacprodan DI-9233, (d) Domovictus 300 HP. See Table 1 for identification of peaks.

Table 1
Identification of peaks in Figs. 2 and 4

Fig.	Peak	t_R (min)	Quantity	Average M_r	Compound
2	1	4.3	++	14178±1	α-Lactalbumin
	2	11.5	++++	18278±1	β-Lactoglobulin B
	3	12.5	++++	18364±1	β-Lactoglobulin A
4a	1	4.3	+++	14179±2	α-Lactalbumin
			+	14341±2	α-Lac+1 Hex
			++	14502±2	α-Lac+1 lactose
	2	11 . . . 12	++++	18278±2	β-Lactoglobulin B
			+	18440±3	β-LgB+1 Hex
			+++	18602±3	β-LgB+1 lactose
			+	18763±3	β-LgB+3 Hex
			++	18927±3	β-LgB+2 lactose
	3	12 . . . 13	++++	18364±2	β-Lactoglobulin A
			+	18525±3	β-LgA+1 Hex
			+++	18687±3	β-LgA+1 lactose
			+	18849±3	β-LgA+3 Hex
			++	19012±3	β-LgA+2 lactose
4b	1	4.1	+	14502±1	α-Lactalbumin+1 lactose
	2	4.3	++	14178±1	α-Lactalbumin
	3	4.8	+	14160±2	Unknown
	4	11.6	+++	18278±2	β-Lactoglobulin B
			+++	18602±2	β-LgB+1 lactose
			++++	18926±2	β-LgB+2 lactose
	5	11.5 . . . 12.5	++++	18363±3	β-Lactoglobulin A
			++	18379±3	β-LgA ox
			++++	18689±3	β-LgA+1 lactose
			++++	19012±3	β-LgA+2 lactose
			++	19337±3	β-LgB+3 lactose
4c	1	4.0	++	14193±2	α-Lactalbumin ox.
	2	8.0	0	18388±3	Unknown
	3	8.8	0	18474±2	Unknown
	4	10.0	0	18309±2	Unknown
	5	11.0	+++	18292±3	β-LgB ox
	6	11.7	+	18277±4	β-Lactoglobulin B
	7	12.0	+++	18378±3	β-LgA ox
	8	12.6	+	18363±2	β-Lactoglobulin A
4d	1	3.0 . . . 4.5	+++	14194±1	α-Lactalbumin ox.?
			+++	14519±2	α-Lac ox.+1 lactose
			+++	14843±2	α-Lac ox.+2 lactose
			++	15168±2	α-Lac ox.+3 lactose
	2	10 . . . 13	+	18294±2	β-LgB ox.
			+++	18618±3	β-LgB ox.+1 lactose
			+++	18942±3	β-LgB ox.+2 lactose
			++	19266±3	β-LgB ox.+3 lactose
			+	18380±3	β-LgA ox
			++	18704±3	β-LgA+1 lactose
			+++	19028±3	β-LgA+2 lactose
			+++	19352±3	β-LgA+3 lactose

Quantities are estimated on a scale from ''0'' (weak) to ''++++'' (very strong), based on both the UV and MS response.

pounds. However, considering the UV response and using averaged data over several neighbouring chromatographic peaks, a rough estimation of the relative abundance of the individual compounds is possible. In Table 1, data for the five samples investigated here are summarised.

3.4. Oxidation products

Another modification often encountered in industrial products is oxidation. An example of a substantially oxidised sample, Lacprodan DI-9224, is given in Fig. 4c. The chromatogram shows multiple peaks that are due to oxidation products. The deconvoluted mass spectrum of this sample (Fig. 5) shows a strong presence of compounds with a difference of +16 u, with only moderate glycosylsation. The mass difference can be attributed to the formation of methionine sulfoxide, which is a common modification of peptides and proteins [13]. Protein oxidation has also been reported to occur during the electrospray process [14], but can be excluded here as the oxidised protein is already present in the UV chromatogram.

The oxidised proteins also elute before the corresponding unmodified protein, usually overlapping with the glycosylated/lactosylated variants mentioned above. From the UV data (not shown) it can be estimated that the quantity of oxidised protein in Fig. 4c is at least twice that of the unmodified lactoglobulin. This sample was described by the manufacturer as being ''heat stable'', which is certainly true as most of the peptide is already oxidised, and no major modifications due to oxidation can be expected.

A combination of both glycosylation and oxidation was observed with Domovictus 300 HP. The product shows very strong oxidation together with lactosylation/glycosylation, leading to multiple, overlapping peaks which are detected as a broad ''hump'' in the UV and the total ion current (TIC) trace (Fig. 4d). In this specific sample, non-oxidised proteins were either present in minor amounts or not detectable at all.

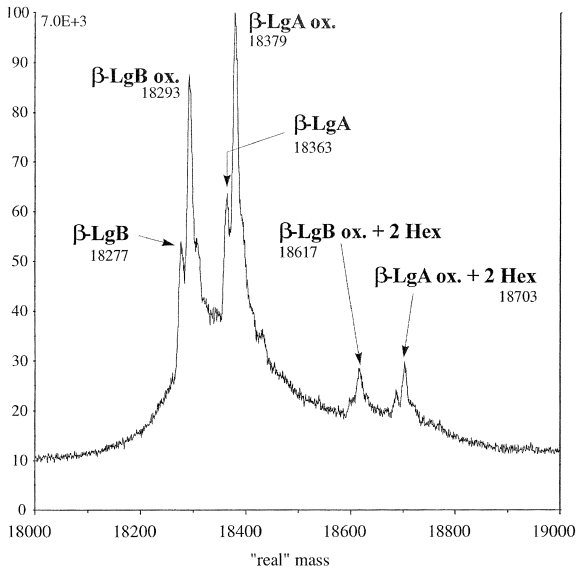

Fig. 5. Averaged and deconvoluted mass spectrum over the lactoglobulin region from the Lacprodan DI-9224 sample. The unmodified protein is present in minor quantities, while the most abundant peaks are attributed to oxidised proteins (+16 mass units) as well as to lactosylated/glycosylated and oxidised species.

4. Conclusions

Lactosylation and oxidation in whey proteins can be identified and characterised by LC–ESI–MS. Although MS ''on its own'' cannot determine the exact nature of a structural modification in a protein, it allows to state clearly if the molecular mass is the expected one or not, and the observed deviations in mass can be used to describe the nature of the modification further. In the commercial samples analysed here, α-lactalbumin, β-lactoglobulin A and β-lactoglobulin B were rarely found in their native state, but mostly with a variety of modifications, such as oxidation and/or single or multiple lactosylation. Lactosylated and oxidised proteins will have conformations that are different from the unmodified proteins [12], which does not only influence their properties in technical processing but might also have an impact on their hydrolysis pattern and biological properties.

Protein modifications induced by processing can be identified and used to evaluate the impact of different processing technologies on whey protein. This information is of value to the dairy industry, as ingredients from different suppliers can be assessed. With today's increasing use of LC–MS, even in factory laboratories, it could be used for the classifi-

cation of whey protein based ingredients or milk powders from different suppliers for a variety of applications.

Acknowledgements

We thank Céline Jordan for expert technical assistance and Elizabeth Prior for critically proof-reading the manuscript. Furthermore, we gratefully acknowledge PerSeptive Biosystems and Paul Bucher AG (Switzerland) for the loan of the MARINER instrument.

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