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Application of liquid chromatography–tandem mass spectrometry for the characterization of galactosylated and tagatosylated β-lactoglobulin peptides derived from *in vitro* gastrointestinal digestion

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ABSTRACT

This article describes a comprehensive characterization of bovine β-lactoglobulin peptides glycated with an aldohexose (galactose) or a ketohexose (tagatose), derived from *in vitro* gastrointestinal digestion, by liquid chromatography coupled to positive electrospray ion trap tandem mass spectrometry. In addition to the dissociation pathway previously described for aldohexoses-derived Amadori compounds, i.e. formation of the pyrylium ($[M+H]^+$-54) and furylium ions ($[M+H]^+$-84) via the oxonium ion ($[M+H]^+$-18), another and more direct fragmentation route involving the formation of the imminium ion ($[M+H]^+$-150) is also reported following extensive glycation rates of β-lactoglobulin with both carbohydrates. These results indicated that the analysis of digested proteins by LC-ESI-MS^2 on a three-dimensional ion trap monitoring neutral losses is an efficient and direct method to identify peptides glycated not only through the Amadori rearrangement but also via the Heyns rearrangement. Nevertheless, as the predominating MS^2 fragmentation pattern of the glycated peptides is derived from the sugar moiety, the sequence-informative *b*- and *y*-ions resulting from peptide backbone cleavage were undetected. To overcome this drawback, and taking advantage of multi-stage fragmentation capabilities of ion traps, the indicative Amadori and Heyns-derived imminium ions were successfully used in MS^3 analyses to identify the peptide backbone, as well as the specific glycation site. In addition, further MS^4 analyses were needed to carry out the characterization of doubly glycated peptides.

1. Introduction

The Maillard reaction is one of the most important and complex reactions that may spontaneously occur in living organisms or during food processing. The first steps of this reaction are normally based on the condensation of reducing sugars with the ε-amino group of lysine residues in proteins to form a Schiff base which rearranges to form either the Amadori rearrangement product, when the sugar is an aldose, or the Heyns rearrangement product, when the sugar is a ketose. Either beneficial or harmful effects have been attributed to the numerous Maillard reaction products formed during the cascade of consecutive reactions [1]. Regarding the latter, mutagenic compounds as heterocyclic aromatic amines and the so-called "advanced glycation end products" (AGEs) can be accumulated in the tissues affecting in an irreversible manner the protein and cellular function. Several studies have shown that the formation of AGEs, during the *in vivo* Maillard reaction, involves structural changes in proteins, which have been related with the development of various pathologies associated with hyperglycemia such as diabetes, cataracts, kidney disorders, arteriosclerosis, neurodegenerative amyloidal diseases such as Alzheimer's and cell aging processes [2].

On the other hand, proteins glycated under controlled conditions are of great interest for their use as food ingredients because of their technological properties [3]. Particularly, bovine β-lactoglobulin (β-lg), which is extensively used in food formulations because of its functional and nutritional properties, has been shown to be prone to reacting with reducing sugars through the Maillard reaction [4–8]. Nowadays, galactose (Gal) and tagatose (Tag) are carbohydrates, widely used in the food industry because of their diverse properties [9,10], which could participate in the glycation of proteins during food processing due to their reducing nature. Understanding the nature of the structure changes that accompany protein glycation could help in the elucidation of the functional glycation consequences in both food and biological systems. In a recent study, we investigated the influence of the sugar carbonyl group on

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the structural changes of undigested bovine β-lg following glycation via Maillard reaction with Gal and Tag [10]. Nevertheless, no further investigation about the nature of such structural changes was carried out. Several reports have shown that the effect of glycation on protein structure and functionality may depend on several factors, including the glycation site on the protein [11].

For the reasons explained above, it is of paramount importance to develop reliable analytical methods which allow the identification of novel glycated proteins and their sites of glycation. The most intense ions present in the MS^2 spectra of glycated peptides arise from losses of water molecules at the sugar moiety, resulting in the formation of oxonium ions such as pyrylium and furylium ions [12–15]. These ions have been proposed to be used as effective markers of peptides glycated with mono- [16], di- [13], and oligosaccharides [17]. Nevertheless, the accurate and direct identification of the specific glycation-sites on an ion trap cannot normally be performed by MS^2 experiments because the sequence-informative *b*- and *y*-ions resulting from peptide backbone cleavage are not formed or are very weak [17–21]. In this sense, chemical treatments such as Schiff base reduction by $NaBH_4$ prior to MS^2 analyses have been performed in order to facilitate the identification of the glycated peptide sequence [22]. On the other hand, to the best of our knowledge, no data regarding the fragmentation of Heyns-derived compounds on an ion trap mass analyzer has been reported to date. In this sense, Frolov et al. [23] proposed a fragmentation pattern of the sugar moiety of fructose-derived Heyns of a synthetic hexapeptide but using a QqTOF mass spectrometer.

The aim of this work was to perform a detailed characterization of bovine β-lg peptides glycated with an aldose (Gal) or a ketose (Tag), derived from *in vitro* gastrointestinal digestion, by using LC-ESI-MS^n on a three-dimensional ion trap analyzer. In order to gain further insight into the peptide sequencing, MS^3 spectra from collisionally generated imminium ion ($[M+H]^+$-150) were performed, stressing the importance of this ion in the accurate identification of the peptide sequence; whilst MS^4 analyses were needed to characterize peptides containing two lysine residues occupied with Gal or Tag.

2. Material and methods

2.1. Materials

Gal, Tag, β-lg from bovine milk (variants A and B), and the synthetic tetrapeptide acetyl-SDKP were purchased from Sigma–Aldrich (St. Louis, MO). All other reagents were of analytical grade.

2.2. Glycation of β-lg and the synthetic tetrapeptide acetyl-SDKP with Gal or Tag

Preparation and purification of β-lg-Gal/Tag conjugates was carried out as indicated by Corzo-Martinez et al. [10]. Briefly, aliquots of a solution consisting of 2.0 mg mL^{-1} β-lg and 2.0 mg mL^{-1} Gal or Tag in 0.1 M sodium phosphate buffer, pH 7 (Merck, Darmstadt, Germany), were lyophilized. These were kept under vacuum in a desiccator at 40 °C for 1 day (for β-lg-Gal/Tag conjugates) and at 50 °C for 2 days (only for β-lg-Tag conjugates) and at water activity of 0.44, achieved with a saturated K_2CO_3 solution (Merck). In addition, control experiments were performed with β-lg stored at 40 °C without reducing sugars during the same period (control heated β-lg). After incubation, the products were reconstituted in distilled water to a protein concentration of 1 mg mL^{-1}. To remove free carbohydrate, 2 mL portions were ultrafiltered through hydrophilic 3 kDa cutoff membranes (Centricon YM-3, Millipore Corp., Bedford, MA) by centrifugation at 1548 × *g* for 2 h. After removal of free Gal or Tag, samples were reconstituted in distilled water at a concentration of 1 mg mL^{-1} for further analysis.

Aliquots of a solution consisting of 0.5 mg mL^{-1} acetyl-SDKP and 0.5 mg mL^{-1} Gal in 10 mM ammonium acetate (Panreac, Barcelona, Spain) pH 6.8, were lyophilized. These were kept under vacuum in a desiccator at 40 °C for 1 day and at water activity of 0.44 as explained above.

2.3. MALDI-MS analysis

The extent of glycation of the undigested β-lg glycated with both carbohydrates was determined by MALDI-MS analyses using a Voyager DE-PRO mass spectrometer (Applied Biosystems, Foster City, CA) equipped with a pulsed nitrogen laser (λ = 337 nm, 3 ns pulse width, and 3 Hz frequency) and a delayed extraction ion source. Ions generated by the laser desorption were introduced into a time of flight analyzer (1.3 m flight path) with an acceleration voltage of 25 kV, a 93% grid voltage, a 0.05% ion guide wire voltage, and a delay time of 350 ns in the linear positive ion mode. Mass spectra were obtained over the *m*/*z* range 10,000–35,000. Apomyoglobin (Calibration Mixture 3 of Sequazyme Peptide Mass Standards Kit, Applied Biosystems) and carbonic anhydrase (Sigma) were used for external calibration. Sinapinic acid (>99%; Fluka, Buchs, Switzerland) at 10 mg mL^{-1} in 0.3% trifluoroacetic acid/acetonitrile, 70:30 (v/v) was used as matrix. Samples were mixed with the matrix at a ratio of approximately 1:15, and 1 μL of this solution was spotted onto a flat stainless-steel sample plate and dried in air.

2.4. In vitro gastrointestinal digestion

The control heated and glycated β-lg were digested *in vitro* by following the simplified procedure described by Moreno et al. [24]. A 3 mg amount of protein was dissolved in 1 mL of Simulated Gastric Fluid (SGF, 0.15 M NaCl, pH 2.5) and the pH was adjusted to 2.5 with 1 M HCl. A solution of 0.32% (w:v) porcine pepsin (EC 3.4.23.1) in SGF, pH 2.5 (Sigma, activity: 3300 U per mg of protein), was added at an approximately physiological ratio of enzyme:substrate (1:20, w:w). The digestion was performed at 37 °C for 2 h.

For the intestinal digestion step, the pH was raised to 7.5 with 40 mM NH_4CO_3 (Panreac) dropwise, and the following was added to adjust the pH to 6.5 and simulate a duodenal environment: (i) a bile salt mixture containing equimolar quantities (0.125 M) of sodium taurocholate (Sigma) and glycodeoxycholic acid (Sigma); (ii) 1 M $CaCl_2$ (Panreac); (iii) 0.25 M Bis-Tris, pH 6.5 (Sigma). Finally, solutions of porcine trypsin (EC 3.4.21.4; 0.05%, w:v, Sigma, Type IX-S, activity: 14 300 U/mg of protein) and bovine α-chymotrypsin (EC 3.4.21.1; 0.1%, w:v, Sigma, Type I-S, activity: 62 U/mg of protein) in water were prepared and added at, approximately, physiological ratios of β-lg:trypsin:chymotrypsin = 1:(1/400):(1/100) (w:w:w). Intestinal digestion of β-lg was carried out at 37 °C for 15 min. After protein hydrolysis, digestive enzymes were inactivated by incubation of the samples in a water bath at 80 °C for 5 min. Digestions were performed without any derivatization of the sulfhydryl groups of cysteine residues in order to remain as close as possible to physiological conditions.

2.5. LC-ESI-MS^n analysis

LC-MS^n experiments were carried out on a Finnigan Surveyor pump with quaternary gradient system coupled to a Finnigan LCQ Deca ion trap mass spectrometer using an ESI interface. Sample injections (10 μL) were carried out by a Finnigan Surveyor autosampler. All instruments from Thermo Fisher Scientific (San José, CA, USA), formic acid of analytical grade (Merck) and Milli-Q water obtained using a Millipore (Bedford, USA) system were used.

The digestion mixtures of both glycated and unglycated β-lg (1.7 mg mL^{-1}) were diluted 1:2 with water, and their separation performed at 25 °C on a Hypersil HyPurity C18 (100 mm × 2.1 mm, 3 μm) column (Thermo Fisher Scientific) at a flow rate of 100 μL min^{-1}. A gradient of two eluents was used: eluent A consisted in 0.1% (v/v) of formic acid (analytical grade, Merck) in water, and eluent B in acetonitrile (LC-MS Chromasolv® grade, Riedel-de Haën, Seelz, Germany) containing 0.1% of formic acid (v/v). The elution program was as described by Moreno et al. [17].

The mass spectrometer spray voltage was set at 4.5 kV, heated capillary temperature at 200 °C, nitrogen (99.5% purity) was used as sheath (0.6 L min^{-1}) and auxiliary (6 L min^{-1}) gas, and helium (99.999% purity) as the collision gas. Mass spectra were recorded in the positive ion mode between *m/z* 450 and 2000. MS^2 data were acquired in the automatic data-dependent mode with a relative collision energy of 35%, using a total cycle time of approximately 5 s. Ion selection threshold was set at 5×10^5 counts. MS^3 and MS^4 experiments were also carried out with a relative collision energy of 35% by selecting the appropriated target ion transitions. Automatic gain control was used to maintain constant ion populations at 5×10^7 and 2×10^7 for full scan MS and MS^n spectra, respectively.

2.6. Data treatment

The LC-MS^n system, data acquisition and processing were managed by Xcalibur software (1.2 version, Thermo Fisher Scientific). The initial assignment of observed ions to the corresponding amino acid sequences was based on the known sequence of β-lg [25] by using the protein database Swiss-Prot and TrEMBL and the tools Peptide Mass and FindPept available at www.expasy.org. Parameters for the search were the following: (i) monoisotopic peptide masses were indicated as $[M+H]^+$ with cysteines treated with nothing; (ii) as enzymes, pepsin and trypsin/chymotrypsin were chosen; (iii) four missed cleavages were allowed; (iv) peptides with a mass larger than 500 u were displayed; (v) the mass tolerance was kept at 0.5 u.

All full scan MS^2 spectral data were searched using the Bioworks version 3.1 suite of programs (Thermo Fisher Scientific). A precursor mass tolerance of 1.4 u was used to search the resulting DTA files against computer-predicted fragments. All Bioworks 3.1 output files were further filtered according to cross-correlation (Xcorr) scores as a function of charge states to increase confidence limits. Specifically, the Xcorr cutoff values used to determine acceptable peptide matches were 1.5 for digest fragments originating from precursor ions with a charge state of one, 2.0 for digest fragments originating from doubly charged precursor ions, and 2.5 for triply charged ions. To confirm the sequence of the glycated peptides, the MS^3 and MS^4 spectra were compared with the theoretical fragmentation of the putative peptides, obtained from the MS-Product software program (Protein Prospector, http://prospector.ucsf.edu/).

3. Results and discussion

3.1. Determination of the extent of glycation

The MALDI-MS analyses of undigested β-lg incubated with Gal or Tag at 40 °C for 1 day indicated that the average number (considering the maximum intensity of the Gaussian peaks) of carbohydrate molecules bound to β-lg were 14 and 3, respectively (Fig. 1), showing the much higher reactivity of Gal as compared to Tag [10,26]. With the aim to achieve similar glycation rates with both carbohydrates, β-lg was also incubated with Tag at a higher temperature (50 °C) and for a longer time (2 days), resulting in the average binding of 15 molecules of Tag per molecule of β-lg (Fig. 1).

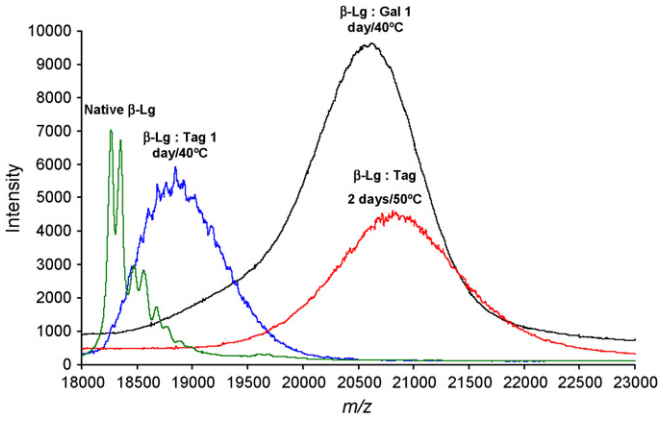

Fig. 1. Superimposed MALDI-MS spectra of native β-lg and β-lg incubated with Gal or Tag at 40 °C for 1 day or at 50 °C for 2 days.

3.2. Characterization of unglycated peptides by LC-ESI-MS^2 derived from in vitro gastrointestinal digestion

The resulting base peak chromatograms for digested unglycated and glycated β-lg, revealed the presence of a complex mixture of peptides eluting between 4 and 35 min in all samples (chromatograms not shown). Control heated and glycated β-lg with Gal and Tag were found to be very resistant to pepsinolysis; however, the protein was rapidly broken down during simulated duodenal digestion (trypsin/chymotrypsin) (results not shown), which is in good agreement with previous results shown for native and β-lg glycated with oligosaccharides [27]. In summary, 56 unglycated peptides were identified by LC-ESI-MS^2 in the digest of native β-lg, whereas 31, 37 and 30 unglycated peptides could be identified in the digests of β-lg glycated with Gal (1 day at 40 °C), Tag (1 day at 40 °C) and Tag (2 days at 50 °C), respectively (Table 1). Around 88% and 77% of the β-lg sequence was covered by the peptides identified in the digests of the native and glycated proteins, respectively. As expected, unglycated peptides produced collisional spectra in which the typical *y*- and *b*-series ions predominated (data not shown).

3.3. Characterization of glycated peptides by LC-ESI-MS^2 derived from in vitro gastrointestinal digestion

3.3.1. Galactosylated peptides

The MS^2 spectra of the peptides glycated with Gal were mostly characterized by two neutral losses of 54 and 84 u, corresponding to the formation of the pyrylium and furylium ions, respectively, via the oxonium ion (Fig. 2A). This dissociation pathway has been well established for aldohexoses-derived Amadori compounds [12,14,15].

Nevertheless, the MS^2 spectra of five galactosylated peptides, ^{83}K-E^{89}, ^{45}E-L^{54}, ^{43}V-L^{54}, ^{9}G-W^{19} and ^{9}G-Y^{20} (Fig. 3A), showed a peak higher than 50% of base peak at $[M+H]^+$-150. This neutral loss could be attributed to the formation of the imminium ion produced by the α-cleavage of the bonds attached to the amino acid nitrogen (Fig. 2B). The imminium ion was previously described as an important diagnostic ion for the identification of Amadori compounds by mass spectrometric techniques based on electron impact ionization [28]. Nevertheless, to the best of our knowledge, this is the first evidence showing the presence of this ion in the spectra of glycated peptides obtained by positive electrospray ion trap tandem mass spectrometric analysis.

Overall, the imminium ion was present in 26 of the 27 galactosylated peptides identified in this study (Table 2). Moreover, the ion corresponding to the unglycated peptide ($[M+H]^+$-162) was much

Table 1
Unglycated peptides identified in native and β-lg glycated with Gal or Tag by LC-ESI-MS^2.

RT (min)	Peptide sequence	Position	Exp. mass [M+H][a]	Theor. mass [M+H][a]	Main charge state	Control heated β-LG	β-LG + Gal 1 day at 40 °C	β-LG + Tag 1 day at 40 °C	β-LG + Tag 2 days at 50 °C
2.7	KVAGT	14–18	475.0	475.2	+1	X	X	X	
3.9	IIAEK	71–75	573.3	573.4	+1	X	X	X	X
3.9	KIDALNENK	83–91	1044.6	1044.6	+1	X	X	X	X
3.9	VRTPEVDD	123–130	930.4	930.5	+1	X	X	X	X
4.4	VLDTDYK	94–100	853.4	853.5	+1	X	X	X	X
4.6	KIDALN	83–88	673.3	673.4	+1	X	X	X	X
5.1	KIDALNE	83–89	802.4	802.4	+1	X	X	X	X
5.7	VFKIDA	81–86	692.2	692.4	+1	X	X	X	X
5.9	DAQSAPL	33–39	701.2	701.5	+1	X	X	X	X
7.0	QSAPLRV	35–41	770.4	770.5	+1	X			
7.0	ELKPTPEGDLE	45–55	1227.5	1227.6	+1	X			
7.0	LKPTPEGDL	46–54	969.4	969.5	+1	X	X	X	X
7.8	YSLA	20–23	453.0	453.2	+1	X	X	X	X
8.2	LIVTQTMK	1–8	933.4	933.5	+1	X	X	X	X
8.5	VTQTMK	3–8	707.2	707.4	+1	X			
10.5	ELKPTPEGDL	45–54	1098.5	1098.6	+1	X	X	X	X
10.5	DAQSAPLRV	33–41	956.4	956.5	+1	X			
11.1	VLVLDTDYK	92–100	1065.5	1065.6	+1	X	X	X	X
11.8	LDAQSAPL	32–40	814.1	814.4	+1	X	X	X	X
13.3	VLVLDTDYKK	92–101	1193.6	1193.7	+2	X			
13.3	DTDYKK	96–101	769.3	769.4	+1	X			
13.3	LDTDYKK	95–101	882.3	882.5	+1	X			
13.3	VLDTDYKK	94–101	981.5	981.5	+1	X			
14.5	TPEVDDEALEK	125–135	1245.5	1245.6	+2	X	X	X	X
15.2	VEELKPTPEGDL	43–54	1326.5	1326.7	+2	X	X	X	X
15.6	DKALKALPMHIRL	137–149	1505.8	1505.9	+2	X			
16.2	VLDTDYKKYL	94–103	1257.5	1257.7	+2	X			
17.1	WENGECAQKK	61–70	1192.4	1192.5	+1	X	X	X	X
17.5	LIVTQTM	1–7	805.2	805.4	+1	X	X	X	X
19.2	VAGTWY	15–20	696.2	696.3	+1	X	X	X	X
19.7	FNPTQL	151–156	719.2	719.4	+1	X		X	
20.9	SFNPTQL	150–156	806.2	806.4	+1	X	X	X	X
21.2	IPAVFKIDALNENKVL	78–93	1784.4	1784.0	+2	X	X	X	X
21.2	LPMHIR	143–148	766.3	766.4	+1	X	X	X	
21.6	ALPMHIR	142–148	837.4	837.5	+1	X		X	
22.8	PEGDLEIL	50–57	885.1	885.5	+1	X	X	X	X
23.3	TPEVDDEALEKFDK	125–138	1635.6	1635.8	+2	X	X	X	X
23.9	KIDALNENKVL	83–93	1256.6	1256.7	+1	X			
23.9	TKIPAVFK	76–83	903.5	903.6	+1	X			
24.3	VRTPEVDDEALEKFDK	123–138	1891.0	1890.9	+3	X			
24.3	AASDISLL	25–32	789.2	789.4	+1	X	X	X	X
24.5	KYLLFCME	101–108	1046.5	1046.5	+1	X			
24.5	APLRVYVE	37–44	946.7	946.5	+1	X			
26.6	TKIPAVF	76–82	775.3	775.5	+1	X	X	X	X
28.0	QKKIIAEKTKI	68–78	1299.4	1299.8	+1	X		X	
28.6	AASDISLLDAQSAPL	25–39	1471.4	1471.8	+1	X		X	
28.8	VEELKPTPEGDLEIL	43–57	1681.9	1681.9	+2	X			
28.9	GLDIQKVAGTWYSLAMAAS DISLLDAQSAPLR	9–40	3361.6	3361.8	+2	X			X
29.2	AMAASDISLL	23–32	991.3	991.5	+1	X	X	X	X
30.5	SLAMAASDISLLDAQSAPLR	21–40	2030.2	2030.1	+2	X	X		
30.5	VEELKPTPEGDLEILLQK	43–60	2051.0	2051.1	+2	X		X	X
30.5	VRTPEVDDEALEKF	123–136	1647.5	1647.8	+2	X		X	
30.8	RVYVEELKPTPEGDLEILLQK	40–60	2469.4	2469.4	+3	X		X	
31.5	VYVEELKPTPEGDLEILLQK	41–60	2313.2	2313.3	+2	X	X	X	X
32.3	RVYVEELKPTPEGDLEILLQ	40–59	2341.0	2341.3	+2	X			
33.0	SLAMAASDISLL	21–32	1191.4	1191.6	+1	X	X	X	X

[a] Monoisotopic mass values.

less abundant than the imminium ion or, even, undetected in many MS^2 spectra. Furthermore, a relationship between the formation of the imminium ion and the glycated peptide chain length was found. Thus, the MS^2 fragmentation of short glycated peptides comprised by 6 or less amino acid residues did not lead to the formation of the imminium ion (^{13}Q-A^{16}) or this was very weak (^{71}I-K^{75}, ^{99}Y-Y^{102}, ^{141}K-M^{145}, ^{136}F-L^{140} and ^{90}N-L^{95}). To confirm this point, a synthetic tetrapeptide, acetyl-SDKP, was glycated with Gal and, then, subjected to MS^2 analysis. Fig. 3B shows the corresponding spectrum characterized by the furylium, pyrylium and oxonium ions whereas the imminium ion was not detected. In this sense, Pallante and Cassady [29] observed that peptide ions undergo proton transfer reactivity more readily as the peptide chain length decreases. It is noteworthy to mention that the locations of these added protons can affect the dissociation of a peptide under MS^2 conditions and can, thus, impact the sequence information obtained.

3.3.2. Tagatosylated peptides

Regarding the glycation of β-lg with Tag, which forms ketose Heyns products, under mild (40 °C for 1 day) and more severe (50 °C for 2 days) conditions, 15 and 24 tagatosylated peptides could be identified in the protein digests, respectively (Table 2). Strikingly, some differences in the fragmentation behaviour between both

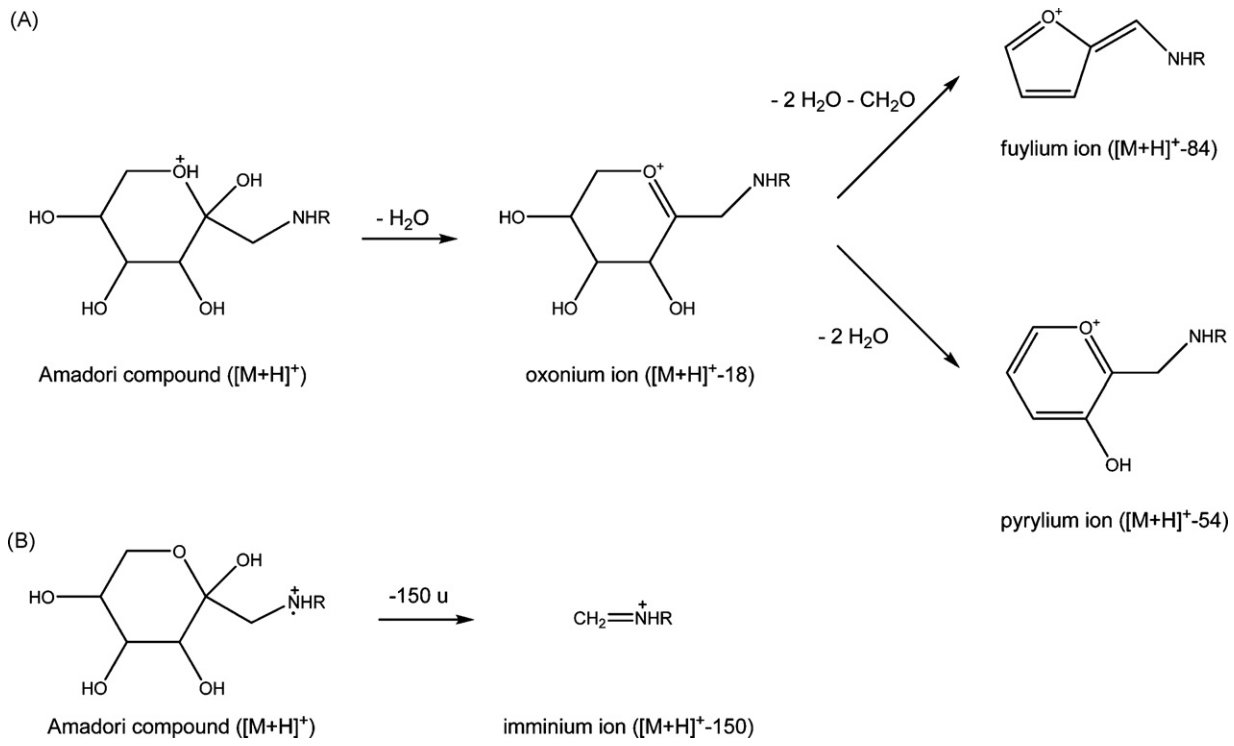

Fig. 2. Proposed dissociation pathways for peptides glycated with aldo- and keto-hexoses following ESI-MS^2 analysis.

samples were found. Thus, the glycated β-lg peptides derived from the incubation with Tag for 1 day at 40 °C produced MS^2 spectra characterized by neutral losses of 18, 36, 54, 72, 84 and 96 u, whereas the corresponding imminium ion was very restricted or undetected. As an example, Fig. 3C shows the MS^2 spectra of the tagatosylated peptide ^{9}G-Y^{20}. This fragmentation behaviour is in good agreement with the fragmentation pattern previously reported for a model hexapeptide, AGGKAA, glycated with fructose and analysed by QqTOF-MS^2 [23]. These authors explained this fragmentation pattern in analogy to Amadori products by the cleavage of one to four water molecules (18, 36, 54 and 72 u), as well as by losses of $[3 \times H_2O + HCHO]$ (neutral loss of 84 u) and $[2 \times H_2O + H_4C_2O_2]$ (neutral loss of 96 u) at the sugar moiety yielding different oxonium ions and, finally, pyrylium and furylium

Table 2
Galactosylated and tagatosylated peptides identified in β-LG by LC-ESI-MS^n. The glycated amino acid is indicated by an asterisk.

RT (min)	Peptide sequence	Position	Exp. mass $[M+H]^+$[a]	Theor. mass $[M+H]^+$[a]	Charge state	Glycated peptide mass $[M+H]^+$[a]	β-Lg:Gal 1 day 40 °C	β-Lg:Tag 1 day 40 °C	β-Lg:Tag 2 days 50 °C
3.97	YK*K*Y	99–102	601.1	601.3	+1	925.4[b]	X		
4.05	K*IDALNENK	83–91	1044.5	1044.6	+1; +2	1206.5	X		X
5.11	K*IDALNE	83–89	802.3	802.4	+1	964.3	X		X
5.56	K*ALPM	141–145	559.3	559.3	+1	721.3	X	X	X
5.56	IIAEK*	71–75	573.3	573.4	+1	735.3	X	X	X
7.18	LK*PTPEGDL	46–54	969.5	969.5	+1; +2	1131.5	X		X
7.92	QK*VA	13–16	445.2	445.3	+1	607.2	X	X	X
8.10	L*IVTQTMK	1–8	933.4	933.5	+1; +2	1095.4	X	X	X
10.45	ELK*PTPEGDL	45–54	1098.5	1098.6	+1; +2	1260.5	X		
10.69	FDK*AL	136–140	593.2	593.3	+1	755.2	X		X
11.09	IQK*VAGTW	12–19	902.4	902.5	+1; +2	1064.4	X		X
14.62	K*IDALNENK*VL	83–93	1256.7	1256.7	+1; +2	1580.6[b]	X		X
15.14	VEELK*PTPEGDL	43–54	1326.7	1326.7	+1; +2	1488.7	X		X
16.80	L*IVTQTM	1–7	805.3	805.4	+1	967.3	X	X	X
17.99	TPEVDDEALEK*FDK	125–138	1635.7	1635.8	+1; +2; +3	1797.6	X	X	X
23.51	ALK*ALPM	139–145	743.4	743.4	+1	905.4	X	X	X
23.94	K*IDALNENKVL	83–93	1256.7	1256.7	+1; +2	1418.7	X	X	X
24.69	TPEVDDEALEK*F	125–136	1392.5	1392.7	+1; +2	1554.5	X		X
24.92	NK*VLVL	90–95	685.4	685.5	+1	847.4	X	X	X
25.30	TK*IPAVF	76–82	775.4	775.5	+1	937.4	X	X	X
26.92	IIAEK*TK*IPAVF	71–82	1329.6	1329.8	+1; +2	1653.6[b]	X		X
27.81	GLDIQK*VAGTW	9–19	1187.5	1187.6	+1; +2	1349.5	X	X	X
29.58	GLDIQK*VAGTWY	9–20	1350.6	1350.7	+1; +2	1512.6	X	X	X
30.92	VEELK*PTPEGDLEILLQK	43–60	2051.2	2051.1	+2; +3	2213.2	X	X	X
31.63	VYVEELK*PTPEGDLEILLQK	41–60	2313.2	2313.3	+2	2475.2	X	X	X
32.49	RVYVEELK*PTPEGDLEILLQ	40–59	2341.4	2341.3	+2; +3	2503.4	X	X	X
32.90	VEELK*PTPEGDLEILLQ	43–59	1922.8	1923.0	+2	2084.8	X		

[a] Monoisotopic mass values.
[b] Glycated with two monosaccharide molecules.

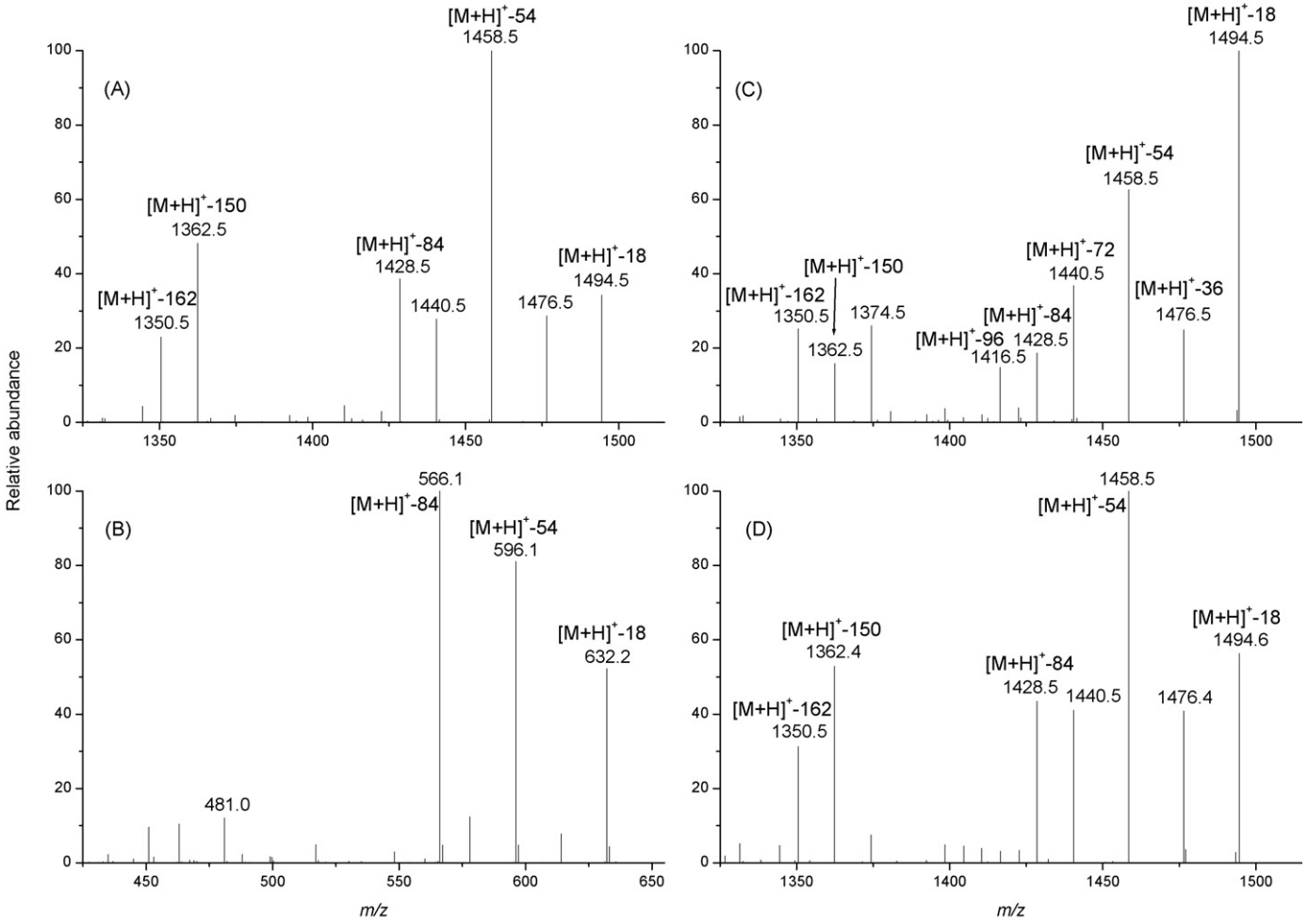

Fig. 3. MS^2 spectra of singly charged ions corresponding to (A) β-lg peptide ^{9}GLDIQK*VAGTWY^{20} glycated with Gal at 40 °C for 1 day, (B) synthetic peptide acetyl-SDK*P glycated with Gal at 40 °C for 1 day, (C) β-lg peptide ^{9}GLDIQK*VAGTWY^{20} glycated with Tag at 40 °C for 1 day, and (D) β-lg peptide ^{9}GLDIQK*VAGTWY^{20} glycated with Tag at 50 °C for 2 days.

ions. Therefore, the fragmentation pattern of the sugar moiety of fructose-derived Heyns compounds proposed by Frolov et al. [23] is compatible with the main ions detected by MS^2 in the tagatosylated peptides following incubation under mild conditions (40 °C for 1 day). Lastly, it should be noted that the neutral loss of 96 u resulted to be distinctive for ketohexoses-derived Heyns compounds as it was not detected in the MS^2 fragmentation pattern of galactosylated peptides.

The MS^2 spectra of the tagatosylated peptides obtained after incubation of β-lg at 50 °C for 2 days were mostly characterized by the neutral losses of 18, 54, 84 and 150 u, in analogy to the galactose-derived Amadori products (Fig. 3D). This difference in the MS^2 fragmentation behaviour between the tagatosylated peptides obtained under different incubation conditions could be supposed to be in part due to: (i) the tautomerization rate of ketoses is highly dependent on temperature [30] and, consequently, the MS^2 fragmentation pattern could be affected by the different composition of the Tag tautomers obtained at 40 and 50 °C; and/or (ii) as ketoses and amino groups can undergo irreversible rearrangements to give not only Heyns products but also Amadori compounds [31,32], a change in temperature might also favour the mechanism of formation of the Amadori compound as a result of tagatosylation. In relation to a change in the tautomer composition of tagatose with temperature, the consecutive loss of up to four water molecules (18, 36, 54, 72 u), which was more extensively observed after incubation of β-lg with Tag at 40 °C than at 50 °C, has previously been associated to a furanose form [23]. Thereby, this fact might indicate a higher degree of the furanose form in the tagatose-derived Heyns compounds obtained at 40 °C than at 50 °C. In good agreement with this, it has been described that the pyranose anomers are favoured thermodynamically [33].

To sum up, it can be concluded that the analysis of digested proteins by LC-ESI-MS^2 on an ion trap monitoring neutral losses is an efficient and direct method to identify peptides glycated either through the Amadori or the Heyns rearrangement. Nevertheless, as the predominating fragmentation pattern is that derived from the sugar moiety, the sequence-informative *b*- and *y*-ions resulting from peptide backbone cleavage are very weak or undetected, impairing the accurate identification of the amino acid sequence and the corresponding glycation site.

3.4. Characterization of glycated peptides by LC-ESI-MS^3 and MS^4 derived from in vitro gastrointestinal digestion

In order to gain further insight into the peptide sequencing, additional runs were carried out to obtain MS^3 spectra from collisionally generated furylium and pyrylium ions located in the MS^2 analysis of tagatosylated and galactosylated peptides. Nevertheless, only a few ions were generated under these conditions and they could not be related to the peptide sequence (data not shown). Lapolla et al. [20] and Moreno et al. [17] found similar results following MS^3 analysis from collisionally generated oxonium and

furylium ions, derived from peptides glycated with glucose and galactooligosaccharides, respectively. Zhang et al. [21], who also used the furylium ion derived from glycated peptides to trigger MS^3 fragmentation, indicated that, although the furylium ion is abundant, the CID process, owing to its limitations (i.e. charge localization and residue effect), may still preferentially cleave certain amide bonds related to the sugar moiety over others on the peptide backbone. Likewise, the mild collision conditions produced on an ion trap greatly favours low critical energy decomposition channels. Hence, water losses, which is a process requiring proton rearrangement, is much more energetically favoured than single bond cleavage [20].

Taking into account that: (i) the imminium ion was normally present in a relative abundance in the MS^2 spectra of the galactosylated peptides and those tagatosylated peptides obtained under more severe conditions (50 °C for 2 days); and (ii) the structure of the imminium ion lacks any sugar pyranose or furanose ring, we proceeded to perform MS^3 spectra from collisionally generated imminium ions with the aim to obtain sufficient fragment ions related to the peptide backbone. As a result, the imminium ion used as a further precursor fragment gave rise to MS^3 spectra preferentially characterized by the *b*-serial ions owing to the free N-terminus (Fig. 4A). This allowed an accurate identification of the peptide backbone, as well as the assignation of the specific glycated lysine (Table 2). Furthermore, it should be noted that several generated *b*-ions exhibited an increase of 12 u, probably due to the CH_2 residue linked to the ε-amino group of the modified lysine, whereas MS^3 spectra obtained from collisionally generated ion corresponding to the unglycated peptide ($[M+H]^+$-162), in those cases where this ion could be detected in a moderately abundance, were dominated by unmodified *b*-ions with their corresponding dehydrations (Fig. 4B).

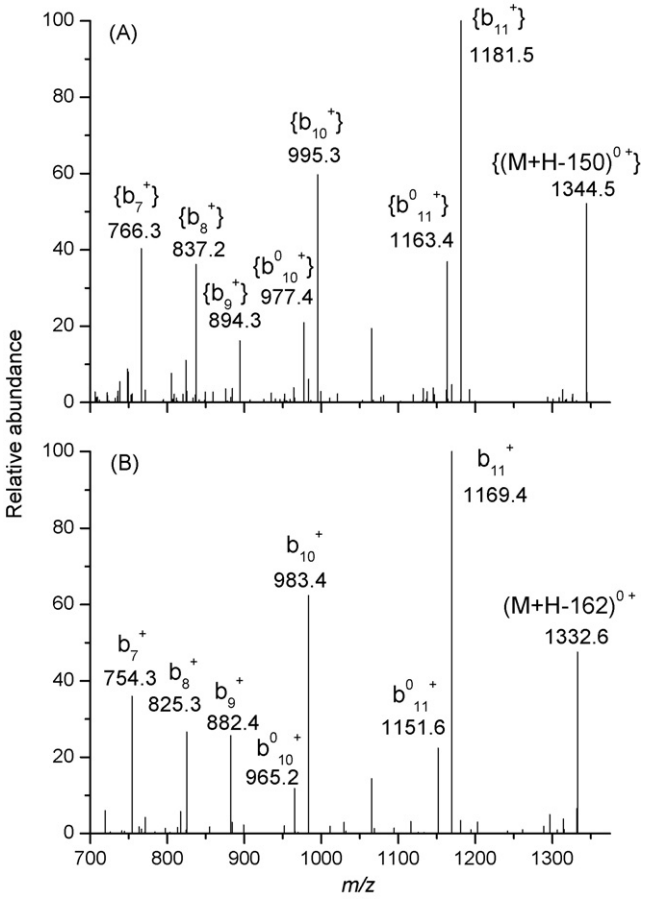

Fig. 4. MS^3 spectra of singly charged ions corresponding to β-lg peptide ^{9}GLDIQK*VAGTWY^{20} glycated with Gal at 40 °C for 1 day from collisionally generated (A) imminium ion ($[M+H]^+$-150) and (B) unglycated peptide ion ($[M+H]^+$-162) derived from the MS^2 analysis. The nomenclature of Roepstorff and Fohlman [34] and Johnson et al. [35] was used for fragment ions; "0" superscript denotes neutral loss of one water molecule.

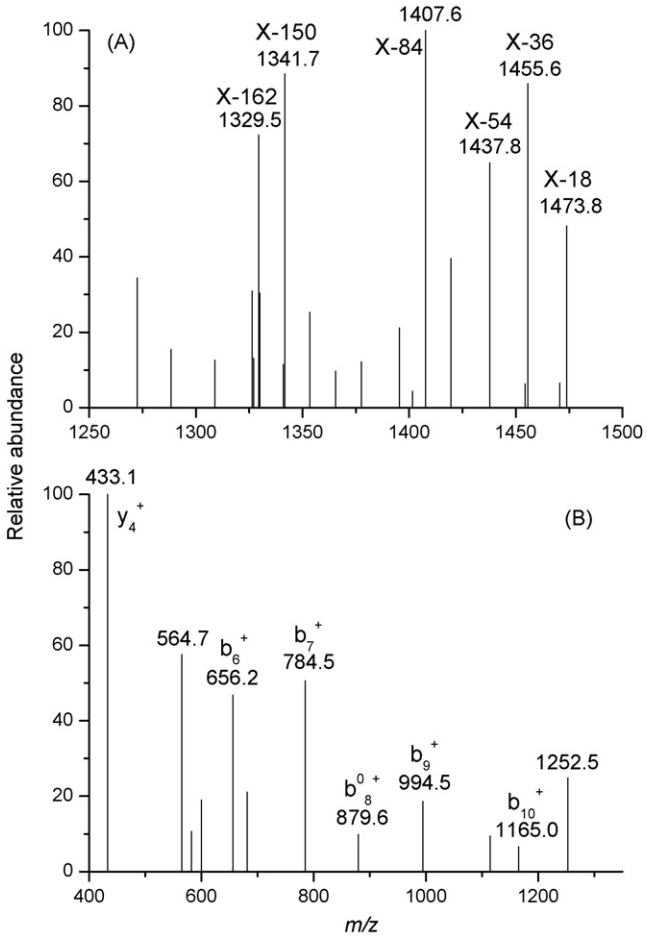

Fig. 5. (A) MS^3 and (B) MS^4 spectra of singly charged ions corresponding to β-lg peptide ^{71}IIAEK*TK*IPAVF^{82} doubly glycated with Gal at 40 °C for 1 day from collisionally generated unglycated peptide ion ($[M+H]^+$-162, denoted as X) derived from the MS^2 and MS^3 analyses, respectively. The nomenclature of Roepstorff and Fohlman [34] and Johnson et al. [35] was used for fragment ions; "0" superscript denotes neutral loss of one water molecule.

Finally, three diglycated peptides containing two lysine residues, ^{99}Y-Y^{102}, ^{83}K-L^{93}, ^{71}I-F^{82}, both occupied with Tag and/or Gal could also be identified (Table 2). In these cases, the MS^3 spectra from either collisionally generated imminium or unglycated peptide ion derived from the MS^2 analysis were characterized by subsequent neutral losses of 54, 84, 150 and, even 162 u, which is indicative of the existence of a second monosaccharide unit into the molecule; whilst no significant detection of the peptide sequence-informative *b*- and *y*-ions was obtained (Fig. 5A). Consequently, it was needed to perform MS^4 spectra from the imminium or unglycated peptide ion derived from the MS^3 analysis to identify the amino acid sequence of these peptides containing two glycated lysine residues (Fig. 5B). This step is only possible, at a reasonable cost, on tandem-in-time analyzers such as ion-traps, but not on tandem-in-space instruments such as triple quadrupole or QqTOF. Once more, the capacity of ion trap analyzers to perform multiple stages of fragmentation (MS^n) is demonstrated as an effective method for the structural characterization of peptides glycated with aldoses and ketoses.

4. Conclusions

This article shows the fragmentation behaviour of galactosylated (through Amadori rearrangement) and tagatosylated (Heyns rearrangement) β-lg peptides by LC-ESI-MS^n analysis on a three-dimensional ion trap spectrometer. Regarding LC-ESI-MS^2 analysis, in addition to the dissociation pathway previously described for Amadori compounds derived from aldohexoses, i.e. formation of the pyrylium ($[M+H]^+$-54) and furylium ions ($[M+H]^+$-84) via the oxonium ion ($[M+H]^+$-18), another and more direct fragmentation route involving the formation of the Amadori and Heyns-derived imminium ion ($[M+H]^+$-150) has been described following extensive glycation rates of β-lg. Whilst the accurate identification of the specific glycation-sites cannot be performed directly by MS^2 on an ion trap due to the sequence-informative *b*- and *y*-ions resulting from peptide backbone cleavage are not normally formed, the indicative Amadori and Heyns-derived imminium ions were successfully used in MS^3 analysis to identify the peptide backbone and, consequently, allowing the accurate assignation of the specific glycation site. Similarly, MS^4 spectra of the imminium/unglycated peptide ions allowed the identification of amino acid sequence in the case of doubly glycated peptides. The importance of the imminium ion relies on its relative abundance and that it retains the characteristic of the original peptide with no sugar moieties attached. In conclusion, the findings presented in this work indicate that MS^3 spectra from collisionally generated imminium ion could allow a direct and easy way for sequencing peptides glycated with aldohexoses and ketohexoses.

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