



Effect of molecular size and charge state of reducing sugars on nonenzymatic glycation of β -lactoglobulin

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ABSTRACT

This study was to investigate the effect of molecular size and charge state of reducing sugars on the nonenzymatic glycation of β -lactoglobulin (β -lg), with emphasis on the variation in glycation extent and glycation site. Five sugars (glucose, galactose, galacturonic acid, maltose and maltotriose) were selected to react with β -lg in dry-state (a_w at 0.53, 45 °C) and the glycated products were analyzed by liquid chromatography–mass spectrometry (LC–MS) and data-independent acquisition LC–MS (LC–MS^E). The results showed that increasing molecular size and negative charge state of reducing sugars would reduce the glycation extent. In addition, the difference of molecular size would result in the variation of glycation on the site of K 8, 70 and 75, and the negative charge state would lead to dissimilarity of glycation on K 77 of β -lg. The analysis of the glycation sites was based on the fragmentation behaviors of the reducing sugars proposed according to the deconvoluted MS^E spectra. Among the five reducing sugars, glucose and galactose had the same dissociation pathway. Galacturonic acid had a distinctive dissociation behavior with a neutral loss of 98. Maltose and maltotriose were comparable to glucose but more complex.

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1. Introduction

The nonenzymatic glycation, which is generally regarded as the initial stage of a Maillard reaction, refers to the reaction between the carbonyl group of reducing sugars and the amino group of proteins, peptides or amino acids (Hodge, 1953; Labuza & Baisier, 1992). Glycation often occurs in foods during heat treatment (Holland, Gupta, Deeth, & Alewood, 2011; Monaci & van Hengel, 2007) or long-term storage (Le, Bhandari, Holland, & Deeth, 2011; Zhou, Guo, Liu, Liu, & Labuza, 2013), which would further lead to a higher stage of a Maillard reaction and will result in changes in texture (Zhou et al., 2013), color (Le et al., 2011; Rao, Rocca-Smith, & Labuza, 2012) and flavor (Boekel, 2006; Ferretti & Flanagan, 1971; Mahajan, Goddik, & Qian, 2004).

As one of the reactants, reducing sugar could affect the glycation process due to their difference in molecular size and charge state. Former studies suggested that large molecular size of reducing sugars would slow down the chemical reaction rate for protein glycation (Aoki, Hiidome, Sugimoto, Ibrahim, & Kato, 2001; Haar, Schols, & Gruppen, 2011; Hiller & Lorenzen, 2010; Laroque et al., 2008; Oliver, Melton, & Stanley, 2006) and the difference in charge state would have an influence on the progress of protein glycation (Haar et al., 2011). However,

most of these studies focused more on the effect on glycation extent, without distinguishing the glycation site preference of various sugars with different molecular size and charge state (Oliver, 2011).

When studying protein glycation, β -lactoglobulin (β -lg) is often selected as the model protein because it is one of the major components of whey proteins, and its sequence and 3-dimensional structure had been extensively studied. It has mainly two variants: A (18,363 Da) and B (18,277 Da). Both of the variants contain 162 amino acids, differing in positions 64 (AspA $\rightarrow$ GlyB) and 118 (ValA $\rightarrow$ AalB). Among the 162 amino acids, each variant has 15 lysyl residues (K 8, 14, 47, 60, 69, 70, 75, 77, 83, 91, 100, 101, 135, 138 and 141) and 1 N-terminal amino acid (L 1), which are generally regarded as the most reactive in glycation. Despite the differences in the primary structures, the secondary and tertiary structures of the two variants are very similar (Qin, Bewley, Creamer, Baker, & Jameson, 1999). The core structure comprises 9 β -strands, among which the first 8 (β -strand A to β -strand B) arrange in an up-down-up-down topology to form a calyx, and the last one (β -strand I), together with the side chain of K 8, loops CD, EF, and GH and part of loop AB, is involved in the formation of β -lg dimers (Qin et al., 1998). The differences in tertiary structures are related to not only the primary sequence but also the pH (Oliveira et al., 2001). According to Qin et al. (1999), the two variants slightly differ in positions 64, 118, and loop EF in the tertiary structures at pH 7.1. When the pH was changed to 7.9, the change in loop EF disappeared (Oliveira et al., 2001).

The approaches for the determination of a glycation vary (Silván, van de Lagemaat, Olano, & del Castillo, 2006), and among which, the mass methods stand out for the accurate and detailed information it provided.

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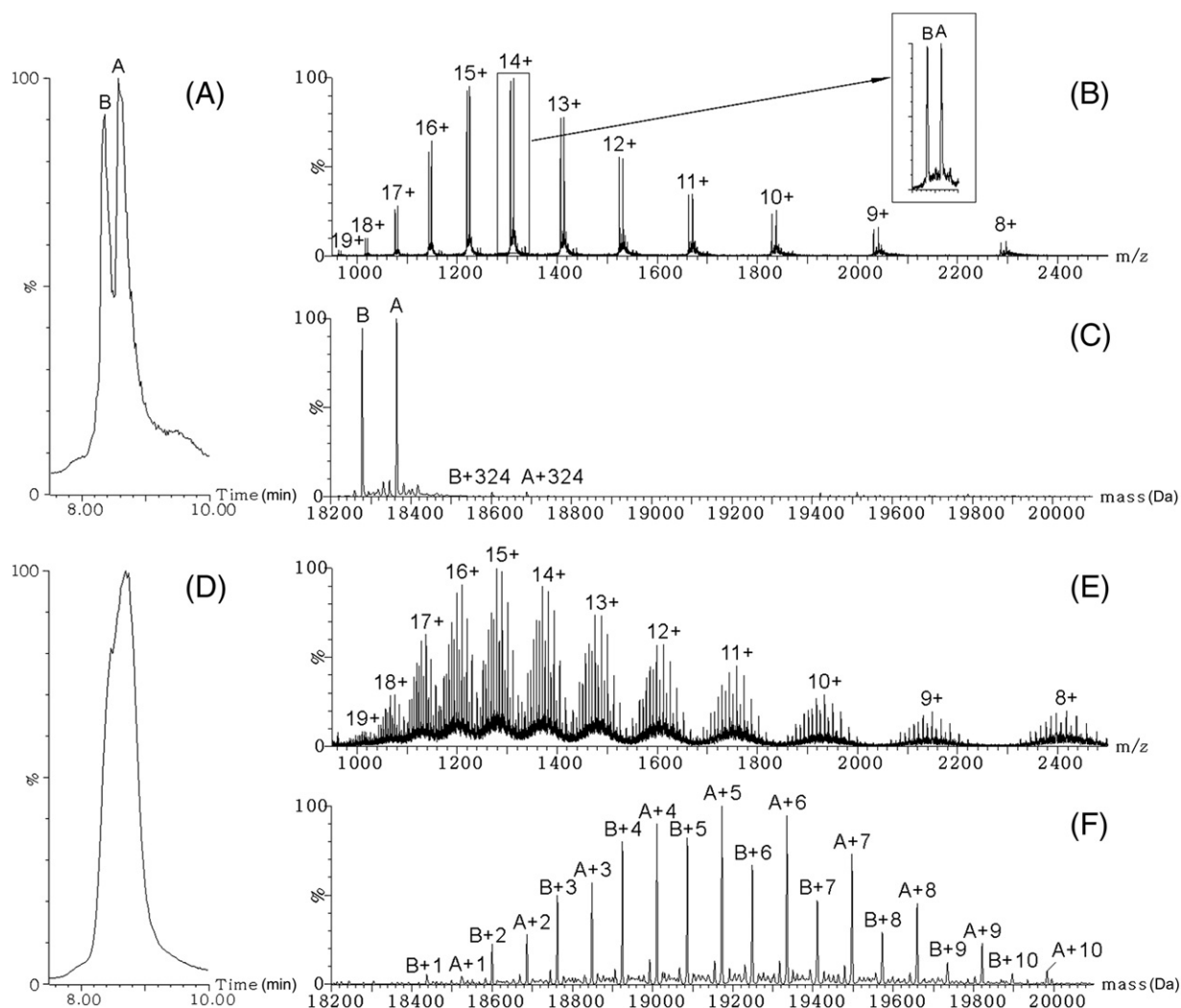


Fig. 1. Total ion current chromatographs (A and D), m/z spectra (B and E) and deconvoluted mass spectra (C and F) of β -lg with glucose prior to storage and stored for 8 h.

Liquid chromatography–mass spectrometry (LC–MS) is often used to show the accurate numbers of sugar adducts for the glycosylated proteins based on the molecular weight change. Moreover, each glycosylated form in one sample could be quantitated by analyzing the mass spectrum. This process had recently been used to evaluate the effect of type and size of the reducing sugars on product dispersion and the degree of substitution on α -lactalbumin (Haar et al., 2011), and the effect of temperatures, water activities and pH on β -lg during glycation (Thomsen et al., 2012). Compared to glycation extent determination, the glycation site determination by LC–MS(/MS) method is more complex. Before it will be submitted to the LC, the sample should be processed such as desalting, reducing, alkylation and enzymatic hydrolysis. The enzymatic hydrolysis is the most important step which cuts the proteins into peptides carrying limited numbers of glycosylated sites. The enzyme could be chymotrypsin (Ledesma-Osuna, Ramos-Clamont, & Vázquez-Moreno, 2008), endoproteinase Asp-N (Meltretter, Becker, & Pischetsrieder, 2008), V8 protease (endoproteinase Glu-C) (Fenaille, Morgan, Parisod, Tabet, & Guy, 2004; Sun, Hayakawa, Ogawa, & Izumori, 2005), pepsin (Fenaille et al., 2004; Moreno, Quintanilla-López, Lebrón-Aguilar, Olano, & Sanz, 2008; Sun et al., 2005), or trypsin (Chevalier, Chobert, Mollé, & Haertlé, 2001; Fenaille et al., 2004; Marvin, Parisod, Fay, & Guy, 2002; Meltretter, Seeber, Humeny, Becker, & Pischetsrieder, 2007; Mollé, Morgan, Bouhallab, & Léonil, 1998; Sun et al., 2005). Trypsin is the most widely used one. It recognizes the Lys-C and Arg-C but would be inhibited when their C-terminals connect to prolines. The enzymatic

hydrolyzed samples would be submitted for the glycation site determination. Data-independent acquisition LC–MS (LC–MS^E) is a newly developed technique for it, which reveals the glycosylated sites by parallel

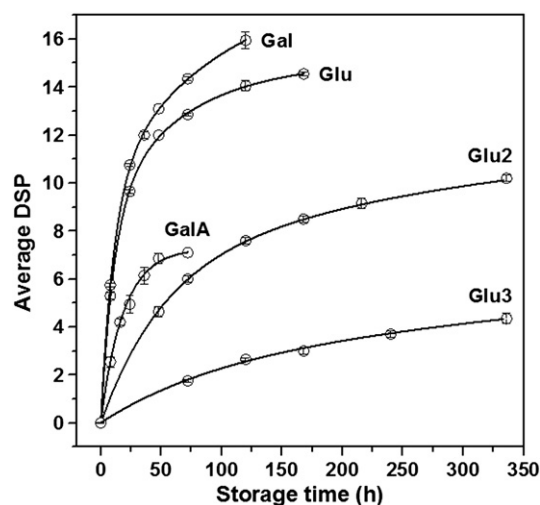


Fig. 2. The average DSP curve of β -lg with various reducing sugars. Gal: galactose; Glu: glucose; GalA: galacturonic acid; Glu2: maltose; Glu3: maltotriose.

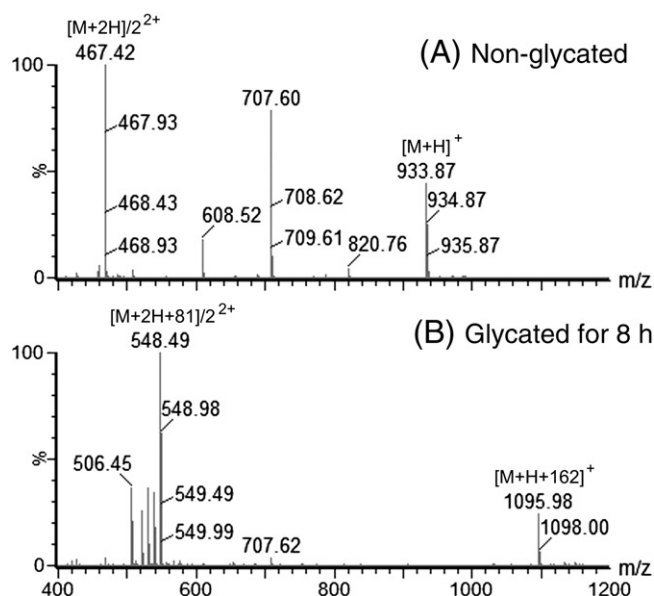


Fig. 3. MS spectra for peptides 1–8 of β -Ig stored with glucose.

generating parent ions by low collision energy and fragment ions by high collision energy (Plumb et al., 2006). This approach is more time-saving and the results are more reproducible compared to tandem mass spectrometry (MS/MS) adopting a data-dependant acquisition (DDA) (Geromanos et al., 2009).

In the present study, the effects of molecular size (glucose, maltose and maltotriose) and negative charge state (galactose and galacturonic acid) of reducing sugars on the glycation extent and glycation sites of β -Ig had been discussed using LC–MS and LC–MS^E methods.

2. Materials and methods

2.1. Materials

β -Ig was obtained from Davisco Foods International, Inc. (Eden Prairie, MN, USA). Glucose, galactose, galacturonic acid, maltose, maltotriose and TPCK-trypsin were obtained from Sigma-Aldrich (St Louis, MO, USA). Ammonia was obtained from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Filter membranes (0.45 μ m, 25 mm) were from Shanghai Bandao Industrial Co., Ltd (Shanghai, China). All the other chemicals were of analytical grade.

2.2. Methods

2.2.1. β -Ig – reducing sugar conjugates preparation

A 10 mg of β -Ig and a proper amount of reducing sugar were dissolved into 1 mL of double distilled water to reach a molar ratio of lysine residues to reducing ends at 1 to 2. The pH of the solution was adjusted to 8.0 using ammonia. The solution was lyophilized and then stored in a desiccator at 45 °C with a_w at 0.53 (saturated aqueous $Mg(NO_3)_2$ solution) for different times depending on the reaction activity of reducing sugars. Then the samples were stored at –20 °C prior to analysis.

2.2.2. Glycation extent determination

The UPLC–ESI–MS (Platform ZMD 4000 system, Waters Co., Milford, MA, USA), equipped with an Acquity UPLC BEH C4 column (2.1 $\times$ 100 mm, 1.7 μ m, Waters Co.), was used to analyze the extent of glycation. A sample containing 10 mg of β -Ig was added to 10 mL of double distilled water. After it dissolved, the solution was filtered through a 0.45 μ m filter membrane. The samples were kept at –20 °C until use, and the thawing

was conducted at 4 °C. Aliquot of 1 μ L was applied to Waters Acquity UPLC system at a column temperature of 45 °C. Solvent A was an aqueous solution containing 0.1% formic acid and solvent B was 100% acetonitrile. The elution was performed at a flow rate of 300 μ L/min using a gradient from 20% B to 40% within 10 min, to 100% in the following 10 min, back to 20% within 1 min and preserving for another 2 min. MS experiments were performed in a positive ionization mode, setting the cone voltage at 40 V and the collision energy at 6 eV. The scan m/z range started at 700 and ended at 2500 with a scan time of 1.5 s and with every 0.02 s interval over the whole course of 23 min.

The obtained data were analyzed with the assistance of the software MassLynx V4.1 and MassEnt (Waters Co.). Assuming all the β -Ig forms in each sample had similar ionization efficiency in mass spectrometry, the intensities of peaks for one variant in each mass spectrum could represent the relative amount of the β -Ig forms (Thomsen et al., 2012). Further, the average degree of substitution per protein molecule β -Ig (average DSP) could be calculated according to the following formulation (Chen, Liang, Liu, Labuza, & Zhou, 2012; Thomsen et al., 2012):

$$\text{Average DSP} = \frac{\sum_{i=0}^n i \times I_{(\beta\text{-Ig X}+i \times \text{reducing sugar})}}{\sum_{i=0}^n I_{(\beta\text{-Ig X}+i \times \text{reducing sugar})}}$$

where I is the peak intensity of one variant of β -Ig for various glycation forms; i is the number of attached reducing sugars; and X is variant A or B.

2.2.3. Glycation sites determination

Glycation sites were determined by UPLC–MS^E performing on a Waters SYNAPT MS system (Waters Co.), equipped with an Acquity UPLC BEH C18 column (2.1 $\times$ 50 mm, 1.7 μ m, Waters Co.). A sample containing 20 mg of β -Ig was added to 20 mL of double distilled water.

The enzyme digestion, HPLC elution and MS^E analysis were done according to our previous work (Chen et al., 2012). Each portion of 12 mL was dialyzed (molecular mass cut-off of 7 kDa, Union Carbide Co., Danbury, CT, USA) against double distilled water at 4 °C to remove the free reducing sugar in the solution. The pH of the solution was adjusted to 8.0 with 0.5 M ammonia, and 0.16 mL of 1 M TPCK-trypsin was added to hydrolyze the glycation proteins at 37 °C for 20 h. Enzyme was then inactivated by lowering the pH to 2.0 with 2 M hydrochloric acid. The sample was filtered through 0.45 μ m filter membrane. The samples were kept at –20 °C before use and thawing was conducted at 4 °C. Aliquot of 10 μ L sample was applied to Waters Acquity UPLC system at a temperature of 45 °C. Solvent A was 100% formic acid and solvent B was 100% acetonitrile. The elution was

Table 1
Glycated amino acid residues of β -Ig.

Glycated amino acid residues	Reducing sugars				
	Glu	Gal	GalA	Glu2	Glu3
L 1	++	++	++	++	++
K 8	++	++	++	++	++
K 14	+	+	+	+	+
K 47	++	++	++	++	++
K 60	+	+	+	+	+
K 69					
K 70	++	++	++	++	+
K 75	++	++	++	+	+
K 77	++	++	++	++	++
K 83	++	++	++	++	++
K 91	++	++	++	++	++
K 100	++	++	++	++	++
K 101					
K 135	++	++	++	++	++
K 138					
K 141					

Symbol “+”: recognized only in MS spectra; symbol “++”: recognized both in MS and MS^E spectrum.

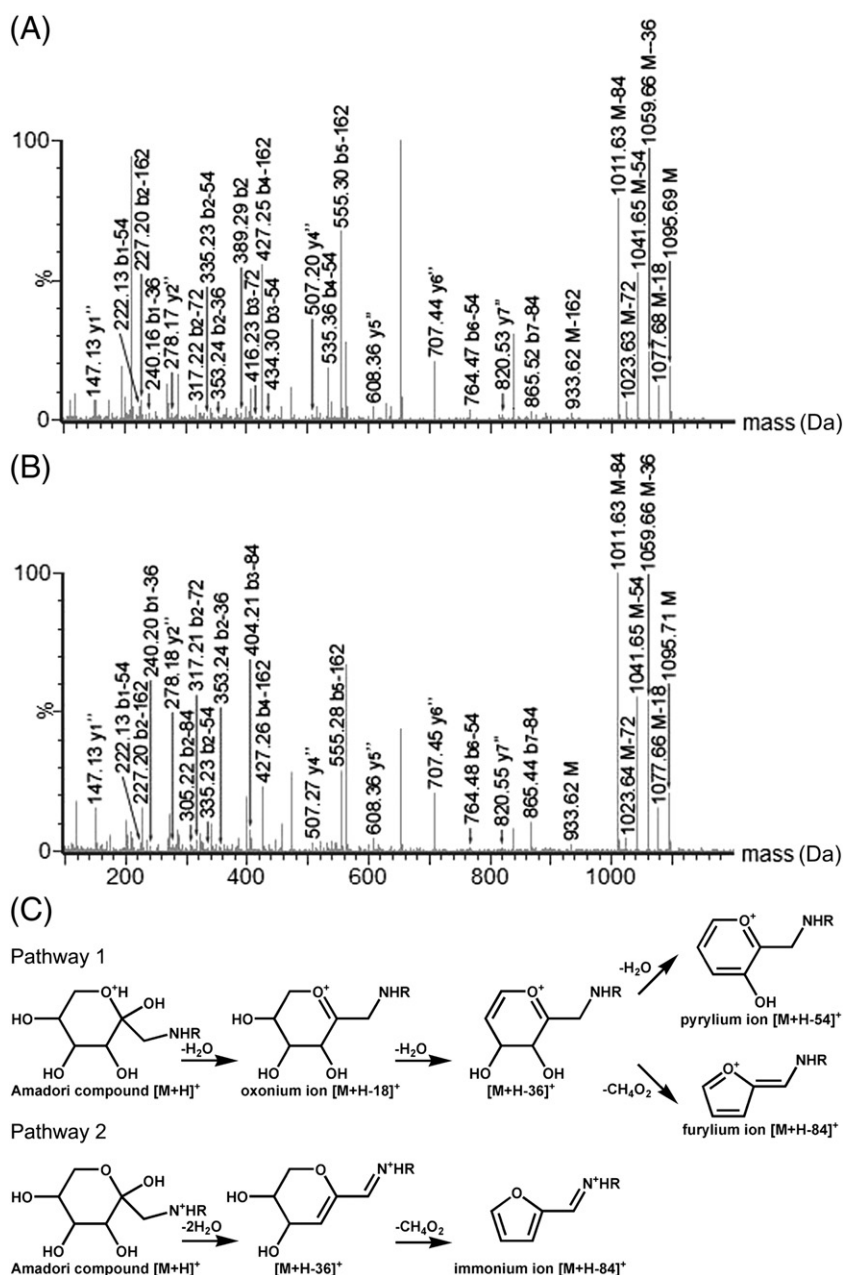


Fig. 4. Deconvoluted MS^E spectrum for peptides 1–8 stored with glucose (A) and galactose (B), and their proposed dissociation pathways (C).

performed at a flow rate of 300 $\mu\text{L}/\text{min}$ using a gradient from 0% B to 40% within 55 min, to 100% in the next 5 min, maintaining at 100% for another 5 min, then back to 0% within 1 min and preserving for another 4 min. The MS^E measurement was performed in a positive ionization mode. The low collision energy was set at 6 V and the high collision energy was set at 25 V. The capillary and sampling cone voltages were 3.5 kV and 30 V, respectively. The source and desolvation temperature were 100 and 400 $^{\circ}\text{C}$, respectively. The cone gas flow and the desolvation gas flow were 50 L/h and 500 L/h. The scan m/z range started at 100 and ended at 3000 with a scan time of 1 s with 0.02 s interval. The results obtained were analyzed with the assistance of the software MassLynx V 4.1 and MassEnt (Waters Co.).

A β -Ig 3-D model was established by the software of Swiss-PDB Viewer V 4.1 (<http://spdbv.vital-it.cn>) to study the glycosylated sites of β -Ig and the distances between nitrogen atoms in the side-chains of lysyl residues were also calculated by this software. The protein structure data was originated from PDB: 2BLG (Qin et al., 1998).

3. Results and discussion

3.1. Glycation extent

Fig. 1(A, B and C) shows the total ion current chromatogram, m/z spectrum and deconvoluted mass spectrum of sample containing β -Ig and glucose prior to storage. There were two peaks eluted by UPLC, indicating that the two variants of β -Ig could be separated by UPLC, although without a baseline separation (Czerwenka, Maier, Pittner, & Lindner, 2006; Monaci & van Hengel, 2007). Its m/z spectrum (incorporating both variants) (Fig. 1B) displayed a charge state from 8+ to 19+, which was similar to a former study (Czerwenka et al., 2006). For a certain number of charges, two peaks appeared, indicating that there are two proteins with different molecular weights in the sample. The MassEnt 1 process gave out its deconvoluted mass spectrum (Fig. 1C) and revealed two peaks at 18,277 and 18,363 Da, corresponding to variants B and A of β -Ig. Besides the peaks for variants B and A, two

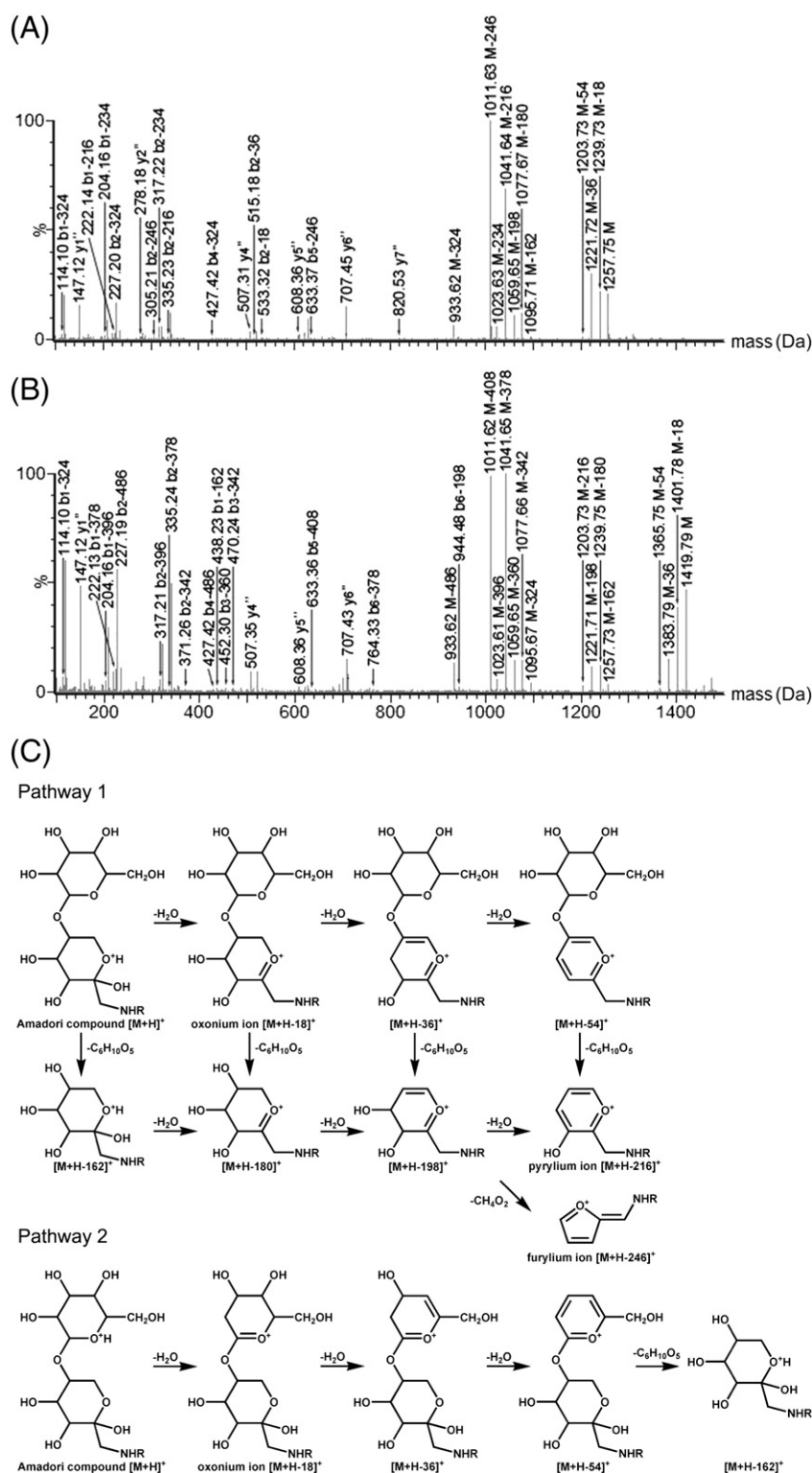


Fig. 5. Deconvoluted MS^E spectra for peptides 1–8 stored with maltose (A) and maltotriose (B), and their proposed dissociation pathways (C).

peaks with weak intensity were also shown. They corresponded to 324 Da mass increase for variants B and A, which might be resulted from the glycation between β -lg and lactose existing in milk (Morgan, Léonil, Mollé, & Bouhallab, 1997). After 8 h of storage at 45 °C, the total ion current chromatogram (Fig. 1D) displayed only one broad peak. It was due to the protein glycation that attached different numbers of glucose to β -lg, resulting in heterogeneous glycation products, which differed slightly in polarity and could not be separated by UPLC

(Corzo-Martínez, Moreno, Olano, & Villamiel, 2008). The m/z spectrum (Fig. 1E) showed a charge state distribution from 8 + to 19 +, indicating that glycation did not change the charge state of the proteins. In addition, the number of the peak with a certain charge increased, indicating the increase in protein species, which were clearly shown in the deconvoluted mass spectrum (Fig. 1F). During protein glycation, once one molecule of glucose was covalent coupled to β -lg, one water molecule was lost, resulting in the mass increase of 162 Da for each modified

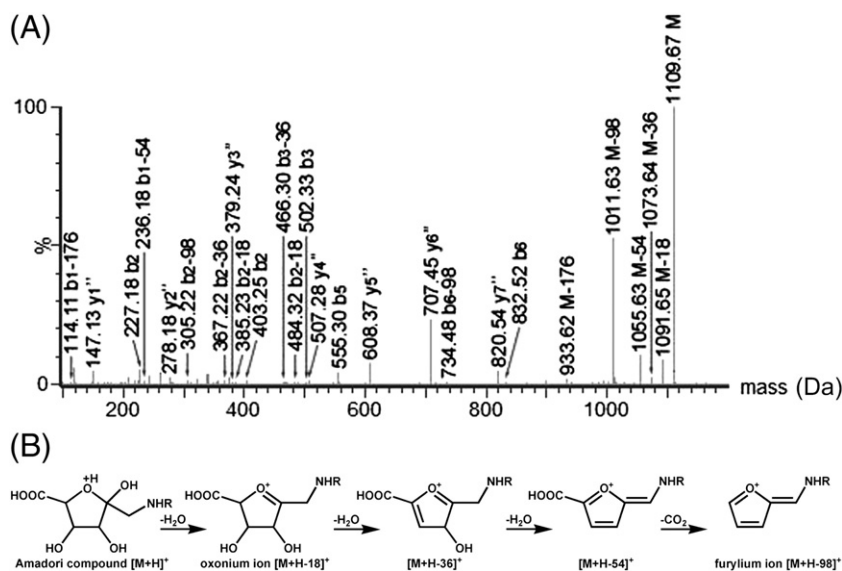


Fig. 6. Deconvoluted MS^E spectrum and proposed dissociation pathway for peptides 1–8 stored with galacturonic acid.

β -lg. After 8 h of storage, the peaks of variants B and A disappeared and twenty new peaks, representing variants B and A glycosylated with 1 to 10 glucose molecules, appeared, suggesting that all the β -lg had been modified by glucose via glycation. For Fig. 1F, the average DSP for variant A was 5.4 and for variant B it was 5.2, suggesting that the two variants have a similar glycation behavior (Thomsen et al., 2012). Therefore, the average value of the two variants was used to evaluate the glycation extent of β -lg.

The other deconvoluted mass spectra for protein glycosylated with various sugars were undergoing the same procedure and the obtained average DSP values were drawn in Fig. 2. The glycation progress of different saccharides could be divided into two stages. In the first stage, the average DSP increased in a linear manner. When glucose and its oligomers, including maltose and maltotriose, were compared, the glycation speed reduced with the increase in sugar size. A similar conclusion was also made when using ovalbumin (Aoki et al., 2001), α -lactalbumin and peptide (Haar et al., 2011). When galactose and galacturonic acid were compared, a negative charge state would reduce the glycation speed in the initial stage. It was probably because the electrostatic repulsion limited the approach of galacturonic acid to β -lg (Haar et al., 2011). This was similar to the results collected by Haar et al. (2011) when comparing the glycation speed of maltotriose and trigalacturonic acid in the initial phase. When glucose and galactose were compared, no similar glycation speed in this stage could be observed. However, the proportion of carbonyl form was traditionally considered as an important reason explaining the glycation reactivity of different sugars. Bunn and Higgins (1981) used the data collected by Hayward and Angyal (1977) to relate the glycation rate to the percentage of carbonyl form of different sugars in a solution. When the sugars were divided into groups of aldose and ketose, a higher percentage of open-ring form contributed to a greater glycation rate in each group in a solution. The data collected by circular dichroism in liquid state fit the result when using α -lactalbumin as a model protein to compare the glycation rate of rhamnose and arabinose to glucose in dry state with a_w of 0.65 (Haar et al., 2011), but not suitable for the dry state with a_w of 0.53. The differences of water content among samples might be the main reason in explaining for the divergence, because the formation of the open-ring form involves the transfer of three electrons and protons from the solvent.

The second stage occurred after a turn of the curve. In this stage, the glycation speed slowed down and the number of attached sugars showed an inclination of saturation. Maltose was a disaccharide and

maltotriose was a trisaccharide, so they had bigger sizes compared to monosaccharides. Therefore, the steric effect became the major factor restricting their approach to potential glycosylated sites. The early occurrence of a platform of galacturonic acid might be related to the electrostatic repulsion because the sugar was negatively charged. A similar phenomenon was also observed when adopting α -lactalbumin as a model protein (Haar et al., 2011).

3.2. Glycation sites

To compare the glycation site preference of reducing sugars with different molecular size and charge state, the glycosylated β -lg samples with average DSP around 5 were treated with TPCK-trypsin and then submitted to LC–MS^E.

The analysis of MS^E data could be divided into 2 steps. The first step was to check the MS spectra to find the glycosylated peptides. If a lysyl residue was glycosylated, the sugar moieties would protect it from trypsin hydrolysis and a new peptide containing a glycosylated site was hence formed, resulting in the novel peaks in the chromatogram (Gadgil, Bondarenko, Treuheit, & Ren, 2007). Therefore, the MS spectra of newly appeared peaks in the total ion current chromatogram for

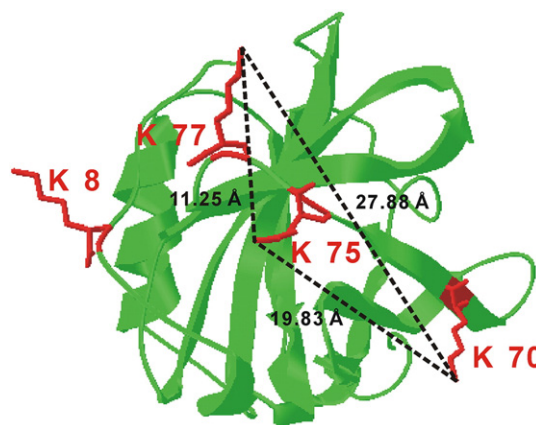


Fig. 7. Three-dimensional model of monomer β -lg. Green: backbone of β -lg; red: K 8, K 70, K 75 and K 77; black: distance from nitrogen atom in the side-chain of one lysyl residue to another.

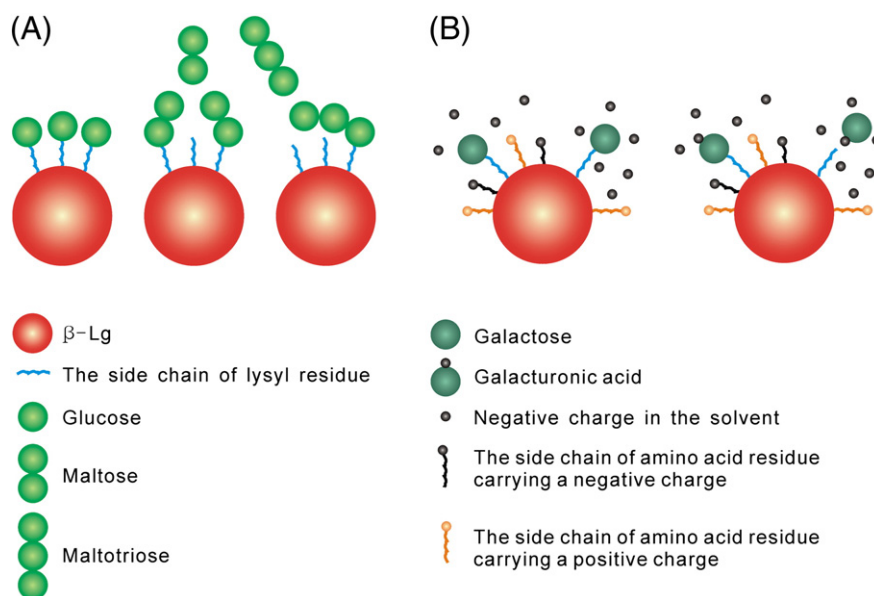


Fig. 8. Schematic representation of site differences affected by molecular size (A) and charge state (B).

glycated samples were checked. After glycation, the m/z peaks for peptides with 1, 2 or 3 ions would display an increase of the molecular weight of sugar-18, (the molecular weight of sugar-18)/2 or (the molecular weight of sugar-18)/3. Take glucose peptides 1–8 as an example (Fig. 3). Peptides 1–8 from non-glycated β -lg displayed m/z peaks at 467.42 for $[M + 2H]/2^{2+}$ and 933.87 for $[M + H]^+$. It agreed with a previous study (Mollé et al., 1998) that tryptic peptides were usually one or two charged in ESI-MS. After reacting with glucose at 45 °C for 8 h, the m/z peaks shifted to 548.49 and 1095.98, representing for ions of $[M + 2H + 81]/2^{2+}$ and $[M + H + 162]^+$, indicating that peptides 1–8 had been glycated by one glucose. All peptides glycated by the five sugars had been checked and the suspected glycated sites had been labeled with + and ++ in Table 1.

To accurately recognize the glycated site, an MS^E spectrum was used. A previous study (Gadgil et al., 2007) reported that attached sugars were more likely to lose neutral fragments compared with amino acid residues with proper collision energy. By checking the b and y" fragment ion series, glycated sites could be confirmed. For example, deconvoluted MS^E spectra of peptides 1–8 from glucose attached β -lg (Fig. 4A) displayed mass peaks at 1095.69 Da, representing peptides 1–8 attached by one molecule of glucose. The peaks at 1077.68, 1059.66, 1041.65, 1023.63, 1011.63 and 933.62 Da were corresponding to mass losses of 18 ($-H_2O$), 36 ($-2 \times H_2O$), 54 ($-3 \times H_2O$), 72 ($-4 \times H_2O$), 84 ($-5 \times H_2O$) and 162 ($-C_6H_{10}O_5$). A well proposed dissociation pathway for glucose was to form the pyrylium and furylium ions via the oxonium ion (Fig. 4C pathway 1) (Frolov, Hoffmann, & Hoffmann, 2006; Jerić, Versluis, Horvat, & Heck, 2002). However, considering the higher peak intensity of 1011.63 (M-84) than 1041.65 (M-54), another pathway originated from a precursor with protonated nitrogen atom should also be taken into account (Fig. 4C pathway 2) (Jerić et al., 2002). A neutral loss of 72 ($-4 \times H_2O$) with low peak intensity was not included in the dissociation pathway. Even though previous studies detected this neutral loss (Corzo-Martínez, Lebrón-Aguilar, Villamiel, Quintanilla-López, & Moreno, 2009; Frolov et al., 2006), no one gave an explanation for pyranose form to lose a fourth water molecule. Frolov et al. (2006) suggested that the consecutive loss of four water molecules might result from the glucose in furanose form. Besides the two mentioned pathways, another one characterized by imminium ion (Corzo-Martínez et al., 2009) was not detected in the present study. These peaks suggested that this peptide was indeed modified by one molecule of glucose. The series of y" ions in the spectra showed that no

modification occurred for amino acids 2–8. In other words, L 1 was modified by glucose. Furthermore, the incomplete series of b ions confirmed this result. Besides L 1, other 9 amino acid residues, namely K 8, 47, 70, 75, 77, 83, 91, 100 and 135, were attached by glucose (Table 1).

The MS^E spectrum of the peptides from samples containing the other four reducing sugars had been checked in order to recognize the glycated sites. Galactose had a similar fragmentation behavior to glucose (Fig. 4B), indicating that its dissociation pathway was also similar to glucose. Furthermore, its modified sites were exactly the same as glucose (Table 1) according to the deconvoluted MS^E spectrum. Maltose and maltotriose consisted of 2 and 3 glucoses, respectively. The deconvoluted MS^E spectrum of peptides 1–8 from the samples containing maltose and maltotriose (Fig. 5A and B) showed that the fragmentation behaviors of these two oligosaccharides were comparable to glucose and two proposed dissociation pathways

Table 2

The solvent accessibility and distances of potential glycated sites to the charges carried by the amino acid residues in β -lg.

Potential glycated sites	Solvent accessibility ($\text{\AA}^2$) ^a	Distance to the nearest positive charge ($\text{\AA}$) ^b	Average distance to the positive charges ($\text{\AA}$) ^b	Distance to the nearest negative charge ($\text{\AA}$) ^b	Average distance to the negative charges ($\text{\AA}$) ^b
L 1	144	5.94 (K 91)	21.89	2.70 (E 108)	24.20
K 8	187	17.32 (K 14)	24.86	13.98 (D 96)	29.64
K 14	118	10.48 (R 124)	23.84	4.71 (D 11)	26.44
K 47	88	4.62 (K 70)	25.39	4.25 (E 45)	23.94
K 60	32	4.83 (K 69)	24.90	3.55 (E 62)	22.01
K 69	87	4.83 (K 60)	24.78	4.40 (E 62)	22.32
K 70	114	4.62 (K 47)	25.11	3.75 (E 55)	23.25
K 75	128	11.25 (K 77)	24.88	9.11 (D 53)	26.40
K 77	211	8.27 (K 83)	27.69	13.62 (E 89)	31.05
K 83	92	6.72 (K 91)	24.32	6.95 (E 89)	26.19
K 91	37	5.94 (L 1)	22.77	6.24 (E 108)	25.06
K 100	142	4.29 (K 101)	23.13	3.02 (D 98)	24.81
K 101	73	4.29 (K 100)	23.18	4.40 (D 98)	24.17
K 135	85	6.24 (K 101)	21.88	6.61 (E 134)	23.89
K 138	138	8.23 (K 135)	24.50	4.55 (E 134)	27.05
K 141	167	6.83 (K 138)	25.21	10.48 (E 134)	27.98

^a The solvent accessibility was determined by Qin et al. (1998).

^b The distances were calculated by Swiss-PDB Viewer V 4.1 (<http://spdbv.vital-it.cn>) using the structure from PDB: 2BLG (Qin et al., 1998).

(Fig. 5C) were suggested according to the fragmentation behaviors. One (Fig. 5C pathway 1) was comparable to a previous study (Moreno et al., 2008), forming an oxonium ion in the six-membered ring linking directly to the amino group, and another (Fig. 5C pathway 2) was to form an oxonium ion far away from the amino group and transfer the electron to the next ring when breaking down the α -1, 4 glycosidic bond. However, inconsistent fragmentation behaviors had been observed regarding different oligosaccharides (Marvin et al., 2002; Moreno et al., 2008). This difference might result from MS acquisitions used or the units forming the oligosaccharides, so further study is necessary before any final conclusion can be reached. After checking the MS^E spectrum, nine sites in total were glycosylated by maltose, namely L 1, K 8, 47, 70, 77, 83, 91, 100 and 135; and 7 sites in total were glycosylated by maltotriose, namely L 1, K 47, 77, 83, 91, 100 and 135 (Table 1). The deconvoluted MS^E spectrum of peptides 1–8 from galacturonic acid attached β -lg showed that galacturonic acid had neutral losses of 18 ($-H_2O$), 36 ($-2 \times H_2O$), 54 ($-3 \times H_2O$), 98 ($-3 \times H_2O-CO_2$), and 176 ($-C_6H_8O_6$) (Fig. 6A), which more resemble to ribose (Frolov et al., 2006) than to glucose. It suggested that galacturonic acid led to the formation of a five-membered Amadori compound rather than a six-membered one in spite of the fact that it is a hexose. This Amadori compound could consecutively lose three molecules of water, followed by a molecule of CO_2 , forming a distinctive neutral loss of 98, to form a furylium ion (Fig. 6B). After checking the MS^E spectrum, nine sites in total were glycosylated by galacturonic acid, namely L 1, K 8, 47, 70, 75, 83, 91, 100 and 135 (Table 1).

The MS and MS^E data (Table 1 labeled with “++”) showed that glucose, maltose and maltotriose had glycation site differences on K 8, 70, and 75. K 8 has low glycation reactivity under the effect of a nearby charge state (Fogliano et al., 1998) and interaction between β -lg molecules (Qin et al., 1998). Meanwhile, maltotriose has a large molecular size. The two factors worked together to interfere the attachment of maltotriose to K 8 in the present experimental condition. The differences on K 70 and 75 were related to the position and glycation reactivity of K 70, 75 and 77. These three sites are spatially centralized: the distance between nitrogen atoms in side-chains of K 75 and 77 is 11.25 Å; the distance between those of K 70 and 75 is 19.83 Å; and the distance between those of K 70 and 77 is 27.88 Å (Fig. 7) (Qin et al., 1998). The diameter of one glucose molecule is around 10 Å. There was a possibility, as shown in Fig. 8A, that once one site with the highest glycation reactivity was attached by a reducing sugar, the attached sugar together with the protein structure would lower the accessibility of the other two sites. The larger the size of the sugar, the bigger the effect is. The result also suggested that among the three potential glycosylated sites, K 77 had the highest reactivity, followed by K 70 and K 75.

Meanwhile, the difference of site specific for galactose and galacturonic acid was on K 77, suggesting that the side chain of K 77 was located in a negatively charged environment built by the charges carried by other amino acid residues and the solvent. When comparing the distances of the 16 potential glycosylated sites to the positive charges carried by K, R or H (Table 2), K 77 had a mediate distance to the nearest positive charge carried by K 83 and the farthest average distance to all the positive charges. As for the negative charges carried by D or E (Haar et al., 2011) (Table 2), K 77 was distant to the nearest negative charge carried by E 89 and the farthest average distance to all the negative charges. These results indicated that the charges carried by other amino acid residues might not be the major reason for the difference of a glycosylated site on K 77 for galactose and galacturonic acid. On the other hand, K 77 showed the highest solvent accessibility among the 16 potential glycosylated sites (Table 2). Considering the pH of the system was 8.0, the alkaline solvent might be responsible for the weak reactivity of K 77 for galacturonic acid in the present study (Fig. 8B).

In conclusion, larger molecular size and negative charge state of reducing sugars would slow down the glycation speed and limit the number of attached sugars during the protein glycation progress of β -lg. Glucose, maltose and maltotriose had differences of glycation

on K 8, 70 and 75, and galactose and galacturonic acid had a difference of glycation on K 77 of β -lg, indicating that increasing molecular size and negative charge state of reducing sugars would lead to the dissimilarity on glycation site preference. The fragmentation results indicated that glucose and galactose had the same dissociation pathways. They could probably form pyrylium and furylium ions via the oxonium ion or form immonium by protonating the nitrogen atom. Galacturonic acid could form a five-membered Amadori compound to produce furylium ion with a distinctive neutral loss of 98. Compared to monosaccharide, maltose and maltotriose displayed a complex dissociation behavior, which was different from previous results.

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