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**The reduction in the IgE-binding ability of  $\beta$ -lactoglobulin by dynamic high pressure microfluidization coupled with glycation treatment revealed by high resolution mass spectrometry**

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**ABSTRACT**

Our previous study indicated that pretreatment by dynamic high pressure microfluidization (DHPM) and glycation with galactose was a promising method for decreasing the immunoglobulin E (IgE) binding ability of  $\beta$ -lactoglobulin ( $\beta$ -LG). In present work, the conformational alteration of  $\beta$ -LG subjected to DHPM and glycation treatment were investigated in relation to IgE binding ability by Orbitrap mass spectrometry. After DHPM pretreatment, lower IgE binding ability of glycated  $\beta$ -LG was observed with increasing pressures. Prior to DHPM pretreatment, 11 glycation sites were identified, while the number of glycation sites was increased to 12 after pretreatment. However, there was no significant difference of the glycation sites at the pressures of 50, 100, and 200 MPa, respectively. Average degree of substitution per peptide molecule of  $\beta$ -LG (DSP) was investigated to assess the degree of glycation for per glycation site. All the samples pretreated by DHPM exhibited higher glycation level than that of without DHPM pretreatment. The shielding effects of epitopes owing to glycation contributed to the reduction of IgE binding capacity. Orbitrap mass spectrometry could provide a comprehensive understanding of the nature of protein glycation.

**KEYWORDS:**  $\beta$ -lactoglobulin, IgE binding ability, dynamic high pressure microfluidization coupled with glycation, mass spectrometry

## INTRODUCTION

Cow milk is considered as a main source of nutrients to feed infants besides breast milk. Cow's milk allergy, mostly triggered by casein,  $\beta$ -lactoglobulin ( $\beta$ -LG), or  $\alpha$ -LA, has attracted more attention to its serious symptoms in health issues<sup>1, 2</sup>.  $\beta$ -LG is a 162-amino-acid globular protein with two disulfide bonds, and a molecular mass of 18.36 kDa<sup>3</sup>. Approximately 90% milk sensitizing individuals possess specific IgE against  $\beta$ -LG<sup>4</sup>. To date, the IgE allergenic epitopes of  $\beta$ -LG including linear or conformational types have been clearly identified. The linear epitopes is a portion of linear sequences along the 162 amino acid molecule, and the conformational epitopes is formed by 3D folding of the secondary and tertiary structure<sup>5, 6</sup>. Based on the different principles, various processing methods, such as high-pressure<sup>7</sup>, glycation<sup>8</sup>, enzymatic hydrolysis<sup>9</sup>, genetic modification<sup>10</sup>, and so on, have been applied for the modification of the  $\beta$ -LG to remove or reduce its allergenicity. However, some limitations exist in their practical application, and due to the protein structure features, a single method is usually insufficient to eliminate or destroy the  $\beta$ -LG allergen to achieve a satisfactory result.

Glycation, via Maillard-induced conjugation of proteins and carbonyl compounds, is an important reaction taking place in industrial food processing<sup>11-13</sup>. Glycation is a promising method for decreasing the allergenicity of  $\beta$ -LG<sup>14</sup>. High hydrostatic pressure is a novel food processing technology, influences the Maillard reaction and changes the allergenicity of protein<sup>15, 16</sup>.

Dynamic high pressure microfluidization (DHPM), which uses collective forces of

59 high-velocity impact, high-frequency vibration, instantaneous pressure drop, intense  
60 shear, cavitation, and ultra-high pressure up to 200 MPa<sup>17</sup>, has been employed for  
61 inactivating pathogens in dairy foods<sup>18</sup>, preparing liposomes<sup>19</sup>, and modifying  
62 protein<sup>20</sup>. In our previous work, we observed that glycation was associated with the  
63 protein tertiary structure<sup>21</sup>. In a recent publication, we demonstrated that the treatment  
64 of DHPM and glycation induced a reduction in the IgE-binding capacity of  $\beta$ -LG<sup>22</sup>. It  
65 should be noticing that glycation of  $\beta$ -LG mainly occurs on the lysyl residues. One or  
66 more lysyl residues were located on the  $\beta$ -LG epitopes, and thus glycation extent is  
67 associated with the IgE binding ability. The shielding effects of epitopes and the  
68 formation of aggregation owing to glycation influence the IgE-binding ability of  $\beta$ -LG.  
69 However, whether the IgE-binding capacity changes of  $\beta$ -LG treated by DHPM and  
70 glycation are due to the glycation extent is still uncertain. Further studies on the  
71 glycation site(s) and glycation extent per site in the  $\beta$ -LG are needed. Mass  
72 spectrometry (MS) was used to obtain a further analysis of the structural  
73 characterization of glycated proteins at the molecular level can be achieved. The  
74 Orbitrap Fusion Tribrid MS, which based on a mass resolving quadrupole, Orbitrap  
75 mass analyzer, collision cell, linear ion trap mass and analyzer architecture, provides a  
76 higher specificity and sensitivity resolution, making it a powerful tool for  
77 characterization of peptides with modification<sup>23, 24</sup>.

78 In present work, we studied the IgE binding ability and glycation site of  $\beta$ -LG  
79 pretreated by DHPM. We initially prepared the  $\beta$ -LG pretreated by DHPM at  
80 pressures 50, 100, and 200 MPa. The pretreated  $\beta$ -LG was then subjected to

81 Maillard-type glycation with galactose in dry state. Inhibition ELISA was employed  
82 to analysis the IgE-binding capacity of  $\beta$ -LG subjected to DHPM and glycation  
83 treatment. The glycation extent of  $\beta$ -LG pretreated or without pretreated by DHPM  
84 were assessed by ultra-high performance liquid chromatography-Orbitrap Fusion  
85 Tribrid MS.

## 86 MATERIALS AND METHODS

### 87 Materials

88  $\beta$ -LG (L3908), galactose (V900922), trypsin (T 8802,  $\geq 10,000$  BAEE units/mg),  
89 and Goat anti-human IgE-HRP conjugate (A9667, 1:2000) were purchased from  
90 Sigma-Aldrich Co. (St. Louis, MO). All other reagents used were of analytical grade.

91 Human sera: A series of 10 sera from patients having milk allergy were purchased  
92 from PlasmaLab International (Everett, W.A., USA) and frozen ( $-80$  °C) until  
93 analysis. Their specific IgE levels measured by ImmunoCAP (Phadia 100) in milk  
94 allergic human sera are 5.74-78.6 KUA/L. According to the documented clinical  
95 history, all patients had the milk related clinical symptoms (such as hives) begin in  
96 childhood. For the inhibition ELISA, the mixed serum pool (equal volume of ten  
97 patients' sera) was used.

### 98 Sample Preparation

99 According to our previous report<sup>25</sup>, DHPM and glycation treatment were carried  
100 out by the following conditions.  $\beta$ -LG (1 mg/mL) was dissolved in sodium phosphate  
101 buffer (50 mM, pH 8.0).  $\beta$ -LG solutions were treated thrice at pressures of 0, 50, 100,

102 and 200 MPa by Microfluidizer processor (M-110EH; Microfluidics Co., Newton,  
103 MA). The samples were collected, followed by the addition of the same mass of  
104 galactose. After lyophilization, the sample were incubated at 55 °C and 65% relative  
105 humidity for 6 h. After terminating glycation reaction, 100  $\mu$ L of distilled water was  
106 added into each sample in the tube and the sample solution was filtered by a Centricon  
107 centrifugal filter unit (3000 Da; Slariob, Beijing, China). The final concentration of  
108  $\beta$ -LG was 2 mg/mL and kept at  $-20$  °C until used. Three repeated were performed for  
109 each sample.

#### 110 **Analysis of Inhibition ELISA**

111 The IgE binding ability was performed by inhibition ELISA<sup>14, 22</sup>. 96-well  
112 micro-plates were coated with  $\beta$ -LG samples (100  $\mu$ L/well, 5 $\mu$ g/mL) and were  
113 incubated overnight at 4 °C. After washing, the wells were blocked with 1% fish  
114 gelatin in phosphate buffer solution (PBS) for 1 h at 37 °C. The plates were wash  
115 thrice with PBS/Tween-20 solution (PBST). Equal volumes of antisera samples (1:20  
116 diluted human sera) and glycated  $\beta$ -LG (inhibitors) were mixed and incubated for 1 h  
117 at 37 °C. One hundred microliters of each inhibited antisera sample mixture were  
118 added into the wells, and the plates were then incubated at 37 °C for 2 h. After  
119 washing, the wells was added one hundred microliters of purified goat anti-human  
120 IgE-HRP conjugate diluted 1:2000 in PBST, and incubated at 37 °C for 1 h. The plates  
121 was washed thrice with PBST, and each well was immediately added 100 microliters  
122 of tetramethylbenzidine solution. The reaction was quenched by adding sulfuric acid  
123 (50  $\mu$ L, 2 M). The absorbance was measured at 450/570 nm by the Bio-Rad

124 Microplate Reader (Synergy H1, Bio-Tek, USA). The inhibition rate was calculated  
125 according to the following formulation: % inhibition =  $(1 - B/B_0) \times 100$ , where B and  
126  $B_0$  are the absorbance values of the well with and without the inhibitor, respectively.  
127  $IC_{50}$  is the concentration of inhibitors that induces a 50% inhibition of IgE binding  
128 ( $\mu\text{g/mL}$ ). Duplicate were performed for each sample.

### 129 **Filter-aided sample preparation (FASP)**

130 Protein was digested according to the FASP method<sup>26</sup>. 20  $\mu\text{g}$  protein was mixed  
131 with 200  $\mu\text{L}$  of 150 mM UA buffer (8M urea, 150 mM Tris-HCl pH 8.0) and 20  $\mu\text{L}$  of  
132 100 mM dithiothreitol and incubated at 95 °C for 5 min, followed by cooling in the  
133 ice bath. Then the solution was transferred to ultrafiltration (10 kDa cutoff) and  
134 centrifuged at  $14000 \times g$  for 15 min at 25 °C; UA buffer (200  $\mu\text{L}$ ) was then added and  
135 centrifuged at  $14000 \times g$  for 15 min. After, iodoacetamide in UA buffer (100  $\mu\text{L}$ , 50  
136 mM) was added, followed by incubating in the dark at 25 °C for 30 min, and then  
137 centrifuged at  $14000 \times g$  for 10 min at 25 °C. UA buffer (100  $\mu\text{L}$ ) was added to wash  
138 the filters twice, followed by the additional of one hundred microliters dissolution  
139 buffer (50 mM triethylammonium bicarbonate at pH 8.5), then centrifuged at  $14000 \times$   
140  $g$  for 10 min and repeated this step twice. Forty microliters of trypsin (0.05  $\mu\text{g}/\mu\text{L}$ )  
141 was added to each filter and incubated 37 °C for 16 h. Two microliters of 50%  
142 trifluoroacetic acid was added to terminate reaction. The peptide were collected by  
143 centrifugation at  $14000 \times g$  for 10 min.

### 144 **Analysis by LC-MS**

145 An Orbitrap Fusion Tribrid mass spectrometer (Thermo scientific, Waltham, MA,  
146 USA) was used to analysis samples. Solvent A was 0.1% formic acid (FA) in ultrapure  
147 H<sub>2</sub>O, whereas solvent B consisted of 84% acetonitrile in ultrapure H<sub>2</sub>O, 0.1% FA. The  
148 digested sample was injected onto a 75 µm i.d. × 100 mm 3µm-C18 column  
149 (EASY-column, Thermo scientific, Waltham, MA, USA). After desalting for 20 min  
150 with A, the peptides were eluted at 300 nL/min with a gradient of 4-50% B for 40 min,  
151 50-100% B for 5 min, and 100% B for 1 min, using a Thermo Scientific™ Dionex™  
152 Ultimate™ 3000 system. Orbitrap high-energy C-trap dissociation (HCD)/electron  
153 transfer dissociation (ETD) fragmentation modes with top 10 method was used to  
154 acquire MS/MS spectra. Determination of the target value was enabled with  
155 predictive automatic gain control. The exclusion duration of 40.0 s was used for  
156 dynamic exclusion. The normalized collision energy was defined as twenty-seven eV,  
157 and the under fill ration of 0.1% was enabled.

158 To further compare the glycation extent of each peptide, the average degree of  
159 substitution per peptide molecule β-LG (DSP) was calculated according to the  
160 equation <sup>27</sup>

$$161 \quad \text{DSP} = \frac{\sum_{i=0}^n i \times I(\text{peptide} + i \times \text{galactose})}{\sum_{i=0}^n I(\text{peptide} + i \times \text{galactose})} \quad (1)$$

162 where I is the sum of the intensities of glycated β-LG peptide, and i is the number  
163 of galactose units attached to the peptide in each glycated form.

#### 164 Database Search

165 Mascot 2.2 (Matrix Science) and proteome discoverer were applied to perform

166 searches against the NCBI database. The search parameters were set as followed:  
167 peptide mass tolerance =  $\pm 20$  ppm, MS/MS tolerance of  $\pm 0.5$ , a maximum of 5  
168 missed cleavages. a fixed modification was carbamidomethyl (C), and variable  
169 modification were oxidation (M) and glycation (K and R).

## 170 RESULTS AND DISCUSSION

### 171 IgE-binding ability analysis

172 Figure 1 depicted the IgE-binding ability of glycated  $\beta$ -LG with and without  
173 DHPM pretreatment. Without DHPM pretreatment, the  $IC_{50}$  values of glycated  $\beta$ -LG  
174 was 2.47  $\mu\text{g/mL}$ , whereas the  $IC_{50}$  were 7.06, 11.04, and 19.78  $\mu\text{g/mL}$  when  $\beta$ -LG was  
175 processed by DHPM at 50, 100, and 200 MPa respectively. It has been showed that  
176 the immunogenicity of  $\beta$ -LG glycated by carboxymethyl dextran was reduced<sup>28</sup>. In the  
177 research of the influence of casein glycomacropeptide on the allergenicity of  $\beta$ -LG, a  
178 decrease of IgE-binding ability of  $\beta$ -LG in the presence of casein glycomacropeptide  
179 was found<sup>29</sup>. According to the literature, the IgE-binding ability of glycated  $\beta$ -LG was  
180 dependent on the level of Maillard reaction<sup>30</sup>. In our study, the decreased IgE binding  
181 ability of glycated  $\beta$ -LG under DHPM pretreatment was observed. We hypothesized  
182 the reason for the decreased IgE binding ability was the glycation reaction induced  
183 masking of some epitopes<sup>31</sup>. Hence, we decided to characterize the glycation sites and  
184 the glycation level of per glycated peptide of  $\beta$ -LG by mass spectrometry to test  
185 whether DHPM pretreatment favored the glycation reaction and reducing the IgE  
186 binding ability.

## 187    **The glycation sites of $\beta$ -LG**

188        To fully understand the conformational changes of  $\beta$ -LG conjugated with galactose,  
189    the preference of galactose for glycation site should be identified. The trypsin  
190    digestion followed by liquid chromatography-tandem mass spectrometry  
191    (LC-MS/MS), was employed to determine the glycation sites. We chose trypsin as the  
192    protease to digest protein since it could theoretically cleave the carbon side of amino  
193    acids lysine and arginine but would be not functional if the C-terminal of these two  
194    amino acids were connected with proline.  $\beta$ -LG ( variant A and B) has 15 lysine and 3  
195    arginine, however, a prolyl residue was found on the C-terminal of Lys47<sup>32, 33</sup>.  
196    Therefore,  $\beta$ -LG has seventeen potential glycated sites. It can be easily identified the  
197    glycated forms of the peptides according to the mass shift induced by glycation<sup>34</sup>.  
198    Theoretically, if a peptide was mono-glycated by galactose, the corresponding m/z of  
199    peaks with 1, 2, 3, 4, or 5 charges will show a mass shift of 162.0528 Da, with m/z  
200    change of 162.0528, 81.0264, 54.0176, 40.5132, and 32.4106, respectively. For  
201    example, the m/z peaks of the unglycated peptide 41-60, 41-69, 61-70, 78-91, and  
202    84-100 (Figure 2) were 579.0708<sup>4+</sup>, 683.9494<sup>5+</sup>, 417.1926<sup>3+</sup>, 524.6317<sup>3+</sup> and  
203    655.0180<sup>3+</sup>, while the corresponding m/z peaks of glycated peptides were 619.5839<sup>4+</sup>,  
204    716.3556<sup>5+</sup>, 471.2102<sup>3+</sup>, 578.6499<sup>3+</sup>, and 709.0361<sup>3+</sup>, respectively. These peaks with  
205    m/z alteration were 40.5132, 32.4062, 54.0176, 54.0182, and 54.0181 Da, respectively,  
206    demonstrating that one molecule of galactose was added to all peptides. The glycated  
207    modifications were further confirmed by MS/MS. We also observed that the ions peak  
208    with m/z of 599.9480<sup>3+</sup> and 653.9657<sup>3+</sup> (Figure 2E) had a mass increase of 162.0525

209 and 324.1056 Da, respectively, from the peptide 125-138 with  $m/z$  of 545.9305<sup>3+</sup>,  
210 demonstrating that the peptide 125-138 was glycated by 2 molecules of galactose.

211 The ETD MS/MS was applied to perform a complete map of the glycated sites of  
212  $\beta$ -LG<sup>35, 36</sup>. The determination of glycated sites Lys47, Lys60, Lys75, Lys77, and Lys83  
213 by MS/MS is depicted in Figure 3. All sites were observed to be glycated in the  $\beta$ -LG  
214 with and without DHPM pretreatment. A number of series of c and z ions generated  
215 by the ETD MS<sup>2</sup> was unambiguously confirmed the sequence of the peptide  
216 <sup>41</sup>VYVEELKPTPEGDLEILLQK<sup>60</sup> (Figure 3A), and the glycated site was identified at  
217 Lys47. The ETD fragmentations of glycated peptides with  $m/z$  of 716.3556<sup>5+</sup>,  
218 446.2594<sup>4+</sup>, and 578.6499<sup>3+</sup> were showed in Figure 3B, 3C, 3D. A series of c and z  
219 ions in the figure was detected, which matched well with the fragments of peptides  
220 <sup>41</sup>VYVEELKPTPEGDLEILLQKWENGECQK<sup>69</sup>, <sup>71</sup>IIEKTKIPAVFK<sup>83</sup>, and  
221 <sup>78</sup>IPAVFKIDALNENK<sup>91</sup>. The results confirmed the galactose was linked to Lys60,  
222 Lys75, Lys77, and Lys83. Likewise, the glycation sites for peptides with  
223 corresponding  $m/z$  of 709.0358<sup>3+</sup>, 452.5824<sup>3+</sup>, 568.7792<sup>4+</sup>, and 443.2520<sup>3+</sup> (Figure 4)  
224 were identified to be Lys91, Lys100, Lys135, Lys138, and Lys141, respectively.

225 The reported IgE epitopes of  $\beta$ -LG are AA 1-14, AA 25-60, AA 84-100, AA  
226 102-124, and AA 149-162<sup>37</sup>. Some of the lysines (e.g., K47, K60, K83, and so on) in  
227  $\beta$ -LG are located in these IgE binding areas. In the present study, the damage to lysine  
228 residues during glycation pretreated by DHPM likely resulted in structural changes of  
229 epitopes on  $\beta$ -LG, and thus decreased the IgE binding ability of  $\beta$ -LG.

230 **Analysis of the glycation extent of  $\beta$ -LG pretreated by DHPM**

LC-MS was employed for analysis of the glycation degree of  $\beta$ -LG. The glycation degree of  $\beta$ -LG pretreated by DHPM can be illustrated by (1) the number of glycation sites and (2) the level of glycation for per glycated site. Glycated sites of all the modified peptides before and after DHPM pretreatment was presented in Table 1, which identified by accurate mass and tandem mass spectrometry. Without DHPM pretreatment,  $\beta$ -LG was found to be glycation modified at 11 sites, which were Lys47, Lys69, Lys70, Lys75, Lys77, Lys83, Lys91, Lys100, Lys135, Lys138, and Lys141. After DHPM (50, 100, and 200 MPa) pretreatment, an additional glycated site (Lys60) was detected in the  $\beta$ -LG. The increased glycated site suggested that DHPM pretreatment induced the conformational structure changes of  $\beta$ -LG, exposing more reactive sites. Corzo-Martínez et al.<sup>38</sup> characterized glycated peptides of  $\beta$ -LG glycated with galactose by LC-ESI-MS/MS derived from in vitro gastrointestinal digestion, 13 glycated sites (L1, Lys14, Lys47, Lys75, Lys77, Lys83, Lys91, Lys99, Lys100, Lys101, Lys135, Lys138, and Lys141) were identified.

DSP was chosen as the best indicator to differentiate the degree of per glycation site and was calculated to in-depth understand the level of glycation of  $\beta$ -LG<sup>39</sup>. Figure 5 shows the DSP of most of glycated peptide with and without DHPM pretreatment. After DHPM pretreatment, the DSP value of glycated peptides are improved, compared to those of the untreated  $\beta$ -LG. According to the results of DSP, we found that DHPM pretreatment promoted glycation reaction and improved the extent of glycation of  $\beta$ -LG. This also indicated that  $\beta$ -LG underwent a structure change under the DHPM pretreatment. Interestingly, we found most glycated peptides had a

253 maximal DSP value at 100 MPa.

254  $\beta$ -LG is known to exist as a noncovalent dimer in the native state<sup>40</sup>. The  
255 monomeric and dimeric configuration of  $\beta$ -LG is typically affected by pH,  
256 temperature, and salt concentration<sup>41</sup>. It was reported that the conformational form of  
257  $\beta$ -LG exist as the monomeric form when the temperature was 5-76 °C<sup>42</sup>. To ensure the  
258 mass spectrometry analysis of heating samples performed well, the aggregation will  
259 not be introduced. We selected preparing the  $\beta$ -LG-galactose solution at pH 8.0 and  
260 heating at 55 °C for 6 h in dry-state as a consequence of without apparent protein  
261 aggregation at these conditions.

262 Previous study demonstrated that secondary structure of  $\beta$ -LG treated by pressure  
263 are slightly changed, as suggested by CD analysis, however, the tertiary structure are  
264 partially retained<sup>7, 43</sup>. According to Zhong et al., the tertiary and secondary of  $\beta$ -LG  
265 was distorted by DHPM and glycation treatment.<sup>8</sup> Our mass spectrometric results  
266 were consistent with this notion. The added glycation site demonstrates the partial  
267 occurrence of conformation structure of  $\beta$ -LG unfolding by the DHPM treatments.

268 The potential glycation sites were dependent on some factors, such as the tertiary  
269 structure, neighboring amino acid compositions, and hydrogen bonding. The nearby  
270 charged amino acid, which located close to the lysine, can influence the glycation of a  
271 lysine. For example, although Lys47 had an intermediate level exposure, it had been  
272 confirmed the high reactivity because of its spatial proximity to Lys70<sup>44, 45</sup>. Besides,  
273 another explanation of promoting the glycation of lysines was the exposure of amino  
274 acids. Even though Lys70 and Lys60 were both found on the  $\beta$ -strand D, the side

275 chains of Lys70 was exposed to outside, determining it easy to react with sugar, while  
276 the side chains of Lys60 pointed into the cavity, making it was less likely to be  
277 glycated<sup>39</sup>.

278 According to the research, collision induced dissociation was employed for analysis  
279 of the formation of the pyrylium and furylium during the Maillard reaction<sup>38</sup>.  
280 However, ions corresponding to the neutral loss of water molecules from glycated  
281 peptides were also detected, So we applied ETD analysis to identify the glycated  
282 peptide. In this work, based on the accurate mass and tandem mass spectrometry  
283 analysis, we can infer the conformational changes after DHPM pretreatment from the  
284 increased glycated site and glycation degree. During the DHPM pressure changes, the  
285 unique alteration of glycation was the occurrence of Lys60. Most likely, a more open  
286 conformation of  $\beta$ -LG in response to DHPM made this amino acid residue to react  
287 with galactose. At 200 MPa, the aggregation of  $\beta$ -LG may cause the decrease of DSP  
288 values for most peptides.

289 It is clear that most abnormal immune responses caused by milk protein are  
290 regulated by IgE<sup>46</sup>. In our study, the inhibition ELISA was performed to evaluate the  
291 effect of DHPM and glycation treatment on potential allergenicity of  $\beta$ -LG,  
292 determined as the capacity to bind IgE from the milk allergic patients' sera. It has been  
293 confirmed that the allergenicity of  $\beta$ -LG contains conformational epitopes and linear  
294 epitopes. Selo et al.<sup>37</sup> reported that the major  $\beta$ -LG allergenic epitopes identified are  
295 the fragments of AA 102-124, AA 41-60, and AA 149-162. As we know, the  
296 substitution rates of lysyl residues located in the epitopes are related with the

297 IgE-binding ability of  $\beta$ -LG. When more lysyl  $\epsilon$ -amino groups of  $\beta$ -LG are glycated  
298 by reducing sugar, IgE is less likely to encounter unhindered epitopes, making  
299 decreased binding to  $\beta$ -LG<sup>19</sup>. In our study, the addition of Lys60 and increased  
300 glycation level could explain the increased IC<sub>50</sub> values of glycated  $\beta$ -LG under DHPM  
301 pretreatment. The formed aggregation might mask the epitopes, which made a  
302 contribution to further weak the IgE binding capacity of glycation  $\beta$ -LG in response to  
303 DHPM pretreatment at 200 MPa.

304 In this study, the high-resolution Orbitrap mass spectrometry was applied to  
305 investigate the glycation sites and the extent of glycation, and the IgE-binding  
306 capacity was characterized by inhibition ELISA. The IgE-binding capacity of  $\beta$ -LG  
307 was reduced under DHPM combined with glycation treatment. The glycation reaction  
308 was promoted by DHPM pretreatment both in DSP value and the number of glycation  
309 sites. The addition of Lys60 and the increase of DSP values contributed to the  
310 decrease of IgE binding capacity. Our results demonstrated that mass spectrometry  
311 analysis provided an overall picture of glycated  $\beta$ -LG for a better understanding of  
312 protein structure alteration.

### 313 ABBREVIATIONS USED

314 DHPM, dynamic high pressure microfluidization; IgE, immunoglobulin E;  $\beta$ -LG,  
315  $\beta$ -lactoglobulin; HHP, High hydrostatic pressure; MS, mass spectrometry; PBS,  
316 phosphate buffer solution; PBST, PBS/Tween solution; HCD, high-energy C-trap  
317 dissociation; ETD, electron transfer dissociation; DSP, the average degree of  
318 substitution per peptide molecule  $\beta$ -LG; LC-MS/MS, liquid chromatography-tandem

319 mass spectrometry;

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476 **Figure captions**

477 **Figure 1:** IgE binding ability of glycosylated  $\beta$ -LG pretreated by DHPM was performed  
478 by inhibition ELISA. Anti- $\beta$ -LG patients' pooled sera (50  $\mu$ L/well) were incubated  
479 separately with 0, 1, 5, 30, 60, 100  $\mu$ g/mL (50  $\mu$ L/well) of glycosylated  $\beta$ -LG as  
480 inhibitors.

481 **Figure 2:** Mass spectra for the glycosylated peptides of  $\beta$ -LG-galactose pretreated at 100  
482 MPa. (A) peptide 41-60 at  $m/z$  579.0708<sup>4+</sup>, (B) peptide 41-69 at  $m/z$  683.9472<sup>5+</sup>, (C)  
483 peptide 61-70 at  $m/z$  417.1926<sup>3+</sup>, (D) peptide 78-91 at  $m/z$  524.6317<sup>3+</sup>, (E) peptide  
484 125-138 at  $m/z$  545.9305<sup>3+</sup>. The determined peptides are labelled by residue numbers.  
485 The  $m/z$  differences between glycosylated and unglycosylated peptides are indicated above the  
486 arrows.

487 **Figure 3:** The ETD MS/MS spectra of the glycosylated peptides. (A) The glycosylated  
488 peptides 41-60 (VYVEELKPTPEGDLEILLQK) with  $m/z$  of 619.5844<sup>4+</sup>, (B) the  
489 glycosylated peptide 41-69 (VYVEELKPTPEGDLEILLQKWENGECQAQK) with  $m/z$  of  
490 716.3556<sup>5+</sup>, (C) the glycosylated peptides 71-83 (IIAEKTKIPAVFK) at  $m/z$  of 446.2594<sup>4+</sup>,  
491 and (D) the glycosylated peptides 78-91 (IPAVFKIDALNENK) at  $m/z$  of 578.6491<sup>3+</sup>. The  
492 sequence of per peptide is depicted on the top of the spectrum. The identified glycosylated  
493 sites are indicated by a line with galactose. The c and z ions are shown by the  
494 numbers and lines.

495 **Figure 4:** The ETD MS/MS spectra of the glycosylated peptides. (E) The glycosylated  
496 peptides 84-100 (IDALNENKVLVLDTDYK) with  $m/z$  of 709.0358<sup>3+</sup>, (F) the  
497 glycosylated peptide 92-101 (VLVLDTDYKK) with  $m/z$  of 452.5824<sup>3+</sup>, (G) the glycosylated

498 peptides 125-141 (TPEVDDEALEKFDKALK) at  $m/z$  of 568.7792<sup>4+</sup>, (H) the glycosylated  
499 peptides 139-148 (ALKALPMHIR) at  $m/z$  of 443.2520<sup>3+</sup>, and (I) the glycosylated  
500 peptides 41-70 (WENGECQAQKKIIAEK) at  $m/z$  of 532.7581<sup>4+</sup>. The sequence of per  
501 peptide is depicted on the top of the spectrum. The identified glycosylated sites are  
502 indicated by a line with galactose. The c and z ions are labeled by numbers and lines.  
503 **Figure 5:** DSP values of glycosylated sites pretreated by DHPM at: 0 MPa (white bars),  
504 50 MPa (slashed bars), 100 MPa (black bars), 200 MPa (grey bars).

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**Table 1.** Summary of the glycosylated peptides with 6 h heating at 55 °C after DHPM pretreated at difference pressures.

| peptide<br>location | m/z<br>(glycated)      | [M + H] <sup>+</sup> |           | ΔM<br>[ppm] | sequence <sup>a</sup>                        | glycated<br>site |
|---------------------|------------------------|----------------------|-----------|-------------|----------------------------------------------|------------------|
|                     |                        | Obsd                 | theor     |             |                                              |                  |
| 0 MPa               |                        |                      |           |             |                                              |                  |
| 41-60               | 619.5844 <sup>4+</sup> | 2475.3160            | 2475.3116 | 1.75        | (R)VYVEELKPTPEGD<br>LEILLQK(W)               | K47              |
| 61-70               | 471.2101 <sup>3+</sup> | 1411.6157            | 1411.6648 | -0.07       | (R)WENGEC*AQKK(I)                            | K69              |
| 61-75               | 532.7586 <sup>4+</sup> | 2128.0125            | 2128.0604 | 0.5         | (R)WENGEC*AQKKII<br>AEK(T)                   | K69,k70          |
| 71-83               | 446.2592 <sup>4+</sup> | 1782.0149            | 1782.0147 | 0.1         | (K)IIAEKTKIPAVFK(I)                          | K75,K77          |
| 78-91               | 578.6491 <sup>3+</sup> | 1733.9327            | 1733.9320 | 0.4         | (K)IPAVFKIDALNENK<br>(V)                     | K83              |
| 84-100              | 709.0352 <sup>3+</sup> | 2125.0909            | 2125.0911 | -0.11       | (K)IDALNENKVLVLD<br>TDYK(K)                  | K91              |
| 92-101              | 452.5815 <sup>3+</sup> | 1355.7300            | 1355.7304 | -0.37       | (K)VLVLDTDYKK(Y)                             | K100             |
| 125-138             | 653.9650 <sup>3+</sup> | 1959.8807            | 1959.8805 | 0.08        | (R)TPEVDDEALEKFD<br>K(D)                     | K135             |
| 125-141             | 568.7795 <sup>4+</sup> | 2272.0963            | 2272.0966 | -0.17       | (R)TPEVDDEALEKFD<br>KALK(A)                  | K135,k13<br>8    |
| 139-148             | 443.2518 <sup>3+</sup> | 1327.7408            | 1327.7402 | 0.41        | (K)ALKALPM* HIR(L)                           | K141             |
| 50MPa               |                        |                      |           |             |                                              |                  |
| 41-60               | 619.5832 <sup>4+</sup> | 2475.3111            | 2475.3116 | -0.23       | (R)VYVEELKPTPEGD<br>LEILLQK(W)               | K47              |
| 41-69               | 716.3577 <sup>5+</sup> | 3577.7595            | 3577.8108 | -0.68       | (R)VYVEELKPTPEGD<br>LEILLQKWENGEC*A<br>QK(K) | K60              |
| 61-70               | 471.2102 <sup>3+</sup> | 1411.6162            | 1411.6648 | 0.26        | (R)WENGEC*AQKK(I)                            | K69              |
| 41-70               | 532.7584 <sup>4+</sup> | 2128.0118            | 2128.0604 | 0.15        | (R)WENGEC*AQKKII<br>AEK(T)                   | K69,k70          |
| 71-83               | 446.2594 <sup>4+</sup> | 1782.0158            | 1782.0147 | 0.65        | (K)IIAEKTKIPAVFK(I)                          | K75,K77          |
| 78-91               | 578.6494 <sup>3+</sup> | 1733.9338            | 1733.9320 | 1.04        | (K)IPAVFKIDALNENK<br>(V)                     | K83              |
| 84-100              | 709.0367 <sup>3+</sup> | 2125.0954            | 2125.0911 | 2.04        | (K)IDALNENKVLVLD<br>TDYK(K)                  | K91              |
| 92-101              | 452.5818 <sup>3+</sup> | 1355.7309            | 1355.7304 | 0.31        | (K)VLVLDTDYKK(Y)                             | K100             |
| 125-138             | 653.9659 <sup>3+</sup> | 1959.8832            | 1959.8805 | 1.39        | (R)TPEVDDEALEKFD<br>K(D)                     | K135             |
| 125-141             | 568.7795 <sup>4+</sup> | 2272.0963            | 2272.0966 | -0.17       | (R)TPEVDDEALEKFD<br>KALK(A)                  | K135,k13<br>8    |
| 139-148             | 443.2519 <sup>3+</sup> | 1327.7412            | 1327.7402 | 0.69        | (K)ALKALPM*HIR(L)                            | K141             |
| 100 MPa             |                        |                      |           |             |                                              |                  |

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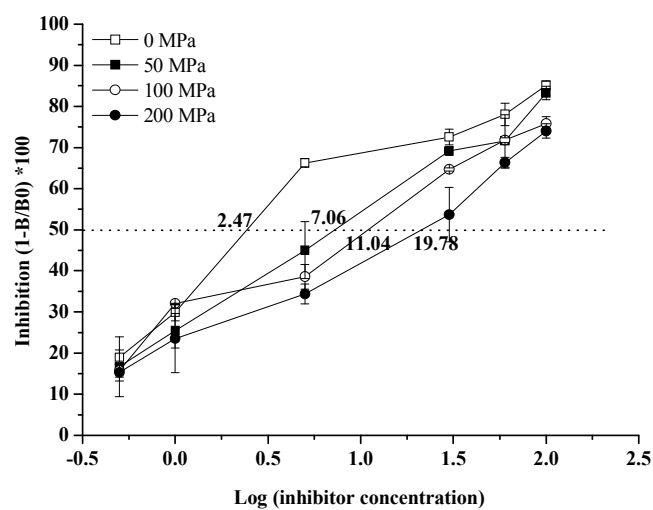
Table 1 (continued)

| peptide<br>location | m/z<br>(glycated)      | [M + H] <sup>+</sup> |           | ΔM<br>[ppm] | sequence <sup>a</sup>                        | Glycated<br>site |
|---------------------|------------------------|----------------------|-----------|-------------|----------------------------------------------|------------------|
|                     |                        | obsd                 | theor     |             |                                              |                  |
| 41-60               | 619.5839 <sup>4+</sup> | 2475.3141            | 2475.3116 | 0.96        | (R)VYVEELKPTPEGD<br>LEILLQK(W)               | K47              |
| 41-69               | 716.3556 <sup>5+</sup> | 3577.7488            | 3577.8108 | -3.67       | (R)VYVEELKPTPEGD<br>LEILLQKWENGEC*A<br>QK(K) | K60              |
| 61-70               | 471.2102 <sup>3+</sup> | 1411.6162            | 1411.6648 | 0.13        | (R)WENGEC*AQKK(I)                            | K69              |
| 41-70               | 532.7581 <sup>4+</sup> | 2128.0104            | 2128.0604 | -0.53       | (R)WENGEC*AQKKII<br>AEK(T)                   | K69,k70          |
| 71-83               | 446.2594 <sup>4+</sup> | 1782.0158            | 1782.0147 | 0.65        | (K)IIAEKTKIPAVFK(I)                          | K75,K77          |
| 78-91               | 578.6499 <sup>3+</sup> | 1733.9351            | 1733.9320 | 1.78        | (K)IPAVFKIDALNENK<br>(V)                     | K83              |
| 84-100              | 709.0358 <sup>3+</sup> | 2125.0927            | 2125.0911 | 0.75        | (K)IDALNENKVLVLD<br>TDYK(K)                  | K91              |
| 92-101              | 452.5824 <sup>3+</sup> | 1355.7326            | 1355.7304 | 1.52        | (K)VLVLDTDYKK(Y)                             | K100             |
| 125-138             | 653.9659 <sup>3+</sup> | 1959.8832            | 1959.8805 | 1.39        | (R)TPEVDDEALEKFD<br>K(D)                     | K135             |
| 125-141             | 568.7792 <sup>4+</sup> | 2272.0951            | 2272.0966 | -0.71       | (R)TPEVDDEALEKFD<br>KALK(A)                  | K135,k13<br>8    |
| 139-148             | 443.2520 <sup>3+</sup> | 1327.7414            | 1327.7402 | 0.90        | (K)ALKALPM*HIR(L)                            | K141             |
| 200MPa              |                        |                      |           |             |                                              |                  |
| 41-60               | 619.5839 <sup>4+</sup> | 2475.3141            | 2475.3116 | 0.96        | (R)VYVEELKPTPEGD<br>LEILLQK(W)               | K47              |
| 41-69               | 895.1974 <sup>4+</sup> | 3577.7677            | 3577.8108 | 1.60        | (R)VYVEELKPTPEGD<br>LEILLQKWENGEC*A<br>QK(K) | K60              |
| 61-70               | 471.2099 <sup>3+</sup> | 1411.6154            | 1411.6648 | -0.32       | (R)WENGEC*AQKK(I)                            | K69              |
| 61-75               | 532.7576 <sup>4+</sup> | 2128.0084            | 2128.0604 | -1.45       | (R)WENGEC*AQKKII<br>AEK(T)                   | K69,k70          |
| 71-83               | 446.2596 <sup>4+</sup> | 1782.0166            | 1782.0147 | 1.06        | (K)IIAEKTKIPAVFK(I)                          | K75,K77          |
| 78-91               | 578.6494 <sup>3+</sup> | 1733.9335            | 1733.9320 | 0.83        | (K)IPAVFKIDALNENK<br>(V)                     | K83              |
| 84-100              | 709.0367 <sup>3+</sup> | 2125.0954            | 2125.0911 | 2.04        | (K)IDALNENKVLVLD<br>TDYK(K)                  | K91              |
| 92-101              | 452.5819 <sup>3+</sup> | 1355.7312            | 1355.7304 | 0.51        | (K)VLVLDTDYKK(Y)                             | K100             |
| 125-138             | 653.9657 <sup>3+</sup> | 1959.8827            | 1959.8805 | 1.10        | (R)TPEVDDEALEKFD<br>K(D)                     | K135             |
| 125-141             | 568.7802 <sup>4+</sup> | 2272.0987            | 2272.0966 | 0.91        | (R)TPEVDDEALEKFD<br>KALK(A)                  | K135,k13<br>8    |
| 139-148             | 443.2524 <sup>3+</sup> | 1327.7425            | 1327.7402 | 1.65        | (K)ALKALPM*HIR(L)                            | K141             |

511

<sup>a</sup>C\* refers to carbamidomethyl. <sup>a</sup>M\* means to oxidantion

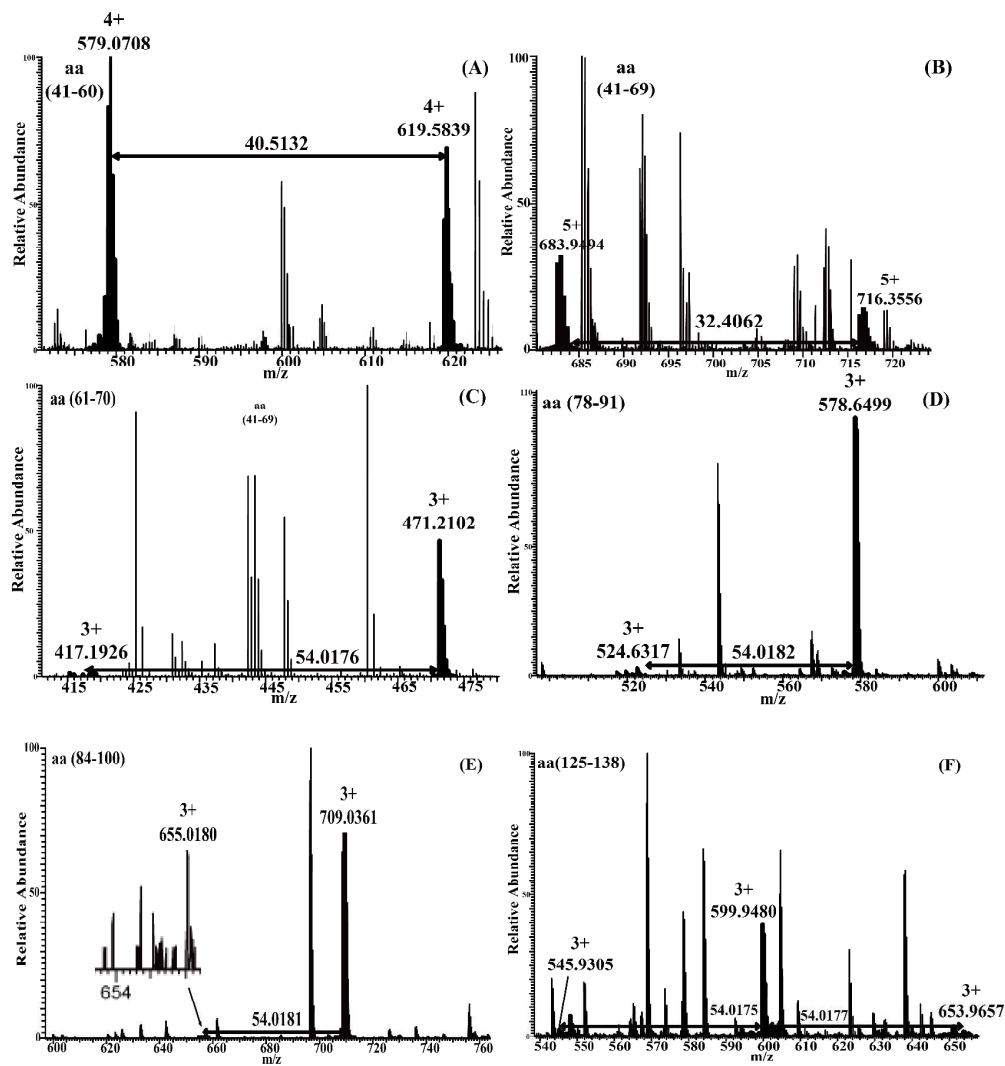
512

513 **Figure 1**

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Figure 2

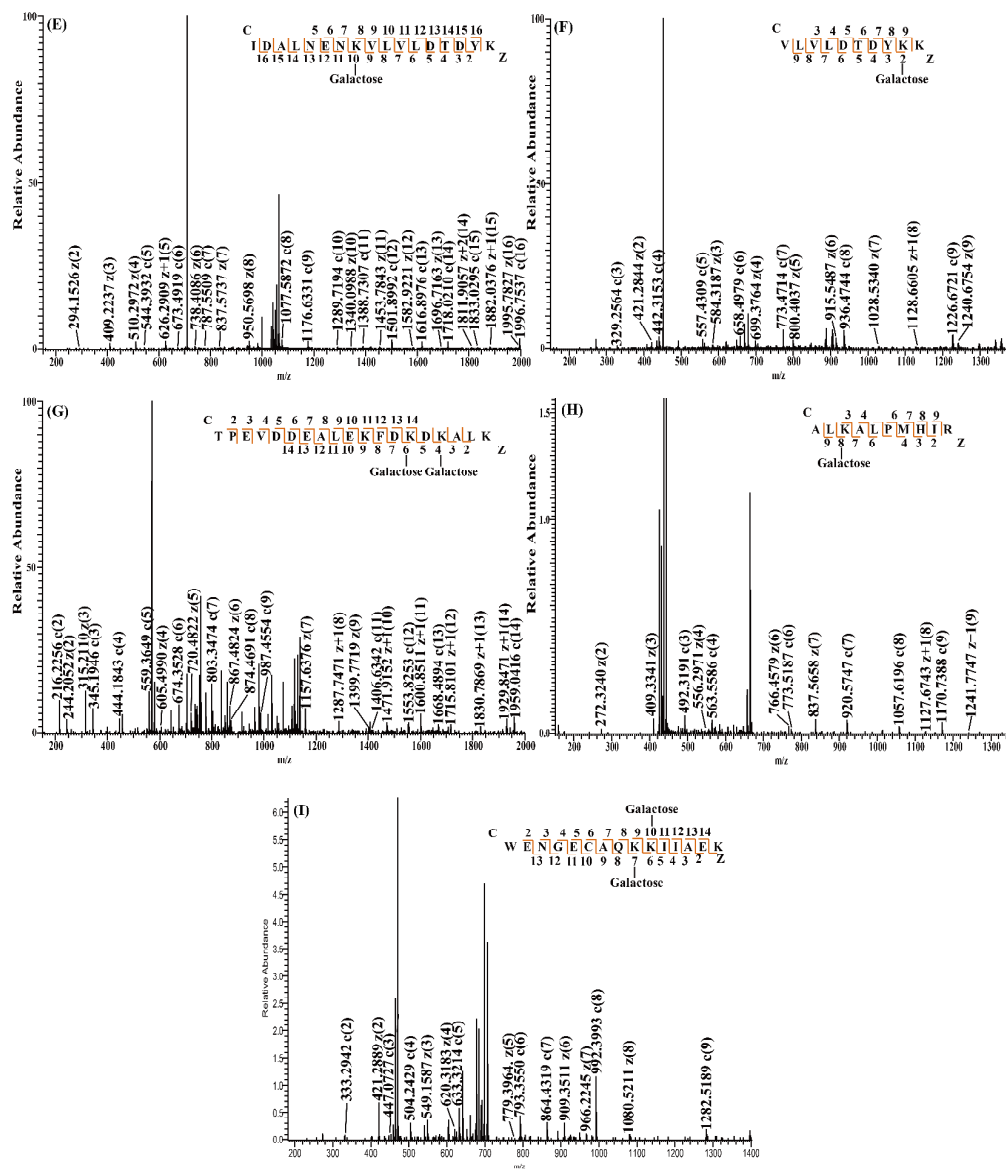


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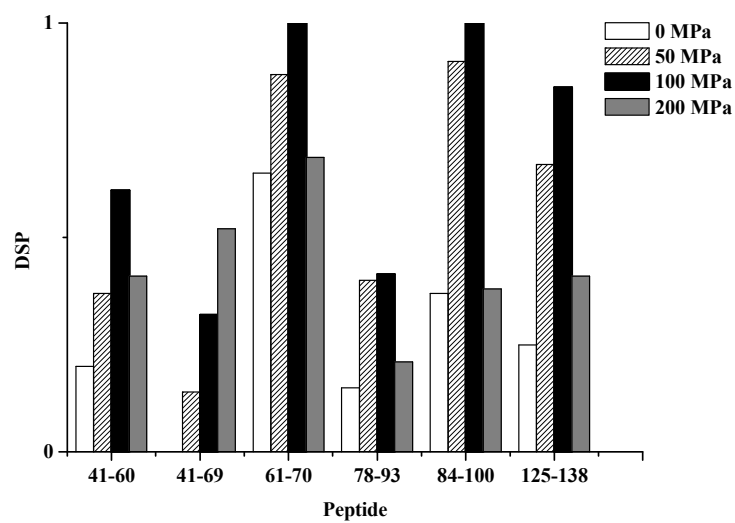


522 **Figure 4**



523

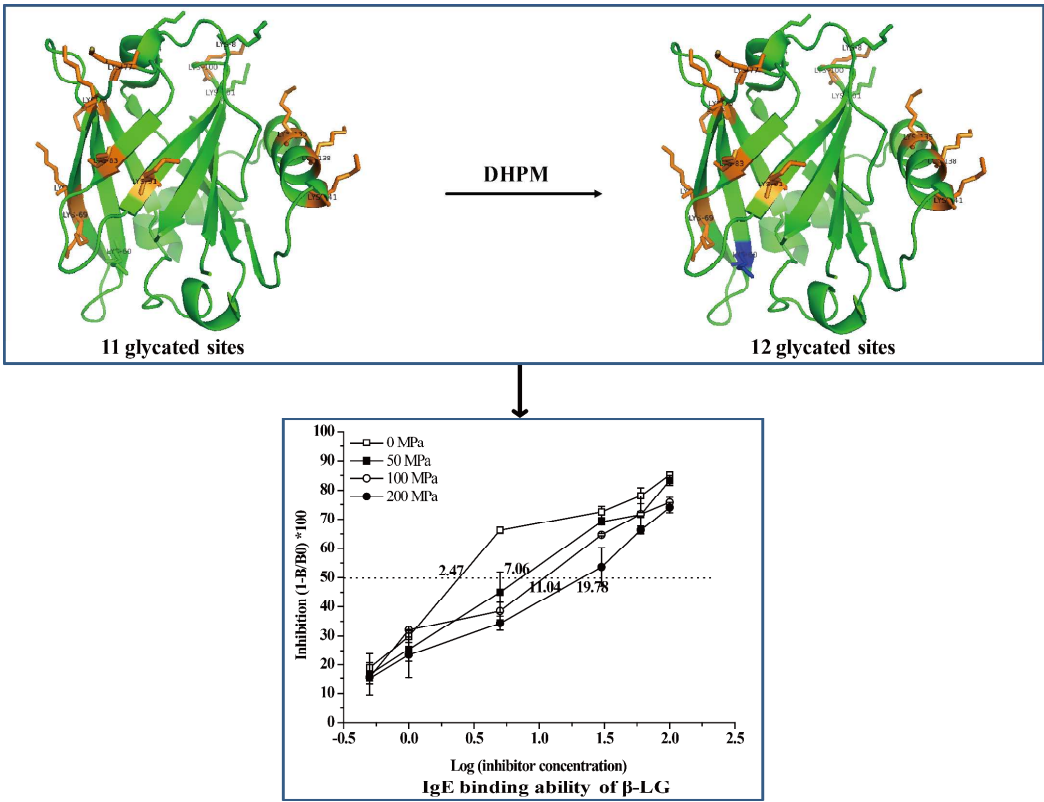
524

525 **Figure 5**

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527

528 TOC Graphic  
529



530

Figure 1

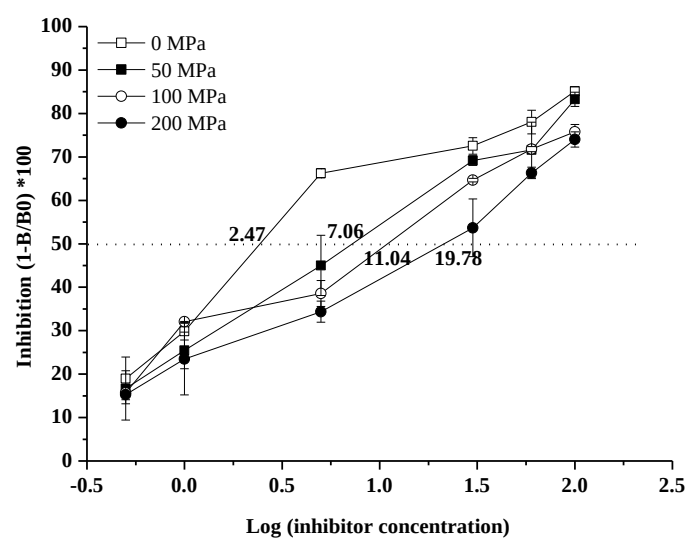


Figure 2

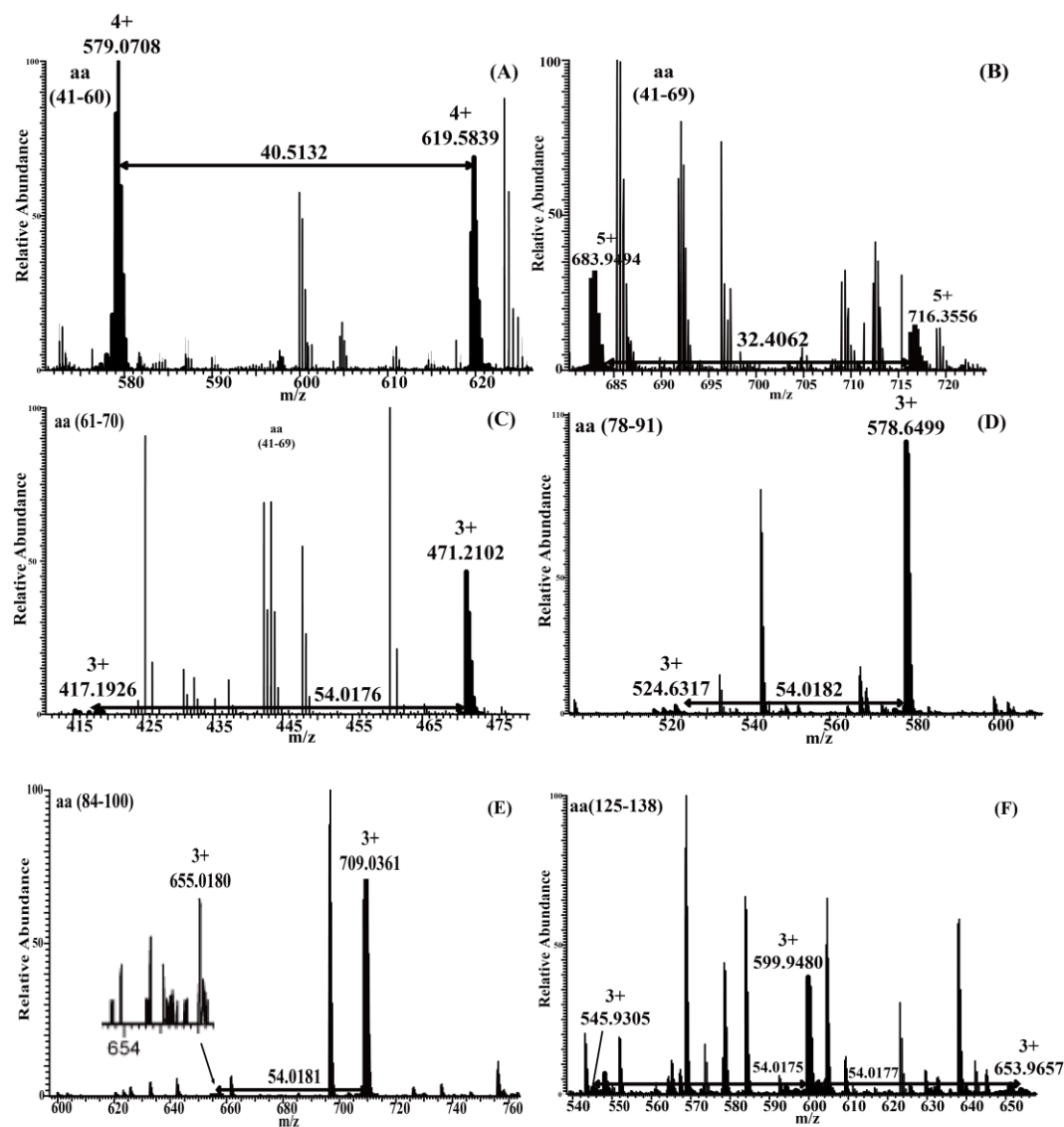


Figure 3

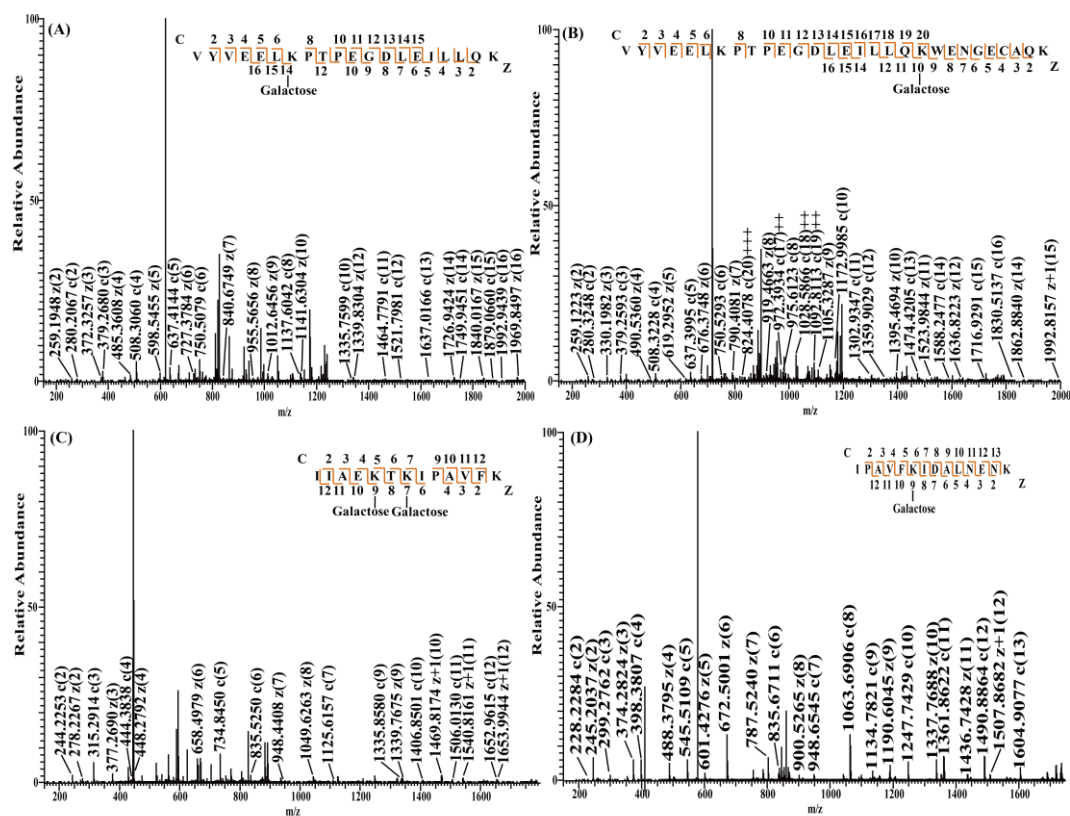


Figure 4

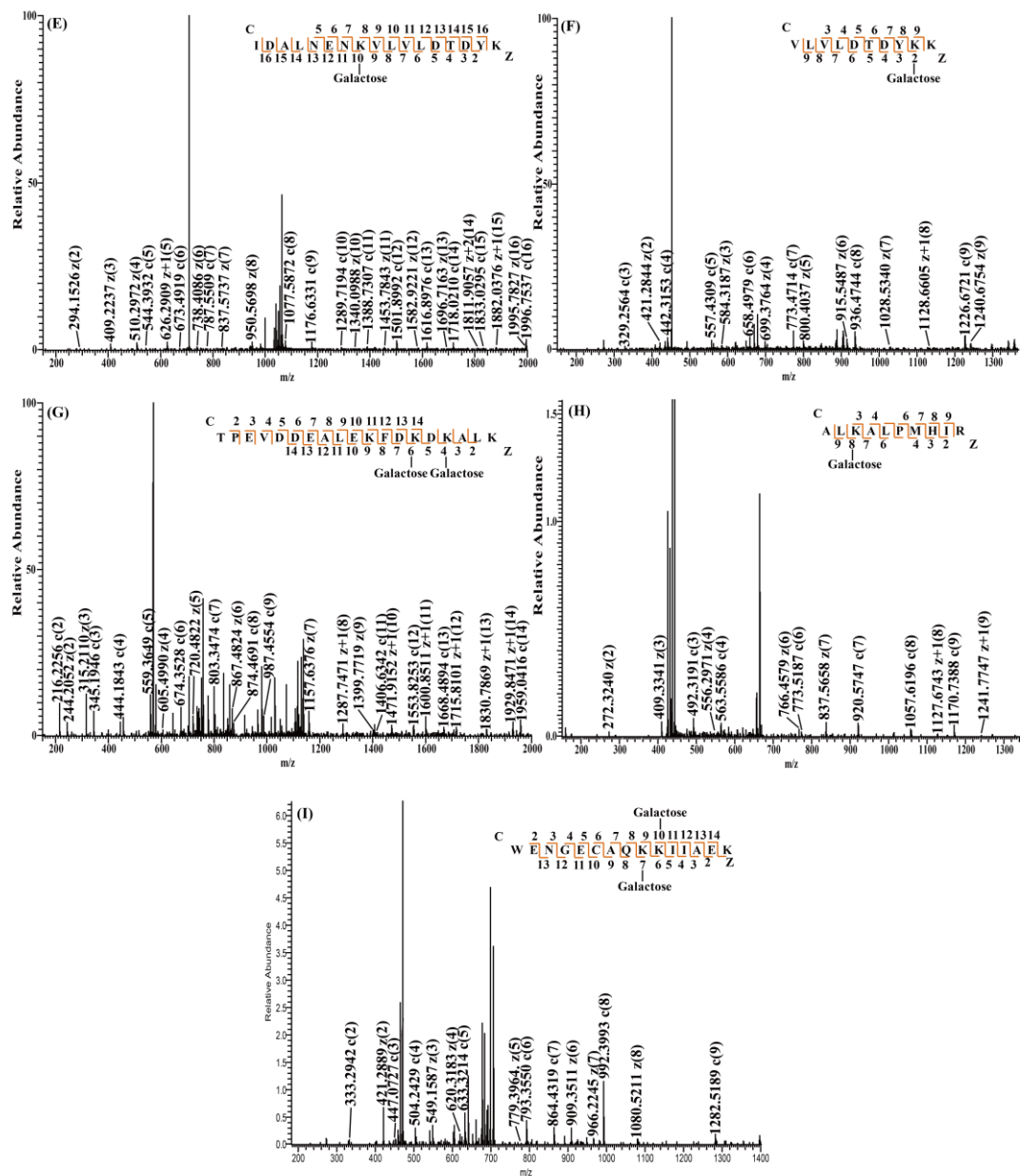


Figure 5

