Structure and antioxidant activity of β-lactoglobulin-glycoconjugates obtained by high-intensity ultrasound induced Maillard reaction in aqueous model systems and neutral conditions

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22 **Abstract**

23 Sonication is a new processing technology in dairy industry. The aim of this study was to test
24 glycation of β-lactoglobulin (BLG) in Maillard reaction (MR) induced by high-intensity
25 ultrasound in aqueous solution under neutral conditions at 10-15 °C, which are not favorable for
26 the MR. BLG was sonicated in the presence of glucose, galactose, lactose, fructose, ribose and
27 arabinose. Formation of Maillard reaction products (MRPs) was monitored by mass
28 spectrometry, spectrophotometry and fluorimetry. Ultrasound treatment resulted in formation of
29 MRPs with all tested carbohydrates. Ribose induced the highest degree of modification resulting
30 in 76% of BLG modified and an average of 3 anhydroribose units attached. Circular dichroism
31 spectra analyses indicated only minor alterations in secondary and tertiary structures. MRP
32 obtained by ultrasound exhibited 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging activity and
33 possessed increased iron chelating activity and reducing power. High-intensity ultrasound
34 efficiently promotes BLG-glycoconjugates formation by MR in aqueous solutions under
35 nondenaturating conditions.

36

37 **Key words:** β-lactoglobulin, ultrasound, Maillard reaction, food processing, antioxidant activity

38 **Running title:** Ultrasound-induced glycation of β-lactoglobulin in Maillard reaction

39 **Abbreviations:** BLG – β-lactoglobulin, MR – Maillard reaction, MRPs - Maillard reaction
40 products

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45 ## 1. Introduction

46 The Maillard reaction (MR) or nonenzymatic glycation is spontaneous reaction between amino
47 groups, usually amino acids or proteins, and reducing compounds, such as reducing saccharides.
48 It represents a set of reactions that result in a variety of early, intermediate and advanced
49 compounds called Maillard reaction products (MRP). The MR is one of the major food protein
50 modifying reactions occurring during thermal food processing and can be effective method to
51 generate pigment, aroma and efficacious antioxidant compounds, which can be widely used in
52 food industry (Amarowicz, 2009). In addition, MR can be utilized for food protein modification
53 in order to obtain proteins with improved functional properties, such as emulsification, gelation,
54 foaming and solubility (Oliver, Melton & Stanley, 2006). However, MRPs have also been
55 demonstrated to cause toxicological effects or health problems (Baynes & Thorpe, 1999).
56 Therefore new emerging technologies, avoiding high temperatures and/or long processing times,
57 for manufacturing MRPs can offer better control of these reactions in order to safely modify food
58 proteins and their functions.

59 β-lactoglobulin (BLG) is the major whey protein present in milk of various ruminant species.
60 Glycation of BLG via Maillard reaction could significantly improve its functional properties
61 (Broersen, Voragen, Hamer & de Jongh, 2004; Chevalier, Chobert, Popineau, Nicolas & Haertlé,
62 2001d; Dunlap & Cote, 2005; Nacka, Chobert, Burova, Leonil & Haertlé, 1998). BLG modified
63 by saccharides showed stronger radical-scavenging activity and/or can influence microorganisms
64 growth therefore having potential to be used as a food preservation additive (Chevalier, Chobert,
65 Genot & Haertlé, 2001b).

66 Sonication is a new processing technology tested in several dairy applications, including
67 inactivation of enzymes and bacteria, homogenization and extraction of enzymes, as well as to

68 alter the physical properties of gels made from milk (Zisu, Bhaskaracharya, Kentish & Ashokkumar, 2010). Ultrasound treatment also enhances the physical and functional properties of whey (Jambrak, Mason, Lelas, Herceg & Herceg, 2008). At low frequencies (20-100 kHz) and higher levels of power, ultrasound generates acoustic cavitation. During acoustic cavitation, micro bubbles that are present in the solution grow in size until a maximum critical size is reached, when they violently implode generating localized temperature hot spots exceeding 5000 K and pressures of several thousand bar (Suslick et al., 1999). The intensity of sonication is proportional to the amplitude of vibration and amplitudes of the order of 100 μm are commonly necessary in order to create high power densities sufficient to facilitate many physical and chemical processes. Unique physical, mechanical or chemical effects of high-intensity ultrasonic waves are capable of altering material properties through generation of immense pressure, shear stresses, turbulence, dynamic agitation, and temperature gradient in the medium through which they propagate (Knorr, Zenker, Heinz & Lee, 2004). High-intensity sonication can modify secondary structure of BLG and lead to increase in surface hydrophobicity (Stanic-Vucinic et al., 2012), as well as to propensity of whey proteins concentrate to aggregate (Chandrapala, Zisu, Palmer, Kentish & Ashokkumar, 2011).

84 Some other non-thermal food processing technologies, such as gamma irradiation (Chawla, Chander & Sharma, 2009) and pulsed electric field (Guan et al., 2010a), were reported to promote MR. Reports on the effects of ultrasound on the Maillard reaction have been done on simple model systems consisting of single amino acid with a reducing sugar and under basic pH conditions which are favorable for MR (Guan, Wang, Yu, Xu & Zhu, 2010b; Guan et al., 2011).

89 In this study we investigated wether high-intensity ultrasound can promote glycation of BLG by MR in aqueous model systems and at neutral conditions. We have shown that glycoconjugation

91 of BLG occurs efficiently in the presence of various sugars, especially ribose, and demonstrated
92 improved functional properties of the obtained glycoconjugates, i.e. antioxidative capacity,
93 reducing power and iron-chelating property with a minor influence on protein's secondary and
94 tertiary structures.

95 ## 2. Materials and Methods

96 ### 2.1 *Materials*

97 D-arabinose, D-lactose, D-glucose, D-ribose, D-galactose and D-fructose monohydrates were
98 obtained from Sigma (Sigma-Aldrich, Traufunken, Germany). DPPH (1,1-diphenyl-2-picryl-
99 hydrazyl), TNBS (trinitrobenzylsulfonic acid), ANS (8-anilino-1-naphtalensulfonic acid) and o-
100 phenantroline were also purchased from Sigma–Aldrich (Taufkirchen, Germany). All other
101 reagents were of analytical grade. Deionized water (DW) used in the experiments was purified in
102 a Milli-Q system (Millipore, Molsheim, France). BLG was purified from raw bovine milk as
103 described previously (Stojadinovic et al., 2011). Protein concentration was determined
104 spectrophotometrically at 280 nm ($\varepsilon = 0.941$ mL mg^{-1} cm^{-1}).

105 ### 2.2 *Preparation of ultrasound induced Maillard reaction products*

106 BLG (4 mg/ml; 216 μM) was mixed with or without 217.5 mM of saccharide in 10 mM
107 potassium phosphate buffer pH 6.5. Sonication of BLG samples (20 kHz frequency, 120 μm
108 amplitude,) was carried out with Branson Sonifier 150 (Branson Ultrasonic Corp., Danbury,
109 Conn., USA) for 60 min with constant cooling (the samples were kept at 10-15 °C by using an
110 ice bath). The ultrasound probe, with output 9.5 W power (135 W/cm2), was immersed into a 1.5
111 ml microtube with 1 ml of sample at a depth of 1.5 cm. For determination of remained amino
112 groups, intrinsic tryptophan fluorescence, hydrophobic ligand binding, mass spectrometry and
113 CD spectrometry, the samples were dialyzed against 10 mM potassium phosphate buffer pH 6.5

114 at 4 °C during 40 hours. For all other experiments undialysed samples were used. After the
115 treatments samples were kept at -20°C until use.

116 **2.3** ***Spectrophotometric analyses and spectrofluorimetry measurements***

117 Absorbance at 294 nm (early MRPs) and 420 nm (late MRPs) was measured (Chawla et al.,
118 2009) on Cintra 5 (GBC, Braeside, Australia). For the A_{294} measurements 10-fold dilution of the
119 treated solutions were made using DW.

120 Fluorescence measurements were performed using HORIBA Scientific Fluoromax-4
121 Spectrofluorometer (Horiba, Kyoto, Japan). The fluorescence of the Maillard reaction products
122 was measured at an excitation wavelength of 350 nm and an emission wavelength 360-600 nm.
123 BLG samples were diluted in 10 mM potassium phosphate buffer (pH 6.5) to give a final
124 concentration of 0.5 mg/mL. For intrinsic tryptophan fluorescence measurements BLG solutions
125 of 0.5 μM in 10 mM potassium phosphate buffer pH 7.2 were used. The emission wavelength
126 was scanned from 290 to 410 nm at the excitation wavelength of 280 nm and 5 nm slit width. For
127 hydrophobic ligand binding experiment the fluorescence spectra of BLG solutions (20 μM)
128 saturated by ANS (100 μM) in 10 mM potassium phosphate buffer pH 7.2 were recorded
129 between 400 and 600 nm with excitation of 350 nm.

130 **2.4** ***CD spectra measurements and CD spectra analysis***

131 CD spectra were recorded on a JASCO J-815 spectropolarimeter (JASCO, Japan) with BLG at
132 concentration of 1 mg/ml in 10 mM potassium phosphate buffer pH 6.5. The spectra were
133 collected over the wavelength range 180-260 nm for far UV and 260-320 nm for near UV. Each
134 spectrum was acquired four times, and the results were averaged. Results were expressed as
135 residue-average molar ellipticity as follows: $[\theta] = \theta / (10 \times n \times C \times d)$, where θ is measured

136 ellipticity, n is number of BLG amino acid residues, C is molar concentration of BLG sample, d
137 is path length of the cell.

138 **2.5 *Electrophoresis and isoelectrofocusing***

139 SDS–PAGE was carried out using a Hoefer Scientific Instruments apparatus with a
140 discontinuous buffer system. Protein components were resolved on 14% polyacrylamide gels
141 according to Laemmli (1970), and stained using Coomassie Brilliant Blue R-250 (Sigma–
142 Aldrich). Reducing conditions were achieved with 5% β-mercaptoethanol (Sigma–Aldrich).
143 Isoelectrofocusing was performed in 7% polyacrylamide slab gel in the presence of 2%
144 Amfolines (Pharmacia, Uppsala, Sweden), pH 3.5–10, in a Multiphor electrophoretic system
145 (Pharmacia) at 200 V for 2 h. The pH gradient was determined by pH measurement after cutting
146 the gel into strips and incubating in 10 mM KCl for 30 min.

147 **2.6 *Mass spectrometry analysis***

148 Mass of modified protein was measured on LTQ Orbitrap XL (Thermo scientific Inc., Waltham,
149 Massachusetts, USA), with temperature of probe 350 C, spray voltage 4.5 kV, temperature of
150 capillary 275 C, and voltage capillary -47 V. Separating of components was done on Accela
151 UHPLC (Thermo scientific) using C18 HYPERSIL GOLD column (2.1 x 50 mm, 1.9 µm
152 particle size, 175 Å pore size) at flow 400 µl/min. Ionisation was done in positive mode on
153 HESI probe. The samples were separated by gradient elution with eluent A (water containing
154 0.1% formic acid) and eluent B (98% acetonitrile containing 0.1 formic acid): 0-1 min, isocratic
155 95% A; 1-3 min, linear 95-65% A; 3-10 min, linear 65-30% A; 10-11 min, linear 30-5% A, 11-
156 14 min, isocratic 5% A, 14-15 min, isocratic 95% A. Data were acquired between m/z 300 and
157 m/z 4000 in continuum mode with scantime of 0.6 s. In elution time range of BLG/modified
158 BLG, about 50 MS scans were combined into one m/z spectrum. The LC/ESI-MS data was

159 acquired with Xcalibur version 2.1 (Thermo Fisher Scientific, Inc.). From intensities of the peaks
160 for the various products present in spectrum the average degree of substitution per BLG
161 molecule were calculated. In degree of substitution per protein molecule (DSP) range indicated,
162 the DSPs representing less than 0.2 % of the total product spectrum were not included.

163 **2.7 Determination of free amino group content**

164 The free amino groups were determined using the TNBS method (Willis & Tu, 1988). The
165 results are expressed as a mean of three different determinations for sonicated BLGs as a
166 percentage of the number of amino groups determined for the native BLG (expressed as 100 %).

167 **2.8 Determination of DPPH radical-scavenging activity, reducing power and iron**
168 **chelating activity**

169 DPPH radical-scavenging activity of the MRPs fractions was determined according to the
170 method of Gu, Kim, Abbas, Zhang, Xia & Chen (2010) with minor modifications. An aliquot of
171 undiluted BLG (4 mg/ml) sample (1.0 ml) was added to 1.0 ml of 0.2 mM DPPH in ethanol.
172 After incubation at room temperature in the dark for 20 min and centrifugation for 10 min at
173 1000 g the absorbance of supernatant was measured at 517 nm. The control was prepared in the
174 same manner except that deionised water was used instead of BLG samples. The blank was
175 done in the same fashion but deionised water was used instead of DPPH solution. The percentage
176 of DPPH radical-scavenging activity was calculated as follows:

177 % radical scavenging activity = (1- (A_{517} sample / A_{517} control)) x 100

178 The reducing power of BLG samples was determined according to the method of Chawla et al.
179 (2009) with a slight modification. BLG sample (300 μl) was mixed with 400 μl ml of 0.2 M
180 sodium phosphate buffer (pH 6.6) and 400 μl of 1% potassium ferricyanide ($K_3Fe(CN_6)$). The
181 reaction mixtures were incubated at 50 C for 20 min, followed by addition of 400 μl of 10%

182 trichloroacetic acid. After centrifugation at 10000 g for 10 min obtained supernatant (1.4 ml) was
183 added to 300 µl of 0.1% $FeCl_3$ and the absorbance was measured at 700 nm. The control was
184 prepared in the same manner except that 10 mM potassium phosphate buffer (pH 6.5) was used
185 instead of BLG samples. Increased A_{700} of the reaction mixture indicated increased reducing
186 power.

187 To examine the iron chelating activity of sonicated BLG samples the o-phenantroline method
188 was used according to Puntel, Nogueira & Rocha (2005). The mixture containing 1 ml of 200
189 µM ferric chloride and 0.5 ml BLG sample (4 mg/ml), was allowed to react for 5 min and form
190 complex(es). After addition of 0.5 ml 0.05 % o-phenantroline colored complex formation
191 between o-phenantroline and remained free Fe^{2+} was recorded by measuring absorbance at 510
192 nm. The control was carried out by addition of 10 mM potassium phosphate buffer pH 6.5
193 instead of BLG sample. Solutions of $FeSO_4$ were made just before use. The ability of sample to
194 chelate ferrous ions was calculated using the following equation:

195 $$\% \text{ iron chelating activity} = (1 - (A_{510} \text{ sample} / A_{510} \text{ control})) \times 100$$

196 **2.9 Statistical analyses**

197 For analysis of remained aminogroups, DPPH scavenging activity, iron chelating activity and
198 reducing power experiments were carried out in duplicates and each measurement was made in
199 triplicate. Differences between the variables were tested for significance by one-way ANOVA
200 accompanied with Tukey`s post test using Origin Pro 8.5.1 (OriginLab, Northampton, MA,
201 USA). Differences at $P < 0.05$ were considered to be significant.

202 **3. Results and discussion**

203 **3.1 *Changes in A294, browning, fluorescence intensity and amino groups content***

204 In the present study neutral aqueous solution of BLG with different common sugar (glucose,
205 fructose, arabinose, ribose, galactose and lactose) was used as model system for natural food
206 protein-sugar mixture, to investigate effect of ultrasound processing on Maillard reaction.
207 Increase of UV absorbance at 294 nm in BLG samples sonicated with sugars (Figure 1A)
208 suggests formation of UV-absorbing intermediate MRPs upon sonication. UV-absorbing
209 compounds were produced to significant extent ($P<0.05$) in the presence of lactose and ribose,
210 with ribose being substantially more efficient then lactose. Formation of UV-absorbing
211 compounds upon sonication of sugar/amino acid in solution has been recently reported (Guan et
212 al., 2010b; Guan et al., 2011). Although thermally-induced Maillard reaction changes pH of the
213 solution, after ultrasonic treatment, pH of all BLG samples did not changed in the presence of
214 sugars (± 0.1, not shown), revealing that there was no substantial production of organic acids
215 during sonication.
216 As a measure of browning effect A420 was monitored after sonication treatment (Figure 1A). In
217 the presence of sugars browning increased by sonication, due to formation of chromophores,
218 with significant increase of A_{420} ($P<0.05$) in case of ribose, arabinose and lactose. During
219 Maillard reaction UV-absorbing intermediate compounds are formed prior to generation of
220 brown pigments due to sugar decomposition by dehydratation and fragmentation (Ajandouz,
221 Tchiakpe, Dalle Ore, Benajiba & Puigserver, 2001). Browning pigments (melanoidins) are
222 formed by polymerization of intermediate products (Wang, Qian & Yao, 2011). The brown
223 pigment development, indicated by A420, coincided with the colorless intermediate formation
224 evidenced by increased A294, suggesting that the brown pigments were formed in parallel to the
225 generated intermediate products. However, higher increase in A294, comparing to increase in
226 A420, suggests domination of early stage of Maillard reaction under applied conditions. Apart

227 from the Maillard reaction, caramelization of sugar could also occur during sonication,
228 contributing to nonenzymatic browning of the mixture (Ajandouz et al., 2001).
229 Development of fluorescent compounds has been reported to be associated with heat-induced
230 Maillard reaction (Jing & Kitts, 2002). In the presence of sugars sonicated BLG samples have
231 shown increased fluorescence with maximum at about 425 nm when excited at 350 nm.
232 Fluorescence of Maillard products was the highest in BLG sample containing ribose (Figure 1B)
233 and therefore related to spectrophotometric properties of tested samples. Different MRPs with
234 fluorescent chemical structures are formed during increasing heating conditions (Morales & van
235 Boekel, 1997).
236 The occurrence of the Maillard reaction was further confirmed by the loss of available $–NH_2$
237 groups after sonication (Figure 1C). Ultrasound itself induced significant ($P< 0.05$) loss of BLG
238 amino groups (10 %), probably due to free radical reactions generated by water sonolysis. Lysine
239 residue is known as prone to oxidation to lysine aldehyde, resulting in loss of its ε-amino group
240 (Meltretter & Pischetsrieder, 2008). These aldehydes are highly reactive, and undergo
241 spontaneous reactions with other free radical-derived aldehyde residues, or with unmodified
242 lysine residues to form intra- and interchain cross-links. In the presence of all sugars further
243 significant reduction of amino groups occurred ($P<0.05$) with loss of up to 38 % in the presence
244 ribose.

245 **3.2 *Characterization of BLG conjugates by SDS-PAGE and isoelectrofocusing***

246 Molecular masses of BLG derivatives formed during ultrasound induced glycation were
247 estimated by SDS PAGE. In non-reducing conditions (Figure 2A) sonicated BLG showed one
248 band at about 18 kDa corresponding to monomeric BLG form, the same as native BLG. In
249 samples sonicated in the presence of sugars a slight shift in the 18 kDa protein bands toward

250 higher molecular masses is attributed to the conjugation of carbohydrate. Upon BLG sonication
251 with sugars, beside monomeric form, also dimer and trimer forms appeared. SDS PAGE under
252 reducing condition (Figure 2B) clearly shows that most of BLG dimers and trimers, formed in
253 the presence of ribose, glucose and fructose, are covalently bonded but not by disulphide
254 linkages. These forms are most likely pentosidine protein cross-links, or protein cross-links from
255 initial Amadori adducts of saccharides and ε-lysyl residues involving an imidazole group
256 (Frye, Degenhardt, Thorpe & Baynes, 1998). Grandhee & Monnier (1991) proposed mechanism
257 of pentosidine formation from D-ribose, D-glucose, D-fructose and ascorbate via the common
258 intermediate. In addition, Maillard reaction can induce Strecker degradation of ε-amino groups
259 of lysine residues and generate substantial amounts of allysine (lysine-derived aldehydes), which
260 can lead to lysyl-lysine protein cross-linking (Monnier et al., 2005). In the case of BLG
261 sonicated in the presence of lactose, polymerization of glycated BLG was essentially due to
262 S-S bonds, similar to results obtained by Chevalier, Chobert, Molle & Haertlé (2001c) in mild
263 heated system.

264 Changes in pI values of glycated BLG was observed by electrofocusing (Figure 2C). Native
265 BLG pattern showed a band near pH 5. After BLG sonication in the presence of sugars several
266 bands ranging pH 4.5 to 5 appeared, indicating heterogeneity of the derivatives. Similar shift in
267 BLG pI toward more acidic values was obtained under mild heating conditions by Chevalier,
268 Chobert, Dalgalarrondo & Haertlé (2001a) and Nacka et al. (1998).

269 **3.3 Mass spectrometry analysis**

270 By ESI-TOF-MS analysis of the intact protein detailed product composition data can be
271 provided, including average extent of glycation, distribution profile of the protein glycoforms
272 and proportion of each glycated species in the product mixture. Figure 3 shows combined m/z

273 spectra as acquired with the charge states of protein generated during ionization ranging from
274 10+ to 19+. The major peak appears at 18 362 Da, which corresponds to expected mass of BLG
275 genetic variant A (Sawyer & Kontopidis, 2000). In the m/z spectrum of BLG sonicated in the
276 presence of glucose (Figure 3B) and ribose (Figure 3C) multiple peaks were observed per charge
277 state indicating the presence of a range of products. Covalent coupling of a saccharide moiety to
278 the protein via the Maillard reaction, accompanied by loss of a water molecule, resulted in a
279 mass increase of 162 Da per anhydroglucose and 132 Da per anhydroribose moiety incorporated.
280 From intensities of the peaks for the various products within one spectrum, the degree of
281 substitution per protein molecule (DSP) range and weighted average DSP was calculated for
282 each BLG sample (Table 1). The product DSP range observed indicated that not each individual
283 protein molecule reacted equally and that DSP range is dependent on the type of saccharide, eg.
284 its reactivity so that maximum DSP range (0-9), as well as glycated BLG content (cca 75%) was
285 obtained in the presence of ribose. Although saccharide degradation products can react with a
286 protein skeleton to form high molecular weight melanoidins (Wang et al., 2011), mass spectra
287 demonstrate that there was no formation of BLG-bound melanoidins, indicating domination of
288 early stages of Maillard reaction, which is in agreement with other results. Ribose is well
289 recognized for its high propensity to react in the MR, and this is usually ascribed to the high
290 proportion of its acyclic form in solution. The kinetics of glycation is dependent on the type of
291 sugar (Chevalier et al., 2001d), the proportion of the reducing sugar existing in the acyclic or
292 active form under the reaction conditions, and the electrophilicity of the sugar carbonyl group
293 (Corzo-Martinez, Moreno, Olano & Villamiel, 2008). The reactivity of reducing sugar was
294 reported to decrease in the following order: aldopentoses > aldohexoses > ketohexoses >
295 disaccharides (Laroque, Inisan, Berger, Vouland, Dufosse & Guerard, 2008). Also, glycation

296 proceeds at a faster rate under dry conditions than in aqueous solution due to higher
297 concentration of reactants, as well as due to elimination of hindering effect of water in reactions
298 in which water is generated as a byproduct (Oliver, 2011).
299 ESI-MS can give better insight into the structure of heterogeneous BLG glycoforms than
300 methods such as CD spectroscopy, which monitors average properties of a heterogeneous
301 sample. The average charge state (ACS) of BLG glycoforms was calculated from relative
302 intensities of each single charge state (Table 1). The ACS of a protein depends on the number of
303 accessible ionization sites, which in turn is influenced by the protein's conformation (Grandori,
304 2003). In the BLG samples sonicated in the presence of sugars ACS of unmodified BLG species
305 was almost unaffected. Only in the presence of ribose ACS slightly increased for modified BLG
306 species indicating slight loosening of tertiary structure with glycosylation. Sonication of BLG
307 with other sugars resulted in lowering of ACS of modified BLG species with number of
308 conjugated saccharide moieties, indicating slightly more compact structure of modified BLG
309 species, the effect observed by Czerwenka, Maier, Pittner & Lindner (2006) with BLG thermally
310 glycated by lactose.

311 **3.4 *Secondary structure changes***

312 The effect of ultrasound promoted glycation on the secondary structure of BLG was studied by
313 far-UV CD spectroscopy. Far-UV CD spectra of control and sonicated BLG (Figure 4A) are
314 almost superimposable indicating negligible change in their secondary structures caused by
315 sonication. However, presence of sugars in ultrasound treated BLG samples resulted in sugar-
316 dependent decrease of negative absorption peak at 215 nm and positive absorption peak at 189
317 nm. Slight shift of peak at 215 nm toward lower wavelengths (blue shift) is noticeable in case of
318 ribose, fructose and lactose. Calculated α-helix, β-sheet, β-turn and random coil from three

319 independent experiments are listed in Table S1 of Supplementary material. BLG sonication in
320 the presence of sugars resulted in minor alteration of secondary structures, reflected in slight
321 decrease in α-helix content with parallel increase in random coil content. However, these
322 changes in secondary structure are not significant ($P < 0.05$). In a study of thermal-promoted
323 BLG glycation Chevalier, Chobert, Dalgalarrondo, Choiset & Haertle (2002) have also observed
324 that BLG glycated with ribose had slightly modified secondary structure by monitoring far-UV
325 CD spectra and showed a similar blue shift. Broersen et al. (2004) also noticed small reduction in
326 CD spectra intensities by BLG glycation with glucose and fructose. Minimal effect on the
327 secondary structure by thermally induced BLG glycation was achieved under water restricted
328 environment (60 °C, 65% relative humidity) with low molecular weight carbohydrates (Broersen
329 et al., 2004; Van Teeffelen, Broersen & De Jongh, 2005). It is evident that, even in solution,
330 ultrasound-induced BLG glycation only slightly change protein secondary structure.

331 **3.5 *Tertiary structure changes***

332 Alterations in BLG tertiary structure due to conjugation of carbohydrate moieties can be
333 observed from near-UV CD spectra (Figure 4B). The near-UV CD spectrum of the control BLG
334 displayed sharp peak at about 293 nm, ascribed to the Trp19. This peak diminished in intensity
335 by sonication treatment of BLG. In the presence of sugars sonication led to further reduction of
336 negative ellipticity of this peak indicating more prominent movement of Trp19 to a less chiral
337 environment and reflecting structural changes that occurred within the calyx of the BLG
338 molecule. Tertiary structure changes corresponded to sugar modification effectiveness so that in
339 the presence of ribose peak at 293 nm almost disappeared suggesting that the tertiary structure of
340 protein surrounding Trp19 has been markedly loosened by ultrasound-induced BLG glycation. In
341 the case of sonication in the presence of ribose well-defined tertiary structure was lost because of

342 the destruction of the interactions responsible for maintaining the rigid native protein tertiary
343 structure.
344 The intrinsic tryptophan fluorescence emission spectra of native, sonicated and glycosylated
345 BLG were examined (Figure 4C). When excited at 280 nm, native BLG exhibited a fluorescence
346 emission maximum (λ_{max}) at 336 nm. Sonication of BLG induced only a small shift of λ_{max} to 338
347 nm. However, in BLG samples sonicated in the presence of sugars noticeable decrease in
348 intensity, as well as red shift of λ_{max} to 348 nm was observed, due to shielding of tryptophan (Trp
349 19 and Trp 61) residues from the aqueous phase by the protein conformational changes. Retained
350 shape of fluorescence spectra of glycated BLG, indicate that glycation affected only partially the
351 side chains of proteins in tertiary structure, without great disruption of native structure, even in
352 the presence of ribose. Similar results were observed during thermal glycation of BLG with
353 galactose and tagatose (Corzo-Martinez et al., 2008) and glucose and fructose (Broersen et al.,
354 2004).
355 In order to examine changes in hydrophobic properties of the protein surface, we tested binding
356 of a hydrophobic fluorescent probe, ANS, to glycoconjugates of BLG. Upon noncovalent
357 binding of ANS to hydrophobic patches on protein surfaces, its fluorescence intensity increases
358 and the wavelength of maximal absorbance shifts. BLG sonicated without presence of sugars
359 have shown increased quantum yield after ANS addition, compared to native protein (Figure
360 4D). Presence of saccharides further increased peak intensity and shifted maximum toward lower
361 wavelengths, indicating that glycated BLG possess newly exposed hydrophobic patches on the
362 protein surface. This effect was the most prominent in the presence of ribose with maximum shift
363 from 481 nm to 465 nm. Morgan, Léonil, Mollé & Bouhallab (1999a) have shown increased

364 ANS binding to BLG thermally glycated in solution, in contrast to dry state glycation which
365 resulted in no change.
366 Other studies have shown that glycation can have variable effects on protein structure. For the
367 heat-induced BLG-lactosylation Morgan et al. (1999a; 1999b) have found higher degree of
368 glycation in the dry state then in aqueous solution, but more significant BLG structural changes
369 in solution. Glycation of BLG in aqueous solution with more reactive sugars (arabinose and
370 ribose) caused protein denaturation, but it was not significant with less reactive sugars (lactose
371 and rhamnose) (Chevalier et al., 2002). Nacka et al. (1998) observed that no significant changes
372 to the tertiary structure of BLG occurred following thermal glycation with aldohexoses and
373 lactose, and complete denaturation of the protein occurred when glycated with ribose.
374 Czerwenka et al. (2006) observed increased hydrophobicity and slight conformational change
375 toward more compact structure in thermally lactosylated BLG.

376 **3.6 *Antioxidative* properties of MRPs estimated as DPPH radical-scavenging activity,**
377 **iron chelation activity and reducing power**

378 Native and sonicated BLG have shown a weak DPPH radical scavenging activity (Figure 5A).
379 Sonication of BLG in the presence of sugars increased DPPH radical-scavenging activity with
380 ribose-containing BLG sample showing significantly ($p \leq 0.05$) highest radical scavenging
381 activity (42%). This study confirms recently reported observation that compounds with
382 antioxidant potential are formed upon sonication of glycine/glucose and glycine/maltose
383 solutions (Guan et al., 2010b; Guan et al., 2011). MRPs obtained by gamma radiation in whey
384 model system also exhibited DPPH scavenging activity (Chawla et al., 2009). Similar relation of
385 radical scavenging activity with nature of the sugar was observed by Chevalier et al. (2001b), in

386 mild heat-induced BLG glycation model system, although free radical scavenging activity of
387 glycated proteins was not directly related to the glycation degree (Chevalier et al., 2001b).
388 Metal chelating activity plays an important role in antioxidant action due to reduction in the
389 concentration of the transition metals required for lipid peroxidation. In the present study we
390 investigated the effect of sonication on ferrous ion chelating activities of BLG in the presence of
391 different sugars (Figure 5A). Native BLG, as well as sonicated BLG have shown noticeable iron
392 chelating activity. Sonication in the presence of all sugars resulted in significant increase of iron
393 chelating capacity ($p < 0.05$), with ribose having the most prominent effect. Our findings are in
394 agreement with an earlier report on iron chelating activity of other model systems, as a result of
395 MRPs formed due to heat treatment (Dong, Wei, Chen, McClements & Decker, 2011; Gu et al.,
396 2010). Gamma-irradiation of whey protein concentrate solutions also generated MRPs with iron
397 chelating activity (Chawla et al., 2009).
398 BLG sonication without saccharides did not have an effect on reducing power of BLG, as shown
399 by an increase in absorbance at 700 nm (Figure 5B). Reducing power of BLG increased
400 significantly ($p \leq 0.05$), after sonication in the presence of glucose, fructose, arabinose, and ribose
401 had the most dramatic effect. In glycin-maltose model system the reducing power of conjugates
402 increased with the time of treatment by high intensity ultrasound (Guan et al., 2010b). Heat-
403 induced MRPs from BLG/ribose aqueous model system also exhibited reducing power (Jiang &
404 Brodkorb, 2012). Our results demonstrated that ultrasound-generated MRPs of BLG-
405 glycoconjugates possessed hydrogen-donating and antioxidant activities mainly related to the
406 mechanism of single electron transfer.

407 ## 4. Conclusion

408 Most of studies on Maillard reaction products preparation are based under dry heating and/or
409 basic pH condition, which are favorable for Maillard reaction. However, dairy products are
410 processed in hydrated or aqueous form and under neutral or acidic conditions. This study
411 demonstrates that even under conditions that are not quite favorable for Maillard reaction, such
412 as neutral pH and reaction in solution, high intensity ultrasound is able to significantly accelerate
413 Maillard reaction in BLG-sugar aqueous systems. Significant increase was found in the early and
414 intermediate Maillard products content, fluorescence, browning intensity and antioxidant activity
415 of solutions. Increase in Maillard reaction degree was in accordance with saccharide reactivity.
416 Ribose showed the most pronounced effect on formation of BLG-glycoconjugates by ultrasound.
417 Glycation of the protein under mild reaction conditions did not have a drastic impact on the
418 secondary and tertiary structures of the BLG protein. Increased temperature and reaction time
419 greatly increase the extent of glycation in Maillard reaction. Therefore, efficient protein
420 glycation in aquaous occurs by treatments with high temperatures (> 100 C) for few hours or at
421 mild temperatures (50-60 C) for few days, and most of methods for obtaining glycated proteins
422 are based on these conditions. Sonication enables short treatment times at low processing
423 temperature, and thus low energy consumption, it is non-polluting, while retaining nutritive and
424 functional properties. In fact, strong sheer forces generated during sonication enable efficient
425 mixing of solution and efficient heat transfer contributing to increased rate and thus increased
426 efficiency of early phases of Maillard reaction. At the same time maintence of low sample
427 temeperature (10-15 C) probably does not substantially increase rate of later phases of Maillard
428 reaction and does not change protein structure to significant level. During conventional thermal
429 processing there is non-uniform heating of sample and the occurrence of over-heating spots in
430 the sample due to limitations of heat transfer. Therefore efficient protein glycation by

431 conventional heating is mainly accompanied by advanced phases of Maillard reaction and
432 protein denaturation, and thus it is more difficult to control. In contrast, ohmic heating, allowing
433 instantaneous and uniform temperature increase could enable more controllable process and high
434 efficiency for short time, similar to sonication. By optimization of ultrasound frequency, power
435 density, time of exposure desired efficiency of protein glycation can be achieved with minimal
436 late phases of Maillard reaction. Therefore, application of high intensity ultrasound could be
437 suitable for preparation of whey protein glycoconjugates in an industrially feasible way, as mild
438 reaction conditions and short reaction times are sufficient to obtain modified proteins. Further
439 optimization of conditions that are more favorable for Maillard reaction may provide reasonable
440 output also with less reactive sugars. From that point of view, ultrasound has potential to become
441 an efficient tool in development of methods for production of glycated food protein with novel
442 functional properties, as well as improved natural antioxidants. Our results imply that in addition
443 to processing by heat, high intensity ultrasound-based food processing technologies can also
444 have a significant impact on the Maillard reaction in susceptible food systems and should
445 not be neglected.

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450 The authors have declared no conflict of interest.

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590

591 **Figure captions**

592

593 **Figure 1.** Effect of ultrasound on (A) A294 and browning intensity (A420), (B) fluorescence and
594 (C) amino group content of BLG solution with and without presence of different sugars. BLG-
595 untreated BLG, sBLG-sonicated BLG, sBLG-Glc – BLG sonicated in the presence of glucose,
596 sBLG-Gal – BLG sonicated in the presence of galactose, sBLG-Lac – BLG sonicated in the
597 presence of lactose, sBLG-Fru – BLG sonicated in the presence of fructose, sBLG-Rib – BLG
598 sonicated in the presence of ribose, sBLG-Ara – BLG sonicated in the presence of arabinose.
599 Different small superscripts (a to c) denote the significant difference ($P < 0.05$).

600

601 **Figure 2.** Molecular weight estimation of native BLG, sonicated BLG without and with the
602 presence of sugars by SDS PAGE : (A) under nonreducing (10 μg of protein per lane) and (B)
603 reducing conditions (20 μg of protein per lane). Isoelectrofocusing (C) of native BLG and
604 sonicated BLG without and with presence of sugars. Figure legend is according to Figure 1. MM
605 – molecular weight markers.

606

607 **Figure 3.** Combined m/z spectra (±50 scans/spectrum) after UPLC-ESI-TOF-MS analysis of
608 native BLG (A), BLG sonicated with glucose (B) and with ribose (C). Intensities are scaled to
609 the highest peak.

610

611 **Figure 4.** Circular dichroism spectra of native BLG and BLG sonicated without and with the
612 presence of sugars in (A) far UV and (B) near UV spectral range. Intrinsic tryptophan

613 fluorescence (C) and ANS binding (D) to native BLG and BLG sonicated without and with the

614 presence of sugars. Figure legend is according to Figure 1.

615

616 **Figure 5.** Antioxidative activity of native BLG and BLG sonicated without and with the

617 presence of sugars measured as DPPH-scavenging and iron chelating activity (A) and reducing

618 power (B). Figure legend is according to Figure 1. Different small superscripts (a to e) denote the

619 significant difference ($P < 0.05$).

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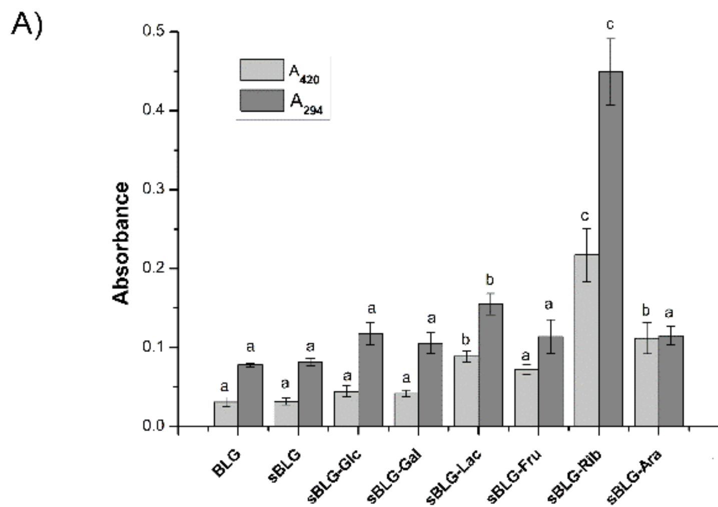
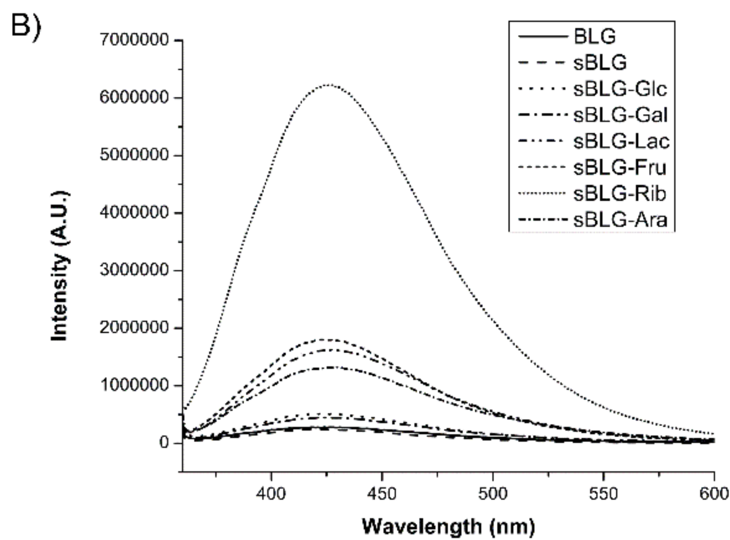
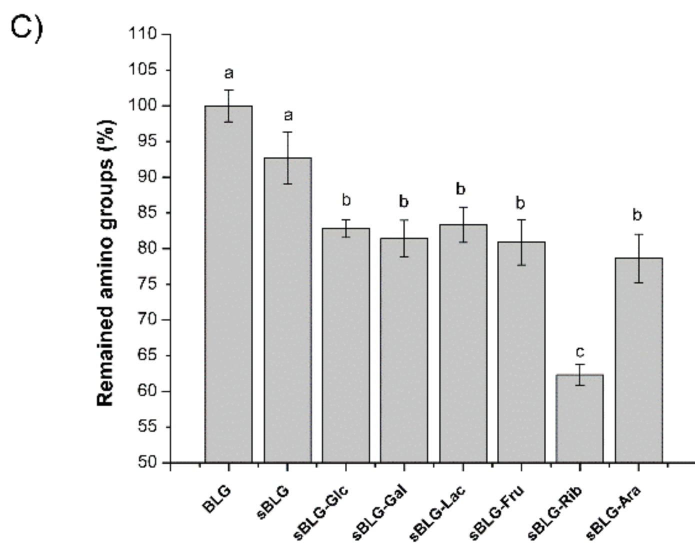
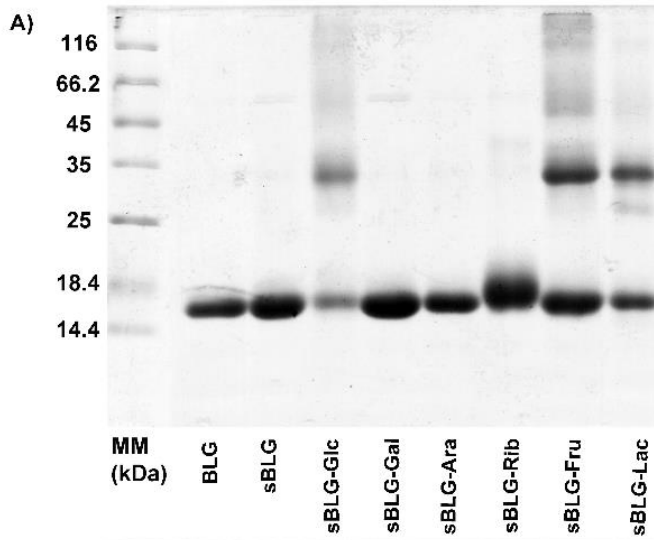
Table caption

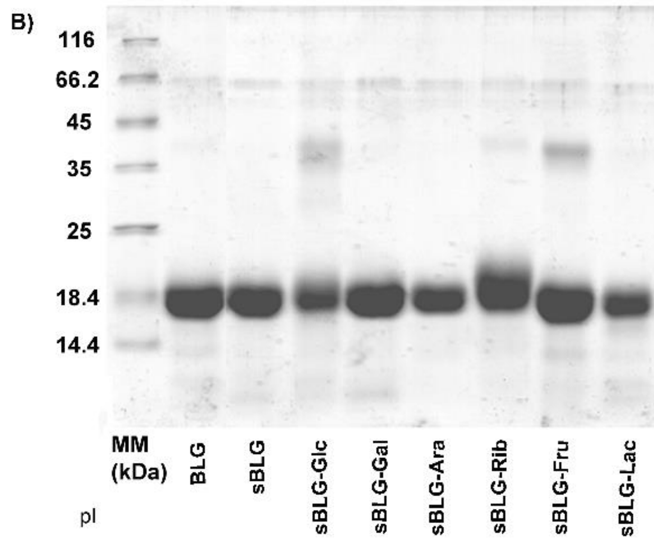
Table 1. Overview of DSP range, weighted average DSP, content of individual BLG species and their average charge state after sonication of BLG in the presence of several saccharides. Average charge state of native BLG and sonicated BLG without presence of saccharide were 14.68 and 14.67, respectively.

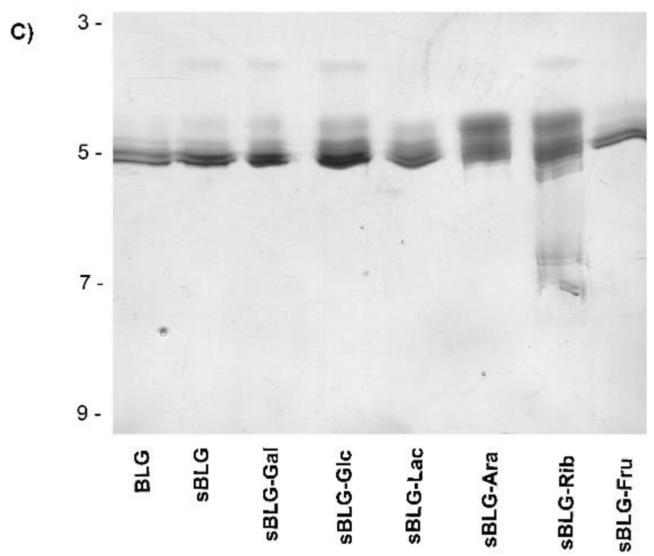

A)
A420
A294
Absorbance
0.0
0.1
0.2
0.3
0.4
0.5
BLG
sBLG
sBLG-Glc
sBLG-Gal
sBLG-Lac
sBLG-Fru
sBLG-Rib
sBLG-Ara

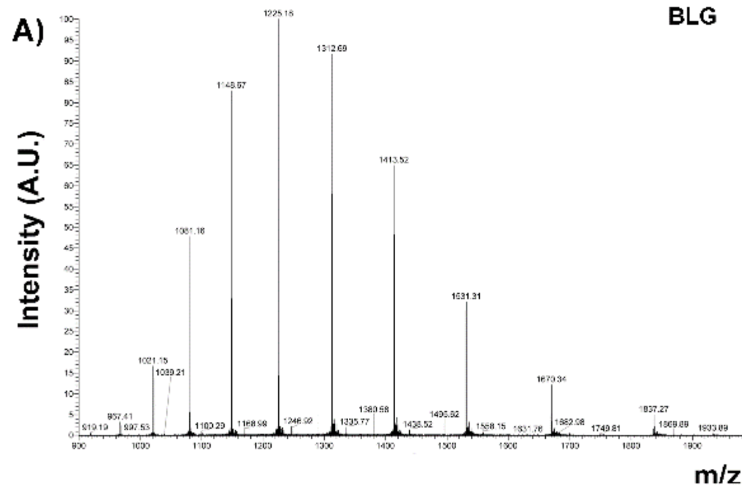

B)
BLG
sBLG
sBLG-Glc
sBLG-Gal
sBLG-Lac
sBLG-Fru
sBLG-Rib
sBLG-Ara
Intensity (A.U.)
Wavelength (nm)

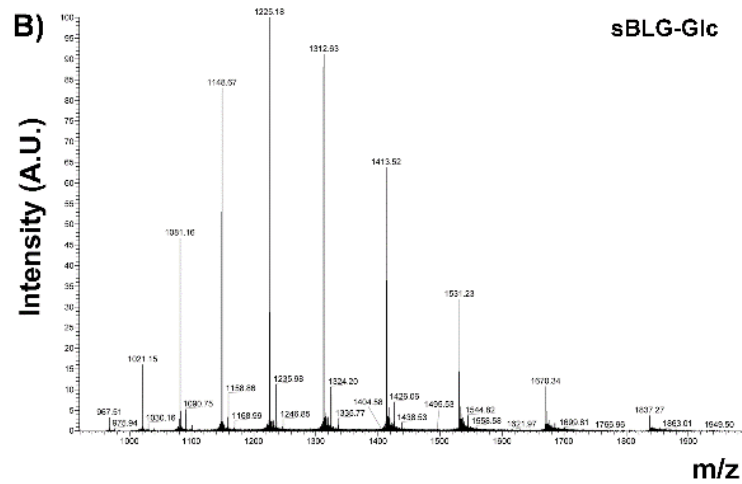

C)
Remained amino groups (%)
BLG
sBLG
sBLG-Glc
sBLG-Gal
sBLG-Lac
sBLG-Fru
sBLG-Rib
sBLG-Ara

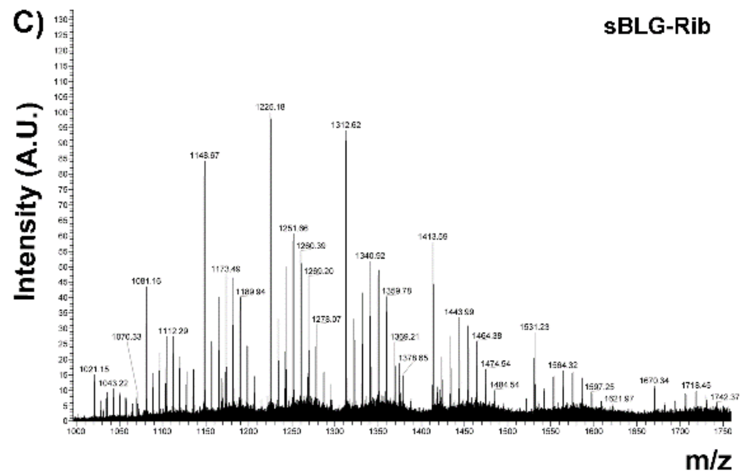

A)
116
66.2
45
35
25
18.4
14.4
MM
(kDa)
BLG
sBLG
sBLG-Glc
sBLG-Gal
sBLG-Ara
sBLG-Rib
sBLG-Fru
sBLG-Lac

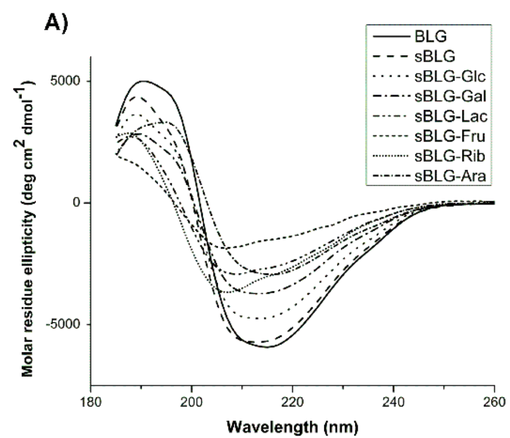

B)
116
66.2
45
35
25
18.4
14.4
MM
(kDa)
BLG
sBLG
sBLG-Glc
sBLG-Gal
sBLG-Ara
sBLG-Rib
sBLG-Fru
sBLG-Lac

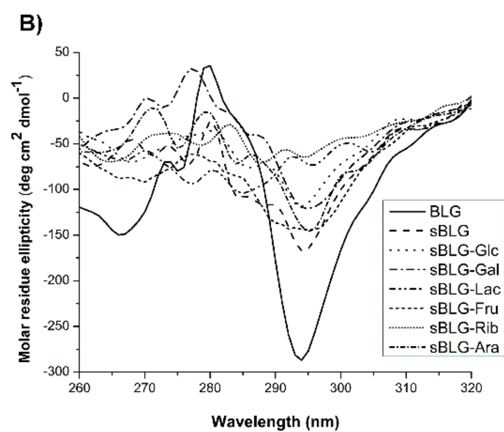

pI
C)
3 -
5 -
7 -
9 -
BLG
sBLG
sBLG-Gal
sBLG-Glc
sBLG-Lac
sBLG-Ara
sBLG-Rib
sBLG-Fru

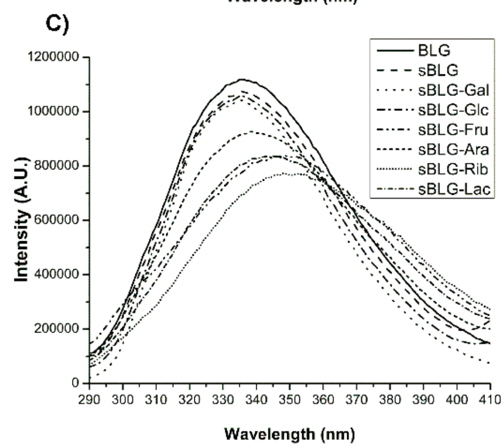

A)
BLG
Intensity (A.U.)
m/z

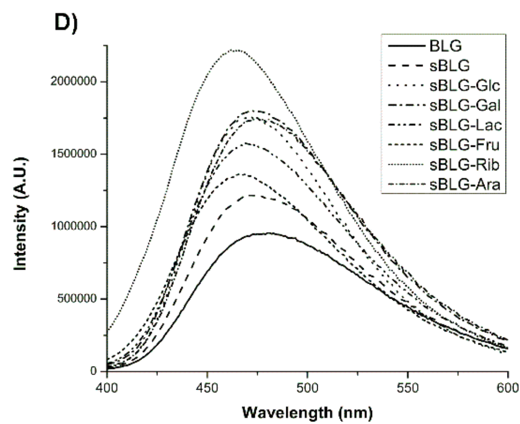

B)
sBLG-Glc
Intensity (A.U.)
m/z

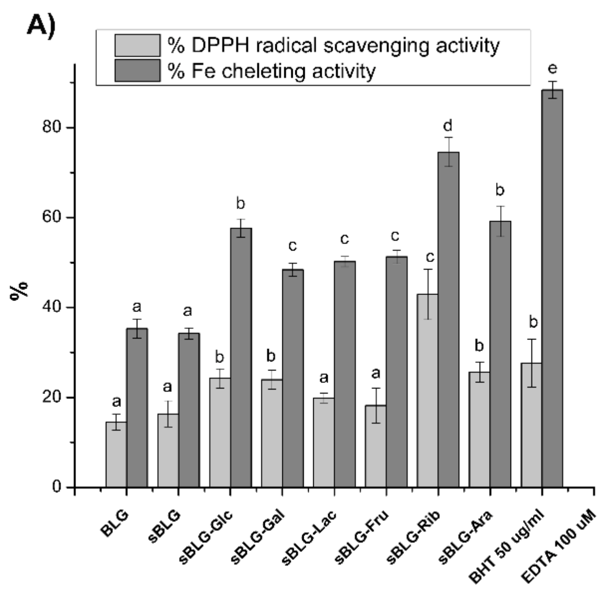

C)
sBLG-Rib
Intensity (A.U.)
m/z

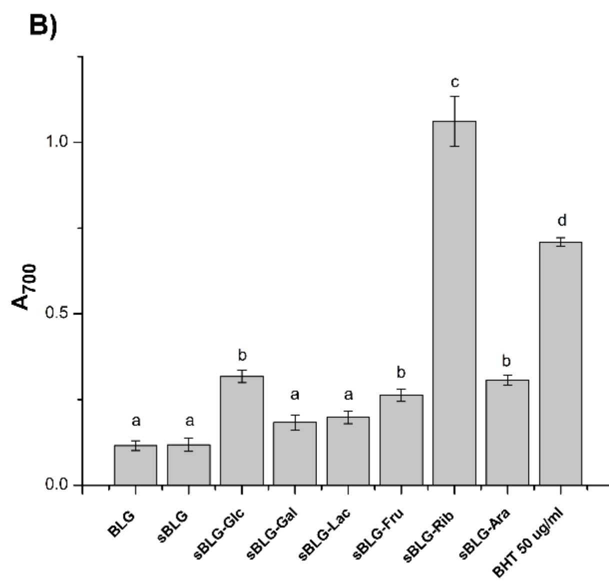

A)
Molar residue ellipticity (deg cm^2 dmol^{-1})
Wavelength (nm)
BLG
sBLG
sBLG-Glc
sBLG-Gal
sBLG-Lac
sBLG-Fru
sBLG-Rib
sBLG-Ara


B)
Molar residue ellipticity (deg cm^2 dmol^{-1})
Wavelength (nm)
BLG
sBLG
sBLG-Glc
sBLG-Gal
sBLG-Lac
sBLG-Fru
sBLG-Rib
sBLG-Ara


C)
Intensity (A.U.)
Wavelength (nm)
BLG
sBLG
sBLG-Gal
sBLG-Glc
sBLG-Fru
sBLG-Ara
sBLG-Rib
sBLG-Lac


D)
Intensity (A.U.)
Wavelength (nm)
BLG
sBLG
sBLG-Glc
sBLG-Gal
sBLG-Lac
sBLG-Fru
sBLG-Rib
sBLG-Ara


A)
% DPPH radical scavenging activity
% Fe cheleting activity
%
0
20
40
60
80
BLG
sBLG
sBLG-Glc
sBLG-Gal
sBLG-Lac
sBLG-Fru
sBLG-Rib
sBLG-Ara
BHT 50 ug/ml
EDTA 100 uM


B)
A700
0.0
0.5
1.0
BLG
sBLG
sBLG-Glc
sBLG-Gal
sBLG-Lac
sBLG-Fru
sBLG-Rib
sBLG-Ara
BHT 50 ug/ml

	sBLG/Rib	sBLG/Ara	sBLG/Glc	sBLG/Gal	sBLG/Lac	sBLG/Fru
DSP range	0-9	0-3	0-3	0-2	0-2	0-3
Weighted average DSP	2.98	0.11	0.17	0.12	0.13	0.08
% of BLG species /average charge state of BLG species						
unmodified	24.33 /14.57	92.51 /14.54	86.78 /14.60	90.45 /14.59	88.99 /14.72	95.01 /14.61
1 unit	8.51 /14.68	5.03 /14.00	10.27 /14.19	6.87 /14.43	8.88 /14.72	2.70 /14.55
2 units	11.82 /14.77	2.46 /13.96	2.94 /13.97	2.68 /14.33	2.13 /14.79	2.29 /14.34
3 units	14.29 /14.76	0.34 /13.71	0.27 /13.83	nd	nd	0.26 /14.20
4 units	13.43 /14.75	nd	nd	nd	nd	nd
5 units	11.17 /14.72	nd	nd	nd	nd	nd
6 units	7.38 /14.77	nd	nd	nd	nd	nd
7 units	4.56 /14.78	nd	nd	nd	nd	nd
8 units	2.81 /14.72	nd	nd	nd	nd	nd
9 units	1.69 /14.77	nd	nd	nd	nd	nd