



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

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ARTICLE INFO

Article history:

Received 20 July 2012

Received in revised form 26 September 2012

Accepted 4 October 2012

Available online 10 November 2012

Keywords:

β -Lactoglobulin

Ultrasound

Maillard reaction

Food processing

Antioxidant activity

ABSTRACT

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

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1. Introduction

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

Abbreviations: BLG, β -lactoglobulin; MR, Maillard reaction; MRPs, Maillard reaction products.

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

Sonication is a new processing technology tested in several dairy applications, including inactivation of enzymes and bacteria, homogenisation and extraction of enzymes, as well as to 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 localised 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 μ m 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 the propensity of whey proteins concentrate to aggregate (Chandrapala, Zisu, Palmer, Kentish, & Ashokkumar, 2011).

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 a single amino acid with a reducing sugar and under basic pH conditions which are favourable for MR (Guan, Wang, Yu, Xu, & Zhu, 2010b; Guan et al., 2011).

In this study we investigated whether high-intensity ultrasound can promote glycation of BLG by MR in aqueous model systems and at neutral conditions. We have shown that glycoconjugation of BLG occurs efficiently in the presence of various sugars, especially ribose, and demonstrated improved functional properties of the obtained glycoconjugates, i.e. antioxidative capacity, reducing power and iron-chelating property with a minor influence on protein secondary and tertiary structures.

2. Materials and methods

2.1. Materials

D-Arabinose, D-lactose, D-glucose, D-ribose, D-galactose and D-fructose monohydrates were obtained from Sigma-Aldrich (Traufunken, Germany). DPPH (1,1-diphenyl-2-picryl-hydrazyl), TNBS (trinitrobenzylsulfonic acid), ANS (8-anilino-1-naphthalensulfonic acid) and o-phenantroline were also purchased from Sigma-Aldrich. All other reagents were of analytical grade. Deionised water (DW) used in the experiments was purified in a Milli-Q system (Millipore, Molsheim, France). BLG was purified from raw bovine milk as described previously (Stojadinovic et al., 2012). Protein concentration was determined spectrophotometrically at 280 nm ($\epsilon = 0.941 \text{ mL mg}^{-1} \text{ cm}^{-1}$).

2.2. Preparation of ultrasound induced Maillard reaction products

BLG (4 mg/ml; 216 μM) was mixed with or without 217.5 mM of saccharide in 10 mM potassium phosphate buffer pH 6.5. Sonication of BLG samples (20 kHz frequency, 120 μm amplitude) was carried out with Branson Sonifier 150 (Branson Ultrasonic Corp., Danbury, CT, USA) for 60 min with constant cooling (the samples were kept at 10–15 °C by using an ice bath). The ultrasound probe, with output 9.5 W power (135 W/cm²), was immersed in a 1.5-ml microtube with 1 ml of sample at a depth of 1.5 cm. For determination of remained amino groups, intrinsic tryptophan fluorescence, hydrophobic ligand binding, mass spectrometry and CD spectrometry, the samples were dialysed against 10 mM potassium phosphate buffer pH 6.5 at 4 °C for 40 h. For all other experiments undialysed samples were used. After the treatments samples were kept at –20 °C until use.

2.3. Spectrophotometric analyses and spectrofluorimetry measurements

Absorbance at 294 nm (early MRPs) and 420 nm (late MRPs) was measured (Chawla et al., 2009) on Cintra 5 (GBC, Braeside,

Australia). For the A₂₉₄ measurements 10-fold dilution of the treated solutions was made using DW.

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

2.4. CD spectra measurements and CD spectra analysis

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

2.5. Electrophoresis and isoelectrofocusing

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

2.6. Mass spectrometry analysis

Mass of modified protein was measured on LTQ Orbitrap XL (Thermo Scientific Inc., Waltham, MA, USA), with temperature of probe 350 °C, spray voltage 4.5 kV, temperature of capillary 275 °C, and voltage capillary –47 V. Separating of components was done on Accela UHPLC (Thermo scientific) using C18 Hypersil Gold column (2.1 $\times$ 50 mm, 1.9 μm particle size, 175 Å pore size) at flow 400 $\mu\text{L}/\text{min}$. Ionisation was done in positive mode on HESI probe. The samples were separated by gradient elution with eluant A (water containing 0.1% formic acid) and eluant B (98% acetonitrile containing 0.1% formic acid): 0–1 min, isocratic 95% A; 1–3 min, linear 95–65% A; 3–10 min, linear 65–30% A; 10–11 min, linear 30–5% A, 11–14 min, isocratic 5% A, 14–15 min, isocratic 95% A. Data were acquired between m/z 300 and m/z 4000 in continuum mode with scan time of 0.6 s. In elution time range of BLG/modified BLG, about 50 MS scans were combined into one spectrum. The LC/ESI-MS data was acquired with Xcalibur version 2.1 (Thermo Fisher Scientific, Inc.). From intensities of the peaks for the various products present in the spectrum the average degree of substitution per BLG molecule was calculated. In degree of

substitution per protein molecule (DSP) range indicated, the DSPs representing less than 0.2% of the total product spectrum were not included.

2.7. Determination of free amino group content

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

2.8. Determination of DPPH radical-scavenging activity, reducing power and iron chelating activity

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

$$\% \text{Radical scavenging activity} = (1 - (A_{517\text{sample}}/A_{517\text{control}})) \times 100$$

The reducing power of BLG samples was determined according to the method of Chawla et al. (2009) with a slight modification. BLG sample (300 µl) was mixed with 400 µl of 0.2 M sodium phosphate buffer (pH 6.6) and 400 µl of 1% potassium ferricyanide ($K_3Fe(CN_6)$). The reaction mixtures were incubated at 50 °C for 20 min, followed by addition of 400 µl of 10% trichloroacetic acid. After centrifugation at 10000g for 10 min the obtained supernatant (1.4 ml) was added to 300 µl of 0.1% $FeCl_3$ and the absorbance was measured at 700 nm. The control was prepared in the same manner except that 10 mM potassium phosphate buffer (pH 6.5) was used instead of BLG samples. Increased A_{700} of the reaction mixture indicated increased reducing power.

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

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

2.9. Statistical analyses

For analysis of remaining amino groups, DPPH scavenging activity, iron chelating activity and reducing power experiments were carried out in duplicates and each measurement was made in triplicate. Differences between the variables were tested for signifi-

cance by one-way ANOVA accompanied with Tukey's post hoc test using Origin Pro 8.5.1 (OriginLab, Northampton, MA, USA). Differences at $p < 0.05$ were considered to be significant.

3. Results and discussion

3.1. Changes in A_{294} , browning, fluorescence intensity and amino groups content

In the present study neutral aqueous solution of BLG with different common sugars (glucose, fructose, arabinose, ribose, galactose and lactose) was used as a model system for natural food protein–sugar mixture, to investigate effect of ultrasound processing on Maillard reaction. Increase of UV absorbance at 294 nm in BLG samples sonicated with sugars (Fig. 1A) suggests formation of UV-absorbing intermediate MRPs upon sonication. UV-absorbing compounds were produced to a significant extent ($p < 0.05$) in the presence of lactose and ribose, with ribose being substantially more efficient than lactose. Formation of UV-absorbing compounds upon sonication of sugar/amino acid in solution has been recently reported (Guan et al., 2010b, 2011). Although thermally-induced Maillard reaction changes pH of the solution, after ultrasonic treatment, pH of all BLG samples did not change in the presence of sugars (± 0.1 , not shown), revealing that there was no substantial production of organic acids during sonication.

As a measure of browning effect A_{420} was monitored after sonication treatment (Fig. 1A). In the presence of sugars, browning increased by sonication, due to formation of chromophores, with significant increase of A_{420} ($p < 0.05$) in the cases of ribose, arabinose and lactose. During Maillard reaction UV-absorbing intermediate compounds are formed prior to generation of brown pigments due to sugar decomposition by dehydration and fragmentation (Ajandouz, Tchiakpe, Dalle Ore, Benajiba, & Puigserver, 2001). Browning pigments (melanoidins) are formed by polymerisation of intermediate products (Wang, Qian, & Yao, 2011). The brown pigment development, indicated by A_{420} , coincided with the colourless intermediate formation evidenced by increased A_{294} , suggesting that the brown pigments were formed in parallel to the generated intermediate products. However, higher increase in A_{294} , comparing to increase in A_{420} , suggests the domination of the early stage of the Maillard reaction under applied conditions. Apart from the Maillard reaction, caramelisation of sugar could also occur during sonication, contributing to non-enzymatic browning of the mixture (Ajandouz et al., 2001).

Development of fluorescent compounds has been reported to be associated with heat-induced Maillard reaction (Jing & Kitts, 2002). In the presence of sugars sonicated BLG samples have shown increased fluorescence with a maximum at about 425 nm when excited at 350 nm. Fluorescence of Maillard products was the highest in BLG sample containing ribose (Fig. 1B) and therefore related to spectrophotometric properties of tested samples. Different MRPs with fluorescent chemical structures are formed during increasing heating conditions (Morales & van Boekel, 1997).

The occurrence of the Maillard reaction was further confirmed by the loss of available $-NH_2$ groups after sonication (Fig. 1C). Ultrasound itself induced significant ($p < 0.05$) loss of BLG amino groups (10%), probably due to free radical reactions generated by water sonolysis. Lysine residue is prone to oxidation to lysine aldehyde, resulting in loss of its ϵ -amino group (Meltretter & Pischetsrieder, 2008). These aldehydes are highly reactive, and undergo spontaneous reactions with other free radical-derived aldehyde residues, or with unmodified lysine residues to form intra- and inter-chain cross-links. In the presence of all sugars further significant reduction of amino groups occurred ($p < 0.05$) with loss of up to 38% in the presence of ribose.

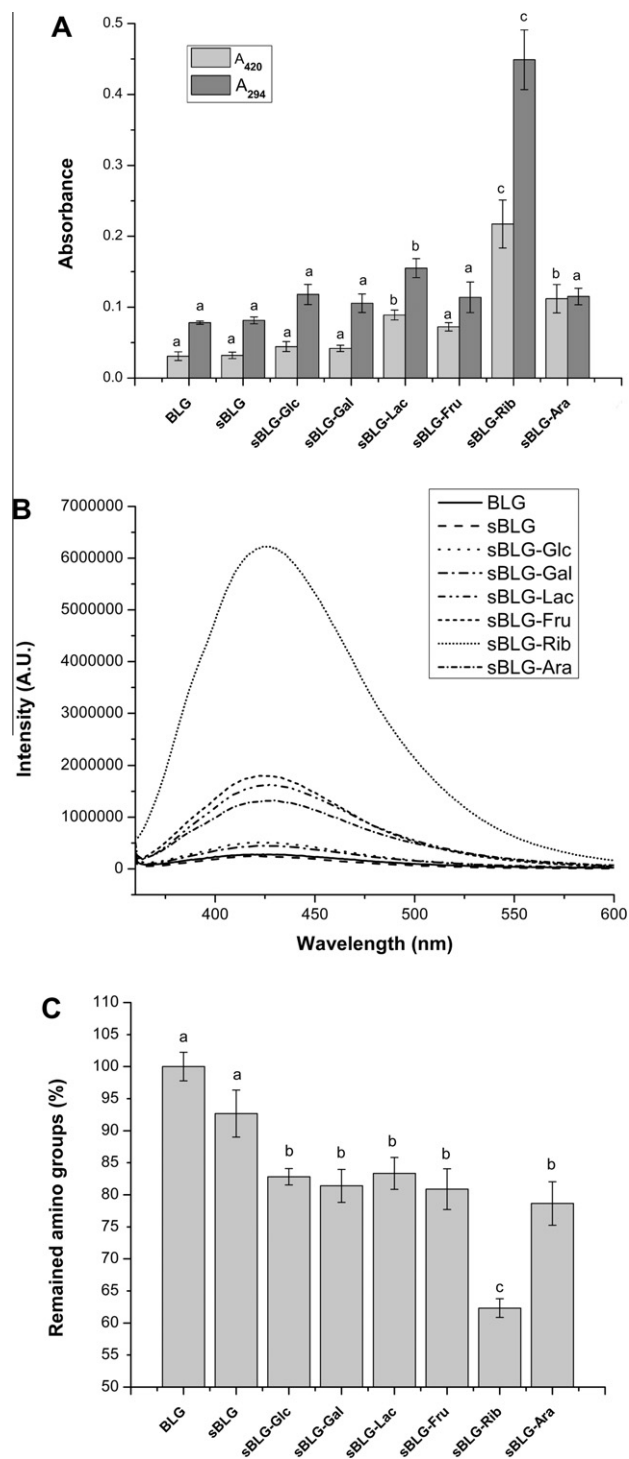


Fig. 1. Effect of ultrasound on (A) A_{294} and browning intensity (A_{420}), (B) fluorescence and (C) amino group content of BLG solution with and without presence of different sugars. BLG-untreated BLG, sBLG-sonicated BLG, sBLG-Glc – BLG sonicated in the presence of glucose, sBLG-Gal – BLG sonicated in the presence of galactose, sBLG-Lac – BLG sonicated in the presence of lactose, sBLG-Fru – BLG sonicated in the presence of fructose, sBLG-Rib – BLG sonicated in the presence of ribose, sBLG-Ara – BLG sonicated in the presence of arabinose. Different small superscripts (a–c) denote significant differences ($p < 0.05$).

3.2. Characterisation of BLG conjugates by SDS-PAGE and isoelectrofocusing

Molecular masses of BLG derivatives formed during ultrasound-induced glycation were estimated by SDS-PAGE. In non-reducing

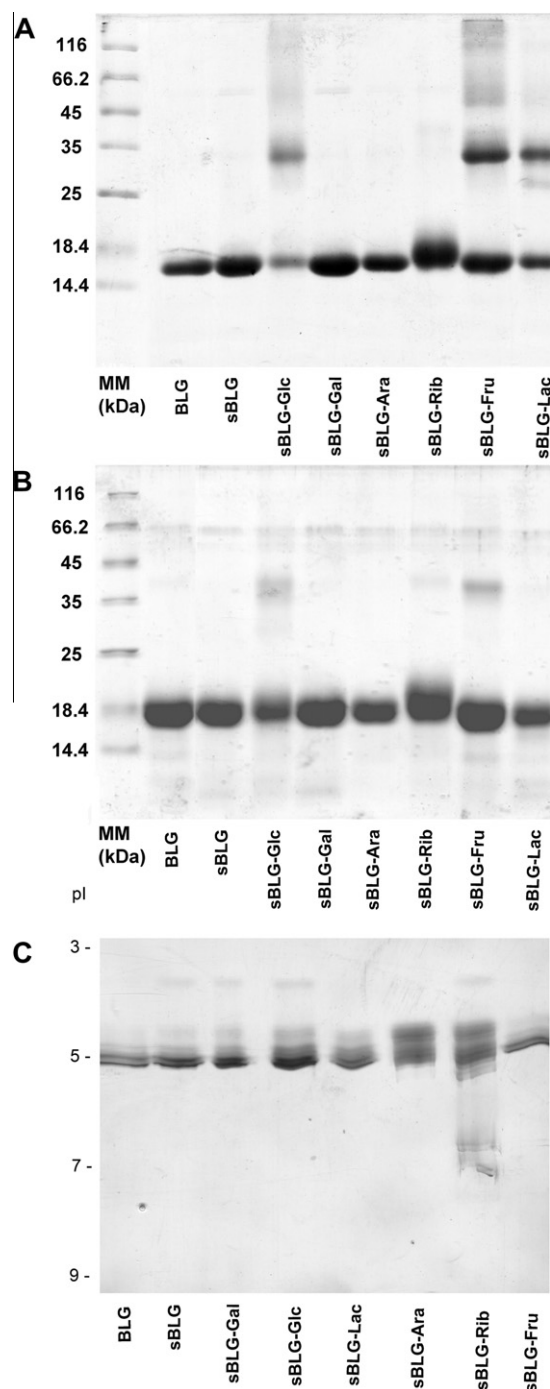


Fig. 2. Molecular weight estimation of native BLG, sonicated BLG without and with the presence of sugars by SDS-PAGE: (A) under non-reducing (10 μ g of protein per lane) and (B) reducing conditions (20 μ g of protein per lane). Isoelectrofocusing (C) of native BLG and sonicated BLG without and with presence of sugars. Figure legend is according to Fig. 1. MM – molecular weight markers.

conditions (Fig. 2A) sonicated BLG showed one band at about 18 kDa corresponding to monomeric BLG form, the same as native BLG. In samples sonicated in the presence of sugars a slight shift in the 18 kDa protein bands toward higher molecular masses is attributed to the conjugation of carbohydrate. Upon BLG sonication with sugars, beside monomeric form, also dimer and trimer forms appeared. SDS-PAGE under reducing conditions (Fig. 2B) clearly shows that most BLG dimers and trimers, formed in the presence of ribose, glucose and fructose, are covalently bonded but not by

disulphide linkages. These forms are most likely pentosidine protein cross-links, or protein cross-links from initial Amadori adducts of saccharides and ϵ -lysyl residues involving an imidazole group (Frye, Degenhardt, Thorpe, & Baynes, 1998). Grandhee and Monnier (1991) proposed a mechanism for pentosidine formation from D-ribose, D-glucose, D-fructose and ascorbate *via* a common intermediate. In addition, Maillard reaction can induce Strecker degradation of ϵ -amino groups of lysine residues and generate substantial amounts of allysine (lysine-derived aldehydes), which can lead to lysyl-lysine protein cross-linking (Monnier et al., 2005). In the case of BLG sonicated in the presence of lactose, polymerisation of glycosylated BLG was essentially due to S–S bonds, similar to results obtained by Chevalier, Chobert, Mollé, and Haertlé (2001c) in a mildly heated system.

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

3.3. Mass spectrometry analysis

By ESI-TOF-MS analysis of the intact protein detailed product composition data can be provided, including average extent of glycation, distribution profile of the protein glycoforms and proportion of each glycosylated species in the product mixture. Fig. 3 shows combined m/z spectra as acquired with the charge states of protein generated during ionisation ranging from 10+ to 19+. The major peak appears at 18,362 Da, which corresponds to the expected mass of BLG genetic variant A (Sawyer & Kontopidis, 2000). In the m/z spectrum of BLG sonicated in the presence of glucose (Fig. 3B) and ribose (Fig. 3C) multiple peaks were observed per charge state indicating the presence of a range of products. Covalent coupling of a saccharide moiety to the protein *via* the Maillard reaction, accompanied by loss of a water molecule, resulted in a mass increase of 162 Da per anhydroglucose and 132 Da per anhydribose moiety incorporated.

From intensities of the peaks for the various products within one spectrum, the degree of substitution per protein molecule (DSP) range and weighted average DSP were calculated for each BLG sample (Table 1). The product DSP range observed indicated that not each individual protein molecule reacted equally and that DSP range is dependent on the type of saccharide, e.g., its reactivity so that maximum DSP range (0–9), as well as glycosylated BLG content (ca. 75%) was obtained in the presence of ribose. Although saccharide degradation products can react with a protein skeleton to form high molecular weight melanoidins (Wang et al., 2011), mass spectra demonstrate that there was no formation of BLG-bound melanoidins, indicating the domination of the early stages of the Maillard reaction, which is in agreement with other results. Ribose is well recognised for its high propensity to react in the MR, and this is usually ascribed to the high proportion of its acyclic form in solution. The kinetics of glycation is dependent on the type of sugar (Chevalier et al., 2001d), the proportion of the reducing sugar existing in the acyclic or active form under the reaction conditions, and the electrophilicity of the sugar carbonyl group (Corzo-Martinez, Moreno, Olano, & Villamiel, 2008). The reactivity of reducing sugar was reported to decrease in the following order: aldopentoses > aldohexoses > ketohexoses > disaccharides (Laroque et al., 2008). Also, glycation proceeds at a faster rate under dry conditions than in aqueous solution due to higher concentration of reactants, as well as due to elimination of hindering effect of water in reactions in which water is generated as a by-product (Oliver, 2011).

ESI-MS can give a better insight into the structure of heterogeneous BLG glycoforms than methods such as CD spectroscopy, which monitors average properties of a heterogeneous sample. The average charge state (ACS) of BLG glycoforms was calculated from relative intensities of each single charge state (Table 1). The ACS of a protein depends on the number of accessible ionisation sites, which in turn is influenced by the protein's conformation (Grandori, 2003). In the BLG samples sonicated in the presence of sugars ACS of unmodified BLG species was almost unaffected. Only in the presence of ribose ACS slightly increased for modified BLG species indicating slight loosening of tertiary structure with glycosylation. Sonication of BLG with other sugars resulted in lowering of ACS of modified BLG species with a number of conjugated saccharide moieties, indicating a slightly more compact structure of the modified BLG species. This effect was observed by Czerwenka, Maier, Pittner, and Lindner (2006) with BLG thermally glycosylated by lactose.

3.4. Secondary structure changes

The effect of ultrasound promoted glycation on the secondary structure of BLG was studied by far-UV CD spectroscopy. Far-UV CD spectra of control and sonicated BLG (Fig. 4A) are almost superimposable, indicating negligible change in their secondary structures caused by sonication. However, the presence of sugars in ultrasound treated BLG samples resulted in sugar-dependent decrease of negative absorption peak at 215 nm and positive absorption peak at 189 nm. Slight shift of peak at 215 nm toward lower wavelengths (blue shift) is noticeable in the case of ribose, fructose and lactose. Calculated α -helix, β -sheet, β -turn and random coil from three independent experiments are listed in Table S1 of Supplementary material. BLG sonication in the presence of sugars resulted in minor alteration of secondary structures, reflected in slight decrease in α -helix content with parallel increase in random coil content. However, these changes in secondary structure are not significant. In a study of thermally-promoted BLG glycation Chevalier, Chobert, Dalgalarondo, Choiset, and Haertlé (2002) have also observed that BLG glycosylated with ribose had slightly modified secondary structure, by monitoring far-UV CD spectra and showing a similar blue shift. Broersen et al. (2004) also noticed small reduction in CD spectra intensities by BLG glycation with glucose and fructose. Minimal effect on the secondary structure by thermally induced BLG glycation was achieved under a water-restricted environment (60 °C, 65% relative humidity) with low molecular weight carbohydrates (Broersen et al., 2004; Van Teeffelen, Broersen, & De Jongh, 2005). It is evident that, even in solution, ultrasound-induced BLG glycation only slightly changes protein secondary structure.

3.5. Tertiary structure changes

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

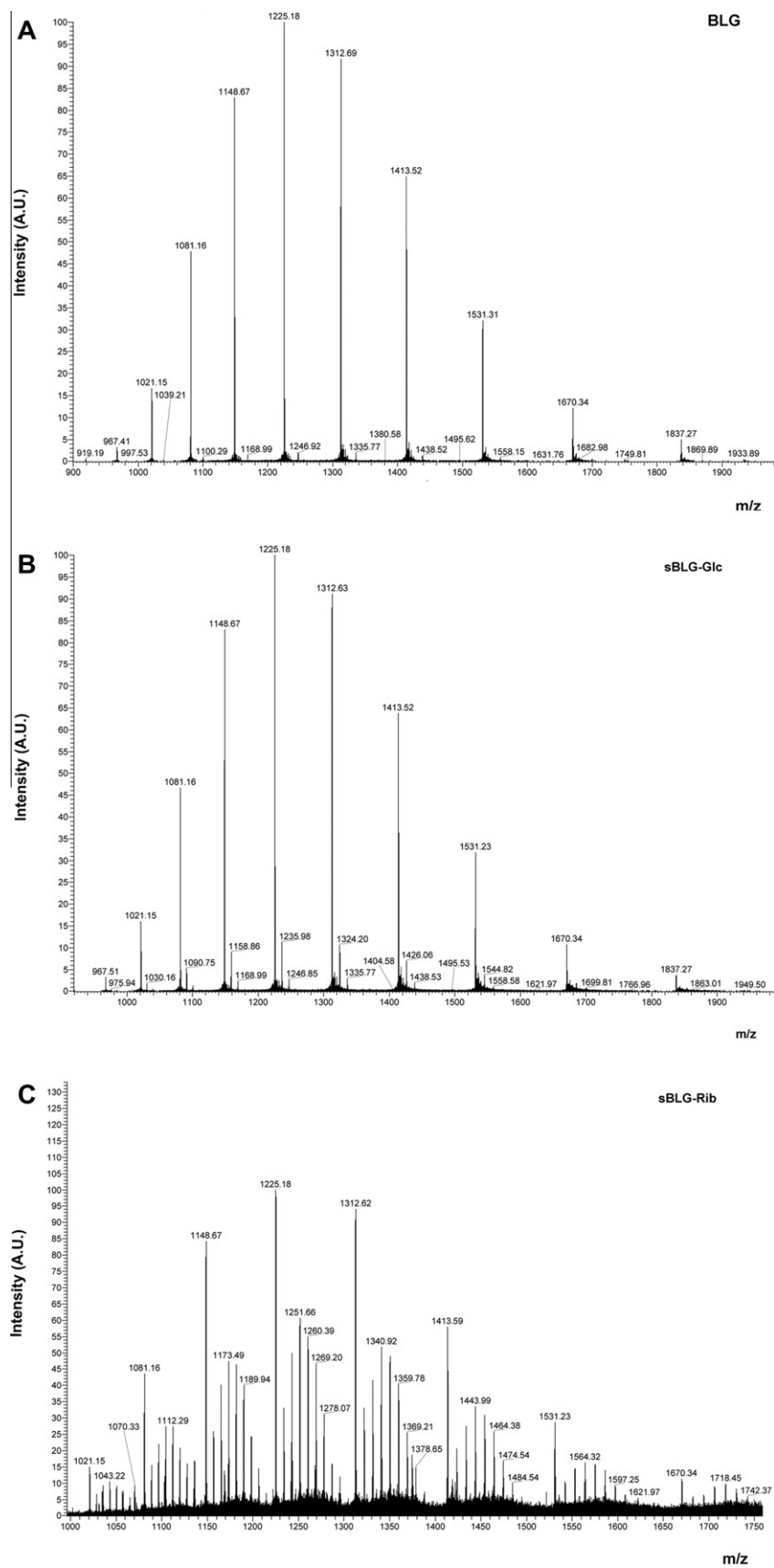


Fig. 3. Combined m/z spectra (± 50 scans/spectrum) after UPLC-ESI-TOF-MS analysis of native BLG (A), BLG sonicated with glucose (B) and with ribose (C). Intensities are scaled to the highest peak.

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.

	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

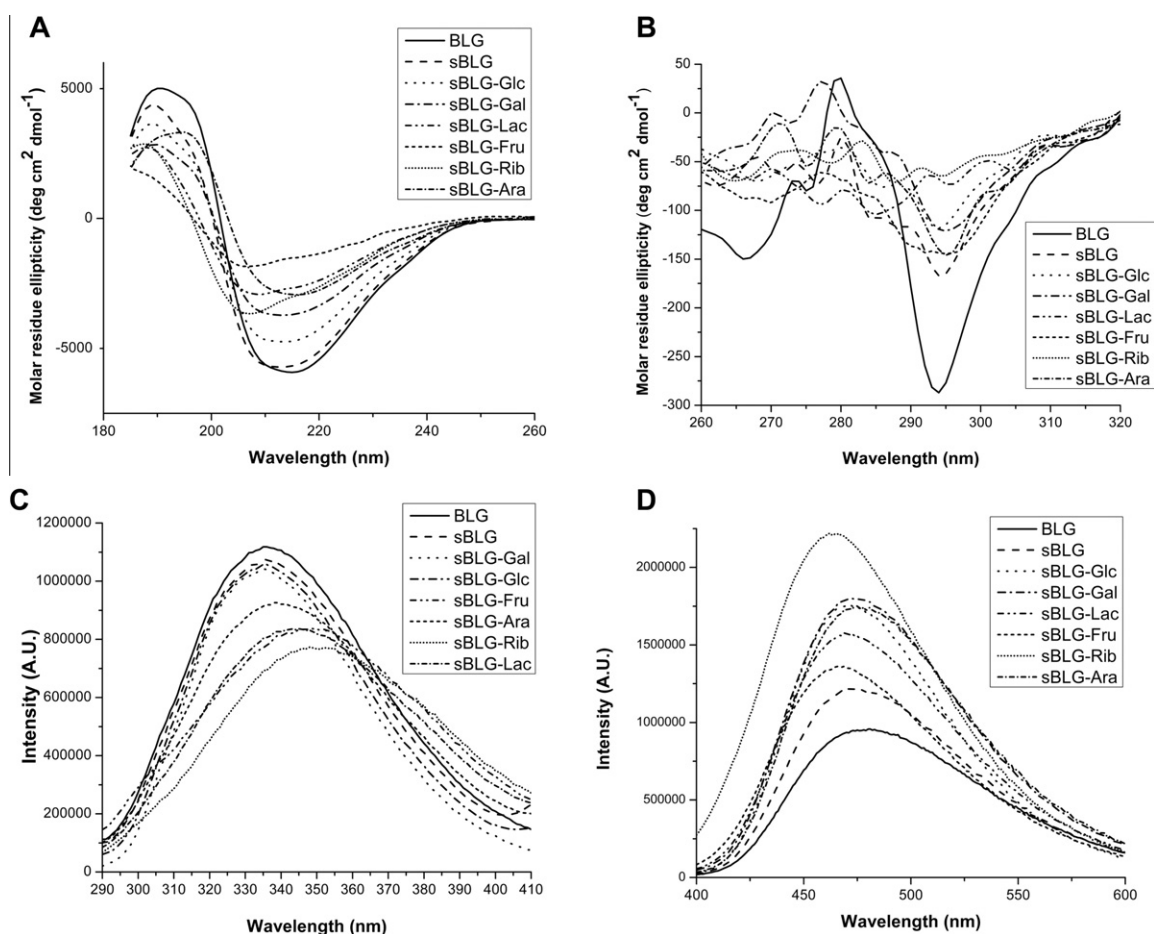


Fig. 4. Circular dichroism spectra of native BLG and BLG sonicated without and with the presence of sugars in (A) far UV and (B) near UV spectral range. Intrinsic tryptophan fluorescence (C) and ANS binding (D) to native BLG and BLG sonicated without and with the presence of sugars. Figure legend is according to Fig. 1.

of the destruction of the interactions responsible for maintaining the rigid native protein tertiary structure.

The intrinsic tryptophan fluorescence emission spectra of native, sonicated and glycosylated BLG were examined (Fig. 4C). When excited at 280 nm, native BLG exhibited a fluorescence emission maximum (λ_{max}) at 336 nm. Sonication of BLG induced only a small shift of λ_{max} to 338 nm. However, in BLG samples sonicated in the presence of sugars noticeable decrease in intensity, as well as red shift of λ_{max} to 348 nm was observed, due to shielding of tryptophan (Trp 19 and Trp 61) residues from the aqueous phase

by the protein conformational changes. Retained shape of fluorescence spectra of glycosylated BLG, indicate that glycation affected only partially the side-chains of proteins in tertiary structure, without great disruption of native structure, even in the presence of ribose. Similar results were observed during thermal glycation of BLG with galactose and tagatose (Corzo-Martinez et al., 2008), and glucose and fructose (Broersen et al., 2004).

In order to examine changes in hydrophobic properties of the protein surface, we tested binding of a hydrophobic fluorescent probe, ANS, to glycoconjugates of BLG. Upon non-covalent binding

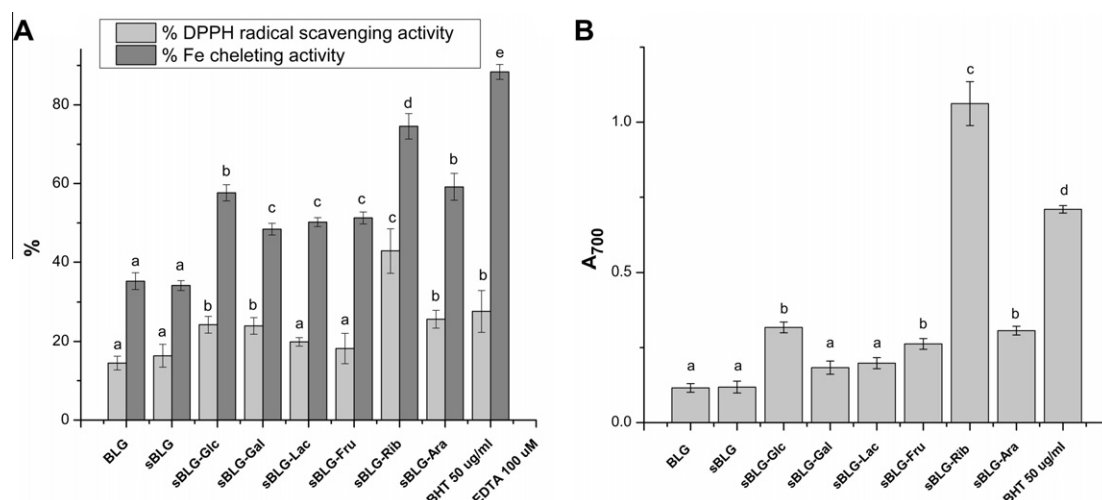


Fig. 5. Antioxidative activity of native BLG and BLG sonicated without and with the presence of sugars measured as DPPH-scavenging and iron chelating activity (A) and reducing power (B). Figure legend is according to Fig. 1. Different small superscripts (a–e) denote significant differences ($p < 0.05$).

of ANS to hydrophobic patches on protein surfaces, its fluorescence intensity increases and the wavelength of maximal absorbance shifts. BLG sonicated without the presence of sugars has shown increased quantum yield after ANS addition, compared to native protein (Fig. 4D). Presence of saccharides further increased peak intensity and shifted the maximum absorbance toward lower wavelengths, indicating that glycosylated BLG possesses newly exposed hydrophobic patches on the protein surface. This effect was the most prominent in the presence of ribose, with maximum shift from 481 to 465 nm. Morgan, Léonil, Mollé, and Bouhallab (1999a) have shown increased ANS binding to BLG thermally glycosylated in solution, in contrast to dry state glycation which resulted in no change.

Other studies have shown that glycation can have variable effects on protein structure. For the heat-induced BLG-lactosylation Morgan et al. (1999a, 1999b) have found higher degree of glycation in the dry state than in aqueous solution, but more significant BLG structural changes in solution. Glycation of BLG in aqueous solution with more reactive sugars (arabinose and ribose) caused protein denaturation, but it was not significant with less reactive sugars (lactose and rhamnose) (Chevalier et al., 2002). Nacka et al. (1998) observed that no significant changes to the tertiary structure of BLG occurred following thermal glycation with aldohexoses and lactose, and complete denaturation of the protein occurred when glycosylated with ribose. Czerwenka et al. (2006) observed increased hydrophobicity and slight conformational change toward more compact structure in thermally lactosylated BLG.

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

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

(2001b), in mild heat-induced BLG glycation model system, although free radical-scavenging activity of glycosylated proteins was not directly related to the glycation degree (Chevalier et al., 2001b).

Metal chelating activity plays an important role in antioxidant action, due to reduction in the concentration of the transition metals required for lipid peroxidation. In the present study we investigated the effect of sonication on ferrous ion chelating activities of BLG in the presence of different sugars (Fig. 5A). Native BLG, as well as sonicated BLG have shown noticeable iron chelating activity. Sonication in the presence of all sugars resulted in significant increase of iron chelating capacity ($p < 0.05$), with ribose having the most prominent effect. Our findings are in agreement with an earlier report on iron chelating activity of other model systems, as a result of MRPs formed due to heat treatment (Dong, Wei, Chen, McClements, & Decker, 2011; Gu et al., 2010). Gamma-irradiation of whey protein concentrate solutions also generated MRPs with iron chelating activity (Chawla et al., 2009).

BLG sonication without saccharides did not have an effect on reducing power of BLG, as shown by an increase in absorbance at 700 nm (Fig. 5B). Reducing power of BLG increased significantly ($p \leq 0.05$), after sonication in the presence of glucose, fructose, arabinose, and ribose, with the last having the most dramatic effect. In glycine–maltose model system the reducing power of conjugates increased with the time of treatment by high intensity ultrasound (Guan et al., 2010b). Heat-induced MRPs from BLG/ribose aqueous model system also exhibited reducing power (Jiang & Brodkorb, 2012). Our results demonstrated that ultrasound-generated MRPs of BLG-glycoconjugates possessed hydrogen-donating and antioxidant activities mainly related to the mechanism of single electron transfer.

4. Conclusion

Most studies on Maillard reaction products preparation are based on dry heating and/or basic pH conditions, which are favourable for Maillard reaction. However, dairy products are processed in hydrated or aqueous form and under neutral or acidic conditions. This study demonstrates that even under conditions that are not quite favourable for Maillard reaction, such as neutral pH and reaction in solution, high intensity ultrasound is able to significantly accelerate Maillard reaction in BLG–sugar aqueous systems. Significant increase was found in the early and intermediate

Maillard products content, fluorescence, browning intensity and antioxidant activity of solutions. Increase in Maillard reaction degree was in accordance with saccharide reactivity. Ribose showed the most pronounced effect on formation of BLG-glycoconjugates by ultrasound. Glycation of the protein under mild reaction conditions did not have a drastic impact on the secondary and tertiary structures of the BLG protein. Increased temperature and reaction time greatly increase the extent of glycation in the Maillard reaction. Therefore, efficient protein glycation in aqueous solution occurs by treatments with high temperatures (>100 °C) for a few hours or at mild temperatures (50–60 °C) for a few days, and most of methods for obtaining glycated proteins are based on these conditions. Sonication enables short treatment times at low processing temperature, and thus low energy consumption; it is non-polluting, while retaining nutritive and functional properties. In fact, strong sheer forces generated during sonication enable efficient mixing of solution and efficient heat transfer, contributing to increased rate and thus increased efficiency of the early phases of the Maillard reaction. At the same time maintenance of low sample temperature (10–15 °C) probably does not substantially increase the rate of the later phases of the Maillard reaction and does not change protein structure to a significant level. During conventional thermal processing there is non-uniform heating of sample and the occurrence of over-heating spots in the sample due to limitations of heat transfer. Therefore efficient protein glycation by conventional heating is mainly accompanied by advanced phases of Maillard reaction and protein denaturation, and thus it is more difficult to control. In contrast, ohmic heating, allowing instantaneous and uniform temperature increase could enable more controllable process and high efficiency for short time, similar to sonication. By optimisation of ultrasound frequency, power density, and time of exposure a desired efficiency of protein glycation can be achieved with minimal late phases of Maillard reaction. Therefore, application of high intensity ultrasound could be suitable for preparation of whey protein glycoconjugates in an industrially feasible way, as mild reaction conditions and short reaction times are sufficient to obtain modified proteins. Further optimisation of conditions that are more favourable for Maillard reaction may provide reasonable output also with less reactive sugars. From that point of view, ultrasound has potential to become an efficient tool in development of methods for production of glycated food protein with novel functional properties, as well as improved natural antioxidants. Our results imply that in addition to processing by heat, high-intensity ultrasound-based food processing technologies can also have a significant impact on the Maillard reaction in susceptible food systems and should not be neglected.

5. Conflict of interest statement

The authors have declared no conflict of interest.

Acknowledgments

The authors acknowledge support of the GA No. 172024 of the Ministry of Education and Science of the Republic of Serbia, COST Action FA 1005 and FP7 RegPot project FCUB ERA GA No. 256716. The EC does not share responsibility for the content of the article.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.foodchem.2012.10.087>.

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Supplementary material

Table S1. Determination of the secondary structure fractions of native BLG and sonicated BLG without and with presence of sugars. Far UV spectra were analyzed by CONTIN software to determine the percentage of β -sheet, α -helical and random coil structures using CDPro software package. For the calculations reference protein base set SP37 was used.

% secondary structures	α - helix	β - sheet	Turn	Random
BLG	11 ± 5	39 ± 4	23 ± 0.9	27 ± 1
sBLG	14 ± 5	35 ± 5	23 ± 1	27 ± 0.6
sBLG/Rib	8 ± 3	41 ± 4	20.1 ± 0.8	31 ± 2
sBLG/Glc	7 ± 1	40 ± 2	22 ± 1	30 ± 2
sBLG/Gal	7 ± 2	41.8 ± 0.6	21.3 ± 0.2	30 ± 1
sBLG/Lac	7 ± 2	41 ± 2	20 ± 1	32 ± 2
sBLG/Ara	7.1 ± 0.6	42 ± 2	22 ± 1	29.4 ± 0.6
sBLG/Fru	9 ± 6	39 ± 7	21.1 ± 0.3	31 ± 1