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Hydrophobicity and aggregation, but not glycation, are key determinants for uptake of thermally processed β-lactoglobulin by THP-1 macrophages

Ying Deng[a,b], Coen Govers[a], Shanna Bastiaan-Net[a], Nina van der Hulst[a], Kasper Hettinga[c], Harry J. Wichers[a,b,*]

[a] Food and Biobased Research, Wageningen University and Research, P.O.Box 17, 6700 AA Wageningen, The Netherlands
[b] Laboratory of Food Chemistry, Wageningen University and Research, Wageningen, The Netherlands
[c] Food Quality and Design, Wageningen University and Research, Wageningen, The Netherlands

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ABSTRACT

The aim of this study is to investigate the immunological relevance of modifications of food protein structure due to thermal processing. We investigated the uptake of β-lactoglobulin, treated with 3 different processing methods, by THP-1 macrophages: wet heating (60 °C in solution) and high- or low-temperature (130 °C or 50 °C, respectively) dry heating, combined with either of 8 types of saccharides or without saccharide. The processing method that was applied significantly affected the uptake of BLG by THP-1 macrophages, while the type of saccharide only had an influence in high-temperature dry heated samples. A set of physicochemical parameters of processed samples was determined, to determine the samples' molecular weight, hydrophobicity, amyloid-like structure, surface charge and secondary structure. Analysis of protein structure alterations indicated the uptake to be linked to the wet heating processing method and percentage of α-helix structure, amyloid-like structures, polymers, and hydrophobicity. We hypothesize that both amyloid-like structures and molecular weight were related to the increased hydrophobicity and therefore postulate that the exposure of hydrophobic regions is the leading physicochemical characteristic for the observed uptake of wet heated BLG samples by THP-1 macrophages. This work demonstrates how differential thermal processing of foods, through protein modification, can have an impact on its interaction with the immune system.

1. Introduction

Bovine milk is a common component of the human diet, especially in western countries. It contains nutrients such as lactose, fat, oligosaccharides, minerals and proteins. Heat treatments like pasteurization and ultra-high temperature (UHT) are widely used to increase the safety of raw milk and extend its shelf life. Such thermal treatments will also lead to structural changes of the milk proteins. In fact, protein aggregation and glycation were observed as a consequence of the usage of high temperature in the production of infant formula (Puig et al., 2003). An intriguing question is whether such heat modifications could impact the development of the, still maturing, infant's immune system.

During food processing, protein is often heat-treated in the presence of saccharides, with which it can chemically react. This reaction, called the Maillard reaction or glycation, leads to modifications of protein structure, ranging from the formation of Amadori products in an early stage to protein crosslinking and degradation in advanced stages of glycation (Zhang et al., 2009). Heating processes in the presence of saccharides and the structural changes caused by this heating were reported to affect the immunological properties of proteins. For instance, glycation of ovalbumin enhanced its uptake by dendritic cells and its immunogenicity compared to the native protein (Hilmenyuk et al., 2010; Ilchmann et al., 2010). Dry heating of whey protein at 130 °C in the presence of lactose led to intense glycation with generation of higher particle diameter and sulfhydryl group accessibility. As a result, the glycated whey protein demonstrated an increased affinity for the receptor for advanced glycation end products (RAGE), and anti-whey IgG binding was reduced (Liu et al., 2016a, 2016b). Heat denaturation of the globular whey protein β-lactoglobulin (BLG), the major protein component of whey protein, was also observed to induce increased infiltration of mononuclear inflammatory cells and eosinophils in the gastro-intestinal mucosa and a lowered IgE response compared to native BLG, in an animal trial (Rytkonen et al., 2002). Also, the binding of rabbit antiserum to BLG was shown to depend on the intensity of its prior heat treatment (Bu et al., 2009).

To clearly link protein structural properties with immunogenicity,

* Corresponding author at: Food and Biobased Research, Wageningen University and Research, P.O.Box 17, 6700 AA Wageningen, The Netherlands.
E-mail address: harry.wichers@wur.nl (H.J. Wichers).

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BLG represents an interesting and versatile model, as it is a bovine-milk-derived protein, which is consumed in various processed forms, from mainly native in pasteurized dairy products to denatured and glycated in high-temperature dried products. BLG is one of the major allergens in cow's milk (Bleumink & Berrens, 1966). It consists of 162 amino acids including 16 lysine residues, 3 arginine's and 1 free cysteine residue (Alexander et al., 1989), which all allow for irreversible tertiary structure changes such as aggregation upon heat treatment and glycation when this is done in presence of reducing saccharides (Chevalier et al., 2002; Chobert et al., 2006; Mousavi et al., 2008; Palazolo et al., 2000).

In this study we applied three different heat-treatments to BLG in the presence, or absence, of various reducing saccharides. The nature of the saccharide used for BLG glycation was reported to determine the type and extent of glycation-induced changes potentially resulting in differences in physiochemical properties of BLG (Chevalier et al., 2001a, 2001b). Dry heating at 130 °C will severely change the protein structure and induces aggregation and glycation as well as lead to the formation of advanced glycation end products (Uribarri et al., 2010). A mild in-solution heating process for a prolonged time is a common alternative method to induce limited glycation (Chevalier et al., 2001a, 2001b; Chun et al., 2016), where the potential formation of aggregates with this method is often overlooked. Finally, heating below the denaturation temperature of BLG in dry form limits the structural changes of the protein itself (Deng et al., 2017) and induces only the early stage of glycation. The resulting glycated and/or aggregated BLG samples were analyzed for their physicochemical properties, which was then related to their uptake by THP-1-derived macrophage immune cells.

2. Materials and methods

2.1. Chemicals

All chemicals were purchased from Sigma Aldrich (St Louis, Missouri, USA) unless otherwise stated.

2.2. BLG purification

Raw cow's milk was collected at the Research Facility 'Carus' of the Department of Animal Sciences, Wageningen UR. β-lactoglobulin (BLG) was isolated from raw cow's milk by a mild anion exchange gel DEAE Sepharose C−6B (GE healthcare, Chicago, Illinois, USA) purification procedure described by De Jongh and colleagues (de Jongh et al., 2001). Isolated BLG was lyophilised (Alpha 1-4 LD, Christ, Osterode, Germany) and stored at −20 °C. The resulting native BLG content was higher than 94% according to size exclusion chromatography.

2.3. Sample preparation

2.3.1. Heat processing of BLG

Purified BLG was dissolved in sodium phosphate buffer (10 mM, pH 7.4, same below) to a protein concentration of 13 mg/mL. To this BLG solution, eight different types of saccharides were added, *i.e.* D-glucose, D-galactose, D-fructose, D-lactose, D-maltose, maltodextrin (dextrose equivalent 16.5–19.5), fructo-oligosaccharide (FOS; Nutricia Research, Utrecht, the Netherlands), and galacto-oligosaccharide (GOS; Royal FrieslandCampina, Wageningen, the Netherlands) in a molar ratio of 1:80 (protein:saccharide) which is similar to raw milk, corresponding to 4 reducing saccharide ends *versus* 1 free amino group or no saccharide (control). Solutions of BLG and saccharides were treated as follows: 1. High-temperature dry heating (H): according to the protocol described by Liu and colleagues (Liu et al., 2016a, 2016b). Sample solutions were lyophilized, incubated in a desiccator with saturated sodium bromide solution for 14 days at room temperature to achieve a water activity (Aw) of 0.53, and heated at 130 °C for 10 min; 2. Wet heating (W): heated in solution at 60 °C for 72 h; 3. Low-temperature dry heating (L): lyophilized and heated at 50 °C, 9 h at a relative humidity of 65% (equivalent to Aw 0.65) using a humidity control chamber (HCP108, Memmert, Schwabach, Germany). After heat processing, samples were dissolved in water to starting concentration (if in dry form) and centrifuged at 2900 ×*g* for 30 min to remove insoluble particles. Supernatants were dialyzed at 4 °C against MQ-water using a membrane with a cut-off of 12–14 kDa (Thermo Fisher, Waltham, Massachusetts, USA), changing water twice every 2 h before dialyzing overnight. Biological duplicates of each protein sample treatment were prepared and stored at −20 °C. Samples were coded by treatment followed by the saccharide that was used for glycation. Saccharides were abbreviated to glu, gal, fru, lac, mal, md, FOS and GOS respectively. For example, "W-glu- BLG" represents the BLG sample mixed with glucose and heated in solution. The final protein concentration of each sample was determined by the Dumas method using Flash EA 1112 Nitrogen and Carbon Analyzer (Thermo Fischer, Waltham, Massachusetts, USA) with 6.29 as conversion factor.

2.4. Fluorescent labelling

Fluorescein isothiocyanate isomer I (FITC) was dissolved in DMSO to a concentration of 1 mg/mL and mixed with 1 mg/mL protein samples in borate buffer (10 mM, pH 9) with a volume ratio 1:10. Mixtures were stored at 4 °C overnight and passed through a NAP-5 column (GE healthcare, Chicago, Illinois, USA) to remove unbound FITC. Protein samples were eluted from the column using sodium phosphate buffer. For cellular experiments, the protein samples were diluted in RPMI 1640 medium without phenol red (Gibco, Thermo Fisher, Waltham, Massachusetts, USA) to 0.1 mg/mL. Fluorescence intensity in arbitrary units (a.u.) of each diluted sample was recorded by Infinite® 200 PRO NanoQuant with i-control software (Tecan, Männedorf, Switzerland) using 495 nm and 525 nm as excitation and emission wavelength (Table S2), to correct for differences in fluorescence intensity between samples.

2.5. THP-1 macrophage cellular uptake

The human monocytic leukemia cell line THP-1 (ATCC, Manassas, Virginia, USA) was differentiated into cells characteristic for resting macrophages (M0) as described by Chanput and colleagues (Chanput et al., 2013) in Corning® 96 Well TC-Treated Microplates (Life Sciences, Tewksbury, Massachusetts, USA) with 100 uL cell suspension (1×10^5 cells) in each well. After exposure to 100 µL sample for 2 h in a humidified incubator (37 °C, 5% CO_2), cells were washed twice with PBS without Ca^{2+} and Mg^{2+} (Gibco, Thermo Fisher, Waltham, Massachusetts, USA) and incubated with 100 µL of 0.25% Trypsin-EDTA (Gibco, Thermo Fisher, Waltham, Massachusetts, USA) for 5 min. Cell suspension in Trypsin-EDTA was centrifuged for 5 min at 450 ×*g*, then the cell pellet was re-suspended in 150 µL 1% paraformaldehyde solution. The mean fluorescence intensity (MFI) of the FITC signal was recorded by flow cytometry (Accuri C6, BD Biosciences, Franklin Lakes, New Jersey, USA) of at least 5,000 cells for each sample using BD Accuri C6 software. For the calculation of relative protein uptake, the background fluorescence intensity of unstimulated cells was subtracted from the obtained MFI after sample exposure, was subsequently corrected for the sample FITC fluorescent signal measured by Tecan after labelling: $(MFI_{sample\ cell}\text{-}MFI_{unstimulated\ cell})/Fluorecence_{sample}$, and finally normalized to untreated BLG signals.

2.6. Advance glycation end product (AGE) related fluorescence

Protein samples were diluted to 1 mg/mL in sodium phosphate buffer and 100 µL of each sample was added to a 96-well black polystyrene plate (Greiner CELLSTAR®, Kremsmünster, Austria). The autofluorescence (in a.u.) of AGEs was measured by Infinite® 200 PRO NanoQuant with i-control software (Tecan, Männedorf, Switzerland)

using 370 nm as excitation wavelength and 440 nm as emission wavelength. All samples were measured in duplicate and corrected against a sodium phosphate buffer control.

2.7. o-Phtaldialdehyde (OPA) method

The amount of free amino groups in untreated and treated BLG was determined by OPA based on Nielsen and colleagues (Nielsen et al., 2001). Briefly, freshly prepared 200 µL OPA reagent was added to 30 µL of 1 mg/mL sample solution and incubated for 20 min at RT, after which the absorbance at 340 nm was measured with Infinite® 200 PRO NanoQuant (Tecan, Männedorf, Switzerland). Sodium phosphate buffer was used as a control and each measurement was performed in duplicate. The amount of free amino groups was determined using an L-leucine standard curve (0.078–20 mM). The loss of amino groups was calculated as the percentage of blocked amino groups per protein relative to the free amino groups in untreated BLG.

2.8. Amyloid structure detection

The protein solution at 0.5 mg/mL was mixed with 50 µM Thioflavin T (ThT) in sodium phosphate buffer using a volume ratio of 1:2. After 1 min incubation at RT, 150 µL of each sample mixture was measured in 96-well black polystyrene plate (Greiner CELLSTAR®, Kremsmünster, Austria) by Infinite® 200 PRO NanoQuant with i-control software (Tecan, Männedorf, Switzerland) in duplicate using 450 nm and 480 nm as excitation and emission wavelengths respectively. The fluorescence intensity (a.u.) of each sample was corrected for a sodium phosphate buffer control and divided by the value of untreated BLG.

2.9. Secondary structure prediction

Using Circular Dichroism spectra (Supplementary Fig. 4), percentages of secondary structure elements were estimated using BeStSel (http://bestsel.elte.hu/) which is specifically useful for detecting β-sheet-rich proteins like BLG (Micsonai et al., 2015).

2.10. Classification of processed BLG in samples according to molecular weight

Based on size exclusion chromatography (SEC) (Supplementary Fig. 5), retention volume and area of peaks for each sample were analyzed using Unicorn 5.31 workstation software (GE healthcare, Chicago, Illinois, USA) while the molecular weight was calculated using a standard curve made by ovalbumin (43 kDa), conalbumin (75 kDa), aldolase (158 kDa), catalase (232 kDa), ferritin (440 kDa) and blue dextran (2000 kDa) from Gel Filtration Calibration Kits (GE healthcare, Chicago, Illinois, USA)Molecular weight of peaks were divided by the molecular weight of monomeric BLG (18.4 kDa) and values were categorized into monomer/dimer (1,2), oligomer (3–9), and polymer (≥10). Sample's molecular weight proportion of each category was calculated according to peak areas.

2.11. Hydrophobicity measurement

To determine the surface hydrophobicity of the samples using the 8-anilino-1-naphthalenesulfonic acid ammonium salt (ANS) analysis, the method of Alizadeh-Pasdar and colleagues (Alizadeh-Pasdar & Li-Chan, 2001) was modified. One mL of protein solution at 0.1 mg/mL in sodium phosphate buffer was mixed with 5 µL ANS stock solution (8 mM in sodium phosphate buffer, prepared in the dark). After incubation in the dark for 15 min, 100 µL of each sample mixture was transferred to a 96-well black polystyrene plate (Greiner CELLSTAR®, Kremsmünster, Austria) and measured by Infinite® 200 PRO NanoQuant with i-control software (Tecan, Männedorf, Switzerland) using 390 nm and 470 nm as excitation and emission wavelengths respectively. The fluorescence intensity (a.u.) of each sample was corrected for the sodium phosphate buffer control, divided by the value of untreated BLG, and shown as relative hydrophobicity (RH).

2.12. Surface charge measurement

The Zetasizer Nano ZS and Zetasizer software (Malvern Panalytica, Malvern, UK) were used to measure the zeta potential, which allows the inference of surface charge of molecules. Prior to measurement, samples were diluted in MQ to 1 mg/mL and injected into a matched disposable capillary cell (DTS1070). The measurements were performed at 25 °C and 40 V and three sequential runs were averaged to obtain the results.

2.13. Statistics

All parameters are presented as means ± standard error of mean (SD) and differences between parameters were assessed for their significance with ANOVA using Prism 6 software. Differences with $p < .05$ were considered significant. The Pearson correlation coefficient (r) andtwo-tailed p value were analyzed using Prism 6 software.Unscrambler software (CAMO, Oslo, Norway) was used to generate PCA plots integrating data from various measurements. For PCA, the dataset of variables was mean centered and weighted by 1/standard deviation to limit the bias from the numerical value.

3. Results

3.1. Heat treatment did not affect crucial parameters for cell cultures experimentation

To link protein structural properties to immunogenicity, we treated BLG in the presence or absence of reducing saccharides with heating methods that variably induce glycation and aggregation. BLG was treated with the wet heating (W) or dry heating at low-temperature (L) or high-temperature (H) methods in the presence of monosaccharides [glucose (glu), galactose (gal) or fructose (fru)], disaccharides [lactose (lac) or maltose (mal)], polysaccharides [maltodextrin (md), fructooligosaccharide (FOS) or galactooligosaccharide (GOS)] or in the absence of saccharides. We firstly analyzed solubility, pH and osmolality of the resulting BLG samples as these properties not only describe the physicochemical structure but are also critical cell culture parameters.

High-temperature dry heating of BLG reduced its solubility. When this treatment was performed in presence of monosaccharides the solubility was further and significantly reduced. In contrast, the addition of saccharides did not alter the solubility of low-temperature dry heated or wet heated BLG (Fig. S1). When dissolved in cell culture medium (*i.e.* 0.1 mg/mL protein), no significant differences were observed between medium and protein samples regarding pH and osmolality (Fig. S2).

3.2. Wet heating most strongly increased BLG uptake by THP-1 macrophages

We analyzed whether heat treatment of BLG affected the uptake of BLG by macrophages. To ensure that the observed effects were due to treatment-induced protein changes, we analyzed lipopolysaccharide (LPS) contamination in our samples as this may influence results in cellular immune assays. We observed a maximum of 0.155 ng LPS contamination per 0.1 mg of protein in all samples (Table S1) which is well below the threshold at which LPS significantly influenced the uptake of fluorescent beads by THP-1 macrophages (*i.e.* 1000 ng/mL; Fig. S3).

All heat-treated BLG samples were fluorescently labelled and uptake by THP-1 macrophages was measured with flow cytometry and reported in mean fluorescence intensity (MFI) following 2 h incubation. The heating method was found to exert a more pronounced effect on the

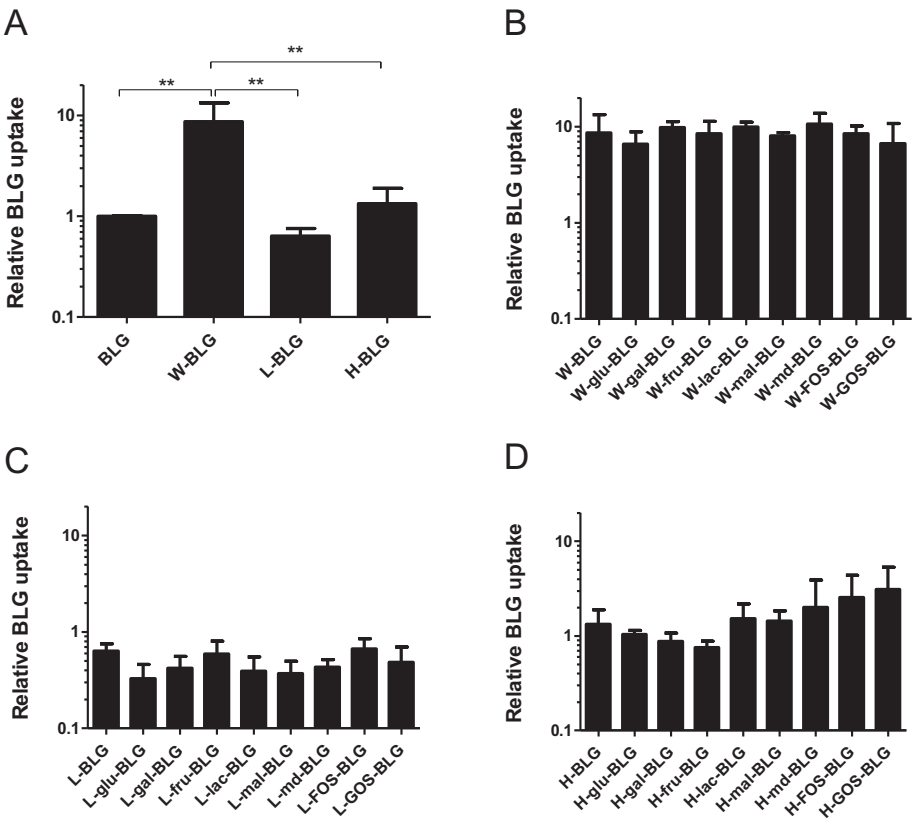

Fig. 1. BLG phagocytosis by THP-1 macrophages is increased upon wet heating. BLG was untreated or heated (W, L or H) in absence or presence of saccharides (glu, gal, fru, lac, mal, md, FOS or GOS) and subsequently fluorescently labelled. BLG uptake by THP-1 macrophages was determined by flow cytometry as mean fluorescence intensity (MFI). BLG uptake was calculated for each sample and normalized for untreated BLG uptake. THP-1 macrophages were tested for uptake of untreated BLG or BLG heat-treated in absence of sugars (A) and wet-heated (B), low-temperature dry-heated (C) and high-temperature dry-heated (D) BLG in presence of saccharides. Bars represent mean value ± SD of 4 independent experiments with 2 independent sample sets. Statistically differences were calculated with Tukey's Multiple Comparison Test (A) or Dunnett's Test (B-D): $^{**}p < .01$.

uptake of BLG than the glycation with various saccharides. Wet-heated BLG, independent of the presence of saccharides, demonstrated significantly increased levels of uptake by the THP-1 macrophages (Fig. 1A and B). Within the dry-heated sample sets, a trend of lowered uptake of BLG was observed for samples exposed to low-temperature dry heating in the presence of saccharides and of increased uptake of BLG when treated with high-temperature dry heating in the presence of oligosaccharides (Fig. 1C and D).

3.3. Physico-chemical characterization of BLG samples after heating

3.3.1. Heat treatment, especially in presence of saccharides, led to loss of free amino groups and formation of AGE-related fluorescence

Protein heat treatments in the presence of reducing saccharides is known to cause glycation which we measured using the OPA method to determine the loss of free amino groups. A significant loss of free amino groups, in comparison to untreated BLG, was detected when wet heating BLG, but not high-temperature or low-temperature dry heating in the absence of saccharides (Fig. 2A). For wet heated samples, no further loss of free amino groups occurred upon incubation with any of the saccharides (Fig. 2B). Contrastingly, when treating BLG with high- and low-temperature dry heating in the presence of any saccharides the loss of free amino groups was significantly increased, except md, FOS and GOS for low- temperature- and FOS for high-temperature dry heating (Fig. 2C and D), with a higher loss of free amino groups compared to wet heated samples. A next step in the chemical process is the formation of advanced glycation end products (AGEs)(Goldberg et al., 2004). Fluorescence intensities at 370/440 nm (exc./em.) were used as an indicator for AGEs formation in the Maillard reaction (Nakamura et al., 1997; Sero et al., 2013; Yamaguchi et al., 2012). In the absence of saccharides, wet-heating but also high-temperature dry-heating of BLG induced a significant increase of the fluorescent signal compared to untreated BLG which suggested glycoxidation (Fig. 3A). This, however, was negligible compared to the observed signals upon the addition of saccharides during heat-treatment. The addition of specific saccharides strongly and significantly increased the fluorescent signal of BLG following wet heating (*i.e.* gal, fru, FOS and GOS) or low-temperature dry heating (*i.e.* glu, gal, lac, FOS and GOS; Fig. 3B and C). Addition of any saccharide in the high-temperature dry heating treatment of BLG significantly increased this signal to levels much higher than with either of the other two heating methods (Fig. 3D).

3.3.2. Wet heating reduced β-sheet formation in BLG but increases amyloid-like structures and α-helices

We analyzed changes in protein structures by determining amyloid-like structure formation (Fig. 4) and presence of α-helix and β-sheets (Fig. 5). Amyloid-like structure formation was measured with ThT staining which revealed a significant increase compared to untreated BLG only upon wet heating (Fig. 4A). The addition of saccharides during the heat treatments did not significantly affect the formation of amyloid structures compared to treatment of untreated BLG (Fig. 4B-C). The prediction of α-helix and β-sheet proportion of the BLG samples was calculated with a software package using the far UV protein spectrum acquired with CD (Fig. S4). The spectra of BLG samples treated with low-temperature dry heating were similar to untreated BLG, whereas wet heating of BLG alters the spectra in a saccharide-independent manner. High-temperature dry heating of BLG altered the spectrum compared to untreated BLG and this change was exacerbated upon the addition of saccharides.

Quantification of α-helix and β-sheet revealed some significant differences (Fig. 5). Wet heating significantly increased the percentages of α-helices and reduced the percentages of β-sheets in BLG (Fig. 5A, B, E and F). Saccharide addition during treatment did not affect the percentages of α-helix or β-sheet in the BLG samples, except for glucose which increased α-helix and lowered β-sheet percentages upon high-temperature dry heating and maltodextrose which lowered α-helix percentages upon low-temperature dry heating (Fig. 5C, D, G and H).

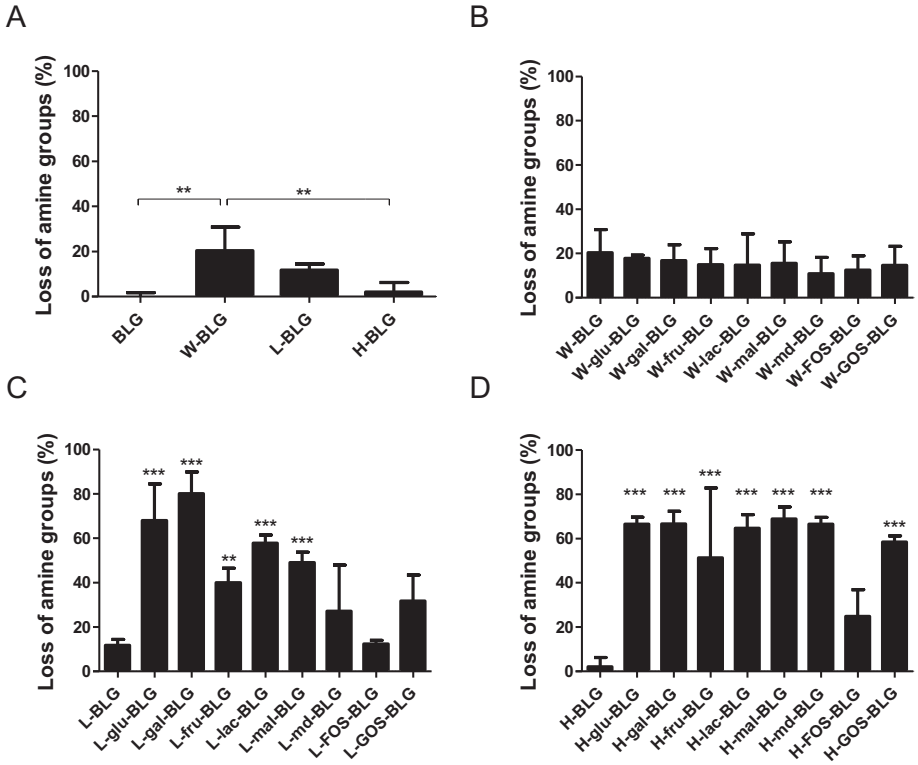

Fig. 2. High- but also low-temperature dry heating in presence of saccharides of BLG led to a loss of free amine-groups. BLG was untreated or heated (W, L or H) in absence or presence of saccharides (glu, gal, fru, lac, mal, md, FOS or GOS). Glycation of untreated BLG or BLG heat-treated in absence of sugars (A) and wet-heated (B), low-temperature dry-heated (C) and high-temperature dry-heated (D) BLG in presence of saccharides was determined by loss of free amine-groups using OPA. Bars represent mean value ± SD of 4 independent experiments with 2 independent sample sets. Statistically differences were calculated with Tukey's Multiple Comparison Test (A) or Dunnett's Test (B-D): **p < .01; ***p < .001.

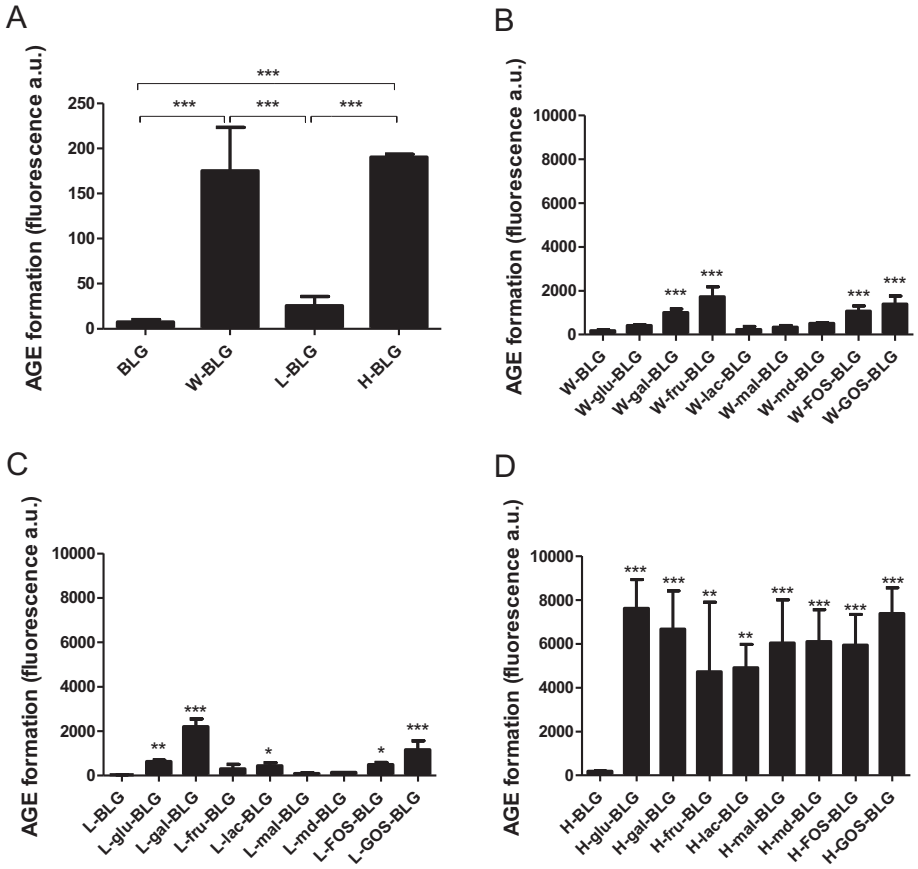

Fig. 3. High-temperature dry heating in presence of saccharides increased BLG fluorescence which is correlated to advanced glycation end products.BLG was untreated or heated (W, L or H) in absence or presence of saccharides (glu, gal, fru, lac, mal, md, FOS or GOS). Advanced glycation end-products in untreated BLG or BLG heat-treated in absence of sugars (A) and wet-heated (B), low-temperature dry-heated (C) and high-temperature dry-heated (D) BLG in presence of saccharides was determined by measuring fluorescence (370 nm exc./440 nm em.). Bars represent mean value ± SD of 4 independent experiments with 2 independent sample sets. Statistically differences were calculated with Tukey's Multiple Comparison Test (A) or Dunnett's Test (B-D): * p < .05; ** p < .01; *** p < .001.

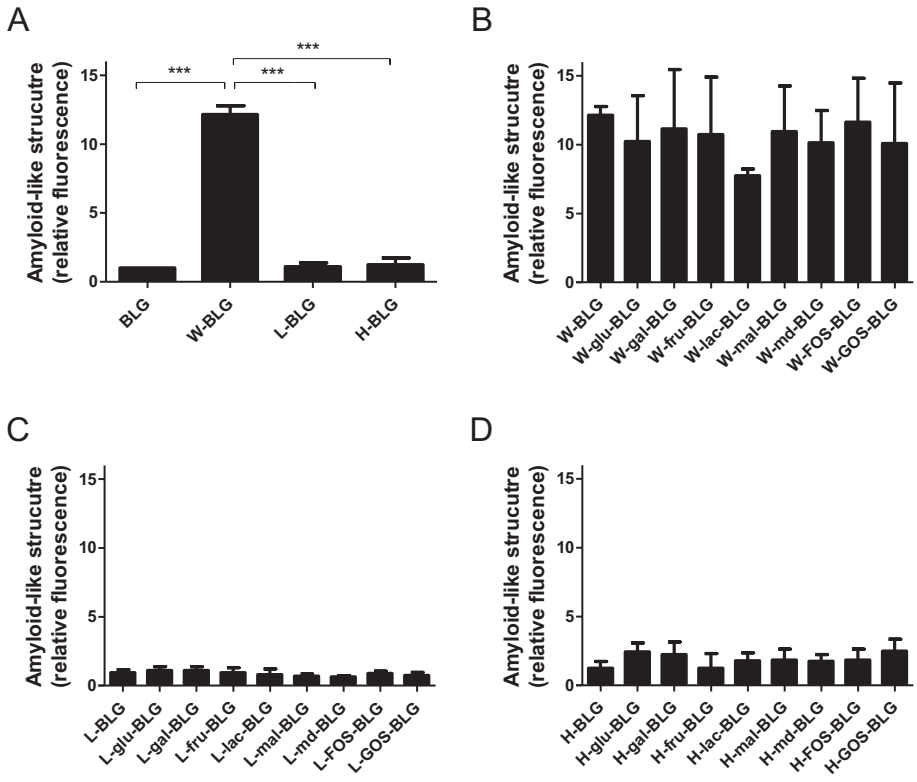

Fig. 4. Wet heating of BLG increased the presence of amyloid-like structures. BLG was untreated or heated (W, L or H) in absence or presence of saccharides (glu, gal, fru, lac, mal, md, FOS or GOS). The presence of amyloid-like structures in untreated BLG or BLG heat-treated in absence of sugars (A) and wet-heated (B), low-temperature dry-heated (C) and high-temperature dry-heated (D) BLG in presence of saccharides was determined by ThT staining. Bars represent mean value ± SD of 4 independent experiments with 2 independent sample sets. Statistically differences were calculated with Tukey's Multiple Comparison Test (A) or Dunnett's Test (B-D): *** $p < .001$.

3.3.3. Wet heating, but also high-temperature dry heating in presence of saccharides, affected the formation of soluble aggregates in BLG samples

Molecular weights were categorized into monomeric or dimeric BLG, oligomeric (3–9 BLG molecules) and polymeric (> 10 BLG molecules) (Fig. 6). Low-temperature dry heated BLG samples consisted mainly of dimers which was similar to untreated BLG, whereas wet heated BLG samples mainly consisted of polymers. For both treatments the addition of saccharides did not affect the aggregation of BLG. Besides insoluble aggregates, high-temperature dry heating induced a relative high amount of soluble oligomers compared to untreated BLG, which appeared to increase with the addition of saccharides in the order of monosaccharide < polysaccharides < oligosaccharide. High-temperature dry heating also appeared to induce the dissociation of the normal dimeric form of BLG in solution (Fig. S5).

3.3.4. Heat treatments but also the presence of saccharides affected the zeta potential and exposure of hydrophobic regions of BLG

The changes in tertiary and quaternary structuremight also result in exposure of hydrophobic regions which could trigger immune responses (Moyano et al., 2012). Hydrophobicity was determined with the ANS measurement which revealed a significant increase upon wet heating of untreated BLG (Fig. 7A). The addition of saccharides did not alter the exposure of hydrophobic regions of the BLG protein for any of the three treatments (Fig. 7B–D).

Finally, we determined the zeta potential as protein surface property. Wet heating of BLG significantly decreased the zeta potential compared to untreated BLG (Fig. 8A). This effect, however, was counteracted by the addition of any of the saccharides during heating, which increased the zeta potential, except glucose and GOS (Fig. 8B). In contrast to wet heating, high-temperature dry heating showed a trend towards an increased zeta potential of untreated BLG, which further and significantly increased upon the addition glucose, galactose, fructose, lactose and FOS (Fig. 8D). Finally, low-temperature dry heating in absence or presence of saccharides did not alter the zeta potential compared to untreated BLG (Fig. 8C).

3.4. Correlation analysis& principle component analysis (PCA)

Heat treatments as well as addition of saccharides during the processing have shown to variably affect the physicochemical characteristics of BLG. We performed a correlation analysis which included all samples and measurements. The correlation analysis (Fig. 9) demonstrated that the transition from monomer/dimer topolymers, percentage of α-helix and amyloid-like structures, and hydrophobicity correlated strongly and significantly with uptake of BLG by THP-1 macrophages ($|r| > 0.8$, $p < .0001$). There was a moderate inverse correlation between uptake and percentage of β-sheet structures, zeta potential, and loss of amino groups (Fig. S6). We also performed a principle component analysis (PCA) (Fig. 10A) which demonstrated that the type of processing, according to PC1 and PC2 was strongly related to the uptake of BLG. Wet heated samples were taken up most efficiently (Fig. 1B), whereas the type of saccharide did not influence the clustering of wet heated BLG samples in the PCA. In the PCA, untreated BLG clustered together with BLG that was treated with low-temperature dry heating in absence or presence of the saccharides and with high-temperature dry heated BLG in absence of saccharides, indicating that these samples were modified the least. The low- and high-temperature dry heated BLG in the presence of saccharides, however, clustered separately.

The type of sugar only influenced clustering for the high-temperature dry heated samples. Detailed analysis including only high-temperature dry heated BLG and untreated BLG (Fig. 10B) confirmed that high-temperature dry heated BLG in presence of either monomeric, dimeric and oligomeric saccharides resulted in separate clusters based on their physicochemical characteristics. Uptake seemed higher for BLG that was glycated with longer saccharides (Fig. 1D), but also in this case the uptake correlated with the occurrence of larger soluble aggregates (Fig. 6).

4. Discussion

Processing of food affects the immunogenicity of proteins contained

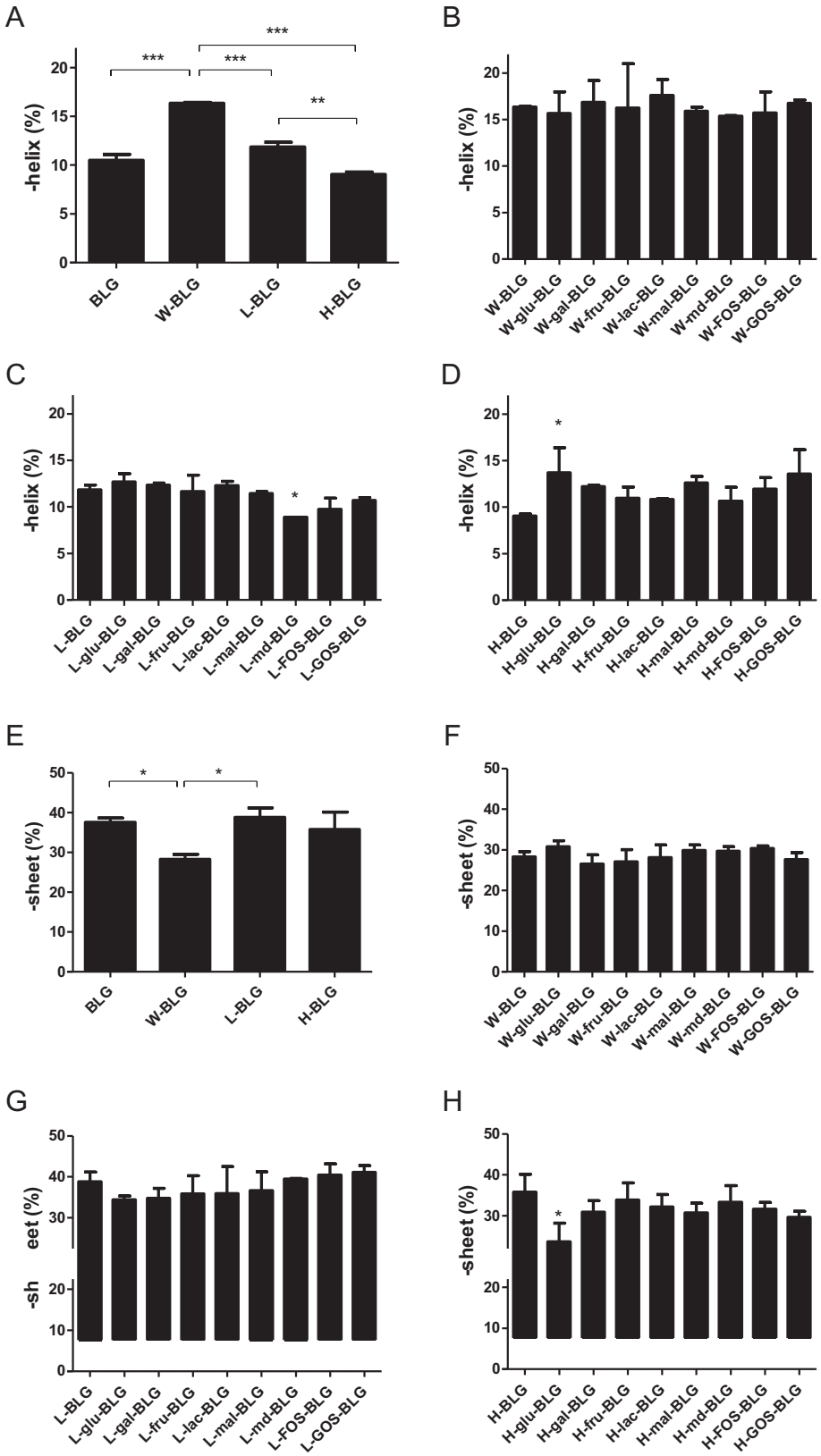

Fig. 5. Wet heating increased α-helix- and lowered β-sheet proportions in BLG. BLG was untreated or heated (W, L or H) in absence or presence of saccharides (glu, gal, fru, lac, mal, md, FOS or GOS). The percentages of α-helix (A-D) and β-sheet (*E*-H) structures in untreated BLG or BLG heat-treated in absence of sugars (A, E) and wet-heated (B, F), low-temperature dry-heated (C, G) and high-temperature dry-heated (D, H) BLG in presence of saccharides were determined using BeStSel software to analyse UV-CD spectra. Bars represent mean value ± SD of 2 independent sample sets, each averaging 10 repeat measurements. Statistically differences were calculated with Tukey's Multiple Comparison Test (A, E) or Dunnett's Test (B-D, F–H): * $p < .05$; ** $p < .01$; *** $p < .001$.

therein, as shown *e.g.* for the increased allergenic potential of peanuts upon dry roasting (Moghaddam et al., 2014) and of BLG upon cross-linking (Stojadinovic et al., 2014). The suggestion is imminent that structural changes, resulting from the processing of such proteins, form the molecular basis of this increased immuno- or allergenicity. Structural damage to proteins in relation to their immunogenicity are already apparent from the DAMP-concept as postulated by Matzinger (Seong & Matzinger, 2004) or from the precautions that are taken to reduce shear-related damage and aggregate formation in the preparation of proteins for pharmaceutical applications (Bee et al., 2012). In

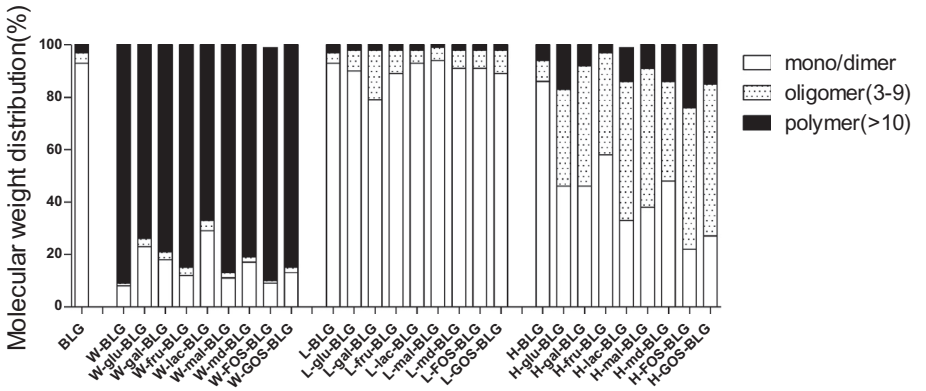

Fig. 6. An increase in BLG multimers was observed upon high-temperature dry heating in presence of sugars but particularly upon wet heating. BLG was untreated or heated (W, L or H) in absence or presence of saccharides (glu, gal, fru, lac, mal, md, FOS or GOS) and measured for aggregation using SEC chromatography. Stacked bars represent averages following single measurements of 2 independent sample sets.

this paper, we described how wet heating and low- or high-temperature dry heating affects the uptake of β-lactoglobulin by THP-1-derived macrophages. BLG was chosen as model because it is a known allergen that is relevant in the context of cow milk allergy. In addition, it is not digested in the human gastric compartment and stable for at least one hour in the jejunal compartment (Sanchon et al., 2018) and therefore relatively undigested protein fragments can interact with intestinal immune cells.

We expected the physicochemical characteristics of glycation and AGE formation to most strongly impact protein uptake by macrophages as these properties have been demonstrated to affect protein immunogenicity (Buttari et al., 2011; Haucke et al., 2014; Nedic et al., 2013; Ott et al., 2014; Price et al., 2004). Low- and high-temperature dry heating of BLG in presence of saccharides, as also previously observed (Deng et al., 2017; Liu et al., 2016a, 2016b), resulted in loss of free amino groups suggestive of glycation (Fig. 2).High-temperature dry heating also induced the formation of AGE-related fluorescence (Fig. 3) although AGEs were not directly measured quantitatively. Both heating methods, however, did not significantly increase BLG uptake by THP-1 macrophages (Fig. 1). Thiscontrasts with previous studies which demonstrated that glycation of OVA drives the uptake by dendritic cells (Heilmann et al., 2014; Hilmenyuk et al., 2010; Ilchmann et al., 2010). Wet heating also significantly increased the fluorescent signal linked to AGE formation and demonstrated a loss of free amino groups when compared to untreated BLG. Both signals were, however, lower compared to low- or high-temperature dry heated BLG in presence of sugars and could result from oxidation or other secondary structural changes (Beisswenger et al., 2012; Soladoye et al., 2015). Surprisingly, analysis towards receptors involved in the uptake of glycated OVA (AGE-OVA) revealed that not the documented receptors for glycated proteins (*i.e.,* RAGE, galectin-3 and CD36 (Ott et al., 2014)) but the scavenger receptor class A I/II,recognized as DAMP-receptor, was important for the uptake by DCs. Further comparison showed that, in these studies, AGE-OVA was obtained by heating at 50 °C for 6 weeks in solution (Gasic-

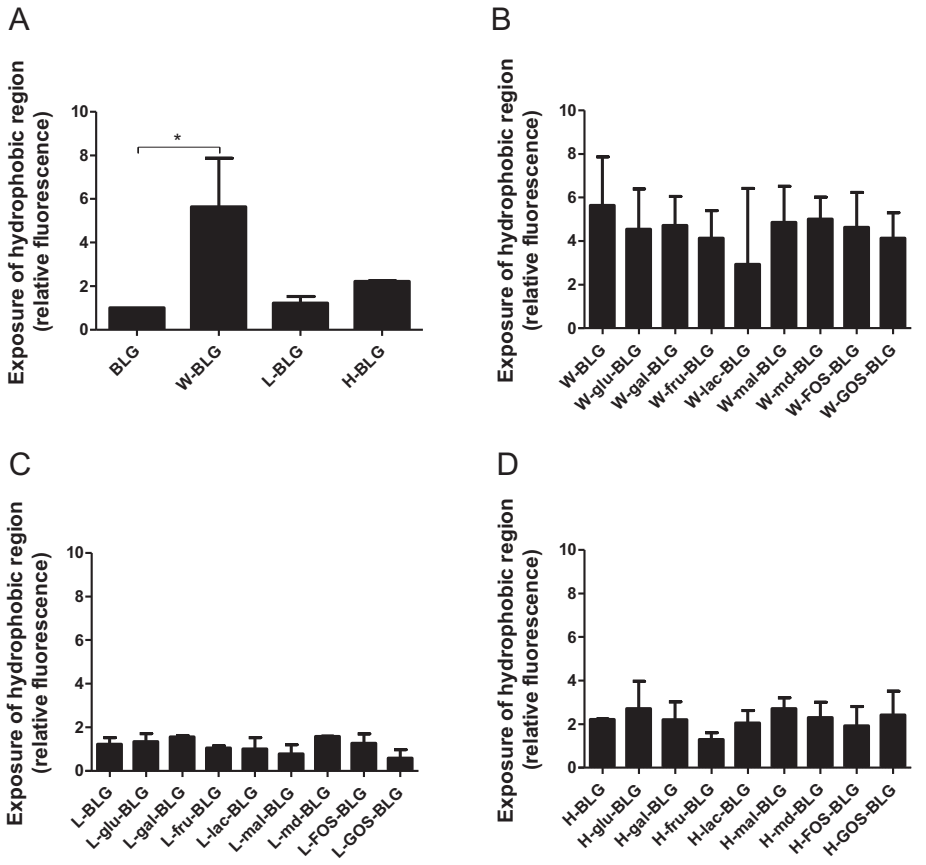

Fig. 7. Wet heating increased exposure of hydrophobic regions of BLG. BLG was untreated or heated (W, L or H) in absence or presence of saccharides (glu, gal, fru, lac, mal, md, FOS or GOS). The exposure of hydrophobic regions in untreated BLG or BLG heat-treated in absence of sugars (A) and wet-heated (B), low-temperature dry-heated (C) and high-temperature dry-heated (D) BLG in presence of saccharides was determined by applying ANS fluorescence normalized to untreated BLG. Bars represent mean value ± SD of 4 independent experiments with 2 independent sample sets. Statistically differences were calculated with Tukey's Multiple Comparison Test (A) or Dunnett's Test (B-D): * $p < .05$.

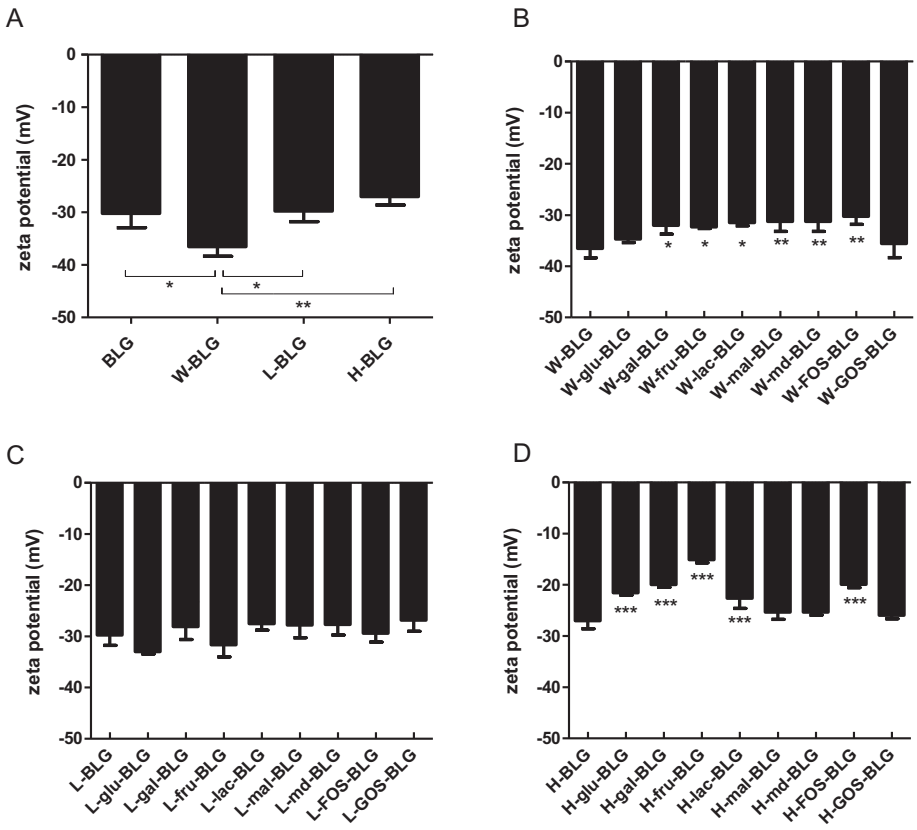

Fig. 8. Wet heating alone or wet heating or high-temperature dry heating in the presence of saccharides affected the zeta potential of BLG. BLG was untreated or heated (W, L or H) in absence or presence of saccharides (glu, gal, fru, lac, mal, md, FOS or GOS). The zeta potential in untreated BLG or BLG heat-treated in absence of sugars (A) and wet-heated (B), low-temperature dry-heated (C) and high-temperature dry-heated (D) BLG in presence of saccharides was measured using the Zetasizer. Bars represent mean value ± SD of 2 independent sample sets, each averaging 3 repeat measurements. Statistically differences were calculated with Tukey's Multiple Comparison Test (A) or Dunnett's Test (B-D): * $p < .05$; ** $p < .01$; *** $p < .001$.

Milenkovic et al., 2003) in which the lengthy incubation period will cause more extensive glycation. As a result, this method holds the middle between our wet heating and dry heating method. Taken together, it appears possible that the increased uptake of AGE-OVA should not be attributed solely to the glycation and AGE formation (*i.e.* typical physicochemical characteristics of dry heated proteins) and that other wet heating-induced physicochemical changes played a role as well.

We found that the process of wet heating of BLG, independent of saccharide presence, induced a significant increase in protein uptake by THP-1 macrophages. Using a correlation and PCA analysis, we demonstrated that the uptake of BLG following wet heating positively

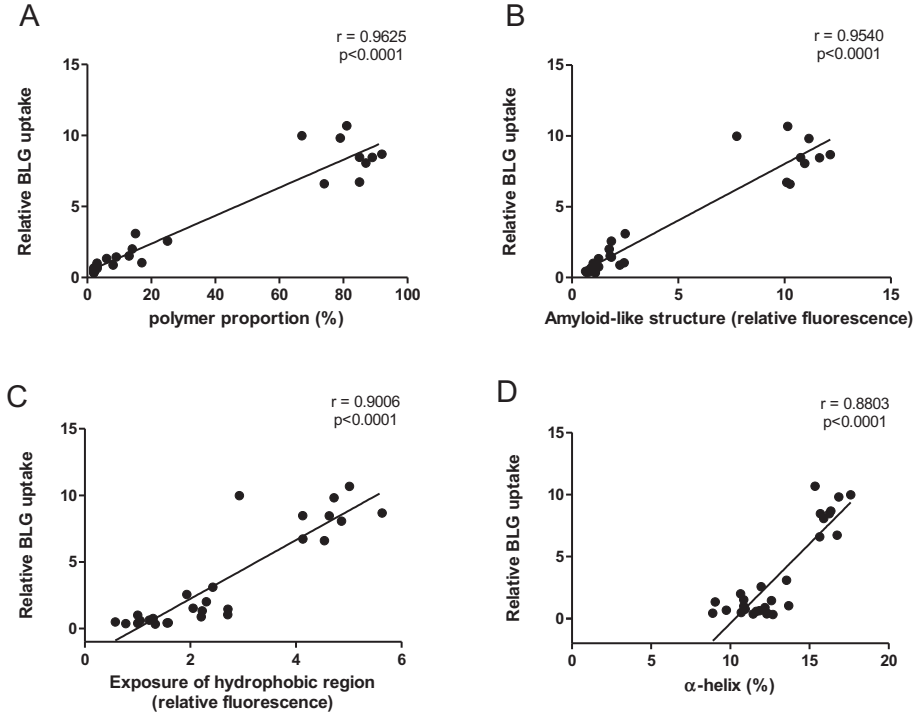

Fig. 9. Uptake of BLG by THP-1 macrophages correlates with aggregation, presence of amyloid-like structure, hydrophobicity, and percentage of α-helix. Uptake of thermally treated BLG in the presence or absence of saccharides was plotted against polymer proportion, amyloid-like structure, exposure of hydrophobic region and α-helix. The correlations were strong with $|r| > 0.8$ and significant with $p < .0001$.

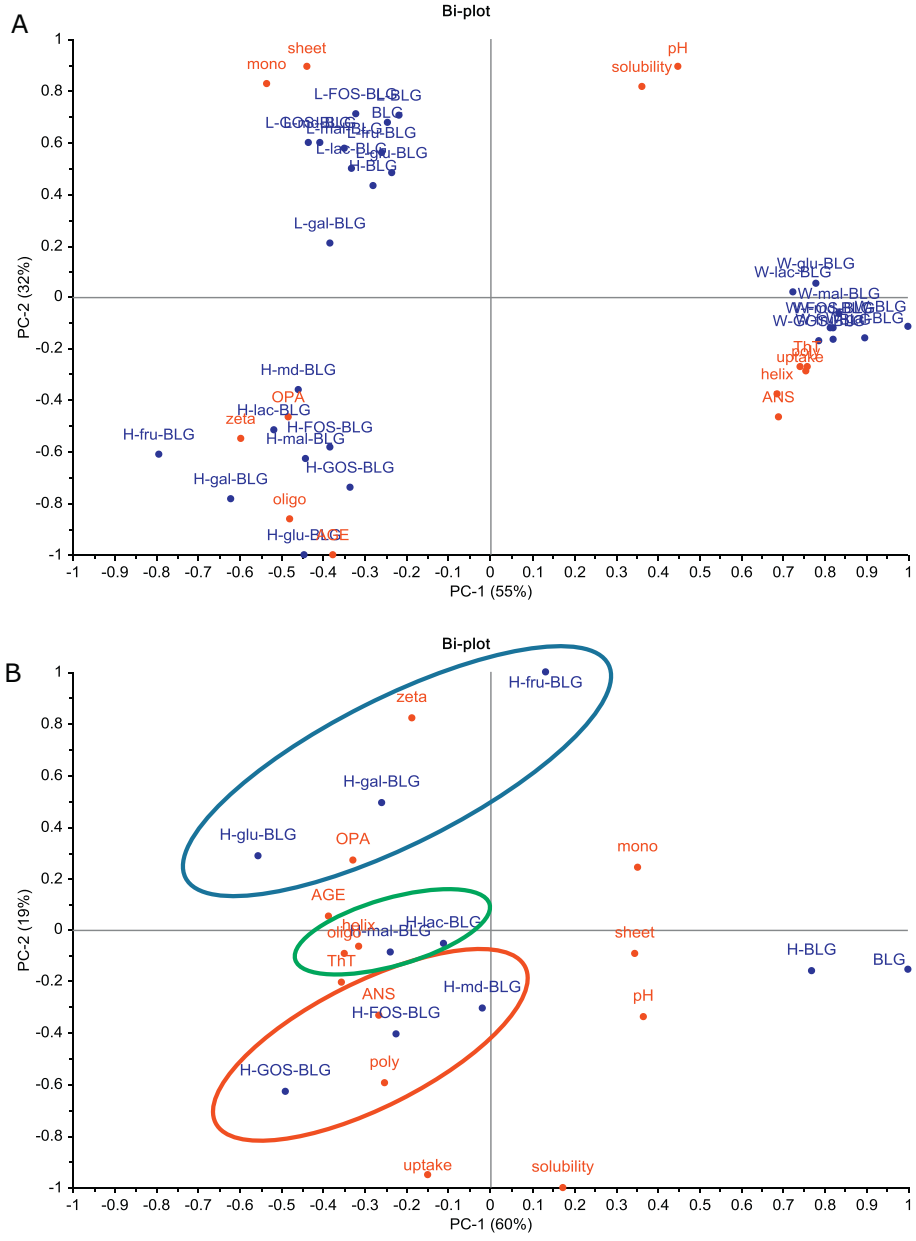

Fig. 10. Uptake of BLG correlated with wet heating and BLG exposure of hydrophobic regions, percentage of α-helix, amyloid-like structures and polymers. BLG was untreated or heated (W, L or H) in absence or presence of saccharides (glu, gal, fru, lac, mal, md, FOS or GOS) and tested for solubility, pH, uptake by THP-1 macrophages (uptake), AGE formation (AGE), glycation (OPA), amyloid-like structure formation (ThT), percentage of α-helix (helix) or β-sheet (sheet), aggregation (monomer and dimer (mono), oligomers (oligo), polymers (poly)), exposure of hydrophobic regions (ANS) and surface charge (zeta). All parameters (A) or all high-temperature dry heating-related parameters (B) were included in a principle component analysis bi-plot using the Unscrambler software and scattered according to heat treatment and saccharides, respectively. In fig. B the clustering of monosaccharides (blue circle), disaccharides (green circle) and oligosaccharides (red circle) is indicated. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

correlated with physicochemical parameters of polymerization, exposure of hydrophobic regions, presence of amyloid-like structures and α-helices and, moderate, inversely correlated with β-sheets, surface charge and loss of amino groups (Fig. 9 and Fig. S6). Most of these physicochemical protein characteristics have individually been linked to immune responses and/or increased phagocytosis by macrophages. Seong and Matzinger concluded that almost all immuno-stimulatory molecules were either entirely hydrophobic or contained exposed hydrophobic regions which can trigger various receptors such as CD14, toll-like receptors (TLRs) or scavenger receptors that recognize danger-associated molecular patterns (DAMPs) (Seong & Matzinger, 2004). Furthermore, they also point out that hydrophobic structures are prone to aggregation into (insoluble) particles. Aggregation itself has also been linked to adverse immune responses through stimulating TLRs *via* intrinsic factors such as size, concentration and generation of neoepitopes (Joubert et al., 2012; Moussa et al., 2016). Of note, these immune responses appear to result from stimulation with soluble aggregates and not insoluble large aggregates (Weltzien & Pachter, 2000) as shown with fractions of aggregated amyloid beta. Amyloid beta may be the best-known example of aggregated protein interacting with the immune system. It is cleared in the brain by macrophages and microglia *via* binding by scavenger receptors and TLRs (Lai & McLaurin, 2012; Mohamed & Posse de Chaves, 2011), which forms a rationale for the observed relation between amyloid-like structures in BLG and uptake by THP-1 macrophages. From literature, the relation between uptake and the increased presence of α-helix structures is less apparent. Similar to wet heating, high-temperature dry heating in the presence of glucose increased the proportion of α-helices in BLG (Fig. 5D). However, this sample did not show increased protein uptake which dismisses the importance of α-helix structures for uptake of BLG by THP-1 macrophages.

Correlation of exposure of hydrophobic regions, protein polymerization (> 10 monomers) and presence of amyloid-like structures to wet heating-mediated enhanced uptake of BLG was also most apparent from the individual figures. Of interest, these parameters are all linked to receptor mediated uptake in contrast to α-helix and surface charge. The fluorescent dye ThT has been used for decades to detect amyloid structures for a variety of applications. More recently, the molecular

base for this interaction has been studied. This revealed that in addition to detecting amyloid fibrils, a loss of protein structure and exposure of hydrophobic regions can also lead to ThT binding in hydrophobic pockets of globular proteins, which putatively requires 5 aromatic and/or hydrophobic residues (Biancalana & Koide, 2010; Reinke & Gestwicki, 2011). The observed ThT signal for wet heated BLG samples might therefore result from exposed hydrophobic regions and provide a false-positive ThT signal. As already discussed above and shown in relation to ANS measurements (Bolognesi et al., 2010), aggregation can be a result of exposure of hydrophobic regions. We therefore postulate that the exposure of hydrophobic regions is the leading physicochemical characteristic for the observed uptake by THP-1 macrophages of wet heated BLG samples. This is supported by multiple studies, using various types of particles, that correlated a hydrophobic particle surface to increased and more rapid uptake by immune cells (Kawaguchi et al., 1986; Sahoo et al., 2002; Simon & Schmidschonbein, 1988; Torche et al., 2000).

We have demonstrated that wet heating of BLG increased the exposure of hydrophobic regions and led to the formation of soluble polymeric aggregates, which we hypothesize are at the basis of the observed increased uptake by macrophages. As indicated above, thermally processed proteins have more widely been demonstrated to be able to activate the immune system. The physiological consequences of such a trigger to the immune system remains to be established but may be relevant to explain the increased incidences of inflammation-associated health effects.

Competing interests

There are no competing interests for any of the authors.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2019.01.038.

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