Impact of the Maillard reaction on the antioxidant capacity of bovine lactoferrin

Y. Joubran[a], A. Mackie[b] & U. Lesmes[a] *

Highlights

- Glucose and fructose used to form lactoferrin-based Maillard conjugates
- Controlled processing had mild effects on lactoferrin structure
- Lactoferrin conjugates exhibit altered colloidal responsiveness to pH
- Glucose increased antioxidant capacity of lactoferrin more than fructose

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Running title: Maillard reaction impact on antioxidant capacity of lactoferrin

Journal: *Food chemistry*

Submitted: *February 2013*

Abstract

Studies raise the notion that the Maillard reaction may be harnessed to modify the antioxidant capacity of proteins. However, little is known on the impact of the Maillard reaction on known bioactive proteins, such as lactoferrin (LF). Glucose and fructose were used as model monosaccharide moieties reacting with lactoferrin in a water restricted environment. UV absorbance and SDS-PAGE analyses using Coomassie and glycoprotein dyes were used to monitor the progression of the Maillard reaction. FTIR and CD indicated minor changes in LF structure while DLS colloidal stability analysis under varying pH showed marked changes. DPPH and FRAP antioxidant assays elucidated marked increase in antioxidant capacity of Maillard conjugates affected by reaction times (12h and 36h), protein: monosaccharide mole ratio (1:1 or 1:3) and moiety type compared to unprocessed LF. Overall, a link between conjugates antioxidant capacity and processing parameters was found, and could assist in the prospective development of healthier processed foods.

1. Introduction

It is commonly accepted that the physicochemical and structural properties of food ingredients underlie their ability to affect human health even beyond their mere nutritional value. In particular, both research and commercial efforts have focused on introducing bioactive ingredients into processed functional foods. Amongst the various physical and chemical changes occurring during processing, non-enzymatic food browning, also termed the Maillard reaction, remains a challenge to scientists because of its diverse reactions, pathways and products. For example, it is accepted that aldoses are intrinsically more reactive than ketoses (Yeboah, Alli & Yaylayan, 1999) and yet numerous reports provide conflicting results on the reactivity of glucose and fructose (Suarez, Etlinger, Maturana & Weitman, 1995; Naranjo, Malec & Vigo, 1998; Yeboah et al., 1999; Jing & Kitts, 2002). The lack of consistency in the literature could be partially attributed to the different conditions used to produce the Maillard reaction products.

On one hand, the Maillard reaction in protein-carbohydrate mixtures are important for the development of characteristic food properties (namely color and flavor) and in some cases may also enhance protein functionality (Oliver, Melton & Stanley, 2006; Gu, Kim, Abbas, Zhang, Xia & Chen, 2010; Wang, Qian & Yao, 2011). On the other hand, the reaction may unintentionally reduce the nutritional value of foods (e.g. loss of essential amino acids) or result in the formation of hazardous compounds (Somoza, 2005; Nasirpour, Scher & Desobry, 2006; Wang et al., 2011). Altogether, various studies have provided evidence against the notion that Maillard reaction products pose a risk to human health (Somoza, 2005; Ames, 2007). The natural, non-extraneous and sometimes uncontrolled nature of this reaction has driven researchers to attempt to rationally harness it to fabricate functional food ingredients (Augustin, Sanguansri & Bode, 2006; Oliver et al., 2006).

63 To this end, bioactive milk proteins and peptides are gaining increasing attention for a
64 myriad of potential beneficial effects, including antimicrobial, anticarcinogenic,
65 antihypertensive and immunomodulatory activities (Madureira, Pereira, Gomes, Pintado &
66 Xavier Malcata, 2007; Hernández-Ledesma, del Mar Contreras & Recio, 2011; Nagpal et
67 al., 2011; Agyei & Danquah, 2012). Different studies have also shown that alimentary
68 proteins and peptides can interfere with radical reactions and act as antioxidants (Elias,
69 Kellerby & Decker, 2008). In this respect, proteins may function as primary or secondary
70 antioxidants. In the case of Primary antioxidants, the proteins display their inactivation
71 mechanisms, such as, electron or hydrogen atom transferring functionality. In the case of
72 secondary or preventive antioxidants, the proteins may retard the rate of oxidation through
73 chelation of prooxidant transition metals (e.g. iron and copper) (Huang, Ou & Prior, 2005).
74 In this respect, Maillard reaction products (MRPs) entail antioxidant activity which has been
75 shown to be affected by intrinsic reactant characteristics, temperature, pH and moisture as
76 well as the physical environment of the reaction (solution or powder) (Ames, 1992;
77 Yaylayan, 1997; Oliver et al., 2006). Such parameters as well as reaction times,
78 protein:sugar ratio and levels of unreacted lysine have been linked to glycate functionality
79 as a texture modifier, gelling agent, emulsifier and even as delivery vehicles for lipophilic
80 nutraceuticals (Augustin et al., 2006; Oliver et al., 2006; Markman & Livney, 2012). These
81 and other studies have revealed that the glycation primarily occurs at the ε-amino group of
82 lysine but can also occur on histidine, tryptophan and arginine but to a lesser extent.
83 Numerous studies demonstrate that MRPs show improved radical scavenging properties
84 and help retard emulsion oxidation, however, little work has been done on bioactive milk
85 proteins (Augustin et al., 2006; Drusch et al., 2009; Gu et al., 2010; Dong, Wei, Chen,
86 McClements & Decker, 2011; Dong, Panya, Zeng, Chen, McClements & Decker, 2012).

Various known bioactive whey proteins have been identified in literature, including glycomacropeptide, alpha lactalbumin, different immunoglobulins and lactoferrin (Madureira et al., 2007). In this respect, bovine lactoferrin (LF), a ~80kDa protein is drawing increasing attention for its various roles in biological functions and as a precursor to potent peptides that may form during digestion (Kuwata, Yip, Tomita & Hutchens, 1998; Troost, Steijns, Saris & Brummer, 2001; Conesa, Rota, Castillo, Perez, Calvo & Sanchez, 2010; Del Olmo, Calzada & Nunez, 2010; Elbarbary, Abdou, Park, Nakamura, Mohamed & Sato, 2010; Flores-Villasenor et al., 2010). An early report indicated LF promotes emulsion oxidation which was attributed to its inherent iron content (Nielsen, Petersen, Meyer, Timm-Heinrich & Jacobsen, 2004). However, a recent study showed that native LF (with little free metal content) can have antioxidant effects on emulsions even when LF was used as an emulsifier (Lesmes, Sandra, Decker & McClements, 2009). Overall, it was identified that fundamental research was needed to establish structure-function relationships for LF that might enable harnessing of the Maillard reaction towards modulating its antioxidant capacity. Thus, this study focused on MRPs of bovine lactoferrin and common food reducing monosaccharides.

2. Materials and methods

2.1. Materials

Food grade bovine lactoferrin (Vivinal lactoferrin FD, 95.6% protein) (LF) was kindly donated by DMV International (Delhi, NY, USA). D-glucose, D-fructose, 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) and ammonium thiocyanate, 2,2-diphenyl-1-picrylhydrazyl (DPPH), were purchased from Sigma-Aldrich Co. (Rehovot, Israel). All solutions were prepared with double-distilled water (DDW) (filtration unit on site) and reagents, such as Hydrochloric acid and sodium hydroxide were of analytical grade.

2.2. Methods

2.2.1. Preparation of Maillard reaction products (MRPs)

Maillard conjugates were prepared by mixing LF with glucose or fructose. Protein and monosaccharaides were dissolved in DDW at ambient temperature for 4 hours using 1:1 and 1:3 molar ratios. Solutions were adjusted to pH 7.0 with 1 M NaOH, lyophilized and ground into fine powders. The resulting powders were incubated at 60^0C under controlled humidity (79% RH achieved over saturated potassium bromide) for 12 or 36 hours. Control samples were produced in a similar way but without being incubated. Heated LF–glucose or LF-fructose mixtures were termed as Maillard reaction products (MRPs) of LF and indexed LF-Glu and LF-Fru respectively with numerical indices denoting mole ratio and the heating duration. For example, a 1:3 mole mixture of LF and Glucose heated for 36h was indexed as LF-Glu 1:3 36h. Finally, mixtures were dialyzed at 4℃ over 12h against 5 volumes of 2L DDW, to remove unreacted monosaccharide (dialysis tubing 12-14 KDa Mw cut off, Thomas Scientific, USA). Dialyzed samples were then lyophilized, pulverized and kept in a desiccator until further analysis.

2.2.2. Evaluation of MRP properties

The occurrence of the Maillard reaction is generally accompanied by physicochemical changes in protein properties and hence can be monitored through various proteomic methods, from simple methods such as UV absorbance and SDS-PAGE to state of the art LC-MS/MS proteomic analyses.

UV absorbance measurements. The UV absorbance at 304 nm of sample solutions was determined using UV/visible spectrophotometer (OPTIZEN POP, MECASYS) and using double distilled water as a blank reference, as previously reported (Zhu, Damodaran & Lucey, 2008). Samples were produced by dissolving native LF or MRPs powders at 0.2% (w/w) in double distilled water and pH was adjusted to 7.0 prior to measurement.

Gel Electrophoresis. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) was carried out using gradient gel (4–15% Mini-PROTEAN® TGX™ Precast Gel) purchased from Bio-Rad Laboratories (Rishon LeZion, Israel). Electrophoresis was carried out at 145 V for 1h in Tris/Glycine/SDS running buffer. After electrophoresis the gels were stained for proteins and glycoproteins by Coomassie brilliant blue R250 and Pierce Glycoprotein stain (Pierce Biotechnology, Rockford, IL) respectively. Individual gels were imaged with a Microtek 9800XL Plus scanner (Microtek,Carson, CA).

2.2.3. Advanced physicochemical characterization.

The protein structure of formed conjugates and control samples was evaluated through FT-IR in dry state form and CD in wet state form. The secondary structure of the samples was assessed using Fourier transform infrared spectroscopy (FTIR) in the Attenuated Total Reflection mode (ATR-FTIR) using a BioRad FTS175C FTIR spectrometer (Bio-Rad Laboratories Inc., Cambridge, MA). Samples were analyzed on a Golden Gate single reflection diamond ATR sampling unit (Specac Ltd, Orpington, Kent, UK). For each spectrum 128 scans at 2 cm^{-1} resolution were averaged and referenced against the empty ATR crystal. Results are presented, after the buffer blank has been subtracted, as molar ellipticity. Circular dichroism (CD) spectra were recorded using a JASCO J-710 spectropolarimeter (Jasco Corp., Tokyo, Japan), under the following conditions: 50 nm/min scan speed, bandwidth = 1 nm, response = 0.5 s, 5 points/nm, and 4 accumulations. Far-UV spectra were recorded with a 0.5 mm path length. The spectropolarimeter was calibrated using camphorsulfonic acid. Samples (0.5 mg/mL protein) were dissolved in deionized water and spectra were collected in the wavelength range 180 and 260 nm and subsequently analyzed using CDPro software.

2.2.4. Colloidal characterization using dynamic light scattering.

The colloidal behavior of proteins is known to be affected by pH and consequently affects the techno-functionality of proteins and their incorporation into various foods. Therefore, experiments were undertaken to monitor LF colloidal behavior under varying pH conditions. This was achieved through monitoring the dynamic light scattering (DLS) of samples using a Nano-ZS DLS instrument (Malvern Instruments, Worcestershire, UK). Dynamic light backscattering (detection angle = 173º) data was collected over at least 12 sequential readings after 2min of sample equilibration within the instrument. Data collected was used to calculate the particle size distribution of samples and the mean particle sizes using the Stokes–Einstein equation. For these experiments, samples were dissolved with double distilled water to 0.2% (w/w), pH was adjusted to desired values using 1 M NaOH or 1 M HCl and then left to equilibrate overnight at ambient temperature before analysis.

2.2.5. Evaluating the antioxidant capacity of Maillard conjugates

The ability of food ingredients to interfere with oxidation reactions and act as antioxidants stems from various mechanisms and hence dictates the use of more than a single assay to adequately evaluate the effectiveness of food antioxidants (Zhu et al., 2008). Therefore, this study made use of two indirect electron transfer methods, namely DPPH radical scavenging assay and Ferric reducing antioxidant power assay.

Determination of DPPH radical-scavenging activity. Radical-scavenging activity of the MRP fractions was determined according to an adapted DPPH method previously described (Dong et al., 2012). An aliquot of MRPs or native LF sample (1.0 ml) was added to 1.0 ml of 0.25 mM DPPH in ethanol. The solution was then mixed vigorously and allowed to stand covered at room temperature in the dark until measurement. The absorbance was measured using a UV/visible spectrophotometer (OPTIZEN POP, MECASYS) at 517 nm over 48 hours. Results were the average of three measurements and expressed as radical-

scavenging activity. The percentage of DPPH radical-scavenging activity was calculated as follows:

Radical-scavenging activity (%)= [1 – (A517 nm_{sample} - A517 $nm_{control}$) / A517 nm_{blank}] x 100.

Ferric reducing/antioxidant power (FRAP). The reducing ability of samples was measured using the ferric reducing/antioxidant power (FRAP) assay (Benzie & Strain, 1996). In the assay, 200 µL of freshly prepared FRAP reagent (mixture of 1 mL of 10 mM TPTZ, 2.5 mL of 20 mM $FeCl_3 \cdot 6H_2O$, 2.4 mL H_2O and 10 mL of 300 mM acetate, pH 3.6) and 10 µL of sample. Absorbances (593 nm) of samples and control solutions were measured after 10 min using a 96-well plate (Microplate reader, Optimax, California, USA). Ascorbic acid stock solutions 0-1000µM were used to generate the calibration curves. Results were expressed as µmol equivalents of Ascorbic acid per gram protein.

3. Results and Discussion

Various studies have shown that the Maillard reaction between proteins and carbohydrates affects a multitude of protein functionalities (Oliver et al., 2006; Drusch et al., 2009; Lesmes & McClements, 2012). This study aimed to link process parameters to the physicochemical properties of lactoferrin, as a model bioactive protein. In particular, the antioxidant capacity of LF-based Maillard conjugates was investigated in an attempt to establish a link between process, structure and functionality, as previously advocated (Ubbink, Burbidge & Mezzenga, 2008; Lesmes & McClements, 2009).

3.1. Evaluation of the Maillard reaction and impact of thermal processing

The progression of the Maillard reaction is accompanied by the formation and breakdown of covalent bonds and the conjugation between proteins and carbohydrates (Yaylayan, 1997; Liu, Ru & Ding, 2012). These have been monitored through various methods which provide indications as to the occurrence and extent of the Maillard reaction. In this study, analyses focused on UV absorbance at 304nm and two different SDS-PAGE staining methods, based on previous reports (Martins, Jongen & van Boekel, 2000; Oliver et al., 2006; Zhu et al., 2008). The data collected for LF-based samples is summarized in **Figure 1**. Control samples verified that lyophilization did not have marked impact on LF properties and thus, analyses focused on native LF versus LF Maillard conjugates. UV absorbance data presented in **Figure 1A** indicates that increasing the duration of thermal treatment slightly increased sample UV absorbance which is attributable to an increasing extent of the Maillard reaction. However, these differences were subtle and SDS-PAGE analyses (**Figure 1B and 1C**) provided further evidence that higher molecular weight (MW) species were formed, as denoted by the appearance of new bands at MW exceeding that of native LF. Further, SDS-PAGE analyses made use of Coomassie (**Figure 1B**) and glycoprotein (**Figure 1C**) stains to facilitate better monitoring of glycated proteins and the screening out

222 of protein smears noted in **Figure 1B** but not in **Figure 1C**. Interestingly, glycoprotein
223 staining provided evidence that new MW species of MW of ~150kDa were formed in all
224 samples. This may indicate that the production of a covalently linked protein dimer was due
225 to some cross linking, as shown previously (Oliver et al., 2006; Ajandouz, Desseaux, Tazi &
226 Puigserver, 2008). These analyses also revealed that the rate and extent of formation of
227 higher MW species was more rapid and pronounced when using glucose as the reactive
228 moiety and at higher protein:monosacchride mole ratio. This noted difference between the
229 reactivity of fructose and glucose in a "dry" reaction is in accordance with previous reports
230 which suggested that this arises from the different electrophlicity of the monosaccharides
231 during early stage Maillard reaction (Naranjo et al., 1998; Yeboah et al., 1999). Altogether,
232 experiments showed the time of glycation, mole ratio of the reactants and monosaccharide
233 moiety are major factors that affect the progression and therefore products of the Maillard
234 reaction. Overall, results indicate that Maillard reaction occurred to a limited extent and
235 apparently reached the early stages of the reaction.

236 **3.2. Advanced physicochemical characterization**

237 To gain more insight into the structural changes arising from Maillard-type conjugation,
238 Fourier Transform infrared (FTIR) and circular dichroism (CD) spectra were recorded for all
239 samples (**Figure 2**). In general, FTIR spectra of modified protein MRP's are accompanied
240 by secondary structure changes which are expressed in the amide I (1600-1690 cm^{-1}) and
241 amide II (1480-1575 cm^{-1}) bands in the spectra (Kong & Yu, 2007; Xavier, Chaudhari,
242 Verma, Pal & Pradeep, 2010). As can be seen in **Figure 2A**, no marked changes were
243 noted in the FTIR spectra, except for a slight shift ($2cm^{-1}$) in the amide I peak and in band
244 intensity levels for both amide I and II regions. Such changes in FTIR spectra may be
245 attributed to conformational changes possibly arising from the pressure of the ATR cell, as
246 previously reported (Lin, Chu & Wei, 2002). Concomitantly, the unsmoothed CD spectra of

native LF and conjugates given in **Figure 2B** did not give rise to any significant differences between the various samples. CD spectra analysis using CDPro software (data not shown) also corroborated this observation. These findings pointed out that the "dry heating" conjugation with monosaccharides applied in this study had little effect on the structure of the protein samples. Studies on β-lactoglobulins and whey protein isolate have shown that under similar reaction conditions changes in protein structure are significantly less pronounced than in studies performed in aqueous solution (Morgan, Léonil, Mollé & Bouhallab, 1998; Morgan et al., 1999; Wooster & Augustin, 2007; Wong, Day, McNaughton & Augustin, 2009). Moreover, such studies have shown that low MW carbohydrate moieties are highly reactive; however, they tend to induce very subtle changes in protein structure and folding. Overall, the effect of glycation in a water restricted environment was found to have little effect on LF structure, thus, further experiments sought to screen for possible ramifications for LF functionality.

3.3. Influence of pH on protein colloid stability

Bioactive lactoferrin, as many functional proteins, may be exposed to a variety of conditions when incorporated into realistic food products and formulations. Thus, the pH responsiveness of LF is an important techno-functional aspect that needs to be addressed in the case of LF based Maillard conjugates. This was studied by monitoring solution appearance and protein size under various pH conditions using direct observations and DLS (**Figure 3**), respectively. These results showed that while native LF solutions had mean sizes not exceeding 100nm over the pH range 5-10. All Maillard conjugates showed decreased colloid stability compared to LF as denoted by pronounced changes in colloid size. This was also accompanied by notable changes in sample turbidity (see inserts **Figure 3**). These marked changes in colloid behavior were confined to pH values of $3<pH<5$ and between $8<pH<9$ and were expressed in one to three orders of magnitude

increase in colloid diameter compared to corresponding native LF samples. For the latter pH range, changes in size and turbidity were expected due to their proximity to the reported isoelectric point of LF (Peinado, Lesmes, Andrés & McClements, 2010). It is worth noting that the electrokinetic charge (ζ-potential) of all the colloids was measured (data not shown) and the findings indicated that glycation did not affect the isoelectric point of LF concurring with a previous report (Pan, Mu, Hu, Yao & Jiang, 2006). The results highlight that increasing the amount of glycation increases LF susceptibility to what appears to be pH induced aggregation or some form of self-assembly. This may be a result of altered inter-molecular interactions altering the ability of molecules to associate, as was proposed by others to occur for other Maillard conjugates tending to form micellar structures (Pan et al., 2006; Markman et al., 2012).

Independent of protein: monosaccharide ratio and reaction time, it was found that fructose affects conjugate stability to a larger extent than glucose. The difference between the impact of fructose and glucose on these properties may arise from different patterns of glycation. Fructose is known to react through its initial breakdown products, while glucose reacts directly with proteins through the Amadori rearrangements (Yeboah et al., 1999; Brands & van Boekel, 2002). Thus, one can postulate that these may lead to different patterns of glycation in which conjugation heavily tend to occur at the outer rim or the inner regions of the protein. Consequently, such differences in glycation sites could affect the inter-molecular and association behavior of conjugates but these require further investigation. Overall, such changes in LF susceptibility to pH are of technical importance as they are linked to solution appearance which highly affects consumer acceptability and appeal of most liquid formulations (McClements, 2005). In the light of these differences in conjugate functionality, other important functional aspects, namely antioxidant activity, were addressed.

3.4. Evaluating the antioxidant capacity of Maillard conjugates

In order to measure the performance of MRP's as antioxidants, two complementary electron transfer *in vitro* antioxidant assays were used: the FRAP assay and the DPPH assay (Decker, Warner, Richards & Shahidi, 2005; Huang et al., 2005). Results obtained from DPPH analyses (**Figure 4**) gave rise to two key observations. First, native LF was found to inhibit radicals formed by DPPH in a gradual and slow acting process compared to vitamin C, used as a standard control. Second, process parameters (protein: monosaccharide ratio and monosaccharide moiety) were found to modulate the antioxidant activity of LF. In particular, the use of glucose as the reacting monosaccharide led to an increase in the rate and extent of DPPH scavenging activity. The difference between glucose based MRP's and native LF was noticeable from the start of the scavenging reaction and became larger and more distinct over the course of 48 hours. Moreover, results indicate a strong link between the amount of glucose used in the reaction and the radical scavenging activity of the corresponding conjugates. MRP's formed using 1:3 mole ratio (**Figure 4B**) presented a much faster inhibition rate than MRP's formed using 1:1 mole ratio (**Figure 4A**). No marked differences were noted for LF-based conjugates formed using fructose. These findings indicate that only glucose conjugates had the ability to scavenge radicals through transferring a single electron to DPPH-derived radicals, thereby, forming stable forms.

FRAP assay findings (**Figure 5**) revealed a similar trend to that observed using DPPH assay where glucose yielded the most active species. Also, the findings concur with previous reports indicating FRAP assay provides enhanced sensitivity for hydrophilic antioxidants (Gil, Tomas-Barberan, Hess-Pierce, Holcroft & Kader, 2000; Ozgen, Reese, Tulio, Scheerens & Miller, 2006). This highlighted the impact of heating times on the antioxidant activity of the conjugates. A significant ($p<0.05$) increase in the FRAP values was seen as the time of heating was extended. Furthermore, the FRAP assay unlike the

DPPH assay, revealed that fructose based MRP's also exhibit a significant increase in radical scavenging values linked to extended glycation time and/or different protein:carbohydrate ratio. However, this activity was consistently lower than all conjugates formed using glucose.

In general, the mechanisms by which proteins and protein-based Maillard conjugates exert their antioxidant activity is yet to be fully understood (Elias et al., 2008). Mutarotation speed or electrophelicity of the reacting monosaccharides are reported to have diverse effects on Maillard conjugates' activity depending on the conditions under which the Maillard reaction took place (Yeboah et al., 1999). Under "wet" reaction conditions, fructose possesses a much faster mutarotation speed which enables it to react more rapidly than glucose and consequently advance the Maillard reaction to a higher extent (Brands et al., 2002). In contrast, this study and others (Wijewickreme & Kitts, 1997; Naranjo et al., 1998; Yeboah et al., 1999) reveal that, under "dry" conditions, glucose advances the Maillard reaction to a higher extent than fructose. One can postulate that this observed change in reactivity could arise from an altered determinant factor guiding the reaction in a water restricted environment. These differences could stem from the different electrophilicity of the carbonyl functions of the reducing moieties under "dry" conditions compared to differences in mutarotation speed guiding moiety reactivity under "wet" conditions.

Conclusion

This study investigated the impact of moderate Maillard modifications of bioactive lactoferrin on the proteins properties and antioxidant capacity. The findings have shown that reaction time, protein: monosaccharide ratio and reacting moiety type have diverse effects on the physicochemical properties of the corresponding conjugates. Radical scavenging assays indicate that glucose has a more pronounced impact on conjugate antioxidant capacity than fructose when conjugates are formed in a water restricted environment. This observed trend

could not be attributed to the monosaccharides themselves or possible small MW caramelization products as those were removed by dialysis. Lyophilization process didn't affect the colloidal stability or antioxidant activity of the resulting MRPs. Two electron transfer antioxidant assays highlighted the different reactivity of the conjugates formed and pointed towards the possible importance of the reaction environment polarity. Together with differences in the colloid stability of the conjugates, it is important to note that conjugate functionality could be affected by environmental pH and/or polarity of the reaction environment. This could suggest that conjugates might have different reactivity in various foodstuffs. Indeed, a recent report demonstrated that antioxidant localization in foods could affect their effectiveness (Alamed, Chaiyasit, McClements & Decker, 2009). Further investigation is needed to evaluate the performance of Maillard conjugates as antioxidants in real food products and to quantitatively monitor such effects. Moreover, the observed differences in the reactivity of glucose and fructose raise the need to better understand the mechanisms by which the Maillard reaction impacts the antioxidant capacity of the formed MRPs. Altogether, this raises the need for further investigations looking to lay the scientific principles that would facilitate optimization between the intent to maximize protein functionality while minimizing changes in its structure, biological activity and digestive fate. These will be significant milestones on the path to ensure development of wholesome processed foods containing bioactive proteins.

Acknowledgements. The authors would like to thank EU COST action FA1001 which facilitated this collaboration. This research was supported by The Wilbush Research Fund and the Russel Berrie Nanotechnology Institute at the Technion. Mr. Y. Joubran would like to acknowledge the financial support of EU COST action FA1001 STSM grant (ECOST-STSM-FA1001-200812-019674). Also, the authors would like to thank Dr. Neil Rigby and Ms. Alice Moscovici for their technical assistance and fruitful discussions.

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Figure captions

Figure 1. Changes in lactoferrin properties arising from Maillard processing in the presence of glucose or fructose under water restricted conditions. (A) UV Absorbance at 304 nm of 0/2% (w/w) control or conjugate solutions (B) SDS-PAGE of LF-based samples stained with Coomassie brilliant blue-R250 (C) corresponding SDS-PAGE analysis obtained using Pierce Glycoprotein stain.

Figure 2. Spectral characterization of lactoferrin and its Millard reaction products. (A) ATR-FTIR spectra highlighting the amide I (1600-1690 cm^{-1}) and amide II (1480-1575 cm^{-1}) bands (B) Circular dichroism spectra of corresponding aqueous solutions in the far UV region.

Figure 3. pH responsiveness of lactoferrin and its Maillard conjugates in terms of colloid size (determined through dynamic light scattering). (A) lactoferrin and its glucose-based Maillard reaction products (B) lactoferrin and its fructose-based Maillard reaction products. Insert images demonstrate the apparent changes in solution appearance of LF-glucose conjugates under pH values between 7 to 10.

Figure 4. DPPH radical scavenging activity of lactoferrin (LF) and LF-based Maillard reaction products prepared using (A) 1:1 protein:monosacchride mole ratio or (B) 1:3 protein:monosacchride mole ratio under various heating times. Data presented as triplicate means with bars indicating standard deviation (error bars may lie within the data points).

532 **Figure 5.** Ferric Reducing/Antioxidant Power (FRAP) activity of lactoferrin (LF) and LF-
533 based Maillard reaction products prepared using (A) 1:1 protein:monosacchride mole ratio
534 or (B) 1:3 protein:monosacchride mole ratio under various heating times. Data presented as
535 triplicate means with bars indicating standard deviation.
536

Color figures

Impact of the Maillard reaction on the antioxidant capacity of bovine lactoferrin

Y. Joubran[a], A. Mackie[b] & U. Lesmes[a] *

Figure 1

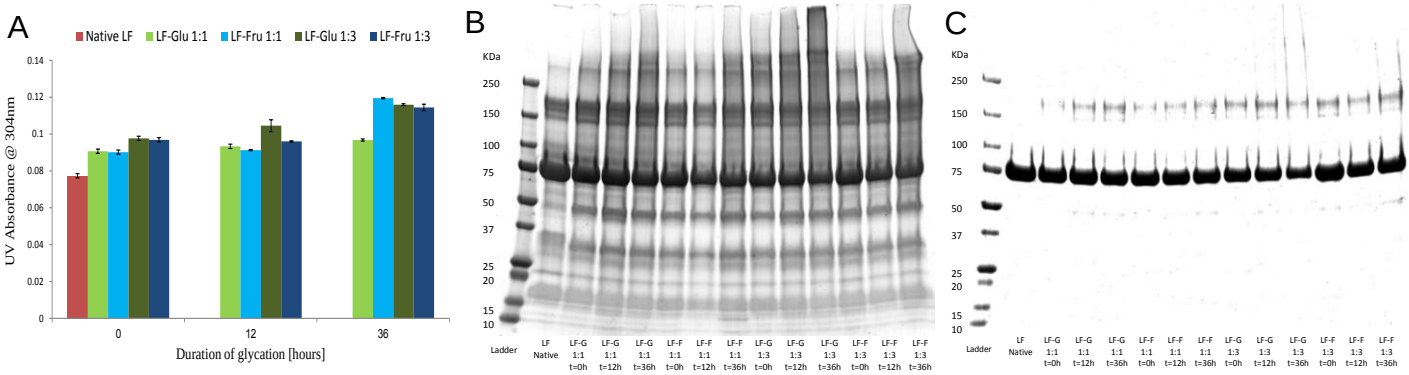
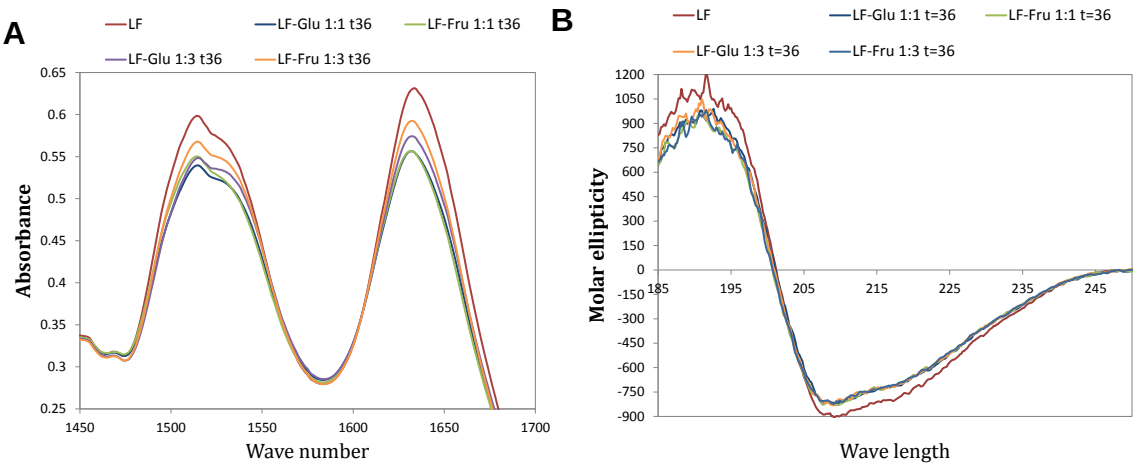


Figure 2


A
LF
LF-Glu 1:1 t36
LF-Fru 1:1 t36
LF-Glu 1:3 t36
LF-Fru 1:3 t36
Absorbance
0.65
0.6
0.55
0.5
0.45
0.4
0.35
0.3
0.25
1450
1500
1550
1600
1650
1700
Wave number
B
LF
LF-Glu 1:1 t=36
LF-Fru 1:1 t=36
LF-Glu 1:3 t=36
LF-Fru 1:3 t=36
Molar ellipticity
1200
1050
900
750
600
450
300
150
0
-150
-300
-450
-600
-750
-900
185
195
205
215
225
235
245
Wave length

23 **Figure 3**

24

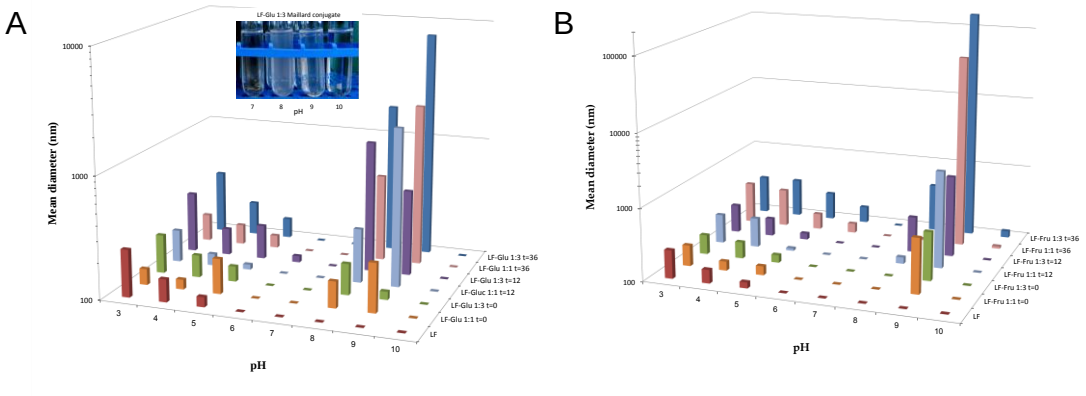


Figure 4

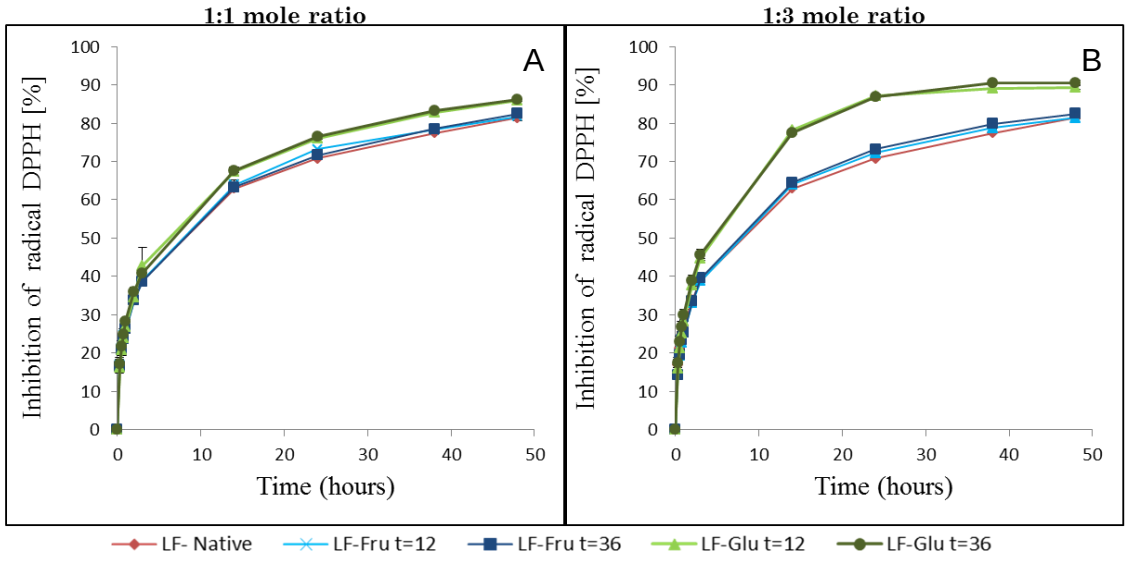
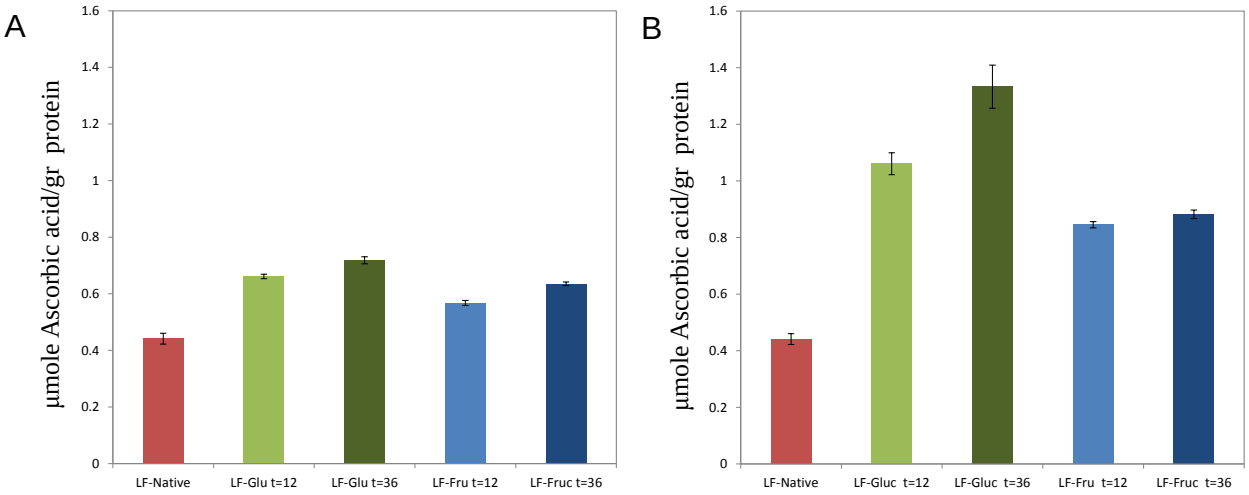

1:1 mole ratio
1:3 mole ratio
A
B
Inhibition of radical DPPH [%]
Time (hours)
LF- Native
LF-Fru t=12
LF-Fru t=36
LF-Glu t=12
LF-Glu t=36

Figure 5


A
µmole Ascorbic acid/gr protein
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
LF-Native
LF-Glu t=12
LF-Glu t=36
LF-Fru t=12
LF-Fruc t=36
B
µmole Ascorbic acid/gr protein
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
LF-Native
LF-Gluc t=12
LF-Gluc t=36
LF-Fru t=12
LF-Fruc t=36