**Use of soluble chitosans in Maillard reaction products with β -lactoglobulin.
Emulsifying and antioxidant properties.**

Marian Mengíbar ¹⁺, Beatriz Miralles ²⁺ and Ángeles Heras ^{*1}

¹Instituto de Estudios Biofuncionales/ Dpto. Química Física II. Facultad de Farmacia. Paseo Juan XXIII, nº1. Universidad Complutense de Madrid. 28040 Madrid, Spain.

²Instituto de Investigación en Ciencias de la Alimentación, CIAL (CSIC-UAM). C/ Nicolás Cabrera nº 9, Campus de la Universidad Autónoma de Madrid, 28049 Madrid. Spain

⁺ Both authors contributed equally to this paper

Highlights

Chitosans (56 to 1.3 kDa) can generate Maillard reaction products with β -lactoglobulin

Chitosan molecular weight has decisive influence on the Maillard reaction development

Conjugates with greater molecular weight improved the emulsion stability of protein

Reaction products with chitosan (1.3 kDa) presented remarkable antioxidant activity

*Corresponding author: Instituto de Estudios Biofuncionales. / Dpto. Química Física II. Facultad de Farmacia. Universidad Complutense de Madrid. Paseo Juan XXIII, nº 1. 28040 Madrid. Spain. Tel/Fax: +34-913943284.

e-mail: aheras@ucm.es (A. Heras).

Abstract

The reaction of commercially available soluble chitosans of equal acetylation degree (11-12%) and differing in their molecular weight (56 to 1.3 kDa) and β -lactoglobulin by Maillard reaction has been conducted in order to evaluate the resulting products and their functional properties. The characterization of the reaction products was performed by measurement of z-potential interactions, solubility, size exclusion chromatography, electrophoretic pattern, and absorbance and fluorescence spectroscopic compounds formation. Higher molecular weight chitosans (39 and 56 kDa) showed a slow progression and a gelled insoluble material appeared. In filtered products, emulsifying properties of the conjugates were improved with regard to those of the unreacted protein. Conjugates formed with the enzymatically depolymerized chitosan (1.3 kDa) displayed a sharp formation of advanced and final products of Maillard reaction. Products with all three chitosans gave rise to antioxidant activity superior to the protein after 2 days of reaction (2 and 3 times in ferric reducing power for 39 and 56 kDa chitosans, and 7 times for the 1.3 kDa chitosan, respectively), and the values correlated well with the spectroscopic and fluorescent compounds from the Maillard reaction. The results are useful with a view to selecting chitosans for development of new ingredients with tailored functional properties.

Keywords: chitosan; conjugate; emulsifying activity index; DPPH radical scavenging; reducing power ability.

1. Introduction

Since proteins and polysaccharides are used in a wide variety of technological applications, the study of their conjugation for functional improvement in conditions industrially feasible results attractive. The Maillard reaction produces browning of compounds due to the initial condensation between a non-protonated amino group of aminoacids, peptides or proteins and a carbonyl group, usually a reducing sugar residue, to form a Schiff base. The reaction continues with cyclization to form the corresponding N-glycosylamine which undergoes an irreversible rearrangement to give the Amadori product. The process continues with a complex mechanism where the Amadori product is degraded and the advanced products that are formed are referred to as advanced glycation end products (AGEs) and brown pigments (Oliver, Melton, & Stanley, 2006). This spontaneous reaction was proposed as an effective and alternative method to chemical reactions involving crosslinking agents (Darewicz & Dziuba, 2001).

The Maillard reaction between dietary proteins and polysaccharides or sugars has been reported in a large number of publications where solubility, emulsifying activity, foaming capacity, and antimicrobial activity have been assessed in relation to the polysaccharide structure, the reaction time or the overall net charge (Yang et al., 2015, Wang & Zhong, 2014, Liu, Kong, Han, Sun, & Li, 2014, Wang, Bao, & Chen, 2013). The versatile physico-chemical characteristics of chitosan due to the variable amine group proportion in the chain (degree of acetylation, DA) and the chain length make suitable the modification of the polymer to get distinctive technological and biological functions.

Enhancing the Maillard reaction for an intended use requires an insight into the structure, including the size and charge, of both the protein and carbohydrate moiety starting material (Evans, Ratcliffe, & Williams, 2013). The number of cationized groups in chitosan plays a key role in inhibiting the coalescence of oil droplets to produce a stable emulsion (Song, Babiker,

Usui, Saito, & Kato, 2002). Satisfactory results have been achieved with high (Laplanche, Turgeon, & Paquin, 2005) and medium molecular weight chitosans (400-600 kDa) (Li & Xia, 2011), (Zinoviadou, Scholten, Moschakis, & Biliaderis, 2012). Also, the exposure of electrostatic binding sites of low acetylation degree (below 10%) chitosan contributes to the disruption of cell membranes in antimicrobial Maillard conjugates (Liang, Yuan, Liu, Wang, & Gao, 2014).

On the other hand, the formation of beneficial compounds through the Maillard reaction is currently gaining a lot of attention (Pastoriza & Rufián-Henares, 2014). Among others, *in vitro* studies have demonstrated that these conjugation products may offer substantial health-promoting activity due to scavenging of reactive oxygen species, radical chain-breaking activity and decomposing hydrogen peroxide and metal chelation (Chawla, Chander, & Sharma, 2009)(Gu et al., 2010). Chitosan presents ferrous ion-chelating potency (Xing et al., 2005) and it is documented that it minimizes lipid oxidation itself (Li, Shi, Jin, Ding, & Du, 2013) or modified with glucose (Kanatt, Chander, & Sharma, 2008).

The goal of this study has been to carry out Maillard reaction with soluble chitosans of varying molecular weights and β -lactoglobulin (β -lac) to highlight the importance of the selection of the polymer with regard to its physico-chemical features, in particular the polymerization degree, on the intended use. To this aim, the emulsifying and antioxidant properties have been selected.

2. Materials and methods

2.1 Samples

β -lac (~80% PAGE) from milk was purchased from Sigma Chemicals (St Louis, MO, USA). Chitosan A (DA 12%, M_w 39 kDa) and Chitosan B (DA 11%, M_w 56 kDa) were provided by

Laboratorios Beslan S.L (Madrid, Spain) and Productos Químicos Gonmisol S.L (Barcelona, Spain), respectively. Chitosan C (DA 12%, Mw 1.3 kDa) was prepared from chitosan A by enzymatic depolymerization using chitosanase from *Streptomyces griseus* (EC 3.2.1.132) (Sigma-Aldrich, St. Louis, MO, USA) by dissolving in 0.2 mol/L acetic/acetate buffer at 5 mg/mL (pH 5.7). One milliliter of enzyme (3.48×10^{-3} mg/mL) was employed per 100 mL of substrate to start the reaction at 37°C in an orbital Lab Therm LT-Xshaker (Thermo Fisher Scientific Inc, Massachusetts, USA) at 100 rpm for 4 days. Ultrafiltration with cellulose acetate membrane disks of 30, 10 and 5 kDa cut-off (Millipore Corporation, Massachusetts, USA) was carried out to obtain the corresponding fraction of chitosan C and to separate chitosanase. The DA was determined by first derivative UV-spectrophotometric method (Muzzarelli, Rocchetti, Stanic & Weckx, 1997) using a spectrophotometer Specord 205 (Analytikjena, Jena, Germany) and the weight average molecular weight (M_w) by size exclusion chromatography (SEC-HPLC). The M_w of the different fractions were obtained from the SEC profiles by extrapolation in a calibration curve using different known M_w chitosans as standards (Mengibar et al., 2013).

2.2 Preparation of Maillard reaction products

Freeze-dried Chitosan:β-lac mixtures (weight ratio 2:1), previously dissolved in 0.1 mol/L acetic acid (Chitosan:β-lac 6.6 and 3.3 mg/mL, respectively) and adjusted to pH 6, were incubated for 7 days at 40 °C and 79% relative humidity. Samples were taken at 2, 4 and 7 days, dissolved at 1 mg/mL in deionized water, filtered through 11 μm pore-sized cellulose acetate membrane filters, and freeze-dried. The solubility was determined from the weight difference between the samples after both freeze-drying processes. Solutions of samples in water (2.5

mg/mL) were measured by microelectrophoresis using a Malvern Zetasizer Nanoseries Nano ZS (Malvern Instruments, Herrenberg, Germany).

2.3. Chromatographic separation of products

SEC-HPLC was performed in Waters 625 LC System pump with an Ultrahydrogel column (Waters, i.d = 7.8mm, l = 300mm) thermostated at 35°C. Waters 2414 differential refractometer and Evaporative Light Scattering (ELS Waters 2424 Milford, MA, USA) were connected online. A 0.15 mol/L ammonium acetate/0.2 mol/L acetic acid buffer (pH 4.5) was used as eluent. The flow rate was 0.6 mL/min and 20 µL of samples dissolved in the buffer were injected.

2.4 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE (Laemmli, 1970) was carried out employing a 150 g/L acrylamide separating gel and a 50 g/L acrylamide stacking gel. Samples (15 µL, 5 mg/mL) were dissolved in Tris-HCl buffer (pH 6.8) containing 10 mg/mL 2-mercaptoethanol. Electrophoresis was conducted for 1 h at a constant voltage of 180 V. Subsequently, the gels were stained with 0.5 mg/mL Coomassie brilliant blue-R250.

2.5 Absorbance and fluorescence measurements of Maillard reaction products

Aqueous solutions of the filtered reaction products (1 mg/mL) were prepared, to follow the formation of intermediate products by measuring the absorbance at 294 nm according to the method proposed by (Lerici, Barbanti, Manzano, & Cherubin, 1990). The formation of AGEs was measured by FAST Index method (Fluorescence of Advanced Maillard products and Soluble Tryptophan) with slight modifications as described previously (Birlouez-Aragon, Leclere,

Quedraogo, Birlouez, & Grongnet, 2001). The fluorescence of soluble peptide tryptophan (Trp) and of advanced Maillard products (AMP) were measured at the wavelength $\lambda_{\text{Ex}}=338/\lambda_{\text{Em}}=410$ nm for AMP and $\lambda_{\text{Ex}}=290/\lambda_{\text{Em}}=340$ nm for Trp.

2.6 Emulsifying properties

The emulsifying properties of the filtered reaction products were determined according to the method of Pierce & Kinsella (1978). β -lac, Chitosan or Chitosan: β -lac reaction products were dissolved at 1 mg/mL in 0.1 mol/L acetate buffer (pH 4). Emulsions, consisting of 1 mL sunflower oil and 2 mL of the above solutions, were shaken and then homogenized in an Ultra Turrax instrument (IKA, Staufen, Germany) at 13,000 rpm for 2 min and at 20 °C. To determine the stability of the emulsions, a 50 μ L aliquot of the emulsion was taken from the bottom of the container at different time intervals (0, 2, 4, 6, 8 and 10 min) and diluted with 5 mL of a 1 mg/mL SDS solution. The absorbance of the diluted emulsion was then determined at 500 nm.

2.7 Antioxidant properties

The α,α -Diphenyl- β -picrylhydrazyl DPPH radical-scavenging activity of Maillard reaction products was assayed by the method proposed by Chen, Tsai, Huang, & Chen (2009) with slight modifications. A total of 250 μ L of sample solution in 5 mg/mL acetic acid was mixed with 1 mL of methanolic DPPH solution (100 μ mol/L). The mixture was shaken and kept at room temperature in the dark. After 60 min, the absorbance was measured at 517 nm.

The FRAP (ferric reducing antioxidant power) assay was carried out according to the method proposed by Benzie & Strain (1996) and modified by Pulido, Bravo, & Saura-Calixto,

(2000) using a water-soluble vitamin E analogue, trolox, as standard. The absorbance was measured at 595 nm using UV-vis spectrophotometer after 30 min.

2.8 Statistical analysis

Differences between the variables in the antioxidant activity data were tested for significance by one-way ANOVA with Duncan post tests using Statgraphics Centurion XVI (Warrenton, VA, USA). Differences at $p < 0.05$ were considered to be significant.

3. Results and discussion

3.1 Electrostatic interaction between chitosan and β -lac and solubility of reaction products

Table 1 shows the electrophoretic mobility of solutions of chitosan and β -lac and their freeze-dried mixtures in water. The decrease in the z-potential values in relation to the z-potential of pure chitosan (Table 1) indicated the formation of an electrostatic complex between chitosan and β -lac in the initial mixture. It is known that, due to its polyelectrolyte nature, chitosan and β -lac interact in solution at slightly acidic pH (Guzey & McClements, 2006). The values indicate a stronger interaction for Chitosan B. A higher size in the polymer chain would supply a greater number of charges and a more extensive interaction with the protein. When the mixtures were incubated, insoluble material with a gel aspect was found in the reacted products when water was added, both with Chitosan A and Chitosan B. After 2 days of reaction, Chitosan A and Chitosan B products showed 65% and 73 % of soluble material from the initial value, respectively. At 4 and 7 days, the percentage remained nearly constant in the case of Chitosan B products but decreased up to 55% in Chitosan A products. This reduction in solubility and gel formation is attributed to the formation of electrostatic coacervates when β -lac and chitosan were

first mixed due to the opposite charges that progress to an insoluble network. Some authors have reported a similar behaviour as viscous solution in products formed with pectin and egg proteins being the remaining solubility of 80% (Al-Hakkak & Al-Hakkak, 2010). In the case of products formed with Chitosan C, the material showed no gelification nor solubility loss.

3.2 Chromatographic profiles of reaction products

The distribution profile chromatograms (Figure 1) showed wide peaks corresponding to the original chitosans as a polydisperse mixture. The Chitosan A: β -lac sample at 0 days presented overlapping of the monodisperse peak of β -lac and a wide peak with an elution curve similar to that of Chitosan A. The Chitosan B: β -lac sample at 0 days presented overlapping peaks to a lower extent, corresponding to the greater Mw difference between chitosan and protein. The Chitosan C: β -lac at 0 days presented a unique peak of lower width, its elution time being close to that of Chitosan C. These elution patterns are compatible with the electrostatic complexes evidenced by measurement of z-potential. The incubated products at 2, 4 and 7 days showed a drop in the peak height that can be attributed to the insoluble material formation. In the case of Chitosan A and B, the products appeared with a chromatographic maximum peak displaced to higher elution times respect to the original chitosan which indicates that only lower molecular weight conjugates remain soluble. In the case of Chitosan C, all products showed lower elution time than the initial chitosan, which implies an increase in molecular weight in the yielded conjugated molecules. Among the products of reaction, glyco-conjugates of different polymerization degree formed between intermediate length chitosan chains and protein, and other products of advanced stages could exist, since the formation of intermediate and advanced products is possible as long as the glycosylation continues (Guan et al., 2010).

3.3 Electrophoretic profile of reaction products

Figure 2 shows the electrophoretic separation (SDS-PAGE) of β -lac, Chitosan A, Chitosan B, Chitosan C and the reacted products. The β -lac band was clearly visible in the 0 days products (lane 3) but a decrease in the band intensity occurred with increasing reaction time (lanes 4, 5 and 6). Even though, the band was detected at all times in the case of Chitosan B. On the other hand, the chitosan lane (2) showed the presence of a polydispersed band at the top of the separating gel. Due to the presence of the amino group in its molecule, chitosan attracts the Coomassie anions under the acidic conditions of the staining step, but it is not able to react with SDS and migrate along the gel. The polydispersed band corresponding to reacted products indicated the strengthened formation of heterogeneous glyco-conjugates over time, still more evident for Chitosan A and Chitosan B.

3.4 Emulsifying properties

To achieve emulsifying performance with a protein-polysaccharide conjugate, the M_w must be large enough to provide sufficient steric stabilization, inhibiting the coalescence of oil droplets (Schmidt et al., 2016). As expected, Chitosan C and the corresponding Maillard reaction products did not improve the emulsion capacity respect to the protein (data not shown).

Figure 3a shows the emulsifying activity index (EAI) increment of Maillard reaction products ($p < 0.05$) respect to the protein. It is noticeable that the 0 days samples showed this improved activity, even being assayed at pH 4, where smoother interaction is expected between β -lac and chitosan due to the acidic isoelectric point of the protein (4.8). For the incubated Chitosan A: β -lac products, the 4 days sample showed significantly ($p > 0.05$) lower EAI than the

2 days one, suggesting a decreasing activity over incubation time. Conversely, the Chitosan B reaction products showed a smooth increasing trend over time (Figure 3a). With regard to emulsion stability, higher values were found in all mixtures and Maillard reaction products vs β -lac, although no significant differences among incubation times could be observed (Figure 3b and c).

The emulsifying properties have been reported as being enhanced during the early stages of conjugation, and at longer times, they gradually decrease, by the degradation of initial Maillard reaction products (Li et al., 2009). This is the observed behaviour for the Chitosan A products, which is in accordance to the favoured Maillard reaction observed with this chitosan with regard to Chitosan B.

3.5 Spectroscopic and fluorescent compounds formation

The ChitosanA: β -lac products presented a UV absorbance increase at 294nm from 0 to 2 days of reaction, followed by a decline to an absorbance below the initial value (Figure 4). For Chitosan B: β -lac the absorbance value remained nearly constant between 0 and 4 days and dropped afterwards to negligible values. In contrast, Chitosan C: β -lac compounds increased continuously upon heating time. Thus, the UV-absorbing intermediate products were more favourably formed with the lower Mw corresponding to oligosaccharide size.

Table 2 shows the FAST Index of the reacted products at the analysed times. Chitosan A: β -lac products showed a progressive increase in Fast Index values over time followed by a decrease at 7 days (Table 2). For Chitosan B: β -lac, a smoother but continuous increasing trend was shown. However, in the case of Chitosan C: β -lac products the concentration of fluorescence compounds increased markedly up to 4 days, where it dropped to a still very high value,

indicating a more favoured reaction for the lower Mw chitosan. The less steric hindrance of low Mw chains in chitosan might produce a fast reaction (Huang, Zhao, Hu, Mao, & Mei, 2012). This is in accordance with previous reports where the saccharides with shorter chains exhibited faster reaction rate than those with longer chains (Li et al., 2014). The elevated FAST index in Chitosan C was in agreement with the greater values of spectroscopic and supports the reaction progress towards AMP which have been related to the development of antioxidant activity. By contrast, the low fluorescence intensity in Maillard reaction products with the high molecular weight was probably caused by the rapid transformation of the intermediates to brown compounds (Lertittikul, Benjakul, & Tanaka, 2007).

3.6 Antioxidant properties

Products formed with Chitosan A and B presented increasing DPPH scavenging activity with time, with significantly ($p<0.05$) superior values than β -lac at 2, 4 and 7 days for Chitosan A products and at 7 days for Chitosan B products. In the case of Chitosan C, the incubated products underwent a sharp increase from 2 days of incubation. The value observed at 7 days was lower but still superior to that of the protein (Figure 5a).

All the Maillard reaction products presented a significantly higher reducing activity than β -lac (Figure 5b). The original chitosans presented in this case a much higher activity than the protein. Maillard reaction products from Chitosan A and B showed a reducing activity between 2 and 3 times increased with regard to the protein but no significant changes were observed with time. Again, the Maillard reaction products from Chitosan C showed a steep increase from 0 to 2 days of incubation and antioxidant values around 7 times higher than those of protein were reached.

The Maillard reaction products antioxidant activity is attributed to the formation of melanoidins which present capacity for trapping free radicals, scavenging reactive oxygen species and metal chelation, in the final stages of the Maillard reaction (Silvan, van de Lagemaat, Olano, & del Castillo, 2006). Some authors have related the Maillard reaction products antioxidant activity with the development of browning intensity and absorbance UV (Kosaraju, Shantha Lakshmi., Weerakkody, Rangika., Augustin, Mary Ann., 2010). Other authors have found a good correlation between Maillard reaction products antioxidant activity and the concentration of fluorescence compounds (347nm/415nm) (Yu, Zhao, Hu, Zeng, & Bai, 2012).

The observed DPPH radical scavenging correlated well with the FAST index values measured and, to a lesser extent, with the UV absorbance values. This indicates that chitosan with low Mw (Chitosan C) in combination with β -lac is able to reach a more advanced reaction state and elicit a scavenging capacity superior to that of the polymer alone. This is in agreement with the results shown by (Gu et al., 2009) who reported that the radical-scavenging activity of low molecular weight Maillard reaction products was greater than that of high molecular weight Maillard reaction products. According to that, (Ying, Xiong, Wang, Sun, & Liu, 2011) have demonstrated the higher DPPH radical scavenging in a water soluble chitosan fructose derivative compared with a N-alkylation chitosan derivative.

Some researchers have reported that compounds responsible for reducing capacity are produced during degradation of Amadori compound in the initial stages (Chang, Chen, & Tan, 2011). In this regard, although the Maillard reaction products showed increased reducing power respect to the protein, the highest activity was found for Chitosan C: β -lac, and no significant differences were observed over time of reaction. On the other hand, in both assays the highest values were recorded after 2 days of incubation. Therefore a short time, which is favorable for

industrial purposes, is enough for the Maillard reaction products to develop their maximum antioxidant capacity.

4. Conclusion

This study showed that soluble chitosans are able to form Maillard reaction products with β -lac though the Chitosan Mw influences the progress of reaction. The reaction with higher Mw chitosans (39 and 56 kDa) showed a slow progression and gave rise to a gelled insoluble material. On the other hand, the depolymerized chitosan (1.3KDa) and β -lac products displayed a sharp formation of advanced and final products of Maillard reaction and remained soluble. Only conjugates recovered from high Mw chitosans showed emulsifying capacity and would be suitable to improve the β -lac emulsifying properties at acidic pH. All the conjugates presented DPPH radical scavenging activity and ferric reducing power superior to the protein, the products formed with the low Mw chitosan being substantially more antioxidant.

In this study a relationship between the different functional properties as a function of the stage the reaction reached with regard to the chitosan molecular weight has been intended. The results are valuable with a view to selecting chitosans for the development of new ingredients with tailored functional properties that facilitate the development of new products in the food industry.

Acknowledgements

This work was partially funded through Project MAT2010-21621-C02-01 and MAT2013-46692-C2-1-R of the Spanish Ministry of Science. M. Mengíbar wants to thank the Ministry of Science (Spain) for a FPU grant.

References

- Al-Hakkak, J., & Al-Hakkak, F. (2010). Functional egg white–pectin conjugates prepared by controlled maillard reaction. *Journal of Food Engineering*, 100(1), 152-159.
- Benzie, I. F. F., & Strain, J. J. (1996). The ferric reducing ability of plasma (FRAP) as a measure of “Antioxidant power”: The FRAP assay. *Analytical Biochemistry*, 239(1), 70-76.
- Birlouez-Aragon, I., Leclere, J., Quedraogo, C. L., Birlouez, E., & Grongnet, J. -. (2001). The FAST method, a rapid approach of the nutritional quality of heat-treated foods. *Food / Nahrung*, 45(3), 201-205.
- Chang, H.L., Y.C. Chen & F.J. Tan. (2011). Antioxidative properties of a chitosan–glucose Maillard reaction product and its effect on pork qualities during refrigerated storage. *Food Chemistry*, 124 (2), 589-595.
- Chawla, S. P., Chander, R., & Sharma, A. (2009). Antioxidant properties of maillard reaction products obtained by gamma-irradiation of whey proteins. *Food Chemistry*, 116(1), 122-128.
- Chen, S. K., Tsai, M. L., Huang, J. R., & Chen, R. H. (2009). In vitro antioxidant activities of low-molecular-weight polysaccharides with various functional groups. *Journal of Agricultural and Food Chemistry*, 57(7), 2699-2704.
- Darewicz, M., & Dziuba, J. (2001). The effect of glycosylation on emulsifying and structural properties of bovine beta-casein. *Food / Nahrung*, 45(1), 15-20.

339 Evans, M., Ratcliffe, I., & Williams, P. A. (2013). Emulsion stabilisation using polysaccharide–
 340 protein complexes. *Current Opinion in Colloid & Interface Science*, 18(4), 272-282.

341 Gu, F., Kim, J. M., Hayat, K., Xia, S., Feng, B., & Zhang, X. (2009). Characteristics and
 342 antioxidant activity of ultrafiltrated maillard reaction products from a casein–glucose model
 343 system. *Food Chemistry*, 117(1), 48-54.

344 Gu, F., Kim, J. M., Abbas, S., Zhang, X., Xia, S., & Chen, Z. (2010). Structure and antioxidant
 345 activity of high molecular weight maillard reaction products from casein–glucose. *Food*
 346 *Chemistry*, 120(2), 505-511.

347 Gu, F., Kim, J. M., Abbas, S., Zhang, X., Xia, S., & Chen, Z. (2010). Structure and antioxidant
 348 activity of high molecular weight maillard reaction products from casein–glucose. *Food*
 349 *Chemistry*, 120(2), 505-511.

350 Guan, Y., Lin, H., Han, Z., Wang, J., Yu, S., Zeng, X., Sun, W. (2010). Effects of pulsed electric
 351 field treatment on a bovine serum albumin–dextran model system, a means of promoting the
 352 maillard reaction. *Food Chemistry*, 123(2), 275-280.

353 Guzey, D., & McClements, D. J. (2006). Characterization of [beta]-lactoglobulin-chitosan
 354 interactions in aqueous solutions: A calorimetry, light scattering, electrophoretic mobility
 355 and solubility study. *Food Hydrocolloids*, 20(1), 124-131.

356 Huang, J., Zhao, D., Hu, S., Mao, J., & Mei, L. (2012). Biochemical activities of low molecular
 357 weight chitosans derived from squid pens. *Carbohydrate Polymers*, 87(3), 2231-2236.

358 Kanatt, S. R., Chander, R., & Sharma, A. (2008). Chitosan glucose complex – A novel food
 359 preservative. *Food Chemistry*, 106(2), 521-528.

360 Kosaraju, Shantha Lakshmi., Weerakkody, Rangika., Augustin, Mary Ann. (2010). Chitosan-
 361 glucose conjugates: Influence of extent of maillard reaction on antioxidant properties.
 362 *Journal of Agricultural and Food Chemistry*, 58, 12449-12449-12455.

363 Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of
 364 bacteriophage T4. *Nature*, 227, 680-685.

365 Laplante, S., Turgeon, S. L., & Paquin, P. (2005). Emulsion stabilizing properties of various
 366 chitosans in the presence of whey protein isolate. *Carbohydrate Polymers*, 59(4), 425-434.

367 Lerici, C. R., Barbanti, D., Manzano, M., & Cherubin, S. (1990). Early indicators of chemical
 368 changes in foods due to enzymic or non enzymic browning reactions. 1: Study on heat
 369 treated model systems. *Lebensmittel-Wissenschaft Und Technologie*, 23, 289-294.

370 Lertittikul, W., Benjakul, S., & Tanaka, M. (2007). Characteristics and antioxidative activity of
 371 maillard reaction products from a porcine plasma protein–glucose model system as
 372 influenced by pH. *Food Chemistry*, 100(2), 669-677.

373 Li, X., Shi, X., Jin, Y., Ding, F., & Du, Y. (2013). Controllable antioxidative xylan–chitosan
 374 maillard reaction products used for lipid food storage. *Carbohydrate Polymers*, 91(1), 428-
 375 433.

376 Li, X., & Xia, W. (2011). Effects of concentration, degree of deacetylation and molecular weight
 377 on emulsifying properties of chitosan. *International Journal of Biological Macromolecules*,
 378 48(5), 768-772.

379 Li, Y., Lu, F., Luo, C., Chen, Z., Mao, J., Shoemaker, C., & Zhong, F. (2009). Functional
 380 properties of the maillard reaction products of rice protein with sugar. *Food Chemistry*,
 381 117(1), 69-74.

382 Liang, C., Yuan, F., Liu, F., Wang, Y., & Gao, Y. (2014). Structure and antimicrobial
 383 mechanism of ϵ -polylysine-chitosan conjugates through Maillard reaction. *International*
 384 *Journal of Biological Macromolecules*, 70, 427-434.

385 Liu, Q., Kong, B., Han, J., Sun, C., & Li, P. (2014). Structure and antioxidant activity of whey
 386 protein isolate conjugated with glucose via the maillard reaction under dry-heating. *Food*
 387 *Structure*, 1(2), 145-154.

388 Mengfbar, M., Mateos-Aparicio, I., Miralles, B., Heras, A. (2013) Influence of the physico-
 389 chemical characteristics of chito-oligosaccharides (COS) on antioxidant activity.
 390 *Carbohydrate Polymers*, 97 (2), 776-782.

391 Oliver, C. M., Melton, L. D., & Stanley, R. A. (2006). Creating proteins with novel functionality
 392 via the maillard reaction: A review. *Critical Reviews in Food Science & Nutrition*, 46(4),
 393 337-350.

394 Pastoriza, S., & Rufián-Henares, J. A. (2014). Contribution of melanoidins to the antioxidant
 395 capacity of the Spanish diet. *Food Chemistry*, 164, 438-445.

396 Pierce, K. N & Kinsella, J.E. (1978). Emulsifying properties of proteins: Evaluation of a
 397 turbidimetric technique. *Journal of Agricultural and Food Chemistry*., 26, 716.

398 Pulido, R., Bravo, L., & Saura-Calixto, F. (2000). Antioxidant activity of dietary polyphenols as
 399 determined by a modified ferric Reducing/Antioxidant power assay. *Journal of Agricultural*
 400 *and Food Chemistry*, 48(8), 3396-3402.

401 Schmidt, U. S., Pietsch, V. L., Rentschler, C., Kurz, T., Endreß, H. U., & Schuchmann, H. P.
 402 (2016). Influence of the degree of esterification on the emulsifying performance of
 403 conjugates formed between whey protein isolate and citrus pectin. *Food Hydrocolloids*, 56,
 404 1-8.

405 Silván, J. M., van de Lagemaat, J., Olano, A., & del Castillo, M. D. (2006). Analysis and
 406 biological properties of amino acid derivates formed by maillard reaction in foods. *Journal*
 407 *of Pharmaceutical and Biomedical Analysis Nutraceuticals Analysis*, 41(5), 1543-1551.

408 Song, Y., Babiker, E. E., Usui, M., Saito, A., & Kato, A. (2002). Emulsifying properties and
 409 bactericidal action of chitosan-lysozyme conjugates. *Food Research International*, 35(5),
 410 459-466.

411 Wang, W., & Zhong, Q. (2014). Properties of whey protein–maltodextrin conjugates as impacted
 412 by powder acidity during the maillard reaction. *Food Hydrocolloids*, 38, 85-94.

413 Wang, W., Bao, Y., & Chen, Y. (2013). Characteristics and antioxidant activity of water-soluble
 414 maillard reaction products from interactions in a whey protein isolate and sugars system.
 415 *Food Chemistry*, 139(1–4), 355-361.

416 Xing, R., Liu, S., Guo, Z., Yu, H., Wang, P., Li, C., Li, P. (2005). Relevance of molecular weight
 417 of chitosan and its derivatives and their antioxidant activities in vitro. *Bioorganic &*
 418 *Medicinal Chemistry*, 13(5), 1573-1577.

419 Yang, Y., Cui, S. W., Gong, J., Guo, Q., Wang, Q., & Hua, Y. (2015). A soy protein-
 420 polysaccharides maillard reaction product enhanced the physical stability of oil-in-water
 421 emulsions containing citral. *Food Hydrocolloids*, 48, 155-164.

422 Ying, G., Xiong, W., Wang, H., Sun, Y., & Liu, H. (2011a). Preparation, water solubility and
 423 antioxidant activity of branched-chain chitosan derivatives. *Carbohydrate Polymers*, 83(4),
 424 1787-1796.

425 Yu, X., Zhao, M., Hu, J., Zeng, S., & Bai, X. (2012). Correspondence analysis of antioxidant
 426 activity and UV–Vis absorbance of maillard reaction products as related to reactants. *LWT -*
 427 *Food Science and Technology*, 46(1), 1-9.

428 Zinoviadou, K. G., Scholten, E., Moschakis, T., & Biliaderis, C. G. (2012). Properties of
 429 emulsions stabilised by sodium caseinate–chitosan complexes. *International Dairy Journal*,
 430 26(1), 94-101.

431

432

Figure captions

Figure 1. SEC-HPLC patterns of a) Chitosan A; b) Chitosan B; c) Chitosan C and the resulting Maillard reaction products (Chitosan: β -lac) at 0, 2, 4 and 7 days. Size exclusion chromatography (SEC-HPLC) has been performed in a Waters 625 LC System with an Ultrahydrogel (Waters, i.d = 7.8mm, l = 300 mm) column thermostated at 35°C. Waters 2414 differential refractometer and Evaporative Light Scattering (ELS Waters 2424 Milford, MA, USA) have been connected online.

Figure 2. SDS-PAGE pattern of the Chitosan: β -lac resulting Maillard reaction products for different times: a) Chitosan A: β -lac, b) Chitosan B: β -lac, b) Chitosan C: β -lac. Lane MM Mw: Molecular marker, lane 1: β -lac, lane 2: Chitosan, lane 3: Chitosan: β -lac 0 days, lane 4: Chitosan: β -lac 2 days, lane 5: Chitosan: β -lac 4 days, lane 6: Chitosan: β -lac 7 days. Electrophoresis was conducted for 1 h at a constant voltage of 180 V in a 150 g/L acrylamide separating gel and a 50 g/L acrylamide stacking gel.

Figure 3. a) Emulsifying activity index of β -lac, Chitosan A, Chitosan B and the resulting Maillard reaction products at 0, 2, 4 and 7 days. β -lac Chitosan A Chitosan B Chitosan A o B: β -lac. The different letters (a, b, c, d, e and f) indicate significant difference ($p < 0.05$). b) and c) Emulsion stability of β -lac, Chitosan A, Chitosan B and the corresponding Maillard reaction products at 0, 2, 4 and 7 days of reaction ($n=3$). β -lac Chitosan A Chitosan B Chitosan A o B: β -lac 0days $\times$ Chitosan A o B: β -lac 2 days Chitosan A o B: β -lac 4days Chitosan A o B: β -lac 7days. * indicates significant difference ($p < 0.05$).

Figure 4. Analysis of intermediate compounds formed during Maillard reaction with Chitosan and β -lac by absorbance measurement at 294nm. Chitosan A: β -lac Chitosan B: β -lac * Chitosan C: β -lac. Data are mean $\pm$ SD ($n=3$).

Figure 5. Determination of DPPH radical scavenging activity (a) and ferric reducing power (b) in β -lac, Chitosan A, B and C and the resulting Maillard reaction products at 0, 2, 4 and 7 days.

 Significant differences between average values (n=3) in different samples by one-way ANOVA (Duncan test). The different letters (a, b, c, d, e, f, g and h) indicate significant difference (p<0.05) between different samples.

Figure 1

Figure 2

Figure 3

Figure 4

482 **Figure 5**

483