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The occurrence of a Maillard-type protein-polysaccharide reaction between β-lactoglobulin and chitosan

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Abstract

Reaction mixtures containing β-lactoglobulin and chitosan (1:4 weight ratio) were dry-heated at 40 °C and 79% relative humidity for 2 weeks. Absorbance measurements and SDS-PAGE analysis indicated the occurrence of the Maillard reaction and conjugate formation, respectively. Some β-lactoglobulin and chitosan properties were modified. For example, the emulsifying capacity at pH 4 and bactericidal activity against *Escherichia coli* of the Maillard reaction products (MRPs) increased with incubation period up to 2 days, after which these properties deteriorated. The latter was explained by MRPs degradation, which was confirmed by the increased appearance of degradation products in electrophoresis gels at longer incubation times. Data show that the Maillard reaction, under the studied conditions, can be successfully employed to generate MRPs from β-lactoglobulin and chitosan, which exhibit improved properties with respect to β–lactoglobulin alone.

Keywords: Chitosan; Maillard reaction; β-Lactoglobulin; Emulsifying properties; Antimicrobial activity

1. Introduction

Whey protein can be used by the food industry as a functional additive in products in which air–water dispersions are desirable. The major protein present in whey is β-lactoglobulin (β-LG), which constitutes approximately 50% of the total whey proteins. β-LG is a valuable protein in terms of technological food characteristics as it has useful emulsifying, foaming and gelling properties (Foegeding, Kuhn, & Hardin, 1992; Shimizu, Saito, & Yamauchi, 1985; Waniska, 1988).

The most important limitation concerning the functional properties of β-LG is that it loses its emulsifying properties in the acidic pH region or in the presence of salt. Therefore, it would be beneficial to create enhanced emulsifying properties under these unfavourable conditions. Conjugation of proteins with polysaccharides has been demonstrated to introduce functional improvements to food proteins (Dickinson, 1993; Hattori, Numamoto, Kobayashi, & Takahashi, 2000; Kato, 2002). If charged polysaccharides are employed for conjugation, ion-exchange activity is provided to the protein and can lead to better emulsifying properties in the presence of salt. Endowment of charge alters the isoelectric point and is also expected to contribute to the improvement of the emulsifying properties in the acidic region.

Chitosan (CHT) is a natural, non-toxic and biodegradable biopolymer. This polysaccharide is a partially deacetylated polymer of N-acetyl glucosamine prepared from chitin. The latter is found in a wide range of natural sources, such as crustaceans, fungi and insects. CHT has been shown to improve the emulsifying properties and

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bactericidal action of lysozyme, gluten peptides and soy protein when it was conjugated with these by Maillard reaction (Babiker, 2002; Song, Babiker, Usui, Saito, & Kato, 2002; Usui et al., 2004). CHT was also conjugated with β-LG by means of a water-soluble carbodiimide in an attempt to lower the antigenicity and immunogenicity of the protein (Hattori et al., 2000). The purpose of this work was to conjugate β-LG with a low molecular weight CHT (prepared in our laboratory), via the Maillard reaction, to improve some β-LG properties.

The purpose of this work was to investigate the occurrence of the Maillard reaction between β-LG and a low molecular weight CHT (prepared in our laboratory) and examine its effects on various properties of the obtained products.

2. Materials and methods

2.1. Materials

β-Lactoglobulin (β-LG) (~80% PAGE) from milk was purchased from Sigma Chemicals (St Louis, MO, USA). Chitosan (CHT), from snow crab, was prepared in the laboratory. The deacetylation degree (87%) was calculated by ^{1}H NMR spectroscopy (Hirai, Odani, & Nakajama, 1991) and UV-spectroscopy (Muzzarelli, Rocchetti, Stanic, & Weckx, 1997). CHT was depolymerized with nitrous acid (Allan & Peyron, 1995) and the resulting average molecular weight was 67,700 Da. All other chemicals used were of analytical reagent grade.

2.2. Preparation of CHT–β-LG conjugates

Freeze-dried CHT–β-LG mixtures (weight ratio 4:1), previously dissolved in 0.1 M acetic acid and adjusted to pH 6, were incubated for 2 weeks at 40 °C and 79% relative humidity in a desiccator containing a saturated KBr solution. CHT incubated under the same conditions was used as a control. Samples were taken at 0, 0.5, 1, 2, 3, 5, 7 and 14 days and kept at −20 °C prior to further analysis. After the incubation, reaction mixtures were dissolved in deionized water at a concentration of 0.25% (w/v) and filtered through 0.45 μm pore-sized cellulose acetate membrane filters (Albet, Barcelona, Spain) for the removal of undissolved aggregate. The reaction products were subsequently freeze-dried. Aqueous solutions of the reaction products (0.1% (w/v)) were prepared, to follow the browning reaction by measuring the absorbance at 420 nm.

SDS-PAGE (Laemmli, 1970) was carried out employing a 15% acrylamide separating gel and a 5% acrylamide stacking gel to confirm conjugate formation. Samples (15 μl, 0.5% (w/v)) were dissolved in Tris–HCl buffer (pH 6.8) containing 1% (w/v) 2-mercaptoethanol. Electrophoresis was conducted for 1 h at a constant voltage of 180 V. Subsequently, the gels were stained with 0.05% (w/v) Coomassie brilliant blue-R250.

2.3. Measurement of the emulsifying properties

The emulsifying properties of the reaction products were determined according to the method of Pierce and Kinsella (1978). 0.1% (w/v) β-LG, CHT or CHT–β-LG MRPs were dissolved in 0.1 M acetate buffer (pH 4). Emulsions, consisting of 2.0 ml sunflower oil and 6.0 ml of the above β-LG, CHT or CHT–β-LG solutions, were shaken and then homogenized in an Ultra Turrax instrument (IKA, Staufen, Germany) at 12,000 rpm for 2 min and at 20 °C. A 50 μl 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 0.1% SDS solution. The absorbance of the diluted emulsion was then determined at 500 nm. The emulsifying activity index (EAI) was calculated by means of the following equation:

$$\mathrm{EAI} = 2T/\Phi C, \quad (1)$$

where T (turbidity) $= 2.3A/L$ (A is the absorbance at 500 nm at time 0 and L is the light path (10^{-2} m), Φ (oil phase volume) $= 0.2$, and C is the concentration of sample (10^3 g/m^3). Values obtained are the means of triplicate samples.

2.4. Antimicrobial activity against Escherichia coli

Strain *E. coli* ATCC 25922 was used as test microorganism. Single colonies of the bacterium, cultivated on Trypticase Soy Agar (TSA) (Scharlau, Barcelona, Spain) plates were inoculated in 50 ml of Trypticase Soy Broth (TSB) (Scharlau, Barcelona, Spain) and grown overnight at 37 °C. One milliliter of the bacterial suspension was diluted 1/50 with TBS and this suspension was then incubated at 37 °C until a concentration of 1–4 × 10^8 colony forming units (CFU/ml) was reached. The obtained culture was diluted with TSB diluted (2%) medium to achieve approximately 10^5 CFU/ml. This suspension was used for inoculation of the assay. Four hundred and fifty microliters of the suspension were mixed with 50 μl of the sample solution (β-LG, CHT, CHT–β-LG mixture and MRPs) with a final concentration of 0.1% (w/v). The mixtures were incubated in sterile 96-well microplates (Sterilin Limited, Hounslow, UK) at 37 °C for 3 h. At the end of the incubation period, serial decimal dilutions were prepared, using 20 mM phospate buffer (pH 7.0), and 20 μl aliquots of these dilutions were spread onto fresh TSA plates. The number of colony-forming units was determined after incubation at 37 °C for 24 h. The assays were conducted in triplicate.

3. Results and discussion

3.1. Formation of MRPs

An increase in absorbance at 420 nm was observed for the CHT–β-LG mixtures from 0.5-day of incubation onwards (data not shown), which indicates that the Maillard reaction was taking place.

SDS-PAGE was performed to look at the formation of the CHT–β-LG MRPs. When the protein and polysaccharide react, forming a covalent bond, the ε-amino residue of the protein and the reducing end of the polysaccharide are expected to attach (Kato, Minaki, & Kobayashi, 1993). Fig. 1 shows the SDS-PAGE patterns of the CHT–β-LG mixture and the reaction products of 0.5, 1, 2 and 7 days. The covalent bonding of the polysaccharide to the protein is suggested by the fact that the β-LG band (lowest visible band) fades with increasing reaction time. In addition, the intensity of the band, just above the boundary of the stacking and separating gel, increases over time, which may indicate the formation of higher molecular weight compounds. This band is also present in the CHT–β-LG reaction mixture (Fig. 1, lane 2) and can, moreover, be seen when only CHT is applied to the gel (results not shown). CHT, due to the presence of the amino group in its molecule, attracts the Coomassie anions under the acidic conditions of the staining step, even though it is not able to react with SDS and migrate along the gel (De St. Groth, Webster, & Datyner,. 1963). Thus, the band just above the boundary of the stacking and separating gels represents both the chitosan and high molecular weight reaction products formed. The polydispersed bands at the top of the separating gel could be products formed in the advanced stages of the Maillard reaction (Tanaka, Huang, Chiu, Ishizaki, & Taguchi, 1993).

3.2. Emulsifying properties

Table 1 presents the EAI of β-LG, CHT and the CHT–β-LG reaction products. The emulsifying properties were enhanced with the progress of the conjugation reaction up to 2 days. However, after this time, the EAI gradually decreased. This loss of emulsifying power is presumably caused by the degradation of the MRPS, which is confirmed by the low molecular weight products found in the electrophoresis gel at longer reaction times. The MRPs of 2 days presented a 20% and 50% increase in emulsifying activity as compared to β-LG and CHT, respectively.

Table 1
Emulsifying activity as EAI of β-LG, CHT, CHT–β-LG mixture and MRPs dry-heated for different times

Sample	EAI
β-LG	74.5 ± 1.95
CHT	44.6 ± 0.52
Mixture CHT–β-LG	77.9 ± 2.34
MRPs 0.5d	85.5 ± 2.87
MRPs 1d	91.2 ± 1.56
MRPs 2d	93.6 ± 1.04
MRPs 3d	88.6 ± 3.39
MRPs 5d	75.2 ± 4.07
MRPs 7d	72.1 ± 1.17
MRPs 14d	69.9 ± 2.10

Changes in the turbidity of the emulsions containing β-LG, CHT, the CHT–β-LG mixture or the 2-day MRPs are shown in Fig. 2. As can be observed, the emulsifying properties of β-LG and CHT are enhanced by Maillard reaction. Other authors have pointed out that, upon conjugation of β-LG with cationic saccharides, the most hydrophobic region might be exposed or the high mobility of the saccharides might improve the flexibility of the protein (Hattori, 2002). Additionally, the increase in saccharide content and net charge resulting from non-enzymatic glycation (Maillard reaction) with a high molecular weight polysaccharide is very effective in improving the emulsifying properties of β-LG under unfavourable conditions (Nagasawa, Ohgata, Takahashi, & Hattori, 1996.) The CHT used in this study has a high net charge, which can greatly facilitate the interaction with the oil phase at the time of emulsification.

3.3. Antimicrobial activity

The antimicrobial effect of β-LG, CHT, CHT–β-LG mixture and the conjugates on *E. coli* ATCC 25922 was investigated. Fig. 3 demonstrates that CHT alone (0.1%

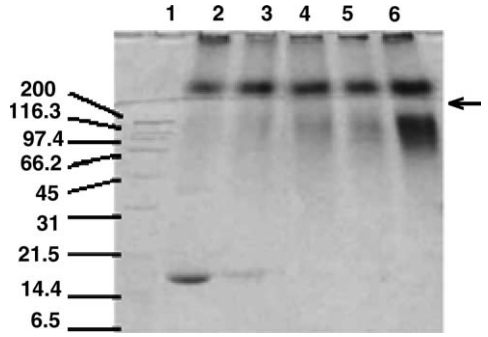

Fig. 1. SDS-PAGE pattern of the CHT–β-LG mixture and MRPs dry-heated for different times. Lane 1: molecular weight standards: myosin, 200 kDa; β-galactosidase, 116 kDa; phosphorylase B, 97 kDa; bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa; trypsin inhibitor, 21.5 kDa; lysozyme, 14.4 kDa; aprotinin, 6.5 kDa; lane 2: CHT–β-LG mixture; lane 3: 0.5-day MRPs; lane 4: 1-day MRPs; lane 5: 2-day MRPs; lane 6: 7-day MRPs. The arrow indicates the boundary between the stacking and separating gel.

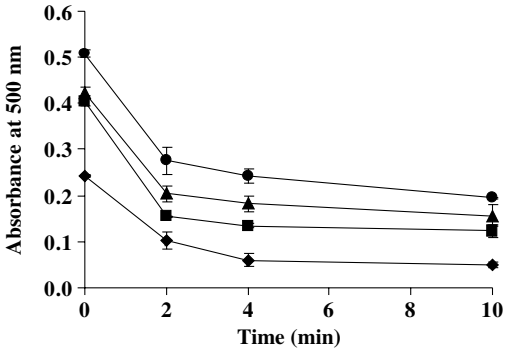

Fig. 2. Changes in the turbidity of emulsions containing CHT (—◆—), β-LG (—■—), CHT–β-LG mixture (—▲—) or the 2-day MRPs (—●—). Values are the means of triplicates ± standard deviations.

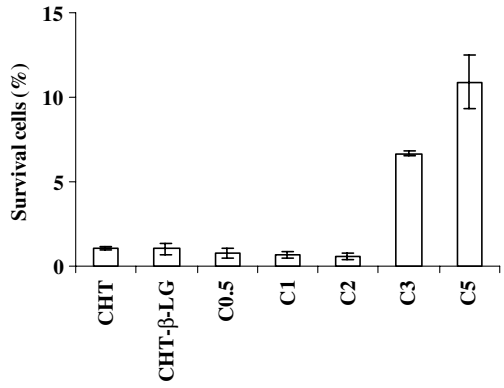

Fig. 3. Antimicrobial activities of CHT, CHT–β-LG mixture and the MRPs corresponding to 0.5 (C0.5), 1 (C1), 2 (C2), 3 (C3), and 5 (C5) days against *E. coli* ATCC 25922. Values are the means of triplicates ± standard deviations.

solution) reduced the percentage of surviving microorganisms greatly (1.08% survival). Although β-LG itself does not affect the viability of *E. coli*, the CHT–β-LG mixture gave an inhibition profile similar to CHT by reducing the *E. coli* viability to 1.03%. This reduction was therefore presumed to be caused mainly by CHT. The 0.5, 1 and 2-day MRPs demonstrated an increased antimicrobial activity against *E. coli*. The 2-day MRPs presented the best antimicrobial activity (0.54% survival), being two times higher than the activity obtained for CHT alone. Similar results were obtained for soy protein-CHT complex (Usui et al., 2004) and gluten peptides–CHT complex (Babiker, 2002). These authors reported that CHT was very effective in improving the bactericidal activity of the original compounds. Although the mechanisms of the enhancement of the bactericidal activity by non-enzymatic glycation are not fully understood, it is believed that this phenomenon contributes to an increase in the net cationized charge of the molecule. This, in turn, improves the growth inhibition of the microorganisms (Babiker, 2002). The polycationic nature of the MRPs might be responsible for binding to the anionic cell surface of the microorganism, resulting in changes in permeability and provoking the loss of the membrane function (Helander, 2001).

From 2 days onwards, antimicrobial activity decreased with reaction time, with the 3- and 5-day MRPs having a highly increased survival rate for *E. coli.* This behaviour is in accordance with the above SDS-PAGE and EAI results. These indicated the degradation of the initial MRPs as a consequence of the advancement of the Maillard reaction, which coincided with the loss of emulsifying capacity.

It can be concluded that the Maillard reaction can be successfully used as a coupling method for β-LG and CHT and thereby improves some of β-LG's functional and biological properties. To the best of our knowledge, this is the first reported attempt. The 2-day CHT–β-LG MRPs demonstrated the best emulsifying capacity and antimicrobial activity against *E. coli*. Dry-heating of a reaction mixture of CHT–β-LG (4:1 weight ratio) for 2 days at 40 °C and 79% relative humidity constituted the most favourable conditions for Maillard reaction. Further investigations are needed in order to elucidate the structure of MRPs responsible for the improvement of β-LG properties here found.

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