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Glycation of caseinmacropeptide

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Abstract

Glycation of caseinmacropeptide (CMP) during storage with lactose at 40 and 50 °C and water activity 0.33–0.65 was studied by measurement of furosine. At pH 8.0 and a_w0.44, maximum levels of furosine up to 1.9 mg/100 mg CMP were obtained within five days at 50 °C, while this value had not been reached at 40 °C after 13 days of storage. Increasing pH up to 11 caused considerable increase in the rate of furosine formation and maximum values were obtained after 9 h at 50 °C. The rate of furosine formation was also enhanced with increasing a_w up to 0.65. These results showed that lactosylated CMP can be efficiently prepared during short time storage of CMP–lactose mixtures under appropriate pH, water activity and temperature conditions.

Keywords: CMP; Lactose; Maillard reaction; Storage

1. Introduction

The Maillard reaction is of primary importance in food processing since is responsible for changes in flavour, colour or nutritive value. The initial step of this complex reaction is the protein glycation that takes place through a condensation of unprotonated amino groups of proteins and the carbonyl groups of reducing sugars, to form a Schiff base which is subsequently rearranged to form the more stable ketoamine derivative commonly referred to as an Amadori product. Furosine (ε-*N*-(2-furoylmethyl-lysine)), generated during acid hydrolysis of the Amadori compound, can be used as an indicator of glycation of lysine residues in proteins and peptides (Olano & Martinez-Castro, 1996).

For many years, the study of the Maillard reaction has been mainly focussed on nutritional and toxicological aspects, development of flavours, tastes and colours and mechanisms of the complex chemistry of the reaction. Recently, protein glycation has received growing attention as a means to improve functional and/or biological properties for food and non-food uses, since the reaction does not require a chemical catalyst and, under well-controlled conditions, it is possible to avoid the formation of advanced Maillard reaction products (Haertle & Chobert, 1999; Chevalier, Chobert, Dalgalarrondo, Choiset, & Haertle, 2002).

k-Caseinmacropeptide (CMP), the C-terminal fragment of k-casein released by chymosin, has lately received much attention as a functional ingredient due to its nutritional and biological properties (Dziuba & Minkiewicz, 1996; Abd El-Salam, El-Shibiny, & Buchheim, 1996). CMP contains four potentially reactive amino groups to be glycated: three lysine residues, situated in the 6, 7 and 11 positions, and the α-amino group of the NH_2-terminal methionine. Formation of lactosylated forms of CMP, through the Maillard reaction in a dry state, has recently been reported (Moreno, López-Fandiño, & Olano, 2002). It was observed that lactosylation improved the emulsifying activity without impairing the great solubility and heat stability of native CMP. Under the assayed conditions, maximum extent

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of lactosylation was attained after 9 days of incubation. The aim of the present work was to study the glycation of CMP with lactose in the dry state under controlled conditions, to establish suitable synthesis procedures of products with controlled degrees of glycation in relatively short periods of time.

2. Materials and methods

2.1. Preparation of whole casein

Ovine milks were provided by a local dairy farm from the central region of Spain. Whole casein was prepared by precipitation from skim milk by adjusting the pH to 4.6 with 1 M HCl, followed by centrifugation at 4500*g* and 5 °C for 15 min. The casein precipitate was washed three times with 1 M sodium acetate acetic acid buffer, pH 4.6, thoroughly dialyzed against water and lyophilized.

2.2. Preparation of casein macropeptide

Commercial rennet powder, containing 85% chymosin (EC 3.4.23.4), and 15% bovine pepsin (EC 3.4.23.1), was obtained from Chr. Hansen's Laboratorium (DK-1250 Copenhagen, Denmark). Rennet solution (1 ml, 4 mg/ml) was added to ovine casein solution (25 g/l) in 0.1 M sodium phosphate buffer, pH 6.5 (100 ml) and the mixture incubated at 35 °C for 20 min. To inactivate chymosin, 0.2 M NaOH was added to pH 9.0–9.5, followed by heating at 60 °C for 15 min (Léonil & Mollé, 1991).The sample was adjusted to pH 4.6 with 1 M HCl and centrifuged at 4500*g* and 5 °C for 15 min. The supernatant was filtered through glass wool, subjected to exhaustive dialysis against water at 4 °C and finally lyophilized.

2.3. Preparation of lactosylated CMPs

Aliquots of a solution consisting of 2.0 mg/ml of CMP and 2.5 mg/ml of lactose (Scharlau Chemie) (lactose/CMP molar ratio = 24) in 0.1 M sodium phosphate buffer, pH 8 (McKenzie & Dawson, 1969), or pH 11 (0.1 M Na_2HPO_4 in 0.05 N NaOH), were lyophilized. These were kept at vacuum in desiccators at 40 and 50 °C at different water activities and then different time intervals. In order to obtain the required water activities (0.33, 0.44 and 0.65), saturated solutions of $MgCl_2$, K_2CO_3, and KI (Labuza & Saltmarch, 1981), respectively, were prepared. In addition, control experiments were performed with CMP stored under the same conditions but without lactose. Storage was performed in duplicate.

2.4. Furosine determination

Lactosylation of CMP was measured through furosine determination by an ion-pair HPLC-RP method (Resmini, Pellegrino, & Batelli, 1990) after acid hydrolysis of the stored powders. Briefly, 400 μl of 8 N HCl was added to 2 mg of CMP in hydrolysis tubes and heated at 110 °C for 23 h under inert conditions; after that, 2 ml of 8 N HCl were added and the hydrolyzate was filtered through Whatman no. 40 filter paper. For determination of furosine, 500 μl of the filtered hydrolyzate were applied to a previously activated (with 5 ml of methanol and 10 ml of water) Sep-Pak C18 cartridge (Millipore). Furosine was eluted with 3 ml of 3 N HCl and 50 μl were injected. HPLC analysis of furosine was performed in a C8 (Alltech furosine-dedicated) column (250 mm × 4.6 mm i.d.) with a variable wavelength detector at 280 nm (LDC Analytical, SM 4000). Operating conditions were as follows: column temperature 35 °C; flow rate, 1.2 ml/min; solvent A, 0.4% HPLC grade acetic acid (Scharlau Chemie) in double-distilled water; solvent B, 0.3% KCl (Merck) in solvent A.

Calibration was performed by the external standard method, using solutions of known concentrations (from 0.52 to 5.2 mg/l) of commercial pure standard of furosine (Neosystem Laboratories). The corresponding calibration curve was $y = 0.037x + 0.1104$ ($R^2 = 0.993$)

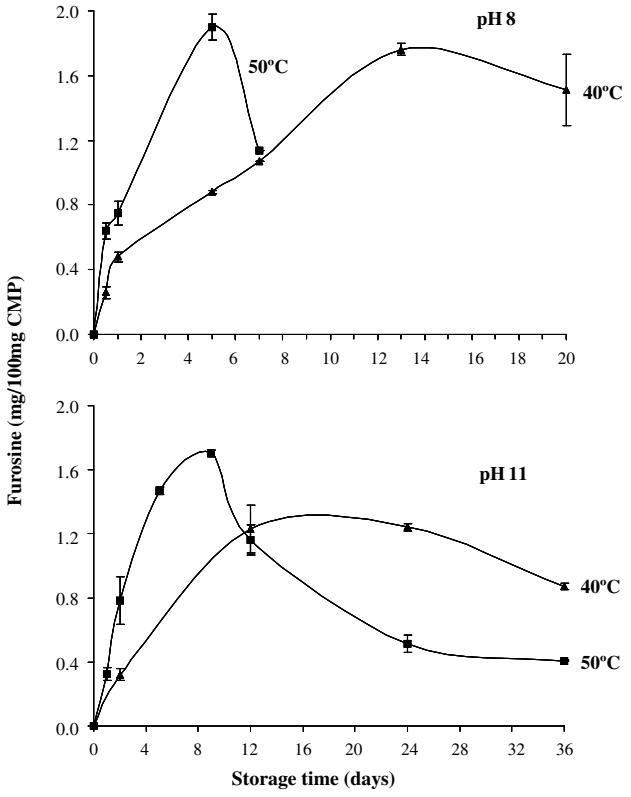

Fig. 1. Evolution of the furosine content in the CMP samples stored with lactose at 40 °C (▲) and 50 °C (■), a_w 0.44 and pH 8 and 11. Bars represent SD (storage was performed in duplicate).

being y, peak area and x, μg injected. Accuracy of the chromatographic method was checked by spiking known concentrations of furosine in a hydrolysed CMP sample, in which the furosine content had been previously determined, and the percent of recovery was 87%.

3. Results

Storage of CMP with lactose under low water activity conditions caused nonenzymic glycosylation. It proceeded through a Schiff base intermediate, followed by Amadori rearrangement to yield a more stable Amadori product. Hydrolysis of glycated proteins results in transformation of about 30% of the Amadori compound, lactulosyllysine, to furosine (Friedman, 1996), thus the progress of the glycation of CMP can be evaluated by measurement of furosine.

Fig. 1 shows the evolution of furosine in CMP incubated at pH 8 and pH 11 with lactose at 40 and 50 °C. As expected, furosine formation increased with temperature. Optimum furosine formation, 1.9 mg/100 mg CMP, was achieved within 5 days of storage while this value had not been reached at 40 °C after 13 days. Increasing pH up to 11 caused considerable increase in the rate of furosine formation and maximum values were obtained after nine hours of storage at 50 °C. It is generally accepted that glycation by the Maillard reaction involves condensation of unprotonated amino groups with the reducing carbohydrate; thus, the observed increase of furosine formation may be due to the increase of unprotonated amino groups with increasing pH.

The results of the study on the formation of furosine at 50 °C and pH 11 at a_w 0.33, 0.44 and 0.65 are shown in Fig. 2. The rate of formation increases with a_w. The maximal amount of 1.6 mg/100 mg was achieved after 2 h of incubation at a_w 0.65. The enhanced reaction rate may be explained in terms of more rapid diffusion of reactants in the mixture at very low water activity, the system becomes so concentrated that diffusion of

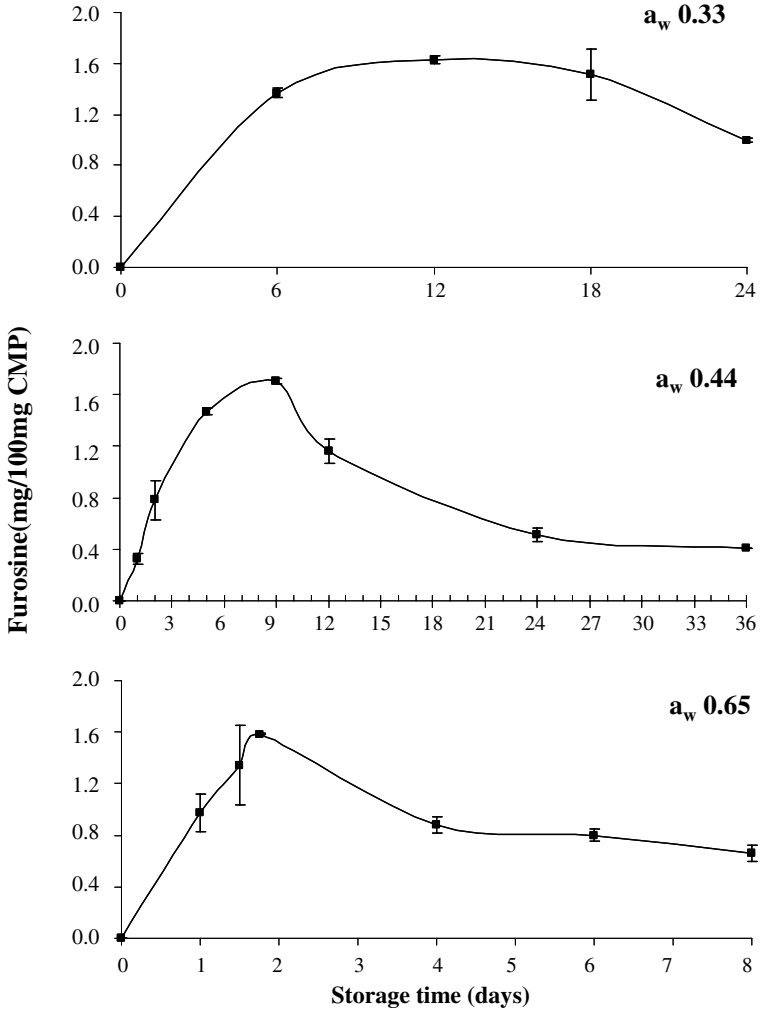

Fig. 2. Effect of a_w on the evolution of the furosine content in the CMP samples stored with lactose at 50 °C and pH 11. Bars represent SD (storage was performed in duplicate).

reactants becomes difficult. Previous studies on molecular mechanisms, involving the role of water in governing the reaction rate in low-to intermediate-moisture foods, showed that solubilization of reactants may contribute to the observed increase of the reaction rate (Sherwin & Labuza, 2003). These results showed that lactosylated CMP can be efficiently prepared during short time storage of freeze-dried powders of CMP–lactose mixtures under appropriate pH water activity and temperature conditions.

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