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International Dairy Journal 9 (1999) 255–260

INTERNATIONAL DAIRY JOURNAL

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Heat-induced aggregation and covalent linkages in β-casein model systems

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Abstract

Conditions and mechanisms leading to covalent aggregation of β-casein (βCN) were studied in protein solutions heat treated in the absence or presence of glucose. Under all the tested heating conditions, covalent aggregation of βCN occurred only in the presence of glucose. Only high-MW aggregates (≫100 kDa) were detectable by polyacrylamide-gel electrophoresis in the presence of SDS, whereas gel permeation chromatography in the presence of urea showed that aggregates with lower MW were formed as well. A characteristic unordered structure was observed using transmission electron microscopy for the covalent aggregates of βCN obtained in the presence of glucose, in contrast to the mostly spherical ones due to hydrophobic interactions only. In addition, lysinoalanine (LAL), lysylpyrraline (LPA) and pentosidine (PTD), as possible crosslinking molecules in proteins, were quantified by HPLC. The highest amounts of LAL (∼150 mmol/mol βCN) were found in the unglycosylated βCN, suggesting that this molecule is mainly involved in intra-molecular reactions. Very small amounts of LPA (<2 μmol/mol βCN) were found in extensively aggregated βCN. The behaviour of PTD formation followed that of βCN aggregation but the low values found (few mmol/mol of protein) suggest that it can only play a minor role. Although the covalent aggregation of βCN is advanced Maillard reaction dependent, molecules other than those considered here are responsible for intermolecular crosslinking but their nature is presently unknown.

Keywords: β-Casein aggregation; Protein crosslinking; Maillard reaction

1. Introduction

Covalent crosslinking has an impact on structure and functional properties of proteins. Polymerization of proteins in food is a recognized effect of both processing and storage leading to increased resistance to proteolytic enzymes. Direct covalent protein–protein interactions are mainly due to rearrangement of disulphide bridges following protein unfolding as discussed by Dalgleish and Hunt (1995). However, formation of xenobiotic crosslinks arising from β-elimination and condensation reactions can occur as well. When reducing sugars are present, proteins can also interact via advanced Maillard reaction (MR) products. One or more of these mechanisms can take place in foods depending on characteristics of the proteins, environment and processing conditions as reviewed by Friedman (1996). The progressive covalent polymerization of casein occurring on heating reconstituted skim milk (Singh & Latham, 1993) and in UHT milk (Henle, Schwarzenbolz & Klostermeyer, 1996) was reported to be due to molecules deriving both from β-elimination reactions, like lysinoalanine (LAL) and histidinoalanine, and from MR. However, it was not possible to distinguish between crosslinks formed at intramolecular and intermolecular level.

Taking into account the possible interferences, the mechanisms involved in covalent polymerization of proteins are better investigated in simple model systems than in complex mixtures such as milk. Bovine casein fractions are well characterized food proteins, are non-globular and have little regular secondary or tertiary structure to disrupt (Walstra & Jenness, 1984). In particular, β-casein (βCN) in solution is close to a random coil and shows no transition between 30 and 100°C in differential scanning calorimetry (Paulsson & Dejmek, 1990). Moreover, it lacks cysteine and intermolecular bonds due to disulphide bonds are not possible. Provided that the strong self-aggregation properties (Buchheim & Schmidt, 1979)

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0958-6946/99/$ - see front matter
PII: S0958-6946(99)00071-0

are carefully considered, βCN represents a useful model-protein for a study of heat-induced covalent aggregation, as carried out in the present work.

2. Materials and methods

Chromatographically pure βCN was prepared from raw bulk milk according to the method of Hipp and co-workers described by Lorenzen and Reimerdes (1992), including purification on DEAE cellulose. Pure pentosidine (PTD) was provided by Dr V. Monnier (Case Western Reserve University, Cleveland, Ohio).

Model solutions at the specified concentrations of βCN and glucose (GLU) in 0.1 M sodium phosphate buffer at pH 7.0 were aseptically filtered using a 0.2 μm membrane and heated in screw-cap vials under the specified conditions. Both before and after heating, samples were incubated at 2°C for 12 h. SDS-PAGE was performed using a phast-system (Pharmacia) on 8–25% gradient gels. Gel filtration chromatography (GPC) of the model solutions, previously diluted with urea up to 5 M and kept at 4°C overnight, was performed at 4°C on a Superose 6 HR10/30 column (Pharmacia) with 0.05 M sodium phosphate and 0.1 M sodium chloride solution as eluant, monitoring at 280 nm. The model solutions were freeze-fractured and observed by transmission electron microscopy (TEM) as reported by Schmidt, Walstra and Buchheim (1973). Preparative ultracentrifugation (UC) was performed using a L12-65B ultracentrifuge and a SW50L swinging-bucket rotor (Beckman) at 200 000 g for 24 h at 1°C in the presence of 1% SDS. The amount of sedimented protein was calculated by difference between concentrations of total protein and protein in the supernatant, both evaluated by micro-Kjeldahl.

Previously reported methods were adopted for measuring furosine (Resmini, Pellegrino & Battelli, 1990) converted to fructoselysine (FL) by the factor 3.12 (mol to mol), lysyl pyrraline (LPA) (Resmini & Pellegrino, 1994) and LAL (Pellegrino, Resmini, De Noni, & Masotti, 1996). PTD was determined after acid hydrolysis (Monnier & Sell, 1994) by RP-HPLC as described for LPA analysis with an additional step with solvent C (Methanol : solvent B = 25 : 75, v/v) in the following linear elution gradient: 0–5 min, 100% A; 5–10 min, 100% B; 10–15 min, 100% B; 15–35 min, 100% C; 35–36 min, 100% A; 36–40 min, 100% A. Fluorescence detection: 328 nm excitation, 378 nm emission, retention time: 32 min. The model solutions were directly submitted to acid hydrolysis for FL, LAL and PTD analysis. A previous precipitation of the protein in presence of 12% TCA (v/v), followed by washing with water, was introduced for LPA analysis before enzymatic proteolysis.

3. Results and discussion

The heat-induced covalent polymerization of βCN was first studied either in the absence or presence of GLU at high concentration of protein (30 mg ml^{-1}) and under severe heating conditions (110°C for 6 or 12 h) in order to induce formation of polymers.

A heating-time dependent decrease of the intensity of the band corresponding to native βCN was found by SDS-PAGE in the solutions heated in the presence of GLU, while large aggregates progressively accumulated at the top of the lane (Fig. 1). As hydrophobic bonds in the heated samples were disrupted by dissociating agents and cooling, covalent bonds must be responsible for crosslinking. No aggregates were formed in the absence of sugar. A similar behaviour was found by GPC (Fig. 2). Noticeable reduction of the peak height of monomeric protein was observed in both heated solutions, probably owing to conformational changes of protein itself, but

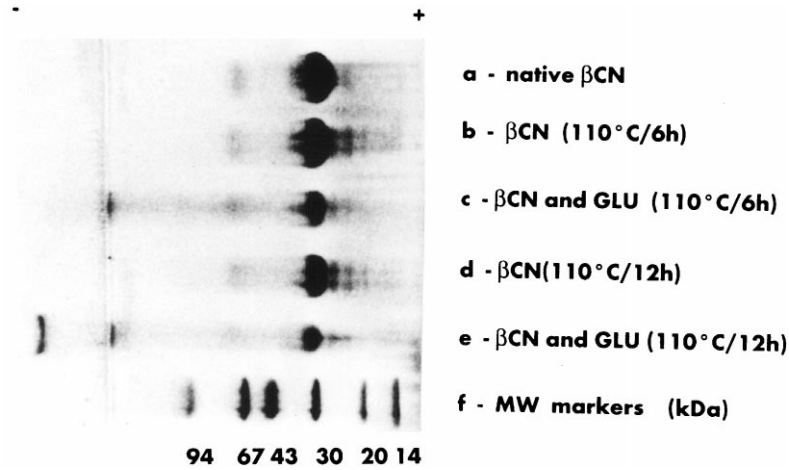

Fig. 1. SDS-PAGE of model systems containing 1.25 μmol β-casein (βCN)/ml unheated (a) or heated in the absence (b, d) or presence (c, e) of glucose (150 μmol ml^{-1}).

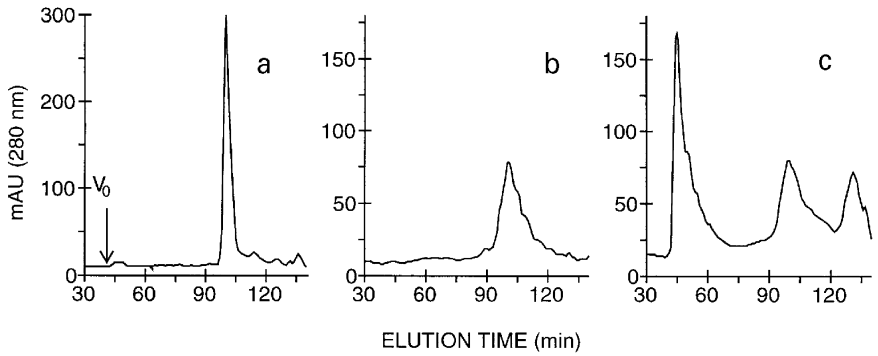

Fig. 2. β-casein (βCN) aggregation evaluated by GPC in model systems containing 1.25 μmol βCN/ml and unheated (a) or heated at 110°C for 12 h in the absence (b) or presence (c) of glucose (150 μmol ml^{-1}).

aggregates, mainly eluting at the void volume (MW > 5×10^6 Da), were observed in the presence of GLU only.

A solution of βCN under TEM was reported to show spherical particles with diameter of about 10 nm for monomers and about 34 nm for polymers with a maximum degree of polymerization of 38 (Buchheim & Schmidt, 1976). Although at 2°C βCN exists as species not larger than trimers (Takase, Niki & Arima, 1980), we observed significant self-association upon prolonged cooling. Particles with diameters ranging from 10 to 30 nm were present in the unheated solution of βCN (Fig. 3a) and no relevant changes in size distribution were observed after heating in the absence of GLU (Fig. 3b). In both cases, the roughly spherical shape of the particles suggests an ordered structure comparable to that of soap micelles due to hydrophobic associations of monomers (Schmidt & Payens, 1972). Some particles had an irregular shape (Fig. 3b), probably caused by heat-induced modification. GLU-reacted βCN was mainly present as large and unordered compact (size up to 60 nm) or chain-like (up to 100 nm length) aggregates (possibly of a fractal nature). Sizes of the largest clusters indicate a MW of several millions of Da and confirm the results found with SDS-PAGE and GPC.

As shown above, the presence of sugar is needed to promote detectable heat-induced covalent aggregation of βCN and this is not in agreement with a possible role for LAL as an intermolecular crosslink. The amount of LAL in heated βCN increased with holding time at each temperature tested, but was always 2–3 fold lower in the presence of GLU (Fig. 4, top). The sugar competes with dehydroalanine for Lys residues and this has been reported to depress LAL formation in milk proteins (Pellegrino et al., 1996). The GPC patterns (Fig. 4, bottom) indicate that aggregation was independent of formation of LAL, the highest amounts of which were always found in aggregate-free systems. These results indicate that LAL is probably formed at an intramolecular level only. Because of the sequence of the N-terminal region of βCN which contains all SerP and 4 Lys residues, the highly reactive dehydroalanine residues (Otterburn, 1989) can readily combine with nearby Lys residues on the same βCN molecule rather than with those on other molecules.

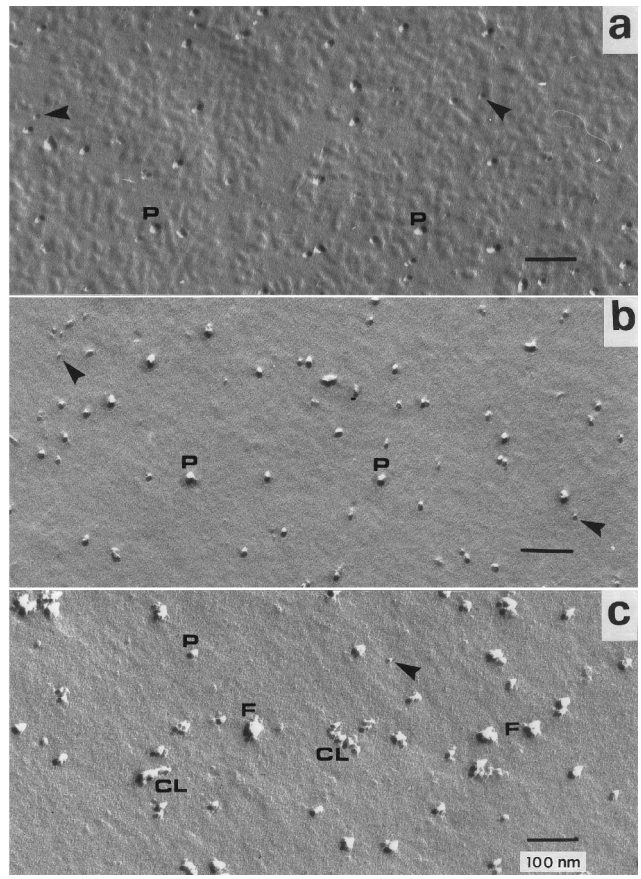

Fig. 3. Freeze-fracture electron micrographs of model systems containing 1.25 μmol β-casein/ml unheated (a) or heated at 110°C for 12 h in the absence (b) or presence (c) of glucose (150 μmol ml^{-1}). Monomers (arrow), self-associated polymers (P), compact (F) and chain-like (CL) clusters are observed. Bars: 100 nm.

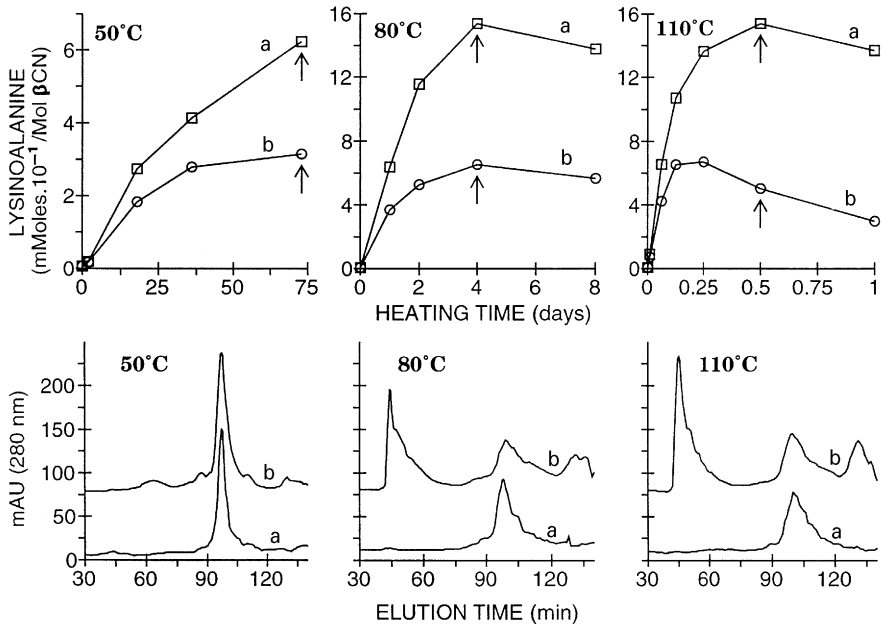

Fig. 4. Formation of lysinoalanine (top) in model systems containing β-casein (1.25 μmol ml^{-1}) and heated in the absence (a) or presence (b) of glucose (150 μmol ml^{-1}). Arrows, model systems submitted to GPC (bottom).

Because of the amphiphilic nature of the molecule, βCN forms micelles with a dense hydrophobic core surrounded by a hydrophilic shell of much lower density (Leclerc & Calmettes, 1997). As previously reported by Dickinson, Horne, Phipps and Richardson (1993) for βCN layers at interfaces, this structure suggests that it is mainly the protruding N-terminal region which is reactive. Accordingly, micellization can interfere with covalent aggregation of monomers and was minimized by using highly diluted solutions (1 mg βCN ml^{-1}), but could not be excluded during heating. The relationship between behaviour of the MR and progress of aggregation was studied at different concentrations of GLU, maintaining the heating conditions constant. The amount of FL was taken as an indicator of the extent of glycation. Formation of PTD, a recognized Lys–Arg crosslink (Monnier & Sell, 1994) and LPA, a putative Lys–Lys crosslink (Ledl & Schleicher, 1990), both derived from advanced MR, was followed and compared to that of LAL (Fig. 5, top). As expected, the increase in GLU/βCN molar ratio increased the amount of Maillard-reacted protein and decreased the formation of LAL. βCN aggregation as measured by GPC (Fig. 5, bottom) was detectable in the presence of very low levels of FL and PTD and proceeded with the accumulation of these two molecules but not with that of LPA. Thus, βCN aggregation seemed to occur even at low extent of glycation and did not need intensive degradation of early MR products.

In a second experiment, the progress of aggregation was studied under milder reaction conditions and evaluated by both GPC and preparative UC. No protein sedimented during UC of model solutions heated in absence of GLU (not shown); hence, the relative amount of protein in the pellet was used to quantify the extent of aggregation. The initial stages of aggregation were detected by GPC even under the mildest conditions tested and a heating-time dependent increase in size and quantity of polymers was observed (Fig. 6, left). In contrast, the quantity of sedimented protein levelled off (Fig. 6, right). When heating time was increased from 64 to 128 h, the peak height of the largest aggregates increased by a factor 2, but the relative amount of sedimented protein only increased from 45 to 50%. GPC patterns describe the progress of aggregation giving a rough estimate of polymerization. Nevertheless, quantification of polymerized protein is unreliable because UV absorbance is affected by progressive rearrangement of the sugar-moieties as well as by conformational changes of the chain (Shaw & Crabbe, 1994), both processes being promoted by severe heating. βCN aggregation was better quantified by UC and levelled off at about 55% of total protein. Significant amounts of unaggregated βCN were detected in all cases by SDS-PAGE, GPC and TEM. The accumulation of PTD in total protein paralleled the aggregation behaviour (Fig. 6, right) and the concentration was about a factor of 2 lower than in the pellet (not shown). PTD was preferentially formed at intermolecular level, but the low level found could not explain the extensive aggregation and other crosslinks must therefore be considered.

Heat-induced covalent aggregation of βCN seems to coincide with that of PTD formation, which involves

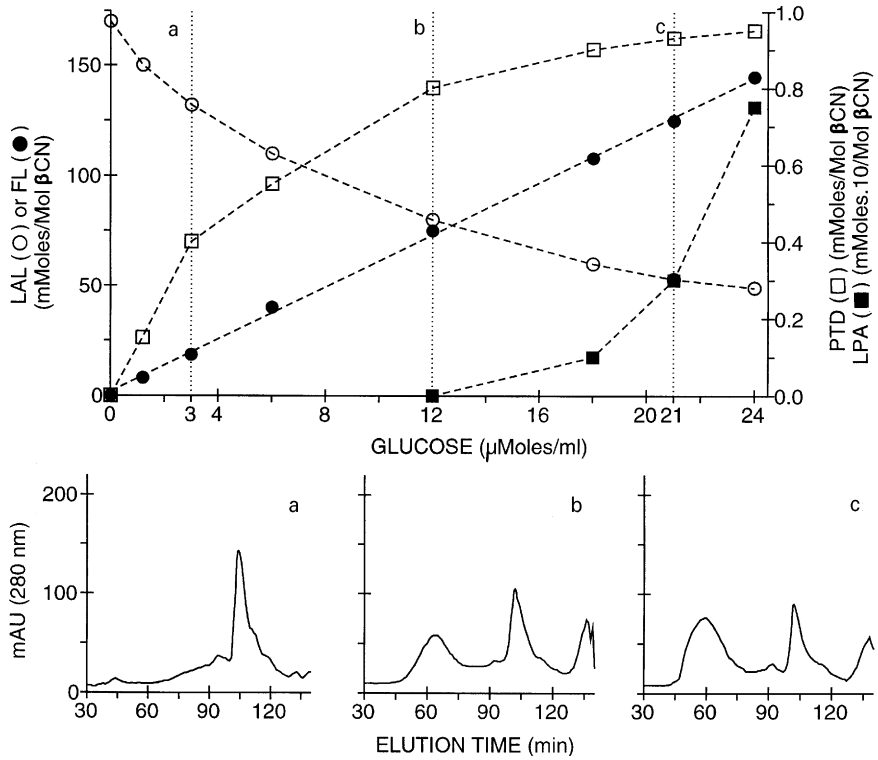

Fig. 5. Formation of lysinoalanine (LAL), fructoselysine (FL), pentosidine (PTD) and lysyl pyrraline (LPA) in model systems containing β-casein (47 nmol ml^{-1}) and heated at 70°C for 48 h in the presence of various amounts of glucose (top). a, b and c, model systems submitted to GPC (bottom).

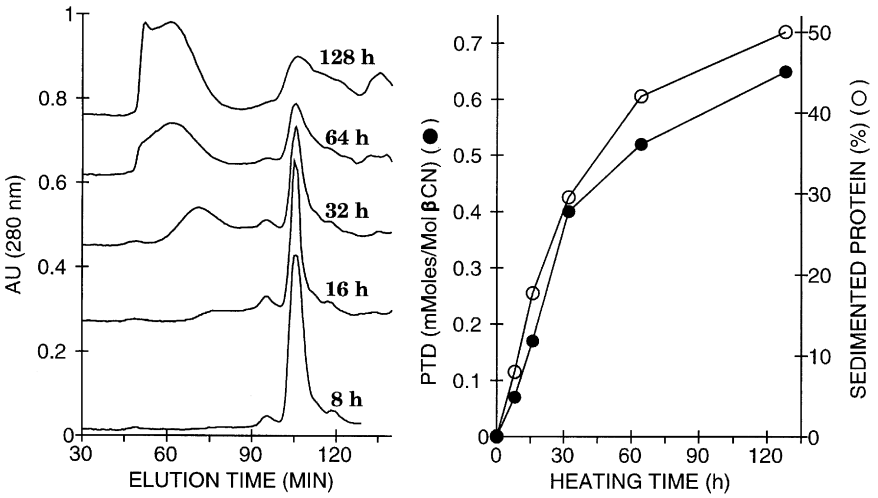

Fig. 6. Aggregation behaviour of β-casein (βCN) and formation of pentosidine (PTD) in model systems containing βCN (47 nmol ml^{-1}) and glucose (7.5 μmol ml^{-1}) heated at 70°C for different times. (left) GPC patterns and (right) accumulation of PTD in the bulk protein and amount of protein (% on bulk protein) sedimented by ultracentrifugation.

mutual interactions of Arg and Lys residues via sugar. However, a comparable mechanism causing aggregation remains to be established. Due to effects of protein conformation and micelle structure, the residues located in the extended hydrophilic N-terminal regions are the most susceptible sites for intermolecular cross linking and can be readily involved, even at low levels of MR. Under severe heating, these residues are irreversibly blocked by extensive glycation and further rearrangement of sugar-moieties, thus possibly explaining the levelling-off of βCN aggregation.

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