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Copper modulates the heat-induced sulfhydryl/disulfide interchange reactions of β-Lactoglobulin

Gulzar Muhammad, Croguennec Thomas *, Jardin Julien, Piot Michel, Bouhallab Saïd

Inra-Agrocampus Ouest, Food Science and Technology, UMR 1253, Université de Bretagne, F35000 Rennes, France

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ABSTRACT

This study describes the effect of copper on the heat-denaturation/aggregation of β-Lactoglobulin AB at neutral pH. The kinetics of disappearance of native β-Lactoglobulin under different ionic strength and Cu^{2+}/β-Lactoglobulin molar ratio conditions were followed and the type of interactions (covalent or non-covalent) shared between non-native structures during the heating process were examined. On heating, the rate of disappearance of native β-Lactoglobulin was accelerated by increasing the Cu^{2+}/β-Lactoglobulin molar ratio. Copper induces oxidation of the free sulfhydryl group of β-Lactoglobulin resulting mainly in the formation of covalent dimers, which were further associated into large non-covalent aggregates under high ionic strength conditions. Characterisation of the β-Lactoglobulin dimers reveals the existence of three different molecular species arising randomly (dimers A–A, A–B and B–B), in which tertiary structure was completely lost. The quantity of added copper constitutes a powerful way to control the heat-denaturation/aggregation process of β-Lactoglobulin in particular regarding the relative proportion of covalent and non-covalent interactions into formed aggregates.

1. Introduction

Whey proteins are widely used as ingredients in food applications. The control of the denaturation/aggregation of whey proteins during processing such as heating is of outstanding importance for the acceptance of the final quality of the products. For instances, in protein-rich beverages, added whey proteins have to remain soluble after heating in order to limit deposition during storage (Bhattacharyya & Das, 1999; O'Kennedy & Mounsey, 2006), whilst large aggregates of whey proteins more or less interconnected to each others are required for texturising agent (Bryant & McClements, 1998). Although the mechanism of heat-denaturation/aggregation of β-Lactoglobulin (β-Lg), the main whey protein in bovine milk, has been extensively studied and reviewed (Considine, Patel, Singh, & Creamer, 2007; De la Fuente, Singh, & Hemar, 2002; de Wit, 2008; Hoffmann & van Mil, 1997; Qi et al., 1997; Roefs & de Kruif, 1994), it is still not completely understood and controlled. Accurate control of the heat-induced denaturation/aggregation process is difficult because the kinetics as well as the nature of involved chemical reactions depend on various physico-chemical conditions (Baussay, Le Bon, Nicolai, Durand, & Busnel, 2004; Totosaus, Montejano, Salazar, & Guerrero, 2002; Weijers et al., 2009).

Native β-Lg is composed of nine β-strands organised in two antiparallel β-sheets facing each other and forming a central cavity, site for the fixation of small hydrophobic molecules (Brownlow et al., 1997; Kontopidis, Holt, & Sawyer, 2004). Its structure is stabilised by two disulfide bonds (Cys66–Cys160 and Cys106–Cys119), which seem to play an important role in the reversibility of β-Lg denaturation (Kitabatake, Wada, & Fujita, 2001). In addition, β-Lg has one free sulfhydryl group (Cys121) buried within the interior of the native protein. Under physiological conditions, the sulfhydryl group is inaccessible to the solvent and chemically unreactive. However, structural changes consecutive to β-Lg denaturation expose the free sulfhydryl group on the protein surface and increase its reactivity (D'Alfonso, Collini, & Baldini, 2002; Iametti, Degregori, Vecchio, & Bonomi, 1996; Monahan, German, & Kinsella, 1995). Then, it is available for sulfhydryl/disulfide interchange reactions and sulfhydryl oxidation reactions leading to β-Lg aggregation (Roefs & de Kruif, 1994; Schokker, Singh, Pinder, Norris, & Creamer, 1999). The balance of these two chemical reactions and the contribution of non-covalent interactions in the aggregates formed on heating is affected by the mineral contents of the medium.

The effect of cations, such as sodium and calcium, on the denaturation/aggregation of proteins has been extensively studied (Caussin, Famelart, Maubois, & Bouhallab, 2003; Croguennec, O'Kennedy, & Mehra, 2004; Hoffmann, Roefs, Verheul, van Mil, & de Kruif, 1996; Simons, Kosters, Visschers, & de Jongh, 2002); In contrast, the influence of trace elements such as copper, zinc or iron is less documented. Metal ions promote β-Lg aggregation

* Corresponding author. Tel.: +33 (0) 2 23 48 59 27; fax: +33 (0) 2 23 48 55 78.
E-mail address: Thomas.croguennec@agrocampus-ouest.fr (T. Croguennec).

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but they have different actions on the general mechanism of denaturation/aggregation (Navarra, Leone, & Militello, 2007; Stirpe et al., 2008). Copper has strong affinity toward sulfhydryl groups in a deprotonated state and promotes disulfide bond formation through oxidation reactions (Bouhallab et al., 2004; Floris, Bondar, Weinbreck, & Alting, 2008; Mateo Marti, Methivier, & Pradier, 2004). Hence, by a rapid oxidation of sulfhydryl groups previously exposed on β-Lg surface, copper is expected to shift the balance between sulfhydryl/disulfide exchange reactions and sulfhydryl oxidation reactions towards the latter. In this study, we investigated the influence of copper on the heat-denaturation/aggregation of β-Lg under different physicochemical conditions (ionic strength and copper concentration) with a special attention given to the nature of the interactions involved in formed aggregates.

2. Material and methods

2.1. Materials

β-Lg AB was prepared from cow milk according to Maubois, Fauquant, Famelart, and Caussin (2001). The powder contains 89 ± 1% proteins (including 97% of β-Lg and less than 1% of both α-lactalbumin and immunoglobulin), 7.5% moisture, 2.3% minerals and 0.04% lactose. β-Lg variants A (β-Lg A) and B (β-Lg B) were separated in the laboratory by anion-exchange chromatography by a Q-Sepharose Fast Flow Exchanger (Amersham, Orsay, France) according to the method described by Croguennec et al. (2004). The final purity for variant A and B were 92% and 97%, respectively. Sodium acetate was from Panreac (Barcelona, Spain), acetic acid from Fischer Scientific (UK) and Glycine from Acros Organics (Geel, Belgium). All other chemicals were from Sigma Aldrich (Saint-Quentin-Fallavier, France).

2.2. Sample preparation

Solutions of β-lg A, B and AB, were prepared by dissolving freeze dried powders in 5 mM Bis–Tris buffer pH 6.7 (in the absence or presence of NaCl up to 100 mM). The protein dispersions were gently stirred for 30 min and then filtered through 0.22 μm non-protein adsorbing filter (sartorius AG, Goettingen, Germany). The protein concentration was determined from the absorbance at 278 nm using the specific extinction coefficient 0.96 L g^{-1} m^{-1}. In all β-Lg samples, the final protein concentration was adjusted to 5 g L^{-1} with the buffer used to dissolved the β-Lg powder. To study the effect of copper on heat-denaturation/aggregation of β-Lg, a stock solution of $CuCl_2$ (27 mM) was used to adjust the final concentration of copper in protein solutions in order to reach the desired Cu^{2+}/β-Lg molar ratio (0.1, 0.3, 0.6, 1 or 2). All samples were duplicate and all analysis were performed at least twice.

2.3. Heat treatment

A series of protein solutions (1 ml) in tightly capped plastic tubes were heated in a water bath adjusted at 78 °C. The tubes were then removed after various time periods (0, 5, 15 and 30 min) and put into ice for 2 min in order to rapidly stop the heat-induced reactions. Samples were stored at 4 °C overnight before subsequent analysis.

2.4. Determination of soluble proteins at pH 4.7

A volume of 50 μL of acetic acid/sodium acetate buffer 0.5 M, pH 4.7 was added to 500 μL of protein samples pre-equilibrated at room temperature. Then, the mixture was placed in a water bath equilibrated at 30 °C for one minute in order to standardise the conditions for the precipitation of denatured/aggregated proteins. The samples were then centrifuged at 10000*g* for 20 min by an eppendorf 5415C Micro Centrifuge (Scientific Support, Hayward, California) to remove the denatured/aggregated protein. Optical Density of the supernatant was read at 278 nm using a Spectrophotometer UV/Visible JENWAY Seri 6505 (Voilab, France) to measure the amount of residual soluble protein at pH 4.7 (considered as native proteins) by using specific extinction coefficient 0.96 L g^{-1} m^{-1}.

2.5. Characterisation of intermolecular interactions in protein samples

2.5.1. Gel permeation chromatography

Samples (50 μL of protein samples diluted 10 times in the eluting buffer) were analysed by high pressure-gel permeation chromatography (HP-GPC) using a TSK G3000 SWXL (300 × 7.8 mm i.d.) column (Phenomenex, France) connected to a Waters chromatography system, consisting of a Waters 2695 Separation Module, a Waters 2487 Dual λ Absorbance Detector and a Empower chromatography application software to acquire, process and report chromatographic information. A phosphate buffer 0.05 M, pH 6.8 containing 0.1 M NaCl was used to equilibrate the column and to elute the proteins at a flow rate of 0.8 mL min^{-1}. Proteins were monitored at 214 nm.

2.5.2. SDS–PAGE

SDS–PAGE was performed by using a Mini Protean II system (Bio-Rad Laboratories, Alpha Technologies, Dublin, Ireland) as described by Laemmli (Laemmli, 1970), using 12% acrylamide separating gel and 4% gel of concentration under reducing (with β-mercaptoethanol) and non-reducing conditions (without β-mercaptoethanol). Protein samples (5 g L^{-1}) were diluted 5 fold with the denaturing buffer (77,975% 0.08 M Tris–HCL pH 6.8; 20% glycerol; 2% SDS; 0.025% bromophenol blue). Ten μg of proteins were loaded in the sample slots and the separation was performed at 150 V for 90 min. Gels were stained with Coomassie Brillant Blue G250. A low molecular weight marker kit (14.4–94 kg mol^{-1}, Amersham Biosciences, France) was used for calibration.

2.6. Characterisation of the copper-induced covalent dimer

2.6.1. Mass spectrometry

Mass spectra were obtained on a hybrid quadrupole time of flight (Q/TOF) mass spectrometer QStar XL, fitted with a Nanospray TM source (Applied Biosystems/MDS Sciex Toronto, Canada). The instrument was calibrated using a multi-point calibration with fragment ions resulting from the collision-induced decomposition of peptide β-CN (193-209). Protein samples, diluted in 50% acetonitrile containing 0.1% formic acid, were infused with the help of a nano ES needle (Proxeon Biosystems, Staermosegaardsvej Odense, Denmark) with an optimised ion spray voltage 1.5 ± 0.1 kV Volts. Data were collected and processed using the Analyst 1.1 Sciex software.

2.6.2. Sulfhydryl quantification

The quantification of sulfhydryl groups was done by the method of Ellman (1959). A volume of 100 μL of protein samples (5 g L^{-1}) were diluted with 900 μL of Tris–glycine buffer (0.05 M, pH 8) containing SDS (0.5%) and then 25 μl of 2,2′-dinitro-5,5′-dithiodibenzoate (DTNB, Merck, Darmstadt, Germany) was added. Accessible sulfhydryl (SH) groups of the proteins reacted with DTNB and released thionitrobenzoate, which was quantified at 412 nm after 180 min of reaction.

2.6.3. Circular dichroism

β-Lg samples were placed in a 2 mm thick quartz vat and their circular dichroism spectra were acquired in a dichrograph Jasco

810 (Jasco, France) at a temperature of 25 °C. The near-UV spectra were recorded between 260 and 320 nm using a β-Lg concentration of 5 g L^{-1}, whilst far-UV spectra were recorded between 190 and 260 nm at a β-Lg concentration of 0.5 g L^{-1}. The speed of scanning was 50 nm min^{-1} and each spectrum was an average of two readings. The molar ellipticity ($[\theta_\lambda]$) was expressed by moles of amino acid from the observed ellipticity (θ_λ) using the following equation:

$$[\theta_\lambda] = \mathrm{MRW} \times \theta_\lambda / 10 \times d \times C$$

With d, the path length (cm); C, the concentration (g mL^{-1}) and MRW, the mean residue weight calculated from MRW = $M_W/(N-1)$, where M_W is the molecular mass of the protein and N the number of amino acids in the polypeptide chain.

3. Results

3.1. Effect of copper on the heat denaturation/aggregation of β-Lg AB

The heat-denaturation/aggregation of β-Lg AB (5 g L^{-1} in Bis–Tris buffer) was determined in the absence and presence of copper at Cu^{2+} to β-Lg molar ratio of 1. Protein samples were heated at 78 °C up to 30 min and were analysed simultaneously by HP-GPC and SDS–PAGE under non-reducing conditions. HP-GPC preserves covalent and non-covalent associations between proteins in the aggregates whilst SDS–PAGE conducted under non-reducing conditions preserves only covalent associations. In non-heated samples, a major chromatographic peak corresponding to the native β-Lg AB (equilibrium between monomer and dimer at this pH and protein concentration conditions) was eluted at a retention time of 13 min (Fig. 1). In addition, a peak eluted at 12 min, representing around 10–15% of total protein content was also observed in non-heated samples. The presence of such material was already reported (Bouhallab et al., 2004; Croguennec et al., 2004) and was attributed to β-Lg covalent dimers occurring naturally during the preparation and storage of protein powders. Heating induced the disappearance of native β-Lg AB and concomitantly the formation of new non-native structures mainly eluted at retention time of 12 min, corresponding to dimers of β-lg. In the absence of copper the disappearance of native β-Lg AB was gentle and account for about 40% of initial native β-Lg after 30 min of heating (insert on Fig. 1B). The addition of copper induced a faster decrease in the native β-Lg AB (insert on Fig. 1A) and concomitant increase in the intensity of dimers of β-Lg AB (Fig. 1). After 15 min of heating at 78 °C, almost 80% of native β-Lg was converted into dimeric species, and prolonged heating did not modify further the HP-GPC profile.

To further characterise the molecular species in β-Lg samples, SDS–PAGE analysis under non-reducing conditions was performed. As shown in Fig. 1B, unheated samples contained mainly monomers of β-Lg AB with the presence of small quantity of dimers independently of the presence or absence of copper, confirming the HP-GPC results. Heating induced a slight increase of dimers in the absence of copper. In contrast, the presence of copper promoted a significant decrease of the β-Lg monomer intensity and concomitant formation of covalent dimers, which became the main molecular species identified earlier during heating.

3.2. Characterisation of copper catalysed dimers of β-Lg AB

The type of bonding in the dimers of β-Lg formed on heating in the presence of copper were analysed through SDS–PAGE under non-reducing and reducing conditions i.e. in the presence of β-ME. The results shown in Fig. 2A indicates that the covalent dimers formed on heating, observed in the absence of β-ME (line 2), were

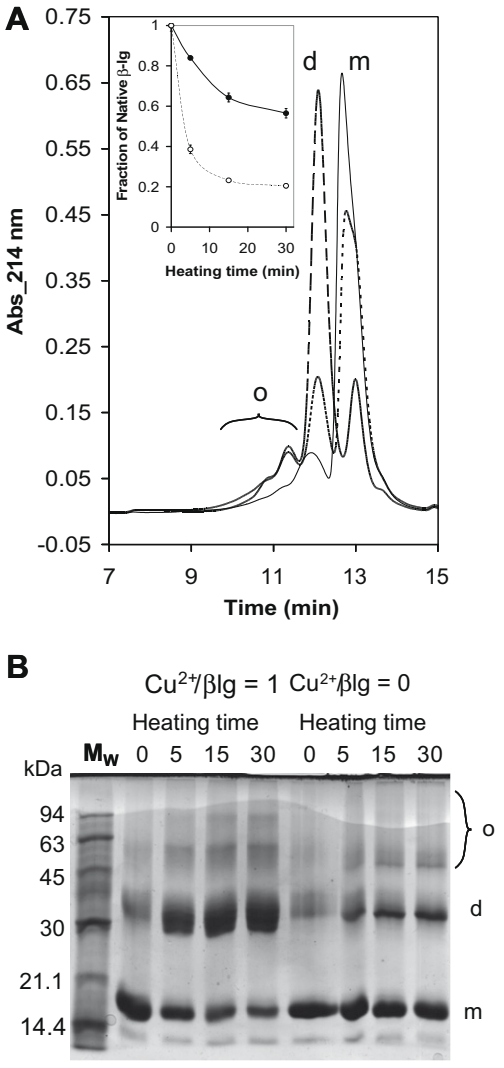

Fig. 1. (A) HP-GPC profiles of β-Lg AB unheated (full line) and heated 30 min at 78 °C in the presence (dashed line) and absence (dotted line) of copper (Cu^{2+}/β-Lg molar ratio of 1). Inserts represent the kinetics of disappearance of residual soluble proteins at pH 4.7 in the presence (dotted line) and absence (full line) of copper. m, monomer/dimer equilibrium; d, dimers; o, oligomers. (B) SDS–PAGE under non-reducing conditions of unheated and heated samples (5, 15 and 30 min at 78 °C) in the presence and absence of copper (Cu^{2+}/β-Lg molar ratio was 1). m, monomer; d, dimers; o, oligomers; M_w, low molecular weight markers.

dissociated in the presence of β-ME (line 4) demonstrating the presence of an intermolecular disulfide bond between β-Lg monomers.

To further specify the type of reaction caused by copper for β-Lg dimerisation (sulfhydryl/disulfide exchange reaction or oxidation reaction), the quantification of free sulfhydryl groups was done by the use of Ellman's reagent (Ellman, 1959). Fig. 2B shows that for unheated samples, the sulfhydryl/β-Lg AB molar ratio was 0.92 ± 0.04 in the absence of copper. This value is slightly below 1, value expected for one free sulfhydryl group per β-Lg molecule. After heating in the absence of copper, the sulfhydryl/β-Lg molar ratio slightly decreased (0.71 ± 0.03), indicating the oxidation of some sulfhydryl groups. In the presence of copper the quantity of sulfhydryl groups in unheated samples was less than in the sample without copper (0.83 ± 0.03). This decrease results from a reaction between copper and β-Lg sulfhydryl groups in the presence of SDS as indicated by Bouhallab et al. (2004). It was observed that in the samples heated in the presence of copper very slight or almost no sulfhydryl groups (0.02 ± 0.01) were still free after 30 min of

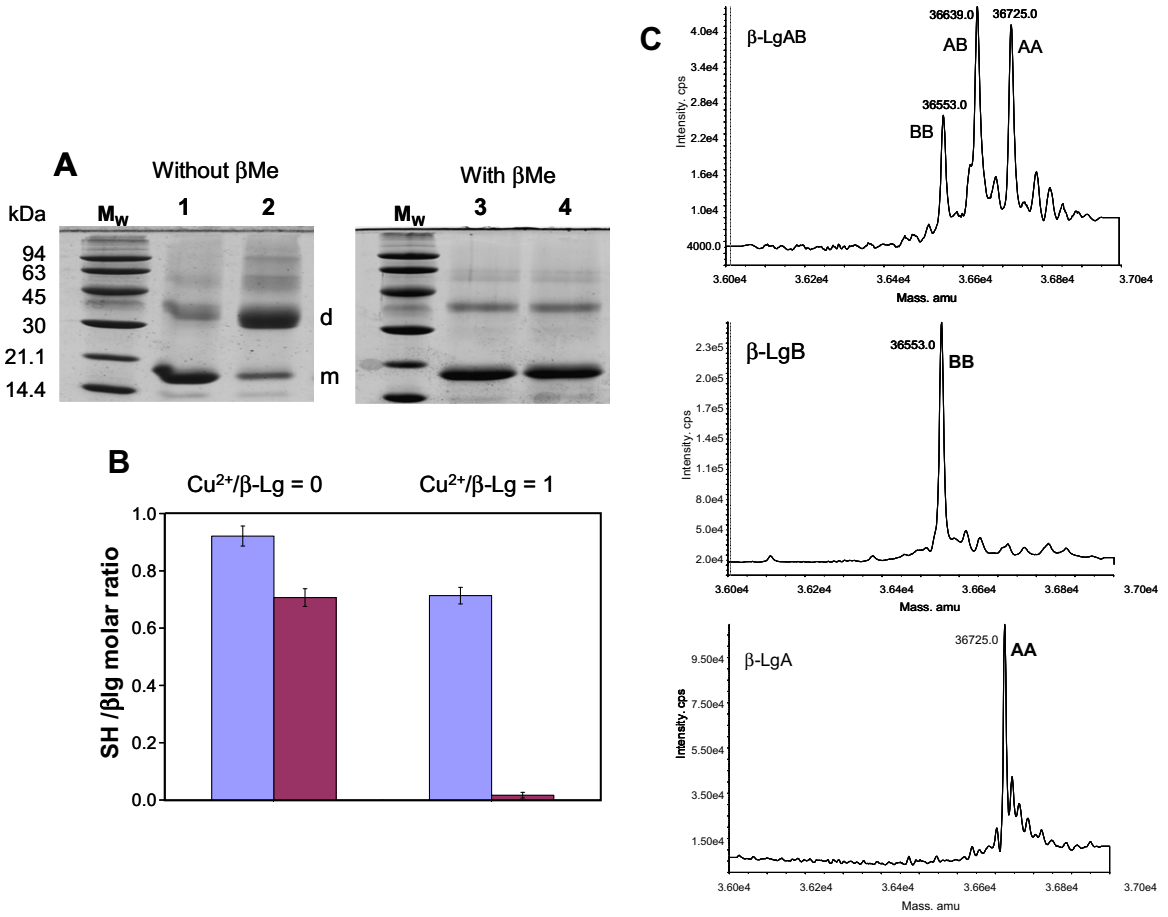

Fig. 2. (A) SDS–PAGE analysis of unheated (line 1 and 3) and heated samples of β-Lg for 30 min at 78 °C with copper (Cu^{2+}/β-Lg molar ratio of 1) (lines 2 and 4) in the absence (lines 1 and 2) and presence (lines 3 and 4) of β-Mercaptoethanol (β-Me). m, monomer; d, dimers; M_W, low molecular weight markers. (B) Quantification of free sulfhydryl groups in unheated and heated samples of β-Lg in the absence and presence of copper at a Cu^{2+}/β-Lg molar ratio of 1 by Ellman's method. (C) Mass spectrometry of copper-catalysed dimers of β-Lg AB, β-Lg B, and β-Lg A obtained after 30 min of heat-treatment at 78 °C in the presence of copper at a Cu^{2+}/β-Lg molar ratio of 1.

heating. Hence, the copper catalysed the covalent dimerisation of β-Lg AB only through oxidation of its free sulfhydryl groups.

The molecular composition of copper-catalysed dimers was determined using mass spectrometry taking advantage of the molecular mass difference between the two β-Lg variants A and B. From the profile of mass spectrometry (Fig. 2C) three different peaks with different molecular masses (36553, 36639 and 36725 g mol^{-1}) were observed. Copper-catalysed dimers formed from purified samples of β-Lg A and β-Lg B prepared under the same conditions and used as standards gave molecular masses of 36725 and 36553 g mol^{-1}, respectively. Consequently, the copper-catalysed dimerisation of β-Lg in a mixture containing both variants A and B is a random reaction leading to the formation of homogeneous dimers A–A (36725 g mol^{-1}) and B–B (36553 g mol^{-1}) and a heterogeneous dimer A–B with a molecular mass of 36639 g mol^{-1}.

The tertiary and secondary structure of copper-catalysed dimers of β-Lg AB were analysed by Circular Dichroism (CD) and compared to the CD spectra of native β-Lg and heated β-Lg in the absence of copper (Fig. 3). As expected, the near-UV CD spectra (Fig. 3B) of native β-Lg AB is characterised by deep troughs at 283 and 293 nm attributed to chiral environment of aromatic Trp 61 and Trp 19, respectively. As compared to Trp 19, the surface located Trp 61 in native β-Lg AB provides only a minor contribution to the CD signal (Fessas, Iametti, Schiraldi, & Bonomi, 2001). The same near-UV CD mark was observed in the presence of copper, suggesting an absence of copper-induced structural change for unheated β-Lg AB. The near-UV CD spectrum of copper-catalysed dimers of β-Lg exhibited profound modifications compared to the tertiary structure of native β-Lg. The large decrease in the CD signal intensity indicates that the tertiary structure of copper-catalysed dimers of β-Lg, at least close to Trp residues, is completely lost. An intermediate CD spectrum was obtained for heat-treated β-Lg in the absence of copper corresponding to the signal of residual native β-Lg after 30 min of heating at 78 °C (Fig. 1). The far-UV CD spectra (Fig. 3A) of native β-Lg AB exhibited a large negative maximum between 210 and 220 nm typical of a protein mainly composed of anti-parallel β-sheets (Kelly, Jess, & Price, 2005). The far-UV CD of copper-catalysed β-Lg dimers showed both an increase of the negative maximum of the CD signal and a shift of this maximum towards the lower wavelengths. This result shows that the copper-catalysed dimerisation induced also irreversible modifications in the secondary structure of β-Lg. Heat treatment per see unveiled less but significant modifications in the far-UV CD spectrum of β-Lg compared to unheated sample.

3.3. Effect of NaCl on copper catalysed denaturation/aggregation of heated β-Lg AB

The solubility of β-Lg AB at pH 4.7 after heating at 78 °C decreased upon increasing the ionic strength from 0 to 0.1 M (Fig. 4). In the absence of copper, we observed that after 30 min of heating the residual soluble proteins at pH 4.7 decreased from 55% (absence of NaCl) to 40% (0.1 M NaCl). The same trend was ob-

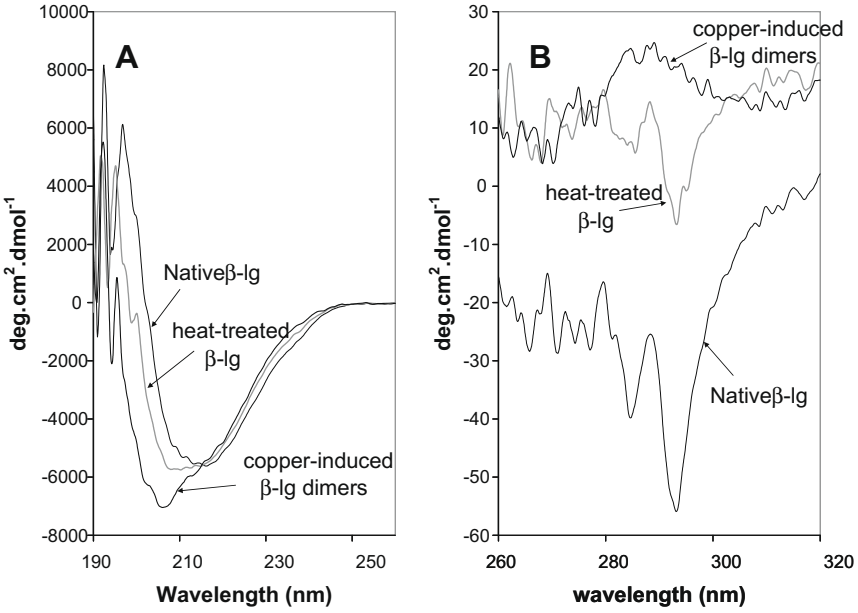

Fig. 3. Far-UV CD (A) and Near-UV CD (B) spectra of native β-Lg (normal line), copper-catalysed dimers of β-Lg (thick line) and β-Lg heated 30 min at 78 °C in the absence of copper (thin line).

served in the presence of copper, with a decrease of residual soluble proteins at pH 4.7 from 19% (absence of NaCl) to 6% (0.1 M NaCl). Though in the presence of copper the denaturation/aggregation of β-Lg AB was very pronounced, the NaCl effect was in the same order of magnitude in the absence and presence of copper. This shows that copper, even at a concentration as low as 0.27 mM has a dominant impact on the denaturation/aggregation of β-Lg compared to 100 mM NaCl. In addition, copper and NaCl do not seem to have a synergic effect on the denaturation/aggregation of β-Lg.

Molecular structures formed on heating β-Lg samples with increasing concentration of NaCl were further analysed by HP-GPC and SDS–PAGE under non-reducing conditions. In the presence of NaCl (0.1 M on Fig. 4B and C), HP-GPC profiles of heated samples were characterised by the presence of soluble aggregates eluted at retention time between 7 and 8 min. Soluble aggregates

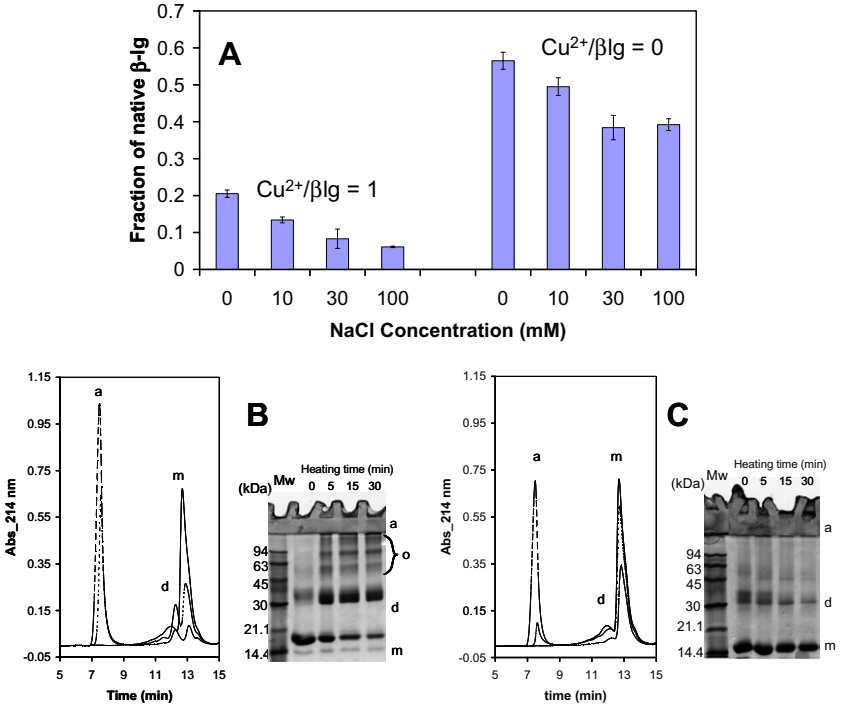

Fig. 4. Effect of NaCl (from 0 to 100 mM NaCl) on the heat-denaturation/aggregation of β-Lg in the absence and presence of copper at a Cu^{2+}/β-Lg molar ratio of 1. (A) Fraction of residual soluble proteins at pH 4.7 after heating β-Lg samples containing 0, 10, 30 and 100 mM of added salt for 30 min at 78 °C. (B) and (C) HP-GPC and SDS–PAGE under non-reducing conditions of β-Lg samples containing 100 mM NaCl unheated or heated in the presence (B) and absence (C) of copper (Cu^{2+}/β-Lg molar ratio of 1). For HP-GPC, heating time at 78 °C (0 min, full line; 5 min, dotted line; 30 min, dashed line) increases from top to bottom for the absorbance at retention time 13 min (m, monomer/dimer equilibrium; d, dimers; a, aggregates). For SDS–PAGE, m refers to monomer; d, to covalent dimers; o, to oligomers and a, to aggregates of β-Lg; M_w, low molecular weight markers.

were formed as soon as 10 mM of NaCl was added (data not shown) and their formation arose mainly in place of the dimers observed in the absence of NaCl (Fig. 1). Addition of copper increased the amount of soluble aggregates for all NaCl concentrations tested. In the meanwhile, results of SDS–PAGE indicated large differences in the electrophoretic bands of β-Lg samples heated in the absence or presence of copper. In the presence of copper (Fig. 4B), the predominant molecular species was a covalent dimer of β-Lg indicating that the soluble aggregates observed by HP-GPC were formed throughout non-covalent associations of covalent dimers. Though SDS–PAGE revealed electrophoretic bands corresponding to molecular species of β-Lg larger than dimers their intensity was weak compared to the one of covalent dimers. In contrast, intensity of the electrophoretic bands corresponding to the β-Lg dimers or larger oligomers was weak in the absence of copper (Fig. 4C). Soluble aggregates observed on HP-GPC were trapped on the top of the resolving gel, suggesting that they were mainly linked by covalent interactions.

3.4. Effect of copper/β-Lactoglobulin molar ratio on the heat denaturation/aggregation of β-Lg AB

The effect of copper/β-Lg molar ratio on the denaturation/aggregation of β-Lg AB was examined for Cu^{2+}/β-Lg molar ratio from 0 to 2. β-Lg samples contained 0.1 M NaCl and were heated at 78 °C up to 30 min. The results reported in Fig. 5A shows that increasing Cu^{2+}/β-Lg molar ratio from 0 to 2, promoted the heat-denaturation/aggregation of β-Lg AB. After 30 min of heating, the residual soluble proteins at pH 4.7 decreases from 40% in the absence of copper to less than 5% in the presence of copper at a Cu^{2+}/β-Lg molar ratio higher than 1. From the disappearance of soluble proteins at pH 4.7 as a function of heating time, the rate constant (k) for the denaturation/aggregation of β-Lg at 78 °C was deduced using the general equation:

$$-dC/dt = kC^n$$

Assuming that copper does not modify the overall reaction order, a value of 1.5 (Roefs & de Kruif, 1994) was taken for all kinetics. After integration with respect to time, the general equation became:

$$(C_t/C_0)^{-0.5} = 1 + (0.5)C_0^{0.5} \times k \times t$$

With C_0 and C_t, the concentration of residual soluble proteins at pH 4.7 at time ($t = 0$) and time t, respectively; n, the overall reaction order; k, the rate constant; t, the heating time.

Increasing the Cu^{2+}/β-Lg molar ratio induced a faster decrease in the residual native β-Lg AB on heating (Fig. 5A) and in the meanwhile a faster increase in the formation of soluble aggregates (Fig. 5B). The rate constant for the disappearance of native β-Lg AB increased ten fold from $0.041 \pm 0.003\ M^{-0.5}\ s^{-1}$ in the absence of copper to $0.461 \pm 0.006\ M^{-0.5}\ s^{-1}$ at a Cu^{2+}/β-Lg molar ratio of 2 (Fig. 5A, insert). SDS–PAGE under non-reducing conditions confirmed that in the absence of copper, β-Lg molecules are mainly included into soluble aggregates unable to go through the resolving gel. This indicated covalent intermolecular interactions between β-Lg molecules in the formed aggregates. Increasing Cu^{2+}/β-Lg molar ratio strengthens the band intensity corresponding to β-lg dimers. In the same time the electrophoretic band corresponding to soluble aggregates disappeared and smaller entities (oligomers) were able to penetrate into the resolving gel. Hence, soluble aggregates formed in the presence of copper were constituted of covalent dimers and oligomers of β-lg held together throughout non-covalent association. Higher the Cu^{2+}/β-Lg molar ratio, lower the molecular size of these oligomers and higher the proportion of dimers indicating a competition between sulfhydryl/disulfide interchange and sulfhydryl oxidation reactions which was shifted to the latter with increasing copper concentration.

4. Discussion

Sulfhydryl oxidation reactions and sulfhydryl/disulfide exchange reactions are the main reactions leading to intermolecular covalent bond during the heat-treatment of proteins containing free sulfhydryl groups. Controlling the balance between both reactions is a way to control the reactivity of the non-native molecular structures resulting from the aggregation process. When sulfhydryl/disulfide exchange reactions prevail, non-native molecular structures still contain free sulfhydryl group for continuing the propagation reactions whilst sulfhydryl oxidation stop the propagation process (termination reaction) through the progressive disappearance of free reactive sulfhydryl groups (Roefs & de Kruif, 1994). The physicochemical conditions selected during processing affect the balance between both reactions. Sulfhydryl oxidation is favoured with the exposure of β-Lg free sulfhydryl groups between 65 and 75 °C leading to small covalent oligomers, whilst higher temperatures favour sulfhydryl/disulfide exchange reactions and the formation of large aggregates mainly stabilised by intermolecular disulfide bonds (Hoffmann & van Mil, 1997; de Wit, 2008; Schokker et al., 1999).

Transition metal ions were reported to be key elements that influence the denaturation/aggregation processes of β-Lg (Bouhallab et al., 2004; Navarra et al., 2007; Pantaloni, 1965). In particular, sulfhydryl oxidation was shown to be promoted by copper when sulfhydryl groups are already exposed on protein surface (Bouhallab et al., 2004; Floris et al., 2008). In the present study, we showed that sulfhydryl oxidation reactions prevailed and occurred rapidly as soon as copper was present in the solution. The large covalent aggregates formed on heating in the absence of copper were substituted by smaller molecular species (covalent dimers and covalent oligomers) in the presence of copper. Indeed, the effect of low concentration of copper (even below 0.1 mM) on the denaturation/aggregation process of β-Lg was found to be higher than the one obtained with high concentration of NaCl (up to 100 mM).

Increasing the Cu^{2+}/β-Lg molar ratio reduces the size of the small covalent oligomers formed on heating and accumulation of dimeric species was predominant above Cu^{2+}/β-Lg molar ratio of 0.3. The formation of β-Lg dimers through sulfhydryl oxidation reactions was expected as there is only one sulfhydryl group per β-Lg monomer. The rate of copper-induced dimerisation of β-Lg increased with increasing the concentration of added copper in accordance with Bouhallab et al. (2004). Compared to the native β-Lg dimers (equilibrium between monomer and dimer), the copper-catalysed covalent dimers exhibited different structures, with slight differences in secondary structure and a complete loss of tertiary structure. The structure obtained for the copper-catalysed β-Lg dimers differs significantly from the structure of molecular species obtained for heated β-Lg in the absence of copper in accordance with the results of Navarra et al. (2007).

Sulfhydryl oxidation reaction is able to take place only when the free sulfhydryl group of β-Lg is exposed on the protein surface, i.e. above denaturation temperature of β-Lg. In experiments up to 65 °C, we were unable to detect any disappearance of native β-Lg even with an excess of copper (data not shown). This result is in accordance with a previous work in which β-Lg AB conserved very similar behaviour on heating up to 60 °C in the absence and presence of copper (Navarra et al., 2007). In a recent work, Stirpe et al. (2008) reported that copper ions destabilised the native conformational state of β-Lg and speeded up the aggregation process. Our re-

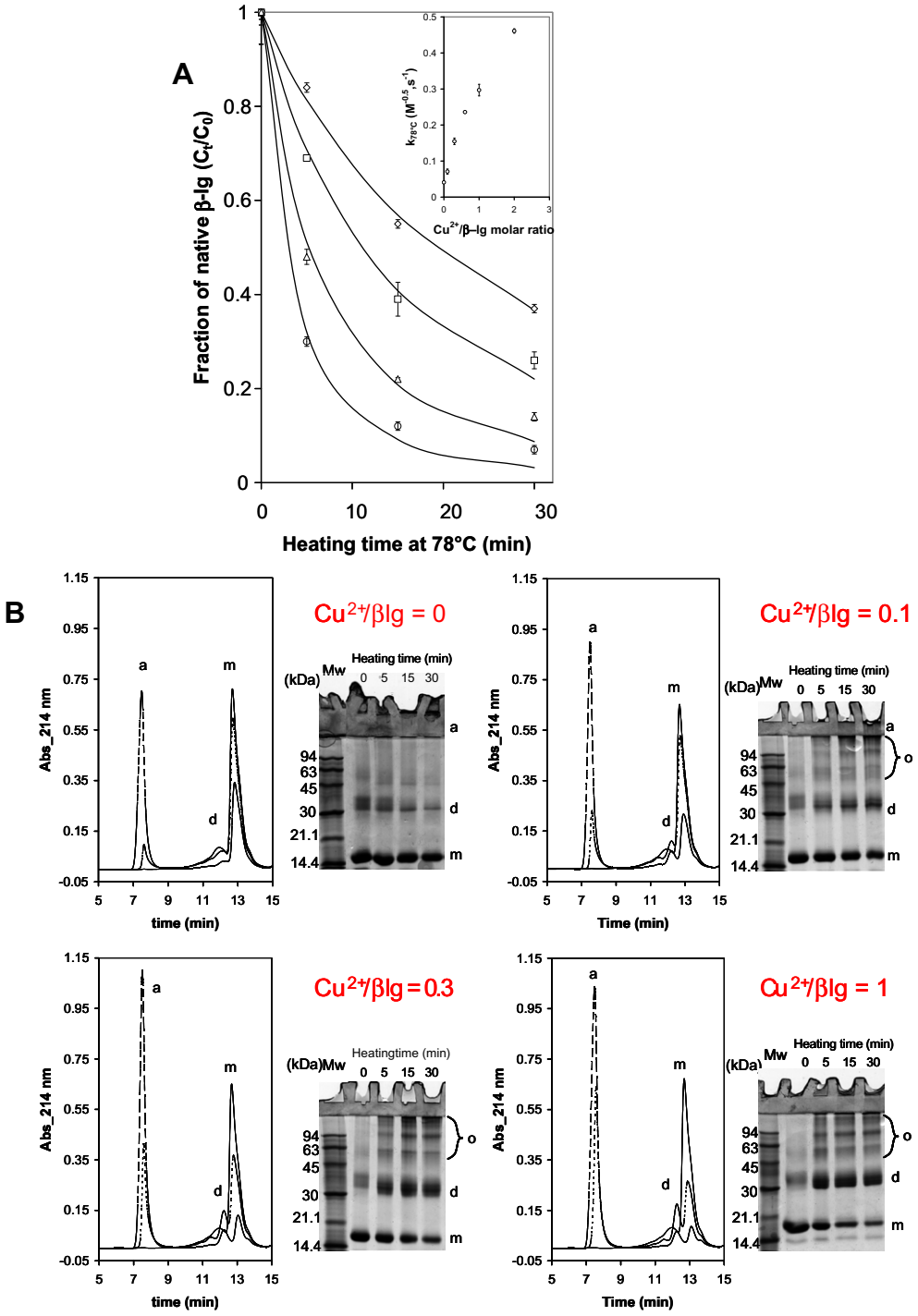

Fig. 5. Effect of Cu^{2+}/β-Lg molar ratio on the heat-denaturation/aggregation of β-Lg. (A) Kinetics of disappearance of residual soluble proteins at pH 4.7 according to the Cu^{2+}/β-Lg molar ratio: 0 (diamond), 0.1 (square), 0.3 (triangle) and 1 (circle). Insert represents the rate constant versus the Cu^{2+}/β-Lg molar ratio. (B) HP-GPC and SDS–PAGE under non-reducing conditions of β-Lg samples containing different Cu^{2+}/β-Lg molar ratio. For HP-GPC, heating time at 78 °C (0 min, full line; 5 min, dotted line; 30 min, dashed line) increases from top to bottom for the absorbance at retention time 13 min (m, monomer/dimer equilibrium; d, dimers; a, aggregates). For SDS–PAGE, m refers to monomer; d, to covalent dimers; o, to oligomers and a, to aggregates of β-Lg. M_w, low molecular weight markers.

sults demonstrated the catalytic role of copper in the aggregation process but confirmed neither copper potential binding to native β-Lg nor its ability to destabilise the native β-Lg structure.

NaCl is known to affect greatly the type of molecular species formed on heating and the nature of the interactions in the formed aggregates (Aymard, Durand, & Nicolai, 1996; Baussay et al., 2004). NaCl favours non-covalent associations between denatured, covalently aggregated molecules throughout the screening of protein surface charges. The presence of copper and high NaCl concentration modifies the final nature of formed aggregates; the copper-catalysed covalent dimers were aggregated into larger particles in the presence of 100 mM NaCl. In these aggregates, the covalent dimers were linked to each others through non-covalent associations. Hence, controlling Cu^{2+}/β-Lg molar ratio and NaCl concentration enable the formation of specific non-native molecular structures characterised by their size, degree of covalent and non-covalent intermolecular interactions, and reactivity towards sulfhydryl agents. In the absence of NaCl,

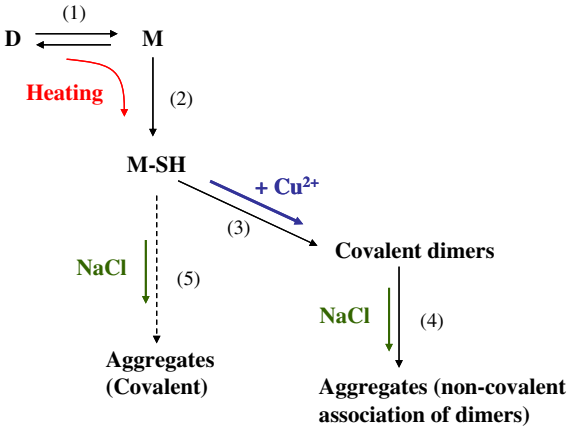

Fig. 6. Proposed mechanism on how copper modulates the heat induced denaturation/aggregation process of β-Lg AB. D, native dimer of β-Lg; M, native monomer of β-Lg; M-SH, non-native monomers of β-Lg with exposed sulfhydryl group available for chemical reactions.

mainly covalent dimers of β-Lg are formed in the presence of copper whilst they form non-covalent aggregates in the presence of NaCl. In contrast, aggregates are mainly covalent in the absence of copper.

5. Conclusion

Metal ions such as copper constitute a powerful mean to control the denaturation/aggregation reactions of β-Lg. We propose the following general mechanism to describe how copper affects the heat induced denaturation/aggregation of β-Lg AB (Fig. 6). In native state, β-Lg exists in monomer/non-covalent dimer equilibrium (1). Heating causes a shift of the equilibrium to the monomeric state of β-Lg AB, which unfolds and leads to reactive monomers characterised by an exposed sulfhydryl group available for chemical reactions (2). In the presence of copper, oxidation of free sulfhydryl group of β-Lg occurs, stabilising covalent dimers (3). Then, these dimers constitute elementary building blocks for further associations into non-covalent aggregates under favourable ionic strength (4). In contrast, in the absence of copper the reactive monomers are mainly directed towards the formation of covalent aggregates under the effect of ionic strength through sulfhydryl/disulfide interchange reaction (5).

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