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Change in conformational, digestive and immunological characteristics of bovine allergen β-lactoglobulin induced by metal ions in combination with heating

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ABSTRACT

Aggregation of bovine β-lactoglobulin is affected easily by external factors. In this study, effects of metal ions combining with temperature on aggregation of β-lactoglobulin were explored. The conformational characteristics of aggregates were detected by environment scanning electron microscope, CD spectrum and free sulfhydryl group, respectively. Digestive and immunological characteristics were assessed by simulated digestion *in vitro* and ELISA respectively. The results showed that the morphology of β-lactoglobulin aggregates became more amorphous in Cu^{2+} and Mg^{2+} treated samples and more constricted in Zu^{2+}-induced protein. Among them, Cu^{2+} altered the secondary structure of β-lactoglobulin aggregates and free sulfhydryl content most as well as that in gastric digestion. However, all ion-treated groups had similar digestive stability in intestinal digestion. Specially, Ca^{2+} and Mg^{2+} made the antigenicity and potential allergenicity of β-lactoglobulin aggregates decrease, which helps us understand the role of metal ions in immunological characteristics.

1. Introduction

Bovine β-lactoglobulin is a major essentially globular protein and has no homologous counterpart in human milk (Wal, 2001). β-lactoglobulin comprises 162 amino acids with a molecular weight of 18.3 kDa and presents a dimeric structure at physiological pH. The secondary structure of native β-lactoglobulin is composed of eight strands of antiparallel β-sheets and one α-helix facing each other and forming a core structural pattern (Brownlow et al., 1997; Kontopidis et al., 2004). In the natural state, β-lactoglobulin presents as a mixture of dimers and monomers, as proven by Townend et al. (1960). Aggregates of β-lactoglobulin can be induced by disulfide bonds, hydrogen bonds, electrostatic forces and van der Waals forces (Gulzar et al., 2011).

Cow's milk allergy is a health problem of great importance, and it is very prevalent among children under the age of three (Damodaran & Li, 2017; Verhoeckx et al., 2015). β-lactoglobulin is a major allergen in cow's milk, and approximately 82% of milk allergy patients are sensitive to β-lactoglobulin (Li et al., 2008). Modification of milk protein structure and changes in conformational epitopes were associated with immunomodulatory properties (Bogahawaththa et al., 2017). Upon aggregation of β-lactoglobulin, the conformational structure changes, leading to changes in certain characteristics, such as thermal stability, turbidity, rheological characteristics, aggregate morphology and particle size (Bolisetty et al., 2012; Mehalebi et al., 2008; Sardar et al., 2014; Zuniga et al., 2010), weakening or intensifying the binding capacity of antibodies (Kleber et al., 2004).

Aggregation of β-lactoglobulin is affected by pH (Mudgal et al., 2009), temperature (Donovan & Mulvihill, 1987), protein concentration

Abbreviations: HRP, Horseradish peroxidase; AP, Ammonium persulfate; SDS-PAGE, Sodium dodecyl sulfate–polyacrylamide gel electrophoresis; CD, Circular dichroism; DTNB, Dinitrobenzoic acid; SGF, Simulated gastric fluid; SIF, Simulated intestinal fluid; ELISA, Enzyme-linked immunosorbent assay; PBS, Phosphate-buffered saline; OPD, O-Phenylenediamine; ESEM, Environmental scanning electron microscopy.

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(Mudgal et al., 2009), salt concentration (Kinsella & Whitehead, 1989), metal ions (Muhammad et al., 2009) and other coexisting factors (Croguennoc et al., 2001). Interestingly, metal ions have a significant impact on aggregation of β-lactoglobulin. At pH 3.5, Ca^{2+} improved aggregation of β-lactoglobulin, which formed a polymer with a molecular weight of >106 kDa (Mudgal et al., 2011). Cu^{2+} could change the tertiary structure of β-lactoglobulin, leading to the formation of supramolecular aggregates (Divsalar et al., 2012). What's more, Zn^{2+} could catalyze the formation of large β-lactoglobulin aggregates rapidly (Navarra et al., 2007). It is known that metal ions are considered a major factor for aggregation. However, the physicochemical characteristics and immunological characteristics of β-lactoglobulin aggregation induced by four similar divalent metal ions (Cu^{2+}, Zn^{2+}, Mg^{2+}, and Ca^{2+}) have not been clearly stated. Of note, Ca^{2+}, Zn^{2+}, Mg^{2+} and Cu^{2+} are important essential trace elements for human nutrition and play different physiological roles in the human body (Brown et al., 2001; Dawson-Hughes et al., 2005; Joint & World Health, 2005). Metal ions can bind at centers of highly hydrophobic groups and influence the formation of supramolecular aggregates by β-lactoglobulin, and ionic strength can affect the aggregation of β-lactoglobulin (Navarra et al., 2007, 2014). Our study explored the changes in structure, digestibility and immunoreactivity of the cow's milk allergen β-lactoglobulin after metal-induced aggregation.

2. Materials and methods

2.1. Materials

Cow's raw milk was collected from the YangGuang Dairy Corporation (Jiangxi, China). Sephadex G-75 and DEAE-Sepharose Fast Flow columns were purchased from GE (Boston, USA). Protein Marker was purchased from the Fermentas Corporation (Burlington, USA). Horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG, biotin-labeled goat anti-human IgE; HRP-labeled avidin, commercial β-lactoglobulin (purity > 90%), pepsin (≥2500 U/mg, from porcine gastric mucosa), pancreatin (8 × USP specifications, from porcine pancreas) and ammonium sulfate (AP) were purchased from Sigma (St. Louis, Mo, USA). Human sera that specifically binds to cow's milk protein was provided by the First Affiliated Hospital of Guangxi Medical University (Guangxi, China) and relative information about bovine milk allergy patients was displayed in Table S1. Metal ions, and other biochemical reagents were purchased from the Sangon Biotech Corporation (Shanghai, China).

2.2. Preparation of β-lactoglobulin

Fresh milk was filtered with four layers of sterile gauze and skimmed by a cream separator. The pH of the milk was adjusted to 4.6 by using 5 M HCl. Subsequently, casein was separated and filtered with four layers of sterile gauze after depositing. Bovine whey (supernatant) was collected after centrifuging at 8000×*g* for 15 min at 4 °C (Sorvall LYNX 4000 centrifuge, Thermo, USA). Bovine β-lactoglobulin was prepared by combining a Sephadex G-75 gel chromatography column (12 mm × 120 cm) and anion-exchange chromatography on a DEAE-Sepharose Fast Flow column in tandem operation column (16 mm × 30 cm) chromatography, according to a previous study (Li et al., 2008). The identity and purity of the prepared β-lactoglobulin was identified by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and mass spectrometry (data not show), and the protein purity (99%) was significantly higher than that of commercial β-lactoglobulin (purity > 90%). β-lactoglobulin was determined quantitatively using Coomassie Brilliant Blue staining analyses. In this research, we chose the whole variants of β-lactoglobulin to carry out our experiment.

2.3. Optimal condition for aggregation of β-lactoglobulin

Firstly, the protein was diluted to 5 mg/mL with phosphate buffer (pH 6.8). Then, the protein was transferred 2-mL polypropylene tubes for subsequent experiments. $CuCl_2$, $CaCl_2$, $ZnCl_2$ and $MgCl_2$ were dissolved in ultrapure water, and their final concentration was 0.5 M. All of the prepared protein solution were filtered through a 0.22-μm filter. At pH 6.8, 2 mL of prepared protein solution was mixed with 1 mL of $CuCl_2$, $CaCl_2$, $ZnCl_2$ and $MgCl_2$ solution. Afterwards, protein-metal ions samples underwent with different treatments, which referred to Navarra's (Navarra et al., 2014), Rahaman's (Rahaman et al., 2015) and Yuchen's (Yuchen and Paula, 2018) studies with some modification. The protein-metal ions samples were treated with different heating phase (40 °C, 50 °C, 60 °C, 65 °C, 70 °C and 75 °C for 1 h, pH 7.0), different pH value (pH 6.5, 7.0, or 7.5 at 70 °C for 1 h) and different heating time (0.5 h, 1 h, or 1.5 h at 70 °C, pH 7.0). Then, prepared protein solution was mixed with different volumes of metal ions ($CuCl_2$, $CaCl_2$, $ZnCl_2$ and $MgCl_2$) to obtain different mole ratios of protein-metal ion (2:1, 1:1, and 1:2 at 70 °C for 30 min, pH 6.8). The control group was treated by ultrapure water (no metal ions). After treatment at different temperatures, pH, heating time and protein-metal ion mole ratios, all samples were put into ice for 20 min and centrifuged (4000×*g*) for 5 min. The aggregates in supernatant induced by metal ions were detected by Native-PAGE, and the amounts of oligomer were regarded as the indicator for evaluation of optimal conditions. The induced aggregates were analyzed by Native-PAGE and mass spectroscopy. All induced samples were collected in sterile bottles and stored at −20 °C for subsequent assays.

2.4. Page

The changes of protein aggregation were detected by SDS-PAGE and Native-PAGE according to the method of our previous study (Meng et al., 2017) and H Schägger (Schägger & von Jagow, 1991) For SDS-PAGE, a 4% stacking gel and a 12% separating gel were used with a constant current (6 ~ 12 mA). 10 μL sample was mixed with 10 μL of SDS sample buffer (2% [w/v] SDS, 50 mM Tris-HCl, 0.02% [w/v] bromophenol blue, 25% [v/v] glycerin, and 3% [v/v] beta-mercaptoethanol). The gel was run by using a Mini Protean system (Bio-Rad, USA), followed by staining with Coomassie brilliant blue R-250 for 15 min and destained by incubation with 7.5% acetic acid and 5% methanol for at least 30 min. Finally, the images were recorded and the band intensity was then calculated with Quantity One software (Bio-Rad). For Native-PAGE, an 8% stacking gel and a 15% separating gel were used with a constant current (60 V ~ 80 V). For Tricine-SDS-PAGE, a 4% stacking gel, 10% spacer gel and 16.5% separating gel were used with a constant current (30 V, 1 h; 100 V, 2–3 h). The other steps were the same as those for SDS-PAGE.

2.5. Determination of particle size and shape after aggregation of β-lactoglobulin

The control and experimental samples were diluted to 3 mg/mL with ultrapure water. Then, the particle sizes were analyzed by a Nanoptic 90 (Bettersize Cooperation, Liaoning, China). The parameters of the laser scattering instrument were as follows. The laser wavelength and the scattering angle was 635 nm and 90°, respectively. The measuring time was 120 s and the medium temperature was 20 °C. For particle shape analysis, the samples were detected in the Institute for Advanced Study, Nanchang University. The samples were freeze-dried firstly (Freeze-mobile 12EL, Virtis, USA), and particle shape was detected by Environmental Scanning Electron Microscopy. (FEI Quanta200F, Bettersizes, China)

2.6. Secondary structure determination

Secondary structures of different aggregates were monitored by

using a Circular Dichroism (CD) Spectropolarimeter (MOS-450/AF-CD, ASCO, Japan). The spectrum for detection ranged from 190 to 250 nm, and the speed of scanning was 60 nm/min with a 1-nm bandwidth. Samples (0.1 mg/mL) were loaded and scanned for three times. The value was the average of the three scores after deducting the blank. The result was showed by the average molar ellipticity [θ] (deg·cm^2/dmol), which was produced by Jasco secondary structure software (http://dichroweb.cryst.bbk.ac.uk/html/process.shtml) to obtain the secondary structure content.

2.7. Content of free sulfhydryl group

The free thiol for the proteins was titrated with dinitrobenzoic acid (DTNB) by manually adding a DTNB solution to the protein solution. Different cysteine concentration solutions (0, 0.025, 0.05, 0.1, 0.15 and 0.2 mM) were prepared and dissolved in 5 mL of 0.25 M Tris-HCl buffer as the standard solution. Then, 1 mL of standard solution was mixed with 5 mL preheated DTNB solution at 25 °C, following by incubation for 10 min. Then, the absorbance of mixed liquor was read at 412 nm for a standard curve. The absorbance of all samples were detected in the same way and the free thiol of β-lactoglobulin aggregates induced by four different metal ions was calculated by the standard curve formula.

2.8. In vitro digestion by simulated gastric fluid and intestinal fluid

The solution and method of simulated gastric and intestinal digestion *in vitro* were prepared and performed according to the instructions of US Pharmacopeia (Inc & USPC, 1995), with minor revision. 800 μL reaction solution was composed with simulated gastric fluid (1.28% [w/v] pepsin, 0.8% [w/v] NaCl, pH 1.2) and metal ions induced samples at a ratio of 1:3 (v/v). The gastric digestion was reacted at 37 °C, pH 1.2 with continuous shaking and 20 μL of the digestion mixtures was periodically taken out at 15, 30, 60 and 120 min. Intestinal digestion *in vitro* was carried out by simulated intestinal fluid (4% [w/v] pancreatin, 2.8% [w/v] KH_2PO_4, pH 7.5, SIF). The simulated intestinal digestion process was performed at 37 °C, pH 7.5. Meanwhile, 20 μL of the digestion mixtures was periodically withdrawn at 5, 15, 30, 40 and 60 min. At the end of digestion, 10 μL of the simulated gastric and intestinal digested samples were analyzed by Tricine-SDS-PAGE. At the same time, a control group without the addition of pepsin was performed in the same way. The mixtures of sample and intestinal fluid at a ratio of 3:1 (v/v) were incubated at 37 °C after preheating the reaction for 5 min for the same amount of time. For detection, 20 μL of solution was removed at 5, 15, 30, 45 and 60 min. Finally, the samples were analyzed using Tricine-SDS-PAGE.

2.9. IgG- and IgE-binding ability

The IgG- and IgE-binding ability of the samples were evaluated with competitive enzyme-linked immunosorbent assay (competitive ELISA), which were evaluated by IC_{50} and inhibition rate after aggregation induced by different ions, respectively.

The procedure of IgG-binding ability detection was as follow. Native β-lactoglobulin were diluted to 2.5 μg/mL with 50 mM sodium carbonate buffer (pH 9.6), followed by coating into a 96-well microtiter plate overnight at 4 °C. The plates were washed three times with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBST), followed by blocking with gelatin in PBS (pH 7.4) for 1 h at 37 °C. At the same time, the other plate was also blocked without coated protein. Aggregated proteins were diluted to six different concentrations (0.3125 μg/mL, 0.625 μg/mL, 1.25 μg/mL, 2.50 μg/mL, 5.00 μg/mL and 10.00 μg/mL) and anti-β-lactoglobulin rabbit sera prepared in previous study (Meng et al., 2017) were diluted to 1:200,000. After that, 60 μL diluted anti-β-lactoglobulin rabbit sera was mixed with 60 μL aggregated protein solution at six different concentrations, following by incubation in the blank wells for 1 h at 37 °C. Then the mixed solutions (100 μL) were transferred to the coated wells and incubated at 37 °C for 1 h. After washing with PBST, 100 μL of HRP-conjugated goat anti-rabbit IgG (1:5000) was added to each well, followed by incubation for 1 h at 37 °C. For detection, 100 μL of an O-phenylenediamine (OPD, Sigma, St. Louis, Mo, USA) substrate solution was added to each well and incubated for 15 min at 37 °C. The reaction was stopped by adding 50 μL of 2 M H_2SO_4. Absorbance was determined at 490 nm by using a microplate reader (Model 1860, Bio-Rad, USA). IC_{50} is the competitive concentration when the binding ability rate was 50%. IC_{50} for IgG-binding ability was calculated by following formula. The higher IC_{50} meant more loss of IgG-binding ability for treated protein.

$$\text{Bindingability}(\%) = \frac{B}{B_0} \times 100\%$$

(B: The optical density of antisera with treated proteins; B_0: the optical density of antisera with PBS)

The detection of IgE-binding ability was similar to that of IgG-binding ability. Because of less contents IgE in sera, we just chose one competitive concentration (2.5 μg/mL) for detection of binding ability changes. Specific antibody was collected from human sera with a dilution of 1:20 and biotin-labeled goat anti-human IgE was diluted to 1:5,000. Then, 100 μL HRP-labeled avidin was added into each coat for the enhancement of chromogenic signal. IgE-binding ability changes was calculated by their inhibition rate as the following formula. The higher inhibition rate, the stronger IgE-binding ability of the treated proteins.

$$\text{Inhibitionrate}(\%) = \frac{1 - OD_{sample}}{OD_0} \times 100\%$$

(OD_{sample}: the optical density of antisera with 2.5 μg/mL samples induced by different concentration; OD_0: the optical density of antisera with PBS)

2.10. Statistical analysis

Each experiment was repeated three times and the results are expressed as the means ± standard deviations (SDs). Statistical significance was analyzed using ANOVA procedures (SPSS for Windows 2003, Microsoft Corp, version 13.0, Chicago, IL, USA) and Fisher's least significant difference (LSD, $^*p < 0.05$) test.

3. Results and discussions

3.1. Aggregation induced by different metal ions

The optimal condition for formation of β-lactoglobulin aggregates was identified: 5 mg/mL β-lactoglobulin heated for 30 min at 70 °C, pH 7 with a protein-metal ion ratio of 1:1. Then, β-lactoglobulin aggregates used in the following experiments were induced by Cu^{2+}, Zn^{2+}, Ca^{2+} and Mg^{2+} under this condition. We could find from Fig. 1, the most aggregates were formed by induced with Cu^{2+} compared with those induced by other three metal ions (Ca^{2+}, Zn^{2+}, Mg^{2+}). As shown in Fig. 1A and 1B, more aggregates were formed with the temperature increasing. However, the ratio of protein to metal ions and pH had a little effect on the aggregation of β-lactoglobulin. Moreover, the oligomer content was the highest at 70 °C and 75 °C in the Cu^{2+}-treated group (Fig. 1B). Accordingly, 70 °C was chosen as the optimal temperature. In Fig. 1C and D, heating time and pH also affected the processing of aggregation.

Here we explored aggregation of β-lactoglobulin induced by metal -ions in combination with heating, since aggregation of β-lactoglobulin by heating has been widely studied in recent decades. Heat treatment was reported to change the functionality, structure and immune reactivity of β-lactoglobulin (Kehoe & Foegeding, 2014; Rahaman et al., 2015), which is the similar to our results as shown in Fig. 1A and 1B. It was reported that changes of external factors could cause reversible or

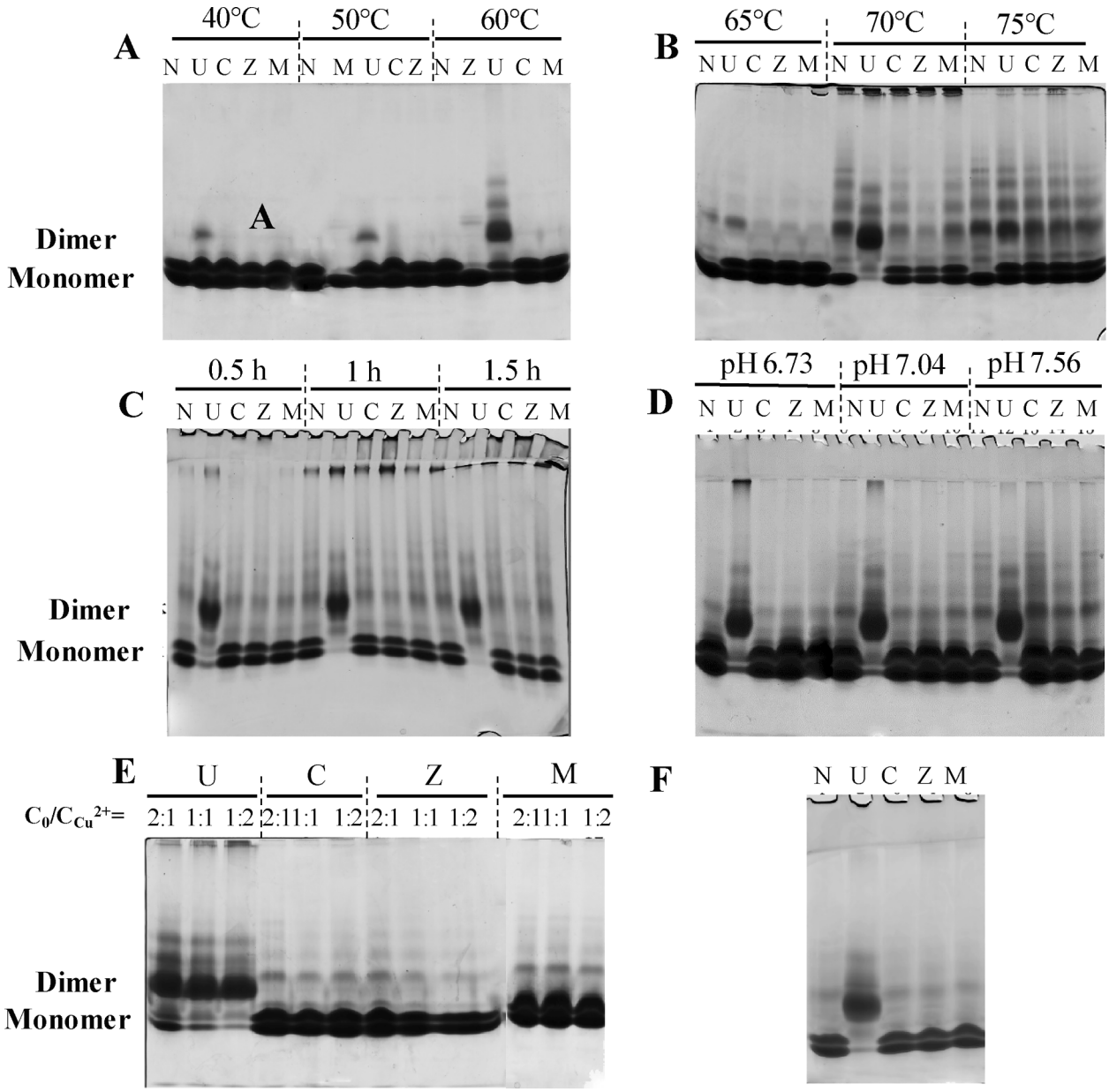

Fig. 1. Native-PAGE patterns of β-lactoglobulin aggregates at different temperatures, times, pH levels and molar ratios. A-B: Impact of temperature (40 °C, 50 °C, 60 °C, 65 °C, 70 °C, 75 °C, 1 h, pH 7.0) on metal ion-induced aggregation. C: Impact of heating time (0.5 h, 1 h, 1.5 h, 70 °C, pH 7.0) on metal ion-induced aggregation of β-lactoglobulin. D. Impact of different pH values (pH 6.5, pH 7.0, pH 7.5, 1 h, 70 °C,) on aggregation of β-lactoglobulin. E: Impact of the protein-metal ion ratio on aggregation of β-lactoglobulin (70 °C, 30 min, pH6.8). F: Native-PAGE patterns of samples at optimal conditions. N: no metal ions; U: Cu^{2+}; C: Ca^{2+}; Z: Zn^{2+}; M: Mg^{2+}.

irreversible protein modification, leading to aggregation (Petit et al., 2012). However, pH, the ratio of protein to metal ions and heating time in our work did not alter aggregation a lot. Although Sawyer's research (Sawyer & Kontopidis, 2000) strongly supported that the formation of β-lactoglobulin aggregation was closely associated with the pH, it was not the same situation in Fig. 1D, which might be the presence of metal ions.

3.2. Particle size and protein shape of aggregation oligomers

The particle size of aggregates induced by the four metal ions were detected by Laser Particle Sizer in Fig. 2. The particle diameter of native protein was 300 nm, while the particle diameters of aggregates induced by Ca^{2+}, Zn^{2+}, Mg^{2+} and Cu^{2+} were 1236, 1167, 379 and 372 nm, respectively. In Fig. 2B, particles smaller than 10 nm decreased sharply when the solution was heated to 70 °C which meant that monomer were disappeared to form aggregates, there were sharp peaks during 1000–10000 nm in Ca^{2+} and Zn^{2+} treated group, while larger particles might be induced by Mg^{2+} and Cu^{2+}, since we did not find in Fig. 2, but their small particles disappeared. Among all the metal ions, Ca^{2+} induced the largest particles, but they are not visualized in Fig. 1F, which might be because these large aggregates too big to enter into gels as shown in Fig. 1B.

Changes in protein shape were monitored by Environmental Scanning Electron Microscopy (ESEM). It was shown that β-lactoglobulin in the natural state displayed an ellipsoid state (Fig. 3A), which was similar to results in previous reports (Qin et al., 1998; Sawyer et al., 1999). Compared with that of native β-lactoglobulin, the morphology of β-lactoglobulin changed obviously after induction of metal ions. ESEM results demonstrated that heating could convert β-lactoglobulin into an

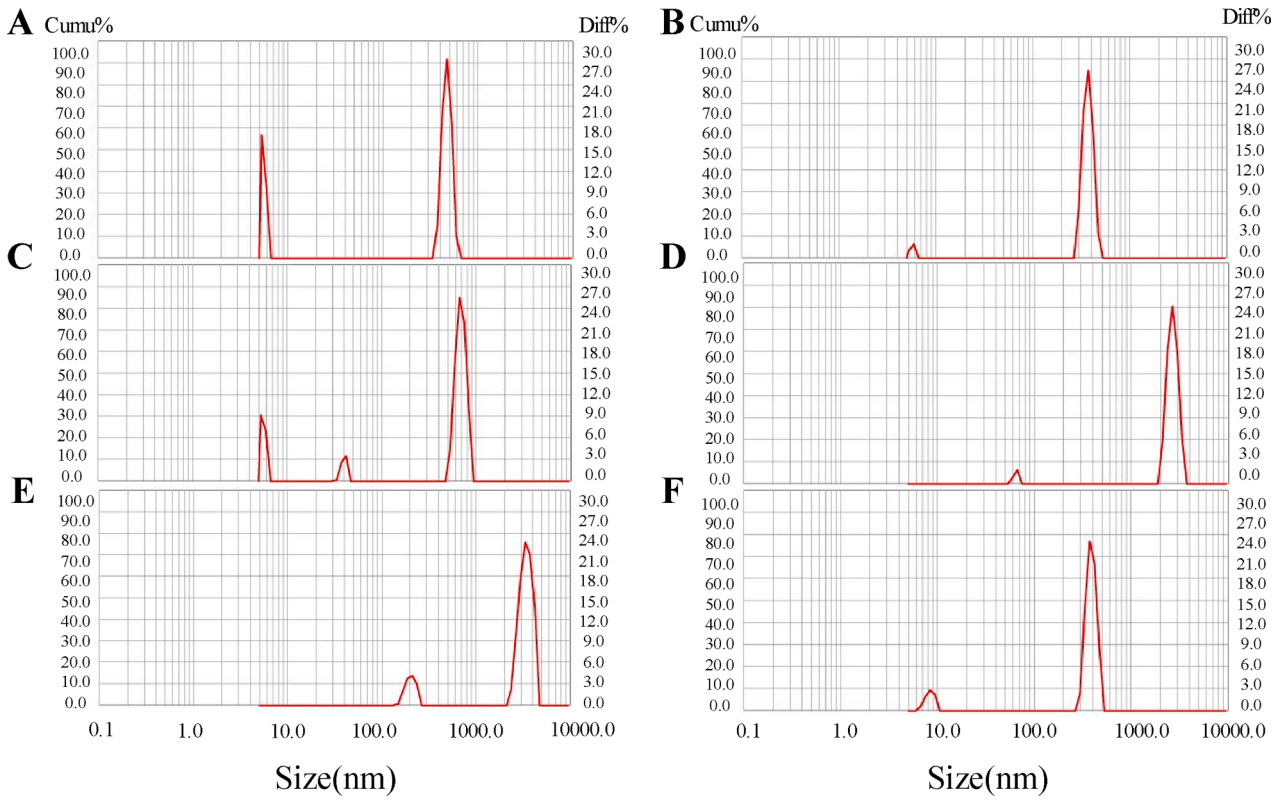

Fig. 2. Particle diameter distribution of β-lactoglobulin aggregates. A: β-lactoglobulin without any treatment; B: heat treatment without metal; C: Cu^{2+}-induced group D: Ca^{2+}-induced group; E: Zn^{2+}-induced group; F: Mg^{2+}-induced group.

expanded melting state (Fig. 3B) and an amorphous polymer of β-lactoglobulin was formed to different extents after induction by Cu^{2+} and Mg^{2+} (Fig. 3C–F). Specifically, under the impact of Zu^{2+}, the structure of β-lactoglobulin became constricted and spheroidal (Fig. 3E). Moreover, the globular structure of β-lactoglobulin treated with Ca^{2+} maintained the major nature state as shown in Fig. 3D.

3.3. Changes in secondary structure and free sulfhydryl content

Secondary structural changes of β-lactoglobulin induced by metal ions were monitored by CD spectrum determination in Fig. 4A. The CD profile of protein induced by Cu^{2+} showed an obvious alteration compared to that of the native protein. The results clearly demonstrated that Cu^{2+} treatment caused an increase in α-helix and a decrease in β-sheet contents, which is the most change in secondary structure. β-Lactoglobulin can be prone to aggregate when its nonnative α-helix structure converts to a β-sheet (Downs & Taylor, 2010; Pourjabbar et al., 2015) which in accord with our results that there were not so much changes of α-helix and β-sheet after treatment of Ca^{2+}, $Mg^{2+}$and Zn^{2+}. With respect to random coil content of aggregates, it was reduced quite obviously in the sample induced by Mg^{2+}.

We can find in Fig. 4B the free sulfhydryl contents in all the treated groups were lower than that in the control group. Among them, the free sulfhydryl content was the least in the aggregates induced by Cu^{2+}, which indicated that Cu^{2+}-induced β-lactoglobulin aggregates was formed by intermolecular pairs of free sulfhydryl groups as shown in Fig. 1F. In general, Cu^{2+} controls a diverse series of biochemical and regulatory events, conferring changes in protein structure and protein–protein interactions (Kim et al., 2008). Hane et al. (2012) reported that copper ions acted as a bridge between peptide molecules, resulting in increased stability of peptide-peptide complexes. For the other metal ions, they were totally different. The free sulfhydryl contents in Ca^{2+}- and Mg^{2+}-induced group were higher than that in no metal ions group. According to the results of Native-PAGE, there was no apparent different content of BLG aggregates induced by Ca^{2+}, Zn^{2+} and Mg^{2+} in Fig. 1F. However, there was a significant difference in the content of sulfhydryl groups as shown in Fig. 4B. It suggested that the formation of BLG aggregates induced by Zn^{2+} partly through the free thiol while more exchange of free sulfhydryl and disulfide bonds contributed to the aggregation induced by Ca^{2+} and Mg^{2+}.

A free sulfhydryl group is necessary for protein aggregation since it can make monomers bind together and package into the β-barrel structure of β-lactoglobulin in its native state. All of these results indicated that Cu^{2+} tended to induce β-lactoglobulin aggregation. Cu^{2+} could destroy β-sheets to expose free sulfhydryl groups for protein aggregation which was contradictory to the result in Pourjabbar's research (Pourjabbar et al., 2015) that α-helical state is resistant to aggregation in BLG native state. It should be mentioned that the free sulfhydryl content of Mg^{2+}-β-lactoglobulin was similar to that of native β-lactoglobulin and it implied that the conformational changes induced by Mg^{2+} were not responsible for the β-lactoglobulin aggregation, but due to the intermolecular cross-linking or intramolecular electrostatic shielding. Moreover, the free sulfhydryl group and β-turn contents of the Zn^{2+}-induced sample decreased by approximately 50%, while the β-sheet and random coil contents increased. The microstructure of Zn^{2+}-β-lactoglobulin aggregate samples was similar to that of native samples, while there was less aggregation during treatment with Zn^{2+} than in the absence of treatment, indicating that Zn^{2+} tended to change the β-lactoglobulin structure but cannot interfere with aggregation progress.

3.4. Digestibility

Simulated gastric and intestinal digestion of native β-lactoglobulin and aggregates induced by metal ions reflected the effects of metal ions on the digestive susceptibility of β-lactoglobulin. According to Chicón et al. (2008), β-lactoglobulin has the characteristics of resistance to

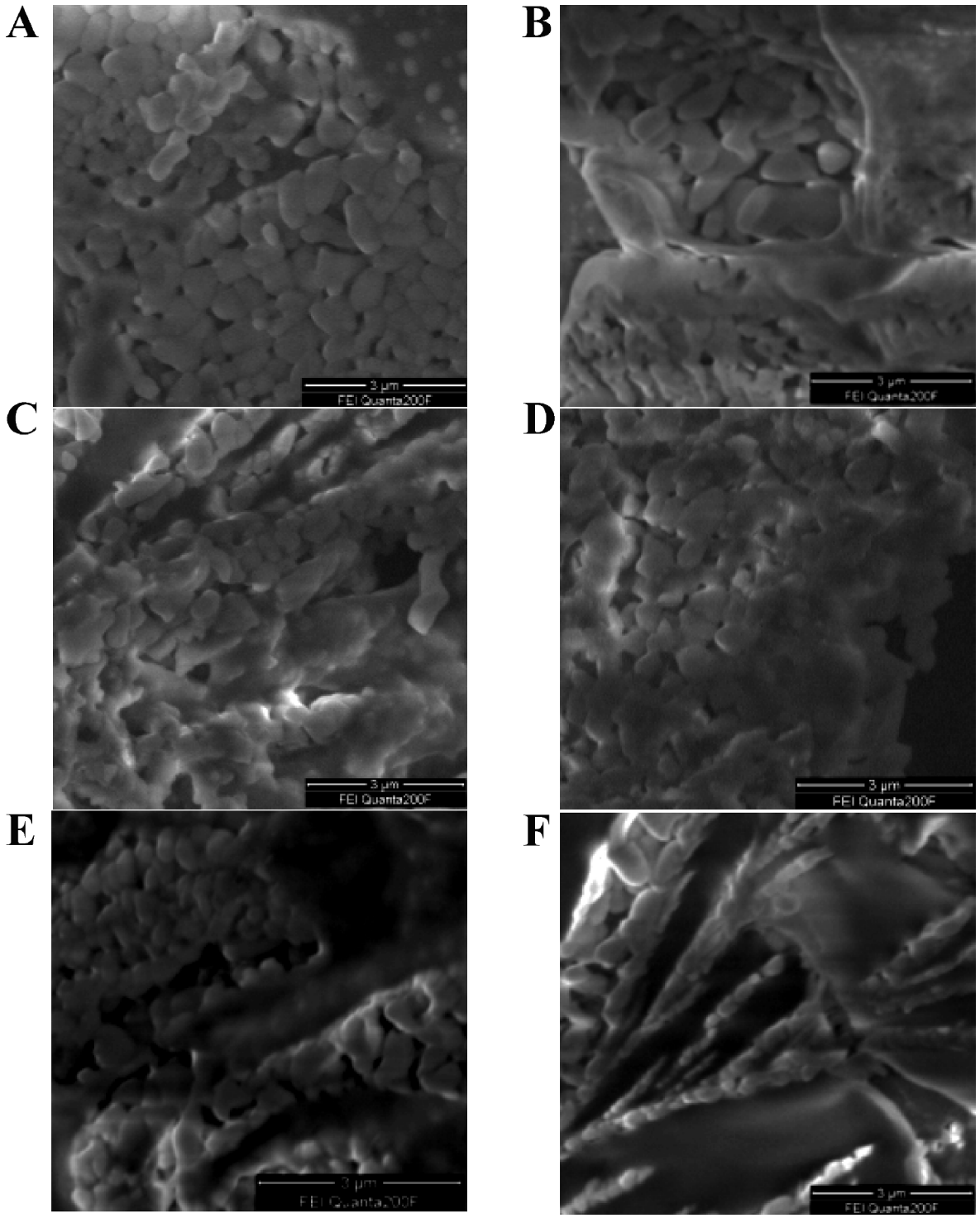

Fig. 3. Environmental scanning electron microscopy (ESEM) of β-lactoglobulin aggregates. A: β-lactoglobulin without any treatment; B: heat treatment without metal; C: Cu^{2+}-induced group D: Ca^{2+}-induced group; E: Zn^{2+}-induced group; F: Mg^{2+}-induced group.

gastric acid and to proteolytic attack. In this study, the digestive stability of aggregates induced by the four metal ions was analyzed by Tricine-SDS-PAGE. Metal ions could not improve the degree of hydrolysis of β-lactoglobulin, and the resistance to gastric digestion may be due to low pH (Breiteneder & Mills, 2005).

After 30 min of digestion in SGF *in vitro*, there were no obvious changes, and a lot of monomers and dimers remained (Fig. 5A), indicating that different forms of β-lactoglobulin were very stable and difficult to digest in gastric juice. At 1 h–2 h, there were some small molecular proteins (10 kDa) emerged excepted β-lactoglobulin aggregates treated with Cu^{2+} (Fig. 5B, line U). Then band in line U at 1 h (Fig. 5B) had a higher content of dimer than those of β-lactoglobulin treated with the other three ions, demonstrating an increased resistance of Cu^{2+}-induced aggregates to gastric digestion. It was suggested that secondary structure and intermolecular forces make it more stability.

During digestion by SIF *in vitro*, the solution was adjusted to pH 7.6 for simulating intestinal digestion. Compared with the control group, the ion-treated groups had similar intestinal digestive stability, and all of

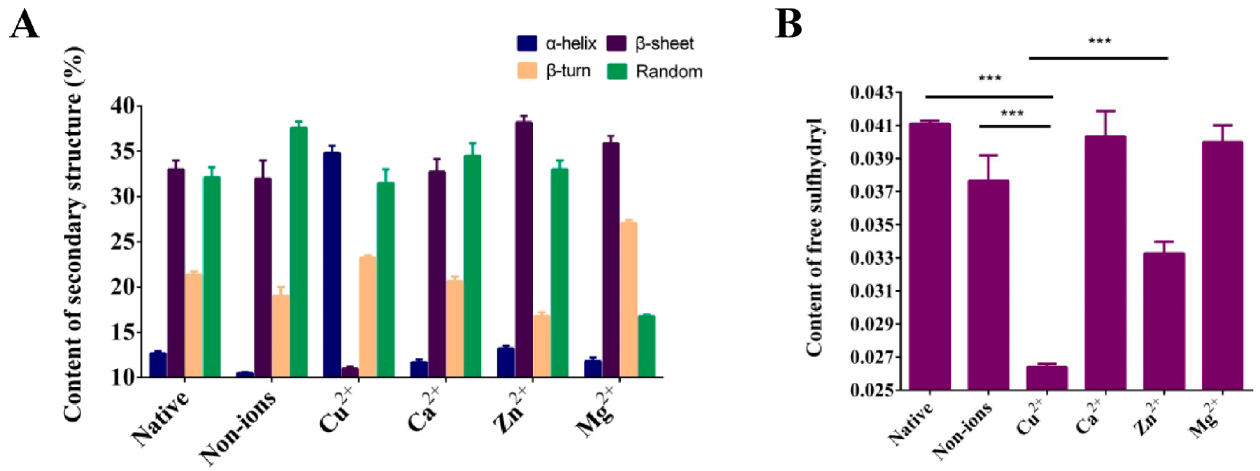

Fig. 4. The secondary structure (A) and free sulfhydryl contents (B) of samples induced by metal ions. Native: β-lactoglobulin without any treatment); No metal ions: β-lactoglobulin only heat treatment; Cu^{2+}: β-lactoglobulin aggregate induced by Cu^{2+}; Ca^{2+}: β-lactoglobulin aggregate induced by Ca^{2+}; Zn^{2+}: β-lactoglobulin aggregate induced by Zn^{2+}; Mg^{2+}: β-lactoglobulin aggregate induced by Mg^{2+}.

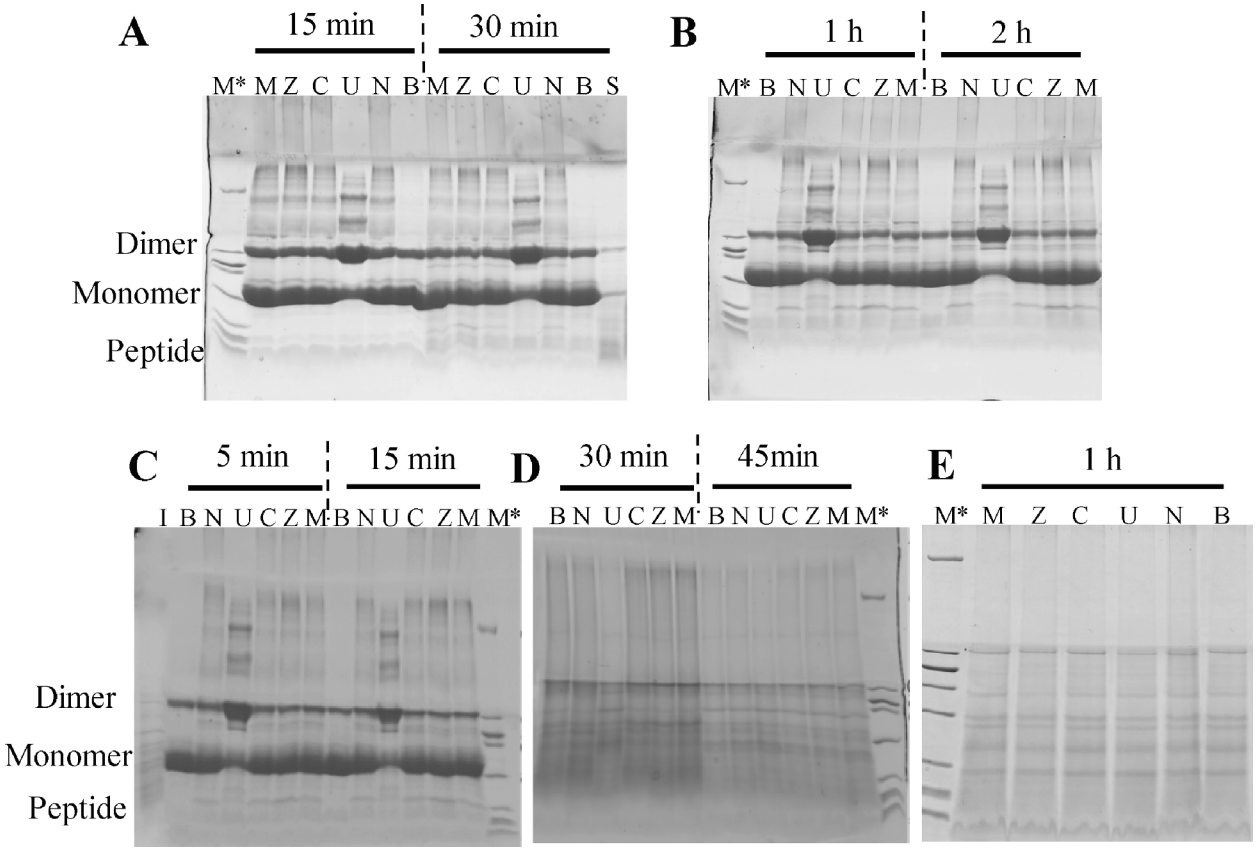

Fig. 5. Tricine-SDS-PAGE patterns of digested β-lactoglobulin aggregates treated with simulated gastric (A-B) and intestinal (C-E) fluid. M*: marker; M: Mg^{2+}-induced group; Z: Zn^{2+}-induced group; C: Ca^{2+}-induced group; U: Cu^{2+}-induced group; N: heat treatment without metal ions as negative control; B: β-lactoglobulin without any treatment as blank control; S: stimulated gastric fluid as negative control; I: stimulated intestinal digestive fluid as negative control; 5 min, 15 min, 30 min, 45 min, 1 h, 2 h: digestion time.

the samples were degraded into small peptides with the decline of 10–15 kDa protein. As shown in Fig. 5D, some monomers and dimers were broken into small peptides after digestion for 15 min and 30 min, respectively. Then there was little band at 45 min with intestinal digestion continuing. Most of the bands disappeared without distinct degradation product bands until 45 min and 60 min (data not shown). The largest aggregates disappeared after intestinal digestion for 30 min, and there was no band after 60 min of intestinal digestion (Fig. 5D and E).

3.5. Changes in IgG- and IgE-binding ability

We found from the Fig. 6 that changes of IgG and IgE binding abilities for aggregates induced by different ions showed diverse extents, and there was a negative correlation between the antigenicity and allergenicity in the Zn^{2+}-induced aggregate.

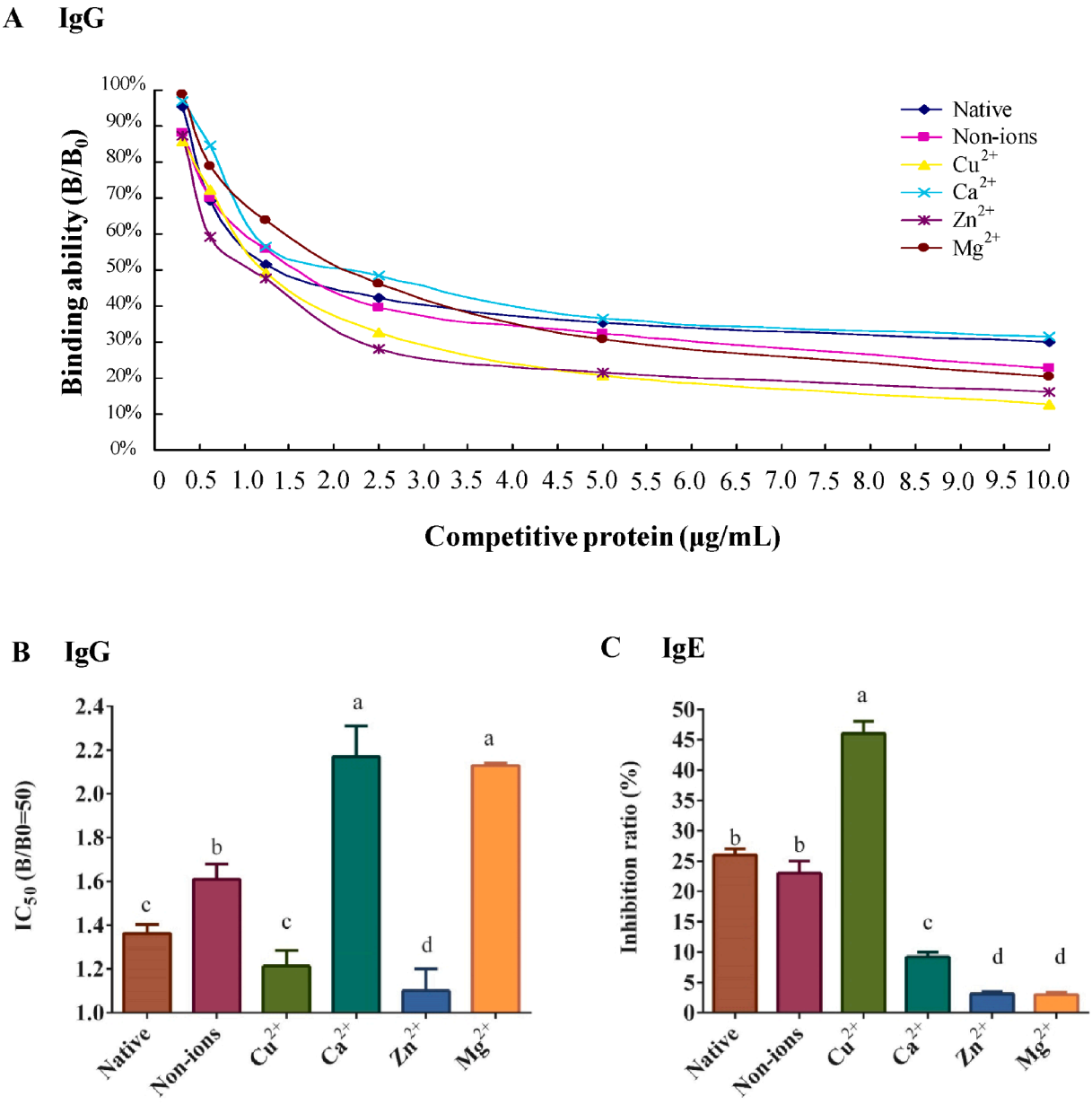

Fig. 6. ELISA of β-lactoglobulin aggregate binding to IgG and IgE. A: Standard curve defined by competitive ELISA of β-lactoglobulin aggregate samples (IgG); B: The IC_{50} value of β-lactoglobulin aggregate samples (IgG); C: The binding rate of β-lactoglobulin aggregate to the serum patients (IgE). Native: β-lactoglobulin without any treatment); No metal ions: β-lactoglobulin only heat treatment; Cu^{2+}: β-lactoglobulin aggregate induced by Cu^{2+}; Ca^{2+}: β-lactoglobulin aggregate induced by Ca^{2+}; Zn^{2+}: β-lactoglobulin aggregate induced by Zn^{2+}; Mg^{2+}: β-lactoglobulin aggregate induced by Mg^{2+}.

For IgG detection, the samples without metal ion was used to confirm whether temperature was involved in a change in antigenicity of β-lactoglobulin during aggregation. The antigenicity (IgG-binding ability) of aggregates induced by Zn^{2+} and Cu^{2+} was significantly increased compared with that of the native group as shown in Fig. 6A. In particular, the samples induced by Zn^{2+} showed the highest antigenicity in all the groups. IgG-binding ability was ordered as follows: Zn^{2+}>Cu^{2+}>native > no metal ion > Mg^{2+}>Ca^{2+} (Fig. 6B).

In Fig. 6C, the result in the no metal ion group indicated that temperature had little impact on the potential allergenicity (IgE-binding ability) of β-lactoglobulin. The allergenicity of aggregates were changed after aggregation, which may due to the conformational changes induced by metal ions. As shown in Fig. 6C, allergenicity of β-lactoglobulin induced by Ca^{2+}, Zn^{2+} and Mg^{2+} were decreased obviously. On the contrary, Cu^{2+} treatment had negative impact on the allergenicity of β-lactoglobulin which was increased evidently. IgE-binding ability was ordered as follows: Cu^{2+}>native > no metal ion > Ca^{2+}> Zn^{2+}> Mg^{2+} (Fig. 6C).

The allergy impacts associated with bovine β-lactoglobulin are a worldwide problem. In our previous work (Meng et al., 2017), we demonstrated that conformational changes of food allergens contribute to altered structure and allergenicity. According to Katarzyna's latest report (Kurpiewska et al., 2019), the antigenicity of β-lactoglobulin may be associated with the fact that the exposed and accessible antibody epitopes are hidden due to structural changes and aggregation. Regarding allergenicity assessment, the allergenicity of β-lactoglobulin was different after aggregation by metal ion induction. Peptides may change after gastrointestinal digestion, which may also change the allergenicity of proteins due to differences in epitopes (Chicón et al., 2008). The antigenicity of β-lactoglobulin aggregates was significantly affected by all four kinds of metal ions (Cu^{2+}, Zn^{2+}, Mg^{2+}, and Ca^{2+}). β-Lactoglobulin aggregates induced by Ca^{2+} and Mg^{2+} showed a lower

level of antigenic reaction than native β-lactoglobulin aggregates. A higher level of antigenic reaction was recorded in Cu^{2+}- and Zn^{2+}-treated β-lactoglobulin aggregates than in Ca^{2+}- and Mg^{2+}-treated aggregates. Those results bring positive outcomes which can reduce the burden due to milk allergy. A series of irreversible modifications induced by metal ions led to altered conformational epitopes and altered IgE-binding capacity and IgG-binding capacity. The free thiol of Cys 121 is of importance because it provides the potential for intermolecular disulfide bridging (Macierzanka et al., 2012), and the free sulfhydryl content in Cu^{2+}- and Zn^{2+}-induced aggregates had the same downward tendency, which may be closely related to the change in allergenicity. Aggregation of β-lactoglobulin may hide some epitopes, resulting in reduced allergenicity of the protein (Kleber et al., 2004).

4. Conclusion

The aggregation of β-lactoglobulin is tightly related by the external environment. In our results, Cu^{2+} induced the greatest amount of β-lactoglobulin dimer, and the aggregates were formed by mainly a combination of disulfide bonds and noncovalent bonds, which may be a major reason for the distinctive structure of aggregates. Moreover, Cu^{2+} considerably enhanced peptic digestion of β-lactoglobulin due to conformational alteration which resulted in increased allergenicity. In terms of Zn^{2+}, it strongly altered the secondary structure, and the specific antibody-binding capability of Zn^{2+}-induced β-lactoglobulin aggregates increased most among the aggregates induced by the four metal ions. β-lactoglobulin aggregates induced by Ca^{2+} and Mg^{2+} displayed lower antigenicity and potential allergenicity compared with native β-lactoglobulin aggregates and no metal ions-β-lactoglobulin aggregates. All of these data provide fundamental information and can be a useful reference for food processing technology, which find a robust way to explore the digestion, immunoactivity and allergenicity *in vitro* through different treatments.

Author contributions

Shuangwen Fei was responsible for doing a part of the experiments and writing the manuscript.

Jianwen Zhou did parts of the experiments.

Yong Wu and Pong Tong helped to collect protein samples during 12 months.

Jingyan Gao helped to detect the digestibility of experimental protein.

Hongbing chen helped to conduct the experimental design.

Xin Li (corresponding author) conducted the experimental design and revised the manuscript.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2021.130030.

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Table S1. Information of bovine milk allergic patients

No.	Gender	Age(years)	Clinical symptom	Total IgE Level (kU_A/L)	Specific IgE to milk protein level (kU_A/L)
1	female	ND*	ND*	>100	2.7
2	male	2	ND*	>100	3.7
3	male	4	Urticarial	>200	5.9
4	male	2	Acute upper respiratory tract infection	>200	2.4
5	female	3	ND*	>200	1.1
6	male	20	Urticarial	>200	1.9
7	male	1	Bronchial asthma	>200	2.0
8	female	4	ND*	100-200	9.7
9	male	3	Chronic rhinitis	>200	2.4
10	male	2.5	Asthma Bronchitis	>200	1.8
11	male	2	Bronchial asthma	>200	1.3

Note: those patients were allergic to milk protein. CM: Cow’s milk