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Effect of 2,2-azobis (2-amidinopropane) dihydrochloride oxidized casein on the microstructure and microrheology properties of emulsions

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Abstract The impacts of protein oxidation on the droplet size and microrheology properties of casein emulsions with 20% oil content were investigated. The degree of protein oxidation was indicated by carbonyl concentration. The droplets in the emulsions of different-oxidation-degree casein had bimodal distribution, but their size altered due to oxidation. The effects of protein oxidation on the morphology, motion type, viscoelasticity, and stability of droplets were also investigated by microrheology analysis. The droplet motion was blocked by protein oxidation due to mean square displacement slope results. Solid–liquid balance values provided the liquid behavior dominating these emulsions. Oxidation of carbonyl concentration 16.72 raised the primary droplets, increased the elasticity, decreased the viscosity, and promoted the droplet motion rate, resulting in better stability of emulsions. Further oxidation promoted the aggregation of droplets and resulted in poor stability.

Keywords: casein, protein oxidation, emulsion stability, microrheology, microstructure

Introduction

Emulsion properties are related to a wide range of protein applications (1). Casein is an important food ingredient due to its high nutritional value and amphiphilic nature (2). Casein comprises αs1, αs2, β, and κ, which are rich in residues impacting the α-helix and β-sheet, leading to the peptides stretch shaped (3). In the suspension of low concentration, casein molecules mainly exist in the form of monomer. As the protein concentration increases, casein molecules can be self-assembled, driven by hydrophobic interaction, hydrogen bonding, and electrostatic interaction to form micelle structure (4). In emulsions, casein could act to form viscoelastic films around dispersed droplets to reduce the interfacial tension at the oil–water interface, which keeps the emulsions stable over time (5). Therefore, the properties of protein emulsion are related to the structure and properties of the adsorbed casein film at the oil–water interface.

A crucial factor related to emulsion property deterioration is oxidation. Various lipid oxidation initiators have been used to study the emulsion. Lipid peroxides derived from lipid oxidation upon storage for up to 2 weeks were evaluated to describe the stability characteristics of protein-stabilized emulsions (6). Oxidation models were also built in emulsion studies. Iron is one of the widely used pro-oxidants to induce protein oxidation (7). Reaction with ammonium thiocyanate and ferrous iron solution in the dark can accelerate the oxidation of protein-stabilized emulsions (8).

Protein in emulsion is also sensitive to oxidation. Protein oxidation either in raw milk or dairy products occurs along with physiological activities and environmental changes (9). Besides, improper processing conditions during manufacture can induce and promote protein oxidative modification, and occasional exposure to UV during storage can also initiate these reactions (10). Interfacial proteins can interact with lipid oxidation products (11). Due to oxidation together with lipid, structural modification occurs in backbone, aliphatic side chain, and aromatic side chains of protein (7). Increasing the intensity of 2,2-azobis (2-amidinopropane) dihydrochloride (AAPH) could result in gradual accumulation of protein carbonyl groups and derivatives, in addition to loss of protein-free amino acid and sulfhydryl groups. Exposure of protein to AAPH led to the transformation of secondary structures such as loss of α-helix structure and increase of β-sheet (12). Both the degree of protein structure modification and the content of protein aggregation with high molecular weight cross-linked by covalent or non-covalent bonds increased because of protein oxidation (10), and the gradual aggregation of protein was finally induced due to oxidation (13). As emulsification is the ability of protein on forming highly elastic films on the synergistic interfacial

preventing the aggregation of dispersion phases (9), oxidized modification of structural may impact the emulsification of proteins. Therefore, investigating the impact of protein oxidation on emulsion properties may supplement the study of oxidation in emulsions.

Changes on macroscopic properties are attributed to microscopic transformation. Thus, the objective of this work was to investigate the impact of AAPH-induced protein oxidation on the microstructure and microrheology properties of 20% oil casein emulsions. The protein carbonyl groups concentration, size distribution, morphology, motion type, viscoelasticity of droplets, and stability of casein-based emulsions were determined to provide some supplement to the oxidation prevention for casein production and processing.

Materials and Methods

Materials 2,2-azobis (2-amidinopropane) dihydrochloride (AAPH) and 5,5′-ddithiobis (2-nitrobenzoic acid) (DTNB) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium azide and 2,4-dinitrophenyl hydrazine were obtained from Sinopharm (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China). Bovine serum albumin (BSA) was purchased from Dingguo Biota Technology Co., Ltd. (Beijing, China). Edible-grade casein was provided by Saishang Dairy (Saishang Dairy Co., Ltd., Ningxia, China). Soybean oil was purchased from the local supermarket (Tianjin, China). All other chemicals were of analytical grade and used without further purification.

Oxidation of casein by AAPH Casein solution (10 mg/mL containing 0.5 mg/mL sodium azide, suspended in 0.1 mol/L sodium phosphate buffer, pH 7.0) was mixed with 20 mmol/L AAPH (final concentration) and then hermetically incubated and shaken at 45°C (7) for 1, 2, 3, and 4 h, which were named as C1, C2, C3, and C4, respectively. Nature casein was named C0 for control. Ice bath was used to adjust the temperature after incubation.

Preparation of emulsion with oxidized casein Native and oxidized casein (C0, C1, C2, C3, and C4) were dispersed in deionized water (pH 7.0) for 2 h at 25°C, and were then attenuated to a fixed suspended casein concentration (1%, w/w). The emulsions containing 20% (w/w) soybean oil were prepared by mixing the protein suspension and the oil with an APV-2000 homogenizer (APV Gaulin; SPX Flow Technology, Crawley, UK) at 22,000 rpm for 3 min at 25°C and then homogenizing at 30 MPa. The emulsion type was to be validated by the following detection of fluorescence microscope in 2.3.3. Sodium azide of 0.02% (w/w) was added to prevent microbial growth during the determination.

Determination and evaluation

***Characterization of oxidized casein*:** Native and oxidized casein were suspended in deionized water, and the supernate was attenuated to a protein concentration of 3.5 mg/mL Protein carbonyl groups were quantified according to the method described by Chen *et al*. (14).

Native and oxidized casein was dissolved in phosphate buffer (0.01 M, pH 8.0). Magnetic stirred 2 h at room temperature and then centrifuge the solution at 10,000× *g* for 30 min. Attenuated the supernate to protein concentration of 3.5 mg/mL. Surface hydrophobicity index of native and oxidized casein were measured according to the method described by Wu *et al*. (15) with the RF-5301pc fluorescence spectrophotometer (Shimadzu, Kyoto, Japan).

The absorbency was evaluated using SP-721 UV spectrophotometer (Shanghai, China). Soluble protein concentration was detected by the Biuret method with BSA as standard.

***Validation of emulsion type*:** Fluorescence microscope was used to validate the emulsion type of casein emulsions. The microstructures of emulsions were observed by an inverted fluorescence microscope (Olympus CKX41; Olympus Co. Ltd., Tokyo, Japan). The samples were quickly stained with a solution of Rhodamine B directly after emulsion preparation. The observation at the microscope (10×100) was completed in 20 min to prevent the fluorescence quenching of protein.

***Determination of droplet size of oxidized casein emulsions*:** The size distribution of the emulsion droplets was measured by a laser analyzer (BT-2001) (Dandong Bettersize Instruments Ltd., Liaoning, China) at 25°C according to the method described by Sun *et al*. (16). The measurement was taken directly after a low intensity ultrasound treatment when the particles were still dispersed in the collection fluid preventing the settlement of casein in the unstable system. The results were analyzed with Bettersize analysis software and transformed into a size distribution using a scattering model for the analysis of the raw data.

***Measurement of microrheology of oxidized casein emulsions*:** The microrheology properties of emulsions were measured by Rheolaser Master (Formulaction, l'Union, France) based on the diffusing wave spectroscopy theory. The mean square displacement (MSD) curves were calculated from the dynamic speckle images, scattering by the sample as a function of time (17). After preparation, the emulsion samples were quickly measured the backscattered light. The tracing particles of emulsions were the droplets. MSD slopes, solid–liquid balance (SLB) values, elasticity index (EI), and macroscopic viscosity index (MVI) values were calculated from the slope, the slope of the platform area, the reciprocal of the value of the platform area height, and the slope behind platform area of the MSD curves calculated by software of Rheolaser Master (Formulaction).

***Determination of emulsion stability*:** The kinetic stability of emulsions was monitored by Turbiscan ASG (Formulaction). The measurement principle of the kinetic stability was that the pulse near-infrared light was utilized to scan the suspension or emulsion system, and the light beam was multiple scattering by the particles or droplets. The transmitted and scattered lights were detected by a synchronous optical detector so that the suspension states of the particles or droplets were obtained by scanning of the image processing system. The transformation of the particle or droplet states in the suspension

could be analyzed via the continuous scanning for a set period of time.

Immediately after preparation, the emulsions were placed into flat-bottomed cylindrical glass tubes (140 mm height, 16 mm diameter) to obtain the initial measurement of the backscattered light. Tubes were stored at 25°C and hourly measurements were taken for 12 h.

The stability of the whole system was characterized by a stability index. The stability index kinetic curve is given by the following equation:

$$\mathrm{di} = \frac{\sum_h \left|\mathrm{scan}_i(h) - \mathrm{scan}_{i-1}(h)\right|}{\mathrm{H}} \quad (1)$$

where scan_i (h) indicates the intensity values of transmission at time i.

***Experimental design and statistical analyses*:** Tests were performed in triplicate to obtain parallel data shown in the illustrated figures, and the results were presented as mean±SD. The experimental data were analyzed by one-way AVONA followed by Student's T-test, and the significant differences among the means were determined at a 95.0% confidence level. Treatment means were considered to be significantly different at p<0.05. The graphs were prepared by Origin 9 and Adobe Photoshop CS6.

Results and Discussion

Characterization of oxidized casein Carbonyl is formed by protein oxidation and is the most commonly used indicator of protein oxidation (13). In this research, casein oxidation induced by AAPH exposure was measured by the formation of carbonyl groups. Results (Table 1) indicated a significant (p<0.05) increase in the protein carbonyls content for casein samples subjected to AAPH with increasing oxidation time of AAPH at 45°C. Carbonyl groups concentration was increased with the oxidizing time. Compared with the control, the carbonyl groups concentration of 4 h of oxidized casein was indicated to a three-fold increase. The trend of carbonyl concentration change was identical to previous reports of soybean protein as well (10,18). The formation of protein carbonyls implies the changes induced by oxidation was related to the transformation of individual amino acid residues (10), and they have the tendency to form covalent linkages with electron dense groups. Thus, after 4 h of oxidization, more amino acid residue was formed in casein, which may interact and crosslink with each other, thus lead to protein polymerization or aggregation, and further affect the properties of proteins.

Surface hydrophobicity of protein is one of the characteristics that can evaluate the protein conformation transformation. Oxidized casein with increasing oxidizing time of AAPH led to a gradual decrease of surface hydrophobicity index compared with unoxidized sample (p<0.05) (Table 1). After 1 h of oxidation, surface hydrophobicity index reduced 13.81% compared with control. After 4 h of oxidation, the decrease was 44.66%. Wu *et al*. (13) also reported that protein oxidation resulted in decrease of surface hydrophobicity. The result could indicate the combined effect of conformational transformation of casein. According to the previous research on whey protein (19) and soybean protein (20), it was suspected that oxidation induced structural unraveling and reaggregating of casein, allowing a greater embedding of hydrophobic residues and the formation of new hydrophilic components (such as carbonyls groups), which might lead to the decrease of casein surface hydrophobicity.

Table 1. Protein carbonyl groups and surface hydrophobicity index of casein incubated with increasing oxidation time in 20 mmol/L AAPH at 45°C

Oxidation time (h)	Carbonyl groups (nmol/mg)	Surface hydrophobicity index
0	6.27±0.16a[1)]	678.44±2.81a
1	10.08±0.50b	584.72±4.79b
2	16.72±0.31c	512.36±2.76c
3	19.09±0.62d	479.14±5.61d
4	20.73±0.34e	375.43±1.72e

[1)]Least-squares difference was used for comparison of mean values among treatments and to identify significant differences. Values in the same column signed by different letters (a–e) are significant difference (p<0.05).

Micromorphology of emulsions Sets of fluorescence microscopy of emulsion droplets were captured to distinguish and verify the emulsion type. Emulsion containing 20% oil and oxidized casein samples were investigated at the same shear rate (Fig. 1A) compare the oxidation effects on the micromorphology of the droplets and the interface aggregation of casein. Protein and aqueous phase appeared fluorescence and oil phase appeared non-fluorescence in the image of emulsions. In emulsions, the non-fluorescence areas were encompassed in the fluorescence areas, indicating that the emulsion type might be O/W. Casein existed within aqueous phase and aggregated at the interfaces but not in oil phase in both emulsions. As oxidation degree increased, the droplets grew larger and the protein aggregation content increased, indicating that oxidation impacted the uniform distribution of casein at the surface of droplets.

The results agreed with the report that only 30-40% of the interface was covered with proteins in emulsions because only a part of the hydrophobic group were attached at the water–oil interface, and the other protein peptide fragments were located in the aqueous phase (7). Emulsifying process was the molecular rearrangement and reaggregation of protein at the water–oil interfaces drove by the interfacial tension. In suspension, the casein existed in the form of micelles via the hydrophobic effect and hydrogen bonding (21). In emulsions, due to the interfacial tension exceed the hydrophobic effect, the casein with surface active were forced to the interface and the original conformation changed (22), molecular refolding taken place so that the casein rearrangement with hydrophobic grouping

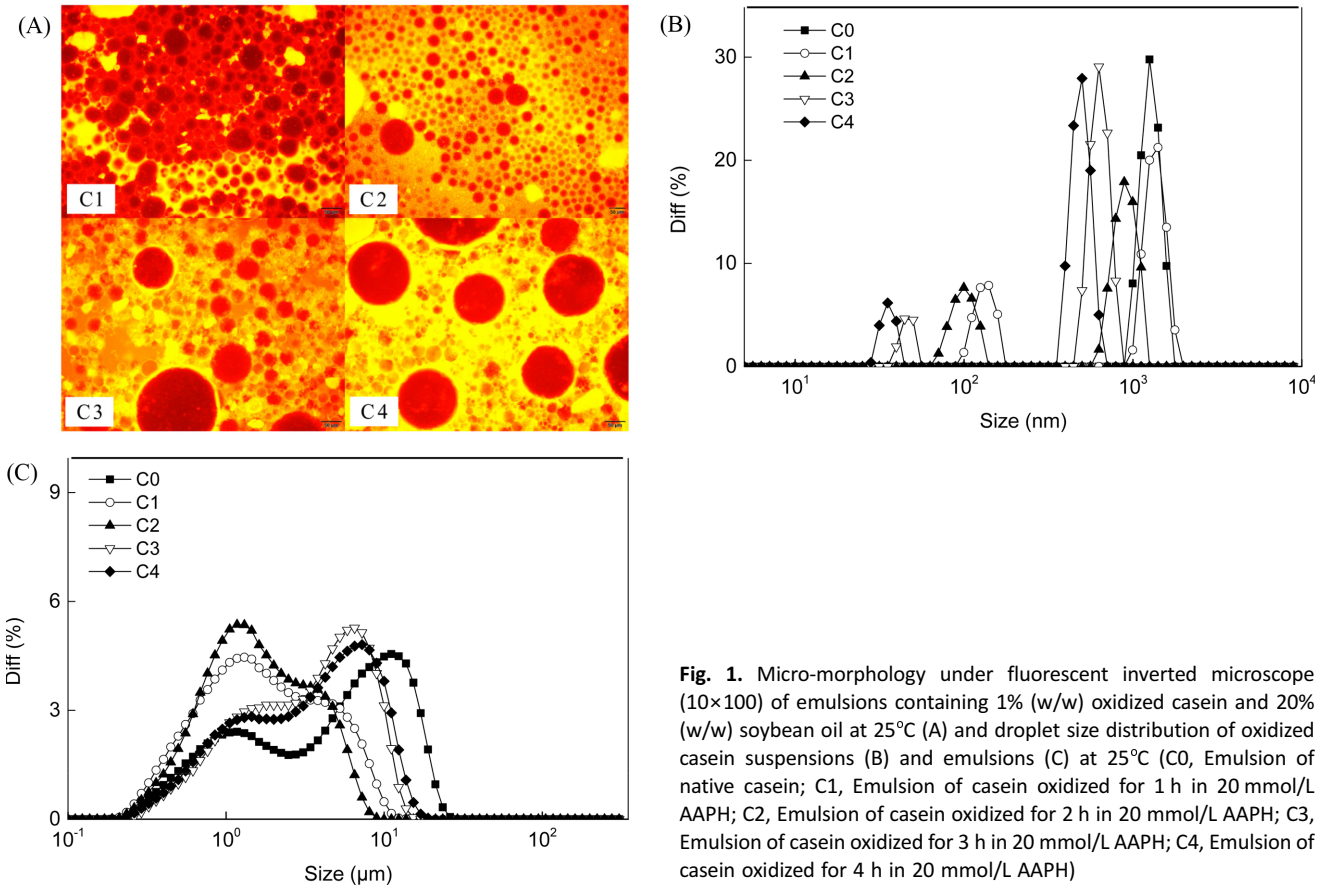

Fig. 1. Micro-morphology under fluorescent inverted microscope (10×100) of emulsions containing 1% (w/w) oxidized casein and 20% (w/w) soybean oil at 25°C (A) and droplet size distribution of oxidized casein suspensions (B) and emulsions (C) at 25°C (C0, Emulsion of native casein; C1, Emulsion of casein oxidized for 1 h in 20 mmol/L AAPH; C2, Emulsion of casein oxidized for 2 h in 20 mmol/L AAPH; C3, Emulsion of casein oxidized for 3 h in 20 mmol/L AAPH; C4, Emulsion of casein oxidized for 4 h in 20 mmol/L AAPH)

outside the aqueous phase (23), and reaggregated to a protein film. Then the micellar structure no more existed at the interface. The content of casein segments attached on the interface was probably due to the surface hydrophobicity content as the surface hydrophobicity index decreased by casein oxidation from 678 to 373. Furthermore, oxidation promoted covalent cross-linking-induced aggregation of casein, the chemical energy of which was higher than the surface tension of the oil-water interface, so that the rearrangement of casein molecule at the interface during emulsifying was prevented and the casein aggregation existed.

Effect of casein oxidation on droplet size distributions of emulsion

Droplet size of the emulsions influences the emulsion stability, rheological properties, and shelf stability of protein-based formulations (24). Not all protein in the emulsions was absorbed to the droplet surface (25), so how casein free of oil behaved was necessary to be examined (Fig. 1B). The particle size of native casein suspension was unimodal distribution while of oxidized casein suspensions were with bimodal distribution. After oxidation, the distribution divided into two peaks of mean droplet size at 0.1 and 1 μm. In this conation, oxidation of AAPH altered the particles of casein in two ways: partly decrease caused by breakage of molecule backbone and partly increase caused by cross linking of amino acid residue. The two ways occurred simultaneously, which was in accord with studies of oxidation on soy protein isolate oxidized by hydroxyl radical-generating systems (19) and milk protein concentrate oxidized by UV (10).

According to Fig. 1C, the droplet size of native casein emulsion was unimodal distribution while of different oxidation-degree caseins emulsions fell in a bimodal distribution. Compared with control, the emulsion droplets of C1 and C2 (carbonyl groups concentration <16.72 nmol/mg) increased in the first peak (with mean droplet size about 1 μm). Whereas, further protein oxidation (C3, C4) increased the last peak (with mean droplet size about 8-10 μm) in droplet size distribution curves. Nevertheless, the droplet size of oxidized casein emulsions decreased compared with the native, which agreed to studies of soy protein emulsions (14). Therefore, oxidation of certain degree (carbonyl groups concentration <16.72 nmol/mg) increased the formation of smaller droplets in casein emulsions.

The status of droplet size is associated with the amount of protein existed in the aqueous phase and at the water–oil interface (26). Therefore, the changes of the two peaks in oxidized-casein emulsion droplet size distribution may be explained probably by two reasons: the smaller casein particle formed due to oxidation absorbed and rearranged at the water–oil interface facilitated the formation of the smaller droplet and contributed to the former peak of droplet size, while the decreased surface hydrophobicity of casein by oxidation reduced the casein content at the water–oil interface so that the

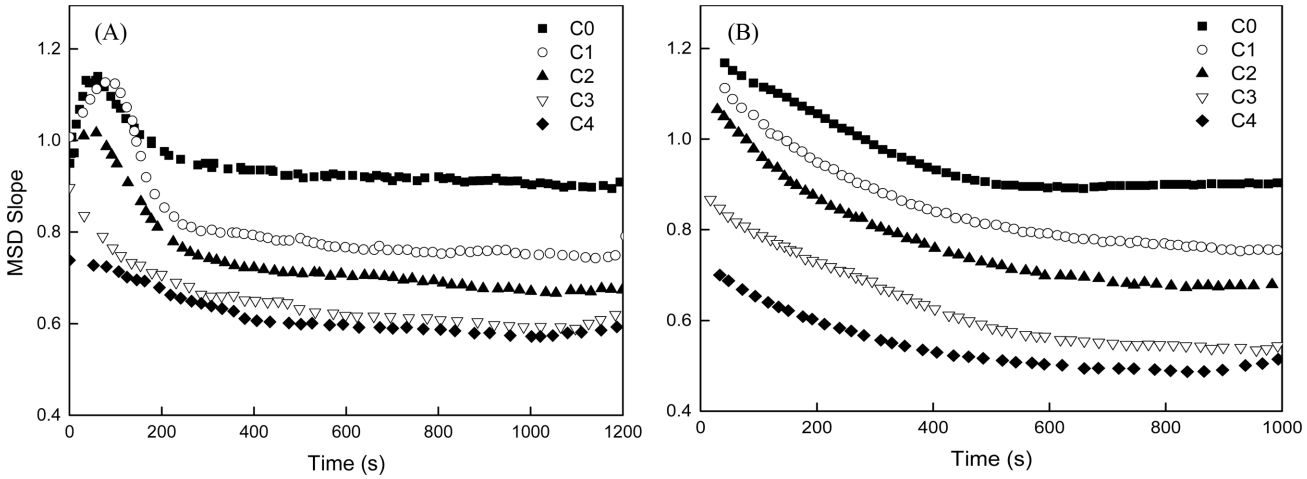

Fig. 2. Mean Square Displacement (MSD) slopes of oxidized casein suspensions (A) and emulsions (B) at 25°C (C0, Native casein emulsion; C1, Emulsion of casein oxidized for 1 h in 20 mmol/L AAPH; C2, Emulsion of casein oxidized for 2 h in 20 mmol/L AAPH; C3, Emulsion of casein oxidized for 3 h in 20 mmol/L AAPH; C4, Emulsion of casein oxidized for 4 h in 20 mmol/L AAPH)

droplet of emulsions aggregated and grew, thus, the latter peak increased. As the aggregation process continues, the number of primary droplets in the system rose until droplet-droplet aggregation dominant, which was similar to the whey protein fluid gels (27). Changes in sizes of casein particles probably also affected droplet sizes of emulsion samples, but the exact reason of the changes with oxidation may not be identical.

Effect of casein oxidation on Mean Square Displacement (MSD) slope of emulsion There are two approaches for microrheology measurement: the active microrheology and the passive microrheology. The active microrheology is to measure the local deformation with micron sized particles due to an applied stress while the passive microrheology from thermal energy (~k_bT) (17). The instrument used in this work was the passive one, performed by measuring the displacement of particles resulted from thermal energy, and no mechanical or external stress was applied, namely the Brownian motion. The speckle patterns were performed with a patented algorithm obtaining the MSD of the particles, which can provide the information on the motion types of the samples.

The ensemble-averaged MSD was calculated as a function of the decorrelation time in order to quantify the dynamics of the casein-oil emulsion particles. The slop of MSD at a decorrelation time provides motion information (28). In emulsion system, motion types of the droplets are of two situations: Brownian and Ballistic. When droplets randomly move without any particular destination or regulation, it is belonged to Brownian motion. A ballistic motion corresponds to a specific motion of the particles, often corresponded to emulsions. Over some period of observation, the droplets in the emulsions were free to move in the continuous phase for the nature of the motion, and then they changed to different states (17): When the MSD slop less than 1, it means that the motion of the particles or droplets were blocked, when it equals to 1, it illustrates a motion type of Brownian, and when it is greater than 1, it illustrates a motion type of Ballistic. In Fig. 2 the information of motions were provided. The native and oxidized casein suspensions free of oil showed difference in MSD slop (Fig. 2A). The initial values of MSD slops of C0, C1, C2 particles were less than 1, then grew to greater than 1 and fell back at very short time, after that the MSD slopes kept approximately stabile, meaning that the particle motion fell to ballistic motion firstly, and then reached a stable state. The MSD slops were decline with the oxidation degree of casein, indicating that oxidation promoted casein to form the micelle or some kinds of gel-like structure in aqueous phase so that the motion of casein colloid particles were blocked.

In the emulsions (Fig. 2B), the MSD slopes of C0 fell firstly and kept stable after about 500 s at the value lower than 1, meaning that the droplets of casein emulsion acted the ballistic motion and then the motion was blocked, which might be caused by the formation of some structure drove by a weak interaction. The initial values MSD slopes of oxidized casein emulsions decreased with the oxidation degree increased, and the MSD slopes declined with detecting time, indicating that the motion of droplets were blocked more, which might be resulted from the stronger interaction of droplets.

Since protein in emulsions acted as isolations around the disperse phase droplets at the interface to reduce the interfacial tension (26), the motion of droplets depended on the structure and the amount of casein existed in the aqueous phase and at the oil-water interface. One of the possible mechanisms for the transformation of droplet motion type would be structural rearrangement of the casein at the water–oil interface, which may be affected by protein oxidation via the carbonylation and sulfhydryl groups degradation of casein, and promoted decrease flexible structures of casein molecular, to the disadvantage of casein molecular rearrangement during emulsifying process. Thus, oxidative modification of protein molecular decreased the casein content at the surface of droplets via the decrease of the hydrophobic grouping and the increase of covalent aggregation in

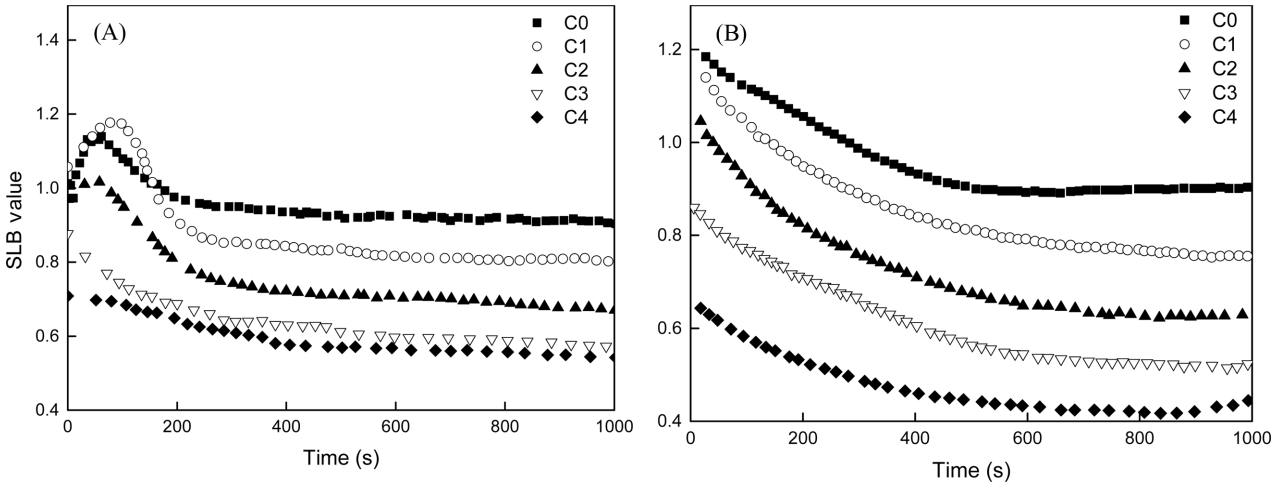

Fig. 3. Solid Liquid Balance (SLB) values of oxidized casein suspensions (A) and emulsions (B) at 25°C (C0, Native casein emulsion; C1, Emulsion of casein oxidized for 1h in 20 mmol/L AAPH; C2, Emulsion of casein oxidized for 2 h in 20 mmol/L AAPH; C3, Emulsion of casein oxidized for 3 h in 20 mmol/L AAPH; C4, Emulsion of casein oxidized for 4 h in 20 mmol/L AAPH)

casein, so that the interaction of droplets was increased and there was a tendency to form the non-covalent structures (29).

Effect of casein oxidation on Elasticity Index (EI) and Viscosity Index (MVI) value of emulsion At the interface of the emulsion, proteins realign themselves to position their surface hydrophobic residues within the oil phase and hydrophilic residues within the aqueous phase (30), and then strong viscoelastic films can be developed. The film at the interface can resist mechanical stress, provides electrostatic and steric stabilisation. Protein aggregation occured under conditions where the net attractive forces outweigh the net repulsive forces (25). Due to formation of the diminutive droplets in oxidized casein emulsions (discussed in 3.2. Droplet size distributions of the emulsions), the area of oil-water interface increased. Thus, droplets interactions and aggregation probably be more depended on viscoelasticity.

According to Tisserand *et al*. (17), the viscoelasticity of the films was measured by the EI and MVI values. EI is the reciprocal of the value of the platform area height in the MSD curve, consistent with the transformation of the elasticity of the emulsion system, indicating the elastic property of samples. MVI is the slope of the MSD curve behind platform area, consistent with the transformation of the viscosity of the emulsion system, indicating the viscous property of samples.

The EI and MVI value of solutions and emulsions of oxidized casein at the 20^{th} min were showed in Table 2. Oxidation reduced the elastic of the casein particles and increased the viscosity in suspensions free of oil. After oxidation (carbonyl concentration >20.73 nmol/mg), the EI value of oxidized casein particle decreased 31.71% compared with the native casein, while the MVI value increased 5-fold. The trend of EI value of emulsions was contrary to the suspensions, increased from 1.39×10^4 (control) to 1.94×10^4 (C3) and decreased to 1.88×10^4 due to further oxidation. The MVI values of oxidized casein emulsions increased from 2.26×10^5 (control) to 5.63×10^5 (C4), the trend of which was agreed with the suspensions.

The viscoelasticity of droplets in emulsions was 10-fold less than the casein particles in suspensions, which might due to attributed to the micellar structure of casein in suspensions. Casein molecules formed aggregated via the non-covalent interaction and formed the micellar structure, which was an amphiphilic comb-shape block copolymer, and the "coat" structure of the casein micellar had higher viscoelasticity. Oxidation declined the hydrophobic index of casein and damaged the "coat" structure, resulted in the EI value decreased. In emulsions, the micellar structure was not existed so that the viscoelasticity decreased. The surface protein aggregation contributed to the thicker protein interfacial layer, thereby helping to protect the droplets against creaming and aggregation (31). Therefore, the elasticity and viscosity properties of droplets in emulsions were related to the surface hydrophobicity of casein, which altered due to the oxidation-induced aggregation. As the coalescence of droplets was closely related to the viscoelasticity of interfacial protein films (32), protein film with low elasticity value could not prevent the droplets from destroying and aggregating, resulting in emulsion phase separation. Larger protein aggregates were more effective at lower interfacial tension, and possible increment of the emulsion droplets might increase the viscosity (20). Thus, EI value was related to the rheology of the samples such as mesh-size, hardness, gelation velocity, and the recovery rate after shearing, and the MVI value was due to the kinetic velocity (33), describing the fluid properties of emulsion. Therefore, the results of EI and MVI value indicated that, low degree of casein oxidation in emulsion probably promoted thicker protein film, resulted in prevention of droplet aggregation.

Effect of casein oxidation on Solid Liquid Balance (SLB) value of emulsion SLB value is the slope of the platform area in MSD curved in a logarithmic coordinate, indicating the equilibrium state of the

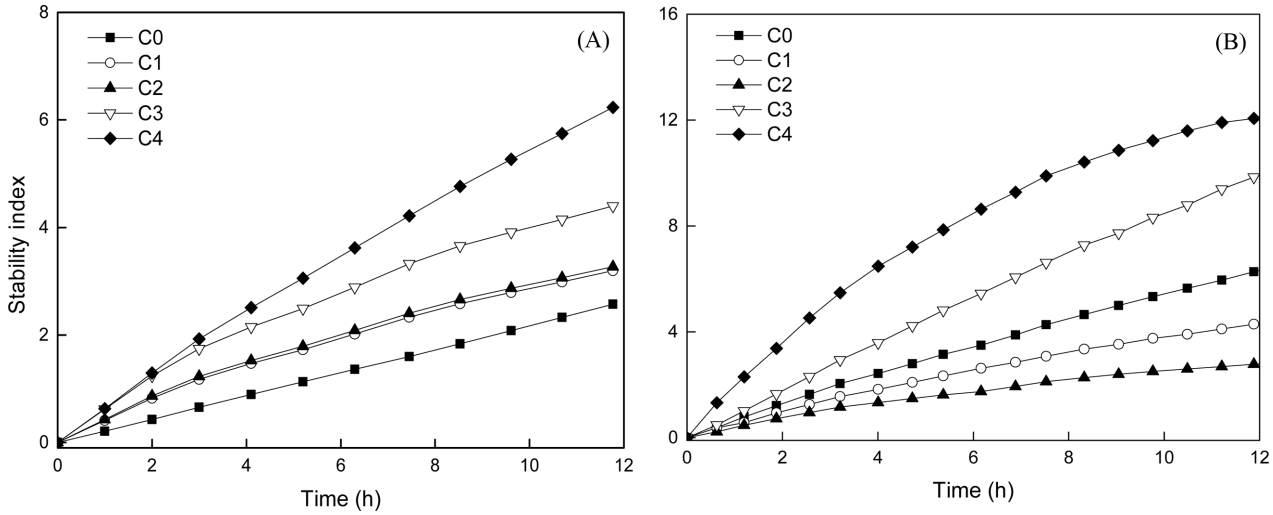

Fig. 4. Stability indexes (SI) of oxidized casein suspensions (A) and emulsions (B) at 25°C (C0, Native casein emulsion; C1, Emulsion of casein oxidized for 1h in 20 mmol/L AAPH; C2, Emulsion of casein oxidized for 2 h in 20 mmol/L AAPH; C3, Emulsion of casein oxidized for 3 h in 20 mmol/L AAPH; C4, Emulsion of casein oxidized for 4 h in 20 mmol/L AAPH)

emulsion (17). SLB value characterizes the rheological properties intuitively. A SLB value lower than 0.5 illustrates a less degree of particle movement, which indicated the emulsion greater solid characteristics. When the slope of the curved increased to higher than 0.5 but lower than 1, it meant that the rate of particle movement was not prevented or reduced, and the sample had greater liquid characteristics (34). When the SLB value is higher than 1, it illustrated a viscous liquid (17). The SLB value could also be used for comparing the similar characteristics of samples or monitoring the maturing of the samples (35).

As was shown in the suspensions of casein (Fig. 3A), the SLB values decreased with oxidation. The initial values of C0, C1, and C2 were equals to 1 while C3 and C4 higher than 0.5 but lower than 1, indicated that C0, C1, and C2 suspensions were viscous liquid and C3 and C4 suspensions were greater liquid after shearing. The SLB values of identical sample decreased with time but were not lower than 0.5, meaning that the balance was not stable and the liquid characteristics got greater. In emulsions (Fig. 3B), the SLB value decreased with time until a balance reached after 500s. At the balance time, the C1, C2, and C3 emulsions tended to be greater liquid according to the SLB value closed to about 0.6, and the C4 emulsion tended to be greater solid according to the SLB value fell below 0.5. Therefore, protein oxidation altered the equilibrium state and motion rate of the droplets in casein emulsions. The motion rate and fluidity of droplets were promoted by the certain casein oxidation.

Effect of casein oxidation on stability of emulsion Stability evaluation proceeded with Near-infrared light as a light source, scanning the sample cell from the bottom to the top. The Stability Index (SI) was a formula of calculation with valuable, representing the comprehensive property of transformations in concentration and particle size in the specific time. A greater rangeability of the SI indicated a higher degree in the instability of a system. The SI measurement was taken directly after shearing, which dispersed the droplets in the emulsion.

The stability of the oxidized casein suspensions free of oil according to the slope of SI values (Fig. 4A) indicated that oxidation might change the transformations in concentration and particle size of casein. The value change of SI indicated that oxidation decreased the particle stability of casein in suspensions. In emulsions (Fig. 4B), there shown a larger rangeability of SI than in suspensions, implying a less stability of the emulsions than suspensions. The value change of emulsions firstly decreased with oxidation, and then increased, indicating that the kinetics stability of emulsion was promoted by a certain degree of protein oxidation, and further oxidation declined the stability.

Due to the unfolding and refolding occurred in protein via oxidation (9), the hydrophilic and hydrophobic groups on the surface of casein molecule altered: groups embedded inside exposed via the unfolding of the protein, while groups exposed outside might converted to other groups. Moreover, due to oxidation, breakage occured in the side chains and backbone of proteins, covalent bonds were unfolded (13), and molecular weight altered. Therefore, the stability of concentration and particle size in casein emulsions might be affected by the structural transformation of the hydrophilic and hydrophobic groups, protein chains and backbone breakage, covalent bonds, and molecular weight.

The rapid SI transformation in emulsions indicated that the certain oxidized samples, C1 and C2, got well stability in emulsions, of which emulsions primary particles rose and particle-particle aggregation were blocked. The emulsions with well stability also had high elasticity and motion rate. Thus, the stability of casein emulsions might also be related to the size distribution, viscoelasticity, and

motion rate of droplets. These combined action induced by oxidation altered the properties of casein emulsions to a better stability or worse.

Thus, according to the results above, oxidation impacted the properties of casein in respects of droplet size distribution, morphological characteristics, and microrheology properties. Carbonyl concentration indicated the degree of protein oxidation, increased from 6.27±0.16 to 20.73±0.34 when exposed to 20 mmol/L AAPH for 4 h, and the surface hydrophobicity index decreased from 1 to 2 at the same time. The 4 oxidation-degree casein emulsion droplets were all with bimodal distribution but size altered due to oxidation. The motion of oxidized casein emulsion droplets was blocked with the oxidation degree, and liquid behavior dominates in these emulsions. Oxidation of carbonyl concentration 16.72 raised the primary droplets, increased the elasticity and decreased the viscosity of droplets, and promoted motion rate of droplets, resulted in better stability of casein emulsions. While further oxidation promoted droplets aggregation and resulted in poor stability.

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