## Abstract of thesis:

# Studies on the structural modification of protein aggregate induced by freezing process

**Fang Bowen**

### Introduction

The characteristics and the functions of protein suspensions are based on the higher-order structure of proteins. Since protein structure will change under various conditions, we intended to modify microstructures of the protein aggregates via freezing. In this research, egg and milk proteins were frozen at conditions with different temperature, pH and ionic strength. After being thawed, the characteristics and functions of the aggregated proteins were analyzed, not only for figuring out the effect of freezing on protein aggregates, but also for understanding the mechanism of protein aggregation. In addition, possible applications of protein freezing were also discussed in this research.

### 1. Modification of casein aggregate microstructures under frozen conditions: a study using tunable resistive pulse sensing

Sodium caseinate, the high-proportion protein in milk, is a good additive in food industry. When sodium caseinate is dissolved in water, the dispersed casein self-aggregates to form particles. In this study, sodium caseinate suspensions with different ionic strength (0.01 M or 0.1 M NaCl) and different pH (5.5 or 8.0) were frozen at −35 °C. After being thawed, the sodium caseinate suspensions were analyzed by a tunable resistive pulse sensing (TRPS) device (Fig. 1). In the TRPS device, particles were pushed to pass through a nanopore and were recorded as electric signals. By analyzing the electric signals, particle characteristics such as particle size, particle number concentration and particle surface characteristics could be obtained.

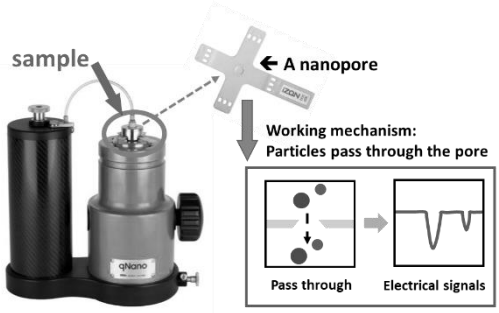


**Fig. 1. Working mechanism of the TRPS device**

As shown in Fig. 2, particle diameter and particle number concentration of 4 samples with different NaCl concentration (0.01 M or 0.1 M) and pH (5.5 or 8.0) were measured. After being frozen for five days, particle diameters of sample were stable. While the influence of ionic strength on particles were obvious. The average particle diameter of 0.1 M NaCl suspensions (3.2-3.6 μm) was larger than that of 0.01 M NaCl suspensions (2.6-3.0 μm). On the other hand, it was found that the particle number concentration for most of the samples decreased after being frozen for more than 2 days. Moreover, both ionic strength and pH affected the particle number concentration. Besides these results, the influence of different

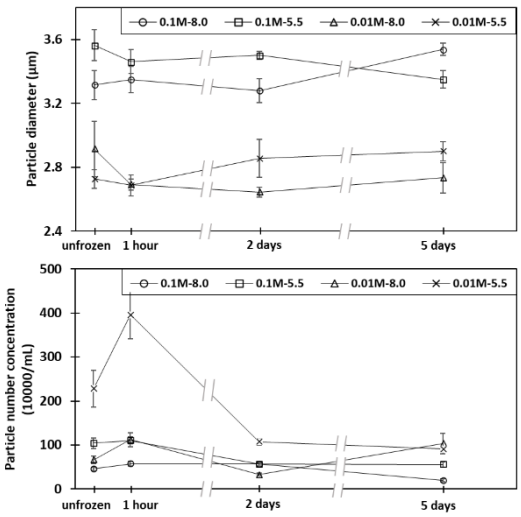


**Fig. 2. Particle diameter and particle number concentration of samples with different NaCl concentration (0.01 M and 0.1 M) and pH (5.5 and 8.0)**

freezing temperatures and the effect of repeated freezing-thawing were also studied, and information about particle surface characteristic were also analyzed in this chapter.

## 2. Effect of pH, ionic strength and freezing treatment on aggregates in egg white powder suspension

Egg white was suspended in NaCl solutions (0.01 M or 0.1 M) and was frozen at −35 °C. The change of particle diameter and particle number concentration were shown in Fig. 3. In the solution with 0.1 M NaCl, protein aggregates were in larger size (*ca* 3.5 μm) than the aggregates in the solution with 0.01 M NaCl (*ca* 2.5 μm). This effect could be explained by the change of electric double layer. When electrolyte strength raised up, the electric double layers surrounding particle became denser and thinner. As a result, proteins were easier to aggregate in a solution with high ionic strength. In this way, particle diameter increased at higher ionic strength conditions.

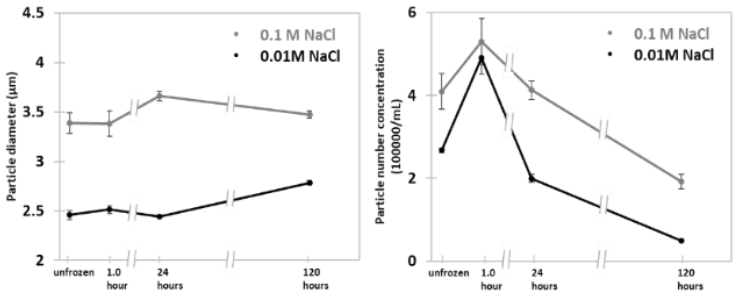


**Fig. 3. The changes of particle diameter and number upon frozen egg white**

Another visible phenomenon in Fig. 3 was the increase of particle number concentration during the first hour of freezing. This effect might be caused by the growth of ice. When ice grew up, the space between ices decreased. In a frozen system, the interspace between ice crystals could be smaller than a few micrometers, comparable to the average diameter of protein aggregates (2.5-3.5 μm). In this narrow space with concentrated solution, protein aggregates would be crushed into small pieces by mechanical and/or osmotic forces.

An important function of egg white suspensions is making foams. In this study, the stability of foam made by different egg white samples were analyzed (Fig. 4). Based on the results, egg white suspensions with 0.1 M NaCl and pH 5.5 had the highest foam stability, while the foam made from 0.01 M NaCl and pH 8.0 suspensions had the lowest foam stability. These results concerning foam stability were consistent with those of the aggregate concentration. The sample with higher protein aggregate concentration would have better performance in foam stability. While the effect of freezing to foam stability was affected by pH and ionic strength of the solutions.

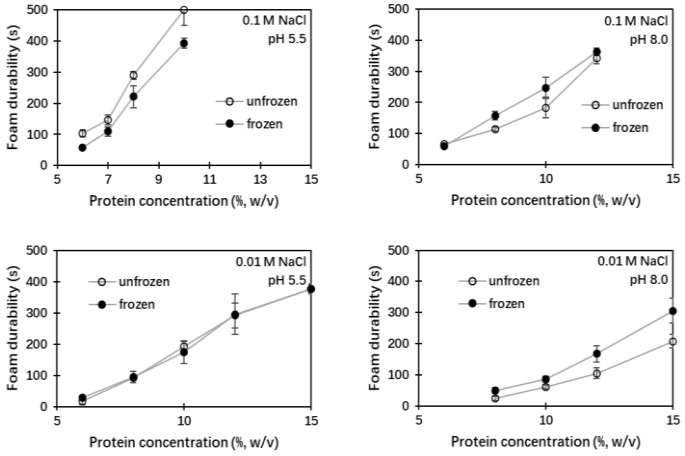


**Fig. 4. Foam durability of protein suspensions at different concentrations**

Besides, the structure of egg white aggregate was analyzed by a small-angle X-ray scattering (SAXS) device. The results showed that freezing and thawing did not significantly affect the nanostructure of egg white aggregates. Based on these results, egg white aggregates formed due

to freezing would be a cluster of the primary nanoparticles.

In this chapter, the change of egg white suspension was analyzed by a TRPS device, and the insights of aggregate structures were given by a SAXS analysis. Moreover, the function of foams produced from frozen egg white suspensions were studied, the results showed that the foam stability of the egg white suspensions could be improved by freezing at selected pH and ionic strength.

## 3. Microstructure change in whole egg protein aggregates upon freezing: Effects of freezing time and sucrose addition

Whole egg solutions were frozen at −35 °C for 5 days. After thawing, the samples were analyzed by a TRPS device. Results showed that both the size and the number of whole egg aggregates increased during freezing process. Quantitative analysis of protein content by bicinchoninic acid showed that protein content in the fraction of pellet increased for approximately 50 μg/mL during the 5 days of freezing (Fig. 5). The increase of protein content in the pellet indicated that the generation of precipitated protein in frozen state. Furthermore, this was simultaneously induced by the protein unfolding and the formation of aggregates (Fig. 6).

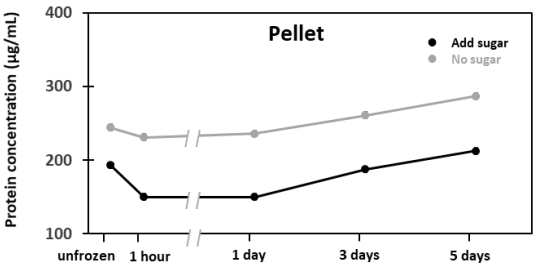


**Fig. 5. Protein content in pellet of whole egg samples**

However, when sucrose was added to whole egg suspension, the protein content in the pelleted were reduced for approximately 80 μg/mL (Fig. 5). This result indicated that the protective effect of sucrose to protein molecules. When sucrose was added to whole egg protein suspension, the sugar molecules surrounded over the protein molecules and formed a protection layer. This protection layer restricted the direct contact between the protein aggregates and hydrophobic ice surfaces. Hence, the surface-induced protein denaturation could be prevented, which simultaneously reduced the formation of new aggregates. In another aspect, the sucrose layer restricted the loss of water molecule that associated with inner structure of the protein molecule (the water molecule was important for the maintenance of protein structure). These indicated that the structure of protein aggregates was protected by sucrose.

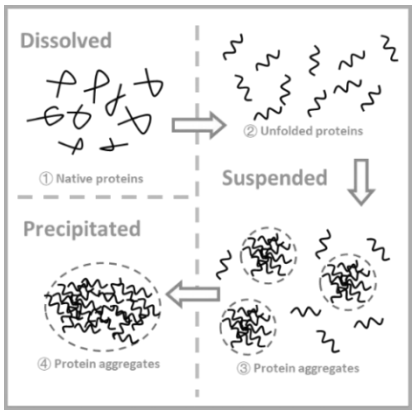


**Fig. 6. The changes of whole egg protein during freezing process**

Besides, the function of sucrose to the structure of aggregates was observed by a SAXS device. As exhibited in Fig. 7, in the absence of sugar, the intensity curves are not identical between unfrozen and frozen samples. On the other hand, in the presence

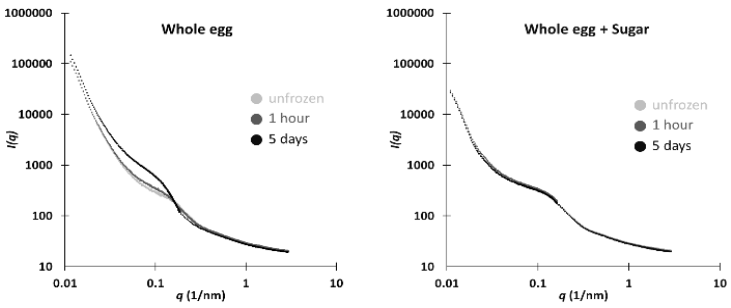


**Fig. 7. Whole egg protein structure changed after being frozen**

of sugar, the intensity curves overlapped well, suggesting that sugar prevent the effect of freezing process.

In this chapter, the aggregation mechanism of whole egg protein under frozen condition were studied. It was confirmed that the aggregate formation in a frozen solution was accelerated by the formation of newly unfolded proteins and that aggregates change their structures by the exchange of water molecule. Sucrose, known as a preservant for the protein denaturation during freezing process, could inhibit not only the formation of aggregates, but also the change on protein structure under frozen conditions.

**4. The manufacturing of *Lactobacillus* microcapsules by freezing with egg yolk**

*Lactobacillus* was mixed with egg yolk and was frozen at −20 °C or −35 °C for 1 hour. In control group, egg yolk was replaced by 0.4% NaCl buffer. After being thawed, the suspensions were treated at HCl buffer (pH 2.5) for 10 minutes. After the acid treatment, suspensions were spread on agar plates and were incubated at 37 °C for 2 days. Afterwards, the number of colonies were recorded and analyzed. The benefit of freezing *Lactobacillus* with egg yolk would be due to the formation of an encapsulated structure by the aggregated egg yolk matrix, which would prevent the bacteria from external stresses, such as acid and freezing.

As shown in Fig. 8, in the absence of egg yolk, the survivability of *Lactobacillus* was reduced by acid treatment (e.g., S1 vs. S3) and freezing process (e.g., S3 vs. S7). While the addition of egg yolk prevented the decrease of the bacterial survivability by acid treatment (e.g., S2 vs. S4). A thing worthy to note was that freezing bacteria with egg yolk at −20 °C (S6) markedly rescued the bacterial survivability against the deteriorative effects of acid treatment. These results indicated that egg yolk protected *Lactobacillus* from acid and freezing treatment. In addition, frozen with egg yolk at −20 °C provided the best preservation effect.

Based on this research, the egg yolk encapsulation by freezing could be a potential method for the preservation of *Lactobacillus* against acid treatment and freezing process. It might be a good technology in food and drug storage to improve the survivability and accessibility of bio-products.

**Summary**

1. Particle diameter, particle concentration and surface characteristics of sodium caseinate and egg white aggregates were modified by freezing process, and the modifications of aggregates under freezing condition were influenced by ionic strength and pH.
2. The stability of egg white foams was influenced by the freezing-induced modifications on protein aggregates.
3. Freezing treatment unfolded whole egg protein, thus induced the increase of diameter and concentration on protein aggregates. While sucrose prevented the influence of freezing on whole egg aggregates.
4. Freezing with egg yolk is a good way to prevent *Lactobacillus* from the deteriorative effects by acid and low-temperature treatments. It could be a potential technology to preserve bio-products.