Effect of pH on Freeze-thaw Stability of Glycated Soy Protein Isolate

Xiaodan Wang+, Shuang Chen+, Qiang Cui, Rui Li, Xibo Wang*, and Lianzhou Jiang

College of Food Science, Northeast Agricultural University, Harbin 150030, CHINA

* Indicates that the two authors contribute consistently to this article

Abstract: The effects of pH on the freeze-thaw stability of glycated soy protein isolate (SPI) and soy protein isolate hydrolysate (SPH) were studied. The covalent compounds were prepared by conjugating SPI, SPH and dextran under heated Maillard reaction, which the macromolecules were named SPI-D and SPH-D. SDS-PAGE analysis verified that SPI-D and SPH-D form a covalent bond through the Maillard reaction. Afterwards, the effects of pH on the freeze-thaw stability of SPI, SPI-D and SPH-D emulsions were evaluated. The covalent conjugate stabilized emulsions improved the stability of the emulsions to pH stress. After freeze-thaw cycles, SPH-D revealed the lowest particle size, degree of coalescence (CD) and oiling off. The results above were also supported by optical microscopy analysis.

Key words: Maillard reaction, SPI-D, SPH-D, emulsions, freeze-thaw stability, pH

1 Introduction

Many proteins are used as high-efficiency emulsifiers because they contain both hydrophobic and charged hydrophilic regions, which reduce the surface tension and interact at the emulsions interface1, they are widely used in food, nutrition, and pharmaceutical industries. In order to maintain microbiological and chemical stability and extending the shelf life of food products, freezing becomes one of the most important preservation methods. When the emulsions are stored at -20°C, a variety of phenomenon can occur including creaming, sedimentation, oiling off and phase separation of the emulsions2, which limit its application in the food industry. And protein emulsions stability is sensitive to environmental stress, such as pH, ionic strength, and temperature3. Degner et al.4 reported that the freeze-thaw instability of emulsions was mainly related to the crystallization of oil-water phase and the change in droplet microenvironment conditions (pH, viscosity, osmotic pressure and ionic strength).

Both proteins and polysaccharides play an important role in terms of structure and stability5. Polysaccharides and proteins can be used in the preparation of edible film applications in the food field6. Polysaccharides can also act as catalysts to promote the amination of 1,2-epoxycyclohexane7. In addition, polysaccharides can be used in the medical field due to their biological activity8. The enhanced emulsifying properties of protein-polysaccharide conjugates had been attributed to the formation of a bulky polymeric layer on the surface of the droplet by conjugated protein molecules, with the polysaccharide portion entering into the continuous phase, thereby enhancing the spatial stability of the emulsified droplet9. These conjugates also have improved functional properties, including enhanced emulsifying properties10,11 and increased heat stability compared to protein itself12,13.

Soy protein-polysaccharide conjugates could improve emulsifying properties, especially in terms of reducing oil droplet size and emulsions stabilization against creaming14-16. In addition, these complexes can effectively enhance environmental changes such as pH and ionic strength17,18. Zhang et al.19 used dry-heated glycation to prepared β-conglycinin-dextran conjugates, and the conjugations effectively enhanced the hydrophilicity of the oil droplet surface and increased the spatial repulsive force between oil droplets, the emulsions was still stable for environmental changes such as pH, ionic strength and heat treatment. At present, the influence of environmental changes on the freeze-thaw stability of soy protein isolate and dextran conjugates emulsions has rarely been reported.

In this study, the effect of pH on freeze-thaw stability of glycated products was investigated. The two products were prepared: SPI-D and SPH-D. The freeze-thaw stability of the emulsions was characterized by testing its particle size, degree of coalescence, oiling off and microstructure. The
effect mechanism of pH on the freeze-thaw stability of glycated product emulsions was discussed.

2 Materials and methods

2.1 Materials

Defatted soybean meal was purchased from Yuwang Co., Ltd. (Shandong, China). Dextran (40kDa) and trypsin were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China) and Beijing Biotopped Science & Technology Co., Ltd. (Beijing, China), respectively. All chemical reagents used in this research were of analytical grade.

2.2 Extraction of soy protein isolate (SPI)

The defatted soybean meal was milled with passing 60 mesh. SPI was extracted according to experimental procedure reported by Sorgentini et al. The defatted soybean flour was stirred for 2 h in deionized water (1:10, w/v, pH 8.5). The mixture was centrifuged at 4000 g for 20 min at 4°C (GSA-rotor, Sorvall RC 5B refrigerated Superspeed Centrifuge). The supernatant was adjusted to pH 4.5 with 2M HCl, then stored for 2 h at 4°C and subsequently centrifuged at 4000 g for 15 min in the same conditions. The precipitate was washed with deionized water and was adjusted to pH 7.0 with 2M NaOH, freeze-dried. The protein content was 90.87% of SPI power, and then was stored as a freeze-dried powder at −20°C.

2.3 Protein hydrolysis

According to the described of Adlemisenss21, the degree of hydrolysis was determined by the pH-stat method. SPI (2 g / 100 mL) was dispersed in 10 mM PBS (pH 8.0) and stirring for 2 h. Subsequently, trypsin was added at 0.25 g/100 mL, the dispersion was incubated at 37°C for 200 minutes with constant stirring. After the reaction, the enzyme was inactivated by heating at 95°C for 10 minutes. The hydrolysis degree of 3% of SPI was obtained and lyophilized.

2.4 Preparation of glycation conjugates

SPI, SPH (4 g / 100 mL) and dextran (12 g / 100 mL, 6 g / 100 mL, respectively) were dissolved in 10 mM PBS (pH 8.0) and stirred for 4 h. Then, store overnight at 4°C. Thereafter, SPI and dextran were heated in a constant temperature water bath at 95°C for 4 h, and SPH and dextran were incubated in a constant temperature water bath at 85°C for 1 h and cooled in an ice bath. Samples were obtained by lyophilized and stored at −20°C.

2.5 SDS-PAGE

SDS-PAGE was performed according to the method of Laemmli22. The separating gel concentration was 13% and the stacking gel was 5%. The samples were boiled for 5 min then samples (7 μL) were loaded into each well. The voltage was set to 90 V in the stacking gel and 120 V in separating gel. When the electrophoresis was run to about 1 cm from the bottom edge of the electrophoresis tank rubber, the power was turned off. Two pieces of gels were subjected to protein staining using G-250 reagent and carbohydrate staining by PAS staining, respectively.

2.6 Fluorescence spectrometry

The protein samples were dissolved in citrate having different pH—phosphate buffer solution (0.25 mg / mL) in. It was then obtained using an excitation wavelength of 290 nm intrinsic fluorescence spectrum, and the obtained emission spectrum between 300 and 400 nm. During these measurements, the excitation and emission slits were adjusted to 5 nm.

2.7 Emulsifying activity and emulsifying stability

According to Kinsella and Pearce23, the emulsifying activity index (EAI) and emulsifying stability index (ESI) of protein samples were determined by the turbidimetric method. The EAI and ESI values were calculated using the following equations:

\[
EAI = \frac{A_0 \times N}{\phi \times L \times C \times 10000}
\]

\[
ESI = \frac{A_0 \times \Delta T}{A_{10} - A_{10}}
\]

where \(T = 2.303, A_0 \) and \(A_{10} \) represent the absorbance at 0 and 10 min, \(N \) is the concentration of protein (g/mL), \(\phi \) is the oil volume fraction of the emulsions, \(L \) is the width of the optical path (0.01 m). Measurements were performed in triplicate.

2.8 Preparation of oil-in-water emulsions

According to Sarkar24, the soy oil was mixed with a samples dispersion (1% protein) to prepare an oil-in-water emulsions (the ratio of solution and soybean oil was adjusted to 9:1). The pre-emulsions was prepared by homogenization at 10000 rpm for 1 minute in a high speed stirrer (IKA, Staufen, Germany). Then resulting emulsions were prepared at pressure level (40 MPa) with a high pressure homogenizer (Stansted Fluid Power Co., UK). Thereafter, the pH of the emulsions was changed by adding 0.5 M HCl or NaOH under continuous stirring to obtain a series of samples (pH 3.0 – 9.0).

2.9 Freeze-thaw treatment

Each emulsions was froze for 22 h at the temperature of −20°C. After freezing, emulsions were thawed for 2 h in a constant temperature water bath at 25°C. Then the freeze-thaw stability of protein emulsions was evaluated. The sample underwent three cycles.

2.10 Particle size measurements of emulsions

The particle size of emulsions was determined by a Mas-
2.11 Coalescence degree (CD)
According to the method reported by Palazolo, calculate the degree of coalescence using volume average particle size data as follows:

\[
\text{CD} = \frac{D_{4,3} - D_{4,3}^{\text{ref}}}{D_{4,3}} \times 100
\]

where \(D_{4,3}\) represents the volume average particle size of initial emulsions diluted in SDS, \(D_{4,3}^{\text{ref}}\) represents the volume average particle size of freeze-thaw emulsions diluted in SDS.

2.12 Oiling off
The amount of oiling off was evaluated according to the description reported by Palanuwet et al. The oiling off was calculated as:

\[
\phi = \frac{m_0 \times (\alpha - 1)}{m_e \times \phi_t} \times 100
\]

where \(m_0\) is the mass of dye solution, \(\alpha\) is the ratio of the measured absorbances of the dye before and after extraction process, \(m_e\) and \(\phi_t\) were the emulsions mass and oil volume fraction of emulsions, respectively.

2.13 Optical microscopy
The emulsion was vortexed for 30 s before the measurement. A drop of emulsions was made into a slide then the microstructure of the emulsions was observed by an Olympus UIS2 optical microscope (Olympus Co., Ltd, Japan).

2.14 Statistical analysis
ANOVA of date was performed with Duncan’s test \((p < 0.05)\) using SPSS 19 Statistical software. The data are expressed as means values ± standard deviation. Each treatment was performed in at least triplicate.

3 Results and discussion
3.1 SDS-PAGE
SDS-PAGE for staining proteins and carbohydrates absolutely demonstrates the covalent bonding of SPI-D and SPH-D. According to Fig. 1, line 2 exhibited typical characteristic bands of SPI. For SPI-D and SPH-D, the new bands were discovered at the top of the separation gel, indicated higher molecular weights were formed (lanes 3, 4). However, the SPI-D band at the top of the separation gel became more visible than SPH-D, which can be contacted with increased levels of protein cross-linking.

Stained for carbohydrate could further confirm the formation of covalent complex, SPI-D and SPH-D showed significant staining of the carbohydrate bands on top of the separation gel (lanes 5, 6), indicated the covalent complex of protein and dextran were successfully linked. It was also reported that glycation of protein and polysaccharide caused polymer bands to appear on the top of the separation gel according to the reported of Zhang et al.

3.2 Endogenous fluorescence spectrometry
Fluorescence spectroscopy could be used to analyze conformational changes of proteins. The tryptophan residue of soy protein isolate was highly fluorescent and sensitive to environmental changes, and was capable of a relatively sensitive change in the conformation of the reactive protein at the tertiary structure level. The maximum absorption wavelength \(\lambda_{\text{max}}\) of the protein increased, which was called the red shift of \(\lambda_{\text{max}}\), indicating that the fluorescent emitting group was exposed to the solvent and the protein molecules were unfolded. When \(\lambda_{\text{max}}\) was decreased, it was called blue shift of \(\lambda_{\text{max}}\), indicating that the soy protein or its subunit undergoes aggregation and the like. The magnitude of the red shift or blue shift could indirectly reflect the extent of protein conformational change.

The fluorescence spectrum was shown in the Fig. 2. The fluorescence spectrum of samples changed in a similar trend. When the pH was close to the isoelectric point, the maximum absorption wavelength of each sample was blue-shifted (SPI, SPI-D SPH-D was 340.8, 343.4, 343.8 nm at pH 5.0, respectively), indicated that the protein was polymerized near the isoelectric point, and the maximum absorption wavelength was red-shifted as the pH away from isoelectric point, shifted by 3.4, 3.8, 3.8 nm at pH 9.0.
respectively, the molecules were turned on. However, SPI-D was significantly red-shifted compared to SPI alone at the same pH, indicated that the glycation reaction changed the surrounding environment of the protein tryptophan residue, and the tryptophan residue was exposed to a more hydrophilic environment\textsuperscript{31}. At the same time, it could also be seen that SPH-D was more red-shifted, indicated that enzymatic hydrolysis could unfold the SPI structure and be exposed by the internal tryptophan group to increase its surface hydrophobicity\textsuperscript{32}.

### 3.3 Emulsifying activity and emulsifying stability

The emulsifying properties of the emulsions formed by SPI, SPI-D and SPH-D conjugates were shown in Fig. 3. The emulsifying activity and emulsifying stability decreased as the pH changed from 3.0 to 5.0, and then increased as the pH adjusted to 9.0. The emulsions stabilized by SPI-D, SPH-D, showed a similar changed of EAI and ESI at pH tested. However, the SPI-D and SPH-D conjugates had higher EAI and ESI compared with SPI alone. It was because that the Maillard reaction between the polysaccharide and the protein forms a covalent substance, inhibited the polymerization of the protein through steric repulsion and contribution to the formation of a macromolecular stabilizing film around oil droplets, the emulsifying property was improved\textsuperscript{10,33}. At the same time, the emulsifying properties were enhanced greatly by enzymatic hydrolysis. The reason was that the soy protein isolate globular molecule was enzymatically decomposed, the molecular flexibility increased\textsuperscript{34}, the hydrophobic group was exposed, the solubility increased after the polysaccharide chain was inserted.

### 3.4 Particle size measurements of emulsions

The average droplet size of the protein emulsions directly affects the stability of the emulsions. The smaller the particle size, the more stable the emulsion. In general, large droplet aggregation can be reflected by $D_{4,3}$\textsuperscript{35}. As shown in Fig. 4, the $D_{4,3}$ values of all emulsions changed in a similar trend, the volume average particle size increased with the pH changing from 9.0 to 5.0, and then decreased as the pH adjusted from 5.0 to 3.0. After freeze-thaw cycles, the particle size of the emulsions was increased at all pH tested. The largest oil droplets were discovered when the pH approached the isoelectric point, which was attributable to the reduction in electrostatic repulsion between the droplets. The $D_{4,3}$ values change of SPI was most obvious after the freeze-thaw cycle (from 11.02 um to 475.76 um), while SPI-D increased from 21.89 um to 294.97 um (Fig. 4a, d). The SPI-D particle size was smaller than SPI alone. There results were similar to Zhang et al.\textsuperscript{26} The reason was that the addition of polysaccharide had a thickening effect, so that the stretched protein molecules were in a relatively stable state, and the glycated molecules...
could be quickly adsorbed to the oil-water interface to form a protein interface film, which could prevent part of the droplets coalesce.

The particle size of SPH-D increased from 20.77 μm to 272.44 μm at pH 5.0 after freeze-thaw cycles (Fig. 4a, d), and the particle size was the smallest at all pH tested. Studies had shown that SPI would expose more hydrophobic groups after enzymatic hydrolysis and produce beneficial interfacial activity polypeptides. However, it could not completely prevent pH-induced aggregation.

When the pH of the emulsions was far from the isoelectric point, the protein carried more positive or negative charges, which could increase the electrostatic repulsion between the emulsions droplets, thereby reducing the aggregation between the droplets. As pH was adjusted to 9.0, the particle size of the emulsions was significantly reduced, and the particle size of samples were 70.08 μm, 67.28 μm, and 25.86 μm after freeze-thaw cycles (Fig. 4d). The particle size of SPH-D was smaller than other samples, indicated that the enzymatic hydrolysis and glycation could greatly enhance the freeze-thaw stability, but still affected by the isoelectric point. This might be the demulsification of emulsions undergone freeze-thaw cycle, resulting in protein coalescence to be severe near the isoelectric point and a larger particle size.

3.5 Degree of coalescence

The coalescence degree (CD) was calculated from droplet diameters (D₄₃) in Fig. 5. All the coalescence degree of the emulsions persistently increased with increasing cycles of freeze-thaw treatment. The coalescence degree of SPI increased to 2775.59 after a freeze-thaw cycle at pH 5.0. While the coalescence degree of SPI-D and SPH-D aggrandized to 1110.30 and 1129.44. When the pH reached 9.0, the degree of coalescence was significantly reduced, and the degree of coalescence of the samples after three freeze-thaw cycles were 3206.25, 805.12, and 724.69 (Fig. 5c), respectively. The addition of polysaccharide could reduce the amount of ice crystals formed during the freezing process, increased the amount of unfrozen water in the emulsion and the viscosity of the aqueous phase, and could form an interfacial layer around the oil droplets, reduced the occurrence of oil droplet coalescence.

In addition, the degree of coalescence of SPH-D was lower than SPI-D. On the one hand, the might be attribute to SPI-D that much unadsorbed or no covalently linked polysaccharides would reduce the stability of the emulsions due to the depletion effect. On the other hand, there was enzymatic hydrolysis of a large molecular weight protein into a relatively small molecular weight peptide fragment to change its flexibility and spatial structure, easier to combine sugar to form an interface film, improved the stability of the emulsions.

3.6 Oiling off

According to Palazolo reported, the oiling off of emulsions was increased when it was treated by repeated freeze-thaw cycles. As shown in Fig. 6, the amount of oiling off of the samples after the freeze-thaw cycles were 66.28%, 35.26%, and 17.75% at pH 5.0, respectively. However, it rated were 22.83%, 10.01%, and 6.07% at pH 9.0 (Fig. 6c), respectively. The oiling off was decreased as the pH moved away from the isoelectric point of protein. Among the all the emulsions, SPH-D had the lowest oiling off, which was reduced by 49.66% than SPI-D, respectively (pH 5.0). The indicated enzymatic hydrolysis combined with Maillard reaction could significantly improve the freeze-thaw stability of SPI, but it was still affected by pH, and the oiling off was higher near the isoelectric point.
Fig. 4 Effect of pH on volume average particle size of emulsions after freeze-thaw cycles, (a): Cycle 0, (b): Cycle 1, (c): Cycle 2, (d): Cycle 3.

Fig. 5 Effect of pH on the degree of coalescence of emulsions after freeze-thaw cycles, (a): Cycle 1, (b): Cycle 2, (c): Cycle 3.
3.7 Microstructure

When the emulsion is frozen, the moisture in the emulsion system is redistributed, and the ice crystals are continuously increased, causing serious damage to the structure of the emulsions. The fat crystals permeate between the oil droplets, resulting in the generation of irregular masses. The droplets in the block fuse with each other during thawing, causing coalescence and even oil precipitation. As shown in Fig. 7, the fresh emulsions were uniformly dispersed in a single drop at different pH, but the emulsions significantly changed after freeze-thaw cycles. When the emulsion was changed at pH 5.0, the emulsions exhibited an unstable microstructure with the increase of the number freeze-thaw cycles, and the emulsions undergone obvious coalescence and large oil droplets were produced. However, the stability of the emulsions was significantly improved at pH 9.0, which the solubility of the pH away from the isoelectric point of protein became better, resulting in a more stable interfacial film. In addition, the stability of SPI-D was better than SPI, which could improve the stability of oil-water interface and prevent droplet aggregation and coalescence through glycation. SPH-D had the best stability in all samples. The moderate enzymatic hydrolysis could reduce the number of spherical molecules in the protein, increased the molecular flexibility of the protein, and made the ordering of protein molecules at the oil-water interface more orderly. Tang et al. also found that the increased flexibility of protein molecules allowed them to stretch in the interfacial layer and form an interfacial film with good strain resistance, which improved the freeze-thaw stability of the emulsions. This result was consistent with the previous results.

4 Conclusions

This study is the evaluation of the particle size, the degree of coalescence (CD), and the microstructure of the SPI, SPI-D and SPH-D emulsions when they were treated by three freeze-thaw cycles. As a final result, the emulsion has the worst freeze-thaw stability near the isoelectric point, and the freeze-thaw stability away from the isoelectric point is stability. The order of increase in freeze-thaw stability of the oil-in-water emulsions is SPI<SPH-D<SPH-D. The current work results show that glycation is an effective method which can greatly improve the freeze-thaw stability of SPI emulsions, but it still affected by pH, the emulsion is unstable near the isoelectric point. Therefore, it is necessary to improve the freeze-thaw stability of the emulsions near the isoelectric point, which needs further study.

In addition, the dissociation constant (pKa) is a very important property of organic compounds, which determines the presence of a compound in a medium. The pKa value of the component decreases and its acidity increases.

Fig. 6 Oiling off of emulsions after freeze-thaw cycles at different pH, (a): Cycle 1, (b): Cycle 2, (c): Cycle 3.
When the pH decreases close to the isoelectric point, the concentration of ionized hydrogen ion increases, and the surface net charge of the protein is almost zero, causing polymerization.

Overall, this study is important for providing information on the role of pH in the freeze-thaw stability of emulsions stabilized by protein-polysaccharide, and broadens the application of soy protein in the food industry, such as vegetable cream, ice cream and quick-frozen food industries, thus providing a theoretical basis for frozen foods.

Acknowledgements
This work was supported by the National Key R&D Program of China (2018YFD0400600) and National Soybean Industrial Technology System of China (CARS-04-PS28).

References
1) Kasran, M.; Cui, S.W.; Goff, H.D. Emulsifying properties of soy whey protein isolate–fenugreek gum conjugates in oil-in-water emulsion model system. Food Hydrocoll. 30, 691-697 (2013).
2) Magnusson, E.; Christer Rosén; Nilsson, L. Freeze-thaw stability of mayonnaise type oil-in-water emul-
Freeze-thaw Stability of Emulsions

3) Pongsawatmanit, R.; Harmislawat, T.; Mcclements, D.J. Influence of alginate, pH and ultrasound treatment on palm oil-in-water emulsions stabilized by β-lactoglobulin. *Colloids Surf.*, A 287, 59-67 (2006).

4) Degner, B.M.; Chung, C.; Schlegel, V.; Hutkins, R.; Mcclements, D.J. Factors influencing the freeze-thaw stability of emulsion-based foods. *Compre. Rev. Food Sci. Food Saf.* 13, 98-113 (2014).

5) Dickinson, E. Hydrocolloids as emulsifiers and emulsion stabilizers. *Food Hydrocoll.* 23, 1473-1482 (2009).

6) Hassan, B.; Chatha, S.A.S.; Hussain, A.I.; Zia, K.M.; Akhtar, M.; Dickinson, E. Whey protein–maltodextrin conjugates as emulsifying agents: an alternative to gum arabic. *Food Hydrocoll.* 21, 607-616 (2007).

8) Yu, Y.; Shen, M.; Song, Q. Xie, J. Biological activities and pharmaceutical applications of polysaccharide from natural resources: a review. *Carbohydr. Polym.* 183, 91-101 (2018).

9) Dickinson, E.; Galazka, V.B. Emulsion stabilization by ionic and covalent complexes of β-lactoglobulin with polysaccharides. *Food Hydrocoll.* 5, 281-296 (1991).

10) Akhtar, M.; Dickinson, E. Whey protein–maltodextrin conjugates as emulsifying agents: an alternative to gum arabic. *Food Hydrocoll.* 21, 607-616 (2007).

11) Liu, J.; Ru, Q.; Ding, Y. Glycation a promising method for food protein modification: physicochemical properties and structure, a review. *Food Res. Int.* 49, 170-183 (2012).

12) Aoki, T.; Hidome, Y.; Kitahata, K. Improvement of heat stability and emulsifying activity of ovalbumin by conjugation with glucuronic acid through the maillard reaction. *Food Res. Int.* 32, 129-133 (1999).

13) Chevalier, F.; Chobert, J.M.; Popineau, Y. Improvement of functional properties of β-lactoglobulin glycated through the Maillard reaction is related to the nature of the sugar. *Int. Dairy J.* 11, 145-152 (2001).

14) Diftis, N.G.; Biladeris, C.G.; Kiosseoglou, V.D. Rheological properties and stability of model salad dressing emulsions prepared with a dry-heated soybean protein isolate–dextran mixture. *Food Hydrocoll.* 19, 1025-1031 (2005).

15) Diftis, N.; Kiosseoglou, V. Improvement of emulsifying properties of soybean protein isolate by conjugation with carboxymethyl cellulose. *Food Chem.* 81, 1-6 (2003).

16) Diftis, N.; Kiosseoglou, V. Physicochemical properties of dry-heated soy protein isolate–dextran mixtures. *Food Chem.* 96, 228-233 (2006).

17) Dickinson, E.; Euston, S.R. Stability of food emulsions containing both protein and polysaccharide. in *Food Polymers Gels & Colloids* (Dickinson E. ed.) 1st ed. pp. 132-146 (1991).

18) Schmitt, C.; Sanchez, C.; Desobry-Banon, S.; Hardy, J. Structure and technofunctional properties of protein-polysaccharide complexes: A review. *Crit. Rev. Food Sci. Nutr.* 38, 689-753 (1998).

19) Zhang, J.B.; Wu, N.N.; Yang, X.Q.; He, X.T.; Wang, L.J. Improvement of emulsifying properties of maillard reaction products from β-conglycinin and dextran using controlled enzymatic hydrolysis. *Food Hydrocoll.* 28, 301-312 (2012).

20) Sorgentini, D.A.; Wagner, J.R. Comparative study of structural characteristics and thermal behavior of whey and isolate soybean proteins. *J. Food Biochem.* 23, 19 (1999).

21) Åldernissen, J. Enzymic hydrolysis of food proteins. *Can. Med. Assoc. J.* 172, 1783-1785 (1986).

22) Laemmli, U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage t4. *Nature* 227, 680-685 (1970).

23) Kinsella, J.; Pearce, K. Emulsifying properties of proteins: evaluation of a turbidimetric technique. *J. Agric. Food Chem.* 26, 716-723 (1978).

24) Sarkar, A.; Kamaruddin, H.; Bentley, A.; Wang, S. Emulsion stabilization by tomato seed protein isolate: influence of pH, ionic strength and thermal treatment. *Food Hydrocoll.* 57, 160-168 (2016).

25) Ariyaprakai, S.; Tananuwong, K. Freeze-thaw stability of edible oil-in-water emulsions stabilized by sucrose esters and tweens. *J. Food Eng.* 152, 57-64 (2015).

26) Zhang, B.; Guo, X.; Zhu, K.; Peng, W.; Zhou, H. Improvement of emulsifying properties of oat protein isolate-dextran conjugates by glycation. *Carbohydr. Polym.* 127, 168-175 (2015).

27) Palazolo, G.G.; Sorgentini, D.A.; Wagner, J.R. Coalescence and flocculation in o/w emulsions of native and denatured whey soy proteins in comparison with soy protein isolates. *Food Hydrocoll.* 19, 595-604 (2005).

28) Palanuwech, J.; Potineni, R.; Roberts, R.F.; Coupland, J.N. A method to determine free fat in emulsions. *Food Hydrocoll.* 17, 55-62 (2003).

29) Viseu, M.I.; Carvalho, T.I.; Costa, S.M. Conformational transitions in beta-lactoglobulin induced by cationic amphiphiles: equilibrium studies. *Biophys. J.* 86, 2392-2402 (2004).

30) Shen, L.; Tang, C.H. Microfluidization as a potential technique to modify surface properties of soy protein isolate. *Food Res. Int.* 48, 108-118 (2012).

31) Sponton, O.E.; Perez, A.A.; Carrara, C.R.; Santiago, L.G. Impact of environment conditions on physicochemical characteristics of ovalbumin heat-induced nanoparticles and on their ability to bind pufas. *Food
Hydrocoll. 48, 165-173 (2015).

32) Stânciuc, N.; Aprodu, I.; Ioniță, E.; Bahrim, G.; Răpeanu, G. Exploring the process-structure-function relationship of horseradish peroxidase through investigation of ph- and heat induced conformational changes. Spectrochim. Acta, Part A 147, 43-50 (2015).

33) Diftis, N.; Kiosseoglou, V. Stability against heat-induced aggregation of emulsions prepared with a dry-heated soy protein isolate–dextran mixture. Food Hydrocoll. 20, 787-792 (2006).

34) Ipsen, R.; Otte, J.; Sharma, R.; Nielsen, A.; Hansen, L.G.; Qvist, K.B. Effect of limited hydrolysis on the interfacial rheology and foaming properties of β-lactoglobulin A. Colloids Surf., B 21, 173-178 (2001).

35) Fioramonti, S.A.; Arzeni, C.; Pilosof, A.M.R.; Rubiolo, A.C.; Santiago, L.G. Influence of freezing temperature and maltodextrin concentration on stability of linseed oil-in-water multilayer emulsions. J. Food Eng. 156, 31-38 (2015).

36) Panyam, D.; Kilara, A. Enhancing the functionality of food proteins by enzymatic modification. Trends Food Sci. Tech. 7, 120-125 (1996).

37) Ghosh, S.; Cramp, G.L.; Coupland, J.N. Effect of aqueous composition on the freeze-thaw stability of emulsions. Colloids Surf., A 272, 82-88 (2006).

38) Degner, B.M.; Olson, K.M.; Rose, D.; Schlegel, V.; Huttin, R.; Mcclements, D.J. Influence of freezing rate variation on the microstructure and physicochemical properties of food emulsions. J. Food Eng. 119, 244-253 (2013).

39) Lam, R.S.H.; Nickerson, M.T. Food proteins: a review on their emulsifying properties using a structure–function approach. Food Chem. 141, 975-984 (2013).

40) Chobert, J.M.; Sitohy, M.; Whitaker, J.R. Specific limited hydrolysis and phosphorylation of food proteins for improvement of functional and nutritional properties. J. Am. Oil Chem. Soc. 64, 1704-1711 (1987).

41) Palazolo, G.G.; Sobral, P.A.; Wagner, J.R. Freeze-thaw stability of oil-in-water emulsions prepared with native and thermally-denatured soybean isolates. Food Hydrocoll. 25, 398-409 (2011).

42) Yu, J.; Wang, G.; Wang, X.; Xu, Y.; Chen, S.; Wang, X. et al. Improving the freeze-thaw stability of soy protein emulsions via combing limited hydrolysis and maillard-induced glycation. LWT-Food Sci. Technol. 91, 63-69 (2018).

43) Aoki, T.; Decker, E.A.; Mcclements, D.J. Influence of environmental stresses on stability of o/w emulsions containing droplets stabilized by multilayered membranes produced by a layer-by-layer electrostatic deposition technique. Food Hydrocoll. 19, 209-220 (2005).

44) Tang, C.H.; Shen, L. Role of conformational flexibility in the emulsifying properties of bovine serum albumin. J. Agric. Food Chem. 61, 3097-3110 (2013).