Influence of Protein Hydrolysis on the Freeze-thaw Stability of Emulsions Prepared with Soy Protein - Dextran Conjugates

Yuying Wang¹, Jie Yu¹, Ning Xu¹, Guorong Wang², and Xibo Wang¹*
¹ College of Food Science, Northeast Agricultural University, Harbin 150030, CHINA
² Leprino Foods Company, Denver, CO 80211, USA

Abstract: Protein hydrolysis on the freeze-thaw stability of emulsions prepared with soy protein - dextran conjugates were investigated. Soy protein isolate-dextran (SPI-D) and soy protein hydrolysates-dextran (SPH-D) conjugates with different degree of hydrolysis (DH) were formed by Maillard reaction. The formation of protein-polysaccharide conjugates between SPI/SPH and dextran molecules was confirmed by SDS-PAGE; this finding was consistent with the degree of glycation and the browning index. The freeze-thaw emulsion stability was investigated. The results confirmed that the SPH3-D (DH at 3%) emulsion with 3% DH of SPI exhibited the lowest creaming index after experiencing 1, 2, and 3 freeze-thaw cycles, with results of 7.69%, 20.74% and 31.30%, respectively. The SPH3-D emulsion had a significantly lower average particle size, which was reduced by 48.28% compared to the SPI-D emulsion. Meanwhile, the SPH3-D solution had low interfacial tension. The confocal laser scanning microscopy analysis indicated that the SPH3-D emulsions were strongly stable against the freeze-thaw treatment and could be used as effective emulsifiers in frozen foods.

Key words: protein hydrolysates, dextran, Maillard reaction, emulsion, freeze-thaw stability

1 Introduction

Soy protein isolate (SPI) is widely used as an emulsifier in the food industry due to its amphiphilic character¹. The freezing of food products is one of the most common treatments used for the extension of shelf-life and the maintenance of textural attributes. However, the effect of environmental stresses on the stability of soy protein emulsions, such as freeze-thaw cycles that can lead to their destabilization (creaming, coalescence, flocculation, Ostwald ripening and phase inversion), severely limits the application of SPI in frozen foods, such as vegetable fat cream, sauces and desserts²,³.

There are many studies focused on the modification of SPI to enhance the freeze-thaw stability of the resultant emulsions. These findings strongly suggest that the ultrahigh pressure homogenization should have an influence on the structure of adsorbed protein molecules, improving the emulsion stability to freeze-thawing⁴,⁵.

Partial enzymatic hydrolysis is a mild and safe means to make better the functional properties of soy protein, such as protein solubility and its emulsifying capacity⁶,⁷. Many studies have demonstrated that limited hydrolysis can be effective in increasing antioxidant activity and reducing the immunoreactivity of glycosylated soy protein⁸,⁹.

We hypothesized that partial hydrolysis and the Maillard reaction would play an important role in the freeze-thaw stability of soy protein emulsions. However, the freeze-thaw stability characterized by measurements of the synergistic effects of the two modifications is limited.

The purpose of this work was to study influence of protein hydrolysis on the freeze-thaw stability of emulsions prepared with soy protein - dextran conjugates. Maillard reaction between SPH and dextran was measured by degree of glycation (DG), browning index and SDS-PAGE. The freeze-thaw stability of the emulsions made from SPH-D was tested for creaming index and average particle size.
2 Materials and Methods

2.1 Materials

The skim soybean powder was provided by the Qinhuangdao Trading Co. LTD. (Qinhuangdao, China). Dextran (40 kDa) was obtained from the Shanghai Zzbio Co., Ltd. (Shanghai, China). Trypsin was provided by Shanghai Guchen Biology Co. (Shanghai, China). Refined soy oil was obtained from a supermarket. The other chemicals used in this study were analytical grade.

2.2 Preparation of soy protein isolate (SPI)

SPI containing 90.96% protein was obtained from the skim soybean powder. The skim soybean powder was dispersed in deionized water (1:10, w/v), and then adjusted to pH 8.5 using 2 M NaOH while incubated in a 50°C water bath for 120 min. The mixture was centrifuged at a speed of 3170 × g at room temperature for 20 min. The supernatant was collected and the pH was adjusted to 4.5 with 2 M HCl solution and then stored at 4°C for 120 min. After, the dispersion was centrifuged again at 3170 × g for 15 min. (At pH 4.5 the storage soy proteins, glycinin and beta-conglycinin, are insolubilized) Deionized water was added to the precipitates, and after washing three times, the precipitates were resolubilized by adjusting the pH to 7.0 with 2 M NaOH. SPI powder was obtained by freeze-drying the resolubilized soy protein solution. Store the powder at room temperature of -20°C. The extracted soy protein isolate had a nitrogen content of 91.23%.

2.3 Preparation of soy protein hydrolysis (SPH)

A 2 wt% SPI solution was prepared by dispersing SPI in a sodium phosphate buffer (10 mM, pH 8.0), and then stirred using a magnetic stirring water bath for 120 min at 37°C to allow complete protein hydration. Trypsin was added at an enzyme/substrate ratio of 0.125 (w/w), and was incubated in a water bath at 37°C for 15 min, 120 min, 200 min, 255 min and 300 min, separately. The reaction was inactivated by heating to 90°C for 10 min and was then cooled to room temperature. The SPI from the freeze-drying was used for further study. DH was determined using the pH-stat method reported by Adler-Nissens10.

2.4 Preparation of soy protein hydrolysis-dextran conjugates (SPH-D)

Lyophilized SPI (4 wt% protein) and dextran (6 wt% sugar) with a protein to sugar ratio of 2:3 were rehydrated in a 10 mM phosphate buffer (pH 8, containing 0.01 wt% sodium azide) by stirring for 4 h at room temperature and were then stored at 4°C overnight to ensure full hydration. The dispersion was heated in a water bath at 95°C for 1.5 h to obtain SPH-D conjugates for analysis.

2.5 Degree of glycation (DG)

The DG was detected using the o-phthalaldehyde (OPA) method described by Wooster & Augustin11. The SPH-D conjugates were diluted in deionized water to a density of 0.2 wt%. Two hundred µL of the dilute SPH-D conjugate solution was mixed with 4 mL OPA in a test tube, and then incubated in a water bath at 35°C for 2 min. The control should be same as the test group in all respects, except for the presence of distilled water in the place of samples. The absorbance was measured using a PG TU-1800 UV-visible spectrophotometer (Purkinje General Instrument Co., LTD., Beijing, China) at 340 nm, and the DG was calculated with the follow formula:

$$DG = (A_0 - A_i)/A_0 \times 100\%$$

where $A_0$ is the absorbance before the Maillard reaction and $A_i$ is the absorbance after the Maillard reaction.

2.6 Browning index

The browning index is an indicator of Maillard reactions12. The SPH-D solutions at 0.2 wt% were dissolved in deionized water and the solutions were clear. Then, the absorbance of the supernatant was measured using a PG TU-1800 UV-visible spectrophotometer (Purkinje General Instrument Co., LTD., Beijing, China) at 420 nm. Meanwhile, deionized water was used as a blank control.

2.7 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE experiments were performed using 5% concentrated gel and 13% separating gel according to Laemmli13 using a Bio-Rad Mini-protein Tetra Electrophoresis System (Bio-Red Laboratories Ltd., California, USA). The sample solution at 0.5 wt% was mixed with a 5 × SDS loading buffer at a volume ratio of 4:1 and was then boiled in a water bath for 5 min. The sample solution (10 µL) was added to each well. Electrophoresis was run at 90 V in the concentrated gel and at 120 V in the separating gel until the bromophenol blue reached probably 1 cm from the bottom of the gel. After electrophoresis, one piece of gel was placed in Coomassie brilliant blue that was stained for 12 h and was then washed 3-4 times with decolorizing solution until the appearance of clear blue bands. The other piece of gel was oxidized for 12 h in a periodic acid solution, followed by soaking 3-5 times with glacial acetic acid-trichloroacetic acid solution (2 h each time). Then, the gel was placed in Schiff reagent and was stored in the darkness for 16 h. Finally, the gel was soaked in an acetic acid solution at 5% v/v until the appearance of pink bands.

2.8 Interfacial tension

The prepared composites were formulated into solutions of different mass concentrations, and the interfacial tension was measured using a TP681 automatic surface tension meter. Small platinum plates (length 10.00 mm, width 9.95 mm, thickness 0.20 mm) were used in the ex-
Influence of Protein Hydrolysis on the Freeze-thaw Stability of Emulsions

2.9 Emulsions preparation

The emulsions were prepared with soy oil (10% v/v) and protein (90% v/v). SPH-D solution was prepared using a T18 Basic high shear rate blender (IKA, Staufen, Germany) at 11,000 rpm for 1 min before further homogenization using an AVP-1000 high pressure homogenizer (Albertslund, Denmark) at 40 MPa.

2.10 Freeze-thaw treatment

All emulsions (20 mL) were immediately diverted to glass tubes (30 mL); then they were stored at −20°C for 22 h. Then, the frozen emulsions were thawed in a room-temperature water bath for 2 h and part of the sample was removed for analysis. The freeze-thaw cycles were repeated up to three times.

2.11 Creaming index

After the freeze-thaw treatment, the emulsions separated into cream layer at the top and serum layer at the bottom. The total height of the emulsions \(H_t\) and the height of the serum layer \(H_s\) were measured. The creaming index (CI) of emulsions was calculated as:

\[
\text{CI} = \frac{H_t}{H_s} \times 100\% 
\]

2.12 Average particle size

A Malvern Master Sizer 2000 (Malvern Instruments Co. Ltd., Worcestershire, UK) was used to measure the average particle size of protein emulsions. The refractive index and adsorption of the dispersed phase were set at 1.460 and 0.001, respectively, and the refractive index of continuous phase was 1.330. Protein emulsions were diluted 5-fold in SDS (1 wt%). The SDS-diluted samples were further diluted with deionized water until a standard obscuration percentage was attained. Then, the volume average particle size \(D_{\text{av}}\) was measured.

2.13 Confocal laser scanning microscopy

Microstructural analysis of emulsions was performed as reported by Drapala, Auty, Mulvihill, & O’Mahony. Add 40 μL Nile Red (0.1 wt%) and 40 μL Nile Blue (0.1 wt%) solution to 1 mL SPH-D emulsion samples with sufficient mixing, and then take 10 μL to the center of the glass slide and covered by a cover glass. The sample was observed under a Leica TCS SP2 AOBS Confocal Laser Scanning Microscope (Leica Microsystems, Heidelberg GmbH, Mannheim, Germany). The excitation was performed at 488 nm (for Nile Red) and 633 nm (for Nile Blue) respectively, and oil immersion lens was used to observe the microstructure of emulsions.

2.14 Statistical analysis

All experiments were performed in triplicate. The results were analyzed according to the analysis of variance (ANOVA) with the significance of differences among the mean values at \(p < 0.05\) by SPSS 19 Statistical software. The figure was drawn using Origin 8.6 software.

3 Results and Discussion

3.1 Effects of protein hydrolysis on the degree of glycation and browning

Results have shown that the higher the degree of the Maillard reaction, the greater the degree of browning. In Fig. 1, SPH1-D~SPH5-D were the conjugates of SPH and dextran with a degree of hydrolysis of 1% to 5%. Figure 1 shows that there was a significant decrease in DG and browning index as the degree of hydrolysis increased to 2%. Therefore, the DG and browning index of the SPH2-D conjugate were both the lowest at 6.32 and 0.524, respectively, which may be due to the combined action of enzymatic hydrolysis and the Maillard reaction, which changed the structure of the modified product. However, the DG and browning index showed significant increases when the degree of hydrolysis increased from 2% to 5%. In addition, the browning index of SPI-D was significantly higher than that of SPH-D, which may be caused by the greater molecular weight of SPI compared to SPH.

3.2 SDS-PAGE of Maillard product

SDS-PAGE is a common technique used for the analysis of protein molecular size. Carbohydrate staining is an important means to confirm the Maillard reaction. The gel
should show pink bands of carbohydrate staining when the protein-polysaccharide conjugates formed. As shown in Fig. 2, all carbohydrate stains (lanes 8 to 13) showed obvious bands of staining, confirming the covalent coupling of protein and dextran. A lighter color was observed in lane 10, indicating that the conjugate of SPH2-D was the lowest with a 2% degree of hydrolysis, which was consistent with the result shown in Fig. 1. Due to the exposition of more lysine residues, which leads to an easy reaction with dextran, thereby increasing the degree of hydrolysis (lanes 11 to 13), the carbohydrate staining gradually became lighter. Lane 2 and lane 3, with darker bands of larger molecules, suggested the formation of higher molecular weight compounds. The same band was also observed in the carbohydrate stained gel (lanes 8 and 9), indicating that a higher degree of the Maillard reaction occurred between SPI/SPH1 and dextran.

3.3 Interfacial tension

Interfacial tension is important for studying the properties of emulsions. The smaller the interfacial tension is, the better the stability of the emulsion is. The composite adsorbs rapidly at the oil-water interface and rapidly reduces the interfacial tension, which can increase the stability of the emulsion and prevent the accumulation of oil droplets. As shown in Fig. 3, the interfacial tension of all composite samples decreased as the mass concentration of the composite increased. The final interfacial tension change tended to be flat, indicating that the composite at the oil-water interface would reach saturation, at which point the interfacial pressure tended to a constant value. The hydrolyzed composite effectively reduced the interfacial tension at the oil-water interface. As the degree of hydrolysis increased, the interfacial tension of the composite decreased continuously, and as the degree of hydrolysis was 3%, it was as low as 4.2 mN/m. This might be due to proper enzymatic hydrolysis to expand the protein structure, increased hydrophobicity to further enhance the interfacial activity, and enhanced emulsion stability. However, excessive hydrolysis might cause the self-polymerization behavior of the composite to weaken the adsorption at the oil-water interface and increased the interfacial tension. Therefore, the emulsifying capacity of the moderately hydrolyzed complex was greatly improved and the emulsion was more stable.

3.4 Creaming Index

The creaming index is an indicator that is used to determine freeze-thaw stability of emulsions. The lower the creaming index is, the better the freeze-thaw stability is. As shown in Fig. 4, as DH increases, the creaming index of...
different protein emulsions tended to decrease and then increase. The enzymatic hydrolysis of protein, due to its flexibility and steric structure, could easily combine with dextran to form a thicker interfacial film, which could effectively inhibit creaming. DH at 3% (SPH3-D) showed the lowest creaming index after each freeze-thaw treatment, which were 7.69%, 20.74% and 31.30% respectively. This indicated that partial hydrolysis combined with Maillard reaction could noticeably improve the freeze-thaw stability of SPI, and the optimum DH was 3%.

3.5 Analysis of average particle size
In general, the smaller the average particle size of droplets, the more stable the emulsions. For SPI-D emulsions, the average particle size of fresh emulsions was 1.37 µm. After triple freeze-thaw cycles, the average particle sizes of SPI-D emulsions were 1.7 µm, 3.67 µm and 4.48 µm, respectively. It was found that the particle size of the SPI-D emulsion increased significantly after the freeze-thaw cycles, indicating that the Maillard reaction between native protein and dextran could not react sufficiently in a short time to produce enough protein-dextran conjugates. Hydrolysis can expose the hydrophobic groups, concealing in proteins, and meanwhile, some peptides with good interfacial activity were produced after enzymatic hydrolysis. Therefore, the average particle size of the SPH-D emulsions increased slightly after each freeze-thaw cycle, exhibiting better stability than the SPI-D emulsions. Specifically, the average particle size of the SPH3-D emulsion was significantly less than that of other samples, which decreased by 48.28% compared to SPI-D, indicating that the freeze-thaw stability of SPH3-D emulsion was the best.

3.6 Confocal laser scanning microscopy analysis
The microstructure of emulsions which were prepared by SPH-D conjugates was observed by confocal laser scanning microscopy. The SPH-D emulsion showed tiny and evenly distributed fat globules in fresh and undergo one freeze-thaw cycle samples with no significant changes. As shown in Fig. 6a, b, an obvious, protein layer surrounded the individual oil droplets was observed. After three freeze-thaw cycles, the oil droplet of SPH-D emulsion was slightly increased and flocculation was slightly occurred, but the oil droplets were still tightly surrounded by protein layers. This was attributed to the higher steric stabilization and interfacial film formed by SPH-D conjugates and it was able to improve freeze-thaw stability of the emulsion by inhibiting coalescence and flocculation. The confocal laser scanning microscopy analysis indicated that the SPH-D emulsion was strongly stable to the freeze-thaw treatment.

4 Conclusions
The SDS-PAGE of the Maillard product with pink bands confirmed that protein-polysaccharide conjugates were formed, which was consistent with the degree of glycation and the browning index. The average index and average particle size were used to evaluated the freeze-thaw emulsion stability of SPI-D and SPH-D. The SPH-D emulsion had a lower creaming index and a smaller average particle size than did the SPI-D emulsion. Specifically, for the DH at 3%, the SPH3-D emulsion exhibited the lowest creaming index and the smallest average particle size. At the same time, it had a lower interfacial tension and could form a
relatively stable interface film at the oil-water interface. The confocal laser scanning microscopy analysis indicated that the optimum SPH-D emulsion was stable against the freeze-thaw treatment and could be used as an effective emulsifier in frozen foods.

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

References
1) Dickinson, E.; Ma, J.; Povey, M.J.W. Crystallization kinetics in oil-in-water emulsions containing a mixture of solid and liquid droplets. J. Chem. Soc. Faraday Trans. 92, 1213-1215 (1996).
2) McClements, D.J. Protein-stabilized emulsions. Curr. Opin. Colloid Interface Sci. 9, 305-313 (2005).
3) Magnusson, E.; Rosén, C.; Nilsson, L. Freeze-thaw stability of mayonnaise type oil-in-water emulsions. Food Hydrocoll. 25, 707-715 (2011).
4) Fernández Ávila, C.; Escrivá, R.; Trujillo, A.J. Ultra-high pressure homogenization enhances physicochemical properties of soy protein isolate-stabilized emulsions. Food Res. Int. 75, 357-366 (2015).
5) Lee, S.H.; Lefèvre, T.; Subirade, M. et al. Effects of ultra-high pressure homogenization on the properties and structure of interfacial protein layer in whey protein-stabilized emulsion. Food Chem. 113, 191-195 (2009).
6) Panyam, D.; Kilara, A. Enhancing the functionality of...
food proteins by enzymatic modification. *Trends Food Sci. Tech.* 7, 120-125 (1996).

7) Jung, S.; Murphy, P.A.; Johnson, L.A. Physicochemical and functional properties of soy protein substrates modified by low levels of protease hydrolysis. *J. Food Sci.* 70, C180-C187 (2010).

8) Zhang, Y.; Tan, C.; Zhang, X. *et al.* Effects of maltodextrin glycosylation following limited enzymatic hydrolysis on the functional and conformational properties of soybean protein isolate. *Eur. Food Res. Technol.* 238, 967-968 (2014).

9) Walter, J.; Greenberg, Y.; Sriramarao, P. *et al.* Limited hydrolysis combined with controlled Maillard-induced glycation does not reduce immunoreactivity of soy protein for all sera tested. *Food Chem.* 213, 742-752 (2016).

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

11) Wooster, T.J.; Augustin, M.A. β-Lactoglobulin–dextran Maillard conjugates: Their effect on interfacial thickness and emulsion stability. *J. Colloid Interface Sci.* 303, 564-572 (2006).

12) Zhong, L.; Ma, N.; Wu, Y. *et al.* Characterization and functional evaluation of oat protein isolate–Pleurotus ostreatus β-glucan conjugates formed via Maillard reaction. *Food Hydrocoll.* 87, 459-469 (2019).

13) Robinson, D.R.; Watts, N.R.; Coombs, D.H. Heat cleavage of bacteriophage T4 gene 23 product produces two peptides previously identified as head proteins. *J. Virol.* 62, 1723-1729 (1988).

14) 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).

15) Drapala, K.P.; Auty, M.a.E.; Mulvihill, D.M. *et al.* Influence of lecithin on the processing stability of model whey protein hydrolysate-based infant formula emulsions. *Int. J. Dairy Technol.* 68, 322-333 (2015).

16) Zhang, B.; Guo, X.; Zhu, K. *et al.* Improvement of emulsifying properties of oat protein isolate–dextran conjugates by glycation. *Carbohydr. Polym.* 127, 168-175 (2015).

17) Nooshkam, M.; Madadlou, A. Microwave-assisted isomerisation of lactose to lactulose and Maillard conjugation of lactulose and lactose with whey proteins and peptides. *Food Chem.* 200, 1-9 (2016).

18) Ipsen, R.; Otte, J.; Sharma, R. *et al.* Effect of limited hydrolysis on the interfacial rheology and foaming properties of β-lactoglobulin A. *Colloids Surf. B* 21, 173-178 (2001).

19) Nakauma, M.; Funami, T.; Noda, S. *et al.* Comparison of sugar beet pectin, soybean soluble polysaccharide, and gum arabic as food emulsifiers. 1. Effect of concentration, pH, and salts on the emulsifying properties. *Food Hydrocoll.* 22, 1254-1267 (2008).

20) 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).

21) Chen, L.; Chen, J.; Ren, J. *et al.* Effects of ultrasound pretreatment on the enzymatic hydrolysis of soy protein isolates and on the emulsifying properties of hydrolysates. *J. Agric. Food Chem.* 59, 2600-2609 (2011).

22) Seko, H.; Shimada, S.; Nakamura, H. *et al.* Three-dimensional distribution of water vapor estimated from tropospheric delay of GPS data in a mesoscale precipitation system of the Baiu front. *Earth Planets Space.* 52, 927-933 (2000).

23) Drapala, K.P.; Mae, A.; Mulvihill, D.M. *et al.* Improving thermal stability of hydrolysed whey protein-based infant formula emulsions by protein-carbohydrate conjugation. *Food Res. Int.* 88 (Pt A), 42-51 (2016).