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REVIEW



## Protein glycosylation: a promising way to modify the functional properties and extend the application in food system

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### ABSTRACT

Modification of functional properties by glycosylating with polysaccharides is an effective solution to improve the internal disadvantages of native proteins. Generally, protein glycosylation belongs to the first stage of the Maillard reaction in essence. Dry-heating, wet-heating, and their combination are the major methods for the preparation of protein-polysaccharide conjugates (PPC). Spectrophotometry, spectroscopy, electrophoresis, calorimetry, chromatography, and mass spectrometry are confirmed to be the most effective methods for the identification of PPC. After glycosylation, functionalities of the native protein, including solubility, rheological properties, emulsifying properties, foaming properties, gel property, film-forming properties, thermal stability, antioxidant activity, allergenicity, and antibacterial properties, are improved. The PPC is extensively used as an encapsulation or a delivering material in order to improve the bioaccessibility of bioactive compounds in food system. Some new applications in food processing could be explored using PPC as an ingredient based on the improved functional properties, such as 3-dimensional printing food, gelled food, and colloid food. Furthermore, the model of protein glycosylation and the application of PPC in food processing could be extended to other protein modification to broaden the exploitation of native protein resource for the processing of novel foods.

### KEYWORDS

Protein glycosylation; protein-polysaccharide conjugates; preparation method; characterization; functional properties; bioactive compound

### Introduction

As a type of biomacromolecule in nature, protein plays an important role in presenting the texture, taste, flavor, color, and nutrition of food. Furthermore, these edible qualities and nutritional value are closely related to the functional properties, which mainly include hydration properties, interface characteristics, and interactions among protein molecules (Foegeding and Davis 2011). The hydration properties are mainly reflected by aqueous solubility, viscosity, and water holding capacity (WHC). The emulsifying and foaming properties are the major interface characteristics. Interactions among proteins includes flocculation, sedimentation, gelation, and molding. Each property has its specific function in food processing, such as aqueous solubility and gelation in soybean curd processing, formation of gluten structure in baking foods, and emulsifying activity (EA) and emulsifying stability (ES) in colloid foods. Inherently, these functional properties are closely related to the amino acid composition, molecular size, molecular structure, charge distribution, and concentration of protein. However, these functional properties of protein would be changed when the environmental conditions changed, such as pH, temperature, ion concentration, and presence of protease. Consequently,

the edible quality of protein-based food is reduced, which restricts the application of native proteins. The variety diversification, high-end oriented quality, and safety of foodstuff promote the increasing requirement of the functional properties of native proteins. Therefore, the modification of protein is considered as the best way to enhance the functional properties of native proteins.

The intention of protein modification is to change a few of chemical groups or molecular structure using appropriate methods to improve the targeted functionality without changing the major functional and nutritional properties. Generally, the modification methods include physical method (Mirmoghtadaie, Aliabadi, and Hosseini 2016), chemical method (Boutureira and Bernardes 2015), and biological method or enzymatic method (Liu et al. 2010). Among these methods, chemical methods, such as acylation, phosphorylation, deamination, alkylation, and glycosylation, exhibit the merits of effectiveness and pertinence. However, most chemical modifications have the drawbacks of excessive reaction and formation of toxic reagents and finally, some uncontrolled hazardous substances would be present in the protein-modified products (Oliver, Melton, and Stanley 2006). Thus, glycosylation is the most promising method for the modification of functional properties of

native protein because of the advantages of moderate reaction process, stable modification products, and free of hazardous products.

Glycosylation of protein assembles the functional properties of both native protein and polysaccharide so as to broaden the application of native protein in food processing. Meanwhile, Since the 1990s, the investigation on protein glycosylation has been extensively carried out and it is still a research hotspot in the field of protein modification nowadays. After glycosylation, the functional properties of whey protein isolate (WPI) (El-Salam and El-Shibiny 2018), ovalbumin (Geng et al. 2014), bovine serum albumin (BSA) (Wang et al. 2016), soybean protein isolate (SPI) (Yang, Cui, Gong, Guo, et al. 2015), and peanut protein isolate (PPI) (Liu et al. 2012), such as solubility, emulsibility, foamability, thermal stability, gel property, antioxidant and antimicrobials activities, were all improved. More importantly, the results of toxicological and nutritional experiments of the glycosylated proteins demonstrated the characteristic of non-toxicity and most amino acids remained unchanged except for the lysine. It is worth mentioning that the carbohydrates used to glycosylate native proteins contain monosaccharides, disaccharides, oligosaccharides, and polysaccharides. Compared to the glycosylation of proteins with monosaccharides, disaccharides, and oligosaccharides, protein glycosylated with polysaccharides exhibited more superior functional properties (Wong, Day, and Augustin 2011; Yadav et al. 2010).

This review summarizes the research progress on the glycosylation of native proteins, including the glycosylation methods, characterization of PPC, modification of functional

properties, and application of PPC in food processing, in order to provide new theoretical guidance and research ideas for the quality improvement of protein-based food.

### Mechanism of food protein glycosylation

The Maillard reaction, also known as the nonenzymic browning reaction, roughly includes the initial, advanced, and final stages. In fact, this complex reaction occurred in food system involves many reactions, such as the condensation between carbonyl compounds and amino compounds, molecular rearrangement, degradation, aldol condensation, and polymerization, to influence the flavor, color, and nutrition of processed food. The specific introduction of the mechanism and characteristics of the Maillard reaction can be seen in the previous classical reviews (Hodge 1953; Martins, Jongen and van Boekel 2000).

Protein glycosylation is commonly regarded as the initial step of the Maillard reaction so that the entire molecular structure of the native protein is not changed and its original functional properties are not lost (de Oliveira et al. 2016). Simply, the protein and polysaccharide are combined by covalent bond(s) to form PPC, illustrated in Figure 1. Thus, after the relatively simple glycosylation with polysaccharides, the native protein not only keeps its functional properties retained but also has enhanced functional properties with the help of polysaccharides. Generally, protein glycosylated with polysaccharide can be easily completed under moderate conditions, such as the temperature below the denaturation temperature of the native protein and a

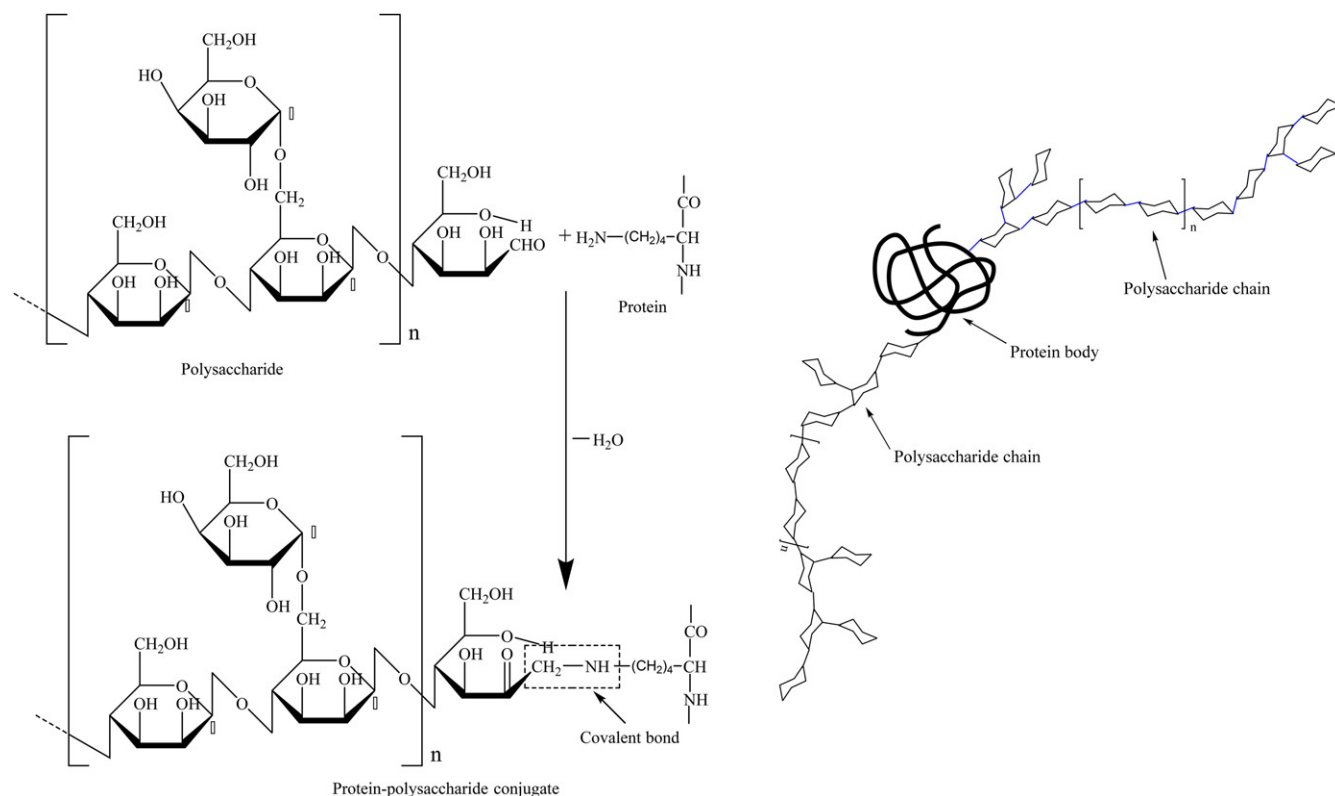


**Figure 1.** The proposed formation mechanism and possible structure of PPC produced via the Maillard reaction (PPC, protein-polysaccharide conjugates) (de Oliveira et al. 2016).

suitable relative humidity (RH). Under the controlled conditions, condensation between the amino groups in protein, especially the  $\epsilon$ -amino group of lysine, and the reducing carbonyl groups in polysaccharide occurs through a covalent bond to form the *N*-glycosylamine with losing a molecule of water. An irreversible Amadori electron rearrangement would occur in this unstable *N*-glycosylamine to form the protein conjugate with the structure of 1-amino-1-deoxyketose. Recently, modification of functional properties of WPI was achieved by glycosylating with sugar beet pectin (SBP) and the fundamental formation mechanism of this protein glycosylation was also elucidated (Qi, Xiao, and Wickham 2017a; 2017b). The contents of free sulfhydryl, primary, and secondary amines of WPI were mitigated by conjugating with SBP accompanying by the proceeding of the initial Maillard reaction, even though the advanced Maillard reaction end products were slightly observed.

Polysaccharides restrict the occurrence of the advanced Maillard reaction and prevent the decomposition of PPC during protein glycosylation due to the relatively weak reducibility and strong molecular steric hindrance compared to monosaccharides or disaccharides (de Oliveira et al. 2016). In addition, the number of polysaccharide molecule linked to protein molecule is closely related to the configuration of protein, also known as the free amino groups, and the number of the reducing end groups of polysaccharide. Thus, the effective formation of PPC depends on the specific types of protein and polysaccharide, as well as the controlling of the involved reaction conditions. Whereas, controlling of these conditions is relatively simpler than those involved in the occurrence of the advanced Maillard reaction. Furthermore, except for the newly formed covalent bond (N–C), no significant changes were observed in the entire structure of the original protein molecule under these moderate conditions. However, introduction of saccharide chains in the protein molecule endows the native protein with more meaningful functions. Denaturation and aggregation of BSA at high temperature were prevented in a certain degree, for example, with the increase in the number of glucose on the BSA molecule during glycosylation (Xia et al. 2015). This might be related to the increased steric hindrance induced by the saccharide chains.

It is worth noting that the reaction substrates of PPC are natural and safe. Specifically, no advanced reaction products of the Maillard reaction are generated under the relatively moderate reaction conditions. Thus, with the enhancement of functional characteristics, no hazardous and toxic compounds are observed during the formation of PPC that is then considered a type of ingredient for the processing of novel food with safe and healthy characteristics.

## Preparation of PPC

As mentioned above, formation of PPC needs an appropriate preparation method with suitable reaction conditions. As common biomacromolecules, protein and polysaccharide are both solid at the ambient temperature and are water soluble. Therefore, the major methods used to prepare PPC are dry-

heating method, wet-heating method, and other methods developed based on the wet-heating method (Liu et al. 2017; Sivapratha and Sarkar 2018). Each method has its own specific operation conditions and characteristics, which are described in Table 1.

### Dry-heating method

Protein glycosylation was first carried out by the dry-heating method that roughly consists of substrates blending, heating treatment, reaction ceasing, and PPC collection (Akhtar and Ding 2017; de Oliveira et al. 2016). Briefly, protein and polysaccharide aqueous solutions are blended in a certain mass ratio and then are dehydrated to perform the glycosylation under appropriate conditions, including time, temperature, and RH. The glycosylation is commonly ceased by instantly cooling to the ambient temperature. Generally, dry-heating method has advantages of free of extra reaction substrates, easily controlling of the reaction conditions, free of poisonous and harmful products, and high grafting degree of the conjugates.

Protein glycosylation using the dry-heating method was first reported by the Kato's research group in the 1990s, who reported the formation of ovalbumin-dextran conjugates via a covalent bond during the glycosylation of ovalbumin with dextran under the conditions of 60 °C and RH of 65% (Kato et al. 1990). In terms of the changes in functional properties, the emulsifying properties were significantly enhanced, indicating that the preparation of macromolecular emulsion using proteins and polysaccharides as the substrates was possible (Kato et al. 1992; Matsudomi et al. 1994). After more than twenty years, the dry-heating method has been regarded as the major method for the preparation of PPC. Meanwhile, optimization of the operation parameters during protein glycosylation has also been extensively carried out to modify the functional characteristics.

Reaction temperature, RH, time, mass ratio of substrates, types of protein and polysaccharide are the most important influence factors involved in the dry-heating method (Jiménez-Castaño et al. 2005). The temperature is one of the most important factors influencing the glycosylation efficiency. Lower temperature results in longer reaction time and harder of the reaction controlling; whereas, higher temperature easily induces the occurrence of the intermediate stage and final stage of the Maillard reaction to increase the complexity and safety of PPC. Generally, the temperature selected during the protein glycosylation is 40–60 °C and 60 °C is the most used reaction temperature (Corzomartínez et al. 2008; Yang, Cui, Gong, Guo, et al. 2015).

The RH of reaction environment can provide an appropriate water activity needed by the occurrence of protein glycosylation. Thus, selection of RH is mainly based on the grafting difficulty between the molecules of protein and polysaccharide or the types of the reaction substrates. Usually, controlling of RH is carried out by putting the saturated salt aqueous solution in a sealed dryer where protein glycosylation taken place using the dry-heating method. For

**Table 1.** The basic information of protein glycosylation methods for the preparation of PPC.

| Preparation methods         | Descriptions                                                                                     | Operation parameters                                                                 | Special requests                                                                                                                                                                               | Characteristics                                                                                                                                                                                                                                         |
|-----------------------------|--------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Dry-heating method          | Protein and polysaccharide were usually heated at 60 °C in solid state for a certain time.       | Types of protein or polysaccharide and their mass ratio, temperature, RH, time       | Protein and polysaccharide are blended in aqueous. Drying treatment, especially lyophilization, is performed.                                                                                  | Direct reaction without extra reagent; Slow reaction and time-consuming, days or weeks; uneasy to purify PPC; high cost for industrial application                                                                                                      |
|                             | Reaction temperature higher than 60 °C is selected to reduce the glycosylation time.             |                                                                                      | In addition to the above two requests, the temperature and time need strict controlling.                                                                                                       | Significantly reduced reaction time, hours; side-effect accompany with high temperature; high cost                                                                                                                                                      |
| Wet-heating method          | Protein and polysaccharide in aqueous were usually heated at 60 °C or higher for a certain time. | Types of protein or polysaccharide and their mass ratio, temperature, pH, time       | Antibacterial material, such as sodium azide, is necessary. Protection of native proteins against thermal denaturation is completed via molecular crowding effect of polysaccharide molecules. | Using water as a reaction medium; reduced reaction time, hours or minutes; easy separation of PPC Glycosylation ceasing due to the presence of water; denaturation of native protein at high temperature in aqueous; occurrence of undesirable products |
|                             | Be catalyzed by microbial transglutaminase (MTGase).                                             | MTGase concentration                                                                 | Selection of appropriate pH and temperature for exerting the optimal enzyme activity                                                                                                           | The disadvantages can be overcome by assistant techniques.                                                                                                                                                                                              |
|                             | Be assisted by ultrasonic technique                                                              | Ultrasonic power and handle time                                                     | Selection of appropriate ultrasonic power and handle time                                                                                                                                      |                                                                                                                                                                                                                                                         |
|                             | Be modified by pulsed electric field or high pressure treatments.                                | Electric field intensity, reaction time<br>Pressure                                  | Selection of specialized equipment and controlling the operation parameters accurately                                                                                                         |                                                                                                                                                                                                                                                         |
| Spinning disc reactor (SDR) | PPC is acquired in a spinning disc with self-heating and rotation.                               | Types of protein or polysaccharide and their mass ratio, rotation speed, temperature | Using a specialized equipment                                                                                                                                                                  | Rapid and cost-effective in practical production                                                                                                                                                                                                        |

Abbreviations: MTGase, microbial transglutaminase; PPC, protein-polysaccharide conjugates; RH, relative humidity.

example, RH values provided by the saturated aqueous solutions of potassium bromide, sodium chloride, and sodium nitrite are 79%, 75%, and 65%, respectively (Bi et al. 2017; Ter Haar et al. 2011; Yang, Cui, Gong, Guo, et al. 2015).

Controlling of reaction time directly influences the grafting degree of protein glycosylation. Generally, reaction time is closely related to the reaction temperature, which means that the higher the reaction temperature selected, the shorter the reaction time was. In addition, the structure of protein and the type of polysaccharide also influence the length of reaction time. Three weeks were needed to perform the glycosylation of lysozyme with galactomannan at 60 °C because the amino groups and carbonyl groups uneasily reacted with each other due to the folded structure of lysozyme molecule (Nakamura, Kato, and Kobayashi 1992). However, the time needed to complete the glycosylation between SPI and soybean soluble polysaccharide (SSP), wheat protein isolate (WHPI) and dextran, and  $\beta$ -lactoglobulin and gum Acacia Seyal were only 0.5–7 days (Bi et al. 2017; Wong, Day, and Augustin 2011; Yang, Cui, Gong, Guo, et al. 2015).

Generally, native protein can be glycosylated with only 1 or 2 polysaccharide molecules because of the limited reaction site of active amino acid residues; however, more polysaccharide molecules can be covalently linked to the exposed amino acid residues because of the denaturation of native proteins. Mass ratio between protein and polysaccharide should be the molar ratio between the amino group and the carbonyl group according to the specific reaction site

between protein and polysaccharide. A series of molar ratio of  $\beta$ -lactoglobulin and maltodextrin, including 1:0.09, 1:0.17, 1:0.25, 1:0.33, 1:0.66, and 1:1.7, were selected to investigate the quantity of amino groups reacted with the carbonyl groups at 60 °C and RH of 76% for 36 h (Wooster and Augustin 2007). As a result, 10.4%, 12.9%, 16%, 25.5%, 35% and 47% of blocked amino groups were observed in the designed experimental groups, respectively. Similarly, the quantity of blocked amino groups of the obtained conjugates increased by 3.5% when the molar ratio of WPI and maltodextrin increased from 1:1 to 1:2 (Martinez-Alvarenga et al. 2014). In other words, the reaction degree was enhanced in conjunction with the increase in the concentration of polysaccharide.

So far, those proteins needed to be glycosylated are animal and vegetable proteins whose functional properties are easily weakened because of the effect of environmental factors, such as pH, temperature, ion concentration, light, and shaking during food processing. Thus, most investigations only focused on the changes in functional properties during protein glycosylation, and the effect of different types of protein on the grafting degree were barely reported. Due to the different structural properties,  $\alpha$ -casein glycosylated with galactomannan using the dry-heating method exhibited much less reaction time than that of glycosylation between lysozyme and galactomannan (Kato 2002). Specifically, only 24 h was needed to make four lysine residues in  $\alpha$ -casein react with galactomannan; however, two lysine residues in

lysozyme was not completely reacted with galactomannan even after two weeks. Proteins extracted from soybean seeds, such as acid-precipitated proteins,  $\beta$ -conglycinin, glycinin, and tofu whey ultrafiltered fractions [molecular weight (MW)  $> 3$  kDa and MW  $< 3$  kDa], were grafted with galactomannan, okara polysaccharides, xyloglucan, chitin, and chitosan oligo under the same dry-heating conditions, respectively (Matemu et al. 2009). Intrinsic differences such as the MW, amino acid profile, molecule structure, and configuration were found in these proteins. Improved EA and ES were observed in tofu whey proteins with MW  $> 3$  kDa conjugated with both polysaccharides and oligosaccharides. However, improvement in ES was only observed in the conjugates that were produced between tofu whey proteins with MW  $< 3$  kDa, acid-precipitated proteins,  $\beta$ -conglycinin, or glycinin and oligosaccharides. The EA and ES of the conjugates between tofu whey proteins with MW  $< 3$  kDa, acid-precipitated proteins,  $\beta$ -conglycinin, or glycinin and chitosan oligo were significantly lower than those of the conjugates between these proteins and xyloglucan or chitin. This might be attributed to the electrostatic interaction between the groups with a negative charge of soybean proteins and the groups with a positive charge of chitosan oligo. Therefore, selection of polysaccharide to perform protein glycosylation should thoroughly consider the structure and the electric charge of protein molecule.

Research on the effect of type of polysaccharide on the grafting degree were extensively carried out (Shu et al. 1996; Xu et al. 2017). Better emulsifying properties were found in the SPI-gum Acacia conjugates compared to the SPI-maltodextrin conjugates obtained under the same dry-heating conditions (Xue et al. 2013). The reason why this difference was observed was that gum Acacia with higher MW than maltodextrin could form a stable layer on the surface of oil particles so as to exhibit better EA and ES. A similar phenomenon was found when PPI was separately glycosylated with dextran (40 kDa) and gum Arabic (240–580 kDa) (Li et al. 2013). In terms of the reaction degree of glycosylation, PPI was more inclined to glycosylate with gum Arabic than dextran; consequently, better emulsifying properties were found in the PPI-gum Arabic conjugates. Relatively best ES was found in the lysozyme-galactooligosaccharide conjugates when lysozyme was separately glycosylated with galactose, galactooligosaccharide, and galactan (Seo et al. 2013). According to the MWs of these three saccharides, more hydrophilic groups were found in the saccharide with high MW so that the hydrophilic property of lysozyme-saccharide conjugates was enhanced. However, overlong polysaccharide chains restricted or prevented the mobility and saturability of lysozyme-polysaccharide conjugates on the emulsion interface. Further, accompanying with the increase in MW of dextran (19.6, 87, 150, 500, and 2000 kDa), the ES of  $\beta$ -lactoglobulin-dextran conjugates were increased (Dunlap and Côté 2005). However, the ES of  $\beta$ -lactoglobulin-dextran conjugates didn't change significantly when the MW of dextran was higher than 150 kDa. Basically, protein glycosylated with polysaccharide with high MW increased the molecule

size of PPC, facilitating to maintain the long-term stability of oil particles of oil-in-water (O/W) emulsion.

Recently, temperature of dry-heating higher than  $60^\circ\text{C}$  has been selected to reduce the reaction time of protein glycosylation. SPI and maltodextrin blended in a mass ratio of 1:2 were used to prepare the SPI-maltodextrin conjugates at a RH of 79% and different temperatures (90, 115, and  $140^\circ\text{C}$ ) for 2 h (Zhang, Wang, et al. 2014). As a result, the highest grafting degree of 26% was found under the condition of  $140^\circ\text{C}$  without the decomposition of the peptide bonds of SPI. Moreover, a maize oil-water emulsion stabilized by the SPI-maltodextrin conjugates obtained at  $140^\circ\text{C}$  exhibited the best storage stability against pH (3–7), thermal treatment ( $90^\circ\text{C}$ ), and ionic strength (0–0.2 mol/L sodium chloride) compared to emulsions stabilized by the SPI, or the SPI-maltodextrin conjugates obtained at both 90 and  $115^\circ\text{C}$ . The WPI glycosylated with maltodextrin at  $80^\circ\text{C}$  for 2 h using the dry-heating method exhibited an excellent transparent and dispersed state under the conditions of pH 3–7, 0–0.15 mol/L sodium chloride and an increased thermal denaturation temperature compared to the native WPI (Liu and Zhong 2013). Afterward, the temperature for the glycosylation of WPI with maltodextrin was increased to  $130^\circ\text{C}$  and the reaction time was reduced to below 30 min (Liu and Zhong 2015). Similarly, the synthesized WPI-maltodextrin conjugates exhibited enhanced aqueous solubility, thermal stability, and salt-tolerance. Furthermore, the color and the content of 5-hydroxymethyl-2-furaldehyde of the WPI-maltodextrin conjugates synthesized at  $130^\circ\text{C}$  for less than 30 min were lighter and lower than those of the WPI-maltodextrin conjugates synthesized at  $80^\circ\text{C}$  for 2 h. Therefore, high temperature and short time did not make the glycosylation of WPI with maltodextrin turn to the advanced step of the Maillard reaction. Dry-heating at high temperature can significantly shorten the reaction time to obtain the targeted grafting degree, which is relatively easily controlled. However, so far, dry-heating method at high temperature was only used to perform the glycosylation of SPI and WPI with polysaccharides, meaning that the universality of this method needs to be further investigated or confirmed.

Glycosylation can directly happen between protein and polysaccharide using the dry-heating method without extra substrates. However, in order to thoroughly blend the reaction substrates, protein and polysaccharide should be initially dissolved in water. Thus, once the protein and polysaccharide blend each other in aqueous, the blended solution would be dehydrated before the heating treatment. The commonly used drying method is lyophilization that avoids the destruction of protein and polysaccharide during the sample preparation at high temperature when hot-air drying, roller drying, and spray drying used. Whereas, lyophilization has disadvantages of relatively high cost compared to these drying methods. In addition, protein glycosylation is usually slow using the dry-heating method because of the moderate reaction conditions ( $60^\circ\text{C}$ ) and lacking of reaction medium, resulting in longer reaction time (several days or weeks) to generate the complex Maillard reaction products. In other words, it is hard to exactly control the

grafting degree during a long-term protein glycosylation using the dry-heating method. Furthermore, when it is necessary, separation or purification of PPC is carried out using proper methods, such as ultrafiltration (Jiménez-Castaño, Villamiel, and López-Fandiño 2007), dialysis (Ter Haar et al. 2011), and anion exchange chromatography (Wooster and Augustin 2006), to remove the unreacted protein and polysaccharide when protein glycosylation partially occurs. These operations increase experimental procedures, which are energy-intensive, tedious, and time-consuming. Based on these disadvantages, the industrialized application of dry-heating method for protein glycosylation is still full of high cost.

### Wet-heating method

Protein glycosylation occurred in aqueous was first reported in a study of the glycosylation of  $\beta$ -lactoglobulin with various types of monosaccharide including arabinose, galactose, glucose, lactose, rhamnose, and ribose (Chevalier et al. 2001). After 72 h of glycosylation in tightly-capped flasks at 60 °C,  $\beta$ -lactoglobulin glycosylated with arabinose or ribose exhibited the best emulsifying properties, and the best foaming properties was observed in  $\beta$ -lactoglobulin grafted with glucose or galactose. The reaction time consumed in this study was almost the same as that of the dry-heating method. Modified wet-heating method was then performed using microwave heating at 90 °C for the glycosylation of SPI with lactose, maltose, dextran, and soluble starch (Guan et al. 2006). The microwave assisted wet-heating increased the reaction speed of the glycosylation of SPI with sugars compared to the dry-heating method. SPI-fructooligosaccharide conjugates synthesized via either dry-heating method or wet-heating method exhibited modified physicochemical properties, especially the largely reduced antigenicity (van de Lagemaat et al. 2007). However, the specific principle and advantages of wet-heating method for protein glycosylation were not discussed.

Systematic research on protein glycosylation in aqueous was reported by Zhu et al. in 2008 (Zhu, Damodaran, and Lucey 2008). WPI and dextran were dissolved in a certain mass ratio in sodium phosphate buffer (pH 6.8) with 0.02% sodium azide for it can prevent the bacterial growth. The sample solution was then fully blended by magnetic stirring for 2 h and was then performed the pH adjustment at the same time. After moderately stirring at 5 °C overnight, the sample solution was heated in a water bath at different temperatures for a certain time. According to the results of single factor experiment, optimal conditions for the WPI glycosylation with dextran were WPI:dextran = 1:3, pH 6.5, 60 °C, and 24 h, resulting in that the WPI-dextran conjugates had the highest content of Schiff base. Thus, reaction time can be significantly reduced because of the sufficient contact between the molecules of proteins and polysaccharides in the water phase, which is in favor of controlling of the Maillard reaction to be the initial stage for the formation of Schiff base. However, native protein easily denatures in aqueous during heating above its denaturation temperature,

resulting in the decreased grafting degree. Thus, the molecular crowding effect was proposed (Zhu, Damodaran, and Lucey 2008). The heat-induced denaturation and aggregation of WPI was prevented by the steric hindrance of dextran molecules so as to form covalent bonds between the amino groups and the carbonyl groups. Therefore, the heat-induced denaturation of native proteins was restricted by the steric hindrance of polysaccharide macromolecules in the water phase, which ensures the efficient occurrence of protein glycosylation.

Recently, protein glycosylation using the wet-heating method is extensively performed to study the influence of polysaccharide on the structure and functionality of native protein. A modified wet-heating at 90 °C was performed for the glycosylation of canola protein isolate (CPI) with gum Arabic (Pirestani, Nasirpour, Keramat, Desobry, et al. 2017; Pirestani et al. 2018). After only 15 min, the content of free amino group of the gum Arabic grafted CPI reduced to 72% compared to the native state. A new band representing a MW of ~90 kDa between the stacking and separation gels and a chromatographic peak shifted to the lower value of retention time were found according to the results of electrophoretic analysis and size exclusion chromatography. A reduction in levels of  $\alpha$ -helix and  $\beta$ -sheet and an increasing in level of random coils were also observed in the secondary structure of the synthesized CPI-gum Arabic conjugates. Based on these results, covalently crosslinking indeed occurred between the molecules of CPI and gum Arabic in the water phase. Furthermore, the solubility in broad pH region of the CPI-gum Arabic conjugates was higher than that of the native CPI. However, although the reaction time of protein glycosylation was significantly reduced in the water phase accompanying with the increase of reaction temperature, the denaturation of native protein also easily occurred. This is the reason why the glycosylation of most proteins cannot be effectively completed by the wet-heating method.

The acyl transfer reaction, deamidation, and crosslinking between glutamine (acyl donor) and lysine (acyl acceptor) peptide residues can be catalyzed by MTGase to form intra- or intermolecular  $\epsilon$ -( $\gamma$ -glutamyl)-lysine isopeptide bonds and finally the crosslinking of proteins (Gaspar and de Góes-Favoni 2015). The lysine peptide residue in a cross-linked protein molecule can be easily substituted by primary amines, which means that MTGase can indirectly catalyze the reaction between the glutamine residue (acyl donor) and the primary amines (acyl acceptor). Thus, MTGase can be used as a catalyst when PPC is prepared by using the wet-heating method. This work was extensively carried out to confirm the application of MTGase on the functionality modification of native proteins, such as SPI and caseinate (Jiang and Zhao 2010; 2011; Zhu, Wang, and Zhao 2015). For example, an oligochitosan-glycosylated and MTGase-crosslinked caseinate with modified functional properties was prepared in aqueous based on the catalytic activity and the reaction activity of primary amine contained in oligochitosan (Song and Zhao 2014a). Briefly, aqueous solutions of caseinate, oligochitosan, and MTGase were first blended

and stored at 37 °C for 3 h. The sample solution was heated at 85 °C for 5 min and then immediately cooled to the ambient temperature. The crosslinked caseinate-oligochitosan conjugates were finally obtained using an isoelectric precipitation at pH 4.5. Under the same conditions, SPI-oligochitosan conjugates were also synthesized (Song and Zhao 2014b). Better WHC, oil-holding capacity, EA, and ES were exhibited by this synthesized SPI-oligochitosan conjugates compared to those of the native SPI or the MTGase-modified SPI. The MTGase catalyzed wet-heating method has advantages of moderate reaction process and the oriented grafting structure of conjugates.

Ultrasonic technique is extensively applied to food processing and food analysis because of the generation of cavitation in solution, such as the extraction of proteins, polysaccharides or micromolecules, degassing of liquid food, passivation of enzyme, and induction of formation of crystal nucleus during freezing process (Knorr et al. 2004). Ultrasonic-assisted wet-heating method was selected to synthesize SPI-gum Acacia conjugates (Zhang, Chi, and Li 2014). The results demonstrated that the grafting degree of SPI-gum Acacia conjugates increased as the heating temperature and ultrasonic power increased. Under the conditions of reaction time of 40 min, heating temperature of 80 °C, and ultrasonic power of 200 W, the SPI-gum Acacia conjugates exhibited the best EA and ES and better solubility at the isoelectric point (pI) than the native SPI or the conjugates synthesized only by the wet-heating method. Moreover, PPI grafted with dextran or gum Arabic was also performed using ultrasonic-assisted wet-heating method (Li, Xue, et al. 2014). By using the ultrasonic-assisted wet-heating method, both the PPI-dextran conjugates and the PPI-gum Arabic conjugates exhibited the increased grafting degree and color lightness but the lower yellow tones compared to the conjugates prepared by the classical wet-heating method. In terms of the characteristics of molecular structure, less  $\alpha$ -helix, more  $\beta$ -sheet,  $\beta$ -turns, and unordered coils, higher surface hydrophobicity, and looser tertiary structure were observed in the PPI-dextran conjugates and PPI-gum Arabic conjugates prepared by the ultrasonic-assisted wet-heating compared to the conjugated prepared by the classical wet-heating. The ultrasonic-assisted wet-heating prepared conjugates also exhibited higher solubility and EA than the classical wet-heating prepared conjugates. Therefore, under the same wet-heating conditions, ultrasonic treatment promoted the glycosylation of proteins to prepare PPC with better functional properties and light color (Zhang, Chi, and Li 2014). Further, selection of ultrasonic treatment to assist protein glycosylation using the classical wet-heating can effectively reduce the reaction time and increase the grafting degree and finally obtain PPC with increased functionality (Qu et al. 2018; Xue et al. 2017).

In addition, some non-thermal processing techniques, such as pulsed electric field treatment and high pressure treatment, were also selected to assist the glycosylation of proteins by the classical wet-heating method. Pulsed electric field treatment was tentatively applied to the preparation of BSA-dextran conjugates (Guan et al. 2010) and WPI-dextran

conjugates (Sun, Yu, Zeng, et al. 2011) using the wet-heating method based on the advantages of high electric intensity (>10 kV/cm), uniform treatment effect, and short time during sterilization and quality enhancement of food. To take an example, WPI and dextran were blended in a mass ratio of 1:1 in sodium phosphate buffer solution and was then placed in a pulsed electric field that was covered with square-wave pulses with a duration of 25  $\mu$ s and a frequency of 1.04 kHz. The major treatment parameters included an electric field intensities of 15 or 30 kV/cm, a reaction time of 7.35 ms, and a temperature of 30 °C. The results demonstrated that glycosylation occurred between the molecules of WPI and dextran and higher grafting degree was observed in the WPI-dextran conjugates synthesized at higher electric field intensity (30 kV/cm). The WPI-dextran conjugates synthesized by the pulsed electric field assisted wet-heating method exhibited higher EA, ES, thermal stability, and solubility around the pI of native WPI compared to the native WPI and the WPI-dextran conjugates synthesized by the classical wet-heating. As reported by Xu, Yu, et al. (2010), high pressure treatment of 10 MPa promoted the occurrence of grafting reaction and prevented the formation of brown products during the glycosylation of  $\beta$ -conglycinin with dextran by wet-heating. The  $\beta$ -conglycinin-dextran conjugates obtained using the high pressure-assisted wet-heating method exhibited better emulsifying properties than the  $\beta$ -conglycinin-dextran conjugates obtained using the classical wet-heating. It can be seen that the glycosylation of proteins by wet-heating at low temperature can effectively prevent the occurrence of brown and harmful products resulted from the advanced Maillard reaction. Other techniques, such as high hydrostatic pressure and dense phase carbon dioxide, also have the ability to modify protein glycosylation with polysaccharides according to the characteristics of non-thermal processing techniques, which would be further confirmed.

The glycosylation of proteins by wet-heating has outstanding merits of short reaction time and easy separation of the synthesized conjugates. However, many problems also exist in this preparation method. Water can inhibit the formation of PPC in a certain degree because water is also a product of the initial stage of the Maillard reaction. The exposure of hydrophobic groups and aggregation of the thermal-induced denaturation of proteins in aqueous also reduce the grafting degree. Furthermore, undesirable products, such as the brown and hazardous products, can be formed during the uncontrolled long wet-heating. However, wet-heating method combined with special treatments, including addition of MTGase, ultrasonic treatment, pulse electric field technique, and high pressure treatment, can effectively promote the glycosylation of proteins.

### **Glycosylation in specialized equipment**

Given that some disadvantages are found in both the dry-heating and the wet-heating methods for the preparation of PPC, some equipment with a potential of application in practical production are developed, such as SDR. According

**Table 2.** The testing principles, key techniques, and analysis characteristics and others of the commonly used methods for the characterization of PPC.

| Characterization method                                              | Testing principle                                                                                                                                                                                                     | Equipment                                                                                                                       | Key technique                                                                                                                                                                                                                                                                          | Analysis characteristic                                                                                                                                                                                                           |
|----------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Spectrophotometry                                                    | Characteristic absorption peaks of the Maillard reaction products during the specific absorption wavelength ranges                                                                                                    | Ultraviolet spectrophotometer                                                                                                   | Calculation of grafting degree by testing the contents of free amino groups using a modified ortho-phthalaldehyde (OPA) method at 340 nm.                                                                                                                                              | Fast, convenient, and of less sample preparation steps<br>Poor accuracy                                                                                                                                                           |
| Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) | Distribution of protein bands in a separation gel based on the different shifting distances due to the different MWs of protein under a certain electricity using protein standards as the marker.                    | Electrophoresis chamber and gel imaging system                                                                                  | Selection of appropriate marker; preparation of spacer gel and separation gel; loading of sample with appropriate quantity; staining with Coomassie brilliant blue (CBB) R-250 and gel imaging                                                                                         | High sensitivity and resolution; no absorption and electroosmosis effect<br>Protein glycosylation is characterized based on the appearance or disappearance and the shade of the protein bands.                                   |
| Circular dichroism (CD)                                              | Changes in the nature of the polarized light in specific absorption wavelengths are observed and quantified based on the Lambert-Beer law due to the different circular dichroisms of the optically active compounds. | CD spectropolarimeter                                                                                                           | Preparation of sample solution with low concentration (1 mg/mL of protein in 10 mmol/L potassium phosphate buffer); Acquisition of CD spectrum during the far ultraviolet region (180–260 nm); The contents of the secondary structures are calculated using a computer program CDPro. | Fast, simple, and accurate<br>The contents of $\alpha$ -helix, $\beta$ -sheet, $\beta$ -turns, and unordered coils can be measured and calculated to analyze the changes in the secondary structure during protein glycosylation. |
| Spectroscopic method                                                 | Characteristic stretching or deformation vibration absorption bands representing the amide I, II, and III of proteins can be reflected in the infrared spectrum.                                                      | Fourier transform infrared (FTIR) spectrometer                                                                                  | Understanding the characteristic functional groups formed after protein glycosylation; Chemometrics methods can effectively distinguish the differences in the infrared spectra of PPC, the native protein, and the mixture of protein and polysaccharide.                             | Fast, convenient, and direct to analyze the covalent bond formed during protein glycosylation; Ambiguous information also exist, especially for the comparison of the heated protein and the conjugated protein                   |
|                                                                      | With the help of noble nanosubstrate with roughened structure or critical desiccation state of analyte, the sensitivity is tremendous enhanced so that the changes in molecular structure can be easily measured.     | Surface-enhanced Raman spectroscopy (SERS) and critical desiccation state Raman spectroscopy (CDSRS)                            | Selection of appropriate noble nanosubstrate or elaborate detector of transition state by a laser confocal microscopy is very important.                                                                                                                                               | Raman spectroscopy is a reliable method for the characterization of protein glycosylation.                                                                                                                                        |
| Thermal analysis                                                     | Changes in denaturation temperature, enthalpy, and weight during heating can be recorded to characterize the thermodynamic properties of protein.                                                                     | Differential scanning calorimeter (DSC)                                                                                         | The PPC is usually thermally-scanned in aqueous with a concentration of 20% (w/v) using 0.01 mol/L phosphate buffer (pH 8.5) in a hermetically sealed aluminum pan.                                                                                                                    | Effective to differentiate the changes in molecular character of native protein during protein glycosylation                                                                                                                      |
| Microstructure analysis                                              | The surface morphology can be recorded in microscale when the test sample is treated by a very finely focused high-power electron beam.                                                                               | Scanning electron microscopy (SEM)                                                                                              | Controlling of the proper layer thickness of gold/palladium onto the surface of sample is very important.                                                                                                                                                                              | The microscale difference in the surface morphologies are effective information to identify the covalently crosslinking between the molecules of protein and polysaccharide.                                                      |
| Chromatographic method                                               | The difference in MWs of macromolecules can be reflected using a size exclusion column in a liquid chromatographic system.                                                                                            | High performance size exclusion chromatography (HPSEC)                                                                          | Selection of appropriate separation column, mobile phase, detector, and standards is very important.                                                                                                                                                                                   | The MW of the PPC is significantly higher than that of the native protein or the polysaccharide, which can be recorded by HPSEC.                                                                                                  |
| Mass spectrometry                                                    | The MW and structure of analyte can be directly measured by the soft ionization mass spectrometry.                                                                                                                    | Electrospray ionization mass spectrometry (ESI-MS) and matrix assisted laser desorption ionization mass spectrometry (MALDI-MS) | These two techniques are usually used in conjunction with the high performance liquid chromatography. The mass spectrum analysis can be completed by consulting the corresponding mass spectral library.                                                                               | Direct and reliable;<br>Because of the powerful analysis ability, the characterization of PPC by these two techniques would be come true.                                                                                         |

Abbreviations: CBB, coomassie brilliant blue; CD, circular dichroism; CDSRS, critical desiccation state Raman spectroscopy; DSC, differential scanning calorimeter; ESI-MS, electrospray ionization mass spectrometry; FTIR, Fourier transform infrared; HPSEC, high performance size exclusion chromatography; MALDI-MS, matrix assisted laser desorption ionization mass spectrometry; OPA, ortho-phthalaldehyde; PPC, protein-polysaccharide conjugates; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SEM, scanning electron microscopy; SERS, surface-enhanced Raman spectroscopy.

to previous review (Akhtar and Ding 2017), a tailor-made inclined plane with a heating ability was designed to promote the formation of thin films ( $\mu\text{m}$  level) of the protein-polysaccharide solution. Further, rapid shifting of this film to the margin of disc was completed by a centrifugal force. These procedures can be repeatedly carried out to obtain a desirable grafting degree. The inclined plane has characteristics of easy controlling of temperature and rotation speed, and convenient cleanup. Thus, the SDR includes the glycosylation of proteins in both water phase and solid phase to reduce the total reaction time and finally the energy cost. However, so far, no publications have reported the changes in functionality of protein glycosylated with polysaccharide using a SDR.

### Characterization of PPC

As mentioned above, the essence of protein glycosylation is the initial stage of the Maillard reaction and PPC is a primary product of the Maillard reaction. Thus, if reaction conditions are not controlled, the glycosylation of protein could easily transfer to the advanced stage or the final stage of the Maillard reaction, resulting in the formation of advanced glycation endproducts. However, the major intention of protein glycosylation is to obtain the PPC with enhanced functionality. Therefore, whether protein glycosylation occurs or how deep the reaction occurs need an appropriate detection technique or analytical method to characterize PPC. Recently, there are many methods adopted to verify the grafting degree of protein glycosylation or to characterize PPC, such as spectrophotometry, gel electrophoretic analysis, chromatography, spectroscopic method, microstructure analysis, calorimetric analysis, and mass spectrometry. The testing principles, key techniques, and analysis characteristics and so on of these methods are described in Table 2.

### Spectrophotometry

Because of the characteristics of fast, convenient, and less sample preparation steps, spectrophotometry is extensively used to characterize the occurrence or degree of glycosylation between a protein and a polysaccharide. This application is mainly based on the formation of color substances that have an absorption peak in a wavelength region of 270–500 nm. After simply dissolving or diluting in water, the centrifuged supernatant was used to study the nature of covalent bonding contained in PPC at a full wavelength scanning mode by an ultraviolet spectrophotometer (Zhu, Damodaran, and Lucey 2008). More effective index expressing the glycosylation extent is the degree of conjugation or grafting degree, which can be calculated by measuring the contents of free amino groups in the conjugates and the native proteins using a modified OPA method (Boostani et al. 2017). The absorbance was commonly measured at a wavelength of 340 nm and *L*-leucine (0.25–2.0 mmol/L) was selected to obtain a standard curve. Although it has an advantage of simple operation without expensive equipment,

spectrophotometry also exhibits a defect of poor accuracy. Thus, spectrophotometry is commonly used to characterize PPC with other methods.

### SDS-PAGE

SDS-PAGE is broadly used to directly detect the occurrence of protein glycosylation and the MW ranges of the obtained conjugates (O'Regan and Mulvihill 2009). Specifically, the formation of PPC is conformed by observing the newly appeared bands and their shade, and the shallowed or disappeared bands in the CBB R-250-stained gels of both the conjugates and native protein or the mixture of protein and polysaccharide. The operation steps of SDS-PAGE was described in detailed by Laemmli (1970), roughly including selection of appropriate marker, dissolution of samples, preparation of spacer gel and separation gel, loading of sample onto the gels, setting of electricity, staining with CBB G-250, and gel imaging. When analyzed, the band assigned to the native protein shallows or disappears and a new band with higher MW appears at the terminal of separation gel, meaning that the content of native protein decreases and the formation of PPC. Based on this directly analytical principle, SDS-PAGE is generally considered the most effective method for the characterization of PPC (Golkar, Nasirpour, and Keramat 2017; Setiowati et al. 2016; Xu et al. 2012).

### CD

As an effective method used to analyze the secondary structure of protein, CD is also extensively used to characterize PPC based on the analyses of changes in contents of  $\alpha$ -helix,  $\beta$ -sheet,  $\beta$ -turns, and unordered coils (Du et al. 2013; Kim and Shin, 2015; Li, Huang, et al. 2014). As reported by Du et al. (2013), rice dreg glutelin- $\kappa$ -carrageenan conjugates exhibited significantly reduced contents of  $\alpha$ -helix and  $\beta$ -sheet and increased contents of  $\beta$ -turns and unordered coils compared to the native rice dreg glutelin and the mixture of rice dreg glutelin and  $\kappa$ -carrageenan. Slightly different results were observed in the study of secondary structure distribution among the PPI, mixture of PPI and glucomannan, and PPI-glucomannan conjugates (Li, Huang, et al. 2014). Significantly reduced content was only found in the  $\alpha$ -helix; however, the contents of  $\beta$ -sheet,  $\beta$ -turns, and unordered coils were significantly increased after the glycosylation of PPI with glucomannan. It was also reported that there were no significant differences between the secondary structures of WPI-dextran conjugate and native WPI analyzed by CD (Fan et al. 2017). This phenomenon was possibly related to the natures of protein and polysaccharide and the grafting degree of glycosylation. However, it is undoubtedly that changes in the secondary structure of the native protein occur when the polysaccharide chains were grafted to the protein molecule. Therefore, CD used to perform the characterization of protein glycosylation has the advantages of strong pertinence and high accuracy.

### Spectroscopic methods

As the most used spectroscopic method, FTIR spectroscopy is also broadly used to analyze the covalent bond formed between the residues of amino group ( $\text{NH}_2$ ) of protein and carbonyl group ( $\text{C=O}$ ) of reducing sugars during protein glycosylation. Theoretically, some typical chemical bonds can be formed because of the formation of  $\text{NH}_2$  and  $\text{C=O}$  during the Maillard reaction, such as the  $\text{C=O}$  in Amadori compounds, the  $\text{C=N}$  in Schiff base, and the  $\text{C-N}$  in pyrazines, which are rightly reflected by the infrared spectrogram. For instance, the absorption bands used to characterize the glycosylation of casein with glucose were the region of  $1650\text{--}1540\text{ cm}^{-1}$  representing the stretching vibration of  $\text{C=O}$  and  $\text{C-N}$  from amide I and II, and the band around  $1300\text{--}1200\text{ cm}^{-1}$  representing the stretching vibration of  $\text{C-N}$  and deformation vibration of  $\text{N-H}$  (Gu et al. 2010). The differences of these absorption bands between the conjugates and native protein or the mixture of protein and polysaccharide can be further analyzed with the help of chemometrics. By virtue of multivariate statistical analysis, principal component analysis, and soft independent modeling of class analogy, a weak absorption band around  $1800\text{--}1400\text{ cm}^{-1}$  assigned to the stretching of amide I and II was used to reflect the difference in the molecular structures between the grafted caseins and the native caseins (Oliver et al. 2009). Wong et al. (2009) reported application of attenuated total reflection (ATR)-FTIR on the characterization of changes in the molecular structure of WHPI grafted with glucose or maltodextrin. As a result, both the WHPI-glucose conjugates and the WHPI-maltodextrin conjugates exhibited different FTIR spectra because of the formation of covalent bond compared to the native WHPI. Gliadin grafted with dextran also exhibited different profiles of absorption bands assigned to amide I and II compared to the native gliadin using ATR-FTIR (Secundo and Guerrieri 2005). However, the changes in molecular structure of native protein during glycosylation are not significantly different to those of the mixture of protein and polysaccharide when FTIR is used. Thus, FTIR is always combined with another method to characterize protein glycosylation.

Another spectroscopy used to characterize protein glycosylation is Raman spectroscopy, which includes the SERS and the CDSRS. Compared to the traditional Raman spectroscopy, sensitivity of SERS is tremendously amplified so as to directly detect the structure of single-molecule with the help of a noble metal nanosubstrate with nanoscale roughened structure (He et al. 2010; Le Ru, Meyer, and Etchegoin 2006). The SERS was used to characterize the structure of WPI-dextran conjugates based on this inspiring characteristic (Wang et al. 2013). As a result, a unique absorption band at  $983\text{ cm}^{-1}$  was observed in the Raman spectrum. This absorption band was assigned to  $\text{C-N}$  formed between the carbonyl carbon of dextran and the amine group of WPI and also reflected the conformational change in the protein backbone that occurred at the glycosylation site. In addition, some absorption bands exhibited in the Raman spectrum of native WPI also weakened or disappeared after glycosylation, such as the bands at  $850$  and  $830\text{ cm}^{-1}$  corresponded to

tyrosine, the band at  $760\text{ cm}^{-1}$  corresponded to tryptophan, and the band around  $550\text{--}510\text{ cm}^{-1}$  corresponded to disulfide bond. Recently, Dai et al. (2017) used CDSRS to characterize the WPI-dextran conjugates and satisfactory characterization results were obtained. First,  $1\text{ }\mu\text{L}$  of sample was dissolved in deionized water and was then placed on the glass slide wrapped in aluminum foil. The Raman spectra of samples were collected using a  $532\text{ nm}$  laser with a power of  $20\text{ mW}$  in a wavelength range of  $2000\text{--}400\text{ cm}^{-1}$  after the acquirement of white light microimages of samples at a magnification of  $50\times$ . During these processes, the sample was gradually dried. Change in white light microimage of the edge of the sample was then observed from an opaque and thick drop to a transparent and thin film when the sample solution was dehydrated in a certain degree. The transition stage from an aqueous dispersion to a solid state was a critical desiccation that could be easily differentiated by a laser confocal microscopy with high resolution. An obvious absorption band at  $1650\text{ cm}^{-1}$  corresponded to the high content of  $\beta$ -sheet was found in the WPI-dextran conjugates comparing with the native WPI according to the Raman spectrum that was obtained within one minute. Raman spectroscopy is demonstrated to be a useful characterization method for protein glycosylation based on the results of these reports.

### Thermal analysis

Thermal analysis focuses on the changes in denaturation temperature, enthalpy, and weight of protein during the transformation of crystal type, fusion, evaporation, and dehydration resulted from temperature variation. Protein glycosylation characterized by thermal analysis using DSC was also reported in previous publications (Liu et al. 2012; Wang, Wu, and Liu 2017). As reported by Liu et al. (2012), both the conarachin-dextran conjugates and the arachin-dextran conjugates exhibited increased denaturation temperatures and reduced enthalpy changes compared to those of the native conarachin and arachin. Furthermore, denaturation temperatures were not changed and the enthalpy changes were reduced as the reaction time increased. After grafting with polysaccharide chains, chemical bonds, such as hydrogen bond, hydrophobic bond, disulfide bond, and electrostatic interaction, are significantly changed in the native protein molecule so as to enhance the integrity and connectivity of PPC. Moreover, protein glycosylation is an endothermic reaction.

For example, denaturation temperatures of  $\beta$ -conglycinin fraction and glycinin fraction of SPI-dextran conjugates were increased from  $73.8$  to  $88.9^\circ\text{C}$  of the native SPI to  $80.5$  and  $97.1^\circ\text{C}$  of the SPI glycosylated with dextran using the dry-heating method at  $60^\circ\text{C}$  and  $\text{pH } 8.5$  for 8 days (Boostani et al. 2017). Enthalpy changes of these two fractions decreased from  $3.2$  and  $4.5\text{ J/g}$  of the native SPI to  $2.1$  and  $2.8\text{ J/g}$  of the conjugates. In terms of the most proteins, increased thermal denaturation temperatures and decreased enthalpy change in different extents were observed in PPC based on the formation of covalent bond during

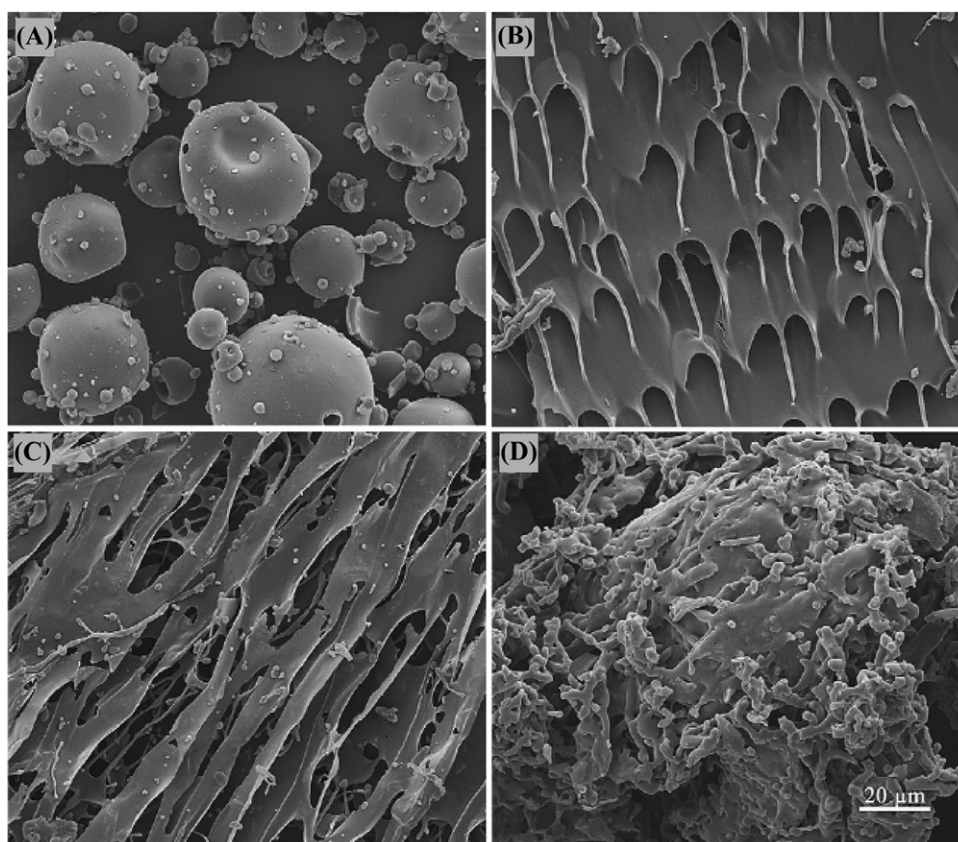


**Figure 2.** Scanning electron micrographs of WPI (A), corn fiber gum (B), WPI-corn fiber gum mixtures (C), and WPI-corn fiber gum conjugates (D) heated at 75 °C and RH of 79% for 2 days (Magnification 1000 $\times$ ; scale bar =20  $\mu$ m; WPI, whey protein isolates) (Yadav et al. 2012).

glycosylation (Ibanoglu 2005; Wang and Zhong 2014). In addition, thermal gravimetric analysis also employed to describe the differences of changes in weight loss and enthalpy of the SPI, soy hull hemicellulose, and SPI-soy hull hemicellulose conjugates (Wang, Wu, and Liu 2017). As a result, the conjugates of SPI and soy hull hemicellulose exhibited the most stable thermal properties with three stages of weight loss at highest temperatures because of the covalently crosslinking between SPI and soy hull hemicellulose. According to these studies, the thermal analysis of PPC can be used to effectively characterize the protein glycosylation from the changes of chemical bonds in molecular level.

### Microstructure analysis

Microstructure analysis performed by electron microscopy technique is also used to characterize PPC based on the study of structure and shape in an atom or molecule level and the relationship between them and functionality. The SEM photos of WPI, mixture of WPI and corn fiber gum, and WPI-corn fiber gum conjugates were illustrated in Figure 2 (Yadav et al. 2012). As illustrated in the figures, the WPI-corn fiber gum conjugates exhibited microstructural characteristics of tough appearance, irregular shape, and compact structure compared to the native WPI or the mixture of WPI and corn fiber gum. These changes in microstructure were attributed to the grafting between protein molecules and polysaccharide chains, which could be used to complement the results of SDS-PAGE and FTIR methods

in a higher scale. In addition, many proteins grafted with polysaccharides were confirmed to have more compact and heterogeneous microstructure compared to the native proteins, such as the SPI-dextran conjugates (Boostani et al. 2017), SPI-gum karaya conjugates (Shekarforoush et al. 2016), and pea protein isolate (PEPI)-pectin conjugates (Tamnak et al. 2016). Thus, SEM is conformed to a useful method to characterize PPC in microscale.

### Chromatographic method

The combination of HPSEC and appropriate detectors, such as refractive index, ultraviolet, multiangle laser-light scattering, intrinsic viscometry, and mass spectrometry, is one of the most used method for the characterization of MW distribution of PPC. Both protein and polysaccharide belong to biological macromolecules and the MW of the synthesized PPC is also large. Thus, the MW distribution of PPC can be detected by HPSEC with an appropriate detector (Kasran, Cui, and Goff 2013a). Specifically, MWs of the mixtures of SWPI and hydrolyzed fenugreek gum with different hydrolysis times of 10, 30, and 50 min were  $2.49 \times 10^5$ ,  $1.08 \times 10^5$ , and  $6.79 \times 10^4$  Da; however, MWs of the corresponding SWPI-hydrolyzed fenugreek gum conjugates were  $3.1 \times 10^5$ ,  $1.71 \times 10^5$ , and  $8.31 \times 10^4$  Da. Chromatographic peaks can be found in lower retention volume ranges in the chromatogram of PPC compared to the chromatogram of native protein or the mixture of protein and polysaccharide, meaning that the conjugates with higher MWs were formed during

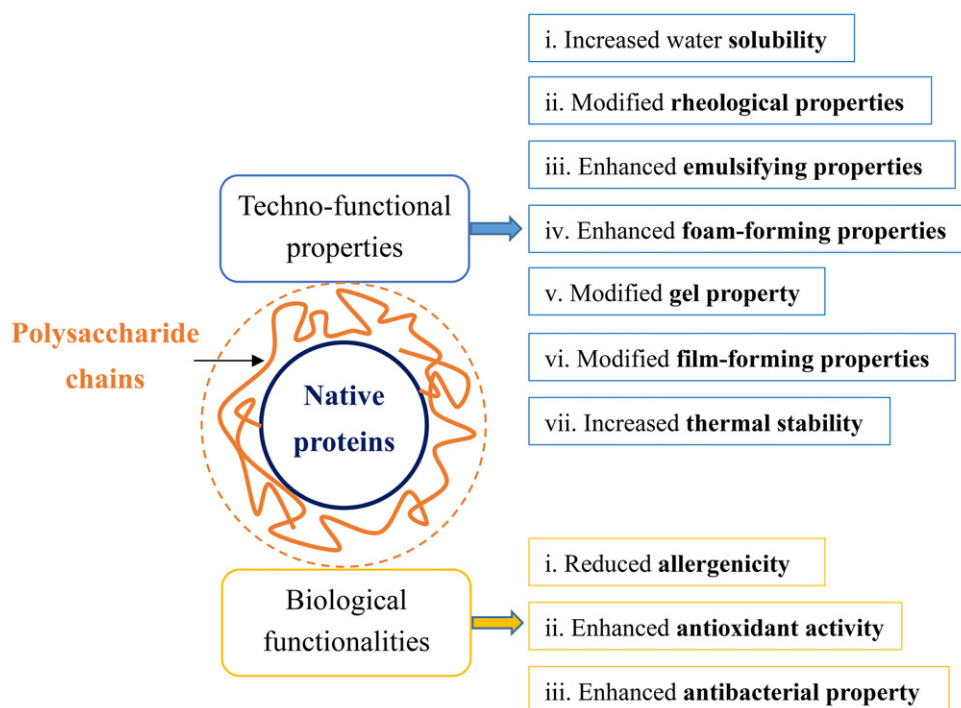


**Figure 3.** The changes in techno-functional properties and biological functionalities of native proteins after glycosylation with polysaccharides.

protein glycosylation (Mengibar, Miralles, and Heras 2017; Yang, Cui, Gong, Guo, et al. 2015). However, HPSEC can only provide the information about the generation of products with higher MWs, the MW distribution of the products, and the relatively qualitative analysis.

### Mass spectrometry

The development of soft ionization mass spectrometry technique offers a reliable method for the accurate qualitative analysis of biological macromolecules, such as protein. Recently, soft ionization mass spectrometry used to analyze the protein or peptide includes ESI-MS and MALDI-MS, which are commonly used in conjunction with high performance liquid chromatography (HPLC) to analyze the complex sample (Oliver 2011). Corzo-Martínez, Soria, et al. (2010) successfully adopted reverse-phase HPLC-ESI-MS to characterize the glycoconjugates of sodium caseinate and various saccharides including galactose, lactose, and dextran. The results demonstrated that several galactose or lactose molecules were grafted to the sodium caseinate molecule according to the changes in MWs of the conjugated sodium caseinates after deconvolution of ESI spectra. However, no evidence of glycosylation of sodium caseinate with dextran was observed after deconvolution of ESI spectra because of the low ionization efficiency of polysaccharide in ESI resulted from the low proton affinity of carbohydrates. In addition, application of MALDI-MS for the characterization of protein glycosylation was also only found in the protein glycosylated with monosaccharides (Medrano et al. 2009). Thus, PPC characterized by ESI-MS or MALDI-MS has been not systemically reported yet. However, accurate analysis of PPC using soft ionization mass spectrometry should

be also achieved by appropriate improved method due to the outstanding advantages in the analysis of proteomics of these two MS techniques.

Compared to the native protein, PPC exhibits different molecule structure and physicochemical properties, such as the content of free amino group, MW, secondary structure, covalent bond (C-N), infrared absorption, microstructure, and thermal denaturation temperature. Therefore, protein glycosylation can be effectively characterized using an appropriate method based on these changes compared to those of the native proteins. However, each method reported in the previous publications had its own detection range and several methods were selected together to characterize the practical protein glycosylation.

### Functional properties of PPC

The modified functionalities exhibited in PPC benefits from the changes in molecular structure during protein glycosylation. The typical changes in molecular structure include disulfide bond, hydrophobic bond, and infrared absorption bands of amide I, amide II, and amide III (Wang et al. 2013). Many publications proof that PPC prepared by the Maillard reaction exhibits high tolerance to the changes of solution conditions, such as temperature, pH and ionic strength, and high resistance against oxidation and proteolytic reaction. Consequently, as exhibited in Figure 3, the modified or enhanced techno-functional properties of PPC, such as solubility, emulsifying property, foaming property, gel property, and thermal stability, are exhibited (Liu and Zhong 2012). In addition, reduced allergenicity and increased antibacterial property and antioxidant activity are also observed in PPC compared to the native protein.

## Solubility

The performance of outstanding functional properties needs the thoroughly dissolution of protein in water. Thus, increase in the solubility of protein is beneficial to the improvement of other physicochemical and properties biological. Generally, most native proteins exhibit poor aqueous solubility around their *pI*s or an extreme pH range. Whereas, the manufacturing of various types of food, such as fruit and vegetable drinks, needs a broad pH range or an acidic environment, resulting in an application limitation of native proteins. Therefore, improvement of solubility of native proteins (especially around their *pI*s) is extensively investigated and promising results are also obtained (Chevalier et al. 2001; Diftis and Kiosseoglou 2006a; Neiryneck et al. 2004; O'Regan and Mulvihill 2009).

After grafting with polysaccharide chains, more hydrophilic groups are found in the molecule of PPC. Moreover, the grafted polysaccharide chains also change the charge characteristics of the native protein. Thus, the prepared PPC exhibits an enhanced hydration capacity and hydrophilism and finally the improved aqueous solubility at the *pI* of the native protein. As a well-known nutritional emulsifier with outstanding emulsifying property, sodium caseinate is broadly applied to manufacture various types of food. However, the emulsifying properties, including the EA and ES, are greatly reduced when the pH is close to its *pI* (4.6), restricting its application in acidic food processing (Neiryneck et al. 2007). Thus, changes in solubility of sodium caseinate after glycosylation with maltodextrin conjugates were studied (O'Regan and Mulvihill 2009). As a result, an improved solubility within a pH range of 2–8 was found in the sodium caseinate-maltodextrin conjugates at 20 or 50 °C compared to that of the sodium caseinate. The characteristic of being independent to the *pI* of sodium caseinate was attributed to the enhanced hydration by sugar residue and the changed amount of electrostatic charge. Kasran et al. (2013b) investigated the changes in solubility of soy whey protein isolates (SWPI) glycosylated with fenugreek gum in a pH range of 3–8 at 22 °C. Almost thorough precipitation of SWPI was observed because of the reduction of net charge when the pH of SWPI solution was adjusted to the *pI* of 4.5. The presence of fenugreek gum in the SWPI solution prevented the aggregation of protein molecules near the *pI* of SWPI in a certain degree so that the SWPI-fenugreek gum mixture exhibited an increased solubility about 28% compared to the SWPI alone. The SWPI-fenugreek gum conjugates exhibited a significantly increased solubility above 90% within the pH range of 3–8.

Therefore, PPC exhibits a significantly improved solubility than the native protein at its *pI* at the ambient temperature or relatively higher temperature. The glycosylated proteins can be kept in a dissolution state in water in a wide pH range to effectively exert other functional properties and nutritional value based on this outstanding enhancement of functionality.

## Rheological property

The rheological properties of fluid food have a great influence on its nature during processing or storage to alter the

final edible quality. Accompanying with the increase in solubility, the rheological properties, including viscosity, viscoelasticity, and type of fluid are also changed in PPC compared to the native protein. Corzo-Martínez, Soria, et al. (2010) comparatively investigated the rheological properties of sodium caseinate glycosylated with galactose, lactose, or dextran (10 kDa). As a result, the monosaccharide-induced glycoconjugates exhibited an increased viscosity and a type of shear-thinning fluid, behaving higher elastic modulus than the native sodium caseinate, especially for the caseinate-galactose conjugates. No significant different rheological properties were found between the caseinate-dextran conjugates and the native caseinate, which might be related to the type and MW of the selected polysaccharide. Rice endosperm protein conjugated with xanthan gum exhibited higher viscosity than that conjugated with glucose, which might be related to the higher molecular size of carbohydrate chain provided by the xanthan gum (Paraman, Hettiarachchy, and Schaefer 2007). The grafting degree of protein glycosylation also exhibited significant impact on the changes in viscosity of the protein conjugates. The viscosity of egg white protein-high methoxyl pectin (50–60 kDa) conjugates was gradually reduced along with the proceeding of glycosylation (0–72h), which was related to its reduced solubility in conjunction with the increase of reaction time (Al-Hakkak and Al-Hakkak 2010).

The modified rheological properties exhibited by PPC are vital to stabilize the O/W emulsion compared to the native protein or polysaccharide. Shekarforoush et al. (2016) investigated the effects of mixing and conjugation of SPI with gum karaya using the dry-heating method on the rheological behavior of both the aqueous systems and the O/W emulsions. Results demonstrated that higher apparent viscosity and stronger shear-thinning behavior were observed in the protein-gum karaya conjugates than the native gum karaya. In addition, the highest elastic modulus and viscous modulus, and the lowest polydispersity were also found in the O/W emulsion stabilized by the conjugates among the native gum karaya, protein-gum karaya mixture, and protein-gum karaya conjugates. However, the apparent viscosity of O/W emulsion stabilized by PPI-pectin conjugates was the lowest among those of O/W emulsions stabilized by the native pectin, the protein-pectin mixture, and the conjugates (Tamnak et al. 2016). Furthermore, substantial effects on the apparent viscosity of an O/W emulsion stabilized by PPI-pectin conjugates were observed by both the reaction time and protein-polysaccharide mass ratio, for instance, with the decrease in apparent viscosity as the reaction time increased. SPI-dextran conjugates prepared using the drying-heating method were added to a salad dressing in order to improve its quality (Diftis, Biliaderis, and Kiosseoglou 2005). As a result, the rheological characteristics, such as the complex viscosity, elastic modulus, and viscous modulus, reflected by the droplet interactions among the simulated salad dressing were closely related to the grafting degree between SPI and dextran. These studies give a practical application direction of glycosylated proteins based on their modified rheological properties in the food industry.

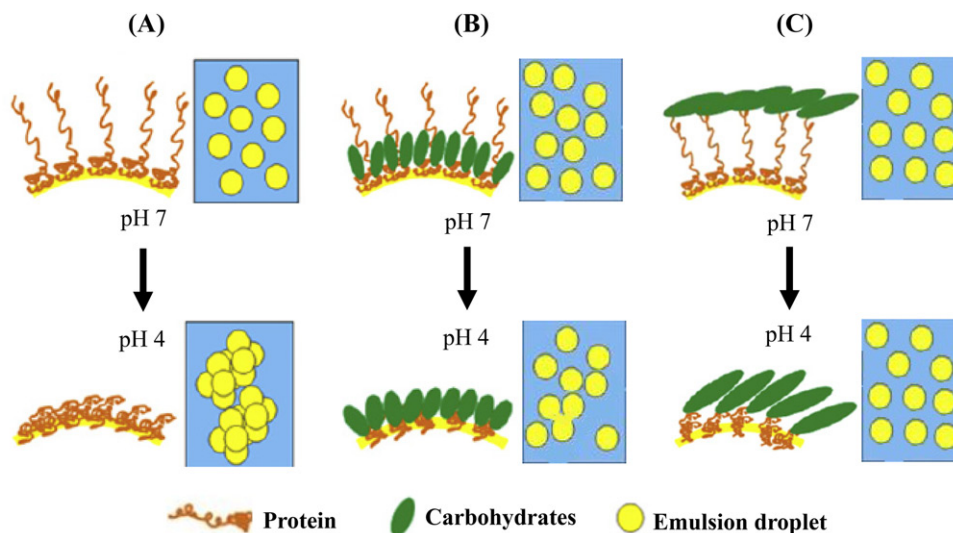


**Figure 4.** The schematic diagrams for the changes in the stability of emulsions stabilized by sIWP (A), sIWP-D10 conjugates (B), and sIWP-D65 conjugates (C) during the decrease of pH (sIWP, deamidated soluble isolated wheat protein; D10, dextrans with MW of 9–11 kDa; D65, dextrans with MW of 64–76 kDa) (Wong et al. 2011).

### Emulsifying properties

Improvement of emulsifying properties, such as the EA and ES, is the most studied functionality modification of native proteins after glycosylation (Chen et al. 2016; Dunlap and Côté 2005; Pirestani, Nasirpour, Keramat, and Desobry 2017; Wooster and Augustin 2006; Xu and Yao 2009). The remarkable improvement of interface characteristics of glycosylated proteins can be reflected by the increased EA index and ES index compared to the native protein or some commercial emulsifier, indicating that PPC has a potential of being used as a new generation of emulsifier in food system. Improvement of EA and ES of PPC benefits from the enhancement of the lipophilicity and hydrophilicity resulted from the moieties of protein and polysaccharide. Thus, the O/W emulsion stabilized by this type of modified proteins has a characteristic of stabilization against high temperature and long-term storage (Diftis, Biliaderis, and Kiosseoglou 2005; Diftis and Kiosseoglou 2006b; Xue et al. 2013). First, the covalently crosslinking between protein and polysaccharide makes the protein structure unfolded and some hydrophobic groups contained in the interior structure of native protein expose to the molecular surface to increase the lipophilicity of conjugates. Secondly, the polysaccharide chains with many hydrophilic groups endow PPC an increased hydrophilicity. For example, a thick protective layer can be formed in the O/W emulsion stabilized by PPC and the phase separation phenomena, such as emulsification, flocculation, and combination of oil droplets, won't occur because of the repulsive effect induced by the steric hindrance of polysaccharide chains. Consequently, the O/W emulsion can remain in a stable state in a long-term storage, high-temperature treatment (such as 80 °C for 20 min), broad concentration range of salt ions, and broad pH range (Chen et al. 2018; Setiowati et al. 2017; Yadav et al. 2010).

The stabilization of emulsion by PPC involves the interface change between the oil phase and the water phase. The specific mechanism includes the reduction of oil-water

interfacial tension by the lipophilicity of protein moiety and the hydrophilicity of polysaccharide moiety (Liu et al. 2011), the formation of a thick protection layer at the oil-water interface (Chen et al. 2014), and the controlling of rheological properties of emulsion by the formation of a stable interface structure (Dickinson 2008). Wong et al. (2011) studied the stabilization of an O/W emulsion by deamidated soluble isolated wheat protein (sIWP) glycosylated with dextran with a MW of 9–11 kDa (D10) or dextran with a MW of 64–76 kDa (D65) using the dry-heating method. Results demonstrated that both the native IWP and the two types of sIWP-dextran conjugates could make O/W emulsions possess good dispersibility and stability at pH 7. However, flocculation and precipitation of oil droplets were found in the O/W emulsion stabilized by the native sIWP and the conjugates-stabilized O/W emulsions remained stable state except a bit of droplet flocculation in the sIWP-D10 conjugates-stabilized O/W emulsion when the pH was adjusted to 4. Thus, higher ES was found in the O/W emulsion stabilized by PPC with higher MW of polysaccharide, which could be also reflected by the formation of a carbohydrate steric layer with a thickness of 5.9 nm at the *N*-terminal amino group of sIWP with D65 (illustrated in Figure 4). In addition, the significant improvement of EA and ES exhibited by PPC in emulsion signified the potential of this type of modified proteins to substitute the traditional commercial emulsifiers in food processing, such as the WPI-maltodextrin conjugates (Akhtar and Dickinson 2007) and SPI-SSP conjugates (Yang, Cui, Gong, Miller, et al. 2015). It follows that the emulsifying properties of glycosylated proteins cannot be reduced by flocculation induced by the effect of pI, broadening the application range of native proteins in food processing.

Another display of the enhanced emulsifying properties of PPC is the remarkable resistance to the pI of native proteins and high salt ion concentration when the conjugates are used to stabilize emulsions. The resistance to high-salt condition of glycosylated protein was first reported by Kato and groups (Kato, Minaki, and Kobayashi 1993; Nakamura,

Kato, and Kobayashi 1992). For example, O/W emulsions stabilized by egg white protein-galactomannan conjugates or lysozyme-galactomannan conjugates exhibited the excellent emulsifying properties at a high salt concentration (i. e. 0.2 mol/L sodium chloride). With the grafting with hydrophilic polysaccharide chains, the glycosylated proteins have both hydrophobicity and hydrophilicity so that the water phase and the oil phase of the conjugates-stabilized emulsion can be effectively combined. Similarly, the O/W emulsions stabilized by WPI-dextran conjugates or WPI-maltodextrin conjugates also exhibited lasting emulsifying properties even at a low pH of 3 and a high salt concentration of 0.2 mol/L (Akhtar and Dickinson 2003; Akhtar and Dickinson 2007). In addition, an O/W emulsion stabilized by SWPI-fenugreek gum conjugates exhibited no significant change in average particle size even after 21 days of storage at 25 °C with a high sodium chloride concentration of 0.5 mol/L (Kasran, Cui, and Goff 2013b). However, an O/W emulsion stabilized by the native SWPI exhibited an increasing average particle size under the same conditions. Thus, the SWPI-fenugreek gum conjugates had an impressive tolerance to salt ionic strength in emulsions.

Zhang et al. (2015) investigated the stabilities of O/W emulsions stabilized by the native oat protein isolate (OPI), heated OPI, and OPI-dextran conjugates within a pH range of 3–9 and a sodium chloride concentration range of 0–0.2 mol/L. The results demonstrated that the average particle size of the conjugates-stabilized emulsion first increased and then decreased as the pH value increased and constantly increased as the salt concentration increased. Nevertheless, an emulsion stabilized by the OPI-dextran conjugates exhibited the lowest average particle size and finally the best ES compared to those stabilized by the native OPI and the heated OPI within either the selected pH range or the ion concentration range. The polysaccharide chains provided a steric layer to prevent the flocculation and precipitation of the protein moieties at the *pI* of the native protein, resulting in the increased steric repulsion and decreased van der Waals attraction among the oil droplets. Consequently, no aggregation occurred among these oil droplets and the average particle size this O/W emulsion remained unchanged. In addition, the thickness of steric layers formed around the oil droplets in glycosylated protein-stabilized O/W emulsion increased as the MW of dextran increased (18.5–440 kDa) (Wooster and Augustin 2006). The thicker the steric layer was, the more distance between the protein charge and the edge of dextran layer formed, and finally reducing the observed emulsion charge. In terms of the salt ion tolerance, the emulsions stabilized by  $\beta$ -lactoglobulin-dextran conjugates remained a similar initial particle size ( $D_{4,3}$ ) of 1.25  $\mu\text{m}$  in a calcium chloride concentration range of 0–0.02 mol/L for 24 h; however, the emulsion stabilized by  $\beta$ -lactoglobulin was unstable when the calcium concentration was above 0.008 mol/L. Therefore, the glycosylated proteins have a wider application in food processing compared to the emulsifiers that are liable to be denatured under the acidic or high salt concentration environments based on the

enhancement of tolerance to low pH and high salt concentration.

### Foaming properties

The foaming ability of proteins can be reflected by the amounts and the stability of foams that formed by the steric repulsion and electric forces of the absorbed protein layer at the air-water interface. The foaming properties of protein are closely influenced by the environment factors, including the concentration of protein, pH, salt concentration, temperature, shaking, as well as the molecular structure of protein (Damodaran 2005). Generally, the excellent solubility of protein is a prerequisite to the foaming properties; thus, the modification of foaming properties of PPC is similar to that of the solubility.  $\beta$ -Lactoglobulin glycosylated with lactose using the dry-heating method exhibited improved foaming properties compared to the native  $\beta$ -lactoglobulin and higher foaming stability than the  $\beta$ -lactoglobulin-glucose conjugates (Medrano et al. 2009). However, among the conjugates formed between the total milk protein, WPI, and sodium caseinate and the glucose, lactose, pectin, and dextran, all of the protein-saccharide conjugates formed outstandingly stable foams compared to the native proteins (Hiller and Lorenzen 2010). The difference between the results reported in these two publications might be attributed to the different types of proteins.

Liu et al. (2012) comparatively investigated the foaming capacity (FC) and foaming stability (FS) of the PPI-dextran (35–45 kDa) conjugates at pH 7 using the dry-heating method for different reaction times (1, 4, and 7 days). Compared to the native PPI and the PPI-dextran conjugates obtained for 1 day, the conjugates obtained for both 4 and 7 days exhibited significant higher FC and FS. More polysaccharide chains were combined to the protein molecules as the reaction time increased, resulting in more enhanced hydrophilicity of the PPI-dextran conjugates. Consequently, the 4 or 7 days-formed conjugates could easily dissolve in water and rapidly transfer to the interface of air-water interface from the bulk so as to form more stable foams than the native PPI or the conjugates obtained for 1 day. In addition, similar to the formation of a thick steric layer at the oil-water interface to stabilize the emulsion, the foaming properties-improved PPC can also form thick viscoelastic layers at the air-water interface to effectively prevent the aggregation and Ostwald ripening of the formed foams (Dunlap and Côté 2005). However, the thickness of the absorption layer of PPC around the air-water interface was also influenced by the MW of the polysaccharide used. WPI glycosylated with alginate of 60 and 180 kDa were comparatively performed to investigate the impact of covalent conjugation on the ability of WPI to resist surfactant-induced competitive displacement from the air-water interface (Cai, Saito, and Ikeda 2018). As a result, the WPI glycosylated with alginate of 180 kDa and the native WPI were more susceptible to Tween-20-induced displacement from the interface than the WPI glycosylated with alginate of 60 kDa. This might be ascribed to the larger interfacial area coverage and

lower interfacial film thickness observed in the lower MW alginate-glycated WPI. Thus, the improvement of foaming properties of glycosylated proteins is also closely influenced by the nature of the selected polysaccharide.

The modification of foaming properties of PPC contributes to overcome the application limitation of native proteins on the food processing under the acidic and high salt concentration conditions and save material cost by reducing the amount of native proteins to obtain the same foaming bubbling effect.

### Gel property

The gel property of protein is an important characteristic to be used to prepare the gelled food (such as the soybean curd) or improve the textural properties of meat products and surimi products. However, the gel property of native proteins is frequently reduced to lose their application value because of the high temperature, low pH, and high salt concentration needed during food processing. Thus, the modification of protein structure by glycosylation for the improvement of gel property can overcome this application limitation of native proteins in the acidic food. The gel strength and appearance of the thermal-induced gels made by the WPI-dextran conjugates were observably better than those of the thermal-induced gels made by the native WPI or the WPI-dextran mixtures (Spotti et al. 2013a). As illustrated in Figure 5, similar appearance was observed among the thermal-induced gels made by the WPI-dextran mixtures with a dextran MW of 6 kDa and concentrations of 0, 3.6, 7.2, and 10.8%. When the MW was 6 kDa and the concentration was higher than 7.2%, the thermal-induced gels of WPI-dextran conjugates exhibited collapsed and dark appearances. When the MW of dextran was 40 or 70 kDa, the WPI-dextran conjugates were capable of forming the steady self-supporting gel with color-lightened appearances. According to the results of a fracture test, all thermal-induced gels made by WPI-dextran conjugates didn't exhibit a fracture compared to the gels made by the WPI-dextran mixture. This might be attributed to the formed covalent bonds between WPI and dextran molecules via the Maillard reaction that could hold the whole gel structure together and prevent the fracture. Similar results could be found in other research on the gel mechanical properties of WPI-dextran conjugates reported by Spotti et al. (2013b). However, the loose state of thermal-induced gel made by WPI-dextran conjugates was closely related to the changed disulfide bond profile because of the dextran chains, influencing the regular formation of protein network (Spotti et al. 2014). In addition, reduced storage modulus and loss modulus, and finally the stiffness were observed in the WPI-dextran conjugate gels compared with those of gels made by WPI or WPI-dextran mixture according to the rheological properties during a thermal-induced gelling process (Sun, Yu, Zeng, et al. 2011).

The rheological and gelling properties during the formation of glucono- $\delta$ -lactone (GDL)-induced gels and the corresponding textural properties were comparatively studied

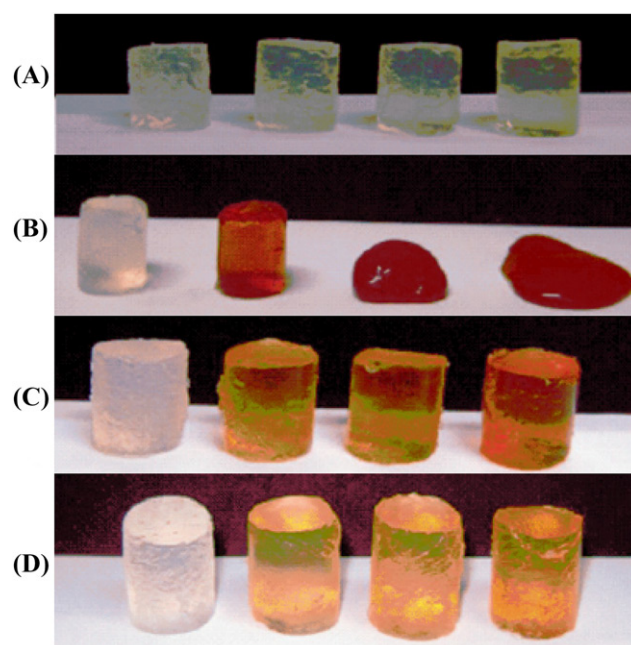


**Figure 5.** The appearances of thermal-induced gels of WPI/dextran mixture with dextran of 6 kDa (A) and WPI-dextran conjugates for different MWs: Dextran of 6 kDa (B), 40 kDa (C) and 70 kDa (D). Dextran concentrations were 0, 3.6%, 7.2% and 10.8% (w/w) from left to right (WPI, whey protein isolates; MW, molecular weight) (Spotti et al. 2013a).

using native caseinate, crosslinked caseinate with MTGase, and oligochitosan-glycosylated and MTGase-crosslinked caseinate as materials (Song and Zhao 2013). The results demonstrated that the glycosylated and crosslinked caseinate formed a GDL-induced gel at pH 5.30 for 95 min, which were higher or shorter than those needed for the formation of gels of native caseinate or crosslinked caseinate. Thus, the glycosylation of caseinate prompted the formation of GDL-induced gels. Moreover, the GDL-induced gel made by the glycosylated and crosslinked caseinate exhibited the enhanced hardness, springiness, and cohesiveness than the other two types of GDL-induced gel. Similar results were found in the research on the properties of GDL-induced gels made by sodium caseinate-maltodextrin conjugates (Zhang et al. 2017). In addition, the grafting degree (0–3 days), reaction temperature (50–70 °C), and the RH (65%, 72%, 79%) also significantly influenced the gel properties of the glycosylated protein, which was reported by Zhang and Chi (2011). The maximum gel strength of the thermal-induced gel made by SPI-glucomannan conjugates were found when the glycosylation conditions were 1.5 days, 60 °C, and 79%. It was worth noting that the decrease tendency of gel strength of the conjugates-formed gel was slower than that of the SPI-formed gel during long-term storage under the simulated shipping conditions.

The controversial conclusions about the modification of gel properties by glycosylation of proteins should be related to the variety differences of the selected proteins or polysaccharides. Due to the abundant variety of protein-based foods, the researches on the improvement of gel property of the native proteins should be further carried out by selecting an appropriate type of protein or polysaccharide to satisfy different requirements of the novel gelled food.

### Film-forming properties

The film-forming properties of protein based on its gel property using a plane carrier, including ductility, barrier property, and breaking strength, make it possess excellent package performance. In addition, the protein-based films and coatings have been extensively studied and applied to the fields of food package and the delivery of bioactive compounds because of the safe, edible, and biodegradable characteristics (Dangaran, Tomasula, and Qi 2009; Vargas et al. 2008). However, the film formed by native proteins is unstable compared to the synthetic film, such as the fracture, breaking, and dissolution during the application on food package. Thus, the protein modification or addition of crosslinking agent to improve the film-forming properties and finally the mechanical properties of fabricated film are the primary means for the production of protein films (Azeredo and Waldron, 2016; Tharanathan 2003). Among the selected methods, the glycosylation of proteins is a safe and effective method.

The PPI glycosylated with xylose using the dry-heating method was blended with glycerol to fabricate films (Lin, Liu, Shi, Liu, Adhikari, et al. 2015). The flexibility, elongation, tensile strength, and water resistance of films made by the mixture of PPI-xylose conjugates and glycerol were significantly improved compared to the mechanical properties of films made by the mixture of PPI and glycerol. The PPI-xylose conjugates used to fabricate a biodegradable film with the best mechanical properties was obtained at pH 9.5 and 91.5 °C for 95 min using the wet-heating method by using the response surface methodology (Lin, Liu, Shi, Liu, Wang, et al. 2015). Films made by the PPI-xylose conjugates exhibited more compact and denser structure than that of the film made by native PPT according to the comparative results of microstructure. Meanwhile, films made by the xylose-glycated PPI exhibited the best mechanical properties among the monosaccharides, including xylose, glucose, mannose, lactose, galactose, and sugar (Lin et al. 2014). The improvement of mechanical properties of protein-fabricated films by glycosylation with monosaccharide imply the possibility of improvement of the film-forming properties of native proteins by glycosylation with polysaccharides.

The SPI glycosylated with glucomannan by the dry-heating method was used to fabricate films and their mechanical properties, including tensile strength, elongation at break, and water vapor permeability, were evaluated compared to those of the films fabricated by the SPI alone and the mixture of SPI and glucomannan (Zhang, Wu, et al. 2014). As expected, the mechanical properties of the conjugate-fabricated film were the best among the three types of films because of the covalent bond formed between SPI and glucomannan at 60 °C and RH of 72% for 48 h. The effect of grafting degree or reaction time (3, 6, and 9 days) on the mechanical properties of a PPC-film was investigated using PPI and gum Arabic as the materials (Li, Wang, et al. 2015). Decreased tensile strength and increased elongation and water vapor permeability of the conjugates film were observed as the glycosylation proceeded from 3 to 9 days, which was attributed to the decreased crystalline and

increased amorphous structure of conjugates film revealed by X-ray diffractometry and SEM. As the predominant interaction forces, disulfide bonds reached an equilibrium level in the PPI-gum Arabic conjugates film networks after 6 days of glycosylation. Thus, the improvement of mechanical properties of a protein film should consider the grafting degree of the native protein and select an appropriate reaction time based on the properties of proteins and polysaccharides. Glycosylation of protein has been proved to be a useful method to modify or improve the mechanical properties of protein-based films.

### Thermal stability

The improvement of thermal stability after glycosylating is manifested in the increased thermal denaturation temperature of native proteins so as to exert their functionalities above their denaturation temperature. The glycosylation of protein can restrict the thermal-induced aggregation and increase the thermal denaturation temperature according to the previous studies. Kim and Shin (2016) investigated the thermal stability of BSA after glycosylation with fucoidan using the dry-heating method. As a result, an increase in more than 20 °C of denaturation temperature and a decreased enthalpy change were observed in the BSA-fucoidan conjugates compared to those of the native BSA. The significant increase in denaturation temperature of glycosylated protein was closely related to the covalently crosslinked polysaccharide chains; however, the reason why the enthalpy change decreased was that the glycosylation of protein was an exothermic reaction. In addition, the denaturation temperatures of the native arachin and conarachin were increased from 101.5 and 89.6 °C to 107.3 and 96.8 °C after glycosylating with dextran (MW 35–45 kDa) using the dry-heating method at 60 °C and RH of 79% for 7 days (Liu et al. 2012). Similarly, the denaturation temperatures of glycinin and  $\beta$ -conglycinin fraction were 97.1 and 80.5 °C, which were significantly higher than 88.9 and 73.8 °C of the native proteins when SPI was glycosylated with dextran (MW 64–76 kDa) using the dry-heating method at 60 °C, pH 8.5, and RH of 79% for 8 days (Boostani et al. 2017). It was worth noting that the reaction time (1, 4, and 7 days) had no significant influence on the changes in denaturation temperature caused by glycosylation (Liu et al. 2012). In addition, the increase degree of denaturation temperature of glycosylated protein first increased and then decreased as the MW of polysaccharide increased (Xu, Yang, et al. 2010). However, gradually increased denaturation temperature in conjunction with the increase of MW of polysaccharide also found in the ovalbumin-carboxymethyl cellulose conjugates (Geng et al. 2014).

The improvement of thermal stability was also reflected by the reduced thermal aggregation of proteins in aqueous. Liu and Zhong (2012) reported that protein aggregation was observed in the WPI-maltodextrin conjugates aqueous solution before and after heating at 88 °C for 2 min at different pH values (3–7) and salt concentrations (0–0.15 mol/L). And the glycosylated WPI was observed in transparent

dispersions with an average particle size lower than 12 nm after heating, which might be mainly ascribed to the steric hindrance supplied by the polysaccharide chains (Liu and Zhong 2013). Sodium caseinate glycosylated with maltodextrin (12–14 kDa) using the dry-heating method for 4 days and its purified fraction, as well as the native sodium caseinate were comparatively used to stabilize O/W emulsions that were then frozen and thawed for several cycles (O'Regan and Mulvihill 2010a). The results demonstrated that the average particle size of oil droplets in the conjugates-stabilized O/W emulsion was decreased in smaller extent than that in the sodium caseinate-stabilized O/W emulsion; moreover, the better frozen-thaw stability was found in the purified conjugates-stabilized O/W emulsion.

As mentioned above, the glycosylated proteins possess higher thermal stability to retain their functionality during high-temperature treatment compared to the native proteins, resulting in broadening the application of PPC in food processing, such as the boiling, thermal sterilization, and cooling or freezing treatment.

### **Biological functionality**

Except for the functional properties needed in food processing, allergenicity, antioxidant activity, and antibacterial property of native proteins are also observed in food matrix. As reported previously, these biological functionalities of protein could also be modified after glycosylation with carbohydrates.

### **Allergenicity**

As a common type of allergen, native proteins and native protein-based foods are carefully considered during processing and consumption. In other words, it is very necessary to reduce the allergenicity of proteins by using an appropriate method for the health of special consumption populations in order to supplement essential amino acids or pursue unique techno-functional characteristics of native proteins. Generally, glycosylation changes the original molecular structure of protein to influence its allergenicity. Reduced allergenicity exhibited in the glycosylated  $\beta$ -lactoglobulin with monosaccharides or oligosaccharides was extensively studied and confirmed (Bu et al. 2010; Hattori et al. 2004; Taheri-Kafrani et al. 2009). Moreover,  $\beta$ -lactoglobulin glycosylated with polysaccharides also exhibited a reduced allergenicity. Kobayashi et al. (2001) investigated the changes in the molecular structure of  $\beta$ -lactoglobulin after glycosylation with carboxymethyl dextran with differing MWs (40 and 162 kDa) and the antibody response to the conjugates by the tested mice. Significantly reduced antibody responses to all  $\beta$ -lactoglobulin-carboxymethyl dextran conjugates were observed in the subjects compared to the native  $\beta$ -lactoglobulin. The greater reduction of antibody response was found in the glycosylated  $\beta$ -lactoglobulin with polysaccharide with higher MW. More epitopes could be masked accompanying with the conjugation with carboxymethyl

dextran of high MW so that the reduced allergenicity of glycosylated  $\beta$ -lactoglobulin was observed.

As a major allergenic protein of buckwheat, Fag e 1 was glycosylated with arabinogalactan or xyloglucan using the dry-heating method (Nakamura et al. 2008). The results demonstrated that remarkable reduction of reactivity against human sera of buckwheat-allergy subjects was found in these two Fag e 1-polysaccharide conjugates accompanying with the modification of solubility and emulsifying properties. The same results were also found in the studies on the changes in allergenicity of whole buckwheat protein glycosylated with dextran (Tazawa et al. 2014) and SPI glycosylated with fructooligosaccharides (van de Lagemaat et al. 2007). However,  $\beta$ -lactoglobulin moderately glycosylated with dextran exhibited an enhanced immunoreactivity and a decreased digestibility, which was related to the decreased reactivity of digestive enzymes toward the conjugates resulted from the dextran chains-induced steric hindrance (Corzo-Martínez, Moreno, et al. 2010). Thus, to study the influence of glycosylation on the allergenicity of native proteins should take into account the level of gastrointestinal digestibility of PPC. Nevertheless, the significant reduction of allergenicity of native proteins after glycosylation offers an important idea to produce high protein foods without protein antigen.

### **Antioxidant activity and antibacterial property**

The antioxidant activity and antibacterial property of proteins may guarantee the stability of protein-based food, such as the preventing of lipid oxidation and the prolonging shelf life of food. It is well known that certain antioxidant activity (Yilmaz and Toledo 2005) and antimicrobials property (Ruriánhenares and Morales 2008) are exhibited by the advanced Maillard reaction products, which are mainly formed at the advance stage and final stage of the Maillard reaction. However, the glycosylated proteins also exhibit antioxidant activity and antibacterial property compared to the native proteins. With respect to the native WPI, the WPI-glucose conjugates synthesized using the dry-heating method exhibited a dramatic enhancement of reducing power and the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical scavenging activity and these antioxidant activities increased as the reaction time increased (Liu et al. 2014). The BSA glycosylated with dextran with the help of pulsed electric field exhibited an enhanced  $\alpha, \alpha$ -diphenyl- $\beta$ -picrylhydrazyl (DPPH) radical-scavenging activity, which was stronger at a higher field intensity (20 kV/cm) (Guan et al. 2010). Similarly, the SPI-dextran conjugates obtained by  $\text{TiO}_2$  photocatalysis treatment also exhibited an enhanced hydroxyl radical scavenging activity and a reducing power; moreover, antioxidant activity increased in a higher degree was observed in the conjugates obtained by using higher photocatalytic power (1000 W) (Jin et al. 2015). All the  $\beta$ -Lactoglobulin-soluble chitosan conjugates with different chitosan MWs (1.3, 39, and 56 kDa) exhibited an enhanced DPPH radical-scavenging activity and a ferric reducing antioxidant power compared to the native  $\beta$ -lactoglobulin (Mengibar,

Miralles, and Heras 2017). Moreover, the highest antioxidant activity was found in the  $\beta$ -lactoglobulin glycosylated with chitosan of 1.3 kDa. This might be related to the highest grafting degree between  $\beta$ -lactoglobulin and chitosan with low MW. The improvement of antioxidant activity of glycosylated proteins is closely related to the abundant hydroxyl group or the formation of hydrogen donor resulted from the linking of polysaccharide chains. Based on the changes in the molecular structure of proteins after glycosylation.

As reported in the early study that focused on the antibacterial property of lysozyme-galactomannan conjugate, 80% lytic activity of the native lysozyme was remained in the glycosylated lysozyme (Nakamura, Kato, and Kobayashi 1992). Further, strong bactericidal effects on the Gram-negative bacteria, such as *E. coli* IF0 12713 and *V. parahaemolyticus* IF0 12711, were observed in conjunction with the improvement of EA. Lysozyme conjugated with dextran exhibited considerable antimicrobial effects on both Gram-negative and Gram-positive bacteria (Nakamura, Kato, and Kobayashi 1991). Almost ten years later, lysozyme glycosylated with chitosan was performed to investigate the bactericidal activity of lysozyme-chitosan conjugates by the same research group (Song et al. 2002). Not surprisingly, an improved bactericidal activity against *Escherichia coli* K-12 was found in the glycosylated lysozyme with chitosan of both low MW (3–30 kDa) and high MW (400 kDa). Recently, lysozyme glycosylated with guar gum (Hamdani et al. 2018), xanthan gum (Hashemi, Aminlari, and Moosavinasab 2014), and pullulan (Sheng et al. 2017) also exhibited a significantly increased or broadened antimicrobial activity to the Gram negative bacteria and functional properties at acidic pHs and different temperatures than their native state. In other words, glycosylation modified the functional properties of lysozyme to extend its application in common sterilization of food.

In addition, SPI glycosylated with chitosan also had an enhanced antimicrobial activity compared to that of the SPI-chitosan mixture (Usui et al. 2004). Recently, chitosan was used to glycosylate  $\epsilon$ -polylysine and the antimicrobial mechanism of the prepared conjugates was analyzed (Liang et al. 2014). As a result, strong antibacterial and antifungal activities against *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis*, and *beer yeast* were observed in the  $\epsilon$ -polylysine-chitosan conjugates compared to those of the  $\epsilon$ -polylysine or chitosan alone. Furthermore, the strongest antimicrobial activity was found in the conjugates prepared between  $\epsilon$ -polylysine and chitosan with a mass ratio of 1:1. The remarkable antimicrobial activity of  $\epsilon$ -polylysine-chitosan conjugates was completed by increasing the permeability of the outer membrane and inner membrane and ultimately disrupting bacterial cell membranes with the release of cellular cytoplasm. Based on the abovementioned results, certain antibacterial activity was exhibited by the native protein or polysaccharide and this antibacterial property was remained or further enhanced by glycosylating.

## Application of PPC in food processing

More extensive use of PPC can be developed compared to the native proteins based on the aforementioned improvement of various techno-technical and biological functionalities. At present, the glycosylated proteins are mainly used to stabilize emulsions in order to protect or increase the utilization of bioactive compounds, prepare microencapsulation or nanogel for the encapsulation or delivering of bioactive compounds, and improve the functionalities of native proteins in food matrix (Liu et al. 2017).

### Stabilization of emulsion system

The emulsifying properties of protein make it be used as an ingredient of multiphase-based food in order to increase their nutritional value, edible quality, and shelf life. However, the emulsifying properties of native protein are commonly weakened under peculiar environmental conditions during the practical food processing, such as the food system with a high acidity or a high salt ion concentration and the cooling or freezing treatment of food. Thus, PPC with enhanced EA and ES compared to the native protein possesses more preponderant application potential in foods (Evans, Ratcliffe, and Williams 2013; Sivapratha and Sarkar 2018).

As a novel and safe amphipathic compound, PPC has more promising application values. Recently, emulsifiers used to protect the unstable nutrients during spray-drying processing mainly include the mixtures of protein and polysaccharide, such as the mixture of PEPI and pectin for the microencapsulation of polyunsaturated fatty acid-rich oil (Aberkane, Roudaut, and Saurel 2014) or food flavors (Gharsallaoui et al. 2012), and the mixture of SPI and gum Arabic for the microencapsulation of paprika oleoresin (Rascón et al. 2011). However, inhomogeneous and unstable characteristics are commonly found in these blended emulsifier so as to obtain the undesirable embedding effect. By using a desolvation method, nanoparticles with spherical structures and enhanced surface hydrophobicity were successfully prepared by both  $\beta$ -conglycinin-dextran conjugate and its amphiphilic hydrolysates with a hydrolysis degree of 2.2% (Wu et al. 2014). Better interfacial pressure and dilatational modulus after long-term absorption at the oil-water interface of O/W emulsion were found in these two types of nanoparticles compared to the nanoparticles prepared by the native  $\beta$ -conglycinin; however, the best stability was found in the amphiphilic hydrolysates-formed nanoparticles. The development of nanoparticles prepared by PPC and its derivatives gives innovative materials for the delivering and protection of bioactive compounds.

As a type of fat-soluble polyphenol extracted from turmeric, curcumin has various biological activities, including antineoplastic activity, antioxidant activity, and anti-inflammatory (Anand et al. 2007). However, the use of curcumin as dietary supplements is restricted because it is water-insoluble and unstable under the neutral or weak alkaline condition. Wang et al. (2016) reported that the BSA-dextran conjugates-stabilized O/W emulsion with curcumin in the

oil phase were observed in a stability state even at 37°C for 10 days. Moreover, the oral bioavailability in mice for the curcumin-contained emulsion stabilized by this conjugate was 4.8 times higher than that for the curcumin-contained emulsion stabilized by Tween 20. Although an outstanding flavor and an antimicrobial activity are found in citral, its application in food is restricted by its physicochemical instability during food processing and digestive and absorption processes after ingesting. Thus, SPI-SSP conjugates were prepared to stabilize an O/W emulsion where the citral was dissolved in the oil phase (Yang, Cui, Gong, Miller, et al. 2015). The citral-loaded O/W emulsion stabilized by SPI-SSP conjugates exhibited a remarkable retention rate of citral under the conditions of long time storage, heating, and simulated gastrointestinal conditions compared to the citral-loaded O/W emulsion stabilized by the SPI or the SPI-SSP mixture. This promising function provided by the prepared conjugates was closely related to its improved emulsifying properties. Therefore, PPC can not only maintain the stability of O/W emulsion, but also enclose the oil particles in a form of protecting layer, which is very important for the protection of polyunsaturated fatty acid or bioactive compounds in the oil particles. Consequently, these fat-soluble components are avoided from oxidation or structural changes because of the surrounding oxygen or ion concentration and finally are delivered or promoted to exert their activity.

Due to the instability of double emulsions, a  $W_1/O/W_2$  emulsion stabilized using sodium caseinate-maltodextrin conjugate as an emulsifier of the outer water phase was fabricated to encapsulate vitamin B<sub>12</sub> in the inner water phase (O'Regan and Mulvihill 2010b). The  $W_1/O/W_2$  emulsion stabilized by the sodium caseinate-maltodextrin 100 (dextrose equivalent of 9–12) exhibited the lowest average particle size and viscosity without significantly changes over a 7 days of storage at 45°C compared to the double emulsion stabilized by the sodium caseinate or the sodium caseinate-maltodextrin 40 (dextrose equivalent of 4–7). The encapsulation efficiency of vitamin B<sub>12</sub> in the inner water phase of  $W_1/O/W_2$  emulsion stabilized by the sodium caseinate-maltodextrin 100 also remained at the highest level under the same accelerated shelf life testing conditions. As a type of promising bioactive food ingredient-delivering material, PPC exhibits not only functions of protection and delivering, but also an excellent controlled release of the encapsulated bioactive compounds under the specific conditions. Fechner et al. (2007) fabricated a double emulsion ( $W_1/O/W_2$ ) stabilized by the caseinate-dextran conjugates for the encapsulation and controlled-release of vitamin B<sub>12</sub>. The caseinate-dextran conjugates-stabilized  $W_1/O/W_2$  emulsion exhibited more coalescence-stable under the acidic condition, such as pH 4 and pH 4.5, compared to the caseinate-stabilized  $W_1/O/W_2$  emulsions. Moreover, the release rate of vitamin B<sub>12</sub> into the surrounding water was significantly lower in the conjugates-stabilized  $W_1/O/W_2$  emulsion (30%) than that in the caseinate-stabilized  $W_1/O/W_2$  emulsion (40%) at pH 4 and ambient temperature after a 7 days of storage.

According to aforementioned studies, PPC, used as a coating material or an emulsifier for the encapsulation of bioactive compounds to protect their nutritional values and functionalities and facilitate the controlled-release and digestive absorption, has a significant actual meaning.

### **Fabrication of microencapsulation or nanogels**

Most bioactive compounds are unstable or easily destroyed during food processing under the conditions of high temperature, high salt concentration, strong acid or strong base, oxygen, and presence of reducing agent. In addition, some of them are also restricted in application in nutrient-fortified foods because of their water solubility or oil solubility. Thus, in order to overcome the thermal sensitivity, volatility, oxidizability, and polarity difference, these bioactive compounds are commonly entrapped by packaging materials that are usually fabricated by using biological macromolecules with film-forming property and gel property (McClements 2017).

Proteins have an ability to form nanoparticles/nanogels with a core-shell structure, which possess excellent size effect, load capacity, and stability. Thus, these novel packaging materials can be used to carry various bioactive compounds in order to prevent the adverse influence resulted from unsuitable environmental factors. Meanwhile, this innovative coating materials of nanoparticle or nanogel are biodegradable, nontoxic, and nonantigenic. This inspiring function can be further modified in the form of stability improvement by protein glycosylation (Li et al. 2008; Qi et al. 2010). Feng, Qi, et al. (2016) successfully fabricated a type of SPI-SSP conjugate-based nanogels using the self-assembly of SPI-SSP conjugates induced by hydrophobic effect and electrostatic interaction. The average particle size of the fabricated nanogels was observed without significant changes during a 120 days of storage at 4°C in a pH range of 2–10 and a sodium chloride concentration range of 0–0.2 mol/L. According to the changes in the tertiary structure of SPI, hydrophobic compartments were formed because of the exposure of hydrophobic groups on the surface of protein molecules, resulting in facilitating the encapsulation of hydrophobic bioactive compounds. In addition, WPI glycosylated with dextran with an average MW of 40 kDa was used to fabricate biopolymer nanoparticles with chondroitin sulfate using the heating electrostatic method above the denaturation temperature and near the pI of WPI (Dai et al. 2015). As a result, the nanoparticles formed via repulsive steric interaction induced by dextran and electrostatic interaction induced by chondroitin sulfate also exhibited a stability in a pH range of 1–8 and in a high sodium chloride concentration of 0.2 mmol/L. These results conformed the possibility to fabricate nanogels for the encapsulation of bioactive compounds using PPC as the coating material.

As a lycopene-rich material, tomato oleoresin has an application potential in food or supplements to increase their nutritional value, functionality, and edible qualities. However, tomato oleoresin is not suitable for the water-based food because of its water insolubility. The SPI-gum

aracia conjugates were successfully used as a coating material to prepare microencapsulation for the encapsulation of tomato oleoresin using a spray-drying method based on the improvement of water solubility of oil-soluble bioactive compounds by encapsulating (Li, Zhu, et al. 2015). As a result, a microencapsulation prepared by the SPI-gum aracia conjugates exhibited an excellent photostability, a thermal stability, and a moisture stability. In addition, the conjugates encapsulated tomato oleoresin exhibited a satisfactory controlled release of lycopene in a simulated gastric fluid, resulting in an effective absorption of this bioactive compound in the small intestine. A eugenol microencapsulation coated by the WPI-maltodextrin conjugate was also prepared using a spray-drying method (Shah, Davidson, and Zhong 2012). The appearance and thermal stability of the nanoscale dispersions formed by dissolving this microencapsulation in water were then studied. The results demonstrated that the nanoscale dispersions constantly kept an excellent transparency even after heating at 80 °C and pH 3–7 for 5 min. However, striking contrast was observed in the formation of thermal-induced gel by heating the eugenol nanoscale dispersions prepared by the WPI-maltodextrin mixture under the same conditions.

$\beta$ -Carotene embedded in an O/W nanoemulsion was performed using the WPI-dextran conjugates as an emulsifier due to the water insolubility and thermal instability (Fan et al. 2017). For example, the average particle size of nanoemulsions was 156 nm when the MW of dextran was 20 kDa. The  $\beta$ -carotene-loaded nanoemulsions stabilized by the WPI-dextran conjugates exhibited an excellent stability at the pI of the native WPI and at 25 or 50 °C for 30 days in a dark environment compared to the  $\beta$ -carotene-loaded nanoemulsions stabilized by WPI. Similar results were found in the study on the formation of nanoparticles encapsulated with vitamin D using the casein-maltodextrin conjugates as a coating material (Markman and Livney 2012). Resveratrol-containing zein nanoparticles were coated with three types of sodium caseinate-polysaccharide conjugates with different polysaccharide MWs to study the influence of the conjugates on the average size and stability of the bioactive compound-containing nanoparticles (Davidov-Pardo et al. 2015). The results demonstrated that the average size of zein nanoparticles remained in nano-level after the coating with sodium caseinate-polysaccharide conjugates; for instance, the average sizes were 198, 176, and 180 nm when MWs of polysaccharides were 2.8, 37, and 150 kDa, respectively. In addition, no significant change was found in the encapsulation efficiency of resveratrol and remarkably enhanced stability was found in the conjugates-coated zein nanoparticles even under environmental stresses of pH 2–9, calcium chloride concentration 0–0.1 mol/L, and 30–90 °C for 30 min. When PPC is used as an emulsifier, the repulsive steric interaction induced by the polysaccharide chains effectively prevents the aggregation among the nanoemulsions or nanoparticles to protect the internal bioactive compounds against changes in pH, high salt concentration, and temperature. However, another theory was concluded to explain the encapsulation of curcumin using the thermal-induced ovalbumin-dextran

conjugates nanogels (Feng, Wu, et al. 2016). Based on the strong hydrophobic force and weak electrostatic repulsion at the pI of ovalbumin, a homogeneous nanogel made by heating and immediately cooling of ovalbumin-dextran conjugates exhibited an excellent stability against a pH range of 2–11, a 30-days storage at ambient temperature, and a freeze-drying treatment, respectively. Thus, this type of ovalbumin-dextran nanogels was used to capsule curcumin using a pH-driven method and the obtained curcumin-containing conjugate nanogels exhibited a better encapsulation efficiency and a similar bioaccessibility compared to the curcumin-containing ovalbumin nanoparticles.

A type of stable nanogels with a core-shell structure fabricated by preparing the amphiphilic grafted copolymers involving  $\beta$ -conglycinin and dextran and self-assembling of the synthesized conjugates via heating treatment exhibited a promising encapsulation and a protection effect of hydrophobic bioactive compounds (Feng et al. 2015). Specifically, the prepared  $\beta$ -conglycinin-dextran conjugate nanogels had an average hydrodynamic diameter of 90 nm and were fairly stable against a long-term storage for 4 months at 4 °C, a pH range of 2–12, and the treatment of lyophilization or dilution. In line with this viewpoint, other PPC could form the similar nanogels to deliver bioactive compounds (illustrated in Figure 6). Nanoparticles prepared by the BSA-dextran conjugates also exhibited similar properties and were successfully incorporated with curcumin (Fan et al. 2018). At the beginning, the BSA-dextran conjugate particles were fabricated with a particle size of 52 nm. After incorporating with curcumin, the particle size increased to 115 nm and the curcumin-loaded nanoparticles was stable even after a month storage (pH 7) at room temperature. The loaded curcumin exhibited an improved cellular antioxidant activity in Caco-2 cells and a better stability in 1 month storage at room temperature compared to the free curcumin. A curcumin-loaded  $\alpha$ -lactalbumin-dextran conjugates nanocomplex with an average particle size of 68.1 nm was fabricated to promote the dispersion of curcumin in water (Yi et al. 2016). Compared to the curcumin-loaded  $\alpha$ -lactalbumin nanocomplex, the curcumin-loaded  $\alpha$ -lactalbumin-dextran conjugates nanocomplex exhibited an almost unchanged average particle size at a temperature range of 30–90 °C for 30 min, a pH range of 3–8, and a calcium chloride concentration range of 0–50 mmol/L, respectively. Meanwhile, curcumin coated in  $\alpha$ -lactalbumin-dextran conjugates exhibited a better stability at pH 7.0 and 95 °C for 4 h compared to the free curcumin or that coated in  $\alpha$ -lactalbumin. In addition, an enhanced DPPH-scavenging activity also observed in the curcumin coated in  $\alpha$ -lactalbumin or  $\alpha$ -lactalbumin-dextran conjugates. Thus, the water solubility and stability of curcumin under various environmental stresses can be enhanced by encapsulating using  $\alpha$ -lactalbumin-dextran conjugates as a nanocarrier to expand the application in a wide range of food products.

As a type of emulsifier or embedding agent, glycosylated proteins exhibit a satisfactory superiority to protect the nutritional value or to exert the best function of native bioactive compounds. However, this characteristic of glycosylated proteins is only observed when the glycosylation of

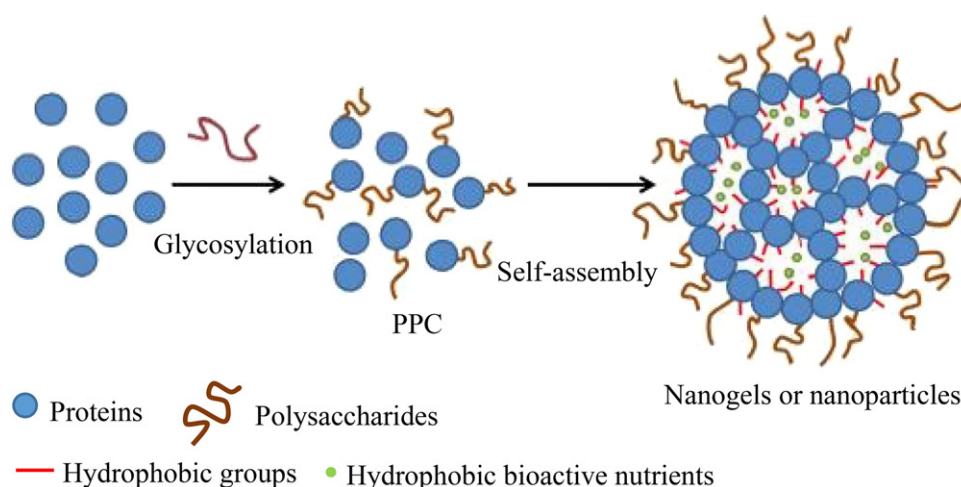


**Figure 6.** The illustration of the preparation of PPC-fabricated nanogels or nanoparticles with core structure for the delivering of bioactive nutrients (PPC, protein-polysaccharide conjugates) (Feng et al. 2015).

native proteins was performed in an appropriate degree. Lesmes and McClements (2012) studied the physicochemical stability and lipase digestibility of corn oil droplets coated by  $\beta$ -lactoglobulin-dextran conjugates under simulated various environmental factors and intestinal digestion conditions. As the dextran MW increased (10, 40, 188, over 400 kDa), the stability of the conjugate-stabilized emulsion increased because of the formation of strong steric repulsion by polysaccharide chains with high MW; however, the digestibility of corn oil reduced. A similar result was found in the study carried out by Fan et al. (2017). As expected, the best DPPH-scavenging activity, lipolysis, and bioaccessibility were found in the  $\beta$ -carotene-loaded nanoemulsions stabilized by the glycosylated WPI with dextran of 5 kDa among the selected MWs (5, 20, and 70 kDa). Thus, the selection of appropriate raw materials, preparation method and its operating parameters to prepare PPC to effectively encapsulate the bioactive compounds should be further studied.

### Other applications in food processing

Apart from the function of protecting the bioactive compounds, the glycosylated proteins also can react with water-insoluble bioactive compounds to indirectly dissolve them in water. For example, complexation can be found in both the resveratrol-deamidated wheat gliadin system and the resveratrol-deamidated wheat gliadin-dextran conjugates system with the help of different interactions (Qiu et al. 2017). However, the poor aqueous solubility of wheat gliadin restricted the application of resveratrol in fluid food system. By virtue of the improved water solubility of deamidated wheat gliadin-dextran conjugates, resveratrol can be extensively used in food matrix with high content of water.

Based on the enhanced thermal stability and solubility of proteins after glycosylation, SPI-dextran conjugates were used to make a spray-dried solid beverage (Boostani et al. 2017). After the glycosylation of SPI with dextran of 6.4–7.6 kDa using the dry-heating method at 60 °C and RH of 79% for 8 days, the conjugates were hydrated with water and blended with other ingredients to prepare solid beverage

using a spray-drying method. The results demonstrated that the solid beverage made by SPI-dextran conjugates had better reconstitution properties than the SPI-based solid beverage. For example, the insolubility index and the wettability or dispersibility of the conjugate-based soy beverage were lower or significantly higher than those of the SPI-based solid beverage. The casein-maltodextrin conjugates prepared using the dry-heating method were used as nanoencapsulation agents for the encapsulation of Vitamin D in clear beverages based on the excellent emulsifying properties (Markman and Livney 2012). The results demonstrated that the lowest particle diameter was observed in the conjugate-Vitamin D nanoparticles and the corresponding solution was the most transparent compared to those of the Vitamin D dispersion and the Vitamin D-containing casein-maltodextrin mixture. Furthermore, the solution of conjugate-Vitamin D nanoparticles remained a colloidal stable state even at the pI of the native casein. Thus, the modification of functionalities of native proteins after glycosylation provides a novel type of protein ingredient to produce food with excellent edible qualities and enhanced nutritional value.

The SPI is usually applied to the quality improvement of meat products. However, the commonly used high temperature treatments, such as boiling and thermal sterilization, significantly weaken the functional properties of SPI. Thus, myofibrillar protein blended with SPI-glucose conjugates exhibited significantly improved emulsifying properties and surface hydrophobicity (Wang et al. 2017). Furthermore, the hardness, springiness, and uniform microstructure of the gel formed by mixing myofibrillar protein with SPI-glucose conjugates were also superior to those of the gel formed by mixing myofibrillar protein with SPI. The application of glycosylated proteins in meat products can obtain quality-improvement effects compared to the native proteins, especially the WHC and tenderness of meat products.

### Conclusion and prospective

According the studies investigating on the modification of proteins by glycosylating for nearly 30 years, mature theories

mainly focusing on the preparation method, characterization technique of conjugates, analysis of changes in functionalities, and application in food system were concluded and performed to provide abundant novel protein ingredients. The food consumption is increasing along with the increase of world population and health or safety awareness. Although it is important to develop a new food resource to increase the food type and improving the food quality, it is also necessary to exploit the existing food resource by using scientific techniques to modify the processing characteristics of food ingredients. One of the most effective technique is protein glycosylation. After an appropriate glycosylation, the solubility, emulsifying properties, foaming properties, gel property, thermal stability, and biological functionalities of the native proteins are significantly improved. Consequently, PPC exhibits excellent processing characteristics even under severe environmental stresses, such as the *pI* of the native protein, high temperature, and high salt concentration. Based on these improved techno-functional characteristics, PPC is extensively used to fabricate nanoemulsions, nanoparticles, and nanogels as vehicles to encapsulate and deliver bioactive compounds for the increase of their stability, function, and bioaccessibility. In other words, dietary supplements would preferably exert their efficacy with the help of these functionality-enhanced delivering materials. Furthermore, the modification of native proteins can also be used to provide techno-functional characteristics-enhanced ingredients for the fabrication of novel foods (such as fluid food, gelled food, and 3-dimensional printing food) and degradable packaging materials.

However, many problems still exist in the modification of native proteins by glycosylating to satisfy the specific demand of modern food, including the selection of proteins, the reasonable design and strict control of glycosylation method and its corresponding operation parameters, and the characterization of PPC. In addition, the nutritional assessment and application form of the glycosylated proteins as well as the quality improvement of the targeted food need to be also further investigated. Thus, it is necessary to know well about the molecular structures of protein and polysaccharide and select appropriate glycosylation conditions to guarantee the precise control of the proceeding and termination of protein glycosylation. Furthermore, application of PPC during food processing should also consider the specific processing requirement, or to realize the intention of functionality improvement of glycosylated proteins under the premise of maintaining the nutritional and functional values of native protein and polysaccharide.

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