Deamidated wheat protein-carbohydrate Maillard conjugates: Effect of size, location and number of carbohydrate conjugated on emulsion steric stabilization at acidic pH and in ionic environment

A thesis submitted in fulfillment of the requirements for the degree of Doctor of Philosophy

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ABSTRACT

This study investigated the effects of conjugation of deamidated wheat protein (sIWP – 40,375 Da) with carbohydrate (180 – 41,000 Da) on the protein’s solution properties (e.g. pI, aggregation behavior at acidic pH, secondary structure, tryptophan local environment), interfacial properties (e.g. interfacial layer thickness) and the ability to stabilize an oil-in-water (O/W) emulsion at acidic pH and in ionic environments. The protein-carbohydrate conjugates were prepared via the Maillard reaction by dry heating at 60 °C/75% relative humidity. The carbohydrates used were glucose (180 Da), maltodextrins (900 – 4,300 Da), and dextrans (6,400 – 41,000 Da). Approximately 3 – 4 moles of glucose or low molecular weight (LMW) carbohydrate fraction in the 900 – 4,300 Da maltodextrins (i.e. < 425 Da) were conjugated per mole of sIWP, whereas approximately 1.3 mole or 0.5 mole of dextran (6,400 Da or 41,000 Da) was conjugated per mole of sIWP.

The zeta-potential and the circular dichroism spectra of the sIWP/protein-conjugates indicate that conjugation did not change the protein’s pI (~pH 4) or the protein’s secondary structure in solution. sIWP and the protein-conjugates attached with glucose or LMW carbohydrates in the maltodextrins became aggregated in solution when the pH approached the pI. Conversely, protein-conjugates attached with dextrans were aggregated to lower extent in solution than sIWP at its pI due to the conjugated dextran acting as a physical barrier.

Dynamic light scattering was used to study the interfacial layer thickness of sIWP/protein-conjugates adsorbed on polystyrene spheres model system. sIWP alone formed a thick protein steric layer of ~18 nm at the interface. Protein-conjugates attached with glucose or LMW carbohydrates in the maltodextrins showed the same
layer thickness as sIWP (i.e. ~18 nm), indicating that the attached carbohydrates did not formed an additional carbohydrate steric layer at the interface due to their small sizes. On the other hand, protein-conjugates attached with dextrans (6,400 Da and 41,000 Da) were able to form a thicker interfacial layer by ~3.5 nm and ~5.9 nm respectively than sIWP. Dextranase digestion on the interfacial layer indicates that an additional carbohydrate steric layer was formed by the protein-conjugate attached with 41,000 Da dextran, but was not with 6,400 Da dextran. The observed difference between the attachments of two dextrans with different MW was due to the difference in the sites of conjugation. The smaller 6,400 Da dextran was attached at the C-terminal domain of sIWP, which is the anchoring point to the interface as suggested by the change in the local environment of the tryptophan residues located at the C-terminal domain of sIWP, whereas the larger 41,000 Da dextran was attached at the N-terminal domain of sIWP, as part of the protein diffuse layer.

Emulsion stabilized by protein-conjugates attached with LMW carbohydrates in the maltodextrin, or the smaller 6,400 Da dextran became flocculated in acidic pH (pH 4) and in CaCl$_2$ environments (0 – 20 mM), similar to the emulsion stabilized by sIWP. However the emulsion stabilized by the protein-conjugate formed with the larger 41,000 Da dextran was stable in acidic pH (pH 4) and in CaCl$_2$ environments (0 – 20 mM). This is due to the additional carbohydrate steric layer formed by the attached dextran which provided adequate steric stability against emulsion droplets flocculation. This study showed that Maillard conjugation can be used to improve the ability of protein (e.g. sIWP) in stabilizing emulsions at acidic pH and in ionic environments by forming an additional carbohydrate steric layer at the interface. However the formation of an effective carbohydrate steric layer is dependent on the size of the carbohydrate, the location of conjugation and the number of carbohydrate conjugated.
DECLARATION

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or in any other institution and, to the best of my knowledge, contains no material previously published or written by another person, except where due reference is made in the text of this thesis.

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CHAPTER 1

Introduction

1.1 General Introduction

Protein-based emulsions are widely used in food products such as beverages, salad dressings, and sauces. Proteins derived from animal sources (e.g. milk proteins: casein and whey) and plant sources (e.g. soy proteins) are examples of protein commonly used to stabilize emulsions. Proteins stabilize emulsions through their adsorption at the interface between the droplets and the dispersing medium. However, protein stabilized emulsions become unstable at pH near their isoelectric point (pI) and/or in ionic environment due to screening of electrostatic charge of the protein adsorbed at the interface (1995; Agboola & Dalgleish 1996a; Demetriades et al. 1997; Srinivasan et al. 2000). This has significantly limited the utility of these proteins to stabilize food emulsions.

One approach to improve the ability of proteins to stabilise emulsions is by direct conjugation of proteins with hydrophilic polysaccharide via the Maillard reaction (Dickinson 2008). Protein-polysaccharide conjugates have been shown to improve the ability of the protein to maintain emulsion stability at acidic pH, in ionic environment or thermal treatment due to the increased in steric repulsion provided by the attached polysaccharide (Jimenez-Castano et al. 2005; Wooster & Augustin 2006; Akhtar & Dickinson 2007; Wooster & Augustin 2007a). Conjugation based on the Maillard
reaction is considered natural and non-toxic, which is therefore suitable for food application.

Most studies examined protein-carbohydrate Maillard conjugates prepared from proteins such as caseins and whey proteins (Dunlap & Cote 2005; Wooster & Augustin 2006; Akhtar & Dickinson 2007; Wooster & Augustin 2007a; Dickinson 2009). However, little attention has been paid to improve the emulsifying properties of wheat gluten proteins using the Maillard reaction. A number of studies have combined the effect from enzymatic hydrolysis and subsequent Maillard conjugation with polysaccharide (e.g. dextran) to improve the emulsifying properties of wheat gluten proteins (Kato & Kobayashi 1991; Babiker 2002). There is no report available in the literature on the combined effect of acid deamidation and Maillard conjugation on the emulsifying properties of wheat gluten proteins. In particular, the effect of Maillard conjugation on improving the ability of deamidated wheat protein to stabilise emulsion at acidic pH/in ionic environment and the underlying mechanisms that are responsible for the improvement in the emulsifying properties.

1.2 Outline of the Chapter

The purpose of this chapter is to provide background and literature review, which covers the background of wheat gluten proteins (section 1.3), food emulsions (section 1.4), mechanisms of emulsion instability (section 1.5), proteins and emulsion stabilisation (section 1.6), proteins versus surfactant and polysaccharide-based emulsifiers (section 1.7), modification of emulsifying properties of protein via the Maillard reaction (section 1.8) and then followed by the hypothesis of this study (section 1.9).
1.3 Background of Wheat Gluten Proteins

Wheat flour is produced from wheat kernels, which consist of two main components, starch (75 – 80%) and protein (10 – 15%). Wheat proteins can be classified into four types based on their solubility, i.e. albumin (water-soluble proteins), globulin (salt-soluble proteins), gliadin (alcohol-soluble proteins) and glutenin (acid- and-alkaline-soluble proteins) as shown in Figure 1.1 (Pomeranz 1999).

![Flour Proteins Diagram](image)

Figure 1.1: Schematic illustration of the main protein fractions of wheat flour proteins, adapted from (Shewry et al. 1986; Pomeranz 1999).

Gliadin and glutenin are the major components (~85%) of the wheat proteins. They form a viscoelastic protein matrix called “gluten” upon hydration with twice its weight of water, which has a significant influence on bread making quality. The gliadin are largely responsible for the extensibility and viscosity of gluten, whereas glutenin are responsible for the elasticity of gluten (Pomeranz 1999).
1.3.1 Structural Characteristic of Monomeric Gliadin

Gliadin are heterogeneous mixtures of single-chained polypeptide that associate with each other via hydrogen bonding and hydrophobic interactions (Beckwith et al. 1963). Gliadin can be classified into three major types, i.e. α/β-, γ- and ω-gliadin based on genetic studies (Payne et al. 1982), amino acid analysis and N-terminal sequencing (Bietz et al. 1977; Kasarda et al. 1983).

α/β- and γ-gliadin are structurally related, sulphur rich gliadin with molecular weight (MW) ranging from 32,000 to 42,000 Da. They have high levels of glutamine and proline residues but are low in ionic amino acids (histidine, arginine, lysine, and free carboxylic groups of aspartic acid and glutamic acid) (Shewry et al. 1986). The α/β-gliadin have six cysteine residues that form three intramolecular disulphide bonds, while γ-gliadin have eight cysteine residues that form four intramolecular disulphide bonds (Kohler et al. 1993; Muller & Wieser 1995). The amino acid sequence showed that the primary structure of α/β- and γ-gliadin can be separated into several domains. The N-terminus contains a short domain with 5-14 amino acid residues and followed by a repetitive domain rich in glutamine- and proline-residues with up to 100 residues, and the C-terminus contains a non-repetitive domain as illustrated in Figure 1.2 (Muller & Wieser 1995). The secondary structure of the repetitive domain adopts β-reverse turn structure, whereas the non-repetitive domain adopts a compact α-helical structure (Tatham & Shewry 1985). Recent studies based on small angle X-ray scattering (SAXS) showed that the α- and γ-gliadin could be modeled as prolate ellipsoids with extended conformation. The SAXS dimension of α- and γ-gliadin is about 15.1 × 3.2 nm and 16.2 × 3.25 nm respectively (Thomson et al. 1999).
The ω-gliadin have large number of glutamine, proline, and phenylalanine residues (~80% of total residues) but little or none of sulphur-containing amino acids (e.g. cysteine and methionine). The ω-gliadin have MW ranging from 44,000 to 72,000 Da. The N-terminal is a short domain with 10 – 11 amino acid residues, followed by a central repetitive domain comprising 90 – 96% of the protein and then finally a short C-terminal domain with 10 – 11 amino acid residues as illustrated in Figure 1.2 (Hsia & Anderson 2001). The ω-gliadin do not have a compact structure, with mainly β-turn structure and low levels of α-helices and β-sheets (Tatham & Shewry 1985). The SAXS dimension of ω-gliadin modeled as rod shape is about 15.5 × 3.25 nm (Thomson et al. 1999).

![Polypeptide structure of α/β-, γ- and ω-gliadin.](image)

**1.3.2 Structural Characteristic of Polymeric Glutenin**

Glutenin are multiple-chain polymeric proteins, which can be divided into two groups, namely high molecular weight glutenin subunit (HMW-GS) and low molecular
weight glutenin subunits (LMW-GS). Based on their separation on SDS-PAGE, the HMW-GS have MW ranging from 95,000 to 136,000 Da, whereas the LMW-GS have MW ranging from 36,000 to 44,000 Da. These glutenin subunits are linked by interchain disulphide bonds and interact through hydrogen bonding to form large macromolecule aggregates as illustrated in Figure 1.3 (Shewry et al. 1986; Tatham et al. 1987).

The HMW-GS consist of a short non-repetitive N-terminal and C-terminal domain (both adopts α-helix/aperiodic structures) separated by a long series of repetitive sequences (~400 – 700 amino acid residues) that rich in glutamine, proline, and glycine residues but poor in cysteine residues as illustrated in Figure 1.4. The repetitive domain adopts a β-turn conformation and the differences in the repetitive sequences can separate HMW-GS into x- and y-types (Tatham et al. 1990). It has been
suggested that the high level of glutamine residues in the repetitive sequence is responsible for the elastic properties of HMW-GS due to their high capacity to form intra- and inter-molecular hydrogen bonds (Belton 1999; Gianibelli et al. 2001; Shewry et al. 2002). Cysteine residues at the N- and C-terminal region provide intermolecular disulphide bonds between HMW-GS and LMW-GS to form protein polymers that could reach up to tens of millions of Daltons in size (Shewry et al. 1992). A SAXS study showed that HMW-GS existed as a highly extended rod-like particle in solution with a dimension of about 69 × 6.4 nm (Thomson et al. 1999).

The LMW-GS are sulfur-rich proteins, containing eight conserved cysteine residues that form intra- and inter-chain disulphide bonds. LMW-GS have similar structure to that of sulfur-rich gliadin. The N-terminal repetitive domain is rich with β-turns (possibly forming regular spiral structure), while the short non-repetitive domain form a compact α-helix structure as illustrated in Figure 1.4 (Thomson et al. 1999).

![Figure 1.4: Polypeptide structures of x-type and y-type high molecular weight-glutenin subunit (HMW-GS) and the low molecular weight-glutenin subunit (LMW-GS).](image)
1.4 Food Emulsions

Many food products are emulsions. They normally contain two immiscible phases, usually oil and water, one of which is dispersed in the other. The dispersed phase is typically fine droplets (up to a few microns) in a continuous phase. Emulsions can be classified according to the relative spatial distribution of the oil and aqueous phase. A system that consists of oil droplets dispersed in an aqueous phase is called an oil-in-water emulsion (or O/W emulsion), for examples, milk, cream, dressings, mayonnaise, and sauces. A system that consists of water droplets dispersed in an oil phase is called a water-in-oil emulsion (or W/O emulsion), for examples, margarine and butter. In addition to the conventional O/W or W/O emulsions described above, it is also possible to prepare various types of multiple emulsions such as oil-in-water-in-oil emulsion (or O/W/O emulsion) or water-in-oil-in-water emulsion (or W/O/W emulsion) (Garti & Bisperink 1998; Garti & Benichou 2004). O/W emulsions are the focus in this study and its formation and factors affecting its stabilization or factors leading to its destabilization will be discussed further.

The process of converting two immiscible liquids into an emulsion or reducing the droplets size in a pre-existing emulsion is called homogenization. This process can be carried out by subjecting the liquids to intense mechanical agitation using equipment such as a high speed mixer, a colloid mill, a high-pressure valve homogenizer, and a microfluidizer. During homogenization new liquid interfaces are created and resulting in increased interfacial area. The work $\Delta G$ (free energy change) required to increase the oil and water surface area $\Delta A$, can be described thermodynamically by:

$$\Delta G = \gamma \Delta A$$

(1)

Where $\gamma$ is the interfacial tension.
In practice, the work required to form an emulsion is significantly greater than that calculated from the equation above, due to the highly curved interfaces of small emulsion droplets, which require additional external pressure gradient to overcome the interfacial forces that holds the larger droplets together (Dickinson 2009).

An O/W emulsion is formed by homogenizing a pre-emulsion mixture containing oil (dispersed phase), water (continuous phase) and emulsifiers. In the absence of emulsifier, the newly formed emulsion system is thermodynamically unstable, and the two incompatible phases separate rapidly into a layer of oil (lower density) on top of a layer of water (higher density) to minimize the interfacial contact and to achieve the global minimum state (Figure 1.5). In the presence of emulsifiers, the emulsion is kinetically stabilized for a period of time (a few days, weeks, months or years) depending on the properties of the emulsifiers (McClements 2005). Although the emulsion is kinetically stabilised, it is still thermodynamically unstable. Essentially, the emulsifier adsorbs onto the interface, lowers the interfacial tension, and creates a free energy barrier $\Delta G^*$ against the transformation from a high to a low free energy state. An emulsion in the high free energy state must acquire a free energy greater than $\Delta G^*$, in order to achieve the global minimum state. The most common emulsifiers used in the food industry are small molecule surfactants, proteins and polysaccharides.
Figure 1.5: Schematic diagram showing the effect of emulsifier addition on the energetic of emulsion stability; adapted from (McClements 2005).

1.5 Mechanisms of Emulsion Instability

Emulsion stability is a term used to describe the ability of an emulsion to resist changes in its properties with time. The properties of a kinetically stabilized emulsion changes through time when exposed to a number of physical forces, such as density difference between the dispersed and the continuous phase, various inter-droplet colloidal interactions and the structural/viscoelastic properties of the emulsifier film (Damodaran 2005). Consequently, these physical forces influence the rates of various emulsion destabilizing mechanisms, such as creaming, flocculation, and coalescence (Figure 1.6). It is important to understand which of these destabilizing mechanisms are affecting a particular emulsion so that effective strategies can be developed to improve its stability.
Figure 1.6: Schematic presentation of various emulsion-destabilising mechanisms that influence emulsion stability (droplets are not to scale; only intended for illustration purposes).

**1.5.1 Creaming**

Creaming is a phenomenon where oil droplets in an emulsion rise to the top. This is due to the lower density of oil droplet compared to the aqueous phase. The creaming rate of an isolated spherical particle in a liquid (e.g. an O/W emulsion droplet) can be calculated using Stokes law:

\[
\nu_{\text{stokes}} = -\frac{2gr^2(\rho_2-\rho_1)}{9\eta_1}
\]  

(2)

Where \( g \) is the acceleration due to gravity, \( r \) is the radius of the particle, \( \rho_2 \) is the density of the oil droplet, \( \rho_1 \) is the density of water and \( \eta_1 \) is the viscosity of water.
According to the Stokes’ law, creaming rate can be lowered by reducing droplet size or increasing the viscosity of the continuous phase. However, Stokes’ law is most applicable to an isolated rigid spherical particle suspended in an ideal liquid. In reality, the liquid surrounding the droplet can move during the rising of the droplet. This reduces the friction force that opposes the movement of the droplet, which in turn increases the creaming rate (Dickinson & Stainsby 1982). The creaming rate is also affected by emulsion droplet concentration. The creaming velocity for emulsions with high droplet concentration is much lower compared to emulsions with low droplet concentration. This is due to the existence of hydrodynamic interactions between the droplets, which slow down the creaming rate (Hunter 1986; Walstra 1996).

In the initial stage of creaming, the emulsion droplets move upwards, leaving a droplet-depleted layer at the bottom. When the droplets reached the top of the emulsion, these droplets packed together to form a creamed layer. The final thickness of the cream layer depends on the initial droplet concentration in the emulsion and the nature of the interactions between the droplets, which in turn affects the effectiveness of the droplet packing. The high concentration of oil droplets in the creamed layer promotes droplet flocculation, aggregation or coalescence (Dalgleish 1997).

The simplest way to monitor creaming is to place an emulsion in a transparent test tube and leave it for a certain length of time, and then measure the height of the interfaces between the different layers formed. Alternatively, the creaming height can be monitored by light scattering technique, which gives a percentage of transmitted and/or scattered light as a function of emulsion height (Chanamai & McClements 2000). An instrument called Turbiscan (MA2000, Formulaction, France) based on a light scattering technique, can monitor the creaming stability of emulsions in situ.
**1.5.2 Flocculation and Aggregation**

Flocculation and aggregation are phenomena where the oil droplets in an emulsion are associating with each other without the rupture of the interfacial membrane. This phenomenon arises due to the inability of the adsorbed emulsifier at the oil droplet interface to prevent the close approach of the droplets. Flocculated or aggregated emulsion droplets may subsequently lead to coalescence or creaming and also changes in emulsion texture by increasing viscosity (Demetriades et al. 1997). Flocculation is generally regarded as being weaker than aggregation. Flocculation is reversible by shaking or stirring. Aggregation is usually irreversible and it is due to attractive inter-atomic force of the adsorbed emulsifier, for example at pH close to protein pI (Dalgleish 1997).

The extent of flocculation can be monitored through microscopic technique (e.g. optical, fluorescence or electron microscopy), light scattering, ultrasonic spectrometry, NMR, or electrical pulse counting methods. However, care must be taken during sample preparation for the measurements because the structure of the flocculated droplets can be easily disrupted (McClements 2005).

The rate of droplet flocculation is linked to the collision frequency of the emulsion droplets. Collision frequency is defined as the total number of droplet encounters per unit of time per unit volume of emulsion. Collision occurs as a result of droplet movement, which is induced by Brownian motion, creaming, or applied mechanical force. Collision frequency can be reduced by increasing the viscosity of the continuous phase of the emulsion, which reduces the droplet movement. However, changing the viscosity of the emulsion can have detrimental effect on the texture of the food product.
The most effective way in controlling the rate or extent of flocculation in an emulsion is to regulate the colloidal interactions (e.g. van der Waals, steric, electrostatic, and hydrophobic) between the droplets. This may be achieved by designing an emulsion in which the repulsive interactions between the droplets are significantly greater than the attractive interactions.

1.5.2.1 Electrostatic Interactions

O/W emulsions can be stabilized against flocculation by using charged emulsifiers (e.g. proteins, some hydrocolloids and small molecule surfactants) that generate an electrostatic repulsion between the droplets. According to DLVO (Derjaguin, Landau, Verwey, and Overbeek) theory, the stability of emulsion droplets stabilized by charged emulsifiers against flocculation is determined by the sum of van-der-Waals attractive force and double layer repulsive force that acts between the two approaching emulsion droplets (Figure 1.7 A & B). If there is net repulsive force acting between the droplets, it will prevent them from adhering together. However, if the droplets are approaching at a high velocity that overcomes the energy barrier (e.g during mechanical shearing), the droplets may flocculate.

The flocculation stability of electrostatically stabilized O/W emulsions is affected by the pH and the ionic environment of the emulsion aqueous phase, which can reduce or remove the energy barrier that prevents the emulsion droplets from adhering together (Damodaran 2005). In a protein stabilized emulsion, the electrical charge on the emulsion droplets changes from positively charge at pH below the pI, to zero net electric charge at pI, and then to negative charge at pH above the pI. Essentially, the concentration of the H⁺ ions in the emulsion aqueous phase changes the degree of ionization of the acidic and basic groups of the proteins. At pH value sufficiently below
or above the pI of the protein, strong repulsive force acting between the emulsion droplets prevent droplet flocculation. On the other hand, at pH close to the protein’s pI, there is no repulsive force acting between the emulsion droplets to prevent droplet flocculation (Demetriades et al. 1997).

In some food products, mineral ions are added for technological or nutritional purposes. As the ionic strength of the emulsion aqueous phase increases, the electrostatic repulsion acting between the emulsion droplets can be progressively screened, until it is no longer sufficient to prevent flocculation (Figure 1.7 C). It also appears that different types of counter ions have different effects on emulsion flocculation stability. Monovalent counterions such as K⁺, Na⁺, and Cl⁻, screen the electrostatic interactions without binding to the surface of emulsion droplets (Kulmyrzaev & Schubert 2004). On the other hand, multivalent counterions like Ca²⁺, Cu²⁺, Fe²⁺, Fe³⁺, Al³⁺ and SO₄²⁻, not only screen the electrostatic interactions, but they may also bind to the adsorbed protein layer via electrostatic interactions, which lead to the alteration of surface charge density and also the pI of the emulsion droplets (Mei et al. 1998; Silvestre et al. 1999; Kulmyrzaev et al. 2000). In addition, multivalent counterions tend to cause flocculation at much lower concentration than monovalent counterions.
Figure 1.7: [A, B & C] DLVO theory describing the dependence of two independent potentials that affects the flocculation stability of emulsion droplets stabilized by electrically charged emulsifiers. [D] The net repulsion potential can be influenced by changes in solution pH or in ionic environment, which significantly reduce or completely remove the energy barrier that prevents the two droplets from adhering together.

1.5.2.2 Steric Interactions

Another factor that can effectively prevent droplets flocculation is the steric repulsion force, which is provided by certain types of emulsifiers (e.g. proteins or hydrocolloids) (Figure 1.8). The steric repulsion force is dependent on the structural configuration of the emulsifier adsorbed at the oil-water interface (Israelachvili 1992).
For example, an adsorbed emulsifier (e.g. caseins) may have a segment that is protruding into the emulsion aqueous phase and a segment that is anchored to the interface (Damodaran & Anand 1997). During the close approach of two emulsion droplets, the overlapping outer segments create a localized high solution concentration that creates an osmotic gradient with opposing force. This opposing force can effectively prevent the emulsion droplets from approaching each other.

A sterically stabilized emulsion is usually less sensitive to changes in pH and in ionic environment than an electrostatically stabilized emulsion, such as those stabilized by non-ionic hydrocolloids (e.g. gum arabic) (Hunter 1986). However, a sterically stabilized emulsion could become unstable if the steric layer is cleaved by acid/enzyme or reduced in thickness due to poor solvation. If the steric repulsion force is provided by charged polymer (e.g. protein), the steric layer thickness may be reduced at pH close to pI or in ionic environment due to a reduction in intramolecular electrostatic repulsive force (McClements 2005).

Figure 1.8: Schematic diagram illustrating the prevention of emulsion droplet flocculation by the adsorbed biopolymer via steric repulsion. Notes: (1) diagram is not to scale, (2) diagram adapted from (Damodaran 2005).
1.5.2.3 Bridging Flocculation

Emulsions stabilised by biopolymers may flocculate due to the formation of bridges between two or more emulsion droplets. This is due to the binding of two separate segments of the same biopolymer directly onto the droplet surface or to the adsorbed emulsifier molecule as a result of hydrophobic or electrostatic interaction (Lips et al. 1991; Dickinson 2003). Bridging flocculation commonly occurs when insufficient emulsifier is available to completely cover the oil-water interface during homogenisation (Walstra 1996). Bridging flocculation could also occur when the biopolymer in the emulsion aqueous phase has an electrical charge that is opposite to that of the emulsion droplets (Dickinson 2003).

1.5.2.4 Depletion Flocculation

Emulsion flocculation can occur when un-adsorbed colloidal particles (e.g. biopolymers) are present in the emulsion continuous phase. The presence of the un-adsorbed colloidal particles leads to an increase in attractive force between the emulsion droplets due to the osmotic effect associated with the exclusion of the colloidal particles from the narrow region between the droplets, particularly when the emulsion contains low concentration of colloidal particles (Dickinson 1995). However, this effect is not observed when the emulsion contains low concentration of colloidal particles and emulsion droplets, due to the free diffusion of the colloidal particles and the emulsion droplets (Dalgleish 1997).
1.5.3 Coalescence

Coalescence is a phenomenon where two or more emulsion droplets are merged into a larger droplet. This will eventually lead to the formation of free oil on top of the emulsion. Coalescence occurs when the thin liquid film separating two emulsion droplets is ruptured as illustrated in Figure 1.9. The stability of this thin liquid film is affected by two opposing forces, namely, capillary pressure and disjoining pressure. Capillary pressure causes drainage and thinning of the thin liquid film. Disjoining force contributed by the electrostatic and/or steric repulsion force from the adsorbed emulsifier, prevents the drainage and thinning of the thin liquid film. When the thin liquid film is being thinned below a critical thickness, hydrodynamic force creates small gaps (or “holes”) in the film and subsequently lead to the merging of the droplets (Walstra 2003; Damodaran 2005). Emulsion droplet coalescence can also occur when insufficient emulsifier is available to cover the entire oil-water interface during homogenization (Fang & Dalgleish 1993).

The emulsifier adsorbed at the interface plays an important role in preventing emulsion droplet coalescence. It provides electrostatic and/or steric repulsion force that prevents the close contact of emulsion droplets. In addition, it reduces the free energy available for the formation of “holes” in the thin liquid film by forming a visco-elastic interfacial membrane (Kabalnov 1998).
1.6 Proteins and Emulsion Stabilisation

Most proteins are amphiphilic in nature because they contain both hydrophilic and hydrophobic groups in their structures. They are able to absorb at the oil-water interface and lower the interfacial tension. At the oil-water interface, a fraction of the protein hydrophobic residues are anchored onto the oil phase, whereas the rest of the protein molecule is suspended into the aqueous phase.

Proteins can be isolated from various sources such as bovine milk, plant, meat and egg. Different types of protein have different primary, secondary and tertiary structures, which in turn influence their behaviour at the interface (e.g. adsorption/packing at the interface, and emulsion stabilising mechanism). Most proteins stabilise O/W emulsions against flocculation via electrostatic and/or steric repulsion (Dalgleish 1997). Proteins containing sulphydryl and disulphide groups are able to...
undergo sulphydryl-disulphide interchange reaction after adsorption at the interface. This makes them irreversibly adsorbed and also provide a highly viscoelastic film that protect against emulsion droplet coalescence (Dickinson & Matsumura 1991; Dickinson 1998). Most understanding on protein stabilised emulsions has come from studies on milk proteins, egg proteins, soy proteins and gelatine. Therefore, backgrounds on these proteins are given as they provide insight into protein behaviour at interfaces and their emulsion stabilisation mechanisms. This information will be used as a reference to compare and understand emulsion stabilised by wheat gluten proteins.

1.6.1 Milk Proteins

Milk proteins are commonly used in emulsion-based food products like beverages, frozen desserts, ice creams, sports supplements, infant formula and salad dressings, etc. Most understanding about protein-stabilized emulsions comes from studies conducted on milk proteins. Two major types of proteins can be separated from bovine milk, caseins and whey proteins. Although they were isolated from the same source, their structure and interfacial properties are different from one another.

1.6.1.1 Caseins

Caseins can be fractionated into four different types of proteins, $\alpha_s1$ (~44%), $\alpha_s2$ (~11%), $\beta$ (~32%) and $\kappa$ (~11%) (Swaisgood 1996). Caseins have limited secondary and tertiary structure in solution, and as such have a random and flexible structure (Leermakers et al. 1996; Caessens et al. 1999). For instance, the lack of ordered secondary structure (e.g. $\alpha$-helix and $\beta$-sheet) of $\beta$-casein is due to the complete absence of cysteine residues and the presence of numerous proline residues distributed along its polypeptide sequence (Swaisgood 1982). Along the casein polypeptide chain, there are
significant numbers of non-uniformly distributed non-polar regions, which favour self-association through hydrophobic interactions. In addition, there are also high amount of negatively charged phosphoseryl residues at the N-terminus region, which favour self-association through electrostatic bridging by multivalent cations (e.g. Ca$^{2+}$) (Oakenfull et al. 1997). Changes in pH and ionic strength influence the electrostatic interaction between casein molecules, which leads to self-association.

Studies showed that casein-stabilised emulsions are vulnerable to droplet flocculation at pH values 3.5 – 5.3, which is close to the protein’s pI at ~pH 4.6 (Agboola & Dalgleish 1996a) and in ionic environment (Agboola & Dalgleish 1996b; Srinivasan et al. 2000). For β-casein, it has a non-uniform distribution of hydrophobic residues along its polypeptide sequence and a hydrophilic segment at the N-terminus (including all of the phosphoserine residues) that is important for stabilising O/W emulsion (Caessens et al. 1999). The conformation of casein molecules adopted at the oil-water interface is dependent on the surface area that the protein is required to cover, and was found to spread widely over the interface forming a loose mobile fluid layer (Fang & Dalgleish 1993; Dickinson 1999). β-casein adsorbed at the interface, can be described by the classical train-loop-tail model as illustrated in Figure 1.10, where the protein regions lying in direct contact with the hydrophobic surface are called train, whereas the regions dangling further into the aqueous phase are called loops and tails (Fleer et al. 1993; Leermakers et al. 1996). β-casein forms a dense inner layer (~2 nm in thickness and 0.9 volume fraction at the interface) and a thick diffuse layer (~5 – 7 nm in thickness and 0.15 in volume fraction at the interface) as measured by neutron scattering (Dickinson et al. 1993). The thick diffuse layer containing the negatively charged phosphoseryl residues (at neutral pH) provides good electrostatic and steric
stabilisation against droplet flocculation (Swaisgood 1982). However, changes to the emulsion conditions such as the pH approach the pl or in ionic environment, there is a decrease in electrostatic repulsion between the emulsion droplets and a decrease in the adsorbed protein layer thickness, which lead to emulsion flocculation (Brooksbank et al. 1993; Leermakers et al. 1996; Dickinson 2008). It should be noted that κ-casein only possesses one phosphoserine residue and is therefore more resistant to calcium bridging. In the presence of sufficiently high concentration of un-adsorbed caseins in the emulsion aqueous phase, depletion flocculation can also occur (Srinivasan et al. 2002). Casein stabilised emulsions appear to be less sensitive to heating than whey protein stabilised emulsion. This is due to the flexible structure of the casein proteins and the lack of cysteine residues, which makes it resistant to heat-induced conformational changes and disulphide cross-linking in comparison to the heating of globular whey proteins (Hunt & Dalgleish 1995; Srinivasan et al. 2002).

Figure 1.10: Schematic diagram illustrating the loop-and-train configuration postulated for the conformation of β-casein adsorbed on a hydrophobic surface. Notes: (1) numbers along the single-chain protein sequence is the amino acid residue numberings, (2) the negative symbols represent negative charge, (3) diagram adapted from (Brooksbank et al. 1993).
1.6.1.2 Whey Proteins

Whey protein can be fractionated into four different types of proteins, β-lactoglobulin (~55%), α-lactoglobulin (~24%), serum albumin (~5%) and immunoglobulins (~15%) (Swaisgoof 1996). Unlike caseins, whey proteins have compact globular structure. Globular whey proteins form much thinner and denser layers at the oil-water interface than caseins (Dickinson 2001). Whey proteins like β-lactoglobulin has been found to form a highly viscoelastic layer surrounding the emulsion droplet due to the high packing density and strong intermolecular interactions between the adsorbed proteins, which can protect the emulsion droplets against coalescence (Dickinson 1999). At the interface, the adsorbed protein may undergo changes in protein conformation to maximise the number of favourable interactions and minimize the number of unfavourable interactions with the oil phase (McClements 2005). Whey proteins stabilises the emulsion droplets mainly though electrostatic repulsions.

Studies showed that whey proteins stabilised emulsions are vulnerable to droplet flocculation at pH values ~4 – 5.5, which is close to the protein’s pI at ~pH 5 (Demetriades et al. 1997), in ionic environment (Agboola & Dalgleish 1995; Hunt & Dalgleish 1995) and on heating above the thermal denaturation temperature of the adsorbed proteins in the presence of salt (Demetriades & McClements 1998; Kim et al. 2002). Studies have suggested that heating of emulsions promote the unfolding of the adsorbed proteins, which leads to the exposure of some hydrophobic regions within the protein molecule to the aqueous phase. When heating in the presence of high salt
concentration, emulsion droplets are in close proximity with each other. This favours hydrophobic interactions between the adsorbed protein from two different droplets and results in flocculation (Kim et al. 2002).

### 1.6.2 Egg Proteins

Egg yolk is commonly used for stabilising emulsions, such as mayonnaise, salad dressings, sauces and cake batters, etc (Mine 1998b; Mine 1998a; Anton et al. 2001; Mine 2002). Hen’s egg yolk can be separated into plasma (supernatant) and granule (precipitate) by centrifugation (Li-Chan et al. 1995). Plasma is composed of 85% low-density lipoproteins (LDL) and 15% livetin. Granules contain 70% high-density lipoproteins (HDL), 16% phosvitin and 12% LDL (McCully et al. 1962).

Studies have suggested that LDL is the main contributor to the emulsifying properties of the plasma constituents (Anton & Gandemer 1997; Anton 1998; Le Denmat et al. 2000). LDL are considered to be large spherical particles with a triacylglycerol core and a surface layer of both phospholipids and proteins (Evans et al. 1968; Schneider et al. 1973). It has been suggested that LDL are disrupted during adsorption at the interface, liberating apo-proteins (or apo-LDL) and phospholipids. Apo-LDL was found to adsorb more readily than other soluble proteins due to their high hydrophobicity and to their flexible structure (Mine 1998a). The pI of apo-LDL is around pH values 6.5 – 7.3 (Nakamura et al. 1977) and it undergoes denaturation and became insoluble at temperature above 70 ºC (Tsutsui 1988).

It was found that pH and ionic environment has little impact on the solubility and the ability of apo-LDL to form small emulsion droplets (Le Denmat et al. 2000). The ability of apo-LDL to prevent emulsion droplet flocculation and subsequent
emulsion creaming is slightly affected at pH near to its pI at ~pH 7 and in high ionic environments (i.e. 0.55 M NaCl). It has been suggested that apo-LDL stabilises O/W emulsion mainly through steric repulsion, although the authors did not measure the layer thickness. The authors also suggested that additional emulsion stability was contributed from electrostatic repulsion when the pH is away from pI at ~pH 3 and in low ionic environment (i.e. 0.15 M NaCl cf. 0.55 M NaCl) (Le Denmat et al. 2000). The authors also found that the stability and rheological properties of emulsions is adversely affected when the emulsion was heated above the thermal denaturation temperature of the protein (i.e. at >70 °C) (Le Denmat et al. 1999).

1.6.3 Soy Proteins

Soy protein can be separated into a number of fractions, namely, 2S, 7S, 11S and 15S. These fractions contains a mixture of different protein subunits that posses different molecular and functional characteristics (Tornberg et al. 1997). Emulsions prepared using soy proteins tend to flocculate extensively, possibly due to bridging of the relatively large soy protein aggregates between the emulsion droplets (Tornberg et al. 1997; Liu et al. 1999). It has been shown that high concentration of soy proteins (> 4 wt. %) were needed to form stable O/W emulsion against creaming by forming a viscous protein network (Roesch & Corredig 2002).

The low surface activity of soy protein is possibly due to the quaternary structure of its two major components, (i.e. 7S and 11S globulins) and the compact tertiary structures of their subunits. It has been suggested that majority of the surface hydrophobic residues is buried at the subunit interfaces within the quaternary structure (Liu et al. 1999). For instance, 11S globulin is a hexamer, with each subunit consists of
a basic polypeptide (~20,000 Da) and an acidic polypeptide (~35,000 Da) joined together by disulphide bond (Staswick et al. 1984).

Acidic subunits of the 11S globulin (AS11S) isolated from soy 11S globulin are able to form small O/W emulsion droplets (i.e. ~0.8 µm) at low protein concentration (i.e 0.2-1 wt. %) (Liu et al. 1999). The authors also found that AS11S has better ability to stabilise emulsions against flocculation compared to intact soy 11S, possibly via steric and electrostatic stabilisation. However, the stability of AS11S stabilised emulsion was found to be affected in ionic environment (i.e. >0.2 M NaCl). Overall, there are limited numbers of systematic studies on the influence of environmental conditions (e.g. pH, ionic strength and temperature) on the stability of soy protein-stabilised emulsions (McClements 2005).

1.6.4 Gelatin

Gelatin is a HMW protein (~ 100, 000 Da) derived from animal collagen (e.g. cow, fish or pig). Gelatin is prepared by hydrolysing collagen through boiling with acid to produce type-A gelatin or with alkaline to produce type-B gelatin. The pI of type-A gelatine is around pH values ~7 – 9, whereas the pI of type-B gelatine is around pH 5. Gelatin exists as a random-coil molecule at relatively high temperature but undergoes coil-helix transition upon cooling to about 10 – 30 ºC for mammalian gelatine, and about 0 – 5 ºC for fish gelatine (Leuenberger 1991). Gelatin forms a thermo-reversible cold-set gel upon cooling below its coil-helix transition temperature, which makes it commonly used as thickening agents and gelling agents to stabilise emulsion based food products such as deserts, sauces, soups (Oakenfull et al. 1997). Nevertheless, gelatin has been shown to be surface active and is able to act as an emulsifier in O/W emulsions
(Dickinson 2001). However, the study showed that emulsion prepared using gelatin at pH 6.8 (20 vol. % triglyceride oil, 1 wt. % protein) has large droplet size (i.e. $D_{4,3}$: 7.1 μm) and the emulsion was prone to flocculation even without the presence of ions.

1.6.5 Wheat Proteins

The use of wheat gluten proteins as emulsifiers is limited by their low water solubility at neutral pH. The poor water solubility of gluten proteins is related to their amino acid composition. Gluten proteins have high levels of glutamine and proline amino acid residues that have uncharged side-chain (> 50%), while low in lysine, arginine, and asparagine residues that have charged side-chain. The glutamine side chains have a tendency to form strong intermolecular interactions via hydrogen bonding that suppress hydration and enhance protein aggregation. A number of studies have focused on improving the solubility and emulsifying properties of wheat gluten proteins by using enzymatic and chemical modifications. These modifications alter protein functionality potentially via two mechanisms: (1) inducing changes to protein structure at the secondary, tertiary, and quaternary structural levels and (2) altering hydrophobicity-hydrophilicity balance (Damodaran 2005).

1.6.5.1 Enzymatic Hydrolysis

Enzymatic hydrolysis using protease has been used to modify the functional properties of plant proteins such as wheat gluten proteins (Verma & McCailla 1966; Larre et al. 2001). After enzymatic proteolysis, the solubility and the emulsifying properties of the protein (e.g. ability to stabilize an emulsion) were improved due to the reduction in protein’s MW resulting from peptide bonds hydrolysis to form lower MW polypeptides and also due to the disruption of the higher structural order of the protein
molecule. However, hydrolysis reactions have to be carefully controlled to avoid excessive proteolysis, which could lead to the release of bitter compounds and the loss of protein’s intrinsic properties such as the viscoelasticity property of wheat gluten proteins. Excessive proteolysis can also result in poor emulsifying properties (Kato et al. 1991).

1.6.5.2 Acid Deamidation

Acid deamidation is another method that has been used to modify the solubility and the emulsifying properties of wheat gluten proteins (William et al. 1996). The deamidation process involves the conversion of the amide group of glutamine and asparagine residues into charged acidic groups (i.e. glutamic acid and aspartic acid) via the liberation of ammonia (William et al. 1996). During acid-catalysed deamidation, the acid (HA) donates a proton to the side chain of the amide-bearing moiety (e.g. glutamine residue). The electron deficient carbonyl group of the amide either attacks the original nucleophile (–A) or reacts with a hydroxyl anion generated by the proton extracting actions of –A (the conjugate base). This step activates the amide side chain, producing the transition state (oxyanion tetrahedral intermediate). This intermediate is further stabilized by the presence of proton donors, which then increases the rate for further deamidation (William et al. 1996).

Deamidated wheat protein was found to have increased charge density and unordered structure, and a decreased in α-helical structure and intermolecular interactions. This is due to an increased in negatively charged glutamic acid residues, which increased intra- and intermolecular repulsion force (Matsudomi et al. 1982). In addition, the authors found that deamidation lead to an increase in surface hydrophobicity due to protein unfolding. In turn, these changes improved the solubility
and emulsifying properties of wheat gluten proteins, including the ability to form small emulsion droplets and maintain emulsion stability (Matsudomi et al. 1981; Matsudomi et al. 1986; Kato et al. 1987; Bollecker et al. 1990; Mimouni et al. 1994; Webb et al. 2002). It has been reported recently that deamidated wheat protein stabilizes O/W emulsions against droplet flocculation via a combination of steric repulsion and electrostatic repulsion (Day et al. 2009). Furthermore, the study also found that emulsion stabilized by deamidated wheat protein was prone to droplet flocculation at acidic pH and in ionic environment.

1.6.5.3 Surface Properties of Wheat Gluten Proteins

A number of studies have examined the surface properties of wheat gluten proteins. These studies were carried out in 70% ethanol for gliadin, urea for glutenin or using hydrolysed wheat gluten peptides. A study based on Wilhelmy plate method showed that gliadin have higher surface activity than glutenin. It has been suggested that competitive adsorption between gliadin and glutenin at air/water interface can also occur (Keller et al. 1997). Although gliadin adsorbed more readily than glutenin at the initial stage of emulsification due to its higher surface activity, but a study has shown that the adsorbed gliadin may be displaced by glutenin (Takeda et al. 2001). When the adsorbed gliadin was displaced by glutenin, the adsorbed glutenin underwent conformational changes to increase intermolecular interaction between the adsorbed glutenin to form a stable protein film at the surface.

The α/β- and γ-gliadins are proteins with high contents of glutamine and proline residues, but with C-terminal regions that are generally rich in hydrophobic amino acids, making them amphiphatic (Okita et al. 1985). It has been found that peptides
derived from the C-terminal domain of protease treated gliadin (presumably from α-, β-, γ-gliadin) were more readily adsorbed to an emulsified oil droplet surface than peptides derived from the N-terminal domain (Chobert et al. 1996). This is because the N-terminal domain contains little hydrophobic residues and many repeating units of glutamine and proline residues. There is also suggestion that protein unfolding may occur upon the adsorption of α-/β- and γ-gliadin onto a hydrophobic surface (Popineau & Pineau 1993). A study based on in-situ ellipsometry showed that the adsorption of β- and γ-gliadin onto a hydrophobic surface switch from a side-on orientation (major axis parallel to the surface) to an end-on orientation (major axis perpendicular to the surface) with increasing protein concentration available for adsorption (Ornebro et al. 1999). ω-gliadin appeared to have different surface properties to the α/β- and γ-gliadin. The ω-gliadin were found to be less surface active than α-/β- and γ-gliadin (Popineau & Pineau 1993). On a hydrophobic surface, ω-gliadin appeared to have side-on orientation at all concentration (Ornebro et al. 1999). It was found that α-, β-, γ-gliadin could block the adsorption of ω-gliadin and also replace the ω-gliadin adsorbed layer. For HMW-GS, based on its amino acid composition, the central repetitive domain is hydrophilic, whereas the short N- and C-terminal domains are hydrophobic (Shewry et al. 1989).

1.7 Comparison of Proteins, Surfactant and Polysaccharide-based Emulsifiers

The term surfactant is used for describing LMW surface active molecules, such as lecithins and monoacylglycerol. These molecules are characterized by having distinct
hydrophilic head group, which has a high affinity for water, and hydrophobic tail group, which has a high affinity for oil (Krog & Sparso 2004). Surfactants can lower interfacial tension more effectively than proteins and stabilize O/W emulsion via short range repulsions. However, surfactant-stabilised emulsions are generally less stable than those stabilized by proteins because they produce interfacial films with poor viscoelastic properties and steric stability (Damodaran 2005). Furthermore, the addition of a surfactant to a food could be constrained by its legal status, due to its chemical composition and its origin (Krog 1997).

Polysaccharides are predominantly hydrophilic in nature, which means that they exhibit little surface activity at the oil-water interface, and therefore are not generally useful as emulsifying agent. Nevertheless, some polysaccharides are surface active, such as gum arabic and sugar beet pectin, due to a small hydrophobic polypeptide moiety that is covalently bound to the highly branched polysaccharide structure (Fincher & Stone 1983; Funami et al. 2007).

Gum arabic is commonly used as an emulsifier to stabilize flavor emulsions (Tan 2004). It has been proposed that gum arabic had a ‘wattle blossom’-type structure as illustrated in Figure 1.11, with a number of polysaccharide units of ~200,000 Da each linked to a common polypeptide chain that only represent ~2 % of the total polysaccharide (Fincher & Stone 1983). Furthermore, it has been postulated that the hydrophobic polypeptide chain anchors to the oil droplet surface, while the hydrophilic polysaccharide blocks attached to the chain protruded out into the solution, providing a strong steric barrier against droplet flocculation and coalescence (Snowden et al. 1987; Ray et al. 1995; Islam et al. 1997). Although gum arabic stabilized emulsion is mainly stabilized through steric repulsion, there is also some contribution from electrostatic
repulsion (Jayme et al. 1999). This electrostatic repulsion is believed to have arisen from the charged amino acid constituent that are sparsely distributed along the adsorbed polypeptide chain of gum arabic. It has been shown that gum arabic stabilised emulsion remains stable to flocculation in various conditions, such as at pH values 3 – 9, in ionic environment (0 – 25 mM CaCl$_2$) and subjected to heating (30 – 90 °C) (Chanamai & McClements 2000).

Although gum arabic appears to be highly effective in stabilizing O/W emulsions, it is relatively less surface active than other surface active biopolymers such as milk proteins. As a result, relatively high gum arabic concentration typically is required (~10 – 30 wt. %) to form a stable emulsion that contains ~15 wt. % triglyceride oil (Funami et al. 2007). On the other hand, sugar beet pectin, which is another type of hydrocolloid, was found to require much lower concentration (~1 – 3 wt. %) to stabilize an O/W emulsion that contains ~15 wt. % triglyceride oil than gum arabic (Funami et al. 2007). The authors suggested that the difference observed between gum arabic and sugar beet pectin was due to the molecular relationships between the proteinaceous moiety and the carbohydrate moeity, which affects the hydrophilic-hydrophobic balance of the hydrocolloid molecule. In addition, the authors also showed that the proteinaceous polypeptide attached to the polysaccharide plays an important role in the surface-activity of the molecule. A study has found that the HMW protein-rich fraction in the gum arabic is preferentially adsorbed at the oil-water interface than other fractions (Randell et al. 1989).
Figure 1.11: Schematic diagram illustrating the “wattle blossom” structure of gum arabic (picture on the left) and adsorbed at the oil-water interface (picture on the right). Note: (1) figures are not to scale, (2) diagram adapted from (Fincher & Stone 1983).

Study has shown that proteins (e.g. caseins and β-lactoglobulins) have higher surface binding affinity, surface activity and lower saturation surface loads (1 – 2 mg m$^{-2}$) than polysaccharide-based emulsifiers (e.g. gum arabic) (Dickinson 2006). However, emulsions stabilised by polysaccharide-based emulsifiers at full surface coverage were shown to have superior stability even in unfavorable conditions such as at acidic pH, in CaCl$_2$ environment or subjected to thermal treatment than protein-stabilised emulsions, which are usually susceptible to destabilization in these unfavourable conditions (Dickinson 2006).
1.8 Modification of Emulsifying Properties of Protein via the Maillard Reaction

As described previously, proteins such as those derived from bovine milk (e.g. casein and whey) or deamidated wheat proteins have excellent ability to form small emulsion droplets and maintain emulsion stability. However, these protein stabilised emulsions became unstable and flocculated in unfavourable conditions, such as at acidic pH, in ionic environment and/or subjected to thermal treatment.

There has been increasing interest to construct protein-polysaccharide conjugates with the motivation to generate a novel amphiphilic entity that exhibits emulsification properties that are equal to or better than gum arabic (Kato 2002; Oliver et al. 2006; Dickinson 2008). Complexation of protein and carbohydrate via covalent bonding is more advantageous than electrostatic interactions, which allow the complex to maintain strongly associated over a wide range of pH and ionic strength without coacervation or precipitation (Dickinson 2003). A novel conjugation method that is regarded as safe for food application was developed by Kato and co-workers (1990) to improve the emulsifying properties of proteins. This method involved the dry heating of an intimate mixture of protein and polysaccharide, which lead to the spontaneous Maillard reaction between the ε-amino groups of the protein and the reducing-end carbonyl group of the polysaccharide. In comparison to other conjugation methods that use chemicals like carbodiimide (Stowell & Lee 1980), sodium cyanoborohydride (Lee et al. 1979) or cyanogens bromide (Kato et al. 1988), these methods may leave toxic reagents and by-products that are difficult to be removed (Kato 2002).
1.8.1 The Chemistry of the Maillard Reaction

The Maillard reaction involves a cascade of complex reactions that forms a number of Maillard reaction products (MRPs) as illustrated in Figure 1.12. The end product of the Maillard reaction is a brown pigment called melanoidin. The Maillard reaction is affected by the nature of the reactants, the combination of temperature and time during heating, water activity, pH, oxygen, metals, and reaction inhibitors such as sulfur dioxide (Ames 1998; Owusu-Apenten 2005).

![Figure 1.12: Schematic diagram illustrating the early and the advanced stage of the Maillard reactions that leads to the formation of various nitrogenous compounds. Note: diagram adapted from (Hodge 1953).](image)

The primary amine that is involved in the Maillard conjugation includes free amino acids, the N-terminal amine of peptides/proteins, and the amino acid side chains of lysine and arginine of proteins (Owusu-Apenten 2005). Protein-conjugate (also known as the Amadori compound) is formed during the early stage of the Maillard
reaction. The extent of the Maillard reaction should be carefully controlled because prolonged reaction can lead to the degradation of the protein-conjugate. It is worth noting that the Maillard reactions can have other desirable effects such as flavor development or undesirable effects such as excessive browning. The Maillard reactions leading to the formation of protein-conjugate can be summarized as follow and in Figure 1.13:

(1) Formation of Schiff’s base – The primary amine of proteins [ANH$_2$] reacts with a reducing carbohydrate [R(CHOH)$_n$CHO] via an addition-elimination reaction. This reaction involves the nucleophilic attack at the carbonyl group of the carbohydrate, which is then followed by the loss of water to form an imine or Schiff’s base.

(2) Amadori Rearrangement – The imine isomerizes to form the Amadori compound and the C$_1$=N double bond migrates to the C$_2$=C$_1$ position forming the 1,2 enol compound. At $< $ pH 4, the Amadori rearrangement involves a further bond migration to C$_3$=C$_2$, which forms the 2,3 enol compound. These Amadori intermediates undergo enol-keto isomerism to form $\alpha$-aminoketones.

(3) Formation of reactive dicarbonyl deoxyones – The Amadori intermediates release an amine [ANH$_2$] to form deoxysone in a pH-dependent fashion. For example, the 1,2 enol compound is decompose to form 3-deoxysones at pH below 4, whereas, the 2,3 enol compound is decomposes to form the 1-deoxysonate at pH 5 – 7.

(4) Formation of flavor compound from deoxysone – The reactive dicarbonyl deoxyones undergo further reactions leading to the formation of flavor and colour compounds. For example, the 1-deoxysonate is cyclised or dehydrated to
form 5-methylfurfural and 5-hydroxy-methylfurfural at pH 6 – 7, whereas, the 3-deoxysone forms 3-furanone at pH 4. The condensation of these ring structures is the first step towards the formation of fluorescence substance and then the formation of colored Maillard reaction products and finally the formation of melanoidins.

(5) Degradation of Amadori products – The Amadori product is fragmented at alkaline pH to form small MW dicarbonyl compounds. The reactive dicarbonyl compounds self-polymerize to form melanoidins.

(6) Streker degradation – A number of 2- and 3-carbon dicarbonyl compounds combine with amino acids and this is then followed by the elimination of RCHO. This leads to the formation of simple heterocyclic ring compounds such as pyrazines, oxazoles, thioloze, pyrrole, and thiophenes.

Figure 1.13: Schematic diagram illustrating the chemical reaction between the ε-amino group of a protein and the reducing-end carbonyl group of a glucose molecule, and the formation of the Amadori compound.
Maillard Conjugates and their Emulsifying Properties

In 1990, Kato and co-workers have successfully prepared an ovalbumin-dextran conjugate using the naturally occurring Maillard reaction by heating an intimate mixture of ovalbumin and dextran (60,000 – 90,000 Da) at 60 °C and 65% relative humidity for 3 weeks. The authors found that the protein-conjugate had improved emulsifying activity and emulsion stability compared to the native ovalbumin. Furthermore, the protein-conjugate maintained its emulsifying activity at pH 3 whereas native ovalbumin has poor emulsifying activity. Further improvements in emulsifying activity were observed at pH 10 and after the protein-conjugate was heated at 100 °C. The observed improvement in emulsifying activity after heating at 100 °C was possibly due to the attached polysaccharide, which protects the unfolded protein resulted from heating from aggregation, while improving the amphiphilic balance of the protein-conjugate moiety (Kato et al. 1993).

Maillard type protein-conjugates has been successfully prepared since using other combinations of protein and polysaccharide, such as phosvitin conjugated with galactomannan (15,000 – 25,000 Da) (Sattar Khan et al. 1999), soy protein hydrolysate conjugated with curdlan (Fan et al. 2006) and bovine serum albumin conjugated with galactomannan (~22,000 Da) (Kim et al. 2003). These authors measured the emulsifying properties of the protein-conjugates according to the method by Pearce and Kinsella (1978), and which were found to have improved emulsifying activity and emulsion stability compared to the native proteins.

Most understanding in relation to the emulsifying properties of the Maillard type protein-conjugates (i.e. emulsifying activity and emulsion stability) and the underlying
mechanisms conferring the improvements came from studies based on dairy proteins. Anionic polysaccharide such as dextran sulphate (Dickinson & Galazka 1991), carboxymethyl dextran (Hattori 2002), methoxyl pectin (Neirynck et al. 2004), carboxymethylcellulose (Kika et al. 2007), cationic polysaccharide such as chitosan (Hattori et al. 2000), and non-ionic polysaccharides such as maltodextrins and dextrans (Dunlap & Cote 2005; Wooster & Augustin 2006; Akhtar & Dickinson 2007; Wooster & Augustin 2007a) have been used to form protein-conjugates with dairy proteins via the Maillard reaction.

Some studies showed that incompatibility between protein and charged polysaccharide affects protein-conjugate formation, such as β-lactoglobulin and dextran sulphate (Dickinson & Galazka 1991) and casein and methoxyl pectin (Einhorn-Stoll et al. 2005). Other studies showed that attachment of charged polysaccharide affects the emulsifying properties of protein, for example, β-lactoglobulin has poor emulsifying activity at neutral pH and at basic pH after conjugation with chitosan, due to the poor solubility of chitosan at these pH (Hattori et al. 2000). In spite of that, β-lactoglobulin conjugated with other type of charged polysaccharides such as carboxymethyl dextrans, has improved emulsifying activity at pH values 3 – 8 and in NaCl environment (0 – 0.5 M) compared to the native protein (Hattori 2002). The authors postulated that the improvement observed on β-lactoglobulin after conjugation with carboxymethyl dextrans was due to the conjugated polysaccharide increasing the hydrophilicity and the amount of negative charges to the protein molecule and also change the protein conformation. Similarly, whey protein concentrate conjugated with carboxymethylcellulose was found to have improved ability to stabilise emulsion against heat induced aggregation at pH 6.5 compared to the native proteins (Kika et al. 2007). This
is due to the attached carboxy-methylcellulose, which provides steric repulsion against emulsion aggregation during heating.

Non-ionic polysaccharides such as dextrans and maltodextrins were the most widely used polysaccharide in preparing Maillard-type protein-conjugates (refer to Table 1.1). $\alpha$-/$\beta$-lactoglobulin conjugated with either 10,000 Da or 20,000 Da dextrans was found to have improved solubility at protein’s pI (~pH 5), and also have enhanced heat stability (60 – 95 °C) at pH 5 compared to the native proteins (Jimenez-Castano et al. 2007). The authors postulated that the improvement in solubility was due to a shift in protein’s pI from pH 5 to pH 4, whereas enhancement in heat stability was due to the attached polysaccharide, which provided steric stabilisation against protein aggregation. However, the authors showed that these improvements were not influenced by the size of the attached dextran or changes to the protein conformation. On the other hand, Dunlap and co-workers (2005) found that emulsions stabilised by $\beta$-lactoglobulin conjugated with various MW dextrans and were stored for 90 days at pH 7 have improved stability as the size of the attached dextran increased from 19,600 Da to 150,000 Da and then levelling off beyond 150,000 Da. In this study, the emulsion stability was determined by monitoring changes in droplet size over time. The authors ascribed the improvement in emulsion stability to the formation of a thick polysaccharide layer at the interface, which provide steric stabilisation. The authors also found that the protein layer density of these protein-conjugates adsorbed at the emulsion interface remained unchanged when the size of the attached dextran was less than 150,000 Da, however the protein layer density decreased as the size of the attached polysaccharide increased beyond 150,000 Da. The authors suggested that the greater density and thickness of the polysaccharide layer formed by the attached dextran (>
150,000 Da) is able to compensate for the decreased protein layer density in maintaining emulsion stability. Similarly, another study found that the emulsifying properties of lysozyme conjugated with galactomannan increase in proportion to the MW of the attached polysaccharide (3,500 – 24,000 Da) (Shu et al. 1996).

Wooster and Augustin (2006) showed that the layer thickness of β-lactoglobulin-dextran conjugates adsorbed at the interface increased according to the MW of the attached dextran, where the layer thickness increased from 5 – 20 nm with the attachment of 18,500 – 440,000 Da dextran. The interfacial layer was measured by adsorbing β-lactoglobulin or the purified protein-conjugates onto latex spheres and then the thickness of the adsorbed layer was measured using dynamic light scattering (DLS). The authors also demonstrated that the attachment of LMW dextran (i.e. 18,500 Da) is sufficient to impart steric stability against emulsion flocculation in calcium environment (5 – 20 mM), even when the emulsion zeta-potential was at minimum. Wooster and Augustin (2007a) also showed that the stability efficacy of emulsions stabilised by β-lactoglobulin conjugated with various MW maltodextrins is a function of both thickness and density of the polysaccharide chains at the interface. The authors found that in order to stabilize an emulsion against flocculation in ionic environment (150 mM NaCl or 0 – 20 mM CaCl₂), β-lactoglobulin conjugated with 900 Da maltodextrin required at least a steric density of one tail per 7.5 nm², whereas with 1900 Da maltodextrin required at least a steric density of one tail per 9.5 nm². The authors also showed that attachment of LMW maltose onto β-lactoglobulin, did not increase the interfacial layer thickness or stabilise the emulsion against flocculation in ionic environment.
Improvement in protein emulsifying properties can arise from changes in protein conformation upon Maillard conjugation. Wooster and Augustin (2007b) found that attachment of ~1 dextran (29,400 Da) per mole of whey protein isolate did not have significant effect on the protein conformation as suggested by tryptophan fluorescence emission and circular dichroism. On the other hand, attachment of ~5.6 dextrans (29,400 Da) per mole of whey protein isolate resulted in substantial protein unfolding. The authors proposed that the unfolding of protein after conjugation was responsible for the increased emulsion capacity observed in other studies. In addition, the authors found that the unfolded whey proteins resulted from conjugation have weaker film strength at the air/water interface than the native protein, and suggested that the interfacial layer formed by this protein-conjugate could be susceptible to droplet coalescence.

Although there have been studies on the use of the Maillard reaction for improving emulsifying properties of a range of proteins, there has been little attention paid in modifying wheat gluten proteins via Maillard conjugation (Table 1.1). Although there are some studies on the Maillard conjugation of wheat gluten proteins, however, all the studies carried out to date were prepared from enzymatic hydrolysed gluten peptides. Pronase digested gluten (PTG) conjugated with dextran was found to have excellent solubility throughout a wide range of pH values 2 – 12 and have enhanced emulsifying properties (i.e. emulsifying activity and emulsion stability) than native wheat gluten proteins and PTG itself (Kato & Kobayashi 1991). On the other hand, other studies found that chymotrypsin digested gluten conjugated with chitosan have poor ability in stabilising emulsions at neutral pH (Babiker 2002).
Table 1.1: Experimental studies of the characterisation and emulsifying properties of Maillard type protein-carbohydrate conjugates.

<table>
<thead>
<tr>
<th>Source</th>
<th>Protein</th>
<th>Polysaccharide</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chicken egg</td>
<td>Ovalbumin</td>
<td>Dextran</td>
<td>Kato et al., 1990; Choi et al., 2005</td>
</tr>
<tr>
<td></td>
<td>Dried egg white</td>
<td>Galactomannan</td>
<td>Kato et al., 1993</td>
</tr>
<tr>
<td></td>
<td>Phosvitin</td>
<td>Galactomannan</td>
<td>Satter Khan et al., 1999</td>
</tr>
<tr>
<td>Bovine plasma</td>
<td>Bovine serum albumin</td>
<td>Galactomannan</td>
<td>Kim et al., 2003</td>
</tr>
<tr>
<td>Soy bean</td>
<td>Soy protein hydrolysate</td>
<td>Curdlan</td>
<td>Fan et al., 2006</td>
</tr>
<tr>
<td></td>
<td>Soy protein</td>
<td>Dextran</td>
<td>Diftis et al., 2005</td>
</tr>
<tr>
<td></td>
<td>β-lactoglobulin</td>
<td>Carboxymethyl dextran</td>
<td>Hattori, 2002</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chitosan</td>
<td>Hattori et al., 2000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dextran</td>
<td>Jimenez-Castano et al., 2005</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Dunlap and Cote, 2005</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Dickinson and Galazka, 1991</td>
</tr>
<tr>
<td>Whey proteins</td>
<td>Maltodextrins</td>
<td>Carboxymethylcellulose</td>
<td>Kika et al., 2007</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Methoxyl pectin</td>
<td>Neirynck et al., 2004</td>
</tr>
<tr>
<td>Bovine milk</td>
<td>Gum arabic</td>
<td>Dextran</td>
<td>Akhtar and Dickinson, 2003; Zhu et al., 2008</td>
</tr>
<tr>
<td></td>
<td>Carboxymethylcellulose</td>
<td></td>
<td>Wooster and Augustin, 2007; Wooster and Augustin, 2007</td>
</tr>
<tr>
<td></td>
<td>Methoxyl pectin</td>
<td></td>
<td>Jimenz-Castano et al., 2007</td>
</tr>
<tr>
<td>Caseins</td>
<td>Maltodextrins</td>
<td></td>
<td>Akhtar and Dickinson, 2007</td>
</tr>
<tr>
<td></td>
<td>Maltodextrins</td>
<td></td>
<td>O’Regan and Mulvihill, 2009</td>
</tr>
<tr>
<td></td>
<td>Methoxyl Pectin</td>
<td></td>
<td>Shepherd et al., 2000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chitosan</td>
<td>Babiker, 2002</td>
</tr>
<tr>
<td></td>
<td></td>
<td>β-carrageenan</td>
<td>Wang et al., 2006</td>
</tr>
</tbody>
</table>

The emulsifying properties of wheat gluten modified via a combination of acid deamidation and Maillard conjugation was examined in this thesis. Of particular interest
is to investigate the ability of deamidated wheat gluten-carbohydrate conjugates in stabilising O/W emulsions at acidic pH and in ionic environment as well as to understand the underlying mechanisms responsible for controlling the emulsifying properties of these protein-conjugates.

1.9 Hypothesis of this Thesis

The hypothesis for this thesis is that conjugation of deamidated wheat protein with non-ionic carbohydrates (e.g. glucose, maltodextrins and dextrans) results in a covalently-linked complex with enhanced ability to maintain emulsion stability at acidic pH and/or in ionic environment compared to non-conjugated deamidated wheat protein. This enhancement is due to steric stabilization that is afforded by the attachment of the polysaccharide to the protein that is anchored at the interface. The effectiveness of this steric layer in providing emulsion stability is dependent on the size, the number and the location of the polysaccharide that is conjugated onto the protein.
CHAPTER 2

The Composition, Solution and Structural Properties of the Soluble Fraction of Isolated Wheat Protein

2.1 Introduction

Protein deamidation involves the removal of the amide groups from the asparagines and glutamine residues to form aspartic acid and glutamic acid respectively as illustrated in Figure 2.1. Wheat gluten proteins contain a high concentration of glutamine residues. Deamidation of these glutamine residues introduces negatively charged glutamic acid residues. This induces protein conformational changes and enhances electrostatic repulsion between protein molecules (Matsudomi et al. 1982). As a consequence, the protein solubility and the emulsifying properties of the wheat gluten proteins are improved (Matsudomi et al. 1982; Webb et al. 2002).

Figure 2.1: This diagram illustrates the deamidation reaction.

A commercially produced deamidated wheat gluten, called isolated wheat protein (IWP) is used as the starting material in this work. In order to remove residue
starch and fibrous materials carried over from the gluten production and any undeamidated insoluble large protein particles (aggregates), a simple purification step was taken to obtain only the soluble fraction of isolated wheat protein (sIWP) for the purpose of this study. The sIWP was the material used as the protein source in this study. The aim of this part of the work is to determine the composition and characterise the sIWP. The solution properties (i.e. protein isoelectric point, solubility, and hydrodynamic size), and the protein conformation (i.e. secondary structure and tryptophan fluorescence emission) of sIWP was compared against vital wheat gluten (VWG), gliadin fraction purified from VWG and a laboratory prepared deamidated gliadin (gliadin purified from VWG). VWG is the non-deamidated wheat gluten from which IWP is produced commercially.

2.2 Materials and Methods

2.2.1 Materials

Commercial VWG and IWP were obtained from Manildra Group (Sydney, Australia). To prepare sIWP, IWP was dispersed in milli-Q water (10 wt. %) under the constant stirring for 2 h and the dispersion was then centrifuged at 15,000 × g for 15 min using a Beckman J2-MC centrifuge. The insoluble material (i.e. the precipitate), and the supernatant were separated and freeze-dried respectively. The resultant sIWP, which represented approximately 65% of the original IWP, was used throughout the study without further purification.
Gliadin was purified from VWG and IWP using 70% ethanol solution for circular dichroism (CD) and tryptophan fluorescence emission measurements. VWG or IWP was dispersed in 70% ethanol (10 wt. %) and then lightly intermixed using an Ultra-turrax at 9,500 rpm for 1 min to assist their solubilisation. The intermixed dispersion was then gently stirred for 1 h at room temperature and then centrifuged at 10,000 × g for 45 min at 10 ºC using a Beckman J2-MC centrifuge. The supernatant was mixed with 1.5 M ammonium chloride solution (2 × volume of the supernatant) and then the protein was allowed to be precipitated out of the solution overnight. The precipitate was then washed with milli-Q water twice to remove residue ammonia chloride salt and then freeze-dried. The resulting gliadin fractions isolated from VWG and IWP are called VWG gliadin and IWP gliadin respectively.

Deamidation was performed on VWG gliadin. VWG gliadin was dispersed in 0.2 M HCl (10 wt. %) and then heated at 70 ºC for 2 h with constant stirring in a sealed container. The dispersion was cooled to room temperature and then neutralised by using a 10 M NaOH. The dispersion was centrifuged at 10,000 × g for 15 min at 10 ºC using a Beckman J2-MC centrifuge and its supernatant was collected. The degree of deamidation was calculated by comparing the ammonia content measured from the supernatant and total deamidated VWG gliadin (prepared at 98 ºC in 2 M HCl for 3 h) using an ammonia test kit (Randox, United Kingdom). The supernatant was dialysed (8,000 Da cut off) exhaustively against milli-Q at 4 ºC overnight and then freeze-dried.

The protein standards used for size exclusion chromatography were myosin (200,000 Da), bovine serum albumin (BSA) (66,300 Da), β-lactoglobulin (18,300 Da), and insulin-B (5,700 Da) which were purchased from Sigma Aldrich (Sydney,
Australia). The protein standards for gel electrophoresis, Mark12™ unstained standard, were purchased from Invitrogen (Australia). The Bio-Rad DC assay kit was purchased from Bio-Rad (Australia). Other analytical grade chemicals were purchased from Sigma Aldrich (Sydney, Australia).

2.2.2 Determination of Protein, Ash and Moisture Content

The percentage of protein content in protein powder was determined using a combustion method. The sample (0.1 g) was weighed into a LECO® boat. The total nitrogen content of the sample was then determined using the LECO® TC400. The total nitrogen weight (mg) obtained was converted to protein weight (mg) by multiplying by 6.25. The multiplication factor was chosen at 6.25 because the protein was deamidated.

The moisture content in protein powder was determined based on AOAC Method 44-19. The sample (2 ± 0.001 g) was evenly distributed on a pre-dried aluminum moisture dish. The sample was dried in an air-forced oven at 135 °C for 2 h and cooled to room temperature (24 ºC) in an air-tight desiccator with reignited CaO. The final weight was measured immediately and the loss in weight as moisture and volatile matter in percentage was calculated.

The ash content in protein powder was determined based on AOAC Method 08-01. Sample (3 ± 0.0001 g) was weighed into an ignited ashing dish. The sample was ignited in a muffle furnace at 670 °C until light gray ash was obtained. The sample was then cooled to room temperature (24 ºC) in an air-tight desiccator with reignited CaO. The final weight was measured immediately and the ash content was calculated.
2.2.3 Size Exclusion-High Performance Liquid Chromatography

Size exclusion-high performance liquid chromatography (SE-HPLC) was conducted using a Shimadzu LC-20 HPLC system equipped with a UV-vis detector (Batey et al. 1991). Protein dispersions were prepared in 50 mM sodium phosphate buffer (pH 6.8) containing 0.5 wt. % SDS (for reduced condition 0.05 M dithiothreitol (DTT) was added) and then mixed for 2 h using a vortex mixer. The solubilisation of VWG or the insoluble fraction of IWP in buffer was assisted by sonicating the dispersion thrice at 10 min each time to disrupt the protein molecules in the aggregates and to allow the binding of SDS. The protein dispersions were centrifuged at 10,000 × g for 5 min and then filtered through a 0.45 µm syringe filter. These samples (20 µL) were then injected into a Phenomenex BIOSEP-SEC-S4000 column and run at 0.5 mL min\(^{-1}\) in a 50 mM sodium phosphate buffer (pH 6.8). The elution profiles were monitored at 214 nm. The estimation of protein average MW was based on the elution profiles of protein standards.

2.2.4 Sodium-Dodecyl-Sulphate Polyacrylamide Gel Electrophoresis

A NuPAGE gradient gel (4–12 % Bis-Tris) purchased from Invitrogen (Sydney, Australia) was used for gel electrophoresis. Samples (10 µL, 3.8 mg/mL) were prepared in sample buffer [3.6 wt. % sodium dodecyl sulfate, 18 wt. % glycerol, 27.2 wt. % Trizma base, 0.009 wt. %, and 0.05 M dithiothreitol (DTT)] at pH 8.6. Electrophoresis was carried out at 100 V for 45 min and then 120 V for 1 h in morpholinoethanesulfonic (MES) running buffer (containing 1.1 wt. % 2-Morpholinoethanesulfonic acid monohydrate, 0.606 wt. % Trizma base, 0.1 wt. % sodium dodecyl sulfate, 0.03 wt. %
ethylenediaminetetraacetic acid (EDTA) and 0.5 mL of NuPage antioxidant. After electrophoresis, the gels were stained for proteins with 0.1 wt. % Coomassie brilliant blue-R250 and then de-stained with 7.5 wt. % acetic acid containing 10 wt. % methanol. The gels were photographed using a Kodak digital camera controlled by Kodak 1D v3.5 software.

2.2.5 **Amino Acid Analysis**

Quantitative amino acid analyses of IWP and sIWP were performed by the Australian Proteome Analysis Facility (Sydney, Australia).

2.2.6 **Protein Zeta-potential, Hydrodynamic Size and Isoelectric Point**

The protein solution (0.1 wt. %) was prepared in milli-Q water and was allowed to disperse for 2 h. The zeta-potential and hydrodynamic size (Z-average) of the proteins was measured using a Zetasizer-Nano ZS (Malvern instruments) coupled with an auto-titrator (Malvern instruments). Three titrants were used, 0.05 M NaOH, 0.05 M HCl and 0.5 M HCl. A total of 10 mL protein dispersion was used as the starting volume. Triplicate measurement of protein zeta-potential and hydrodynamic size (Z-average) was taken at 24 °C using a DTS1060C clear disposable zeta cell. A protein refractive index of 1.45 and water refractive index of 1.33 was used. The protein isoelectric point was identified at 0 mV zeta-potential.

2.2.7 **Determination of Protein Concentration in Solution**

The protein solution (1 wt. %) was diluted 15 times with aqueous or buffer solution. 100 µL of the diluted protein solution was reacted with 500 µL of Reagent A (alkaline copper tartrate solution) and 4 mL of Reagent B (dilute Folin reagent) from the
Bio-Rad DC assay kit (Lowry assay based method) as per the manufacturer’s protocol. After 15 min of incubation, absorbance at 750 nm was taken. The protein concentration of the supernatant was then calculated based on the absorbance (750 nm) of bovine serum albumin (BSA) at known concentration (Figure 2.2). A multiplication factor of 1.26 was applied, as obtained after standardisation of samples using the total nitrogen analysis by the LECO® FP2000 (Figure 2.3). The total nitrogen analysis is considered as a standard method for the determination of protein concentration and was used to standardise against the rapid Lowry assay based method. For total nitrogen analysis, the protein solution was diluted to a series of concentration, and then 2.5 mL of the solution was dried in a LECO® boat at 100 ºC overnight. The total nitrogen content in the LECO® boat was then measured and converted to protein weight as described in section 2.2.2. The concentration of the protein solution that was dried in the LECO® boat was then calculated based on the protein weight and the known solution volume (i.e. 2.5 mL).

![Graph](image)

Figure 2.2: Absorbance measured at 750 nm as a function of bovine serum albumin concentration.
Figure 2.3: Comparison between protein concentration measured using the combustion based method and Lowry based method.

2.2.8 Protein Solubility in Aqueous Solution

The protein solution was prepared in milli-Q water and was allowed to be dispersed for 2 h at room temperature (24 °C). The dispersion was then adjusted to the desirable pH using 0.1 M HCl and 0.1 M NaOH (pH 2.5 – pH 8.5). During the preparation of the solution, the weight of the protein powder and the volume of the aqueous solution were carefully measured and recorded. These were then used to calculate the initial protein concentration. The percentage of protein content in the protein powder was determined based on the total nitrogen content in the powder (see section 2.2.2). The dispersion was centrifuged at 10,000 × g for 15 min using a Beckman J2-MC centrifuge. The supernatant was carefully separated from the pellet and then its protein concentration was determined using a Bio-Rad Dc assay as
described in section 2.2.7. The aqueous solubility of the protein was calculated from the difference in protein concentration before and after the centrifugation.

2.2.9 *Hydrodynamic Size Coupled with SE-HPLC*

The hydrodynamic size of sIWP was analysed using SE-HPLC coupled to a dynamic light scattering (DLS) detector. A Shimadzu LC-20 HPLC system equipped with a UV-Vis detector was connected to a DLS instrument (zeta-sizer Nano ZS, Malvern instruments) via a short path length flow cell (Hellma 3 mm path length, 100 µl volume, 8.5 mm centre height). The DLS correlation functions were collected for 3 s and were analysed using CONTIN analysis (Provencher 1982). sIWP was solubilised in 50 mM sodium phosphate buffer (pH 6.8) containing 0.5 wt. % SDS. The protein sample (50 mL) was then injected into a Phenomenex BIOSEP-SEC-S4000 column and eluted using 50 mM sodium phosphate buffer (pH 6.8) at 0.3 mL/min.

2.2.10 *Attenuated Total Reflectance - Fourier Transform Infrared Spectroscopy and Principal Component Analysis*

Attenuated total reflectance (ATR) spectra of the freeze-dried samples were recorded at 4 cm\(^{-1}\) with a nitrogen purged Bruker Equinox 55 FTIR spectrometer equipped with a liquid nitrogen cooled mercury-cadmium-telluride (MCT) detector and a Golden gate single bounce diamond ATR. Each spectrum was the result of an average of 256 scans which were Fourier-transformed in OPUS 5.5 using Blackmann-Harris 4 term apodization and a zero filling factor of 8. Spectra were converted from OPUS format to JCAMP-DX format and imported into the Unscrambler 9.2, where the extended multiplicative signal correction (EMSC) was applied, followed by
transformation into 2nd derivative using Savitzky-Golay function (3rd order polynomial and 3 smoothing points). After mean centering, the pre-processed spectra were then analysed using principal component analysis (PCA) in Unscrambler 9.2. Changes in secondary structure were determined using the Amide I band (1700-1600 cm\(^{-1}\)) (C=O stretch coupled with C-N stretch).

The typical wavenumber values assigned to Amide I modes were as follows: \(\alpha\)-helix (1650-1657 cm\(^{-1}\)), antiparallel \(\beta\)-sheet (intermolecular) (1612-1640 cm\(^{-1}\) and 1670-1690 cm\(^{-1}\) (weak)), parallel \(\beta\)-sheet (intramolecular) (1626-1640 cm\(^{-1}\) and 1655-1675 cm\(^{-1}\)), turn (1655-1675 cm\(^{-1}\) and 1680-1696 cm\(^{-1}\)) and random coil (1640-1651 cm\(^{-1}\)) (Pelton & McLean 2000).

### 2.2.11 Circular Dichroism

Far-UV CD was used to examine the protein secondary structure in solution. CD spectra were taken from a synchrotron radiation based CD instrument (SRCD) (Figure 2.4), which is located at the institute for storage ring facility (ISA) in Aarhus University (Denmark). The protein solutions (0.1 wt. %) were prepared in 20 mM McIlvaine’s buffer at pH 8 through gentle stirring for 2 h prior to CD measurements. A dispersion containing gliadin purified from VWG was prepared by stirring the protein dispersion in 20 mM McIlvaine’s buffer [20 mM Na\(_2\)HPO\(_4\) (19.45 mL) mixed with 10 mM citric acid (0.55 mL); pH 8] overnight at 4 °C. Then, the dispersion was subjected to centrifugation at top-speed using a mini bench-top centrifuge to remove the insoluble protein.

The protein concentration of the solutions was determined by measuring the absorbance at 280 nm using a 0.1 cm path length quartz cell and 0.9551 as the
extinction coefficient ($\varepsilon$) [Note: $\varepsilon$ was calculated from the absorbance (at 280 nm) of a series of known protein concentrations (0 – 2.2 mg/mL) using $A_{280\text{ nm}} = \varepsilon c l$]. CD spectra were recorded at 24 °C from 260 to 185 nm with a spectral resolution of 1 nm. Spectra were recorded using a 0.1 cm path length quartz suprasil cell (Hellma, Germany) and were baseline corrected for 20 mM McIlvaine’s buffer (pH 8). The CD spectra in ellipticity ($\theta$) units were scaled to delta epsilon ($\Delta\varepsilon$) using 110 as the mean residue weight of protein (Kelly et al. 2005). The proportions of each secondary structure components were determined through a web-based calculation server (DICHROWEB) based on a CONTIN programme with reference set 6 (optimised for 185 – 240 nm), which predicts the proportion of the $\alpha$-helix, $\beta$-sheet, turns, and random coil in the protein (Whitmore & Wallace 2008).

Figure 2.4: The set-up of the synchrotron radiation based circular dichroism (SRCD). Circularly polarised synchrotron radiation was transmitted through the sample cell, and then reached the detector.
2.2.12 Tryptophan fluorescence Emission

The tryptophan fluorescence emission spectra were recorded using a Perkin-Elmer LS-55 spectro-fluorophotometer equipped with a front-face-fluorescence accessory. Protein solutions (0.04 mg/mL) were prepared in 20 mM McIlvaine’s buffer (either at pH 4.5, or pH 8) and allowed to dispersed for 2 h. Spectra were recorded using a excitation wavelength of 280 nm, with 1 nm resolution, 10 nm slit widths and a scanning speed of 100 nm/min.

2.3 Results and Discussion

2.3.1 Compositional Analysis

2.3.1.1 Gross Composition of sIWP

Table 2.1 shows the composition of sIWP in comparison to VWG. sIWP contains 96.25 ± 1.0 % total solids, 89.12 ± 0.8 % protein and 7.51 ± 0.1 % ash. sIWP contains a higher amount of ash compared to VWG. The higher ash content in sIWP is possibly attributed by the use of acid and base during deamidation process, where acid was used as a catalyst for deamidation to occur, whereas base was added for neutralization after deamidation. In addition, sIWP may contain varying amount of starch, fibre and lipid in the sample because these components can be entrapped within the protein matrix (Day et al. 2006).
2.3.1.2 Protein Components in sIWP

The SE-HPLC of reduced and non-reduced sIWP and VWG are given in Figure 2.5. The two major protein fractions in wheat gluten are monomeric gliadin and polymeric glutenin. Polymeric glutenin are composed of HMW and LMW subunits of glutenin joined together by intermolecular disulphide bonds (Wrigley 1996; Gianibelli et al. 2001). These two major fractions can be differentiated using SE-HPLC, based on molecular sizes in the order of large to small proteins. The SE-HPLC chromatograms obtained under non-reducing conditions can be divided into four regions: P1 – HMW-glutenin polymers (> 160,000 Da), P2 – LMW glutenin polymers (75,000 – 160,000 Da), P3 – α-, β-, γ-, ω-gliadin (25,000 – 75,000 Da) and P4 – polypeptides (< 25,000 Da). These assignments are based on that of Linares and co-workers (2000).

---

Table 2.1: The gross composition of VWG and sIWP

<table>
<thead>
<tr>
<th>Composition</th>
<th>VWG Manufacturer specification sheet</th>
<th>sIWP Experimentally determined</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Solids % (g/100g sample)</td>
<td>93</td>
<td>96.3 (± 1)</td>
</tr>
<tr>
<td>Moisture % (g/100g sample)</td>
<td>7</td>
<td>3.8 (± 1)</td>
</tr>
<tr>
<td>Ash % (g/100g solids)</td>
<td>0.8</td>
<td>7.4 (± 0.1)</td>
</tr>
<tr>
<td>Protein % (g/100g solids)</td>
<td>80</td>
<td>89.1 (± 0.8)</td>
</tr>
<tr>
<td>Others % (g/100g solids)</td>
<td>19.2</td>
<td>3.5 (± 0.7)</td>
</tr>
</tbody>
</table>

(e.g. starch, fibre and lipid, etc.)
Figure 2.5: The SE-HPLC chromatogram of sIWP and VWG obtained under non-reduced and reduced conditions. The chromatograms under non-reduced conditions can be separated into four regions according to Linares and co-workers (2000), P1 – HMW-glutenin polymers (> 160,000 Da), P2 – LMW glutenin polymers (75,000 – 160,000 Da), P3 – α-, β-, γ-, ω-gliadin (25,000 – 75,000 Da) and P4 – peptides (< 25,000 Da). In the non-reduced chromatograms, peak A was used to represent polymeric glutenin, while peak B was used to represent monomeric gliadin. The B/A peak area ratio indicates that sIWP contain mostly of monomeric gliadin.

Comparison made between the non-reduced SE-HPLC chromatogram of sIWP and VWG showed that sIWP had an increased amount of LMW polypeptides in the P4 region (MW < 25,000 Da). The deamidation process can lead to limited peptide chain
cleavage reaction. This is similar to the β-aspartyl shift reaction mechanism, where peptide chain cleavage as well as deamidation occurs immediately after an asparagine residue (Wright 1991). It is likely that the LMW polypeptides observed in the P4 region (MW < 25,000 Da) in the non-reduced chromatogram originate from the monomeric gliadin fraction rather than polymeric glutenin because there are limited cleavage sites in wheat gluten protein due to their low level of asparagines residues, which suggests that the protein sizes (both monomeric gliadin and polymeric glutenin) should not decrease significantly. Furthermore, peptide cleavage usually does not reduce the intermolecular disulphide bonds that link between the glutenin subunits, which mean that they should remain polymeric in nature. This is confirmed by SE-HPLC chromatograms of sIWP obtained under reduced conditions, where a shift in peaks to lower MW region is observed upon the disruption of the disulphide bonds by reducing agent. However, the absence of peaks at the P1 and P2 region in the reduced SE-HPLC chromatogram of sIWP compared to VWG suggests that there were peptide cleavage occurred in the glutenin fraction of sIWP. It should be noted that the cleaved peptides is not the main fraction of sIWP, which suggests that peptide hydrolysis occurred during deamidation has minimal contribution to the overall functionality of sIWP.

Using the peak area A (P1 + P2) and B (P3 + P4) shown in the non-reduced SE-HPLC chromatogram of sIWP and VWG (Figure 2.5) to represent polymeric glutenin and monomeric gliadin respectively, the ratio of B/A is 4.3 for sIWP and 1.9 for VWG. This indicates that sIWP contained much higher levels of monomeric gliadin than VWG, or in other words, sIWP contain little glutenin in comparison to VWG. The relative peak area ratio was not calculated for the reduced SE-HPLC chromatogram of sIWP and VWG because reducing agent disintegrates polymeric glutenin into HMW
glutenin and LMW glutenin. Due to the similarity between the MW of LMW glutenin (36,000 – 44,000 Da) and the gliadin (32,000 – 42,000 Da), they cannot be distinguished from each other using SE-HPLC.

The higher level of monomeric gliadin compared to polymeric glutenin in sIWP could be due to the difference in degree of deamidation between these two protein fractions. The rate of deamidation is affected by the accessibility of water molecules to the protein’s glutamine and asparagines residues (Wright 1991). Glutenin subunits are joined together via intermolecular disulphide bonds to form very large glutenin polymers. This is likely to restrict the accessibility of water molecule for deamidation reaction to occur. As a result, a much higher proportion of polymeric glutenin remained as aggregates and low in solubility compared to monomeric gliadin, and subsequently they were removed during the purification step. This is evident as shown in Figure 2.6, the peak B/A ratio calculated from the non-reduced SE-HPLC chromatograms of IWP, sIWP and the precipitate is 3.4, 4.3 and 2.0 respectively indicating that larger amount of glutenin biopolymers was found in the insoluble fraction of IWP. It is worth noting that the solubilisation of the precipitate in the buffer solution was assisted through sonication, which disrupts most of the protein aggregates. The results showed that most of the insoluble proteins removed from IWP during the purification step for the isolation of sIWP are the polymeric glutenin, as indicated by the low B/A ratio of the precipitate and the higher B/A ratio of sIWP compared to IWP.
Figure 2.6: SE-HPLC chromatogram of the isolated wheat protein (IWP), soluble fraction of the isolated wheat protein (sIWP) and insoluble protein fraction of IWP (precipitate).

2.3.1.3 Average Molecular Weight of sIWP

The average MW of sIWP was determined under reducing conditions. This allows the measurement to be based on individual proteins rather than protein clusters. Figure 2.7 shows the reduced SE-HPLC chromatogram of sIWP that was separated into 11 sections. Myosin (200,000 Da), bovine serum albumin (BSA) (66,300 Da), β-lactoglobulin (18,300 Da) and insulin-B (5,700 Da) were used as protein standards and their retention time are indicated in Figure 2.7. The average MW of sIWP is approximately 40,375 Da, which was calculated based on the protein standards and the percentage area of each section. sIWP is composed mainly of gliadin and the average MW of sIWP determined in this study falls within the MW range of gliadin (25,000 – 75,000 Da).
Figure 2.7: The calculation of the average MW of sIWP using SE-HPLC chromatogram obtained under reducing conditions were demonstrated in this figure. Individual protein molecules rather than clusters of proteins were measured. The average MW of sIWP is 40,375 Da. The retention time of the MW standards is shown at the top region of the figure.

The average MW of sIWP was also estimated from SDS-PAGE. Figure 2.8 shows the protein pattern of VWG (lane 2) and sIWP (lane 3) in the SDS-PAGE, which was obtained under reduced conditions. The major protein bands observed in VWG (lane 2) are A – HMW-glutenin subunits (70,000 – 116,000 Da), B – ω-gliadin (55,000 – 65,000 Da) and C – α-gliadin, γ-gliadin and LMW-glutenin subunits (36,000 – 48,000 Da). On the other hand, protein pattern observed in sIWP (lane 3) appeared smeary, which is likely resulted from peptide hydrolysis. A comparison of the intensity (Coomassie blue stain) of the protein pattern and the position in the SDS-PAGE of
sIWP (lane 3) (Figure 2.8) and the peak areas observed in the reduced SE-HPLC chromatograms of sIWP (Figure 2.7) indicated that the two methods concurred. The position where the highest intensity band was observed in the SDS-PAGE protein pattern of sIWP (lane 3) falls between 55,400 Da and 36,500 Da, this was within the range of 40,375 Da, which confirms the average MW estimated from the reduced SE-HPLC chromatogram of sIWP.

Figure 2.8: SDS-PAGE gel of sIWP and VWG were obtained under reducing conditions and stained in Coomassie blue. The major protein bands of VWG observed in lane 2 are A: HMW-glutenin subunits (70,000 – 116,000 Da), B: ω-gliadin (55,000 – 65,000 Da) and C: α-gliadin, γ-gliadin and LMW-glutenin subunits (36,000 – 48,000 Da). (The labelled lanes are, lane 1: MW standards; lane 2: VWG, lane 3: sIWP)
2.3.1.4 Amino Acid Analysis of sIWP

Table 2.2 shows the amino acid composition of sIWP in comparison with those in gliadin and glutenin reported in the literature (Ewart 1967). sIWP contains a high amount of non-polar (aliphatic and aromatic R groups) and polar uncharged amino acids, approximately 32% and 25% respectively, and a low amount of positively charged amino acid residues such as arginine, lysine and histidine, accounting for approximately 5%. Approximately another 38% consists of either glutamine/asparagines (polar uncharged) or their acid form (negatively charged). The proportion of glutamine/asparagine residues and their acid form in sIWP could not be determined because all the glutamine residues were converted to acid form during the amino acid compositional analysis. It has been shown that most of the glutamine residues (~82 – 92%) in native VWG (non-deamidated) are in the amide form based on the determination of total amount of ammonia liberated from the hydrolysed gluten proteins during amino acid compositional analysis (Ewart 1967).

By comparing the amino acid composition of sIWP with both gliadin and glutenin, sIWP is more closely matched to the gliadin fraction rather than the glutenin fraction. This confirms that sIWP is mainly composed of deamidated gliadin, which is in agreement with the SE-HPLC results showing that sIWP is mainly composed of deamidated gliadin. Based on sIWP having an average MW of 40,375 Da, this comprises approximately 267 amino acid residues per mole of sIWP.
Table 2.2: Amino acid composition of sIWP, glutenin and gliadin.

<table>
<thead>
<tr>
<th>Amino Acids</th>
<th>Glutinin* (mole %)</th>
<th>Gliadin* (mole %)</th>
<th>sIWP (mole %)</th>
<th>Amino acid R group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methionine</td>
<td>1.46</td>
<td>1.25</td>
<td>1.22</td>
<td></td>
</tr>
<tr>
<td>Valine</td>
<td>4.99</td>
<td>4.98</td>
<td>4.40</td>
<td></td>
</tr>
<tr>
<td>Isoleucine</td>
<td>3.92</td>
<td>4.47</td>
<td>3.90</td>
<td>24.97</td>
</tr>
<tr>
<td>Leucine</td>
<td>6.76</td>
<td>7.17</td>
<td>7.18</td>
<td></td>
</tr>
<tr>
<td>Alanine</td>
<td>4.57</td>
<td>3.43</td>
<td>3.55</td>
<td></td>
</tr>
<tr>
<td>Glycine</td>
<td>7.8</td>
<td>3.22</td>
<td>4.72</td>
<td></td>
</tr>
<tr>
<td>Aspartic acid /</td>
<td>3.85</td>
<td>2.90</td>
<td>2.69</td>
<td>38.33</td>
</tr>
<tr>
<td>Asparagine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutamic acid/</td>
<td>30.07</td>
<td>35.92</td>
<td>35.64</td>
<td></td>
</tr>
<tr>
<td>Glutamine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Threonine</td>
<td>3.53</td>
<td>2.49</td>
<td>2.70</td>
<td>24.43</td>
</tr>
<tr>
<td>Serine</td>
<td>7.18</td>
<td>6.33</td>
<td>6.10</td>
<td></td>
</tr>
<tr>
<td>Proline</td>
<td>12.38</td>
<td>16.82</td>
<td>15.63</td>
<td></td>
</tr>
<tr>
<td>Arginine</td>
<td>3.12</td>
<td>2.08</td>
<td>2.46</td>
<td>5.35</td>
</tr>
<tr>
<td>Lysine</td>
<td>2.08</td>
<td>0.62</td>
<td>1.19</td>
<td></td>
</tr>
<tr>
<td>Histidine</td>
<td>1.97</td>
<td>1.97</td>
<td>1.70</td>
<td></td>
</tr>
<tr>
<td>Tyrosine</td>
<td>2.60</td>
<td>1.87</td>
<td>2.34</td>
<td>6.92</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>3.70</td>
<td>4.47</td>
<td>4.58</td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>100%</strong></td>
<td><strong>100%</strong></td>
<td><strong>100%</strong></td>
<td><strong>100%</strong></td>
</tr>
</tbody>
</table>

*Data obtained from (Ewart 1967)

The MW of α–gliadin and γ–gliadin range from 32,000 to 42,000 Da (Shewry et al. 1986). They contain approximately 260 amino acid residues based on their amino acid sequences obtained from a protein database (http://www.ncbi.nlm.nih.gov) (Figure 2.9). This is closed matched to the average amino acid residues of sIWP.
Figure 2.9 Amino acid sequence of α-gliadin (ABQ52121) and γ-gliadin (CAC94871) obtained from http://www.ncbi.nlm.nih.gov. N-terminus is indicated by the underlined amino acids. Lysine residues are abbreviated as (k) and tryptophan residues are abbreviated as (w).

As shown in these amino acid sequences, the N-terminal repetitive domain (underlined) is rich in glutamine (q) and proline (p) residues, whereas the C-terminal domain contains many hydrophobic amino acid residues like valine (v), cysteine (c), isoleucine (i), and leucine (l). After deamidation, the N-terminal is likely to be more hydrophilic due to the conversion of the many uncharged glutamine residues to negatively charged glutamic acid residues. The study by Chobert and co-workers (1996) showed that peptides derived from C-terminal region is more readily adsorbed to an emulsified oil droplet surface than peptides derived from N-terminal region because the C-terminal region contains many hydrophobic amino acid residues that can interact with the oil phase.
2.3.1.5 SDS-PAGE Analysis of Gliadin Purified from VWG and IWP

Figure 2.10 shows the SDS-PAGE of VWG and IWP and their purified fractions. According to the protein bands in SDS-PAGE, gliadin enriched fractions were successfully purified from VWG and IWP, as indicated by higher intensity protein bands around 31,000 – 50,000 Da in lane 3 and around 31,000 – 55,400 Da in lane 6 respectively. The 70% ethanol insoluble precipitate removed from VWG and IWP is primarily the glutenin as indicated by the presence of HMW protein bands (> 66,300 Da) in lane 4 and lane 7 respectively. The presence of LMW protein bands (< 66,300 Da) observed in lane 4 and lane 7 are possibly gliadin that were entrapped within the precipitate.

Figure 2.10: SDS-PAGE gel of VWG and IWP and their purified gliadin fractions (soluble in 70% ethanol) were obtained under reducing conditions and stained in Coomassie blue. (The labelled lanes are, lane 1 and 8: MW standards; lane 2: VWG, lane 3: VWG gliadin, lane 4: precipitate from VWG; lane 5: IWP, lane 6: IWP gliadin; and lane 7: precipitate from IWP).
2.3.2 Solution Properties

2.3.2.1 Isoelectric Point of sIWP

Figure 2.11 shows the pH titration curve of sIWP in solution as a function of zeta-potential. At pH 6.5, sIWP exhibited a zeta-potential of approximately – 50 mV. As the pH decreased, the zeta-potential gradually became less negative and reaches 0 mV at approximately pH 4. Further reduction in pH to below pH 4, the zeta-potential of sIWP turned to positive. This is a typical zeta-potential curve for proteins (Bowen, 1996). The results suggest that the pI of sIWP is around pH 4. The pI of sIWP is at much lower pH compared to the pI of VWG, which is around pH 7 (Mejri et al. 2005). The reason that sIWP has lower pI than VWG is due to the conversion of glutamine residues (uncharged) to its acidic form (negatively charged) during deamidation process, which increases the negatity of the overall protein charge density.

The minimum zeta-potential required by protein molecules to produce effective electrostatic repulsion against aggregation is around +/- 30 mV (Hunter 1981). sIWP has a zeta-potential above +/- 30 mV at pH greater than pH 5, which provides sufficient electrostatic repulsion force against aggregation between protein molecules. Furthermore, a charged emulsifier is desirable for stabilising O/W emulsions because when the emulsifier is adsorbed at the interface, it can be provide electrostatic repulsion against droplet flocculation.
2.3.2.2 Solubility of sIWP

It is known that VWG has poor water solubility at pH 7 (VWG’s pI) (Mejri et al. 2005). This is because VWG contains high level of glutamine, non-polar and polar uncharged amino acids in VWG that allows the formation of strong intermolecular interactions via hydrogen bonding, which strongly suppress protein hydration (Beckwith et al. 1963; Krull & Wall 1966). However, after deamidation, many of the glutamine residues (uncharged) were converted to its acidic form (negatively charged) and zeta-potential showed in Figure 2.11 indicate that sIWP should be soluble at neutral pH.

Figure 2.11 shows the water solubility of IWP as a function of pH. IWP exhibits high water solubility (~70 – 90 %) at pH ranging from pH 5 – 8. However, when the pH is decreased down to acidic pH range (2.5 – 4.5), IWP became poorly soluble in water.
(~10 – 20%). In addition, the water solubility of IWP at pH 6.5 was independent of protein concentration (1 – 15 wt. %) as shown in Figure 2.13.

![Graph showing solubility of IWP dispersed in water at 5 wt. % protein concentration measured at pH 2.5 – 8.0.](image)

Figure 2.12: Solubility of IWP dispersed in water at 5 wt. % protein concentration measured at pH 2.5 – 8.0.

![Graph showing solubility of IWP dispersed in water at natural pH (~6.5) measured at protein concentration 1 – 15 wt. %](image)

Figure 2.13: Solubility of IWP dispersed in water at natural pH (~6.5) measured at protein concentration 1 – 15 wt. %.
After the purification of IWP, the resulting sIWP has much higher water solubility (~97 %) at pH 6.5 (cf. IWP ~85%) because most of the insoluble proteins were removed. Since sIWP was purified from IWP, the effect of pH on its water solubility is likely to show a similar trend to that of IWP, where high solubility at above pH 5 and minimum solubility at the pI of sIWP (~pH 4). This correlates well with the zeta-potential of sIWP showed in Figure 2.11, where sIWP exhibit greater than – 30 mV at pH above pH 5 and 0 mV at pH 4 (sIWP’s pI). The highly negative electrostatic charge exhibited by sIWP at pH above pH 5 provides sufficient electrostatic repulsion force against intermolecular interactions between protein molecules, which enhances protein hydration and solubility. It is also expected that there would be minimal effect on the water solubility of sIWP at protein concentration ranging from 1 – 15 wt. % because sIWP was purified from IWP. The difference between the solubility of sIWP and VWG at neutral pH is primarily due to the shift in pI after deamidation, which decreased from pH 6.5 to pH 4.

2.3.2.3 Hydrodynamic Sizes of sIWP

Hydrodynamic size of sIWP in solution was initially analysed using DLS. Figure 2.14 shows the hydrodynamic diameter of sIWP measured as a function of pH. The measured hydrodynamic size of sIWP is quite large (~230 nm at pH 6.5). It has been reported by Thomson and co-workers (1999) that the HMW-glutenin subunit in solution has a dimension of about 69 × 6.4 nm measured by the Small Angle X-ray Scattering technique (SAXS). In addition, glutenin subunits are known to link together via interchain disulphide bonds to form HMW polymeric macromolecule (Shewry et al. 1986). The hydrodynamic sizes shown here is likely to due to the measurements of a few large polymeric glutenin particles present in sIWP, which obscured the smaller
sizes of gliadin. This is because the hydrodynamic size reported here is the intensity weighted mean hydrodynamic size (Z-average) of sIWP, which means that the presence of HMW polymeric glutenin in sIWP can lead to higher calculated Z-average.

Figure 2.14: Hydrodynamic size of sIWP as a function of pH.

As the pH decreased from pH 6.5 to 4.5, a decrease in hydrodynamic diameter from 230 nm to 160 nm was observed for sIWP. The slight decrease in hydrodynamic size is probably due to the loss of charge, which reduces electrostatic repulsion between parts of the protein molecule. This resulted in the formation of a compact protein structure. However, when the pH approached the pI of sIWP, a huge increase in hydrodynamic size was observed, which indicates the formation of protein aggregates. This is because there was insufficient electrostatic repulsion force available to keep the protein particles apart. As the pH decreased further away from the pI of sIWP, the protein became positively charge. This provides electrostatic repulsion force again that separates the protein particles apart, thus the hydrodynamic size began to decrease to smaller size.
In order to more accurately measure the hydrodynamic sizes of major protein in sIWP, which is believed to be gliadin based on the SE-HPLC and SDS-PAGE analysis, the zeta-sizer instrument was coupled with the SE-HPLC. This allows on-line detection of hydrodynamic sizes of proteins in sIWP as they were separated by SE-HPLC and eluted through the DLS detector. Figure 2.15 shows the hydrodynamic size of protein components in sIWP separated by SE-HPLC under non-reduced condition at pH 7 in the order of large to small protein sizes. The hydrodynamic diameters for the gliadin peak (elution time 30 – 40 min) cover a range of 5 to 20 nm. Thomson and co-workers (1999) measured gliadin in solution using SAXS and showed that monomeric gliadin are modeled as prolate ellipsoids with extended conformation, with a dimension of 15 – 16 × 2 – 3 nm. The result confirms that although there are still a few residue large glutenin particles after purification, sIWP is mainly comprised of monomeric gliadin. Slight larger hydrodynamic size obtained for sIWP could be due to slight changes to the protein conformation resulted from the deamidation process.

Figure 2.15: Hydrodynamic size of sIWP protein components separated by SE-HPLC at pH 7.
2.3.3 Structural Properties

2.3.3.1 Fourier Transform Infrared Spectroscopy

Figure 2.16 shows the 2nd derivative spectra of the Amide I bands of sIWP and VWG measured by ATR-FTIR in the dry powder state. Measurements were taken in dry powder state because water has strong absorption of the infrared radiation. The amide I band is commonly used to investigate secondary structure changes of proteins including gluten protein (Wellner et al. 2005; Georget & Belton 2006). The broad band at 1651 cm\(^{-1}\) is attributed to \(\alpha\)-helix and random coil, with some contribution from \(\beta\)-turn. The bands at 1632 cm\(^{-1}\) and 1622/1612 cm\(^{-1}\) are attributed to intra-molecular and intermolecular \(\beta\)-sheets, respectively. The FTIR spectra obtained for VWG is similar to that reported by Georget and Belton (2006) using ATR-FTIR measured in dry state, with major bands observed at 1652, 1632 and 1614 cm\(^{-1}\). The ATR-FTIR spectra showed that sIWP had increased random coil structure as indicated by an increase in the shoulder band at 1647 cm\(^{-1}\) and a decrease in strong inter-molecular \(\beta\)-sheet structure as indicated by the disappearance of the band at 1612 cm\(^{-1}\). However, these spectra were not quantitatively analysed for the proportion of each of the secondary structure components. This is because of the uncertainty about the contribution of multiple signals to the amide I band and also the possibility that the spectra may be affected by the pressure exerted by the ATR cell. It has been shown that pressure causes a shift of the amide I band that leads to apparent decrease in \(\alpha\)-helix and an increase in \(\beta\)-sheet structure (Lin et al. 2002; Wong et al. 2009). Nevertheless, it is expected that there would be an increase in random coil structure and a decrease in strong inter-molecular \(\beta\)-sheet structure after deamidation. This is because the demidation process increases the
number of negatively charged glutamic acid residues that could increase intra- and inter-
molecular repulsion, which promote protein unfolding and also prevent protein-protein
aggregation.

Figure 2.16: The secondary structures of sIWP and VWG was measured using ATR-
FTIR in dry powder state. Comparison between the FTIR spectrum of sIWP to VWG
showed that sIWP has a decrease in strong intermolecular β-sheet content and increase
in random coil structure.

2.3.3.2 Circular Dichroism

The CD spectrum of sIWP in solution was acquired by SRCD at pH 8. SRCD
allowed data to be acquired further down into the far UV (< 195 nm) region in
comparison to a conventional CD instrument because the synchrotron radiation source
have higher flux than conventional Xe arc light. As the characterisation of sIWP
suggests that sIWP is composed mainly of deamidated gliadin, the secondary structure
structure of sIWP was therefore compared against the gliadin fraction purified from VWG and IWP using 70% ethanol as well as the laboratory prepared deamidated VWG gliadin (~29% deamidated).

Figure 2.17 shows the SRCD spectra of sIWP, VWG gliadin, deamidated VWG gliadin (~29% deamidated), and IWP gliadin. The secondary structure composition of these proteins were predicted from their SRCD spectra based on the algorithm and datasets comprising CD spectra of proteins of various fold types whose structures have been solved by X-ray crystallography. The results are summarised in Table 2.3. The SRCD spectrum and the secondary structure composition of sIWP and IWP gliadin were very similar. The results further confirm that sIWP comprises mainly the gliadin fraction and has similar secondary structure as to IWP gliadin prepared using 70% ethanol.

![SRCD Spectra of sIWP, VWG Gliadin, Deamidated VWG Gliadin (~29% deamidation), and IWP Gliadin](image)

Figure 2.17: The SRCD spectra of sIWP, VWG gliadin, deamidated VWG gliadin (~29% deamidated) and IWP gliadin acquired in solution at pH 8.
Table 2.3: The secondary structure composition (% α-helix, β-sheet, turns and unordered) of sIWP, VWG gliadin, deamidated VWG gliadin (~29% deamidated) and IWP predicted from their respective SRCD spectrum.

<table>
<thead>
<tr>
<th>SRCD</th>
<th>Degree of deamidation</th>
<th>α-Helix</th>
<th>β-Sheet</th>
<th>Turns</th>
<th>Unordered</th>
</tr>
</thead>
<tbody>
<tr>
<td>VWG gliadin</td>
<td>0 %</td>
<td>34.3 ± 1.3</td>
<td>10.6 ± 0.6</td>
<td>18.2 ± 0.5</td>
<td>36.9 ± 0.3</td>
</tr>
<tr>
<td>Deamidated</td>
<td>~29 %</td>
<td>24.7 ± 1.3</td>
<td>11.6 ± 2.3</td>
<td>18.1 ± 1.7</td>
<td>45.6 ± 2.7</td>
</tr>
<tr>
<td>VWG gliadin</td>
<td>~35 %</td>
<td>16.9 ± 0.9</td>
<td>16.0 ± 1.1</td>
<td>17.2 ± 0.2</td>
<td>50.1 ± 0.4</td>
</tr>
<tr>
<td>IWP gliadin</td>
<td>~35 %</td>
<td>15.8 ± 0.0</td>
<td>16.6 ± 0.1</td>
<td>18.4 ± 0.6</td>
<td>49.3 ± 0.6</td>
</tr>
</tbody>
</table>

In order to further elucidate the effect of deamidation on the structural confirmation changes of wheat protein (gliadin), a laboratory prepared deamidated gliadin, which has a degree of deamidation (~29%) lower than the commercial processed was also analysed. As the level of deamidation increased from 0 – 35%, there is a decrease in α-helix from ~34 % to ~16% and an increased unordered structures from ~37% to ~50% as shown in Table 2.3. This is in similar range to that reported by Matsudini and co-workers (1982), who studied the secondary structure of VWG at various degree of deamidation (0 – 40%) using circular dichroism. The authors found that approximately 20% of the α-helix content was lost at 35% deamidation. Furthermore, the authors found that there was no further change in secondary structure when the degree of deamidation was above 35%. It should be noted that the authors estimated the α-helical content based on the ellipticity value at 222 nm, which is a different calculation method compared to this study. In this study, the prediction of secondary structure is based on algorithm and datasets comprising CD spectra of proteins of various fold types whose structures have been solved by X-ray crystallography.
The change in sIWP secondary structure is due to increase number of negatively charged glutamic acid residues converted from uncharged glutamine residues during the deamidation process. As a result, intra-molecular repulsion between the negatively charged glutamic acid residues promotes protein unfolding. The N-terminal repetitive domain of gliadin is rich in glutamine residues and adopts a β-reverse turn structure (Tatham & Shewry 1985). As the degree of deamidation increases, many of the glutamine residues located in this domain are converted to glutamic acid residues, which make this domain highly negatively charged. The C-terminal non-repetitive domain of gliadin contains many hydrophobic residues and adopts a compact α-helical structure (Tatham & Shewry 1985). As the degree of deamidation increases, some of the glutamine residues located in this domain were converted to glutamic acid residues, which lead to a decrease in α-helical structure. However, this domain contains about 3 – 4 intramolecular disulphide bonds, which are likely to restrict the structural freedom of this domain.

2.3.3.3 Tryptophan Fluorescence Emission

Gliadin contains about 1 – 2 tryptophan residues located at the C-terminal domain according to the amino acid sequences of α-gliadin (ABQ52121) and γ-gliadin (CAC94871) (as shown above in Figure 2.9). This domain is rich in hydrophobic amino acid residues like valine, cysteine, isoleucine, and leucine, which makes this region hydrophobic. In order to further understand whether the deamidation and changes in secondary structure of gliadin would expose the hydrophobic core of the C-terminal domain to the hydrophilic aqueous environment, tryptophan fluorescence emission was therefore used to investigate the local environment of these tryptophan residues. The polarity of the environment surrounding tryptophan residues affects the fluorescence
emission maximum ($\lambda_{\text{max}}$). A red shift in $\lambda_{\text{max}}$ suggests that the tryptophan residues are in a more hydrophilic environment, whereas, a blue shift in $\lambda_{\text{max}}$ suggests that the tryptophan residues are in a more hydrophobic environment (Lakowicz 1999).

Figure 2.18 shows the tryptophan fluorescence emission spectra of VWG gliadin and IWP gliadin. When excited at 280 nm, VWG gliadin exhibited $\lambda_{\text{max}}$ at 351 nm, whereas IWP gliadin exhibited $\lambda_{\text{max}}$ at 353 nm. A slight red shift of $\lambda_{\text{max}}$ was observed in IWP gliadin. This shift suggests that the tryptophan residues are in a more hydrophilic environment and the hydrophobic core of the protein molecule is exposed to the hydrophilic environment due to protein unfolding. This concurs with the results reported by Matsudomi and co-workers (1982), which showed that deamidation process increases the surface hydrophobicity of wheat gluten proteins.

![Figure 2.18: Tryptophan emission profile of VWG gliadin (not deamidated) and IWP gliadin (~35% deamidated) at pH 8.](image-url)
2.4 Conclusions

The results of SE-HPLC, SDS-PAGE, SRCD showed that sIWP is composed mainly of deamidated monomeric gliadin. The average MW of sIWP is 40,375 Da. The deamidation process converts many of the protein’s glutamine residues into negatively charge glutamic acid residues, reducing the number of glutamine residues that promote protein-protein interactions via hydrogen bonding. Zeta-potential results showed that sIWP is highly negatively charged at neutral pH (~50 mV). The strong negative charge provided repulsion force against protein-protein aggregation, allowing sIWP being highly soluble in aqueous solution. However, when the pH approaches the pl of sIWP (~ pH 4), sIWP became insoluble in aqueous solution. This is because sIWP carries net zero zeta-potential at pH 4, which allow the protein molecules to interact with each other via attractive interactions like hydrophobic interactions and van der Waals forces. The SRCD and tryptophan fluorescence emission results also showed that deamidation process decreases α-helical structure and induces protein unfolding through increasing number of negatively charged glutamic acid residues that promote intramolecular repulsion.

There is no model available for sIWP. Based on these results, a model was proposed for sIWP in comparison to native gliadin (not deamidated) in solution, with the assumption that there is a random conversion of glutamine residues to negatively charged glutamic acid residues across the entire sequence of the protein molecules (Figure 2.19).
Figure 2.19: This diagram illustrates the deamidation of monomeric gliadin, where glutamine residues are converted to its acidic form, assuming average random conversion across entire sequence. Monomeric gliadin contains two distinctive domains, an N-terminal repetitive domain rich in β-turns structure and a C-terminal domain rich in α-helix (Tatham et al. 1990). Deamidation lead to a decreased in α-helix content and induced protein unfolding. Note: (1) figure is not to scale, only intended for illustration purpose only. (2) Structural domain model of monomeric gliadin (not deamidated) illustrated in this diagram is based on that described by Shewry and co-workers (1989).

The model proposed takes into consideration on the structure of native α– and γ– gliadin (Shewry et al. 1989) and applies to pH regions where sIWP is soluble in aqueous solution (> pH 7). The model showed that the increased number of negatively charged glutamic acid residues and a decreased number of polar uncharged glutamine residues lead to an increased in intramolecular repulsion force and a decrease in intramolecular hydrogen bonding interactions. As a result, protein became partially
unfolded, which lead to an increase in protein surface area that exposed to the aqueous environment. The N-terminal domain of gliadin is rich in glutamine residues, which makes this region highly negatively charged and hydrophilic after deamidation. The C-terminal domain of gliadin contains many hydrophobic residues, which makes this region hydrophobic in nature. In addition, this domain also contains 3 – 4 intramolecular disulphide bonds that restrict complete protein unfolding.
CHAPTER 3

The Conjugation of sIWP with Glucose, Maltodextrins and Dextrans via
the Maillard Reaction

3.1 Introduction

The Maillard conjugation involves covalent linkage between the available –NH₂ group of the protein and the reducing group of the polysaccharide as illustrated in Figure 3.1. Various authors have examined Maillard conjugation of different carbohydrates (e.g. dextran, galactomannan, maltodextrin) to proteins (e.g. β-lactoglobulin, ovalbumin, lysozyme, caseins, soy proteins, enzymatically hydrolysed gluten) (Kato et al. 1990; Kato et al. 1991; Babiker et al. 1998; Shepherd et al. 2000; Kim et al. 2003; Dunlap & Cote 2005; Wooster & Augustin 2006; Akhtar & Dickinson 2007). These studies showed that protein conjugated with polysaccharides have improved solubility in water and also the ability to maintain emulsion stability in unfavourable environments such as at acidic pH, in ionic environment and during heating. In comparison to non-conjugated proteins, these protein-conjugates have superior emulsifying properties. The preparation of deamidated wheat protein–carbohydrate conjugate has not been reported in the literature.
Figure 3.1: Schematic illustration of conjugation between the available –NH$_2$ groups of protein and the reducing group of polysaccharide via the Maillard reaction.

In this study, a range of different MW carbohydrates were chosen for conjugation onto sIWP via the Maillard reaction, in an effort to understand the effect of conjugation of different carbohydrate sizes on the structural and solution characteristics for subsequent investigation of emulsifying properties of the modified sIWP (Chapters 4 and 5). These carbohydrates examined include glucose, maltodextrins (900 – 4,300 Da) and dextrans (6,400 – 41,000 Da). Glucose is a monosaccharide, maltodextrin is a linear polysaccharide consisting of variable number of glucose units joined together by $\alpha$–1,4 glycosidic linkage, and dextran is a branched polysaccharide with a straight chain that consists of variable units of glucose joined together by $\alpha$–1,6 glycosidic linkage.
and branched at α-1,4 linkage (Figure 3.2). These carbohydrates are non-ionic in nature, which means that their water solubility are not affected in unfavourable environments like at acidic pH and in ionic environments (Dickinson 2003).

![Chemical structure of glucose, maltodextrin and dextran](image)

**Figure 3.2: Chemical structure of glucose, maltodextrin and dextran**

In Chapter 2, the composition and structural properties of sIWP in solution were determined. In this chapter, sIWP conjugation with carbohydrates via the Maillard reaction will be studied. The extent of conjugation will be investigated via the loss of available –NH₂ groups. The effect of conjugation on protein water solubility will be investigated using a Lowry-based protein assay and the covalent linkage between protein and carbohydrate will be investigated via sodium-dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under reduced conditions. These methods are commonly used to characterise the Maillard conjugates (Morris et al. 2004).
3.2 Materials and Methods

3.2.1 Materials

sIWP was prepared as described in Chapter 2 (section 2.2.1) and used without further purification.

Glucose monohydrate was obtained from Sigma Aldrich (Sydney, Australia). Maltodextrins (M200, M100, and M040) were obtained from Salkat Australia (Melbourne, Australia). The commercial maltodextrins were a mixture of various MW carbohydrates. The relative percentage present for each degree of polymerisation (DP) in each maltodextrin as given by the manufacturer’s specification sheet was as follows: M200 (DP1 – 2.3%, DP2 – 7.4%, DP3 – 9.1%, DP4 – 6.8%, DP5 – 6.3%, DP6 – 11.9%, DP7 – 10%, DP8 – 3.7%, DP9 – 2.1%, DP10 – 1.7%, and >DP10 – 38.7%), M100 (DP1 – 0.8%, DP2 – 2.9%, DP3 – 4.4%, DP4 – 3.8%, DP5 – 3.4%, DP6 – 5.7%, DP7 – 6.8%, DP8 – 4.5%, DP9 – 3.1%, DP10 – 2.5%, and >DP10 – 62.1%), and M040 (DP1 – 0.3%, DP2 – 0.9%, DP3 – 1.4%, DP4 – 1.4%, DP5 – 1.3%, DP6 – 1.8%, DP7 – 2.4%, DP8 – 2.0%, DP9 – 1.8%, DP10 – 1.7%, and >DP10 – 85%). The average theoretical MW, based on these compositions, are 900, 1,800, and 3,600 Da respectively. The measured average MW for M200, M100, M040 were 900, 1,900 and 4,300 Da, as determined using the Somogyi-Nelson reducing sugar assay (Wood & Bhat 1988). Dextrans (D10N and D65N) were obtained from Sigma Aldrich (Sydney, Australia). The measured average MW for D10N and D65N were 6,400 and 41,000 Da, as determined using the Somogyi-Nelson reducing sugar assay (Wood & Bhat 1988).

The protein standards for gel electrophoresis, Mark12™ unstained standard, were purchased from Invitrogen (Australia). Bio-Rad DC assay kit was purchased from Bio-
Rad (Australia). Other analytical grade chemicals were purchased from Sigma Aldrich (Sydney, Australia).

3.2.2 Somogyi-Nelson Reducing Sugar Assay

The sample solution (2 mL) was mixed with 0.4 mL of pre-constituted Somogyi reagent I (288 g of anhydrous sodium sulfate, 24 g of Rochelle salt, 48 g of sodium carbonate, 32 g of sodium bicarbonate diluted to 1.6 L with boiling water) and 1.6 mL of Somogyi reagent II (72 g of sodium sulfate and 8 g of copper sulfate diluted to 400 mL with boiling water). The solution was then boiled for 15 min and cooled to room temperature (24 ºC). Nelson reagent (100 g of ammonium molybdate, 84 mL of concentrated H$_2$SO$_4$, 12 g of sodium arsenate diluted to 100 mL with deionised water; 2 mL), was added into the solution and vortexed. Deionised water (4 mL) was then added and mixed by inversion. The absorbance of the solution at 520 nm was then measured (Wood & Bhat 1988). Glucose (5 – 200 µg) was used as standards.

3.2.3 Determination of Available –NH$_2$ Groups and Extent of Conjugation

The number of available –NH$_2$ groups were determined using the o-phthalaldialdehyde (OPA) assay (Vigo et al. 1992). OPA reagent was prepared by dissolving 80 mg of o-phthalaldialdehyde in 2 mL of absolute ethanol, mixing this solution with 5 mL of 10% sodium dodecyl sulphate (SDS), 0.2 mL of mercapto-ethanol, 50 mL of 0.1 M Borax buffer (pH 9.85) and diluting to 100 mL. The assay consisted of mixing 3 mL of the OPA reagent with 50 µL of 3 wt. % protein dispersion and measuring the absorbance at 340 nm after 3 min of incubation. The number of available –NH$_2$ groups was then determined using a standard curve constructed from
using lysine as the amino acid standard. Each mole of the lysine amino acid has 2 mole of available –NH₂ group. The number of –NH₂ groups available per mole protein was then calculated based on the solution protein concentration determined using the Lowry-based Bio-Rad DC assay (see section 2.2.7) and the average MW of sIWP (40,375 Da). For calculation of the extent of conjugation, measurement of the available –NH₂ groups were taken before and after the Maillard reaction and the difference in the available –NH₂ groups were assumed to have resulted from the attachment of carbohydrate moieties.

3.2.4 Preparation of Maillard Conjugates

The wet reaction was carried out by heating a solution containing a mixture of sIWP (3 wt. %) and glucose (3 wt. %) at 90 °C and at pH 7.5 up to 5 h. After the reaction, the solution was rapidly cooled in ice-cold water and then stored at 4 °C until further analysis.

In the dry reaction, an intimate mixture of sIWP and carbohydrate was prepared in milli-Q water and then freeze-dried. The codes used to represent these freeze-dried mixtures were sIWP-glucose (10 g protein: 0.55 g glucose monohydrate), sIWP-M200 (10 g protein: 2.81 g), sIWP-M100 (10 g protein: 5.28 g), sIWP-M040 (10 g protein: 11.97 g), sIWP-D10N (10g protein: 8.92 g) and sIWP-D65N (10g protein: 57.5 g) for mixtures containing the various carbohydrates. The weight ratios used corresponded to 1:2 mole ratio for sIWP-glucose/maltodextrins mixtures and 1:1 mole ratio for sIWP-dextran mixtures of the measured available –NH₂ (i.e. ~5.5 –NH₂ groups per mole of sIWP) to the reducing group of the various carbohydrates. The available –NH₂ to the reducing group ratio was lowered in sIWP-dextran mixtures so that the mixtures are not
saturated by dextrans, while maintaining sufficient dextran for reaction. The mole of reducing groups was determined based on the measured average MW of the carbohydrate and the available –NH₂ was determined using the method described in section 3.2.3. Maillard conjugation was achieved by incubating freeze-dried sIWP-carbohydrate mixtures at 60 °C and at 75% relative humidity (over saturated potassium bromide). As a control, sIWP without added carbohydrate was also heated under the same conditions.

Soluble protein was used for further characterisation, where there was insoluble material (i.e. < ~90% water solubility), this was removed by centrifugation. This was the case for dry heated sIWP alone for 72 h and sIWP dry heated in the presence of D10N dextran (6,400 Da) for 72 h. The samples were dispersed in milli-Q for 2 h at room temperature (24 °C). The dispersion was then centrifuged at 10,000 × g for 15 min using a Beckman J2-MC centrifuge. The supernatant was carefully removed into a beaker and then freeze-dried.

3.2.5 The Water Solubility of sIWP/Maillard Conjugates

sIWP/ protein-conjugates were dispersed in 50 mM sodium phosphate buffer (pH 7) at 1 wt. % concentration (protein basis) and held at room temperature (24 °C) for 2 h. During the preparation of the solution, the weight of the protein/protein-conjugate powder and the volume of the solution were carefully measured and recorded. These weights were then used to calculate the initial protein concentration. The percentage of protein content in the protein/protein-conjugate powder was determined based on the total nitrogen content in the powders (see section 2.2.2). The dispersion was centrifuged at 10,000 × g for 15 min using a Beckman J2-MC centrifuge. Supernatant was carefully
separated from the pellet and then its protein concentration was determined using Bio-
Rad Dc assay as described in section 2.2.7. The water solubility of the protein/protein-
conjugate was calculated from the difference in protein concentration before and after
the centrifugation.

3.2.6 Sodium-Dodecyl-Sulphate Polyacrylamide Gel Electrophoresis

This is described in Chapter 2 (section 2.2.4). After the gels were photographed,
the MW of the protein band relative to the protein standards were obtained using
Discovery Series™ Quantity One® 4 software distributed by Bio-Rad.

3.3 Results and Discussion

3.3.1 Determination of the Number and Location of –NH₂ Groups in sIWP Available for Conjugation

The formation of protein-carbohydrate conjugate is based on the Amadori
rearrangement steps in the Maillard reaction, where the free amino groups in the protein
is covalently bound to the reducing end carbonyl group in the carbohydrate. The ε–
amino group of lysine residues is most reactive for Maillard conjugation (Owusu-
Apenten 2005). It is important to identify the possible location of these lysine residues
in sIWP in order to understand the effect of conjugation on the structural and functional
properties of sIWP.

The amino acid composition analysis of sIWP (Chapter 2) suggests that it
contains an average of 3 moles of lysine residues per mole of sIWP. This is calculated
based on sIWP having an average MW of 40,375 Da (refer to section 2.3.1.4 in Chapter 2). As suggested in the Chapter 2, sIWP is composed mainly of monomeric gliadin. A search on the amino acid sequences of α– and γ–gliadin from the protein database (http://www.ncbi.nlm.nih.gov) revealed that gliadin contain approximately 2 – 3 lysine residues located at the C-terminal of the protein molecule (refer to Figure 2.9 in Chapter 2). This concurs with the calculations based on the amino acid composition analysis of sIWP. Figure 3.3 shows a model proposed for sIWP with the lysine residues located at the C-terminal end of the protein molecule. It is worth noting that the number of lysine residues available in sIWP is significantly lower than that compared to other proteins, like β-lactoglobulin, α-lactoglobulin, and bovine serum albumin, which contain 15, 12 and 59 moles of lysine residues per mole of protein respectively (Jimenez-Castano et al. 2007).

The number of available –NH₂ groups in sIWP can be measured using the OPA assay, which can then be used to calculate the extent of conjugation. The OPA assay has been used in a number of studies to determine the extent of conjugation of Maillard conjugates prepared from dairy proteins (Chevalier et al. 2001a; Dattatreya & Rankin 2006; Wooster & Augustin 2006). The assay involves the reaction between the chemical compound OPA and the amino groups in the presence of SDS and mercaptoethanol, which forms a complex that absorbs light at 340 nm. The presence of denaturing and reducing agents promote the exposure of all the available –NH₂ groups within the protein molecule to react with the OPA, for example, –NH₂ groups that were located inside the protein molecule or entrapped within protein aggregates.
Figure 3.3: This protein model illustrates the location of N-terminal amino groups and the 3 lysine residues in sIWP. The N-terminal domain is hydrophilic, whereas the C-terminal domain is hydrophobic. Notes: (1) the location of the lysine residues indicated in the model is based on the lysine residues located in the amino acid sequence of γ−gliadin, (2) this figure is not to scale.

In the OPA assay, free lysine was used to construct a standard curve. Free lysine has two amino groups, one ε-amino group and one α-amino group (Figure 3.4). Theoretically, two OPA molecules can be bound to one molecule of free lysine residue, which means that the absorbance would be two times higher than amino acid that contain only one amino group such as leucine residue. Figure 3.5 shows the standard curve constructed using free lysine residues and free leucine residues. Since lysine residue has twice the number of available −NH₂ groups than leucine residue, the slope for the standard curve of lysine residue should be twice the value compared to the slope for the standard curve of leucine residue. However, the slope for lysine residues (0.00408) is slightly lower than 0.00448 (i.e. 0.00224 × 2). This is possibly due to the steric hindrance caused by the attachment of OPA to one of the lysine’s free amino
group that prevents the attachment of another OPA to the other free amino group or due to a slight change in the extinction coefficient of the complex. In this study, it was assumed that OPA has same reactivity towards both the ε-amino and α-amino groups of the amino acids. It was also assumed that one mole of free lysine residue measured in the OPA assay corresponds to the theoretically two mole of −NH₂ groups.

Figure 3.4: The chemical structures of OPA, lysine, and leucine.

![Chemical structures of OPA, lysine, and leucine](image)

\[
y = 0.0041x + 0.0029 \\
R^2 = 0.9999
\]

\[
y = 0.0022x + 0.0016 \\
R^2 = 1
\]

Figure 3.5: Comparison between the lysine and leucine standard curve used for the determination of the available −NH₂ groups in protein. Lysine has two available −NH₂ groups, whereas leucine has one available −NH₂ groups for reaction with OPA.
The initial number of available –NH₂ groups per mole of sIWP were found to be approximately 5.5 moles, based on sIWP having an average MW of 40,375 Da. It appears that the number of available –NH₂ groups per mole of sIWP is greater than the average 3 lysine residues available per mole of sIWP. This is possibly due to the presence of other available amino groups that can interact with the OPA, such as the N-terminal amino group, histidine and arginine. These amino acids are also able to participate in the Maillard reaction but at a much lower extent compared to lysine residues (Ames 1992). It is worth noting that the theoretical maximum number of –NH₂ groups available for Maillard reaction (i.e. lysine + arginine + histidine + N-terminal amino group) is ~16 moles of –NH₂ groups per mole of sIWP, which is calculated based on the amino acid composition analysis and the average MW of sIWP (i.e. 40,375 Da).

3.3.2 Comparison between Wet and Dry Reaction for Maillard Conjugation

There are two reaction conditions that are commonly used to prepare protein-carbohydrate Maillard conjugate, that is the wet reaction (Zhu et al. 2008) and the dry reaction (Kato et al. 1990). The wet reaction is carried out by dispersing protein and carbohydrate in milli-Q water and then heated at high temperature (> 90 °C) for a given time. Conversely, the dry reaction is carried out by incubating a freeze-dried mixture of protein and carbohydrate at 60 °C for a given time at a controlled relative humidity of 75%. The suitability of these two reaction conditions for conjugation was investigated by reacting sIWP with excess amount of glucose (1:1 protein to glucose weight ratio), which correspond to 1:36 mole ratio of the measured available –NH₂ (i.e. ~5.5 –NH₂ groups per mole of sIWP) to the reducing group of the glucose. The presence of excess amount of glucose would increase the kinetics of the reaction.
The formation of Maillard conjugates was monitored through assessment of visual browning and the extent of conjugation. Sample browning is due to the formation of melanoidins at the advanced stage of the Maillard reaction. It is possible that Maillard conjugation may have occurred without any indication of sample browning because it occurs at the early stage of the Maillard reaction, which precedes the formation of brown products. It is worth noting that intensity of sample browning does not necessarily correlate with the extent of conjugation because different types of matrices can give rise to different browning rates, for example sIWP that is reacted with different types of carbohydrates (Bell et al. 1998; Lievonen et al. 2002).

In this study, the wet reaction was carried out in solution (pH 7.5) at 90 °C, whereas the dry reaction was carried out at 60 °C and at 75% relative humidity. Figure 3.6 shows the pictures of sIWP heated alone or heated in the presence of glucose using the wet reaction or the dry reaction. In the wet reaction, no visual browning was observed after sIWP was heated alone or heated in the presence glucose for a period of 5 h. Conversely, in the dry reaction, visual browning was observed after sIWP was dry heated in the presence of glucose for 2 h. Furthermore, the browning intensity increased with incubation time, which indicates that the amount of melanoidins formed was increasing. It is worth noting that glucose dry heated alone for 6 h did not result in any visual browning (result not shown), which confirms that the browning observed was not caused by glucose cameralisation. However, there is slight visual browning observed after sIWP was dry heated alone for 6 h. It is possible that when sIWP was dry heated alone, residual carbohydrate that remained in the sIWP preparation after the purification steps might have conjugated onto the protein.
Figure 3.6 Pictures of sIWP heated alone or heated in the presence of excess amount of glucose (1:36 mole ratio of available –NH₂ to reducing group of glucose) using the wet reaction (heated at > 90 °C in solution) or the dry reaction (heated at 60 °C/75% RH).

Figure 3.7 shows the extent of conjugation for sIWP after heated alone or heated in the presence of glucose using the wet reaction or the dry reaction. In the wet reaction, sIWP heated in the presence of glucose for 1 to 5 h, resulted in approximately 0.5 mole of glucose attached per mole of sIWP. sIWP heated alone in the wet reaction did not result in any glucose attachment. In the dry reaction, sIWP dry heated in the presence of glucose for 1 h, resulted in approximately 2 moles of glucose attached per mole of sIWP. When the reaction time was increased to 6 h, the extent of conjugation increased to 4 moles of glucose attached per mole of sIWP. It appeared that sIWP dry heated alone resulted in low level of conjugation, which was likely resulted from the conjugation of residual carbohydrates that were present in the sIWP preparation.
Figure 3.7 The extent of conjugation for sIWP heated alone or heated in the presence of excess amount of glucose (1:36 mole ratio of available –NH$_2$ to reducing group of glucose) using the wet reaction (heated at > 90 °C in solution) or the dry reaction (heated at 60 °C/75% RH).

The lack of visual browning and low level of conjugation suggest that the conditions used in the wet reaction are not suitable for sIWP to undergo Maillard conjugation with reducing carbohydrate (e.g. glucose). On the other hand, sIWP undergoes spontaneous conjugation with reducing carbohydrate (e.g. glucose) in the dry reaction conditions. The Maillard reaction between the ε-amino groups in protein and the reducing-end carbonyl group in the carbohydrate is accelerated in the low water activity ($a_w$) condition of the dry reaction (Kato 2002). The optimum water activity for the Maillard reaction is between 0.5 and 0.8 (Labuza 1994; Van Boekel 2001).

In addition to the reaction conditions, the conformation of the protein molecule plays an important role in allowing Maillard conjugation to occur. Studies found that
unfolded proteins such as αs-casein form polysaccharide conjugates much faster (within 24 h) than folded protein like lysozyme, which slowly forms polysaccharide conjugate even after 2 weeks (Kato et al. 1992; Nakamura et al. 1994). This has been ascribed to the difference between folded and unfolded proteins and the accessibility/reactivity of the lysine residues. As shown in Chapter 2, the deamidation process caused protein unfolding. Preliminary experiments showed that native gliadin (i.e. purified from VWG) reacted with glucose using the dry reaction resulted in lower extent of conjugation (~22% of available –NH₂ groups were conjugated after reacted for 3 days) than sIWP (~55% of available –NH₂ groups were conjugated after reacted for 2 h). This suggests that some or all of the lysine residues within sIWP molecule were exposed outside after the deamidation process, which in turn, promotes the Maillard conjugation.

3.3.3 Maillard Conjugation of sIWP with Glucose, Maltodextrins or Dextrans

Figure 3.8 shows the pictures of sIWP dry heated alone or dry heated in the presence of glucose, maltodextrins or dextrans. sIWP was given a longer period of time to react with dextran (up to 72 h) than with glucose or maltodextrins (up to 24 h) because HMW carbohydrates are known to react at a slower rate than those with lower MW (Kato 2002). Visual browning was observed after sIWP was dry heated alone or dry heated in the presence of glucose, maltodextrins or dextrans, which indicates that the Maillard reaction had occurred. Since the formation of the protein-conjugate is the first step in the Maillard reaction, the observation of browning is a surrogate indicator of the formation of the protein-conjugate. It is worth noting that glucose, maltodextrins or dextrans dry heated under the same conditions did not result in any visual browning.
(result not shown), which confirmed that the browning observed was not caused by cameralisation or breakdown of sugar units.

Figure 3.8: Pictures of sIWP dry heated alone or [A] dry heated in the presence of glucose, maltodextrins [M200 (900 Da), M100 (1,900 Da) or M040 (4,300 Da)], or [B] dextrans [D10N (6,400 Da) or D65N (41,000 Da)] at 60 °C/75% RH. sIWP-glucose/maltodextrins mixtures are consist of 1:2 mole ratio of available –NH₂ to reducing group of the glucose/various maltodextrins, whereas sIWP-dextrans mixtures are consist of 1:1 mole ratio of available –NH₂ to reducing group of the various dextrans.

Figure 3.9 shows the extent of conjugation for sIWP after dry heated alone or dry heated in the presence of glucose, maltodextrins or dextrans, as estimated by the loss of available –NH₂ groups. sIWP dry heated alone resulted in low level of
conjugation, which was likely due to the conjugation of residual carbohydrates that was present in the sIWP preparation.

Figure 3.9: The extent of conjugation of sIWP dry heated alone or dry heated in the presence of glucose (sIWP-Glc), maltodextrins [sIWP-M200 (900 Da), sIWP-M100 (1,900 Da) or sIWP-M040 (4,300 Da)], or dextrins [sIWP-D10N (6,400 Da) or sIWP-D65N (41,000 Da)] at 60 °C/75% RH. sIWP-glucose/maltodextrins mixtures were consist of 1:2 mole ratio of available –NH$_2$ to reducing group of the glucose/various maltodextrins, whereas sIWP-dextrins mixtures were consist of 1:1 mole ratio of available –NH$_2$ to reducing group of the various dextrans.

The extent of conjugation for sIWP dry heated with glucose and all three maltodextrins appeared to be similar to each other, where approximately 3 – 4 moles of carbohydrates were conjugated per mole of sIWP after reacted for 24 h. Since sIWP has an average of 3 moles of lysine residues per mole of sIWP, these carbohydrates are possibly attached at the three lysine residues and one at the N-terminal amino group (Figure 3.10) as these are the most reactive –NH$_2$ groups for Maillard conjugation
(Owusu-Apenten 2005). However, confirmation of the site of conjugation requires further work (e.g. combination of peptide digestion and MALDI-TOF, etc).

Figure 3.10: This diagram illustrates the conjugation sites for glucose, maltodextrins and dextrans in sIWP (indicated by the red arrows). Based on the extent of conjugation, ~3 – 4 moles of glucose or maltodextrins (900, 1,900, 4,300 Da) were conjugated per mole of sIWP, whereas ~1.3 or 0.5 mole of dextran (6,400 or 41,000 Da) were conjugated per mole of sIWP. Notes: (1) the location of the lysine residues indicated in the model is based on the lysine residues located in the amino acid sequence of \( \gamma \)-gliadin, (2) this figure is not to scale.

The extent of conjugation for sIWP dry heated with dextrans appeared to be much lower compared to that reacted with glucose or maltodextrins, although the mixtures were allowed to react for longer period. sIWP reacted with the smaller D10N dextran for 72 h resulted in approximately 1.3 mole of dextran conjugated per mole of sIWP, which suggests that some of the protein molecules have two dextran attached.
sIWP reacted with the larger D65N dextran for 72 h resulted in approximately 0.5 mole of dextran conjugated per mole of sIWP, which suggests that only half of the protein population were conjugated with 1 mole of dextran. The possible sites for conjugation of dextrans could be at the N-terminal amino group or one of lysine residues located at the C-terminal domain of sIWP (Figure 3.10). However, as discussed previously, confirmation of the conjugation sites requires further work (e.g. combination of peptide digestion and MALDI-TOF, etc).

The difference in the extent of conjugation observed above could be due to the differences in the size and/or the type of the carbohydrate used for conjugation. Other studies have shown that branched chain polysaccharides such as dextran have a slower Maillard conjugation rate than simple sugar e.g. glucose or linear chain polysaccharides maltodextrins (Choi et al. 2005; Dunlap & Cote 2005). Dextran usually requires higher temperature and/or longer reaction time to reach the required number of polysaccharide attached per protein molecule. The size of dextran also affects the Maillard conjugation rate. It has been reported that a higher extent of conjugation could be achieved when lower MW dextran (e.g. 10,000 Da) was used for conjugation with the dairy proteins (e.g. α- or β-lactoglobulin) than the higher MW dextran (e.g. 20,000 Da) (Jimenez-Castano et al. 2007). Furthermore, the attachment of HMW polysaccharides could impose steric hindrance against further conjugation (Kato 2002). This is possibly why the attachment of dextrans (6,400 Da or 41,000 Da) onto sIWP has plateau after the attachement of ~1.3/0.5 moles of dextrans per mole of sIWP (refer to Figure 3.9).
3.3.4 Water Solubility of sIWP-carbohydrate Conjugates

The solubility of protein in water is one of the pre-requisites of an effective emulsifier (Damodaran 2005). In this study, the Maillard reaction was carried out by heating a mixture of protein and carbohydrate at elevated temperature (i.e. 60 °C) under dry conditions for a period of time (i.e. 24 h or 72 h). This condition could lead to protein aggregation via hydrophobic interaction between heat-induced unfolded proteins and/or sulphhydryl-disulphide interchange reactions (Dickinson & Galazka 1991; Diftis et al. 2005). This, in turn, could lead to the loss of protein solubility in water.

As a control, sIWP alone was treated under the same conditions (60 °C/75% RH) as mixtures of sIWP and carbohydrates. The solubility of sIWP in water before and after heating was measured and summarised in Table 3.1.

Table 3.1 Comparison between the initial water solubility of reconstituted sIWP alone and sIWP-carbohydrate mixture at pH 7 and after dry heated at 60 °C/75% relative humidity for 24 h or 72 h.

<table>
<thead>
<tr>
<th></th>
<th>Solubility at pH 7 (%)</th>
<th></th>
<th>Solubility at pH 7 (%)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before heating (0 h)</td>
<td>After heating (24 h)</td>
<td>Before heating (0 h)</td>
<td>After heating (72 h)</td>
</tr>
<tr>
<td>sIWP alone</td>
<td>95.7 ± 4.5</td>
<td>88.4 ± 0.6</td>
<td>sIWP alone</td>
<td>95.7 ± 4.5</td>
</tr>
<tr>
<td>sIWP-Glc (180 Da)</td>
<td>92.9 ± 0.3</td>
<td>86.6 ± 3.4</td>
<td>sIWP-D10N (6,400 Da)</td>
<td>94.4 ± 0.8</td>
</tr>
<tr>
<td>sIWP-M200 (900 Da)</td>
<td>91.4 ± 0.6</td>
<td>93.2 ± 4.3</td>
<td>sIWP-D65N (41,000 Da)</td>
<td>95.5 ± 1.3</td>
</tr>
<tr>
<td>sIWP-M100 (1,900 Da)</td>
<td>92.5 ± 5.5</td>
<td>91.2 ± 7.3</td>
<td>sIWP-M040 (4,300 Da)</td>
<td>90.1 ± 1.5</td>
</tr>
</tbody>
</table>

The solubility of sIWP in water before heating is approximately 95.7 ± 4.5 %. However, the solubility of sIWP in water decreased slightly to 88.4 ± 0.6 % after dry heated alone for 24 h and then decreased significantly to 67.6 ± 4.1 % after dry heated...
alone for 72 h. As shown in Figure 3.11, protein aggregates can be clearly seen precipitated out of dispersions containing sIWP after dry heated alone for 72 – 168 h (indicated by arrows). This showed that the temperature selected for Maillard conjugation in this study have negative impact on the solubility of sIWP in water after prolonged heating under dry conditions (i.e. 60 °C/75% RH).

Figure 3.11: Pictures of protein dispersion containing sIWP and sIWP dry heated alone for 24 h to 168 h. Insoluble protein aggregates can be clearly seen precipitated out of the solution after sIWP was prolonged dry heated for 72 to 168 h as indicated by the yellow arrows.

The loss of solubility after heating is linked to the formation of protein aggregates via sulphydryl-disulphide interchange reactions because these protein aggregates were solubilised in the presence of a reducing agent (data not shown). In Chapter 2, it was shown that the deamidation process leads to protein unfolding. This in turn could have facilitated the sulphydryl/disulphide interchange between the exposed sulphydryl/disulphide groups of the protein molecule. This is probably the reason why
for a compact globular protein like β-lactoglobulin with most of the disulphide bonds buried inside the protein, required more time (> 10 days of heat treatment at 60 °C/75% RH) for protein denaturation and protein aggregation to occur (Chevalier et al. 2002; Wooster & Augustin 2007b). Although, sIWP is composed mainly of gliadin, there are low level of glutenin remained in the sIWP. Schofield and co-workers (1983) showed that the sulphhydryl/disulphide interchange reaction predominantly involves the glutenin fraction rather than the gliadin fraction when wet gluten was heated between 55 °C and 70 °C. However, the participation of the gliadin fraction of the sIWP in the sulphhydryl/disulphide interchange reaction during heating at 60 °C/75% RH should not be discounted.

Table 3.1 shows the solubility of sIWP before and after conjugated with glucose, maltodextrins or dextrans. The solubility of sIWP after reaction with glucose or maltodextrins for 24 h appeared to have slightly decreased. The loss of solubility was similar to that of sIWP that was treated alone under the same reaction conditions for 24 h. This showed that the Maillard reaction conditions used in this study have minimal effects on the solubility of sIWP in water when the reaction time is limited to 24 h.

The water solubility of sIWP reacted with dextrans for 72 h (~81 – 94%) was higher than sIWP treated alone under the same reaction conditions (solubility: ~67%). The solubility of sIWP reacted with D10N dextran decreased from 94.4 ± 0.8 % to 81.0 ± 0.2 %, whereas the solubility of sIWP reacted with D65N dextran maintained at ~94 %. The presence of dextran during the reaction possibly acts as a physical barrier against sulphhydryl/disulphide interchange reaction, where larger MW/size dextran (e.g. D65N) being more effective than the smaller ones (e.g. D10N).
The solubility of sIWP in water is largely unaffected after dry heated alone for 24 h, dry heated with glucose or maltodextrins for 24 h, or dry heated with D65N dextran (41,000 Da) for 72 h at 60 °C/75% RH. Hence, the dry heated protein and the protein-conjugates were used without further purification. However there was some insoluble protein fraction resulted from sIWP dry heated alone for 72 h or dry heated with D10N dextran (6,400 Da) for 72 h, therefore they were removed prior to further analysis of soluble protein/protein-conjugate. This is because the insoluble protein aggregates usually have decreased effectiveness in adsorbing at the fresh oil-water interface and if adsorbed may result in the formation of strongly flocculated emulsion droplets due to the adsorption of the protein aggregates on both sides of the thin liquid film of the neighbouring emulsion droplets (Dickinson & Galazka 1991; Diftis et al. 2005). However, it should be noted that the removal of these insoluble proteins from the samples may change the protein composition (i.e. increase in gliadin/glutenin ratio).

3.3.5 Determination of the Covalent Linkage between sIWP and the Conjugated Carbohydrates

The covalent linkage between sIWP and the conjugated carbohydrates were investigated using the SDS-PAGE under reduced conditions. This is to verify that the association between the sIWP and the non-ionic carbohydrates was not due to physical entanglement of the two components. Non-ionic carbohydrates like glucose, maltodextrins and dextrans do not migrate in SDS-PAGE because they are hydrophilic and do not carry any surface charge. If these non-ionic carbohydrates are covalently attached onto the protein, the protein-carbohydrate complex would migrate through the SDS-PAGE. This will lead to a shift in protein bands to higher MW when compared against the protein bands of the non-conjugated protein.
Glucose/Maltodextrins conjugates

Figure 3.12 shows the SDS-PAGE of sIWP, sIWP dry heated alone for 24 h and sIWP dry heated with glucose, or each of the maltodextrin for 24 h at 60 °C/75% RH. The SDS-PAGE was separated into 3 sections, Box a (36,500 – 66,000 Da), Box b (~26,000 Da) and Box c (~11,500 Da). Based on the distribution/intensity of the bands, the protein fraction with higher molecular weight in Box a represent the major protein fraction of the whole sIWP system, whereas the other two protein fractions with lower molecular weight in Box b & c represent the minor fraction of the whole sIWP system. 

Figure 3.12: SDS-PAGE of sIWP, dry heated sIWP (24 h) and dry heated sIWP-glucose/maltodextrins mixtures (24 h) stained with Coomassie blue (arrows indicate shifted bands). Labelled lanes are: (1 and 8) MW markers; (2) sIWP; (3) dry heated sIWP (24 h); (4) dry heated sIWP-Glc; (5) dry heated sIWP-M200 (900 Da); (6) dry heated sIWP-M100 (1,900 Da); (7) dry heated sIWP-M040 (4,300 Da).
There was no obvious shift in protein bands observed for sIWP dry heated alone for 24 h (lane 3) in comparison to sIWP (lane 2) (Figure 3.12 box a – c). This confirmed that sIWP dry heated alone for 24 h did not result in the formation of protein-conjugates.

It was expected that sIWP conjugated with 3 – 4 moles of glucose/maltodextrins per mole of sIWP with the concomitant loss of a water molecule, would result in a shift of protein bands by ~480 – 640 Da, ~2,640 – 3,520 Da, 5,640 – 7,520 Da, or 12,840 – 17,120 Da for the conjugation of glucose, M200 maltodextrin (900 Da), M100 maltodextrin (1,900 Da), or M040 maltodextrin (4,300 Da) respectively. However, due to the lack of discrete protein bands within the 36,500 – 66,000 Da region (box a, Figure 3.12), it was not possible to distinguish between the conjugated and non-conjugated proteins in this range by SDS-PAGE. This could be due to the change in MW to this protein fraction (box a) was too small to be resolved using the SDS-PAGE.

The most obvious shift of protein to a higher MW after incubation was observed for the fraction containing LMW proteins (< 31,000 Da) (arrows in box b and c, Figure 3.12). The shifts in protein bands in SDS-PAGE gels stained with Coomassie blue were used to assess the number and size of carbohydrate conjugated to the LMW proteins in sIWP (box c, Figure 3.12). In this region, the band for sIWP and dry heated sIWP (24 h) corresponded to a MW of ~11,500 – 11,600 Da. This band is possibly the 0.19 dimeric alpha-amylase inhibitor (AAV39519) of the LMW-albumins, which has approximately 6 lysine residues and 1 N-terminal amino group available for conjugation (DuPont et al. 2005). However, this protein is a very small fraction of the whole sIWP judging from the intensity of the band. For the sIWP-glucose system, this band shifted to ~12,800 Da upon conjugation. The increase in its MW by approx. 1,300 Da corresponds to the
increase in the numbers of glucose (i.e. ~8) conjugated to this protein, with the concomitant loss of a water molecule. This result does not necessarily contradict the extent of conjugation measured by the loss of –NH₂ groups which was ~3 – 4 moles of carbohydrates conjugated per mole of sIWP. This is a measure of an average value for the whole protein system which contains a mixture of various proteins in varying proportions that may conjugated to different extent. For example, these carbohydrates can react with gliadin present in the sIWP as well (box a, Figure 3.12), which contains an average of 3 lysine residues.

For the sIWP-M200, sIWP-M100 and sIWP-M040 systems, the MW of the conjugated protein (box c, Figure 3.12) were 13300, 13600 and 14500 Da, respectively. This gives the increase in MW due to conjugation of carbohydrates of approximately 1800, 2100, and 3000 Da. If assumed that eight carbohydrate moieties are attached to the protein fraction (MW 11,500 Da) visible in box c (Figure 3.12), the average MW of the maltodextrins conjugated to this protein are 243, 281 and 393 Da, respectively in sIWP-M200, sIWP-M100 and sIWP-M040 mixtures (Table 3.2).

An a priori calculation was carried out based on assumptions that (1) the smallest MW carbohydrates of commercial maltodextrins (which are a mixture of simple sugars as well as polysaccharides of different degree of polymerization (DP)) were preferentially attached to sIWP, and (2) the loss of available –NH₂ groups as measured using the OPA assay was due solely to the conjugation of the carbohydrate to the protein. The calculated average size of carbohydrate moieties attached on incubation of sIWP-M200, sIWP-M100, and sIWP M040 mixtures were 286, 378, and 425 Da, respectively (Table 3.2).
Table 3.2: Calculated MW of the attached carbohydrate on sIWP after heated with glucose/maltodextrins

<table>
<thead>
<tr>
<th>sIWP</th>
<th>MW of attached carbohydrate (Da)</th>
<th>Conjugated sIWP Fraction (11,500 Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Average based on a priori calculation</td>
<td>Size range based on a priori calculation</td>
</tr>
<tr>
<td>sIWP-Glc (180 Da)</td>
<td>180</td>
<td>180</td>
</tr>
<tr>
<td>sIWP-M200 (900 Da)</td>
<td>286</td>
<td>180-360</td>
</tr>
<tr>
<td>sIWP-M100 (1,400 Da)</td>
<td>378</td>
<td>180-540</td>
</tr>
<tr>
<td>sIWP-M040 (4,300 Da)</td>
<td>425</td>
<td>180-720</td>
</tr>
</tbody>
</table>

*a priori calculated values based on assumptions that (1) the smallest MW carbohydrates of commercial maltodextrins (which are a mixture of simple sugars as well as maltodextrins of different degrees of polymerisation (DP)) were preferentially attached to the protein, (2) the loss of available –NH$_2$ groups as measured using the OPA assay was due solely to the conjugation of the carbohydrate to the protein.

*b calculated based on protein bands in Box c in figure 4A, assuming 8 carbohydrate moieties are attached to fraction of protein with MW 11,500 Da

These results suggest that LMW carbohydrates are more susceptible to conjugation than the HMW carbohydrates within a commercial preparation of maltodextrin. The LMW carbohydrates are able to conjugate more readily than their HMW counterparts due to their increased mobility within a matrix, their smaller size allowing closer proximity and easier access to available –NH$_2$ groups. The preferential conjugation of the LMW carbohydrate moieties means that it is difficult to observe MW changes to HMW proteins using SDS-PAGE (box a, Figure 3.12), which contains an average of 3 lysine residues. Furthermore, the conjugation of LMW carbohydrates in the
maltodextrins is probably why sIWP reacted with glucose or the various MW of maltodextrins have the same extent of conjugation.

- **Dextran conjugates**

Figure 3.13 shows the SDS-PAGE of sIWP, sIWP dry heated alone for 72 h and sIWP dry heated with D10N dextran (6,400 Da) or D65N dextran (41,000 Da) for 72 h at 60 °C/75% RH. It should be noted that only the soluble protein fraction isolated from the dry heated sIWP (72 h) and the dry heated sIWP-D10N mixture (72 h) were analysed in the SDS-PAGE. The protein bands for the soluble fraction of dry heated sIWP (72 h) appeared similar to sIWP. The most obvious shift to higher MW after incubation was observed for sIWP reacted with D10N dextran and D65N dextran (arrows in Figure 3.13). sIWP reacted with D65N dextran (41,000 Da) lead to greater shift in MW than sIWP reacted with D10N dextran (6,400 Da). However, the presence of protein bands at the 3,500 – 55,400 Da regions indicates the present of non-conjugated proteins.

Figure 3.14 shows the SDS-PAGE of sIWP and physical mixtures containing sIWP and dextrans [D10N dextran (6,400 Da) or D65N dextran (41,000 Da)]. It appeared that the protein bands for sIWP and the sIWP-dextran mixtures are similar to each other. This shows that the shift observed in the sIWP-dextran conjugates (in Figure 3.13) were not caused by the presence of un-reacted dextran.
Figure 3.13 SDS-PAGE of sIWP, sIWP dry heated alone for 72 h and sIWP dry heated with D10N dextran (6,400 Da) or D65N dextran (41,000 Da) for 72 h stained with Coomassie blue (arrows indicate shifted bands). It should be noted that only the soluble protein fraction isolated from the dry heated sIWP (72 h) and the dry heated sIWP-D10N mixture (72 h) were analysed in the SDS-PAGE. Labelled lanes are: (1 and 6) MW markers; (2) sIWP; (3) dry heated sIWP (72 h); (4) dry heated sIWP-D10N mixture; (5) dry heated sIWP-D65N mixture.
3.4 Conclusions

This part of work showed that sIWP-carbohydrate conjugates were successfully prepared using the dry reaction conditions at 60 °C/ 75% RH. The results indicate that approximately 3 – 4 moles of glucose or LMW carbohydrates (i.e. < 425 Da) in the M200 (900 Da), M100 (1,900 Da) or M040 (4,300 Da) maltodextrins were conjugated per mole of sIWP (prepared at 1:2 –NH₂ to reducing group mole ratio and reacted for 24 h), whereas approximately 1.3 or 0.5 mole of dextran [D10N (6,400 Da) or D65N (41,000 Da)] were conjugated per mole of sIWP (prepared at 1:1 –NH₂ to reducing group mole ratio and reacted for 72 h).
CHAPTER 4

*Structural Properties of sIWP Maillard Conjugates in Solution and Adsorbed at Interfaces*

### 4.1 Introduction

There has been increasing interest to construct protein-carbohydrate conjugates with the motivation to generate new entities with enhanced ability to maintain emulsion stability under unfavourable conditions such as at acidic pH or in ionic environment (Kato 2002; Oliver et al. 2006; Dickinson 2008). The enhancement in emulsion stability results from changes in protein conformation in solution or the formation of polysaccharide steric layer at the interface (Darewicz & Dziuba 2001; Dunlap & Cote 2005; Wooster & Augustin 2007a).

Previous studies indicated that β-casein (24,000 Da) conjugated with 6 glucose moieties under wet conditions (0.05 M sodium phosphate buffer (pH 7.4), 37 °C, 24 h) had increased β-turn structure and decreased random coil in solution. The conjugated β-casein had improved emulsion forming and stabilizing properties in comparison to an unmodified control (Darewicz & Dziuba 2001). Whey protein isolates (consisting of β-lactoglobulin and α-lactoglobulin) conjugated with approximately 5 dextran moieties (29,000 or 42,000 Da) per mole protein under dry conditions (75 °C, 75% RH) have increased random coil and an increase in emulsion capacity (Wooster & Augustin 2007b). β-lactoglobulin conjugated with non-ionic polysaccharides such as maltodextrins or dextrans under dry conditions (65/75 °C, 75% RH) forms a polysaccharide steric layer at the emulsion interface, which enhances emulsion stability.
in ionic environment in comparison to non-conjugated β-lactoglobulin (Dunlap & Cote 2005; Wooster & Augustin 2006; Wooster & Augustin 2007a).

The aim of this chapter is to examine and to understand the changes occurring to the sIWP structure in solution and at interface as a result of conjugation with different MW carbohydrates. As shown in Chapter 3, sIWP conjugates were successfully prepared using the dry reaction (65 °C, 75% RH), where approximately 3 – 4 moles of glucose or LMW carbohydrates in the M200 (900 Da), M100 (1,900 Da) or M040 (4,300 Da) maltodextrins (i.e. < 425 Da) were conjugated per mole of sIWP, and approximately 1.3 and 0.5 moles of dextrans (6,400 and 41,000 Da) were conjugated per mole of sIWP. Changes to sIWP properties in solution after conjugation were investigated by measuring the changes in protein’s pI and its conformation (i.e. secondary structure and tryptophan local environment). The effect of conjugation on the protein adsorbed structure at the interface was investigated by measuring the steric layer thickness and surface topography by Atomic Force Microscopy (AFM) using polystyrene particles and polystyrene coated glass surface as the model system.

It was hypothesized that conjugation may lead to a change in protein’s pI as a result of the loss of positively charged lysine residues and to protein unfolding upon conjugation of HMW carbohydrates. It was also hypothesized that formation of an additional carbohydrate steric layer at the interface, which could provide steric stabilization against droplet flocculation at acidic pH or in ionic environment, will depend on the size and the number of carbohydrate conjugated and the location of conjugation.
4.2 Materials and Methods

4.2.1 Materials

The protein-conjugates were prepared according to the dry reaction method described in section 3.2.4 (Chapter 3). Freeze-dried mixtures containing sIWP-glucose/maltodextrins (900, 1,900, and 4,300 Da) were dry heated at 60 °C/75% RH for 24 h, whereas, freeze-dried mixtures containing sIWP-dextrans (6,400 and 41,000 Da) were dry heated at 60 °C/75% RH for 72 h. As a control, sIWP was dry heated alone at 60 °C/75% RH for either 24 h or 72 h. It should be noted that the LMW carbohydrates in the maltodextrins [i.e. 286 Da from M200 (900 Da), 378 Da from M100 (1,900 Da), and 425 Da from M040 (4,300 Da)] were preferentially conjugated onto sIWP (refer to Table 3.2 in Chapter 3) compared to the higher MW components in these maltodextrins. The extent of conjugation for the protein-conjugates were as follows: approximately 3 − 4 moles of glucose or LMW carbohydrates in the maltodextrins (i.e. < 425 Da) were conjugated per mole of sIWP, and approximately 1.3 and 0.5 moles of dextrans (6,400 and 41,000 Da) were conjugated per mole of sIWP.

It should be noted that the water solubility of sIWP dry heated alone for 72 h and sIWP dry heated with D10N dextran (6,400 Da) for 72 h were reduced significantly to ~67% and ~81% respectively (refer to Chapter 3, section 3.3.4). The insoluble proteins in these samples were removed prior to further analysis. Insoluble proteins usually have decreased effectiveness in adsorbing to an interface and may promote flocculation if adsorbed at the interface (Dickinson & Galazka 1991; Diftis et al. 2005). The water solubility of sIWP dry heated alone for 24 h, sIWP dry heated with glucose or maltodextrins for 24 h and sIWP dry heated with D65N dextran (41,000 Da) for 72 h...
were minimally affected (solubility > 90%) and were used without fractionation. It should be noted that the protein-conjugates used for analysis contain a mixture of conjugated and non-conjugated proteins, and unreacted carbohydrates. The names given to the dry heated sIWP and the protein-conjugates are detailed in Table 4.1.

Table 4.1 Sample names for dry heated sIWP and protein-conjugates.

<table>
<thead>
<tr>
<th>Name of samples (Protein or protein-conjugates)</th>
<th>Carbohydrate reacted</th>
<th>Reaction timea</th>
</tr>
</thead>
<tbody>
<tr>
<td>dry heated sIWP (24 h)</td>
<td>None</td>
<td>24 h</td>
</tr>
<tr>
<td>sIWP-Glc conjugate (24 h)</td>
<td>glucose (180 Da)</td>
<td>24 h</td>
</tr>
<tr>
<td>sIWP-M200 conjugate (24 h)</td>
<td>M200 maltodextrin (900 Da)</td>
<td>24 h</td>
</tr>
<tr>
<td>sIWP-M100 conjugate (24 h)</td>
<td>M100 maltodextrin (1,900 Da)</td>
<td>24 h</td>
</tr>
<tr>
<td>sIWP-M040 conjugate (24 h)</td>
<td>M040 maltodextrin (4,300 Da)</td>
<td>24 h</td>
</tr>
<tr>
<td>soluble dry heated sIWP (72 h)b</td>
<td>None</td>
<td>72 h</td>
</tr>
<tr>
<td>soluble sIWP-D10N conjugate (72 h)b</td>
<td>D10N dextran (6,400 Da)</td>
<td>72 h</td>
</tr>
<tr>
<td>sIWP-D65N conjugate (72 h)</td>
<td>D65N dextran (41,000 Da)</td>
<td>72 h</td>
</tr>
</tbody>
</table>

(a) Maillard reaction performed at 60°C/75% RH
(b) only the soluble fractions of dry heated sIWP (72 h) and sIWP-D10N conjugate (72 h) were analysed

IWP gliadin was prepared as described in section 2.2.1 (Chapter 2).

An aqueous suspension (10 wt. %) of polystyrene spheres (100 nm in diameter) and dextranase from Penicillium species was purchased from Sigma-Aldrich (Sydney, Australia). Other analytical grade chemicals were purchased from Sigma Aldrich (Sydney, Australia).

4.2.2 Protein Zeta-potential, Hydrodynamic Size and Isoelectric Point

As described in Chapter 2 (section 2.2.6).
4.2.3 Solution Optical Density

Protein/protein-conjugates were dispersed in milli-Q (1 mg/mL – protein basis) at pH 7 for 2 h. The optical density of the solution was then measured at 600 nm using a spectrophotometer (UV-1700 Pharma Spec, Shimadzu). The pH of the solution was then adjusted to pH 4 using 0.05 M HCl and the optical density of the solution was measured at 600 nm. Photographs of the solutions at pH 7 and at pH 4 were also taken.

4.2.4 Attenuated Total Reflectance-Fourier Transform Infrared (ATR-FTIR) and Principal Component Analysis

Attenuated total reflectance (ATR) spectra of the freeze-dried samples were recorded at 4 cm$^{-1}$ with a nitrogen purged Bruker Equinox 55 FTIR spectrometer equipped with a liquid nitrogen cooled mercury-cadmium-telluride (MCT) detector and a Golden gate single bounce diamond ATR. Each spectrum was the result of an average of 256 scans which were Fourier-transformed in OPUS 5.5 using Blackmann-Harris 4 term apodization and a zero filling factor of 8. Spectra were converted from OPUS format to JCAMP-DX format and imported into the Unscrambler 9.2, where the extended multiplicative signal correction (EMSC) was applied, followed by transformation into 2$^{nd}$ derivative using Savitzsky-Golay function (3$^{rd}$ order polynomial and 3 smoothing points). After mean centring the pre-processed spectra were then analysed using principal component analysis (PCA) in Unscrambler 9.2. Changes in secondary structure were determined using the Amide I band (1700-1600 cm$^{-1}$) (C=O stretch coupled with C-N stretch).
The typical wavenumber values assigned to Amide I modes were as follows: $\alpha$-helix (1650-1657 cm$^{-1}$), antiparallel $\beta$-sheet (intermolecular) (1612-1640 cm$^{-1}$ and 1670-1690 cm$^{-1}$ (weak)), parallel $\beta$-sheet (intramolecular) (1626-1640 cm$^{-1}$ and 1655-1675 cm$^{-1}$), turn (1655-1675 cm$^{-1}$ and 1680-1696 cm$^{-1}$) and random coil (1640-1651 cm$^{-1}$) (Pelton & McLean 2000).

### 4.2.5 Circular Dichroism

Far-UV CD was used to examine the protein secondary structure in solution. The CD spectra of aqueous dispersions of dry heated sIWP (24 h), sIWP-Glc conjugate (24 h), sIWP-M200 conjugate (24 h), sIWP-M100 conjugate (24 h), and sIWP-M040 conjugate (24 h) were obtained using a conventional CD instrument, Jasco J-810 spectropolarimeter (Jasco Corporation Japan). The protein/protein-conjugates were dispersed in milli-Q water (0.25 wt. % - protein basis) and the pH was adjusted to pH 7. The spectra of the solutions were then recorded at 24 °C from 160 to 190 nm with a spectral resolution of 0.1 nm using a 0.1 cm path length quartz cell at a speed of 20 nm/min, a response time of 1 s and a bandwidth of 1 nm. These spectra were baseline corrected with milli-Q water and scaled to delta epsilon ($\Delta\varepsilon$) using 110 as the mean residue weight of protein prior to secondary structure composition analysis (Kelly et al. 2005).

All other CD spectra, including reconstituted aqueous dispersion of soluble dry heated sIWP (72 h), soluble sIWP-D10N conjugate (72 h) and sIWP-D65N conjugate (72 h) were obtained using a synchrotron radiation based CD instrument (SRCD), which is located at the institute for storage ring facility (ISA) in Aarhus University (Denmark). The protein solutions (0.1 wt. %) were prepared in 20 mM McIlvaine’s buffer at pH 7
through gently stirring for 2 h prior to CD measurements. CD spectra were recorded at 24 °C from 260 to 185 nm with a spectral resolution of 1 nm. Spectra were recorded using a 0.1 cm path length quartz suprasil cell (Hellma, Germany) and were baseline corrected for 20 mM McIlvaine’s buffer (pH 7). The CD spectra in ellipticity (θ) units were scaled to delta epsilon (Δε) using 110 as the mean residue weight of protein (Kelly et al. 2005). However, the spectra were truncated to 260 to 190 nm because the presence of dextran appeared to interfere with the CD signal below 190 nm.

The proportions of each secondary structure components for all the CD spectra were determined through a web-based calculation server. This was DICHROWEB, based on a CONTIN programme with reference set 7 (optimised for 190 – 240 nm), which predicts the proportion of the α-helix, β-sheet, turns, and random coil in the protein (Whitmore & Wallace 2008). The use of different reference sets is expected to result in slightly different proportion of each predicted secondary structures.

4.2.6 Tryptophan Fluorescence Emission

As described in Chapter 2 (section 2.2.12).

4.2.7 Measurement of Interfacial Layer Thickness

The size of the polystyrene particles measured using dynamic light scattering (DLS) at pH 7.5 was 111.03 ± 0.64 nm in diameter. The adsorbed layer thickness was estimated from the difference in size between naked polystyrene particles and those adsorbed with proteins as illustrated in Figure 4.1. Solution containing sIWP/protein-conjugate was prepared in 20 mM Tris-HCl (pH 7.5) at 10 mg/mL protein concentration. The solution was filtered through a 0.45 μm filter and then diluted
accordingly to achieve the desirable protein concentration (0.01 – 0.1 mg/mL). The protein solution (1.3 mL) was gently mixed with 100 µl of diluted polystyrene sphere suspension (0.05 wt. %) and the size was measured immediately at 25 ºC by DLS (Nano ZS, Malvern, Worcestershire, UK), at a scattering angle of 173º using a 633 nm laser with each measurement being an average of 15 runs of 10 s duration.

Figure 4.1: The adsorbed layer thickness was estimated from the difference in hydrodynamic size between naked polystyrene particles and those with protein/protein-conjugate adsorbed onto their surface. (Note: protein: orange colour, dextran: green colour, and polystyrene sphere: yellow colour)

Prior to dextranase digestion, protein/protein conjugates (0.1 mg/mL - protein basis) were allowed to be adsorbed onto the polystyrene particles suspended in 20 mM Tris-HCl (pH 7.5). The adsorbed layer were left quiescent at room temperature for 2 h, to allow enough time for the interfacial layer to reach a constant layer thickness. The adsorbed layer were then incubated with either buffer as a control or dextranase (100
μL, 0.1 mg/mL of 170 units/mg) at room temperature and the layer thickness was measured as a function of time. The dextranase catalyse the endohydrolysis of α-(1−6)−D−glucosidic linkages in the dextran.

The layer thickness of sIWP-D65N conjugate (72 h) was investigated as a function of pH. sIWP-D65N conjugate (72 h) (0.1 mg/mL – protein basis) was allowed to be adsorbed onto the polystyrene particles suspended in milli-Q water at pH 7.5. The absorbed layer were left quiescent at room temperature for 2 h. The layer thickness was then measured as the pH was titrated from pH 7.5 to pH 4 using 0.1 M HCl.

4.2.8 Solution Viscosity

The solution viscosity of protein/protein-conjugate at 0.1 mg/mL (protein basis) was measured by using a capillary U-tube viscometer (B.S.S. No.1). The viscometer was placed in a water bath to maintain the temperature at 25 ºC. Solution containing protein/protein-conjugate was prepared in 20 mM Tris buffer (pH 7.5) at 0.1 mg/mL protein concentration. The prepared protein/protein-conjugate solution was then carefully transferred into the viscometer to avoid bubbles formation and was given 5 min for equilibrating to 25 ºC. The time required for the meniscus of the protein solution to pass through the viscometer was recorded. The viscosity of the protein solution was then calculated by comparing against the time required for the meniscus of milli-Q water to pass through the viscometer (372 ± 1 s) and the viscosity of water at 25 ºC (0.89 mPa/s).
4.2.9 Surface Topography Images by Atomic Force Microscopy

4.2.9.1 Preparation of Hydrophobic Polystyrene Surface

A microscopy glass slide was cut into a dimension of 2.5 cm². The glass surface was soaked overnight in a solution containing a mixture of sulphuric acid and hydrogen peroxide mixed in a ratio of 1:3. The glass slide was sonicated 3 times in acetone and then 3 times in ethanol for about 15 minutes for each time. The glass slide was rinsed in deionised water and then blow dried with nitrogen gas.

Polystyrene (~90,000 Da) solution (1.5 wt. %) was prepared in toluene. The polystyrene solution (200 µL) was pipetted onto the centre of the glass surface. The glass slide was spin coated at $2 \times 10^3$ RPM for 1 min. The spin-coated slide was then dried in vacuum oven at 120 ºC for 24 h. The slide was allowed to be cooled to room temperature (24 ºC) and then stored in a clean petri dish until further analysis (Figure 4.2).

4.2.9.2 Protein Adsorption and Atomic Force Microscopy Imaging

Protein/protein-conjugate (10 µg/mL - protein basis) was reconstituted in 20 mM Tris-HCl and then filtered using a 0.45 µm syringe filter. The reconstituted protein/protein-conjugate solution was transferred onto the polystyrene coated glass surface and was allowed to be adsorbed onto the surface for 2 h. The surface was rinsed with de-ionised water 7 times over a period of 4 h to remove unadsorbed proteins and unreacted dextrans and then allowed to air dry at room temperature (24 ºC).

A Dimension 3100 Atomic Force Microscope (Digital Instruments, Santa Barbara, CA) was used to acquire AFM images. The microscope was operated in
tapping mode under air using golden silicon cantilevers (NSG11, NT-MDT) and a scan rate of 0.5 Hz (Figure 4.2). Image analysis was performed using Nanoscope version 6.12 (Digital Instruments, Santa Barbara, CA).

Figure 4.2: Schematic illustration of the preparation of a hydrophobic polystyrene surface on a glass slide and the acquisition of AFM images on a protein adsorbed surface.
4.3 Results and Discussion

4.3.1. Solution Properties of sIWP and Protein-conjugates

4.3.1.1 Effect of Conjugation on Protein Isoelectric Point

➢ Glucose/Maltodextrins conjugates

The zeta-potential of various conjugates [i.e. sIWP-Glu conjugate (24 h), sIWP-M200 conjugate (24 h), sIWP-M100 conjugate (24 h) and sIWP-M040 conjugate (24 h)] with approximately 3 – 4 moles of carbohydrates conjugated per mole of sIWP (refer to table 3.2 of Chapter 3) is shown in Figure 4.3.

![Zeta-potential graph](image)

Figure 4.3: The zeta-potential of sIWP, dry heated sIWP (24 h) and sIWP conjugates with glucose and maltodextrins in solution as a function of pH. Note: (1) protein conjugates were prepared at 60 °C/75% RH, (2) asterisk indicates the calculated average MW of carbohydrates conjugated onto sIWP from glucose or the various MW maltodextrins [M200 (900 Da), M100 (1400 Da), M040 (4300 Da)] (calculation shown in Table 3.2 in Chapter 3).
Dry heated sIWP (24 h) and the protein-conjugates have similar zeta-potential (~ − 50 mV) as sIWP at neutral pH. When the pH was decreased from pH 7.5 to pH 4, the zeta-potential of all samples gradually decreased from ~ − 50 mV to 0 mV, which indicates that they have the same pI (i.e. at ~pH 4). This result suggests that conjugation of sIWP with up to 4 moles of glucose or LMW carbohydrates in the maltodextrins (i.e. < 425 Da) per mole of sIWP did not have any impact on the protein’s pI.

Dextran conjugates

Figure 4.4 shows the zeta-potential of sIWP, soluble dry heated sIWP (72 h), soluble sIWP-D10N conjugate (72 h) and sIWP-D65N conjugate (72 h) as a function of pH. Soluble dry heated sIWP (72 h) and soluble sIWP-D10N conjugate (72 h) have similar zeta-potential (~ − 50 mV) as sIWP at neutral pH. However, the sIWP-D65N conjugate (72 h) exhibited much lower zeta-potential (~ − 30 mV) compared to sIWP (~ − 50 mV). The reduction in zeta-potential of the protein may be attributed to the conjugation of a large dextran molecule (41,000 Da), which can physically screen the protein charge. However, a reduction in zeta-potential was not observed in soluble sIWP-D10N conjugate (72 h), suggesting that conjugation of 6,400 Da dextran is not sufficiently large enough to screen the protein charge. Nevertheless, as the pH decreased from pH 7.5 to pH 4, their zeta-potential preparations gradually decreased from ~ − 30/− 50 mV to 0 mV. This result suggests that conjugation of sIWP with 0.5 − 1.3 moles of dextran (6,400 or 41,000 Da) per mole of sIWP also did not have an impact on the protein’s pI.
Figure 4.4: The zeta-potential of sIWP, soluble dry heated sIWP (72 h), soluble sIWP-D10N conjugate (72 h) and sIWP-D65N conjugate (72 h) in solution as a function of pH. Note: (1) protein-conjugates were prepared at 60 °C/75% RH, (2) asterisk indicates the average MW of dextran assumed to have conjugated onto sIWP from D10N dextran (6,400 Da) or D65N dextran (41,000 Da), (3) only the soluble fractions of dry heated sIWP (72 h) and sIWP-D10N conjugate (72 h) were analysed.

In other studies, the effects of conjugation of carbohydrates with other proteins were inferred from loss in solubility as a function of pH and not by direct measurement of zeta-potential as a function of pH (Chevalier et al. 2001b; Jimenez-Castano et al. 2005; Jimenez-Castano et al. 2007). In these studies, it was suggested that proteins such as β-lactoglobulin and bovine serum albumin conjugated with ~2 – 8 moles of carbohydrates (e.g. glucose or dextran) per mole of protein lead to a shift in protein’s pI to a more acidic pH value (Chevalier et al. 2001b; Jimenez-Castano et al. 2005; Jimenez-Castano et al. 2007). These results contrast with those obtained with protein-conjugates prepared from sIWP. One possible reason for this is the differences in the amino acid profiles and distribution of charges in various proteins. A search on the
protein database (http://www.ncbi.nlm.nih.gov) showed that β-lactoglobulin (CAA32835) has 27 positively charged and 21 negatively charged amino acid residues, whereas, bovine serum albumin (CAA76847) has 101 positively charged and 97 negatively charged amino acid residues. It is possible that for a shift in protein’s pI to a more acidic pH value to occur in proteins that have high amount of positively and negatively charged amino acids that equally distributed along the protein molecule (e.g. β-lactoglobulin and bovine serum albumin), conjugation of low amount of carbohydrate to the positively charged lysine residues may be sufficient to affect overall protein charge. The observation that sIWP conjugated with 1 – 4 moles of carbohydrates [i.e. glucose, LMW carbohydrates in the maltodextrins (i.e. < 425 Da), or dextrans] per mole of sIWP was not sufficient to have any impact on the sIWP’s pI, may be because of the localisation of a large number of negatively charged glutamic acid residues at the N-terminal domain of sIWP as a result of the deamidation process and the presence of low number of positively charged amino acid residues along the protein molecule in sIWP (refer to Table 2.2 & Figure 2.9 in Chapter 2). It is likely that reduction in 1 – 4 moles of positively charged lysine residues per mole of sIWP is not sufficient to affect overall protein charge.

4.3.1.2 Effect of pH on Aggregation Behaviour of the Protein-conjugates

The aggregation behaviour of sIWP, dry heated sIWP and the protein-conjugates was investigated by measuring their particle size in solution as a function of pH. Photographs of these samples in solution at pH 7 and at pH 4 were also taken to support the results obtained from particle size measurements. It is worth noting that the particle size instrument (Zetasizer nano ZS) that was used in this study has a detection range of
0.6 nm to 6 µm. Hence, any particle size reported in the result sections close to or greater than 6 µm is considered as outside the detection range of the instrument.

- Glucose/Maltodextrins conjugates

Figure 4.5A shows the particle size of sIWP, dry heated sIWP (24 h), sIWP-Glc conjugate (24 h), sIWP-M200 conjugate (24 h), sIWP-M100 conjugate (24 h) and sIWP-M040 conjugate (24 h) as a function of pH. A similar trend was observed for all samples, where an average particle size of ~200 nm was detected from pH 7 down to pH 4.5, and followed by a marked increase in particle size at the pI of the protein/protein-conjugates at ~pH 4. The marked increase in particle size is due to the formation of protein aggregates. When the pH was reduced further below the protein’s pI, the particle size decreased, due to re-solubilisation of the aggregates as the surface of the protein particles became positively charged.

It was observed that the particle size for sIWP-M040 conjugate (24 h) was much lower than the other protein-conjugates or sIWP alone at the pI (~pH 4). This can be explained by the average higher MW of carbohydrate attached. As previously determined, all protein-conjugates contain ~3 – 4 moles of carbohydrates conjugated per mole of sIWP, but the average MW of the conjugated carbohydrate range from 180 – 425 Da, when glucose and the various MW maltodextrins (900 – 4,300 Da) were used, with the average MW of the attached carbohydrate increased with increasing average MW of the carbohydrates. The fact that sIWP incubated with the highest MW maltodextrin [i.e. M040 (4300 Da)] had the largest carbohydrate moiety attached (i.e. 425 Da), explains the slight reduction in the extent of aggregation, due to a reduction in protein-protein hydrophobic interactions by the conjugated carbohydrates.
The sIWP and the protein-conjugates [i.e. conjugated with glucose or LMW carbohydrates in the M040 (4,300 Da) maltodextrins (i.e. ~425 Da)] showed a transition from a clear solution at pH 7 to a turbid solution when the pH was adjusted to pH 4 (Figure 4.5B). The optical density of the pH 4 solutions was not reported here because the measurements of these solutions taken at pH 4 were off the scale due to high turbidity. These results suggest that sIWP as well as sIWP conjugated with glucose or LMW carbohydrates in the M040 (4,300 Da) maltodextrins (i.e. up to 425 Da) did not have protection against protein aggregation at the pI of the protein/protein-conjugates (~pH 4).

Figure 4.5: [A] The particle size (Z-average) of sIWP, dry heated sIWP (24 h), protein conjugated with glucose and various MW maltodextrins in solution as a function of pH. [B] Photographs of sIWP, sIWP-Glc conjugate (24 h) and sIWP-M040 conjugate (24 h) in solution taken at pH 7 (top) and after the pH of the solutions was adjusted to pH 4 (bottom). Note: (1) protein conjugates were prepared at 60 °C/75% RH, (2) asterisk indicates the calculated average MW of carbohydrates conjugated onto sIWP from glucose or the various MW maltodextrins [M200 (900 Da), M100 (1400 Da), M040 (4300 Da)] (calculation shown in Table 3.2 in Chapter 3).
Dextran conjugates

Figure 4.6 shows the particle size of sIWP, soluble dry heated sIWP (72 h), soluble sIWP-D10N conjugate (72 h) and sIWP-D65N conjugate (72 h) as a function of pH. An average particle size of ~200 nm was detected for soluble dry heated sIWP (72 h), soluble sIWP-D10N conjugate (72 h) and sIWP-D65N conjugate (72 h) from pH 7 down to pH 4.5, which is similar to that for sIWP. A marked increase in particle size at the pI (~pH 4) was found in sIWP and in soluble dry heated sIWP (72 h) (Figure 4.6). The difference in particle size between sIWP and soluble dry heated sIWP (72 h) observed at pI is due to outside the detection range of the instrument. The increase in particle size at the pI (~pH 4) was not observed in soluble sIWP-D10N conjugate (72 h) or sIWP-D65N conjugate (72 h). This suggests that conjugation with dextrans even with lower numbers of dextran conjugated per mole of protein [i.e. ~1.3 mole of D10N dextran (6,400 Da) or ~0.5 mole of D65N dextran (41,000 Da) per mole of sIWP] compared to the conjugation of ~3 – 4 moles of glucose or LMW carbohydrates in the maltodextrins (i.e. < 425 Da) is able to prevent sIWP from aggregating at protein’s pI. This is despite the fact that the zeta-potential results showed that conjugation with dextrans did not change the pI of sIWP (i.e. ~pH 4). The ability of the dextran-conjugates to prevent protein from aggregation when there is a net zero surface charge (i.e. at pI ~pH 4) is likely due to the conjugated dextran, which acts as a physical barrier against protein aggregation.
Figure 4.6: The particle size of sIWP, soluble dry heated sIWP (72 h), soluble sIWP-D10N conjugate (72 h) and sIWP-D65N conjugate (72 h) in solution as a function of pH. Note: (1) protein conjugates were prepared at 60 °C/75% RH, (2) asterisk indicates the average MW of dextran assumed to have conjugated onto sIWP from D10N dextran (6,400 Da) or D65N dextran (41,000 Da), (3) only the soluble fractions of dry heated sIWP (72 h) and sIWP-D10N conjugate (72 h) were analysed.

Figure 4.7A shows the photographs of sIWP, soluble dry heated sIWP (72 h), soluble sIWP-D10N conjugate (72 h) and sIWP-D65N conjugate (72 h) taken at pH 7 and after the solution pH was adjusted to pH 4. All the solutions appeared clear at pH 7. When the pH was lowered to pH 4, sIWP and soluble dry heated sIWP (72 h) solutions became turbid due to the formation of protein aggregates. Conversely, soluble sIWP-D10N conjugate (72 h) and sIWP-D65N conjugate (72 h) solutions showed less turbid than sIWP and soluble dry heated sIWP (72 h) solutions when the pH was lowered to pH 4. Solutions containing physical mixtures of sIWP and dextran [i.e. D10N (6,400 Da) or D65N (41,000 Da)] became turbid when the pH was lowered from pH 7 to pH 4, which is similar to sIWP alone (Figure 4.7B). This shows that the lower turbidity
observed at pH 4 in the solutions containing the dextran-conjugates were not caused by the presence of un-reacted dextrans.

Figure 4.7: [A] Photographs of sIWP, soluble dry heated sIWP (72 h), soluble sIWP-D10N conjugate (72 h) and sIWP-D65N conjugate (72 h) in solutions taken at pH 7 (top) and after the pH of the solutions was adjusted to pH 4 (bottom). [B] Photographs of sIWP-D10N physical mixture and sIWP-D65N physical mixture in solution taken at pH 7 (top) and after the pH of the solutions was adjusted to pH 4 (bottom). Note: (1) protein conjugates were prepared at 60 °C/75% RH, (2) only the soluble fractions of dry heated sIWP (72 h) and sIWP-D10N conjugate (72 h) were analysed.

Optical density of sIWP, soluble sIWP-D10N conjugate (72 h) and sIWP-D65N conjugate (72 h) solutions at pH 7 and after the pH of the solution was adjusted to pH 4 were measured at 600 nm (Figure 4.8). The optical density of other solutions [i.e. soluble dry heated sIWP (72 h), sIWP-D10N physical mixture, and sIWP-D65N physical mixture] was not reported here because measurements for these solutions taken at pH 4 were off the scale due to high turbidity. On the other hand, optical density
measurements taken for the dextran-conjugate solutions at pH 4 were within the detection limit. All the solutions have low optical density at pH 7 of ~0.01. When the pH of the solutions was lowered to pH 4, the optical density of sIWP, soluble sIWP-D10N conjugate (72 h) and sIWP-D65N conjugate (72 h) increased to ~0.61, ~0.36 and ~0.15 respectively. This confirms that soluble sIWP-D10N conjugate (72 h) and sIWP-D65N conjugate (72 h) aggregated to lower extent than sIWP at pH 4. Furthermore, the sIWP-D65N conjugate (72 h) appeared less aggregated than soluble sIWP-D10N conjugate (72 h) at pH 4. This suggests that the size of the conjugated dextran played an important role in preventing the aggregation of sIWP at protein’s pI. It acts as a physical barrier against protein aggregation when the protein surface charge is net zero. The slight increase in turbidity observed in the dextran-conjugate solutions at pH 4 is likely contributed from the aggregation of un-reacted proteins present in the protein-conjugates. This has been reported by other studies that the increase in optical density in solutions containing non-purified whey protein-maltodextrin conjugates was attributed to the presence of some un-reacted protein that aggregate at a pH close to the protein’s pI (Akhtar & Dickinson 2007).

Particle size measurement (Figure 4.6) and solution turbidity (Figures 4.7 & 4.8), both suggests that sIWP conjugated with D10N dextran (6,400 Da) and D65N dextran (41,000 Da) is less prone to aggregation at the protein’s pI, but the slight aggregation observed in the dextran-conjugates was not detected by the particle size measurement. This is because the hydrodynamic size reported here is the intensity weighted mean hydrodynamic size (Z-average) of the dextran-conjugates. This suggests that the amount of protein aggregates present in the dextran-conjugates at pH 4 is not high enough to increase the Z-average value.
Figure 4.8: Optical density of sIWP, soluble sIWP-D10N conjugate (72 h) and sIWP-D65N conjugate (72 h) in solution were measured at 600 nm at pH 7 and after the pH of the solution was adjusted to pH 4. Note: (1) protein conjugates were prepared at 60 °C/75% RH, (2) only the soluble fractions of sIWP-D10N conjugate (72 h) were analysed.

4.3.1.3 Effect of Conjugation on Protein Secondary Structure

Fourier Transformed Infrared Spectroscopy (FTIR)

- Glucose/Maltodextrins conjugates

FTIR coupled with an attenuated total reflectance (ATR) accessory was used to investigate the effect of conjugation of sIWP with glucose or LMW carbohydrates in the maltodextrins (i.e. < 425 Da) on the protein secondary structure of sIWP. The detail of this study is published in Food Biophysics by Wong et al. (2009) (refer to attached paper in Appendices). The study found that sIWP-M100 conjugate (24 h) and sIWP-M040 conjugate (24 h), conjugated with 3 – 4 moles of LMW carbohydrates (i.e. 378 Da and 425 Da respectively) per mole of sIWP have increased α-helix/random coil and
a decreased in inter-/intra-molecular β-sheet to that of sIWP. However, these changes were found to be an artefact resulting from the presence of un-reacted carbohydrates in the samples and the pressure exerted on the protein samples by the ATR accessory (Wong et al. 2009). Hence, ATR-FTIR was not used for further studies.

- Dextran conjugates

ATR-FTIR was not used to characterise the secondary structure of soluble sIWP-D10N conjugate (72 h) and sIWP-D65N conjugate (72 h) because of the likelihood of an artefact resulted from the presence of un-reacted dextran in the samples as described above.

Circular Dichroism (CD)

The secondary structure of dispersions of protein-conjugates was investigated using circular dichroism and compared against the secondary structure of sIWP and dry heated sIWP. It is worth noting that the protein-conjugates prepared in this study contained un-reacted proteins. Hence, the circular dichroism spectrum obtained from the protein-conjugates is an average signal from both conjugated and non-conjugated proteins.

- Glucose/Maltodextrins conjugates

Figure 4.9 shows the circular dichroism spectra of sIWP, dry heated sIWP (24 h), sIWP-Glc conjugate (24 h), sIWP-M200 conjugate (24 h), sIWP-M100 conjugate (24 h) and sIWP-M040 conjugate (24 h) in solution measured at neutral pH. Table 4.2 shows the proportion of each secondary structure components (i.e. α-helix, β-sheet, turns and unordered structure) predicted from these circular dichroism spectra. There
was no significant difference between the secondary structure of dry heated sIWP (24 h) and the protein-conjugates and that of sIWP. This suggests that sIWP dry heated for 24 h or conjugated with 3 – 4 moles of glucose or LMW carbohydrates in the maltodextrins (i.e. < 425 Da) per mole of sIWP did not result in any changes to the secondary structure.

Figure 4.9: The circular dichroism spectra of sIWP, dry heated sIWP (24 h), protein conjugates with glucose and various MW maltodextrins in solution measured at neutral pH. Note: (1) protein conjugates were prepared at 60 °C/75% RH, (2) asterisk indicates the calculated average MW of carbohydrates conjugated onto sIWP from glucose or the various MW maltodextrins [M200 (900 Da), M100 (1400 Da), M040 (4300 Da)] (calculation shown in Table 3.2 in Chapter 3).
Table 4.2: The secondary structure composition (% α-helix, β-sheet, turns and unordered structure) of sIWP, dry heated sIWP (24 h) and protein conjugates with glucose and various MW maltodextrins dispersions predicted from their respective circular dichroism spectrum.

<table>
<thead>
<tr>
<th>Preparations</th>
<th>α-Helix</th>
<th>β-Sheet</th>
<th>Turns</th>
<th>Unordered</th>
</tr>
</thead>
<tbody>
<tr>
<td>sIWP</td>
<td>21.7 ± 0.4</td>
<td>6.7 ± 3.2</td>
<td>15.0 ± 0.1</td>
<td>56.8 ± 2.7</td>
</tr>
<tr>
<td>Dry heated sIWP (24 h)</td>
<td>19.4 ± 0.8</td>
<td>11.0 ± 1.0</td>
<td>14.5 ± 0.1</td>
<td>55.3 ± 0.1</td>
</tr>
<tr>
<td>sIWP-Glc conjugate (24 h)</td>
<td>20.2 ± 0.8</td>
<td>9.7 ± 0.6</td>
<td>14.2 ± 0.4</td>
<td>55.9 ± 0.6</td>
</tr>
<tr>
<td>sIWP-M200 conjugate (24 h)</td>
<td>19.7 ± 0.2</td>
<td>11.3 ± 0.6</td>
<td>14.9 ± 0.2</td>
<td>54.3 ± 0.2</td>
</tr>
<tr>
<td>sIWP-M100 conjugate (24 h)</td>
<td>20.4 ± 0.9</td>
<td>9.2 ± 0.7</td>
<td>14.4 ± 0.1</td>
<td>56.2 ± 0.6</td>
</tr>
<tr>
<td>sIWP-M040 conjugate (24 h)</td>
<td>20.4 ± 0.3</td>
<td>9.2 ± 0.7</td>
<td>14.3 ± 0.1</td>
<td>56.2 ± 0.6</td>
</tr>
</tbody>
</table>

Dextran conjugates

The secondary structures of sIWP, soluble dry heated sIWP (72 h), soluble sIWP-D10N conjugate (72 h) and sIWP-D65N conjugate (72 h) in solution were also measured using CD at neutral pH (Figure 4.10). The proportion of each secondary structure components (i.e. α-helix, β-sheet, turns and unordered structure) predicted from the circular dichroism spectra of the protein/protein-conjugates are summarised in Table 4.3. The results suggest that there is no significant difference in their secondary structure of sIWP, soluble dry heated sIWP (72 h), soluble sIWP-D10N conjugate (72 h) and the sIWP-D65N conjugate (72 h). This suggests that sIWP dry heated for 72 h or conjugated with 1.3 or 0.5 mole of dextran [D10N (6,400 Da) or D65N (41,000 Da)] per mole of sIWP did not result in any changes to the secondary structure.

A study by Wooster and Augustin (2007b) suggest that the effect of conjugation on the protein secondary structure is dependent on the size and the number of
carbohydrate conjugated onto the protein. The authors found that whey protein isolate (WPI) conjugated with approximately 1 mole of dextran (29,400 Da) per mole of WPI did not change the protein structure. However, when the number of dextran (29,400 Da) attached increased to approximately 5.5 moles of dextran per mole of WPI, considerable loss of protein secondary structure was observed. The authors also found that when WPI was conjugated with approximately 1 mole of higher MW dextran (42,200 Da) per mole of WPI, slight protein unfolding was observed.

Figure 4.10: The circular dichroism spectra of sIWP, soluble dry heated sIWP (72 h), soluble sIWP-D10N conjugate (72 h) and sIWP-D65N conjugate (72 h) in solution measured at neutral pH. Note: (1) protein conjugates were prepared at 60 ºC/75% RH, (2) asterisk indicates the average MW of dextran assumed to have conjugated onto sIWP from D10N dextran (6,400 Da) or D65N dextran (41,000 Da), (3) only the soluble fractions of dry heated sIWP (72 h) and sIWP-D10N conjugate (72 h) were analysed.
Table 4.3: The secondary structure composition (i.e. % α-helix, β-sheet, turns and unordered structure) of sIWP, soluble dry heated sIWP (72 h), soluble sIWP-D10N conjugate (72 h) and sIWP-D65N conjugate (72 h) dispersions predicted from their respective circular dichroism spectrum.

<table>
<thead>
<tr>
<th>Preparations</th>
<th>α-Helix</th>
<th>β-Sheet</th>
<th>Turns</th>
<th>Unordered</th>
</tr>
</thead>
<tbody>
<tr>
<td>sIWP</td>
<td>21.2 ± 0.4</td>
<td>9.7 ± 0.4</td>
<td>14.0 ± 0.3</td>
<td>55.1 ± 0.2</td>
</tr>
<tr>
<td>Soluble dry heated sIWP (72 h)</td>
<td>22.6 ± 0.0</td>
<td>6.4 ± 0.5</td>
<td>13.4 ± 0.4</td>
<td>57.7 ± 0.1</td>
</tr>
<tr>
<td>Soluble sIWP-D10N conjugate (72 h)</td>
<td>20.9 ± 0.0</td>
<td>9.8 ± 0.0</td>
<td>14.0 ± 0.3</td>
<td>55.3 ± 0.3</td>
</tr>
<tr>
<td>sIWP-D65N conjugate (72 h)</td>
<td>20.3 ± 0.0</td>
<td>10.8 ± 0.1</td>
<td>14.2 ± 0.3</td>
<td>54.8 ± 0.3</td>
</tr>
</tbody>
</table>

Other studies showed that the effect of conjugation on the protein secondary structure is dependent on the type of protein used in the conjugation. For example, Wooster and Augustin (2007b) found that conjugation of whey proteins [5.5 moles of dextran (29,400 Da) conjugated per mole of WPI] that has compact globular structure lead to protein unfolding, whereas, Darewicz and co-workers (2001) found that conjugation of β-casein [6 moles of glucose conjugated per mole of β-casein] that has disordered flexible structure, lead to an increased in β-turn structure and a decreased in random coil.

A study by Kim and co-workers (2003) found that the effect of conjugation on the protein secondary structure is dependent on the site of conjugation. The authors found that bovine serum albumin (BSA) conjugated with 3 – 7 moles of galactomannan (22,000 Da) per mole of BSA did not change the protein structure. The authors postulated that the galactomannan were conjugated on the surface of BSA without changing the protein secondary structure.
This study showed that sIWP conjugated with 3 – 4 moles of glucose or LMW carbohydrates in the maltodextrins (i.e. < 425 Da) per mole of sIWP, or 1.3 or 0.5 mole of dextrans [D10N (6,400 Da) or D65N (41,000 Da)] per mole of sIWP, did not change the protein secondary structure. In Chapter 2, it has been shown that the deamidation process caused protein unfolding. In Chapter 3, it was proposed that the lysine residues located within the sIWP molecule were exposed to the surface of the protein molecule as a result of protein unfolding. This allows the lysine residues of sIWP to be more accessible for carbohydrates to be conjugated. It is therefore suggested that the conjugation of carbohydrate onto sIWP occurred at the surface of the protein molecule to the lysine residues located at the C-terminal domain. Hence, this may account for why there is no change in protein secondary structure upon conjugation, regardless of the size/number of carbohydrate conjugated onto sIWP. Furthermore, the C-terminal domain of sIWP contains ~3 – 4 intramolecular disulphide bonds that could restrict changes in protein structure (e.g. protein unfolding).

4.3.1.4 Effect of Conjugation on Tryptophan Fluorescence Emission

Tryptophan fluorescence emission can be used to detect changes to the microenvironment of the tryptophan residue that usually buried in the hydrophobic core of the protein molecule. The polarity of the environment surrounding tryptophan residues affects the fluorescence emission maximum ($\lambda_{\text{max}}$). A red shift in $\lambda_{\text{max}}$ suggests that the tryptophan residues are in a more hydrophilic environment, whereas, a blue shift in $\lambda_{\text{max}}$ suggests that the tryptophan residues are in a more hydrophobic environment (Lakowicz 1999). According to the amino acid sequences of $\gamma$-gliadin, it contains 2 tryptophan residues located at the C-terminal domain of the protein molecule and these tryptophan residues are located close to the lysine residues (Figure 4.11A).
Figure 4.11: (A) A model showing the location of the 2 tryptophan residues located at the C-terminal domain of sIWP, (B & C) Models showing possible conjugation site of D10N dextran (6,400 Da) and D65N dextran (41,000 Da) in sIWP. Notes: (1) the location of the lysine residues indicated in the model is based on the lysine residues located in the amino acid sequence of γ-gliadin, (2) Figures are not to scale.

Figure 4.12 shows the tryptophan fluorescence emission spectrum of sIWP, soluble dry heated sIWP (72 h), soluble sIWP-D10N conjugate (72 h) and sIWP-D65N conjugate (72 h) in solution measured at neutral pH. The tryptophan fluorescence emission maximum (λ max) for sIWP is at 350.7 nm. There is a red shift in λ max observed for soluble dry heated sIWP (72 h) and soluble sIWP-D10N conjugate (72 h) to 353.7 nm and 352.6 nm respectively. The λ max for sIWP-D65N conjugate is at 350.7 nm, similar to the λ max of sIWP. A red shift observed for soluble dry heated sIWP (72 h) is unexpected and the reason for this shift could not be readily explained.
Figure 4.12: Tryptophan fluorescence emission spectra of sIWP, soluble dry heated sIWP (72 h), soluble sIWP-D10N conjugate (72 h) and sIWP-D65N conjugate (72 h) in solution measured at neutral pH. Note: (1) protein conjugates were prepared at 60 °C/75% RH, (2) asterisk indicates the average MW of dextran assumed to have conjugated onto sIWP from D10N dextran (6,400 Da) or D65N dextran (41,000 Da), (3) only the soluble fractions of dry heated sIWP (72 h) and sIWP-D10N conjugate (72 h) were analysed.

A red shift in $\lambda_{\text{max}}$ observed for soluble sIWP-D10N conjugate (72 h) indicates that the tryptophan residues within the protein molecule are in a more hydrophilic environment. This is possibly due to the conjugation of hydrophilic D10N dextran (6,400 Da) to the lysine residues, which are located within the vicinity of the tryptophan residues at the C-terminal domain of sIWP (Figure 4.11 B). As a result, the conjugated D10N dextran (6,400 Da) changed the microenvironment of these tryptophan residues to a more hydrophilic environment without changing the overall protein secondary structure dramatically. The lack of change in $\lambda_{\text{max}}$ for sIWP-D65N conjugate (72 h) to
that of sIWP, indicates that the microenvironment of the tryptophan residues were not affected after the protein was conjugated with D65N dextran (41,000 Da). This indicates that the site of conjugation for D65N dextran (41,000 Da) on sIWP is unlikely to be located within the vicinity of the tryptophan residues. It is possible that due to the larger hydrodynamic size of D65N dextran (d$_{nm}$: 13.8 nm) in comparison to D10N dextran (d$_{nm}$: 5.6 nm), the larger D65N dextran was preferentially conjugated at the N-terminal amino group of the protein molecule, rather than any of the lysine residues located at the C-terminal domain. The conjugation of D65N dextran (41,000 Da) at the N-terminal domain would keep the dextran far away from the tryptophan residues (Figure 4.11 C).

The site of conjugation for the dextrans proposed here provide further insight into why there was a reduction in zeta-potential observed for sIWP-D65N conjugate (72 h) but not for soluble sIWP-D10N conjugate (72 h) (as shown previously in Figure 4.4). This is possibly due to the difference in the site of conjugation, where D65N dextran (41,000 Da) was conjugated at the N-terminal domain of sIWP, which contains high level of negatively charged glutamic acid residues that lead to the shielding of protein charge. On the other hand, D10N dextran (6,400 Da) was conjugated at the C-terminal domain of sIWP, which contains mainly hydrophobic residues and little negatively charged glutamic acid residues.

Tryptophan fluorescence emission spectrum of sIWP, soluble dry heated sIWP (72 h), soluble sIWP-D10N conjugate (72 h) and sIWP-D65N conjugate (72 h) in solution were also measured at pH 4 (Figure 4.13). A blue shift in λ max to ~347 nm was observed for all the samples when the pH was adjusted from pH 7 to pH 4. This indicates that the tryptophan residues are in a more hydrophobic environment (Lakowicz 1999). This suggests that the protein molecules were aggregated at the
protein’s pI (~pH 4). However, as shown previously in section 4.3.1.2 that soluble sIWP-D10N conjugate (72 h) and sIWP-D65N conjugate (72 h) have lower extent of aggregation in solution at pH 4 in comparison to sIWP. It is possibly that the D10N dextran (6,400 Da) or the D65N dextran (41,000 Da) conjugated onto the sIWP could act as a physical barrier that prevent intermolecular protein aggregation, even though it could not prevent intramolecular protein aggregation when the pH is close to the protein’s pI (~pH 4).

Figure 4.13: Tryptophan fluorescence emission spectra of sIWP, soluble dry heated sIWP (72 h), soluble sIWP-D10N conjugate (72 h) and sIWP-D65N conjugate (72 h) in solution measured at pH 4. Note: (1) protein conjugates were prepared at 60 °C/75% RH, (2) asterisk indicates the average MW of dextran assumed to have conjugated onto sIWP from D10N dextran (6,400 Da) or D65N dextran (41,000 Da), (3) only the soluble fractions of dry heated sIWP (72 h) and sIWP-D10N conjugate (72 h) were analysed.
4.3.2 Interfacial Properties of Protein/Protein-conjugates

4.3.2.1 Adsorption Behaviour of sIWP

Figure 4.14 shows the interfacial layer thickness of sIWP adsorbed onto polystyrene particles as a function of protein concentration. The layer thickness of sIWP increased gradually with increasing protein concentration and reached a plateau of 18 nm at a protein concentration of 0.1 mg/mL. As discussed in Chapter 2, sIWP is composed mainly of monomeric gliadin. α/β- and γ-gliadin in particular have higher surface activity than ω-gliadin and polymeric glutenin (Keller et al. 1997). The long axis of α/β- and γ-gliadin is approximately 15 – 16 nm (Thomson et al. 1999). The slight difference between the measured layer thickness (i.e. ~18 nm) and the long axis of gliadin (i.e. 15 – 16 nm) is possibly due to changes in dynamic structure to the proteins by the deamidation process. The reduction in intramolecular hydrogen bonding and protein unfolding after the deamidation caused sIWP to be longer/bigger in its hydrated dynamic size than native gliadin. The difference in layer thickness observed between the different applied protein concentrations is possibly due to the orientation of the adsorbed sIWP at the polystyrene surface (Ornebro et al. 1999; Day et al. 2009). When the protein concentration is low, it would adopt a conformation that allows it to cover a larger surface area. With the increase in available protein molecules to the interface, the orientation of the adsorbed sIWP at the polystyrene surface could switch from a side-on orientation (major axis parallel to the surface) (Figure 4.14 B) to an end-on orientation (major axis perpendicular to the surface) (Figure 4.14 C) to allow more close packing of protein molecules at the interface. This transition was observed as the protein concentration increased from 0.00125 – 0.1 mg/mL.
Figure 4.14: Interfacial layer thickness of sIWP as a function of protein concentration.

[A] A model illustrating the hydrophilic and hydrophobic regions of sIWP. sIWP adsorbed on the polystyrene particles surface is postulated to switch from [B] a side-on orientation to [C] an end-on orientation as the protein concentration increased from 0.00125 – 0.1 mg/mL. Note: Figures are not to scale.

Chobert and co-workers (1996) found that the C-terminal peptides obtained from protease treated gliadin were more readily adsorbed to an emulsified oil droplet surface than the N-terminal peptides. Furthermore, many of the glutamine residues located at the N-terminal of sIWP would have been converted to negatively charged glutamic acid residues during the deamidation process. Hence, the N-terminal domain of sIWP would be largely negatively charged and will therefore prefer a hydrophilic environment, whereas the C-terminal domain contains many hydrophobic amino acids and little glutamic acid residues and would be likely to prefer hydrophobic environment. Therefore, when sIWP is adsorbed at an end-on orientation (i.e. at 0.1 mg/mL), its C-
terminal is postulated to anchor at the polystyrene surface, while the N-terminal forms a diffuse outer layer (Figure 4.14 A & C).

One of the important mechanisms by which a protein stabilises an emulsion droplet is through the formation of a thick layer at the interface, thus, providing steric repulsion against droplet flocculation (Brooksbank et al. 1993). The entropic stabilization theory that explains the steric stabilization mechanism, assumes that the droplet surface is impenetrable (Sato & Ruch 1980). When the steric layer of two individual droplets encounter with each other and are compressed, the polymer segments lose configurational entropy. This means that the polymer segments occupy fewer possible configurations in the compressed state than in the uncompressed state. The reduction in configurational entropy increases the Gibbs free energy change $\Delta G$, which produces a net repulsion between the two droplets and prevents them from flocculating (Sato & Ruch 1980). In this theory, the enthalpic interaction between the adsorbed layers and the dispersion medium is negligible so $\Delta G$ is reduced to:

$$\Delta G = -T \Delta S$$

The hydrodynamic layer thickness of the adsorbed protein at the interface is generally estimated by employing polystyrene particles as model emulsion droplets (Brooksbank et al. 1993; Wooster & Augustin 2006; Wooster & Augustin 2007a; Day et al. 2009). This is because it is not possible to produce and measure the size of a “bare” emulsion droplet onto which protein can be subsequently layered and measured using dynamic light scattering. This is because newly formed emulsion droplets rapidly coalesce to achieve global minimum state in the absence of sufficient protein emulsifier. In addition, homogenised emulsions are polydisperse. On the other hand, spherical
polystyrene particles can be obtained in monodisperse form and they are intrinsically stable, which is ideal for studying the hydrodynamic properties of the adsorbed protein layer. The primary mechanism involved in the adsorption of protein onto the surface of polystyrene particles is hydrophobic interactions (Brooksbank et al. 1993). However, it should be noted that protein molecule can penetrate further into a liquid surface (e.g. oil) and the protein can move more freely, whereas, adsorption on the polystyrene particles is on a solid surface. It has been suggested that processes such as diffusion, reorientation and conformational reorganisation of the adsorbed protein molecule will occur much faster at oil-water interfaces than on solid substrates (Dickinson 1999).

These results suggest that the thick steric layer formed by sIWP (i.e. ~18 nm) at the interface at neutral pH could prevent emulsion droplet from flocculation via steric repulsion. Day and co-workers (2009) showed that deamidated wheat protein prevents the flocculation of O/W emulsion (1 wt. % protein basis, 25 wt. % oil) at neutral pH via a combination of electrostatic and steric repulsion. However, the authors found that when the environment of the emulsion was at acidic pH (pH 4) or the ionic environment contained > 10 mM CaCl₂, the emulsion became flocculated. The emulsion instability under these conditions has been associated with the loss of protein charge and the collapse of steric layer (Day et al. 2009). In this study, one of the aims is to enhance the ability of sIWP to stabilise emulsions under these conditions by the conjugation of non-ionic carbohydrates onto sIWP to form an additional carbohydrate steric layer at the interface and to understand the effect of the size of the conjugated non-ionic carbohydrate on the carbohydrate steric layer thickness.
4.3.2.2 Effect of Conjugation on Interfacial Layer Thickness

- Glucose/Maltodextrin conjugates

Figure 4.15A shows the interfacial layer thickness of sIWP-Glc conjugate (24 h) and sIWP-M040 conjugate (24 h) adsorbed onto polystyrene particles as a function of protein concentration in comparison to the layer thickness of sIWP. The result shows that the protein-conjugates have the same layer thickness as that of sIWP throughout the whole range of protein concentration. This indicates that conjugation of 3 – 4 moles of glucose or LMW carbohydrates in the M040 maltodextrin (i.e. ~ 425 Da) per mole of sIWP is not sufficient to form an additional carbohydrate steric layer at the interface.

As shown in Chapter 3, the sIWP-Glc conjugate (24 h) was conjugated with approximately 4 moles of glucose per mole of sIWP, whereas the sIWP-M040 conjugate (24 h) was conjugated with approximately 3.5 moles of LMW carbohydrates in the M040 maltodextrin (i.e. ~ 425 Da) per mole of sIWP. The glucose molecule has a long axis of ~ 0.86 nm and a short axis of ~ 0.84 nm (Lourvanij & Rorrer 1994). Assuming that the LMW carbohydrate from the M040 maltodextrin (i.e. ~425 Da) that conjugated on sIWP-M040 conjugate (24 h) is a linear chain of 3 glucose units, it would have a long axis of ~2.6 nm and a short axis of ~0.84 nm. Figure 4.15 B illustrate the location of the N-terminal amino group and the 3 lysine residues located at the C-terminal domain of sIWP based on the amino acid sequence of γ-gliadin (CAC94871). The most reactive site for conjugation is the lysine residues, and followed by N-terminal amino group.
Figure 4.15: [A] Interfacial layer thickness of sIWP, sIWP-Glc conjugate (24 h) and sIWP-M040 conjugate (24 h) as a function of protein concentration. [B] A model of sIWP adsorbed at the polystyrene surface with the illustration of the location of the N-terminal amino group and the 3 lysine residues located at the C-terminal domain, based on the amino acid sequence of γ-gliadin (CAC94871). Note: (1) protein conjugates were prepared at 60 °C/75% RH, (2) asterisk indicates the calculated average MW of carbohydrates conjugated onto sIWP from glucose or the M040 maltodextrin (4300 Da) (calculation shown in Table 3.2 in Chapter 3). (3) Figures are not to scale.

If the glucose or the LMW carbohydrate from the M040 maltodextrin (i.e. ~425 Da) was conjugated at the 3 lysine residues located at the C-terminal domain of sIWP, these conjugated carbohydrates are too small to impart a carbohydrate steric layer because they would be shielded by the thick N-terminal diffuse layer of sIWP (i.e. ~18 nm). If the glucose or LMW carbohydrate from the M040 maltodextrin (i.e. ~425 Da) was conjugated at the N-terminal amino group of sIWP without imparting an additional carbohydrate steric layer as shown by the results above, this suggests either (a) that the N-terminal amino group is not located at the edge of the sIWP protein steric layer and
the conjugated carbohydrate is too small to impart a carbohydrate steric layer and/or (b) that the long axis of the conjugated carbohydrate is not projecting out of the sIWP protein steric layer into the aqueous phase to form a carbohydrate steric layer. Therefore, irrespective of where these carbohydrates were bound, these conjugated carbohydrates were too small to form an additional carbohydrate steric layer on top of the sIWP protein steric layer.

Wooster and Augustin (2007a) showed that β-lactoglobulin conjugated with ~6 maltodextrins of various MW (i.e. 900, 1900 and 3800 Da) per protein molecule, could form a carbohydrate steric layer that is 1.1, 2.5 and 7.3 nm thick respectively. The authors suggested that these protein-conjugates have a diblock copolymer architecture, where the outer layer is comprised of maltodextrin and the inner layer is comprised of β-lactoglobulin. The authors found that a minimum of 1.1 nm thick carbohydrate steric layer with a steric layer density of one tail per 7.5 nm² is required to provide steric stabilization against the flocculation of O/W emulsion (1 wt. % protein basis, 20 wt. % oil) in ionic environment that contained 150 mM NaCl. However, the results with sIWP showed that sIWP-Glc conjugate (24 h) and sIWP-M040 conjugate (24 h) did not form an additional carbohydrate steric layer at the interface due to the small size of the conjugated carbohydrates [i.e. glucose (180 Da) or LMW carbohydrates in the M040 maltodextrin (i.e. ~425 Da)]. These results suggest that sIWP-Glc conjugate (24 h) and sIWP-M040 conjugate (24 h) would not have any impact on the ability of sIWP to maintain emulsion stability in unfavourable conditions such as in CaCl₂ environment.
Dextran conjugates

Figure 4.16 shows the interfacial layer thickness of soluble sIWP-D10N conjugate (72 h) and sIWP-D65N conjugate (72 h) on the surface of the polystyrene particles as a function of protein concentration in comparison to the interfacial layer thickness of sIWP. A gradual increase in the interfacial layer thickness with increasing protein concentration was observed for all the samples and reached a plateau at a protein concentration of 0.1 mg/mL. At 0.1 mg/mL, soluble sIWP-D10N conjugate (72 h) and sIWP-D65N conjugate (72 h) formed a thicker interfacial layer than sIWP (i.e. ~18 nm) by approximately 3.5 nm and 5.9 nm respectively.

![Graph showing interfacial layer thickness vs protein concentration](image_url)

Figure 4.16: Adsorbed layer thickness of sIWP, soluble sIWP-D10N conjugate (72 h), and sIWP-D65N conjugate (72 h) as a function of protein concentration. Adsorbed layer thickness was determined from the difference between naked and protein adsorbed polystyrene particles. Note: (1) Protein conjugates were prepared at 60 °C/75% RH, (2) asterisk indicates the average MW of dextran assumed to have conjugated onto sIWP from D10N dextran (6,400 Da) or D65N dextran (41,000 Da), (3) only the soluble fractions of sIWP-D10N conjugate (72 h) were analysed.
The difference in interfacial layer thickness observed between sIWP and the protein-conjugates was not due to the presence of un-reacted dextrans in the samples as discussed below. Dynamic light scattering relies on the velocity of the Brownian motion of the particles in the solution to determine their particle size. It is possible that the velocity of the particles can be reduced if the un-reacted dextran increases the viscosity of the solution, which results in larger particle size measurements. The solution viscosity of sIWP, soluble sIWP-D10N conjugate (72 h) and sIWP-D65N conjugate (72 h) were measured at 0.1 mg/mL protein concentration. All the solutions showed similar viscosity of ~0.87 mPa/s at 24 °C. Furthermore, physical mixtures containing sIWP and D10N dextran (6,400 Da) or D65N dextran (41,000 Da) have the same interfacial layer thickness as that of sIWP (Figure 4.17). These results suggest that the presence of un-reacted dextrans in the protein-conjugates did not cause the thicker interfacial layer thickness observed in the soluble sIWP-D10N conjugate (72 h) and sIWP-D65N conjugate (72 h).

![Interfacial Layer Thickness](image)

Figure 4.17: Interfacial layer thickness of sIWP, and physical mixtures of sIWP and D10N dextran (6,400 Da) or D65N dextran (41,000 Da) as a function of protein concentration.
In order to further investigate whether the thicker interfacial layer formed by the soluble sIWP-D10N conjugate (72 h) and sIWP-D65N conjugate (72 h) was a carbohydrate steric layer, the interfacial layer of these protein-conjugates were subjected to dextranase digestion. As a control, the interfacial layer of sIWP was also subjected to dextranase digestion. The dextranase used in this study was a 1,6-glycosidase, which catalyses the endohydrolysis of dextran.

Prior to the dextranase experiment, the interfacial layer thickness of sIWP, soluble sIWP-D10N conjugate (72 h) and sIWP-D65N conjugate (72 h) were found to be increased by approximately 3 – 5 nm when the polystyrene particles adsorbed with the protein/protein-conjugates were left quiescent at room temperature for 2 h. After the 2 h period, no further increase in interfacial layer thickness was observed. One plausible explanation for the increased interfacial layer observed, is the occurrence of uneven packing of the adsorbed protein/protein-conjugates on the polystyrene surface. sIWP adsorbed at the air/water interface was shown to exhibit a weak fluid-like behaviour that is similar in nature to open rheomorphic caseins (Day et al. 2009). It is possible that the adsorbed protein/protein-conjugates on the polystyrene surface may have migrated slowly at the interface, which then allowed further adsorption of protein/protein-conjugates to the interface. This may have lead to uneven packing of the protein/protein-conjugates at the interface, which caused an artificially higher layer thickness as illustrated in Figure 4.18. It has been reported that sequentially adsorbed β-lactoglobulin-dextran conjugate on polystyrene particles lead to an artificially higher layer thickness due to uneven packing of the protein-conjugates on the polystyrene particle surface (Wooster & Augustin 2006).
Figure 4.18: This diagram illustrates the migration of the adsorbed protein on the polystyrene surface. This allowed [A] the adsorption of additional protein to the interface, which then [B] lead to uneven packing of protein at the interface. Note: Figures are not to scale.

Figure 4.19 shows the interfacial layer thickness of sIWP, soluble sIWP-D10N conjugate (72 h) and sIWP-D65N conjugate (72 h) that was left quiescent at room temperature (24 °C) for 2 h and then incubated with either (A) buffer as a control or (B) dextranase at room temperature (24 °C). The interfacial layer thickness was then measured as a function of time. In the buffer control, the interfacial layer thickness for all the samples remained constant throughout the whole measurement period. When dextranase was added, the thicker layer formed by sIWP-D65N conjugate (72 h) was reduced to that same layer thickness as sIWP alone. This suggests that the thicker layer observed in sIWP-D65N conjugate (72 h) was a dextran steric layer and confirms that the protein-conjugate was adsorbed at the interface. However, no reduction in adsorbed layer thickness was observed for the adsorbed layer by the soluble sIWP-D10N conjugate (72 h) after dextranase digestion.
Figure 4.19: The interfacial layer of sIWP, soluble sIWP-D10N conjugate (72 h) and sIWP-D65N conjugate (72 h) adsorbed on polystyrene particles that was left quiescent at room temperature (24 °C) for 2 h and then incubated with either (A) buffer as a control or (B) dextranase at room temperature (24 °C). The interfacial layer thickness was then measured as a function of time. Note: (1) Protein-conjugates were prepared at 60 °C/75% RH, (2) asterisk indicates the average MW of dextran assumed to have conjugated onto sIWP from D10N dextran (6,400 Da) or D65N dextran (41,000 Da), (3) only the soluble fractions of sIWP-D10N conjugate (72 h) were analysed.
The interfacial layer thickness of soluble sIWP-D10N conjugate (72 h) did not reduced to same thickness as sIWP alone after dextranase digestion, which suggests that the conjugated D10N dextran (6,400 Da) did not form a carbohydrate steric layer despite the fact that there was an increase in interfacial layer thickness as shown in Figure 4.16. As shown in Chapter 3, the soluble sIWP-D10N conjugate (72 h) was conjugated with approximately 1.3 mole of D10N dextran (6,400 Da) per mole of sIWP. The hydrodynamic size of the D10N dextran in solution determined by dynamic light scattering is ~5.6 nm. As discussed earlier in section 4.3.1.4 that the D10N dextran is conjugated at one of the three lysine residues located at the C-terminal domain of sIWP. It is worth reiterating that the C-terminal domain of sIWP anchors the protein molecule onto a hydrophobic surface, whereas the N-terminal domain of sIWP forms a thick diffuse layer (~18 nm) (Figure 4.20 A). Hence, the conjugated D10N dextran with a hydrodynamic size of ~5.6 nm would not be large enough to form a carbohydrate steric layer because it would be completely shielded by the diffused layer of sIWP as illustrated in Figure 4.20 B. The increased in interfacial layer thickness (~3.5 nm) observed for the soluble sIWP-D10N conjugate (72 h) in comparison to that of sIWP is possibly due to a change in adsorbed secondary structure caused by the conjugated D10N dextran (6,400 Da). Although the conjugation of D10N dextran on sIWP did not change the secondary structure of sIWP in solution, but it is possible that the conjugated D10N dextran affects the secondary structure of sIWP when adsorbed on a hydrophobic surface, which lead to an increase in interfacial layer thickness.
Figure 4.20: [A] Schematic illustration of the N-terminal domain (diffuse layer) and the C-terminal domain (anchor protein molecule onto hydrophobic surface) of sIWP adsorbed on a hydrophobic surface. [B] Schematic illustration of the site where D10N dextran (6,400 Da) was conjugated on sIWP. The conjugated D10N dextran with hydrodynamic size of ~5.6 nm is completely shielded by the N-terminal diffuse layer of sIWP. Note: Figures are not to scale.

The interfacial layer thickness of sIWP-D65N conjugate (72 h) was reduced to same thickness as sIWP alone after dextranase digestion, which suggests that the conjugated D65N dextran (41,000 Da) on sIWP forms a carbohydrate steric layer of ~5.9 nm thick. As shown in Chapter 3, sIWP-D65N conjugate (72 h) was conjugated with approximately 0.5 mole of D65N dextran (41,000 Da) per mole of sIWP. The
hydrodynamic size of the D65N dextran in solution determined by DLS is ~13.8 nm. Dextran behaves as an ellipsoid in solution with one axis longer than the other two. As discussed earlier in section 4.3.1.4, the D65N dextran could be conjugated at the N-terminal amino group located at the N-terminal domain of sIWP, rather than any lysine residues located at the C-terminal domain due to its size. Assuming that the long axis of the D65N dextran is ~13.8 nm, the increase in layer thickness of ~5.9 nm suggests that the long axis of the conjugated D65N dextran is horizontal to the polystyrene surface as illustrated in Figure 4.21 B. Alternatively, the conjugated D65N dextran could be conjugated at one of the three lysine residues located at the C-terminal domain of sIWP. Assuming that the long axis of the conjugated D65N dextran (~13.8 nm) is perpendicular to the polystyrene surface, this would mean that part of the D65N dextran (~7.9 nm) is shielded by the N-terminal diffuse layer of sIWP and forms a ~5.9 nm thick carbohydrate steric layer as illustrated in Figure 4.21 C. These results suggest that sIWP-D65N conjugate (72 h) may stabilize an emulsion against flocculation in unfavourable conditions such as in CaCl₂ environment because a minimum of 1.1 nm thick carbohydrate steric layer is sufficient to provide steric stabilization (Wooster & Augustin 2007a).
Figure 4.21: Schematic illustration of the possible conjugation sites for D65N dextran (hydrodynamic size: ~13.8 nm) on sIWP: [A] sIWP adsorbed on a hydrophobic surface with its C-terminal domain anchored onto hydrophobic surface and the N-terminal domain forms a diffuse layer, [B] D65N dextran conjugated at the N-terminal amino group located at the N-terminal domain of sIWP, [C] D65N dextran conjugated at one of the three lysine residues located at the C-terminal domain of sIWP. Note: Figures are not to scale.

4.3.2.3 Effect of pH on the Interfacial Layer Thickness

Results presented earlier in this chapter (section 4.3.1.2) showed that the sIWP-D65N conjugate (72 h) was able to prevent protein aggregation in solution at pH 4, therefore, it was speculated that it would also prevent particle aggregation when it was adsorbed onto polystyrene particles. This would allow the sIWP-D65N conjugate (72 h) adsorbed layer on polystyrene particles to be measured at a wider range of pH (pH 7.5 – 4) to understand the behaviour of the adsorbed layer as a function of pH.
The sIWP-D65N conjugate (72 h) adsorbed layer on polystyrene particles was left quiescent at room temperature (24 °C) for 2 h and then the pH was titrated from ~pH 7.5 to ~pH 4. As the pH was reduced from ~pH 7.5 to ~pH 4.5, the interfacial layer thickness was gradually reduced by ~7 nm (Figure 4.22). This is attributed to the collapse of the protein layer as the pH approached the protein’s pI as illustrated in Figure 4.22. This result concurs with earlier suggestion in section 4.3.1.4 that the conjugated D65N dextran (41,000 Da) does not prevent intramolecular aggregation of sIWP when the pH is close to the protein’s pI (~pH 4). However, an increased in interfacial layer thickness was observed when the pH reached ~pH 4 (protein’s pI). This is attributed to the aggregation of the small amount of un-reacted proteins in the sIWP-D65N conjugate (72 h).

![Figure 4.22](image)

Figure 4.22: [A] The sIWP-D65N conjugate (72 h) adsorbed layer on polystyrene particles was left quiescent at room temperature (24 °C) for 2 h and then the pH of the solution was titrated from ~pH 7.5 to ~pH 4. The interfacial layer thickness was measured as a function of pH. Illustration of the interfacial layer formed by sIWP-D65N conjugate (72 h) [B] at pH 7 and [C] collapsed at pH 4.5. Notes: Figures are not to scale.
4.3.2.3 Effect of Conjugation on Surface Topography

The surface topology of sIWP, soluble sIWP-D10N conjugate (72 h) and sIWP-D65N conjugate (72 h) was assessed using AFM via tapping mode in air. A planar polystyrene surface was used as model hydrophobic surface. The planar polystyrene surface was created via spin-coating a layer of polystyrene onto a cleaned glass surface. The hydrophobicity of the polystyrene coated glass surface was determined by measuring the contact angle of a water droplet. Figure 4.23A shows a freshly prepared clean glass surface with 0° contact angle, which indicates complete wettability (i.e. hydrophilic). After the glass surface was coated with a layer of polystyrene, the water contact angle increased to 88.0 ± 0.4° (Figure 4.23B). This shows that the surface was successfully coated with hydrophobic polystyrene layer. AFM image of the polystyrene coated surface showed that the surface is flat and featureless with surface roughness of approximately 1 – 2 nm (Figure 4.23 C and D).

Figure 4.23: The contact angle of a droplet of milli-Q water on [A] a freshly prepared clean glass surface and [B] a polystyrene spin-coated glass surface. [C] An AFM topographic image of a polystyrene spin-coated glass surface was taken and [D] the surface roughness was measured.
In order to test whether the buffer used for dispersing sIWP and the protein-conjugates would contribute any artefactual features on the AFM image, a polystyrene coated surface was incubated with buffer alone for 2 h and then rinsed exhaustively with milli-Q water. AFM image taken on the buffer treated surface remained flat and featureless as shown in Figure 4.24. Furthermore, the hydrophobicity of the buffer treated surface remained unchanged.

Figure 4.24: The [A] topographic image, [B] surface roughness and [C] water contact angle of a polystyrene spin-coated glass surface that was incubated with buffer solution for 2 h and rinsed exhaustively with milli-Q water.

A preliminary study was carried out to identify the optimal protein concentration that was required for the adsorption of protein onto the polystyrene surface. Figure 4.25 shows the AFM images taken on polystyrene surfaces adsorbed with sIWP (A) at 0.1 µg/mL and (B) at 10 µg/mL protein concentration. These AFM images showed that at lower protein concentration (i.e. 0.1 µg/mL), there were little protein particles adsorbed onto the polystyrene surface. When the protein concentration was increased to 10 µg/mL, the polystyrene surface was fully covered with protein particles.
Figure 4.25: AFM images taken on a polystyrene surface adsorbed with sIWP [A] at 0.1 µg/mL and [B] at 10 µg/mL protein concentration.

sIWP, soluble sIWP-D10N conjugate (72 h) and sIWP-D65N conjugate (72 h) was allowed to be adsorbed onto the hydrophobic polystyrene surface at 10 µg/mL protein concentration for 2 h and then rinsed exhaustively with milli-Q water to remove the non-adsorbed protein/protein-conjugates and un-reacted dextrans. AFM images of these surfaces were then taken and were shown in Figure 4.26.

Figure 4.26: AFM images taken on a polystyrene surface adsorbed with [A] sIWP, [B] soluble sIWP-D10N conjugate (72 h) and [C] sIWP-D65N conjugate (72 h) at 10 µg/mL protein concentration. Note: D10N dextran has an average MW of 6,400 Da and D65N dextran has an average MW of 41,000 Da.
Cross-section analysis was performed on the AFM images, where the heights of the protein or protein-conjugate particles were measured from the valley to the peak (Figure 4.27). The protein particle heights for sIWP, soluble sIWP-D10N conjugate (72 h) and sIWP-D65N conjugate (72 h) varies from 1 – 5 nm, 1 – 5 nm and 4 – 12 nm respectively. AFM has been used to study gluten proteins (e.g. gliadin) deposited on surfaces such as highly oriented pyrolytic graphite (HOPG) and freshly cleaved mica plate (McMaster et al. 1999; Tatham et al. 1999). However, there is no study reported in the literature involved the study of deamidated wheat protein and protein-conjugates adsorbed on polystyrene surface using AFM. McMaster and co-workers (1999) reported that α-gliadin formed a linked protein network with a height of 2 – 2.5 nm, based on AFM image taken in air using tapping mode on α-gliadin deposited on a freshly cleaved mica plate. The topography of α-gliadin deposited on the mica plate is similar to that of sIWP adsorbed on polystyrene surface reported here. The slight difference between the protein network height reported here (i.e. sIWP on polystyrene surface: 1 – 5 nm) to that of α-gliadin on mica plate (i.e. 2 – 2.5 nm) is possibly due to the deamidation process, which caused sIWP to appear longer/bigger than native gliadin due to the reduction in intramolecular hydrogen bonding and protein unfolding.

The results showed that soluble sIWP-D10N conjugate (72 h) has similar particle heights as sIWP, which suggests that the conjugated D10N dextran (6,400 Da) is not large enough to form a dextran steric layer. It is likely that the dextran was conjugated at one of the lysine residues located at the C-terminal domain, thus, residing at the side of the protein when it was adsorbed onto the hydrophobic surface. On the other hand, sIWP-D65N conjugate (72 h) formed much larger particles with taller particle heights as well as more spatially separated on the polystyrene surface than the
others. This indicates the formation of a dextran steric layer by the conjugated D65N dextran (41,000 Da). These results corroborate with those of the measured values for the interfacial layer thickness using DLS, where sIWP-D65N conjugate (72 h) was found to form a thicker layer thickness than sIWP. Differences observed between the particle heights measured from the AFM and the interfacial layer thickness measured from DLS and that there was no increased in particle height observed for soluble sIWP-D10N conjugate (72 h) to that of sIWP in AFM study is because the AFM study was performed in dry state, whereas the DLS study was performed in solution.

Figure 4.27: Cross-section analysis on the AFM images taken on a polystyrene surface adsorbed with [A] sIWP, [B] soluble sIWP-D10N conjugate (72 h) and [C] sIWP-D65N conjugate (72 h) at 10 µg/mL protein concentration. Note: D10N dextran has an average MW of 6,400 Da and D65N dextran has an average MW of 41,000 Da.
4.4 Conclusions

This part of the study showed that sIWP conjugated with ~3 – 4 moles of glucose or LMW carbohydrates in the M200 (900 Da), M100 (1,900 Da) or M040 (4,300 Da) maltodextrins (i.e. < 425 Da) per mole of sIWP, or ~1.3 or ~0.5 mole of dextran [D10N (6,400 Da) or D65N (41,000 Da)] per mole of sIWP did not change the protein’s pI, which remained at ~pH 4. However, the sIWP conjugated with dextrans appeared to aggregate to a lower extent than sIWP and other protein-conjugates (i.e. sIWP conjugated with glucose/ LMW carbohydrates in the maltodextrins) in solution at the protein’s pI. This is attributed to the conjugated dextran, which acts as a physical barrier against intermolecular protein aggregation at protein’s pI, although the conjugated dextran does not prevent intramolecular protein aggregation at protein’s pI.

This study also showed that conjugation of glucose, LMW carbohydrates in the maltodextrins (i.e. < 425 Da) or dextrans did not change the secondary structure of sIWP as determined by circular dichroism. Results from tryptophan fluorescence emission suggests that the D10N dextran (6,400 Da) was conjugated at one of the three lysine residues located at the C-terminal domain of sIWP, whereas D65N dextran (41,000 Da) was conjugated at the N-terminal amino group located at the N-terminal domain of sIWP.

This study also showed that conjugation of glucose, LMW carbohydrates in the M040 maltodextrin (i.e. ~425 Da) or D10N dextran (6,400 Da) was not sufficient to form a carbohydrate steric layer. This is because these carbohydrates are too small to form a carbohydrate steric layer and possibly shielded by the N-terminal diffusive layer of sIWP. On the other hand, this study showed that sIWP conjugated with D65N dextran (41,000 Da) is sufficient to form a ~5.9 nm thick carbohydrate steric layer, due
to its larger hydrodynamic size (i.e. ~13.8 nm) compared to the hydrodynamic size of the conjugated carbohydrates of other protein-conjugates (i.e. < 5.6 nm). Hence, these results suggest that the prevision of additional carbohydrate steric layer on top of the protein steric layer at the interface is dependent on the size of the carbohydrate and the site of conjugation.
CHAPTER 5

The Effect of Conjugation on the Ability of sIWP to Maintain Emulsion Stability at Acidic pH and in Ionic Environments

5.1 Introduction

Emulsions are stabilised by proteins via a combination of electrostatic and steric mechanisms (Damodaran 2005). Protein-stabilised emulsions flocculate when the pH environment is near to the protein’s isoelectric point (pI) or in ionic environments. This is due to the loss of electrostatic charge and/or the collapse of the protein steric layer (Dalgleish 1997). A number of studies have shown that protein conjugated with non-ionic carbohydrates via the Maillard reactions can enhance emulsion stability, even in unfavourable conditions (Akhtar & Dickinson 2003; Dunlap & Cote 2005; Wooster & Augustin 2006). This enhancement has been attributed to the steric stabilisation provided by the conjugated hydrophilic carbohydrate moiety (Akhtar & Dickinson 2003; Wooster & Augustin 2006; Wooster & Augustin 2007a).

The results in Chapter 3 showed that approximately 3 – 4 moles of glucose or LMW carbohydrate fraction in the maltodextrins (< 425 Da) were conjugated per mole of sIWP, whereas, approximately 1.3 or 0.5 moles of dextrans (6,400 or 41,000 Da) were conjugated per mole of sIWP. In Chapter 4, the results presented showed that sIWP conjugated with glucose or LMW carbohydrate fraction in the 4,300 Da maltodextrin (i.e. ~425 Da) or 6,400 Da dextran did not form an additional carbohydrate steric layer. On the other hand, sIWP conjugated with 41,000 Da dextran is sufficient to
increase the steric layer thickness beyond that of sIWP alone, where the increase is due to carbohydrate steric layer (~6 nm thick) formed.

The aim of this chapter was to investigate the ability of sIWP and the protein-conjugates to stabilise O/W emulsion at acidic pH and in ionic environments. It was hypothesized that conjugation of sIWP with non-ionic carbohydrates results in a covalently-linked complex which could enhance emulsion stability at acidic pH and/or in ionic environment compared to sIWP (non-conjugated). Furthermore, it was hypothesized that the effectiveness of conjugation to improve emulsion stability is dependent on the size of the carbohydrate that was conjugated onto the protein.

5.2 Materials and Methods

5.2.1 Materials

The protein-conjugates were prepared according to the dry reaction method described in section 3.2.4 (Chapter 3). Freeze-dried mixture containing sIWP and M040 maltodextrin (4,300 Da) was dry heated at 60 °C/75% RH for 24 h, whereas freeze-dried mixture containing sIWP and dextran [D10N dextran (6,400 Da) or D65N dextran (41,000 Da)] were dry heated at 60 °C/75% RH for 72 h. Soluble fraction of sIWP dry heated with D10N dextran (6,400 Da) for 72 h was used for this study. sIWP-M040 conjugate (24 h) and sIWP-D65N conjugate (72 h) were used as it is. The names given to the protein-conjugates were described in Table 4.1 (Chapter 4). It should be noted that the protein-conjugates used for analysis contain a mixture of conjugated and non-conjugated proteins, and unreacted carbohydrates.
Crisco canola oil (Goodman Fielder Ltd, Australia) was purchased from a local supermarket and used as the emulsion oil phase throughout. Other analytical grade chemicals were purchased from Sigma Aldrich (Sydney, Australia).

5.2.2 Emulsion Preparation

Emulsions were prepared by homogenising 20 wt. % canola oil and 80 wt. % aqueous sIWP/sIWP-dextran conjugate solution (pH 7) at room temperature (24 °C). In all cases, the final protein concentration is 1 wt. % (on a protein basis) in emulsion. A coarse emulsion was first prepared by mixing the oil and protein solution with an Ultra-turrax mixer at 11,500 rpm for 2 min. A fine emulsion was then prepared by passing the coarse emulsion through a valve homogeniser (2 passes at 80 bar). Sodium azide (0.02 wt. %) was added to all emulsions after formation to prevent microbial contamination.

5.2.3 Emulsion Zeta-potential

The zeta-potential measurements were conducted using a Malvern Nano ZS instrument (Worcestershire, UK). Prior to analysis, emulsions were diluted 10,000 fold with milli-Q water. The effect of pH on zeta-potential of the emulsion was assessed by adjusting the emulsion pH using 0.1 M HCl. Then an aliquout of the emulsion was transferred into a folded capillary cell for zeta-potential measurement. The zeta-potential of the emulsions were obtained by measuring the direction and velocity of droplet movement when subjected to an applied electric field. The results reported are an average of three measurements.
5.2.4 Emulsion Droplet Size

The droplet size of the emulsion was measured by laser light scattering using a Malvern Mastersizer 2000 instrument (Malvern Instruments Ltd, Worcestershire, UK). The effect of pH on emulsion droplet size was assessed by adjusting the emulsion pH using 0.5 M HCl. The effect of CaCl$_2$ on the emulsion droplet size was assessed by exposing the emulsion to a range of CaCl$_2$ concentrations (0 – 20 mM) for 24 h. An aliquot of the emulsion sample was then transferred into the recirculating water of a Hydro SM sample dispersion unit until an obscuration rate of 10 – 20% was obtained. A differential refractive index of 1.102 (1.466 for oil/1.33 for water) and the absorption of 0.001 were used as the optical properties of the emulsion. Emulsion droplet sizes were then obtained via a best fit between light scattering (Mie) theory and the measured light scattering pattern. The mean emulsion droplet size is quoted the volume-length mean diameter $d_{4,3}$ ($d_{4,3} = \Sigma n_i d_i^4 / \Sigma n_i d_i^3$). The results reported are an average of three measurements.

5.2.5 Confocal Microscopy

The emulsion microstructure was examined using a Leica SP5 Confocal Laser Scanning Microscope (CLSM) equipped with an argon ion laser (Leica Microsystems, Wetzlar, Germany). The emulsion sample (0.4 mL) was then mixed with 20 µl of fluorescence dye Nile Red (0.025% dissolved in 0.1% dimethyl sulfoxide/ 99.9% ethanol) in a 1.5 mL Eppendorf tube and then gently mixed using a pipette. The sample was then transferred onto a cavity slide and covered with a cover slide with minimum presence of air bubbles. The emulsion was then observed under the CLSM.
5.3 Results and Discussion

5.3.1 Effect of Conjugation on sIWP’s Ability to Maintain Emulsion Stability at Acidic pH

sIWP and protein-conjugates emulsions were first prepared at pH 7. The stability of these emulsions at acidic conditions was then assessed by monitoring changes in emulsion droplet size and confocal microstructure by adjusting the pH of the emulsion from pH 7 to pH 4.

5.3.1.1 sIWP Emulsion Stability at Acidic pH

Figure 5.1A shows the droplet size of sIWP emulsion (20 wt. % oil, 1 wt. % protein basis) as a function of pH. At pH 7, the average droplet size was 1.68 ± 0.01 µm and the confocal image showed a stable emulsion (Figure 5.1C). When the pH of the emulsion was adjusted from pH 7 to pH 4, the average droplet size increased significantly from 1.68 ± 0.01 µm to 142.06 ± 1.56 µm and the confocal image showed that the emulsion has flocculated (Figure 5.1D).

The results presented in Chapter 4 showed that sIWP forms a thick interfacial layer (~18 nm) at pH 7. Figure 5.1 B showed that emulsion stabilised by sIWP had an average zeta-potential of ~ − 50 mV at pH 7. A minimum of ~ − 30 mV zeta-potential is generally required to electrically stabilize colloidal particles, such as emulsion (Hunter 1981). This is similar to other protein stabilised emulsions such as caseins, which has zeta-potential ranging from − 50 to − 70 mV (Dickinson 1998). These results suggest that sIWP stabilises the emulsion at pH 7 through forming a negatively charged thick
interfacial layer that provides electrostatic and steric repulsion against droplet flocculation.

Figure 5.1: Effect of pH on the [A] droplet size and [B] zeta-potential of emulsion stabilised by sIWP. Microstructure of emulsion stabilised by sIWP visualised by confocal microscopy [C] at pH 7 and [D] at pH 4. Notes: Emulsions are composed of 20 wt. % canola oil, 1 wt. % protein basis, y-axis in log scale.

However, emulsion stabilised by sIWP became flocculated when the pH of the emulsion was adjusted from pH 7 down to pH 4. Figure 5.1B shows that the zeta-potential of the emulsion stabilised by sIWP increased slightly from – 50 mV at pH 7 to
− 55 mV at pH 6 and then decreased to 0 mV at pH 4. It should be noted that there is evidence of flocculation even when the zeta-potential reported is greater than −30 mV (Figure 5.1 A & B). This is likely due to the flocculation of some emulsion droplets that exhibit lower emulsion zeta-potential (i.e. less than −30 mV) than the average emulsion zeta-potential reported above. At pH 4, the emulsion stabilised by sIWP has insufficient surface charge to stabilise the emulsion droplets electrostatically against flocculation. This is because the proteins adsorbed at the interface loss their protein charge as the pH decreased from pH 7 to pH 4, due to the conversion of the many α-carboxyl group of the glutamate residues located within the protein molecule from −COO− to −COOH. This is supported by the results presented in Chapter 4 (section 4.3.1.2), which showed that sIWP dispersed in water at pH 7 loss their protein charge when the pH was adjusted from pH 7 to pH 4 (protein’s pI, zeta-potential: 0 mV) and became aggregated. The loss of protein charge and protein aggregation suggests that the thick protein steric layer (~18 nm) formed by sIWP at the emulsion interface collapse at pH 4, due to the reduction in intermolecular and intramolecular protein repulsion force. Therefore, sIWP adsorbed at the interface is unavailable to provide electrostatic and steric repulsion for the emulsion droplets at pH 4. This allowed various attractive interactions between the emulsion droplets such as van der Waals and hydrophobic interactions, which lead to the flocculation of the emulsion droplets (McClements 2005).

It is worth noting that the slight increase in zeta-potential observed for emulsion stabilised by sIWP from −50 mV at pH 7 to −55 mV at pH 6 was not observed during the measurement of the zeta-potential of sIWP in solution from pH 7 to pH 6 (Figure 5.2). The differences in zeta-potential between pH 7 and pH 6 in emulsion stabilised by sIWP and sIWP in solution was not due to changes in protein secondary structure when
sIWP was adsorbed at the emulsion interface (result not shown). Hence, the difference observed is more likely to be due to the specific orientation adopted by sIWP at the interface, where the C-terminal domain of sIWP anchored the protein molecule to the interface, whereas the N-terminal domain of sIWP formed the protein diffuse layer. Zeta-potential is measured at the slipping plane of the particle, which is the outer boundary surrounding the particle (Hunter 1981). This means that for the adsorbed sIWP on the emulsion droplets, the zeta-potential measured is primarily influenced by the charge on the N-terminal domain of sIWP. For sIWP in solution, the zeta-potential measured takes into account the charge on both N-terminal and C-terminal domain of sIWP. However, most of the charged amino acid residues within sIWP (e.g. glutamic acid residues) are located at the hydrophilic N-terminal domain of sIWP, and little located at the hydrophobic C-terminal domain of sIWP. Hence, only slight difference was observed between the zeta-potential of the emulsion stabilised by sIWP to that of sIWP in solution.

![Zeta-potential of emulsion stabilised by sIWP and the zeta-potential of sIWP measured in solution as a function of pH.](image)

Figure 5.2: The zeta-potential of emulsion stabilised by sIWP and the zeta-potential of sIWP measured in solution as a function of pH.
5.3.1.2 Effect of Conjugation of sIWP in the Presence of M040 maltodextrin (4,300 Da) on Emulsion Stability at Acidic pH

The results presented in Chapter 3 showed that sIWP heated with glucose or various MW maltodextrins (i.e. M200 (900 Da), M100 (1,900 Da) and M040 (4,300 Da) for 24 h at 60 °C/75% RH resulted in the attachment of approximately 3 – 4 moles of glucose or LMW carbohydrates in the M200, M100 or M040 maltodextrins (i.e. < 425 Da) conjugated per mole of sIWP. Due to the similarity in the number of carbohydrate conjugated on the protein-conjugates, sIWP-M040 conjugate (24 h) with the largest attached carbohydrate (i.e. ~425 Da) was selected for the emulsion study.

Figure 5.3A shows the droplet size of the emulsion stabilised by sIWP-M040 conjugate (24 h) as a function of pH in comparison to sIWP emulsion. At pH 7, the average emulsion droplet size for sIWP-M040 conjugate is ~1.85 ± 0.02 μm and the confocal image showed a stable emulsion (Figure 5.3C). The results presented in Chapter 4 showed that sIWP-M040 conjugate (24 h) forms a thick interfacial layer (~18 nm) similar to sIWP at pH 7. Figure 5.3B showed that emulsion stabilised by sIWP-M040 conjugate (24 h) had an average zeta-potential of ~ – 50 mV at pH 7. These results suggest that sIWP-M040 conjugate (24 h) stabilises the emulsion at pH 7 through forming a negatively charged thick interfacial layer that provides electrostatic and steric repulsion against droplet flocculation.
Figure 5.3: Effect of pH on the [A] droplet size and [B] zeta-potential of emulsion stabilised by sIWP-M040 conjugate (24 h) in comparison to that stabilised by sIWP. Microstructure of emulsion stabilised by sIWP-M040 conjugate (24 h) visualised by confocal microscopy [C] at pH 7 and [D] at pH 4. Notes: (1) Emulsions are composed of 20 wt. % canola oil, 1 wt. % protein basis. (2) Asterisk indicates the calculated average MW of carbohydrates conjugated onto sIWP from M040 maltodextrin (4300 Da) (calculation shown in Table 3.2 in Chapter 3).

When the pH of the emulsion stabilised by sIWP-M040 conjugate (24 h) was adjusted from pH 7 to pH 4, the emulsion droplet size increased significantly and the confocal image showed strongly flocculated droplets (Figure 5.3D). The zeta-potential of the emulsion stabilised by sIWP-M040 conjugate (24 h) increased slightly from – 50
mV at pH 7 to −55 mV at pH 6 and then decreased to 0 mV at pH 4 similar to sIWP (Figure 5.3B). This shows that at pH 4, the emulsion stabilised by sIWP-M040 conjugate (24 h) has insufficient surface charge to stabilise the emulsion droplets electrostatically against flocculation. Results presented in Chapter 4 showed that sIWP-M040 conjugate (24 h) dispersed in solution loss its protein charge when the pH was adjusted from pH 7 to pH 4 (i.e. at protein’s pI with 0 mV zeta-potential) and aggregated. The loss of protein charge and protein aggregation suggests that the thick protein steric layer (~18 nm) formed by sIWP-M040 conjugate (24 h) at the emulsion interface collapse at pH 4, due to the reduction in intermolecular and intramolecular protein repulsion force. Therefore, emulsion stabilised by sIWP-M040 conjugate (24 h) became flocculated at pH 4 due to the loss of electrostatic and steric repulsion force that prevent the flocculation emulsion droplets. This concurs with the results presented in Chapter 4, which showed that the conjugated 3 – 4 moles of LMW carbohydrate from the M040 maltodextrin (i.e. ~425 Da) per mole of sIWP are not large enough to form a carbohydrate steric layer at the interface, and hence cannot provide steric repulsion against droplet flocculation at acidic pH.

Figure 5.4 shows the creaming stability of emulsions stabilised by sIWP and sIWP-M040 conjugate (24 h) after stored at pH 4 (24 ºC) for 24 h. These emulsions formed a creamed layer, leaving a droplet-depleted layer at the bottom. This happens because flocculated droplets in these emulsions have a larger effective size than the individual droplets, which increases the creaming velocity.
5.3.1.3 Effect of conjugation of dextran on emulsion stability at acidic pH

Results presented in Chapter 3 showed that sIWP heated with D10N dextran (6,400 Da) or D65N dextran (41,000 Da) for 72 h at 60 °C/75% RH resulted in ~1.3 mole and ~0.5 mole of dextran conjugated per mole of sIWP respectively. Figure 5.5 shows the droplet size of emulsions stabilised by sIWP, soluble sIWP-D10N conjugate (72 h) and sIWP-D65N conjugate (72 h) as a function of pH. At pH 7, the average droplet size of emulsions stabilised by sIWP, soluble sIWP-D10N conjugate (72 h) and sIWP-D65N conjugate (72 h) is ~1.68 ± 0.01 µm, ~1.71 ± 0.01 µm and ~1.59 ± 0.01 µm respectively and all emulsions appeared stable as shown in the confocal images (Figure 5.7A – C).
Figure 5.5: The droplet size of emulsions stabilised by sIWP, soluble sIWP-D10N conjugate (72 h) and sIWP-D65N conjugate (72 h) as a function of pH. Notes: (1) Emulsions are composed of 20 wt. % canola oil, 1 wt. % protein basis, (2) asterisk indicates the average MW of dextran assumed to have conjugated onto sIWP from D10N dextran (6,400 Da) or D65N dextran (41,000 Da), (3) only the soluble fractions of sIWP-D10N conjugate (72 h) were analysed.

The results presented in Chapter 4 showed that soluble sIWP-D10N conjugate (72 h) and sIWP-D65N conjugate (72 h) forms a thick interfacial layer (∼21.5 nm and ∼23.9 nm respectively) at pH 7. Figure 5.6 showed that emulsion stabilised by soluble sIWP-D10N conjugate (72 h) and sIWP-D65N conjugate (72 h), both had an average zeta-potential of ∼ – 50 mV at pH 7. These results suggest that soluble sIWP-D10N conjugate (72 h) and sIWP-D65N conjugate (72 h) stabilises the emulsion at pH 7 through formation of a negatively charged thick interfacial layer that provides electrostatic and steric repulsion against droplet flocculation.
Figure 5.6: The zeta-potential of emulsions stabilised by sIWP, soluble sIWP-D10N conjugate (72 h) and sIWP-D65N conjugate (72 h) as a function of pH. Notes: (1) Emulsions are composed of 20 wt. % canola oil, 1 wt. % protein basis, (2) asterisk indicates the average MW of dextran assumed to have conjugated onto sIWP from D10N dextran (6,400 Da) or D65N dextran (41,000 Da), (3) only the soluble fractions of sIWP-D10N conjugate (72 h) were analysed.

When the pH of the emulsion stabilised by soluble sIWP-D10N conjugate (72 h) was adjusted from pH 7 to pH 4, the emulsion droplet size increased from 1.71 ± 0.01 µm to 16.03 ± 1.39 µm (Figure 5.5) and the confocal image showed that the latter emulsion (i.e. at pH 4) has flocculated (Figure 5.7E). The zeta-potential of the emulsion stabilised by soluble sIWP-D10N conjugate (72 h) increased slightly from −50 mV at pH 7 to −55 mV at pH 6 and then decreased to 0 mV at pH 4, which is similar to sIWP (Figure 5.6). This shows that at pH 4, the emulsion stabilised by soluble sIWP-D10N
conjugate (72 h) has insufficient surface charge to stabilise the emulsion droplets electrostatically against flocculation.

Figure 5.7: Microstructure of emulsions stabilised by sIWP, soluble sIWP-D10N conjugate (72 h), and sIWP-D65N conjugate (72 h) visualised by confocal microscopy at pH 7 and at pH 4. Notes: (1) Emulsions are composed of 20 wt. % canola oil, 1 wt. % protein basis, (2) asterisk indicates the average MW of dextran assumed to have conjugated onto sIWP from D10N dextran (6,400 Da) or D65N dextran (41,000 Da), (3) only the soluble fractions of sIWP-D10N conjugate (72 h) were analysed.

Results presented in Chapter 4 showed that soluble sIWP-D10N conjugate (72 h) dispersed in solution loss its protein charge when the pH was adjusted from pH 7 to pH 4 (i.e. at protein’s pI with 0 mV zeta-potential), which is similar to sIWP. However, as shown in Chapter 4, it appeared that the soluble sIWP-D10N conjugate (72 h) dispersed in solution was aggregated at much lower extent than sIWP at pH 4 (Optical
density at 600 nm: ~0.36 and ~0.61 respectively) and this may be attributed to the conjugated D10N dextran (6,400 Da) that contributed to increasing the physical barrier against protein aggregation. It is worth noting that the confocal image of emulsion stabilised by soluble sIWP-D10N conjugate (72 h) showed that the emulsion was weakly flocculated at pH 4, whereas emulsion stabilised by soluble sIWP was strongly flocculated at that pH. It is possible that as the pH of the emulsion stabilised by soluble sIWP-D10N conjugate (72 h) decreased from pH 7 to pH 4, the N-terminal protein diffuse layer became collapsed due to the loss of protein charge. In turn, this exposed the D10N dextran (6,400 Da) conjugated at the C-terminal domain of sIWP that was originally shielded by the N-terminal diffuse layer of sIWP at pH 7. As a result the conjugated D10N dextran (6,400 Da) on sIWP is able to prevent emulsion droplets from being strongly flocculated at pH 4 by acting as a physical barrier.

On the other hand, when the pH of the emulsion stabilised by sIWP-D65N conjugate (72 h) was adjusted from pH 7 to pH 4, the emulsion droplet size remained constant at 1.59 ± 0.01 µm (Figure 5.5) and the confocal image showed stable emulsion at pH 4 (Figure 5.7F). The zeta-potential of the emulsion stabilised by sIWP-D65N conjugate (72 h) decreased gradually from −50 mV at pH 7 to 0 mV at pH 4 (Figure 5.6). This shows that at pH 4, the emulsion stabilised by sIWP-D65N conjugate (72 h) has insufficient surface charge to stabilise the emulsion droplets electrostatically against flocculation. It is worth noting that emulsion stabilised by sIWP-D65N conjugate (72 h) exhibited much lower zeta-potential throughout the pH range (7 – 4) in comparison to emulsion stabilised by sIWP, sIWP-M040 conjugate (24 h) and soluble sIWP-D10N conjugate (72 h). This is likely attributed to the conjugation of a large dextran molecule (i.e. 41,000 Da) that physically screened the surface charge. This is because zeta-
potential is measured at the slip plane of the emulsion droplet, which is at the edge of the dextran layer. It has been reported that emulsions stabilised by β-lactoglobulin conjugated with dextrans (18,500 – 440,000 Da) have decreased surface charge due to physically screening by the addition of dextran layer (Wooster & Augustin 2006). This was not observed on emulsion stabilised by soluble sIWP-D10N conjugate (72 h), which confirms that the conjugated D10N dextran (6,400 Da) is located at the C-terminal domain and was shielded by the N-terminal protein diffuse layer.

Results presented in Chapter 4 showed that sIWP-D65N conjugate (72 h) forms a carbohydrate steric layer of ~5.9 nm thick. It has been reported that a minimum of 1.1 nm thick carbohydrate steric layer with a steric layer density of one tail per 7.5 nm² is required to provide steric stabilization against the flocculation of oil/water emulsion (1 wt. % protein basis, 20 wt. % oil) in ionic environment (150 mM NaCl) (Wooster & Augustin 2007a). Hence, it is likely that the reason that the emulsion stabilized by sIWP-D65N conjugate (72 h) did not flocculate at pH 4 is due to steric repulsion afforded by the thick carbohydrate steric layer (~5.9 nm) formed by the conjugated D65N dextran (41,000 Da) at the interface. It is worth noting that the interfacial layer formed by sIWP-D65N conjugate (72 h) may collapse slightly as shown in results presented in Chapter 4, due to the collapse of the protein layer. This is because the conjugated D65N dextran (41,000 Da) does not prevent intramolecular aggregation of the protein molecules when the pH is close to the protein’s pI (i.e. ~pH 4). However, the collapse of the protein layer does not affect the conjugated D65N dextran from providing steric stabilisation against emulsion flocculation at acidic pH.

The stability of the emulsion stabilised by sIWP-D65N conjugate (72 h) after prolonged storage at pH 4 was also investigated. Figure 5.8 shows the droplet size and
confocal image of emulsion stabilised by sIWP-D65N conjugate (72 h) after storage for up to 39 days at pH 4 (24 ºC). The droplet size of the emulsion remained unchanged throughout the whole storage period from day 1 to day 39. No obvious droplet flocculation/coalescence was observed on the emulsion microstructure after prolonged storage for 39 days. These results showed that emulsion stabilised by sIWP-D65N conjugate (72 h) is stable against flocculation and coalescence for up to 39 days of storage at pH 4.

![Graph](image)

Figure 5.8: [A] The droplet size of emulsion stabilised by sIWP-D65N conjugate (72 h) was monitored for up to 39 days at pH 4 (24 ºC). The emulsion microstructure visualised by confocal microscopy [B] at day 1 and [C] at day 28. Notes: Emulsions are composed of 20 wt. % canola oil, 1 wt. % protein basis.

The improved emulsion stability at pH 4 observed on emulsions stabilised by soluble sIWP-D10N conjugate (72 h) and sIWP-D65N conjugate (72 h) in comparison to emulsions stabilised by sIWP was not due to the presence of un-reacted dextran in the protein-conjugates. Figure 5.9 shows the droplet size and zeta potential of the emulsions stabilised by sIWP and physical mixtures of sIWP and dextran [i.e. D10N dextran...
(6,400 Da) or D65N dextran (41,000 Da)] as a function of pH. It should be noted that the level of dextran contained in these protein-dextran physical mixtures are equivalent to that in the soluble sIWP-D10N conjugate (72 h) and sIWP-D65N conjugate (72 h).

![Figure 5.9: Effect of pH on [A] the droplet size and [B] the zeta-potential of emulsion stabilised by sIWP and physical mixtures containing sIWP and dextran [i.e. D10N dextran (6,400 Da) or D65N dextran (41,000 Da)]. Notes: Emulsions are composed of 20 wt. % canola oil, 1 wt. % protein basis, y-axis in log scale.](image)

The average droplet size of emulsions stabilised by physical mixtures containing sIWP-D10N dextran (6,400 Da) and sIWP-D65N dextran (41,000 Da) at pH 7 is $\sim 1.71 \pm 0.01 \ \mu m$ and $\sim 1.59 \pm 0.01 \ \mu m$ respectively. The droplet size of these emulsions increased significantly as the pH of the emulsions was adjusted from pH 7 to pH 4 (Figure 5.9), and the confocal image showed strongly flocculated droplets at pH4 (Figure 5.10). Furthermore, the zeta-potential of these emulsions increased slightly from $-50 \ \text{mV}$ at pH 7 to $-55 \ \text{mV}$ at pH 6 and then decreased to 0 mV at pH 4. These results showed that physical mixture containing sIWP-D10N dextran (6,400 Da) and sIWP-D65N dextran (41,000 Da) behave similarly to the emulsion stabilised by sIWP. Hence,
these results confirmed that un-reacted dextran present in the protein-conjugate samples does not have any effect on the emulsion stability. This is not surprising because the type of dextran used in this study is hydrophilic and uncharged, which means that there is no electrostatic interaction between the protein component and the dextran component. This is unlike anion polysaccharide such as pectin, which can interact with β-lactoglobulin via electrostatic complexation (Ganzevles, 2006) and may adsorbed onto the oil/water emulsion interface and provide stability at acidic pH.

![Microstructure of emulsions](image)

**Figure 5.10:** Microstructure of emulsions stabilised by sIWP, and physical mixtures containing sIWP and dextran [i.e. D10N dextran (6,400 Da) or D65N dextran (41,000 Da)] visualised by confocal microscopy at pH 7 and at pH 4. Notes: Emulsions are composed of 20 wt. % canola oil, 1 wt. % protein basis.

Figure 5.11 shows the creaming stability of emulsions stabilised by sIWP, soluble sIWP-D10N conjugate (72 h), and sIWP-D65N conjugate (72 h) after stored at
pH 4 (24 °C) for 24 h. Emulsions stabilised by sIWP, soluble sIWP-D10N conjugate (72 h) formed a creamed layer at pH 4, leaving a droplet-depleted layer at the bottom. This happens because flocculated droplets in these emulsions have a larger effective size than the individual droplets, which increase the creaming velocity. On the other hand, no creaming was observed on emulsion stabilised by sIWP-D65N conjugate (72 h) because the conjugated D65N dextran (41,000 Da) prevented the emulsion from flocculation at pH 4 via steric repulsion.

Figure 5.11: Creaming stability of emulsions stabilised by sIWP, soluble sIWP-D10N conjugate (72 h) and sIWP-D65N conjugate (72 h) after stored at pH 4 (24 °C) for 24 h.

A number of studies have reported that the conjugation of whey proteins or caseins with maltodextrins (1,200 – 8,700 Da) formed protein-conjugates with improved ability to maintain emulsion stability at acidic pH close their isoelectric point (pI) (Shepherd et al. 2000; O'Regan & Mulvihill 2009). These studies have suggested that the improvement observed was due to improvement in protein solubility at acidic pH and the formation of a carbohydrate steric layer at the interface that provide steric stabilisation, which is in-line with the results shown in this study for primarily the
sIWP-D65N conjugate (72 h). However, the previous studies did not measure the carbohydrate steric layer thickness formed by the protein-conjugates (Shepherd et al. 2000; O'Regan & Mulvihill 2009). The present study showed that sIWP conjugated with D65N dextran (41,000 Da) is able to form a ~5.9 nm thick carbohydrate steric layer at the interface. Furthermore, the emulsion stabilised by this protein-conjugate (i.e. sIWP-D65N conjugate (72 h)) has 0 mV zeta-potential at pH 4 and there is no emulsion flocculation observed at this pH. This showed that the emulsion stabilised by this protein-conjugate was not due to electrostatic repulsion, and it was due to the conjugated D65N dextran (41,000 Da), which provide steric stabilisation against emulsion flocculation.

5.3.1.4 Proposed Emulsion Stabilising Mechanisms of sIWP and the Protein-conjugates at Acidic pH

Models of sIWP, sIWP-M040 conjugate (24 h), soluble sIWP-D10N conjugate (72 h) and sIWP-D65N conjugate (72 h) adsorbed at the interface of O/W emulsion and the changes occurred to the adsorbed layer as the pH decreased from pH 7 to pH 4 are shown in Figure 5.12:

(A) – At pH 7, sIWP stabilises O/W emulsion by forming a negatively charged thick interfacial layer that provides electrostatic and steric repulsion against droplet flocculation. The emulsion stabilised by sIWP became strongly flocculated as the pH decreased from pH 7 to pH 4, due to the loss of protein charge and the collapse of protein steric layer.
Figure 5.12: A schematic illustration of the adsorbed structure of [A] sIWP, [B] sIWP-M040 conjugate (24 h), [C] soluble sIWP-D10N conjugate (72 h) and [D] sIWP-D65N conjugate (72 h) at oil/water emulsion interface at pH 7 and pH 4, and the state of the emulsion droplets (stable or flocculated). Note: Figures are not to scale.
(B) – At pH 7, sIWP-M040 conjugate (24 h) stabilises O/W emulsion by forming a negatively charged thick interfacial layer that provides electrostatic and steric repulsion against droplet flocculation. The size of the LMW carbohydrates (i.e. ~425 Da) from the M040 maltodextrin (4,300 Da) conjugated onto sIWP was not large enough to form a carbohydrate steric layer at the interface. As a result, the emulsion stabilised by sIWP-M040 conjugate (24 h) became strongly flocculated as the pH decreased from pH 7 to pH 4, due to the loss of electrostatic charge and the collapse of protein steric layer.

(C) – At pH 7, soluble sIWP-D10N conjugate (72 h) stabilises O/W emulsion by forming a negatively charged thick interfacial layer that provides electrostatic and steric repulsion against droplet flocculation. At pH 7, the D10N dextran (6,400 Da) conjugated on sIWP was not large enough to form a carbohydrate steric layer because it was conjugated at the C-terminal domain of sIWP, and was shielded by the N-terminal diffuse protein layer as illustrated in Figure 5.12C, although there was a slight increase in the interfacial layer thickness. As the pH of the emulsion decreased from pH 7 to pH 4, the N-terminal protein diffuse layer collapsed and the conjugated D10N dextran (6,400 Da) became exposed to the aqueous phase. The conjugated D10N dextran (6,400 Da) exposed to the aqueous phase at pH 4 is able to prevent the emulsion from becoming strongly flocculated by acting as a physical barrier, although not as effectively as sIWP-D65N conjugate (72 h) [D].

(D) – At pH 7, sIWP-D65N conjugate (72 h) stabilises O/W emulsion by forming a negatively charged thick interfacial layer that provides electrostatic and steric repulsion against droplet flocculation. The D65N dextran (41,000 Da) conjugated on sIWP was large enough to form a carbohydrate steric layer of approximately 5.9 nm thick at the interface as illustrated in Figure 5.12D. As the pH of the emulsion decreased from pH 7
to pH 4, the protein layer collapsed, while the conjugated D65N dextran (41,000 Da) on sIWP provides steric repulsion against flocculation.

5.3.2 Effect of Conjugation on sIWP’s Ability to Maintain Emulsion Stability in CaCl₂

- **Maltodextrin conjugates**

The effect of CaCl₂ on the stability of emulsions stabilised by sIWP, and sIWP-M040 conjugate (24 h) at pH 7 was investigated. Figure 5.13 shows the droplet size of the emulsions stabilised by sIWP and sIWP-M040 conjugate (24 h) as a function of CaCl₂ concentration (0 – 20 mM).

![Figure 5.13: Effect of the CaCl₂ concentration on the droplet size of emulsions stabilised by sIWP and sIWP-M040 conjugate (24 h). Notes: (1) Emulsions are composed of 20 wt. % canola oil, 1 wt. % protein basis, and prepared at pH 7, (2) asterisk indicates the calculated average MW of carbohydrates conjugated onto sIWP from M040 maltodextrin (4,300 Da) (calculation shown in Table 3.2 in Chapter 3).](image-url)

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The emulsion droplet size of both emulsions increased from ~1 µm to ~5 – 6 µm as the CaCl₂ concentration increased from 0 – 20 mM, which showed that these emulsions were unstable in CaCl₂ environment. These results showed that sIWP conjugated with the LMW carbohydrate fraction (i.e. ~425 Da) in the M040 maltodextrin (4,300 Da) did not have improved ability to maintain emulsion stability in CaCl₂ environment. It has been shown that a minimum of 1.1 nm thick carbohydrate steric layer is required to stabilise an emulsion against flocculation at high CaCl₂ environment (~20 mM) (Wooster & Augustin 2007a). As shown in results presented in Chapter 4, the conjugation of ~3–4 moles of LMW carbohydrates (i.e. ~425 Da) in the M040 maltodextrin (4,300 Da) per mole of sIWP was not large enough to form a carbohydrate steric layer at the interface, particularly those that were conjugated at the lysine residues located at the C-terminal domain, which is the anchoring point of the protein to the interface.

As shown earlier, sIWP and sIWP-M040 conjugate (24 h) stabilises O/W emulsion at pH 7 (without CaCl₂), by forming a negatively charged thick interfacial layer that provides electrostatic and steric repulsion against droplet flocculation. The presence of divalent counter-ion such as Ca²⁺ ions in the emulsion, screens protein charge and also binds to the adsorbed protein layer via electrostatic interactions, which makes Ca²⁺ ions to flocculate emulsions at much lower concentration than monovalent counter-ions such as K⁺ and Na⁺ that only screen protein charges (McClements 2005). These Ca²⁺ ions are likely to bind at the negatively charged α-carboxyl group of the glutamic acid residues located at the N-terminal diffuse layer of sIWP as illustrated in Figure 5.14. This leads to the alteration of surface charge density and the reduction in interfacial layer thickness due to a marked reduction in electrostatic repulsion within the protein interfacial layer (Mei et al. 1998; Silvestre et al. 1999; Kulmyrzaev et al. 2000;
Day et al. 2009). Furthermore, studies showed that Ca\(^{2+}\) ions caused bridging flocculation of emulsions stabilised by β-casein and phosvitin (Dickinson et al. 1992). Hence, these studies suggest that emulsion stabilised by sIWP and sIWP-M040 conjugate (24 h) became unstable and flocculated in CaCl\(_2\) environment due to bridging flocculation and reduction in inter-droplet repulsion force. This study is in agreement with the study reported by Day and co-workers (2009), which showed that deamidated wheat protein stabilised emulsion became unstable and flocculated in CaCl\(_2\) environment.

![Figure 5.14: Illustration of the binding of calcium ions onto the negatively charged α-carboxyl groups of the glutamic acid residues located at the N-terminal diffuse layer of sIWP. Notes: Figures are not to scale.](image)

Dextran conjugates

The effect of CaCl\(_2\) on the stability of emulsions stabilised by sIWP, soluble sIWP-D10N conjugate (72 h) and sIWP-D65N conjugate (72 h) at pH 7 was investigated. Figure 5.15 shows the droplet size of the emulsions stabilised by sIWP, soluble sIWP-D10N conjugate (72 h) and sIWP-D65N conjugate (72 h) as a function of CaCl\(_2\) concentration. The droplet size of emulsions stabilised by soluble sIWP-D10N
conjugate (72 h) increased from ~1.5 µm to ~6 – 7 µm as the CaCl₂ concentration increased from 0 – 20 mM, which showed that these emulsions were unstable in CaCl₂ environment, similar to emulsion stabilised by sIWP. On the other hand, the droplet size of emulsion stabilised by sIWP-D65N conjugate (72 h) remained unchanged throughout the CaCl₂ concentration (0 – 20 mM), which showed that the emulsion was stable in CaCl₂ environment.

As shown in results presented in Chapter 4, the conjugation of ~1.3 mole of D10N dextran (6,400 Da) per mole of sIWP was not large enough to form a carbohydrate steric layer at the interface because it was conjugated at the C-terminal domain of sIWP and was shielded by the N-terminal protein diffuse layer. As shown earlier, soluble sIWP-D10N conjugate (72 h) stabilises O/W emulsion at pH 7 (without CaCl₂), by forming a negatively charged thick interfacial layer that provides electrostatic and steric repulsion against droplet flocculation. It is likely that the emulsion stabilised by soluble sIWP-D10N conjugate (72 h) became unstable in CaCl₂ environment due to reduction in inter-droplet repulsion force caused by the screening of surface charge by Ca²⁺ ions and also the binding of Ca²⁺ ions to the adsorbed layer that lead to bridging flocculation. It is worth noting that there was no slight improvement in emulsion stability in CaCl₂ environment offered by the conjugated D10N dextran (6,400 Da) as observed earlier in Figure 5.5. As discussed in Chapter 4, the conjugation of D10N dextran (6,400 Da) on sIWP could have changed the protein’s adsorbed secondary structure at the interface. This may have enhanced the binding of Ca²⁺ binding on the adsorbed layer and promote bridging flocculation.

On the other hand, the conjugation of ~0.5 mole of D65N dextran (41,000 Da) per mole of sIWP was large enough to form a carbohydrate steric layer of ~5.9 nm
thick, as shown in results presented in Chapter 4. It likely that sIWP-D65N conjugate (72 h) stabilises the emulsion in CaCl$_2$ environment by steric repulsion afforded by the conjugated D65N dextran (41,000 Da) and also act as a physical barrier that prevent the binding of Ca$^{2+}$ ions onto the adsorbed layer. This is in agreement with the study reported by Wooster and Augustin (2006), which showed that β-lactoglobulin conjugated with 18,500 Da dextran is able to form a ~5 nm thick carbohydrate steric layer, and the authors found that the protein-conjugate was able to stabilise an O/W emulsion in CaCl$_2$ environment.

Figure 5.15: Effect of the CaCl$_2$ concentration on the droplet size of emulsions stabilised by sIWP, soluble sIWP-D10N conjugate (72 h) and sIWP-D65N conjugate (72 h). Notes: (1) Emulsions are composed of 20 wt. % canola oil, 1 wt. % protein basis, and prepared at pH 7, (2) asterisk indicates the average MW of dextran assumed to have conjugated onto sIWP from D10N dextran (6,400 Da) or D65N dextran (41,000 Da), (3) only the soluble fractions of sIWP-D10N conjugate (72 h) were analysed.
5.4 Conclusions

This study showed that conjugation of LMW carbohydrates (i.e. ~425 Da) in the M040 maltodextrin (4,300 Da) is not sufficient to give steric stability to emulsions, even though 3 – 4 moles of carbohydrates were conjugated per mole of sIWP. Similarly, conjugation of ~1.3 mole of D10N dextran (6,400 Da) per mole of sIWP is not sufficient to give steric stability to emulsions. On the other hand, the conjugation of ~0.5 mole of D65N dextran (41,000 Da) per mole of sIWP is large enough to give steric stability to emulsions at acidic pH and in ionic environment.

Therefore, in order to enhance the ability of sIWP to maintain emulsion stability at acidic pH or in ionic environments, it is required to select a carbohydrate (e.g. 41,000 Da dextran) that when conjugated onto sIWP can form a carbohydrate steric layer at the interface. This study showed that the formation of a carbohydrate steric layer at the interface depends on the size and the location of the conjugated carbohydrate on sIWP.
CHAPTER 6

Conclusions

Deamidated wheat protein has the potential to be utilised as an emulsifier because it forms stable O/W emulsion at pH 7 (Webb et al. 2002). However, as with dairy proteins that have been used to stabilise food emulsions, the emulsion stabilised by deamidated wheat protein became flocculated at acidic pH or in ionic environment (Demetriades et al. 1997; Day et al. 2009). The soluble fraction of isolated wheat protein (sIWP) was prepared from a commercially produced deamidated wheat protein and used throughout this study.

In this study it was hypothesized that conjugation of sIWP with non-ionic carbohydrates such as glucose, maltodextrins or dextrans results in a covalently-linked complex which could enhance the stability of sIWP emulsions at acidic pH and in ionic environment. It was further hypothesized that: (a) this enhancement is due to the steric stabilization that is afforded by the attachment of the carbohydrate to the protein that is anchored at the interface, and (b) that the effectiveness of this steric layer in providing emulsion stability is dependent on the size of the carbohydrate that is conjugated onto the protein.

In this study, sIWP-carbohydrate complexes were formed via the Maillard reaction by heating a mixture of protein and carbohydrate at 60 °C/75% RH. Changes to protein conformational structure due to conjugation were investigated using circular dichroism and tryptophan fluorescence emission. The interfacial layer thickness of the protein-carbohydrate conjugate adsorbed on a model polystyrene sphere system was
investigated by measuring the difference in hydrodynamic size between a naked polystyrene sphere and a polystyrene sphere adsorbed with protein-carbohydrate using dynamic light scattering. The ability of the protein-carbohydrate conjugate to maintain O/W emulsion stability at acidic pH and in CaCl$_2$ environment (0 − 20 mM), was investigated by monitoring emulsion droplet size and zeta-potential using DLS and changes in emulsion microstructure visualised by confocal microscopy. This study sought to gain insight into the structure-function relationships of the protein and protein-conjugates by understanding how the structure of the protein and protein-conjugates adsorbed at the oil-water interface correlated with their ability to maintain emulsion stability at acidic pH and in ionic environment.

**Characterisation of sIWP (Chapter 2)**

sIWP was characterised to obtain information on its composition, solution properties (i.e. protein isoelectric point (pI), solubility, and hydrodynamic size), and protein conformation in solution (i.e. secondary structure and polarity of the tryptophan local environment).

sIWP is composed mainly of monomeric gliadin based on size-exclusion high performance liquid chromatography chromatogram profile and amino acid composition analysis. The zeta-potential of sIWP determined at pH 7 is approximately − 50 mV. This is due to the deamidation process, where many of the uncharged glutamine residues located within the protein molecule was converted to negatively charge glutamic acid residues. The highly negative protein charge exhibited by sIWP at pH 7 enhanced the electrostatic repulsion between protein molecules and prevented protein aggregation. As a result, sIWP is highly soluble in water at pH 7. The pI of sIWP
measured by DLS is at pH 4, where sIWP became poorly soluble in water due to the loss of protein surface charge, which allowed protein molecules to aggregate.

Circular dichroism results obtained at pH 7 showed that sIWP has lower $\alpha$-helical structure (by ~18 %) and higher unordered structure (by ~13 %) in comparison to native gliadin. Native gliadin has an N-terminal domain rich in uncharged glutamine residue (adopts $\beta$-spiral structure) and a C-terminal domain rich in hydrophobic amino acid residues (adopts $\alpha$-helical structure). A comparison made between the tryptophan fluorescence emission spectra of sIWP and native gliadin showed that the tryptophan residues of sIWP located at the C-terminal domain of the protein molecule are in a more hydrophilic environment. The results obtained in this chapter suggests that the conversion of uncharged glutamine residues to negatively charged glutamic acid residues in sIWP increases intramolecular repulsion force that promote protein unfolding, thus resulting in the reduction of $\beta$-spiral structure at the N-terminal domain and $\alpha$-helical structure at the C-terminal domain and giving rise to a more unordered structure. It is postulated that the N-terminal domain of sIWP is highly negatively charged and hydrophilic (due to the presence of high amount of glutamic acid residues), whereas the C-terminal domain of sIWP is hydrophobic (due to the presence of high amount of hydrophobic residues). The C-terminal domain of sIWP also contains about 3 to 4 intramolecular disulphide bonds, which are likely to restrict the structural freedom of this domain.
Maillard Conjugation of sIWP with Various Molecular Weight Carbohydrates (Chapter 3)

sIWP-carbohydrate conjugates formed via the Maillard reaction were prepared using a range of carbohydrates with MW ranging from 180 – 41,000 Da. The carbohydrates used in this study were: glucose, maltodextrins (900, 1,900 and 4,300 Da) and dextrans (6,400 and 41,000 Da). The Maillard reaction involved dry heating a mixture of sIWP and carbohydrate at 60 ºC and at 75% RH. The formation of protein-conjugate was indicated by visual browning, which was resulted from the formation of melanoidins during the Maillard reaction. The degree of conjugation was assessed via the loss of available –NH$_2$ groups. The covalent bonding between protein and carbohydrate in the protein-conjugates was assessed by sodium-dodecyl-sulphate polyacrylamide gel electrophoresis.

There are four available sites on sIWP for conjugation; these are the N-terminal amino group located at the N-terminal domain and the three lysine residues located at the C-terminal domain. Approximately 3 – 4 moles of glucose or LMW carbohydrates in the M200 (900 Da), M100 (1,900 Da) or M040 (4,300 Da) maltodextrins (i.e. < 425 Da) were conjugated per mole of sIWP after heated for 24 h, indicating that all four sites have been conjugated. Approximately 1.3 mole or 0.5 mole of dextran [D10N (6,400 Da) or D65N (41,000 Da)] were conjugated per mole of sIWP after heated for 72 h, indicating that only one of the four sites have been conjugated. The low level of conjugation observed for Maillard reaction between sIWP and dextran is likely due to steric hindrance imposed by the conjugated dextran against further conjugation.

The study also showed that sIWP underwent conjugation at much faster rate than native gliadin (not deamidated). This is likely due to slight opened protein structure of
sIWP after the deamidation, which makes the lysine residues located at the C-terminal domain of sIWP more accessible for conjugation.

**Characterisation of sIWP Maillard Conjugates (Chapter 4)**

The solution properties and the interfacial adsorbed structure of sIWP-carbohydrate conjugates were investigated. The conjugation of carbohydrates onto sIWP (i.e. sIWP incubated with glucose, maltodextrins or dextrans) did not have an effect on the protein’s pI (i.e. remained at ~pH 4) or the protein secondary structure in solution (pH 7), based on measurements on the zeta-potential and the CD spectra of the protein/protein-conjugates. Protein-conjugates formed with glucose or LMW carbohydrates in the maltodextrins (i.e. < 425 Da) became aggregated in solution at ~pH 4, similar to sIWP alone. However, protein-conjugates formed with dextrans showed minimum protein aggregation in solution at ~pH 4, where sIWP has minimum charge. The aggregated proteins observed in these dextran-conjugates in solution at ~pH 4 were due to the aggregation of non-conjugated proteins in these samples. The conjugation of dextrans on sIWP was able to prevent protein aggregation in solution at ~pH 4 by acting as a physical barrier.

Examination of the absorbed layer formed by sIWP on polystyrene particles showed that sIWP itself forms a ~18 nm thick protein steric layer. It is postulated that the C-terminal domain of sIWP is likely to anchor the protein molecule at the interface because it contains many hydrophobic amino acid residues, whereas the N-terminal domain of sIWP is likely to form the protein diffusive layer because it contains many negatively charged hydrophilic glutamic acid residues.
The conjugation of sIWP with carbohydrates of different MW has different effect on the interfacial layer thickness. sIWP conjugated with ~3 – 4 moles of glucose or LMW carbohydrates in the maltodextrins (i.e. < 425 Da) possessed the same interfacial layer thickness (~18 nm) at the interface as sIWP. This was because the conjugated LMW carbohydrates in these protein-conjugates (i.e. < 425 Da) was too small to form an additional carbohydrate steric layer on top of the protein steric layer at the interface, in particular the LMW carbohydrates that were conjugated at the lysine residues located at the C-terminal, which are shielded by the N-terminal protein diffuse layer.

In the case of sIWP conjugated with ~1.3 mole of D10N dextran (6,400 Da) per mole of sIWP, a thicker interfacial layer than sIWP by ~3.5 nm was formed. However, the extended layer was not digestible by dextranase, which suggests that the extended layer was not an additional carbohydrate steric layer. Tryptophan fluorescence emission spectra of the dextran-conjugate indicate that the local environment of the tryptophan residues located at the C-terminal domain became more hydrophilic after conjugated with D10N dextran. These tryptophan residues are located close to the lysine residues, which suggest that the D10N dextran was conjugated at the C-terminal domain of sIWP. As a result, it is possible that the conjugated D10N dextran is being shielded by the N-terminal protein diffuse layer. The extended layer observed for sIWP conjugated with D10N dextran was linked to a change in adsorbed secondary structure at the interface.

On the other hand, sIWP conjugated with ~0.5 mole of D65N dextran (41,000 Da) per mole of sIWP, increased the interfacial layer thickness by ~6 nm. Dextranase digestion of this interfacial layer reduced the thickness to the same thickness as sIWP. This suggests that the extended layer thickness observed was due to the formation of a carbohydrate steric layer in addition to the protein steric layer at the interface.
Tryptophan fluorescence emission spectra of this dextran-conjugate did not show any change in comparison to sIWP. This indicates that the D65N dextran was likely to be conjugated at the N-terminal amino group of sIWP, which is located at the N-terminal domain. These results suggest that the prevision of additional carbohydrate steric layer on top of the protein steric layer at the interface is dependent on the size of the carbohydrate and the site of conjugation.

➢ *Ability of the Protein-conjugates to Maintain Emulsion Stability at Acidic pH and in Ionic Environment (Chapter 5)*

The ability of the protein-conjugates to maintain O/W emulsion stability at acidic pH (~pH 4) and in CaCl$_2$ environment (0 – 20 mM) was investigated by monitoring changes in emulsion droplet size and the microstructure of the emulsion visualised by confocal microscopy. Like many other food proteins (e.g. milk proteins), the sIWP stabilised emulsion became unstable and flocculated at acidic pH (i.e. ~pH 4 for sIWP) or in the presence of Ca$^{2+}$ ions in the emulsion continuous phase.

Protein-conjugates formed with ~3 – 4 moles of LMW carbohydrates (i.e. ~425 Da) in the M040 maltodextrin (4,300 Da) or with ~1.3 mole of D10N dextran (6,400 Da) per mole of sIWP had little or minimal ability to enhance the stability of sIWP-based emulsion at acidic pH (~pH 4) and up to 20 mM CaCl$_2$. This is not surprising as it has been shown in Chapter 4 that the size of the conjugated carbohydrates in these protein-conjugates was not large enough to form an additional carbohydrate steric layer on top of the protein layer to provide steric stabilisation. On the other hand, sIWP conjugated with the larger size D65N dextran (41,000 Da) was able to maintain emulsion stability at acidic pH (~pH 4) and up to 20 mM CaCl$_2$. This improvement is linked to the formation of an additional carbohydrate steric layer (~6 nm) on top of the
protein steric layer at the interface, which provides steric repulsion against droplet flocculation, even when the protein layer was collapsed due to the loss of protein charge at ~pH 4, or the surface charge of the protein adsorbed at the interface was shielded by the binding of Ca\(^{2+}\) ions.

**Overall Conclusions**

The hypothesis of this research study was that conjugation of sIWP with non-ionic carbohydrates such as glucose, maltodextrins or dextrans results in a covalently-linked complex that could enhance the ability of sIWP to maintain emulsion stability at acidic pH and in ionic environment:

(a) due to steric stabilization that is afforded by the attachment of the carbohydrate to the protein that is anchored at the interface, and

(b) is dependent on the size, location and the number of the carbohydrate that is conjugated onto the protein.

This study showed that carbohydrates from small glucose to large dextrans (up to 41,000 Da) can be conjugated onto sIWP via the Maillard reaction to form Maillard type complexes; the number of carbohydrates conjugated per mole of sIWP varied with the nature of the carbohydrate. Although all three available lysine residues and the N-terminal amino group were likely to be conjugated with glucose or LMW carbohydrates in the maltodextrins (i.e. < 425 Da), the conjugated complex did not contribute to an additional carbohydrate steric layer or the prevention of emulsion flocculation at acidic pH (~pH 4) or in CaCl\(_2\) environment (20 mM). Only one of the four sites was conjugated with D10N or D65N dextrans (6,400 or 41,000 Da), which are much larger sizes of carbohydrates, but the conjugated complex did contribute to an additional steric
layer thickness in different ways depending on the site of conjugation. The dextran-conjugate with the largest dextran (i.e. 41,000 Da) studied, although it has the lowest in terms of the number of carbohydrate conjugated per mole of protein, is the most effective in maintaining emulsion stability at acidic pH (~pH 4) and in CaCl$_2$ environment (20 mM). This is due to the formation of a ~6 nm steric layer in addition to the protein layer at the interface. The different levels of conjugation, the size and/or the location of the conjugated carbohydrate on sIWP is possibly contributed to the different effect observed on the interfacial layer thickness and the ability of protein to maintain emulsion stability at acidic pH (~pH 4) and in ionic environment (up to 20 mM CaCl$_2$).

**Future Directions**

Although the hypothesis of the study has been confirmed through the experimental results presented in this study, however, there were a number of questions arise during the study that needs further clarification:

a) **Purification of the Protein-conjugates:**

In this study, the purification of the protein-conjugates was not successful because there was no clear separation between the size of the conjugated and non-conjugated proteins. Although the study has showed that the non-conjugated proteins did not influence the adsorption of the conjugated proteins at the interface, however, it did make a difference for studying the effect of conjugation on the possible changes to the protein folding structure in solution.

One method that can be used to isolate pure conjugated proteins is by adjusting the pH of the solutions dispersed with the protein-conjugates to pH 4 and then isolate
the soluble protein fraction. This is because results presented in Chapter 4 suggest that sIWP conjugated with dextrans do not aggregate at pH 4, and hence soluble at this pH.

b) **Confirmation of the Site of Conjugation of Dextran on sIWP:**

Further understanding on site of conjugation for sIWP conjugated with D10N dextran (6,400 Da) or D65N dextran (41,000 Da) is required to validate the models proposed on the structures of the dextran-conjugates adsorbed at the interface (as presented in Figure 4.20 and Figure 4.21). These models are based on the tryptophan emission profiles of the dextran-conjugates, which suggests that the D10N dextran (6,400 Da) was conjugated at one of the lysine residues located at the C-terminal domain of sIWP, whereas the D65N dextran (41,000 Da) was conjugated at the N-terminal amino group located at the N-terminal domain of sIWP. In order to strengthen these models, methods such as systematic enzymatic digestion on the dextran-conjugates followed by the detection of the peptide mass using Maldi-TOF to identify peptides with increased mass (i.e. conjugated with dextran) can be used to elucidate the conjugation site of the dextran. Furthermore, this method can be used to confirm the size of the dextran conjugated onto the protein.

c) **Secondary Structure of the Protein-conjugates Adsorbed at Interface:**

Further study to understand the adsorbed secondary structure of sIWP conjugated with D10N dextran (6,400 Da) at the emulsion interface is needed to confirm whether if the change in adsorbed secondary structure have caused this dextran-conjugate to exhibit a thicker interfacacial layer thickness than sIWP at the interface. A study based on synchrotron radiation circular dichroism has been developed recently to study the secondary structure of IWP gliadin (purified from sIWP) adsorbed at the O/W emulsion interface (water/tricaprin). Conventional CD instrument could not obtain CD
signal from a turbid solution such as an emulsion. The synchrotron radiation source have higher flux than conventional Xe arc light, which allowed sufficient CD radiation to be collected at the detector after interact with the emulsion sample. Thus, SRCD can be used to study emulsion stabilised by sIWP conjugated with D10N dextran (6,400 Da), to investigate whether there is any changes occurred after the dextran-conjugate was adsorbed at the emulsion interface.
References


