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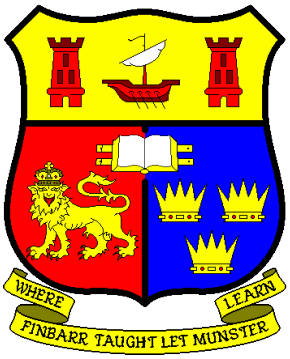

PHYSICO-CHEMICAL PROPERTIES AND COMPONENT INTERACTIONS IN HIGH SOLIDS FOOD SYSTEMS

Thesis presented by

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DEDICATION

To "Potes and Roos" families with love, respect,

and gratitude.

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ABSTRACT

Typical reactions of components in biological materials (proteins, lipids, and carbohydrates) such as nonenzymatic browning reaction (NEB) or Maillard reaction or glycation (nonenzymatic glycosylation), and oxidation result in changes of dynamic mechanical, physicochemical and thermal properties. In food and pharmaceutical materials such component interactions may reduce stability and shelf life.

The present study investigated high solids systems containing (*i*) lactose–maltodextrin (MD) with various dextrose equivalents (DE) and at different ratios of the components as a model of typical carbohydrates in dairy ingredients to investigate effects of molecular weight and size of MD and glass transition on lactose crystallization; high solids systems were also designed to represent dairy ingredient powders and high protein foods and included (*ii*) whey protein isolate (WPI, 0.31 a_w)–oil [olive oil (OO) or sunflower oil (SO)] at 75:25 ratio (0.35 or 0.34 a_w); and (*iii*) WPI–OO– or WPI–SO–[glucose–fructose (G–F) 1:1 syrup (70% total solids, w/w, 0.75 a_w)] at a component ratio of 45:15:40 (0.62 or 0.63 a_w) systems. The systems (*ii*) and (*iii*) were stored at 20 and 40°C for up to 14 weeks to investigate effects of hydrophobic and hydrophilic interactions on the properties of the proteins and systems during storage at low water activity (a_w). The properties of the amorphous freeze-dried lactose–MD systems were investigated using a water sorption study, differential scanning calorimetry (DSC), and dielectric (DEA) and dynamic mechanical analyses (DMA). Data on hydrophobic and hydrophilic interactions in protein–oil and/or sugar were derived from carbonyl (protein oxidation) and total sulfhydryl (disulfide bonding) contents, DSC and DMA measurements, reducing and non-reducing SDS-PAGE electrophoresis, cryo-scanning electron microscopy (SEM), texture measurements, colorimetry, and variations in a_w.

Crystallization of lactose in lactose-MD systems was delayed and increasingly inhibited with increasing MD contents and higher DE values (small molecular size or low molecular weight). Amorphous lactose–MD systems had glass

transition temperatures (T_g) close to that of lactose as anhydrous materials and at 0.11 up to 0.44 a_w, but showed differences in T_α and inhibition of lactose crystallization. Lactose crystallization in lactose–MD systems was more affected by MD components hindering the mobility of lactose molecules than their T_g. Our study proved that the number average molecular weight, molecular size, and lattice interference of components had significant effects on nucleation or crystal growth during lactose crystallization. We found that sorbed water contents for non-crystalline lactose and lactose–MD up to 0.76 a_w could be derived from that of lactose in the non-crystalline lactose–MD at 40:60 ratio sorption data with the GAB equation.

The protein–oil and protein–oil–sugar materials showed changes in physicochemical and thermal properties during storage. Protein oxidation and disulfide bonding in high solids systems were most pronounced after 2 weeks of storage. Oil with WPI before storage showed an increased onset temperature (T_{onset}) of protein [10% solids (w/w) in water] denaturation. These systems also showed more pronounced protein aggregation exotherms. After storage for 14 weeks protein sensitivity to heat was increased. Protein aggregation occurred during storage as measured from a decrease in post-storage heat of aggregation, especially for the system with OO. Oil with WPI–sugar reduced the sizes of protein aggregation exotherms. The presence of G–F in WPI–oil increased T_{onset} and T_{peak} of protein aggregation and oxidative damage of the protein. Storage at 40°C showed more pronounced changes of protein conformation in the high solids systems. Lipid oxidation and glycation products in the systems containing sugar promoted oxidation of proteins, increased changes in protein conformation and aggregation of proteins, and resulted in insolubility of solids or increased hydrophobicity concomitantly with hardening of structure, covalent cross-linking of proteins, and formation of stable polymerized solids, especially after storage at 40°C. There were differences in visual appearance in systems prepared using OO or SO. The WPI–oil–(G–F) after 14 weeks of storage showed no phase separation of oil and G-F syrup from -140 to 45°C. We found that protein hydration (reversible and time-dependent) gave an endotherm preceding denaturation (irreversible) transitions in WPI–oil and WPI–oil–sugar systems using DSC. Our DMA measurements also found the glass transition of confined

water in protein systems at temperatures over the range from -105 to -75°C, which conversely has been reported as the glass transition of protein in earlier studies.

Our study found new data of water sorption of non-crystalline lactose and lactose–MD systems, protein hydration transition (in the dry materials), protein and lipid oxidation, glycation involving oil as well as hydrophobic interactions, which explain the roles of proteins, lipids, and carbohydrates of various molecular weights or sizes, and loss of physical stability and nutritional quality of high solids foods; e.g., confectionary products, dairy (infant formulae and hard cheese products) and meat products, high-protein nutritional bars, sport foods, supplements; cosmetics; and medicinal products during storage at low water contents.

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INTRODUCTION

High solids foods and particularly high protein formulations are widely studied because of their relevance as models of formulated foods [infant formulae, yoghurt powder, milk tablets, high protein bars, sport foods and supplements (reconstituted powder as energy fuel drinks and food mixed powder for body building, sport training, and athlete)], cosmetics, and medicinal foods and pharmaceuticals. The components of edible high solids and protein systems consist of carbohydrates (e.g., fibre, starch, maltodextrins, saccharides and sugars, and gum arabic), proteins [e.g., milk (whey proteins and caseins), meat (e.g., gelatin, collagen, and taurine), egg (albumin), beans (soy proteins) and nuts], and oil [solid (hydrogenated vegetable oils, coconut oil, milk fat) and liquid fats (vegetable oils, *n*-3 fatty acids) including flavouring (essential natural oils), fat soluble vitamins (retinols, tocopherols), colourings (carotenes), humectants or lubricants (glycerol or glycerin) and emulsifying (lecithins, tweens) agents]. The interactions of such components affect physical stability and physicochemical properties of the formulated systems during processing and storage. Component interactions often depend on a_w and water content (Labuza *et al.*, 1972; Labuza and Saltmarch, 1982; Bell, 1996; Acevedo *et al.*, 2008); matrix or structure (Buera and Karel, 1995; Lievonen *et al.*, 1998); pH (Hodge, 1953; Wolfrom *et al.*, 1974; Ashoor and Zent, 1984; Ajandouz and Puigserver, 1999; Richards and Hultin, 2000; Ajandouz *et al.*, 2001); temperature (Labuza and Saltmarch, 1982; Bell, 1996; Roos *et al.*, 1996; Lievonen *et al.*, 1998; Schebor *et al.*, 1999; Akhtar and Dickinson, 2007); time under processing and storage (Labuza and Saltmarch, 1982; Kato *et al.*, 1989); type and concentration of reactants (Wolfrom *et al.*, 1974; Ashoor and Zent, 1984; Kato *et al.*, 1989; Kwak and Lim, 2004); the presence of others components such as acids and bases (Ortwerth and Olesen, 1988), buffer (Bell, 1997), and salt (Kwak and Lim, 2004); and environmental conditions [light and oxygen (Ahmed *et al.*, 1986)]. Noncrystalline amorphous solids often show structural changes (collapse or shrinkage or loss of shape, crystallization, stickiness, softening, and hardening) during dehydration processes and storage at temperatures above the relevant glass transition temperature (T_g). The physical state of the components also

affects rates of chemical reactions. Collapse and deformation of the matrix (Buera and Karel, 1995), crystallization of sugars (Shimada *et al.*, 1991; Buera *et al.*, 2005; Drusch *et al.*, 2006), and phase separation and diffusion or mobility of reactants in the matrix (Karmas *et al.*, 1992; Roos *et al.*, 1996; Lievonen *et al.*, 1998; Schebor *et al.*, 1999) were shown to increase rates of nonenzymatic browning and lipid oxidation during storage above the T_g. The T_g and water content of the systems govern molecular mobility and diffusion that relate to physical stability and occurrence of chemical reactions in foods during storage (Slade *et al.*, 1991; Roos, 1995a; b).

Our studies presented in the present thesis investigated component interactions (carbohydrate, protein, and oil) and their impact on properties of high solids systems at low water activities during storage. The systems served as models for studies of carbohydrate-carbohydrate, protein–lipid, protein–sugar, and protein–lipid–sugar interactions typical of dehydrated food and pharmaceutical materials and formulations.

The main hypotheses of the present study were the following:

(*i*) Maltodextrin (MD) components in amorphous sugar systems and molecular size and weight of MD inhibit and affect crystallization of sugars.

(*ii*) A lipid phase accelerates oxidation and changes protein properties in systems containing protein and oil at low a_w or water content after prolonged storage at a high temperature.

(*iii*) Chemical reactions such as Maillard reaction or glycation and oxidation increase hydrophobicity, polymerization, browning, hardening of structure, and decrease solubility of solids in systems containing proteins, oil, and sugars during storage at a high temperature.

(*iv*) The hydration properties of proteins contribute to changes of properties of high solids systems during storage at a high temperature.

The above hypotheses were tested using a series with the following objectives:

(*i*) Determination of the effects of DE (molecular weight or molecular size), concentration or content, and glass transition of MD with lactose on water sorption and lactose crystallization behaviour in amorphous lactose–MD systems;

(*ii*) Establishment of relationships between the DE and concentration of MD and loss of water sorbed to determine rates of lactose crystallization in lactose–MD systems at various mixture ratios;

(*iii*) Measurements of dielectric and dynamic mechanical properties at crystallization of lactose in amorphous lactose–MD systems;

(*iv*) Measurements of relaxation times to describe mobility of lactose and MD molecules and crystallization in amorphous lactose–MD systems.

(*v*) Analysis of the effects of temperature and storage time on physicochemical and thermal properties of high protein systems (WPI–oil and WPI–oil–sugars) as both reconstituted and solid systems;

(*vi*) Determination of the effects of oil and sugar on physicochemical and thermal properties of WPI proteins in both reconstituted and solid systems;

(*vii*) Analysis of thermal transitions of WPI proteins and the glass transition of sugars in high protein systems at low a_w during storage at different temperatures;

(*ix*) Determination of hydration of proteins in WPI at various a_w and effects of hydrophilic and hydrophobic surroundings on the hydration behaviour of proteins in high protein systems;

(*x*) Measurement of the dynamic transition or α-relaxation of water, oil, and sugar components and hydration behaviour of WPI proteins in the high protein systems during storage at different temperatures;

(*xi*) Establishment of relationships between chemical reactions (protein oxidation and Maillard reaction or glycation), physical stability (colour and morphology), physicochemical changes (a_w, protein conformation,

protein oxidation and disulfide bonding, size distribution or molecular weight of protein, structure of proteins, texture), and thermal properties (denaturation and aggregation of proteins for reconstituted material, hydration and denaturation of proteins for solid material, glass transition of sugars and water) of high protein systems at low a_w during storage at different temperatures.

CHAPTER I

LITERATURE REVIEW

1.1 MAILLARD REACTION IN HIGH SOLIDS SYSTEMS

The Maillard reaction (nonenzymatic browning reaction, NEB) results from interactions of amines, amino acids, peptides, and proteins with carbonyl groups of reducing sugars (aldehydes and ketones) and leads to the formation of brown pigments or coloured polymers (Fig. 1.1) (Hodge, 1953). This chemical reaction is the most common type of browning reactions in heated foods during their processing and storage, and is relevant to human health as the reaction occurs *in vivo* during ageing (Hodge, 1953; Monnier, 1990). Maximum browning rates of the Maillard reaction occur at a_w between 0.60 and 0.90 (Labuza and Dugan, 1971). The Maillard reaction can be divided into an initial (sugar-amine condensation, Amadori rearrangement), intermediate (dehydration and fragmentation of sugar and degradation of amino acid), and final stage (aldol condensation and aldehyde-amine polymerization), as shown in Fig. 1.1.

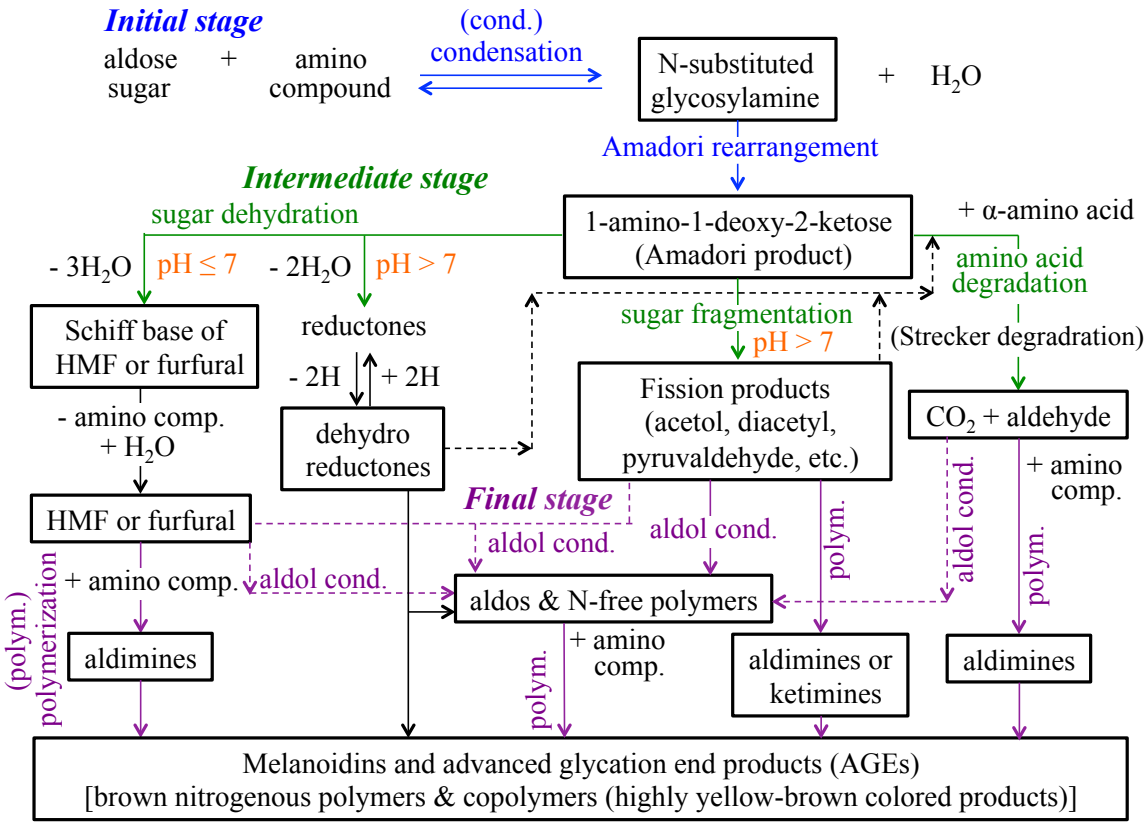

Fig. 1.1 Schematic of Maillard reaction pathways in foods (HMF = hydroxymethylfurfural) (Hodge, 1953).

The first reaction of aldose or ketose sugar with amine groups in proteins and other molecules leads to N-substituted glycosylamine or N-substituted

ketosylamine, respectively. This first step is followed by the formation of an Amadori (1-amino-1-deoxy-2-ketose) or Heyns rearrangement product (2-amino-2-deoxyaldoses), respectively (Hodge, 1953; Monnier, 1990; Dills Jr., 1993). The Amadori or Heyns rearrangement step is generally referred to as "glycation" although various terms such as glucation, fructation, and ribation are used to indicate or specific type of the sugar component of the reaction, i.e., glucose, fructose, and ribose, respectively (Monnier, 1990). Formation of Amadori products (colourless compounds) is followed by formation of an increased quantity of the unsaturated carbonyl compounds produced by sugar dehydration (furfural formation) and sugar fragmentation (degradation of sugar) reactions, and Strecker degradation (degradation of amino acid) (Hodge, 1953; Nursten, 1981). The degradation of sugars and amino acids is a major source of low molecular weight carbonyl compounds (Wells-Knecht *et al.*, 1995; Anderson *et al.*, 1997). The carbonyl compounds can be precursors of both oxidative advance glucation end products (AGEs) [N^{ε}-(carboxymethyl)lysine (CML) and pentosidine] and nonoxidative AGEs derived from 3-deoxyglucosone (deoxyglucasone-lysine dimer) and methyl glyoxal (methyl glyoxal-lysine dimer) (Reddy *et al.*, 1995; Liggins and Furth, 1997; Miyata *et al.*, 1998; Singh *et al.*, 2001).

Reducing sugars can react non-enzymatically with lipids and nucleic acids forming Schiff bases and Amadori products, and subsequently AGEs [irreversible chemical modification and covalent cross-linking of proteins, and browning (Makita *et al.*, 1991; Dyer *et al.*, 1993; Wells-Knecht *et al.*, 1995)] typical of the later stages of the Maillard reaction pathways (Singh *et al.*, 2001). If oxidation accompanies glycation or Amadori compound formation then the products formed are known as glycoxidation products, e.g., N^{ε}-(carboxymethyl)lysine (CML) (Ahmed *et al.*, 1986; Dyer *et al.*, 1993) and pentosidine (fluorescence cross-linking between arginine and lysine residues) (Sell and Monnier, 1989; Grandhee and Monnier, 1991; Dyer *et al.*, 1991; Dyer *et al.*, 1993). The AGE in the non-oxidative pathway is pyrraline (Hayase *et al.*, 1989). The lipid peroxidation forms glyoxal that can be formed in the Maillard reaction as the AGE (Thornalley *et al.*, 1999; Singh *et al.*, 2001) (Fig. 1.2). The AGE formation pathways in lived proteins (connective tissue, membrane, skin

collagen) in *vivo* are shown in Fig. 1.2, which is similar to Maillard reaction in foods (Fig. 1.1). The structure of AGEs is shown in Fig. 1.3. Formation and accumulation of AGEs in *vivo* are involved in the development of chronic complicated diseases [diabetes (McCance *et al.*, 1993; Beisswenger *et al.*, 1993)] and age-related diseases [e.g., atherosclerosis (Lyons, 1993; Colaco and Roser, 1994; Horiuchi, 1996), and neurodegenerative diseases (Smith *et al.*, 1994; Sasaki *et al.*; 1998)]. Many studies also used the CML and pentosidine as biochemical markers for assessing the advanced glycation reaction in cumulative damages of proteins (Dyer *et al.*, 1991), diabetes (Ahmed *et al.*, 1986; Tenada and Monnier, 1994) and diabetic vascular complications [diabetic nephropathy (Hirata and Kubo, 2004), diabetic retinopathy (Salman *et al.*, 2009; Ghanem *et al.*, 2011)], and uremia (Tenada and Monnier, 1994; Degenhardt *et al.*, 1997) in *vivo*.

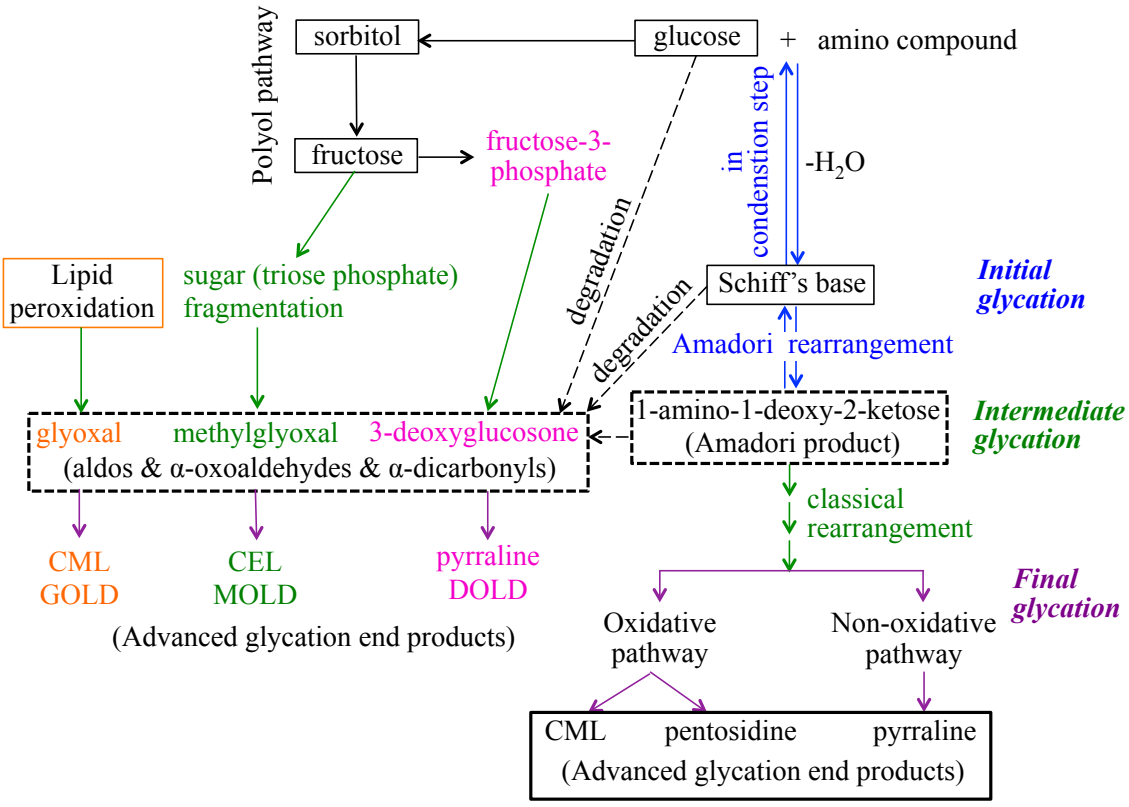

Fig. 1.2 The advanced glycation end products (AGEs) formation pathways in *vivo* (Singh *et al.*, 2001). The AGEs are N^{ϵ}-(carboxymethyl)lysine (CML), pentosidine, pyrraline, N^{ϵ}-(carboxyethyl)lysine (CEL), glyoxal-lysine dimer (GOLD), methyl gloxal-lysine dimer (MOLD), and deoxyglucasone-lysine dimer (DOLD).

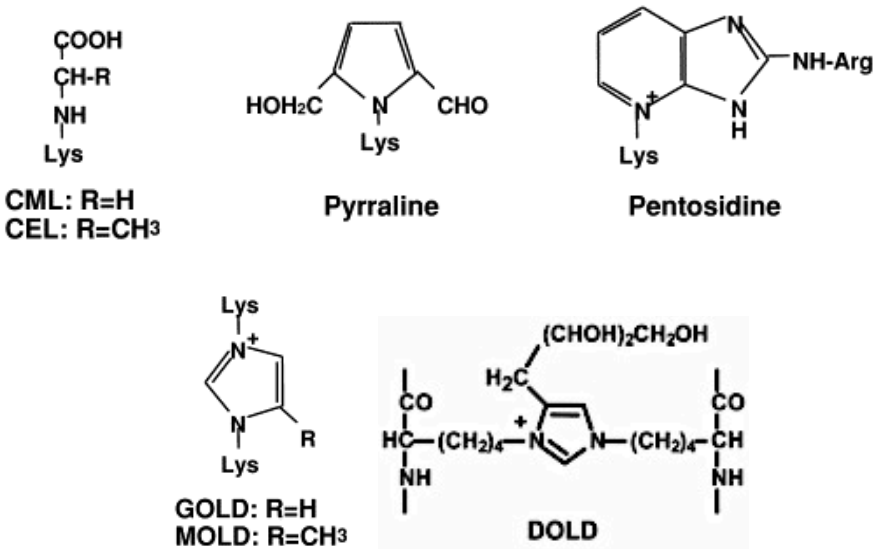

Fig. 1.3 Structure of the N^{ε}-(carboxymethyl)lysine (CML), pentosidine, pyrraline, N^{ε}-(carboxyethyl)lysine (CEL), glyoxal-lysine dimer (GOLD), methyl gloxal-lysine dimer (MOLD), and deoxyglucasone-lysine dimer (DOLD).

1.1.1 Impact of the Maillard reaction on properties of high solids systems

The Maillard reaction or NEB affects the colour, flavour, odour, texture, and nutritional value of foods. The interaction between proteins and reducing sugars contributes both positively and negatively on properties, quality, and safety of foods, and biological and medicinal systems. The Maillard reaction forms volatile flavour components and brown melanoidins as the high molecular weight products. These compounds are often desirable in cooking, baking, and roasting. Formation of volatile substances and brown polymers during food storage can be undesirable and lead to reduced stability, quality, and safety [e.g., formation of acrylamide (carcinogen compound) during the Maillard reaction (Stadler *et al.*, 2002)].

Maillard reaction and physical and physicochemical properties

Sugars are used as stabilising agents for the retention of biological activity, and stabilisation of protein conformation in freezing, freeze-thawing, dehydration, thermal processes, and storage. Carpenter *et al.* (1987a) showed that 200 to 500 mM maltose with phosphofructokinase (sensitive enzyme in rabbit skeletal muscle) in freeze-drying improved recovery of enzyme activity up to 80% of the original activity, and 0.9 mM ionic zinc (Zn^{2+}) with the enzyme-monosaccharide

(80 to 100 mM glucose and 60 to 200 mM galactose) or -disaccharide (30 to 500 mM maltose) mixtures greatly enhanced stability after freeze-drying. The results of the study indicated that the enzyme activity was not only dependent on the type of the sugar moiety present in the system, but the subunit and orientation of sugar molecules was also an important factor affecting enzyme stabilisation. Carpenter and Crowe (1989) and Allison *et al.* (1999) demonstrated that the direct interactions of sugars (glucose and lactose) and polar groups of lysozyme protein (from egg white) via hydrogen bonding protected the protein against unfolding which preserved the native protein structure during freeze-drying and rehydration. The Maillard reaction at the later stage (advanced Maillard reaction) caused modification of protein structure (Zhou *et al.*, 2013), formation of nondisulfide covalently cross-linked aggregates of proteins (McPherson *et al.*, 1988; Kato *et al.*, 1989) and fluorescent compounds (McPherson *et al.*, 1988; Kato *et al.*, 1989; Yeboah *et al.*, 1999), formation of yellow-brown colour (Lewis and Lea, 1950; Kato *et al.*, 1986; Kato *et al.*, 1989), protein insolubility (Kato *et al.*, 1986; Stapelfeldt *et al.*, 1997; Yeboah *et al.*, 1999), protein indigestibility *in vitro* (Öste *et al.*, 1986), protein polymerization (Kaanane and Labuza, 1989; Kato *et al.*, 1989; Stapelfeldt *et al.*, 1997), and loss of amino acid residues (Lewis and Lea, 1950; Fry and Stegink, 1982; Kato *et al.*, 1986) in food systems during storage.

Loveday *et al.* (2009) found that the initial and intermediate stages of Maillard reaction (no browning formation) in milk protein concentrate-cocoa butter-glucose-glycerol-water mixtures at a component ratio of 2:1:4:1.5:1.5, as a model of a protein bar, after storage at 20°C for 50 days decreased the reactive lysine content, clustered protein particles, and precipitated protein that led to hardening of protein bars. Chen *et al.* (2012) showed that glucose had a higher reactivity with β-lactoglobulin for glycation than fructose in intermediate-moisture food model systems (β-lactoglobulin-glucose- or β-lactoglobulin-fructose-glycerol-water at a component ratio of 4.5:1.25:3:1.25, 0.54 or 0.52 a_w). Their systems after storage at 35°C for up to 49 days showed an increase in the molecular weight of the β-lactoglobulin fraction, aggregated proteins, and formation of high molecular weight protein polymers as a result of the Maillard reaction during storage. Zhou *et al.* (2013) studied effects of Maillard reaction on the properties

of high protein bars [whey protein isolate (WPI)-fructose- or WPI-sorbitol-glycerol-water, 0.60 or 0.59 a_w). Their results showed that formation of protein aggregates by nondisulfide covalent cross-linking after storage at 25, 35, 45°C for 45, 14, and 3 days, respectively, occurred in systems containing fructose. The solubility of WPI in that system decreased after storage at 45°C for 7 days and formation of insoluble protein aggregates (high molecular weight polymers) via nondisulfide covalent cross-linking through the final stage of Maillard reaction was obvious after 45 days of storage. Also changes in texture (hardening) of the systems containing fructose correlated with the development of brown colour during storage, particularly during storage at 35 and 45°C.

Maillard reaction and thermal properties

The Maillard reaction affects thermal properties of proteins, including hydration, denaturation, and aggregation, and the glass transition of amorphous food solids. Hydration is used to refer to the interactions of protein with water (Kinsella and Whitehead, 1989). The definition of denaturation is alteration in the original native structure of protein, which is involved with the structural stability of the native protein (Mulvihill and Donovan, 1987). These changes in native structure are restricted to those occurring in the secondary or higher structure of proteins (Mulvihill and Donovan, 1987). The typical thermal denaturation of proteins is unfolding of the protein structure. Unfolding of protein conformation exposes side-chain groups of amino acid residues that are buried within the native structure, which enhances hydrophobic interactions (Kinsella and Whitehead, 1989). The unfolded protein molecules associate to form aggregates of denatured molecules, which can reduce protein solubility and result in coagulation, and/or gelation. Aggregation or thermal gelation of proteins (irreversible phenomenon) results from intermolecular association of partially denatured protein chains (Mulvihill and Donovan, 1987; Mulvihill and Kinsella, 1987), and modifications of structure and subsequent exposure of the hydrophobic surface. The denaturation and aggregation of proteins can be induced by a number of factors such as pH, protein concentration, time and temperature of heating, and chemical reactions (e.g., Maillard reaction and lipid oxidation) (Kinsella and Whitehead, 1989).

Addition of sugars to protein systems can stabilize proteins against thermal denaturation [increased temperature of protein denaturation (Ball *et al.*, 1943; Back *et al.*, 1979; Kato *et al.*, 1981; Boye and Alli, 2000; Rich and Foegeding, 2000)] and aggregation [increased time and temperature required for aggregation (Ball *et al.*, 1943; Kato *et al.*, 1981; Rich and Foegeding, 2000)]. Back *et al.* (1979) showed that the presence of 28% and 50% (w/w) of monosaccharides (arabinose, fructose, galactose, glucose, mannose, rhamnose, and ribose), disaccharide (maltose), trisaccharides (raffinose and melezitose), and polysaccharide (dextran) in ovalbumin dispersion at pH 7 increased the peak temperature of protein denaturation measured from a differential scanning calorimetry (DSC) heating scan. The study concluded that the stabilisation of protein was due to the effects of sugars on hydrophobic interactions of hydrophobic groups of protein. Arakawa and Timasheff (1982) suggested that sugars (glucose and lactose) in aqueous solutions increased surface tension of water that promoted preferential interaction of proteins with solvent components resulting in thermal stabilisation of proteins. Kella and Poola (1985) found that arachin (protein in peanut) became more compact (reduced viscosity) in the presence of D-xylose or D-glucose at pH 3.6. An increased hydroxyl content of the sugar (D-xylose < D-glucose) was more effective reducing thermal denaturation and aggregation of the protein. Rich and Foegeding (2000) found that 500mM ribose or 500mM lactose increased the peak temperature of protein denaturation [14% (w/w) whey protein isolate dispersion] and also that both sugars showed an ability to inhibit the heat-induced aggregation of proteins.

The extent of interactions between proteins and sugars through Maillard reaction affects thermal properties of the proteins in dry systems. Kato *et al.* (1981) found that freeze-dried ovalbumin-glucose (at 65% relative humidity) at 50°C for up to 6 days of storage increased denaturation (4 days) and aggregation (6 days) temperatures of the protein. Kato et al. (1981) found increased solubility of the protein at the initial stage of the reaction, but a decreased soluble protein content at the final stages of the reaction, and increased formation of aggregated proteins through the Maillard reaction. Their results also showed that ovalbumin after storage with glucose for 6 days became more heat sensitive (lower temperature and heat enthalpy of protein denaturation) than the systems (with and without

glucose) before storage and the system without glucose after storage for 6 days. The formation of protein-glucose complexes and cross-linking as a result of the Maillard reaction promoted the unfolding and insolubilization, respectively, of the proteins. Easa *et al.* (1996) showed that the production of acidic products from Maillard reaction during heating at 90°C for up to 75 min decreased the pH of bovine serum albumin-ribose or -xylose systems, which influenced the thermal gelation or aggregation of the protein.

The Maillard reaction affects glass transition of amorphous foods and systems containing amorphous solids were related to physical stability (stickiness, collapse, crystallization, and oxidation) of the materials. Roos *et al.* (1996) found that the Maillard reaction (exothermal phenomenon and temperature dependent) in freeze-dried amorphous skim milk [4.9% (w/w) lactose and 3.4% (w/w) protein] and lactose-hydrolyzed skim milk [2.45% (w/w) glucose, 2.45% (w/w) galactose, 3.4% (w/w) protein] produced water that plasticized the amorphous material and consequently decreased the glass transition temperature (T_g). Their results also showed that the glass transition of the systems became more broadened with increasing isothermal (at 100, 110, 120, 130, and 140°C) holding time in the differential scanning calorimetry because of the formation of high molecular weight polymeric compounds. The formation of polymeric compounds was likely to increase T_g of the material. Increased water content and decreased T_g of the materials, as the result of the Maillard reaction, at each temperature were linear against heating time.

Maillard reaction and protein structure

The effects of Maillard on the chemical properties of proteins have been related to the sulfhydryl groups content, and conformational and structural changes of proteins. Watanabe *et al.* (1980) showed that the Maillard reaction promoted unfolding of ovalbumin and reduced α-helix conformation in the secondary structure of the proteins. The system contained lysine and arginine residues in freeze-dried ovalbumin with glucose (65% relative humidity) during storage at 50°C for 18 days. Kato *et al.* (1987) showed that 3-deoxyglucosone (dicarbonyl compound from Maillard reaction between butylamine and glucose) induced polymerisation of proteins by intermolecular cross-linking of lysine, arginine,

and tryptophan residues in freeze-dried mixtures of lysozyme or acetylated lysozyme with glucose during storage for 3 days at 50°C and 75% relative humidity. The 3-deoxyglucosone was a cross-linker for protein polymerization through Maillard reaction. Handa and Kuroda (1999) found that spray-dried egg white (6.5% water content) with glucose (35% relative humidity) after storage at 55°C for up to 12 days showed the formation of protein-glucose complexes in the initial stages of the Maillard reaction (decrease of glucose and available lysine contents); an increase of browning, sulfhydryl groups content, polymerization and aggregation of proteins at the later stage of the reaction; and a decrease of pH and α-helix content with increasing thermal storage time. Their results showed that the proteins in egg albumin were unfolded through the Maillard reaction. The polymerization or aggregation of proteins via Maillard reaction involved the covalent bonds rather than the disulfide bonds.

1.1.2 Control and inhibition of the Maillard reaction

The Maillard reaction is a_w and water content dependent (Labuza and Dugan, 1971), and it is affected by numerous factors including the chemical structure of sugars and amino acids (Lewis and Lea, 1950; Pomeranz *et al.*, 1962; Ashoor and Zent, 1984; Kato *et al.*, 1986; Carpenter *et al.*, 1987a), concentration or quantitative ratio of amino groups to reducing sugar (Kato *et al.*, 1986), pH (Pomeranz *et al.*, 1962; Ashoor and Zent, 1984; Morita and Kashimura, 1991; Baxter, 1995), storage time (Kato *et al.*, 1986), temperature (Fry and Stegink, 1982; Baxter, 1995), oxygen (Fu *et al.*, 1990; Hayase *et al.*, 1996), porosity and collapsed structure of the matrix (Karmas *et al.*, 1992; Buera and Karel, 1995; Schebor *et al.*, 1999; Burin *et al.*, 2004), crystallization of sugar in the systems (Karmas *et al.*, 1992; Burin *et al.*, 2004; Buera *et al.*, 2005; Drusch *et al.*, 2006).

Lewis and Lea (1950) showed that aldopentoses (xylose and arabinose) reacted with casein proteins at 25°C over a 16 days period more rapidly than aldohexose (glucose), followed by aldodisaccharides (lactose and maltose) and ketohexose (fructose). The development of brown colour in the casein-sugar mixtures at 37°C followed the order xylose > arabinose > glucose > lactose, maltose, and fructose; which showed that pentoses cased a more rapid damage to the amino groups of casein proteins than aldohexose and ketohexose. Bunn and Higgins

(1981) showed that the reactivity of sugars with proteins in glycation was more dependent on the open (carbonyl) structure than on the ring (hemiacetal or hemiketal) structure due to the high stability of the ring structure, and aldoses showed higher reactivities than ketoses. Pilková *et al.* (1990) showed that the rate of browning of Heyns products was slower that that of Amadori products. Morita and Kashimura (1991) suggested that phosphorylated monosaccharides produced higher levels of browning and fluorescence than their monosaccharide units. Baxter (1995) reported results in agreement with Lewis and Lea (1950) that an aldohexose (glucose) was more reactive to interact with amino acids and formed browning at pH 7.5 after heating at 128°C for 225 s than a ketohexose (fructose) and aldodisaccharides (lactose and maltose). Kwak and Lim (2004) showed that the intensities of browning from interactions between sugars (maltose, fructose, glucose, arabinose, and xylose) and amino acids (aspartic acid, glutamic acid, alanine, leucine, isoleucine, valine, proline, serine, cysteine, phenylalanine, arginine, and lysine) was in the order xylose > arabinose > glucose > maltose > fructose, and lysine had the highest reactivity with sugars followed by arginine and cysteine. The sulfur containing amino acids and peptides such as cysteine and glutathione were effective in preventing long-term food browning (both enzymatic and nonenzymatic browning reactions) under typical food storage and processing conditions (Molnar-Perl and Friedman, 1990).

Ashoor and Zent (1984) found that the maximum pH value for formation of brown colour in 0.005 M L-amino acids (lysine, alanine, and arginine) and 0.005 M glucose or fructose or α-lactose at 1:1 ratio in 8 mL of 0.05 M carbonate buffer solutions (pH 8 to 12) after heating at 121°C for 10 min was pH 10.0. They classified common amino acids and amides into three groups according to formation of brown colour during heating with reducing sugars (D-glucose, D-fructose, D-fructose, D-ribose, and α-lactose). The first group showed strong colour and included L-amino acids lysine, glycine, tryptophan, and tyrosine. The second group showed intermediate browning and included L-amino acids proline, leucine, isoleucine, alanine, hydroxyproline, phenylalanine, methioninine, valine, and the amides L-glutamine and L-asparagine. The third group was the least browning and included L-amino acids histidine, threonine, aspartic acid, arginine, glutamic acid, and cysteine. However, the conclusions of

these studies used results of studies conducted using buffer solutions and the results may vary in systems under dry heating or in solid systems. Bell (1997) showed that using different types (citrate and phosphate buffers) and concentrations (0.02 up to 0.5 M) of buffer to control the pH at 7.0 of the glucose-glycine (at 1:1 ratio) systems affected the initial degradation of amino acids and formation of brown pigment in the Maillard reaction. His results showed that the glucose-glycine in phosphate buffer showed a higher rate of glycine loss and formation of brown pigment than in the citrate buffer, especially at a high reactant concentration. Yeboah *et al.* (1999) found that the initial rate of utilization of amino groups (bovine serum albumin) by D-glucose in glycation was higher than by D-fructose under dry heating conditions [heated freeze-dried protein-sugar mixture (0.65 a_w) at 50°C]. The study was carried out in the presence of oxygen or nitrogen, which showed that the interaction of sugars and amino groups was slower in the presence of nitrogen than oxygen at the initial stage of Maillard reaction. D-fructose was more sensitive than D-glucose to the presence or absence of oxygen.

Burin *et al.* (2004) showed that the brown colour development in Maillard reaction between whey protein and lactose in a compressed structure of the system was higher than in the porous structure, which was accounted for the diffusion of water formed in the browning reaction. Their results showed that crystallization of lactose also accelerated browning, and, therefore, of the use of a lactose-maltodextrin (MD) mix in the systems retarded lactose crystallization (reduced molecular mobility of lactose) and also the brown colour development.

Nonenzymatic browning reaction can be inhibited and controlled by several means, e.g., using the following principles:

(*i*) The addition of reagents that can combine with or eliminate carbonyl groups such as aminoguanidine (prevented formation of fluorophores in the reaction), antioxidants [phenolic compounds, butylated hydroxyltoluene (BHT)], sodium bisulfite, metal chelating agents [e.g., diethylenetriamine pentaacetic acid (DETAPAC), citrate, deferoxamine mesylate, and superoxide dismutase; inhibited the browning and the

formation of fluorophores], sodium cyanoborohydride ($NaCNBH_3$; reduced Schiff Bases, inhibited Amadori rearrangement and formation of fluorecence), pyridoxamine [one form of vitamin B_6; inhibited post-Amadori steps, blocking oxidative degradation of Amadori intermediate, and scavenged reactive carbonyl compounds (from degradation of sugars, lipids, and amino acids)] (Molnar-Perl and Friedman, 1990; Pilková *et al.*, 1990; Morita and Kashimura, 1991; Voziyan and Hudson, 2005; Uribarri *et al.*, 2010).

(*ii*) Lowering of a_w or water content and temperature during processing and storage of products (Karmas *et al.*, 1992; Schebor *et al.*, 1999; Burin *et al.*, 2004).

(*iii*) Lowering of pH to the acidic condition below pH 7 (Pomeranz *et al.*, 1962; Molnar-Perl and Friedman, 1990; Pilková *et al.*, 1990; Kwak and Lim, 2004; Uribarri *et al.*, 2010).

(*iv*) Removal or conversion of one of the reactants of the Maillard reaction such as replacement of reducing sugars with nonreducing ingredients [sugar alcohols such as glycerol, sorbitiol, and maltitol (Liu *et al.*, 2009; Zhou *et al.*, 2013)].

(*v*) Removal of metal ions such as Zn^{2+}, Mn^{2+}, Fe^{2+}, Fe^{3+} and Cu^{2+} that may accelerate formation of Maillard reaction products such as chromophores and fluorophores by the oxidative pathway in the systems (Fry and Stegink, 1982; Pilkova *et al.*, 1990; Morita and Kashimura, 1991; Kwak and Lim, 2004).

(*vi*) Addition of mono- and divalent salt ions such as Na^+, K^+, Ca^{2+}, and Mg^{2+}, which can delay and inhibit the development of browning and prevent formation of acrylamide (Kwak and Lim, 2004; Gökmen and Şenyuva, 2007).

(*vii*) Removal and replacement of oxygen with an inert gas to reduce the rate of oxidation reactions that promote the Maillard reaction and thereby to decrease the formation of the browning products (Ahmed *et al.*, 1986; Fu *et al.*, 1990).

(*viii*) Reduction of structural changes (maintenance physical stability) and deteriorative reactions that can accelerate the Maillard reaction such as collapse or shrinkage (Karmas *et al.*, 1992; Buera and Karel, 1995; Schebor *et al.*, 1999), sugar crystallization (Karmas *et al.*, 1992; Burin *et al.*, 2004; Buera *et al.*, 2005; Drusch *et al.*, 2006), state of the matrix (Lievonen *et al.*, 1998), phase separation (Lievonen *et al.*, 1998), and lipid oxidation (Hidalgo and Zamora, 2000; Zamora and Hidalgo, 2005).

1.2 LIPID OXIDATION IN HIGH SOLIDS SYSTEMS

Lipid oxidation is a deteriorative reaction that often occurs during food processing and storage, and ageing *in vivo*. The breakdown or decomposition reactions of unsaturated fatty acids lead to the development of undesirable off flavours (rancidity); formation of toxic compounds; loss of flavour, nutritional value, and physical stability (colour changes) of foods (Greene, 1969; Labuza and Dugan, 1971). Lipid oxidation shows highest rates at a_w between 0.60 and 0.80. That a_w range agrees with the highest rates reported for the nonenzymatic browning reaction, but oxidation reactions also show high rates at low a_w (a_w < 0.1) (Labuza and Dugan, 1971) (Fig. 1.4). Lipid oxidaton reactions can be divided into three stages (initiation, propagation, and termination) following the free radical mechanism shown in Fig. 1.5. The initiation stage is for the formation of a free radical (an unpaired electron compound). Formation of the free radicals is initiated by catalysts such as light, oxygen, and water (Labuza *et al.*, 1969). The propagation stage is a chain of reactions of free radicals with oxygen or unsaturated fatty acids. Propagation produces more free radicals (initiating a chain reaction or autocatalyic process) that become responsible for producing compounds causing rancidity [volatile aldehydes and ketones (hydroperoxides)]. The termination stage includes reactions between free radicals and non-radical end products. The general pathways of lipid oxidation and the extent of the reaction against time are shown in Fig. 1.6 and 1.7, respectively.

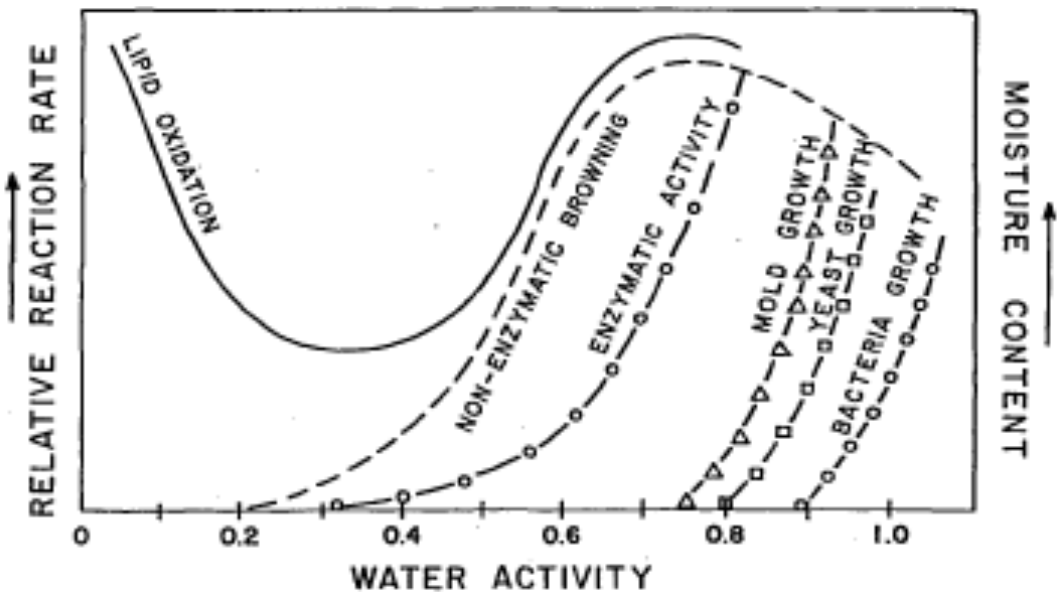

Fig. 1.4 Stability map of foods showing relative rates of reactions against water activity (Labuza and Dugan, 1971).

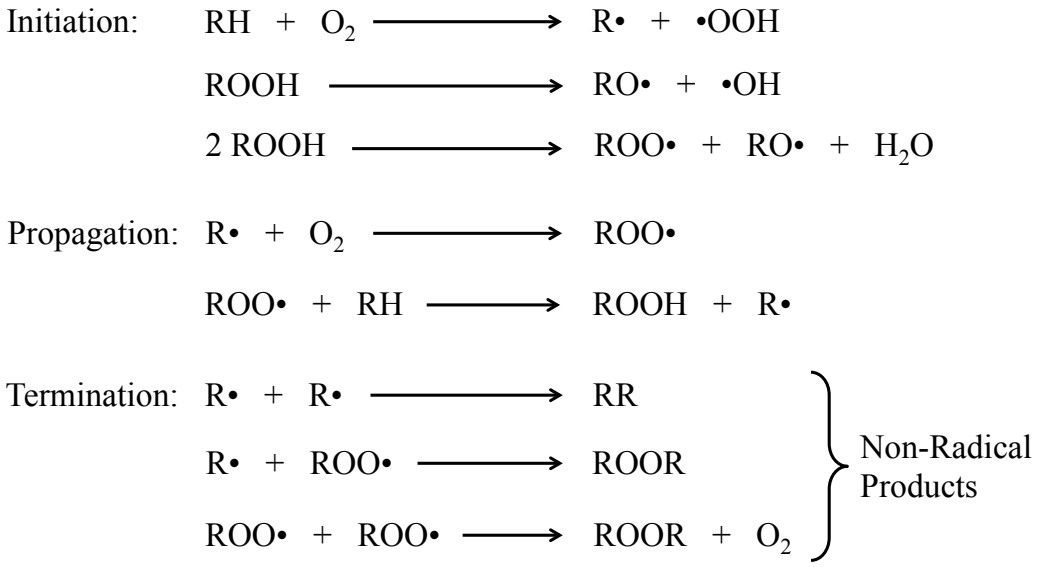

Fig. 1.5 The free radical mechanism in lipid oxidation [RH = unsaturated fatty acid or substrate, R• = alkyl radical (substrate), ROOH = hydroperoxide (e.g., hexanal, pentanal, and malonaldehyde), RO• = alkoxyl radical (break down product of lipid hydroperoxides), •OOH = hydroperoxyl radical, •OH = hydroxyl radical, ROO• = peroxyl radical, and RR and ROOR = stable end product with no free radical] [modified from Labuza and Dugan (1971), and Karel (1973)].

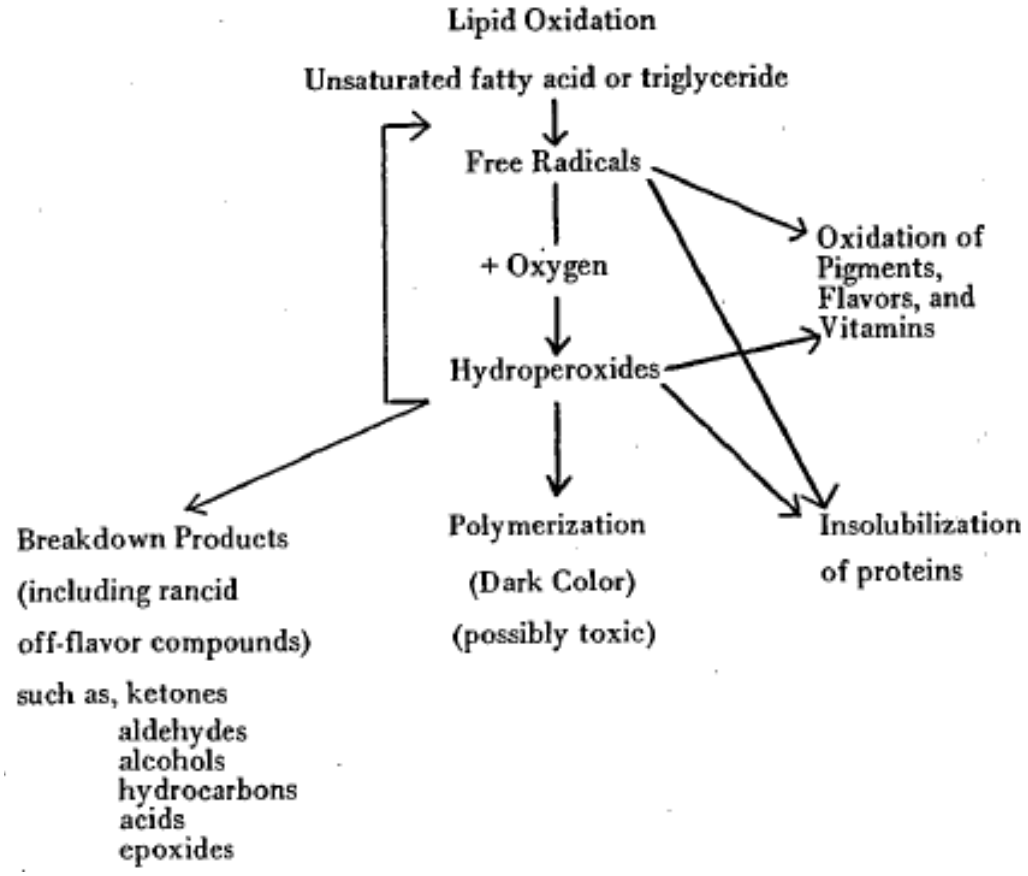

Fig. 1.6 The general pathways of lipid oxidation (Labuza and Dugan, 1971).

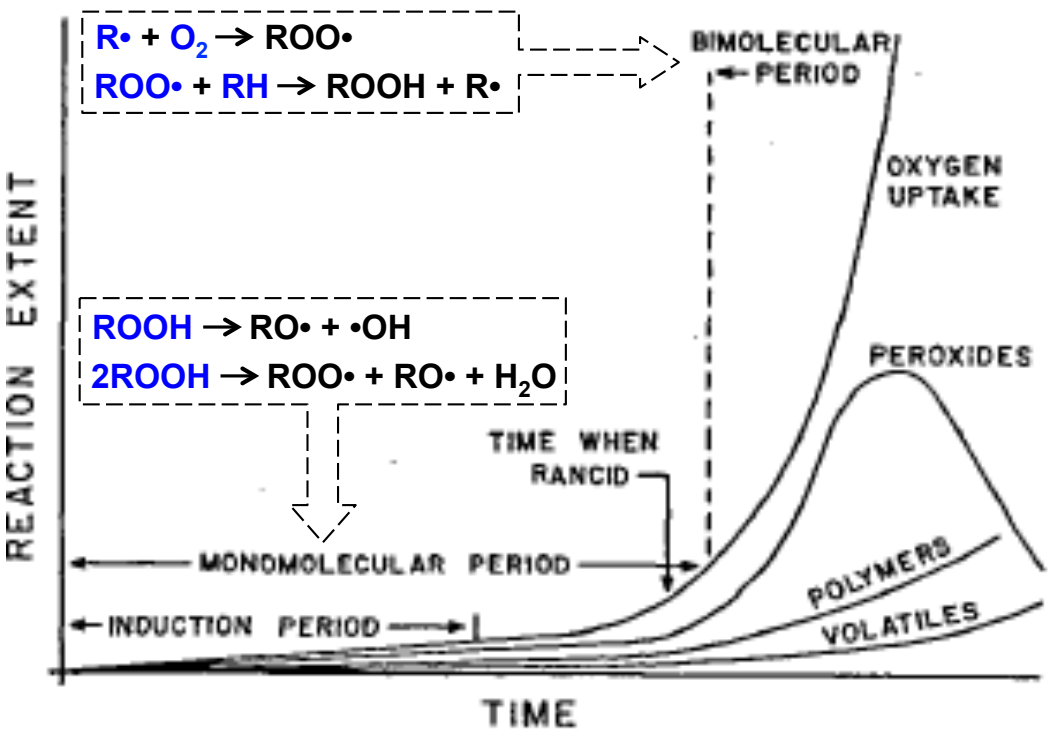

Fig. 1.7 Extent of lipid oxidation reactions as a function of time [modified from Labuza and Dugan (1971) and Karel (1973)].

The consumption of lipid oxidation products in diet, especially products from lipid peroxidation (e.g., malonaldehyde and 4-hydroxynonenal), can cause apoptosis or damage to phospholipid (lipoprotein) membranes (Karel, 1973; Spiteller, 2005), Alzheimer's disease (neuron degeneration in brain) (Sayre *et al.*, 1997; Markesbery and Lovell, 1998), atherosclerosis and coronary artery diseases (Addis, 1986; Stringer *et al.*, 1989; Plachta *et al.*, 1992), liver diseases (Yagi, 1987; Rouach *et al.*, 1997), formation of ageing pigments or fluorescent complexes (Karel, 1973), rheumatoid arthritis (Baskol *et al.*, 2006), and skin inflammation and acne (Briganti and Picardo, 2003). The lipid peroxidation products (including free radicals) can attack the endothelial cells of vessels, intact organs and tissues (dysfunction of the membrane), increasing platelets aggregation, and accumulate in the blood (attack the blood vessels) *in vivo* (Yagi, 1987). The malonaldehyde (dicarbonyl compound) and 4-hydroxynonenal (polar lipid) can interact with amino acids (e.g., arginine, lysine, cysteine, histidine, methionine, and tyrosine) in proteins via either intra- or intermolecular cross-linking leading to chemical modification of proteins (oxidative protein damage, increased protein carbonyl content), polymerization, browning, and formation of fluorescent complexes (Karel, 1973; Requena *et al.*, 1996; Rouach *et al.*, 1997) in ageing and neurodegenerative and chronic diseases. The mechanisms of free-radicals transfer to the proteins and amino acids via lipid peroxidation products are given below (Schaich and Karel, 1976):

$$\mathrm{ROOH + PH \longrightarrow ROOH\text{ ---- }HP} \begin{cases} \nearrow \mathrm{RO\bullet + P\bullet + H_2O} \\ \searrow \mathrm{RO\bullet + \bullet OH + PH} \end{cases}$$

$$\mathrm{PH + RO\bullet \longrightarrow P\bullet + ROH}$$

where ROOH = lipid hydroperoxide

PH = nitrogen or sulfur centers of reactive amino acid residues of the protein

RO• = alkoxyl radical (break down product of lipid hydroperoxides)

P• = protein radical

•OH = hydroxyl radical

ROH = lipid hydroxide

It should be noted, however, that lipid peroxidation reactions could produce carbonyl compounds (e.g., glyoxal, CML, and GOLD), as shown in Fig. 1.2. Such carbonyl compounds are typical products of the Maillard reaction in food and biological systems (Requena *et al.*, 1996). Indeed, oxidation and the Maillard reaction can be interrelated and concomitant occur.

1.2.1 Impact of lipid oxidation

The effects of lipid oxidation products on properties of high solid foods have been studied by several authors. Labuza *et al.* (1969) and Zirlin and Karel (1969) investigated effects of oxidized lipids on proteins during storage at various relative humidities. Their studies revealed that aggregation [decreased protein solubility in water and acetate buffer (pH 4.8); increased protein hydrophobicity] and oxidation of the protein in a freeze-dried gelatin-methyl linoleate system increased with increasing relative humidity (up to 60% relative humidity) during 6 days of storage at 50°C, although less lipid oxidation occurred at high relative humidities. The system stored at a low relative humidity (maximum for lipid oxidation) showed a lower melting temperature for a gel prepared using the system. That is the degraded protein had lower molecular weight fragments (protein scission), which gave an increased solubility of the gelatin in ethanol-0.8M NaCl mixtures. Zirlin and Karel (1969) concluded that at the high relative humidities cross-linking of protein radicals [from interaction of lipid peroxyl radical (ROO•) with protein (PH); ROO• + PH → ROOH + P•] was favoured and predominated over the oxidative scission of the protein. Conversely, in the dry state (low relative humidity) protein radicals interacted with oxygen to form protein peroxyl radicals (P• + O_2 → POO•), and subsequent scission of the –N–C– bonds.

The interaction of lipid peroxidation products (free radicals, lipid hydroperoxides, and aldehyde derivatives) and proteins in dehydrated food systems may result in losses of amino acids (cysteine, histidine, lysine, methionine, tryptophan, tyrosine) (damaged protein) (Roubal and Tappel, 1966a;

Zirlin and Karel, 1969; Roubal, 1971; Nielsen *et al.*, 1985; Refsgaard *et al.*, 2000; Wu *et al.*, 2010), browning (Zirlin and Karel, 1969), copolymerization of peroxidized lipids and proteins (lipid-protein complexes) (Roubal and Tappel, 1966b; Kanner and Karel, 1976; Funes *et al.*, 1982), formation of free protein radicals (Roubal and Tappel, 1966a; b; Zirlin and Karel, 1969; Karel *et al.*, 1975), formation of disulfide bonds (Hidalgo and Kinsella, 1989), protein oxidation [formation of protein-carbonyl derivatives (Stadtman, 1992; Dalle-Donne *et al.*, 2003)] (Labuza *et al.*, 1969; Zirlin and Karel, 1969; Refsgaard *et al.*, 2000), denaturation and aggregation [protein insolubility increasing at high a_w (0.75 a_w)] (Zirlin and Karel, 1969; Karel, 1973; Kanner and Karel, 1976; Leake and Karel, 1982), and scission of proteins (increased protein solubility and decreased viscosity of protein dispersions) (Roubal and Tappel, 1966b; Labuza *et al.*, 1969; Zirlin and Karel, 1969) as well as production of covalently cross-linked protein-protein polymers (Roubal and Tappel, 1966b; Karel, 1973; Kanner and Karel, 1976; Leake and Karel, 1982). The polymerization, cross-linking, and scission of proteins depend on water activity of the system. Zirlin and Karel (1969) showed that the protein scission (decreased molecular weight and increased content of protein amide groups) occurred when proteins were exposed to peroxidized lipids, but the reaction was inhibited after increasing the a_w of the system. Kanner and Karel (1976) showed that a high a_w (0.75 a_w) promoted cross-linking and aggregation or insolubilization of proteins in system containing lipid peroxides and proteins.

Karel (1973) reported that lipid-protein complexes in food systems affected stiffness (rigor mortis) in aging of meat, and loss of protein solubility and browning during storage of frozen fish and dried muscle foods. The interactions of radicals produced by lipid oxidation with proteins are shown in Fig. 1.8. Wu *et al.* (2010) found that the structure and properties of a soy protein isolate were modified by acrolein (aldehyde derivative of a product of lipid peroxidation) in a sodium phosphate buffer (pH 7.4) dispersion during incubation at 25°C in dark for 24 h. Acrolein caused denaturation (unfolding and exposed hydrophobic residues) and aggregation (hydrophobic interactions) of the protein, decreased protein solubility in water (pH 7), increased protein carbonyls, decreased total sulfhydryl (S-H) content (formation of disulfide bonds and sulfur-containing

oxidation products), formation of covalently cross-linked proteins (with no disulfide bonds), loss of α-helix structure, and increased β-sheet structure. Cucu *et al.* (2011) showed that incubation of solutions of whey protein isolate with oils (fish, sunflower, soybean, and olive oil) at 70°C for up to 48 h resulted in an increase of protein bound carbonyls [amino acids react with lipid hydroperoxides (e.g., 4-hydroxy-2-nonenal and malonaldehyde) or with reactive carbonyl derivatives (e.g., ketoamines, ketoaldehydes, deoxyosones) generated from the Maillard reaction (Berlett and Stadtman, 1997; Refsgaard *et al.*, 2000)]. The carbonyl content was dependent on the oil used and the carbonyl content decreased in the order fish oil > soybean oil > sunflower and soybean oil > olive oil. The formation of high molecular weight aggregates of the protein and covalent cross-linking of the protein as well as increased lipid oxidation increased with storage time.

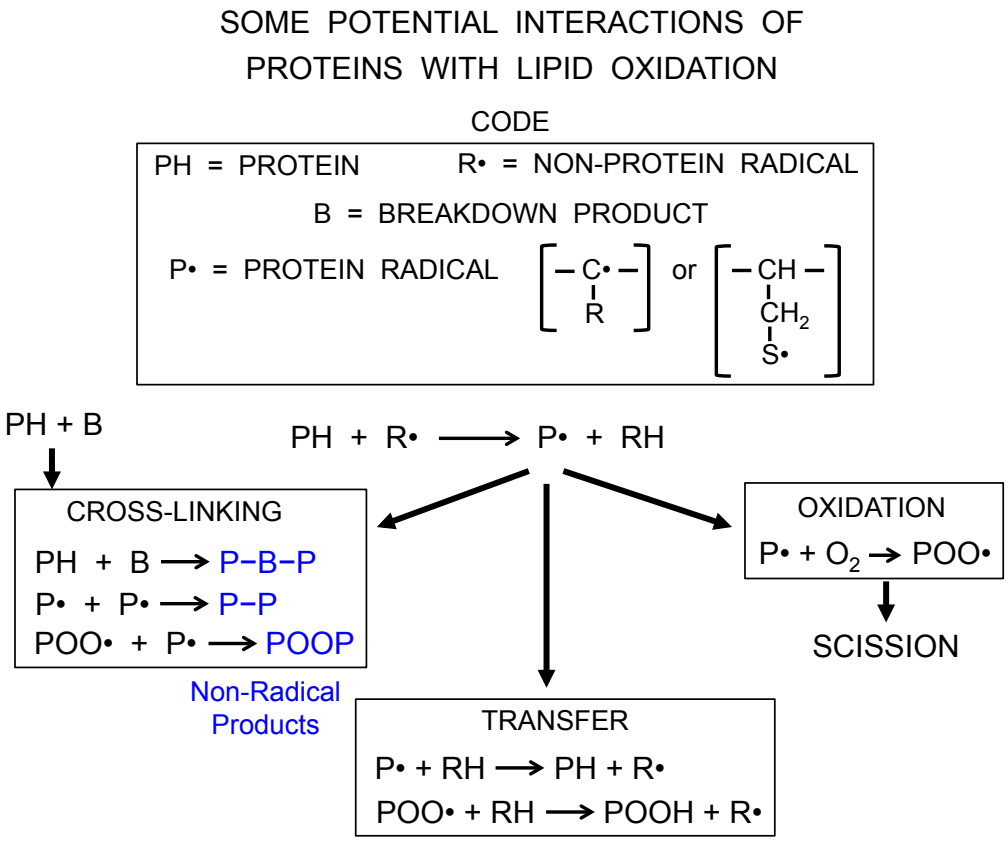

Fig. 1.8 Schematic representation of reactions of proteins with peroxidizing lipids (R• and B) (Karel *et al.*, 1975).

1.2.2 Occurrence and inhibition of lipid oxidation

The rate of lipid oxidation in high solids systems depends on the amount of unsaturated fatty acid moieties or double bonds in the lipid molecules (not on the total fat content) (Labuza and Dugan, 1971), a_w or water content (Labuza *et al.*, 1969; Labuza and Dugan, 1971; Labuza *et al.*, 1972), enzymes (Rhee *et al.*, 1987), food components (Labuza *et al.*, 1969; Sakanaka *et al.*, 2004; Peña-Ramos and Xiong, 2003), glass transition temperature (Shimada *et al.*; 1991), oxygen (Labuza and Dugan, 1971; Ordonez and Ledward, 1977), pH (Richards and Hultin, 2000), processing and storage temperature (Labuza and Dugan, 1971; Morcira *et al.*, 1997; Krokida *et al.*, 2000; Jakobsen and Bertelsen, 2000), physical structure (e.g., porosity, size, and surface area) of the material (Labuza *et al.*, 1969; Pinthus *et al.*, 1995; Morcira *et al.*, 1997; Krokida *et al.*, 2000), crystallization of components (Shimada *et al.*, 1991; Drusch *et al.*, 2006), and Maillard reaction (Kirigaya *et al.*, 1968; Bucala *et al.*, 1993; Yen and Hsieh, 1995; Mastrocola and Munari, 2000; Breitling-Utzmann *et al.*, 2001).

Labuza *et al.* (1969) demonstrated that the gelatin-methyl linoleate systems (60% relative humidity) at 50°C showed enhanced oxidation of protein (aggregation and reduced solubility of protein), although the lipid oxidation was reduced. They reported that the protein reacted with the peroxides through a free radical mechanism, which reduced the lipid oxidation reaction rate. Labuza and Dugan (1971) found both positive and negative effects of water on the rate of lipid oxidation. Water as solvent mobilized hydrophilic reactants. Water could interact with metal catalysts making them less effective as well as hydrogen bond with hydroperoxides with a consequent reduction of the rate of lipid oxidation at the initiation stage. The solvent and mobilization properties of water are pronounced at high water activities (0.55 to 0.85 a_w) as a result of mobilization of catalysts (increasing rate of lipid oxidation).

Foods containing proteins {e.g., egg proteins (Sakanaka *et al.*, 2004), gelatin (Labuza *et al.*, 1969), dairy and other proteins [e.g., caseins, soy protein isolates, and whey protein isolates (Hu *et al.*, 2003)], protein hydrolysates (Peña-Ramos and Xiong, 2003; Sakanaka *et al.*, 2004)} can retard or inhibit lipid oxidation because of their chelating properties and free radical scavenging by amino acids.

Systems containing carbohydrates, sugars, and caramelization products can also retard lipid oxidation (Sims *et al.*, 1979; Benjakul *et al.*, 2005a; Drusch *et al.*, 2006). In a wet emulsion system, the use of a sugar at a high concentration (up to 67%) decreased the concentration of oxygen in the aqueous phase, which decreased the diffusion of oxygen in the matrix (because of the increased viscosity of the aqueous phase) (Sims *et al.*, 1979). In an amorphous system, retarded lipid oxidation was attributed to the unique binding properties of sugars to dienes (Drusch *et al.*, 2006). The caramelization products from heated sugars (D-ribose, D-fructose, D-glucose, and D-xylose) acted as antioxidants (free radical scavenging and chelating activities). Such compounds effectively retard and inhibit lipid oxidation in food systems (Benjakul *et al.*, 2005a).

Products from the Maillard reaction either promote or reduce lipid oxidation. Amadori products from the Maillard reaction can react with phospholipids (biological membranes) leading to increased lipid oxidation *in vivo* (Bucala *et al.*, 1993; Breitling-Utzmann *et al.*, 2001). Several authors reported that formation of brown pigments in the Maillard reaction reduced lipid oxidation (Kirigaya *et al.*, 1968; Yen and Hsieh, 1995; Mastrocola and Munari, 2000). Such inhibition of lipid oxidation was dependent upon brown colour intensity (Kirigaya *et al.*, 1968; Yen and Hsieh, 1995). Water-soluble products (from a condensation step at the early stage), hydroxymethylfurfural (intermediate product), and melanoidins (brown polymers at final stage) of the Maillard reaction can act as free radical scavengers and reducing (donation of hydrogen atoms) and chelating agents (Gomyo and Horikoshi, 1975; Kirigaya *et al.*, 1968; Ames, 2001; Yilmaz and Toledo, 2005; Chen *et al.*, 2009). Conversely, the Maillard reaction can be promoted by the presence of oil and its oxidation products (Mastrocola *et al.*, 2000).

The lipid oxidation can be controlled and prevented by the following ways:

(*i*) Addition of antioxidants (radical-scavenging activity) {e.g., butylated hydroxyl anisole (BHA), butylated hydroxyl toluene (BHT), tertiary-butylated hydroquinone (TBHQ), propyl gallate (PG), lecithin, phenolic compounds [ascorbic acid (vitamin C), α-tocopherol (vitamin E),

xanthones, carotenoids (β-carotene, lutein), gallic acid, flavonoids (e.g., anthocyanidins, quercitin, and catechins)]} for scavenging free radicals (Labuza *et al.*, 1969; Sherwin, 1978; Rice-Evans *et al.*, 1997; Balasundram *et al.*, 2006).

(*ii*) Control factors that increase mass transfer of oil (water loss and oil uptake) into the products during processing such as low initial water content, low viscosity of oil, physical structure (low porosity or low pore size distribution, low surface area or small size, and high thickness of materials), high heating temperature and short heating time (Pinthus *et al.*, 1995; Morcira *et al.*, 1997; Krokida *et al.*, 2000).

(*iii*) Control a_w or water content, temperature, and storage time (Labuza *et al.*, 1969; Labuza and Dugan, 1971; Nielsen *et al.*, 1985; Sun *et al.*, 2002).

(*iv*) Removal of metals and minerals (the predominant prooxidant materials) such as cobalt, copper, iron, magnesium, manganese (Labuza *et al.*, 1969; Sherwin, 1978).

(*v*) Removal of enzymes (e.g., lipases and lipoxygenases) in animal and plant tissues. Enzymes strongly catalyze oxidative decomposition of fats and oils (Sherwin, 1978).

(*vi*) Removal (anaerobic packaging) and replacement of oxygen with an inert gas (nitrogen) to reduce the rate of oxidation reactions in packages (Greene, 1969; Labuza and Dugan, 1971; Nielsen *et al.*, 1985).

(*vii*) Minimize light and UV exposure during storage (Wishner, 1964; Sattar *et al.*, 1975).

(*viii*) The use of fats and oils that contain low levels of unsaturated fatty acids.

(*ix*) Reduction of unsaturated fatty acids by hydrogenation (addition of pairs of hydrogen atoms to the double bonds) to improve flavor and oxidative stability (increased oil mellting temperature), although this reaction converts liquid oils to semisolid fats (Coenen, 1976; Emken, 1984). The hydrogenated oil can be used in a variety of foods such as margarines, mayonnaise, potato chips, shortenings, and bakery products (e.g., breads, cakes, cookies, crackers) (Emken, 1984).

(*x*) Reduction of structural changes (maintenance physical stability). The liquid-like structure was a catalytic condition for lipid oxidation (Labuza *et al.*, 1969).

1.3 PROTEIN OXIDATION

Protein oxidation results in covalent modification of the protein (irreversible modification) structure. Protein oxidation is induced either directly by reactive oxygen species, especially free radicals of oxygen [i.e., superoxide ($O_2^{\bullet -}$), hydroxyl radical (•OH), hydroperoxyl radical (HOO•), alkoxyl radical (RO•), peroxyl radical (ROO•), nitric oxide (NO•), sulfinyl radical (RSO•), and thioperoxyl radical (RSOO•). (Stadtman and Berlett, 1998)] or indirectly by reactions with secondary products of oxidation (Rivett *et al.*, 1985; Starke-Reed and Oliver, 1989; Stadtman, 1992; Shacter, 2000). The reactive oxygen species are generated in glycation or glycoxidation or Maillard reaction, irradiation (γ-rays, X-rays, and UV), lipid peroxidation and from free radical break down products (e.g., hydroxyl radical, alkoxyl radical, and peroxyl radical), and inflammatory reactions (neutrophil macropharges) (Stadtman and Levine, 2000). The lipid peroxidation products including free radicals, lipid hydroperoxides, and reactive aldehyde derivatives [e.g., 4-hydroxy-2-alkenals (4-hydroxynonenal and 4-hydroxyhexenal), dicarbonyls (gloxal, methylglyoxal and malonaldehyde), unsaturated aldehydes (acrolein), and saturated aldehydes (ethanal, propanal, and hexanal) (Fig. 1.9)] are most probably responsible for the damage of protein and biological membranes (Chio and Tappel, 1969; Uchida and Stadtman, 1992; Stadtman and Berlett, 1998). The gloxal and methylglyoxal are also produced in the Maillard reaction as shown in Fig. 1.2.

The lipid peroxidation products and the increase in protein oxidation can inactivate the sulfhydryl groups and protease (e.g., ribonuclease A, brain, and liver enzymes) enzymes or inhibit the specific (binding) activity of enzymes (Chio and Tappel, 1969; Rivett *et al.*, 1985; Starke-Reed and Oliver, 1989; Carney *et al.*, 1991). Such enzymes are responsible for the degradation of the oxidized (modified or denatured) forms of proteins (Chio and Tappel, 1969; Starke-Reed and Oliver, 1989; Stadtman *et al.*, 1992). Protease (enzyme) activities are not responsible for the increase of oxidized proteins during aging (Starke-Reed and Oliver, 1989). A number of amino acid residues of proteins such as arginine, cysteine, histidine, leucine, lysine, phenylalanine, methionine, proline, threonine, tryptophan, and tyrosine are highly sensitive to attacks by reactive oxygen in free radical species and lipid peroxidation products (Creeth *et*

al., 1983; Uchida and Stadtman, 1992; Stadtmen and Berlett, 1998; Stadtman and Levine, 2003; Lund *et al.*, 2008). The oxygen free radical-mediated oxidation of methionine forms methionine sulfoxide and methionine sulfone, and cysteine forms cysteine disulfides and sulfenic acid (Garrison, 1987).

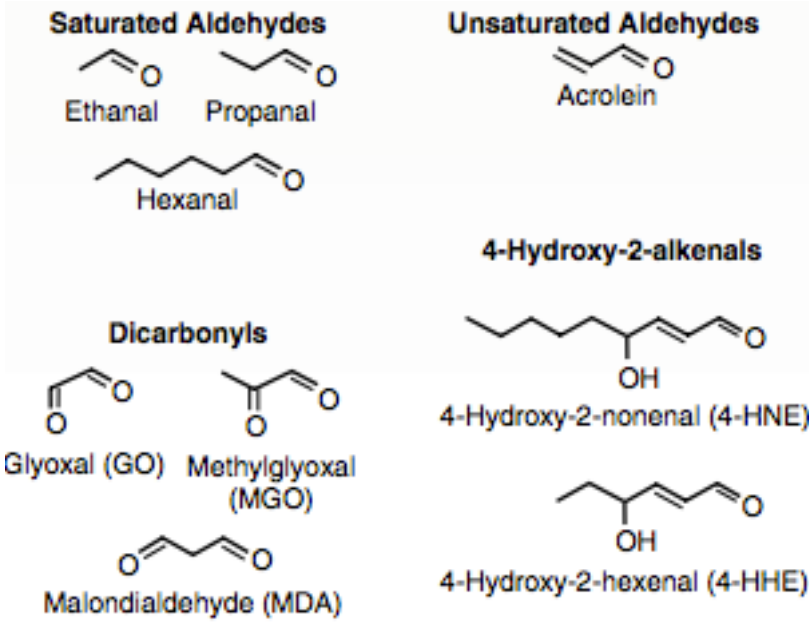

Fig. 1.9 Structure of some aldehydic compounds derived from lipid peroxidation (Negre-Salvayre *et al.*, 2008).

Oxidation of enzymes and some amino acids (lysine, arginine, threonine, and proline) leads to the formation of carbonyl (aldehydes and ketones) derivatives (Amici *et al.*, 1989; Stadtman and Berlett, 1998; Bedell-Hogan *et al.*, 1993; Requena *et al.*, 2001; Stadtman, 2001). Amici *et al.* (1989) and Requena *et al.* (2001) found that glutamic semialdehyde was a major product of both oxidized proline and arginine residues. Oxidized proline also produced 2-pyrrolidone (Uchida *et al.*, 1990), hydroxyproline (Stadtman and Levine, 2003), and pyroglutamic acid (carbonyl derivatives) (Stadtman and Levine, 2003). Pinnell and Martin (1968) and Bedell-Hogan *et al.* (1993) found that aminoadipic semialdehyde was a carbonyl product of oxidized lysine. Oxidized threonine produces 2-amino-3-ketobutyric acid (Dalle-Donne *et al.*, 2003). Oliver *et al.* (1987) found that the protein carbonyl derivatives and the loss of enzyme activity were increased with age. An increased carbonyl content of proteins with age and time could be explained by first-order reaction kinetics (Oliver *et al.*, 1987;

Carney *et al.*, 1991; Smith *et al.*, 1991; Wells-Knecht *et al.*, 1995). The protein carbonyl derivatives were formed by interaction of proteins with reducing sugars and dicarbonyl compounds or with products of lipid peroxidation. The carbonyl groups were major products of reactive oxygen free radical-mediated oxidation reaction. Carbonyl groups in proteins are widely accepted as an indicator or marker of oxidative protein damage *in vivo* and in foods (Carney *et al.*, 1991; Stadtman, 1992; Dalle-Donne *et al.*, 2003). The presence of carbonyl derivatives of proteins reflects the extent of damages induced by multiple forms of reactive oxygen species and free radicals as a result of oxidation. The accumulation of proteins with oxidative damages *in vivo* is associated with ageing, ischemia-reperfusion injury, and a number of age-related diseases including diabetes, Alzheimer's disease, Parkinson's disease, amyotropic lateral sclerosis, cataractogenesis, atherosclerosis, Huntington's disease, rheumatoid arthritis, chronic renal failure, and many other disorders (Berlett and Stadtman, 1997; Stadtman and Berlett, 1998; Butterfield and Kanski, 2001; Dalle-Donne *et al.*, 2003).

1.3.1 Impact of oxidation on proteins

The covalent modification of proteins during ageing as well as food processing has been shown to result in losses of protein functionality or a specific activity of enzymes (Rivett *et al.*, 1985; Meucci *et al.*, 1991); loss of amino acids (Decker *et al.*, 1993); structural, conformational, and functional alteration of amino acids and proteins [e.g., gel strength (rigid gel), elasticity, water holding capacity, gel-formation ability, heat resistance] (Meucci *et al.*, 1991; Decker *et al.*, 1993; Liu *et al.*, 2000; Requena *et al.*, 2001). Protein fragmentation (increased carbonyl groups) (Davies, 1987; Liu and Xiong, 2000; Liu *et al.*, 2000; Lund *et al.*, 2008), formation of disulfide bonds (Bhoite-Solomon *et al.*, 1992; Liu and Xiong, 2000; Liu *et al.*, 2000; Lund *et al.*, 2008), protein-protein cross-linking (Roubal and Tappel, 1966b; Pinnell and Martin, 1968; Kanner and Karel, 1976; Bhoite-Solomon *et al.*, 1992; Bedell-Hogan *et al.*, 1993; Lund *et al.*, 2008), decreased protein denaturation (unfolding) temperature (Liu and Xiong, 2000), protein aggregation (Kanner and Karel, 1976; Butterfield and Kanski, 2001), increased protein hydrophobicity (Stadtman, 2001), insolubilization and indigestibility of proteins (Roubal and Tappel, 1966b; Kanner and Karel, 1976; Meucci *et al.*,

1991; Bedell-Hogan *et al.*, 1993; Liu and Xiong, 2000), polymerization of proteins (Decker *et al.*, 1993; Liu and Xiong, 2000; Liu *et al.*, 2000), and increased surface hydrophobicity of proteins (Meucci *et al.*, 1991; Chao *et al.*, 1997). Decker *et al.* (1993) demonstrated that oxidative damage of myofibrillar protein in white turkey muscle by iron and copper increased protein carbonyl content, protein hydrophobicity (lower solubility), and loss of myosin and actin proteins with concomitant protein polymerization. Liu *et al.* (2000) found that the amino acid side chains of whey protein isolate (WPI) and soy protein isolates (SPI) were modified during metal-catalyzed oxidation by $FeCl_3$, H_2O_2, and ascorbate. Such oxidation increased formation of disulfide bonds, protein carbonyls, and free amines in WPI, SPI, and myofibril-protein isolate (1:1) mixtures; and increased elasticity of an SPI gel and interactions of myofibrils with SPI.

Several pathways of protein-protein cross-linking in oxidative modification of proteins were reported by Stadtman and Levine (2003), including: (1) oxidation of cysteine sulfhydryl groups (disulfide bonds formation); (2) interaction of carbonyl groups of oxidized proteins with the amino groups of a lysine residues in the same or different protein molecules; (3) interaction of glycation derived protein carbonyls with lysine or arginine residues in the same or different protein molecules; (4) interaction of dicarbonyl groups (e.g., malonaldehyde and glyoxal) with two lysine residues in the same or different protein molecules; and (5) interaction of amino groups of lysine residues with protein carbonyls produced by lipid peroxidation reactions. The type of cross-linking in the systems was dependent on the oxidizing agents and interaction time (Bhoite-Solomon *et al.*, 1992; Lund *et al.*, 2008). Bhoite-Solomon *et al.* (1992) found that myoglobin and H_2O_2 (hydroxyl free radicals) induced formation of intermolecular disulfide cross-links of myosins (protein in heart and skeletal muscle) and myosin formed covalently aggregated protein (intermolecular covalent bonds). The latter aggregates of myosin resulted from pairing of myosin radicals formed by the H_2O_2. Lund *et al.* (2008) showed that oxidation of myosin by myoglobin and H_2O_2 produced oxidized tyrosine and cysteine residues (thiyl, tyrosyl, and other unidentified radical species) of myosin, and formation of both disulfide linkages and covalent cross-linking. These studies have shown that

formation of disulfide bonds and covalent cross-linkages occur in concomitant processes.

1.3.2 Control and reduction of protein oxidation

The oxidative damage of proteins is associated with chemical reactions and various environmental factors *in vivo* and in food processing, which can be governed by following factors:

(*i*) Use of antioxidants to prevent protein from oxidative damage by conversion of reactive free radical species to unreactive derivatives.

(*ii*) Removal of metal ions that can catalyze oxidation such as Fe^{2+}, Fe^{3+}, and Cu^{+} (Decker *et al.*, 1993; Liu *et al.*, 2000).

(*iii*) Minimize irradiation and UV exposure.

(*iv*) Removal of oxygen, reactive oxygen species, and free radicals.

(*v*) Control of relevance factors that induce development of protein oxidation such as glycation or Maillard reaction and lipid oxidation.

1.4 CONCLUSIONS

The interactions of carbohydrate, protein, and lipid components in high solids systems affect physical, physicochemical, thermal, and molecular properties, including losses of nutrients (e.g., vitamins, amino acids, proteins, and enzymes), physical stability (e.g., formation of unfavourable colour and flavour, deformed and collapsed structure, softened and hardened texture), and functional properties (solubility, indigestibility, heat and pH sensitivity, polymerization, cross-linking of proteins via covalent and disulfide bonds). Such complex phenomena are important factors that must be considered and controlled during processing and storage of high solids food systems and pharmaceutical formulations. Furthermore, consequences of chemical changes resulting from component interactions in the products have been found as risk factors for degenerative diseases *in vivo* as well as ageing.

CHAPTER II

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ADDITIVITY OF WATER SORPTION, α-RELAXATIONS AND CRYSTALLIZATION INHIBITION IN LACTOSE–MALTODEXTRIN SYSTEMS

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ABSTRACT

Water sorption of lactose–maltodextrin (MD) systems, structural relaxations and lactose crystallization were studied. Accurate water sorption data for non-crystalline lactose previously not available over a wide range of water activity, a_w (< 0.76 a_w) were derived from lactose–MD systems data. Structural relaxations and crystallization of lactose in lactose–maltodextrin (MD) systems were strongly affected by water and MD. At high MD contents, inhibition of crystallization was significant. Inhibition with a high dextrose equivalent (DE) MD was more pronounced possibly because of molecular number and size effects. At 0.55–0.76 a_w, inhibition increased with increasing MD content. At a_w > 0.66, the rate of lactose crystallization decreased at increasing MD contents. Different MDs with similar T_g in lactose–MD systems showed different crystallization inhibition effects. The results of the present study showed that the DE in selection of MD for applications has important effects on component crystallization characteristics.

KEYWORDS: crystallization, glass transition temperature, lactose, maltodextrin, molecular size, water sorption

2.1. INTRODUCTION

Crystallization is an important factor affecting physical stability of materials containing amorphous sugars during storage. It often results from an increase in molecular mobility (decrease of viscosity) above glass transition allowing formation of the highly ordered crystalline, equilibrium state, and leads to release of water sorbed by the amorphous material prior to crystallization (Roos, 1995b). The rate of crystallization of amorphous sugars is governed by water content and temperature of storage above the glass transition temperature, T-T_g (Roos and Karel, 1991a). Increasing water content leads to water plasticization and increased molecular mobility, which accelerate crystallization of amorphous sugars by decreasing the Tg (Roos and Karel, 1991a, 1992).

Previous studies showed that crystallization of amorphous sugars was delayed in mixtures with high molecular weight carbohydrate components, e.g., starch (Iglesias and Chirife, 1978; Roos and Karel, 1991a), MD (Labrousse *et al.*, 1992;

Iglesias *et al.*, 1997; Mazzobre *et al.*, 1997; Kouassi and Roos, 2001) and corn syrup solids or MD with the DE above 20 (Gabarra and Hartel, 1998). Miscible high molecular weight components generally increase viscosity, and the average molecular weight, and decrease molecular mobility of amorphous systems (Roos and Karel, 1991b; Sillick and Gregson, 2009). They may also increase the T_g value. Below the T_g, molecular mobility of amorphous materials is restricted to vibrations and rotations due to decreased free volume and packing of molecules. However, the effect of addition of high molecular weight components to decrease the rate of crystallization is not entirely a result of the increased T_g of the amorphous system (Roos and Karel, 1991a; Gabarra and Hartel, 1998) depending on the type of sugars. Roos and Karel (1992) suggested that the rate of crystallization of lactose at varying water contents and temperatures was controlled by the T_g. Crystallization of amorphous sugars can be reduced or delayed by increasing viscosity, decreasing diffusion, and reduced molecular mobility above the T_g (Roos and Karel, 1991a, 1991c), including effects of the presence of various other molecular species and impurities. Iglesias and Chirife (1978) reported that changes in physical state and the apparent rate of crystallization of freeze-dried sucrose was reduced by the presence of others components (starch, carboxymethylcellulose, microcrystalline, cellulose, guar gum, garrofin gum, and sodium alginate). In the same way, as reported of Gabarra and Hartel (1998), the crystallization of freeze-dried sucrose was interfered by the addition of 10 and 20% (w/w) and prevented by the addition above 50% (w/w) of DE 20 and 42 corn syrup solids. Mazzobre *et al.* (2001) reported that the addition of a second sugar component, such as trehalose, to an amorphous lactose system delayed crystallization, without affecting the T_g value. This may be related to interactions between sugars and other components which reduced or delayed the rate of diffusion or mobility of sugar molecules to form crystals (nucleation) or crystal growth, lattice interference or steric hindrance effects, above T_g. It should, however, be noted that the T_g values of lactose and trehalose are similar and the sugars may be mixed in any ratio with no significant effects on the T_g of the blend.

Dielectric (DEA) and dynamic-mechanical analyses (DMA) may be used in studies of molecular mobility, including secondary relaxations (γ- and β-

relaxations) below the calorimetric T_g and the α-relaxation (primary relaxation) of glass forming carbohydrate polymers (Kilmartin *et al.*, 2004; Pagnotta *et al.*, 2009) and amorphous solids formulations (Kalichevsky and Blanshard, 1993; Noel *et al.*, 2000; Lievonen and Roos, 2003; Ermolina *et al.*, 2007; Silalai and Roos, 2011). Such measurements have been used to study dynamic (Mijovic, 1998; Massalska-Arodz *et al.*, 1999; Talja and Roos, 2001; Wurm *et al.*, 2003) and isothermal crystallization (Mijovic, 1998; Massalska-Arodz *et al.*, 1999; Wurm *et al.*, 2003; Alie *et al.*, 2004; Bhugra *et al.*, 2007) of sugars and polymers. Crystallization behavior of amorphous materials may be observed from a sharp frequency-independent change of permittivity (dielectric constant, ε′) and dielectric loss (ε″) from dielectric analysis data, and storage modulus (E′; mechanical energy storage) and loss modulus (E″; mechanical energy dissipation) from dynamic mechanical analysis measurements above the α-relaxation temperature. Rapid changes in dielectric and mechanical properties result from the transformation of the amorphous components into crystals, growth of crystalline regions, and migration of charges and orientation of dipoles (Mijovic, 1998; Talja and Roos, 2001; Gonnet *et al.*, 2002). Gonnet *et al.* (2002) reported that the crystallization process of Kynar 710 poly (vinylidene fluoride) (semi-crystalline polymer) showed an increase in ε′ when measured using DEA corresponding to a sharp increase of the E′ measured by DMA. The onset temperature of crystallization found by DEA and DMA was approximately the same as the onset temperature of crystallization recorded in DSC measurements. Thus, the DEA and DMA were powerful and suitable techniques for monitoring of the crystallization of polymers.

The objectives of the present study were to determine effects of DE and glass transition of MD with lactose on water sorption, lactose crystallization behavior and to investigate dielectric and dynamic mechanical properties at crystallization of lactose in lactose–MD systems. This study is useful for understanding effects of MD on the crystallization of lactose in food and pharmaceutical materials as the DE of MD may have an important role in preventing sugar crystallization.

2.2. MATERIALS AND METHODS

2.2.1. PREPARATION OF AMORPHOUS FREEZE-DRIED MATERIALS

MDs were of low DE of 4–7, 9–12, and high DE of 23–27 (Maltrin M040, M100, and M250, respectively; Grain Processing Corp., IA, USA). Freeze-dried amorphous solids of MD DE 4–7, 9–12, and 23–27, α-lactose (monohydrate, Sigma Chemical Co., St. Louis, MO, USA), and lactose–MD were prepared from solutions containing 20% (w/w) total solids. The ratios of lactose:MD DE 4–7 in solid was 40:60 and lactose:MD DE 9–12 and 23–27 in solids were 90:10, 80:20, 70:30, and 40:60. Aliquots of 5 mL (approximately 1 g of freeze-dried material) of MD, lactose, and lactose–MD solutions in preweighed 10 mL glass vials (semi-closed with septum) were frozen at -20°C for 24 h, followed by -80°C for 3 h, and then freeze-dried for 60 h at pressure (p) < 0.1 mbar (Lyovac GT 2, Steris®, Hürth, Germany) to obtain amorphous materials. All vials were hermetically closed with vacuum inside the freeze dryer at p < 0.1 mbar and kept over P_2O_5 in vacuum desiccators (Roos and Karel, 1990) at room temperature (20±2°C) to protect samples from water uptake.

2.2.2. WATER SORPTION STUDY

Sorbed water of stored freeze-dried MD DE 9–12 and 23–27, lactose, and lactose–MD DE 9–12 and 23–27 at 90:10, 80:20, and 70:30 ratios were monitored for 5 days (non-crystallizing components) and 25 days (crystallizing components) over saturated solutions of LiCl, CH_3COOK, $MgCl_2$, K_2CO_3, $Mg(NO_3)_2$, $NaNO_2$, and NaCl (Sigma Chemical Co., St. Louis, MO, U.S.A.) at 0.11, 0.23, 0.33, 0.44, 0.55, 0.66, and 0.76 a_w, respectively, in vacuum desiccators at room temperature. Freeze-dried lactose–MD DE 4–7, 9–12, and 23–27 at 40:60 ratios were monitored for 25 days over a series of saturated salt solutions with a_w from 0.55 to 0.76. Samples were weighed at intervals during storage. All vials, when removed and during transfers for weighing, were closed with septums. The crystallization of lactose was investigated from the loss of sorbed water. Water contents of the materials were measured as a function of time, and the average weight of triplicate samples was used in calculations. The average water contents at 5 days for freeze-dried MD and at 25 days for freeze-dried lactose and lactose–MD systems were used as water contents at steady state

for water sorption isotherms. The Guggenheim–Anderson-de Boer equation (GAB) (Eq. 2.1) was fitted to experimental data to model water sorption isotherm.

$$\frac{m}{m_0} = \frac{Cka_w}{(1-ka_w)(1-ka_w+Cka_w)} \tag{2.1}$$

2.2.3. DIFFERENTIAL SCANNING CALORIMETRY (DSC)

Freeze-dried "anhydrous" materials (MD DE 9–12 and 23–27, lactose, and lactose–MD DE 9–12 and 23–27 at 90:10, 80:20, and 70:30 ratios) were transferred to preweighed DSC aluminium pans (40 μL, Mettler Toledo Schwerzenbach, Switzerland), hermetically sealed before weighing, and analyzed using punctured pans. At varying a_w, the freeze-dried materials were transferred to preweighed DSC aluminum pans and weighed. These unsealed pans with samples were rehumidified for 72 h over a series of saturated salt solutions with a_w from 0.11 to 0.44 in vacuum desiccators at room temperature. After equilibration the pans were hermetically sealed and reweighed. An empty punctured pan was used as a reference and the instrument was calibrated for temperature and heat flow as reported by Roos and Karel (1991a). The lids of DSC aluminum pans of anhydrous samples were punctured to allow evaporation of any residual water during the measurement. Freeze-dried materials with varying water contents were scanned in DSC using hermetically sealed pans. Samples were scanned from ~30°C below to over the T_g region at 5°C/min and then cooled at 10°C/min to initial temperature. The second heating scan was run to well above the T_g. The onset T_g values were recorded using STARe software, version 8.10 (Mettler Toledo Schwerzenbach, Switzerland). Triplicate samples were analyzed and average values of onset T_g were calculated. The Gordon and Taylor equation (Eq. 2.2) was fitted to the average values of onset T_g.

$$T_g = \frac{w_1 T_{g1} + k w_2 T_{g2}}{w_1 + k w_2} \tag{2.2}$$

2.2.4. DIELECTRIC (DEA) AND DYMAMIC MECHANICAL ANALYSES (DMA)

Dielectric properties of freeze-dried anhydrous materials (lactose and lactose–MD DE 9–12 and 23–27 at 90:10, and 70:30 ratios) were analyzed using a dielectric spectrometer, DEA (DS6000, Triton Technology Ltd., UK), with titanium sample holders as the electrodes. Before starting an experiment, the LCR meter (LCR-819) and DEA instrument were calibrated at frequency 0.103 kHz with open-short circuit of the electrodes and zeroed in bridge RQ on a regular basis at 1 kHz with open-short circuit of the electrodes to ensure that the electrodes were clean (resistance value < 2Ω). Anhydrous materials were rehumidified for 5 days over a saturated salt solution with a_w 0.33 (MgCl2) in vacuum desiccators at room temperature. Humidified samples were ground and approximately 100 mg samples were transferred onto the lower cup electrode (40 mm diameter) and then pressed with the upper electrode (33 mm diameter). Two electrodes were placed into a dielectric cell. The sample thickness was less than 2 mm. Triplicate samples of each material were analyzed using dynamic measurements and recorded using Triton Laboratory software, version 1.0.330. Samples were scanned from -50 to 200°C with cooling rate of 5°C/min and heating rate of 3°C/min at frequencies of 0.012–10 kHz (Silalai and Roos, 2011). The measuring head was connected to a liquid nitrogen tank [1 litre; Cryogun, Brymill cryogenic systems, Labquip (Ireland) Ltd., Dublin, Ireland]. The α-relaxation temperature (T_α) was determined from the peak temperature above glass transition temperature of dielectric loss (ε'') and the crystallization temperature (T_{cr}) was determined from the onset temperature of increasing ε'' above the T_α region. Average values of triplicate samples for peak T_α and onset T_{cr} of ε'' were calculated. The ε'' describes an energy that is required to align ions and dipoles in an altering electric field.

Dynamic-mechanical properties of freeze-dried anhydrous materials (lactose and lactose–MD DE 9–12 and 23–27 at 90:10, and 70:30 ratios) were studied using a dynamic mechanical analyzer, DMA (Tritec 2000 DMA, Triton Technology Ltd., UK), by measuring the loss modulus (E'') as a function of temperature. The DMA instrument was balanced or set at zero to determine the zero displacement position and return the force to the zero position before starting an experiment. Anhydrous materials were rehumidified to 0.33 a_w for 5 days as described for

DEA experiments, and samples were ground to powder. Approximately 60 mg of the grinded samples were spread on a metal pocket-forming sheet (Triton Technology Ltd., UK). The sheet with sample was crimped along a pre-scored line to form a thin sandwich pocket. This pocket was attached directly between the clamps (the fixed clamp and the driveshaft clamp) inside the measuring head of DMA. The length, width, and thickness (< 2 mm) of sample and pocket between the clamps were measured. Triplicate samples of each material were analyzed using dynamic measurements and recorded using DMA control software version 1.43.00. Samples were scanned from -50 to 180°C with cooling rate of 5°C/min and heating rate of 3°C/min at frequencies of 0.5 to 20 Hz by using the single cantilever bending mode (Silalai and Roos, 2011). The measuring head was connected to a liquid nitrogen tank (1 litre; Cryogun, Brymill Cryogenic Systems, Labquip (Ireland) Ltd., Dublin, Ireland). During dynamic heating, the samples were analyzed for T_α determined from the peak temperature of loss modulus (E'') above the glass transition, and the T_{cr} determined from the onset temperature of E''. Average values for triplicate measurements of peak T_α and onset T_{cr} of E'' were calculated.

The relaxation times of average values for peak T_α and onset T_{cr} measuring by DMA and DEA at various frequencies were calculated using the relationship; $\tau = 1/(2\pi f)$ (Noel *et al.*, 2000). The peak T_α and onset T_{cr} were modeled using the Vogel–Tammann–Fulcher (VTF) equation (Eq. 2.3) (Angell, 1997).

$$\tau = \tau_0 \exp [DT_0 / (T-T_0)] \quad (2.3)$$

2.3. RESULTS AND DISCUSSION

2.3.1. WATER SORPTION OF COMPONENTS AND CARBOHYDRATE MIXTURES

The GAB sorption isotherms, experimental data for the amorphous MD DE 9–12 and 23–27 (0.11–0.76 a_w), lactose (0.11–0.44 a_w), lactose–MD DE 9–12 and 23–27 at 70:30 ratio (0.11–0.55 a_w), and predicted water contents for non-crystalline, amorphous lactose (0.55–0.76 a_w) and lactose–MD DE 9–12 and 23–27 at 70:30 ratio (0.66–0.76 a_w) are shown in Fig. 2.1 and Table 2.1.

Table 2.1 Water contents and water activity (a_w) for non-crystalline lactose (NCL), amorphous MD DE 9–12 (MD 9) and 23–27 (MD 23), and NCL–MD 9 and 23 at 70:30 ratio. The water content of NCL at 0.55 to 0.76 a_w was derived from experimental NCL–MD 23 at 40:60 ratio. Water contents of NCL–MD systems were obtained from experimental data at 0.11 to 0.55 a_w and fractional water contents calculated for NCL and measured for MD 9 and 23 to predict sorbed water contents for lactose–MD mixtures at 0.66 and 0.76 a_w.

a_w	Water content (g/100 g of dried solids)									
	NCL by experiment and weight fraction	70% lactose [a] by weight fraction	Amorphous MD by experiment		30% MD [a] by weight fraction		NCL–MD [a] by weight fraction		Lactose–MD by experiment	
			MD 9	MD 23	MD 9	MD 23	MD 9	MD 23	MD 9	MD23
0.11	2.57	1.80	4.57	2.95	1.37	0.89	3.17	2.69	2.95	2.77
0.23	4.39	3.07	6.64	4.96	1.99	1.49	5.06	4.56	4.43	4.38
0.33	5.53	3.87	8.20	6.24	2.46	1.87	6.33	5.74	6.22	5.70
0.44	9.03	6.32	9.58	7.60	2.87	2.28	9.19	8.60	8.26	7.90
0.55	12.50	8.75	11.55	9.32	3.47	2.80	12.21	11.54	11.76	11.50
0.66	16.71	11.70	13.73	13.46	4.12	4.04	15.82	15.74	7.63	7.51
0.76	20.28	14.20	15.59	17.02	4.68	5.11	18.87	19.30	7.83	8.48

[a] Fractional water contents of 70 and 30% of lactose and MD components, respectively, giving water sorption of NCL–MD mixtures (70:30).

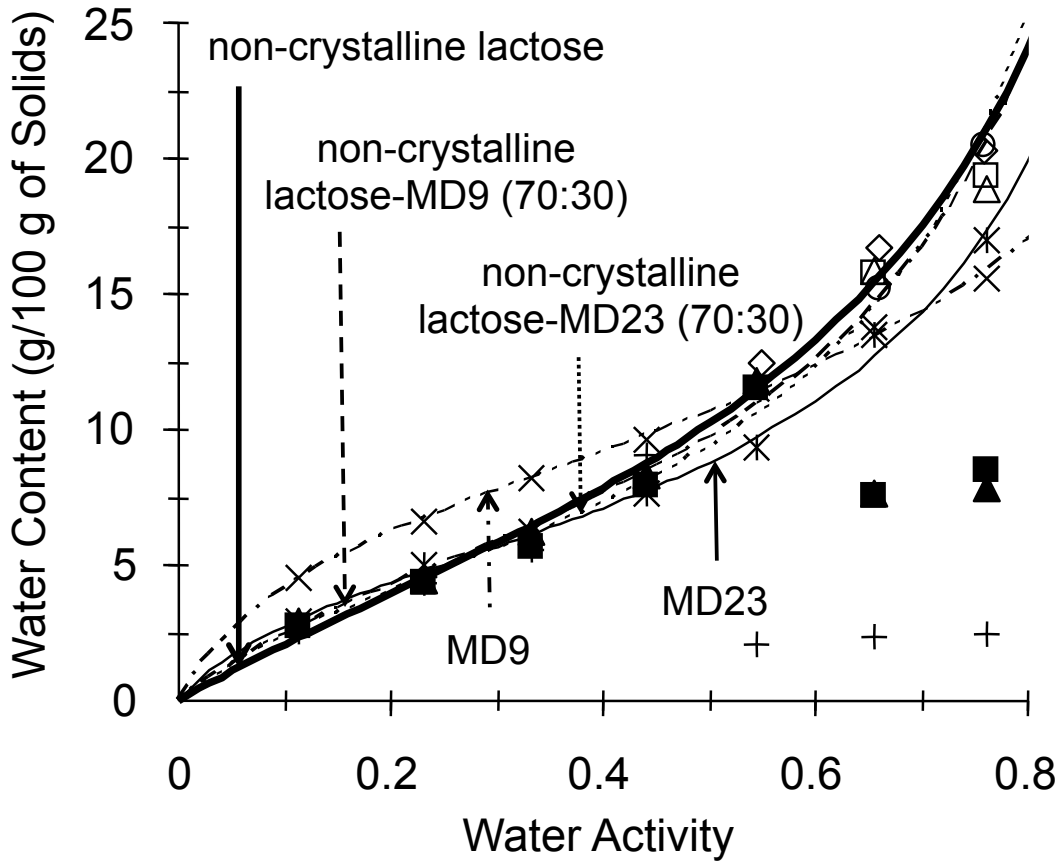

Fig. 2.1 The experimental data (solid symbols) and calculated water contents (clear symbols) for amorphous lactose, lactose–MD DE 9–12 (MD 9) at 70:30 ratio (▲, △), and lactose–MD DE 23–27 (MD 23) at 70:30 ratio (■, □) systems were compared to experimental data of MD 9 (×) and 23 (∗). The thick solid (amorphous lactose), dashed (lactose–MD 9 (70:30)), dotted [lactose–MD 23 (70:30)], dashed–dotted (MD 9), and solid lines (MD 23) correspond to the GAB sorption isotherms. The GAB sorption isotherm of non-crystalline lactose was obtained using experimental data at 0.11 to 0.44 a_w (+) and water contents derived from lactose–MD 9 (○) and lactose–MD 23 (◇) at 40:60 ratio at 0.55 to 0.76 a_w. The GAB sorption isotherms for non-crystalline lactose–MD systems at 70:30 ratio used experimental data at 0.11 to 0.55 a_w and fractional water contents calculated for non-crystalline lactose and measured for MD 9 and 23 to predict sorbed water contents for lactose–MD mixtures at 0.66 and 0.76 a_w.

Previous studies showed that the GAB relationship could be fitted to experimental data of amorphous lactose below 0.55 a_w (Roos and Karel, 1990; Jouppila and Roos, 1994; Bronlund and Paterson, 2004; Shrestha *et al.*, 2007; Silalai and Roos, 2010; Zhou and Roos, 2011) at 20–25°C due to the limitation of crystallization of lactose at intermediate and high water activities. The maximum extent of lactose crystallization occurred at 0.70 a_w at 24°C, shown by a parabolic relationship with storage water activity (Jouppila *et al.*, 1997). At

higher water activities, the extent of lactose crystallization decreased due to increased solubilisation of lactose in sorbed water (Jouppila *et al.*, 1997). However, the extrapolated sorption data above 0.44 a_w extensively exceed true sorbed water contents and are not valid. *In the present study we found that the experimental data at 0.11–0.44 a_w and water contents at 0.55–0.76 a_w, derived from lactose–MD DE 9–12 or 23–27 at 40:60 ratio (steady state sorbed water contents of non-crystalline components) water sorption data for non-crystalline lactose could be used in the GAB equation (Fig. 2.1). Our result showed the first time experimental water sorption isotherm for non-crystalline lactose over a broad a_w range of 0.11 to 0.76. Also water sorption data of non-crystalline lactose–MD systems could be derived from the sum of water contents of amorphous components over a wide range of a_w as shown in Eq. 2.4 (where M = water content of amorphous or non-crystalline solid), including high a_w (0.55–0.76 a_w), i.e., the sorbed water contents of lactose–MD mixtures were additive and agreed with the sum of the equilibrium water contents of individual amorphous components at each a_w*. This was in agreement with Shamblin and Zografi (1999), who found that the water vapour absortion behaviour of binary amorphous mixtures of sucrose–poly(vinyl pyrrolidone) (PVP) and sucrose–poly(vinylpyrrolidone-co-vinylacetate) (PVP/VA) at 80:20 and 50:50 ratios were equal to the weighted sums of the individual isotherms where no sucrose crystallization occurred.

$$M_{TOTAL} = M_{LACTOSE} + M_{MD} \quad (2.4)$$

Therefore, this result could be used to obtain the GAB sorption isotherms for non-crystalline lactose–MD DE 9–12 and 23–27 mixes at all ratios. Water contents of non-crystalline lactose–MD at 70:30 ratio systems were available from experimental data for systems in the absence of crystallization of lactose (0.11–0.55 a_w) and calculated water content at higher a_w (0.66–0.76 a_w) were total water contents of non-crystalline lactose, given by non-crystallizing lactose–MD DE 23–27 at 40:60 ratio (0.66–0.76 a_w), and amorphous MD (Fig. 2.1 and Table 2.1). These results for the first time make available sorbed water contents for non-crystalline lactose up to 0.76 a_w and the GAB isotherm can be used for lactose systems to predict their water sorption. The additivity principle

of sorbed water contents of components is likely to apply similarly to other carbohydrate mixtures.

2.3.2. GLASS TRANSITION

The T_g values and aw for anhydrous and humidified freeze-dried MD DE 9–12 and 23–27, lactose, and lactose–MD systems are shown in Fig. 2.2. The amorphous lactose showed similar T_g values to lactose–MD DE 9–12 and 23–27 at all ratios from 0 to 0.44 a_w, and MD DE 9–12 and 23–27 did not significantly affect the T_g of the systems. The T_g values of anhydrous and humidified lactose at 0.11, 0.23, 0.33, and 0.44 a_w were 105, 52, 42, 32, and 13°C, respectively, in agreement with Haque and Roos (2006). The MD DE 9–12 had significantly higher T_g than MD DE 23–27, which was close to the T_g of lactose at 0.11 to 0.44 a_w. Lactose–MD DE 9–12 and 23–27 systems at all ratios showed similar T_g and water contents to those of lactose at corresponding a_w up to 0.44 a_w. These results showed that the addition of MD DE 9–12 and 23–27 at ratios 90:10, 80:20, and 70:30 had minor effects on the T_g values and water contents of the systems. This was in accordance with Roos and Karel (1991b), who reported that sucrose–MD mixtures with less than 50% (w/w) MD showed no significant increase of the T_g. Gabarra and Hartel (1998) also reported that the mixtures of amorphous sucrose:corn syrup solids with DE 20 and 42 at 80:20 and 90:10 ratios showed similar T_g. The T_g values of the mixture systems were mainly dependent on number average rather than weight average molecular weight of components. The relationship between number average molecular weight and T_g was linear (Roos and Karel, 1991b; d; Gabarra and Hartel, 1998; Avaltroni *et al.*, 2004).

Sugar–MD mixtures in previous studies were used to increase the T_g to control and inhibit crystallization of amorphous sugars (Iglesias *et al.*, 1997; Mazzobre *et al.*, 1997; Kouassi and Roos, 2001; Mazzobre *et al.*, 2003). For crystallization inhibition, however, the presence of small molecular weight components described by the DE value of maltodextrins may be more important than the T_g of a sugar–MD alone as also found by Gabarra and Hartel (1998). The freeze-dried lactose–MD DE 9–12 systems at 90:10, 80:20, and 70:30 ratios showed similar T_g to the amorphous lactose–MD DE 23–27, but the inhibition of lactose

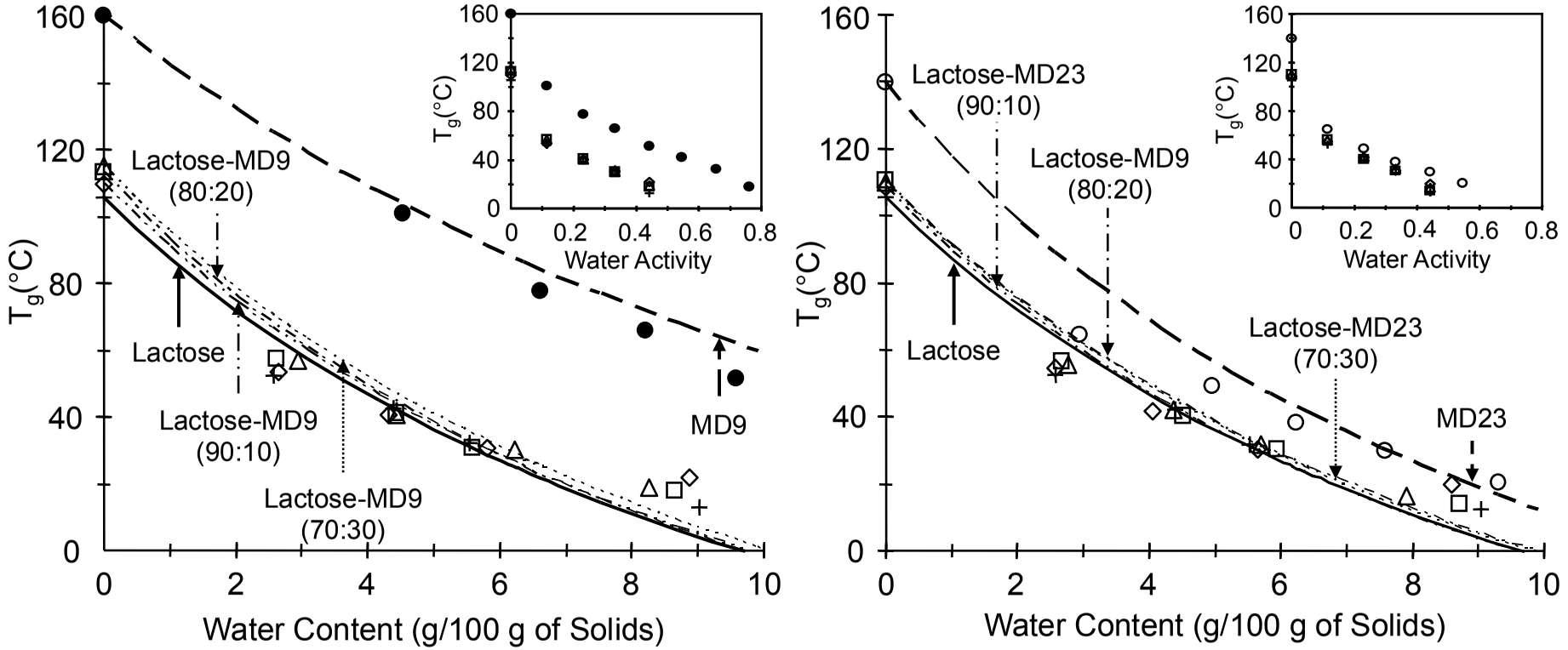

Fig. 2.2 Glass transition temperature (T_g), water content, and water activity (a_w) for the freeze-dried MD DE 9–12 (MD 9) and 23–27 (MD 23), lactose, and lactose–MD systems at 90:10, 80:20, and 70:30 ratios. Experimental data are shown for MD 9 (●), MD 23 (○), lactose (+), lactose–MD (90:10) (◇), lactose–MD (80:20) (□), and lactose–MD (70:30) (△). The solid (lactose), dashed (MD), long dashed dotted dotted [lactose–MD (90:10)], long dashed dotted [lactose–MD (80:20)], and dotted lines [lactose–MD (70:30)] correspond to the T_g predicted by the Gordon-Taylor equation. Data for T_g of anhydrous MD 9 are from Roos and Karel (1991b).

crystallization above the T_g, as assessed from the water sorption data (Fig. 2.3), was different. It is well known that the crystallization kinetics of amorphous sugars is governed by $T-T_g$. Adding a second component is a general principle to control crystallization by disturbing the mobility of sugar molecules, and a carbohydrate mixture may show an increased T_g of the system depending on the molecular weight and concentration of the second component. Mazzobre *et al.* (2001) found that lactose crystallization was inhibited in freeze-dried lactose–trehalose systems with respective ratios of 80:20 and 70:30 of solids, but the T_g values did not differ from that of lactose. However, our systems emphasized that the crystallization inhibition of lactose was affected by the glass transition of the systems as well as the number average molecular weight of the MD while the lactose–trehalose system of Mazzobre *et al.* (2001) had a composition independent T_g.

2.3.3. CRYSTALLIZATION KINETICS

No crystallization of amorphous lactose in lactose and lactose–MD systems occurred at low a_w (a_w 0.11–0.44) during 25 days of storage. At intermediate and high a_w (a_w 0.55–0.76), lactose crystallization was affected by MD at all ratios with the rate of crystallization decreasing with increasing MD content at the same a_w. Freeze-dried lactose lost sorbed water most rapidly at a_w 0.55, 0.66, and 0.76 at 5, 2, and 2 days of storage, respectively. At 0.55 a_w, crystallization of lactose–MD DE 23-27 at 90:10 and 80:20 ratios was complete with loss of sorbed water at 11 and 19 days, respectively, but lactose–MD DE 9–12 at 90:10 and 80:20 ratios showed high variation of the time of loss of sorbed water. The MD DE 23–27 was the strongest crystallization inhibitor in lactose–MD systems at 40:60 ratio within 25 days of storage at 0.55 a_w. The lactose–MD DE 4–7 and 9–12 at 40:60 ratio systems showed a minor loss of sorbed water (partial crystallization) after storage at 0.55 a_w for 1 and 3 days, respectively. This result proved that lower molecular weight carbohydrates present in the high DE MD could exhibit molecular motions and diffusion rates exceeding those of the higher molecular weight (low DE) MD carbohydrates, as described by Gabarra and Hartel (1998). The smaller carbohydrates could also retard lactose mobility, reduce and disturb lactose nucleation and crystal growth. The MD DE 23–27 prevented crystallization of lactose in lactose–MD at 40:60 ratio up to 0.55 a_w.

The effective concentration of MD in crystallization prevention in this study was lower than found in previous studies. Iglesias *et al.* (1997), and Gabarra and Hartel (1998) reported that above or equal to 50% MD DE 10 and corn syrup solid DE 20 could prevent crystallization of trehalose (up to 0.52 a_w for 15 days of storage), and sucrose (dry mixtures), respectively. According to the present study, MD at ratios up to 70:30 of lactose–MD could delay crystallization of lactose up to 0.55 a_w during storage at room temperature without being substantially affected by storage close to the T_g of the systems.

Water sorption of amorphous freeze-dried lactose and lactose–MD systems at 0.66 and 0.76 a_w for 25 days is shown in Fig. 2.3. The amorphous freeze-dried lactose showed water contents of 2.0–2.4 g/100 g of dried solids at 0.55 to 0.76 a_w, which showed that amorphous lactose did not crystallize as α-lactose monohydrate (Fig. 2.3A and B). Haque and Roos (2005a) reported that amorphous freeze-dried lactose crystallized as mixtures of α-lactose monohydrate, anhydrous β-lactose, and anhydrous forms of crystals with α- and β-lactose in a molar of 4:1 ratio at $a_w > 0.55$, but also that recrystallization to α-monohydrate took place. Final water contents of lactose–MD DE 9–12 and 23–27 systems increased with increasing MD content at 0.66 and 0.76 a_w (Fig. 3A), and corresponded to water retained in the amorphous MD proportion (Roos and Karel, 1992). This result was in accordance with Nowakowski and Hartel (2002), who reported that mixtures of sucrose–corn syrup solid with DE 43 showed higher water contents with increasing contents of corn syrup solid. At 0.66 a_w, the lactose–MD DE 9–12 and 23–27 at 40:60 ratios seemed to have no crystallization of lactose during 25 days of storage, but lactose–MD DE 9–12 and 23–27 showed partial crystallization of lactose after storage for 9 and 15 days, respectively. Lactose–MD DE 4–7 at 40:60 ratio showed partial crystallization after 5 days of storage. This result showed that MD DE 23–27 inhibited crystallization of lactose more strongly than MD DE 9–12 and 4–12 in lactose-MD systems at 40:60 ratio at 0.66 a_w, which indicated that the crystallization of lactose was affected by the number average molecular weight of the MD in lactose–MD systems. At 0.76 a_w, crystallization of lactose in lactose–MD DE 4–7, 9–12, and 23–27 at 40:60 ratio occurred after 7 days of storage in all

systems (Fig. 2.3B). The systems of lactose–MD DE 23–27 lost sorbed water after reaching a maximum water uptake less rapidly than lactose-MD DE 9–12 at 80:20, 70:30, and 40:60 ratios and lactose-MD DE 4–7 at 40:60 ratio with a_w 0.76 (Fig. 2.3B). It should be noted that the use of MD DE 9–12 in lactose–MD systems at 90:10 to 70:30 ratios increased the rate of water uptake from 0.10 (lactose) up to 0.27 day^{-1} at 0.66 a_w and from 0.20 (lactose) up to 0.32 day^{-1} at 0.76 a_w, but lactose–MD DE 23–27 at 90:10 to 70:30 ratios did not show differences in water uptake from lactose.

Kinetics of loss of sorbed water used as an indicator for the rate of lactose crystallization from amorphous lactose and lactose–MD DE 9–12 and 23–27 systems at all ratios are shown in Fig. 2.4. The rate of loss of water sorbed by lactose-MD systems decreased with increasing MD content at 0.66 and 0.76 a_w. The lactose–MD DE 9–12 and 23–27 systems at all ratios showed rate constants for loss of sorbed water (k_1) from -1.47 (lactose) up to 0 and 0 day^{-1} (no loss of water sorbed), respectively, at 0.66 a_w and -0.79 up to -0.04 and -0.02 day^{-1}, respectively, at 0.76 a_w. Therefore lactose–MD DE 23–27 showed a smaller rate of loss of water sorbed than lactose–MD DE 9–12 and lactose systems at 0.76 a_w. The relations between the k_1 and content of lactose in lactose–MD DE 9–12 and 23–27 systems at 0.66 and 0.76 a_w are shown in Fig. 2.5. At 0.66 and 0.76 a_w, the rate of loss of sorbed water of lactose–MD systems decreased (close to zero) with increasing MD content. Systems containing MD DE 23–27 showed a stronger effect on the rate of loss of sorbed water than MD DE 9–12. Also lactose–MD systems at 90:10 ratio showed that MD DE 23–27 (k_1 = -0.19 day^{-1}) gave a lower rate of loss of sorbed water than MD DE 9–12 (k_1 = -0.40 day^{-1}) at 0.66 a_w. The rate of loss of sorbed water of lactose-MD DE 9–12 system did not show significant differences from lactose–MD DE 23–27 (k_1 = -0.20 day^{-1}) system at 0.76 a_w (Fig. 2.5). Therefore, the effect of DE of the MD on lactose crystallization was dependent on a_w. This further confirmed that the MD with the higher DE more effectively delayed lactose crystallization from lactose–MD systems.

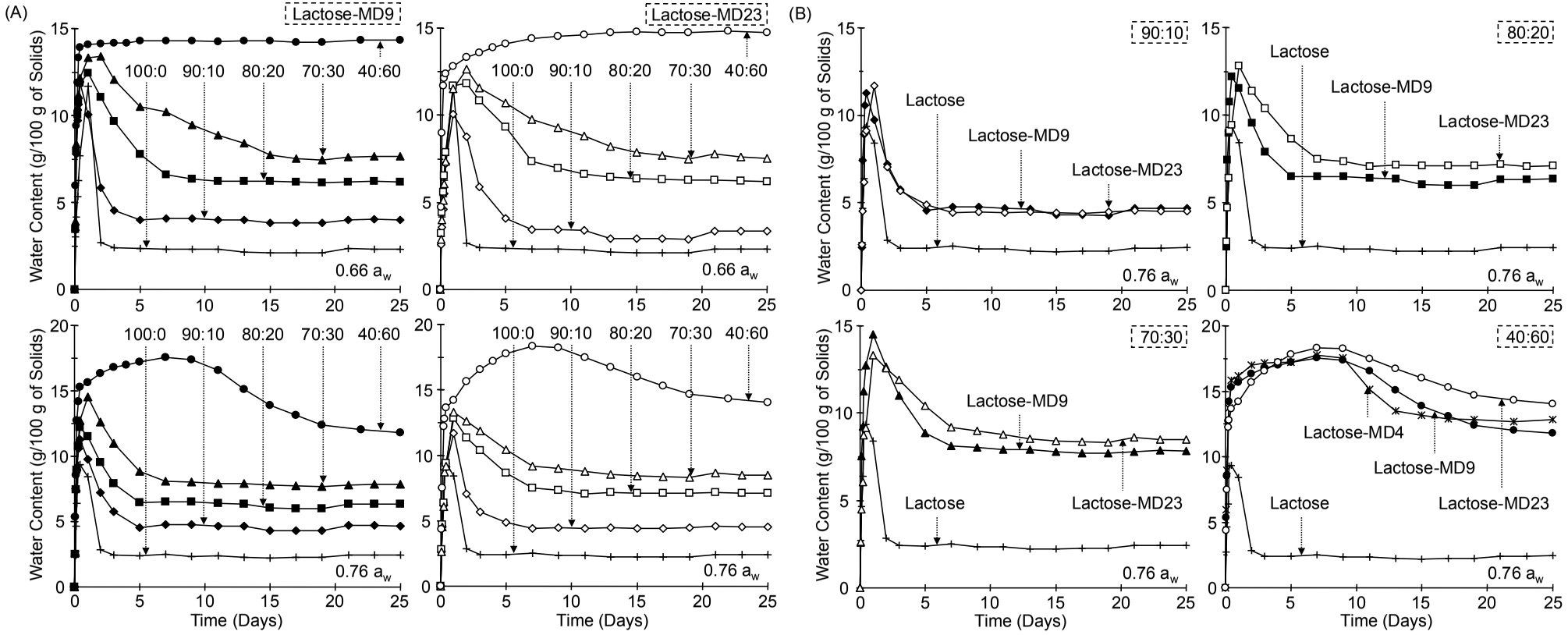

Fig. 2.3 Water sorption for (A) freeze-dried lactose and lactose–MD DE 9–12 (MD 9), 23–27 (MD 23) systems at all ratios (90:10, 80:20, 70:30, and 40:60) at a_w of 0.66 and 0.76 and (B) freeze-dried lactose–MD DE 4–7 (MD 4) at 40:60 ratio, 9–12, and 23–27 at all ratios at a_w of 0.76 for 25 days.

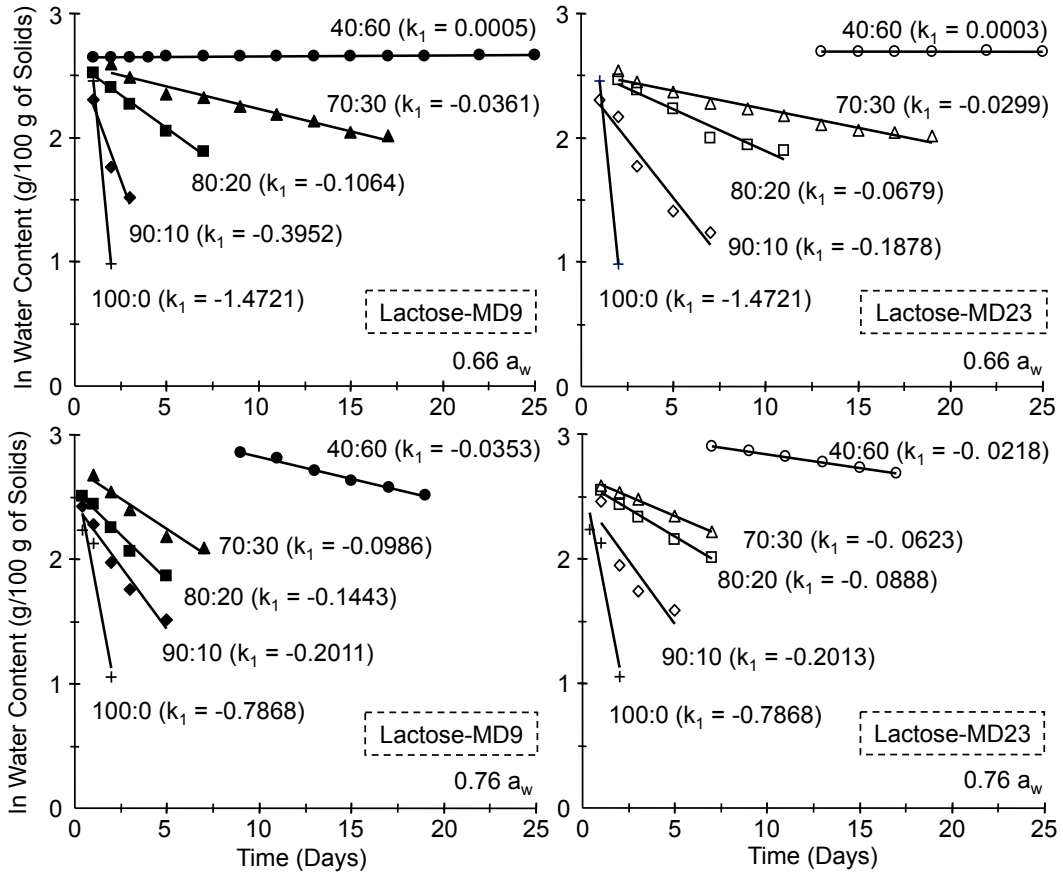

Fig. 2.4 Kinetics of loss of water sorbed by lactose (+) and lactose–MD DE 9–12 (MD 9), 23–27 (MD 23) systems at ratios 90:10 (◆, ◇), 80:20 (□, ■), 70:30 (▲, △), and 40:60 (●, ○) at 0.66 and 0.76 a_w.

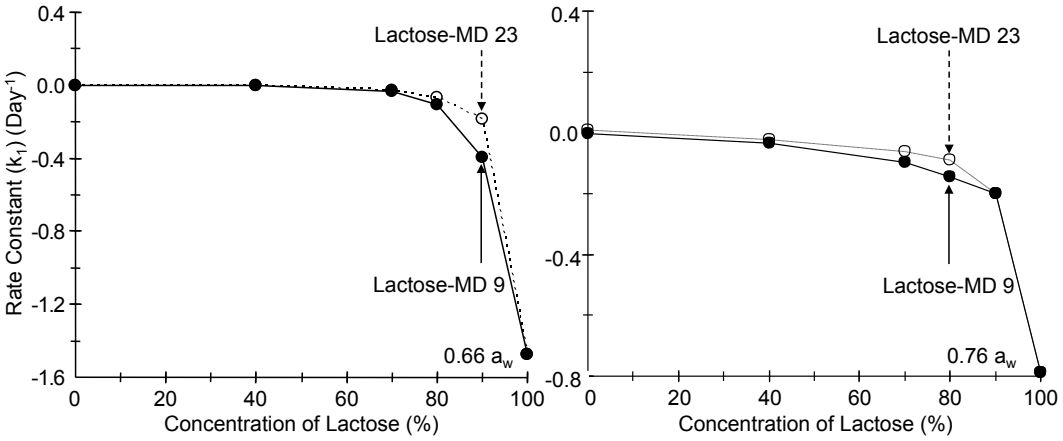

Fig. 2.5 Rate constants (k_1) from kinetics of loss of water sorbed from freeze-dried lactose, MD DE 9–12 (MD 9) and 23–27 (MD 23), and lactose–MD 9 (—●—) and 23 (--⊖--) systems at 90:10, 80:20, 70:30, and 40:60 ratios at 0.66 and 0.76 a_w (from Fig. 2.4).

During 25 days of storage, we noted that freeze-dried lactose–MD systems except DE 4–7 at 40:60 ratio at 0.66 and 0.76 a_w had collapsed structures. The collapse phenomenon of dehydrated materials occurs above T_g and before complete crystallization of amorphous compounds (Roos and Karel, 1991c). This occurred as a result of a lower T_g and viscous flow. The collapsed structure formed at the end of water uptake was likely to reduce water loss, it caused thickening of the microstructure, and the materials could retain higher water contents. Lactose–MD DE 4–7 at 40:60 ratio had highest water sorption of MD components and no collapse or changes in volume during storage at 0.76 a_w, and it showed a higher water content than lactose–MD DE 9–12 (Fig. 2.3B). The highest residual water content of the lactose–MD DE 23–27 system could be related to the slow desorption from the collapsed structure during lactose crystallization. The lactose–MD DE 9–12 at 40:60 ratio appeared more opaque (higher crystallinity) than amorphous lactose–MD DE 23–27 with a_w 0.66 and 0.76 at 7 and 2 days, respectively. Our results showed that lactose crystallization in lactose–MD systems was more affected by MD components hindering lactose movement than their glass transition. This finding emphasized the importance of concentration, molecular size effects, molecular interactions, lattice interference or steric hindrance effects of the mixed components that disturbed nucleation or crystal growth (Iglesias and Chirife, 1978; Roos and Karel, 1991a; c; Mazzobre *et al.*, 2001).

2.3.4. DIELECTRIC AND DYNAMIC MECHANICAL PROPERTIES

The T_α of amorphous lactose and lactose–MD DE 9–12 and 23–27 at 90:10 and 70:30 ratios at 0.33 a_w above the onset T_g measured by DSC was taken from peak temperature of ε'' and E'' of dielectric and dynamic mechanical analyses, and the T_{cr} was the onset temperature of a subsequent increase in ε'' and E'' giving a higher temperature peak above T_α (Figs. 2.6 and 2.7). The peak temperatures of ε'' at 0.5 kHz for freeze-dried lactose and lactose–MD DE 9–12 and 23–27 at 90:10 and 70:30 ratios (Fig. 2.6) were not significantly different in accordance with the glass transition data and the peak temperature of E'' (Fig. 2.6). The T_α of amorphous lactose and lactose–MD systems at 0.33 a_w occurred at ~30°C and ~20°C above the onset T_g measured by DSC in dielectric analysis at 0.5 kHz and

dynamic mechanical analysis at 0.5 Hz, respectively, in agreement with Silalai and Roos (2011). It should be noted that the T_α from dielectric and dynamic mechanical analyses are highly dependent on the frequencies of the measurements (Talja and Roos, 2001). The lactose–MD at 70:30 ratio showed higher magnitudes of dielectric loss and loss modulus peaks in the region of T_α than lactose–MD at 90:10 ratio. Increasing MD content affected the water content of the amorphous systems. This was dependent on the DE of MD and water sorption, which increased the number of dipoles responding to the electric field. Lactose–MD DE 9–12 at 90:10 and 70:30 ratios showed a higher magnitude of the dielectric loss and loss modulus peaks at T_α than lactose–MD DE 23–27 at the same ratios. The height of magnitude of dielectric loss and loss modulus correlated directly to water content of lactose–MD systems at 0.33 a_w. This result agreed with Silalai and Roos (2011), who found that dielectric loss and loss modulus peaks of skim milk powders increased in magnitude with increasing water content.

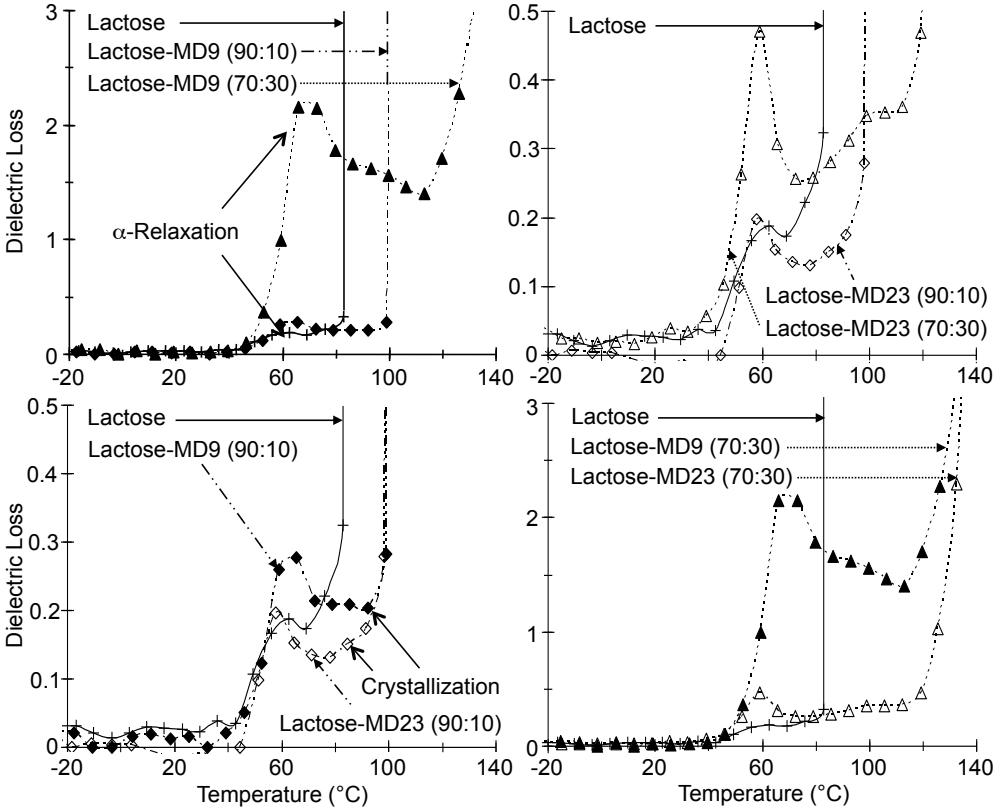

Fig. 2.6 Dielectric loss and onset temperature of crystallization for freeze-dried lactose, lactose–MD DE 9–12 (MD 9) and 23–27 (MD 23) at 90:10 and 70:30 ratios at 0.5 kHz. Experimental data are shown for lactose (—+—), lactose–MD 9 (90:10) (−·◆·−) and (70:30) (···▲···), and lactose–MD 23 (90:10) (−·◇·−) and (70:30) (···△···).

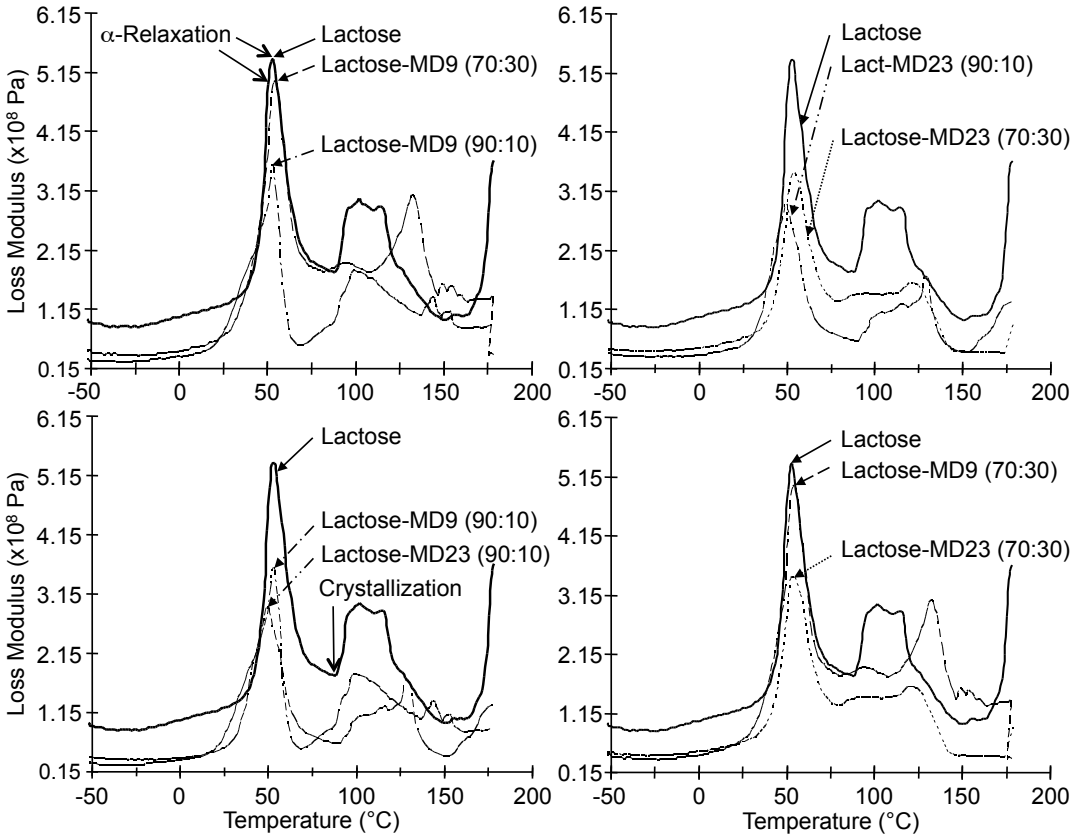

Fig. 2.7 Loss modulus and onset temperature of crystallization for freeze-dried lactose, lactose–MD DE 9–12 (MD 9) and 23–27 (MD 23) at 90:10 and 70:30 ratios at 0.5 Hz. Experimental data are shown for lactose (——), lactose-MD 9 (90:10) (−·−·−) and (70:30) (−−−−), and lactose–MD 23 (90:10) (−··−··) and (70:30) (·····).

The intensity of the loss modulus peak of amorphous lactose showed water content independence (Fig. 2.7), which was different from dielectric loss peak of lactose (Fig. 2.6). The amorphous lactose–MD DE 9–12 at 90:10 and 70:30 ratios agreed with onset T_{cr} of ε'' with lactose–MD DE 23–27 at same ratio (Fig. 2.6). The onset T_{cr} of E'' of amorphous lactose and lactose–MD systems at 90:10 and 70:30 ratios did not differ at same ratio, but systems containing MD with high DE showed bigger changes in crystallization behavior of lactose than MD with low DE (Fig. 2.7). These results showed that increasing MD content in lactose–MD systems showed changes in crystallization behavior and the onset T_{cr} of ε'' and E'' was at higher temperatures and also the magnitudes of changes in dielectric loss and loss modulus were increased. The sharp increase or sudden variation of dielectric loss and loss modulus above the T_{α} in accordance with the results of Talja and Roos (2001) for xylitol and Gonnet, Guillet, Sirakov,

Fulchiron, and Seytre (2002) for Kynar 710 poly (vinylidene fluoride) was frequency independent indicating crystallization of lactose (Fig. 2.7 and 2.8). The rate of lactose crystallization in lactose–MD might depend on content and DE of MD in the systems. Lactose–MD DE 9–12 and 23–27 systems with high contents of MD showed a frequency dependent change and a slower growth or progression rate of lactose crystallization.

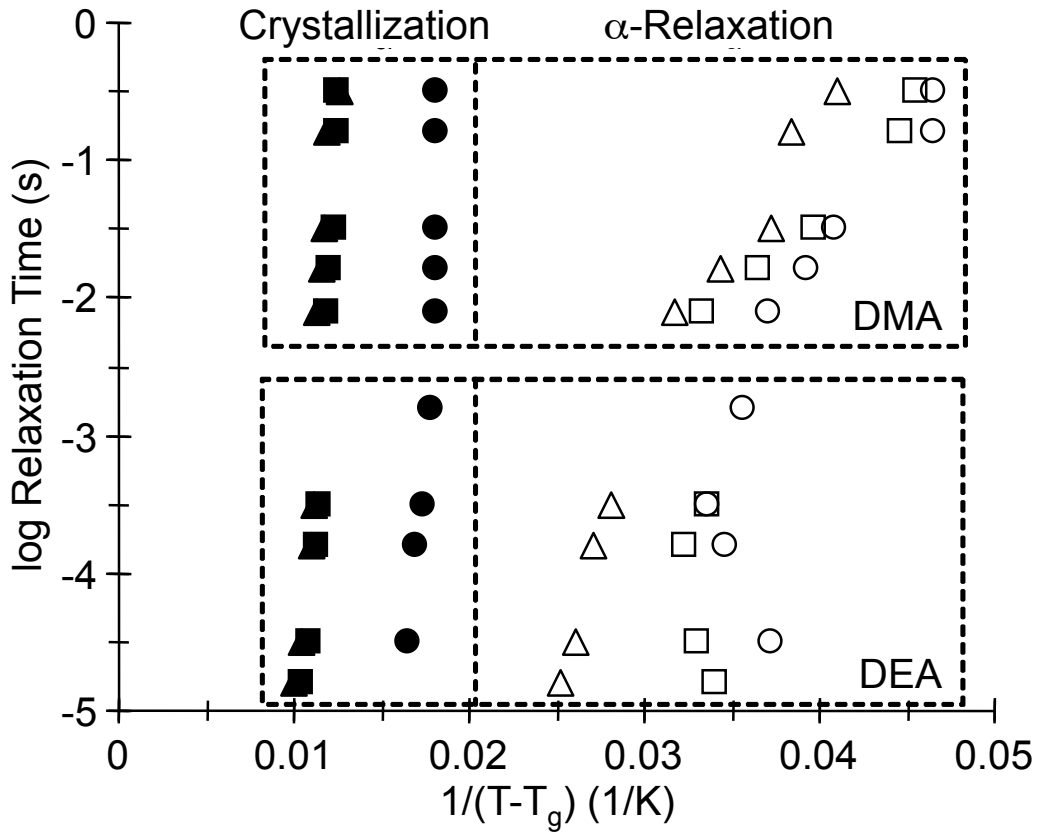

Fig. 2.8 The plots of relaxation times at peak temperature of α-relaxation (clear symbols) and onset temperature of crystallization (solid symbols) derived from dielectric loss and loss moduli measured by DEA and DMA for freeze-dried lactose (○, ●) and lactose–MD DE 9–12 (△, ▲) and 23-27 (□, ■) at 70:30 ratio. The VTF (Vogel-Fulcher-Tammann) model was fitted to all experimental data at 0.33 a_w.

The VTF model using $T_0 = T_g$ was fitted to experimental data of peak T_α and onset T_{cr} of dielectric loss and loss modulus for freeze-dried lactose and lactose-MD systems at 70:30 ratios at 0.33 a_w as shown in Fig. 2.8. The retarded mobility of lactose–MD systems was shown by increasing τ_α at corresponding T-T_g, also increasing for the lower DE MD 9–12. The τ_α at high frequencies and corresponding high temperatures (DEA data for lactose and lactose–MD DE 23–

27) showed decreasing T-T_g probably because of some crystallization releasing water and consequent increase in mobility. Increasing MD content in lactose–MD systems showed increasing onset T_{cr} for ε'' and E'' (Fig. 2.6, 2.7, and 2.8), but the crystallization temperature was almost frequency independent (Fig. 2.8). These results showed that small sugar molecules in MD with high DE gave a higher 'fragility', but disturbed crystallization of lactose more than MD with lower DE, which was in accordance with results of water sorption studies. It also appeared that instant lactose crystallization required about a constant mobility as shown by the frequency independent T-T_g. These results showed that the crystallization of lactose was inhibited with increasing MD content and the MD with high DE showed a stronger inhibition of crystallization of lactose than lower DE MD. Therefore an addition of a second component may delay crystallization of lactose, as it disturbs nucleation and crystal growth by hindering diffusion of lactose molecules.

2.4. CONCLUSIONS

We have shown that amorphous carbohydrate mixtures have additive water sorption properties, and the crystallization of lactose was delayed by the addition and increasing contents of MD. The DE of MD has an important role in preventing lactose crystallization. A smaller molecular size (high DE) of MD components increased the delay or was a stronger inhibitor of crystallization of lactose and also MD showed an individual property/role in water sorption of lactose–MD systems. Despite a similar T_g of amorphous lactose–MD systems, they showed differences in τ_α and different inhibition effects on lactose crystallization. The capability of MD in preventing crystallization of lactose was water activity dependent, and systems containing smaller molecular size or lower molecular weight components showed stronger hindering effect and affected the rate of loss of water sorbed. These results proved that in amorphous systems the number average molecular weight, molecular size effects, and steric hindrance effects of components have significant effects on sugar crystallization.

CHAPTER III

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OIL AS REACTION MEDIUM FOR GLYCATION, OXIDATION, DENATURATION, AND AGGREGATION OF WHEY PROTEIN SYSTEMS OF LOW WATER ACTIVITY

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ABSTRACT

Whey protein isolate (WPI)–oil (75:25) and WPI–oil–(glucose–fructose) (45:15:40) as models of high-protein systems containing either olive (OO) or sunflower oil (SO) were stored at 20 or 40°C to investigate component interactions. The indicators of protein oxidation (carbonyl content) and aggregation (total sulfhydryl content) and heats of protein denaturation and aggregation were investigated. Highest levels of disulfide bonding and carbonyls in WPI-OO formed during the first 2 weeks of storage concomitantly with enhanced protein aggregation. WPI-OO and WPI-SO systems (prestorage) showed increased protein denaturation temperature. The WPI proteins showed higher heat sensitivity with OO or SO at 40°C, and the system with OO showed preaggregated protein as found from decreased heats of protein aggregation. OO or SO in WPI–oil–(glucose–fructose) systems reduced heats of protein aggregation. Lipid oxidation products and nonenzymatic browning reactions in glucose–fructose-containing systems decreased the solubility of solids and increased protein aggregation, hydrophobicity, and hardening of structure.

KEYWORDS: aggregation, denaturation, differential scanning calorimetry, disulfide bond, protein oxidation, nonenzymatic browning, water, whey proteins

3.1. INTRODUCTION

Molecular interactions of protein–lipid and protein–sugar systems are of significant interest to various areas (biological, food, medicine, nutritional, and pharmaceutical sciences), but co-interactions of protein–lipid–sugar systems are poorly understood, especially in dry and low water content systems. The properties of proteins depend on their molecular environment (concentration, pH, temperature) and the presence of other components (acids and bases, antioxidants, buffers, lipid, proteins, saccharides, salt, water). Products of lipid oxidation and interactions of proteins with other molecules may accelerate changes of protein properties and functionality.

Lipid oxidation produces reactive oxygen groups and free radicals that attack proteins and amino acids in aqueous solutions or dispersions, anhydrous and low water systems (Zirlin and Karel, 1969; Chipault and Hawkins, 1971; Kanner and Karel, 1976), and the frozen state and accelerate oxidation of proteins. These

reactions decrease the nutritional quality of proteins due to browning (Tappel, 1955; Zirlin and Karel, 1969; Karel, 1973; Pokorný *et al.*, 1974), insolubilization (increasing hydrophobicity of proteins) (Roubal and Tappel, 1966a; Funes and Karel, 1981), loss of enzyme activity (Roubal and Tappel, 1966a; Leake and Karel, 1982; Oliver *et al.*, 1987), and loss of cellular membrane integrity *in vivo* (Menzel, 1967; Gardner, 1979; Stadtman, 1992). Furthermore, the carbonyl groups of oxidized lipids may participate in covalent bonding to exposed amino groups of protein, leading to the formation of stable protein–lipid complexes (Davies, 1987; Hidalgo and Kinsella, 1989; Howell *et al.*, 2001). Methionine, cysteine, tryptophan, lysine, histidine, and tyrosine residues in proteins were reported as the most sensitive amino acids to lipid oxidation products. Transient free radicals from lipid peroxidation may accelerate major protein-damaging reactions and the human aging process (Roubal and Tappel, 1966a). In living tissues, oxidation of proteins is known to play an essential role in the pathogenesis of degenerative diseases, for example, Alzheimer's disease, Huntington's disease, muscular dystrophy, progeria, Parkinson's disease, rheumatoid arthritis, and Werner's syndrome. The accumulation of unrepaired or oxidized protein occurs during aging, affecting cellular integrity that accounts for the age-related loss of important physiological functions (Stadtman, 1992). Also, increased carbonyl contents of proteins appear in human brain tissue (Smith *et al.*, 1991; Butterfield and Kanski, 2001), eye lenses (Garland *et al.*, 1988), and red blood cells (Oliver *et al.*, 1987). The carbonyl groups (aldehydes and ketones) are produced on protein side chains (especially proline, arginine, lysine, and threonine) when they are oxidized, and carbonyls may be introduced into proteins by secondary reactions of the nucleophilic side chains (cysteine, histidine, and lysine residues) with aldehydes [4-hydroxy-2-nonenal, malondialdehyde, 2-propenal (acrolein)] produced during lipid peroxidation or with reactive carbonyl derivatives (ketoamines, ketoaldehydes, deoxyosones) generated as a consequence of the reaction of reducing sugars, or their oxidation products, with lysine residues of proteins (glycation and glycoxidation reactions) (Dalle-Donne *et al.*, 2003).

Addition of saccharides to protein systems affects the functionality of proteins. Many previous studies used reducing and nonreducing saccharides, such as

glucose (Back *et al.*, 1979; Arakawa and Timasheff, 1982), lactose (Arakawa and Timasheff, 1982), sorbitol (Back *et al.*, 1979), and sucrose (Back *et al.*, 1979; Lee and Timasheff, 1981; Baier and McClements, 2001), to stabilize proteins without covalent bonding in solutions. Saccharides, such as maltose, lactose, sorbitol, sucrose, and trehalose, provided cryoprotection of enzymes (Hellman *et al.*, 1983; Carpenter *et al.*, 1987a; Carpenter *et al.*, 1987b) and proteins (Levine and Slade, 1992; Imamura *et al.*, 2003; Chang *et al.*, 2005) during freezing, freeze-drying and other dehydration processes. Mixtures of proteins and saccharides after dehydration form an amorphous matrix, in which protein molecules are embedded and stabilized by surrounding saccharides. The reactions of amino acids, peptides, and proteins with aldehydes, ketones, and a reducing sugar are known as carbonyl–amino reactions, Maillard reaction, nonenzymatic browning (NEB) reaction, and glycation or nonenzymatic glycosylation. This reaction, referred to here as NEB, often occurs in both aqueous solutions and dehydrated foods during processing and storage depending on the concentration and structure of proteins, sugar content, type of reactants, pH, water activity (a_w), temperature (T), and other related parameters. Several authors reported that products from Maillard reaction can decrease the lipid oxidation rate or act as antioxidants. For example, Mastrocola and Munari (2000) demonstrated that antioxidant activity of Maillard reaction products developed with increased browning of preheated systems containing pregelatinized corn starch, water, glucose, and lysine with or without soybean oil. Conversely, the Maillard reaction may be initiated and the final composition affected by lipid oxidation products (Thorpe and Baynes, 1996; Hidalgo and Zamora, 2000). Few studies, however, have used low water and nonaqueous media to investigate interactions of carbohydrates, lipids, and proteins that may take place in food and pharmaceutical materials.

The objectives of the present study were to investigate the effects of temperature and storage on physicochemical and thermal properties of systems containing whey protein isolate (WPI) proteins with oil and WPI with oil and reducing sugars to understand component interactions and behavior in the dry state or at a low water content in highly concentrated systems during storage at different temperatures and to determine effects of oil and reducing sugars on

physicochemical and thermal properties of WPI proteins. Our systems served as models for protein–lipid, protein–sugar, and protein–oil–sugar interactions in dehydrated foods and pharmaceuticals as well as confectionary products, high-protein foods, and supplements, including high protein nutritional bars. Furthermore, the data are useful in understanding protein denaturation and aggregation in food, nutritional, pharmaceutical, and *in vivo* studies involving lipid–protein oxidation, nonenzymatic browning and glycation, and aging.

3.2. MATERIALS AND METHODS

3.2.1 MATERIALS

The present study used WPI (Isolac®) from Carbery Food Ingredients (Ballineen, Cork, Ireland). Olive oil (OO; Don Carlos®, Hacienda Don Carlos, Sevilla, Spain) and sunflower oil (SO; Mediterani, Pan Euro Foods, Dublin, Ireland) were purchased from local suppliers. D-(+)-Glucose (G; ≥ 99.5% GC), D-(–)-fructose (F; ≥ 99%), and other chemicals were of analytical grade purchased from Sigma-Aldrich, Co. (St. Louis, MO, USA) except trichloroacetic acid and hydrochloric acid, which were purchased from Merck (Damstadt, Germany). Deionized (DI) water was a product of KB Scientific Ltd. (Cork, Ireland).

3.2.2. SAMPLE PREPARATION

This study used two high-protein systems: (i) WPI–oil systems with WPI (water content was 5 g/100 g of dry solid; 0.31 a_w) mixed with OO or SO at a 75:25 ratio (w/w) for 15.5 min (0.35 or 0.34 a_w, respectively, after mixing); and (ii) WPI–oil–sugar systems with WPI mixed with OO or SO and G–F (at 1:1 ratio) syrup with 30% (w/w) DI–water (0.75 a_w) at a component ratio of 45:15:40 (w/w) for 31 min (0.62 or 0.63 a_w, respectively, after mixing). Both systems were prepared using a Kenwood mixer (KM330; Kenwood Limited, Hampshire, UK) at minimum speed. The WPI–oil (2.5 to 3 g) and WPI–oil–sugar (4 to 4.5 g) systems were transferred to 10 mL clear glass vials (Schott, Müllheim, Germany). Vials with samples were closed with septa under vacuum in a freeze-dryer (Lyovac GT 2, Steris®, Hürth, Germany). Closed and vacumized vials were subsequently sealed in plastic packages (PA/PE 90, Fispak Ltd., Dublin, Ireland) under vacuum at 99% using a vacuum packaging machine (Polar 80 KL,

Henkelman B.V., Den Bosch, The Netherlands). Samples were protected from water loss and uptake from the environment during storage, and the packages with vials retained vacuum during storage. All systems were stored in temperature-controlled incubators at 20°C (Cooling Incubator, KBP 6151, Series 6000, Termaks, Bergen, Norway) and 40°C (TS 8136, Termaks). Samples were analyzed at intervals during storage for up to 14 weeks.

3.2.3. CARBONYL CONTENT

The carbonyl content of protein is the most commonly used marker of protein oxidation. The method for determination of the carbonyl content was modified from Levine *et al.* (1990) and Cucu *et al*. (2011). Samples of WPI–oil and WPI–oil–sugar systems before and during storage at 20 and 40°C for up to 14 weeks were dispersed and mixed at room temperature (20±2°C) using a magnetic bar for 30 min in DI–water to obtain 10% (w/w) protein dispersions. All sample dispersions were diluted to 5% (w/w) protein in DI–water and defatted four times with hexane (sample dispersion/hexane at 1:1 ratio) to remove free lipids. Aliquots of 0.35 mL of defatted protein dispersions were mixed with 1 mL of 2,4-dinitrophenylhydrazine hydrochloric acid solution (0.005M in ethanol, 18189 Fluka® Analytical, Switzerland) and incubated at 20°C for 1 h. Trichloroacetic acid (10% (w/v), 0.45 mL) was added to each sample to precipitate the protein. The protein dispersion was centrifuged at 14,000 rpm for 5 min using a microcentrifuge (1-15, Sigma Laborzentrifugen, Osterode am Harz, Germany). The precipitated protein sediment was collected, washed with 1 mL of ethanol/ethyl acetate (at 1:1 ratio) three times (centrifuged at 14,000 rpm for 10 min at each time) to remove any free reagent, and redissolved in 1 mL of 6 M guanidine hydrochloride solution at 20°C in a temperature-controlled incubator for 16 h. The samples were centrifuged at 14,000 rpm for 10 min to obtain a clear supernatant. The supernatant of all samples was used to determine the carbonyl content from absorbance at maximum wavelength, λ_{max} = 365 nm, read against 5% (w/v) hydrochloric acid solution (blank) using an UV–visible spectrophotometer (Varian Cary 1E, Varian Australia Pty Ltd., Victoria, Australia). The absorbance values were recorded using Cary Win UV software, version 01.00(9) (Varian Australia Pty Ltd., Victoria, Australia). Average values of carbonyl contents from triplicate samples were calculated using a molar

absorption coefficient of 22,00 $M^{-1}cm^{-1}$ and expressed as nanomoles of carbonyl per milligram of protein.

3.2.4. TOTAL SULFHYDRYL GROUPS

The total sulfhydryl groups of the protein were determined using the method of Sedlak and Lindsay (1968). Dispersions, 5% (w/v) protein in DI–water, of WPI–oil and WPI–oil–sugar were prepared in Tris-HCl buffer, pH 8.2 [30 mM Tris-HCl (Trizma® hydrochloride), 3 mM ethylenediamine-tetraacetic acid (EDTA), then pH to 8.2 with 0.1 M sodium hydroxide]. Samples, 0.2 mL, of dispersions were mixed with 0.8 mL of the Tris-HCl buffer, 0.25 mL of 3 mM 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB or Ellman's reagent) in methanol, and 0.4 mL of methanol. The samples were centrifuged at 14,000 rpm for 5 min. The supernatant of all samples was collected, and the absorbance at 412 nm was measured using an UV–visible spectrophotometer (Varian Cary 1E, Varian Australia Pty Ltd., Victoria, Australia). The optical density was calculated using a linear relationship of a standard curve measured for N-acetyl-L-cysteine as a sulfhydryl reactant at various concentrations (0 to 2.0 mM) in Tris-HCl buffer. The Tris-HCl buffer was used as a blank. Average values of total sulfhydryl groups of triplicate samples were calculated and expressed as millimoles of total sulfhydryl groups per gram of protein.

3.2.5. DIFFERENTIAL SCANNING CALORIMETRY (DSC)

Dispersions of WPI and WPI–sugar as control materials and of WPI–oil and WPI–oil–sugar systems adjusted to 10% (w/w) protein in DI–water before and during storage at 20 and 40°C for up to 14 weeks were prepared, as described for carbonyl content measurement, and used to investigate thermal properties. Glass Pasteur pipets (Corning®, Corning Inc., NY, USA) were used to transfer 2 to 4 mg of dispersion of each sample in a preweighed DSC aluminium pan (40 μL, Mettler Toledo, Schwerzenbach, Switzerland). The DSC pans were hermetically sealed, reweighed, and analyzed in triplicate. The thermograms were analyzed for temperatures and heats associated with endothermic [denaturation (Rüegg *et al.*, 1977; de Wit and Swinkels, 1980; Huang *et al.*, 1994; Bellavia *et al.*, 2011)] and exothermic [aggregation (Barone *et al.*, 1992; Dzwolak *et al.*, 2003;

Unterhaslberger *et al.*, 2006; Fitzsimons *et al.*, 2007)] transitions. All samples were scanned from 0 to 110°C at 5°C/min.

An empty punctured pan was used as a reference, and the DSC instrument (Mettler Toledo 821e, Schwerzenbach, Switzerland) was calibrated for temperature and heat flow as reported by Haque and Roos (2004). The thermograms were analyzed using STARe software, version 8.10 (Mettler Toledo), and average data were reported.

3.3. Results and Discussion

3.3.1. Protein oxidation and sulfhydryl content

Protein oxidation results in an increase of carbonyl groups (aldehydes and ketones) (Smith *et al.*, 1991; Stadtman, 1992; Stadtman and Berlett, 1998; Dalle-Donne *et al.*, 2003). In the present study, proteins were mixed with OO or SO at a components ratio of 75:25 (w/w), which showed oxidation during storage and resulted in accelerated protein oxidation (Zirlin and Karel, 1969; Chipault and Hawkins, 1971; Karel, 1973; Karel *et al.*, 1975; Kanner and Karel, 1976). The protein-bound carbonyl content of WPI–oil and WPI–oil–(G–F) systems at 20 and 40°C for 14 weeks is shown in Fig. 3.1. The carbonyl content of WPI–oil systems showed a rapid increase and then a decrease after 2 weeks of storage, and this was followed by a second slow increase and a decrease that was more pronounced for storage at 40°C. The initial increase was more pronounced for the WPI–OO systems at 20 and 40°C than for the WPI–SO systems. These results were in agreement with an initial decrease of total sulfhydryl content of the WPI–oil systems at 20 and 40°C (Fig. 3.2), which indicated formation of disulfide linkages leading to polymerization (Watanabe and Klostermeyer, 1976; Li-Chan, 1983).

The native structures of the globular whey proteins are stabilized by intermolecular disulfide bonds, and β-lactoglobulin also exhibits one free sulfhydryl or thiol group (cysteine^{121}) per monomer (Mulvihill and Donovan, 1987; Kinsella and Whitehead, 1989). Our results showed that the maximum formation of disulfide linkages and the highest protein oxidation occurred in

WPI–OO systems at 2 weeks of storage, and after 2 weeks of storage, the free thiol groups in β-lactoglobulin were oxidized completely. This was in agreement with Hidalgo and Kinsella (1989), who found that the presence of linoleic acid 13-hydroperoxide in the system caused formation of disulfide linkages following with covalent cross-linking of β-lactoglobulin B [containing one free thiol group, high binding affinity with non-polar compounds (O'Neill and Kinsella, 1987)] proteins during incubation at 37°C for up to 24 h. The WPI–SO systems showed less pronounced changes of the carbonyl and total sulfhydryl contents, which could account for the antioxidant activities of natural tocopherols in SO. Disulfide bonds form through oxidation of thiol groups (Shimada and Cheftel, 1988), and their formation can be markly influenced by the antioxidant activities of oil components. The total sulfhydryl contents of WPI–OO or –SO systems at 20 and 40°C over the storage period, as shown in Fig. 3.2, were higher than those reported by Cucu *et al.* (2011). They found that total sulfhydryl contents of dispersions of 2% (w/v) WPI with 1% (w/v) OO or SO during storage at 70°C for up to 50 h were within the range of 0.18 to 0.21 mmol/g protein, and the results for WPI with OO or SO incubation for up to 50 h were not significantly different. The increased total sulfhydryl content after 2 weeks of storage could result from further reactions, such as breakdown of disulfide bonds with release of hydrogen sulfide (H_2S) and ammonia (NH_3) from amide groups (Watanabe and Klostermeyer, 1976; Li-Chan, 1983; Shimada and Cheftel, 1988).

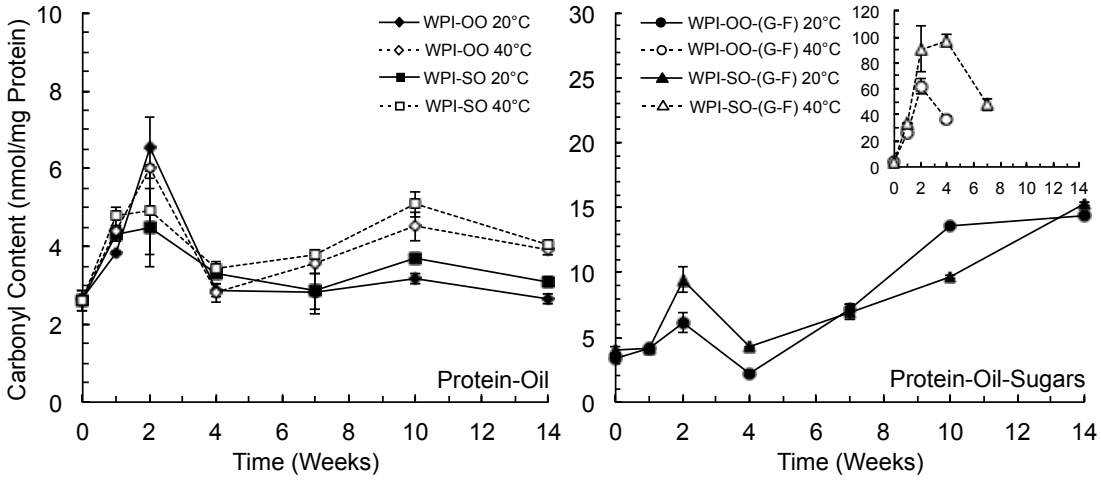

Fig. 3.1 Carbonyl content (protein oxidation) of 5% (w/w) protein dispersions of WPI–OO or –SO at 75:25 ratio, and WPI–OO– or WPI–SO–(G–F) at a component ratio of 45:15:40 during storage at 20 and 40°C for up to 14 weeks.

The loss of carbonyl groups of WPI–oil systems after 2 weeks of storage could result from degradation of oxidized protein by protease (Berlett and Stadtman, 1997), but more likely from products of lipid oxidation. The reactive oxygen groups and free radicals produced by oxidation of fatty acids can attack proteins and amino acids in anhydrous and low water content systems and induce formation of free radicals in protein, leading to proteins oxidation (Zirlin and Karel, 1969; Chipault and Hawkins, 1971; Kanner and Karel, 1976; Karel *et al.*, 1975). Leake and Karel (1982) found that freeze-dried lysozyme with oxidized methyl linoleate at a 1:1 ratio during incubation at 22°C and a_w of 0.75 for up to 20 days showed partial protein denaturation or fragmentation caused by the opening of disulfide bonds. The results of Leake and Karel (1982) showed that the denatured fraction of protein (tryptophan, nonsulfhydryl protein) produced dimer and trimer fractions. Fragmentation and polymerization of oxidized protein were independent processes. The overall kinetics of polymer formation in the oxidized protein–oil system was consistent with a free radical protein polymerization mechanism. Such fragmentation in our study could cause the increase of total sulfhydryl contents and the decrease of carbonyl contents of the WPI–oil systems after 2 weeks of storage at 20 and 40°C.

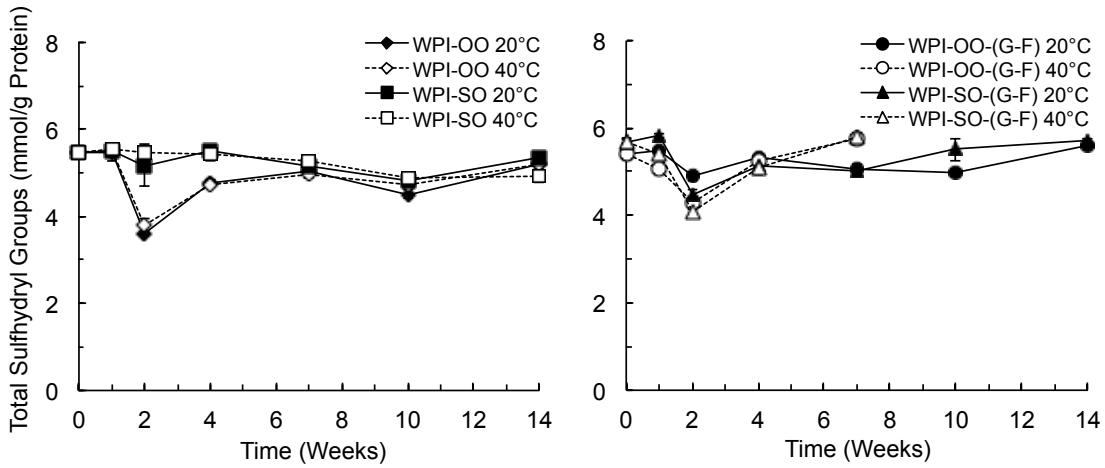

Fig. 3.2 Total sulfhydryl groups of 5% (w/w) protein dispersions of WPI–OO or –SO at 75:25 ratio, and WPI–OO– or WPI–SO–(G–F) at a component ratio of 45:15:40 during storage at 20 and 40°C for up to 14 weeks.

After 4 weeks of storage at 40°C, WPI–oil systems (0.11 to 0.16 a_w) showed higher carbonyl contents than systems at 20°C (0.36 to 0.39 a_w). The carbonyl content of WPI–SO was higher than that of WPI–OO at 40°C (Fig. 3.1). This result was in agreement with Cucu *et al.* (2011), who found that the ability of oils to promote carbonyl formation depended on the level of unsaturation of oil (in the order OO < soybean oil and SO < oxidized soybean oil < fish oil) and its initial oxidation. The probable explanation for the second slow increase of carbonyl contents of WPI–OO and –SO systems, particularly during storage at 40°C with low a_w (< 0.2 a_w), was lipid oxidation (Labuza *et al.*, 1972) with aldehydes, carboxylic acids, and ketones as the end products. The WPI–oil systems at 20 and 40°C during prolonged storage showed constant total sulfhydryl contents at the same level (Fig. 3.2). Steady sulfhydryl contents showed that most changes reflecting protein aggregation in WPI–oil systems occurred within the first 2 weeks, and no additional disulfide bonds were formed thereafter.

A rapid increase of carbonyl content during 2 weeks of storage followed by a decrease in the WPI–oil–sugar systems at 20°C was in agreement with an initial decrease of total sulfhydryl content. The total sulfhydryl contents of the WPI–oil systems and the WPI–oil–sugar systems were not significantly different (Fig. 3.2). The WPI–OO– and WPI–SO–(G–F) at 20°C showed dramatic increases of carbonyl contents after 4 weeks of storage, which was very different from the data of WPI–oil systems (Fig. 3.1). Such increases of the total sulfhydryl contents were not in agreement with the levelling-off of total sulfhydryl contents of WPI–oil–sugar systems after 4 weeks of storage at 20°C. The levelling-off of total sulfhydryl contents of WPI–oil–sugar systems were similar to WPI–oil systems at 20°C. Carbonyl contents of the WPI–oil–sugar systems after 4 weeks of storage at 20°C, and during storage for up to 7 weeks at 40°C, were significantly higher than in the WPI–oil systems at both temperatures, especially in the WPI–SO–(G–F) systems (Fig. 3.1). This indicated that the presence of glucose and fructose in the WPI–oil systems could accelerate protein oxidation with resultant carbonyl formation. On the other hand, the carbonyl groups (aldehydes and ketones) of glucose and fructose were present, and glycation of the protein by the reducing sugars could cause the substantial increase of

carbonyls during storage. Glycation forms reactive carbonyl derivatives (ketoamines, ketoaldehydes, and deoxyosones), which promote oxidation of proteins (Berlett and Stadtman, 1997). Our result was in accordance with Takagi *et al.* (1995), who found that 150 μM of fatty-acid-free bovine serum albumin (BSA) dispersion incubated with 50 or 100 mM of glucose or fructose at 37°C for up to 2 weeks increased carbonyls and fluorescent albumin (advanced glycation end products), which did not occur when BSA was incubated in the absence of the reducing sugars. Chen *et al.* (2012) found that glucose (aldose) was more reactive in the Maillard reaction than fructose (ketose) in intermediate-moisture food systems composed of β-lactoglobulin with glucose or fructose and glycerol during storage at 25 and 35°C for 7 weeks, although these two sugars have similar preferences for glycation sites in proteins. The highest carbonyl contents of WPI–OO– and WPI–SO–(G–F) systems were found at 2 or 4 weeks, respectively, during storage at 40°C. Overall, the carbonyl concentrations in the systems containing SO were higher than in the systems containing OO. This result suggested that during storage at 40°C a substantially higher level of protein oxidation occurred in systems with a higher level of unsaturated fatty acids. A rapid decrease of the carbonyl contents of WPI–oil–sugar systems occurred after 2 to 4 weeks of storage at both temperatures. This decrease accounted for further reactions of aldehydes, ketones, and dicarbonyls at the advanced stage of the nonenzymatic browning (Maillard) reaction, which produced higher molecular weight polymers and fluorescent compounds (Kato *et al.*, 1989; Dills, 1993).

The WPI–oil systems stored at 40°C for 14 weeks showed less extractable, hydrophilic protein (increased hydrophobicity). This was in accordance with Nielsen *et al.* (1985), who reported that storage of dry whey protein mixtures (100 to 300 g) with methyl linoleate (half-weight of protein) for 4 weeks at 37 and 55°C showed a decrease of extractable protein. We presumed that the decrease of extractable protein in the WPI–oil systems at 40°C in vacuum or in limited-oxygen systems was a result of hydrophobic interactions with oil, which increased protein surface hydrophobicity and protein insolubility. The extractable protein from a protein–oil system was dependent on a_w, oxygen, temperature, and time (Nielsen, 1985). Proteins in contact with peroxidized lipid or their secondary break down products changed functional properties of proteins and

amino acids (insolubilization, polymerization or cross-linking, and formation of lipid–protein complexes). The fatty acids in lipid molecules showed a surfactant effect on protein surfaces leading to hydrophobic interactions and protein unfolding, thus exposing protein interior groups to hydrophobic reactions. The carbonyl groups of oxidized lipids may participate in covalent bonding leading to the formation of stable protein–lipid aggregates (Saeed and Howell, 2002).

Carbonyl contents of WPI–OO– or WPI–SO–(G–F) systems showed a decrease of extractable hydrophilic protein-sugar dispersions at 2 or 4 weeks, respectively, of storage at 40°C. According to results shown in Fig. 3.1, decreases of carbonyl contents of WPI–OO– and WPI–SO–(G–F) occurred in storage and carbonyls could not be determined after 4 and 7 weeks, respectively, of storage at 40°C. The materials showed insolubility in water caused by polymerization and hydrophobic interactions in the hardened and brittle material. Within such materials, oil could also form a hydrophobic layer around protein particles and decrease its accessibility to solvents. Our results showed that the presence of G–F in the WPI–oil systems could induce protein hydrophobicity and hydrophobic interactions of components. Insolubilisation and hardening of WPI–oil–(G–F) systems resulted from complex reactions between oxidized protein–lipid compounds (Roubal and Tappel, 1966a; Funes and Karel, 1981) and products of Maillard reaction (at the advanced stage of the reaction) or advanced glycation end products (Kato *et al.*, 1986; Liang and Rossi, 1990).

3.3.2. DENATURATION AND AGGREGATION

The thermograms of the 10% (w/w) protein dispersions of WPI, WPI–(G–F), and WPI–oil and WPI–oil–sugar systems at 20 and 40°C before and after 14 weeks of storage are shown in Fig. 3.3. The first heating scan of 10% (w/w) protein dispersions of WPI, WPI–oil, and WPI–oil–sugar systems before storage showed protein denaturation endotherms with onset and peak temperatures ranging from 55 to 61°C and from 72 to 75°C, respectively, and protein aggregation exotherms with onset and peak temperatures ranging from 81 to 92°C and from 94 to 104°C, respectively (Table 3.1). The denaturation peak temperatures for α-lactalbumin and β-lactoglobulin were above 60°C and ~70 to 80°C, respectively (de Wit and Klarenbeek, 1984; Boye and Alli, 2000; Fitzsimons and Mulvihill,

2007). The 10% (w/w) protein dispersion of WPI showed a lower onset temperature of protein denaturation than was found for the WPI–oil systems before and after storage at 20 and 40°C (Table 3.1). The exothermic peak of 10% (w/w) protein dispersion of WPI showed a smaller heat of aggregation than was found for the systems containing oil before storage (0 day). This difference in aggregation behavior showed that mixing WPI with OO or SO increased the thermal stability of the proteins (increased onset temperature of protein denaturation), but enhanced protein aggregation (increased size of the exotherm), as shown in Fig. 3.3. Jones *et al.* (2005) found in agreement with our study that the presence of 0.5% silicone oil (for coating disposable plastic syringes and stoppers to facilitate processing) in a protein dispersion at a concentration of 0.5 mg protein/mL buffer caused conformational changes, alterations in thermal stability, and acceleration of protein aggregation [bovine serum albumin (BSA) and ribonuclease A] at pH 4.5, 6.5, and 7.2 before and after storage at 45°C for 5 h. The WPI–oil systems after storage at both temperatures for 14 weeks showed broader but smaller aggregation exotherms (Fig. 3.3). Storage effects on denaturation and aggregation behavior were more substantial for storage at 40°C, probably because protein could undergo more rapid denaturation and aggregation (Li-Chan, 1983) as a result of changes of protein conformation during storage (de Wit and Klarenbeek, 1984; Jones *et al.*, 2005). The WPI–oil systems during storage for up to 14 weeks at 20 and 40°C showed a lower onset temperature for protein denaturation and a higher peak temperature of protein aggregation, especially at week 10 of storage, than systems before storage (Table 3.1), indicating changes in protein conformation and induced aggregation during storage, that is, decreased poststorage heat of aggregation. The heat of protein denaturation of WPI–OO decreased after storage at both 20 and 40°C for 14 weeks. The WPI–OO system stored at 40°C for 4 weeks gave the highest heat of protein aggregation (Fig. 3.4), which showed that preaggregation (nucleation) during storage could enhance instant poststorage aggregation upon heating in water, as found by DSC. As a result of such preaggregation, the heat of protein aggregation of WPI–OO during storage at 20 and 40°C for 14 weeks against time showed a parabolic relationship. Hsu and Fennema (1989) found that temperature, time, and a_w were the most important factors that affected changes of structural and physicochemical properties of proteins. The WPI–SO system

showed less changes of heats of protein denaturation and aggregation during storage at both temperatures (Fig. 3.4). This finding showed that the type of oil and possibly the presence of natural antioxidants (tocopherols) in SO affected interactions of protein molecules that reduced oxidative aggregation (disulfide bonds) (Fig. 3.4).

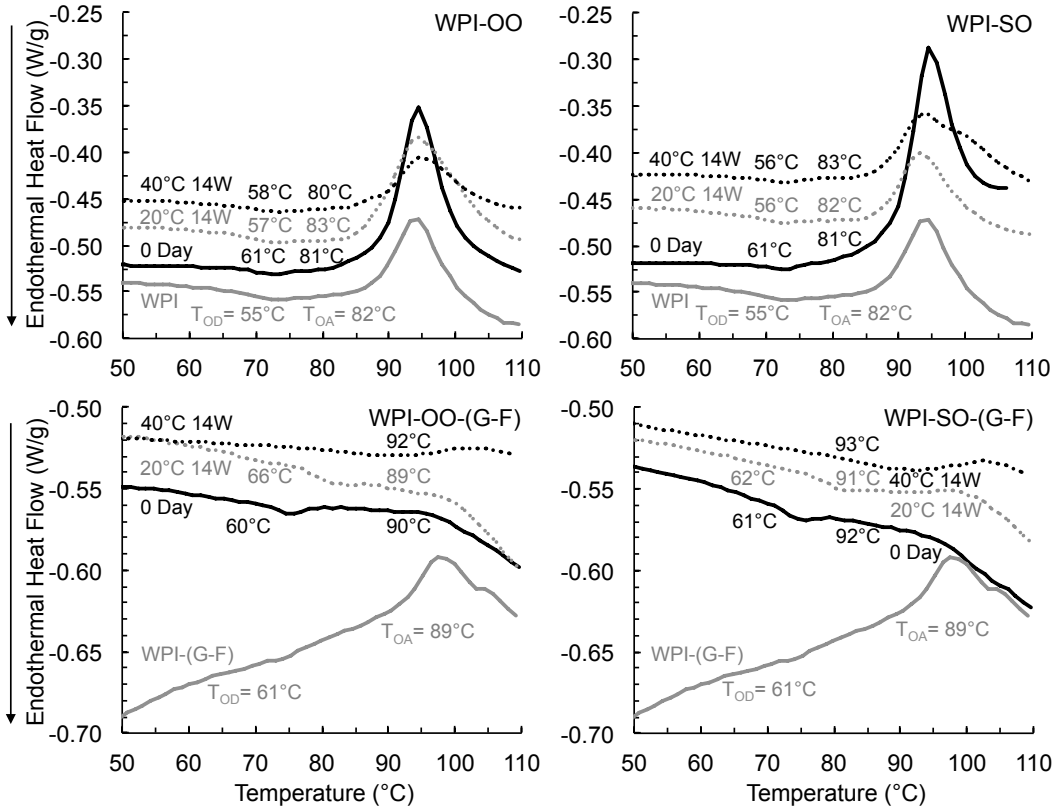

Fig. 3.3 Differential scanning calorimetry thermograms of first heating scan of 10% (w/w) protein dispersions of WPI–OO or –SO at 75:25 ratio and WPI–OO– or WPI–SO–(G–F) at a component ratio of 45:15:40 before (0 Day) and after 14 weeks (W) of storage at 20 and 40°C compared with 10% (w/w) protein solutions of WPI alone and WPI–(G–F) at 45:40 ratio. The temperatures shown on endothermic and exothermic peaks were onset temperature of protein denaturation (T_{OD}) and aggregation (T_{OA}), respectively.

Table 3.1 Onset and peak temperature of protein denaturation and aggregation of 10% (w/w) protein dispersions of WPI–OO or –SO and WPI–OO– or WPI–SO–(G–F) systems before and during storage at 20 and 40°C for up to 14 weeks compared with 10% (w/w) protein dispersions of nonstored WPI and WPI–(G–F) using DSC[a].

Materials	Time (Weeks)	Temperature (°C)									
		20°C					40°C				
		Denaturation			Aggregation		Denaturation			Aggregation	
		Onset	Peak	Endset	Onset	Peak	Onset	Peak	Endset	Onset	Peak
WPI	0	55	72	77	82	95	55	72	77	82	95
WPI–OO	0	61	73	80	81	94	61	73	80	81	94
	2	61	73	80	81	94	61	73	80	81	96
	4	63	73	81	82	96	61	74	78	83	96
	7	60	73	78	84	97	61	73	81	82	95
	10	58	72	79	84	100	60	72	79	82	99
	14	57	72	80	83	95	58	73	80	80	94
WPI–SO	0	61	72	78	81	94	61	72	78	81	94
	2	61	72	78	80	94	61	73	79	81	93
	4	64	73	78	80	94	58	72	79	81	95
	7	60	73	78	82	95	58	72	78	83	95
	10	60	72	78	85	100	61	72	78	86	100
	14	56	72	77	82	94	56	73	78	83	94
WPI–(G–F)	0	61	75	79	89	98, 104	61	75	79	89	98, 104

Table 3.1 (continued)

Materials	Time (Weeks)	Temperature (°C)									
		20°C					40°C				
		Denaturation			Aggregation		Denaturation			Aggregation	
		Onset	Peak	Endset	Onset	Peak	Onset	Peak	Endset	Onset	Peak
WPI–OO–(G–F)	0	60	75	80	90	97	60	75	80	90	97
	2	62	76	82	N/A	N/A	N/A	N/A	N/A	85	98
	4	65	78	87	N/A	N/A	N/A	N/A	N/A	88	98
	7	61	79	87	N/A	N/A	N/A	N/A	N/A	85	99
	10	65	82	89	N/A	N/A	N/A	N/A	N/A	93	105
	14	66	81	88	89	98	N/A	N/A	N/A	92	101
WPI–SO–(G–F)	0	61	75	80	92	97	61	75	79	92	97
	2	60	76	83	92	98	N/A	N/A	N/A	84	96
	4	62	78	85	N/A	N/A	N/A	N/A	N/A	84	98
	7	65	79	86	N/A	N/A	N/A	N/A	N/A	85	98
	10	69	81	89	N/A	N/A	N/A	N/A	N/A	94	104
	14	62	81	90	91	98	N/A	N/A	N/A	93	103

[a] N/A = no endothermic and exothermic peaks occurred, hardly define, or specific temperature.

WPI with G–F showed increased onset and peak temperatures of protein denaturation and aggregation in 10% (w/w) WPI dispersions (Table 3.1). The increased denaturation temperatures demonstrated that G–F promoted thermal stability (higher temperature of protein denaturation and aggregation) of WPI. Hydrophobic interactions of hydrophobic groups of proteins can become strengthened in the presence of sugars (Back *et al.*, 1979). Sugars in aqueous solutions increase surface tension and promote preferential interaction of proteins (Arakawa and Timasheff, 1982). Our result was in agreement with Boye and Alli (2000), who found that α-lactalbumin/β-lactoglobulin [at 1:1 ratio, 40% (w/v)] mixtures in 20% (w/v) solutions of glucose or fructose had the greatest effect in stabilizing proteins against thermal denaturation. Their results showed that the peak temperature of protein denaturation increased in the presence of the sugars. The peak temperature of β-lactoglobulin denaturation increased from 69 to 73 and 77°C with fructose and glucose, respectively. In the present study, the 10% (w/w) aqueous dispersions of WPI–(G–F) and WPI–oil–(G–F) systems showed the same onset temperatures of protein denaturation. The WPI–oil–(G–F) systems, however, showed smaller aggregation exotherms than the WPI–(G–F) systems before storage (Fig. 3.3). Accordingly, in protein–sugar systems OO and SO reduced protein aggregation.

The WPI–oil–sugar systems during storage at 20°C showed an increase of onset, peak, and endset temperatures of protein denaturation endotherms, but no denaturation endotherms were found for WPI–oil–sugar systems stored at 40°C. The exclusion of protein denaturation after storage at 40°C was a strong indication of substantial conformational changes in WPI caused by glycation, nonenzymatic browning, and possible protein oxidation, which agreed with the substantial increase in carbonyl contents (Fig. 3.1). An increase of the peak temperature of protein aggregation occurred during storage at 40°C (Table 3.1) in line with the increased carbonyl contents and decreased sulfhydryl groups. The WPI–OO–(G–F) had a substantially increased heat of protein denaturation at week 4 of storage at 20°C, but the WPI–SO–(G–F) system had a fairly constant heat of protein denaturation over the storage period (Fig. 3.5), which agreed with no significant change of the heat of protein denaturation of the WPI–SO system during storage (Fig. 3.4). Conversely, the WPI–OO–(G–F) system showed

significant effects of the oil on the thermal properties of WPI during storage. The increased heat of denaturation of the WPI–OO–(G–F) system agreed with increased exothermic heat of aggregation, lower carbonyl content, and higher total sulfhydryl groups and could relate to differences in changes in protein conformation, oxidation, aggregation, and hydrophobic interactions during storage at 20°C, as compared to the WPI–SO–(G–F) system. The protein aggregation exotherm was absent in thermograms of WPI–OO–(G–F) and WPI–SO–(G–F) during storage at 20°C after 2 to 10 and 4 to 10 weeks, respectively (Table 3.1). The exotherms in both systems, however, reappeared after 14 weeks of storage (Fig. 3.3 and Table 3.1).

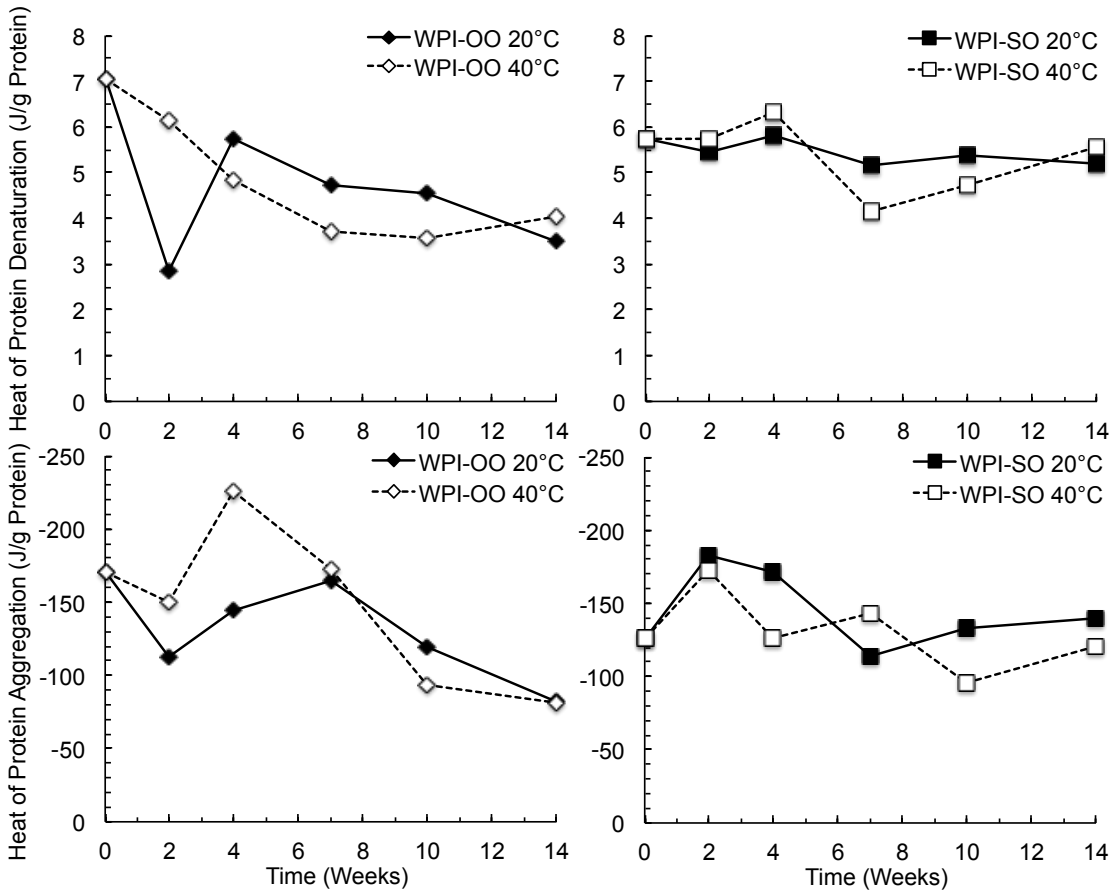

Fig. 3.4 Heat of protein denaturation and aggregation of 10% (w/w) protein dispersions of WPI–OO or –SO at 75:25 ratio during storage at 20 and 40°C for up to 14 weeks.

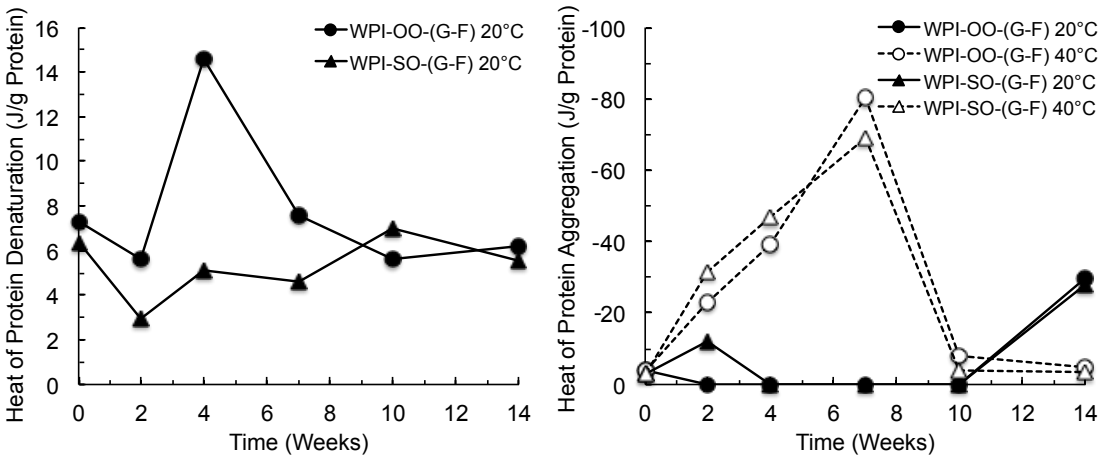

Fig. 3.5 Heat of protein denaturation and aggregation of 10% (w/w) protein dispersions of WPI–OO– or WPI–SO–(G–F) at a component ratio of 45:15:40 during storage at 20°C and 40°C for up to 14 weeks.

The WPI–oil–(G–F) systems during storage at 40°C for 14 weeks showed increased peak temperatures of protein aggregation, especially at week 10, which agreed with the results for the WPI–oil systems in storage at 40°C (Table 3.1). The heat of protein aggregation of WPI–oil–(G–F) increased dramatically during storage at 40°C from 0 to 7 weeks, but there was a dramatic decrease during subsequent storage (Fig. 3.5). A subsequent decrease of the heat of protein aggregation after 7 weeks of storage could result from significant polymerization in the protein–sugar–oil systems, forming complex structures and loss of protein conformation needed for aggregation when heated in water. Protein aggregation was induced by glycation and oxidation that often change physicochemical properties of protein, such as hydrophobicity, secondary/tertiary structure, and inhibition of protein unfolding (Wang *et al.*, 2010). Such changes presumably occurred more slowly at 20°C than at 40°C, and a prolonged storage at 20°C could result in similar preaggregation and increase in the heat of protein aggregation as was found at 40°C from the beginning of storage (Fig. 3.5).

3.4. CONCLUSIONS

The protein–oil and protein–oil–sugar systems in dry and highly concentrated systems showed component interactions during storage. These interactions affected the physicochemical and thermal properties of the high–protein systems. The disulfide bonds reflecting protein aggregation were at the highest level in

WPI–oil systems within 2 weeks of storage at 20 and 40°C and coincided with the highest carbonyl contents. Possible protein fragmentation occurred in WPI–oil systems at 20 and 40°C, which caused the increase of total sulfhydryl contents and the decrease of carbonyl contents after 2 weeks of storage. Addition of OO and SO to WPI increased the thermal stability of protein (increased onset temperature of protein denaturation) but increased the size of protein aggregation exotherms, suggesting preaggregation reactions in the oil medium. During storage at 20 and 40°C for up to 14 weeks, WPI with oil showed increased protein sensitivity to heat (decreased onset temperature of denaturation and heat of denaturation) and induced protein aggregation (increased peak temperature of aggregation and decreased poststorage heat of aggregation), especially when OO was used in the systems. WPI–OO showed the preaggregation of protein (nucleation) during storage at 40°C that enhanced poststorage aggregation. Storage effects on thermal properties were more pronounced at 40°C than at 20°C, and they led to more rapid denaturation and aggregation because of likely changes of protein conformation. We found that the type of oil and the natural antioxidants in the oil medium affected interactions of proteins (decreased disulfide bonds formation or protein aggregation). The presence of OO and SO in WPI–sugar systems reduced the sizes of protein aggregation exotherms. The glucose–fructose syrup in WPI–oil enhanced protein resistance to heat, protein oxidative damage (increased of carbonyls), and hydrophobic interactions of components. An increase of carbonyl contents could result from aldehydes and ketones of glucose and fructose and glycation of the protein by reducing sugars. The glycation forms reactive carbonyl derivatives that promote protein oxidation. The lipid oxidation and Maillard reaction promote oxidation of proteins as was shown by increased carbonyl contents during storage, especially in the systems containing SO or a higher level of unsaturated fatty acids during storage at 40°C. Products from lipid oxidation (oxidized protein–lipid) and nonenzymatic browning reactions (advanced glycation end products) resulted in decreased solubility and increased aggregation, hydrophobicity, and hardening, especially during storage at 40°C. The thermal properties of WPI–oil–sugar systems during storage showed less rapid changes at 20°C than at 40°C, which after prolonged storage at 20°C reached levels found at the beginning of storage at 40°C (preaggregation of protein and increased heat of protein aggregation).

CHAPTER IV

Food Biophysics
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PROTEIN MODIFICATIONS IN HIGH PROTEIN–OIL AND PROTEIN–OIL–SUGAR SYSTEMS AT LOW WATER ACTIVITY

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ABSTRACT

Physicochemical and thermal properties of high protein systems during storage at 20 and 40°C were investigated for 14 weeks. Component interactions of whey protein isolate (WPI)–olive oil (OO), WPI–sunflower oil (SO) (75:25), WPI–(glucose–fructose; G–F) (45:40), WPI–OO–(G–F), and WPI–SO–(G–F) (45:15:40) systems at low water contents during storage were derived from differential scanning calorimetry (DSC), colorimetric, water activity (a_w), reducing and nonreducing SDS-PAGE electrophoresis data. The degree of unsaturation of oil affected colour (yellowness) and microstructure of the systems as well as variations in water migration and nonenzymatic browning kinetics (NEB) during storage. These effects were evident in the SO systems. All systems at 40°C showed changes in protein conformation to those favoring hydrophobic interactions with oil. These systems showed decreased a_w, insolubilization, hardening as a result of carbonyl–amine polymerization and covalent cross-linking of proteins in the NEB. The DSC data showed a protein hydration transition for rehumidified-WPI, WPI–oil, WPI–sugar, and WPI–oil–sugar. The rehumidified-WPI and WPI–oil also showed a_w-dependent denaturation endotherms (irreversible transition) for α-lactalbumin and β-lactoglobulin at higher temperatures (T). The WPI–sugar and WPI–oil–sugar showed an exotherm for the browning reaction (irreversible transition) at T_{onset} ~ 90°C. An exothermic protein hydration in the systems containing sugar was storage time-dependent, and indicated changes of protein conformation. The presence of oil in WPI–oil–sugar caused an increase in the glass transition of sugars during storage, especially for SO. The WPI–(G–F) and WPI–oil–(G–F) showed broadened glass transition during a reheating scan in DSC that was a result of polymerization in protein, oil, and sugar components mixture. Stability of high protein systems is dependent on hydration and reactions in both hydrophilic and hydrophobic phases.

KEYWORDS: cross-linked protein, denaturation, glass transition, hydration, nonenzymatic browning, oil reactant

4.1. INTRODUCTION

Materials with high protein and solids contents are common and of significant interest in the food and pharmaceutical industries as ingredients and final products. Such ingredients and products include dehydrated dairy products, protein ingredients, and foods, e.g., meat and plant proteins, as well as many novel high protein foods, sports foods, and medicinal foods. The properties of high protein systems are often altered by complex physicochemical phenomena during storage. Several interactions of protein and nonprotein components (acids and bases, antioxidants, buffers, lipid, saccharides, salt, water), such as glycation (Eichner and Karel, 1972; Labuza *et al.*, 1972; Warmbier *et al.*, 1976; Labuza and Saltmarch, 1981; Franzen *et al.*, 1990; Baisier and Labuza, 1992; Mastrocola and Munari, 2000; Benjakul *et al.*, 2005b; Potes *et al.*, 2013a) and hydrophobic interactions (Zirlin and Karel, 1969; Sun *et al.*, 2002; Potes *et al.*, 2013a), may depend on environmental conditions [water activity (a_w) and water content, concentration, pH, temperature] and storage time. Product acceptability and shelf life is reduced by deteriorative changes in physicochemical properties (colour, flavors, solubility, and structure or texture) (Roubal and Tappel, 1966a; Waletzko and Labuza, 1976; Burin *et al.*, 2000; Roos, 2002; Saeed and Howell, 2002), and modifications of protein properties and functionality (Roubal and Tappel, 1966a; Leake and Karel, 1982; Saeed and Howell, 2002; Anema *et al.*, 2006; Cucu *et al.*, 2011), loss in nutritional quality (Waletzko and Labuza, 1976; Labuza and Saltmarch, 1981; Nielsen *et al.*, 1985; Naranjo *et al.*, 2013; Schmitz-Schug *et al.*, 2013), and deterioration (lower stability) of the organoleptic (Karel, 1973; Waletzko and Labuza, 1976; Saeed and Howell, 2002) and overall quality of the materials (Karel, 1973; Ukhun and Izi, 1991; Friedman, 1996; Roos, 2002; Saeed and Howell, 2002; Schmitz-Schug *et al.*, 2013).

Typical component interactions or reactions in high protein systems at low a_w or water content includes nonenzymatic browning (NEB) or Maillard reaction or glycation (nonenzymatic glycosylation reaction), and lipid and protein oxidation. The Maillard reaction involves amines, amino acids, peptides, and proteins with carbonyls including aldehydes, ketones, and reducing sugars promoting the formation of free radicals (cation) and coloured compounds (Hodge, 1953; Namiki *et al.*, 1973; Wolfrom *et al.*, 1974; Roberts and Lloyd, 1997; Hofmann *et*

al., 1999), water in condensation steps (Hodge, 1953), polymeric aggregates (cross-linking of proteins) and fluorescent compounds in the advanced stage (Hodge, 1953; Friedman, 1996), and fragmentation or fission of sugar moieties (glycolaldehyde, glyceroldehyde, pyruvaldehyde, acetol, dihydroxyacetone, acetoin, and diacetyl) (Hodge, 1953). Another browning reaction may occur as protein-oxidized fatty acids reaction (hydrophobic interaction) via formation of covalent bonding and formation of stable protein-lipid complexes (Tappel, 1955; Tannenbaum *et al.*, 1969; Pokorný *et al.*, 1974; Davies, 1987; Hidalgo and Kinsella, 1989; Friedman, 1996; Howell *et al.*, 2001). Transient free radicals from lipid peroxidation may accelerate major protein damaging reactions (Roubal and Tappel, 1966a). The lipid oxidation can be promoted (Thorpe and Baynes, 1996; Hidalgo and Zamora, 2000) and reduced (Mastrocola and Munari, 2000) by products from the Maillard reaction acting as intermediates and antioxidants. The rate of these NEB reactions depends on a_w and water content, concentration and structure of proteins, content and type of sugar, degree of unsaturated fatty acids, pH, temperature, and other related parameters.

The structure and conformation of a protein may vary according its water content, temperature, and storage time. A better understanding of the behavior and properties of proteins at various conditions with other food components may be used to control the NEB reactions or interactions of components to reduce the instability of high protein foods and drugs at high solids contents. Such materials, e.g., dehydrated ingredients, infant and nutritional formulations, high protein bars, and confectionary, are typically expected to have a long shelf life. The present study investigated the effects of temperature and storage time on the physical, physicochemical, and thermal properties of proteins and their transitions in high protein systems to understand interactions of components and the behavior of proteins in the dry state or at a low a_w during storage at different temperatures. The study used oil and reducing sugars to obtain data for systems at conditions enhancing hydrophobic interactions and glycation at various a_w and temperature environments for the investigation of variations in physical, physicochemical, and thermal properties of mixed formulations during storage. The systems contained protein with oil, protein with sugar, and protein with oil and sugar as the models for dehydrated or low a_w biological, and food and

pharmaceutical materials. The data from our study are useful in understanding protein behavior (hydration and denaturation transitions) and interactions of components (protein, oil, and sugar) at low water contents and in the high solids systems, which has not received much attention, especially the nonenzymatic browning reaction involving oil. The results from our study can be used to explain variations in physical, physicochemical, and chemical properties (colours, solubility, texture, and protein conformation) in high protein nutritional bars (Loveday et al., 2009; Potes et al., 2013a; Zhou *et al.*, 2013), infant formulae (Le *et al.*, 2011a; b), and hard cheese products (low water content) (Lobato-Calleros *et al.*, 2007) as common examples.

4.2. MATERIALS AND METHODS

4.2.1. MATERIALS

Whey Protein Isolate, WPI (Isolac®), used in the present study was purchased from Carbery Food Ingredients (Ballineen, Cork, Ireland). Olive oil (OO; Don Carlos®, Hacienda Don Carlos, Sevilla, Spain) and Sunflower Oil (SO; Mediterani, Pan Euro Foods, Dublin, Ireland) were purchased from local suppliers. D-(+)-Glucose (G; ≥ 99.5% GC), D-(–)-fructose (F; ≥ 99%), and other chemicals were of analytical grade purchased from Sigma-Aldrich, Co. (St. Louis, MO, USA). De-ionized (DI) water was a product of KB Scientific Ltd. (Cork, Ireland).

4.2.2. SAMPLE PREPARATION

This study used two high protein systems: (i) WPI–oil system with WPI (water content 5g/100 g of dry solid; 0.31 a_w) mixed to OO or SO at 75:25 ratio (w/w) for 15.5 min (0.35 or 0.34 a_w, respectively, after mixing); (ii) and WPI–oil–sugar with WPI mixed to OO or SO and G–F (at 1:1 ratio) syrup with 30% (w/w) DI-water (0.75 a_w) at a component ratio of 45:15:40 (w/w) for 31 min (0.62 or 0.63 a_w, respectively, after mixing). Both systems were prepared using a Kenwood mixer (KM330; Kenwood Limited, Hampshire, UK) at minimum speed. Samples of the WPI–oil and WPI–oil–sugar systems were transferred to 10 mL clear glass vials (Schott, Müllheim, Germany) (2.5 to 3 g and 4 to 4.5 g, respectively) and placed in plastic cups (diameter 37 mm, volume 15 mL; AquaLab, Decagon

Devices, Inc., WA, USA) (2 to 2.5 g and 5 to 5.5 g, respectively). Vials with samples were closed with rubber septa under vacuum using a freeze-dryer (Lyovac GT 2, Steris®, Hürth, Germany). Samples in cups were pressed with a titanium plate (diameter 33 mm; Triton Technology Ltd., Lincolnshire, UK) to obtain a smooth surface and homogeneous structure, and the cups were closed with plastic, airtight lids (AquaLab, Decagon Devices, Inc., WA, USA). Closed and vacumized vials, and closed cups were subsequently sealed in plastic packages (PA/PE 90, Fispak Ltd., Dublin, Ireland) under vacuum at 99 and 75%, respectively, using a vacuum packaging machine (Polar 80 KL, Henkelman B.V., Den Bosch, The Netherlands). Samples were protected from water loss and uptake from the environment during storage. The vacuum in the packages retained hermetic conditions during storage and served as an indicator of the closed system. All systems were stored in temperature controlled incubators at 20°C (Cooling Incubator, KBP 6151, Series 6000, Termaks, Bergen, Norway) and 40°C (TS 8136, Termaks, Bergen, Norway). Samples were analyzed at intervals during storage for 14 weeks.

4.2.3. COLOUR AND a_w

Colour and a_w of WPI–oil and WPI–oil–sugar samples was monitored at intervals during storage at 20 and 40°C. Colour of samples in vials was measured using a colorimeter (CR300 chroma meter, Konica Minolta Holdings, Inc., Japan) and measured from outside of the clear glass vials. Colour parameters used the CIE Lab colour space [L^* = lightness (0 to 100), a^* = greenness/redness (+/-), and b^* = blueness/yellowness (+/-)]. The samples in cups stored in vacuumized and sealed plastic packages were equilibrated at room temperature (20±2°C) for 1 h before a_w was measured using a water activity meter (4TE, AquaLab, Decagon Devices, Inc., WA, USA). Triplicate vials and a minimum of duplicate cups were used in measurements and average values of L^*, a^*, b^*, and a_w were calculated.

4.2.4. X-RAY DIFFRACTION (XRD)

The method for XRD measurement was similar to that described by Jouppila *et al.* (1997) and Haque and Roos (2005b). The XRD patterns were recorded for the high protein systems containing sugar before and after storage at 20 and 40°C for 19 weeks using a Philips X-ray diffractometer (XPERT PRO, PW 3830

generator, PW 3710 MPD diffractometer and PC-MPD software for automatic sample diffraction version 3.0; Philips Analytical B.V., Almelo, The Netherlands) to confirm noncrystallinity of suga components during storage. The X-ray diffractometer was operated with an anode current of 40 mA and an accelerating voltage of 40 kV. Samples from plastic cups were transferred to an aluminium tray and exposed to CuKα radiation at diffraction angles (2θ) from 5 to 30° (step size 0.02; time per step 40.01 s). The peak search program of the X'Pert HighScore (Version 1.0a, Phillips Analytical B.V., Almelo, The Netherlands) software was used to locate possible peaks in XRD patterns by detecting the minima from the second derivative of the diffractogram. Intensity maxima were given as Kα1 net peak height in counts (per step measurement time) at Kα1 position in degrees. The WPI–OO–(G–F) systems at 20 and 40°C after 14 weeks of storage (19 weeks) were chosen as a representative of WPI–oil–sugar systems. The XRD patterns of WPI–OO–(G–F) systems obtained in this study were compared with XRD pattern of D-(+)-glucose (≥ 99.5% GC).

4.2.5. DIFFERENTIAL SCANNING CALORIMETRY (DSC)

The nonhumidified (0.31 a_w)-WPI, rehumidified (0.76 and 0.85 a_w)-WPI, WPI–oil, WPI–oil–(G–F), and WPI–(G–F, 1:1) at 45:40 ratio (w/w) (0.66 a_w) samples were freshly prepared and transferred to preweighed DSC aluminium pans (40 μL, Mettler Toledo, Schwerzenbach, Switzerland). To vary a_w, WPI at a_w of 0.31 in preweighed DSC pans were weighed, humidified over saturated solutions of NaCl and KCl at 0.76 and 0.85 a_w, respectively, in vacuum desiccators at room temperature (20±2°C) for 3 days, then the pans were hermetically sealed and reweighed. The DSC pans of WPI at 0.31 a_w, WPI–oil, WPI–oil–(G–F), and WPI–(G–F) systems were hermitically sealed and reweighed. Duplicate samples were analysed and scanned in the DSC from -80°C [WPI at 0.76 and 0.85 a_w, WPI–oil, WPI–oil–(G–F), and WPI–(G–F)] or from 0°C (WPI at 0.31 a_w) to 110°C at 5°C/min, then cooled at 10°C/min to initial temperature, and rescanned in second heating to ≥ 100°C at 5°C/min. The thermograms were analysed for temperatures associated with glass transition (T_g), and endothermic and exothermic transitions.

A number of experiments were carried out to investigate reversible and irreversible endothermic and exothermic transitions found in the DSC heating and reheating scans of high protein systems (Farahnaky *et al.*, 2005). Denatured-WPI was prepared by slow heating (30 min) of WPI dispersions, 10% (w/w) hydrated WPI in DI–water (30 min), from room temperature to 80°C. Holding at 80°C was applied for 30 sec. Aliquots of denatured-WPI dispersion (25 mL) were transferred to plastic petri dishes (92×16 mm, Sarstedt AG & Co., Nümbrecht, Germany), then frozen at -20°C for 3 h, followed by -80°C (Icebird/Mini Freeze 80, Heto, Jouan Nordic A/S, Allerød, Denmark) for 3.5 h, and freeze-dried for 70 h at pressure, $p < 0.1$ mbar (Lyovac GT2, Steris®, Hürth, Germany). The freeze-dried denatured-WPI (anhydrous) was stored over P_2O_5 in vacuum desiccators at room temperature to protect the material from water uptake. Storage was followed by humidification of samples over a saturated solution of NaCl at 0.76 a_w in vacuum desiccators at room temperature for 3 days. Duplicate anhydrous and humidified (0.76 a_w) denatured-WPI samples were scanned in the DSC (Mettler Toledo 821e, Schwerzenbach, Switzerland) from 0 to 110°C at 5°C/min. After heating, these sealed pans containing denatured-WPI samples at 0.76 a_w were further stored in vacuum desiccators (0.76 a_w) at room temperature for 7 days. Thereafter the samples were rescanned in the DSC (0 to 110°C, 5°C/min), re-stored in sealed DSC pans at 0.76 a_w for 29 days, and scanned for the third time in the DSC (0 to 110°C; 5°C/min) to investigate reversible and the time-dependence of endothermic transitions. The thermograms of anhydrous and humidified denatured-WPI at 0.76 a_w were analysed for the onset (T_{onset}) and peak temperatures (T_{peak}) of the endothermic transitions.

Duplicate samples of the systems containing sugar (WPI–oil–sugar) in vials were taken at intervals from storage at 20 and 40°C and transferred to preweighed DSC pans, hermitically sealed, reweighed, and scanned from -120 to ≥100°C at 5°C/min. The glass transition of the G–F syrup, and transitions of protein were analyzed at intervals during storage at 20 and 40°C for 14 weeks.

An empty punctured pan was used as a reference and the DSC was calibrated for temperature and heat flow as reported by Haque and Roos (2004). The

thermograms were analyzed using STARe software, version 8.10 (Mettler Toledo, Schwerzenbach, Switzerland) and average data were reported.

4.2.6. SODIUM DODECYL SULFATE-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

Proteins of the high protein systems were separated on a 15% acrylamide solution according to method of Laemmli (1970). All WPI systems in approx. 10 mg aliquots were weighted and transferred to 2 mL Eppendorf Protein "LoBind" Tubes (Eppendorf AG, Hamburg, Germany). The SDS buffer [50 mL of DI-water, 12.5 mL of 0.5 M Tris-HCl (pH 6.8), 10mL of glycerol, 20 mL of 10% (w/v) SDS in DI–water, and 2.5 mL of 0.05% (w/w) bromophenol blue] with (reducing condition) or without (non-reducing condition) 5 mL of 2-β-mercaptoethanol was added to each sample tube. Dispersions of samples under reducing condition were heated at 99°C for 5 min. The resolving and stacking gels contained 40 mL and 3.9 mL, respectively, of 3% (w/v) acrylamide. The 7 µL of WPI (63.06 µg protein) and WPI–oil (47.25 µg protein), 12 µL of WPI–oil–(G–F) (48.6 µg protein) systems, and 10 and 15 µL of marker suspension (30 µg protein) were injected into the SDS-PAGE gels. The standard molecular weight marker used for protein profile study ranged from 6.5 to 200 kDa (S8445 SigmaMarker^{TM}, Sigma Life Science, Steinheim, Germany). Electrophoresis was pre-run at 200V for 30 min and run after samples injection at 200V for 8 h (the tracking dye front was at the bottom of the gel slab). The reducing and non-reducing gels were stained with 0.25% (w/v) Coomassie brilliant blue R-250 for 15 h at room temperature and destained for few times with 10% (v/v) acetic acid plus 40% (v/v) methanol in DI–water.

4.3. RESULTS AND DISCUSSION

4.3.1. PHYSICAL AND PHYSICOCHEMICAL PROPERTIES

The WPI–oil systems at 20°C up to 14 weeks of storage showed constant L^*, a^*, b^*, and a_w values, as shown in Fig. 1. WPI–OO and WPI–SO stored at 40°C for 14 weeks had constant L^* and a^*, but the a_w values of these systems decreased from 0.35 to 0.18 and 0.34 to 0.15, respectively, during 4 weeks of storage and remained constant thereafter (Fig. 4.1). The WPI–OO showed a constant b^*

value, but the WPI–SO system showed increased *b** value at 40°C (Fig. 4.1). This increased yellowness could result from oxidation of lipids (unsaturated fatty acids) and protein which may also have led to the a_w changes at 40°C. The reactive oxygen groups and free radicals produced by oxidation of fatty acids attack proteins and amino acids in anhydrous and low water systems (Zirlin and Karel, 1969; Chipault and Hawkins, 1971; Kanner and Karel, 1976), and accelerate oxidation of proteins. These reactions cause browning (Tappel, 1955; Zirlin and Karel, 1969; Karel, 1973; Pokorný *et al.*, 1974), insolubility (increased hydrophobicity of proteins) (Roubal and Tappel, 1966a; Funes and Karel, 1981), polymerization or cross-linking (Roubal and Tappel, 1966b; Funes and Karel, 1981; Leake and Karel, 1982), scission (Schaich and Karel, 1975), loss of enzyme activity (Roubal and Tappel, 1966a; Leake and Karel, 1982; Oliver *et al.*, 1987) and loss of biological membrane integrity (leading to loss of cellular functions) *in vivo* (Menzel, 1967; Gardner, 1979; Stadtman, 1992), and formation of lipid–protein complexes (Davies, 1987; Howell *et al.*, 2001). The polymerization or cross-linking and formation of lipid–protein complexes alter water sorption properties, physical structure and physicochemical properties of dried foods (Karel, 1973; Koch, 1962). The higher molecular weight materials exhibit higher water content at low a_w than the lower molecular weight components (Roos, 1993; Potes *et al.*, 2012). Our results showed decreasing a_w values during the first 4 weeks of storage, which possibly resulted from polymerization or formation of lipid–protein complexes at 40°C. The formation of polymeric components may result in a higher sorbed water content, i.e., decreasing the a_w values during storage of the WPI–oil systems at a constant water content. It should be noted that all samples were in vacuumized vials and vacuum was maintained throughout the storage. Most likely changes responsible for lowering of the a_w values of the WPI–oil systems could include changes in protein conformation favoring hydrophobic interactions with oil and subsequent hydrogen bonding of water to opened hydrogen bonding sites. Degradation of lipids (lipolysis) by lipase enzymes or in the presence of radicals and the thermal treatment could also release fatty acids from triglycerides (consumption of water). Rüegg *et al.* (1975) found that thermal stability of β-lactoglobulin over the water content range from 0 to 0.75 g/g was strongly dependent on the degree of hydration. We found dark surface browning of the WPI–SO system during

storage at 40°C in plastic cups for 7 to 14 weeks. The coloured surface also showed a harder texture than the colourless area (Fig. 4.2a). Such browning could result from highly oxidized unsaturated fatty acids and impurities of the whey protein and sunflower oil.

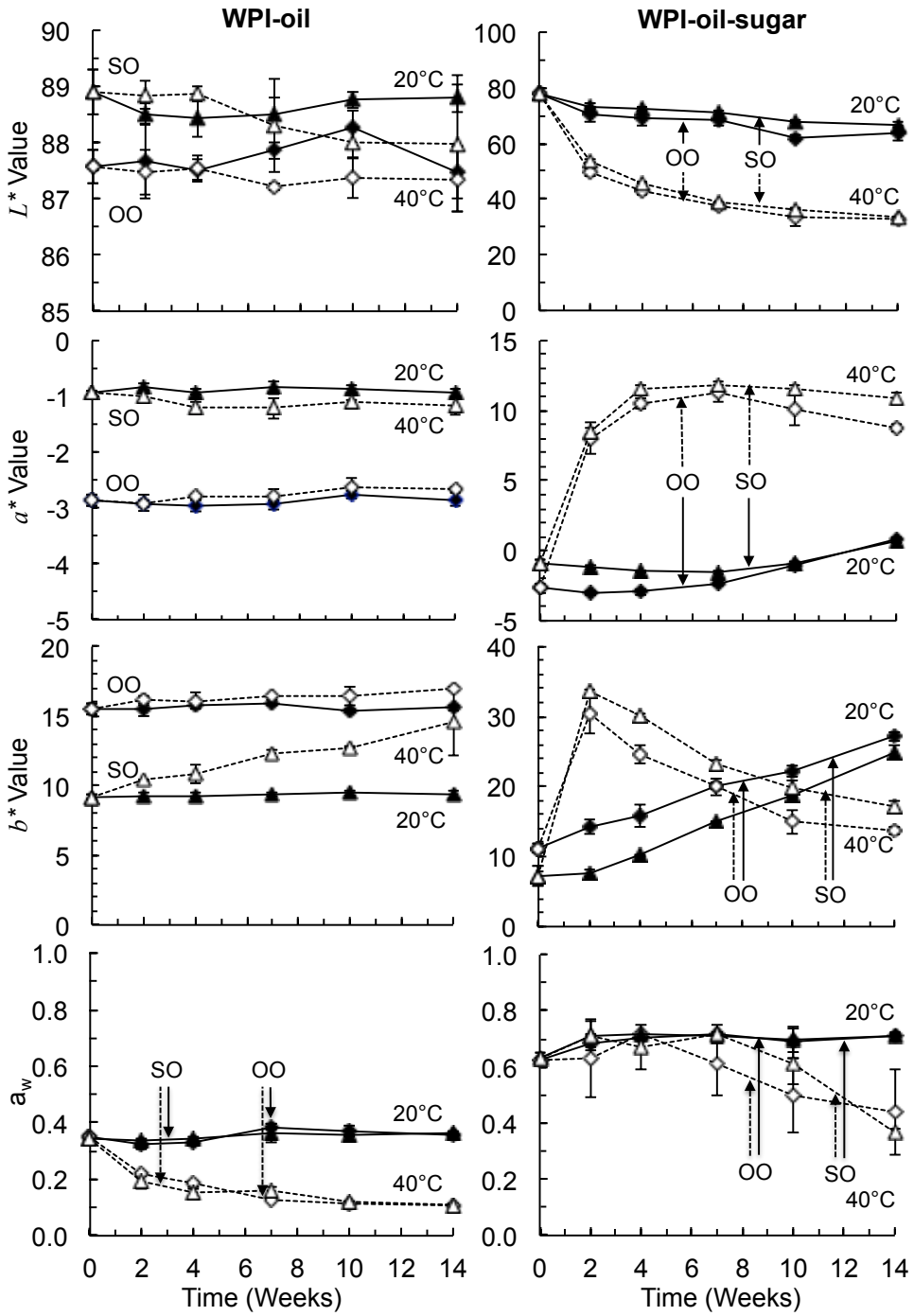

Fig. 4.1 The *L**, *a**, *b**, and water activity (a_w) values of WPI–olive oil (OO) or –sunflower oil (SO) at 75:25 ratio, and WPI–OO– or WPI–SO–(glucose–fructose; G–F) at a component ratio of 45:15:40 during storage at 20 and 40°C for up to 14 weeks.

The WPI–oil–sugar systems for up to 14 weeks of storage at 40°C showed dramatic changes in colour, and progressive hardening (observed from the appearance and texture of the systems) with slightly increased a_w as shown in Fig. 4.1 and Fig. 4.2b. These results agreed with McMahon *et al.* (2009), who reported that high protein bars made of hydrolyzed WPI or WPI with high fructose corn syrup or sorbitol syrup mixes showed darker colour, hardening, and increasing water activity during storage at 32°C for 42 days. The changes in colour of the bars were dependent on the presence of Maillard browning reactants. In the present study, the WPI–SO–sugar system, but not the WPI–OO–sugar system, showed surface flattening and smoothening during storage at 20 and 40°C for 14 weeks. This finding showed that the type of oil could affect the structure or other physical properties that could affect the diffusion of water or reactants and thereby the NEB of protein-oil-sugar systems during storage. Karmas *et al.* (1992), and Buera and Karel (1995) found that structural or physical changes (collapse and shrinkage) of freeze-dried model systems of mixtures of sugar and high molecular weight polymers, as non-crystallizing matrices, with reacting sugar and amino acid increased the rate of NEB reaction and the sensitivity of the reaction to temperature during storage.

The L^* value of the WPI–oil–sugar systems dramatically decreased during storage at 40°C during the first 7 weeks as a result of NEB (Maillard reaction), while the a^* value dramatically increased at 40°C of storage within 4 weeks. After 7 weeks of storage at 40°C, the L^* value leveled-off but the a^* value showed a decrease, especially for the WPI–OO–sugar system (Fig. 4.1). The b^* value increased sharply during storage at 40°C for 2 weeks, yellow colour occurred in all systems containing sugar as shown in Fig. 4.2b, and a dramatic decrease of b^* value occurred after 2 weeks of storage (Fig. 4.1). The WPI–SO–sugar showed higher b^* value than WPI–OO–sugar at 40°C, which was in accordance with the data for the WPI–SO system. These results showed that the NEB reactions of the WPI–oil and WPI–oil–sugar systems at 40°C were most likely affected by the fatty acid composition, and lipid and protein oxidation of the oil.

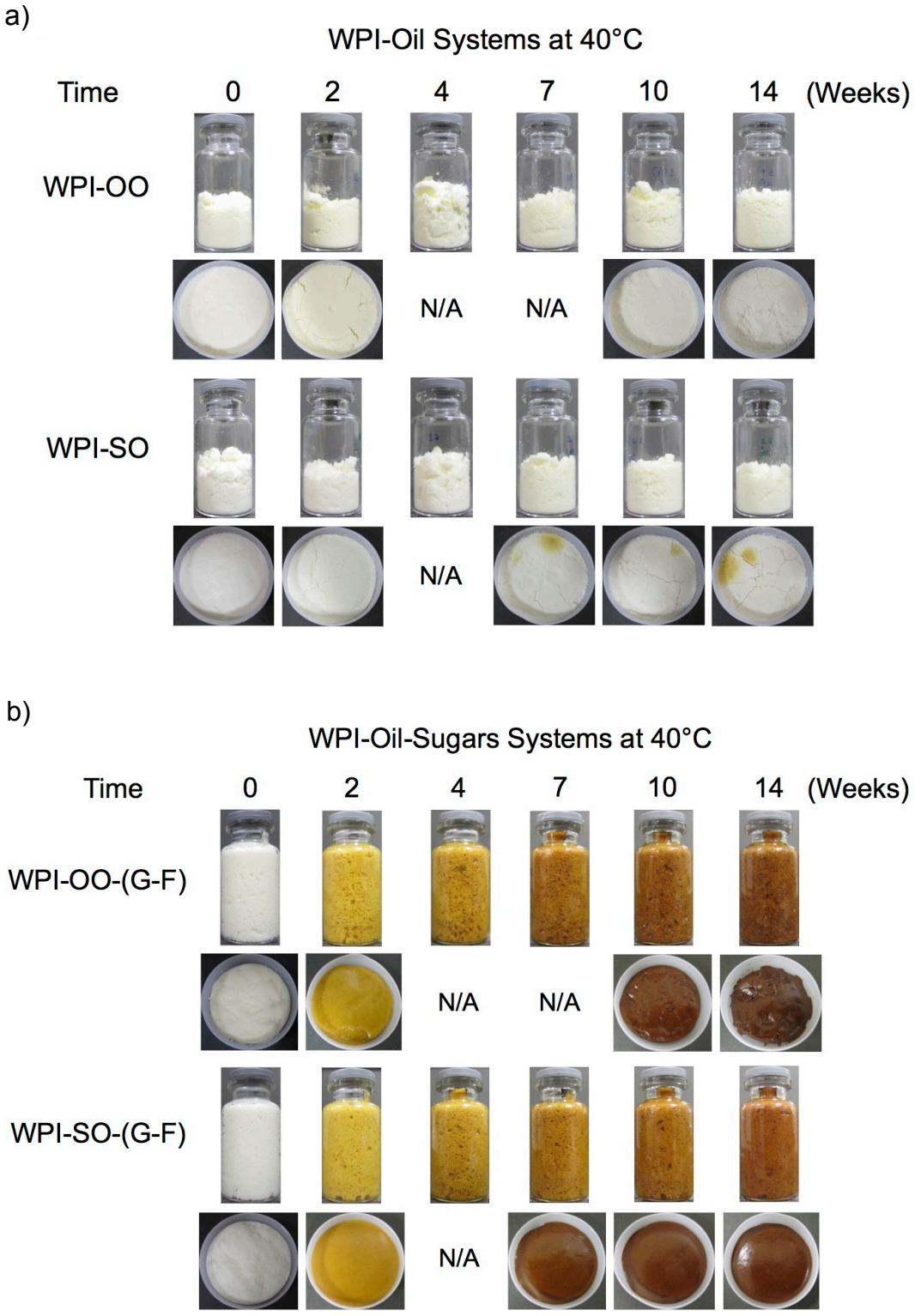

Fig. 4.2 Appearance of a) WPI–olive oil (OO) or –sunflower oil (SO) at 75:25 ratio, and b) WPI–OO– or WPI–SO–(glucose–fructose; G–F) systems at a component ratio of 45:15:40 at 40°C of storage for up to 14 weeks.

a_w values of WPI–OO–sugar and WPI–SO–sugar systems decreased after 7 weeks of storage at 40°C. Water is a product of several condensation steps of the NEB between amino acids or proteins and reducing sugars as increased covalent bonding produces water causing an increase of a_w (Hodge, 1953; Eichner and Karel, 1972). In the present study, the NEB could contribute to a_w values and hardening of WPI–oil–sugar systems during the first 7 weeks, in agreement with Gonzales *et al.* (2010). Carbonyl–amine polymerization, formation of stable coloured compounds, and cross-linking of proteins could result in the lowering of the a_w during prolonged storage.

During storage at 20°C for 14 weeks the systems containing WPI–oil–sugar, showed decreased L^* and increased a^* values, but these changes were significantly less than those that occurred in the systems at 40°C. The b^* value showed a dramatic increase during storage, but that increase was more rapid in the WPI–SO–sugar than WPI–OO–sugar systems as was also found for the WPI–SO at 40°C. Mastrocola and Munari (2000) reported that the Maillard reaction had a significant rate after pre-heating (at 100°C for 90 min) of mixtures of pregelatinized corn starch, water, glucose, lysine, and soybean oil at a component ratio of 32:30:16:4:18 at 25°C for 180 days, and also the rate of the Maillard reaction was increased by the presence of the oil and its oxidation products in the system. a_w values of WPI–oil–sugar systems increased over storage time for 14 weeks at 20°C, but were stable thereafter. The WPI–oil–sugar systems developed only a dark yellowish colour during storage at 20°C, which meant a slow formation of pigments and polymerization.

X-ray diffraction of the WPI–OO–(G–F) system, as a representative of WPI–oil–sugar systems, showed no crystallization of glucose or fructose during up to 19 weeks (Fig. 4.3). Sugar crystallization was neither likely in the WPI–SO–(G–F) system during storage up to 19 weeks. Therefore, changes of physical and physicochemical properties and a_w of WPI–oil–sugar during storage at both temperatures could not relate to crystallization of sugars and their hydrate forms.

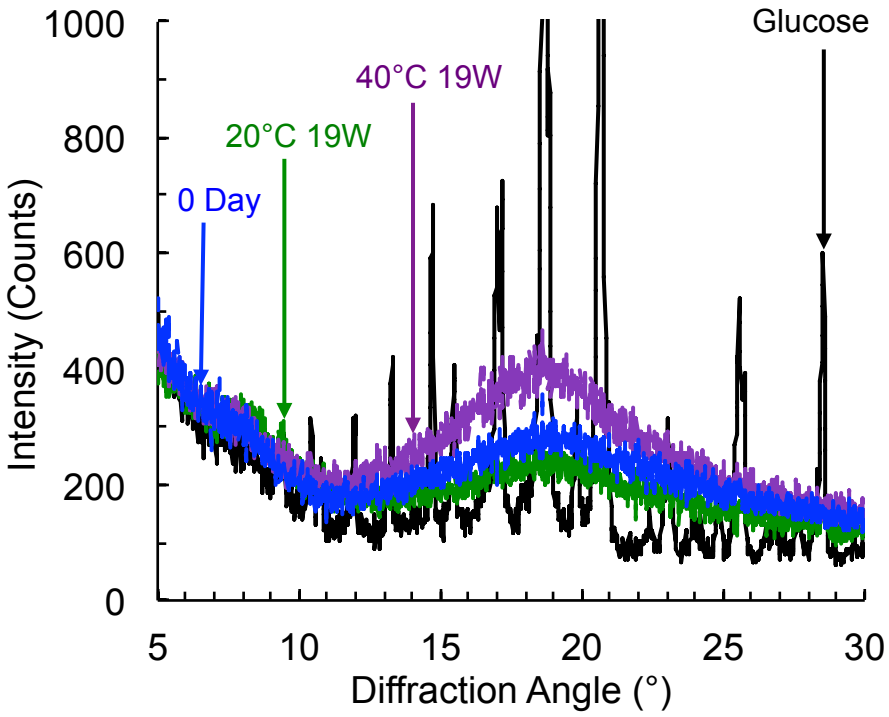

Fig. 4.3 X-ray diffraction patterns for WPI-olive oil (OO)-(glucose-fructose; G-F) system before (0 day, —) and after storage at 20 (—) and 40°C (—) for 19 weeks (W) compared with reference peaks of x-ray diffraction pattern of an anhydrous glucose (—). The height of the line gives intensity of a peak as percentage of the intensity of the highest peak, which is taken as 100%.

4.3.2. THERMAL PROPERTIES AND PROTEIN TRANSITIONS

The DSC thermograms of the first and second heating scans of WPI at 0.31, 0.76, and 0.85 a_w, and WPI–OO (0.35 a_w), WPI–OO–(G–F) (0.62 a_w), and WPI– (G–F) (0.66 a_w) are shown in Fig. 4.4. The WPI–OO and WPI–OO–(G–F) systems were chosen as models for WPI–oil and WPI–oil–(G–F) systems, respectively, as shown in Fig. 4.4. The WPI at 0.31, 0.76 and 0.85 a_w, and WPI–OO systems showed endotherms with T_{peak} above 47°C in the first heating, but these endotherms were absent in an immediate reheating scan (Fig. 4.4). This result agreed with Farahnaky *et al.* (2005), who found endotherms (35 to 60°C and 90 to 180°C) in the first heating scan of bovine serum albumin. They found that the endotherm reappeared in heating (130°C) after storage of samples at 25, 40, and 60°C for up to 7 days, but the endotherm was absent from an immediate rerun of samples. Our results showed no glass transitions in WPI at 0.31, 0.76, and 0.85 a_w, and WPI–OO systems in the first or subsequent reheating scans (Fig. 4.4). These systems showed no colour change in a dynamic reheating at 5°C/min up to

110°C. It should be noted that the WPI at 0.31 a_w, however, had an increase in yellow colour in heating to ≥130°C.

WPI at 0.76 and 0.85 a_w when heated up to 110°C showed two endotherms in the first heating scan. The lower temperature endotherm had a_w independent onset (36 and 35°C) and peak temperatures (50 and 48°C). The higher temperature endotherm was a_w-dependent (0.76 to 0.85 a_w) and occurred at a lower temperature with increasing a_w (WPI at 0.76 and 0.85 a_w, T_{onset} = 75 and 65°C, respectively, and T_{peak} = 104 and 91°C, respectively) as shown in Fig. 4.4. Corresponding a_w dependence of the higher temperature endotherm was also reported by Zhou and Labuza (2007). Farahnaky *et al.* (2005), and Zhou and Labuza (2007) assumed that the higher temperature endotherm of WPI at intermediate and high a_w systems resulted from protein denaturation. Further experiments using anhydrous and humidified (at 0.76 a_w) denatured-WPI were carried out to investigate the endothermic transitions, and to compare the thermograms with those of WPI at different a_w. Anhydrous denatured-WPI did not show any endothermic transition, but the humidified denatured-WPI at 0.76 a_w for 3 days showed only one endothermic transition at the low temperature (T_{onset} and T_{peak} at 35 and 46°C, respectively), which was not significantly different from WPI at 0.76 and 0.85 a_w. This low temperature endotherm of the denatured-WPI at 0.76 a_w reappeared after heating (up to 110°C) in the DSC and storage at room temperature for 7 (T_{onset} = 33°C, T_{peak} = 44°C) and 29 days (T_{onset} = 32°C, T_{peak} = 44°C). Our result demonstrated that the low temperature endotherm was a reversible transition in agreement with Farahnaky *et al.* (2005). Also this endothermic transition at low temperature appeared only in the systems that had sufficient water content in the protein matrix. Our results confirmed that the endothermic transition at the higher temperature was protein denaturation as previously reported by Farahnaky *et al.* (2005), and Zhou and Labuza (2007). The denaturation endotherms were absent in the DSC scan of the anhydrous and humidified denatured-WPI. The denaturation endotherm, as found for WPI at 0.76 and 0.85 a_w belonged to α-lactalbumin (on shoulder of the peak, dashed dark-blue arrow) and β-lactoglobulin (at peak of the endotherm, dark-blue arrow) (Fig. 4.4). A somewhat similar endothermic transition was found in the 3% WPI

(w/w) dispersion in distilled water at a heating rate of 1°C/min in DSC by Fitzsimons *et al.* (2007).

The WPI at 0.31 a_w and WPI–OO showed the lower temperature endotherm at T_{onset} of 36 and 38°C and T_{peak} of 60 and 57°C, respectively. A second endothermic transition appeared above 110°C. The WPI at 0.31 a_w after heating in the DSC (110°C) and storage at 4°C and room temperature for 5 days showed the low temperature endotherm. This low temperature endotherm was referred to as enthalpy relaxation of protein by several authors (Farahnaky *et al.*, 2005; Zhou and Labuza, 2007; Haque *et al.*, 2012), but it was neither a_w-dependent nor associated with a glass transition. The endothermic low temperature transition of WPI at 0.31, 0.76 and, 0.85 a_w showed no significant changes for the T_{onset} with increasing a_w and water content (Fig. 4.4). Therefore we propose that the low temperature endothermic transition of the first heating scan of WPI at 0.31, 0.76, and 0.85 a_w, WPI–OO, and freeze-dried denatured-WPI at 0.76 a_w at T_{onset} in range of 33 to 38°C (broadened area with low a_w) showing time-dependent recovery occurs as a result of changes in protein hydration and concomitant changes in protein conformation. The lower temperature endothermic transitions of all WPI systems in the first DSC heating scan could result from breaking of hydrogen bonds between proteins and water molecules, which required heat input to the systems. This explanation agreed with the lowering of the a_w values of the WPI-oil systems during storage at 40°C which has not been reported in earlier studies.

The systems containing sugars [WPI–OO–(G–F) (0.62 a_w) and WPI–(G–F) (0.66 a_w)] showed the glass transition of sugars (G–F) and two exothermic transitions (T_{peak} = 65 and 100°C, 64 and 100°C, respectively) during the first heating scan in the DSC (Fig. 4.4). The behavior of glass transition of the sugar component of high protein systems in the first and second heating scans differed. The glass transition was broadened in the second heating scan, which showed carbohydrate-protein interactions, especially in the WPI–(G–F) system. The systems were prepared by mixing aqueous sugars with strong hydrogen bonding of water to sugar molecules while the protein had low water content. These exotherms in the WPI–oil–sugar systems were irreversible, as they did not occur

in the reheating scans of the samples after storage at room temperature for 5 days. There are no earlier reports of the lower temperature exotherm in the first heating which presumably indicated increased hydrogen bonding of the protein to the G–F components, i.e., an increase in temperature induced rapid hydrogen bonding of water and sugar molecules to the dehydrated protein molecules as an exothermic process. The sugar component was also hydrogen bonded to water and the assumption of protein hydration as an exothermic process was supported by a resultant broadening of the glass transition. It was important to note that WPI–oil–(G–F) and WPI–(G–F) systems showed no colour change at 80°C (above first exotherm), but at temperatures ≥100°C the materials showed brownish-yellow colour (about second endotherm). Thus the higher temperature exotherm in the first heating scan of the systems containing sugars resulted from the NEB reaction between proteins and sugars. Raemy *et al.* (1983) and Roos *et al.* (1996) reported that an exothermic transition at T_{peak} of 100 to 160°C observed in DSC during heating scans for casein–lactose and skim milk–lactose systems indicated NEB. The interaction of proteins or amino acids and reducing sugars produced water that generated heat, became plasticized, and showed a reduced T_g (early stage of Maillard reaction), and increased T_g (advanced stage of Maillard reaction) of the amorphous material (Raemy *et al.*, 1983; Roos *et al.*, 1996). The presence of oil crystallization exotherms (OO or SO) in WPI–oil and WPI–oil–sugar systems in the cooling scan at -10°C/min after the first heating scan and subsequent melting of the oil crystals in reheating confirmed that the oil had limited interactions with the carbohydrate–protein components and it crystallized to a separate phase within the protein–sugar network.

The DSC thermograms of WPI–oil–(G–F) before and after storage at 20 and 40°C for 14 weeks compared to WPI–oil and WPI–(G–F) are shown in Fig. 4.5. The WPI–OO– and WPI–SO–(G–F) before storage showed a lower temperature exotherm corresponding to the exotherm of the WPI–(G–F) (Fig. 4.5). Endotherms preceding exotherms were found in WPI–oil–(G–F) systems after 2 weeks of storage for up to 14 weeks at 20°C of storage. The WPI–oil–(G–F) showed a single endotherm during storage at 40°C from 2 to 7 weeks. But at longer storage times neither endothermic nor exothermic transitions were present as shown in Fig. 4.5. The absence of thermal transitions was accounted for

complete changes in protein conformation. Changes in protein conformation could increase hydrophobicity of the proteins. The WPI–(G–F) showed the lowest T_g, increased onset T_g of sugar was found for the WPI–oil–(G–F) especially with SO. It is known that at normal pH of milk β-lactoglobulin is present as dimers with hydrophobic linkages, and it has the capability to bind with nonpolar molecules including fatty acids (Madadlou and Azarikia, 2013). It may be assumed that in a WPI–(G–F) system the dimer structure and internal protein-protein hydrophobic interactions limited WPI–(G–F) interactions. That was detected from the T_g occurring around that measured for the G–F syrup alone. The WPI–SO–(G–F) included oil droplets on which protein could assembly hydrophobically towards the oil. In these systems, the water content was sufficient to allow for conformational changes of the proteins and exposure of hydrogen bonding sites towards the carbohydrate phase. As a result, the T_g occurred at a higher temperature confirming stronger hydrophilic protein–sugar interactions.

The onset T_g values of the sugars in WPI–oil–(G–F) increased during storage at 20°C, especially in the system containing OO (Table 4.1), which could result from polymerization of proteins and reducing sugars (Maillard reaction), or formation of advanced glycation end products. The WPI–OO–(G–F) showed significantly broadened glass transition of sugars during 4 to 14 weeks of storage at 40°C, but no changes of glass transition occurred in WPI–SO–(G–F) systems. This meant protein-sugar in WPI–OO–(G–F) was slowly hydrated during storage at 40°C as shown by the broadened glass transition and lower T_g (Table 4.1). Our results demonstrated that WPI–oil–sugar systems showed component interactions during storage, and oil induced changes to the glass transition of sugars in high protein systems, especially for the systems containing OO. The systems containing OO showed more pronounced changes of thermal properties of the high protein systems than SO. However, WPI–OO– or WPI–SO–(G–F) before and after storage at 20 and 40°C for up to 14 weeks showed no changes of T_{onset} (at -15 or -30°C, respectively) and T_{peak} (at -3 or -24°C, respectively) of oil melting. Therefore, the type of oil affected interactions of protein molecules and glass transition of sugars in low a_w systems.

Table 4.1 The onset, midpoint, and endpoint temperatures of glass transition in the first heating scan of DSC for glucose-fructose (G-F) in WPI-olive oil (OO)- or WPI-sunflower oil (SO)-(G-F) systems during storage at 20 and 40°C for up to 14 weeks[a].

Materials	Time (Weeks)	Temperature (°C)					
		20			40		
		Onset	Midpoint	Endpoint	Onset	Midpoint	Endpoint
WPI-(G-F)	0	-57±1	-52±1	-48±1	-57±1	-52±1	-48±1
WPI-OO-(G-F)	0	-53±1	-45±0	-39±1	-53±1	-45±0	-39±1
	2	-50±1	-42±0	-35±0	-50±1	-43±1	-37±2
	4	-50±0	-42±0	-36±1	-51±0	-43±1	-35±1
	7	-50±0	-42±1	-34±1	-51±3	-43±3	-34±1
	10	-48±1	-40±2	-34±1	-55±1	-44±1	-34±0
	14	-48±0	-41±2	-34±2	-54±1	-44±1	-37±1
WPI-SO-(G-F)	0	-47±1	-45±1	-38±2	-47±1	-45±1	-38±2
	2	-47±0	-44±0	-35±0	-43±0	-39±0	-34±1
	4	-47±0	N/A	-35±1	-43±0	N/A	-35±1
	7	-45±0	-41±2	-35±1	-42±1	-39±0	-34±0
	10	-44±1	-40±0	-34±0	-40±0	N/A	-34±0
	14	-45±0	N/A	-34±1	-42±0	N/A	N/A

[a] N/A = hardly define or specific temperature.

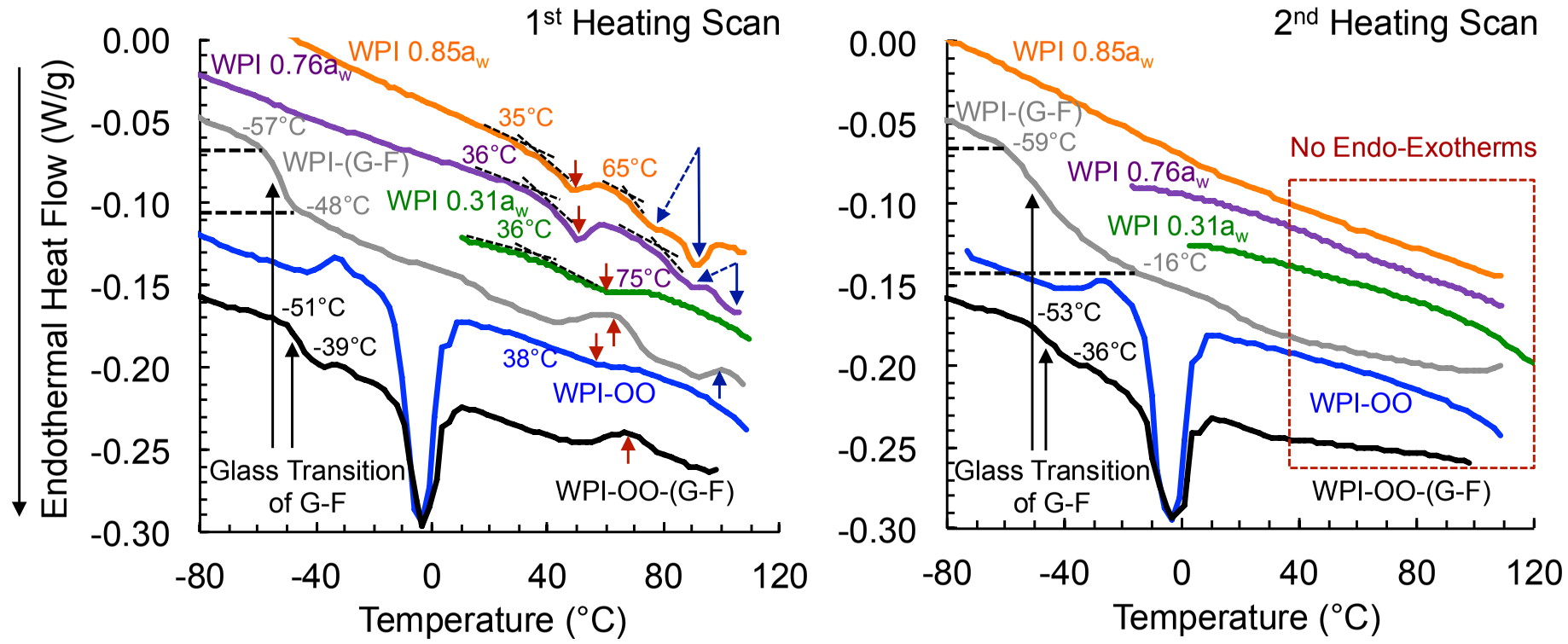

Fig. 4.4 Differential scanning calorimetry thermograms of the first and second heatig scans of WPI at a_w of 0.31, 0.76, and 0.86, WPI–olive oil (OO) at 75:25 ratio, WPI–OO–(glucose–fructose; G–F) at a component ratio of 45:15:40 (0.62 a_w), and WPI–(G–F) at 45:40 ratio (0.66 a_w) before storage. The onset temperatures of endotherms for the systems with no sugar are shown in first heating scan. The onset and endpoint temperatures of glass transition of G–F of the systems containing sugar are shown in both heating scans. The red and dark blue arrows indicated first and second, respectively, endotherm or exotherm in first heating scan of DSC.

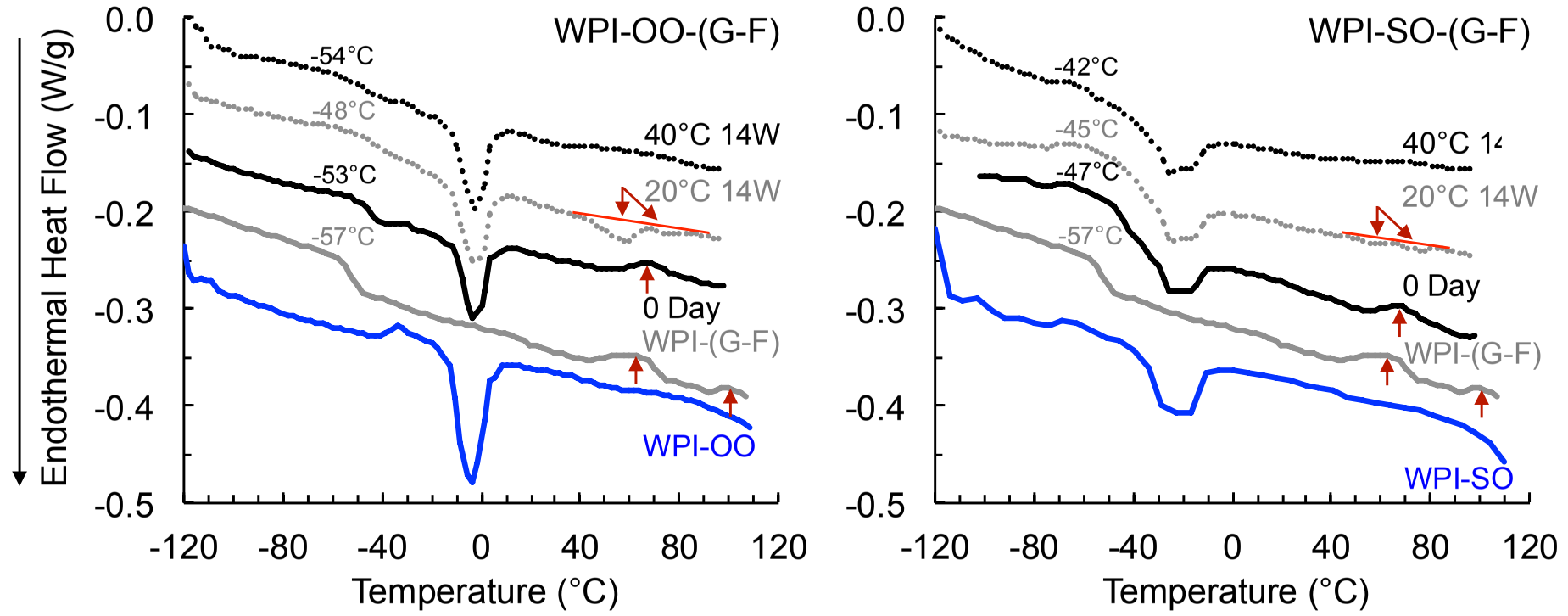

Fig. 4.5 Differential scanning calorimetry thermograms of the first heating scan of WPI–olive oil (OO)– or WPI–sunflower oil (SO)–(glucose–fructose; G–F) systems before (0 day; ——) and after storage at 20 (·····) and 40°C (·····) for 14 weeks (W) compared to WPI–OO or –SO (——) and WPI– (G–F). The red arrows indicated endotherms and exotherms in the first heating scan of DSC.

4.3.3. SDS-PAGE

The protein profiles of WPI, WPI–oil and WPI–oil–sugar systems before storage (non-storage), and WPI–oil and WPI–oil–sugar systems after storage at 20 and 40°C for 19 weeks under reducing and non-reducing conditions compared to wide range molecular weights of proteins standard marker are shown in Fig. 4.6. The protein profiles for reducing and non-reducing gels of WPI, WPI–oil, and WPI–oil–sugar systems before storage were similar, showing intrinsic proteins in WP, such as, α-lactalbumin (molecular weight 14.2 kDa), β-lactoglobulin (18.4 kDa), bovine serum albumin or BSA (66.2 kDa), and immunoglobulin G (160 kDa) (Shimada and Cheftel, 1989; Xiong and Kinsella, 1990; Monahan *et al.*, 1993; Monahan *et al.*, 1995). The WPI–oil after storage at 40°C showed formation of higher molecular weight components in the range of 29 to 36 kDa by the non-reducing gel, especially for WPI–OO (lane 5). The WPI–OO–(G–F) (lane 10) and WPI–SO–(G–F) (lane 13) after storage at 20°C showed lower solubility (decreased mobility) of α-lactalbumin, β-lactoglobulin, and other proteins in the range of molecular weight of 29 to 36 kDa. Bands of principal intrinsic proteins of WPI became more diffuse compared to those of the systems before storage, and there was indication of formation of high molecular weight protein polymers in the range of 36 to 45 kDa in both reducing and non-reducing gels (Fig. 4.6). These results indicated protein modification, polymerization, component interactions via glycation (nonenzymatic glycosylation) or Maillard reaction leading to aggregation and covalent cross-linking (nondisulfide covalent bonds) of proteins, and insolubilization during storage of the WPI–oil–sugar systems as was described for physical and physicochemical properties. Our results were in accordance with Rich and Foegeding (2000), who demonstrated that the Maillard gel of 14% (w/v) WPI with 0.5 M ribose at pH 9 showed lower solubility, and diffused bands of protein in SDS-PAGE under reducing condition. Mohammed *et al.* (2000) reported that solubility (in water and mixed solvents of 1% SDS with 1% β-mercaptoethanol) of whey isolate powder decreased continuously with increasing heating temperature (105 to 145°C, 16 h) and water content [0, 5 (12% RH), and 30 g/100 g dry solids (95% RH)], and β-lactoglobulin showed a very low solubility after heat treatment as a result of increased covalent crosslinks. Their results showed that isoelectric point of the whey isolate was lowered from pH 4.8 to 3.5 (positive charge) after heat

treatment, and suggested that the important reactions were the loss of amide groups and the Maillard reaction. The WPI–oil–sugar systems at 40°C of storage for 19 weeks were not soluble in SDS buffers with and without 2-β-mercaptoethanol, so lanes 11 and 14 for both gels showed no protein profile. Insolubilisation of WPI–oil–sugars after storage at 40°C could result from complex reactions between oxidized protein–lipid compounds (Roubal and Tappel, 1966a; Funes and Karel, 1981) and products of Maillard reaction (at the advanced stage of reaction) or dominance of advanced glycation end products (Kato *et al.*, 1986; Liang and Rossi, 1990). It should be noted that no samples from all systems retained the top of both gels. From our results we confirmed that protein, sugar, and oil had strong intermolecular hydrophobic interactions, which resulted in hydrophobicity of the WPI–oil–sugar systems that increased with storage time.

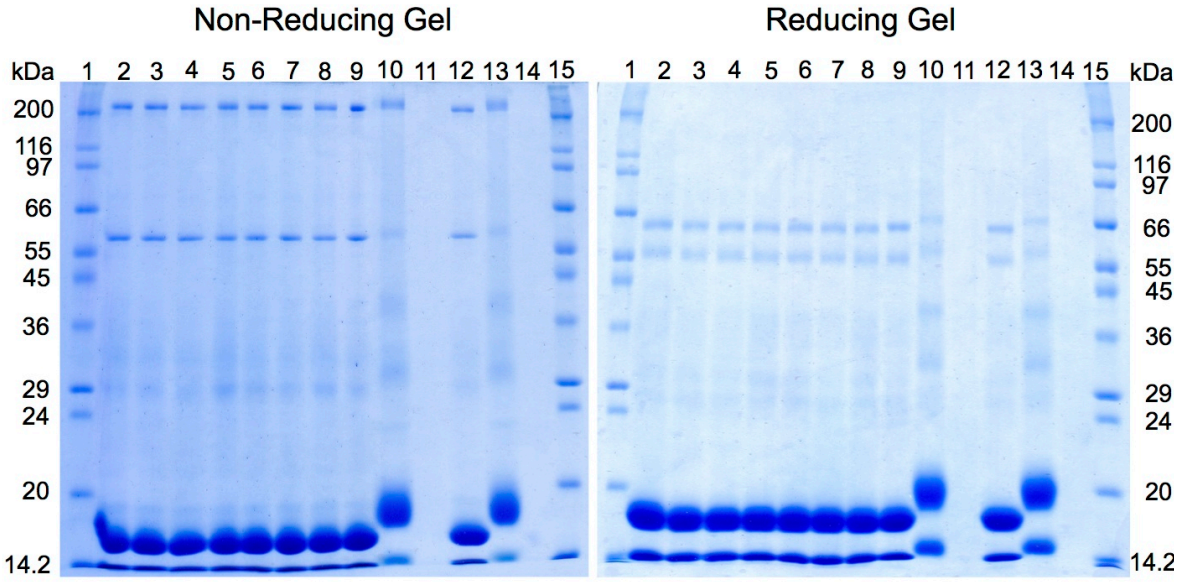

Fig. 4.6 SDS-PAGE gels for non-reducing and reducing gels (without and with 2-β-mercaptoethanol, respectively) contained protein standard marker (lanes 1 and 15), WPI (lanes 2), WPI–olive oil (OO; lanes 3 = non-storage, 4 = 20°C for 19 weeks, 5 = 40°C for 19 weeks), WPI–sunflower oil (SO; lanes 6 = non-storage, 7 = 20°C for 19 weeks, 8 = 40°C for 19 weeks), WPI–OO–(glucose–fructose; G–F) (lanes 9 = non-storage, 10 = 20°C for 19 weeks, 11 = 40°C for 19 weeks), WPI–SO–(G–F) (lanes 12 = non-storage, 13 = 20°C for 19 weeks, 14 = 40°C for 19 weeks).

4.4. CONCLUSIONS

The protein–oil and protein–oil–sugar systems at low a_w showed interactions of components during storage at 20 and 40°C for up to 14 weeks affecting physical, physicochemical, and thermal properties of the high protein systems. The oxidation of unsaturated fatty acids increased yellowish colour in WPI–oil systems, especially for WPI–SO, and caused changes of a_w during storage at 40°C. The lowered a_w values during storage at 40°C resulted from polymerization of lipid-protein complexes (hydrophobic interaction), which showed an increase of water sorbed by the polymers. The systems containing sugar showed browning and hardening with time that resulted from carbonyl-amine polymerization via glycation (nonenzymatic glycosylation) or Maillard reaction (NEB) leading to aggregation and covalent cross-linking of proteins, and increased hydrophobicity (less solubility) of the α-lactalbumin and β-lactoglobulin proteins. The strong intermolecular hydrophobic interactions of components or insolubilization of the systems containing sugars occurred during storage at 40°C due to complex reactions between oxidized protein-lipid compounds and the advanced glycation end products. The type of oil or the fatty acid composition played an important role on the surface and the structure, which affected the diffusion of water or reactants and NEB in protein–oil–sugar systems during storage. The rehumidified-WPI and WPI–oil systems showed endotherms for protein hydration (reversible and time-dependent recovery transition) followed by denaturation of α-lactalbumin and β-lactoglobulin proteins (a_w-dependent). The systems containing sugar showed two irreversible exotherms (protein hydration and browning reaction) before storage, but these exotherms had changed to endotherms and were not present after storage at 20 and 40°C, respectively, for 14 weeks. This indicated conformational changes (denaturation and aggregation) of proteins during storage. The presence of oil in WPI–oil–(G–F) systems increased T_g of sugar, especially for SO. The broadened glass transition of the sugar components occurred during storage indicating slow hydration of the WPI–oil–sugar systems. The results of the present study showed for the first time that high protein foods and pharmaceuticals, including high protein bars, infant formulae, dairy powders, protein drugs, and medical foods, exhibit physicochemical changes related to protein hydration, protein and lipid oxidation, and nonenzymatic browning and water migration as well as

hydrophobic interactions that explain changes in rehydration properties, hardening of texture, and loss of quality of such materials during storage.

CHAPTER V

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PROTEIN HYDRATION, HYDROPHILIC AND HYDROPHOBIC INTERACTIONS, AND THE GLASS TRANSITION OF CONFINED WATER IN HIGH PROTEIN SYSTEMS

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ABSTRACT

Hydration behavior of whey protein isolate (WPI), components (protein, oil, and sugar) interactions, and dynamic mechanical and physicochemical properties of WPI–oil [olive oil (OO) or sunflower oil (SO)] (75:25) and WPI–oil (OO or SO) –sugar [glucose-fructose (G–F), 70% (w/w) solids] (45:15:40) systems at low water activity (a_w) during storage at 20 and 40°C were investigated. An endothermic change in protein conformation occurred at 35 to 45°C. The WPI–oil–sugar showed an exothermic process as sugar molecules replaced hydration water. The dynamic mechanical properties of rehumidified WPI, WPI–oil, and WPI–oil–sugar systems showed glass transition of the hydration water (unfrozen, confined water) at temperatures over the range from -105 to -75°C. The conformational transformations of protein (a_w- and water content-independent) were also found from storage (E') and loss moduli (E'') of dynamic mechanical analysis. After storage DMA data confirmed polymerization resulting from protein, oil, and sugar interactions (glycation or Maillard reaction). The systems containing sugar after prolonged storage at 40°C showed no phase separation, and an aggregated and polymerized solid with stability over a wide range of temperature (-140 to 45°C) was formed.

KEYWORDS: α-relaxation, antiplasticizer, glass transition, glycation, protein hydration, protein–oil oxidation

5.1. INTRODUCTION

Hydration properties of globular proteins have received a wide interest in biochemistry, food, pharmaceutical, and medical sciences. The hydration of a protein surface was assumed to affect protein conformation or structural stability, unfolding, intramolecular interactions, and the thermal denaturation of proteins (Ooi and Oobatake, 1988; Oobatake and Ooi, 1993; Khechinashvili *et al.*, 1995). Electrostatic properties of the protein surface (Nadig *et al.*, 1998) and functionality of proteins (Rupley and Careri, 1991; Chen *et al.*, 2006; Bhattacharjee and Biswas, 2011) have also depended on protein hydration (the presence of a hydration shell) or the number of molecular layers of water around the protein molecules (Rupley and Careri, 1991; Bellissent-Funel, 2000). Hydration properties of individual globular proteins may depend on their type and amino acid composition and the nature of internal groups (aliphatic,

aromatic, and polar groups). These compositional differences relate to the thermodynamic functions, such as heat of non-polar group hydration, enthalpy of intramolecular interactions within protein, entropy of conformational changes of protein, and Gibbs free energy of protein unfolding (Ooi and Oobatake, 1988; Makhatadze and Privalov, 1994; Khechinashvili *et al.*, 1995). The structure of globular proteins is stabilized by the thermodynamic interactions of the aliphatic, aromatic, and polar groups (internal groups) of amino acids while the hydration effects on the aromatic and polar groups that are exposed upon unfolding or destabilization define the state of a protein (Makhatadze and Privalov, 1994). Chemical reactions (Roubal and Tappel, 1966a; Funes and Karel, 1981; Potes *et al.*, 2013a; b), dehydration (Prestrelski *et al.*, 1993; DePaz *et al.*, 2002), hydration of polar and non-polar groups (Privalov and Makhatadze, 1992), pH (Monahan *et al.*, 1995), pressure (Kharakoz, 1997; Hummer *et al.*, 1998), temperature (Ooi and Oobatake, 1988; Privalov and Makhatadze, 1990; Khechinashvili *et al.*, 1995), storage in water and aqueous solutions (DePaz *et al.*, 2002) could cause unfolding (exposure of hydrophobic amino acids) and destabilization of native globular proteins. Water molecules around the hydration sites of protein molecules were often classified into three categories: (*i*) the bound internal water (water molecules that are buried and exists in channels inside the cavities of interiors in protein structures or confined water); (*ii*) the surface water or hydration water (water molecules which interact with molecular units of the protein surface); and (*iii*) the bulk water (water which is not in the direct contact with the protein surface but continuously exchanges with the water on the protein surface) (Mattos, 2002; Chen *et al.*, 2006). In hydrophobic or water-immiscible solvents, any water around protein molecules may remain at the protein surface due to the hydrophilic nature of the protein (Ru *et al.*, 1999). Proteins in hydrophobic conditions were capable to retain their native structure due to strong internal hydrogen bonding and a more rigid structure in the absence of excess water (Mattos and Ringe, 2001). Sirotkin *et al.* (2012) reported that changes of protein hydration could be attributed mainly to excess water, which was associated with the protein transition from glassy to a flexible state. That transition occurred when water molecules covered charged groups of proteins.

Relaxation rates of water associated with protein molecules as a function of hydration or hydrodynamic properties have been studied using nuclear magnetic resonance (NMR) spectroscopy (Grossfield *et al.*, 2008); dielectric spectroscopy (Shinyashiki *et al.*, 2009; Jansson and Swenson, 2010); light, X-ray, and neutron scattering (Doster *et al.*, 1989; Giordano *et al.*, 1995; Dellerue and Bellissent-Funel, 2000; Combet *et al.*, 2011; Magazù *et al.*, 2011; Paciaroni *et al.*, 2013); infrared and neutron spectroscopy (Chen *et al.*, 2006); Raman spectroscopy (Blanch *et al.*, 1999); fluorescence spectroscopy (Ladokhin, 2000); molecular dynamics simulations (Rasmussen *et al.*, 1992; Nadig *et al.* 1998; Tarek and Tobias, 2000; Bhattacharjee and Biswas, 2011; Paciaroni *et al.*, 2013); X-ray and neutron diffractions (Rasmussen *et al.*, 1992; Jiang and Brünger, 1994; Tarek and Tobias, 2000); and X-ray and neutron crystallography (Chou and Morr, 1979; Squire and Himmel, 1979; Mattos, 2002). The information from these measurements explained the behavior of water around the hydration sites of protein or interactions between water and protein molecules, which could be used to describe the effects of hydration on equilibrium protein structures.

Most previous studies of protein hydration used dilute systems or aqueous solutions (wet system) (Klotz, 1958; Berendsen *et al.*, 1981; Ooi and Oobatake, 1988; Otting *et al.*, 1991; Rupley and Careri, 1991; Denisov and Halle, 1996; Valdez *et al.*, 2001; Timasheff, 2002a; Halle, 2004; Russo *et al.*, 2004; Sirotkin *et al.*, 2012) and solvation (Jiang and Brünger, 1994; Timasheff, 2002b), and data of protein hydration at low a_w or low water contents are limited. The objectives of the present study were to investigate protein hydration using whey protein isolate (WPI, natural blend of native bovine lactalbumin, lactoglobulin, and lactoferrin) and properties of the hydration water in the presence of nonprotein components, glucose–fructose, and oil. Moreover, the effects of chemical changes (protein and lipid oxidation and Maillard reaction or glycation) on the physicochemical stability of the high-protein solid systems and protein conformation during storage at different temperatures were monitored. Our results advance understanding of effects of protein hydration on the texture and shelf life of numerous food and pharmaceutical formulations, such as dairy powders, pharmaceutical preparations, infant formula, high protein bars, and confectionery. We used dynamic water activity measurements, dynamic

mechanical analysis, and Fourier transform infrared spectroscopy (FTIR) to investigate hydration of whey proteins at various water activities (a_w), the effects of hydrophilic (sugar) and hydrophobic (oil) surroundings on the hydration behavior and structural transformations of proteins during storage at different temperatures. Furthermore, composite texture measurements and cryo-scanning electron microscopy were used to gain better understanding of the effects of component interactions on the properties and structure of high solid systems at low a_w during storage.

5.2. MATERIALS AND METHODS

5.2.1. MATERIALS

Whey protein isolate (WPI; Isolac®) was purchased from Carbery Food Ingredients (Ballineen, Cork, Ireland). Olive oil (OO; Don Carlos®, Hacienda Don Carlos, Sevilla, Spain) and sunflower oil (SO; Mediterani, Pan Euro Foods, Dublin, Ireland) were purchased from local suppliers. D-(+)-glucose (G; ≥ 99.5% GC), D-(–)-fructose (F; ≥ 99%), and 2-propanol (isopropanol; ≥ 99.8% GC) were purchased from Sigma-Aldrich, Co. (St. Louis, MO, U.S.A.). Deionoized (DI) water was purchased from KB Scientific Ltd. (Cork, Ireland).

5.2.2. PREPARATION OF HIGH PROTEIN SYSTEMS

The high protein materials in this study were (i) WPI–oil system, WPI (water content was 5g/100 g of dry solid; 0.31 a_w) mixed with OO or SO at 75:25 ratio (w/w) for 15.5 min (0.35 or 0.34 a_w, respectively, after mixing), and (ii) WPI–oil–sugar system, WPI mixed with OO or SO following with G–F (at 1:1 ratio) syrup (0.75 a_w) at a component ratio of 45:15:40 (w/w) for 31 min (0.62 or 0.63 a_w, respectively, after mixing). The G and F sugars were dispersed in DI–water (70% w/w of 1:1 sugars) at 50°C for 30 min to obtain clear syrups. Each high protein system was mixed using a Kenwood mixer (KM330; Kenwood Limited, Hampshire, UK) at minimum speed. Samples of WPI–oil and WPI–oil–sugar systems were transferred (*i*) into 10 mL clear glass vials (Schott, Müllheim, Germany) (2.5 to 3 g and 4 to 4.5 g, respectively) and (*ii*) placed in plastic cups (diameter 37 mm, volume 15 mL; AquaLab, Decagon Devices, Inc., WA, USA) (2 to 2.5 g and 5 to 5.5 g, respectively). Vials with samples were closed with

rubber septa under vacuum in a freeze-dryer equipped with a stoppering device (Lyovac GT2, Steris®, Hürth, Germany). Samples in cups were pressed with a metal disk (titanium plate, diameter 33 mm; Triton Technology Ltd., Lincolnshire, UK) to obtain a smooth surface and homogeneous structure, and the cups were closed with plastic, airtight lids (AquaLab, Decagon Devices, Inc., WA, USA). Closed and vacumized vials, and closed cups were subsequently sealed in plastic packages (PA/PE 90, Fispak Ltd., Dublin, Ireland) under vacuum at 99 and 75%, respectively, using a vacuum packaging machine (Polar 80 KL, Henkelman B.V., Den Bosch, The Netherlands). Samples were protected from water loss and uptake from the environment during storage. The vacuum in the packages retained hermetic conditions during storage and served as an indicator of the closed system. All systems were stored in temperature controlled incubators at 20°C (Cooling Incubator, KBP 6151, Series 6000, Termaks, Bergen, Norway) and 40°C (TS 8136, Termaks, Bergen, Norway). Samples were analyzed at intervals during storage for 14 weeks.

5.2.3. DYNAMIC WATER ACTIVITY

The WPI at a_w of 0.31 was dehumidified over saturated solutions of LiCl and CH_3COOK at 0.11 and 0.23 a_w, respectively, and rehumidified over series of saturated solutions of K_2CO_3, $Mg(NO_3)_2$, $NaNO_2$, NaCl, and KCl at 0.44, 0.55, 0.66, 0.76, and 0.85 a_w, respectively, in vacuum desiccators at room temperature (20±2°C) for 3 days. The WPI-oil at 75:25 ratio and WPI–oil–sugar at a component ratio of 45:15:40 systems were freshly prepared before measurements. Duplicate samples of WPI (0.31 a_w), WPI at various a_w, WPI–oil, and WPI–oil–sugar systems were transferred to cups (volume = 15 mL, diameter = 38.9 mm, height = 11.4 mm, thickness = 0.7 mm; AquaLab, Decagon Devices, Inc., WA, USA) and a_w values were measured over the temperature range of 20 to 50°C (heating) followed by a ramp from 50 to 20°C (cooling) using a water activity meter (Series 4TE, AquaLab, Decagon Devices, Inc., WA, USA). The a_w values during the cooling ramp were continuously measured after the heating ramp using the same sample. All samples were weighed before and after the measurement to monitor any water uptake or loss during the measurement. Average a_w values from duplicate samples at each temperature during the heating

and cooling ramps were calculated. The temperature accuracy inside the sample chamber was < ±0.5°C.

5.2.4. TEXTURE MEASUREMENT

A texture analyzer (TA-XT2*i*, Stable Micro Systems Ltd., Godalming, UK) with a cylindrical stainless probe (3 mm diameter; probe number = P3) in compression mode for 70% of deformation [modified from Zhou *et al.* (2008)] was used to analyze the texture of WPI–oil–sugar systems at intervals during storage. The test speed of deformation was 1 mm/s and the trigger force was 0.5 N. The initial and alternate gradients (slope values, N/s), and maximum force (N) during deformation were determined as hardness. The pattern or shape of the deformation curve for each material was considered as textural indicator of the internal structure monitored during storage at 20 and 40°C. The Exponent software, version 2.64 (Stable Micro Systems Ltd., Godalming, UK) was used. The initial and alternate gradients were obtained by dividing the compressive force (N) by time (s). All samples from the cups in the vacuum plastic package were transferred to room temperature (20±2°C) at minimum for 1 hour before the measurement. Average values of initial and alternate slopes, and maximum force of at least seven replicate samples of each system were calculated.

5.2.5. DYNAMIC MECHANICAL ANALYSIS (DMA)

Dynamic-mechanical properties of the high protein systems [WPI–OO, –SO, WPI–OO–, and WPI–SO–(G–F)] and WPI 0.31 and 0.66 a_w were studied at intervals during storage at 20 and 40°C for up to 14 weeks using a dynamic mechanical analyzer, DMA (Tritec 2000 DMA, Triton Technology Ltd., Lincolnshire, UK). The equipment was used to measure the storage (E') and loss moduli (E'') as a function of temperature. The DMA instrument was set at zero as described by Potes *et al.* (2012). Samples of high protein systems from vials were spread on a metal pocket-forming sheet (Triton Technology Ltd., UK). The metal pockets were cleaned with 2-propanol to remove residual fat and oil before use. The metal sheet with sample was crimped along a pre-scored line to form a thin sandwich pocket. This pocket was attached directly between the clamps (the fixed clamp and the driveshaft clamp) inside the measuring head of the DMA. The length, width, and thickness (≤ 2 mm) of the sample and pocket between the

clamps were measured. Duplicate samples of each system were analyzed using dynamic measurements and recorded using DMA control software version 1.43.00. Samples were scanned from -150 to 120°C with a cooling rate of 5°C/min and heating rate of 3°C/min at frequencies over the range from 0.1 to 10 Hz by using the single cantilever bending mode (Silalai and Roos, 2011). The measuring head was connected to a liquid nitrogen tank (1 litre; Cryogun, Brymill Cryogenic Systems, Labquip (Ireland) Ltd., Dublin, Ireland). During dynamic heating, all samples were analyzed for the α-relaxation temperature (T_α), determined from the onset temperature (T_{onset}) of E' and the peak temperature (T_{peak}) of E''. Average values of the T_α, T_{onset} of E', and T_{peak} of E'' from duplicate measurements of each system were calculated.

5.2.6. FOURIER TRANSFORM INFRARED SPECTROSCOPY (FTIR)

The WPI–OO and WPI–OO–(G–F) before and after storage at 20 and 40°C for 19 weeks were analyzed, and compared with WPI, OO, and G-F to investigate variations in spectra that involved molecules and functional groups during storage. The FTIR spectra were measured and recorded using the Varian 660-IR series FT-IR spectrometer (Varian, Inc., Victoria, Australia) and Agilent ResolutionsPro software, version 5.2.0 (CD 846) (Agilent Technologies, Inc., Australia). The deuterated triglycine sulphate detector was used in the FTIR measurements. All measurements were carried out at 27.5°C and 44.8% RH. Each sample from the vials was transferred onto the crystal cell, and the sample was pressed and covered with manual screw tight lid inside the FTIR spectrometer. The surface of the crystal cell was cleaned with DI–water (for WPI–oil–sugar system) and/or 2-propanol before and after each measurement. The background spectrum was recorded with no sample on the cleaned crystal cell before each measurement. Samples were scanned and absorbance was recorded over the wavelength range from 400 to 4000 cm^{-1}, and the spectra data were collected in 16 scans with a resolution of 4 cm^{-1}. At least triplicate samples of each material were analyzed and average spectra were reported.

5.2.7. CRYO-SCANNING ELECTRON MICROSCOPY (CRYO-SEM)

The WPI–OO–(G–F) was used to investigate structural changes and phase separation from images taken before and after storage at 20 and 40°C for 19 weeks using cryo-scanning electron microscope (Helios NanoLab 600I, DualBeams FIB, FEI company, Eindhoven, The Netherlands). Three samples were mounted onto the sample holder and immersed into liquid nitrogen. The holder with samples was transferred to the preparation chamber, which was vacuumized and pre-chilled at -140°C, for coating with platinum for 60 s at 10 mA at pressure (p) < 0.1 mbar, and then loaded into the SEM chamber. The sample stage inside the SEM unit was pre-chilled at -140°C before loading the samples and the holder. The temperature of the samples was maintained at -140, -100, -80, -20, and 45°C during imaging using xT software (FEI company, Eindhoven, The Netherlands). Each image before and after storage at each holding temperature during heating from -140 to 45°C was taken from the same area and at the similar scale of magnification.

5.3. RESULTS AND DISCUSSION

5.3.1. DYNAMIC WATER ACTIVITY

Protein hydration in WPI–OO (0.35 a_w) and WPI–OO–(G–F) (0.62 a_w) before storage was compared to hydration of WPI as detected from a_w (initial value 0.11 to 0.85 a_w at 25°C) measured during heating (20 to 50°C) and cooling (50 to 20°C) (Fig. 5.1). The WPI–OO showed similar hydration in both heating and cooling scans to that of WPI 0.31 a_w, as shown in Fig. 5.1A. The WPI–OO and WPI 0.31 a_w showed increased a_w values during the heating scan up to 45°C (maximum value of a_w), and then a decrease at 50°C. Such decrease in a_w indicated a conformational change allowing an increase of hydration of the protein molecules. Consequently the increase in hydration of the WPI 0.31 a_w and WPI–OO systems was a result of this transition that occurred in the vicinity of 45°C and reduced a_w values in cooling were also recorded. These results agreed with Potes *et al.* (2013b), who found an endothermic transition (a_w or water content independent, and reversible transition) that suggested a change in conformation of WPI at various a_w and WPI–OO at T_{onset} of 36 and 38°C, respectively. The WPI–OO–(G–F) system showed a significant difference in

hydration behavior during the heating and cooling ramps from the hydration of the WPI–OO 0.35 a_w and WPI 0.66 a_w, especially during cooling from 50 to 20°C (Fig. 1A). The WPI–OO–(G–F) showed increasing a_w values during heating from 20 to 50°C, but the a_w showed a consistent decrease at 35°C. Such decrease of a_w at 35°C was a result of an exchange of water molecules between the protein and carbohydrate components. Chou and Morr (1979) showed that the binding of protein and water molecules caused a decrease in vapor pressure and chemical potential of the water. An increase in temperature induced rapid decrease in hydrogen bonding of water and sugar molecules and concomitant hydration of protein molecules (Potes *et al.*, 2013b). During cooling WPI–OO–(G–F) showed an increase of a_w at 50 to 45°C being almost constant at a high a_w level during further cooling [at 45 to 20°C (0.720 to 0.715 a_w)]. This showed hydrogen bonding of small carbohydrate molecules and WPI, which decreased hydrogen bonding of water molecules to the carbohydrate and protein molecules. Potes *et al.* (2013b) found an exothermic transition during heating with T_{onset} ~40°C and T_{peak} 65°C in high protein systems containing sugar [WPI–(G–F) at ratio of 45:40 and WPI–OO–(G–F) at a component ratio of 45:15:40]. Their results showed that the glass transition of sugar in the systems after heating was broadened in an immediate reheating scan, which suggested increased interactions of the sugar and protein components.

The WPI 0.11 a_w showed a typical increase of a_w values during heating (Fig. 5.1B). WPI at 0.23 and 0.66 a_w showed similar changes in a_w for heating and cooling (Fig. 5.1B). The WPI at 0.44, 0.55, 0.76 and 0.85 a_w showed concavity with increasing temperature that could relate to hydration of proteins. A decrease of a_w values over the temperatures range from 35 to 45°C during heating agreed with the location of the endotherm found for proteins (Potes *et al.*, 2013b) (Fig. 5.1B). WPI initially at 0.44 and 0.55 a_w showed increased hydration (lower a_w) after heating at 50°C. WPI initially at 0.76 a_w had similar temperature dependence of a_w to WPI initially at 0.66 a_w. The WPI initially at 0.85 a_w showed released water or decrease in hydration during cooling. It is important to note that the WPI–OO, WPI–OO–(G–F), and WPI systems at various a_w showed no weight changes that could have indicated variations in water contents. Our results confirmed that conformational changes occur in proteins around 35 to

45°C, which affected protein hydration and hydrogen bonding with small carbohydrates. No earlier studies have reported variations in water activity (vapour pressure) as an indicator of hydration behaviour of proteins over temperatures ranging from 20 to 50°C.

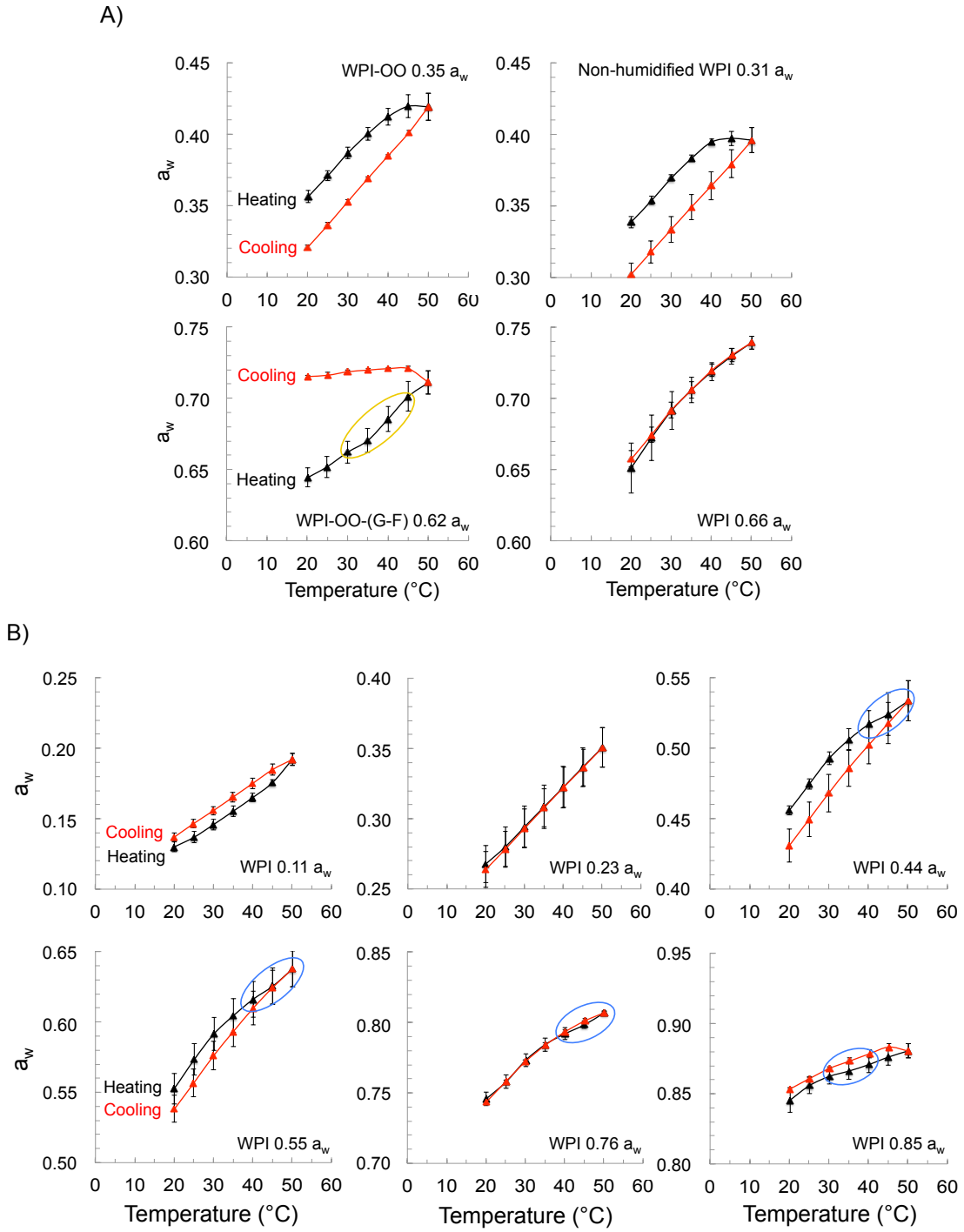


Fig. 5.1 Water activity (a_w) values of (A) WPI–olive oil (OO) and WPI–OO–(glucose–fructose, G–F) before storage compared to similar a_w of WPI systems (non-humidified WPI and humidified WPI at 0.66 a_w), and (B) humidified WPI at various a_w by the dynamic a_w measurements.

5.3.2. TEXTURE MEASUREMENT

A penetration test measured textural and physical changes of the high protein systems containing G–F during storage at 20 and 40°C for up to 14 weeks from gradients (initial and alternate slopes) and maximum force. These parameters quantified hardening of the texture as shown in Fig. 5.2. Textural properties were divided into 3 stages derived from initial and alternate gradients. The first stage (soft materials) was that of 0 to 2 weeks of storage, especially for the systems at 40°C, where the WPI–OO– and WPI–SO–(G–F) systems showed a steep increase of the initial gradient values. This result showed that significant hardening of high protein systems may occur rapidly, and possibly as a result of water migration. Zhou *et al.* (2008) found a correlation between hardening of a protein/buffer matrix [WPI, phosphate buffer (10mM, pH 7), sodium azide (0.05%)] and protein aggregation. They found a dramatic change of texture or hardening at 23, 34, and 45°C to coincide with a 12 (58 days of storage), 15 (30 days of storage), and 25% (3 days of storage) increase of insoluble whey proteins, respectively. The aggregated network of proteins was concluded to cause a significant hardening of texture during storage of the high protein system. The increase in hardening found during the first stage was also in line with the change in protein conformation that could enhance hardening at the higher temperature storage.

The second stage (film or elastic structure formation) occurred during 2 to 14 weeks and 2 to 7 weeks at 20 and 40°C, respectively. Over the second stage period the systems showed little or no observable changes of the initial and alternate gradients. The maximum force of the WPI–oil–(G–F) systems at 20°C after 2 weeks were constant, but at 40°C there was a continuing increased the maximum force values and the texture became harder during storage for up to 7 weeks.

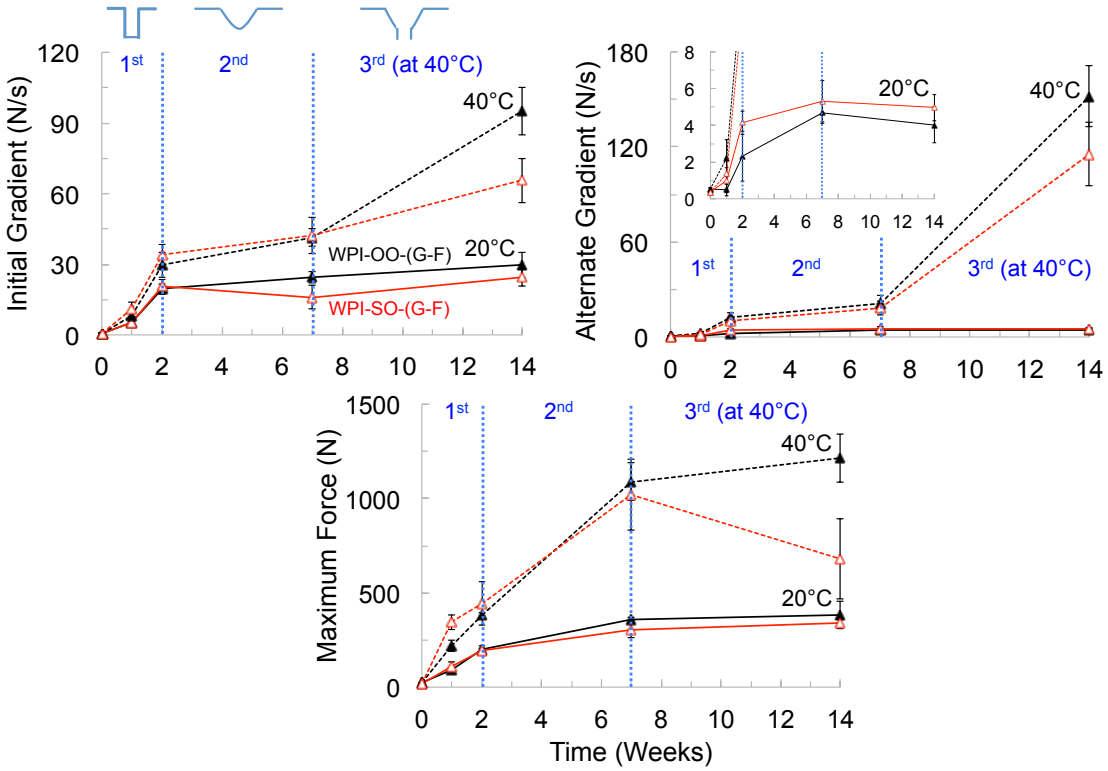


Fig. 5.2 The textural and physical changes of WPI–olive oil (OO)– and WPI–sunflower oil (SO)–(glucose–fructose, G–F) during storage at 20 (solid line) and 40°C (dash line) for up to 14 weeks using initial and alternate gradients, and maximum force as parameters for hardening from texture measurements.

At the third stage (hard and brittle solid structure) there was a sharp increase of the initial and alternate gradients, especially for the WPI–OO–(G–F) system. After 7 weeks of storage a dark brown colour, hardened texture, and brittleness (like glassy solid) were typical of the materials that also showed breaking during the measurements. These changes of the colour from white (before storage) to yellow (2 weeks) and to dark brown (7 weeks) colour in systems containing sugar coincided with hardening and loss of solubility [after 4 and 7 weeks of storage for WPI–OO– and WPI–SO–(G–F), respectively], especially during storage at 40°C. These changes were typical of extensive nonenzymatic browning reactions (NEB) or glycation (nonenzymatic glycosylation) and protein–oxidized fatty acids reactions (hydrophobic interaction) causing polymerization (protein aggregation or crosslinking) and formation of stable protein-lipid complexes or protein-lipid aggregates via covalent bonding (Hodge, 1953; Tappel, 1955; Tannenbaum *et al.*, 1969; Pokorný *et al.*, 1974; Davies, 1987; Hidalgo and Kinsella, 1989; Friedman, 1996; Howell *et al.*, 2001; Potes *et*

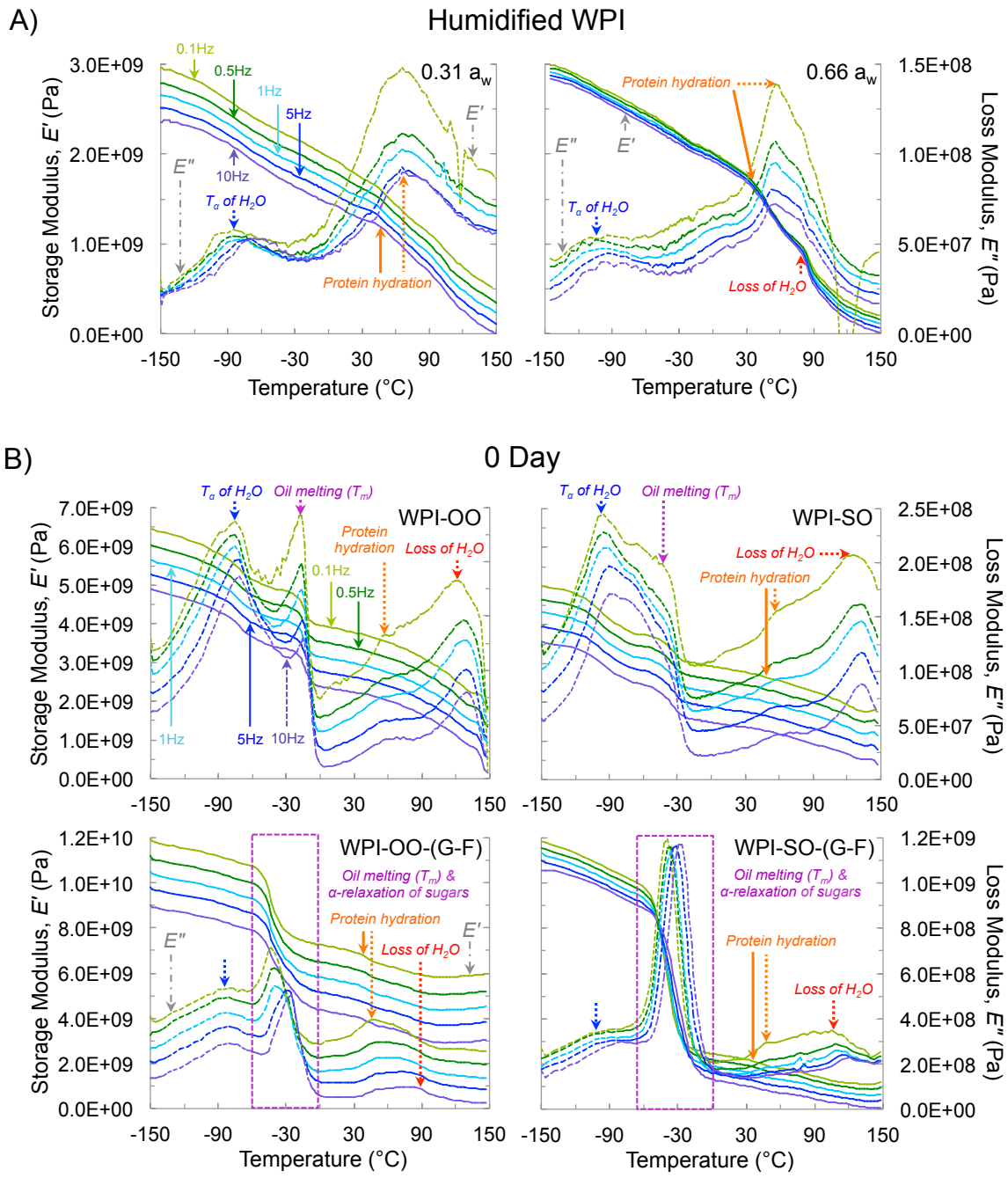
al., 2013b). It appeared that textural and physicochemical changes of the WPI–oil–(G–F) systems during storage at both temperatures, including hardening, darkening, and decrease in solubility (increased hydrophobicity of protein) were more pronounced in the systems containing OO, particularly during storage at 40°C. The presence of natural antioxidants (tocopherols or vitamin E) in SO could reduce textural and physical changes. Potes *et al.* (2013a, b) found that the interactions of protein molecules leading to oxidative aggregation or disulfides bond formation and NEB or Maillard reaction or glycation were reduced in the systems containing WPI, oil with tocopherols, and glucose–fructose syrup. The present results for WPI–SO–(G–F) and WPI–OO–(G–F) systems at both temperatures agreed with those of Potes *et al.* (2013a, b). We conclude that the type of oil or the degree of its unsaturation can play an important role in hardening and physicochemical reactions of high protein systems during storage.

5.3.3. DYNAMIC MECHANICAL ANALYSIS

The moduli, E', and E'' of WPI at 0.31 and 0.66 a_w, WPI–OO (0.35 a_w) or –SO (0.34 a_w), and WPI–OO– (0.62 a_w) or WPI–SO–(G–F) (0.63 a_w) systems at frequencies of 0.1 to 10 Hz before and during storage at 20 and 40°C are shown in Fig. 5.3. The frequency dependent T_{peak} of E'' at -85 to -66°C and -105 to -94°C, -76 to -72°C, -95 to -90°C, -81 to -81°C, and -88 to -79°C for the WPI at a_w of 0.31 and 0.66, WPI–OO, WPI–SO, WPI–OO–(G–F), and WPI–SO–(G–F) systems, respectively, before storage was interpreted as the **α-relaxation of hydration water**. The repeatability of the relaxation at the low temperature and its frequency dependence showed that hydration (or confined water) water in protein structures is a glass forming substance or a vitrifying network of hydrogen-bonded water molecules associated with the proteins. This conclusion was further supported by the frequency dependent T_{onset} of E' and subsequent decrease of the storage modulus (Fig. 5.3A and B) typical of an α-relaxation. The α-relaxation of hydration water in the WPI–SO–(G–F) coincided with a broadened relaxation and approached softening associated with SO melting and the α-relaxation of the G–F syrup. Numerous researchers (Doster *et al.*, 1989; Rasmussen *et al.*, 1992; Vitkup *et al.*, 2000; Teeter *et al.* 2001; Chen *et al.*, 2006; Ngai *et al.*, 2008; Shinyashiki *et al.*, 2009; Jansson and Swenson, 2010; Magazù *et al.*, 2011) have interpreted the α-relaxation of hydration water in fully

hydrated dispersions [30 to 100 g of water or deuterium oxide ($2H_2O$; D_2O; heavy water) or mixtures of glycerol and water in 100 g of dry protein] of globular proteins [including bovine serum albumin, myoglobin, lysozyme, and ribonuclease A (RNase A)] at ~200 to 220 K (-73.15 to -53.15°C) as "the glass transition or the α-relaxation or enthalpy relaxation of protein molecules". These studies also showed that the relaxation was temperature-dependent, it was restored with time, and it was not present in the absence of water or solvent or in the dehydrated globular proteins. However, these studies reported the predominant role of water or solvent mobility required for the presence of the relaxation. Chen *et al.* (2006) reported that the relaxation of hydrated lysozyme attributed to the translational mobility of the water molecules in contact with the hydroxyl group (hydration water) of protein surface (hydrophilic region), and that this relaxation resulted from the glass transition of the protein. Our results agreed with Chen *et al*. (2006), but since the addition of oil and oil-sugar to the WPI affected the T_α and the magnitude of the E'' at all frequencies (Fig. 5.3A and B) the α-relaxation must be that of hydration water rather than that of the protein. Conversely, protein molecules cause confinement of water molecules and thereby act as an "antiplasticizer" for the water associated with the protein structure.

The T_α at various frequencies in systems containing OO were less frequency-dependent (Fig. 5.3B). The magnitudes of the E'' peaks at T_α of hydration water for the WPI-oil and WPI–oil–(G–F) systems were higher than those of WPI at 0.31 and 0.66 a_w and a sharp decrease of the E' was typical of the α-relaxation. The T_α of hydration water and the magnitude of the E'' peak at T_α were a_w- and water content-independent (Fig. 5.3B). Anhydrous WPI systems showed no α-relaxation in agreement with the reports of Doster *et al.* (1989), Rasmussen *et al.* (1992), Vitkup *et al.* (2000), Teeter *et al.* (2001), Chen *et al.* (2006), Ngai *et al.*, (2008), Shinyashiki *et al.* (2009), Jansson and Swenson (2010), and Magazù *et al.* (2011). The presence of SO in the high protein systems showed lower values of T_α for the hydration water than systems containing OO at the same concentration. This difference could result from the hydrophobicity and lower melting temperature (level of unsaturated fatty acids) of the oil.


A)
Humidified WPI
0.31 a_w
0.66 a_w
0.1Hz
0.5Hz
1Hz
5Hz
10Hz
E′
E″
T_α of H_2O
Protein hydration
Loss of H_2O
Storage Modulus, E′ (Pa)
Loss Modulus, E″ (Pa)
Temperature (°C)
B)
0 Day
WPI-OO
WPI-SO
WPI-OO-(G-F)
WPI-SO-(G-F)
T_α of H_2O
Oil melting (T_m)
Protein hydration
Loss of H_2O
Oil melting (T_m) & α-relaxation of sugars
0.1Hz
0.5Hz
1Hz
5Hz
10Hz
E′
E″
Storage Modulus, E′ (Pa)
Loss Modulus, E″ (Pa)
Temperature (°C)

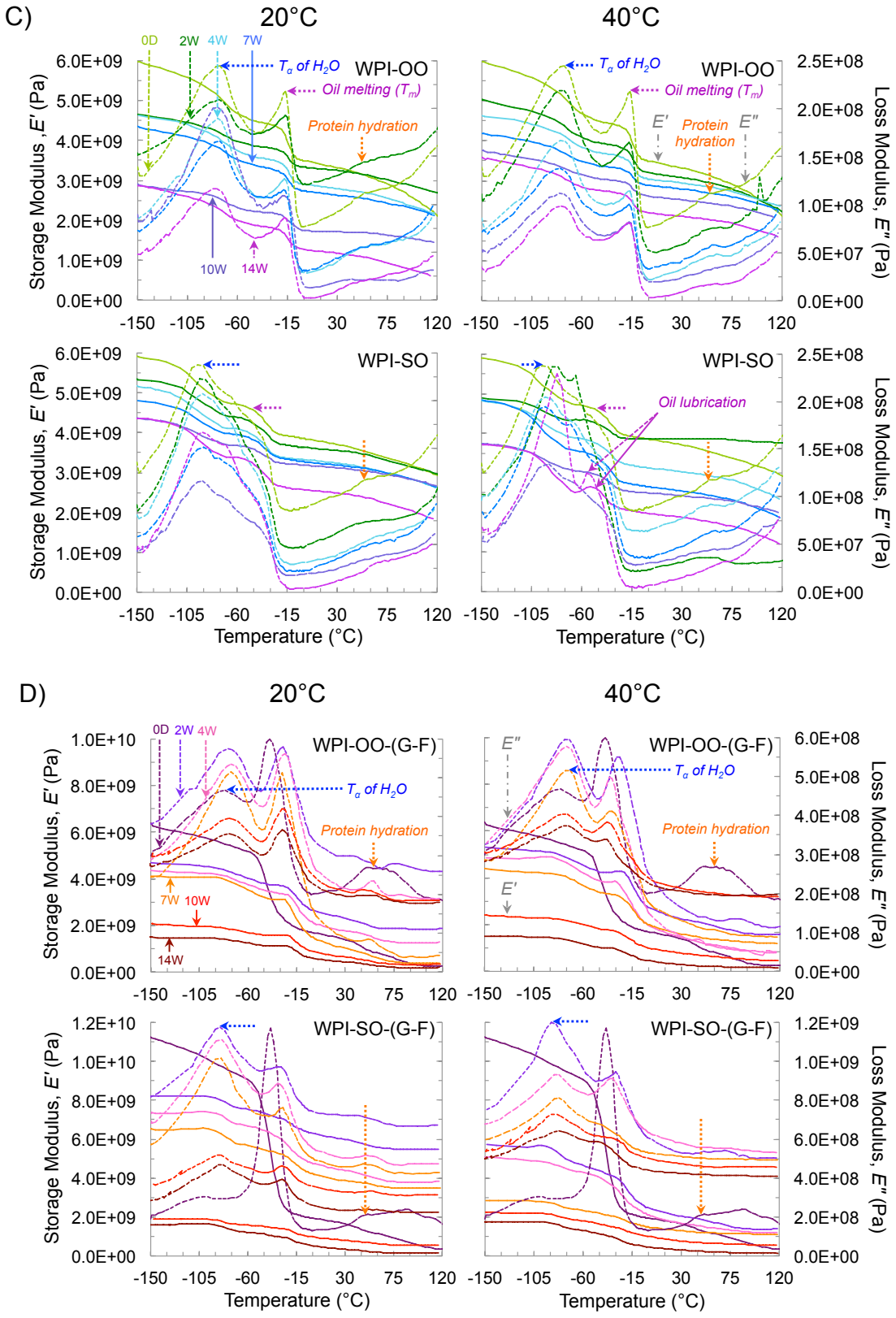


Fig. 5.3 The storage (E', solid line) and loss moduli (E'', dashed line) of (A) WPI 0.31 and 0.66 a_w, (B) WPI–olive oil (OO) or –sunflower oil (SO) and WPI–OO– and WPI–SO–(glucose–fructose, G–F) before storage (0 day) in the frequencies range of 0.1 to 10 Hz, (C) WPI–OO or –SO, and (D) WPI–OO– and WPI–SO–(G–F) systems during storage at 20 and 40°C for up to 14 weeks (W) at frequency of 0.5 Hz using the dynamic mechanical analysis.

The T_α of hydration water in the high protein systems [except for WPI–OO–(G–F)] before storage as derived from the E' and E'' data was dependent on the frequency of the measurement (Fig. 5.3A and B). The WPI at 0.31 and 0.66 a_w; WPI–OO or –SO; and WPI–SO–(G–F) systems had T_α of hydration water that became increasingly frequency-dependent at higher frequencies (1, 5, 10 Hz; 5, 10 Hz; and 10 Hz) while the WPI–OO–(G–F) relaxation was frequency-independent and possibly included effects of other molecular interactions of the components. Chou and Morr (1979) considered the protein-water interaction to show water associated with the protein molecules, including physically held water, i.e., capillary water or water held within the protein matrix, and such water was restricted in mobility and was difficult to remove out from proteins. Dellerue and Bellissent-Funel (2000) demonstrated that the protein surface (hydrophilic region) retarded mobility of water molecules. Our data showed that water molecules in the WPI systems at T_α showed a higher stiffness and could become reduced in mobility in the presence of others components, especially in systems containing OO and G–F, or protein at a lower initial a_w (WPI 0.66 a_w) (Fig. 5.3A and B). The components (protein, sugar, oil, and water) became increasingly segregated and formed separate phases in the systems below -60°C, including the separate glassy state of the G–F (Roos, 2012). Mattos (2002) reported that confined water molecules that used all the available hydrogen bonds within hydrophilic or water binding sites of proteins might be involved in facilitating low-frequency vibrations associated with large scale protein motions, whereas the surface water molecules might be more important for higher-frequency motions. The results clearly showed that the dynamic transition at a low temperature related to the molecular mobility of the unfrozen water or glass forming water molecules in the system than to the mobility of the protein molecules. Our results demonstrated that *the dynamic transition at a low temperature was the α-relaxation at the glass transition of water and not the glass transition of the globular proteins.*

The E'' for the WPI–OO showed the oil melting at -19 to -15°C with a corresponding decrease of E', and the E'' peak at ~120°C for WPI–oil systems indicated the loss of water (frequency-independent). The T_{peak} of SO melting of E'' for the WPI–SO was overlapping with the α-relaxation of hydration water

transition at the lower temperature. The high protein systems containing oil and sugar showed overlapping phenomena at oil melting and α-relaxation of the G–F (frequency-dependent) syrup, as shown in Fig. 5.3B. The E′ and E″ for WPI 0.31 and 0.66 a_w, WPI–OO, and WPI–oil–(G–F) systems showed also the conformational change of proteins around 40°C, as shown in Fig. 5.3A and B. This change in protein conformation was independent of frequency. The WPI 0.31 and 0.66 a_w showed conformational changes at 38 and 36°C, respectively, for T_{onset} of E'; and 59 and 56°C, respectively, for T_{peak} of E'' (Fig. 5.3A). Before storage the change in protein conformation in systems containing OO and SO occurred at T_{peak} of E'' of 52 and 51°C, respectively. The WPI–OO– and WPI–SO–(G–F) showed a change at 44 and 44°C, respectively, for T_{peak} of E'', and at 34 and 34°C, respectively, for T_{onset} of E'. The change in protein conformation in WPI-oil systems was not dependent on a_w and water content. These results were in agreement with Potes *et al.* (2013b), who found such changes of proteins in WPI at various a_w, WPI–OO, and WPI–OO–(G–F) at T_{onset} ~36, 38, and 45°C, respectively, and it was a_w-independent.

During storage at 20 and 40°C for up to 14 weeks of WPI-oil systems, the values of T_α of hydration water at 0.5 Hz for the WPI-SO system became higher after storage, especially at 40°C (-95 to -82°C). The E'' peaks of OO melting in WPI–oil system at both temperatures showed no changes during storage, but the E'' peaks of SO melting became more pronounced as there was phase separation of SO after storage, especially at 40°C (Fig. 5.3C). The WPI–SO system after storage at 40°C showed merging transitions for the α-relaxation of water and oil melting [the higher T_α of water approached SO melting with storage time] (Fig. 5.3C). The conformational change of proteins in the WPI-oil systems was not apparent, especially at 40°C. This could result from hydrophobic interaction between protein and oil during storage, which could increase surface hydrophobicity of the protein, and result in changes of hydration properties of the protein. The OO and SO could also become oxidized as a result of autoxidation reactions in which the residual or trapped oxygen in the systems was available to the reaction. The proteins in contact with peroxidized lipid or their secondary products show differences in functional properties of proteins and amino acids and increased hydrophobicity of proteins (insolubilization) (Roubal and Tappel,

1966a; Funes and Karel, 1981), polymerization or cross-linking (Roubal and Tappel, 1966b; Funes and Karel, 1981; Leake and Karel, 1982), and formation of lipid-protein complexes (Davies, 1987; Howell *et al.*, 2001). The polymerization or cross-linking and formation of lipid–protein complexes alter water sorption properties, physical structure, and physicochemical properties of dried food systems (Koch, 1962; Karel, 1973). The fatty acids in lipid molecules had a surfactant effect on protein surfaces [leading to hydrophobic interaction and protein unfolding or exposing interior amino groups of protein (Saeed and Howell, 2002)], which could be observed by the oil lubrication behavior affecting E' and E'' at T_{peak} -55°C for WPI–SO at 40°C of storage for 14 weeks (Fig. 5.3C). A higher level of unsaturated fatty acids in oil could induce or promote oxidation of proteins leading to hydrophobic interactions of components and increased surface hydrophobicity of protein particles.

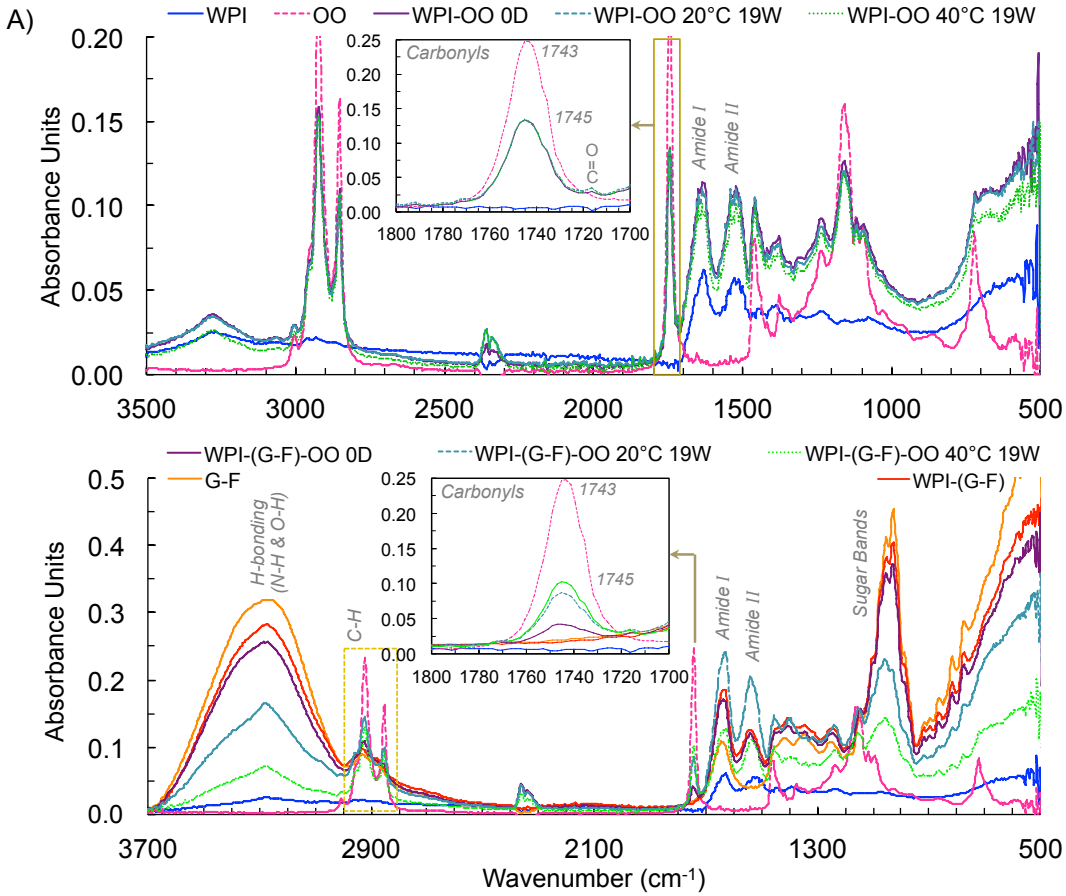
The WPI–OO– and WPI–SO–(G–F) showed slightly increased T_α of hydration water from E'' at 0.5 Hz during storage at 20 (-81 to -78°C and -97 to -83°C, respectively) and 40°C (-81 to -74°C and -97 to -82°C, respectively) for up to 14 weeks, especially in the SO systems (Fig. 5.3D). The systems containing G–F showed overlapping transitions for oil melting and α-relaxation of the G–F phase for E' and E'' during storage for 14 weeks. The oil melting and G–F relaxation from E'' peak at 0.5 Hz for the WPI–OO– (-40 to -28°C) and WPI–SO–(G–F) (-39 to -28°C) systems increased during storage at 20°C for up to 14 weeks. At 40°C the systems containing G–F showed increased temperature for oil melting and G–F relaxation at 2 weeks of storage and the temperatures decreased thereafter. This behaviour of oil and sugar at 40°C resulted in the merge of the α-relaxation of hydration water, and the changes in moduli were broadened during storage (Fig. 3D). Increasing oil melting and G–F relaxation temperatures in the high protein systems during storage at 20 and 40°C for 14 and 2 weeks, respectively, could result from carbonyl–amine polymerization (protein and reducing sugars interaction) via nonenzymatic browning (NEB) (Maillard reaction), or formation of advanced glycation end products [covalent cross-linking and polymeric aggregates between proteins or amino groups with the Amadori products (1-amino-1-deoxy-2-ketose or 1,2-enol form) (Friedman, 1996)]. The formation of polymeric or high molecular weight components may

increase the glass transition temperature (T_g) of amorphous materials (Roos and Karel, 1991d; Buera *et al.*, 1992). The increased temperatures of oil melting and G–F relaxation in WPI–oil–sugar systems during storage were also due to the migration of water from G–F syrup to the dry proteins. The lower temperature of the oil melting and G–F relaxation corresponded to the broadening of the temperature range with storage time at 40°C could show effects of water as a reaction product [from several condensation steps of NEB (Hodge, 1953; Eichner and Karel, 1972)] causing a lowered T_g of G–F. These changes also coincided with increased hydrophobicity (insolubilization) (Roubal and Tappel, 1966a; Funes and Karel, 1981; Smith *et al.*, 1994; Zhou *et al.*, 2008; Le *et al.*, 2011b) and aggregation of proteins (Zhou *et al.*, 2013). Our data from texture measurements confirmed the hardened structure of the high protein systems. We also observed the disappearance of the conformational change of the proteins in WPI–oil–sugar systems during storage, especially at 40°C after 2 weeks of storage (Fig. 5.3D).

5.3.4. STRUCTURE AND CONFORMATION OF PROTEINS

The FTIR spectra of high protein systems containing WPI–oil and WPI–oil–(G–F) systems before and after storage at 20 and 40°C for 19 weeks as compared to WPI 0.31 a_w, OO, and G–F syrup are shown in Fig. 5.4. The OO showed typical spectra of unsaturated fatty acids, such as C-H stretching (2800-3000 cm^{-1}), carbonyl groups at 1743 cm^{-1} (ester = 1725 to 1750 cm^{-1}, aldehyde = 1725-1740 cm^{-1}), and methyl C-H bending (1430-1470 cm^{-1}) (Coats, 2000), as shown in Fig. 5.4A. The bands sensitive to the carbonyl groups could be used as an index of lipid and protein oxidation during storage. The WPI–OO showed no significant differences of the carbonyl peaks at 1745 cm^{-1} from the system before storage, but the WPI–OO–(G–F) after storage at 40°C showed more pronounced effects on the bands sensitive to carbonyl groups than the system at 20°C and before storage (Fig. 5.4A). These results agreed with the lesser pronounced changes of bands sensitive to hydrogen bonding [N-H and O-H stretching = 3400-3070 cm^{-1} (Farhat *et al.*, 1998)] of WPI–OO–(G–F) at 40°C. The data showed an increased intensity of carbonyls band in the order of G–F > WPI–(G–F) > WPI–OO–(G–F) before storage > WPI–OO–(G–F) stored at 20°C for 19 weeks > WPI–OO–(G–F) stored at 40°C for 19 weeks (Fig. 5.4A). Farhat *et al.* (1998) found an

influence of the hydrogen bonding of water on hydration sites, C=O and N-H, resulting in changes of peak intensities corresponding to IR bands. Our results demonstrated the relationship of lipid and protein oxidation and hydrophobicity of protein at low a_w during storage, as the lipid and protein oxidation lowered the hydration properties of protein (increased protein hydrophobicity or insolubility) with time and temperature. This was in accordance with the results from texture measurement and dynamic mechanical properties of high protein systems containing G–F.



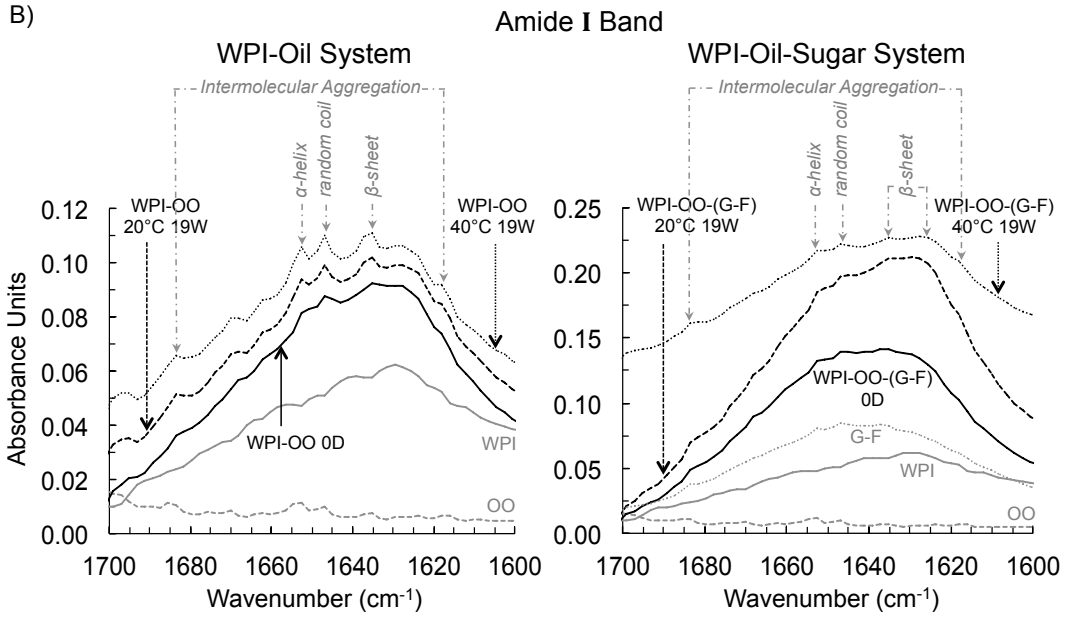


Fig. 5.4 FTIR spectra of WPI–olive oil (OO) and WPI–OO–(glucose–fructose; G–F) systems before (0 day; 0D) and after storage at 20 and 40°C for 19 weeks (W) compared to WPI 0.31 a_w, OO, G–F, and WPI–(G–F), as control systems, in the frequencies range of (A) 500 to 3700 cm^{-1} and (B) 1600 to 1700 cm^{-1}.

The amide I band (vibration of three dimensional structure for peptides, polypeptides, and proteins) for proteins absorb in the region between approximately 1600 and 1700 cm^{-1} (Byler and Susi, 1986; Krimm and Bandekar, 1986; Surewicz *et al.*, 1993; Farhat *et al.*, 1998; Jung, 2000; Kong and Yu, 2007). This band was used to investigate the secondary structure and intermolecular interactions of globular proteins (in aqueous and nonaqueous or organic solvent systems) (Byler and Susi, 1986; Krimm and Bandekar, 1986; Dong *et al.*, 1990; Kumosinski and Farrell, 1993; Haris and Severcan, 1999; Souillac *et al.*, 2002; Liu *et al.*, 2009). The WPI–OO and WPI–OO–(G–F) systems showed increased intensity of IR bands at 1684 and 1618 cm^{-1} after storage at both temperatures for 19 weeks, especially for the WPI–OO at 40°C (Fig. 5.4B). These bands attribute to intermolecular β-sheet formation resulting from aggregation (intermolecular aggregation or β-aggregation) of denatured α-lactalbumin (Boye *et al.*, 1997a), β-lactoglobulin (Boye *et al.*, 1997b; Panick *et al.*, 1999), bovine serum albumin (Militello *et al.*, 2004), and whey proteins (Boye *et al.*, 1995; Liu *et al.*, 2009). These two bands confirmed the increased surface hydrophobicity and irreversible aggregation of proteins (Lala and Kaul, 1992). The band at 1653 cm^{-1} attributed to α-helix (Byler and Susi, 1986; Susi

and Byler; 1988; Boye *et al.*, 1995; Boye et al., 1997a; b) and increased sharply in size for the WPI–OO after storage at both temperatures, especially at 40°C. This α-helix band increased together with the bands at 1647 [random coil (Byler and Susi, 1986; Boye *et al.*, 1997a; Kong and Yu, 2007; Kealley *et al.*, 2008) and disordered structures (Panick *et al.*, 1999)] and 1635 cm^{-1} [β-sheet, (Byler and Susi, 1986; Susi and Byler; 1988; Boye *et al.*, 1997a; Kealley *et al.*, 2008)], and the bands sensitive to intermolecular aggregation of proteins for all high protein systems after storage at both temperatures, especially for the WPI–OO system at 40°C. It appeared that the presence of oil in the WPI induced aggregation and conformational changes of proteins in WPI, which showed changes of secondary structure (α-helix, random coil, and β-sheet) of proteins after storage at 20 and 40°C for 19 weeks. The IR spectra for systems containing G–F in the region of amide I were broadened and of a lower intensity than those of the systems before and after storage at 20°C (Fig. 5.4B), which confirmed changes in hydration of the protein (Jackson and Mantsch, 1995). The absorption bands at 1624 and 1627 cm^{-1} were more pronounced for the systems containing G–F after storage at 40 and 20°C, respectively, which corresponded to the extended chains of β-sheet structure for globular proteins (α-lactalbumin and β-lactoglobulin) (Byler and Susi, 1986; Dong *et al.*, 1990).

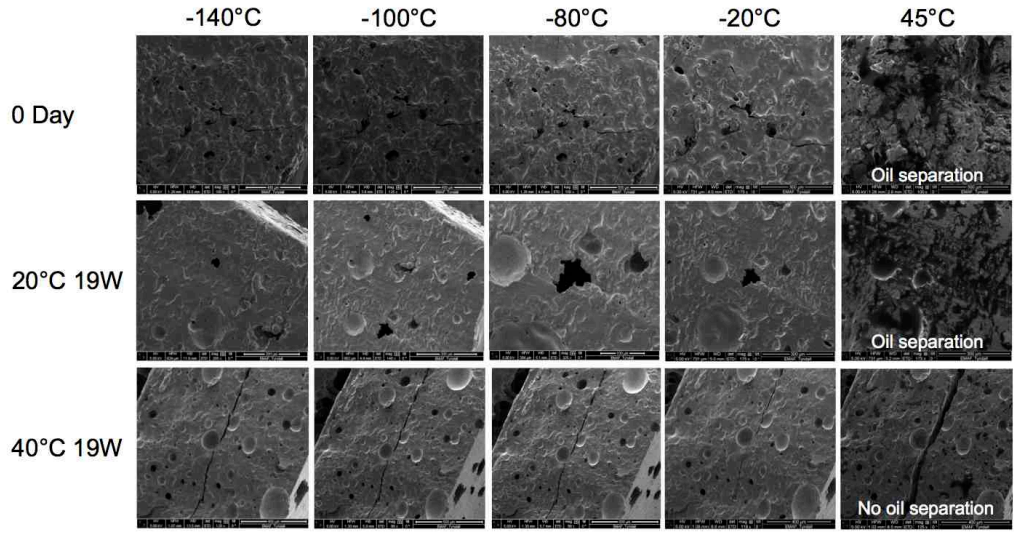


Fig. 5.5 Morphology of WPI–olive oil (OO)–(glucose–fructose; G–F) before (0 day) and after storage at 20 and 40°C for 19 weeks (W) during heating from -140 to 45°C using Cryo-SEM.

The WPI–OO–(G–F) after storage at 40°C for 19 weeks showed hard, brittle, and insoluble dark brown solid structure, which was stable over a wide temperature (-140 to 45°C) range with no oil separation present in the cryo-stage SEM images at the surface of the material after storage at 45°C and above the melting point of OO (Fig. 5.5). Polymerization of protein, oil, and G–F occurred due to the lipid and protein oxidation and glycation during storage at the higher temperature. At 45°C the oil separation in the WPI–OO–(G–F) stored at 20°C was less than in the system before storage. The proteins were clearly observed as a circular particle (from spray dryer) in the WPI–OO–(G–F) systems before storage, and the system remained uniform after storage at both temperatures. The porosity inside the structure of the systems containing G–F showed air entrapped within the viscous structure during mixing, which could also affect oxidation and glycation reactions and physicochemical stability of the systems during storage.

5.4. CONCLUSIONS

The WPI at 0.31, 0.44, 0.55, 0.76, and 0.85 a_w, and WPI–OO showed conformational changes of proteins over the temperature range of 35 to 45°C. WPI–oil–(G–F) systems showed significant differences in hydrogen bonding of water and small carbohydrates and protein and associated water migration between sugars and protein components. The polymerization (aggregation or crosslinking of proteins) and formation of stable protein-lipid complexes or protein-lipid aggregates from glycation and protein-oxidized fatty acids reaction decreased physical stability and changed physicochemical properties of the WPI–oil–(G–F) during storage, which depended on the type of oil, degree of unsaturation of fatty acids, temperature, and storage time. The dynamic mechanical properties of WPI at 0.31 and 0.66 a_w, WPI–oil, and WPI–oil–sugar systems demonstrated the glass transition of hydration water (unfrozen water) of proteins. Oil containing high level of unsaturated fatty acids could induce or promote oxidation of proteins leading to hydrophobic interactions of components and increased surface hydrophobicity of protein particles (lowered water hydration of proteins). The WPI–oil–(G–F) during storage at both temperatures showed migration of water from G–F syrup to the dry proteins and component interactions leading to carbonyl-amine polymerization and formation of advanced glycation end products, which affected dynamic transition of G–F and

oil, and protein hydration. The results from FTIR showed the relation of lipid and protein oxidation with hydrophobicity of protein during storage. The lipid and protein oxidation increased protein hydrophobicity with time and temperature with resultant lowering of hydration of proteins. The WPI–oil and WPI–oil–sugar systems showed intermolecular aggregation of proteins at 20 and 40°C after storage for 19 weeks. Addition of oil to the WPI could induce aggregation and conformational changes of secondary structure of proteins during storage at both temperatures. The systems containing sugar after storage at 40°C was hardened, stable over a wide range of temperatures from -140 to 45°C with no phase separation of oil during heating to above T_{melt}. The result indicated that polymerization of protein, oil, and sugar occurred during storage largely because of the lipid and protein oxidation and glycation.

CHAPTER VI

GENERAL DISCUSSION

6.1. CARBOHYDRATE−CARBOHYDRATE INTERACTIONS IN HIGH SOLIDS SYSTEMS

6.1.1 IMPACT ON CRYSTALLIZATION OF LACTOSE

Concentration, average molecular weight, and molecular size distribution of MD showed a significant impact on crystallization of lactose in lactose−MD systems. Crystallization of lactose was affected by MD at all concentrations at a_w of 0.55 to 0.76. Increasing MD concentration decreased the rate of lactose crystallization and the corresponding loss of sorbed water (Fig. 2.4). The non-crystalline MD increased final sorbed water contents of lactose−MD systems, especially for lactose−MD DE 23-27, at high a_w (> 0.44 a_w) (Fig. 2.3A and B). Increasing quantities of final water contents of lactose−MD corresponded to water sorbed by the amorphous MD fraction (Roos and Karel, 1992). The lactose and lactose−MD with medium (9−12) and high (23−27) DE values at 40:60 ratios were viscous fluids and showed a collapsed structure (liquid-like behavior) at high a_w values and the T_g during storage below the storage temperature (Fig. 6.1). Structural collapse occurred because of the significant decrease of viscosity and viscous flow above the critical a_w ($T > T_g$) indicating that the material was not able to carry its own weight (Levine and Slade, 1986; Roos, 1995a). As shown in Fig. 6.1, collapse was average molecular weight, molecular size, and molecular mobility dependent above the T_g.

The lactose−MD DE 4−7 at 40:60 ratio at 0.76 a_w showed crystallization of lactose after 7 days of storage with no collapse or shrinkage for up to 25 days (Fig. 6.1). Levine and Slade (1986) reported that collapse could be prevented at $T < T_g$. Accordingly formulations that increase the T_g to above the storage temperature may increase the overall molecular weight of the water-soluble carbohydrate solutes and resist structural changes during storage. Temperatures above the T_g enhance molecular mobility and enhance diffusion, which promotes crystallization of sugars (Cardona *et al.*, 1997). Our study clearly showed that higher numbers of smaller carbohydrate molecules as components of the MD with high DE (high number of small sugar molecules) had a high mobility around the lactose molecules resulting in delayed and inhibited lactose crystallization.

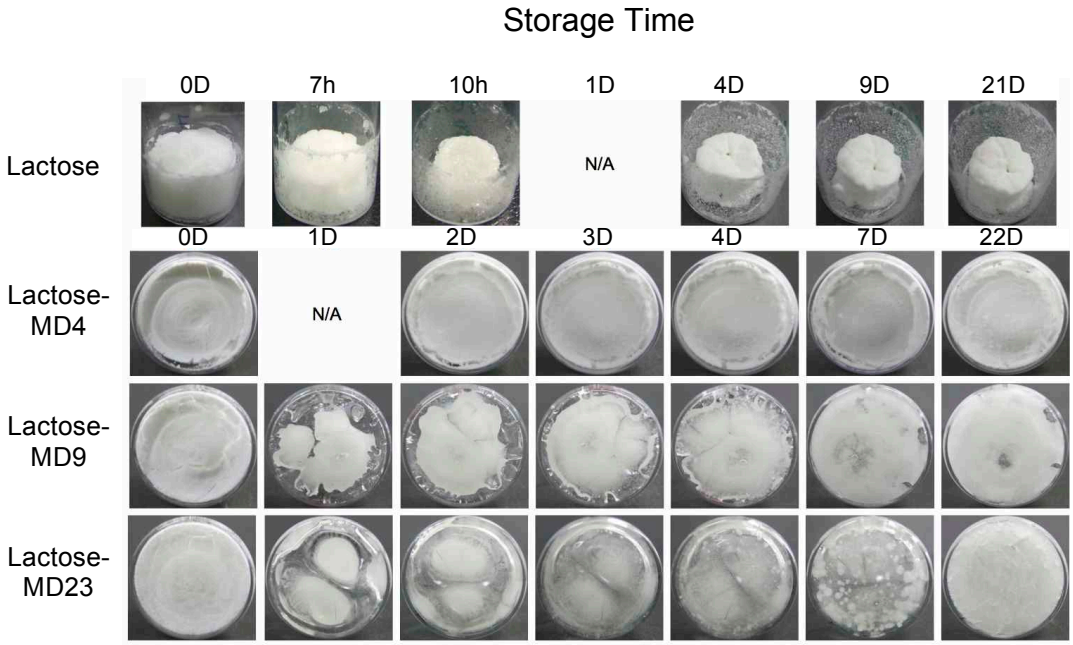


Fig. 6.1 Appearance of amorphous lactose and lactose−MD DE 4−7 (MD4), 9−12 (MD9), and 23−27 (MD23) at 40:60 ratio during storage at room temperature at 0.76 a_w for up to 22 days (D).

In the present study, MD with DE 9−12 and 23−27 at 10 to 30% (w/w) total solids with lactose showed no significant affects on the T_g of the systems at 0 to 0.44 a_w (Fig. 2.2). However, the inhibition of lactose crystallization at each lactose−MD ratio above the T_g was different (Fig. 2.3). Gabarra and Hartel (1998) found that the presence of small molecular weight components described by the DE value of the MD and corn syrup solids was more important factor affecting sucrose crystallization in mixed solids than the T_g of the sucrose−MD DE 20 or sucrose−corn syrup solids DE 42 system. The results from our studies emphasized the importance of concentration, molecular size, number average molecular weight, and molecular interactions as factors affecting crystal growth and lactose diffusion besides the glass transition of the amorphous solids. The systems of lactose and MD with low to high DEs at high a_w and above the T_g are shown schematically in Fig. 6.2.

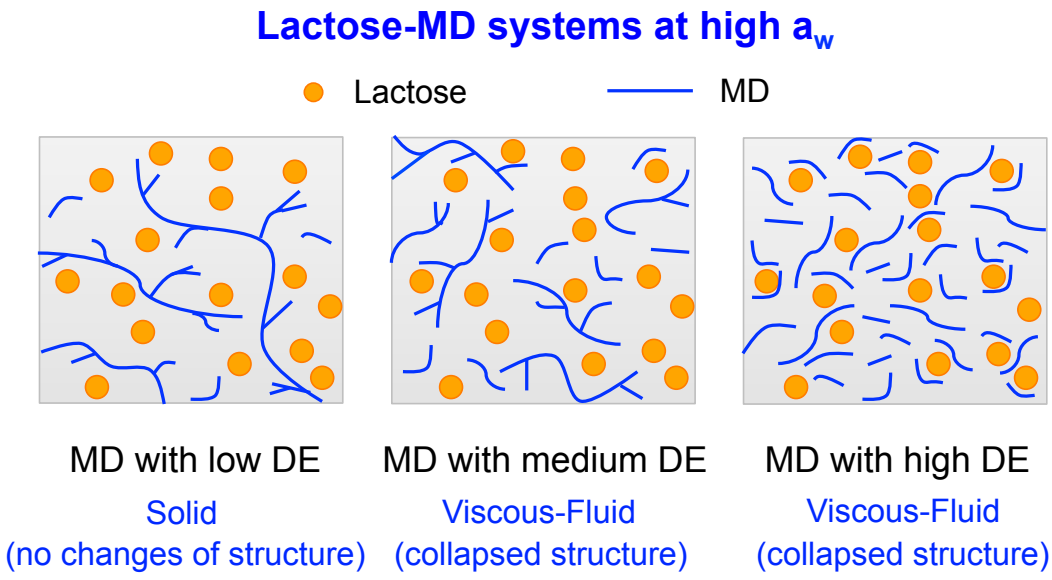


Fig. 6.2 Schematic presentation of effects of maltodextrin (MD) with various molecular weights and their dextrose equivalents (DEs) on the crystallization of lactose at high water activity (a_w).

Our water sorption study showed that the sorbed water contents of lactose−MD systems were proportional to quantities sorbed by the system components. The sum of water contents of lactose and MD (MD DE 9−12 or MD DE 23−27) at each a_w and for all lactose−MD mixture ratios from components sorption data agreed with experimental results. Our findings agreed with Shamblin and Zografi (1999), who showed the additivity of water sorption data of sucrose with long chain polymers [poly (vinyl pyrrolidone) or poly (vinylpyrrolidone-co-vinylacetate)] systems. In the present study we also showed that the sorbed water contents of lactose could be derived from water sorption data of lactose−MD systems at high a_w or in the critical a_w range of lactose crystallization using the non-crystalline lactose−MD at 40:60 ratios (Fig. 2.1). The GAB sorption isotherm was fitted to the experimental lactose (at 0.11 up to 0.55 a_w) and lactose water content in lactose−MD (at higher a_w) to model water sorption of lactose and lactose–MD systems. The results showed that the additivity principle could be applied in general to binary and multicomponent amorphous systems, and to produce sorption isotherms for crystallizing materials over a wide water activity range. We also established a full sorption isotherm from 0 to 0.8 a_w, which was not available in the literature.

6.1.2. DIELECTRIC AND DYNAMIC MECHANICAL PROPERTIES

The T_α and lactose crystallization behaviour of lactose−MD systems were studied by dielectric and dynamic mechanical analyses. The T_α is the primary or α-relaxation (frequency-dependent) temperature of glass forming amorphous materials, including carbohydrate polymers. α-relaxation occurs as a result of the increase in molecular mobility upon heating through the glass transition (Noel *et al.*, 1992). The T_α was taken from the peak temperature of ε'' and E'' in the present study. The T_α derived from ε'' and E'' data of amorphous lactose and lactose−MD DE 9−12 and 23−27 systems at 0.33 a_w were frequency-dependent in agreement with Noel *et al.* (1992), Laaksonen and Roos (2000), and Talja and Roos (2001), and it occurred at a higher temperature than the onset T_g. Our results corresponded to those of Williams *et al.* (1955) and Noel *et al.* (1992), who demonstrated that sugars (D-glucose, D-galactose, D-xylose, D- arabinose, and L-arabinose), sugar alcohols (glycerol, sorbitol, xylitol, and propylene glycol), and polymers (polyisobutylene, butadiene-styrene, polymethyl acrylate, polyvinyl chloroacetate, polyvinyl acetate, polystyrene, polymethyl methacrylate) showed a primary relaxation at a temperature above the T_g. At increasing concentrations of MD in lactose−MD systems to levels up to 30% (w/w) of the total solids the T_α values were not significantly different from that of lactose in accordance with the glass transition temperature data.

Crystallization of lactose at temperatures well above the T_α resulted in a sharp frequency-independent change of ε'' in dielectric analysis data and E'' in dynamic mechanical analysis data of the lactose−MD systems (Fig. 2.6 and 2.7). The sharp increase or sudden change of dielectric loss and loss modulus due to crystallization of sugars was also shown in previous studies (Talja and Roos, 2001; Gonnet *et al.*, 2002). The systems containing MD with high DE showed more pronounced changes in crystallization behaviour of lactose than systems containing MD with low DE, especially at high MD concentrations. Lactose−MD systems at high concentrations of MD showed a frequency-dependent change, a higher onset temperature of crystallization (T_{cr}) of lactose, and a slower progression of the crystallization in the systems. These data were dependent on the rate of lactose crystallization as it was dependent on the concentration and DE of the MD in the systems.

6.1.3. RELAXATION TIMES AND CRYSTALLIZATION OF LACTOSE

The relaxation times in amorphous materials can be calculated and modeled using the Vogel-Tammann-Fulcher (VTF) (Eq. 2.3) and Williams-Landel-Ferry (WLF) (Eq. 6.1) relationships (Williams *et al.*, 1955). The WLF equation is often applied to amorphous polymers at and above the T_g to model the temperature dependence of structural relaxation times or corresponding relationships derived from viscosity, mechanical or dielectric properties (Williams *et al.*, 1955). The VTF and WLF equations are interconvertible, although the VTF model was reported as suitable for modeling the temperature dependence of relaxation phenomena in non-polymeric liquids or sugar systems (Angell *et al.*, 1994; Recondo *et al.*, 2006).

$$\log\left(\frac{\tau}{\tau_g}\right) = -\frac{C_1 (T - T_g)}{C_2 + (T - T_g)} \quad (6.1)$$

where τ = relaxation time at temperature T

τ_g = structural relaxation time at T_g [$\tau_g \sim 100$ s (Angell, 2002)]

C_1 and C_2 = 17.44 and 51.6 (universal constants)

T = temperature

T_g = glass transition temperature

The VTF model was fitted to the dielectric and dynamic mechanical data of peak T_α and onset T_{cr} (Fig. 2.8). The presence of 30% (w/w) MD DE 9−12 and 23−27 in lactose systems was found affect apparent mobility of lactose molecules. This was shown by increasing T_{cr}. The dielectric loss peaks of lactose−MD DE 23−27 at high frequencies showed decreasing T_α-T_g, which indicated a higher molecular mobility than in lactose−MD DE 9−12. Increasing molecular mobility of lactose−MD 23−27 could result from the presence of the small carbohydrate molecules or some crystallization releasing water and plasticizing the system. The WLF model was fitted to a_w using the temperature of storage above the glass transition temperature (T-T_g) and correlated to the crystallization rate constants (k_1) derived from kinetics of loss of water sorbed from lactose and lactose−MD systems at 70: 30 ratio (Fig. 6.3). Lactose−MD DE 9−12 and 23−27 showed no

differences in relaxation times, but the rate constants of loss of water indicating lactose crystallization differed (as shown by orange dotted lines). From these data we could find that at constant value of τ or a_w, the systems containing MD with high DE showed a lower k_1 than systems MD with low DE. This confirmed that the MD with high DE could inhibit or retard crystallization of lactose in lactose−MD systems. The presence of small carbohydrate molecules in lactose−MD with high DE required longer time before lactose molecules were able to diffuse to appropriate locations on the crystal lattice. Such diffusion could be retarded more by collisions and competitive diffusion of the small MD components than by the mobility of the lactose molecules.

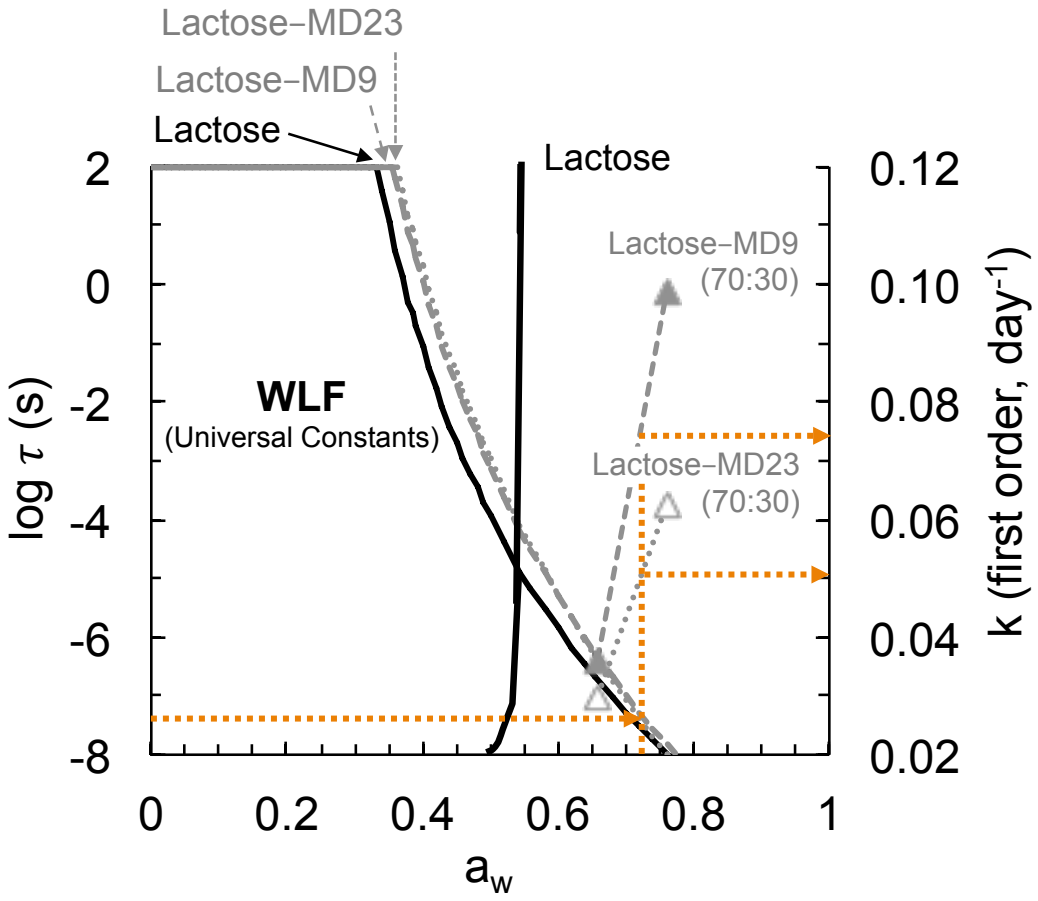


Fig. 6.3 Effects of correlation of WLF predicted relaxation time, τ, and rate constants (k_1) from kinetics of loss of water sorbed from freeze-dried lactose and lactose−MD DE 9−12 (MD9) or 23−27 (MD23) at 70:30 ratio against water activity (a_w).

6.2. PROTEIN−OIL INTERACTIONS IN HIGH SOLIDS SYSTEMS

6.2.1. PHYSICAL AND PHYSICOCHEMICAL PROPERTIES

Storage temperature showed significant effects on the colour and a_w of the WPI−oil systems. Our results showed higher yellowness, especially for WPI−SO, and decreasing a_w values during storage at 40°C (Fig. 4.1). The decrease in a_w could result from cooperative oxidation of lipids and proteins, including oxidation (break down reaction) of water molecules. The reactive oxygen groups and free radicals produced by oxidation of unsaturated fatty acids attack proteins and amino acids in anhydrous and low water systems (Zirlin and Karel, 1969; Chipault and Hawkins, 1971; Kanner and Karel, 1976), and accelerate oxidation of proteins. These reactions cause browning (Tappel, 1955; Zirlin and Karel, 1969; Karel, 1973; Pokorný *et al.*, 1974), insolubilisation (increased hydrophobicity of proteins) (Roubal and Tappel, 1966a; Funes and Karel, 1981), polymerization or cross-linking (Roubal and Tappel, 1966b; Funes and Karel, 1981; Leake and Karel, 1982), scission (Schaich and Karel, 1975), and formation of lipid–protein complexes (Davies, 1987; Howell *et al.*, 2001). The polymerization or cross-linking and formation of lipid–protein complexes alter water sorption properties, physical structure and physicochemical properties of dried foods (Karel, 1973; Koch, 1962). The higher molecular weight materials exhibit higher sorbed water content at low a_w than the lower molecular weight components (Roos, 1993; Potes *et al.*, 2012). Decreasing of a_w values of WPI−oil systems possibly resulted from oxidation of water and polymerization or formation of lipid−protein complexes. The formation of polymeric components also results in a higher sorbed water content, i.e., decreasing the a_w values during storage of the WPI–oil systems at a constant water content. Changes in protein conformation favouring hydrophobic interactions with oil and subsequent increase in hydrogen bonding of water to exposed hydrogen bonding sites can be another explanation for lowering of the a_w values. Degradation of lipids (lipolysis) by lipase enzymes or in the presence of radicals and the thermal treatment could also release fatty acids from triglycerides (consumption of water in the systems). Highly oxidized unsaturated fatty acids and impurities of the whey protein and SO could lead to hardening and brown coloured surface of WPI−SO after prolong storage at 40°C (Fig. 4.2A).

6.2.2. THERMAL PROPERTIES

The thermal properties of protein−oil systems before and during storage were determined from 10% (w/w) WPI dispersion (protein denaturation and aggregation) of samples. The temperatures of protein denaturation (endothermic transition) and heats of aggregation (exothermic transition) of WPI increased after mixing with oil before storage (Table 3.1 and Fig. 3.3). The presence of oil (OO and SO) in the WPI systems increased the thermal stability of the proteins, but enhanced protein aggregation. Jones *et al.* (2005) found that the presence of silicone oil in a protein dispersion caused conformational changes, alterations in thermal stability, and acceleration of protein [bovine serum albumin (BSA) and ribonuclease A] aggregation at pH 4.5, 6.5, and 7.2 before and after storage at 45°C. Storage at the higher temperature showed stronger effects on protein denaturation and aggregation than at 20°C (Fig. 3.4), especially for WPI−OO. During storage of WPI−OO for up to 14 weeks the protein showed decreased denaturation temperatures and heats of protein aggregation (size of exotherms) (Table 3.1 and Fig. 3.3 and 3.4), indicating changes in protein conformation and aggregation of the protein during storage (reduced poststorage heat of protein aggregation). Our results showed that the type of oil and the presence of natural antioxidants (e.g., tocopherols in SO) affected interactions of protein molecules that reduced oxidative aggregation (disulfide bonds). In accordance, the SO systems showed less changes of heats of protein denaturation and aggregation during storage as shown in Fig. 3.4.

6.2.3. PROTEIN STRUCTURE

Protein−oil systems showed a maximum formation of disulfide linkages (decreased total sulfhydryl content) and the highest protein oxidation (increased carbonyl content) in WPI–OO systems at 2 weeks of storage (Fig. 3.1 and 3.2). Our results agreed with Hidalgo and Kinsella (1989), who found that the presence of linoleic acid 13-hydroperoxide in β-lactoglobulin B in the buffer (pH 7.4) system caused formation of disulfide linkages of β-lactoglobulin B during incubation at 37°C for 24 h. Disulfide bonds form through oxidation of thiol groups (Shimada and Cheftel, 1988), and their formation can be markly influenced by the antioxidant activities of oil components. The systems containing SO showed less changes of carbonyl and total sulfhydryl contents

because of antioxidant activities of natural tocopherols typical of SO (Speek *et al.*, 1985) and found in our laboratory using high performance liquid chromatography (data not included). After 7 weeks of storage at both temperatures SO systems showed higher carbonyl contents than OO systems. Cucu *et al.* (2011) suggested that the ability of oils to promote carbonyl formation depended on the level of unsaturation of oil (in the order fish oil > oxidized soy bean oil > SO and soybean oil > OO) and its initial oxidation. Storage of protein−oil systems at 40°C for 14 weeks decreased solubilisation of the protein. This was a result of hydrophobic interactions of protein with oil, which increased protein surface hydrophobicity (Fig. 5.4B) and insolubilisation of the protein. Roubal and Tappel (1966b), Zirlin and Karel (1969), Schaich and Karel (1975), and Leake and Karel (1982) found that proteins in contact with peroxidized lipid or their secondary break down products changed functional properties of proteins and amino acids [insolubilisation, polymerization (addition of monomers from both native and denatured proteins) or cross-linking (at 0.60 to 0.75 a_w), and formation of lipid–protein complexes]. The carbonyl groups of oxidized lipids may participate in covalent bonding leading to the formation of stable protein–lipid aggregates (Saeed and Howell, 2002).

The protein–oil after 14 weeks of storage at 40°C showed formation of high molecular weights components via disulfide bonds (in the range of 29 to 36 kDa) in the non-reducing gel electrophoresis, especially for WPI–OO (Fig. 4.6). These compounds resulted from the aggregation (intermolecular aggregation) of denatured proteins in WPI (Fig. 5.4B), protein polymerization and lipid–protein complex formation from lipid and protein oxidation products during storage. The intermolecular aggregation of proteins increased surface hydrophobicity and irreversible aggregation of proteins (Lala and Kaul, 1992). The presence of oil in the WPI induced aggregation and conformational changes of proteins in WPI, which showed changes of secondary structure (α-helix, random coil, and β-sheet) of proteins after 14 weeks of storage at both temperatures (Fig. 5.4B). The lipid and protein oxidation lowered the hydration properties of protein (increased protein hydrophobicity or insolubility) in WPI–OO during storage at both temperatures (Fig. 5.3C and 5.4A).

6.3. PROTEIN−OIL−SUGAR INTERACTIONS IN HIGH SOLIDS SYSTEMS

6.3.1. PHYSICAL AND PHYSICOCHEMICAL PROPERTIES

Physical and physicochemical changes of systems containing sugar were strongly affected by temperature and storage time. The systems showed dramatic changes in colour, a_w, and texture with time at 40°C (Fig. 4.1, 4.2B, and 5.2). The changes of colour (dark brown) were dependent on the Maillard browning reactants. The systems containing SO showed more pronounced browning than OO systems, in accordance with the systems with no sugar. This confirmed that the NEB reactions of all systems containing oil at 40°C were affected by the fatty acid composition, and lipid and protein oxidation of the oil. The systems at the 40°C temperature showed slightly increased a_w during half time of storage and decreased thereafter. Water was a product of several condensation steps of the NEB between amino acids or proteins and reducing sugars increased covalent bonding that produced water causing an increase of a_w (Hodge, 1953; Eichner and Karel, 1972). The NEB could contribute to a_w values and hardening of the systems during the first period, in agreement with Gonzales *et al.* (2010). Carbonyl–amine polymerization, formation of stable coloured compounds, and cross-linking of proteins as well as oxidation of water could result in the lowering of the a_w during prolonged storage at 40°C. Mastrocola and Munari (2000) reported that the Maillard reaction was increased by the presence of the oil and its oxidation products in their systems (mixtures of pregelatinized corn starch, water, glucose, lysine, and soybean oil at a component ratio of 32:30:16:4:18) after storage at 25°C. In the present study, the Maillard reaction that occurred slowly at 20°C as shown in Fig. 4.1 increased yellowness.

The type of oil in the systems containing sugar affected the physical properties (appearance and structure) of the systems during storage at both temperatures, which was observed from the surface characteristic as shown in Fig. 4.2B. Especially SO systems showed surface flattening and smoothening. Karmas *et al.* (1992) and Buera and Karel (1995) found that structural or physical changes (collapse and shrinkage) of freeze-dried model systems of mixtures of sugar and high molecular weight polymers, as non-crystallizing matrices, with reacting sugar and amino acid increased the rate of NEB reaction and the sensitivity of the

reaction to temperature during storage. The differences of structure or texture could affect the diffusion of water or reactants and the rate of NEB of the WPI−oil−(G−F) systems during storage. The structural changes of the systems were divided into 3 stages including soft (0−2 weeks), formation of film or elastic (after 2−7 weeks), and hard and brittle or glassy solid (insoluble polymeric compounds) (after 7 weeks) structures (Fig. 5.2). The critical period of textural changes leading to hardening was in the first stage, which could relate to water migration. Zhou *et al.* (2008) found that a dramatic change of texture (hardening) at various temperatures coincided with increases of insoluble whey proteins. The aggregated network of proteins was reported as the cause of a significant hardening of texture during storage of the high protein system. The increase in hardening found during the first stage was also in line with the change in protein conformation and properties that could enhance hardening during storage at 40°C (Fig. 3.1, 3.2, and 5.2). The changes during prolong storage at 40°C were typical of extensive nonenzymatic browning reactions (NEB) or glycation (nonenzymatic glycosylation) and protein–oxidized fatty acids reactions (hydrophobic interaction) causing polymerization (protein aggregation or crosslinking) and formation of stable protein-lipid complexes or protein-lipid aggregates via covalent bonding (Hodge, 1953; Tappel, 1955; Tannenbaum *et al.*, 1969; Pokorný *et al.*, 1974; Davies, 1987; Hidalgo and Kinsella, 1989; Friedman, 1996; Howell *et al.*, 2001; Potes *et al.*, 2013b). The presence of OO enhanced hardening, darkening, and insolubilisation (increased hydrophobicity of protein), which were more obvious than in WPI−SO−(G–F). The presence of natural antioxidants (tocopherols or vitamin E) in SO could reduce textural and physical changes. This agreed with Potes *et al.* (2013a, b), who found that the interactions of protein molecules leading to oxidative aggregation or disulfides bond formation and NEB or Maillard reaction or glycation were reduced in the systems containing WPI, oil with tocopherols, and glucose–fructose syrup. The type of oil or the degree of its unsaturation can play an important role in hardening and physicochemical reactions of high protein systems during storage.

6.3.2. THERMAL PROPERTIES

Thermal properties of the systems containing sugar before and during storage were determined for 10% (w/w) WPI dispersions (protein denaturation and aggregation) and solid (protein hydration and denaturation) samples. The mixing of sugar with WPI (no oil) increased the T_{onset} and T_{peak} of protein denaturation and aggregation in protein dispersions (promoted thermal stability) (Table 3.1 and Fig. 3.3). Hydrophobic interactions of hydrophobic groups of proteins can become strengthened in the presence of sugars (Back *et al.*, 1979). Sugars in aqueous solutions increased surface tension and promoted preferential interaction of proteins (Arakawa and Timasheff, 1982). Boye and Alli (2000) found that glucose or fructose solution [20% (w/v)] stabilized α-lactalbumin/β-lactoglobulin proteins against thermal denaturation (increased denaturation temperature). The presence of sugar in WPI–oil systems showed smaller size of protein aggregation exotherms than the WPI–oil systems before storage (Fig. 3.3).

During storage for up to 14 weeks at 40°C the denaturation of protein in the 10% (w/w) protein of WPI−oil−(G−F) dispersions did not occur, indicating substantial conformational changes in WPI caused by glycation, nonenzymatic browning, and possible protein oxidation (increased carbonyl contents as shown in Fig. 3.1). The WPI−oil−(G−F) showed increased heat of protein aggregation during 7 weeks of storage, but aggregation decreased thereafter. Protein aggregation was induced by glycation and oxidation that often change physicochemical properties of proteins, such as hydrophobicity, secondary/tertiary structure, and inhibition of protein unfolding (Wang *et al.*, 2010). Our systems were insoluble in water after storage at 40°C, especially for the systems containing OO. A decrease of the heat of protein aggregation could result from polymerization of components in the systems, and formation of complex structures and loss of protein conformation needed for aggregation when heated in excess water.

The WPI−oil−(G−F) materials before storage showed the glass transition of the sugars and two irreversible exothermic transitions (Fig. 4.4). The systems showed broadening of the glass transition in an immediate reheating scan, which confirmed carbohydrate–protein interactions during the first heating, especially in the WPI–(G–F) system. The lower temperature exotherm indicated increased

hydrogen bonding of the protein to the G–F components, i.e., an increase in temperature induced rapid hydrogen bonding of water and sugar molecules to the dehydrated protein molecules (water migration from syrup to dry protein) as an exothermic process. The sugar component was hydrogen bonded to water and the assumption of protein hydration as an exothermic process was supported by a resultant broadening of the glass transition. The high temperature exotherm of the systems containing sugar was due to browning that resulted from the NEB reaction, in agreement with Raemy *et al.* (1983) and Roos *et al.* (1996).

The transition exotherms of the WPI−oil−(G−F) system found before storage appeared as endotherms after a long time of storage at 20°C or no transition (complete changes in protein conformation) after storage at 40°C (Fig. 4.5). The apparent changes in protein conformation during storage increased hydrophobicity of the system with insolubilisation of the protein. The oil phase in the WPI–(G−F) increased the glass transition of the sugars before storage, especially in the SO system (Fig. 4.5). It is known that at normal pH of milk β-lactoglobulin is present as dimers with hydrophobic linkages, and it has the capability to bind with nonpolar molecules including fatty acids (Madadlou and Azarikia, 2013). It may be assumed that in a WPI–(G–F) system the dimer structure and internal protein–protein hydrophobic interactions limited WPI–(G–F) interactions. That was detected from the T_g occurring around that measured for the G–F syrup alone. The WPI–SO–(G–F) included oil droplets on which protein could assembly hydrophobically towards the oil. In these systems, the water content was sufficient to allow for conformational changes of the proteins and exposure of hydrogen bonding sites towards the carbohydrate phase. As a result, the T_g occurred at a higher temperature confirming stronger hydrophilic protein–sugar interactions. The systems after storage demonstrated component interactions (broadened glass transition), and the oil induced changes to the glass transition in high protein systems, especially for the systems containing OO. Therefore the systems containing OO showed more pronounced changes of thermal properties during storage than SO systems, in agreement with the thermal properties of the protein–oil systems. The type of oil was concluded to affect interactions of protein molecules and the glass transition of the sugar phase of the systems.

6.3.3. PROTEIN STRUCTURE

The protein–oil–sugar systems showed the maximum formation of disulfide linkages at 2 weeks of storage. The carbonyl contents (protein oxidation) of the protein–oil–sugar systems after 4 weeks of storage at 20°C, and during storage for up to 7 weeks at 40°C, were higher than in the systems with no sugars at both temperatures (Fig. 3.1). The presence of glucose and fructose in the protein–oil systems could accelerate protein oxidation with resultant carbonyl formation. On the other hand, the carbonyl groups (aldehydes and ketones) of glucose and fructose were present and glycation of the protein by the reducing sugars could cause the substantial increase of carbonyls during storage. Glycation forms reactive carbonyl derivatives (ketoamines, ketoaldehydes, and deoxyosones), which promote oxidation of proteins (Berlett and Stadtman, 1997). Takagi *et al.* (1995) found that fatty-acid-free bovine serum albumin (BSA) dispersion incubated with glucose or fructose at 37°C for up to 2 weeks increased carbonyls and fluorescent albumin (advanced glycation end products), which did not occur when BSA was incubated in the absence of reducing sugars. The results in Fig. 3.1 suggested that during storage at 40°C a higher level of protein oxidation occurred in systems with a higher level of unsaturated fatty acids. The WPI–OO– or WPI–SO–(G–F) system after 4 or 7 weeks, respectively, of storage at 40°C became insoluble in water as a result of polymerization, hydrophobic interactions, and hardened and brittle material was obtained. The presence of G–F in the WPI–oil systems could induce protein hydrophobicity and hydrophobic interactions of components leading to insolubilisation. Insolubilisation resulted from complex reactions between oxidized protein–lipid compounds (Roubal and Tappel, 1966a; Funes and Karel, 1981) and products of Maillard reaction (at the advanced stage of the reaction) or advanced glycation end products (Kato *et al.*, 1986; Liang and Rossi, 1990).

The protein–oil–sugar after 14 weeks of storage at low temperature showed formation of high molecular weight protein polymers over the range of 29 to 45 kDa in both reducing and non-reducing SDS PAGE gels (Fig.4.6). Our results indicated protein modifications (lower solubility or decreased mobility of proteins on the gels), polymerization, component interactions via glycation (nonenzymatic glycosylation) or Maillard reaction leading to aggregation and

covalent cross-linking (nondisulfide covalent bonds) of the proteins, and insolubilization during storage of the WPI–oil–(G–F) systems. Rich and Foegeding (2000) demonstrated that the Maillard gel of WPI and ribose at pH 9 showed lower solubility, and diffused bands of protein in SDS-PAGE under reducing condition. Mohammed *et al.* (2000) found that increasing heating temperature decreased solubility of WPI and increased covalent crosslinks of β-lactoglobulin. The systems at 40°C after 14 weeks of storage were insoluble in buffers with and without 2-β-mercaptoethanol. The study confirmed that protein–oil–sugar system had strong intermolecular hydrophobic interactions, which resulted in hydrophobicity of the WPI–oil–(G–F) systems that increased with storage time and temperature. These results were in accordance with a smaller size of the protein hydration relaxation magnitudes (Fig. 5.3D), and lowered the hydration properties of protein with time and temperature [in order of WPI–OO–(G–F) 40°C, 19 weeks < WPI–OO–(G–F) 20°C, 19 weeks < WPI–OO–(G–F) before storage < WPI–(G–F) before storage] in the FTIR spectra (Fig. 5.4A). The hydration properties of protein depended on a_w or water content and the presence of others non-protein components in the systems. This corresponded to lowered T_α for the protein hydration in the DMA analysis with increasing a_w and presence of the sugars in the systems (Fig. 5.3A and B). The WPI–OO–(G–F) after 14 weeks of storage showed loss of protein hydration according to the DMA data (Fig. 5.3D), and intermolecular aggregation of denatured proteins and conformational changes of β-sheet structure of globular proteins (α-lactalbumin and β-lactoglobulin) (Fig. 5.4B). The polymerization of protein–oil–sugar systems after storage at 40°C caused merged relaxations of the sugar and oil components and confined water within the proteins of WPI (Fig. 5.3D). This possibly was a result from the NEB or Maillard reaction or glycation. The dramatic changes in dynamic mechanical properties corresponded to browning and hardening of the protein–oil–sugar systems during storage at 40°C (Fig. 4.2 and 5.2).

6.4. APPLICATIONS OF THE RESEARCH OUTCOMES

The results from our studies are useful in understanding interactions of food components such as carbohydrate–carbohydrate, protein–oil, protein–sugar, and protein–oil–sugar; and protein behaviour (hydration, denaturation, and

aggregation) in models of high solids systems at various conditions during storage. This can be used to explain physical, physicochemical, and thermal properties, and structural changes of proteins that are affected by chemical reactions [lipid and protein oxidation, and Maillard reaction) in foods (e.g., confectionary products, hard cheese products, infant formulae, dairy powders and ingredients, high protein snack bars and high protein foods (included sausages, dried-meat, frozen fish and meat products), supplements (reconstituted and food mixed powder as energy fuel beverages and muscle building)] and pharmaceutical products during thermal storage, and *in vivo* {development of chronic diseases (e.g., diabetes, renal failure, and rheumatoid arthritis) and age-related diseases [atherosclerosis and coronary artery, neurodegenerative (e.g., Alzheimer, Huntington, and Parkinson diseases), and uremia diseases]}. In conclusion, the outcomes of our studies can be applied in various areas such as food, medicine, membrane, and nutritional and pharmaceutical sciences to design and develop high solids formulations suitable as foods and drugs, drug and food delivery systems, and to improve quality and nutrient delivery in diet.

6.5. OVERALL CONCLUSIONS

The present study investigated factors that in food systems may result in time-dependent loss of quality. The research included studies of various physicochemical, structural, and thermal changes giving the following overall conclusions:

- A smaller molecular size (high DE and high number average molecular weight) and a high concentration of MD decreased the rate of lactose crystallization and loss of sorbed water in amorphous systems.
- The sorbed water contents of amorphous lactose-MD systems were proportional to quantities sorbed by the individual component.
- Crystallization of lactose at $T > T_{\alpha}$ showed a sharp frequency-independent change of ε'' in dielectric analysis data and E'' in dynamic mechanical analysis data. Increasing the MD concentration increased the temperature of lactose crystallization in dielectric and dynamic mechanical analyses.

- The smaller carbohydrate molecules of high DE MD components showed a higher molecular mobility in lactose−MD systems in the dielectric analysis data at high frequencies.
- Systems that showed similar relaxation times could exhibit different rates of lactose crystallization, i.e., the MD with high DE as the system component showed a lower rate of loss of water during lactose crystallization.
- Storage at 40°C promoted changes of colour and a_w, decrease of solubility, denaturation and aggregation of protein as measured in reconstituted WPI–oil and WPI–oil–sugars systems, and altered hydration properties of protein (changes of protein conformation) and broadened glass transition temperature of sugars (component interactions) of WPI–oil–sugars.
- Dynamic mechanical properties of WPI systems in the presence of oil (hydrophobic) or/and sugars (hydrophilic) showed altered hydration properties of proteins, increased protein hydrophobicity and hydrophobic interactions of components, especially after storage at 40°C, in accordance with differential scanning calorimetry data and FTIR spectra.
- The dynamic mechanical analysis data showed the α-relaxation of confined water of the protein molecules.
- The protein oxidation and Maillard reaction or glycation showed significant effects on colour (browning) and a_w, protein conformation and hydrophobicity, cooperative protein oxidation and disulfide bonding, resultant size distribution or molecular weight of proteins, hardening of texture, and broadened glass transition of sugars during storage, which for storage at 40°C were more pronounced than at 20°C.
- The type of oil affected physical appearance and structure, interactions of protein molecules [the systems containing oil with natural antioxidants reduced oxidative aggregation (disulfide bonds)], thermal properties (heats of protein denaturation and aggregation), the glass transition of the sugar phase, solubility, and interactions of water–protein–sugars–oil components of the systems during storage.

BIBLIOGRAPHY

Acevedo, N.C.; Schebor, C.; Buera, P. Non-enzymatic browning kinetics analysed through water-solid interactions and water mobility in dehydrated potato. *Food Chem.* **2008**, *108*, 900-906.

Addis, P.B. Occurrence of lipid oxidation products in foods. *Food Chem. Toxic.* **1986**, *24*, 1021-1030.

Ahmed, M.U.; Thorpe, S.R.; Baynes, J.W. Identification of N^{ε}-carboxylethyllysine as a degradation product of fructoselysine in glycated protein. *J. Biol. Chem.* **1986**, *261*, 4889-4894.

Ajandouz, E.H.; Puigserver, A. Nonenzymatic browning reaction of essential amino acids: effect of pH on caramelization and Maillard reaction kinetics. *J. Agric. Food Chem.* **1999**, *47*, 1786-1793.

Ajandouz, E.H.; Tchiakpe, L.S.; Dalle Ore, F.; Benajiba, A.; Puigserver, A. Effects of pH on caramelization and Maillard reaction kinetics in fructose-lysine model systems. *J. Food Sci.* **2001**, *66*, 926-931.

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

Alie, J.; Menegotto, J.; Cardon, P.; Duplaa, H.; Caron, A.; Lacabanne, C.; Bauer, M. Dielectric study of the molecular mobility and the isothermal crystallization kinetics of an amorphous pharmaceutical drug substance. *J. Pharm. Sci.* **2004**, *93*, 218-233.

Allison, S.D.; Chang, B.; Randolph, T.W.; Carpenter, J.F. Hydrogen bonding between sugar and protein is responsible for inhibition of dehydration-induced protein unfolding. *Arch. Biochem. Biophys.* **1999**, *365*, 289-298.

Ames, J.M. Melanoidins as pro- or antioxidants. *Cerevisia* **2001**, *26*, 210-216.

Amici, A.; Levine, R.L.; Tsai, L.; Stadtman, E.R. Conversion of amino acid residues in proteins and amino acid homopolymers to carbonyl derivatives by metal-catalyzed oxidation reactions. *J. Biol. Chem.* **1989**, *264*, 3341-3346.

Anderson, M.M.; Hazen, S.L.; Hsu, F.F.; Heinecke, J.W. Human neutrophils employ the myeloperoxidase-hydrogen peroxide-chloride system to convert hydroxyl-amino acids into glycolaldehyde, 2-hydroxypropanol, and acrolein. *J. Clin. Invest.* **1997**, *99*, 424-432.

Anema, S.G.; Pinder, D.N.; Hunter, R.J.; Hemar, Y. Effects of storage temperature on the solubility of milk protein concentrate (MPC85). *Food Hydrocoll.* **2006**, *20*, 386-393.

Angell, C.A.; Bressel, R.D.; Green, J.L.; Kanno, H.; Oguni, M.; Sare, E.J. Liquid fragility and the glass transition in water and aqueous solutions. *J. Food Eng.* **1994**, *22*, 115-142.

Angell, C.A. Why C_1 = 16-17 in the WLF equation is physical-and the fragility of polymers. *Polymer* **1997**, *38*, 6261-6266.

Arakawa, T.; Timasheff, S.N. Stabilization of protein structure by sugars. *Biochemistry* **1982**, *21*, 6536-6544.

Ashoor, S.H.; Zent, J.B. Maillard browning of common amino acids and sugars. *J. Food Sci.* **1984**, *49*, 1206-1207.

Avaltroni, F.; Bouquerand, P.E.; Normand, V. Maltodextrin molecular weight distribution influence on the glass transition temperature and viscosity in aqueous solutions. *Carbohydr. Polym.* **2004**, *58*, 323-334.

Back, J.F.; Oakenfull, D.; Smith, M.B. Increased thermal stability of proteins in the presence of sugars and polyols. *Biochemistry* **1979**, *18*, 5191-5196.

Baier, S.; McClements, D.J. Impact of preferential interactions on thermal stability and gelation of bovine serum albumin in aqueous sucrose solutions. *J. Agric. Food Chem.* **2001**, *49*, 2600-2608.

Baisier, W.M.; Labuza, T.P. Maillard browning kinetics in a liquid model system. *J. Agric. Food Chem.* **1992**, *40*, 707-713.

Balasundram, N.; Sundram, K.; Samman, S. Phenolic compounds in plants and agri-industrial by-products: Antioxidant activity, occurrence, and potential uses. *Food Chem.* **2006**, *99*, 191-203.

Ball, C.D.; Hardt, C.R.; Duddles, W.J. The influence of sugars on the formation of sulfhydryl groups in heat denaturation and heat coagulation of egg albumin. *J. Biol. Chem.* **1943**, *151*, 163-169.

Barone, G.; Giancola, C.; Verdoliva, A. DSC studies on the denaturation and aggregation of serum albumins. *Thermochim. Acta* **1992**, *199*, 197-205.

Baskol, G.; Demir, H.; Baskol, M.; Kilic, E.; Ates, F.; Karakukcu, C.; Ustdal, M. Investigation of protein oxidation and lipid peroxidation in patients with rheumatoid arthritis. *Cell Biochem. Funct.* **2006**, *24*, 307-311.

Baxter, J.H. Free amino acid stability in reducing sugar systems. *J. Food Sci.* **1995**, *60*, 405-408.

Bedell-Hogan, D.; Trackman, P.; Abrams, W.; Rosenbloom, J.; Kagan, H. Oxidation, cross-linking, and insolubilization of recombinant tropoelastin by purified lysyl oxidase. *J. Biol. Chem.* **1993**, *268*, 10345-10350.

Beisswenger, P.J.; Moore, L.L.; Brinck-Johnsen, T.; Curphey, T.J. Increased collagen-linked pentosidine levels and advanced glycosylation end products in early diabetic nephropathy. *J. Clin. Invest.* **1993**, *92*, 212-217.

Bell, L.N. Kinetics of non-enzymatic browning in amorphous solids systems: distinguishing the effects of water activity and the glass transition. *Food Res. Int.* **1996**, *28*, 591-597.

Bell, L.N. Maillard reaction as influenced by buffer type and concentration. *Food Chem.* **1997**, *59*, 143-147.

Bellavia, G.; Giuffrida, S.; Cottone, G.; Cupane, A.; Cordone, L. Protein thermal denaturation and matrix glass transition in different protein-trehalose-water systems. *J. Phys. Chem. B* **2011**, *115*, 6340-6346.

Bellissent-Funel, M.-C. Hydration in protein dynamics and function. *J. Mol. Liq.* **2000**, *84*, 39-52.

Benjakul, S.; Lertittikul, W.; Bauer, F. Antioxidant activity of Maillard reaction products from a porcine plasma protein-sugar model system. *Food Chem.* **2005b**, *93*, 189-196.

Benjakul, S.; Visessanguan, W.; Phongkanpai, V.; Tanaka, M. Antioxidative activity of caramelisation products and their preventive effect on lipid oxidation in fish mince. *Food Chem.* **2005a**, *90*, 231-239.

Berendsen, H.J.C.; Postma, J.P.M.; van Gunsteren, W.F.; Hermans, J. Interaction Models for water in relation to protein hydration. In *Intermolecular Forces*; Pullman, B., Ed.; D. Reidel Publishing Co.: Dordrecht, The Netherlands, 1981; pp 331-342.

Berlett, B.S.; Stadtman, E.R. Protein oxidation in aging, disease, and oxidative stress. *J. Biol. Chem.* **1997**, *272*, 20313-20316.

Bhattacharjee, N.; Biswas, P. Structure of hydration water in proteins: a comparison of molecular dynamics simulations and database analysis. *Biophys. Chem.* **2011**, *158*, 73-80.

Bhoit-Solomon, V.; Kessler-Icekson, G.; Shaklai, N. Peroxidative crosslinking of myosins. *Biochem. Int.* **1992**, *26*, 181-189.

Bhugra, C.; Rambhatla, S.; Bakri, A.; Duddu, S.P.; Miller, D.P.; Pikal, M.J.; Lechuga-Ballesteros, D. Prediction of the onset of crystallization of amorphous sucrose below the calorimetric glass transition temperature from correlations with mobility. *J. Pharm. Sci.* **2007**, *96*, 1258-1269.

Blanch, E.W.; Bell, A.F.; Hecht, L.; Day, L.A.; Barron, L.D. Raman optical activity of filamentous bacteriopharges: hydration of α-helices. *J. Mol. Biol.* **1999**, *290*, 1-7.

Boye, J.I.; Alli, I.; Ismali, A.A. Use of differential scanning calorimetry and infrared spectroscopy in the study of thermal and structural stability of α-lactalbumin. *J. Agric. Food Chem.* **1997a**, *45*, 1116-1125.

Boye, J.I.; Alli, I.; Ismali, A.A.; Gibbs, B.F.; Konishi, Y. Factors affecting molecular characteristics of whey protein gelation. *Int. Dairy J.* **1995**, *5*, 337-353.

Boye, J.I.; Ma C.-Y.; Ismail, A.; Harwalkar, V.R.; Kalab, M. Molecular and microstructural studies of thermal denaturation and gelation of β-lactoglobulins A and B. *J. Agric. Food Chem.* **1997b**, *45*, 1608-1618.

Boye, J.I.; Alli, I. Thermal denaturation of mixtures of α-lactalbumin and β-lactoglobulin: a differential scanning calorimetric study. *Food Res. Int.* **2000**, *33*, 673-682.

Breitling-Utzmann, C.M.; Unger, A.; Friedl, D.A.; Lederer, M.O. Identification and quantification of phosphatidylethanolamine-derived glucosylamines and aminoketoses from human erythrocytes-influence of glycation products on lipid peroxidation. *Arch. Biochem. Biophys.* **2001**, *391*, 245-254.

Briganti, S.; Picardo, M. Antioxidant activity, lipid peroxidation and skin diseases. What's new. *J. Eur. Acad. Dermatol. Venereol.* **2003**, *17*, 663-669.

Bronlund, J.; Paterson, T. Moisture sorption isotherms for crystalline, amorphous and predominantly crystalline lactose powders. *Int. Dairy J.* **2004**, *14*, 247-254.

Bucala, R.; Makita, Z.; Koschinsky, T.; Cerami, A.; Vlassara, H. Lipid advanced glycosylation: Pathway of lipid oxidation in *vivo*. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 6434-6438.

Buera, M.P.; Karel, M. Effect of physical changes on the rates of nonenzymatic browning and related reactions. *Food Chem.* **1995**, *52*, 167-173.

Buera, M.P.; Levi, G.; Karel, M. Glass transition in poly(vinylpyrrolidone): effect of molecular weight and diluents. *Biotechnol. Prog.* **1992**, *8*, 144-148.

Buera, P.; Schebor, C.; Elizalde, B. Effects of carbohydrate crystallization on stability of dehydrated foods and ingredient formulations. *J. Food Eng.* **2005**, *67*, 157-165.

Bunn, H.F.; Higgins, P.J. Reaction of monosaccharides with proteins: possible evolutionary significances. *Science* **1981**, *213*, 222-224.

Burin, L.; Jouppila, K.; Roos, Y.; Kansikas, J.; Buera, M.P. Color formation in dehydrated modified whey powder systems as affected by compression and T_g. *J. Agric. Food Chem.* **2000**, *48*, 5263-5268.

Burin, L.; Jouppila, K.; Roos, Y.H.; Kansikas, J.; Buera, M.P. Retention of β-galactosidase activity as realated to Maillard reaction, lactose crystallization, collapse and glass transition in low moisture whey systems. *Int. Dairy J.* **2004**, *14*, 517-525.

Buss, H.; Chan, T.P.; Sluis, K.B.; Domigan, N.M.; Winterbourn, C.C. Protein carbonyl measurement by a sensitive ELISA method. *Free Radic. Biol. Med.* **1997**, *23*, 361-366.

Butterfield, D.A.; Kanski, J. Brain protein oxidation in age-related neurodegenerative disorders that are associated with aggregated proteins. *Mech. Ageing Dev.* **2001**, *122*, 945-962.

Byler, D.M.; Susi, H. Examination of the secondary structure of proteins by deconvolved FTIR spectra. *Biopolymers* **1986**, *25*, 469-487.

Cardona, S.; Schebor, C.; Buera, M.P.; Karel, M.; Chirife, J. Thermal stability of invertase in reduced-moisture amorphous matrices in relation to glassy state and trehalose crystallization. *J. Food Sci.* **1997**, *62*, 105-112.

Carney, J.M.; Starke-Reed, P.E.; Oliver, C.N.; Landum, R.W.; Cheng, M.S.; Wu, J.F.; Floyd, R.A. Reversal of age-related increase in brain protein oxidation, decrease in enzyme activity, and loss in temporal and spatial memory by chronic administration of the spin-trapping compound N-tert-butyl-alpha-phenylnitrone. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 3633-3636.

Carpenter, J.F.; Crowe, J.H. An infrared spectroscopic study of the interactions of carbohydrates with dried proteins. *Biochemistry* **1989**, *28*, 3916-3922.

Carpenter, J.F.; Crowe, L.M.; Crowe, J.H. Stabilization of phosphofructokinase with sugars during freeze-drying: Characterization of enhanced protection in the presence of divalent cations. *Biochim. Biophys. Acta* **1987a**, *923*, 109-115.

Carpenter, J.F.; Martin, B.; Crowe, L.M.; Crowe, J.H. Stabilization of phosphofructokinase during air-drying with sugars and sugar/transition metal mixtures. *Cryobiology* **1987b**, *24*, 455-464.

Chang, L.; Shepherd, D.; Sun, J.; Ouellette, D.; Grant, K.L.; Tang, X.; Pikal, M.J. Mechanism of protein stabilization by sugars during freeze-drying and storage: Native structure preservation, specific interaction, and/or immobilization in a glassy matrix? *J. Pharm. Sci.* **2005**, *94*, 1427-1444.

Chao, C.-C.; Ma, Y.-S.; Stadtman, E.R. Modification of protein surface hydrophobicity and methionine oxidation by oxidative systems. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 2969-2974.

Chen, S.-H.; Liu, L.; Fratini, E.; Baglioni, P.; Faraone, A.; Mamontov, E. Observation of fragile-to-strong dynamic crossover in protein hydration water. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 9012-9016.

Chen, S.-L.; Yang, D.-J.; Chen, H.-Y.; Liu, S.-C. Effect of hot acidic fructose solution on caramelisation intermediates including colour, hydroxymethylfurfural and antioxidative activity changes. *Food Chem.* **2009**, *114*, 582-588.

Chen, Y.-J.; Liang, L.; Liu, X.-M.; Labuza, T.P.; Zhou, P. Effect of fructose and glucose on glycation of β-lactoglobulin in an intermediate-moisture food model system: analysis by liquid chromatography-mass spectrometry (LC-MS) and data-independent acquisition LC-MS (LC-MS^E). *J. Agric. Food Chem.* **2012**, *60*, 10674-10682.

Chipault, J.R.; Hawkins, J.M. Lipid autoxidation in freeze-dried meats. *J. Agric. Food Chem.* **1971**, *19*, 495-499.

Chio, K.S.; Tappel, A.L. Inactivation of ribonuclease and other enzymes by peroxidizing lipids and by malonaldehyde. *Biochemistry* **1969**, *8*, 2827-2832.

Chou, D.H.; Morr, C.V. Protein-water interactions and functional properties. *J. Am. Oil Chem. Soc.* **1979**, *56*, 53-62.

Coates, J. Interpretation of infrared spectra, A practical approach. In *Encyclopedia of Analytical Chemistry*; Meyers, R.A., Ed.; John Wiley & Sons, Ltd.: Chichester, U.K., 2000; pp 10815-10837.

Coenen, J.W.E. Hydrogenation of edible oils. *J. Am. Oil Chemists' Soc.* **1976**, *53*, 382-389.

Colaco, C.A.; Roser, B.J. Atherosclerosis and glycation. *Bioessays.* **1994**, *16*, 145-147.

Combet, S.; Zanotti, J.-M.; Bellissent-Funel, M.-C. Temperature- and hydration-dependent internal dynamics of stripped human erythrocyte vesicles studied by incoherent neutron scattering. *Biochim. Biophys. Acta* **2011**, *1810*, 202-210.

Creeth, J.M.; Cooper, B.; Donald, A.S.; Clamp, J.R. Studies of the limited degradation of mucus glycoproteins. The effect of dilute hydrogen peroxide. *Biochem. J.* **1983**, *211*, 323-332.

Cucu, T.; Devreese B.; Mestdagh, F.; Kerkaert, B.; Meulenaer, B.D. Protein-lipid interactions during the incubation of whey proteins with autoxidizing lipids. *Int. Dairy J.* **2011**, *21*, 427-433.

Dalle-Donne, I.; Rossi, R.; Giustarini, D.; Milzani, A.; Colombo, R. Protein carbonyl groups as biomarkers of oxidative stress. *Clin. Chim. Acta* **2003**, *329*, 23-38.

Davies, K.J.A. Protein damage and degradation by oxygen radicals. *J. Biol. Chem.* **1987**, *262*, 9895-9901.

Decker, E.A.; Xiong, Y.L.; Calvert, J.T.; Crum, A.D.; Blanchard, S.P. Chemical, physical, and functional properties of oxidized turkey white muscle myofibrillar proteins. *J. Agric. Food Chem.* **1993**, *41*, 186-189.

Degenhardt, T.P.; Grass, L.; Reddy, S.; Thorpe, S.R.; Diamandis, E.P.; Baynes, J.W. The serum concentration of the advanced glycation end product N^{ε}-(carboxymethyl)lysine is increased in uremia. *Kidney Int.* **1997**, *52*, 1064-1067.

Dellerue, S.; Bellissent-Funel, M.-C. Relaxational dynamics of water molecules at protein surface. *Chem. Phys.* **2000**, *258*, 315-325.

Denisov, V.P.; Halle, B. Protein hydration dynamics in aqueous solution. *Faraday Discuss.* **1996**, *103*, 227-244.

DePaz, R.A.; Dale, D.A.; Barnett, C.C.; Carpenter, J.F.; Gaertner, A.L.; Randolph, T.W. Effects of drying methods and additives on the structure, function, and storage stability of subtilisin: Role of protein conformation and molecular mobility. *Enzyme Microb. Technol.* **2002**, *31*, 765-774.

Dills, W.L. Protein fructosylation: Fructose and the Maillard reaction. *Am. J. Clin. Nutr.* **1993**, *58*, 779S-787S.

Dong, A.; Huang, P.; Caughey, W.S. Protein secondary structures in water from second-derivative amide I infrared spectra. *Biochemistry* **1990**, *29*, 3303-3308.

Doster, W.; Cusack, S.; Petry, W. Dynamical transition of myoglobin revealed by inelastic neutron scattering. *Nature* **1989**, *337*, 754-756.

Drusch, S.; Serfert, Y.; Van Den Heuvel, A.; Schwarz, K. Physicochemical characterization and oxidative stability of fish oil encapsulated in an amorphous matrix containing trehalose. *Food Res. Int.* **2006**, *39*, 807-815.

Dyer, D.G.; Blackledge, J.A.; Thorpe, S.R.; Baynes, J.W. Formation of pentosidine during nonenzymatic browning of proteins by glucose. Identification of glucose and other carbohydrates as possible precursors of pentosidine in vivo. *J. Biol. Chem.* **1991**, *266*, 11654-11660.

Dyer, D.G.; Dunn, J.A.; Thorpe, S.R.; Bailie, K.E.; Lyons, T.J.; McCance, D.R.; Baynes, J.W. Accumulation of Maillard reaction products in skin collagen in diabetes and aging. *J. Clin. Invest.* **1993**, *91*, 2463-2469.

Dzwolak, W.; Ravindra, R.; Lendermann, J.; Winter, R. Aggregation of bovine insulin probed by DSC/PPC calorimetry and FTIR spectroscopy. *Biochemistry* **2003**, *42*, 11347-11355.

Easa, A.M.; Hill, S.E.; Mitchell, J.R.; Taylor, A.J. Bovine serum albumin gelation as a result of the Maillard reaction. *Food Hydrocoll.* **1996**, *10*, 199-202.

Emken, E.A. Nutrition and biochemistry of trans and positional fatty acid isomers in hydrogenated oils. *Ann. Rev. Nutr.* **1984**, *4*, 339-376.

Ermolina, I.; Polygalov, E.; Bland, C.; Smith, G. Dielectric spectroscopy of low-loss sugar lophiles: II. Relaxation mechanisms in freeze-dried lactose and lactose monohydrate. *J. Non-Cryst. Solids* **2007**, *353*, 4485-4491.

Eichner, K.; Karel, M. The influence of water content and water activity on the sugar-amino browning reaction in model systems under various conditions. *J. Agric. Food Chem.* **1972**, *20*, 218-223.

Farahnaky, A.; Badii, F.; Farhat, I.A.; Mitchell, J.R.; Hill, S.E. Enthalpy relaxation of bovine serum albumin and implications for its storage in the glassy stage. *Biopolymer* **2005**, *78*, 69-77.

Farhat, I.A.; Orset, S.; Moreau, P.; Blanshard, J.M.V. FTIR study of hydration phenomena in protein-sugar systems. *J. Colloid Interface Sci.* **1998**, *207*, 200-208.

Fitzsimons, S.M.; Mulvihill, D.M.; Morris, E.R. Denaturation and aggregation processes in thermal gelation of whey proteins resolved by differential scanning calorimetry. *Food Hydrocoll.* **2007**, *21*, 638-644.

Franzen, K.; Singh, R.K.; Okos, M.R. Kinetics of nonenzymatic browning in dried skim milk. *J. Food Eng.* **1990**, *11*, 225-239.

Friedman, M. Food browning and its prevention: an overview. *J. Agric. Food Chem.* **1996**, *44*, 631-653.

Fry, L.K.; Stegink, L.D. Formation of Maillard reaction products in parenteral alimentation solutions. *J. Nutr.* **1982**, *112*, 1631-1637.

Fu, M.-X.; Knecht, K.J.; Thorpe, S.R.; Baynes, J.W. Role of oxygen in cross-linking and chemical modification of collagen by glucose. *Diabetes* **1992**, *41*, 42-48.

Funes, J.; Karel, M. Free radical polymerization and lipid binding of Lysozyme reacted with peroxidizing linoleic acid. *Lipids* **1981**, *16*, 347-350.

Funes, J.A.; Weiss, U.; Karel, M. Effects of reaction conditions and reactant concentrations on polymerization of lysozyme reacted with peroxidizing lipids. *J. Agric. Food Chem.* **1982**, *30*, 1204-1208.

Gabarra, P.; Hartel, R.W. Corn syrup solids and their saccharide fractions affect crystallization of amorphous sucrose. *J. Food Sci.* **1998**, *63*, 523-528.

Gardner, H.W. Lipid hydroperoxides reactivity with proteins and amino acids: A review. *J. Agric. Food Chem.* **1979**, *27*, 220-229.

Garland, D.; Russel, P.; Zigler, J.S. 1988. The oxidative modification of lens proteins. *Basic Life Sci.* **1988**, *49*, 347-352.

Garrison, W.M. Reaction mechanisms in the radiolysis of peptides, polypeptides, and proteins. *Chem. Rev.* **1987**, *87*, 381-398.

Ghanem, A.A.; Elewa, A.; Arafa, L.F. Pentosidine and N-carboxymethyl-lysine: Biomarkers for type 2 diabetic retinopathy. *Eur. J. Opthalmol.* **2011**, *21*, 48-54.

Giordano, R.; Teixeira, J.; Wanderlingh, U. Dynamics of water on protein surfaces. *Phys. B* **1995**, *213&214*, 769-771.

Gökmen, V.; Şenyuva, H.Z. Acrylamide formation is prevented by divalent cations during the Maillard reaction. *Food Chem.* **2007**, *103*, 196-203.

Gomyo, T.; Horikoshi, M. On the interaction of melanoidin with metallic ions. *Agr. Biol. Chem.* **1976**, *40*, 33-40.

Gonnet, J-M.; Guillet, J.; Sirakov, I.; Fulchiron, R.; Seytre, G. "In-situ" monitoring of the non-isothermal crystallization of polymers by dielectric spectroscopy. *Polym. Eng. Sci.* **2002**, *42*, 1159-1170.

Gonzales, A.S.P.; Naranjo, G.B.; Leiva, G.E.; Malec, L.S. Maillard reaction kinetics in milk powder: Effect of water activity at mild temperatures. *Int. Dairy J.* **2010**, *20*, 40-45.

Grandhee, S.K.; Monnier, V.M. Mechanism of formation of the Maillard protein cross-link pentosidine. *J. Biol. Chem.* **1991**, 11649-11653.

Greene, B.E. Lipid oxidation and pigment changes in raw beef. *J. Food Sci.* **1969**, *34*, 110-113.

Grossfield, A.; Pitman, M.C.; Feller, S.E.; Soubias, O.; Gawrisch, K. Internal hydration increases during activation of the G-protein-coupled receptor rhodopsin. *J. Mol. Biol.* **2008**, *381*, 478-486.

Halle, B. Protein hydration dynamics in solution: a critical survey. *Phil. Trans. R. Soc. Lond. B* **2004**, *359*, 1207-1224.

Handa, A.; Kuroda, N. Functional improvements in dried egg white through Maillard reaction. *J. Agric. Food Chem.* **1999**, *47*, 1845-1850.

Haque, E.; Whittaker, A.K.; Gidley, M.J.; Deeth, H.C.; Fibrianto, K.; Bhandari, B.R. Kinetics of enthaipy relaxation of milk protein concentrate powder upon ageing and its effect on solubility. *Food Chem.* **2012**, *134*, 1368-1373.

Haque, MD.K.; Roos, Y.H. Water plasticization and crystallization of lactose in spray-dried lactose/protein mixtures. *J. Food Sci.* **2004**, *69*, 23-29.

Haque, MD.K.; Roos, Y.H. Crystallization and X-ray diffraction of spray-dried and freeze-dried amorphous lactose. *Carbohydr. Res.* **2005a**, *340*, 293-301.

Haque, MD.K.; Roos, Y.H. Crystallization and X-ray diffraction of crystals formed in water-plasticized amorphous spray-dried and freeze-dried lactose/protein mixtures. *J. Food Sci.* **2005b**, *70*, 359-366.

Haque, MD.K.; Roos, Y.H. Differences in the physical state and thermal behavior of spray-dried and freeze-dried lactose and lactose/protein mixtures. *Innov. Food Sci. Emerg. Technol.* **2006**, *7*, 62-73.

Haris, P.I.; Severcan, F. FTIR spectroscopic characterization of protein structure in aqueous and non-aqueous media. *J. Mol. Catal. B: Enzym.* **1999**, *7*, 207-221.

Hayase, F.; Nagarai, R.H.; Miyata, S.; Njoroge, F.G.; Monnier, V.M. Aging of proteins: immunological detection of a glucose-derived pyrrole frmed during Maillard reaction in vivo. *J. Biol. Chem.* **1989**, *263*, 3758-3764.

Hayase, F.; Shibuya, T.; Sato, J.; Yamamoto, M. Effects of oxygen and transition metals on the advanced Maillard reaction of proteins with glucose. *Biosci. Biotech. Biochem.* **1996**, *60*, 1820-1825.

Hellman, K.; Miller, D.S.; Cammack, K.A. The effect of freeze-drying on the quaternary structure of L-asparaginase from *Erwinia carotovora*. *Biochim. Biophys. Acta* **1983**, *749*, 133-142.

Hidalgo, F.J.; Kinsella, J.E. Changes induced in β-lactoglobulin B following interactions with linoleic acid 13-hydroperoxide. *J. Agric. Food Chem.* **1989**, *37*, 860-866.

Hidalgo, F.J.; Zamora, R. The role of lipids in nonenzymatic browning. *Grasas Aceites* **2000**, *51*, 35-49.

Hirata, K.; Kubo, K. Relationship between blood levels of N-carboxymethyl-lysine and pentosidine and the severity of microangiopathy in type 2 diabetes. *Endocr. J.* **2004**, *51*, 537-544.

Hodge, J.E. Dehydrated foods, chemistry of browning reactions in model systems. *J. Agric. Food Chem.* **1953**, *1*, 928-943.

Hofmann, T.; Bors, W.; Stettmaier, K. Studies on radical intermediates in the early stage of the nonenzymatic browning reaction of carbohydrates and amino acids. *J. Agric. Food Chem.* **1999**, *47*, 379-390.

Horiuchi, S.; Advanced glycation end products (AGE)-modified proteins and their potential relevance to atherosclerosis. *Trends Cardiovasc. Med.* **1996**, *6*, 163-168.

Howell, N.K.; Herman, H.; Li-Chan, E.C.Y. Elucidation of protein-lipid interactions in a lysozyme-corn oil system by Fourier Transform Raman Spectroscopy. *J. Agric. Food Chem.* **2001**, *49*, 1529-1533.

Hsu, K.H.; Fennema, O. Changes in the functionality of dry whey protein concentrate during storage. *J. Dairy Sci.* **1989**, *72*, 829-837.

Hu, M.; McClements D.J.; Decker, E.A. Lipid oxidation in corn oil-in-water emulsions stabilized by casein, whey protein isolate, and soy protein isolate. *J. Agric. Food Chem.* **2003**, *51*, 1696-1700.

Huang, X.L.; Catignani, G.L.; Swaisgood, H.E. Relative structural stabilities of β-lactoglobulins A and B as determined by proteolytic susceptibility and differential scanning calorimetry. *J. Agric. Food Chem.* **1994**, *42*, 1276-1280.

Hummer, G.; Garde, S.; García, A.E.; Paulaitis, M.E.; Pratt, L.R. The pressure dependence of hydrophobic interactions is consistent with the observed pressure denaturation of proteins. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 1552-1555.

Iglesias, H.A.; Chirife, J. Delayed crystallization of amorphous sucrose in humidified freeze dried model systems. *Int. J. Food Sci. Technol.* **1978**, *13*, 137-144.

Iglesias, H.A.; Chirife, J.; Buera, M.P. Adsorption isotherm of amorphous trehalose. *J. Sci. Food Agric.* **1997**, *75*, 183-186.

Imamura, K.; Ogawa, T.; Sakiyama, T.; Nakanishi, K. Effects of types of sugar on the stabilization of protein in the dried state. *J. Pharm. Sci.* **2003**, *92*, 266-274.

Jackson, M.; Mantsch, H.H. The use and misuse of FTIR spectroscopy in the determination of protein structure. *Crit. Rev. Biochem. Mol. Biol.* **1995**, *30*, 95-120.

Jakobsen, M.; Bertelsen, G. Colour stability and lipid oxidation of fresh beef. Development of a response surface model for predicting the effects of temperature, storage time, and modified atmosphere composition. *Meat Sci.* **2000**, *54*, 49-57.

Jansson, H.; Swenson, J. The protein glass transition as measured by dielectric spectroscopy and differential scanning calorimetry. *Biochim. Biophys. Acta* **2010**, *1804*, 20-26.

Jiang, J.-S.; Brünger, A.T. Protein hydration observed by X-ray diffraction: Solvation properties of penicillopepsin and neuraminidase crystal structures. *J. Mol. Biol.* **1994**, *243*, 100-115.

Jones, L.S.; Kaufmann, A.; Middaugh, C.R. Silicone oil induced aggregation of proteins. *J. Pharm. Sci.* **2005**, *94*, 918-927.

Jouppila, K.; Kansikas, J.; Roos, Y.H. Glass transition, water plasticization, and lactose crystallization in skim milk powder. *J. Dairy Sci.* **1997**, *80*, 3152-3160.

Jouppila, K.; Roos, Y.H. Water sorption and time dependent phenomena of milk powders. *J. Dairy Sci.* **1994**, *77*, 1798-1808.

Jung, C. Insight into protein structure and protein-ligand recognition by Fourier transform infrared spectroscopy. *J. Mol. Recognit.* **2000**, *13*, 325-351.

Kalichevsky, M.T.; Blanshard, J.M.V. The effect of fructose and water on the glass transition of amylopectin. *Carbohydr. Polym.* **1993**, *20*, 107-113.

Kanner, J.; Karel, M. Changes in lysozyme due to reactions with peroxidizing methyl linoleate in a dehydrated model system. *J. Agric. Food Chem.* **1976**, *24*, 468-472.

Karel, M. Protein-lipid interactions. *J. Food Sci.* **1973**, *38*, 756-763.

Karel, M.; Schaich, K.; Roy, R.B. Interaction of peroxidizing methyl linoleate with some proteins and amino acids. *J. Agric. Food Chem.* **1975**, *23*, 159-163.

Karmas, R.; Buera, M.P.; Karel, M. Effect of glass transition on rates of nonenzymatic browning in food systems. *J. Agric. Food Chem.* **1992**, *40*, 873-879.

Kato, H.; Cho, R.K.; Okitani, A.; Hayase, F. Responsibility of 3-deoxyglucosone for the glucose-induced polymerization of proteins. *J. Agric. Biol. Chem.* **1987**, *51*, 683-689.

Kato, Y.; Matsuda, T.; Kato, N.; Nakamura, R. Maillard reaction of disaccharides with protein: suppressive effect of nonreducing end pyranoside groups on browning and protein polymerization. *J. Agric. Food Chem.* **1989**, *37*, 1077-1081.

Kato, Y.; Matsuda, T.; Kato, N.; Watanabe, K.; Nakamura, R. Browning and insolubilization of ovalbumin by the Maillard reaction with some aldohexoses. *J. Agric. Food Chem.* **1986**, *34*, 351-355.

Kato, Y.; Watanabe, K.; Sato, Y. Effect of Maillard reaction on some physical properties of ovalbumin. *J. Food Sci.* **1981**, *46*, 1835-1839.

Kealley, C.S.; Rout, M.K.; Dezfouli, M.R.; Strounina, E.; Whittaker, A.K.; Appelqvist, I.A.M.; Lillford, P.J.; Gilbert, E.P.; Gidley, M.J. Structure and molecular mobility of soy glycinin in the solid state. *Biomacromolecules* **2008**, *9*, 2937-2946.

Kella, N.K.D.; Poola, I. Sugars decrease the thermal denaturation and aggregation of arachin. *Int. J. Pept. Protein Res.* **1985**, *26*, 390-399.

Kharakoz, D.P. Partial volumes and compressibilities of extended polypeptide chains in aqueous solution: Additivity scheme and implication of protein unfolding at normal and high pressure. *Biochemistry* **1997**, *36*, 10276-10285.

Khechinashvili, N.N.; Janin, J.; Rodier, F. Thermodynamics of the temperature-induced unfolding of globular proteins. *Protein Sci.* **1995**, *4*, 1315-1324.

Kilmartin, P.A.; Reid, D.S.; Samson, I. Dielectric properties of frozen maltodextrin solutions with added NaCl across the glass transition. *J. Sci. Food Agric.* **2004**, *84*, 1277-1284.

Kinsella, J.E.; Whitehead, D.M. Proteins in whey: Chemical, physical, and functional properties. *Adv. Food Nutr. Res.* **1989**, *33*, 343-438.

Kirigaya, N.; Kato, H.; Fujimaki, M. Studies on antioxidant activity of nonenzymatic browning reaction products. *Agr. Biol. Chem.* **1968**, *32*, 287-290.

Klotz, I.M. Protein hydration and behavior. *Science* **1958**, *128*, 815-822.

Koch, R.B. Dehydrated foods and model systems. In *Symposium on Foods: Lipids and Their Oxidation*; Shultz, H.W.; Day, E.A.; Sinnhuber, R.O. Eds.; AVI, Westport, Connecticut, U.S.A., 1962; Chapter 13, pp. 230-251.

Kong, J.; Yu, S. Fourier transform infrared spectroscopic analysis of protein secondary structures. *Acta Biochim. Biophys. Sin.* **2007**, *39*, 549-559.

Kouassi, K.; Roos, Y.H. Glass transition and water effects on sucrose inversion in noncrystalline carbohydrate food systems. *Food Res. Int.* **2001**, *34*, 895-901.

Krimm, S.; Bandekar, J. Vibrational spectroscopy and conformation of peptides, polypeptides, and proteins. *Adv. Protein Chem.* **1986**, *38*, 181-364.

Krokida, M.K.; Oreopoulou, V.; Maroulis, Z.B. Water loss and oil uptake as a function of frying time. *J. Food Eng.* **2000**, *44*, 39-46.

Kumosinski, T.F.; Farrell, Jr.H.M. Determination of the global secondary structure of proteins by Fourier transform infrared (FTIR) spectroscopy. *Trends Food Sci. Technol.* **1993**, *4*, 169-175.

Kwak, E.,-J.; Lim, S.,-I. The effect of sugar, amino acid, metal ion, and NaCl on model Maillard reaction under pH control. *Amino Acids* **2004**, *27*, 85-90.

Laaksonen, T.J.; Roos, Y.H. Thermal, dynamic-mechanical, and dielectric analysis of phase and state transitions of frozen wheat doughs. *J. Cereal Sci.* **2000**, *32*, 281-292.

Labrousse, S.; Roos, Y.; Karel, M. Collapse and crystallization in amorphous matrices with encapsulated compounds. *Sci. Aliment.* **1992**, *12*, 757-759.

Labuza, T.P.; Dugan, Jr.L.R. Kinetics of lipid oxidation in foods. *Crit. Rev. Food Technol.* **1971**, *2*, 355-405.

Labuza, T.P.; McNally, L.; Gallagher, D.; Hawkes, J.; Hurtado, F. Stability of intermediate moisture foods. 1. Lipid oxidation. *J. Food Sci.* **1972**, *37*, 154-159.

Labuza, T.P.; Saltmarch, M. Kinetics of browning and protein quality loss in whey powders during steady state and nonsteady state storage conditions. *J. Food Sci.* **1981**, 47, 92-96.

Labuza, T.P.; Tsuyuki, H.; Karel, M. Kinetics of linoleate oxidation in model systems. *J. Am. Oil Chem. Soc.* **1969**, *46*, 409-416.

Ladokhin, A.S. Fluorescence spectroscopy in peptide and protein analysis. In *Encyclopedia of Analytical Chemistry*; Meyers, R.A., Ed.; John Wiley & Sons, Ltd.: New York, 2000; pp 5762-5779.

Laemmli, U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680-685.

Lala, A.K.; Kaul, P. Increased exposure of hydrophobic surface in molten globule state of α-lactalbumin. *J. Biol. Chem.* **1992**, *267*, 19914-19918.

Le, T.T.; Bhandari, B.; Deeth, H.C. Chemical and physical changes in milk protein concentrate (MPC80) powder during storage. *J. Agric. Food Chem.* **2011a**, *59*, 5465-5473.

Le, T.T.; Bhandari, B.; Holland, J.W.; Deeth, H.C. Maillard reaction and protein cross-linking in relation to the solubility of milk powders. *J. Agric. Food Chem.* **2011b**, *59*, 12473-12479.

Leake, L.; Karel, M. Polymerization and denaturation of lysozyme exposed to peroxidizing lipids. *J. Food Sci.* **1982**, *47*, 737-739.

Lee, J.C.; Timasheff, S.N. The stabilization of proteins by sucrose. *J. Biol. Chem.* **1981**, *256*, 7193-7201.

Levine, H.; Slade, L. A polymer physico-chemical approach to the study of commercial starch hydrolysis products (SHPs). *Carbohydr. Polym.* **1986**, *6*, 213-244.

Levine, H.; Slade, L. Another view of trehalose for drying and stabilizing biological materials. *Biopharm.* **1992**, *5*, 36-40.

Levine, R.L.; Garland, D.; Oliver, C.N.; Amici. A.; Climent, I.; Lenz, A.G.; Ahn, B.W.; Shatiel, S.; Stadtman, E.R. Determination of carbonyl content in oxidatively modified protein. *Methods Enzymol.* **1990**, *186*, 464-478.

Lewis, V.M.; Lea, C.H. A note on the relative rates of reaction of several reducing sugars and sugar derivatives with casein. *Biochim. Biophys. Acta* **1950**, *4*, 532-534.

Liang, J.N.; Rossi, M.T. In vitro non-enzymatic glycation and formation of browning products in the bovine lens alpha-crystallin. *Exp. Eye Res.* **1990**, *50*, 367-371.

Li-Chan, E. Heat-induced changes in the proteins of whey protein concentrate. *J. Food Sci.* **1983**, *48*, 47-56.

Lievonen, S.M.; Laaksonen, T.J.; Roos, Y.H. Glass transition and reaction rates: Nonenzymatic browning in glassy and liquid systems. *J. Agric. Food Chem.* **1998**, *46*, 2778-2784.

Lievonen, S.M.; Roos, Y.H. Comparison of dielectric properties and non-enzymatic browning kinetics around glass transition. *Innov. Food Sci. Emerg. Technol.* **2003**, *4*, 297-305.

Liggins, J.; Furth, A.J. Role of protein-bound carbonyl groups in the formation of advanced glycation endproducts. *Biochim. Biophys. Acta* **1997**, *1361*, 123-130.

Liu, G.; Xiong, Y.L. Electrophoretic pattern, thermal denaturation, and in vitro digestibility of oxidized myosin. *J. Agric. Food Chem.* **2000**, *48*, 624-630.

Liu, G.; Xiong, Y.L.; Butterfield, D.A. Chemical, physical, and gel-forming properties of oxidized myofibrils and whey- and soy-protein isolates. *J. Food Sci.* **2000**, *65*, 811-818.

Liu, X.; Zhou, P.; Tran, A.; Labuza, T.P. Effects of polyols on the stability of whey proteins in intermediate-moisture food model systems. *J. Agric. Food Chem.* **2009**, *57*, 2339-2345.

Lobato-Calleros, C.; Reyes-Hernández, J.; Beristain, C.I.; Hornelas-Uribe, Y.; Sánchez-García, J.E.; Vernon-Carter, E.J. Microstructure and texture of white fresh cheese made with canola oil and whey protein concentrate in partial or total replacement of milk fat. *Food Res. Int.* **2007**, *40*, 529-537.

Loveday, S.M.; Hindmarsh, J.P.; Creamer, L.K.; Singh, H. Physicochemical changes in a model protein bar during storage. *Food Res. Int.* **2009**, *42*, 798-806.

Lund, M.N.; Luxford, C.; Skibsted, L.H.; Davies, M.J. Oxidation of myosin by haem proteins generates myosin radicals and protein cross-links. *Biochem. J.* **2008**, *410*, 565-574.

Lyons, T.J. Glycation and oxidation: A role in the pathogenesis of atherosclerosis. *Am. J. Cardiol.* **1993**, *71*, 26-31.

Madadlou, A.; Azarikia, F. Chapter 6: Nano carriers, films and composites based on milk proteins. In *Advances in Natural Polymers: Composites and nanocomposites*; Thomas, S.; Visakh, P.M.; Mathew, Aji.P., Eds.; Springer Science+Business, LLC.: New York, 2013; pp 169-191.

Magazù, S.; Migliardo, F.; Benedetto, A. Puzzle of protein dynamical transition. *J. Phys. Chem. B* **2011**, *115*, 7736-7743.

Makhatadze, G.I.; Privalov, P.L. Hydration effects in protein unfolding. *Biophys. Chem.* **1994**, *51*, 291-309.

Makita, Z.; Radoff, S.; Rayfield, E.J.; Yang, Z.; Skolnik, E.; Delaney, V.; Friedman, E.A.; Cerami, A.; Vlassara, H. Advanced glycosylation end products in patients with diabetic nephropathy. *N. Engl. J. Med.* **1991**, *325*, 836-842.

Markesbery, W.R.; Lovell, M.A. Four-hydroxynonenal, a product of lipid peroxidation, is increased in the brain in Alzheimer's disease. *Neurobiol. Aging* **1998**, *19*, 33-36.

Massalska-Arodz, M.; Williams, G.; Thomas, D.K.; Jones, W.J.; Dabrowski, R. Molecular dynamics and crystallization behavior of chiral isooctyloxycyanobiphenyl as studied by dielectric relaxation spectroscopy. *J. Phys. Chem. B* **1999**, *103*, 4197-4205.

Mastrocola, D.; Munari, M. Progress of the Maillard reaction and antioxidant action of Maillard reaction products in preheated model systems during storage. *J. Agric. Food Chem.* **2000**, *48*, 3555-3559.

Mastrocola, D.; Munari, M.; Cioroi, M.; Lerici, C.R. Interaction between Maillard reaction products and lipid oxidation in starch-based model systems. *J. Sci. Food Agric.* **2000**, *80*, 684-690.

Mattos, C. Protein-water interactions in a dynamic world. *Trends Biochem. Sci.* **2002**, *27*, 203-208.

Mattos, C.; Ringe, D. Proteins in organic solvents. *Curr. Opin. in Struct. Biol.* **2001**, *11*, 761-764.

Mazzobre, M.F.; Buera, M.P.; Chirife, J. Protective role of trehalose on thermal stability of lactase in relation to its glass and crystal forming properties and effect of delaying crystallization. *Lebensm. Wiss. Technol*. **1997**, *30*, 324-329.

Mazzobre, M.F.; Hough, G.; Buera, M.P. Phase transitions and functionality of enzymes and yeasts in dehydrated matrices. *Food Sci. Technol. Int.* **2003**, *9*, 163-172.

Mazzobre, M.F.; Soto, G.; Aquilera, J.M.; Buera, M.P. Crystallization kinetics of lactose in systems co-lyofilized with trehalose. Analysis by differential scanning calorimetry. *Food Res. Int.* **2001**, *34*, 903-911.

McCance, D.R.; Dyer, D.G.; Dunn, J.A.; Bailie, K.E.; Thorpe, S.R.; Baynes, J.W.; Lyons, T.J. Maillard reaction products and their relation to complications in insulin-dependent diabetes mellitus. *J. Clin. Invest.* **1993**, *91*, 2470-2478.

McMahon, D.J.; Adams, S.L.; McManus, W.R. Hardening of high-protein nutrition bars and sugars/polyol-protein phase separation. *J. Food Sci.* **2009**, *74*, 312-321.

McPherson, J.D.; Shilton, B.H.; Walton, D.J. Role of fructose in glycation and cross-linking of proteins. *Biochemistry* **1988**, *27*, 1901-1907.

Menzel, D.B. Reaction of oxidizing lipids with ribonuclease. *Lipids* **1967**, *2*, 83-84.

Meucci, E.; Mordente, A.; Martorana, G.E. Metal-catalyzed oxidation of human serum albumin: conformational and functional changes. Implications in protein aging. *J. Biol. Chem.* **1991**, *266*, 4692-4699.

Mijovic, J. Monitoring Crystallization by dielectric spectroscopy. *Dielectrics Newsletter* **1998**, *1-3*.

Militello, V.; Casarino, C.; Emanuele, A.; Giostra, A.; Pullara, F.; Leone, M. Aggregation kinetics of bovine serum albumin studied by FTIR spectroscopy and light scattering. *Biphys. Chem.* **2004**, *107*, 175-187.

Miyata, T.; Ueda, Y.; Yamada, Y.; Izuhara, Y.; Wada, T.; Jadoul, M.; Saito, A.; Kurokawa, K.; van Ypersele de Strihou, C. Accumulation of carbonyls accelerates the formation of pentosidine, an advanced glycation end product: carbonyl stress in uremia. *J. Am. Soc. Nephrol.* **1998**, *9*, 2349-2356.

Mohammed, Z.H.; Hill, S.E.; Mitchell, J.R. Covalent crosslinking in heated protein systems. *J. Food Sci.* **2000**, *65*, 221-226.

Molnar-Perl, I.; Friedman, M. Inhibition of browning by sulfur amino acids. 2. Fruit juices and protein-containing foods. *J. Agric. Food Chem.* **1990**, *38*, 1648-1651.

Monahan, F.J.; German, J.B.; Kinsella, J.E. Effect of pH and temperature on protein unfolding and thiol/disulfide interchange reactions during heat-induced gelation of whey proteins. *J. Agric. Food Chem.* **1995**, *43*, 46-52.

Monahan, F.J.; McClements, D.J.; Kinsella, J.E. Polymerization of whey proteins in whey protein-stabilized emulsions. *J. Agric. Food Chem.* **1993**, *41*, 1826-1829.

Monnier, V.M. Nonenzymatic glycosylation, the Maillard reaction and the aging process. *J. Gerontol.* **1990**, *45*, 105-111.

Morcira, R.G.; Sun, X.; Chen, Y. Factors affecting oil uptake in tortilla chips in deep-fat frying. *J. Food Eng.* **1997**, *31*, 485-498.

Morita, J.; Kashimura, N. The Maillard reaction of DNA with D-fructose 6-phosphate. *Agric. Biol. Chem.* **1991**, *55*, 1359-1366.

Mulvihill, D.M.; Donovan, M. Whey proteins and their thermal denaturation - a review. *Ir. J. Food Sci. Technol.* **1987**, *11*, 43-75.

Mulvihill, D.M.; Kinsella, J.E. Gelation characteristics of whey proteins and beta-lactoglobulin. *Food Technol.* **1987**, *41*, 102-111.

Nadig, G.; Van Zant, L.C.; Dixon, S.L.; Merz, Jr.K.M. Charge-transfer interactions in macromolecular systems: a new view of the protein/water interface. *J. Am. Chem. Soc.* **1998**, *120*, 5593-5594.

Namiki, M.; Hayashi, T.; Kawakishi, S. Free radicals developed in the amino-carbonyl reaction of sugars with amino acids. *Agric. Biol. Chem.* **1973**, *37*, 2935-2936.

Naranjo, G.B.; Gonzales, A.S.P.; Leiva, G.E.; Malec, L.S. The kinetics of Maillard reaction in lactose-hydrolysed milk powder and related systems containing carbohydrate mixtures. *Food Chem.* **2013**, *141*, 3790-3795.

Negre-Salvayre, A.; Coatrieux, C.; Ingueneau, C.; Salvayre, R. Advanced lipid peroxidation end products in oxidative damage to proteins. Potential role in diseases and therapeutic prospects for the inhibitors. *Br. J. Pharmacol.* **2008**, *153*, 6-20.

Ngai, K.L.; Capaccioli, S.; Shinyashiki, N. The protein “glass” transition and the role of the solvent. *J. Phys. Chem. B* **2008**, *112*, 3826-3832.

Nielsen, H.K.; Löliger, J.; Hurrell, R.F. Reactions of proteins with oxidizing lipids. *Br. J. Nutr.* **1985**, *53*, 61-73.

Nowakowski, C.M.; Hartel, R.W. Moisture sorption of amorphous sugar products. *J. Food Sci.* **2002**, *67*, 1419-1425.

Noel, T.R.; Parker, R.; Ring, S.G. Effect of molecular structure and water content on the dielectric relaxation behaviour of amorphous low molecular weight carbohydrates above and below their glass transition. *Carbohydr. Res.* **2000**, *329*, 839-845.

Noel, T.R.; Ring, S.G.; Whittam, M.A. Dielectric relaxations of small carbohydrate molecules in the liquid and glassy states. *J. Phys. Chem.* **1992**, *96*, 5662-5667.

Nursten, H.E. Recent developments in studies of the Maillard reaction. *Food Chem.* **1981**, *6*, 263-277.

Oliver, C.N.; Ahn, B.-W.; Moerman, E.J.; Goldstein, S.; Stadtman E.R. Age-related changes in oxidized proteins. *J. Biol. Chem.* **1987**, *262*, 5488-5491.

O'Neil, T.E.; Kinsella, J.E. Binding of alkanone flavors to β-lactoglobulin: Effects of conformational and chemical modification. *J. Agric. Food Chem.* **1987**, *35*, 770.

Oobatake, M.; Ooi, T. Hydration and heat stability effects on protein unfolding. *Prog. Biophys. Mol. Biol.* **1993**, *59*, 237-284.

Ooi, T.; Oobatake, M. Effects of hydrated water on protein unfolding. *J. Biochem.* **1988**, *103*, 114-120.

Ordonez, J.A.; Ledward, D.A. Lipid and myoglobin oxidation in pork stored in oxygen- and carbon dioxide-enriched atmospheres. *Meat Sci.* **1977**, *1*, 41-48.

Ortwerth, B.J.; Olesen, P.R. Ascorbic acid-induced crosslinking of lens proteins: evidence supporting a Maillard reaction. *Biochim. Biophys. Acta* **1988**, *956*, 10-22.

Öste, R.E.; Dahlqvist, A.; Sjöström, H.; Norén, O.; Miller, R. Effect of Maillard reaction products on protein digestion. In *vitro* studies. *J. Agric. Food Chem.* **1986**, *34*, 355-358.

Otting, G.; Liepinsh, E.; Wuthrich, K. Protein hydration in aqueous solution. *Science* **1991**, *254*, 974-980.

Paciaroni, A.; Nibali, V.C.; Orecchini, A.; Petrillo, C.; Haertlein, M.; Moulin, M.; Tarek, M.; D'Angelo, G.; Sacchetti, F. Vibrational excitations of proteins and their hydration water in the far-infrared range. *Chem. Phys.* **2013**, *424*, 80-83.

Pagnotta, S.E.; Cerveny, S.; Alegria, A.; Colmenero, J. Dielectric relaxtions in ribose and deoxyribose supercooled water solutions. *J. Chem. Phys.* **2009**, *131*, 851021-851027.

Panick, G.; Malessa, R.; Winter, R. Differences between the pressure- and temperature-induced denaturation and aggregation of β-lactoglobulin A, B, and AB monitored by FT-IR spectroscopy and small-angle X-ray scattering. *Biochemistry* **1999**, *38*, 6512-6519.

Pantke, U.; Volk, T.; Schmutzler, M.; Kox, W.J.; Sitte, N.; Grune, T. Oxidized proteins as a marker of oxidative stress during coronary heart surgery. *Free Radic. Biol. Med.* **1999**, *27*, 1080-1086.

Peña-Ramos, E.A.; Xiong, Y.L. Whey and soy protein hydrolysates inhibit lipid oxidation in cooked pork patties. *Meat Sci.* **2003**, *64*, 259-263.

Pilková, L.; Pokorný, J.; Davídek, J. Browning reactions of Heyns rearrangement products. *Nahrung.* **1990**, *34*, 759-764.

Pinnell, S.R.; Martin, G.R. The cross-linking of collagen and elastin: Enzymatic conversion of lysine in peptide linkage to α-aminoadipic-δ-semialdehyde (allysine) by an extract from bone. *Proc. Natl. Acad. Sci. U.S.A.* **1968**, *61*, 708-716.

Pinthus, E.J.; Weinberg, P.; Saguy, I.S. Oil uptake in deep fat frying as affected by porosity. *J. Food Sci.* **1995**, *60*, 767-769.

Plachta, H.; Bartnikowska, E.; Obara, A. Lipid peroxides in blood from patients with atherosclerosis of coronary and peripheral arteries. *Clin. Chem. Acta.***1992**, *211*, 101-112.

Pokorný, J.; El-Zeany, B.A., Kolakowska, A.; Janiček, G. Nonenzymic browning. IX. Correlation of autoxidation and browning reactions in lipid-protein mixtures. *Z. Lebensm. -Unters. -Forsch.* **1974**, *155*, 287-291.

Pomeranz, Y.; Johnson, J.A.; Shellenberger, J.A. Effect of various sugars on browning. *J. Food Sci.* **1962**, *27*, 350-354.

Potes, N.; Kerry, J.P.; Roos, Y.H. Additivity of water sorption, alpha-relaxations and crystallization inhibition in lactose-maltodextrin systems. *Carbohydr. Polym.* **2012**, *89*, 1050-1059.

Potes, N.; Kerry, J.P.; Roos, Y.H. Oil as reaction medium for glycation, oxidation, denaturation, and aggregation of whey protein systems of low water activity. *J. Agric. Food Chem.* **2013a**, *61*, 3748-3756.

Potes, N.; Kerry, J.P.; Roos, Y.H. Protein modifications in high protein-oil and protein-oil-sugar systems at low water activity. *Food Biophys.* **2013b**, DOI: 10.1007/s11483-013-9316-1.

Prestrelski, S.J.; Tedeschi, N.; Arakawa, T.; Carpenter, J.F. Dehydration-induced conformational transitions in proteins and their inhibition by stabilizers. *Biophys. J.* **1993**, *65*, 661-671.

Privalov, P.L.; Makhatadze, G.I. Heat capacity of proteins: II. Partial molar heat capacity of the unfolded polypeptide chain of proteins: Protein unfolding effects. *J. Mol. Biol.* **1990**, *213*, 385-391.

Privalov, P.L.; Makhatadze, G.I. Contribution of hydration and non-covalent interactions to the heat capacity effect on protein unfolding. *J. Mol. Biol.* **1992**, *224*, 715-723.

Raemy, A.; Hurrell, R.F.; Löliger, J. Thermal behaviour of milk powders studied by differential thermal analysis and heat flow calorimetry. *Thermochim. Acta* **1983**, *65*, 81-92.

Rasmussen, B.F.; Stock, A.M.; Ringe, D.; Petsko, G.A. Crystalline ribonuclease A loses function below the dynamical transition at 220 K. *Nature* **1992**, *357*, 423-424.

Recondo, M.P.; Elizalde, B.E.; Buera, M.P. Modeling temperature dependence of honey viscosity and of related supersaturated model carbohydrate systems. *J. Food Eng.* **2006**, *77*, 126-134.

Reddy, S.; Bichler, J.; Wells-Knecht, K.J.; Thorpe, S.R.; Baynes, J.W. N^{ε}-(carboxymethyl)lysine is a dominant advanced glycation end product (AGE) antigen in tissue proteins. *Biochemistry* **1995**, *34*, 10872-10878.

Refsgaard, H.H.F.; Tsai, T.; Stadtman, E.R. Modifications of proteins by polyunsaturated fatty acid peroxidation products. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 611-616.

Requena, J.R.; Fu, M.-X.; Ahmed, M.U.; Jenkins, A.J.; Lyons, T.J.; Thorpe, S.R. Lipoxidation products as biomarkers of oxidative damage to proteins during lipid peroxidation reactions. *Nephrol. Dial. Transplant.* **1996**, *11*, 48-53.

Requena, J.R.; Chao, C.-C.; Levine, R.L.; Stadtman, E.R. Glutamic and aminoadipic semialdehydes are the main carbonyl products of metal-catalyzed oxidation of proteins. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 69-74.

Rhee, K.S.; Ziprin, Y.A.; Ordonez, G. Catalysis of lipid oxidation in raw and cooked beef by metmyoglobin-H_2O_2, nonheme iron, and enzyme systems. *J. Agric. Food Chem.* **1987**, *35*, 1013-1017.

Rice-Evans, C.A.; Miller, N.J.; Paganga, G. Antioxidant properties of phenolic compounds. *Trends Plant Sci.* **1997**, *2*, 152-159.

Rich, L.M.; Foegeding, E.A. Effects of sugars on whey protein isolate gelation. *J. Agric. Food Chem.* **2000**, *48*, 5046-5052.

Richards, M.P.; Hultin, H.O. Effect of pH on lipid oxidation using trout hemolysate as a catalyst: a possible role for deoxyhemoglobin. *J. Agric. Food Chem.* **2000**, *48*, 3141-3147.

Rivett, A.J.; Roseman, J.E.; Oliver, C.N.; Levine, R.L.; Stadtman, E.R. Covalent modification of proteins by mixed-function oxidation: Recognition by intracellular proteases. *Prog. Clin. Biol. Res.* **1985**, *180*, 317-328.

Roberts, R.L.; Lloyd, R.V. Free radical formation from secondary amines in the Maillard reaction. *J. Agric. Food Chem.* **1997**, *45*, 2413-2418.

Roos, Y.H. Water activity and physical state effects on amorphous food stability. *J. Food Process. Preserv.* **1993**, *16*, 433-447.

Roos, Y.H. Glass transition-related physicochemical changes in foods. *Food Technol.* **1995a**, *49*, 97-102.

Roos, Y.H. *Phase transitions in foods*. Academic Press, Inc.: California, U.S.A., 1995b.

Roos Y.H. Importance of glass transition and water activity to spray drying to spray drying and stability of dairy powders. *Le Lait.* **2002**, *82*, 475-484.

Roos, Y.H. Materials science of freezing and frozen foods. In *Food Materials Science and Engineering*; Bhandari, B., Roos, Y.H., Eds.; John Wiley & Sons, Ltd.: Chichester, U.K., 2012; pp 373-386.

Roos, Y.H.; Jouppila, K.; Zielasko, B. Non-enzymatic browning-induced water plasticization. *J. Therm. Anal.* **1996**, *47*, 1437-1450.

Roos, Y.; Karel, M. Differential scanning calorimetry study of phase transitions affecting the quality of dehydrated materials. *Biotechnol. Prog.* **1990**, *6*, 159-163.

Roos, Y.; Karel, M. Plasticizing effect of water on thermal behavior and crystallization of amorphous food models. *J. Food Sci.* **1991a**, *56*, 38-43.

Roos, Y.; Karel, M. Phase transitions of mixtures of amorphous polysaccharides and sugars. *Biotechnol. Prog.* **1991b**, *7*, 49-53.

Roos, Y.; Karel, M. Applying state diagrams to food processing and development. *Food Technol.* **1991c**, *45*, 66, 68-71, 107.

Roos, Y.; Karel, M. Water and molecular weight effects on glass transition in amorphous carbohydrates and carbohydrate solutions. *J. Food Sci.* **1991d**, *56*, 1676-1681.

Roos, Y.; Karel, M. Crystallization of amorphous lactose. *J. Food Sci.* **1992**, *57*, 775-777.

Rouach, H.; Fataccioli, V.; Gentil, M.; French, S.W.; Morimoto, M.; Nordmann, R. Effect of chronic ethanol feeding on lipid peroxidation and protein oxidation in relation to liver pathology. *Hepatology* **1997**, *25*, 351-355.

Roubal, W.T. Free radicals, malonaldehyde and protein damage in lipid-protein systems. *Lipids* **1971**, *6*, 62-64.

Roubal, W.T.; Tappel, A.L. Damage to proteins, enzymes, and amino acids by peroxidizing lipids. *Arch. Biochem. Biophys.* **1966a**, *113*, 5-8.

Roubal, W.T.; Tappel, A.L. Polymerization of proteins induced by free radical lipid peroxidation. *Arch. Biochem. Biophys.* **1966b**, *113*, 150-155.

Ru, M.T.; Dordick, J.S.; Reimer, J.A.; Clark, D.S. Optimizing the salt-induced activation of enzyme in organic solvents: Effects of lyophilization time and water content. *Biotechnol. Bioeng.* **1999**, *63*, 233-241.

Rupley, J.A.; Careri, G. Protein hydration and function. *Adv. protein chem.* **1991**, *41*, 37-172.

Russo, D.; Hura, G.; Head-Gordon, T. Hydration dynamics near a model protein surface. *Biophys. J.* **2004**, *86*, 1852-1862.

Rüegg, M.; Moor, U; Blanc, B. Hydration and thermal denaturation of beta-lactoglobulin. A calorimetric study. *Biochim. Biophys. Acta* **1975**, 400, 334-342.

Rüegg, M.; Moor, U; Blanc, B. A calorimetric study of the thermal denaturation of whey proteins in simulated milk ultrafiltrate. *J. Dairy Res.* **1977**, *44*, 509-520.

Saeed, S.; Howell, N.K. Effect of lipid oxidation and frozen storage on muscle proteins of Atlantic mackerel (*Scomber scombrus*). *J. Sci. Food Agric.* **2002**, *82*, 579-586.

Sakanaka, S.; Tachibana, Y.; Ishihara, N.; Juneja, L.R. Antioxidant activity of egg-yolk hydrolysates in a linoleic acid oxidation system. *Food Chem.* **2004**, *86*, 99-103.

Salman, A.G.; Mansour, D.E.; Swelem, A.H.; AI-Zawahary, W.M.; Radwan, A.A. Pentosidine - a new biochemical marker in diabetic retinopathy. *Ophthalmic Res.* **2009**, *42*, 96-98.

Sasaki, N.; Fukatsu, R.; Tsuzuki, K.; Hayashi, Y.; Yoshida, T.; Fujii, N.; Koike, T.; Wakayama, I.; Yanagihara, R.; Garruto, R.; Amano, N.; Makita, Z. Advanced glycation end products in Alzheimer's disease and other neurodegenerative diseases. *Am. J. Pathol.* **1998**, *153*, 1149-1155.

Sattar, A.; deMan, J.M.; Furia, T.E. Photooxidation of milk and milk products: A review. *Crit. Rev. Food Sci. Nutr.* **1975**, *7*, 13-37.

Sayre, L.M.; Zelasko, D.A.; Harris, P.L.R.; Perry, G.; Salomon, R.G.; Smith, M.A. 4-hydroxynonenal-derived advanced lipid peroxidation end products are increased in Alzheimer's disease. *J. Neurochem.* **1997**, *68*, 2092-2097.

Schaich, K.M.; Karel, M. Free radicals in lysozyme reacted with peroxidizing methyl linoleate. *J.Food Sci.* **1975**, *40*, 456-459.

Schaich, K.M.; Karel, M. Free radical reactions of peroxidizing lipids with amino acids and proteins: An ESR study. *Lipids* **1976**, *11*, 392-400.

Schebor, C.; Buera, M.P.; Karel, M.; Chirife, J. Color formation due to non-enzymatic browning in amorphous, glassy, anhydrous, model systems. *Food Chem.* **1999**, *65*, 427-432.

Schmitz-Schug, I.; Gianfrancesco, A.; Kulozik, U.; Foerst, P. Physical state, molecular mobility and chemical stability of powdered dairy formulations. *Food Res. Int.* **2013**, *53*, 268-277.

Sedlak, J.; Lindsay, R.H. Estimation of total, protein-bound, and nonprotein sulfhydryl groups in tissue with Ellman's reagent. *Anal. Biochem.* **1968**, *25*, 192-205.

Sell, D.R.; Monnier, V.M. Structure elucidation of a senescence cross-link from human extracellular matrix. *J. Biol. Chem.* **1989**, *264*, 21597-21602.

Shacter, E. Quantification and significance of protein oxidation in biological samples. *Drug Metab. Rev.* **2000**, *32*, 307-326.

Shamblin, S.L.; Zografi, G. The effects of absorbed water on the properties of amorphous mixtures containing sucrose. *Pharm. Res.* **1999**, *16*, 1119-1124.

Sherwin, E.R. Oxidation and antioxidants in fat and oil processing. *J. Am. Oil Chem. Soc.* **1978**, *55*, 809-814.

Shimada, K.; Cheftel, J.C. Determination of sulfhydryl groups and disulfide bonds in heat-induced gels of soy protein isolate. *J. Agric. Food Chem.* **1988**, *36*, 147-153.

Shimada, K.; Cheftel, J.C. Sulfhydryl group/disulfide bond interchange reactions during heat-induced gelation of whey protein isolate. *J. Agric. Food Chem.* **1989**, *37*, 161-168.

Shimada, Y.; Roos, Y.; Karel, M. Oxidation of methyl linoleate encapsulated in amorphous lactose-based food model. *J. Agric. Food Chem.* **1991**, *39*, 637-641.

Shinyashiki, N.; Yamamoto, W.; Yokoyama, A.; Yoshinari, T.; Yagihara, S.; Kita, R.; Ngai, K.L.; Capaccioli, S. Glass transitions in aqueous solutions of protein (Bovine Serum Albumin). *J. Phys. Chem. B* **2009**, *113*, 14448-14456.

Shrestha, A.K.; Howes, T.; Adhikari, B.P.; Bhandari, B.R. Water sorption and glass transition properties of spray-dried lactose hydrolysed skim milk powder. *Lebensm. Wiss. Technol.* **2007**, *40*, 1593-1600.

Silalai, N.; Roos, Y.H. Roles of water and solids composition in the control of glass transition and stickiness of milk powders. *J. Food Sci.* **2010**, *75*, 285-296.

Silalai, N.; Roos, Y.H. Coupling of dielectric and mechanical relaxations with glass transition and stickiness of milk solids. *J. Food Eng.* **2011**, *104*, 445-454.

Sillick, M.; Gregson, C.M. Viscous fragility of concentrated maltopolymer/sucrose mixtures. *Carbohydr. Polym.* **2009**, *78*, 879-887.

Singh, R.; Barden, A.; Mori, T.; Beilin, L. Advanced glycation end-products: A review. *Diabetologia* **2001**, *44*, 129-146.

Sirotkin, V.A.; Komissarov, I.A.; Khadiullina, A.V. Hydration of proteins: Excess partial volumes of water and proteins. *J. Phys. Chem. B* **2012**, *116*, 4098-4105.

Slade, L.; Levine, H.; Reid, D.S. Beyond water activity: Recent advances based on an alternative approach to the assessment of food quality and safety. *Crit. Rev. Food Sci. Nutr.* **1991**, *30*, 115-360.

Smith, C.D.; Carney, J.M.; Starke-Reed, P.E.; Oliver, C.N.; Stadtman, E.R.; Floyd, R.A.; Markesbery, W.R. Excess brain protein oxidation and enzyme dysfunction in normal aging and in Alzheimer disease. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 10540-10543.

Smith, M.A.; Taneda, S.; Richey, P.L.; Miyata, S.; Yan, S.-D.; Stern, D.; Sayre, L.M.; Monnier, V.M.; Perry, G. Advanced Maillard reaction end products are associated with Alzheimer disease pathology. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 5710-5714.

Speek, A.J.; Schrijver, J.; Schreurs, W.H.P. Vitamin E composition of some seed oils as determined by high performance liquid chromatography with fluorometric detection. *J. Food Sci.* **1985**, *50*, 121-124.

Spiteller, G. The relation of lipid peroxidation processes with atherogenesis: A new theory on atherogenesis. *Mol. Nutr. Res.* **2005**, *49*, 999-1013.

Souillac, P.O.; Middaugh, C.R.; Rytting, J.H. Investigation of protein/ carbohydrate interactions in the dried state. 2. Diffused reflectance FTIR studies. *Int. J. Pharm.* **2002**, *235*, 207-218.

Squire, P.G.; Himmel, M.E. Hydrodynamics and protein hydration. *Arch. Biochem. Biophys.* **1979**, *196*, 165-177.

Stadler, R.H.; Blank, I.; Varga, N.; Robert, F.; Hau, J.; Guy, P.A.; Robert, M.-C.; Riediker, S. Food chemistry: Acrylamide from Maillard reaction products. *Nature* **2002**, *419*, 449-450.

Stadtman, E.R. Protein oxidation and aging. *Science* **1992**, *257*, 1220-1224.

Stadtman, E.R. Protein oxidation in aging and age-related diseases. *Ann. N.Y. Acad. Sci.* **2001**, *928*, 22-38.

Stadtman, E.R.; Berlett, B.S. Reactive oxygen-mediated protein oxidation in aging and disease. *Drug Metab. Rev.* **1998**, *30*, 225-243.

Stadtman, E.R.; Levine, R.L. Protein oxidation. *Ann. N.Y. Acad. Sci.* **2000**, *899*, 191-208.

Stadtman, E.R.; Levine, R.L. Free radical-mediated oxidation of free amino acids and amino acid residues in proteins. *Amino Acids* **2003**, *25*, 207-218.

Stadtman, E.R.; Starke-Reed, P.E.; Oliver, C.N.; Carney, J.M.; Floyd, R.A. Protein modification in aging. *EXS.* **1992**, *62*, 64-72.

Starke-Reed, P.E.; Oliver, C.N. Protein oxidation and proteolysis during aging and oxidative stress. *Arch. Biochem. Biophys.* **1989**, *275*, 559-567.

Stapelfeldt, H.; Nielsen, Bo.R.; Skibsted, L.H. Effect of heat treatment, water activity and storage temperature on the oxidative stability of whole milk powder. *Int. Dairy J.* **1997**, *7*, 331-339.

Stringer, M.D.; Görög, P.G.; Freeman, A.; Kakkar, V.V. Lipid peroxides and atherosclerosis. *Br. Med. J.* **1989**, *298*, 281-284.

Sun, Q.; Senecal, A.; Chinachoti, P.; Faustman, C. Effect of water activity on lipid oxidation and protein solubility in freeze-dried beef during storage. *J. Food Sci.* **2002**, *67*, 2512-2516.

Surewicz, W.K.; Mantsch, H.H.; Chapman, D. Determination of protein secondary structure by Fourier transform infrared spectroscopy: A critical assessment. *Biochemistry* **1993**, *32*, 389-394.

Susi, H.; Byler, D.M. Chapter 14: Fourier transform infrared spectroscopy in protein conformation studies. In *Methods for protein analysis*; Cherry, J.P., Barford, R.A., Eds; American Oil Chemists' Society: Champaign, Illinois, 1988; pp 235-250.

Takagi, Y.; Kashiwagi, A.; Tanaka, Y.; Asahina, T.; Kikkawa, R.; Shigeta, Y. Significance of fructose-induced protein oxidation and formation of advanced glycation end products. *J. Diabetes Complicat.* **1995**, *9*, 87-91.

Talja, R.A., & Roos, Y.H. Phase and state transition effects on dielectric, mechanical, and thermal properties of polyols. *Thermochim. Acta.* **2001**, *380*, 109-121.

Tannenbaum, S.R.; Barth, H.; Le Roux, J.P. Loss of methionine in casein during storage with autoxidizing methyl linoleate. *J. Agric Food Chem.* **1969**, *17*, 1353-1354.

Tappel, A.L. Studies of the mechanism of vitamin E action. III. *In vitro* copolymerization of oxidized fats with protein. *Arch. Biochem. Biophys.* **1955,** *54*, 266-280.

Tarek, M.; Tobias, D.J. The dynamics of protein hydration water: A quantitative comparison of molecular dynamics simulations and neutron-scattering experiments. *Biophys. J.* **2000**, *79*, 3244-3257.

Teeter, M.M.; Yamano, A.; Stec, B.; Mohanty, U. On the nature of a glassy state of matter in a hydrated protein: Relation to protein function. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 11242-11247.

Tenada, S.; Monnier, V.M. ELISA of pentosidine, an advanced glycation end product, in biological specimens. *Clin. Chem.* **1994**, *40*, 1766-1773.

Thornalley, P.J.; Langborg, A.; Minhas, H.S. Formation of glyoxal, methylglyoxal and 3-deoxyglucosone in the glycation of proteins by glucose. *Biochem. J.* **1999**, *344*, 109-116.

Thorpe, S.R.; Baynes, J.W. Role of Maillard reaction in diabetes mellitus and diseases of aging. *Drugs Aging* **1996**, *9*, 69-77.

Timasheff, S.N. Protein hydration, thermodynamic binding, and preferential hydration. *Biochemistry* **2002a**, *41*, 13473-13482.

Timasheff, S.N. Protein-solvent preferential interactions, protein hydration, and the modulation of biochemical reactions by solvent components. *Proc. Natl. Acad. Sci. U.S.A.* **2002b**, *99*, 9721-9726.

Uchida, K.; Kato, Y.; Kawakishi, S. A novel mechanism for oxidative cleavage of prolyl peptides induced by the hydroxyl radical. *Biochem. Biophys, Res. Commun.* **1990**, *169*, 265-271.

Uchida, K.; Stadtman, E.R. Modification of histidine residues in proteins by reaction with 4-hydroxynonenal. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 4544-4548.

Ukhun, M.E.; Izi, U. Effects of storage on some chemical indices of beef quality. *Food Chem.* **1991**, *41*, 55-62.

Unterhaslberger, G.; Schmitt, C.; Sanchez, C.; Appolonia-Nouzille, C.; Raemy, A. Heat denaturation and aggregation of β-lactoglobulin enriched WPI in the presence of arginine HCl, NaCl and guanidium HCl at pH 4.0 and 7.0. *Food Hydrocoll.* **2006**, *20*, 1006-1019.

Uribarri, J.; Woodruff, S.; Goodman, S.; Cai, W.; Chen, X.; Pyzik, R.; Yong, A.; Striker, G.E.; Vlassara, H. Advanced glycation end products in foods and a practical guide to their reduction in the diet. *J. Am. Diet. Assoc.* **2010**, *110*, 911-916.

Valdez, D.; Le Huérou, J.-Y.; Gindre, M.; Urbach, W.; Waks, M. Hydration and protein folding in water and in reverse micelles: Compressibility and volume changes. *Biophys. J.* **2001**, *80*, 2751-2760.

Vitkup, D.; Ringe, D.; Petsko, G.A.; Karplus, M. Solvent mobility and the protein 'glass' transition. *Nat. Struct. Biol.* **2000**, *7*, 34-38.

Voziyan, P.A.; Hudson, B.G. Pyridoxamine: The many virtues of a Maillard reaction inhibitor. *Ann. N.Y. Acad. Sci.* **2005**, *1043*, 807-816.

Waletzko, P.; Labuza, T.P. Accelerated shelf-life testing of intermediate moisture food in air and in an oxygen-free atmosphere. *J. Food Sci.* **1976**, *41*, 1338-1344.

Wang, W.; Nema, S.; Teagarden, D. Protein aggregation-pathways and influencing factors. *Int. J. Pharm.* **2010**, *390*, 89-99.

Watanabe, K.; Klostermeyer, H. Heat-induced changes in sulfhydryl and disulfide levels of β-lactoglobulin A and the formation of polymers. *J. Dairy Res.* **1976**, *43*, 411-418.

Watanabe, K.; Sato, Y.; Kato, Y. Chemical and conformational changes of ovalbumin due to the Maillard reaction. *J. Food Process. Preserv.* **1980**, *3*, 263-274.

Warmbier, H.C.; Schnickels, R.A.; Labuza, T.P. Nonenzymatic browning kinetics in an intermediate moisture model system: effect of glucose to lysine ratio. *J. Food Sci.* **1976**, *41*, 981-983.

Wells-Knecht, K.J.; Zyzak, D.V.; Litchfield, J.E.; Thorpe, S.R.; Baynes, J.W. Mechanism of autoxidative glycosylation: Identification of glyoxal and arabinose as intermediates in the autoxidative modification of proteins by glucose. *Biochemistry* **1995**, *34*, 3702-3709.

Williams, M.L.; Landel, R.F.; Ferry, J.D. The temperature dependence of relaxation mechanisms in amorphous polymers and other glass-forming liquids. *J. Am. Chem. Soc.* **1955**, *77*, 3701-3707.

Wishner, L.A. Light-induced oxidations in milk. *J. Dairy Sci.* **1964**, *47*, 216-221.

de Wit, J.N.; Klarenbeek, G. Effects of various heat treatments on structure and solubility of whey proteins. *J. Dairy Sci.* **1984**, *67*, 2701-2710.

de Wit, J.N.; Swinkels, G.A. A differential scanning calorimetric study of the thermal denaturation of bovine beta-lactoglobulin thermal behavior at temperatures up to 100 degrees C. *Biochim. Biophys. Acta* **1980**, *624*, 40-50.

Wolfrom, M.L.; Kashimura, N.; Horton, D. Factors affecting the Maillard browning reaction between sugars and amino acids. Studies on the nonenzymic browning of dehydrated orange juice. *J. Agric. Food Chem.* **1974**, *22*, 796-800.

Wu, W.; Wu, X.; Hua, Y. Structural modification of soy protein by the lipid peroxidation product acrolein. *Lebensm. Wiss. Technol.* **2010**, *43*, 133-140.

Wurm, A.; Soliman, R.; Schick, C. Early stages of polymer crystallization–A dielectric study. *Polymer* **2003**, *44*, 7467-7476.

Xiong, Y.L.; Kinsella, J.E. Mechanism of urea-induced whey protein gelation. *J. Agric. Food Chem.* **1990**, *38*, 1887-1891.

Yagi, K.; Lipid peroxides and human diseases. *Chem. Phys. Lipids* **1987**, *45*, 337-351.

Yeboah, F.K.; Alli, I.; Yaylayan, V.A. Reactivities of D-glucose and D-fructose during glycation of bovine serum albumin. *J. Agric. Food Chem.* **1999**, *47*, 3164-3172.

Yen, G.-C.; Hsieh, P.-P. Antioxidant activity and scavenging effects on active oxygen of xylose-lysine Maillard reaction products. *J. Sci. Food Agric.* **1995**, *67*, 415-420.

Yilmaz, Y.; Toledo, R. Antioxidant activity of water-soluble Maillard reaction products. *Food Chem.* **2005**, *93*, 273-278.

Zamora, R.; Hidalgo, F.J. Coordinate contribution of lipid oxidation and Maillard reaction to the nonenzymatic food browning. *Crit. Rev. Food Sci. Nutr.* **2005**, *45*, 49-59.

Zhou, P.; Guo, M.; Liu, D.; Liu, X.; Labuza, T.P. Maillard-reaction-induced modification and aggregation of proteins and hardening of texture in protein bar model systems. *J. Food Sci.* **2013**, *78*, 437-444.

Zhou, P.; Labuza, T.P. Effect of water content on glass transition and protein aggregation of whey protein powders during short-term storage. *Food Biophys.* **2007**, *2*, 108-116.

Zhou, P.; Liu, X.; Labuza, T.P. Effects of moisture-induced whey protein aggregation on protein conformation, the state of water molecules, and the microstructures and texture of high-protein-containing matrix. *J. Agric. Food Chem.* **2008**, *56*, 4534-4540.

Zhou, Y.; Roos, Y.H. Characterization of carbohydrate-protein matrices for nutrient delivery. *J. Food Sci.* **2001**, *76*, 368-376.

Zirlin, A.; Karel, M. Oxidation effects in a freeze-dried gelatin-methyl linoleate system. *J. Food Sci.* **1969**, *34*, 160-164.

APPENDIX

LIST OF PUBLICATIONS

Potes, N.; Kerry, J.P.; Roos, Y.H. Additivity of water sorption, alpha-relaxations and crystallization inhibition in lactose-maltodextrin systems. *Carbohydr. Polym.* **2012**, *89(4)*, 1050-1059.

Potes, N.; Kerry, J.P.; Roos, Y.H. Oil as reaction medium for glycation, oxidation, denaturation, and aggregation of whey protein systems of low water activity. *J. Agric. Food Chem.* **2013**, *61(15)*, 3748-3756.

Potes, N.; Kerry, J.P.; Roos, Y.H. Protein modifications in high protein-oil and protein-oil-sugar Systems at low water activity. *Food Biophys.* **2013**, Published Online: 25 August 2013 (DOI: 10.1007/s11483-013-9316-1).

Potes, N.; Kerry, J.P.; Roos, Y.H. Protein hydrations, hydrophilic and hydrophobic interactions, and the glass transition of confined water in high protein systems. *(Manuscript to be submitted)*

APPENDIX

LIST OF CONFERENCES

Published Proceedings

Potes, N.; Roos, Y.H. Crystallization in Amorphous Lactose-Maltodextrin Mixtures. *International Congress on Engineering and Food (ICEF11)*, May 22-26, **2011**, Athens, Greece, 785-786.

Potes, N.; Febrer, C.M.; Roos, Y.H. Composition and Storage Effects on Mechanical Relaxations in High-Protein Systems. *International Congress on Food Engineering and Technology (IFET2012)*, March 28-30, **2012**, Bangkok, Thailand, 121-123.

Oral Presentations

Potes, N.; Roos, Y.H. Crystallization in Amorphous Lactose-Maltodextrin Mixtures. *International Congress on Engineering and Food (ICEF11)*, May 22-26, **2011**, Athens, Greece. *(One in four selected exceptional research papers in session: The Marcus Karel Symposium on Food Materials Science.)*

Potes, N.; Cuxac, M.C.; Roos, Y.H. Thermally Induced Changes in Protein-Oil Systems. *11th International Hydrocolloids Conference*, May 14-18, **2012**, Purdue University, Lafayette, Indiana, U.S.A.

Potes, N.; Roos, Y.H. Glass Transition and Lactose Crystallization by Water Plasticization in Lactose-Maltodextrin Systems. 7*th International Conference on Water in Food (EUROFOODWATER),* June 3-5, **2012**, Helsinki, Finland.

Potes, N.; Roos, Y.H. Crystallization and Fluidness of Lactose-Maltodextrin Systems. *5th IDF/INRA International Symposium on Spray Dried Dairy Products (SDDP)*, June 19-21, **2012**, St-Malo, France.

Potes, N.; Kerry, J.P.; Roos, Y.H. Formulation Engineering Challenges in Stabilisation of High Solid Systems. *7th European Workshop on Food Engineering and Technology*, May 7-8, **2013**, Parma, Italy.

Potes, N.; Roos, Y.H. Effects of Hydrophilic and Hydrophobic Phases on Structural, Physicochemical, and Thermal Properties of High Solids Systems at Low Water Activity or in the Dry State. *International Conference on Food Properties (ICFP2014)*, January 24-26, **2014**, Kuala Lumpur, Malaysia.

Poster Presentations

Potes, N.; Roos, Y.H. Crystallization Kinetics of Lactose in Freeze-Dried Lactose-Maltodextrin Systems. *Institute of Food Technology Annual Meeting & Food Expo (IFT11),* June 11-14, **2011**, New Orleans, Louisiana, U.S.A.

Potes, N.; Febrer, C.M.; Roos, Y.H. Composition and Storage Effects on Mechanical Relaxations in High-Protein Systems. *International Congress on Food Engineering and Technology 2012 (IFET)*, March 28-30, **2012**, Bangkok, Thailand.

Potes, N.; Febrer, C.M.; Roos, Y.H. Thermal and Mechanical Properties of High Protein Systems Containing Sugars and Lipids. *Institute of Food Technology Annual Meeting & Food Expo (IFT12),* June 25-28, **2012**, Las Vegas, Nevada, U.S.A.

Potes, N.; Kerry, J.P.; Roos, Y.H. Storage Effects on Thermal Properties of High-Protein Systems. São Paulo School of Advanced Science in University of São Paulo, April 1-5, **2013**, Pirassununga, São Paulo, Brazil.

Roos, Y.H.; Yingchen, L.; Potes, N. Thermal Complexity of Whey Proteins at High Solids. *Institute of Food Technology Annual Meeting & Food Expo (IFT13)*, July 13-16, **2013**, Chicago, Illinois, U.S.A.