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Application Potential of Food Protein Modification

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1. Introduction

Proteins are essential in foods, not only for their nutritional value, but also as modulator of structure and perception of a food product. The functional behavior of a protein is inherently susceptible to physico-chemical conditions as pH, ionic strength, temperature, or pressure, making them also an unpredictable, and at the same time, opportune component in food production. Proteins are generally also industrially costly, and with increasing world population and welfare the pressure on protein-availability for food purposes gives rise to some concerns. In view of a more sustainable use of protein-sources a number of routes have been followed in the past decades that provided big steps forward in protein availability: (i) more efficient production or protein refinery methods, (ii) use of alternative protein sources, and (iii) optimized usage of protein functionality. Especially in wheat production correlations between genetic expression and functional product behavior allowed breeders to optimize cultivars for geographic location (e.g. Payne et al., 1984). Alternatively, one has the ability to express specific proteins in non-original sources, for example human milk proteins in plants, such as rice (e.g. Lönnerdal, 2002). Directed alterations in the genome of food-producing organisms can lead to changes in the primary sequences of relevant proteins and thereby introduce potentially new functionality. If sufficient quantities of the novel protein are synthesized and become admixed with the basal levels of protein in the food, the functional properties of the food system (texture-formation) may become improved. Alternatively, the modified protein can be isolated for use as food ingredient. More recently, a number of proteins from less-conventional origin have been identified as human food ingredients that one has started to exploit, e.g. algae, leafs, insects, and various seeds. Successful utilization of these new proteinaceous materials has thus far been rather limited, requiring breakthroughs in extractability, their digestibility, nutritive value, and overall functional and organoleptic properties. More downstream in the process is the modulation of protein functional behavior at an ingredient level. This can be physical-chemically, enzymatically, or via chemical engineering.

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This review will focus on the use of chemical engineering to study or better exploit protein functionality in food products. Reasons to employ chemical modification of proteins will be discussed in the context of its relevance in understanding the fundamental principles of proteins as structuring agents in food. These include improving shelf life and sensory properties as well as the development of new functionalities of food proteins, such as the application of plant proteins as meat-texturizers. Further we discuss how these insights have contributed thus far to a more sustainable utilization of protein, including aspects as consumer acceptance and existing/changing legislations for the use as ingredients.

2. Functional role of proteins as food ingredient

This paragraph will briefly summarize the functional properties of proteins as food ingredients. The molecular mechanisms of their roles in food products will be highlighted. In the next paragraph these molecular mechanisms will be discussed as target for chemical engineering. On each of these topics extensive reviews have been published which will be pointed out.

2.1 Surface properties: Emulsions and foams

Surfaces or interfaces in food products are abundantly present in terms of emulsions (oil-water interface) or foams (air-water interface). Examples of foams and emulsions in food products include ice cream, dressings or margarine. In foams and emulsions respectively air cells or oil droplets are dispersed or in an aqueous phase; the latter case also water in oil emulsions exist. As a result of their amphiphilic character, proteins, being composed of polar and non-polar amino acids, can contribute to the formation and stability of such dispersions by adsorbing to the interface and developing stabilizing films by coating the oil or air droplets and hence act as emulsifiers. The ability of proteins to induce film formation depends on a number of parameters of molecular nature which have been studied into detail in the past and is collectively governed by a net energy gain from absorbing at an interface. Milk proteins have been identified as good foaming agents as a result of their aggregation state, molecular stability and flexibility, electrostatics, and (surface) exposed hydrophobicity (Hunter et al., 1991; Luey et al., 1991; Shirahama et al., 1990; Suttiprasit et al., 1992; Waniska & Kinsella, 1985). Chemical modifications have been employed in the past to improve surface activity of less performing proteins. These studies and consequences of the used modifications will be discussed in more detail in the following paragraph. A wide range of methods has been employed in literature to study the chemical and molecular properties of proteins adsorbed at interfaces which lead to a detailed understanding of the principle forces of importance to surface activity. These methods include ellipsometry and infrared reflection absorption spectroscopy (IRRAS) which provide information on denaturation and concentration of adsorbed proteins (de Jongh & Wierenga, 2006; Grigoriev et al., 2007; Martin et al., 2003; McClellan et al., 2003). Stabilization of an air-water or oil-water interface is governed by a multiple step process. First, absorption at the interface requires proteins to diffuse to the interface and their retention at the interface is governed by the kinetic barrier of absorbance which, in turn, is influenced by factors such as exposed hydrophobicity (Wierenga et al., 2003), and net charge (Wierenga et al., 2005). Effective absorption onto the interface is followed by rearrangement of the protein molecules to form a thermodynamically stable but dynamic monolayer of molecules coating the droplets (de

Jongh & Wierenga, 2006; Graham & Phillips, 1979a, 1979b). Some publications report that the rearrangement process coincides with structural extension of the protein molecules (reviewed in MacRitchie, 1978) while other results suggest that secondary structure does not undergo variation subsequent to adsorption onto the interface (de Jongh & Wierenga, 2006; Graham & Phillips, 1979c). Despite the finding that local concentrations of 150-300 mg/ml can be reached at the interface (Meinders et al., 2001), proteins are still able to laterally diffuse as was shown by thiolated ovalbumin (de Jongh & Wierenga, 2006), illustrating that chemical modification has been used to underpin the molecular mechanisms of the surface activity of proteins.

2.1.1 Emulsions

Emulsions consist of two immiscible liquids, oil and water, in which the droplets are termed dispersed phase and the liquid surrounding the droplets is called continuous phase. Depending on the concentrations of each liquid and the environmental conditions, oil-in-water emulsions or water-in-oil emulsions can be formed. These consist of oil droplets in a continuous water phase and water droplets in a continuous oil phase, respectively. Examples of food related emulsions are milk, vinaigrette, and mayonnaise. Emulsions are often unstable by nature and will phase separate or coalesce over extended time, or storage. To stabilize emulsions, so-called emulsifiers can be used which form a small layer on the surface of the dispersed phase, thereby physically separating the continuous phase from the dispersed phase. Such treatment will allow the incorporation of emulsified solutions in food products which can be stored over an extended time frame. Amphiphilic proteins, containing both hydrophilic and hydrophobic regions, are effective emulsifiers. These proteins adsorb onto the interface between the oil and water phase, and stabilize the oil and water phase by selective interaction with both surfaces, thereby preventing the individual droplets from coalescing (reviewed in Rodríguez Patino et al., 2008). Other types of emulsifiers used in food products include lipids, phospholipids, surfactants or polysaccharides (Dickinson, 1992; McClements, 2005). Apart from a texture perspective, the absorption of proteins at the oil-water interface is of interest to the delivery of nutrients (reviewed in Malaki Nik et al., 2010). Upon digestion, changes occur at the oil-water interface of emulsified food products as a function of emulsifier stability, affecting the digestibility and subsequently the availability of encapsulated nutrients. The amphiphilic character of proteins can be modulated for example by the covalent attachment of lipid chains (see paragraph 3.2). Attachment of lipid chains of various lengths to a protein renders it more hydrophobic resulting in an improved ability to stabilize emulsions and/or foams (Aewsiri et al., 2011a, 2011b). Apart from exposed hydrophobicity, other factors have also been identified to determine the affinity of a protein for an oil-water interface. Aggregation or molecular weight of proteins for example adversely affects the emulsifying activity of proteins (Baldursdottir et al., 2010; Corzo-Martínez, 2011). That the aggregation effect is more complex than originally postulated was shown by single molecule total internal reflectance fluorescence microscopy of bovine serum albumin showing that the orientation at which the aggregates absorb to the interface largely determine the rate of absorbance to the interface (Walder & Schwartz, 2010). The identification of factors contributing to the surface activity of proteins allows the improvement of emulsifying activity by means of targeted chemical modification.

2.1.2 Foams

Foams consist of gas bubbles dispersed into a liquid. The stability of the air bubbles in a foam is determined by the foaming agent which forms a layer of adsorbed molecules separating the air bubbles from the continuous liquid phase, similar to the emulsifying activity described in paragraph 2.1.1 (reviewed in Halling, 1981; Wilde, 2000). Adsorption of a protein to the air-water interface induces partial dehydration of the molecule promoting protein-protein interactions. This effect is further amplified by the finding that local protein concentrations at the air-water interface can reach up to 150 to 300 mg/ml (Meinders et al., 2001). The rate of absorption to the air-water interface has been reported to depend largely on the hydrophobic nature of the protein under investigation (Kudryashova et al., 2003; Wierenga et al., 2003). Increasing the exposed hydrophobicity of proteins by means of conjugation with lipid chains was shown to increase the adsorption rate to the air-water interface (Wierenga et al., 2003). Net charge is a second parameter of interest determining adsorption kinetics, in which higher net charge slows down the adsorption process due to the electric repulsive forces involved (de Jongh et al., 2004; Kudryashova et al., 2005; Le Floch-Fouéré et al., 2011). Highly aggregated heat-treated ovalbumin was further shown to induce a ten-fold decrease the diffusion rate of proteins to the interface compared to the native protein (Kudryashova et al., 2005). However, the tendency of aggregated ovalbumin to remain adhered to the interface upon first interaction is significantly larger than for non-aggregated protein, which was found to rapidly desorb from the surface after absorption (Kudryashova et al., 2005). Collectively, it has been shown that surface activity of proteins is not determined by a single molecular characteristic but rather depends on a combination of factors. Hence, several types of chemical modification can be employed to improve the air-water interface activity of proteins.

2.2 Aggregation and gelation

Protein aggregation is a major topic in the field of food science, the regulation of which is believed to markedly affect the texture of food products (Zhou et al., 2008; reviewed in Doi, 1993). Aggregated protein can act as a nucleation prerequisite to induce gelation, albeit at high protein concentration (Alting et al., 2003; Barbut & Foegeding, 1993; Ju & Kilara, 1998). Processing conditions and storage can induce protein aggregation, even at ambient temperatures (Promeyrat et al., 2010; Santé-Lhoutellier et al., 2008). The resulting protein aggregates can vary widely in size and morphology as a result of the environmental conditions under which they were formed, among other factors. For example, upon inspection using electron microscopy, amylin (Patil et al., 2011), hen egg white lysozyme (Arnaudov & de Vries, 2005), and β-lactoglobulin (Arnaudov et al., 2003; Veerman et al. 2002) were found to form negatively stained and long fibrillar aggregates at pH values far away from the isoelectric point of the respective proteins. Near the isoelectric pH (Arnaudov & de Vries, 2005), or at high salt concentration (Arnaudov & de Vries, 2006; Veerman et al. 2002), spherical or amorphous aggregates are formed. That aggregate morphologies cannot always be categorized as either fibrillar or amorphous in a clear-cut manner was shown by various groups observing substantial heterogeneity in aggregate morphology, also called polymorphism, within the same preparation (Bauer et al., 1995; Jiménez et al., 2002, 1999). As a consequence, gels formed under these conditions of β-lactoglobulin are particulate and the particle size depends on heating temperature and heating rate (Bromley et al., 2006).

These observations lead to the understanding that net charge plays a major role in determining aggregate and gel morphology (Krebs et al., 2009; Langton & Hermansson, 1992). Cryo-EM investigation of aggregates formed from ovalbumin which had been succinylated to various degrees provided further evidence that net charge dominantly determines aggregate morphology (Weijers et al., 2008). The propensity of proteins to aggregate, or rate of aggregation, has been shown to vary as a function of protein conformational stability (Chiti et al., 2000; Hurle et al., 1994; Kelly, 1998; Quintas et al., 1997; Ramirez-Alvarado et al., 2000; Siepen & Westhead, 2002), rate of unfolding (Broersen et al., 2007a), net charge (Calamai et al., 2003; DuBay et al., 2004), and secondary structure propensity (Fernandez-Escamilla et al., 2004). Exposed hydrophobicity (Calamai et al., 2003) and the possibility to form disulfide bonds naturally affect the aggregation and gelation propensity of proteins as these two forces are primarily driving the assembly process. This has been exemplified in a study which showed that β-lactoglobulin A modified with *N*-ethylmaleimide largely resisted aggregation induced by heating (Kitabatake et al., 2001). Extensive knowledge of the molecular factors driving the aggregation process of proteins has lead to the development of a number of algorithms able to predict protein aggregation with high fidelity (Chiti et al., 2003; DuBay et al., 2004; Fernandez-Escamilla et al., 2004; Maurer-Stroh et al., 2010).

The energetics and kinetics of protein aggregation have been subject of many publications to date and has been reviewed on numerous occasions (e.g. Luheshi & Dobson, 2009; Straub & Thirumalai, 2011). It has been recognized that, regardless of primary sequence or physicochemical properties, all proteins have an inherent tendency to form aggregates *in vitro* under certain conditions (reviewed in Chiti & Dobson, 2006; Dobson, 1999). Protein aggregation is a multiple step complex process which can be viewed as a cascade of steps of assembly which may vary in molecular detail as a function of the protein studied or the environmental conditions. Nevertheless, the aggregate growth mechanisms of many different proteins share essential characteristics which have been elucidated both by experimental and computational methods (Teplow et al., 2006). The onset of aggregation often requires the (partial) unfolding or conformational rearrangement of proteins (Calamai et al., 2003; Dobson, 1999; Kelly, 1996; Rochet & Lansbury, 2000). Using human lysozyme (Canet et al., 1999) and HypF-N (Marcon et al., 2005) it has been shown that a population of less than 1% of partially folded protein can be sufficient to trigger the onset of the aggregation process. The resulting exposure of hydrophobic regions which normally reside in the core of a folded protein drives the self-assembly process to form small oligomers. These oligomers, or nuclei, are metastable and their transient and short-lived nature dictates that they can dissociate into monomeric protein, which has been shown for various proteins. Many efforts in the field of protein aggregation suggest that the nucleus has to reach a critical size which then allows further assembly by monomer addition to ultimately form mature fibrils or aggregated networks (Jarrett & Lansbury, 1993; Lomakin et al., 1996, 1997; Sorci et al., 2011). An alternative scenario is the formation of intermediate protofibrils along the pathway which subsequently assemble into fibrils (Harper et al., 1997; Walsh et al., 1997). Mature fibrils have classically been viewed as the stable end-stage of the aggregation process which are not susceptible for dissociating conditions. Recently, using calorimetric methods (Morel et al., 2010), molecular dynamics simulations (Zidar & Merzel, 2011), and mechanical deformation studies (Paparcone & Buehler, 2011; Paparcone et al., 2010; Xu et al., 2010) it has been observed that fibrils can be dissociated albeit at high temperature.

Detailed knowledge of the molecular parameters determining aggregation propensity, rate and morphology resulted in the ability to tune protein aggregation through chemical engineering. The attachment of sugar chains to proteins has been shown to inhibit self-association (Marquardt & Helenius, 1992; reviewed in Helenius et al., 1997; Land & Braakman, 2001; Song et al., 2001). This effect was largely attributed to covalently linked sugar moieties affecting kinetic partitioning between folding and aggregation from an (partially) unfolded state. For example, glycosylation was found to increase the folding rate of the protein rapidly shielding exposed hydrophobic regions which could potentially act as a driving force for aggregation at ambient temperature (Broersen et al., 2007b; Shental-Bechor & Levy, 2008; Wang et al., 1996). Interestingly, high temperatures induced more rapid aggregation of glycosylated proteins compared to their non-glycosylated counterparts (Broersen et al., 2007b; Chobert et al., 2006). However, another study involving glycated bovine serum albumin concluded that glycation of the protein inhibited its aggregation upon incubation at moderate temperatures (Rondeau et al., 2010, 2007). Glycosylation of proteins has also been shown to affect gel properties: attachment of a ketohexose to ovalbumin by means of the Maillard reaction resulted in the formation of gels with enhanced breaking strength (Sun et al., 2004). The effects of various types of chemical protein engineering on the physico-chemical functionality of proteins will be discussed in more detail in paragraph 3 of this review.

2.3 Protein structural integrity

Proteins in food products can lose their native structure as a result of processing conditions including storage, heat treatment, acidification, dehydration, mechanical processing or shear, and microbial hydrolysis. For example, long-term storage of milk powder has been found to induce lactosylation of the proteins present in the preparation which, in turn, results in affected powder solubility and emulsifying and foaming properties (reviewed in Thomas et al., 2004). Unfolded or hydrolyzed protein molecules can exert very different functionality to food products compared to folded proteins, a classical example being the boiling of an egg which converts the liquid-like transparent egg white into an opaque semi-solid structure with very different textural properties. This paragraph will shortly discuss the principles of protein folding and structure and the forces that are implied. It was first recognized by Anfinsen (1973) that the primary sequence of a protein dictates the specific folded, or native, conformation a protein will assume to allow functional activity. Following urea-induced denaturation of ribonuclease A, the protein was allowed to refold by removal of urea. The protein was found to regain its native structure and functionality after this treatment suggesting that proteins can adopt their native conformation spontaneously (Anfinsen, 1973). This finding was awarded with the Nobel Prize in Chemistry in 1972 and opened up an avenue of experimental and theoretical work in the field of protein folding and unfolding. The structural insights into the folding and unfolding processes of many proteins have since then been explored using a vast range of biophysical instrumentation, both at the ensemble (reviewed in Buchner et al., 2011; Sanchez-Ruiz, 2011) as well as at single molecule (reviewed in Borgia et al., 2008; Ferreon & Deniz, 2011) level. Protein conformational stability can be defined as the ability of the natively folded structure of a protein to resist unfolding. Two types of stability can be distinguished: the difference in energy content between the folded state and unfolded state of a protein is termed thermodynamic stability. Boltzmann's distribution law defines the distribution of folded

and unfolded protein molecules at a certain time. The rate at which the unfolded protein collapses to a folded state is reflected by the folding rate and represents the kinetic stability of a protein. Energy landscape theories and the folding funnel hypothesis have both been used as models to understand the energetic barriers of a protein separating the folded from the unfolded state (reviewed in Onuchic et al., 1997; Plotkin & Onuchic, 2002; Wolynes, 2005). Both models start from a similar principle in which there is an energy difference between the folded state of a protein and its unfolded state and that the folded state is defined as the favored entropic state of a protein. Local energy minima in the folding process can result in the accumulation of transient intermediate structures, which are neither folded, nor unfolded, to a larger or lesser extent, depending on the environmental conditions of folding or the primary sequence of the protein (reviewed in Baldwin, 2008; Englander et al., 2007). These intermediate structures, which often lack biological activity, are sometimes sufficiently stable to allow substantial accumulation (reviewed in Englander et al., 2007). Stable folding intermediates are related to a high propensity of aggregation as the hydrophobic core is not sufficiently shielded while opposing assembly forces are absent (reviewed in Ferreira et al., 2006). Kinetic protein stability is defined by a variety of molecular parameters including the proximity of native contacts in the primary sequence of a protein (Cieplak et al., 2004; Plaxco et al., 1998, 2000), internal friction or the energetic of intrachain interactions, energy barriers to backbone rotations and long-range residue interactions (Pabit et al., 2004; Qiu & Hagen, 2004), rate of diffusional motion of an unfolded peptide chain through the solvent (Pabit et al., 2004), and the presence or absence of intermediate state(s) (Baumketner, 2003; Onuchic et al., 1997). Forces that contribute to thermodynamic stability are the strength of intramolecular hydrogen bonds and solvent-protein interactions, both enthalpic in nature. The entropic contribution is mainly defined by the hydrophobic effect of folding through an increase in disorder of water molecules upon folding. Many of the forces retaining a protein structure intact can be disrupted, removed or introduced by chemical modification. For example, reaction of proteins with sugars by means of the Maillard reaction can lead to distinct changes in protein stability. The stability of proteins in food products can be affected by using proteins which have been modified by means of glycosylation or charge modification. Succinylation with the aim to increase net charge of soy protein hydrolysate lead to improved digestibility of the protein as investigated by a multienzyme method involving trypsin, chymotrypsin, and peptidase (Achouri & Zhang, 2001). This finding suggests that the protein had undergone structural rearrangement as a result of the succinylation process. Succinylation also lead to destabilization of Faba bean legumin (Schwenke et al., 1998). This group used differential scanning calorimetry (DSC) to study protein stability of legumin and found a decreased specific enthalpy for unfolding upon succinylation. Succinylation also resulted in an increased surface hydrophobicity of the protein suggesting at least partial unfolding of the molecule. Kosters and colleagues (2003) compared the effects of many different types of chemical modification on protein stability, including lipophilization using capric acid, glycosylation and succinylation of ovalbumin. Ovalbumin stability was probed by DSC, tryptophan fluorescence and circular dichroism (CD). Lipophilization resulted in a decreased denaturation temperature of ovalbumin reflected in an enthalpy decrease and a lower stability upon guanidine titration. Glycosylation was found to stabilize structural integrity of ovalbumin. A similar finding using CD and DSC to study temperature-induced unfolding has been reported upon glucosylation (Broersen et al., 2004; van Teeffelen et al., 2005) and fructosylation (Broersen et al., 2004) of β-lactoglobulin or glucosylation of codfish parvalbumin (de Jongh et al., 2011). Interestingly, assays performed at ambient temperature but involving denaturant-induced unfolding

reported a decrease in protein stability upon conjugation of glucose to β-lactoglobulin. This phenomenon, which appeared unique for a glycosylation reaction, has been studied in further detail by van Teeffelen and colleagues (2005). The observations could be explained in terms of a decreased change in heat capacity upon unfolding as a result of glucosylation indicating that the hydration pattern of proteins upon glycosylation is significantly affected.

2.4 Shelf life

Proteins can affect shelf life and stability of food products by enhancing antioxidant activity, affecting gas exchange, antimicrobial activity or by stabilization of emulsion or foam-based food products (del Rosario Moreira et al., 2011; Emmambux et al., 2004; Mendis et al., 2005). Protein films can be used as as packaging biomaterials as a result of their ability to form networks with rheologically advantageous characteristics (Arvanitoyannis, 1999; Audic & Chaufer, 2005, Longares et al., 2005). However, mixtures of for example proteins and polysaccharides have been found to exert superior functional properties compared to proteins or polysaccharides in isolation (reviewed by Pogaku et al., 2007). For example, the application of an edible coating of storage proteins obtained from sorghum, called kafirins, has been shown to extend the shelf life of freshly harvested pears (Buchner et al., 2011). The shelf life of meat was shown to be extended upon application of a collagen and gelatin coating and led to reduced decoloration, antioxidant activity, and reduction of microbial spoilage (Havard & Harmony, 1869). Such coatings extend quality and shelf-life by acting as a slow-release gas barrier (Baldwin, 1994; Buchner et al., 2011; Park, 1999). Nanobiocomposites of maize prolamin protein zein have also been employed as a gas barrier by coating tomatoes (Park et al., 1994), and apples (Bai et al., 2003). Even though collagen and gelatin coatings were reported to both effectively retain water in meat products (Antoniewski et al., 2007; Farouk et al., 1990), extensive moisture loss of kafirin coated pears compared to the uncoated product left them unacceptable toward consumers (Buchner et al., 2011). The authors (Buchner et al., 2011) therefore suggested to prepare wax or triglycerides/kafirin mixtures instead of pure kafirin coatings to prevent moisture loss as kafirin films themselves do not function effectively as water barriers (Emmambux et al., 2004; Gillgren & Stading, 2008). Because lipids form a very suitable moisture barrier as a result of their hydrophobic character, lipophilized proteins possibly form more effective coatings for fruit. However, to date, no work has been published to demonstrate the effect of lipid-incorporation into proteins to prepare stable coatings for fruit.

Some proteins and peptides are known to have antimicrobial activity (Nizet, 2006; reviewed by Wimley, 2010). Some of these are also applied as food preservatives such as nisin, which is a potent antibacterial 34 amino acid peptide containing a number of uncommon amino acids. Nisin has been employed as an approved food preservative in cheese (Martins et al., 2010), fish, meat, and beverages (reviewed in Lubelski et al., 2008). Another known antimicrobial peptide is ε-poly-L-lysine which exhibits antimicrobial activity against bacteria and fungi and is used as a food preservative (reviewed in Hamano, 2011). This asset has also been explored within a food based environment, for example by applying mixed formulations of chitosan, a linear polysaccharide, and casein polymers to a number of food products including carrot, cheese, and salami (del Rosario Moreira et al., 2011). Pure caseinate films applied to squash slices showed limited antimicrobial activity (Ponce et al., 2008). While chitosan alone exerts significant anti-microbial activity, the inclusion of casein polymers into the formulation

improved bactericidal properties even more (del Rosario Moreira et al., 2011) as a result of the ionic interaction between the two biopolymers (Pereda et al., 2008, 2009). Cao-Hoang and colleagues (2010) produced a nisin-containing sodium caseinate film to investigate the antimicrobial activity of both surface- and in-depth *Listeria innocua* inoculated soft cheese. The presence of the film reduced surface contamination with *L. Innocua* significantly, while antimicrobial activity within the cheese matrix depended on the distance from the film-coated surface. Antimicrobial films prepared from a mixture of oregano oil and whey protein isolate showed inhibition of growth of lactic acid bacteria, reduction of pseudomonads, total flora and growth rates when applied to fresh beef (Zinoviadou et al., 2009). Even though in many cases complex formulae have been employed, containing both a protein component as well as a carbohydrate or oil component, no publications are known that show the effects of covalently linked lipid or sugar to protein films as a potential edible film.

2.5 Sensory: Color, flavor, odor, texture

Sensory aspects of food products include sensation of flavor, odor, color, and texture. These factors play a large role in consumer acceptation of food products and the effects of various types of protein chemical engineering and their applications will be discussed in this paragraph. Many types of modification target the amino groups of lysine residues, including succinylation, lipidation and glycosylation through the Maillard reaction. Textural properties, including emulsifying, foam and gelling capacities, have been discussed in detail in paragraphs 2.1 and 2.2.

2.5.1 Flavor

Even though most proteins are tasteless, ingestion of a small number of proteins is perceived as sweet. These include thaumatin (Ohta et al., 2008; van der Wel & Loeve, 1972), monellin (Morris & Cagan, 1972), brazein (Ming & Hellekant, 1994), and lysozyme (Masuda et al., 2001). The sweetness of lysozyme results from the abundance of lysine residues which was shown by alanine substitution in lysozyme (Masuda et al., 2005a). It is therefore perceivable that modification of lysine residues by conjugation of a chemical group has consequences for the sweetness of the protein. Extensive acetylation and phosphopyridoxylation of lysine residues of lysozyme decreased the perceived sweetness of the protein further demonstrating that lysine residues play a major role in sensory aspects of this protein (Masuda et al., 2005, Kaneko & Kitabatake, 2001). No other reports on flavor modulating aspects as a result of chemical protein engineering have been reported.

2.5.2 Odor

One publication which studied the effect of acylation by acetic and succinic anhydride of flaxseed protein isolates reported no off-odors upon modification, although no results were presented (Wanasundara & Shahidi, 1997).

2.5.3 Color

Three types of modifications have been reported to affect the color of the protein preparation. First, succinylation was reported to convert the color of soy isolate from tan to chalk-white upon visual inspection (Franzen & Kinsella, 1976a). Upon measurement of

surface reflectance using a colorimeter, increasing degrees of succinylation of flax seed protein lead to brighter protein preparations (Wanasundara & Shahidi, 1997). A similar observation has been reported for succinylation of fish muscle (Groninger, 1973), alfa-alfa leaf protein (Franzen & Kinsella, 1976b), and soy bean protein preparations in a U.S. patent for coffee whitener (Melnychym & Stapley, 1973). Acylation by treatment of protein with acetic anhydride also lead to *brighter* flaxseed protein isolate, although the effect of succinylation of the same protein was stronger (Wanasundara & Shahidi, 1997). Franzen and Kinsella (1976a) showed no effect on color upon acetylation of soy isolate. A third type of modification with a strong effect on color is glycosylation through the Maillard reaction. This reaction is a complex cascade of reactions initiated by the interaction of a reducing sugar with an amino group. Colored products are formed only at later stages of the reaction pathway and include aldols and melanoidins which are high molecular weight compounds (reviewed in Zamora & Hidalgo, 2006). Paragraph 3.1 further extends on the formation of browning products and Amadori compounds related to the Maillard reaction.

2.5.4 Texture

Texture is a complex consumer perception of mouthfeel, tastants and afterfeel. A number of most relevant texture attributes, like 'spreadability' (essential for attributes like 'creamy'), 'crumbliness' or 'separating/wateriness' have been shown to be directly related to the energy household in protein-based products when energy is exerted onto the system. This applied energy may arise from oral processing, exposure to heat, gravity or applied pressure during for example industrial processing. The energy balance in protein-based food structures has been proposed by van Vliet and Walstra (1995): $W_{applied} = W_{stored} + W_{dissipated} + W_{fracture.}$ This model implies that all energy applied to the gel can be used either for fracture, can dissipate or can be stored in the network (and regained after release of exerted forces). At a microstructural level this translates into fracture nucleation points and fracture propagation modes, whereas energy dissipation is often assumed to be controlled by serum flow properties. This latter factor is directly determined by the porosity of the gel as set-up by the microstructure and the pore deformation propensity when the system is put under strain. Van den Berg and colleagues (2008) showed that an attribute like 'spreadability' is directly related to directing as much energy as possible to fracture. When energy can be efficiently stored in the protein matrix, this directs the attribute 'crumbliness'. The effective interaction between protein-building blocks that make up the product matrix can be engineered. Strengthening this interaction, using for example transglutaminase (e.g. Dondero et al., 2006), will direct the energy flow from fracture to storage and gels will become less spreadable and more crumbly. Sala and co-workers (2008) showed that by modulating the interaction between a filler (like a fat-particle) and the protein matrix the texture of these protein gels could be strongly affected. This balance between active-inactive property of the filler could be delicately controlled by means of chemical modification.

3. Types of chemical protein engineering – Exploring potential functionality

Proteins can be chemically modified *in vitro* by covalently cross-linking the protein with a molecule of interest. Many of these reactions will also take place post-translationally in the strictly regulated environment of the cell, such as phosphorylation of cAMP-dependent protein kinases which plays a role in the enhancement of glycogen degradation (Soderling,

1975). However, this paragraph will entirely focus on the kind of chemical engineering intentionally brought about to link specific molecules to proteins which act as functional ingredients in food. These molecules change the behavior of the protein and are largely hypothesized to infer characteristics to the protein which are little present in the unmodified protein, such as improved foaming properties, inhibition of aggregation or enhanced surface activity. Rationale for chemical modification of proteins is multiple but can be categorized into four main reasons.

i. Waste control: For example the re-use of fish gelatin from waste requires less natural resources for their production. Other examples include the production of a peptide with anti-oxidant activity from algae (Sheih et al., 2009). Protein rich by-products are also recovered upon electrocoagulation of wastewater resulting from egg processing (Xu et al., 2002). Chemical modification can be used to increase the functional properties of these proteins.
ii. Health considerations: An example of this is the replacement of meat or soy proteins by (other) vegetable proteins (O'Kane et al., 2004; Pedroche et al., 2004; Vioque et al., 1999; reviewed in Moure et al., 2006). Similar to proteins obtained as by-product from waste material, unmodified plant proteins often have limited functional applicability. Hence, chemical modification of plant proteins can be used to improve molecular functionality.
iii. Cost effectivity by extending molecular functionality of a protein. Chemical modification can be employed to enhance the functional properties of a protein, such that less material is required to obtain a product with similar structural characteristics.
iv. Structure-function relationships: Chemical modification is often used to investigate the contribution of specific molecular parameters, such as surface hydrophobicity, to functionality of the protein at ingredient level. Ample examples of protein functional extension are discussed in the rest of this paragraph.

The types of modification described in literature are extensive and include phosphorylation (attachment of a phosphate to serine, tyrosine or threonine), methylation (attachment attachment of a methyl group to arginine or the N-terminus of the protein), glycosylation (attachment of carbohydrates to lysine or the N-terminus), acetylation (attachment of acetyl to an amino group such as lysine or the N-terminus), and many more. The various types of modification and their impact on molecular behavior of proteins have been reviewed in a vast number of publications and book chapters (Feeney et al., 1982; Means & Feeney, 1971; Tawfik, 2002). This paragraph aims to shortly discuss the current state-of-the-art of the various reactive groups in proteins which can be targeted by chemical engineering. The chemical basis of these modifications will be discussed and applications from literature will be reviewed merely to illustrate the wide variety of applications of the chemical modification procedure.

3.1 Glycosylation and deglycosylation

Food storage and preparation processes such as heating by pasteurization or sterilization often provide for conditions which induce spontaneous and uncontrolled reaction of a reducing carbohydrate present in the food matrix with proteins. This reaction, termed the Maillard reaction, which is actually a complex cascade of reactions, is responsible for the formation of browning products and can have substantial impact on the flavor and color of food products. The Maillard reaction is initiated by a condensation reaction between the ε-

amino group of lysine and the reducing group of a sugar to form Amadori or Heyn's rearrangement products via *N*-substituted glycosylamine. During the advanced stages of this reaction, the Amadori and Heyn's rearrangement products are degraded via a number of pathways (Mossine et al., 1994; Röper et al., 1983). The last stages of the Maillard reaction involve extensive protein cross-linking reactions and the formation of so-called melanoidins (Pellegrino et al., 1999). As a result of the wide range of intermediate chemical structures formed, several of these intermediates can be employed as indicators of the Maillard reaction to monitor the extent of the reaction in food products as a measure of quality control. For example, ε-*N*-(furoylmethyl)-L-lysine (furosine) formation, an intermediate in the Maillard reaction, was shown to be the result of lactosylation upon storage of milk powder (Le et al., 2011). Also prolonged storage of high-protein nutrition bars showed non-enzymatic Maillard browning as a result of interaction between whey protein isolate and high-fructose corn syrup or sorbitol syrup (McMahon et al., 2009). The baking process of bread was found to affect color formation determined by furosine and hydroxymethylfurfural concentrations (Ramírez-Jiménez et al., 2000). Variation of baking temperature and dough composition determines the extent of furosine formation and loss, acid-released lysine, and carboxymethyllysine formation of cookies (Charissou et al., 2007). Glycosylation via the Maillard reaction has also been brought about intentionally to study the effects of covalent sugar linkage to proteins in terms of e.g. protein stability and aggregation (Feeney et al., 1975). Glycosylation of proteins by means of the Maillard reaction has been observed to both induce and protect against aggregation. Incubation of hazelnut proteins with glucose resulted in the formation of high molecular weight protein aggregates detected by SDS-PAGE (Cucu et al., 2011). At the same time, others have shown that Maillardation with glucose inhibited the aggregation of cod fish parvalbumin (de Jongh et al., 2011). As the Maillard reaction is a reaction involving many steps, one possibility which can be raised to explain the discrepancy between these observations is that the various intermediates may display differences in resistance against aggregation, some of which may be protective, others which may be inducing aggregation. Increased thermal stability upon glycosylation was found for many proteins including the apple allergen Mal d3 upon reaction with glucose (Sancho et al., 2005), and β-lactoglobulin reaction with glucose and fructose (Broersen et al., 2005). Alternatively, deglycosylation was shown to induce denaturation and aggregation of ovalbumin (de Groot et al., 2007). Apart from the Maillard reaction, protein glycosylation can be achieved by several other routes. *N*-glycosylation takes place by modification of the side chains of asparagine or arginine (Kornfeld & Kornfeld, 1985). *O*-glycosylation is brought about by modification of serine, threonine, or tyrosine (Hart, 1992). Many different glycan structures have been identified to be involved in these two types of modification and they are often necessarily involved in biological function of the protein (Rudd et al., 2001). These last two types of glycosylation take place mainly as a result of post-translational processing of proteins *in vivo* and are not used as means to induce glycosylation of proteins applied in the food industry. Hence, *N*- and *O*-glycosylation will not be discussed in this paragraph.

3.2 Lipophilization

Covalent linkage of lipids to proteins results in increased hydrophobic exposure of a protein with interesting applications related to altered surface properties. Increased exposed hydrophobicity of proteins has for example been related to an improved capacity to form

and stabilize emulsions and foams which is the result of improved potential to interact with hydrophobic surfaces, both the air-water and oil-water interface, and including (model)membranes (Nakai, 1983; Wierenga et al., 2003; reviewed in Wilde, 2000; Wilde et al., 2004). Various saturated and unsaturated fatty acids have been employed to induce lipophilization of proteins including caproic acid (Liu et al., 2000), capric acid (Aewsiri et al., 2010; Kosters et al., 2003; Liu et al., 2000), lauric acid (Aewsiri et al., 2010), myristic acid (Aewsiri et al., 2010; Ibrahim et al., 1993; Liu et al., 2000), palmitic acid (Haque et al., 1982; Haque & Kito, 1983a, 1983b; Ibrahim et al., 1991), stearic acid (Djagny et al., 2001; Ibrahim et al., 1993), and oxidized forms of linoleic acid (Aewsiri et al., 2011a, 2011b), and the efficiency of the lipophilization reaction was found to be inversely proportional to the length of the lipid chains used (Liu et al., 2000). Reaction of 28% of the available free amino groups of ovalbumin with activated capric acid was shown to result in retained secondary structure while inducing oligomerization and destabilization of the protein structure as a result of lowering the enthalpy for unfolding (Kosters et al., 2003). The presence of acyl chains was thought to cause significant dehydration of the protein. In another study, hen egg white lysozyme was lipophilized with short and middle chain saturated fatty acids including caproic (C6:0), capric (C10:0), and myristic (C14:0) acid (Liu et al., 2000). Lipophilization of lysozyme was reported to decrease the thermal stability of lysozyme as a result of partial loss of α-helical content of the protein, and this molecular destabilization appeared to be proportionally related to the chain length and the number of bound fatty acids. The lysine residues involved in the modification were thought to be located in the helical region and to subsequently induce partial unfolding of the α-helical region surrounding these residues (Liu et al., 2000). Lysozyme has also been chemically modified using palmitic acid (Ibrahim et al., 1991, 1993) with the primary aim to study the effect of lipophilization on the antimicrobial effect of the protein. Even though increasing extents of covalent linkage with palmitoyl residues lead to insoluble protein, as spectrophotometrically determined by solution turbidity at 500 nm, foaming stability and emulsifying activity were progressively improved by linkage of palmitic acid to the protein molecule. More groups showed that the foaming or emulsifying activities of a wide range of proteins could be improved upon lipophilization, including soybean glycinin (Haque et al., 1982), α_{s1}-casein (Haque & Kito, 1983b), and cuttlefish skin gelatin (Awesiri et al., 2011a, 2011b). A further effect resulting from the incorporation of myristic and stearic acids into lysozyme was related to antimicrobial activity and stearic and palmitic acid conjugation resulted in more effective antimicrobial agents against *E. coli*, than the attachment of myristic acid or the unmodified protein (Ibrahim, 1993). Myristoylation was found to induce lysozyme aggregation resulting in concurrent loss of antimicrobial function. The effects of palmitoylation on the structural and functional properties of α_{s1}-casein have also been explored (Haque & Kito, 1983a, 1983b). The conjugation of the ε-amino groups of α_{s1}-casein with palmitic acid lead to micelle formation as a result of increased hydrophobicity while negative net charge was increased (Haque & Kito 1983a). Further work by this group showed that palmitic acid linkage to α_{s1}-casein did not lead to large scale structural rearrangement of the molecule, both at a secondary and a tertiary structure level using circular dichroism. Interestingly, Aewsiri and colleagues have also investigated the antioxidative activity of cuttlefish skin gelatin modified with a combination of oxidized linoleic acid and oxidized tannic acid, a potent antioxidant (Aewsiri et al., 2011a). Oxidation of lipids and proteins in foams primarily takes place at the air-water interface and the addition of a hydrophilic anti-oxidant alone reduces surface activity (Aewsiri et al., 2011a). Co-conjugation of tannic acid

and linoleic acid to gelatin both improves migration of the protein to the air-water or oil-water interface improving foaming and emulsifying activity, respectively, while retaining anti-oxidant activity (Aewsiri et al., 2011a).

3.3 Chemical-reactive groups

Sulfhydryl groups play an important role in regulating the self-assembly of proteins as well as their stability driven by disulfide interchange reactions (Sawyer, 1968). Hence, the presence of these groups has substantial impact on the aggregation and gelation behavior of a wide variety of proteins which has been confirmed by many researchers (Arntfield et al., 1991; Broersen et al., 2006; Graña-Montes et al., 2011; Hayakawa & Nakai, 1985; Hoffmann & van Mil, 1997; Margoshes, 1990; Mine, 1992; Sawyer, 1968; Shimada & Cheftel, 1989). A variety of modifications can be performed targeting sulfhydryl groups, which are part of the cysteine residues. Sulfhydryl groups are highly reactive against various reactants and are thus suitable targets for modification. Sulfhydryls can be blocked to prevent cross-linking by S-methyl methanethiosulfonate (MMTS), *N*-ethylmaleimide (NEM) (Kitabatake et al., 2001), or iodoacetamide (Anson, 1940; Huggins & Jensen, 1949; Smythe, 1936), or additional sulfhydryl-groups can be attached to primary amines (SATA) of proteins. Further, *N*-hydroxysuccinimide esters can react irreversibly with primary amines releasing *N*-hydroxysuccinimide.

Disulfide bonds are thought to play a crucial role determining the stability of proteins (Betz, 1993; Zavodszky et al., 2001; reviewed by Creighton, 1988) as well as to impact on the aggregation process and gel formation of various proteins including ovalbumin (Broersen et al., 2006; Kato et al., 1983), vicilin (Arntfield et al., 1991), and β-lactoglobulin (Sawyer, 1968). Aggregates and gel networks are often the result of combined action of hydrophobic and electrostatic interactions and covalent interactions, in the form of disulfide bonds, are sometimes present (Kato et al., 1983; Koseki et al., 1989; Sun & Hayakawa, 2002). Thiolation of ovalbumin mediated through the reaction of S-acetylmercaptosuccinic anhydride (S-AMSA) with primary amines results in the formation of acetylthio groups and the acetyl group can be cleaved off to yield reactive sulfhydryl groups by the addition of hydroxylamine (Klotz & Heiney, 1962). A range of modification degrees can be obtained by varying the S-AMSA:lysine ratio (Broersen et al., 2006). Next to the linkage of a sulfhydryl group, additional carboxyl groups are conjugated through this reaction introducing additional charge variation which can lead to an additional parameter which can induce variation in aggregation, gelation or stability of a protein. To circumvent this variation, proteins with activated sulfhydryl groups are best compared with similarly modified proteins with blocked (i.e. not reacted with hydroxylamine) acetylthio groups, rather than directly with the unmodified protein (Broersen et al., 2006). Thiolation of ovalbumin in this way lead to limited changes at a secondary and tertiary structure level at high degrees of modification suggesting that the original molecular fold was largely retained upon modification. High degrees of thiolation resulted in a decrease of thermal stability of ovalbumin while fibril morphology was affected. Interestingly, the rate of aggregate formation was not modified by the presence of additional sulfhydryl groups available for disulfide formation. It was concluded that disulfide formation does not represent the prime driving force for aggregation of ovalbumin which was further illustrated by the finding that at room temperature, where significant sulfhydryl groups are already exposed upon

modification, no disulfide bond aggregation was observed. Final aggregate morphology, gel formation and stability are affected as a result of rapid covalent network formation which does not allow rearrangement into more stable networks, as illustrated by lower gel Young's moduli obtained upon thiolation (Broersen et al., 2006).

3.4 Charge modification by methylation and succinylation

Net charge and local charge densities of proteins have been implicated in the regulation of protein stability, aggregation, and aggregate morphology affecting the visual appearance of food products. These hypotheses have been substantiated by a range of observations which involved charge introduction, removal or reversal through succinylation and methylation reactions (Broersen et al., 2007a; Weijers et al., 2008). The reactions of succinylation and acetylation both lead to blockage of the reactive amino groups of proteins with an acyl residue and are hence collectively termed acylation reactions. The rate of acylation reaction depends on the rate of nucleophilic attack. Succinylation leads to increased net negative charge by the covalent linkage of succinate anions to the cationic amino groups of a protein thereby converting a cationic group into an anionic residue having implications for the distribution of net charge of a protein. Upon acetylation, ammonium cations are replaced by neutral acetyl groups resulting in electrostatically neutral groups. Large extents of succinylation have been reported to affect the integrity of secondary and tertiary structure of soy protein hydrolysate as shown by intrinsic tryptophan fluorescence and circular dichroism (Achouri & Zhang, 2001). Similar conformational rearrangements have been reported upon succinylation of whey protein isolate (Gruener & Ismond, 1997), bovine serum albumin (Jonas & Weber, 1970), canola protein (Lakkis & Villota, 1992), Faba bean legumin (Schwenke et al., 1998), rapeseed 12S globulin (Gueguen et al., 1990), and winged bean protein (Narayana & Rao, 1991). As a result of co-incubation of soy protein hydrolysate with succinic anhydride, which is a common compound used to succinylate proteins, heterogeneous reaction mixtures were obtained. Next to the aimed amine groups, this method of succinylation also commonly results in *O*-succinylation, involving threonine or serine hydroxyl groups or tyrosine succinylation (Achouri & Zhang, 2001; Chang & Sun, 1978; Schwenke et al., 1998). This latter reaction was found to be reversible upon treatment with hydroxylamine (Habeeb & Atassi, 1969), but, when present, to induce substantial expansion of Faba bean legumin as observed by viscometric studies (Schwenke et al., 1998). It was postulated that the high accumulation of negative charge upon extensive succinylation leads to dissociation and expansion of the individual subunits legumin is composed of (Schwenke et al., 1998). Other functional properties are equally affected by succinylation. For example, protein solubility has been reported to increase upon succinylation as has been demonstrated for rapeseed preparations (Dua et al., 1996), flax protein isolate (Wanasundara & Shahidi, 1997), oat protein isolate (Mirmoghatadaie et al., 2009), and soy protein isolate (Franzen & Kinsella, 1976a). Improved solubility has been related to the ability of proteins to perform more efficiently as stabilizers in emulsions and foams (Nakai & Li-Chan, 1988; Waniska & Kinsella, 1979), which, in turn, is greatly affected by their ability to absorb at the air-water interface (Wierenga et al., 2005). It has indeed been shown that treatment of a variety of proteins with succinic anhydride leads to increased foam capacity (Dua et al., 1996; Franzen & Kinsella, 1976a; Mirmoghatadaie et al., 2009), although others suggest that succinylation leads to decreased foam expansion capacity (Wanasundara & Shahidi, 1997). These seemingly contradictive findings may be explained

by the demonstration by Wierenga and colleagues (2005) that the likelihood of a protein molecule to adsorb at an interface is the result of a balance between hydrophobic and steric effects: highly charged molecules may be adsorbing to the interface as a result of hydrophobic interaction, but the density at which protein molecules continue adsorbing to the interface is mainly determined by the repulsive nature of the charged proteins. Other effects observed upon charge modification of proteins are related to emulsification properties: methylation increased while succinylation decreased the emulsifying activity of rapeseed preparations (Dua et al., 1996). Other studies show an increased emulsifying activity and stability upon succinylation of soy protein (Franzen & Kinsella, 1976a), and oat protein isolate (Mirmoghatadaie et al., 2009). In terms of gelation, an increase in net charge lead to more transparent gels upon gelation of ovalbumin which is related to the morphology of the aggregated network making up the gel structure (Weijers et al., 2008). Overall, many and detailed efforts have been made employing net charge modification of proteins in the field of food science. These studies have lead to in-depth knowledge of the role of electrostatics to common protein functionalities such as emulsification, foaming, and aggregation propensity.

3.5 PEGylation

The covalent attachment of a polyethylene glycol (PEG) polymer chain to a protein, also termed 'PEGylation' is mostly applied in the field of pharmaceutics as the conjugation of non-toxic PEG imparts substantial advantages to support drug delivery (reviewed in Damodaran & Fee, 2010; DeSantis & Jones, 1999; Francis et al., 1998). The protein targets for PEG modification are regarded as non-specific and include the ε-amino groups of lysine and other nucleophilic groups such as glutamic acid, aspartic acid, threonine, serine or tyrosine on the surface of the protein resulting in highly heterogeneous protein-PEG conjugates upon modification (Losso & Nakai, 2002). Commercially available PEG is available as mixtures of different oligomer sizes in various molecular weight ranges enabling the variation of exposed hydrophobicity of proteins. Conjugation of hydrophilic PEG to a hydrophobic protein generally results in an increase in hydrodynamic size and water solubility (Damodaran & Fee, 2010). From a pharmaceutical viewpoint, PEGylation has been reported to enhance circulation life of bovine liver catalase in the blood of mice while the presence of PEG does not induce an immune response upon injection (Abuchowski et al., 1977). Modification of peroxidase from turnip was shown to enhance catalytic activity of the enzyme with increased stability in organic solvents as well as increased temperature resistance (Quintanilla-Guerrero et al., 2008). Similar results were found upon PEGylation of trichosanthin, which showed prolonged plasma half-life and reduced immunogenicity (He et al., 1999). PEGylation of lysozyme similarly lead to stabilization of the protein against pH and temperature variation as well as resistance against proteolysis (Silva Freitas & Abrahão-Neto, 2010). Most of these studies have been carried out in the context of pharmaceutical application. The study of PEGylation as a potential route to bring about modification of physicochemical parameters of proteins applied in food products has been less well explored. The only known effort in the field of food science combined oxidative sulfitolysis with conjugation of 5000 dalton activated PEG to investigate the impact on the emulsifying properties of β-lactoglobulin A (Losso & Nakai, 2002). PEG molecules were found to cover the entire surface of unmodified and sulfitolyzed β-lactoglobulin and PEGylation alone did not improve emulsifying activity or emulsion stability. The combination of sulfitolysis and

PEGylation however lead to more stable emulsions and improved emulsion activity index as a result of better stabilization of individual droplets against coalescence by the absorption of PEG onto the surface of the sulfitolyzed β-lactoglobulin (Losso & Nakai, 2002). From the limited number of studies available it is not possible to derive direct conclusions on the applicability of PEGylation on the advancement of functionality of proteins in food products.

3.6 Deamidation

Deamidation involves the hydrolysis of the amino acids glutamine and asparagine into glutamic and aspartic acid and is achieved by acid, alkaline, or enzymatic treatment (Liao et al., 2009; Shih, 1990; reviewed in Wright & Urry, 1991). Consequently, deamidated protein is often obtained as a by-product of food processing. For example, the extrusion of wheat flour induces deamidation of wheat proteins (Izzo et al., 1993). Deamidation has been shown to affect protein functionality. Functional properties, such as solubility, emulsifying and foaming properties, of gluten have been reported to improve upon low levels of deamidation brought about by mild acid hydrolysis (Hamada & Marshall, 1989; Matsudomi et al., 1982, 1985). Deamidation also was reported to increase exposed hydrophobicity of gluten induced by a conformational change and, subsequently, to increase surface activity (Matsudomi et al., 1982). A number of studies investigated the molecular mechanism for protein structural destabilization upon deamidation. For example, the deamidation treatment has direct implications for charge density and, in turn, affects electrostatic interactions the protein may undergo by interacting with water or upon self-assembly (Finley, 1975; reviewed in Riha et al., 1996). This role of electrostatics to deamidation-induced disruption of protein structure was supported by further observations on wheat gluten: both acetic acid and HCl induced deamidation had substantial consequences for the secondary structure of wheat gluten. It was thus postulated that strong deamidation induced protein unfolding as a result of electrostatic repulsion (Liao et al., 2010). Acetic acid induced deamidation of wheat gluten was further found to inhibit SDS-stable aggregate formation whilst largely retaining its ability to form disulfide bonds (Liao et al., 2010). The rate of the deamidation reaction has been found to depend on primary sequence and pH under which the reaction takes place, but was independent of ionic strength for model peptides (Patel & Borchardt, 1990a, 1990b; Robinson & Rudd, 1974; Tyler-Cross & Schirch, 1991), soy protein and egg white lysozyme (Zhang et al., 1993).

3.7 Cross-linking

A variety of aldehydes, including gluteraldehyde, formaldehyde, and α-hydroxyadipaldehyde, have been used to induce chemical cross-linking of proteins. Between these reagents, gluteraldehyde was found to cross-link bovine serum albumin most efficiently forming large insoluble networks (Hopwood, 1969). Also for other proteins gluteraldehyde has been reported as the most efficient cross-linking agent yielding thermally and chemically stable cross-links (Bowes & Cater, 1968; Nimni et al., 1987). Primary amino groups have been reported to act as prime target to initiate the aldehyde-induced cross-linking reaction (Quiocho & Richards, 1966), next to aromatic amino acids (Hopwood et al., 1970). Depending on environmental conditions, gluteraldehyde can bring about cross-linking through a wide variety of reaction mechanisms (reviewed in Migneault

et al., 2004). This is caused by the large number of different molecular structures gluteraldehyde can assume in solution (Hardy et al., 1969; Korn et al., 1972; Richard & Knowles, 1968; reviewed in Migneault et al., 2004) although the mechanistic details for this is unknown. As a result of its reported toxicity, gluteraldehyde-induced cross-linking has not been employed in the field of food technology, other than as a tool to enable the investigation of intermediates in the aggregation pathway or to immobilize proteins onto a surface to allow further investigation.

3.8 Measuring the degree of modification

To evaluate the effect of a specific type of modification on the functional aspects under study, the success of the chemical engineering process on proteins is evaluated by most researchers. To this end, targeted chemical and biophysical assays have been developed which are now widely used. These quantitative assays are mostly based on the formation of a chromogenic or fluorogenic product upon specific interaction with reactive groups of a protein. Some assays will provide information on the average degree of modification in the entire ensemble of protein molecules in a solution. Examples of such assays are Ellman's reagent (Ellman, 1959) or the sulfhydryl-disulfide exchange (SEI) index (Owusu Apenten et al., 2003). Others are also useful to obtain information on the distribution of the degree of modification obtained, such as mass spectrometry. The type of assays developed can be categorized by the type of aimed conjugated chemical group of the protein they probe, such as amine groups, thiol groups or carboxyl groups. Some other researchers use methods which rather probe for the attached molecule, such as the use of gas liquid chromatography (GLC) to determine the degree of lipid incorporation (Haque et al., 1982).

3.8.1 Amine groups

Amine groups in proteins originate either from free amino groups of proteins or from N-terminal residues of proteins (Skraup & Kaas, 1906; Chibnall, 1942). From 1906 it was recognized that lysines in proteins were largely responsible for the free amino groups present in proteins (Skraup & Kaas, 1906). This finding triggered the development of various assays to study accessible ε-amino groups of lysines in proteins (Gurin & Clarke, 1934; Sanger, 1945). Three of the most commonly used assays in the field of protein engineering to determine the number of available amino groups of a chemically modified protein involve the use of chemicals 2,4,6-trinitrobenzenesulphonic acid (TNBS) (Fields, 1971), ninhydrin (Yemm & Cocking, 1955; Schilling et al.; 1963; Samejima et al., 1971), and ortho-phtaldialdehyde (OPA) (Roth, 1971). The latter assay is based on the reaction of the OPA compound with free amino groups in proteins in the presence of β-mercaptoethanol under alkaline conditions. This reaction results in the formation of highly fluorescent alkyl-iso-indole derivatives which emit at a wavelength of 455 nm upon excitation at 340 nm. High concentrations (*i.e.* 10%) of sodium dodecyl sulfate (SDS) are often added to the protein solution to aid the exposure of all amino groups which are sometimes buried in the folded protein. The extinction coefficient for the formed adducts of both α- and ε-amino groups are similar with an absorptivity of 6000 M^{-1} cm^{-1}. The OPA assay was further developed to evaluate the degree of proteolysis of dairy proteins by determining the number of α-amino groups released upon hydrolysis (Church et al., 1983). The assay can also be used to quantify all types of reactions involving the modification of lysine and,

hence, includes lipidation of ovalbumin (Kosters et al., 2003; Wierenga et al., 2003), lysozyme (Liu et al., 2000), and gelatin (Aewsiri et al., 2010, 2011a, 2011b). Other modifications which can be quantified using the OPA assay are succinylation (Kosters et al., 2003; Wierenga et al., 2005), methylation (Kosters et al., 2003), glycosylation (Broersen et al., 2004; Kosters et al., 2003), and thiolation of proteins (Broersen et al., 2006).

The second frequently used method to quantify available amino groups upon modification involving the chemical TNBS was developed by Okuyama and Satake (1960) and Satake and colleagues (1960). This method was first employed to study free amino acid groups in trypsin and chymotrypsin inhibitors (Haynes et al., 1967) and for routine screening of protein concentrates for animal feeds (Hall et al., 1973). The chemical TNBS reacts with high preference to free amino groups resulting in the formation of trinitrophenyl derivatives. The reaction product can be quantified spectrophotometrically at 335 nm. A disadvantage of this method is that TNBS also reacts with free sulfhydryl groups, albeit at a slower rate than with amino groups and to form a labile product (Kotaki et al., 1964). The TNBS assay has been used for various types of protein modification including fatty acid incorporation (Andersson et al., 1971; Ibrahim et al., 1991, 1993), glycosylation of proteins as a result of the Maillard reaction (Sun et al., 2004), succinylation (El-Adawy, 2000; Schwenke et al., 1998; Zhao et al., 2004a, 2004b), and acetylation of faba bean legumin (Krause et al., 1996).

The compound 2,2-dihydroxyindane-1,3-dione (ninhydrin) reacts with ε-amino groups and ammonium ions into a blue-purple Schiff base product called Ruhemann's purple, that can be colorimetrically detected at a wavelength of 440 nm (Yemm & Cocking, 1955; Schilling et al., 1963; Samejima et al., 1971). The ninhydrin assay has been used to quantify degrees of succinylation of soy protein (Franzen & Kinsella, 1976a), and acylation of flax protein isolates (Wanasundara & Shahidi, 1997).

Comparison of TNBS, OPA or ninhydrin to determine α-amino groups in pea protein isolates and hydrolysates thereof lead to the conclusion that TNBS and OPA produced comparable results while ninhydrin detected only half of the amino groups that were detected by TNBS and OPA (Panasiuk et al., 1998).

3.8.2 Sulfhydryl groups

Two commonly used assays are available to evaluate the successful conjugation or blockage of sulfhydryl groups in proteins. The assay which was developed first by Ellman (1959) involves the reaction of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) or Ellman's reagent with free sulfhydryl groups yielding colored 3-carboxylato-4-nitrothiophenolate (CNT). Spectrophotometric absorbance intensity at 412 nm provides for a direct measure of the concentration of CNT in solution and cysteine is commonly used as calibration standard. Ellman's assay has been used before to determine the extent of *N*-ethylmaleimide modification of β-lactoglobulin A (Kitabatake et al., 2001; Wada & Kitabatake, 2001), thiolation of ovalbumin (Broersen et al., 2006), or acylation of soy protein sulfhydryl groups (Franzen & Kinsella, 1976a). However, the sizeable DTNB at 400 dalton may not be able to detect sulfhydryl groups which are buried inside the folded structure of intact proteins or aggregated proteins (reviewed by Visschers & de Jongh, 2005). A useful alternative for the Ellman's assay has been developed by Owusu Apenten and colleagues (2003) for β-

lactoglobulin and bovine serum albumin. Chemical reactivity of thiol groups is an absolute requirement to enable covalent cross-linking through disulfide bond formation (Hillier et al., 1980). This assay, called the sulfhydryl-disulfide exchange (SEI) index, therefore provides a direct measure of the chemical reactivity of thiol groups as it determines the conversion of substrate in time and kinetically relates the conversion to that of fully exposed thiol groups (Owusu Apenten et al., 2003). Chemical activity of introduced sulfhydryl groups by modification of ovalbumin using the SEI index has been verified for example by Wierenga and colleagues (2006).

3.8.3 Carboxylic acid groups

The carboxylic acid content of proteins is primarily investigated using the compound 2-ethyl-5-phenylisoxazolium-3′-sulfonate or Woodward's reagent K (Woodward & Olofson, 1961; Woodward et al., 1961; Sinha & Brewer, 1985). The activity of Woodward's reagent K is the result of a multistep process. First, Woodward's reagent K is converted into ketoketenimine at neutral pH. The intermediate compound is then either further disintegrated to form ketoamide or interacts with carboxylic acid groups of a protein. The latter interaction results in the formation of an enol ester (Pétra, 1971) which absorbs at 340 nm with a molar extinction coefficient of 7000 M^{-1} cm^{-1} (Sinha & Brewer, 1985). At a later stage, Kosters and de Jongh (2003) revised the extinction coefficient of the product to 3150 M^{-1} cm^{-1} at 269 nm to improve specificity of the reaction and eliminate the substantial contribution of side reactions with other nucleophiles in proteins (Llamas et al., 1986), and histidine and cysteine (Bustos et al., 1996; Johnson & Dekker, 1996) to the absorbance at 269 nm. This revised version of the assay employing Woodward's reagent K to estimate the number of carboxylic acid groups of chemically modified proteins has been used by a number of researchers. Wierenga and colleagues (2005) used Woodward's reagent K to estimate the degree of modification of succinylated ovalbumin to study the relation between protein net charge and adsorption to air-water interfaces. Similar net charge variation induced by succinylation was used to investigate colloidal versus conformational stability of ovalbumin to aggregation (Broersen et al., 2007a). To investigate the stability of ovalbumin, the protein was modified by succinylation, methylation, glycosylation, and lipophilization and the degrees of modification were validated using Woodward's reagent K and the OPA assay (paragraph 3.8.1).

3.8.4 Conjugated groups

An alternative route to obtain information on the degree of protein modification is to selectively probe the conjugated group. This can be achieved for example by incorporating an isotopically labeled reagent or inclusion of a chromophore or fluorophore which can then be quantified by read-out of fluorescence intensity using a fluorimeter or simple absorbance measurements using a standard spectrophotometer. Raman spectroscopy was shown to provide direct insight into degrees of succinylation and acetylation of a range of proteins originating from soy, egg white or whey by distinct contributions of the conjugated groups at 1737 cm^{-1} and 1420 cm^{-1} (Zhao et al., 2004a, 2004b). The peak intensities at these wavelengths could be directly converted to obtain information on the degree of modification. Degrees of palmitoylation of soybean glycinin (Haque et al., 1982) and α_{s1}-casein (Haque & Kito, 1983a) have been determined using gas liquid chromatography.

Chromatography has been further explored in the shape of cation exchange chromatography to validate the degree of methylation of ovalbumin which was found to provide comparable read-outs as the revised version of Woodward's reagent K method to determine carboxylic acid groups (Kosters & de Jongh, 2003). All these techniques provide insight in the ensemble average degree of modification. Chemical engineering inherently implies the rise of heterogeneous species of proteins. Mass spectrometry, often employed as Matrix-assisted laser desorption/ionization-time of flight (MALDI-tof) mass spectrometry, has proven a powerful method to specifically obtain insight into the distribution of the modification reactions. This method has been employed to derive information on modification distributions of *N*-ethylmaleimide modified β-lactoglobulin A (Wada & Kitabatake, 2001), and glycosylation of β-lactoglobulin (Broersen et al., 2004; van Teeffelen et al., 2005). These studies demonstrated that degrees of modification obtained upon chemical engineering of proteins are rather broad and show a Gauss distribution profile rather than a single well-defined modification degree (Broersen et al., 2004; van Teeffelen et al., 2005). Some attempts have been made to isolate modified protein fractions with more defined degrees of modification, for example by using ion exchange chromatography of succinylated ovalbumin (Wierenga et al., 2005).

4. Application potential of food protein modification

From the above it is clear that in the past decades a lot of effort has been spend on better understanding and controlling protein behavior and protein-based microstructure formation by making use of chemical engineering approaches. But how much impact have these insights had on the development of new food applications? There are a number of well-known product categories where engineered protein functionality has led to improved product properties. In the early seventies Unilever produced new lines of margarines that showed better performance in aspects like spreadability, prolonged storage stability and during baking, caused by acetylation of milk proteins leading to better fat emulsification (Evans & Irons, 1970). Also for mayonnaises and salad dressings modification of egg yolk proteins (via *N*-succinylation) provided improved product quality (Evans & Irons 1970). Another example of an application of improved protein functionality is that of the use of succinylation to improve the solubility/dispersability properties of soy proteins in the extraction and refinery process (Melnychyn & Stapley, 1973). It is interesting to evaluate via a patent-literature screening how frequent the wide variety of technological possibilities as described in section 3 to better control protein behavior have led to unique market-propositions.

4.1 Current application of protein modifications in food/feed-related products

Figure 1 illustrates a landscape representation of patents (worldwide), filed in the last decade, in the area of food and feed where protein modification has played a crucial role in deriving a new type of product functionality. The height of the contour indicates the activity in that particular area. The distance between patents reflects their commonality. In total only 445 relevant patents (grouped in 157 families) can be found. In comparison, a search on any biobased-product (including products with protein-based technical polymers as in coatings, paints, paper, etc.) revealed more than 8600 patents. Clearly the role of protein engineering to derive new food product specifications are very limited, especially in view of the

potential commercial impact. The observation that the patent-families found are spread rather constant over the plot illustrates that these patents are not directly linked to each other in terms of engineering approach or application area. When evaluating the patent filings of the last ten years five areas can be distinguished with a relatively high patenting activity and these will be discussed below in more detail. These are numbered 1 to 5 in the figure.

1. *Refinery of seed storage proteins (12 patents).* There is an increasing interest to use readily abundant and relatively cheap seed storage proteins as nutritional component in food and feed. The major difficulty of this protein-source is to obtain functional proteins after the refinery steps. Loss of functionality occurs especially when the protein is used as powdered ingredient, because of difficulties in resolubilization and unpredictable caking of the powder during storage/transport. A number of patents have been filed that use mild engineering tools, like Maillardation, or enzyme-treatments to preserve functional proteins during refinery steps (e.g. patent EP1370157B1: “Highly soluble, high molecular weight soy protein”). Interestingly, all patents filed in this area pay attention to the in principle reversibility of the modification applied.
2. *Nano-particles (23 patents).* To better direct properties of protein-based nano-particles enzymatic introduction of lipidic groups like small fatty acids or PEGylation has been employed to encapsulate bioactives, typically as microemulsions (e.g. patent US20070154907A1: ”Microemulsions as precursors to solid nanoparticles”). These applications are considered food-grade or have passed medical-ethical approval in their testing/and or usage. There are no indications that these application have found their way into the food/feed product market.
3. *Nutritional availability in feeds (13 patents).* A considerable number of patents can be found in the application area to increase the nutritional availability of amino acids in feed (14 patents). Most patent-positions are dealing with destabilizing protein-structures to promote their digestibility and their (proposed) nutritional uptake. Typical patented approaches are de-amidation and Maillardation (e.g. patent WO2004020977A3: “De-amidation to promote nutritional value of rumen in feed”). No patents can be found that link nutritional value and protein modification in foods.
4. *Protein-based emulsion-stabilizers (two times 7 patents).* A few patents are found where specific protein modification is used to improve emulsification of (oil in water) products. Especially enzymatic glycosylation and lipidation approaches have been used (e.g. patent US7126042B1: “Recombinant oleosins from cacao and their use as flavoring or emulsifying agents”). In view of the small scale examples provided in these patents, it is not likely that these inventions have been implemented in a commercial product.
5. *Edible coatings (12 patents).* A whole family of patents is present on enzymatic protein cross-linking (mainly by transglutaminase) in relation to the production of edible coatings. Modifications typically act on a microstructural level, and not so much on the protein molecular level, to strengthen the spatial network formed (e.g. patent EP963704B1: “Food containing proteinaceous material treated with a transglutaminase and an oxidoreductase“). As these interventions in a product do not necessarily need to be labeled on the product, it is difficult to evaluate whether they have resulted in product development.

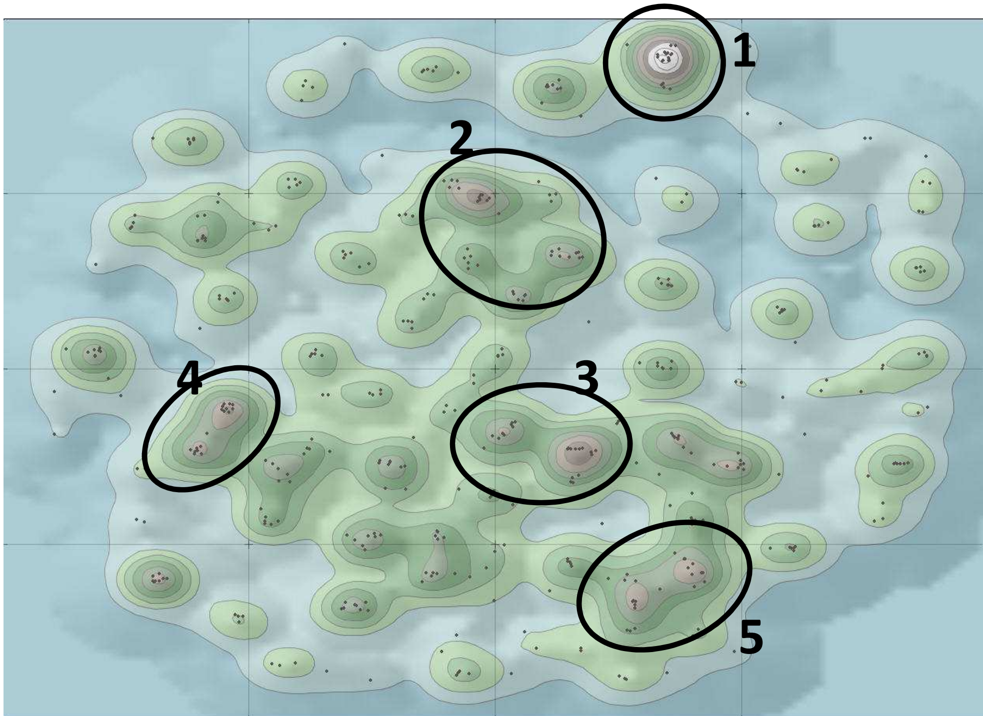


Fig. 1. Landscape representation of patent (families) filed between 2000 and 2011 where engineering/modification of protein functionality has been used to derive new material/product characteristics. The small black dots indicate the position of a patent-family. The numbered circles represent the areas with the highest activity in patents. The numbers are explained in the body text.

4.2 Food legislation

Whereas in paragraph 3 it was demonstrated how active and progressing the understanding of protein functionality in complex systems has been in the past decades, it is striking to see how minor the contributions to new applications these insights apparently have been. To understand this better one needs to take into account that the technological developments coincided with an increasing level of legislation. In the United States this is embodied in the Food and Drug Administration (FDA). The European Food Safety Authority (EFSA) is its European counterpart. The FDA is responsible for protecting and promoting public health through the regulation and supervision of food safety. It does so by formulating acts that set the boundaries for implementation of new ingredients, processes or compositions related to foods. There are a few acts that have had a strong impact on the food sector. In 1990 the Nutrition Labeling and Education Act was launched. This required food products to be labeled in terms of composition, allowing traceability of its ingredients to their source. This act also amended that all nutrient content claims (e.g. 'high fiber', 'low fat', etc.) would meet the standards set by the FDA. In the end it meant that every engineered protein would require a new label and would need to be recognized and approved first by the FDA.

Exceptions were those modified ingredients that could be considered as 'occurring from a natural process' or that were 'reversibly modified' (so temporary). The FDA Modernization Act of 1997 was designed to reduce the time for the approval of new pharmaceutical drugs, but also had an impact on food technology by the acknowledgment of the advancement of technological, trade, and public health complexities. Basically, a 'new' food ingredient needed to be seen and evaluated in the complex role it had considering its production up to its digestion in the food. This act was further refined by the Amendments Act of 2007, leading to much sharper defined criteria in what was considered as safe in food products. Recently, in 2010, The Food Safety Modernization Act was signed. Sections of this act require food producers to enable tracking and tracing of all ingredients used. The use of engineered proteins (either genetically or chemically) requires a separate approval for market-clearance. Summarizing, the FDA (and EFSA) have acknowledged that (future) food production requires innovations at the ingredient level, but also via processing routes, and they are in principle open to protein engineering routes. At the same time it enforces that functionally improved ingredients are checked along the full chain from refinery to nutritional value and human health within the complexity of the product.

4.3 Potentials in the area of food product sustainability

With increasing world-population and welfare the demand for protein as food-nutritional component is rising sharply. Also the identification of proteins as building blocks in non-food applications in view of a more sustainable economy, has led to increasing pressure on innovations in production, refinery, and application of proteins from wider sources than in the current economy is provided. For the development of a vision on food quality and especially the role of nutritional impact needs to be seen in the context of the basic requirements set not only by consumer demands, but also by participating industries. This can be presented by a so-called Pyramid of Food Innovation, as shown in figure 2. On top one finds the foods that need to be developed in a most sustainable way; to achieve this prospect one needs to comply with lower levels of restrictions, limitations and concomitant scientific challenges.

Food *safety* forms the most fundamental aspect here. Terminology like for example 'nanotechnology' cannot count on consumer acceptance and also the inclusion of genetically modified ingredients gives rise within Europe to hesitance in applicability by food-producing companies. Especially for traceability and chemical characterization the demands become exceedingly higher. This is the level though where new protein engineering routes could be contributing most. A major bottleneck today in reformulation-strategies is the occurrence of (sensory) differentiations relative to the original product. Exceptions are reduced sugar or salt products, but from a marketing-technical perspective moderations of structuring components are preferably performed within the frame as novel food. A continuously on-going drive to elucidate structure-texture-product acceptance relations is essential, just like innovations in the area of optimized processing tools to deliver products that are *acceptable* with retained food safety. Typically the efforts on applying protein modifications are focusing in deriving food structures more efficiently or to provide new functional building blocks to create new food structures. In an economically global society there is a strong pressure on both ingredient-prices and commercial acceptable processing. Industrial entrepreneurship is essential and the availability of second-line ingredients for products hampers the implementation of new food production strategies. Especially within

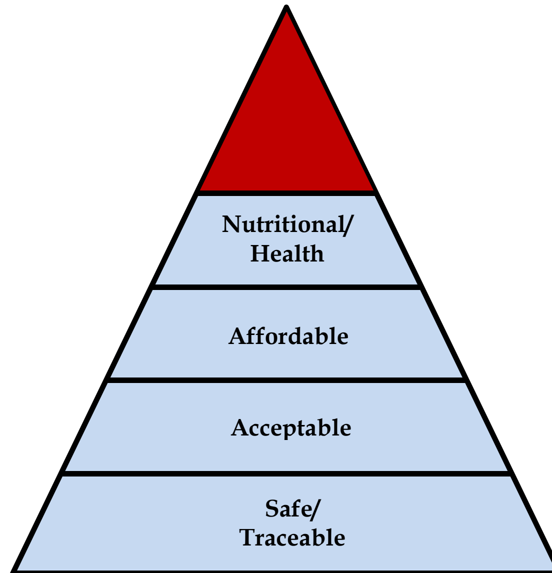


Fig. 2. The Pyramid of Food Innovation

the EU there is an increasing attention to social inequality issues. From a governmental regulatory point of view there is increasing support for open innovations and shared responsibilities to produce products of good quality that are *affordable* to all social classes. It is unclear what the role of optimized protein behavior by engineering approaches can be. Consumers are susceptible to additional *health*-aspects when safe and acceptable good quality products are available for reasonable prices. In this information technology era consumers are capable to evaluate the added value of products far better, setting higher demands for health marks of food products. The use of added value to products, like bioactives, has not led to major winners in the food sector yet. The desire (and need) to innovate in food product developments could be fed by the wealth of information on how product functionality relates to microstructural morphologies and how these in turn are dictated by molecular properties of specific proteins. On the other hand there are robust approaches in the field of protein engineering that allows us to direct protein behaviour and their propensities to (self)assemble into spatial networks. Still, one has to recognize that these two aspects have not come together yet to contribute to a more sustainable production and use of proteins in foods. This will be further discussed in section 5.

4.4 Considerations of chemical modification of proteins

While genetic modification of proteins generally results in a homogeneous product, chemical engineering approaches to modify proteins are well-known to result in heterogeneity. A good example is the application of the Maillard reaction to β-lactoglobulin (Broersen et al., 2004) or fish parvalbumin (de Jongh et al., 2011). On average one may find that for example 6 of the 12 available lysine residues has become glycosylated, where mass analysis will demonstrate that a significant population of protein molecules is present with 5 or 7 sugar groups, some molecules may contain 4 or 8 groups and even traces of molecules with 3 and 9 groups are

present. This is commonly the result of variation in reactivity between the different amino groups present in a specific protein structure: i.e. some amino acids are more exposed and hence are more prone to rapid modification. Buried amino acids do not present a straight forward target for modification as most chemicals are unable to access the folded or aggregated protein structure. Another effect which likely contributes to the extent of heterogeneity is the location of target amino acids in the primary sequence. For example, modification of an amino acid located next to a negatively charged amino acid by succinic anhydride will be hampered as a result of steric hindrance. Some attempts have been made to fractionate protein molecules with different degrees of modification. Five ovalbumin preparations with different degrees of modification have been purified using ion exchange chromatography (Wierenga et al., 2005). The heterogeneous nature of chemically modified proteins has marked implications on the acceptance of these novel ingredients under current food law. Chemical heterogeneity would imply that each obtained modified species would have to be tested separately on toxicity in order to become regarded as safe to use as novel food ingredient.

4.5 Health risks

In this paragraph we will discuss potential health implications induced by chemically modified proteins used in food products including allergenic response, the presence of anti-nutritional factors linked to proteins and the development of potential toxic compounds as a side-effect of the modification reaction. Concerns can be raised regarding the impact of protein modification on the potential health risks from products containing modified protein ingredients. Bernstein and colleagues (2003) identified three possible modes for novel food ingredients to result in adverse health effects. These include toxicity, impaired nutrition and food allergy.

4.5.1 Toxicity

One of the known toxic compounds to be formed upon chemical engineering of proteins is acrylamide, which results from the Maillard reaction between reducing sugars and asparagine or methionine (Mottram et al., 2002; Stadler et al., 2002). The high solubility of this compound induces rapid absorption and metabolism in the body (reviewed in Dearfield et al., 1988). Following absorption, acrylamide can bind to DNA aiding a genotoxic and carcinogenic response which has been demonstrated in animals (Rudén, 2004). The occurrence of carcinogenic acrylamide in foods has been related to frying and cooking of food products (Rosen & Hellenäs, 2002; Tareke et al., 2002). Two communications published in Nature in 2002 demonstrated the requirement of the reaction between asparagine or methionine and a reducing sugar to intermediate formation of the dicarbonyl reactant followed by Strecker degradation (Mottram et al., 2002; Stadler et al., 2002). Particularly plant proteins are rich in asparagine suggesting that glycosylation of proteins from plant-origin using the Maillard reaction should be carefully considered in terms of the known toxic effects of acrylamide. Other toxic side effects of chemical modification of proteins have not been reported.

4.5.2 Impaired nutrition

Chemical engineering can theoretically have far-reaching consequences for the nutritional value of proteins. In a limited number of cases protein engineering has been intentionally

employed to modify the nutritional quality, for example to increase the nutritional value of plant proteins (Liao et al., 2010). However, the main objective of most studies employing protein engineering is to investigate the consequences of the modification procedure for a range of functional properties. Only a small number of studies investigates nutritional aspects of chemical protein engineering which is mostly regarded as a convenient or inconvenient side effect of the modification procedure. Nutritional aspects covered in literature are exclusively based on *in vitro* studies and cover protein digestibility, availability of essential amino acids or the presence of anti-nutritional factors.

4.5.2.1 Anti-nutritional factors

Anti-nutritional factors are related to reduced protein digestibility and amino acid availability (reviewed by Gilani et al., 2005; Salunkhe et al., 1982) and are commonly present in large concentrations in plant products (Kay, 1979; Liener, 1980). One of the studies in this field reports on the nutritional quality of mung bean isolate following the exposure to varying concentrations of acetic or succinic anhydride to induce acylation (El-Adawy, 2000). The concentrations of anti-nutritional factors tannin, phytic acid, and trypsin inhibitor showed a significant loss with increasing degrees of modification suggesting that this type of modification can positively impact the effect of anti-nutritional factors. The concentration of trypsin inhibitor even decreased with 70% of the original level of trypsin inhibitor in unmodified protein. The introduction of bulky and/or negatively charged side groups was postulated to affect the extent of protein-tannin (El-Adawy, 2000), protein-mineral-phytic acid (Dua et al., 1996; El-Adawy, 2000), or protein-phenol (Loomis (1974) interactions. Loomis (1974) further showed that the flour and protein production processes provide for optimal conditions for the conversion of polyphenols into quinone oxidation products which, in turn, may bind covalently with sulfhydryl groups of cysteine and ε-amino groups of lysine and N-terminal amino groups. Further support was provided by Dua and colleagues (1996) who showed that acylation and methylation of rapeseed meal and its water-soluble fraction resulted in loss of anti-nutritional factors polyphenol, glucosinolates and phytic acid. However, the methylation procedure employed by Dua and colleagues (1996) resulted in very limited degrees of modification compared to the succinylation and acetylation process suggesting that other factors than the chemical conjugation itself may play a role in the loss of anti-nutritional factors upon chemical modification. As El-Adawy (2000) comments, the extensive dialysis of the protein following the acylation procedure may well be primarily responsible for the loss of water-soluble anti-nutritional factors upon modification. No studies are known to date that report on the loss of anti-nutritional factors upon dialysis.

4.5.2.2 *In vitro* digestibility

In vitro digestibility of modified proteins is often assayed through exposure of the proteins to a single or a mixture of enzymes including trypsin and pancreatin (Salgó et al., 1984), a combination of trypsin, chymotrypsin and peptidase (Hsu et al., 1977), or pepsin-pancreatin mixtures (Haque et al., 1982) to simulate (post)gastrointestinal digestion of food proteins. The small increase in digestibility of acylated mung bean protein isolate reported by El-Adawy (2000) was primarily correlated to the concurrent loss of tannin; tannins have been shown to play an important role in the reduction of protein digestibility (Barroga et al., 1985). Alternative factors proposed to induce increased digestibility of modified proteins

include the improved access of sites susceptible to enzymatic cleavage as a result of the dissociation of quaternary complexes of proteins or partial unfolding induced by the modification procedure (Achouri & Zhang, 2001). Protein unfolding as a result of modification has been shown for soy protein hydrolysate using techniques to study the secondary and tertiary structure content of the protein upon succinylation (Achouri & Zhang, 2001). However, the acylation of cotton seed flour did not improve *in vitro* protein digestibility (Rahma & Narasinga Rao, 1983) suggesting that results in this area are somewhat controversial and perhaps other factors play a role (see paragraph 4.5.2.1). *In vitro* protein digestibility using a multienzyme system containing trypsin, chymotrypsin and peptidase was not impaired for pea proteins upon acetylation (Johnson & Brekke, 1983) while Ma (1984) reported increased digestibility for acylated pea protein, similar to low degrees of succinylation of soy protein hydrolysate (Achouri & Zhang, 2001). Loss of *in vitro* digestibility has also been reported upon succinylation of a variety of proteins, particularly affecting the release of lysine (Matoba & Doi, 1979; Siu & Thompson, 1982; Wanasundara & Shahidi, 1997) or lipophilization of soy bean glycinin with palmitic acid (Haque et al., 1982). Data on *in vivo* digestibility of ingested proteins upon modification have not been reported.

4.5.2.3 Availability of essential amino acids

A commonly used target for protein modification are the lysine or cysteine residues, lysine being classified as an essential amino acid, i.e. this amino acid cannot be synthesized *de novo* by humans and should therefore be ingested. Extensive modification of these amino acids can therefore result in a lower availability. Few studies report on the impact of lysine or cysteine modification on the availability of these amino acids, usually assayed through amino acid analysis. Overall only small decreases in lysine were reported upon succinylation of soy protein hydrolysate (Achouri & Zhang, 2001). Similar findings were reported for acylated soy proteins (Franzen & Kinsella, 1976a), acylated sunflower proteins (Kabirullah & Wills, 1982), and succinylation of oat proteins (Ma & Wood, 1987). It is not clear whether these reported effects of chemical engineering inducing the loss of lysine availability ultimately result in a noticeable and substantial loss of nutritional quality for the human population.

4.5.3 Food allergy and intolerance

An estimated 3-4% of the children and 1-2% of adults in the industrialized world exert an allergenic response to one or more ingested food proteins (Baral & Hourihane, 2005; Jansen et al., 1994). Not all ingested proteins behave as allergens but proteins implicated in allergenic response often share features such as unusual resilience against heat, acid or protease digestion, propensity to bind to lipids and are glycosylated to some degree (Lehrer et al., 2002; Metcalfe et al., 1996). Also posttranslational modifications including *N*-glycosylation, and hydroxylation of proline residues have been postulated to affect IgE reactivity to Phl p 1 present in timothy grass pollen (Petersen et al., 1998). The precise mechanism of the effects of glycosylation on the allergenic response are not clear. Attempts have been made to develop and evaluate algorithms which predict protein allergenic response based on sequence homology (Aalberse & Stapel, 2001; Jameson & Wolf, 1988), structural identity, and evolutionary relationship (Jenkins et al., 2007), albeit with limited predicting power. The Food and Agriculture Organization (FAO) of the United Nations together with the World Health Organization (WHO) developed a decision tree to assess the

allergenic potential of genetically engineered food ingredients based on sequence homology followed by serum screens (FAO/WHO 2001, reviewed by Bernstein et al., 2003). Based on these findings and the fact that to date there is no indication for a common sequence motif of linear IgE epitopes, it can be concluded that the underlying mechanisms of immunological sensitization to food proteins remain elusive. To be able to assess the potential of chemically modified food proteins to induce allergenic reactions implies extensive knowledge of the underlying mechanical aspects of the allergic response. No publications are known which report on the allergenic effect in humans of modified ingested food proteins. In the pharmaceutical field PEGylation is used to modulate drug delivery of proteins or peptides. An early paper in the late 1970s reported that, upon covalent attachment of methoxypolyethylene glycols of 1900 daltons or 5000 daltons to bovine liver catalase, no evidence was observed of a modulated immune response following repeated injection of the modified enzymes into mice (Abuchowski et al., 1977).

5. Future prospects

There is a clear ambition to implement fundamental insights obtained on protein behavior in complex systems more effectively in sustainable food production in the future. There is also the need, considering that one should become more flexible in protein sources and more effective in utilizing the proteins structuring and nutritional potential. The technology to enable this is largely present, as demonstrated by the diversity of literature presented in paragraph 3. The mechanistic insight that has been derived in underlying principles on the relation between (engineered) protein functionality is impressive. And still, the development of new applications using chemical protein engineering to make a better usage of protein functionality or to promote protein source exchangeability is poor. There are a number of reasons to identify. The use of food protein modification has been limited to the domain of food chemistry or food physics. There is no significant literature available where the fine-tuning of microstructure formation as part of product development has been studied at a sensory or (human) physiological level. Compared to genetic modification, chemically engineered proteins pose an inherent heterogeneity. Instead of a single ingredient with an altered functionality, the FDA needs to consider all levels of heterogeneity in their approvals. This is an enormous laborious task as it requires fractionation of derived materials and sufficient stability of the formed products. Moreover, many attempts to reduce heterogeneity in the protein material will lead to non-food grade materials that cannot be studied in relation to e.g. sensory or human physiological aspects. The food safety authorities have amended (see paragraph 4.2) that the protein needs to be seen in the complex context of the food product. This is not just chemical analysis, but also includes product stability, consumer acceptance (sensory) and human physiological data. Especially in these latter two domains there is no public literature readily available to help these authorities to establish objective views on tolerance. This is where the scientific community has a highly needed role in bringing different disciplines together to produce literature relevant for authorities to base their legislations on.

6. References

Aalberse, R.C. & Stapel, S.O. (2001). Structure of food allergens in relation to allergenicity. *Pediatr. Allergy Immunol.* 12: 10-14.

Abuchowski, A.; McCoy, J.R.; Palczuk, N.C.; van Es, T. & Davis, F.F. (1977). Effect of covalent attachment of polyethylene glycol on immunogenicity and circulating life of bovine liver catalase. *J. Biol. Chem.* 252: 3582-3586.

Achouri, A. & Zhang, W. (2001). Effect of succinylation on the physicochemical properties of soy protein hydrolysate. *Food Res. Int.* 34: 507-514.

Aewsiri, T.; Benjakul, S.; Visessanguan W.; Wierenga, P.A. & Gruppen, H. (2010). Improvement of foaming properties of cuttlefish skin gelatin by modification with *N*-hydroxysuccinimide esters of fatty acid. *Food Hydrocoll.* In press.

Aewsiri, T.; Benjakul, S.; Visessanguan, W.; Wierenga, P.A. & Gruppen, H. (2011a). Emulsifying property and antioxidative activity of cuttlefish skin gelatin modified with oxidized linoleic acid and oxidized tannic acid. *Food Bioprocess. Technol.* In press.

Aewsiri, T.; Benjakul, S.; Visessanguan, W.; Wierenga, P.A. & Gruppen, H. (2011b). Surface activity and molecular characteristics of cuttlefish skin gelatin modified by oxidized linoleic acid. *Int. J. Biol. Macromol.* In press.

Alting, A.C.; Hamer, R.J.; de Kruif, C.G. & Visschers, R.W. (2003). Cold-set globular protein gels: interactions, structure and rheology as a function of protein concentration. *J. Agric. Food Chem.* 51: 3150-3156.

Andersson, L.-O.; Brandt, J. & Johansson, S. (1971). The use of trinitrobenezenesulfonic acid in studies on the binding of fatty acid anions to bovine serum albumin. *Arch. Biochem. Biophys.* 146: 428-440.

Anfinsen, C.B. (1973). Principles that govern the folding of protein chains. *Science* 181: 223-230.

Anson, M.L. (1940). The reactions of iodine and iodoacetamide with native egg albumin. *J. Gen. Physiol.* 23: 321-331.

Antoniewski, M.N.; Barringer, S.A.; Knipe, L. & Zerby, H. (2007). The effect of a gelatin coating on the shelf-life of fresh meat. *J. Food Sci.* 72: E382-E387.

Arnaudov, L.N.; de Vries, R.; Ippel, H. & van Mierlo, C.P. (2003). Multiple steps during the formation of β-lactoglobulin fibrils. *Biomacromolecules* 4: 1614-1622.

Arnaudov, L.N. & de Vries, R. (2005). Thermally induced fibrillar aggregation of hen egg white lysozyme. *Biophys. J.* 88: 515-526.

Arnaudov, L.N. & de Vries, R. (2006). Strong impact of ionic strength on the kinetics of fibrilar aggregation of bovine β-lactoglobulin. *Biomacromolecules* 7: 3490-3498.

Arntfield, S.D.; Murray, E.D. & Ismond, M.A.H. (1991). Role of disulfide bonds in determining the rheological and microstructural properties of heat-induced protein networks from ovalbumin and vicilin. *J. Agric. Food Chem.* 39: 1378-1385.

Arvanitoyannis, I.S. (1999). Totally and partially biodegradable polymer blends based on natural and synthetic macromolecules: preparation, physical properties, and potential as food packaging materials. *Polym. Rev.* 39: 205-271.

Audic, J.L. & Chaufer, B. (2005). Influence of plasticizers and crosslinking on the properties of biodegradable films made from sodium caseinate. *Eur. Polym. J.* 41: 1934-1942.

Bai, J.; Alleyne, V.; Hagenmaier, R.D.; Mattheis, J.P. & Baldwin, E.A. (2003). Formulation of zein coatings for apples (*Malus domestica* Borkh). *Postharv. Biol. Technol.* 28: 259-268.

Baldursdottir, S.G.; Fullerton, M.S.; Nielsen, S.H. & Jorgensen, L. (2010). Adsorption of proteins at the oil/water interface – observation of protein adsorption by interfacial shear stress measurements. *Colloids Surf. B Biointerfaces* 79: 41-46.

Baldwin, R.L. (2008). The search for folding intermediates and the mechanism of protein folding. *Annu. Rev. Biophys.* 37: 1-21.

Baldwin, E.A. (1994). Edible coatings for fresh fruits and vegetables: past, present, and future. In: Edible Coatings and Films to Improve Food Quality. Ed. By Krochta, J.M., Bandwin, E.A., Nisperos-Carriedo, M.O., Technomic Publishing, Lancaster PA, pp. 25-64.

Baral, V.R. & Hourihane, J.O. (2005). Food allergy in children. *Postgrad. Med. J.* 81: 693-701.

Barbut, S. & Foegeding, E.A. (1993). Ca^{2+}-induced gelation of preheated whey protein solutions. *J. Food Sci.* 58: 867-871.

Barroga, C.F.; Laurena, A.C. & Mendoza, E.M.T. (1985). Polyphenol in mung bean (*Vigna radiata* L. Wilczek) determination and removal. *J. Agric. Food Chem.* 33: 1006-1009.

Bauer, H.H.; Aebi, U.; Häner, M.; Hermann, R.; Müller, M.; Arvinte, T. & Merkle, H.P. (1995). Architecture and polymorphism of fibrillar supramolecular assemblies produced by *in vitro* aggregation of human calcitonin. *J. Struct. Biol.* 115: 1-15.

Baumketner, A.; Jewett, A. & Shea, J.E. (2003). Effects of confinement in chaperonin assisted protein folding: rate enhancement by decreasing the roughness of the folding energy landscape. *J. Mol. Biol.* 332: 701-713.

Bernstein, J.A.; Bernstein, I.L.; Bucchini, L.; Goldman, L.R.; Hamilton, R.G.; Lehrer, S.; Rubin, C. & Sampson, H.A. (2003). Clinical and laboratory investigation of allergy to genetically modified foods. *Environ. Health Persp.* 111: 1114-1121.

Betz, S.F. (1993). Disulfide bonds and the stability of globular proteins. *Protein Sci.* 2: 1551-1558.

Borgia, A.; Williams, P.M. & Clarke, J. (2008). Single-molecule studies of protein folding. *Annu. Rev. Biochem.* 77: 101-125.

Bowes, J.H. & Cater, C.W. (1968). The interaction of aldehydes with collagen. *Biochim. Biophys. Acta* 168: 341-352.

Broersen, K.; Weijers, M.; de Groot, J.; Hamer, R.J. & de Jongh, H.H. (2007a). Effect of protein charge on the generation of aggregation-prone conformers. *Biomacromolecules* 8: 1648-1656.

Broersen, K.; Elshof, M.; de Groot, J.; Voragen, A.G.J.; Hamer, R.J. & de Jongh, H.H.J. (2007b). Aggregation of β-lactoglobulin regulated by glucosylation. *J. Agric. Food Chem.* 55: 2431-2437.

Broersen, K.; Voragen, A.G.; Hamer, R.J. & de Jongh, H.H. (2004). Glycoforms of beta-lactoglobulin with improved thermostability and preserved structural packing. *Biotechnol. Bioeng.* 86: 78-87.

Broersen, K.; van Teeffelen, A.M.; Vries, A.; Voragen, A.G.; Hamer, R.J. & de Jongh, H.H. (2006). Do sulfhydryl groups affect aggregation and gelation properties of ovalbumin? *J. Agric. Food Chem.* 54: 5166-5174.

Bromley, E.H.C.; Krebs, M.R.H. & Donald, A.M. (2006). Mechanisms of structure formation in particulate gels of β-lactoglobulin formed near the isoelectric point. *Eur. Phys. J. E.* 21: 145-152.

Buchner, S.; Kinnear, M.; Crouch, I.J.; Taylor, J. & Minnaar, A. (2011). Effect of kafirin protein coating on sensory quality and shelf-life of 'Packham's Triumph' pears during ripening. *J. Sci. Food Agric.* In press.

Buchner, G.S.; Murphy, R.D.; Buchete, N.V. & Kubelka, J. (2011). Dynamics of protein folding: probing the kinetic network of folding-unfolding transitions with experiment and theory. *Biochim. Biophys. Acta* 1814: 1001-1020.

Bustos, P.; Gajardo, M.I.; Gómez, C.; Hughes, G.; Cardemil, E. & Jabalquinto, A.M. (1996). Woodward's reagent K reacts with histidine and Cysteine residues in *Escherichia coli* and *Saccharomyces cerevisiae* phosphoenolpyruvate carboxykinases. *J. Protein Chem.* 15: 467-472.

Calamai, M.; Taddei, N.; Stefani, M., Ramponi, G. & Chiti, F. (2003). Relative influence of hydrophobicity and net charge in the aggregation of two homologous proteins. *Biochemistry* 42: 15078-15083.

Canet, D.; Sunde, M.; Last, A.M.; Miranker, A.; Spencer, A.; Robinson, C.V. & Dobson, C.M. (1999). Mechanistic studies of the folding of human lysozyme and the origin of amyloidogenic behavior of its disease-related variants. *Biochemistry* 38: 6419-6427.

Cao-Hoang, L.; Chaine, A.; Grégoire, L. & Waché, Y. (2010). Potential of nisin-incorporated sodium caseinate films to control *Listeria* in artificially contaminated cheese. *Food Microbiol.* 27: 940-944.

Chang, T.-S. & Sun, S.F. (1978). Structural studies on the succinylated bovine serum albumin. *Int. J. Peptide Protein Res.* 11: 65-72.

Charissou, A.; Ait-Ameur, L. & Birlouez-Aragon, I. (2007). Kinetics of formation of three indicators of the Maillard reaction in model cookies: influence of baking temperature and type of sugar. *J. Agric. Food Chem.* 55: 4532-4539.

Chibnall, A.C. (1942). Bakerian lecture: amino-acid analysis and the structure of proteins. *Proc. Roy. Soc. B* 131: 136-160.

Chiti, F. & Dobson, C.M. (2006). Protein misfolding, functional amyloid, and human disease. *Annu. Rev. Biochem.* 75: 333-366.

Chiti, F.; Stefani, M.; Taddei, N.; Ramponi, G. & Dobson, C.M. (2003). Rationalization of the effects of mutations on peptide and protein aggregation rates. *Nature* 424: 805-808.

Chiti, F.; Taddei, N.; Bucciantini, M.; White, P.; Ramponi, G. & Dobson, C.M. (2000). Mutational analysis of the propensity for amyloid formation by a globular protein. *EMBO J.* 19: 1441-1449.

Church, F.C.; Swaisgood, H.E.; Porter, D.H. & Catignani, G.L. (1983). Spectrophotometric assay using *o*-phthaldialdehyde for determination of proteolysis in milk and isolated milk proteins. J. Dairy Sci. 66: 1219-1227.

Cieplak, M. (2004). Cooperativity and contact order in protein folding. *Phys. Rev. E Stat. Nonlin. Soft Matter Phys.* 69: 031907.

Corzo-Martínez, M.; Soria, A.C.; Villamiel, M.; Olano, A.; Harte, F.M. & Moreno, F.J. (2011). Effect of glycation on sodium caseinate-stabilized emulsions obtained by ultrasound. *J. Dairy Sci.* 94: 51-58.

Creighton, T.E. (1988). Disulphide bonds and protein stability. *Bioessays* 8: 57-63.

Cucu, T.; Platteau, C.; Taverniers, I.; Devreese, B.; de Loose, M. & de Meulenaer, B. (2011). ELISA detection of hazelnut proteins: effect of protein glycation in the presence or absence of wheat proteins. *Food Addit. Contam. Part A Chem. Anal. Control Expo. Risk Assess.* 28: 1-10.

Damodaran, V.B. & Fee, C. (2010). Protein PEGylation: an overview of chemistry and process considerations. *Eur. Pharm. Rev* 15: 18-26.

Dearfield, K.L.; Abernathy, C.O.; Ottley, M.S.; Brantner, J.H. & Hayes, P.F. (1988). Acrylamide: its metabolism, developmental and reproductive effects, genotoxicity, and carcinogenicity. *Mutat. Res.* 195: 45-77.

De Groot, J.; Kosters, H.A. & de Jongh, H.H. (2007). Deglycosylation of ovalbumin prohibits formation of a heat-stable conformer. *Biotechnol. Bioeng.* 97: 735-741.

De Jongh, H.H.J.; Kosters, H.A.; Kudryashova, E.; Meinders, M.B.J.; Trofimova, D. & Wierenga, P.A. (2004). Protein adsorption at air-water interfaces; a combination of details. *Biopolymers* 74: 131-135.

De Jongh, H.H.J. & Wierenga, P.A. (2006). Assessing the extent of protein intermolecular interactions at air-water interfaces using spectroscopic techniques. *Biopolymers* 82: 384-389.

De Jongh, H.H.; Taylor, S.L. & Koppelman, S.J. (2011). Controlling the aggregation propensity and thereby digestibility of allergens by Maillardation as illustrated for cod fish parvalbumin. *J. Biosci. Bioeng.* 111: 204-211.

Del Rosario Moreira, M.; Pereda, M.; Marcovich, N.E. & Roura, S.I. (2011). Antimicrobial effectiveness of bioactive packaging materials from edible chitosan and casein polymers: assessment on carrot, cheese, and salami. *J. Food Sci.* 76: M54-M63.

DeSantis, G. & Jones, J.B. (1999). Chemical modification of enzymes for enhanced functionality. *Curr. Opin. Biotechnol.* 10: 324-330.

Dickinson, E. (1992). An introduction to food colloids. Oxford, UK: Oxford University Press.

Djagny, K.B.; Wang, Z. & Xu, S. (2001). Conformational changes and some functional characteristics of gelatin esterified with fatty acid. *J. Agric. Food Chem.* 49: 2987-2991.

Dobson, C.M. (1999). Protein misfolding, evolution and disease. *Trends Biochem. Sci.* 24: 329-332.

Doi, E. (1993). Gels and gelling of globular proteins. *Trends Food Sci. Technol.* 4: 1-5.

Dondero, M.; Figueroa, V.; Morales, X. & Curotto, E. (2006). Transglutaminase effects on gelation capacity of thermally induced beef protein gels. *Food Chemistry* 99, 546-554.

Dua, S.; Mahajan, A. & Mahajan, A. (1996). Improvement of functional properties of rapeseed (*Brassica campestris* Var. Toria) preparations by chemical modification. *J. Agric. Food Chem.* 44: 706-710.

DuBay, K.F.; Pawar, A.P.; Chiti, F.; Zurdo, J.; Dobson, C.M. & Vendrusculo, M. (2004). Prediction of the absolute aggregation rates of amyloidogenic polypeptide chains. *J. Mol. Biol.* 341: 1317-1326.

El-Adawy, T.A. (2000). Functional properties and nutritional quality of acetylated and succinylated mung bean protein isolate. *Food Chem.* 70: 83-91.

Ellman, G.L. (1959). Tissue sulfhydryl groups. *Arch. Biochem. Biophys.* 82: 70-77.

Emmambux, M.N.; Stading, M. & Taylor, J.R.N. (2004). Sorghum kafirin film property modification with hydrolysable and condensed tannins. *J. Cereal Sci.* 40: 127-135.

Englander, S.W.; Mayne, L. & Krishna, M.M. (2007). Protein folding and misfolding: mechanisms and principles. *Q. Rev. Biophys.* 40: 287-326.

Evans, M.T.A. & Irons, L. (1970). N-succinylated egg yolk proteins. German Patent 1 951 247.

FAO/WHO (2001). Evaluation of allergenicity of genetically modified foods. Report of a Joint FAO/WHO Expert Consultation of Allergenicity of Foods Derived from Biotechnology. Available: ftp://ftp.fao.org/es/esn/food/allergygm.pdf [accessed 08 August 2011].

Farouk, M.M.; Price, J.F. & Salih, A.M. (1990). Effect of an edible film overwrap on exudation and lipid oxidation in beef round steak. *J. Food Sci.* 55: 1510-1512, 1563.

Feeney, R.E.; Blankenhorn, G. & Dixon, H.B. (1975). Carbonyl-amine reactions in protein chemistry. *Adv. Protein Chem.* 29: 135-203.

Feeney, R.E.; Yamasaki, R.B. & Geoghegan, K.F. (1982). Chapter 1: Chemical modification of proteins: an overview. In 'Modification of Proteins', Advances in Chemistry; American Chemical Society, Washington DC. pp 3-55.

Fernandez-Escamilla, A.M.; Rousseau, F.; Schymkowitz, J. & Serrano, L. (2004). Prediction of sequence-dependent and mutational effects on the aggregation of peptides and proteins. *Nat. Biotechnol.* 22: 1302-1306.

Ferreira, S.T.; De Felice, F.G. & Chapeaurouge, A. (2006). Metastable, partially folded states in the productive folding and in the misfolding and amyloid aggregation of proteins. *Cell Biochem. Biophys.* 44: 539-548.

Ferreon, A.C. & Deniz, A.A. (2011). Protein folding at single-molecule resolution. *Biochim. Biophys. Acta* 1814: 1021-1029.

Fields, R. (1971). The measurement of amino groups in proteins and peptides. *Biochem. J.* 124: 581-590.

Fraenkel-Conrat, H. (1957). Methods for investigating the essential groups for enzyme activity. *Methods Enzymol.* 4: 247-269.

Finley, J.W. (1975). Deamidated gluten: a potential fortifier for fruit juices. *J. Food Sci.* 40: 1283-1285.

Francis, G.E.; Fisher, D.; Delgado, C.; Malik, F.; Gardiner, A. & Neale, D. (1998). PEGylation of cytokines and other therapeutic proteins and peptides: the importance of biological optimisation of coupling techniques. *Int. J. Hematol.* 68: 1-18.

Franzen, K.L. & Kinsella, J.E. (1976a). Functional properties of succinylated and acetylated soy protein. *J. Agric. Food Chem.* 24: 788-795.

Franzen, K.L. & Kinsella, J.E. (1976b). Functional properties of succinylated and acetylated leaf proteins. *J. Agric. Food Chem.* 24: 914-919.

Frister, H.; Meisel, H. & Schlimme, E. (1988). OPA method modified by use of *N,N*-dimethyl-2-mercaptoethylammonium chloride as thiol component. *Fresenius'Z. Anal. Chem.* 330: 631-633.

Gerbanowski, A.; Rabiller, C.; Larré, C. & Guéguen, J. (1999a). Grafting of aliphatic and aromatic probes to bovine serum albumin: influence on its structural and physicochemical characteristics. *J. Protein Chem.* 18: 325-335.

Gerbanowski, A.; Malabat, C.; Rabiller, C. & Guéguen, J. (1999b). Grafting of aliphatic and aromatic probes on rapeseed 2S and 12S proteins: influence on their structural and physicochemical properties. *J. Agric. Food Chem.* 47: 5218-5226.

Gilani, G.S.; Cockell, K.A. & Sepehr, E. (2005). Effects of antinutritional factors on protein digestibility and amino acid availability in foods. *J. AOAC Int* 88: 967-987.

Gillgren, T. & Stading, M. (2008). Mechanical and barrier properties of avenin, kafirin and zein films. *Food Biophys.* 3: 287-294.

Graham, D.E. & Phillips, M.C. (1979a). Proteins at liquid interfaces. I. Kinetics of adsorption and surface denaturation. *J. Colloid Interface Sci.* 70: 403-414.

Graham, D.E. & Phillips, M.C. (1979b). Proteins at liquid interfaces. II. Adsorption isotherms. *J. Colloid Interface Sci.* 70: 415-426.

Graham, D.E. & Phillips, M.C. (1979c). Proteins at liquid interfaces. III. Molecular structures at adsorbed films. *J. Colloid Interface Sci.* 70: 427-439.

Graña-Montes, R.; de Groot, N.S.; Castillo, V.; Sancho, J.; Velazquez-Campoy, A. & Ventura, S. (2011). Contribution of disulfide bonds to stability, folding and amyloid fibril formation: the PI3-SH3 domain case. *Antioxid. Redox Signal.* In press.

Grigoriev, D.O.; Derkatch, S.; Krägel, J. & Miller, R. (2007). Relationship between structure and rheological properties of mixed BSA/Tween 80 adsorption layers at the air/water interface. *Food Hydrocoll.* 21: 823-830.

Groninger, H.S. Jr. (1973). Preparation and properties of succinylated fish myofibrillar protein. *J. Agric. Food Chem.* 21: 978-981.

Gruener, L. & Ismond, M.A.H. (1997). Effects of acetylation and succinylation on the physicochemical properties of the canola 12S globulin. *Food Chem.* 60: 357-363.

Gueguen, J.; Bollecker, S.; Schwenke, K.D. & Raab, B. (1990). Effect of succinylation on some physicochemical and functional properties of the 12S storage protein from rapeseed (*Brassica napus* L.). *J. Agric. Food Chem.* 38: 61-69.

Gurin, S. & Clarke, H.T. (1934). Allocation of the free amino groups in proteins and peptides. *J. Biol. Chem.* 107: 395-419.

Habeeb, A.F.S.A. & Atassi, M.Z. (1969). Enzymic and immunochemical properties of lysozyme II. Conformation, immunochemistry and enzymic activity of a derivative modified at tryptophan. *Immunohistochemistry* 6: 555-566.

Hall, R.J.; Trinder, N. & Givens, D.I. (1973). Observations on the use of 2,4,6-trinitrobenzenesulphonic acid for the determination of available lysine in animal protein concentrates. *Analyst* 98: 673-686.

Halling, P.J. (1981). Protein-stabilized foams and emulsions. *Crit. Rev. Food Sci. Nutr.* 15: 155-203.

Hamada, J.S. & Marshall, W.E. (1989). Preparation and functional properties of enzymatically deamidated soy proteins. *J. Food Sci.* 54: 598-601.

Hamano, Y. (2011). Occurrence, biosynthesis, biodegradation, and industrial and medical applications of a naturally occurring ε-poly-L-lysine. *Biosci. Biotechnol. Biochem.* 75: 1226-1233.

Haque, Z.; Matoba, T. & Kito, M. (1982). Incorporation of fatty acid into food protein: palmitoyl soybean glycinin. *J. Agric. Food Chem.* 30: 481-486.

Haque, Z. & Kito, M. (1983a). Lipophilization of α_{s1}-casein. 1. Covalent attachment of palmitoyl residue. *J. Agric. Food Chem.* 31: 1225-1230.

Haque, Z. & Kito, M. (1983b). Lipophilization of α_{s1}-casein. 2. Conformational and functional effects. *J. Agric. Food Chem.* 31: 1231-1237.

Hardy, P.M.; Nicholls, A.C. & Rydon, H.N. (1969). The nature of gluteraldehyde in aqueous solution. *J. Chem. Soc. Chem. Commun.* 10: 565-566.

Harper, J.D.; Wong, S.S.; Lieber, C.M. & Lansbury, P.T. Jr. (1997). Atomic force microscopy imaging of seeded fibril formation and fibril branching by the Alzheimer's disease amyloid-β protein. *Chem. Biol.* 4: 951-959.

Hart, G.W. (1992). Glycosylation. *Curr. Opin. Cell Biol.* 4: 1017-1723.

Havard, C. & Harmony, M.X. (1869). Improved process of preserving meat, fowls, fish. June 8, U.S. Patent 90: 944.

Hayakawa, S. & Nakai, S. (1985). Contribution of the hydrophobicity, net charge and sulfhydryl groups to thermal properties of ovalbumin. *Can. Inst. Food Sci. Technol. J.* 18: 290-295.

Haynes, R.; Osuga, D.T. & Feeney, R.E. (1967). Modification of amino groups in inhibitors of proteolytic enzymes. *Biochemistry* 6: 541-547.

He, X.-H.; Shaw, P.-C. & Tam, S.-C. (1999). Reducing the immunogenicity and improving the inv vivo activity of trichosanthin by site-directed PEGylation. *Life Sci.* 65: 355-368.

Helenius, A.; Trombetta, E.S.; Hebert, D.N. & Simons, J.F. (1997) Calnexin, calreticulin and the folding of glycoproteins. *Trends Cell Biology* 7: 193-200.

Hillier, R.M.; Lyster, R.L.J. & Cheeseman, G.C. (1980). Gelation of reconstituted whey powders by heat. *J. Sci. Food Agric.* 31: 1152-1157.

Hoagland, P.D. (1968). Acylated β-caseins. Effect of alkyl group size on calcium ion sensitivity and on aggregation. *Biochemistry* 7: 2542-2546.

Hoffmann, M.A.M. & van Mil, P.J.J.M. (1997). Heat-induced aggregation of β-lactoglobulin: role of the free thiol group and disulfide bonds. *J. Agric. Food Chem.* 45: 2942-2948.

Hopwood, D. (1969). A comparison of the crosslinking abilities of gluteraldehyde, formaldehyde and α-hydroxyadipaldehyde with bovine serum albumin and casein. *Histochemie* 17: 151-161.

Hopwood, D., Callen, C.R. & McCabe, M. (1970). The reactions between gluteraldehyde and various proteins. An investigation of their kinetics. *Histochem. J.* 2: 137-150.

Hsu, H.W.; Vavak, D.L.; Satterlee, L.S. & Miller, G.A. (1977). A multienzyme technique for estimating protein digestibility. *J. Food Sci.* 42: 1269-1273.

Huggins, C. & Jensen, E.V. (1949). Thermal coagulation of serum proteins; the effects of iodoacetate, iodoacetamide, and thiol compounds on coagulation. *J. Biol. Chem.* 179: 645-654.

Hunter, J.R.; Carbonell, R.G. & Kilpatrick, P.K. (1991). Coadsorption and exchange of lysozyme/β-casein mixtures at the air/water interface. *J. Colloid Interface Sci.* 143: 37-53.

Hurle, M.R.; Helms, L.R.; Li, L.; Chan, W. & Wetzel, R. (1994). A role for destabilizing amino acid replacements in light-chain amyloidosis. *Proc. Natl. Acad. Sci. U.S.A.* 91: 5446-5450.

Ibrahim, H.R.; Kato, A. & Kobayashi, K. (1991). Antimicrobial effects of lysozyme against gram-negative bacteria due to covalent binding of palmitic acid. *J. Agric. Food Chem.* 39: 2077-2082.

Ibrahim, H.R.; Kobayashi, K. & Kato, A. (1993). Length of hydrocarbon chain and antimicrobial action to gram-negative bacteria of fatty acylated lysozyme. *J. Agric. Food Chem.* 41: 1164-1168.

Izzo, H.V.; Lincoln, M.D. & Ho, C.-T. (1993). The effect of temperature, feed moisture, and pH on protein deamidation in an extruded wheat flour. *J. Agric. Food Chem.* 41: 199-202.

Jameson, B.A. & Wolf, H. (1988). The antigenic index: a novel algorithm for predicting antigenic determinants. *Comp. Appl. Biosci.* 4: 181-186.

Jansen, J.J.N.; Kardinaal, A.F.M.; Huijbers, G.; Vlieg-Boerstra, B.J.; Martens, B.P.M. & Ockhuizen, T. (1994). Prevalence of food allergy and intolerance in the adult Dutch population. *J. Allergy Clin. Immun.* 93: 446-456.

Jarrett, J.T. & Lansbury, P.T. Jr. (1993). Seeding "one-dimensional crystallization" of amyloid: a pathogenic mechanism in Alzheimer's disease and scrapie? *Cell* 73: 1055-1058.

Jenkins, J.A.; Breiteneder, H. & Mills, E.N.C. (2007). Evolutionary distance from human homologs reflects allergenicity of animal food proteins. *J. Allergy Clin. Immunol.* 120: 1399-1405.

Jiménez, J.; Nettleton, E.J.; Bouchard, M.; Robinson, C.V.; Dobson, C.M. & Saibil, H. (2002). The protofilament structure of insulin amyloid fibrils. *Proc. Natl. Acad. Sci. U.S.A.* 99: 9196-9201.

Jiménez, J.; Guijarro, J.I.; Orlova, E.; Zurdo, J.; Dobson, C.M.; Sunde, M. & Saibil, H.R. (1999). Cryo-electron microscopy structure of an SH3 amyloid fibril and model of the molecular packing. *EMBO J.* 18: 815-821.

Johnson, E.A. & Brekke, J. (1983). Functional properties of acylated pea protein isolates. *J. Food Sci.* 48: 722-725.

Johnson, A.R. & Dekker, E.E. (1996). Woodward's reagent K inactivation of *Escherichia coli* L-threonine dehydrogenase: increased absorbance at 340-350 nm is due to modification of cysteine and histidine residues, not aspartate or glutamate carboxyl groups. *Protein Sci.* 5: 382-390.

Jonas, A. & Weber, G. (1970). Partial modification of bovine serum albumin with dicarboxylic anhydrides. Physical properties of the modified species. *Biochemistry* 9: 4729-4735.

Ju, Z.Y. & Kilara, A. (1998). Aggregation induced by calcium chloride and subsequent thermal gelation of whey protein isolate. *J. Dairy Sci.* 81: 925-931.

Kabirullah, M. & Wills, R.B.H. (1982). Functional properties of acetylated and succinylated sunflower protein isolate. *J. Food Technol.* 17: 235-249.

Kaneko, R. & Kitabatake, N. (2001). Structure-sweetness relationship in thaumatin: importance of lysine residues. *Chem. Senses* 26: 167-177.

Kato, A.; Ibrahim, H.; Takagi, T. & Kobayashi, K. (1990). Excellent gelation of egg white preheated in the dry state is due to the decreasing degree of aggregation. *J. Agric. Food Chem.* 38: 1868-1872.

Kato, A.; Nagase, Y.; Matsudomi, N. & Kobayashi, K. (1983). Determination of molecular-weight of soluble ovalbumin aggregates during heat denaturation using low-angle laser-light scattering technique. *Agric. Biol. Chem.* 47: 1829-1834.

Kay, D.E. (1979). Crop and product digest. No. 3. Food legumes. London, Tropical Products Institute, 435 p.

Kelly, J.W. (1998). The alternative conformations of amyloidogenic proteins and their multi-step assembly pathways. *Curr. Opin. Struct. Biol.* 8: 101-106.

Kitabatake, N.; Wada, R. & Fujita, Y. (2001). Reversible conformational change in β-lactoglobulin A modified with *N*-ethylmaleimide and resistance to molecular aggregation on heating. *J. Agric. Food Chem.* 49: 4011-4018.

Klotz, I.M. & Heiney, R.E. (1962). Introduction of sulfhydryl groups into proteins using acetylmercaptosuccinic anhydride. *Arch. Biochem. Biophys.* 96: 605-612.

Korn, A.H.; Feairheller, S.H. & Filachione, E.M. (1972). Gluteraldehyde: nature of the reagent. *J. Mol. Biol.* 65: 525-529.

Kornfeld, R. & Kornfeld, S. (1985). Assembly of asparagines-linked oligosaccharides. *Annu. Rev. Biochem.* 54: 631-664.

Koseki, T.; Kitabatake, N. & Doi, E. (1989). Irreversible thermal denaturation and formation of linear aggregates of ovalbumin. *Food Hydrocoll.* 3: 123-134.

Kosters, H.A. & de Jongh, H.H. (2003). Spectrophotometric tool for the determination of the total carboxylate content in proteins; molar extinction coefficient of the enol ester from Woodward's reagent K reacted with protein carboxylates. *Anal. Chem.* 75: 2512-2516.

Kosters, H.A.; Broersen, K.; de Groot, J.; Simons, J.W.; Wierenga, P. & de Jongh, H.H. (2003). Chemical processing as a tool to generate ovalbumin variants with changed stability. *Biotechnol. Bioeng.* 84: 61-70.

Kotaki, A.; Harada, M. & Yagi, K. (1964). Reaction between sulfhydryl compounds and 2,4,6-trinitrobenzene-1-sulfonic acid. *J. Biochem.* 55: 553-561.

Krause, J.-P.; Mothes, R. & Schwenke, K.D. (1996). Some physicochemical and interfacial properties of native and acetylated legumin from faba beans (*Vicia faba* L.). *J. Agric. Food Chem.* 44: 429-437.

Krebs, M.R.H.; Devlin, G.L. & Donald, A.M. (2009). Amyloid fibril-like structure underlies the aggregate structure across the pH range for β-lactoglobulin. *Biophys. J.* 96: 5013-5019.

Kudryashova, E.V.; Meinders, M.B.J.; Visser, A.J.W.G.; van Hoek, A. & de Jongh, H.H.J (2003). Structural properties and rotational dynamics of egg white ovalbumin adsorbed at the air/water interaface. *Eur. J. Biophys.* 32: 553-562.

Kudryashova, E.V.; Visser, A.J.W.G., & de Jongh, H.H.J. (2005). Reversible self-association of ovalbumin at air-water interfaces and the consequences for the exerted surface pressure. Protein Science 14: 483-493.

Lakkis, J. & Villota, R. (1992). Effect of acylation on substructural properties of proteins: a study using fluorescence and circular dichroism. *J. Agric. Food Chem.* 40: 553-560.

Land, A. & Braakman, I. (2001). Folding of the human immunodeficiency virus type 1 envelope glycoprotein in the endoplasmatic reticulum. *Biochimie* 83: 783-790.

Langton, M. & Hermansson, A.-M. (1992). Fine-stranded and particulate gels of β-lactoglobulin and whey protein at varying pH. Food Hydrocoll. 5: 523-539.

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

Le Floch-Fouéré, C.; Pezennec,S.; Pézolet, M.; Rioux-Dubé, J.F.; Renault, A. & Beaufils, S. (2011). Unexpected differences in the behavior of ovotransferrin at the air-water interface at pH 6.5 and 8.0. *J. Colloid Interface Sci.* 356: 614-623.

Lehrer, S.B.; Ayuso, R. & Reese, G. (2002). Current understanding of food allergens. *Ann. N. Y. Acad. Sci.* 964: 69-85.

Liao, L.; Zhao, M.; Ren, J.; Zhao, H.; Cui, C. & Hu, X. (2010). Effect of acetic acid deamidation-induced modification on functional and nutritional properties and conformation of wheat gluten. *J. Sci. Food Agric.* 90: 409-417.

Liener, I.E. (1980). Toxic constituents of plant foodstuffs. New York and London, Academic Press, 502 p.

Liu, S.-T.; Sugimoto, T.; Azakami, H. & Kato, A. (2000). Lipophilization of lysozyme by short and middle chain fatty acids. *J. Agric. Food Chem.* 48: 265-269.

Llamas, K.; Ownes, M.; Blakeley, L. & Zerner, B. (1986). *N*-ethyl-5-phenylisoxazolium-3'-sulfonate (Woodward's reagent K) as a reagent for nucleophilic side chains of proteins. *J. Am. Chem. Soc.* 108: 5543-5548.

Lomakin, A.; Chung, D.S.; Benedek, G.B.; Kirscher, D.A. & Teplow, D.B. (1996). On the nucleation and growth of amyloid β-protein fibrils: detection of nuclei and quantitation of rate constants. *Proc. Natl. Acad. Sci. U.S.A.* 93: 1125-1129.

Lomakin, A.; Teplow, D.B.; Kirschner, D.A. & Benedek, G.B. (1997). Kinetic theory of fibrillogenesis of amyloid β-protein. *Proc. Natl. Acad. Sci. U.S.A.* 94: 7942-7947.

Longares, A.; Monahan, F.J.; O'Riordan, E.D. & O'Sullivan, M. (2005). Physical properties of edible films made from mixtures of sodium caseinate and WPI. *Int. Dairy J.* 15: 1255-1260.

Lönnerdal, B. (2002). Expression of human milk proteins in plants. *J. Am. Coll. Nutr.* 21: 218–221

Loomis, W.D. (1974). Overcoming problems of phenolics and quinones in the isolation of plant enzymes organelles. In: *Methods in Enzymology*; Fleischer, S., Packer, L., Eds.: Academic Press: New York, p. 528.

Losso, J.N. & Nakai, S. (2002). Stabilization of oil-in-water emulsions by β-lactoglobulin-polyethylene glycol conjugates. *J. Agric. Food Chem.* 50: 1207-1212.

Lubelski, J.; Rink, R.; Khusainov, R.; Moll, G.N. & Kuipers, O.P. (2008). Biosynthesis, immunity, regulation, mode of action and engineering of the model lantibiotic nisin. *Cell. Mol. Life Sci.* 65: 455-476.

Luey, J.-K.; McGuire, J. & Sproull, R.D. (1991). The effect of pH and NaCl concentration on adsorption of β-lactoglobulin at hydrophilic and hydrophobic silicon surfaces. *J. Colloid Interface Sci.* 143: 489-500.

Luheshi, L.M. & Dobson, C.M. (2009). Bridging the gap: from protein misfolding to protein misfolding diseases. *FEBS Lett.* 583: 2581-2586.

Ma, C.-Y. (1984). Functional properties of acylated oat protein. *J. Food Sci.* 49: 1128-1131.

Ma, C.-Y. & Wood, D.F. (1987). Functional properties of oat proteins modified by acylation, trypsin hydrolysis or linoleate treatment. *J. Am. Oil Chem. Soc.* 64: 1726-1731.

MacRitchie, F. (1978). Proteins at interfaces. *Adv. Protein Chem.* 32: 283-326.

Malaki Nik, A.; Wright, A.J. & Corredig, M. (2010). Interfacial design of protein-stabilized emulsions for optimal delivery of nutrients. *Food Funct.* 1: 141-148.

Marcon, G.; Plakoutsi, G.; Canale, C.; Relini, A.; Taddei, N.; Dobson, C.M.; Ramponi, G. & Chiti, F. (2005). Amyloid formation from HypF-N under conditions in which the protein is initially in its native state. *J. Mol. Biol.* 347: 323-335.

Margoshes, B.A. (1990). Correlation of protein sulfhydryls with the strength of heat-formed egg white gels. *J. Food Sci.* 55: 1753.

Martin, A.H.; Meinders, M.B.J.; Bos, M.A.; Cohen Stuart, M.A. & van Vliet, T. (2003). Conformational aspects of proteins at the air/water interface studied by infrared reflection-absorption spectroscopy. *Langmuir* 19: 2922-2928.

Martins, J.T.; Cerqueira, M.A.; Souza, B.W.; Carmo Avides, M. & Vicente, A.A. (2010). Shelf life extension of ricotta cheese using coatings of galactomannans from nonconventional sources incorporating nisin against *Listeria monocytogenes*. *J. Agric. Food Chem.* 58: 1884-1891.

Masuda, T.; Ueno, Y. & Kitabatake, N. (2001). Sweetness and enzymatic activity of lysozyme. *J. Agric. Food Chem.* 49: 4937-4941.

Masuda, T.; Ide, N. & Kitabatake, N. (2005a). Structure-sweetness relationship in egg white lysozyme: role of lysine and arginine on the elicitation of lysozyme sweetness. *Chem. Senses* 30: 667-681.

Masuda, T.; Ide, N. & Kitabatake, N. (2005b). Effects of chemical modification of lysine residues on the sweetness of lysozyme. *Chem. Senses* 30: 253-264.

Matoba, T. & Doi, E. (1979). In vitro digestibility of succinylated protein by pepsin and pancreatic proteases. *J. Food Sci.* 44: 537-539.

Matsudomi, N.; Kato, A. & Kobayashi, K. (1982). Conformation and surface properties of deamidated gluten. *Agric. Biol. Chem.* 46: 1583-1586.

Matsudomi, N.; Sasaki, T.; Kato, A. & Kobayashi, K. (1985). Conformational changes and functional properties of acid-modified soy protein. *Agric. Biol. Chem.* 49: 1251-1256.

Maurer-Stroh, S.; Debulpaep, M.; Kuemmerer, N.; Lopez de la Paz, M.; Martins, I.C.; Reumers, J.; Morris, K.L.; Copland, A.; Serpell, L.C.; Serrano, L.; Schymkowitz, J.W.H. & Rousseau, F. (2010). Exploring the sequence determinants of amyloid structure using position-specific scoring matrices. *Nature Methods* 7: 237-242.

Marquardt, T. & Helenius, A. (1992). Misfolding and aggregation of newly synthesized proteins in the endoplasmatic reticulum. *J. Cell Biol.* 117: 505-513.

McClellan, S.J. & Franses, E.I. (2003). Effect of concentration and denaturation on adsorption and surface tension of bovine serum albumin. *Colloids Surf, B Biointerfaces* 28: 63-75.

McClements, J. (2005). Food emulsions: principles, practice, and techniques. 2nd Ed. Boca Raton, FL: CRC Press.

McMahon, D.J.; Adams, S.L. & McManus, W.R. (2009). Hardening of high-protein nutrition bars and sugar/polyol-protein phase separation. *J. Food Sci.* 74: E312-E321.

Means, G.E. & Feeney, R.E. (1971). Chemical modifications of proteins. Holden-Day, San Francisco.

Meinders, M.B.J.; van den Bosch, G.G.M. & de Jongh, H.H.J. (2001). Adsorption properties of proteins at and near the air/water interface from IRRAS spectra of protein solutions. *Eur. Biophys. J.* 30: 256-267.

Melnychyn, P. & Stapley, R. (1973). Acylated protein from coffee whitener formulations. U.S. Patent 3,764,711.

Mendis, E.; Rajapakse, N. & Kim, S. (2005). Antioxidant properties of a radical-scavenging peptide purified from enzymatically prepared fish skin gelatin hydrolysate. *J. Agric. Food Chem.* 53: 581-587.

Metcalfe, D.D.; Astwood, J.D.; Townsend, R.; Sampson, H.A.; Taylor, S.L. & Fuchs, R.L. (1996). Assessment of the allergenic potential of foods derived from genetically engineered crop plants. *Crit. Rev. Fd. Sci. Nutr.* 36: S165-S186.

Migneault, I.; Dartiguenave, C.; Bertrand, M.J. & Waldron, K.C. (2004). Gluteraldehyde: behavior in aqueous solution, reaction with proteins, and application to enzyme crosslinking. *BioTechniques* 37: 790-802.

Mine, Y. (1992). Sulfhydryl groups changes in heat-induced soluble egg white aggregates in relation to molecular size. *J. Food Sci.* 57: 254-255.

Ming, D. & Hellekant, G. (1994). Brazzein, a new high-potency thermostable sweet protein from *Pentadiplandra brazzeana* B. *FEBS Lett.* 355: 106-108.

Mirmoghatadaie, L.; Kadivar, M. & Shahedi, M. (2009). Effects of succinylation and deamidation on functional properties of oat protein isolate. *Food Chem.* 114: 127-131.

Moore, S. & Stein, W.H. (1948). Photometric ninhydrin method for use in the chromatography of amino acids. *J. Biol. Chem.* 176: 367-388.

Morel, B.; Varela, L. & Conejero-Lara, F. (2010). The thermodynamic stability of amyloid fibrils studied by differential scanning calorimetry. *J. Phys. Chem. B* 114: 4010-4019.

Morris, J.A. & Cagan, R.H. (1972). Purification of monellin, the sweet principle of *Dioscoreophyllum cumminsii*. *Biochem. Biophys. Acta* 261: 114-122.

Mossine, V.V.; Glinsky, G.V. & Feather, M.S. (1994). The preparation and characterization of some Amadori compounds (1-amino-1-deoxy-D-fructose derivatives) derived from a series of aliphatic omega-amino acids. *Carbohydr. Res.* 262: 257-270.

Mottram, D.S.; Wedzicha, B.L. & Dodson, A.T. (2002). Acrylamide is formed in the Maillard reaction. *Nature* 419: 448.

Moure, A.; Sineiro, J.; Domínguez, H. & Parajó, J.C. (2006). Functionality of oilseed protein products: a review. *Food Res. Int.* 39: 945-963.

Nakai, S. (1983). Structure-function relationships of food proteins with an emphasis on the importance of protein hydrophobicity. *J. Agric. Food Chem.* 31: 676-683.

Nakai, S. & Li-Chan, E. (1988). Hydrophobic interaction in food systems. CRC Press: Boca Raton, FL.

Narayana, K. & Rao, N. (1991). Effect of acetylation and succinylation on the physicochemical properties of winged bean (Psophocarpus tetragonolobus) proteins. *J. Agric. Food Chem.* 39: 259-261.

Niestijl Jansen J.J.; Kardinaal, A.F.M.; Huijbers, G.; Vlieg-Boerstra, B.J.; Martens, B.P.M. & Ockhuizen, T. (1994). Prevalence of food allergy and intolerance in the adult Dutch population. *J. Allergy Clin. Immunol.* 93: 446-456.

Nimni, M.E., Cheung, D.; Strates, B.; Kodama, M. & Sheikh, K. (1987). Chemically modified collagen: a natural biomaterial for tissue replacement. *J. Biomed. Mater. Res.* 21: 741-771.

Nizet, V. (2006). Antimicrobial peptide resistance mechanisms of human bacterial pathogens. *Curr. Issues Mol. Biol.* 8: 11-26.

Ohta, K.; Masuda, T.; Ide, N. & Kitabatake, N. (2008). Critical molecular regions for elicitation of the sweetness of sweet-tasting protein, thaumatin I. *FEBS J.* 275: 3644-3652.

O'Kane, F.E.; Happe, R.P.; Vereijken, J.M.; Gruppen, H. & van Boekel, M.A.J.S. (2004). Heat-induced gelation of pea legumin: comparison with soy glycinin. *J. Agric. Food Chem.* 52: 5071-5078.

Okuyama, T. & Satake, K. (1960). On the preparation and properties of 2,4,6-trinitrophenyl-amino acids and –peptides. *J. Biochem.* 47: 454-466.

Onuchic, J.N.; Luthey-Schulten, Z. & Wolynes, P.G. (1997). Theory of protein folding: the energy landscape perspective. *Ann. Rev. Phys. Chem.* 48: 545-600.

Owusu Apenten, R.K.; Chee, C. & Hwee, O.P. (2003). Evaluation of a sulphydryl-disulphide exchange index (SEI) for whey proteins – beta-lactoglobulin and bovine serum albumin. *Food Chem.* 83: 541-545.

Pabit, S.A.; Roder, H. & Hagen, S.J. (2004). Internal friction controls the speed of protein folding from a compact configuration. *Biochemistry* 43: 12532-12538.

Paik, W. & Kim, S. (1972). Effect of methylation on susceptibility of proteins to proteolytic enzymes. *Biochemistry* 11: 2589-2594.

Panasiuk, R.; Amarowicz, R.; Kostyra, H. & Sijtsma, L. (1998). Determination of α-amino nitrogen in pea protein hydrolysates: a comparison of three analytical methods. *Food Chem.* 62: 363-367.

Paparcone, R. & Buehler, M.J. (2011). Failure of Aβ(1-40) amyloid fibrils under tensile loading. *Biomaterials* 32: 3367-3374.

Paparcone, R.; Keten, S. & Buehler, M.J. (2010). Atomistic simulation of nanomechanical properties of Alzheimer's Aβ(1-40) amyloid fibrils under compressive and tensile loading. *J. Biomechanics* 43: 1196-1201.

Park, H.J.; Chinnan, M.S. & Shewfelt, R.L. (1994). Edible corn-zein film coatings to extend storage life of tomatoes. *J. Food Process Preserv.* 18: 317-331.

Park, H.J. (1999). Development of advanced edible coatings for fruits. *Trends Food Sci. Technol.* 10: 254-260.

Patel, K. & Borchardt, R.T. (1990a). Chemical pathways of peptide degradation. II. Kinetics of deamidation of an asparaginyl residue in a model hexapeptide. *Pharm. Res.* 7: 703-711.

Patel, K. & Borchardt, R.T. (1990b). Chemical pathways of peptide degradation. III. Effect of primary sequence on the pathways of deamidation of asparaginyl residues in hexapeptides. *Pharm. Res.* 7: 787-793.

Patil, S.M.; Mehta, A.; Jha, S. & Alexandrescu, A.T. (2011). Heterogeneous amylin fibril growth mechanisms imaged by total internal reflection fluorescence microscopy. *Biochemistry* 50: 2808-2819.

Payne, P.I.; Holt, L.M.; Jackson, E.A. & Law, C.N. (1984). Wheat storage proteins: their genetics and their potential for manipulation by plant breeding. *Phil. Trans. R. Soc. Lond. B* 304: 359-371.

Pedroche, J.; Yust, M.M.; Lqari, H.; Giron-Calle, J.; Alaiz, M.; Vioque, J. & Millan, F. (2004). *Brassica carinata* protein isolates: chemical composition, protein characterization and improvement of functional properties by protein hydrolysis. *Food Chem.* 88: 337-346.

Pellegrino, L.; van Boekel, M.A.J.S.; Gruppen, H.; Resmini, P. & Pagani, M.A. (1999). Heat-induced aggregation and covalent linkages in β-casein model systems. *Int. Dairy J.* 9: 255-260.

Pereda, M.; Aranguren, M.I. & Marcovich, N.E. (2008). Characterization of chitosan/caseinate films. *J. Appl. Polymer Sci.* 107: 1080-1090.

Pereda, M.; Aranguren, M.I. & Marcovich, N.E. (2009). Water vapor absorption and permeability of films based on chitosan and sodium caseinate. *J. Appl. Polym. Sci.* 111: 2777-2784.

Petersen, A.; Schramm, G.; Schlaak, M. & Becker, W.M. (1998). Post-translational modifications influence IgE reactivity to the major allergen Phl p 1 of timothy grass pollen. *Clin. Exp. Allergy* 28: 315-321.

Pétra, P.H. (1971). Modification of carboxyl groups in bovine carboxypeptidase A. I. Inactivation of the enzyme by *N*-ethyl-5-phenylisoxazolium-3'-sulfonate (Woodward's reagent K). *Biochemistry* 10: 3163-3170.

Plaxco, K.W.; Simons, K.T.; Ruczinski, I. & Baker, D. (2000). Topology, stability, sequence, and length: defining the determinants of two-state protein folding kinetics. *Biochemistry* 39: 11177-11183.

Plaxco, K.W.; Simons, K.T. & Baker, D. (1998). Contact order, transition state placement and the refolding rates of single protein domains. *J. Mol. Biol.* 277: 985-994.

Plotkin, S.S. & Onuchic, J.N. (2002). Understanding protein folding with energy landscape theory. Part I: Basic concepts. *Quart. Rev. Biophys.* 35: 111-167.

Pogaku, R.; Eng Seng, C.; Boonbeng, L. & Kallu, U. (2007). Whey protein isolate-starch system – a critical review. *Intl. J. Food Eng.* 3: 104-119.

Ponce, A.G.; Roura, S.I.; Del Valle, C.E. & Moreira, M. (2008). Antimicrobial and antioxidant activities of edible coatings enriched with natural plant extracts: in vitro and in vivo studies. *Postharvest. Biol. Technol.* 49: 294-300.

Promeyrat, A.; Bax, M.L.; Traoré, S.; Abry, L.; Santé-Lhoutellier, V. & Gatellier, Ph. (2010). Changed dynamics in myofibrillar protein aggregation as a consequence of heating time and temperature. *Meat Sci.* 85: 625-631.

Qiu, L. & Hagen, S.J. (2004). A limiting speed for protein folding at low solvent viscosity. *J. Am. Chem. Soc.* 126: 3398-3399.

Quintanilla-Guerrero, F.; Duarte-Vázquez, M.A.; Tinoco, R.; Gómez-Suárez, M.; García-Almendárez, B.E.; Vazquez-Duhalt, R. & Regalado, C. (2008). Chemical modification of turnip peroxidase with methoxypolyethylene glycol enhances activity and stability for phenol removal using the immobilized enzyme. *J. Agric. Food Chem.* 56: 8058-8065.

Quintas, A.; Saraiva M.J. & Brito, R.M. (1997). The amyloidogenic potential of transthyretin variants correlates with their tendency to aggregate in solution. *FEBS Lett.* 418: 297-300.

Quiocho, F.A. & Richards, F.M. (1966). The enzymic behaviour of carboxypeptidase-A in the solid state. *Biochemistry* 5: 4062-4076.

Rahma, E.H. & Narasinga Rao (1983). Effect of acetylation and succinylation of cottonseed flour on its functional properties. *J. Agric. Food Chem.* 31: 351-355.

Ramírez-Alvarado, M.; Merkel, J.S. & Regan, L. (2000). A systematic exploration of the influence of protein stability on amyloid fibril formation in vitro. *Proc. Natl. Acad. Sci. U.S.A.* 97: 8979-8984.

Ramírez-Jiménez, A.; Guerra-Hernández, E. & García-Villanova, B. (2000). Browning indicators in bread. *J. Agric. Food Chem.* 48: 4176-4181.

Richard, F.M. & Knowles, J.R. (1968). Gluteraldehyde as a protein cross-linkage reagent. *J. Mol. Biol.* 37: 231-233.

Riha, W.E.; Izzo, H.V.; Zhang, J. & Ho, C.T. (1996). Nonenzymatic deamidation of food proteins. *Crit. Rev. Food Sci. Nutr.* 6: 225-255.

Robinson, A.B. & Rudd, C. (1974). Deamidation of glutaminyl and asparaginyl residues in peptides and proteins. *Curr. Top. Cell. Regul.* 8: 247-295.

Rodríguez Patino, J.M.; Carrera Sánchez, C. & Rodríguez Niño, M.R. (2008). Implications of interfacial characteristics of food foaming agents in foam formulations. *Adv. Colloid Interf. Sci.* 140: 95-113.

Rondeau, P.; Navarra, G.; Cacciabaudo, F.; Leone, M.; Bourdon, E. & Militello, V. (2010). Thermal aggregation of glycated bovine serum albumin. *Biochim. Biophys. Acta* 1804: 789-798.

Rondeau, P.; Armenta, S.; Caillens, H.; Chesne, S. & Bourdon, E. (2007). Assessment of temperature effects on β-aggregation of native and glycated albumin by FTIR spectroscopy and PAGE: relations between structural changes and antioxidant properties. *Arch. Biochem. Biophys.* 460: 141-150.

Röper, H.; Röper, S.; Heyns, K. & Meyer, B. (1983). N.M.R. spectroscopy of N-(1-deoxy-D-fructos-1-yl)-L amino acids (“fructose-amino acids”). *Carbohydr. Res.* 116: 183-195.

Rosén, J. & Hellenäs, K.E. (2002). Analysis of acrylamide in cooked foods by liquid chromatography tandem mass spectrometry. *Analyst* 127: 880-882.

Roth, M. (1971). Fluorescence reaction for amino acids. *Anal. Chem.* 43: 880-882.

Rudd, P.M.; Colominas, C.; Royle, L.; Murphy, N.; Hart, E.; Merry, A.H.; Hebestreit, H.F. & Dwek, R.A. (2001). A high-performance liquid chromatography based strategy for rapid, sensitive sequencing of N-linked oligosaccharide modifications to proteins in sodium dodecyl sulphate polyacrylamide electrophoresis gel bands. *Proteomics* 1: 285-294.

Rudén, C. (2004). Acrylamide and cancer risk – expert risk assessments and the public debate. *Food Chem. Tox.* 42: 335-349.

Sala, G.; de Wijk, R.A.; van de Velde, F. & van Aken G.A. (2008). Matrix properties affect the sensory perception of emulsion-filled gels. *Food Hydrocolloids* 22: 353-363.

Salgó, A.; Granzler, K. & Jecsai, J. (1984). Simple enzymatic methods for prediction of plant protein digestibility. In: R. Lásztity, M. Hidvé. Proc. Int. Assoc. Cereal Chem. Symp. (pp. 311-321). Budapest: Akadémiai Kiadó.

Salunkhe, D.K.; Jadhav, S.J.; Kadam, S.S. & Chavan, J.K. (1982). Chemical, biochemical, and biological significance of polyphenols in cereals and legumes. *Crit. Rev. Food Sci. Nutr.* 17: 277-305.

Samejima, K.; Dairman, W. & Underfriend, S. (1971). Condensation of ninhydrin with aldehydes and primary amines to yield highly fluorescent ternary products. *Anal. Biochem.* 42: 222-247.

Sanchez-Ruiz, J.M. (2011). Probing free-energy surfaces with differential scanning calorimetry. *Annu. Rev. Phys. Chem.* 62: 231-255.

Sancho, A.I.;. Rigby, N.M.; Zuidmeer, L.; Asero, R.; Mistrello, G.; Amato, S.; González-Mancebo, E.; Fernández-Rivas, M.; van Ree, R. & Mills, E.N.C. (2005). The effect of thermal processing on the IgE reactivity of the non-specific lipid transfer protein from apple, Mal d 3. *Allergy* 60: 1262-1268.

Sanger, F. (1945). The free amino groups of insulin. *Biochem. J.* 39: 507-515.

Santé-Lhoutellier, V.; Astruc, T.; Marinova, P.; Greve, E. & Gatellier, P. (2008). Effect of meat cooking on physicochemical state and in vitro digestibility of myofibrillar proteins. *J. Agric. Food Chem.* 56: 1488-1494.

Satake, K.; Okuyama, T.; Ohashi, M. & Shinoda, T. (1960). The spectrophotometric determination of amine, amino acid and peptide with 2,4,6-trinitrobenzene-1-sulfonic acid. *J. Biochem.* 47: 654.

Sawyer, W.H. (1968). Heat denaturation of bovine β-lactoglobulins and relevance of disulfide aggregation. *J. Dairy Sci.* 51: 323-329.

Schilling, E.D.; Burchill, P.I. & Clayton, R.A. (1963). Anomalous reactions of ninhydrin. *Anal. Biochem.* 5: 1-6.

Schwenke, K.D.; Knopfe, C.; Mikheeva, L.M. & Grinberg, V.Y. (1998). Structural changes of legumin from Faba beans (*Vicia faba L.*) by succinylation. *J. Agric. Food Chem.* 46: 2080-2086.

Sheih, I.C.; Wu, T.K. & Fang, T.J. (2009). Antioxidant properties of a new antioxidative peptide from algae protein waste hydrolysate in different oxidation systems. *Bioresour. Technol.* 100: 3419-3425.

Shental-Bechor, D. & Levy, Y. (2008). Effect of glycosylation on protein folding: a close look at thermodynamic stabilization. *Proc. Natl. Acad. Sci. U.S.A.* 105: 8256-8261.

Shih, F.F. (1990). Deamidation during treatment of soy protein with protease. *J. Food Sci.* 55: 127-129.

Shimada, K. & Cheftel, J.C. (1989). Sulfhydryl group/disulfide bond interchange reactions during heat-induced gelation of whey protein isolate. *J. Agric. Food Chem.* 3: 161-168.

Shirahama, H.; Lyklema, J. & Norde, W. (1990). Comparative protein adsorption in model systems. *J. Colloid Interf. Sci.* 139: 177-187.

Siepen, J.A. & Westhead, D.R. (2002). The fibril_one on-line database: mutations, experimental conditions, and trends associated with amyloid fibril formation. *Protein Sci.* 11: 1862-1866.

Silva Freitas, D. & Abrahão-Neto, J. (2010). Batch purification of high-purity lysozyme from egg white and characterization of the enzyme modified by PEGylation. *Pharm. Biol.* 48: 554-562.

Sinha, U. & Brewer, J.M. (1985). A spectrophotometric method for quantitation of carboxyl group modification of proteins using Woodward's reagent K. *Anal. Biochem.* 151: 327-333.

Siu, M. & Thompson, L.U. (1982). In vitro and in vivo digestibilities of succinylated cheese protein concentrates. *J. Agric. Food Chem.* 30: 743-747.

Skraup, Z.H. & Kaas, K. (1906). Über die Einwirkung von salpetriger Säure auf Ovalbumin. *Justus Liebigs. Ann. Chemie* 351: 379-389.

Smythe, C.V. (1936). The reaction of iodoacetate and of iodoacetamide with various sulfhydryl groups, with urease, and with yeast preparations. *J. Biol. Chem.* 114: 601-612.

Soderling, T.R. (1975). Regulation of glycogen synthetase. Specificity and stoichiometry of phosphorylation of the skeletal muscle enzyme by cyclic 3′:5′-AMP-dependent protein kinase. *J. Biol. Chem.* 250: 5407-5412.

Song, Y.; Azakami, H.; Hamasu, M. & Kato, A. (2001). In vivo glycosylation suppresses the aggregation of amyloidogenic hen egg white lysozymes expressed in yeast. *FEBS Lett.* 491: 63-66.

Sorci, M.; Silkworth, W.; Gehan, T. & Belfort, G. (2011). Evaluating nuclei concentration in amyloid fibrillation reactions using back-calculation approach. *PLoS One* 6: e20072.

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

Straub, J.E. & Thirumalai, D. (2011). Toward a molecular theory of early and late events in monomer to amyloid fibril formation. *Annu. Rev. Phys. Chem.* 62: 437-463.

Sun, Y.; Hayakawa, S. & Izumori, K. (2004). Modification of ovalbumin with a rare ketohexose through the Maillard reaction: effect on protein structure and gel properties. *J. Agric. Food Chem.* 52: 1293-1299.

Sun, Y. & Hayakawa, S. (2002). Heat-induced gels of egg white/ovalbumins from five avian species: thermal aggregation, molecular forces involved, and rheological properties. *J. Agric. Food Chem.* 50: 1636-1642.

Suttiprasit, P.; Krisdhasima, V. & McGuire, J. (1992). The surface activity of α-lactalbumin, β-lactoglobulin and bovine serum albumin. I. Surface tension measurements with single-component and mixed solutions. *J. Colloid Interface Sci.* 154: 316-326.

Tareke, E.; Rydberg, P.; Karlsson, P.; Eriksson, S. & Törnqvist, M. (2002). Analysis of acrylamide, a carcinogen formed in heated foodstuffs. *J. Agric. Food Chem.* 50: 4998-5006.

Tawfik, D.S. (2002). Chapter 63: Side chain selective chemical modifications of proteins. In: The Protein Protocols Handbook, 2nd Edition, Edited by J.M. Walker, Humana Press Inc. Totowa, NJ, pp 465-467.

Teplow, D.B.; Lazo, N.D.; Bitan, G.; Bernstein, S.; Wyttenbach, T.; Bowers, M.T.; Baumketner, A.; Shea, J.E.; Urbanc, B.; Cruz, L.; Borreguero, J. & Stanley, H.E. (2006). Elucidating amyloid-β protein folding and assembly: a multidisciplinary approach. *Acc. Chem. Res.* 39: 635-645.

Thomas, M.E.C.; Scher, J.; Desobry-Banon, S. & Desobry, S. (2004). Milk powders ageing: effect on physical and functional properties. *Crit. Rev. Food Sci. Nutr.* 44: 297-322.

Tyler-Cross, R. & Schirch, V. (1991). Effects of amino acid sequence, buffers, and ionic strength on the rate and mechanism of deamidation of asparagines residues in small peptides. *J. Biol. Chem.* 266: 22549-22556.

Van den Berg, L.; Carolas, A.L.; van Vliet, T.; van der Linden, E.; van Boekel, M.A.J.S. & van de Velde, F. (2008). Energy storage controls crumbly perception in whey proteins/polysaccharide mixed gels. *Food Hydrocolloids* 22: 1404-1417.

Van der Wel, H. & Loeve, K. (1972). Isolation and characterization of thaumatin I and II, the sweet-tasting proteins from *Thaumatococcus danielli* Benth. *Eur. J. Biochem.* 31: 221-225.

Van Teeffelen, A.M.M.; Broersen, K. & de Jongh, H.H.J. (2005). Glucosylation of β-lactoglobulin lowers the heat capacity change of unfolding; a unique way to affect protein thermodynamics. *Protein Sci.* 14: 2187-2194.

van Vliet, T. & Walstra, P. (1995). Large deformation and fracture behaviour of gels. *Faraday Discussions* 101: 359-370.

Veerman, C.; Ruis, H.; Sagis, L.M.C.; van der Linden, E. (2002). Effect of electrostatic interactions on the percolation concentration of fibrillar β-lactoglobulin gels. *Biomacromolecules* 3: 869-873.

Visschers, R.W. & de Jongh, H.H.J. (2005). Disulphide bond formation in food protein aggregation and gelation. *Biotechnol. Adv.* 23: 75-80.

Vioque, J.; Sánchez-Vioque, R.; Clemente, A.; Pedroche, J.; Bautista, J. & Millán, F.J. (1999). Production and characterization of an extensive rapeseed protein hydrolysate. *J. Am. Oil Chem. Soc.* 76: 819-823.

Wada, R. & Kitabatake, N. (2001). β-Lactoglobulin A with *N*-ethylmaleimide-modified sulfhydryl residue, polymerized through intermolecular disulfide bridge on heating in the presence of dithiothreitol. *J. Agric. Food Chem.* 49: 4971-4976.

Walder, R. & Schwartz, D.K. (2010). Single molecule observations of multiple protein populations at the oil-water interface. *Langmuir* 26: 13364-13367.

Walsh, D.M.; Lomakin, A.; Benedek, G.B.; Condron, M.M. & Teplow, D.B. (1997). Amyloid β-protein fibrillogenesis. Detection of a protofibrillar intermediate. *J. Biol. Chem.* 272: 22364-22372.

Wanasundara, P.K.J.P.D. & Shahidi, F. (1997). Functional properties of acylated flax protein isolates. *J. Agric. Food Chem.* 45: 2431-2441.

Wang, C.; Eufemi, M.; Turano, C. & Giartosio, A. (1996). Influence of the carbohydrate moiety on the stability of glycoproteins. *Biochemistry* 35: 7299-7307.

Waniska, R.D. & Kinsella, J.E. (1979). Foaming properties of proteins: evaluation of a column aeration apparatus using ovalbumin. *J. Food Sci.* 44: 1398-1411.

Waniska, R.D. & Kinsella, J.E. (1985). Surface properties of β-lactoglobulin: adsorption and rearrangement during film formation. *J. Agric. Food Chem.* 33: 1143-1148.

Weijers, M.; Broersen, K.; Barneveld, P.A.; Cohen Stuart, M.A.; Hamer, R.J.; de Jongh, H.H. & Visschers, R.W. (2008). Net charge affects morphology and visual properties of ovalbumin aggregates. *Biomacromolecules* 9: 3165-3172.

Wierenga, P.A.; Kosters, H.; Egmond, M.R.; Voragen, A.G.J. & de Jongh, H.H.J. (2006). Importance of physical vs. chemical interactions in surface shear rheology. *Adv. Colloid Interface Sci.* 119: 131-139.

Wierenga, P.A.; Meinders, M.B.J.; Egmond, M.R.; Voragen, F.A.G.J. & de Jongh, H.H.J. (2003). Protein exposed hydrophobicity reduces the kinetic barrier for adsorption of ovalbumin to the air-water interface. *Langmuir* 19: 8964-8970.

Wierenga, P.A.; Meinders, M.B.; Egmond, M.R.; Voragen, A.G. & de Jongh, H.H. (2005). Quantitative description of the relation between protein net charge and protein adsorption to air-water interface. *J. Phys. Chem. B* 109: 16946-16952.

Wilde, P.J. (2000). Interfaces: their role in foam and emulsion behaviour. *Curr. Opin. Colloid Interface Sci.* 5: 176-181.

Wilde, P.J.; Mackie, A.; Husband, F.; Gunning, P. & Morris, V. (2004). Proteins and emulsifiers at liquid interfaces. *Adv. Colloid Interface Sci.* 108-109: 63-71.

Wimley, W.C. (2010). Describing the mechanism of antimicrobial peptide action with the interfacial activity model. *ACS Chem. Biol.* 5: 905-917.

Wolynes, P.G. (2005). Recent successes of the energy landscape theory of protein folding and function. *Quart. Rev. Biophys.* 38: 405-410.

Woodward, R.B. & Olofson, R.A. (1961). The reaction of isoxazolium salts with bases. *J. Am. Chem. Soc.* 83: 1007-1009.

Woodward, R.B.; Olofson, R.A. & Mayer, H. (1961). A new synthesis of peptides. *J. Am. Chem. Soc.* 83: 1010-1012.

Wright, H.T. & Urry, D.W. (1991). Nonenzymatic deamidation of asparaginyl and glutaminyl residues in proteins. *Crit. Rev. Biochem. Mol. Biol.* 26: 41-52.

Xu, L.J.; Sheldon, B.W.; Larick, D.K. & Carawan, R.E. (2002). Recovery and utilization of useful by-products from egg processing wastewater by electrocoagulation. *Poult. Sci.* 81: 785-792.

Xu, Z.; Paparcone, R. & Buehler, M.J. (2010). Alzheimer's Aβ(1-40) amyloid fibrils feature size-dependent mechanical properties. *Biophys. J.* 96: 2053-2062.

Yemm, E.W. & Cocking, E.C. (1955). The determination of amino acids with ninhydrin. *Analyst* 80: 209-213.

Zamora, R. & Hildalgo, F.J. (2006). Coordinate contribution of lipid oxidation and Maillard reaction to the nonenzymatic food browning. *Crit. Rev. Food Sci. Nutr.* 45: 49-59.

Zavodsky, M.; Chen, C.W.; Huang, J.K.; Zolkiewski, M.; Wen, L. & Krishnamoorthi, R. (2001). Disulfide bond effects on protein stability: designed variants of *Cucurbita maxima* trypsin inhibitor-V. *Protein Sci.* 10: 149-160.

Zhang, J.; Lee, T.C. & Ho, C.-T. (1993). Comparative study on kinetics of nonenzymatic deamidation of soy protein and egg white lysozyme. *J. Agric. Food Chem.* 41: 2286-2290.

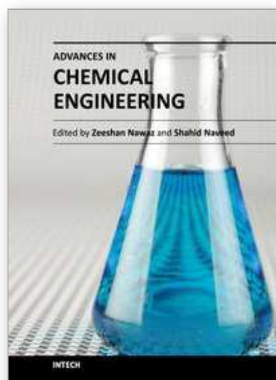
Zhao, Y.; Ma, C.-Y.; Yuen, S.-N. & Phillips, D.L. (2004a). Study of succinylated food proteins by raman spectroscopy. *J. Agric. Food Chem.* 52: 1815-1823.

Zhao, Y.; Ma, C.-Y.; Yuen, S.-N. & Phillips, D.L. (2004b). Study of acetylated food proteins by raman spectroscopy. *J. Food Sci.* 69: 206-213.

Zhou, P.; Liu, X. & Labuza, T.P. (2008). Effects of moisture-induced whey protein aggregation on protein conformation, the state of water molecules, and the microstructure and texture of high-protein-containing matrix. *J. Agric. Food Chem.* 56: 4535-4540.

Zidar, J. & Merzel, F. (2011). Probing amyloid-beta fibril stability by increasing ionic strengths. *J. Phys. Chem. B* 115: 2075-2081.

Zinoviadou, K.G.; Koutsoumanis, K.P. & Biliaderis, C.G. (2009). Physico-chemical properties of whey protein isolate films containing oregano oil and their antimicrobial action against spoilage flora of fresh beef. *Meat Sci.* 82: 338-345.



Advances in Chemical Engineering

Edited by Dr Zeeshan Nawaz

ISBN 978-953-51-0392-9
Hard cover, 584 pages
Publisher InTech
Published online 23, March, 2012
Published in print edition March, 2012

Chemical engineering applications have been a source of challenging optimization problems in terms of economics and technology. The goal of this book is to enable the reader to get instant information on fundamentals and advancements in chemical engineering. This book addresses ongoing evolutions of chemical engineering and provides overview to the sate of the art advancements. Molecular perspective is increasingly important in the refinement of kinetic and thermodynamic molding. As a result, much of the material was revised on industrial problems and their sophisticated solutions from known scientists around the world. These issues were divided in to two sections, fundamental advances and catalysis and reaction engineering. A distinct feature of this text continues to be the emphasis on molecular chemistry, reaction engineering and modeling to achieve rational and robust industrial design. Our perspective is that this background must be made available to undergraduate, graduate and professionals in an integrated manner.

How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Harmen H.J. de Jongh and Kerensa Broersen (2012). Application Potential of Food Protein Modification, Advances in Chemical Engineering, Dr Zeeshan Nawaz (Ed.), ISBN: 978-953-51-0392-9, InTech, Available from: http://www.intechopen.com/books/advances-in-chemical-engineering/chemical-engineering-of-proteins-to-provide-a-toolbox-to-study-functional-properties-of-proteins-in-

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