Developments in the Science of Zein, Kafirin and Gluten Protein Bio-plastic Materials

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ABSTRACT

Despite much research, there are very few commercial prolamin bio-plastics. The major reason, apart from their high cost, is that they have inferior functional properties compared to synthetic polymer plastics. This is because the prolamins are complex, each consisting of several classes and sub-classes and the functional properties of their bio-plastics are greatly affected by water. Prolamin bio-plastics are produced by protein aggregation from a solvent or by thermoplastic processing. Recent research indicates that protein aggregation occurs by polypeptide self-assembly into nanostructures. Protein secondary structure in terms of α-helical and β-sheet structure seems to play a key, but incompletely understood role in assembly. Also, there is inadequate knowledge as to how these nanostructures further assemble and organize into the various forms of prolamin bio-plastics such as films, fibres, microparticles and scaffolds. Some improvements in bio-plastic functionality have been made by better prolamin solvation, plasticization, physical and chemical cross-linking, derivatization and blending with other polymers. The most promising area of

commercialization is the biomedical field where the relative hydrophilicity, compatibility and biodegradability of particularly zein and kafirin are advantageous. With regard to biomedical applications, "supramolecular design" of prolamin bio-plastics through control over inter- and intramolecular weak interactions and SS/SH interchange between and within polypeptides appears to have considerable potential.

Key words: Biomedical, Bio-plastic, Cross-linking, Film, Microparticle, Prolamin, Scaffold, Secondary structure, Self-assembly, Thermoplastic.

INTRODUCTION

There has been on-going interest in using cereal prolamin proteins such as maize zein to make plastics for more than 100 years (Lawton 2002)due to the insolubility of prolamins in aqueous solvents. Since the 1990s research has increased and there have several excellent reviews and book chapters focussing on the technology and applications for prolamin bio-plastics (Anderson and Lamsal 2011; Cuq et al 1998; Fu et al 1999; Guilbert et al 2002; Lagrain et al 2010; Lawton 2002; Padua and Wang 2002; Reddy and Yang 2011; Shukla and Cheryan 2001; Taylor et al 2006). This upsurge in research has been driven by concerns about the finite nature of fossil fuel feedstocks for synthetic polymers and the persistence and non-degradability of synthetic polymer plastics, with the consequent problems of their disposal and environmental pollution. Additionally, vastly increasing quantities of prolamin-rich co-products, particularly from grain biofuel production but also from wet milling and brewing, are now being generated, which are potentially attractive sources of feedstock to produce "prolamin bio-plastics". Here the term prolamin bio-plastic is used to describe any nano-, micro- or macro-structure fabricated from prolamin proteins such as zein (maize),

gluten (wheat), hordein (barley) and kafirin (sorghum) intended for use as a material in food-related and non-food applications.

What is clear, however, is that there are remarkably few actual commercial products made from prolamin bio-plastics. Apart from their higher cost, the major reason for this is that prolamin bio-plastics, such as those from zein and gluten, have inferior functional properties compared to synthetic polymer plastics (Lagrain et al 2010; Lawton 2002), in particular their susceptibility to water. With this in mind, this review focuses on kafirin, which probably has the most superior functional properties (Schober et al 2011) and on zein and gluten which are commercial products (Anderson and Lamsal 2011; Czuchajowska and Paszczyńska 1996). Firstly, the structure of these prolamins is discussed with respect to their functionality in bio-plastics. Then, the state of our knowledge as to how the prolamin polypeptides assemble into nanostructures and their organization in the structures is reviewed. Then, how the various forms of prolamin bio-plastic materials are produced is discussed. Following on from this, the methods being investigated to modify their structures and improve the functional properties of prolamin bio-plastics are reviewed and evaluated. Based on the foregoing, conclusions are drawn as to research directions needed to fulfil the undoubted promise of prolamin bio-plastics.

STRUCTURES AND PROPERTIES OF ZEIN, KAFIRIN AND WHEAT PROLAMINS AS THEY RELATE TO BIO-PLASTIC FUNCTIONALITY

Zein and kafirin show a remarkable degree of homology (DeRose et al 1989) and will be considered together. They are both classified into four classes, α-, β-, γ- and δ- prolamins (Shewry and Tatham 1990; Belton et al 2006). Of these, the cysteine-poor α-prolamins

comprise about 80% of the total and the cysteine-rich γ-prolamins 9-12% (reviewed by Shewry and Tatham 2002). Each class can be further separated into several sub-classes (Shewry and Tatham 1990; Erny et al 2007; Belton et al 2006). All classes of zein and kafirin prolamins are small (≤ 28 k) polypeptides and are insoluble in water but soluble in aqueous ethanol, with the exception of the γ-prolamins which are soluble in water in their monomeric form (Wilson et al 1981; Evans et al 1987).

The α-prolamins contain repeated blocks of repeated sequences of about 20 amino acids rich in glutamine, proline, alanine and leucine (reviewed by Shewry and Tatham 2002). In contrast, the γ-zeins, contain a repetitive sequence based on a hexapeptide repeat (Pro,Pro,Pro,Val,His,Leu) close to the N-terminus (Tatham et al 1993), as does γ-kafirin (Belton et al 2006). Comprehensive reviews of the composition and amino sequences of the different zein and kafirin classes have been published elsewhere (Shewry and Tatham 1990; Belton et al 2006).

The prolamins of wheat can be classified into three groups, the sulfur-rich (S-rich), sulfur-poor (S-poor) and high molecular weight (HMW) (reviewed by Shewry and Tatham 1990). The HMW prolamins consist of the glutetin HMW subunits of 65-90 k and are linked together end to end into macro polymers by disulfide bonding. They account for about 10% of the wheat gluten complex. The γ- and α-gliadins and low molecular weight (LMW) glutenins make up the S-rich prolamins, of molecular weight 30-45 k and account for about 80% of gluten. The S-poor prolamins consist ω-gliadins (monomers) and D-type LMW glutenin subunits, with molecular weights ranging from 30-75k. The HWM, S-rich and S-poor wheat prolamins all share extensive repeated sequences based on proline-rich and glutamine-rich motifs (reviewed by Shewry and Halford 2002). Also, the non-repetitive

domains of the S-rich and HMW prolamins show sequence similarity. Wheat gluten has a major limitation a bio-plastic material in food-related and biomedical applications in that amino acid sequences in its gliadin and glutenin proteins are responsible for celiac toxicity and wheat allergies (reviewed by Weisser and Koehler 2008). There is limited evidence that some people may be allergic to precursors of zein (Pastorello et al 2009) and good evidence that kafirin is non-allergenic (Pontieri et al 2013).

The method of extraction affects the composition of the prolamins, which in turn influences the functional properties of bio-plastics made from them (Schober et al 2011). Commercial zein, used for much of the zein bio-plastic research, is extracted by a number of different methods (reviewed by Shukla and Cheryan 2001; Lawton 2002; Anderson and Lamsal 2011) but consists of mainly α-zein (Lawton 2002). However, there is considerable inter-batch variation even when the same process is used for extraction (Selling et al 2005). These differences appear to be mainly due to differences in molecular mass and may be due to processing conditions or differences in feedstock. Similarly, the functionality of commercially processed vital wheat gluten is also highly variable, due to denaturation with heat during the drying process (reviewed by Czuchajowska and Paszczyńska 1996). Kafirin is not commercially extracted. Kafirin used for bio-plastic material research is laboratory prepared, and so generally contains all the kafirin classes (Schober et al 2011). Schober et al (2011) showed that kafirin and zein extracted by different methods were composed of differing proportions of the different prolamin classes. Zein with a ratio of β+γ/α-zeins of approx. 10% gave maximum functionality as a "gluten-like" visco-elastic substance, whereas film formation was found to be less sensitive to the presence of β- and γ-zeins.

Prolamin polypeptides, as with synthetic polymer plastics (Morrison and Boyd 1983), link together mainly non-covalent (weak) forces including hydrogen bonding, hydrophobic interactions, van der Waals forces and other weak forces. However, when the cysteine-rich β- and γ-prolamins are present, the ability of these prolamin classes to form covalently bonded disulfide (SS) linkages either between or within the polypeptides has an effect on the functional properties of the bio-plastic materials. This was demonstrated by Taylor et al (2005), who showed that kafirin films with all the kafirin classes present were stronger but less extensible than zein films consisting of mainly α-zein. There is also compelling evidence that native kafirin polymerizes by disulfide bonding to a much greater extent than native zein (Emmambux and Taylor 2009), a factor which also influences prolamin bioplastic properties. Concerning the wheat proteins, Lagrain et al (2010) and Kuktaite et al (2011) stress the importance of disulfide bonding and sulfhydryl-disulfide (SH-S) interchange in the formation and structure of gluten bio-plastics.

Of great significance to their application in bio-plastics, is that both zein and kafirin are considered as remarkably hydrophobic proteins, but in fact both have hydrophilic characteristics (Belton et al 2006). The degree of hydrophobicity, as determined by the free energy of hydration values for α-zein and α-kafirin, calculated from their amino acid composition, are almost the same at -139.78 and -140.36 kcal/mol for α-zein and α-kafirin, respectively (Duodu et al 2003). However, values for the γ-prolamins show that γ-kafirin (-113.63 kcal/mol) is more hydrophobic than γ-zein (-124.52 kcal/mol) (Duodu et al 2003). Thus, overall, kafirin is considered to be more hydrophobic than zein (Wall and Paulis 1978; Duodu et al 2003). Related to this is the fact that the glass transition temperature T_g of zein and kafirin also differs. Zein's T_g is very dependent on its moisture content (Lawton 1992). With commercial zein, at a moisture level of 5% its T_g is around 100^{o}C but at 10% moisture

falls to around 60°C and levels off to about 20°C at higher moisture contents. At 40°C, commercial zein with shear will hydrate in water to form a "gluten-like" visco-elastic dough (Schober et al 2008). In contrast, with laboratory prepared kafirin equilibrated in an excess of water no T_g could be found up to 120°C (Adebowale et al 2011). Further, a "gluten-like" dough will only form when hydrated at 60°C and only with kafirin extracted with an unusually hydrophobic solvent (Schober et al 2011). The wheat prolamins are less hydrophobic than either the zeins or kafirins (Belton et al 2006). The T_g of gluten, like zein is highly affected by its moisture content (Hoseney et al 1986) but as would be expected seems to be somewhat lower, at 10% moisture around 50°C. As is very well-known it forms a rubbery mass when hydrated with water at ambient temp.

The barrier and mechanical properties of prolamin bio-plastics are presumably a consequence of not just the number of classes and sub-classes of prolamins present, or their contents of hydrophobic amino acids but also the degree of repetition of sequences of amino acids, and related to this the uniformity of their structures (Kuktaite et al 2011). Clearly, because of these factors they are far less uniform in structure than comparable synthetic plastics such as nylon 66 (Morrison and Boyd 1983).

The secondary structure of α-zein dissolved in 70% aqueous ethanol or methanol has been shown to range from 40-60% α-helical (Argos et al 1982; Cabra et al 2005; Forato et al 2004; Tatham et al 1993) and between 7.1 and 19.5% β-sheet (Cabra et al 2005), whereas, Z19-zein, studied by Fourier transform infrared spectroscopy (FTIR) in the solid state showed 46% α-helix and 22% β-sheet (Forato et al 2004). One of the earliest models for α-zein is based on a group of nine anti-parallel (hairpin) α-helices arranged within a distorted cylinder (Argos et al 1982). The hydrophobic amino acids are hidden within the helices, whereas the

polar amino acids are on the helices surface and so are available to form intra-and inter-molecular hydrogen bonds, allowing the zein molecules to be arranged in planes. The turns at the top and bottom of the cylinder of helices are rich in glutamine residues which allow hydrogen bonding between molecules in different planes. The Argos model was extended and modified by Garratt et al (1993) to include all α-prolamins. Physical data suggested that α-zeins were present in solution as extended structures (Tatham et al 1993). Subsequently, a number of other models have been proposed many of them based on extended helical hairpin, rod or ribbon type structures 17 nm in length, 4.5 nm wide and 1.2 nm thick with clearly defined hydrophilic and hydrophobic domains (Matsushima et al 1997, Bugs et al 2004; Forato et al 2004; Momany et al 2006).

There are no structural models for either β- or δ-zein and neither of these zeins contain repeated sequence motifs (Tatham et al 1993). The secondary structure of β-zein has been shown to consists mainly of β-sheet, β-turn and random coil, with little α-helix present (reviewed by Shewry and Tatham 1990), whereas γ-zein consists of 19-32% α-helix and 11-32% β- sheet (Wu et al 1983). There has been little work done on the secondary structure of kafirin and none on the specific kafirin classes (Belton et al 2006).

Predictions of the structure of HMW subunits of gluten indicate a α-helical structure exists at the N- and C- terminal domains, with a central repetitive domain of repeated β-turns (Shewry and Tatham 1990). In contrast to α-zein, a structural model for HMW gluten subunits based on spectroscopic and hydrodynamic studies gave a rod-shaped structure for the whole protein with a central spiral of regularly repeated β-turns (Shewry and Tatham 1990). The predominance of β-turn structure in native gluten has been confirmed by Fourier transform infra-red spectroscopy (Wellner et al 2005).

ASSEMBLY OF PROLAMIN POLYPEPTIDES INTO NANOSTRUCTURES

Prolamin proteins can be used to make bio-plastic materials of many different forms, including fibers, films and coatings, nanoparticles, microparticles (microspheres) and macrostructures. All appear to be produced by controlled protein aggregation and, depending on the interactions between the prolamin monomers, different structures can be formed (Bolder et al 2006). Protein aggregation can be linear (also referred to as fibrillar) or non-linear (also known as particulate or aggregate) (reviewed by Van der Linden and Venema 2007). According to Krebs et al (2007) the type of protein aggregation depends primarily on the state of the protein when aggregation occurs rather than the specific amino acid sequence of the protein. The state of the protein is dependent on concentration, temperature, pH, salt concentration, salt type and type of solvent added. These differing conditions favor partial unfolding of natively folded proteins or refolding of unfolded proteins and may enable polymerization into β-sheet structures. Beta-sheet structures are thought to be a universal energetic minimum for aggregated proteins (reviewed by Gorbenko and Kinnunen 2006).

Assembly of the prolamin polypeptides into nanostructures seems to be a common origin for formation of many forms of bio-plastics.

Nanostructures

Wang et al (2008) identified a number of different structures including spheres, sponges and lamellae when investigating the amphiphilic nature of zein and its ability to self-assemble when the hydrophilic/hydrophobic balance of the system was varied. Further work concluded that spheres were the base of all other microphases (Wang et al 2010). Using

high-resolution TEM images of different zein nanostructures deposited from aqueous ethanol (a secondary solvent) as solvent evaporated, Wang and Padua (2012) observed a periodicity of 0.35 nm, which is typical of β-sheet. Based on their data and circular dichroism measurements, they have devised a model describing the formation of zein nanostructures by a process of evaporation induced self-assembly as a result of β-sheet orientation, alignment and packing (Fig. 1). Molecular self-assembly is the spontaneous organization of molecules under thermodynamic equilibrium conditions into structurally well-defined and stable arrangements (protein aggregates) through a number of non-covalent interactions (Whitesides et al 1991).

One of the major driving forces of self-assembly is amphiphilicity (Wang and Padua 2012). The amphiphilicity of zein was demonstrated by Kim and Xu (2008) when they showed that zein can adhere to hydrophilic surfaces (glass spheres), in solutions of less than 90% ethanol. Zein micelles formed with a hydrophilic end directed to the solvent. On hydrophobic surfaces (toner particles), in solutions greater than 90% ethanol, zein micelles orientate themselves with the hydrophobic end directed to the solvent.

In Wang and Padua's model the self-assembly process is thought to start with the unfolding of the α-helical structures and transformation into β-sheet (Wang and Padua 2012) (Fig. 1a,b). Antiparallel β-sheets were then proposed to pack side by side to form a long ribbon (Fig 1c,d) which could then be curled into a ring, driven by hydrophobic interactions (Fig. 1e). Addition of further β-sheets is then thought to 'grow' the rings into nanospheres (Fig. 1f). The authors extended the model to include the formation of nanotubes by end to end linking of β-sheets via glutamine bridges and then coiling into three-dimensional columns.

The model proposed by Wang and Padua (2012) is only for α-zein deposited from aqueous ethanol and may not be applicable when bio-plastic materials are made from zein or kafirin where the full complement of prolamin sub-classes are present (Schober et al 2011; Panchapakesa et al 2012; Byaruhanga et al 2005; Taylor et al 2009a,b). Consideration should, however be given to the fact that γ-, β- and δ-prolamins are also amphiphiles and contain many more cysteine residues than the α-prolamins, as described. Disulfide bonding involving these cysteine residues may serve to stabilize bio-plastics once formed, in the same way as zein and kafirin protein bodies are thought to be stabilized (Coleman et al 1996).

If we accept Wang and Padua's model for nanostructure formation, then the question arises, 'How do the nanostructures assemble into the many different final forms of bio-plastic materials?' It is possible that the nanostructures assemble to form microstructures of similar morphology, which in turn form similar macroscale structures during prolamin bio-plastic material formation. This may be considered to be analogous with the filaments of myosin and the structure of muscle (Alais and Linden 1991) or the assembly of blocklets which make up the starch granule (Gallant et al 1997).

FORMATION OF PROLAMIN BIO-PLASTIC MATERIALS

The main processes used to produce the various forms of bio-plastics are casting/phase separation (Zhang and Mittal 2010), thermoplastic processing (Redl et al 1999) and electrospinning (Wang and Chen 2012a). Apart from thermoplastic processing, all these processes begin by dissolving the prolamin protein in an appropriate solvent, such as aqueous ethanol, and then removing or changing the solvent composition. Some workers have proposed models describing how specific bio-plastic materials are formed and the molecular

structure of the bio-plastic materials themselves. However, complex structural and organizational changes seem to be necessary in order to form ordered materials. In some cases, the native α-helical structure has been identified and has been found to be beneficial to the functional properties of the specific material. This includes zein blown thermoplastic films (Oliviero et al 2010), zein resin stretched films (Wang et al 2005), aqueous ethanol cast films of zein (Wang et al 2005) and kafirin films, (Gao et al 2005). Electrospun zein fibers seem to have predominantly α-helical conformation, but there is also a β-sheet contribution (Torres-Giner et al 2008; Wang and Chen 2012b). Certain other forms of prolamin bio-plastic materials do contain a considerable degree of β-sheet structure. These include acetic acid cast kafirin films (Byaruhanga et al 2006), hordein films (Xia et al 2011), gluten films (Kuktaite et al 2011), kafirin microparticles (Taylor et al 2009a), kafirin microparticle films (Taylor et al 2009b), glutaraldehyde cross-linked kafirin microparticles (Anyango et al 2012), kafirin microparticle films (Anyango et al 2011) and zein microspheres (Wang and Padua 2012).

Microparticles

Microparticles is a collective name for microcapsules (a single core surrounded by a layer of wall material) and microspheres (the core dispersed in a continuous matrix network). Both are colloidal microstructures with size 1-250 μm (reviewed by Allemann et al 1998; Reis et al 2006). The most commonly used processes for protein microparticle preparation are spray-drying, solvent extraction/evaporation and phase separation/coacervation (reviewed Sinha and Trehan 2003; Chen and Subirade 2007).

Taylor et al (2009a) formed microparticles from native kafirin containing all the kafirin classes by simple coacervation, where kafirin was dissolved in acetic acid (a primary solvent) and then water added to change the solvent polarity and precipitate out the protein as

microparticles. In practical terms this process is the essentially the opposite of that described by Wang and Padua (2012) but in principle the same. Atomic Force Microscopy showed the surface the kafirin microparticles consisted of nano-sized protuberances (Anyango et al 2012), which may be related to the spherical structures reported by Wang and Padua (2012). On wet heat treatment of these microparticles, the nanostructure looked as if spheres had melted together, in keeping with the model of Wang and Padua (2012).

Taylor (2008) proposed a model for formation of the kafirin microparticles (Fig. 2). The model uses the analogy of protein body formation in maize and sorghum, but noting that protein body membranes are absent. In maize, protein body assembly is thought to occur by an initial interaction between the hexapeptide repeat of γ-zein and the endoplasmic reticulum (ER) membrane (Kogan et al 2001). The self-assembly mechanism of this protein then coats the inner face of the ER membrane and is stabilized by intermolecular disulfide bonds (Coleman et al 1996). Subsequently, the hydrophobic α-zein deposits within the γ-zein coat. According to the microparticle formation model (Taylor 2008), as water is added to a concentration solution of kafirin in glacial acetic acid, microparticles form by precipitation of α- and β-kafirin around small particles of undissolved kafirin and air bubbles (Fig. 2a,b),which act as nucleation sites. Lastly, the γ-kafirin would form a layer on the surface of the partially formed microparticle and stabilize the microparticle by disulfide bonds (Fig. 2c).

Films and Coatings

Prolamin bio-plastic films and coatings are generally formed by casting (reviewed by Zhang and Mittal 2010). The process involves dissolving or dispersing the prolamin in a suitable solvent, with heat if necessary, and then drying the casting the mixture to form free-standing films or coatings (reviewed by Cuq et al 1998). Film formation is based on separation of

proteins from the solvent phase by precipitation or phase changes caused by changes in solvent conditions (polarity or pH changes, electrolyte additions) thermal treatments or solvent removal (Cuq et al 1998). Solvent removal results in increased polymer concentration in the medium, inducing bonding and the formation a three-dimensional network. Surprisingly, there is an absence of satisfactory models as to how the polypeptides are organized in the films. Further, none of the models address the issues of the effects on film structure of the various prolamin classes.

Resin-drawn films can be made by dispersing zein with the plasticizer oleic acid in aqueous alcohol and precipitating out the resin in cold water (Lai et al 1999). These authors proposed a model for such a zein resin-drawn film, based on their own x-ray data and molecular measurements determined by Matsushima et al (1997). Lai et al (1999) suggested that the oleic acid adsorbed onto the charged residues on the zein surface by electrostatic forces. Precipitating the resin in water would result in hydrophobic aggregation of zein-oleic acid units to form films. It was suggest that subsequent kneading of the resin and hot-rolling it to form sheets would result in unfolding of the prism-like zein molecule, exposing new binding sites for the oleic acid to bond. The model shows layers of double stacked zein units alternated with bilayers of oleic acid which conferred film flexibility (Fig.3).

Kafirin microparticles can be cast into very uniform films by evaporation from a suspension in acetic acid-water solution (Taylor et al 2009b). Taylor (2008) proposed when such microparticles are suspended in acetic-water, the prolamin amphipathic helix would have its hydrophilic side towards the solvent (Fig. 4a). As the water preferentially evaporates, the environment would become more hydrophobic and the amphipathic helix would invert and its hydrophobic face would be towards the solvent (Fig. 4b). As the acetic acid concentration

increases further the secondary structure of the individual kafirin molecules would begin to unfold becoming a more open β-sheet conformation (Fig. 4c,d). The hydrophobic side of the molecule could then adhere to the hydrophobic film casting surface by hydrophobic interactions (Fig. 4e). This would leave a mainly hydrophilic surface exposed, which could then bond by electrostatic interactions or hydrogen bonding to the hydrophilic side of another kafirin molecule. It was further suggested that the kafirin molecules would be deposited into film as a series of alternating hydrophobic and hydrophilic layers (Fig. 4e).

Shi et al (2009) suggested a model for the organization of zein molecules in films, based on the model of Matsushima et al (1997) for zein in solution where rectangular prisms of zein molecules are hexagonally aligned parallel to each other. Shi et al (2009) proposed that the molecules either stack in layers with their α-helices at right angles to the surface when aqueous ethanol is used as solvent in parallel to the surface when glacial acetic acid was the solvent, but in both cases the basic organization of the prolamin polypeptides was the same as in solution, unlike the models proposed by Taylor (2008) and Wang and Padua (2012).

In this regard, Kuktaite et al (2011) describing a model for extruded wheat gluten films, highlighted the complexities of the supramolecular structures and conformations of the wheat prolamins in films and how these are influenced by the film preparation conditions. They identified tetragonal prolamin structures when sodium hydroxide was present in the film forming process, whereas in the presence of ammonium hydroxide, a highly polymerized bidimensional hexagonal structure was formed with glutenins and gliadins intimately mixed. The films contained a large amount of β-sheet structures and the film structure was orientated in the direction of extrusion. The results showed the incorporated gliadins along the direction

of extrusion favored intermolecular β-sheet formation, whereas intramolecular β-sheet may increase due to changes in the globular non-repetitive domains of the gluten proteins.

Sponges, Hydrogels and Three-dimensional Scaffolds

A sponge is a form of foam, where there is interconnected gas cells entrapped in a solid polymer. Hydrogels are three-dimensional networks of chemically or physically cross-linked hydrophilic polymers that have high water content and a soft and rubbery consistency (Hoffman 2002; reviewed by Reddy and Yang 2011). Both are forms of three-dimensional scaffolds. Cross-linking can be by covalent bonds, hydrogen bonding, van der Waals interactions or physical entanglement (reviewed by Qiu and Park 2001).

Sponges can be prepared from prolamins by phase separation. Taylor et al (2009a) precipitated kafirin microparticles from a viscous solution of acetic acid using water. The resulting microparticles contained interconnected holes or vacuoles, which were thought to be the footprint of air bubbles entrapped within the viscous acetic acid solution. Padua and Wang (2010) prepared zein sponge, with a pore size range between tens to hundreds of nanometers, using evaporation induced self-assembly as described above under Nanostructures.. Anyango et al (2011) formed a kafirin microparticle sponge/hydrogel by washing out the plasticizer (porogram) from a kafirin microparticle film. The sponge/hydrogel retained its integrity on drying and when hydrated the sponge was very flexible..

Physically-formed hydrogels generally have poor strength and stability in physiological environments, whereas chemically cross-linked hydrogels have better mechanical properties but may possibly be immunogenic and the cross-linkers used could be toxic (reviewed by

Reddy and Yang 2011). Sun et al (2009) produced a gliadin gel from aqueous propanol under alkaline conditions. A stable gel structure was obtained but this was not retained after freeze-drying. This instability was thought to be related to protein aggregation during the formation of the gel network composing of cross-linked strands.

Three-dimensional scaffolds can also be formed by molding (Tsang and Bhatia, 2004). Gong et al (2006) developed cylindrical zein scaffolds with 75–79% porosity by molding followed by a salt-leaching (porogram) technique. The scaffolds had good mechanical properties and were degradable by mammalian proteases. In contrast, Gillgren et al (2010) used molding to produce a solid zein foam by heating a zein resin in a mold. This foam had moderate mechanical properties when compared to commercially available petroleum- and bio-based foams.

Fibers and Fibrils

Prolamin fibers can be produced by dry and wet spinning (the Swallen process) (reviewed by Lawton 2002) and electrospinning (Miyoshi et al 2005). Dry spinning involves extruding a prolamin solution into the air, where coagulation takes place due to evaporation of solvent, while in wet spinning the prolamin solution is extruded into water or some other coagulation medium (Lawton 2002). In a dry spinning version patented by Uy (1998) zein fibers are prepared by dissolving zein in a volatile solvent system and extruding in the air. An example, wet spinning was reported by Reddy et al (2008) on spun gluten fibers. In this process, gluten solution in 8 M aqueous urea solution with reducing agent sodium sulfite was allowed to age at ambient temperature to form a viscous dope suitable for spinning. The spinning dope was extruded as fibers into a coagulation bath consisting of 10% sodium sulfate. Electrospinning is a fabrication process that uses an electric field to control the deposition of polymer fibers

onto a target substrate (reviewed by Matthews et al 2002). The technique involves applying a high voltage to create electrically charged jets of a polymer solution, which dry to form fibers and are collected on a target as a nonwoven fabric (Miyoshi et al 2005. Electrospun prolamin fibers have a high surface area-to-volume ratio and morphology similar to natural tissues (reviewed by Wang and Chen 2012a).

With regard to assembly of prolamin polypeptides into fibers by electrospinning, Torres-Giner et al (2008) found that when zein was electrospun from an aqueous ethanol solution, pH and viscosity played a major role the quality fibers produced. They proposed that under alkaline conditions, as a result of hydrolysis of glutamine residues to glutamate, oligomerization of zein is strongly favored, resulting a low viscosity solution which did not produce fibers. In contrast, acidic conditions produced ribbon-like fibers with high T_g (163^oC). They attributed this to protein aggregation/polymerization, as a result of strong intermolecular hydrogen bonding. Wang and Chen (2012a) found that hordein, gliadin and zein exhibited a different electrospinning behavior from solution/colloidal suspension in acetic acid. In acetic acid, the hordein molecules were apparently unfolded and reorganized so that they could easily combine together by intermolecular hydrogen bonding. However, this transformation apparently resulted in brittle fibers with reduced mechanical strength. Gliadin formed the strongest fibers, which they attributed to its higher glutamic acid (glutamine) content causing stronger intermolecular interactions.

Zein can also form fibrils/strands in aqueous environments above its T_g, which are analogous to gluten fibrils associated with dough formation (Schober et al 2008; Erickson et al 2012). Erickson et al (2012) proposed that the mechanism for zein fibril formation is possibly akin to the self-assembly of amyloid fibrils, typically associated with neurodegenerative diseases.

The formation of amorphous aggregates and rapid precipitation of insoluble β-sheet structures, which are typical of amyloid fibrils, are also characteristic of zein's behavior in aqueous ethanol, when the polarity of the solvent is changed.

Thermoplastic Materials

These are produced by a dry process that uses thermal and mechanical energy, due to the thermoplastic properties of prolamins when plasticized and heated above their T_g under low water content (reviewed by Redl et al 1999). Prolamin bio-plastics can be processed using existing plastic processing machinery such as extrusion, thermoforming, various types of injection moulding, compression moulding and film blowing (reviewed by Lagrain et al 2010; Oliviero et al 2010). Prolamin polymerization, in particular disulfide bonding, can also occur depending on the processing conditions and the nature of the plasticizer used (reviewed by Lagrain et al 2010). Prolamin rigid plastics are stiff, glassy bio-plastics with mechanical properties approaching those of synthetic rigid plastics, such as polystyrene (reviewed by Jansens et al 2012). The properties prolamin thermoplastics differ from those produced by phase separation. For example, thermo-pressed gluten films had higher stress and lower strain values than cast films, suggesting that the dry process resulted in a more densely cross-linked network (Mangavel et al 2004).

Using thermoplastic technology prolamins can also be blown into films, like polyethylene. Oliviero et al (2010) produced zein films (80-150 μm thick) using blowing technology. The ability of zein to be blown into a film appeared to be very dependent on the proportion of α-helical structure. Zein with higher proportion of native-like α-helical structure was more suitable for blowing and resulted in films with higher modulus of elasticity and breaking stress. Thermoplastic processed prolamin bio-plastic films generally have a higher modulus

of elasticity than cast prolamin films, probably because of the higher water content used in casting resulting in a plasticization effect.

The different prolamins have very different thermoplastic behavior. Di Maio et al (2010) found that laboratory-prepared kafirin had a much higher melt temperature and higher viscosity than commercial zein when they were plasticized with polyethylene glycol 400. This may be attributable to the greater degree of disulfide cross-linking in kafirin.

IMPROVEMENT IN THE FUNCTIONAL PROPERTIES OF PROLAMIN BIO-PLASTICS

Prolamin bio-plastics have relatively poor functional properties when compared to similar synthetic polymer materials, as stated (reviewed by Reddy et al 2008). Improvements in their mechanical strength, flexibility and extensibility (prevention of brittleness) and water stability are needed. This is being attempted through improving prolamin solvation (Taylor et al 2005a), plasticization (McHugh and Krochta 1994), various cross-linking treatments and blending with synthetic or other natural polymers (reviewed by Ayres et al 2010; Reddy and Yang 2011).

Prolamin Solvation

The solubility of prolamin in a solvent and the resultant viscosity of the prolamin solution have an effect on prolamin bio-plastic functional properties and these may in some cases also affect the protein secondary structure. Bicudo et al (2008) working with γ-zein found major conformational changes which were solvent dependent. The two most commonly used solvents for prolamin bio-plastic material formation are aqueous ethanol and glacial acetic

acid. Shewry et al (2003) suggested that solvation of prolamin proteins depends on hydrophobicity, hydrophilicity, secondary structure and protein-protein interactions. Solvation occurs when there is a net decrease in free energy of the protein and solvent on mixing. These workers also suggested that the solubility of prolamins in aqueous alcohols is possible when there is a favorable balance of hydrophobic and hydrophilic forces.

However, Li et al (2012) reported that in aqueous ethanol, α-zein behaves like polymer but can be fully dissolved in acetic acid, and acts as a polyelectrolyte, being protonated and associated through acetate salt bridges. These authors found that zein was larger and more unfolded in acetic acid resulting in a greater solvent accessible area and hydration of the zein surface than in aqueous ethanol. They suggested that protein protonation by association with acetic acid may prevent protein aggregation and allow better dissolution. They further observed that if acetic acid is added to water (a zein non-solvent), the protonation of zein helps stabilization by long range electrostatic repulsion forming a colloidal dispersion. Taylor et al (2005b) showed that kafirin films cast in glacial acetic acid were more uniform than those cast from ethanol and attributed this to the better solubility of kafirin in glacial acetic acid. These differences may be directly related to different film morphologies (Shi et al 2009). Lately, glacial acetic acid has been used for electro-spinning of zein (Selling et al 2007), hordein and gliadin (Wang and Chen 2012a).

Plasticization

Most plastics, including prolamin bio-plastics are very brittle and must therefore be plasticized to improve the functionality. Plasticizers are non-volatile, small molecular weight-compounds that when added to a bio-plastic materials improve the flexibility and mechanical properties (Banker 1966). Plasticizers achieve this by reducing interactions between protein chains and increasing free volume. However, due to a decrease in the T_g,

there is a corresponding decrease in film mechanical strength and permeability (McHugh and Krochta 1994). Plasticizers can be external or internal. External plasticizers solvate and lubricate the protein chains (Fig. 3), lowering the T_g of the proteins and increasing the free volume (Banker 1966). Generally, external plasticization is used for cast prolamin films (Park et al 1994). Probably the most commonly used external plasticizer for prolamin bio-plastics is glycerol. Others include propylene glycol, polypropylene glycol, lactic acid, dibutyl tartrate, sorbitol, sucrose and fatty acids (Lawton 2002; reviewed by Dangaran et al 2009).

Water has a considerable plasticization effect on prolamins (Gontard et al 1993). Therefore, a very hygroscopic plasticizer such as glycerol may increase water uptake and thus influence film tensile and barrier properties (Banker 1966). The effectiveness of plasticizers depends on size, shape and compatibility with the protein matrix (Banker 1996). Many external plasticizers such as glycerol and polyethylene glycol because they are not well-bound to the prolamin as well as being hydrophilic leach out, changing tensile properties and increasing permeability to water (Anyango et al 2011; Xu et al 2012). However, if glycerol is used at low levels it may adsorb onto and possibly into the protein's structure (Gao et al 2006; Gillgren et al 2009).

Internal plasticizers attempt to overcome these problems by chemically modifying the protein chains (Banker 1966). This can be through addition of covalently linked substituent groups by derivatization reactions such as acetylation, succinylation and Maillard–type (carbonyl) reactions (reviewed by Dangaran et al 2009). Common internal plasticizers are acetic anhydride and succinic anhydride. Internal plasticizers create steric hindrance between the protein chains resulting into increased free volume and improved flexibility (reviewed Dangaran et al 2009). Gómez-Martinez et al (2012) used a combination of glycerol and citric acid to plasticize zein and pennisetin (pearl millet prolamin) melts. Both proteins formed

melts which formed cross-linkages above 60°C. Pennisetin showed more thermocomplex properties, probably due to its high cysteine and methionine content. When Xu et al (2012) used a mixture of glycerol and oleic acid to plasticize cast zein films, they found a synergistic effect. The glycerol acted as an external (structural) plasticizer and the oleic acid, apparently acted as an internal (molecular) plasticizer.

Cross-linking

As described, in prolamin bio-plastics the individual polypeptides are generally held together by weak forces and disulfide polymerization of the polypeptides is limited. Additional covalent cross-linking can produce proteins with altered thermal stability, surface reactivity, lipophilicity, molecular weight, charge, shear stability and resistance to proteases (reviewed by Stark and Gross 1994).

Physical Treatments

Heat treatment can result in protein cross-linking through the formation of disulfide and hydrophobic bonds. For example, work by Byaruhanga et al (2005) on kafirin cast films showed that microwave heating wet kafirin before film making increased film tensile strength but decreased both the film extensibility and film water vapour permeability (WVP). Cross-linking using microwave heating reduced the rate of kafirin film degradation under composting conditions (Byaruhanga et al 2007). These effects of disulfide bond mediated polymerization of the kafirin polypeptides (Byaruhanga et al (2005, 2006, 2007) were associated with increase in β-sheet structure in the kafirin (Byaruhanga et al 2006). Similarly, Anyango et al (2012) working on kafirin microparticles produced by coacervation, found that wet-heating the formed microparticles substantially increased their both their size and vacuolation.

Cross-linking through γ-irradiation has been found to improve both barrier and mechanical properties of protein-based edible films (reviewed by Zang and Mittal 2010). The γ-irradiation affects proteins by causing conformational changes, oxidation of amino acids, rupture of covalent bonds, and the formation of protein free radicals (reviewed by Wittaya 2012). Proteins can be converted to higher molecular weight aggregates through the generation of inter-protein cross-linking reactions, hydrophobic and electrostatic interactions, and the formation of disulfide bonds. Gamma-irradiation of gluten film forming solutions has been found to increase tensile strength of the films (Lee et al 2005). Similarly, Soliman et al (2009) working on zein-based films found a reduction in WVP and increase in light transmission with γ-irradiation treatment. The reduction in the WVP was probably due to protein aggregation reducing the rate of diffusion through the film, while the increase in film light transmission was probably as a result of destruction of yellow pigments by irradiation.

Chemical Cross-linking

Aldehydes such as glutaraldehyde (Lee and Rosenberg 1999, 2000), formaldehyde and glyceraldehyde (Vandelli et al 2001) have been frequently investigated as a means of cross-linking prolamin bio-plastics. Cross-linking of protein with an aldehyde involves the reaction of free amino groups of peptide chains with the aldehyde (carbonyl) groups through Schiff base (imine) formation (Bigi et al 2002; reviewed by Migneault et al 2004). Sessa et al (2007) working on zein films reported glutaraldehyde reaction with zein generated films with improved tensile strength, ductility, stiffness and water resistance when compared with zein control films. Similarly, working with kafirin microparticle films Anyango et al (2011) found that glutaraldehyde cross-linking resulted in films that maintained their integrity and flexibility in ambient temperature water, despite total loss of external plasticizer through solubilization. Other studies that have demonstrated improvement in either physical strength

and/or water stability of prolamin bio-plastics with aldehyde treatments include those on gliadin films (Hernández-Muñoz et al 2005), zein fibers (Selling et al 2007) and gluten fibers (Reddy et al 2008). The water stability of glutaraldehyde-treated prolamin bio-plastics is probably due to increase in covalent bonding within the bio-plastic matrices through formation of covalent glutaraldehyde-polypeptide linkages. Because glutaraldehyde cross-linked prolamin bio-plastics are quite stable in water, they have good potential for use as biomaterials in aqueous applications. Glutaraldehyde cross-linking has also been shown to increase the size of microparticles formed from kafirin by coacervation (Anyango et al 2012).

Carboxylic acids can cross-link prolamins simarly. Reddy et al (2009) developed a wet alkali-catalyzed process, which they demonstrated by cross-linking gliadin and zein fibers with malic acid, citric acid, and butanetetracarboxylic acid. A modified process by Jiang et al (2010) doubled the strength of zein fibers and reduced their swelling by about half. These authors reported an increase in fibroblast cell attachment on cross-linked zein fibers, which was mainly due to an increase in their stiffness. Increase in scaffold stiffness has been shown to promote cell attachment and growth (Genes et al 2004).

The acylation reaction through treatment with lauryl chloride has been found to result in a reduction in zein film T_g with increase in content of the lauryl chloride, probably due to plasticization by the lauryl moiety (Shi et al 2011). Similarly, electrospun zein nanofiber mats cross-linked by hexamethylene diisocyanate had an increased tensile strength (Yao et al 2006). Recently, Sessa et al (2012) showed that treatment of zein by isocyanate and diisocyanate reduced moisture uptake, probably due to decreased zein hydrophilicity.

Crosslinking with natural compounds such as amino acids and tannins offers an alternative to potentially toxic chemical cross-linkers in food and biomedical applications. The amino acid cysteine can be used to disulfide cross-link prolamin bio-plastics. Hernandez-Munoz et al

(2004a,b) treated gliadin films with cysteine. The treated films maintained their integrity in water and became less extensible, and their tensile strength and T_g increased. The changes in film properties was attributed to development of a more protein rigid network, through formation of both inter- and intra-molecular disulfide bonding.

Tannins are plant polyphenols with an exceptional ability to cross-link with prolamins (Emmambux and Taylor 2003). Unlike cysteine, cross-linking is probably through hydrogen bonding with proline-rich regions in the prolamin (Emmambux et al 2004). Hence, the γ-kafirin class, which contains some 23 mole% proline, binds preferentially with condensed tannins (Taylor et al 2007). Emmambux et al (2004) found that modification of kafirin films with tannins resulted in films with better mechanical stability at a higher relative humidity, attributed to the decrease of kafirin polypeptide chain mobility and free volume caused by tannin cross-linking. Further, Taylor et al (2007) reported that films made from tannin-bound kafirin were less digestible and had slower degradability. As the plant polyphenols bound to prolamin bio-plastics have been shown to exhibit antioxidant effects (Taylor et al 2009c), cross-linking prolamin bio-plastics with the polyphenols would enhance their potential for biomedical applications.

Enzymes that catalyze intra- and/or inter-molecular protein cross-linking can improve the functional properties of prolamin bio-plastics (reviewed by Wittaya 2012). Such enzymes include transglutaminase, lipoxygenase, lysyl oxidase, polyphenol oxidase and peroxidase. Transglutaminase (protein-glutamine:amine γ-glutamyl transferase) catalyzes an acyl transfer reaction in which carboxyamide groups of peptide-bound glutamine residues are the acyl donors and a variety of primary amines are the acyl acceptors resulting in ε-(γ-glutamyl)-lysine bridges (Ohtsuka et al 2000). The polymers so formed may be chemically, enzymatically resistant and mechanically strong. For example, Larre et al (2000) reported

that cross-linking with transglutaminase introduced covalent bonds into gluten films. These films had increased integrity, strength and extensibility. However, Anyango et al (2011) working on kafirin microparticle films found that treatment with transglutaminase did not improve the mechanical strength and water stability of the films, probably because kafirin's very low lysine content hindered transglutaminase-catalyzed cross-linking reaction.

The potential of cross-linking with peroxidases to improve the functional properties of prolamin films has also been investigated. Protein cross-linking occurs through intermolecular dityrosine bond formation (reviewed by Michon et al 1999). These authors working with horseradish peroxidase and soybean peroxidase found that treating gliadin films increased the tensile strength accompanied by a reduction in elongation.

Chemical Derivatization

In a somewhat different approach, Biswas et al (2009) modified the surface properties of cast zein films by reaction with octenyl succinic anhydride and alkyl and alkenyl ketene dimers to form derivatized zein by formation of an alkyl β-ketone ester. The surface derivatized films showed a higher water contact angle (similar to waxed paper) and substantially reduced water absorption. As chemical derivatization can be carried out after the bio-plastic is made, this modification technique is potentially suitable for surface dressing treatment of prolamin bio-plastic films such as packaging materials.

Blending with Synthetic Polymers or other Natural Polymers

The aim of blending polymers is to achieve improved performance which is not possible by either of the components alone. For example, Tihminlioglu et al (2010) investigated zein-coating polypropylene films and found significant improvements in WVP and oxygen barrier properties. However, zein has been found to be incompatible with natural polymers such as

starch and gelatin and synthetic polymers such as poly ε-caprolactone (reviewed by Sessa et al 2011). ,Also, for example low density polyethylene-zein co-polymer has inferior mechanical properties to zein alone (Herald et al 2002). However, Sessa et al (2011) have successfully melt-processed blended zein with the synthetic polymer polyvinylpyrrolidone (PVP). Surprisingly, even at PVP levels up to 20%, there was a remarkably little change in material properties (Sessa et al 2011). Unfortunately, however, at high relative humidity (70%) there was a decrease in tensile strength. Interestingly, it has been found that electrospinning zein with dodecane, a phase-changing alkane, resulted in bi-component material with supercooling properties (Pérez-Masiá et al 2013).

Lipids have been investigated to improve the water barrier of prolamin bio-plastics. Weller et al (1998) showed that sorghum and carnauba waxes could be used to produce bilayer zein films improved WVP properties. Adding different types of lipids to zein microparticles, Önal and Landon (2005) found a substantial improvement in riboflavin encapsulation, delivery and retention efficiencies.

Concerning non-starch polysaccharides, one of the few widely used commercial applications for zein is a coating for bamboo fiber textiles, in order to improve surface finish (Alibaba 2012). Conversely, cellulose microfibrils have been added to zein to produce a cast nanocomposite film with improved tensile strength (Phiriyawirut and Maniaw 2012). With regard to thermoplastic processing, Oliviero et al (2011) investigated the addition of lignins to produce zein-lignin nanocomposites. They found that even at low lignin concentration (1%), there was major de-structuring of inter- and intramolecular interactions in the zein α-helix, β-sheet and β-turn secondary structures. The authors suggested that the lignins were inserted within the inter-helix layers of zein induced by hydrogen bonding between the zein amino acids and functional groups of the lignin.

Lui et al (2006) developed a complex hydrogel bead by combining zein with pectin through evaporation from aqueous ethanol. The hydrogel did not swell under physiological conditions, but could be hydrolyzed in the presence of pectinases, probably because a a portion of zein may have been bound to the pectin by molecular entanglement. Bobkalonov et al (2012) showed, using a simulated gastrointestinal tract system, that pectin-zein hydrogel could give controlled drug delivery of up to 36 hr.

With regard to other proteins, silk fibroin (SF) has been used to fabricate a bicomponent nanofibrous scaffolds with zein for biomedical applications, using electrospinning (Yao et al 2009). The zein/SF electrospun nanofibers had a smaller diameter and narrower diameter distribution than pure zein nanofibers and improved tensile strength. The improvement in mechanical strength of the fibers was stated to be probably due to entanglements and physical interactions among partial chains of the blend polymers.

CONCLUSIONS

It is clear that we need to know far more about the very complex processes of how prolamin polypeptides assemble into nanostructures, including the role of protein secondary structure, and especially about how these structures further assemble into the organizational structures of the various prolamin bio-plastic materials. Such knowledge should enable us to manipulate and direct the process to improve functionality. Literature on protein biomaterials emphasizes the importance of control over inter- and intramolecular weak interactions, and of SS/SH interchange, as these play a profound role defining material performance because they are responsible for arrangement at the macromolecular level (reviewed by Guan 2007; Kuktaite et al 2011). This is referred to as “supramolecular design” and offers the potential

to produce bio-plastics, such as those which exist in nature, that have better mechanical properties than synthetic polymers.

However, it is evident that given the more complex primary structure and the numbers of different classes and sub-classes of proteins making up any prolamin, the situation is infinitely more complex than with synthetic polymers. Thus, despite the vast amount of research that has already been carried out into physical and chemical methods of improving prolamin bio-plastic structures, it seems unlikely that we will find a simple practical method that is going radically raise their general functionality to the level of synthetic polymer plastics. Also, any such treatment adds to the already high cost of these materials and "non-natural" chemical modifications undermine a major competitive edge of prolamins. One avenue to obviate these problems seems to use the sulfur-rich prolamin classes such as γ-zein and γ-kafirin for polymerization. Obviously these can be preferentially extracted but perhaps a better way is the use of mutants with enhanced levels such as Quality Protein Maize (Wu et al 2010).

Probably the biggest potential for zein and kafirin bio-plastics lies in biomedical applications, in particular as biomaterials (materials that can be introduced into living tissues). Here the relative hydrophilicity, bio- and cyto-compatibility and biodegradability of such proteins highly advantageous (reviewed by Reddy and Yang 2011) and their high cost is not such a drawback. As can be seen from Table I there is already much promising research into biomedical applications for the various types of prolamin bio-plastics discussed. However, a major and very costly hurdle still to be overcome will be to ensure that such prolamin-based biomaterials are safe (non-immunogenic) when used, for example, as implants such as scaffolds in living tissues.

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