1. INTRODUCTION

Whilst working on a Masters degree on alternative solvents and extractants for sorghum kafirin, (Taylor, 2003) serendipitously, it was discovered that when water was added to a solution of kafirin in glacial acetic acid a 'colloidal suspension' was formed. When this 'colloidal suspension' dried a clear, transparent film was formed. Thus it appears that using this type of 'colloidal suspension' may be an ethanol-free method of making cast bioplastic films. Aqueous ethanol is the solvent most often used to form free-standing prolamin films, with aqueous acetone being used less frequently (Cuq, Gontard and Gilbert, 1998). Ethanol is flammable, needs a government license for its use and is unacceptable for food use by some religious communities. Thus an alternative to ethanol for the casting of prolamin bioplastic films would be advantageous.

The particles in the kafirin 'colloidal suspension' were subsequently identified as microparticles of kafirin by light microscopy and by comparison with zein literature (Liu, Sun, Wang, Zang and Wang 2005; Parris, Cooke and Hicks, 2005). A few references concerning the preparation and use of zein microparticles have been found in the scientific literature but none at all for kafirin microparticles. The method discovered for preparing kafirin microparticles appears to be novel. There are a number of potential applications for zein microparticles, including encapsulation of pesticides (Demchak and Dybas, 1997), drug delivery (Liu et al., 2005), tissue engineering (Dong, Sun and Wang, 2004), and drug eluting films (Wang, Lin, Liu, Sheng and Wang, 2005). Food applications for zein microparticles include food coatings and glazes (Cook and Shulman, 1998) and the encapsulation of essential oils (Parris et al., 2005).

Kafirin and zein are very similar (DeRose, Ma, Kwon, Hasnain, Klassy and Hall, 1989). However, kafirin has potentially better properties for film formation than zein since it is somewhat more hydrophobic (Belton, Delgadillo, Grant and Taylor, 1997) and more highly cross-linked than zein (El Nour, Peruffo and Curioni, 1998; Duodu, Nunes, Delgadillo, Parker, Mills, Belton and Taylor, 2002). It is thus possible that kafirin microparticles would have better properties for certain applications than those of zein for the same reasons. Research into the preparation, characterisation and functionality of kafirin microparticles is needed before the potential applications of these microparticles can be exploited.

2. LITERATURE REVIEW

The following chapter will review current literature concerning microparticles in general and specifically protein microparticles. It will discuss the definition and characteristics of microparticles, methods of preparation, how their functional properties can be modified by varying the conditions of preparation, suitability of different materials for microparticle preparation, kafirin composition, structure and functional properties, the properties of protein microparticles, the improvement of microparticle resistance to degradation by cross-linking and potential applications of microparticles.

2.1. MICROPARTICLES

2.1.1. Definitions

Microparticles are colloidal particles, which according to Coombes, Lin, O'Hagen and Davis (2003) are mainly spherical and have a diameter in the range 10 nm to 2 mm. Sub-micron microparticles are sometimes referred to as nanoparticles or nanospheres. In the literature microparticles are also referred to as microspheres, microcapsules or colloidal particles but for the purpose of this review the term microparticles will be used throughout. This term has been chosen because the size of the particles is within the micron range and they are particulate in nature.

One of the major applications for microparticles is microencapsulation. Microencapsulation may be defined as the process of surrounding or enveloping one substance within another on a very small scale (Schrooyen, Van der Meer and De Kruif, 2001). The core substance may be considered to be surrounded by a shell or coating of wall material, or the core material may be embedded in a matrix of wall material (Burgess and Hickey, 1994). The objectives of microencapsulation include masking unpleasant tastes of functional ingredients, converting liquids into solids for ease of use and extended storage (Gouin, 2004), protecting the encapsulated agent from the environment (moisture, heat, light, oxidation) (Burgess and Hickey, 1994), decreasing the evaporation or transfer rate of the encapsulated agent to the outside environment (Shahidi and Han, 1993), and more recently for use as agents to allow delayed or controlled release of the encapsulated core material. For the purpose of this review the term microencapsulation will refer to the embedding of a core material within a microparticle. When microparticles are used to form a film or shell around a specific material it will be referred to as a coating.

2.1.2. Characteristics

Microparticles can be made from a number of synthetic and natural biodegradable polymers (reviewed by Sinha and Trehan, 2003; reviewed by Whittlesey and Shey, 2004). Synthetic polymers used for microparticle preparation include silicone, polyethylene vinyl coacetate, poly(lactic acid) (PLA) and poly(lactic-co-glycolic acid) (PLGA), whilst natural polymers include albumin, gelatin, casein, chitosan, collagen, whey protein, silk fibroin and zein. Microparticles are characterised by their small size and volume, large surface area, the ability to form stable dispersions and the ability to diffuse (Kawaguchi, 2000). They can be uniform or variable in size, with variable surface chemistry and morphology. According to Kawaguchi (2000) the total surface area of 1 g of microparticles with a diameter of 0.1 μm is about 60 m^2. This extremely large surface area results in a large number of sites for chemical reaction, adsorption and desorption. The surface area of microparticles can be increased still further by the use of an extractable porogen such as Pluronic F127, a tri block copolymer (Kim, Chung and Park, 2006). A porogen is incorporated during microparticle preparation and results in the formation of microparticles with an open-cellular porous structure. The porogen is removed by leaching out into the aqueous phase during solvent removal.

Extrinsic factors such as pH and temperature affect the apparent volume of microparticles and electrostatic repulsive forces, Van der Waals' attractive forces and steric repulsive forces determine their stability (Kawaguchi, 2000). The large number of sites for chemical reaction, adsorption and desorption enable their potential use in various biomedical applications and food uses.

2.1.3. Microparticle preparation

There are many methods of microparticle preparation but most are modifications of three basic techniques: spray drying, solvent extraction/evaporation and phase separation/coacervation (Whittlesay and Shea, 2004; Sinha and Trehan, 2003). Protein microparticles have been produced by all of the above methodologies. The method of choice is dependant on the stability and solubility of the polymer and any other agent (e.g. drug) that is to be incorporated into the microparticle (Whittlesay and Shea, 2004). According to Tice and Gilley (1985), the process chosen also affects the location of the agent which is to be encapsulated in the microparticle. Some preparation methods allow for the encapsulated agent to be located in a central core, whilst other methods form

microparticles with the encapsulated agent dispersed throughout the polymeric matrix. The method of preparation also affects the size and properties of the microparticles (Sinha and Trehan, 2003) and also the encapsulation efficiency (Whittlesay and Shea, 2004). The process of choice should be simple and reproducible, ensuring different batches of microparticles have the same properties and release characteristics (Sinha and Trehan, 2003).

2.1.3.1. Spray Drying

A biopolymer is dissolved in a solvent and then the agent to be incorporated into the microparticles is dispersed into the polymer solution by high-speed homogenisation (Sinha and Trehan, 2003). This dispersion is then atomised in a stream of hot air. The temperature of the hot air stream is not stated in microparticle preparation literature. However, milk and eggs are generally spray dried with an inlet temperature of 200°C and the temperature of the product usually does not exceed 80°C (Potter and Hotchkiss, 1995). The solvent evaporates instantaneously from the droplets forming microparticles. The size range is between 1 to 100 µm. A cyclone separator is used to collect the microparticles. Residual solvent is removed by vacuum drying. Spray drying is a simple technique with a high throughput but particle size control is difficult and it is not suitable for temperature sensitive polymers (Freitas, Merkle and Gander, 2005).

2.1.3.2. Solvent extraction/evaporation

This method is dependant on the relative solubility of a biopolymer in a mixture of solvents. The biopolymer is dissolved in an organic solvent, which is then emulsified into a second continuous phase with which it is immiscible (Sinha and Trehan, 2003; Freitas et al., 2005). This phase is usually water. As the organic solvent is removed by extraction or evaporation the biopolymer comes out of solution, resulting in the formation of microparticles, which can then be separated and dried. The advantages of this procedure are that no elevated temperatures or phase separating chemicals are required and aqueous solutions of proteins can be used (Sinha and Trehan, 2003; Freitas et al., 2005). With careful selection of encapsulation conditions, good encapsulation efficiencies and high microparticle yields of controlled particle size can be obtained. However, it is a complex process, which is sensitive to the polymer properties (Sinha and Trehan, 2003). The authors did not state in what way the method is sensitive to the polymer properties.

2.1.3.3. Phase separation/coacervation

Also known as the emulsion method, coacervation is based on the separation of a solution of biopolymer into two immiscible liquid phases, a dense coacervate phase rich in the biopolymer and a dilute equilibrium phase (Radwick and Burgess, 2002). The separation occurs when the molecules of biopolymer have a reduced ability to react with the solvent and an increased tendency to react with each other (simple coacervation) or due to ionic interaction between oppositely charged macromolecules (complex coacervation). When only one type of biopolymer molecule is involved the process is known as simple coacervation (Burgess and Hickey, 1994), whereas complex coacervation is when there are two different biopolymer molecules of opposite charge present.

Coacervation as a method of microparticle formation generally begins when the biopolymer is dissolved in an aqueous solvent and then emulsified with a non-miscible organic solvent or oil (Whittlesay and Shea, 2004; Sinha and Trehan, 2003). Alternatively, the biopolymer can be dissolved in an organic solvent and emulsified into water containing the agent to be encapsulated. In some cases the primary emulsion is then transferred to an excess of water containing a coacervation agent resulting in a w/o/w emulsion. Removal of the organic solvent or oil results in a phase separation producing microparticles. The disadvantages of this process are its complexity, sensitivity to polymer properties and the presence of residual solvents and coacervation agent within the microparticles (Sinha and Trehan, 2003). The authors did not state in what way the polymer properties were sensitive to the preparation method. Complex coacervation processes involve a large number of variables which can be manipulated in order to control specific properties of the microparticles (Burgess and Hickey, 1994). These include pH, ionic strength, biopolymer concentration and weight.

2.1.3.4. Methods of zein microparticle preparation

There are no reports in the literature of kafirin microparticles but because of the similarity between kafirin and zein, the methodology used to prepare zein microparticles could be applied to kafirin.

Zein microparticles have been prepared using both solvent extraction/evaporation (Cook and Shulman, 1998) and phase separation/coacervation techniques (Demchak and Dybas, 1997; Dong et al., 2004; Lui et al., 2005; Parris et al., 2005; Wang et al., 2005;

Muthuselvi and Dhathathreyan, 2006; Hurtado-López and Murdan, 2005). Cook and Shulman (2005) dissolved zein in aqueous ethanol and then admixed sufficient aqueous acid (lactic, acetic or citric acid) in order to precipitate the zein as microparticles. The ethanol was then removed by evaporation. When phase separation is used for zein microparticle preparation the zein is generally dissolved in aqueous ethanol (Figure 2.1.). Water or an acid is used as the second solvent, which may include a coacervation agent. This can be an oil or another organic solvent such as acetone or isopropanol (Coombes et al., 2003). Demchak and Dybas (1997) used acetic acid (concentration unstated) as an alternative to ethanol as a zein solvent and emulsified this solution into a solution containing lecithin. The emulsion was diluted and then the acetic acid removed by ion-exchange chromatography.

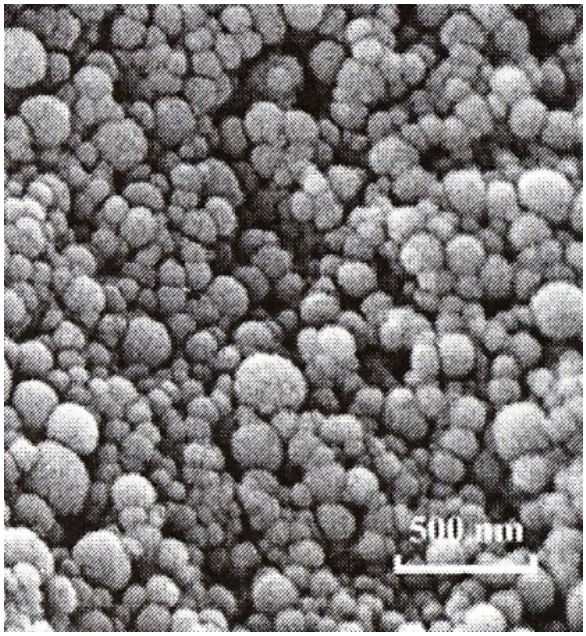

Figure 2.1: Scanning electron micrograph of zein microspheres (Parris et al., 2005)

All these methods except that of Demchak and Dybas (1997), involve dissolving zein in aqueous ethanol and then reducing the ethanol concentration until the zein is no longer soluble and precipitates out in the form of microspheres (Dong et al., 2004; Lui et al., 2005; Parris et al., 2005; Wang et al., 2005; Hurtado-López and Murdan, 2006a; Muthisel and Dhalathreyan, 2006). Hurtado-López and Murdan (2006a) attempted to explain the precipitation of zein microspheres by combining what is known of zein solubility and

structure. They suggested the zein dissolved by fitting its elongated α-helical structure into the three dimensional structure formed when 'polymers' of ethanol formed by intermolecular hydrogen bonds combined with the three dimensional clusters of water molecules. As the amount of water in the ethanol/water mixture increased, the number of hydrogen bonds between the water and ethanol increases changing the solvent three dimensional structure needed to dissolve the zein, resulting in the precipitation of the zein.

2.1.4. Suitability of different materials for microparticle preparation

2.1.4.1. PLGA microparticles

Poly lactic-co-glycolic acid (PLGA), is probably the most widely used biopolymer for microparticle preparation due to its commercial availability, controllable degradation rate (Whittlesay and Shea, 2004), proven animal and human biocompatibility (Athanasiou, Niederauer and Agrawal, 1996) and Food and Drug Administration approval (Whittlesay and Shea, 2004). However, a problem is that during biodegradation an acidic microenvironment is produced within the PLGA microparticles due to the formation of lactic and glycolic acids (Sinha and Trehan, 2003). This is detrimental to the sensitive agents (often proteins) which, are microencapsulated within the PLGA microparticles, resulting in denaturation, formation of aggregates or hydrolytic degradation of these agents. Consequently, alternatives to synthethic biopolymers have been sought and investigation into the use of natural polymers such as proteins and polysaccharides for microparticle preparation has been undertaken (Dong et al., 2004; Wang et al., 2005; Lui et al., 2005; Hurtado-López and Murdan, 2005, 2006a,b). The suitability of proteins for microparticle preparation and use is discussed below.

2.1.4.2. Protein microparticles

Many proteins have been investigated for use in microparticle preparation, including albumin, gelatin, casein, collagen, whey protein, silk fibroin and zein (Sinha and Trehan, 2003; Whittlesey and Shey, 2004). This is because natural polymers have certain advantages that synthetic polymers lack, including being natural products of living organisms, having GRAS (generally recognised as safe) status, being completely biodegradable, have low toxicity of end products, being readily available and replenishable, relatively inexpensive and capable of multiple chemical modifications (Sinha and Trehan, 2003; Chen, Remondetto and Subirade, 2006).

If we consider the suitability of different proteins for microparticle preparation an important issue is hydrophilicity versus hydrophobicity of the protein. Gelatin, casein, soluble monomer collagen and whey protein are hydrophilic proteins, are rapidly soluble in aqueous environments (Liu et al., 2005) and thus show fast release of the encapsulated material. In contrast, when hydrophobic proteins such as zein, are used for encapsulation their hydrophobic property delays the penetration of water into the interior thus the diffusion of the encapsulated material into the release medium is retarded (Liu et al., 2005).

Two other issues which may be pertinent to the choice of protein used for the development of protein microparticles for food use are potential allergenicity and the use of animal products which some religious groups are sensitive to. Albumin, casein and whey protein are known food allergens (Chapman, Pomes, Breiteneder and Ferreira, 2007). Their use for production of protein microparticles and subsequent use as microencapsulating agents may therefore result in allergic responses in some people. Gelatin and collagen are both animal products which unless processed in specific ways as defined by religious authorities may cause ethical problems for some people. Products produced using microparticles made from these proteins would need to be labeled as such to enable individuals to avoid them if necessary.

The two remaining proteins in the above list, zein, the prolamin protein of maize which is commercially available and silk fibrion, a scleroprotein, are not known to be allergenic (Borelli, Stern and Wüthrich, 1999; Mills, Madsen, Shewry and Wichers, 2003). Silk fibrion is insoluble in water (Alais and Linden, 1991) and is reported to have good water vapour permeability (Yeo, Lee, Lee and Kim, 2003). This protein may have potential for protein microparticle preparation but little work has been published so far in this area.

Zein is generally considered as a highly hydrophobic protein due to its high proportion of non-polar amino acids and lack of ionic groups (as reviewed by Fu, Weller and Wehling, 1999). However, it also has hydrophilic characteristics which will be described later in this review. Zein is very resistant to water and grease and has good film forming properties, producing transparent, strong films and fibres (as reviewed by Fu et al., 1999; Lawton, 2002; Padua and Wang, 2002). Films made from zein have the lowest water vapour permeability when compared to other protein films (Krochta, 2002). These films

also exhibit good oxygen barriers at low water contents (Beck, Tomka and Waysek, 1996). Zein also has antioxidant properties (Fu et al., 1999), an added advantage when used as a microencapsulating agent for oxygen sensitive materials such as essential oils. Other properties of zein which may be advantageous for microparticle preparation and use is its capacity to gel under certain conditions (Manley and Evans, 1943) and a property known as changrongxing. Fu et al. (1999) in their review on the properties of zein refer to a paper written in Japanese which describes this phenomenon as zein's resistance to digestion in the stomach. No other references to this property have been found but if true, could be exploited by using zein to encapsulate nutrients for ruminants. The nutrients would then bypass the rumen and be available for digestion and absorption in the intestine thus preventing digestion of nutrients by the rumen microflora. However, since there are many references which demonstrate that zein is readily digestible by pepsin (as reviewed by Duodu, Taylor, Belton and Hamaker, 2003) it is unlikely that zein is resistant to digestion in the stomach.

Kafirin, the prolamin protein of sorghum has potential for microparticle preparation and use. DeRose et al. (1989) demonstrated extensive homology between kafirin and zein. The two prolamins have similar molecular weights and are structurally related (Shull, Watterson and Kirleis, 1991). Although very similar to zein, kafirin has some properties which may enable microparticles to be produced with superior properties to zein microparticles. Kafirin is more hydrophobic than zein (Belton, Delgadillo, Halford and Shewry, 2006), which may result in microparticles with better barrier properties to gases and water vapour than zein microparticles. Kafirin is also less digestible than zein (Axtell, Kirleis, Hassen, D'Crox-Mason, Mertz and Munck, 1981; Hamaker, Kirleis, Mertz and Axtell, 1986; Hamaker, Kirleis, Butler, Axtell and Mertz, 1987; Duodu et al., 2002; Duodu et al., 2003), a factor which may make kafirin microparticles less susceptible to bacterial attack, enabling delivery of the encapsulated agent to the desired site.

A description of the composition and structure of kafirin is required in order to better understand its functional properties which make it a potential alternative to zein for microparticles.

2.2 KAFIRIN

2.2.1. Kafirin chemical composition

Kafirins are the most abundant proteins of sorghum grain making up some 80% of the total endosperm nitrogen (Hamaker, Mohamed, Hadden, Huang and Larkins, 1995). They are endosperm specific (Taylor and Schüssler, 1986), acting as a store for nitrogen, carbon and sulphur in the seed (Shewry, 2002). The kafirins are deposited within discrete protein bodies of the seed storage tissue where they remain inert until required during germination. A full description of the sorghum kernel and its constituent parts is given by Taylor and Belton (2002) and so these issues will not be dealt with here.

The kafirins are rich in glutamine, proline, alanine and leucine but contain little lysine (Taylor and Belton, 2002). Based on their solubility, electrophoretic mobility, amino acid composition and sequences, molecular masses and immunochemical cross-reactions, they have been classified into three groups, α-, β-, and γ-kafirin, equivalent to the α-, β- and γ-zeins of maize (Shull et al., 1991; Mazhar, Chandrashekar and Shetty, 1993). A fourth group, δ-kafirin, (AY834250) (GENPET accession number, Institute for Genome Research, Belton et al., 2006) equivalent to δ-zein has also been identified based on the sequences of cloned DNA (Izquierdo and Godwin, 2005). This protein has not been characterised at the protein level but is predicted as a 16 k polypeptide of 147 amino acids, rich in methionine and is only expressed in developing seeds. Belton et al. (2006) referred to two reports of δ-kafirin (AAK72689 and AAW3236) from the Institute for Genome Research *Sorghum (Sorghum bicolor)* Gene Index which have fewer amino acids and consequently slightly lower molecular masses of 13 and 14 k respectively.

According to Watterson, Shull and Kirleis (1993) α-kafirins constitute some 80-84% of the kafirin fraction in vitreous endosperms and 66-71% in opaque endosperms. The β-kafirins constitute the smallest amounts of the endosperm proteins (7-8% vitreous endosperm and 10-13% opaque endosperm) and the γ-kafirins an intermediate amount of 9-12% vitreous endosperm and 19-22% of the opaque endosperm. The α-kafirins have between 240-250 amino acid residues (Belton et al., 2006) and are resolved into two bands of molecular mass 25 k (α_1-kafirin, Z22) and 23 k (α_2-kafirin, Z19) when analysed by SDS-PAGE under reducing conditions (Shull et al., 1991). More sophisticated separation techniques have shown that α-kafirin can be resolved into more fractions

which vary in number depending on the technique used (reviewed by Belton et al., 2006). According to Chamba, Halford, Forsyth, Wilkinson and Shewry (2005) there is only a single β-kafirin gene which encodes for a protein of 172 amino acids with a molecular mass of 18745 Da. This protein shows as a single band under reducing conditions of SDS-PAGE whereas the γ-kafirins resolve into two bands of molecular mass 28 k and a minor band of 49 k (Evans, Schüssler and Taylor 1987).

El Nour et al. (1998) using SDS-PAGE under non-reducing conditions showed the α-kafirins as being present in defatted whole grain flour as either monomers or as polymers. They showed that α_1-kafirin formed different sized oligomers, linking by disulphide bonds with γ-kafirin. In contrast, α_2-kafirin formed dimers or small oligomers. These workers hypothesised that the different polymerisation behaviour of the two α-kafirins was due to the presence of one cysteine residue in the case of α_2-kafirin or two cysteine residues in α_1-kafirin resulting in differences in terms of functionality. Thus α_1-kafirin could be considered as a 'chain extender' and α_2-kafirin a 'chain terminator.'

Beta-kafirin is rich in methionine and contains 10 cysteines which enables it to form either inter- or intra-molecular disulphide bonds resulting in oligomers and polymers (Belton et al., 2006). El Nour et al. (1998) postulated that β-kafirin can act as a bridge between oligomers of α_1- and γ-kafirin resulting in very large polymers which can only be extracted in the presence of reducing agents. The γ-kafirins contain by far the most cysteine residues (15) and are present in their native state as polymers stabilised by disulphide bonds (Belton et al., 2006) and as previously discussed cross-linked by disulphide bonds with α_1-kafirin (El Nour et al., 1998). However, curiously in their reduced state they are soluble in water (Evans et al., 1987).

The presence of large numbers of cysteine residues in the various kafirins and their ability to form disulphide cross-linkages would be expected to have an influence on microparticle formation and the properties of those microparticles. Another factor which would be expected to influence microparticle formation and properties would be the secondary structure of kafirin which will now be discussed.

2.2.2. Kafirin secondary structure

There is little information on kafirin conformation and apparently none at all on the specific kafirin subunits. Wu, Cluskey and Jones (1971) using kafirin dissolved in 60% tert butanol showed the presence of 40-47% α-helical conformation, some unordered structures and no β-sheet. Gao, Taylor, Wellner, Byaruhanga, Parker, Mills and Belton (2005) using freeze dried kafirin extracted from whole grain flour with 60% tert butanol containing a reducing agent, dithiothreitol (DTT), reported α-helical and β-sheet present in a ratio of 1.39:1 and Belton et al. (2006) quoting unpublished work from his own laboratory showed the presence of approximately 60% α-helical conformation when the same material was examined by circular dichroism (CD) in solution. This is in agreement with Fourier transform infrared reflectance (FTIR) values of 50-60% α-helical conformation found by Duodu, Tang, Wellner, Belton and Taylor (2001) for sorghum protein bodies. One would expect that the ability to form disulphide cross-linkages would have an effect on protein secondary structure. However, according to Taylor and Belton (2002) this is not so in the case of kafirin and zein. When alkylated and unalkylated samples were compared by FTIR it was found that there was only a slight increase in β-sheet structure with no other changes. This confirms the work of Wu, Paulis, Sexson and Wall (1983) who found that zein had an α-helical content of 45% in 70% ethanol regardless of whether disulphide cross-linkages were present or broken. This implies that the presence of disulphide linkages is not important for the maintenance of the conformation of kafirin and zein secondary structure.

There are no secondary structural models for any of the kafirin subunits. Since there is a large degree of homology between the kafirin and zein (DeRose et al., 1989) we can assume that models used to describe the secondary structure of zein will apply to kafirin. The classic structure for zein was proposed by Argos, Pedersen, Marks and Larkins (1982) (Figure 2.2.A). This is based on the two α-zeins and depends on the homologous repeat sequence of 20 or so amino acids present in their primary sequences. The suggested structure is a group of nine anti-parallel α-helices arranged within a distorted cylinder. The hydrophobic amino acids are hidden within the helices, whereas the polar amino acids are on the surface of the helices and are available to form intra-and inter-molecular hydrogen bonds, allowing the zein molecules to be arranged in planes.

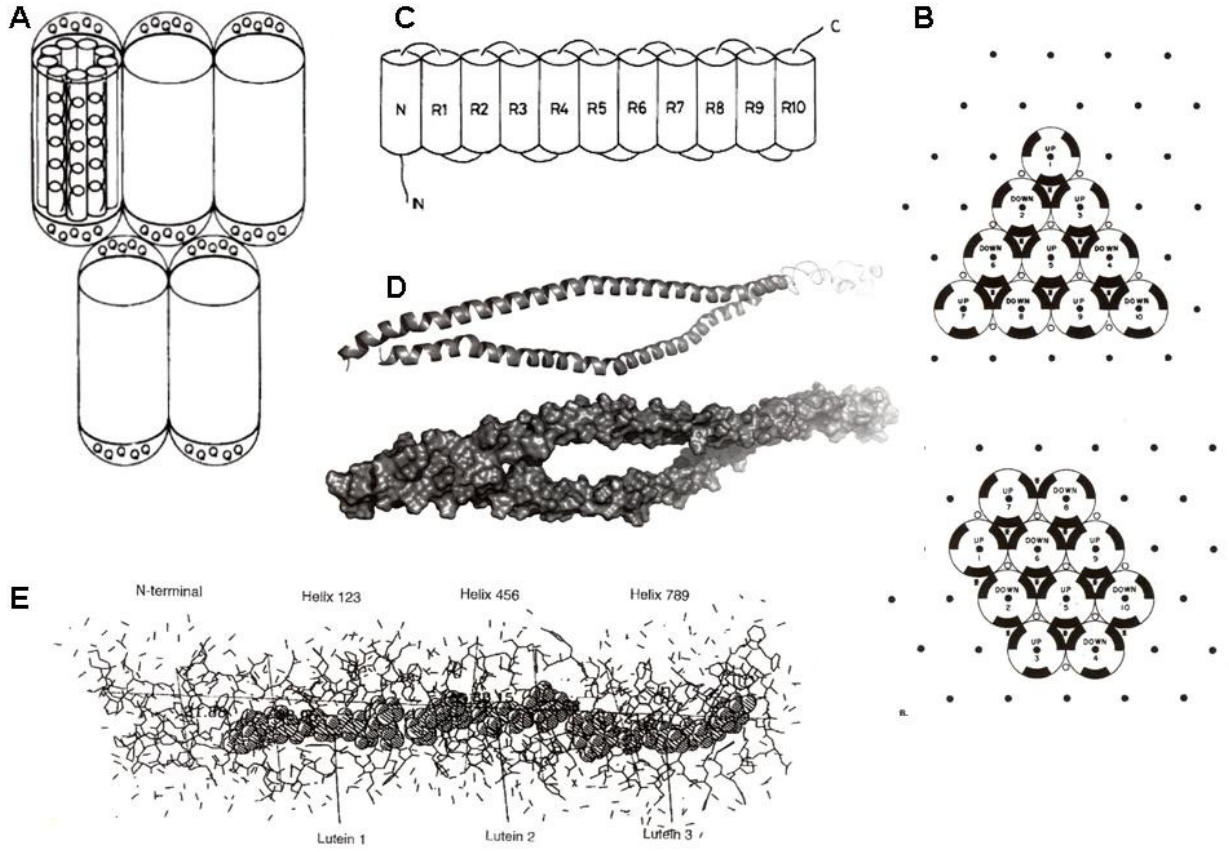

Figure 2.2: Structural models proposed for α-zein. A-Argos et al., (1982) B-Garratt, et al., (1993) C-Matsushima et al., (1997) D- Bugs et al., (2004) E- Momany et. al. (2006) (adapted from Belton et al., 2006)

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. This model fitted the physical measurements available at the time of publication but as measurement techniques have become more sophisticated the Argos model has been modified to better fit the latest physical data.

Garratt, Oliva, Caracelli, Leite and Arruda (1993) extended a modified Argos model to include all α-prolamins including α-kafirin (Figure 2.2.B). This asymmetric structure was based on pairs of the repeat amino acid sequences forming anti-parallel helices arranged in the form of hexagonal wheels with alternating groups of hydrophobic and polar amino acids.

Tatham, Field, Morris, I'Anson, Cardle, Dufton and Shewry (1993) and later Matsushima, Danno, Takezawa and Izumi (1997) provided physical data that suggested that α-zeins were present in solution as extended structures. Tatham et al. (1993) described these structures as 'asymmetric particles approximating to prolate ellipsoids or rods'. Matsushima et al. (1997) revised the Argos et al. (1982) model stacking the nine anti-parallel helices in a linear manner (Figure 2.2.C).

Recent publications by Bugs, Forato, Bortoleto-Bugs, Fischer, Mascarenhas, Ward and Colnago (2004) and Forato, Doriguetto, Fischer, Mascarenhas, Craievich and Colnago (2004) both suggest models based on helical hairpin structures. The Forato et al. (2004) model has short sections of helices arranged in an extended way connected by loops, turns or sheets. This allows the helices to fold back on themselves or to extend depending on the prevailing environment. The Bugs et al. (2004) model consists of two anti-parallel α-helices formed from coiled coils to form a superhelical conformation with polar charged and hydrophobic amino acids distributed along the helical surfaces (Figure 2.2.D). Momany, Sessa, Lawton, Selling, Hamaker and Willett (2006) published the most recent model for the Z19 α-zein which comprises of three interacting coiled coil helices with segments positioned end to end (Figure 2.2.E). The non polar amino acid side chains form a hydrophobic face inside the triple super helix. This model also accommodates lutein, a natural carotenoid pigment of zein which is difficult to extract from α-zein, in the core of the triple helical segments and helps to stablise the configuration.

There are no secondary structure models for either β- or δ-zein and neither of these zeins contain repeated sequence motifs (Tatham et al., 1993). Forato, Bicudo and Colnago (2003) examined a sample of protein bodies rich in β-zein and discovered it to be rich in β-sheets (46%) and low in α-helices. The γ-zeins, however, do contain a repetitive sequence based on a hexapeptide repeat (PPPVHL) close to the N-terminus (Tatham et al., 1993) as does γ-kafirin (Belton et al., 2006). The γ-zeins have been analysed by CD and optical rotary dispersion (OPD) in aqueous solution containing a reducing agent and found to comprise 26% α-helical, 24% β-sheet and 49% unordered structures (Wu et al., 1983). Bicudo, Forato, Batista and Colnago (2005) determined the secondary structure of γ-zein by solid state FTIR and found that in its physiological state it comprises of 33% α-helical and 31% β-sheet conformation. Examination of synthetic peptides based on the hexapeptide repeat (VHLPPP) has shown its structure to be that of an amphipathic polyproline II type conformation in aqueous solution (Pons, Feliz, Celma and Giralt, 1987; Dalcol, Pons, Ludevid and Giralt, 1996). Further work by Kogan, Dalcol, Gorostiza, López-Inlesias, Pons, Sanz, Ludevid and Giralt (2001) and Kogan, Dalcol, Gorostiza, López-Inlesias, Pons, Pons, Sanz and Giralt (2002) using atomic force microscopy and transmission electron microscopy (TEM) has shown that these peptides are able to self assemble into cylindrical micelles. Based on these results the authors suggest a role of the N-terminal domain of γ-zein in protein body formation.

It can be seen that there is a considerable body of evidence that supports the theory that in solution at least α-zein and by inference α-kafirin is present in an extended helical form. However Guo, Liu, An, Li and Hu (2005) showed that when zein was deposited from a solution of ethanol it forms globules. The globules were variable in size, diameter between 150-550 nm and height between 50-150 nm and were thought to be aggregates of many molecules. This interesting observation may have some relevance to the mechanism of microparticle formation.

Two functional properties of kafirin which are influenced by its amino acid composition and secondary structure are hydrophobicity and protein digestibility, both of which would in turn be expected to influence the properties of kafirin microparticles.

2.2.3. Kafirin hydrophobicity

As stated kafirin is a highly hydrophobic protein being rich in hydrophobic amino acids (Belton et al., 2006). Calculation of the free energy of hydration of a protein allows us to quantify the degree of hydrophobicity of a protein. This can be calculated from the amino acid sequence of the protein and the free energy of hydration of each amino acid (Duodu et al., 2003). A hydrophilic protein will have a high negative free energy of hydration, whereas conversely a hydrophobic protein will have low negative free energy of hydration. Table 2.1. shows the free energy of hydration of the kafirin subunits compared with that of an 'average protein' with -164 kcal/mol (Shewry, Halford, Tatham, Popineau, Lafiandra and Belton, 2003). We can see that all the kafirin proteins are more hydrophobic than the 'average protein' and that γ-and δ-kafirins are the most hydrophobic of all. Just how many hydrophobic amino acids are contained in γ-kafirin is illustrated by Figure 2.3. which shows the amino acid sequence of γ-kafirin compared with that of γ-zein. The hydrophobic amino acids are highlighted.

Table 2.1: Free energy of hydration of kafirin subunits compared with that of an 'average protein'. Adapted from Belton et al. (2006)

Kafirin type	**Energy of hydration (kcal/mol of 100 residues)**
Alpha	-140
Beta	-123
Gamma	-100
Delta	-99
Average protein	-164

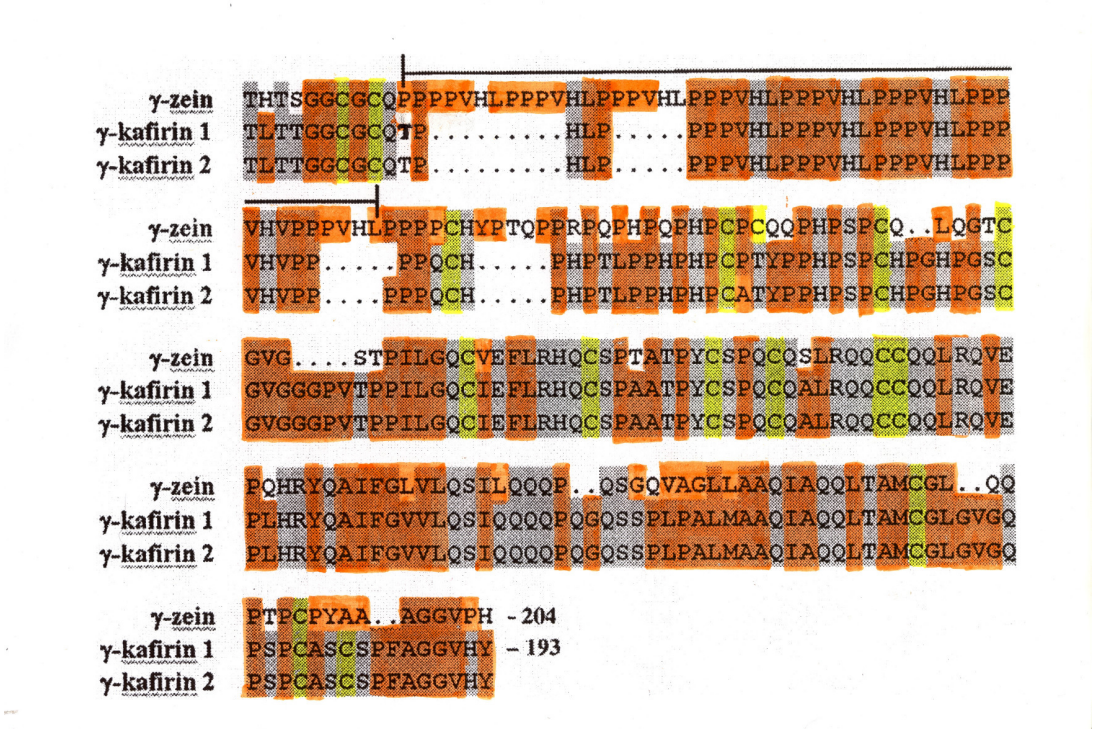

Figure 2.3: Alignment of amino acid sequences of γ-kafirins with those of γ-zein to demonstrate the number of hydrophobic amino acids present. Hydrophobic amino acids are highlighted (adapted from Belton et al., 2006)

Measuring the changes in water absorption is another way of comparing the relative hydrophobicity of proteins. Hydrophilic proteins like gluten in wheat absorb water when heated, whereas hydrophobic proteins expel water under the same conditions (Grant, Belton, Colquhoun, Parker, Plijter, Shewry, Tatham and Wellner, 1999). Taylor and Belton (2002) described experiments where kafirin, zein and wheat proteins were put in contact with excess deuterated water (D_2O) and the amount of D_2O absorbed by the proteins was measured by NMR. On heating, kafirin and zein took in small amounts of water but much less than wheat prolamins. As expected, this shows that they are more hydrophobic than wheat prolamins. However, the fact that kafirin and zein took in water instead of expelling it is indicative of some hydrophilic characteristics, which is not unreasonable since both kafirin and zein contain large amounts of glutamine, a hydrophilic amino acid. It can be concluded that although kafirin and zein have some hydrophilic characteristics they are probably the most hydrophobic of the cereal prolamin proteins.

2.2.4. Kafirin protein digestibility

Kafirin is less digestible than zein and the digestibility decreases for both cereals on wet cooking (reviewed by Duodu et al., 2003). Under these conditions the protein digestibility of kafirin decreases more than that of zein. Causal factors include grain organisational structure, polyphenols, phytic acid, starch and non-starch polysaccharides, disulphide cross-linking, kafirin hydrophobicity and changes in protein secondary structure. These workers suggested that causal factors may vary depending on the state in which the grain is examined for instance whole grain, decorticated grain, high tannin or tannin free.

As would be expected since sorghum is a staple food in large parts of Africa and Asia most of the research into the effect of processing, specifically heating, on the protein digestibility of sorghum has been carried out on whole grain or decorticated sorghum for example Hamaker et al. (1986); Rom, Shull, Chandrashekar and Kirleis (1992); Oria, Hamaker and Shull (1995a,b) and Duodu et al. (2002). Little work has been carried out on the protein digestibility of the isolated proteins. Duodu et al. (2002) showed that sorghum protein body preparations were more digestible than either whole grain sorghum or isolated endosperm. These workers suggested as the preparations were purified, elements responsible for lowering protein digestibility were removed. Factors removed included polyphenols and phytate in the bran and starch and non-starch polysaccharides in the endosperm.

Certainly tannins have long been implicated in the reduction of sorghum protein digestibility (reviewed by Serna-Saldivar and Rooney, 1995). Tannins are thought to bind to sorghum proteins by hydrogen bonding and non-polar hydrophobic interactions forming indigestible sorghum-tannin complexes (Emmambux and Taylor, 2003). Belton et al. (2006) has expressed a different view stating that the removal of tannins could lead to an increase in hydrophobic protein-protein interactions and thus cause a decrease in protein digestibility. As justification they referred to work of Nunes (2004) who extracted polyphenols from sorghum flours with acidified methanol and found it had a negative effect on protein solubility and protein digestibility. In the same PhD dissertation published in Portuguese and referred to by Belton et al. (2006), Nunes (2004) also removed lipids by hexane extraction from sorghum flour and found an increase in protein

digestibility. This suggested that lipids form protein-lipid complexes that also decrease protein digestibility.

Duodu et al. (2003) proposed that protein cross-linking may be the strongest factor influencing sorghum protein digestibility. Oria et al. (1995a, b) compared the differences in pepsin digestibility between α-, β- and γ-kafirins in cooked and uncooked sorghum flour and the effect of treatment with a reducing agent. It was found that α-kafirin was more resistant to digestion than either β- or γ-kafirin in uncooked flour. TEM showed that protein body digestion began on the surface where the β-, and γ-kafirins were located and indicated that these kafirins protected α-kafirin from enzymic attack. Thus whilst apparently α-kafirin appeared less digestible than either β- or γ-kafirin it was actually more easily digested but less accessible to the pepsin enzymes. The fact that isolated α-kafirin is more easily digested than either β- and γ-kafirins both before and after cooking is mentioned by Aboubacar, Axtell, Huang and Hamaker (2001) referring to unpublished work of Hamaker. Addition of a reducing agent during pepsin digestion decreased the residue remaining after digestion of all kafirins and protein bodies were greatly reduced in size. The digestibility of all the kafirins was reduced on wet cooking. The digestibility of β- or γ-kafirin was reduced on wet cooking more than that of α-kafirin and this was reversed but not completely so by the addition of a reducing agent. Oria et al. (1995a,b) concluded that β- or γ-kafirin formed disulphide cross-linkages on cooking which delayed the digestion of α-kafirin. The addition of a reducing agent prevented the formation of these cross-linkages and so exposed α-kafirin to enzymic attack. Thus it appears that the more protease resistant β- and γ-kafirins on the periphery of the protein body with their ability to form disulphide cross-linkages form a protective shell around the more easily digestible α-kafirin which is located in the centre of the protein body.

Interestingly dry cooking of sorghum as in popping (Duodu et al., 2001), or by extrusion (Dahlin and Lorenz, 1993) does not cause the same reduction in protein digestibility as wet cooking. Belton et al. (2006) suggested that water may be necessary for the interaction of the proteins with each other and with other components.

The effect of cooking on the secondary structure of the individual kafirin proteins has not been reported. Duodu et al. (2001) looked at the secondary structure by FTIR and ^{13}C

spectroscopy of protein body enriched preparations. On cooking it was found that there was some β-sheet formation and a corresponding decrease in amount of α-helices. It was thought that the effect of heat would cause some loss of α-helical formation and allow the proteins to associate by intermolecular β-sheets. The same protein body enriched samples and some isolated prolamin samples were examined by SDS-PAGE (Duodu et al., 2002) under both reducing and non-reducing conditions. It was found that under non-reducing conditions in addition to monomers of α-, β- and γ-kafirin there were bands of greater than 66 k, and 45-50 k. In cooked material there also appeared to be reduction-resistant oligomers present. Pepsin indigestible residues from protein body enriched preparations consisted mainly of α-kafirin when uncooked and α-, β- and γ-kafirin and reduction-resistant proteins of 45-50 k when cooked. Duodu et al. (2002) thus concluded that wet cooking lead to the formation of disulphide bonded oligomers that were resistant to pepsin digestion. These results were confirmed by Nunes, Correia, Barros and Delgadillo (2004) who identified two non-reducible and non-digestible 45 and 47 k proteins which appeared to be related to the loss of sorghum digestibility on wet cooking. Further work showed the presence of a 66 k oligomer in cooked sorghum that was not present in maize (Nunes Correia, Barros and Delgadillo, 2005). On reduction, this oligomer resolved into γ-kafirin or γ- and α-kafirin depending on cultivar with some residual oligomer still present.

Recent work on zein by Lee and Hamaker (2006) has identified a 27 k γ-zein as critical to the digestibility characteristics of all zeins due to its high cysteine content. This protein is analogous to γ-kafirin in sorghum. A series of mutations of each of the conserved cysteine residues on this γ-zein were produced in order to investigate their relative contribution to the protein's overall digestibility. Of these the mutants of cysteine 155 was found to have an increased protease digestibility. Thus it was concluded that by eliminating just one disulphide bond a large improvement in protein digestibility could be made.

Having discussed the properties of proteins which make them suitable for microparticle use, the following section will review what is known about the properties of protein microparticles from different types of proteins.

2.3. PROPERTIES OF PROTEIN MICROPARTICLES

The properties of protein microparticles are dependant on the type of protein and their method of preparation. The review will now consider the properties of the major types of proteins used to make microparticles starting with albumin. Albumin microparticles can be made from egg white (ovalbumin), bovine serum or human serum (reviewed by Patil, 2003). They vary greatly in size from 15 nm to 150 μm and their surface and internal characteristics may vary from smooth to slightly roughened and porous to less porous. They have been produced with both hydrophobic and hydrophilic surfaces (MacAdam, Shafi, James, Marriott and Martin, 1997). The surface properties of albumin microparticles plays a role in determining the ability of the protein to bind to and release the encapsulated material and influences the degradation profile of the microparticle (Patil, 2003). Albumin microparticles swell and aggregate when placed in distilled water, a property that is reduced when the particles are stabilized by either heat or chemical treatment.

Gelatin microparticles are smooth spheres of diameter between 0.5-85 μm (Vandelli, Rivasi, Guerra, Forni and Arletti, 2001; Vandelli, Romagnoli, Monti, Gozzi, Guerra, Rivasi and Forni, 2004; Morita, Horikiri, Suzuki and Yoshino, 2001). Like albumin microparticles, gelatin microparticles swell in water. Gelatin microparticles swell less in water when the microparticles are cross-linked compared to uncross-linked gelatin microparticles (Vandelli et al., 2001). This is due to the reduction in mobility of the gelatin macromolecular chains caused by cross-linking. In vitro digestibility of gelatin microparticles occurs within 5 minutes with trypsin even when the microparticles have undergone microwave cross-linking (Vandelli et al., 2004). Mahanty and Bohidar (2005) studied the secondary structure of gelatin microparticles by UV absorbance and CD and found that conformational changes occurred in the gelatin molecules as the microparticles formed from random coil to triple helix state.

Generally whey protein microparticles are larger in size than microparticles made from other proteins and vary in size depending on their method of preparation. They range in size from 3-75 μm when prepared by spray drying (Picot and Lacroix, 2004), 10-100 μm when prepared by double emulsification and heat gelation (Lee and Rosenberg, 2000a), 400-1000 μm by double emulsification and glutaldehyde cross-linking (Lee and

Rosenberg, 1999), 850-1600 μm by emulsification and heat gelation followed by a calcium alginate outer coating (Rosenberg and Lee, 2004) and 1.8-2.1 mm by emulsification and cold gelation using calcium chloride (Beaulieu, Savoie, Paquin and Subirade, 2002). All are spherical, some having smooth surfaces (Rosenberg and Lee, 2004) and others being porous with (Lee and Rosenberg, 1999) or without cracking (Lee and Rosenberg, 2000a,b). Porous microparticles offered significant resistance to diffusion at high moisture levels, restricting core extraction from the microparticle interior (Lee and Rosenberg, 2000a, b).

Beaulieu et al. (2002) examined the ability of whey microparticles prepared by emulsification and cold gelation using calcium chloride to take up water. They found a minimum value at pH 4.5, near the pI 5.2 of the protein. The water uptake increased at pH 7.5 and pH 1.9, the intestinal pH and gastric pH, respectively. These results suggest that the charge on the protein molecules influences the amount of water uptake. Whey protein microparticles made by emulsification and heat gelation followed by coating with calcium alginate were found to be largely water insoluble over a period of 10 days (Rosenberg and Lee, 2004). In addition, these microparticles prevented oxidative deterioration of the paprika oleoresin that they encapsulated over a period of 30 days. Non-encapsulated paprika oleoresin showed the first signs of oxidative deterioration after 10 days of storage under the same conditions. Unfortunately these microparticles were large (0.5-1 mm) which would limit their use for some applications. Beaulieu et al. (2002) found that whey microparticles prepared by emulsification and cold gelation using calcium chloride were resistant to pepsin hydrolytic action but were totally digested by a simulated pancreatic medium. Similar results were also found by Picot and Lacroix (2004) using spray dried whey microparticles.

Microparticles based on pea proteins have been made by a water in oil emulsion method using microwave heating for stablisation (De Graaf, Harmsen, Vereijken and Mőnikes, 2001). These microparticles were spherical and highly porous when stabilized at low temperature (90°C) and less porous when a higher temperature (120°C) was used. No size distribution was given for these microparticles. Finally microparticles made from silk fibroin either by spray drying or by using lipid vesicles as templates for their formation were smooth spheres ranging in size from 2-10 μm (Hino, Tanimoto and Shimbayashi, 2003; Yeo et al., 2003; Wang, Wenk, Matsumoto, Meinel, Li and Kaplan,

2007). Release profiles of horseradish peroxidase, a model core material encapsulated in silk fibroin microparticles could be manipulated by differing processing conditions (Wang et al., 2007). This enzyme maintained its activity and was released over a period of 10-15 days when encapsulated with sodium chloride processed silk microparticles. The same enzyme encapsulated with methanol processed silk microparticles had an even longer release profile.

2.3.1. Properties of zein microparticles

There is very little information in the literature concerning the properties of zein microparticles. Since most of the applications in the literature concern encapsulation and drug release, available information concentrates on encapsulation efficiency and rate of release of the active components which have been encapsulated (Dong et al., 2004; Lui et al., 2005; Parris et al., 2005; Wang et al., 2005; Muthuselvi and Dhathathreyan, 2006; Hurtado-López and Murdan, 2005, 2006a, b).

Reported sizes of zein microparticles are between 0.1 μm (Parris et al., 2005) and 0.5 to 2.5 μm (Dong et al., 2004). Scanning electron microscopy (SEM) showed the surface of the microparticles to be spherical with a smooth surface (Dong et al., 2004; Lui et al., 2005; Parris et al., 2005; Wang et al., 2005). According to Parris et al. (2005) zein microparticles appear to be composed of aggregated spheres.

Dong et al. (2004) showed that zein microparticles could agglomerate together to form a film which they observed by SEM. This film casting suspension of microparticles contained a very low concentration of zein (0.3% w/v) and was used as a matrix to grow cells for use in tissue engineering. Another type of film made from zein microparticles was that used as a coating for a cardiovascular device (Wang et al., 2005). The film thickness was given as 26.3 μm. However, the illustrations of the microparticle film showed very little fusion of the microparticles and so would not be considered as a continuous film. The patent of Cook and Shulman (1998) claims that zein colloidal dispersions can be dried into glossy coatings and films, which had excellent resistance to moisture, lipid and gas permeation and excellent mechanical properties and were superior to their ethanolic counterparts. No details of these properties were given.

Hurtado-López and Murdan (2005, 2006b) exposed zein microparticles to simple buffers in order to determine their degradation profiles. After seven days there was an increase in particle size, possibly caused by Ostwald ripening, but no changes in pH or turbidity. Capillary zone electrophoresis (CZE) showed that at pH 2, the amount of α-zein was reduced, possibly due to acid hydrolysis. At pH 5 and 7.4 there was an increase in the amount of dimers present. The authors attributed this to disulphide cross-linking of the sulphur containing amino acids (cysteine) of the α-zein. This seems unlikely as the amount of cysteine in α-zein is very low (0.4 Cys mole %) (Taylor and Belton, 2002). However, the authors also suggested that polymerisation by non disulphide cross-linkages such as by tyrosine coupling could have occurred.

Parris et al. (2005) determined the stability of zein microparticles under physiological conditions by digestion with pepsin at pH 3.5. They found that the zein microparticles formed aggregates initially on contact with the buffer but gradually dispersed and completely dissolved within 52 hours. Hurtado-López and Murdan (2005, 2006a) showed that zein microparticles made by coacervation were extremely resistant to degradation in the absence of enzymes as described above but were degraded rapidly by simulated gastric and intestinal fluids containing pepsin and pancreatin, respectively. Using pepsin, these authors found that α-zein was completely digested within an hour and that the microparticles disintegrated in spite of small amounts of residual α-zein dimer and tetramer still being present. These pepsin resistant dimers and trimers were still present after one week of incubation with pepsin. Pancreatin digested both α-zein and the α-zein dimers causing the disintegration of the zein microparticles. However, CZE identified four new proteins of molecular weight between 26-30 k and of 50 k which were associated with the formation of elongated tubules in the incubation media.

2.4. ALTERATION OF PROTEIN MICROPARTICLE PROPERTIES

Properties, specifically the size and size distribution of microparticles, as well as the encapsulation efficiency and material release are affected by and can be manipulated by the method and conditions of preparation (Sinha and Trehan, 2003; Patil, 2003; Freitas et al., 2005). Microparticle size is important as it determines the encapsulation rate, the rate of release of capsulated material, and the application to which the microparticles can be used (Feritas et al., 2005). This is especially important in biomedical applications as it

determines whether for example microparticles can be administered by syringe or if they can be taken up by phagocytic cells.

In the case of proteins, higher molecular weight and increased concentration increases the viscosity of the protein solution (Tice and Gilley, 1985; Sinha and Trehan, 2003; Patil, 2003). This necessitates higher shear forces for droplet disruption (Freitas et al., 2005) which in turn results in an increase in microparticle size and size distribution. The speed and duration of agitation during droplet formation also influences microparticle size. Increased speed and longer mixing times causes the formation of smaller emulsion droplets (Patil, 2003; Whittlesey and Shea, 2004; Freitas et al., 2005). Consequently smaller microparticles are formed. This is as a result of stronger shear forces and increased turbulence. Smaller microparticles are formed on addition of stabilisers or surfactants during the emulsification process which prevents droplets coalescing (Patil, 2003; Whittlesey and Shea, 2004; Freitas et al., 2005). However in some cases surfactants cause nucleation and aggregation effects (Patil, 2003). Also of importance is the rate of which the solvent is evaporated (Tice and Gilley, 1985). If the solvent is removed too fast then the walls of the microparticles may be disrupted or damaged during formation resulting in cracks or pinholes. Foaming during microparticle formation may also be detrimental to microparticle quality. The pH of the preparation medium can also affect the size and morphology of microparticles by effecting protein/protein interactions and electrostatic forces (Kawaguchi, 2000). This was shown by Lee and Rosenberg (2000ab) for whey protein microparticles. Whey particles prepared at neutral pH have smooth surfaces (Lee and Rosenberg, 2000ab), whereas those prepared at acid pH (4.2 or 5.5) have wrinkled surfaces, are very porous and are larger in size than those prepared at neutral pH (Lee and Rosenberg, 2000ab). These authors attributed increased size and porous nature of microparticles prepared at low pH to increased protein/protein interactions and aggregation caused by a reduction in electrostatic repulsive forces that occur at low pH.

Release of encapsulated materials is usually slower and over longer time periods from larger microparticles (Chen et al., 2006). Smaller microparticles have the advantage of increased adhesive force and prolonged gastric intestinal transit time which leads to better bioavailability of the encapsulated material. The release rate of a microparticle encapsulated material is dependant on the ratio of the polymer to the material to be

encapsulated (Whittlesey and Shea, 2004). The structure of the polymer used to make the microparticles affects the hydration rate and consequently the degradation rate of the microparticles (Sinha and Trehan, 2003).

Protein microparticles (albumin, gelatin and casein) used for biomedical applications are easily absorbed and their degradation products are of low toxicity (Lui et al., 2005). However, their major disadvantage is that they suffer from fast, unsustained drug release due to their generally hydrophilic nature. Both physical and chemical means have been used to cross-link the microparticles and make them more resistant to degradation (Patil, 2003). Physical cross-linking is usually by the application of heat. Chemical methods involve cross-linking with substances such as glutaraldehyde. Generally the more intense the treatment, the greater the degree of cross-linking is achieved and results in a slower disintegration of the microparticles when exposed to enzymic attack.

Albumin microparticles have been cross-linked using heat, rendering the microparticles insoluble by formation of inter-chain amide links (reviewed by Patil, 2003). The disadvantage of using heat to cross-link microparticles is that heat sensitive compounds which are to be encapsulated by the microparticles may be damaged and lose efficacy by the use of high temperatures (Patil, 2003; Chen et al., 2006). Microwave energy has been used to cross-link gelatin microparticles producing insoluble but swellable microspheres (Vandelli et al., 2004). This has enabled drugs to be loaded into the microparticles by soaking which prevents high temperature drug degradation.

As an alternative to the use of heat as a cross-linking method, chemical cross-linking agents have been developed. Glutaraldehyde, formaldehyde and D-glyceraldehyde have been used in an attempt to make protein microparticles more hydrophobic (Burgess and Hickey, 1994; Latha, Rathinam, Mohanan and Jayakrishnan, 1995; Latha, Lal, Kumary, Sreekumar and Jayakrishnan, 2000). Cross-linking occurs by a condensation reaction between the amino groups of the protein and the aldehyde (Burgess and Hickey, 1994). This has resulted in toxic side effects due to residual cross-linking chemicals and unwanted reactions between the drug and the cross-linking agent (Burgess and Hickey, 1994; Chen et al., 2006). Additionally, the chemicals used are not suitable for food use. Strauss and Gibson (2004) suggested the use of plant derived polyphenols as alternative to chemical cross-linking agents which would be suitable for food use. They used grape

juice and coffee containing unidentified phenolic acids and flavonoids directly to cross-link gelatin-pectin coacervates resulting in microparticles with greater mechanical strength and thermal stability than untreated material.

Lee and Rosenberg (2000a) and Rosenberg and Lee (2004) have published two alternative methods to using chemical cross-linking to improve whey protein microparticle properties. The first involves the use of heat to form an irreversible gel which is insoluble in water and allows high core retention (Lee and Rosenberg, 2000a). Unfortunately this method is unsuitable for heat sensitive core materials. The second is a method using a calcium alginate coating for producing water-insoluble whey protein based microspheres (Rosenberg and Lee, 2004). A further method for stabilizing whey protein microparticles is by emulsification of pre heat-denatured whey protein followed by cold gelation induced by calcium ions as previously described (Beaulieu et al., 2002). This resulted in gastroresistant microparticles which were effective in protecting fat soluble vitamins, such as retinol from oxidation.

A further alternative to either physical or chemical cross-linking methods is the use of enzymes. Gouin (2004) reviewed a patented method where transglutaminase was used to cross-link a protein used to encapsulated a flavour oil. The process was long (16 hours) but had the advantage of using low temperatures ensuring the heat sensitive, volatile oil was not degraded during the process.

2.5. POTENTIAL USES OF MICROPARTICLES

2.5.1. Non food uses

The first commercial use for microparticles was the encapsulation of dyes used for the production of carbonless paper (reviewed by Shahidi and Han, 1993). Currently the predominant uses for microparticles are in the medical and biomedical fields including the microencapsulation of absorbents, latex diagnostics, affinity bioseparators and drug and enzyme carriers (reviewed by Kawaguchi, 2000).

Research and patent literature suggest some potential applications for zein microparticles. This includes encapsulation of pesticides (Demchak and Dybas, 1997), drug delivery (Suzuki, Sato, Matsuda, Tada, Unno and Kato, 1989; Matsuda, Suzuki, Sato, Sato, Koizumi, Unno, Kato and Nakai, 1989; Liu et al., 2005; Muthuselvi and Dhathathreyan,

2006), drug, antigen and vaccine delivery (Hurtado-López and Murdan, 2005, 2006a,b), tissue engineering (Dong et al., 2004; Gong, Wang, Sun, Xue and Wang., 2006), and drug eluting films (Wang et al., 2005). An additional application is the use of zein microparticles for delivery of riboflavin to fish larvae (Önal and Langdon, 2005).

Some success has been reported in biomedical areas using zein microspheres for drug delivery (Suzuki et al., 1989; Matsuda et al., 1989; Dong et al., 2005; Lui et al., 2005; Wang et al., 2005) but early work used toxic chemicals (Suzuki et al., 1989, Matsuda et al., 1989) and drug encapsulation efficiencies are low. Immunogenic problems were also encountered when zein microparticles were administered as particulates intramuscularly. This has lead to concerns on the suitability of zein microparticles for use as drug and vaccine delivery vehicles (Hurtado-López and Murdan, 2006b). Scaffolds and film matrices made from zein have shown to be biocompatible and have good properties for the adhesion and proliferation of cells (Dong et al., 2004; Gong et al., 2006). Cardiovascular devices coated with a zein microsphere film loaded with heparin also had good biocompatibility, suppressed platelet adhesion and allowed the control of heparin at the site of implantation whilst maintaining heparin activity (Wang et al., 2005).

Literature on non-medical uses for zein microparticles is very limited. A pesticide, abamectin, sensitive to air and light has been successfully encapsulated within zein microspheres resulting in an increase in photostability (Demchak and Dubas, 1997). Zein has also been used to bind and coat riboflavin for delivery of micronutrients to fish larvae (Önal and Langdon, 2005). Some treatments showed promise but there were still high leaching losses with time.

2.5.2. Food Uses

A recent review by Chen et al. (2006) claims that protein-based microparticles have found wide and increasing applications in the food industry. However, these authors described only three examples of experimental microparticle use. The first, whey protein was used for encapsulating the fat soluble vitamin, retinol, which has already been described in this review (Beaulieu et al., 2002). Two further examples were of protein-alginate composite beads, one used to encapsulate riboflavin based on work from their own laboratory and the other to encapsulate bifidobacteria. Similar applications will be described below.

After extensive searching in many scientific databases very few additional food related research references have been found. The limited amount of literature concerning the food use of microparticles indicates either a lack of research in this area or possibly proprietary research. The apparent lack of work in this area may be due to the relative expense and inconsistent supply of raw materials for microparticle preparation or because many of the chemicals used for microparticle preparation are not food compatible, for example dichloromethane, methanol and gluteraldehyde (Sinha and Trehan, 2003). In addition, technologies developed and used for microparticle preparation in the pharmaceutical industry are relatively sophisticated, are difficult to scale up and have narrow applicability range and consequently result in a 'high cost in use' (Gouin, 2004). This would make them uneconomical for use in the food industry except in specific instances where the end product is of high monetary value such as some nutraceuticals.

The trend towards healthier lifestyles and the consumer's interest in the benefits of functional foods may drive further research into the use of microparticles for encapsulation of new food ingredients. Interest in the microencapsulation of ingredients such as nutraceuticals (antioxidants), flavours, polyunsaturated fatty acids (PUFA), enzymes and micronutrients (vitamins and minerals) has been shown (Schrooyen et al., 2001). In this review these authors describe examples of microencapsulation using waxes for vitamin C, maltodextrin or gum arabic for vitamin A, D, E, K and β-carotene, starch for polyunsaturated fats, liposomes for antioxidants and a commercial product made from gelatin and a polysaccharide for protecting flavours from shear or temperature.

There is some research literature where protein microparticles have been used for microencapsulation of food ingredients. The methodology for the following applications has already been described. Whey protein microparticles have been used to encapsulate an apolar core of anhydrous milk fat (Lee and Rosenberg, 2000a, b). The same authors then developed an all aqueous microencapsulation process using whey protein to encapsulated paprika oleoresin protecting it against oxidative degradation (Rosenberg and Lee, 2004). Beaulieu et al. (2002) also used whey protein microparticles to encapsulate the fat soluble vitamin, retinol. The microparticles formed protected the vitamin from oxidation and were gastro-resistant, the retinol being released on digestion by intestinal enzymes (trypsin, chymotypsin and elastase). Chen and Subirade (2007) used a similar method to encapsulate riboflavin with microparticles made from a combination of whey

protein and alginate. At a whey protein to alginate ratio of 8:2, a microparticle size of approximately 95 μm was obtained with good release properties.

Whey protein microparticles produced by spray drying have been used to successfully encapsulate probiotic bacteria (Picot and Lacroix, 2004). Probiotic bacteria need protection during the food manufacturing process as well as during the passage through the upper part of the gastrointestinal tract (Suita-Cruz and Goulet, 2001). An effective protective process should then allow the release of viable and metabolically active cells in the intestine. Picot and Lacroix (2004) drew attention to the need to select probiotic strains which could withstand the processing conditions, particularly with respect to heat stability.

Other examples of protein microparticles food applications include the use of pea protein for encapsulating β-carotene (De Graaf et al., 2001) and gelatin coacervates with the potential for use as fat mimics in food formulations (Strauss and Gibson, 2004). These authors suggest that gelatin microparticles could be used as a reduced calorie fat replacer, flavour binder and texturiser.

Food applications for zein microparticles include food coatings and glazes (Cook and Shulman, 1998) and the encapsulation of essential oils (Parris et al., 2005). Essential oils have been encapsulated by phase separation into zein nanospheres (Parris et al., 2005). These particles appear to have limited digestibility in the stomach, slow release in the intestine and rapid release in the large intestine.

2.6. CONCLUSIONS

It appears that the properties of zein and kafirin are more suitable than other proteins used for microparticle preparation. However reagents used to prepare zein microparticles generally still include the use of ethanol or food incompatible solvents. Research into other methods of preparing microparticles, which do not include these solvents is required before food applications of microparticles can be exploited. Also research is needed into the structure and properties of zein and kafirin microparticles. As yet no scientific literature could be found on the formation, structure, functions and film forming abilities of kafirin microparticles.

It is possible that encapsulation properties of zein and kafirin microparticles could be improved by modification of these microparticles by cross-linking. If the cross-linking agent were food compatible then there would be a wider range of applications including food uses. Thus a possibility is the use of natural condensed tannins from sorghum to cross-link zein and kafirin microparticles. Sorghum condensed tannins have already been used successfully to cross-link kafirin and improve the properties of kafirin films (Emmambux, Stading and Taylor, 2004).

3. HYPOTHESES AND OBJECTIVES

3.1. HYPOTHESES

Kafirin microparticles are not homogeneous but consist of aggregates of many different kafirin molecules. Parris, Cooke and Hicks (2005) described zein microparticles as being composed of aggregated spheres of zein. Due to the many similarities of kafirin and zein it is suggested that the same is true of kafirin microparticles. It is proposed that kafirin microparticles form because, when water is added to a solution of kafirin in glacial acetic acid, kafirin solubility decreases resulting in aggregation and precipitation on very tiny particles of undissolved kafirin or on the surface of tiny air bubbles which act as nucleation sites.

Suspensions of kafirin microparticles form a continuous film on drying. This could be because as the dilute acid evaporates the microparticles would coalesce into bigger aggregates. These aggregates would then form a continuous film on evaporation of the solvent.

Kafirin microparticles can encapsulate polyphenolic antioxidants for controlled release in the stomach and gastrointestinal tract. This is due to the large surface area of microparticles (Kawaguchi, 2000) and the ability of polyphenols to bind to kafirin (Emmambux and Taylor, 2003).

3.2. OBJECTIVES

To characterise kafirin microparticles with respect to size, structure and chemical composition.

To determine how kafirin microparticles coalesce to form a continuous film when the dilute acid is evaporated off at elevated temperature.

To determine whether kafirin microparticles can be used for practical food applications, specifically to use kafirin microparticles to encapsulate polyphenolic antioxidants for controlled release of antioxidant activity.