5. GENERAL DISCUSSION

The general discussion will be divided into several parts. The first part will be a critical evaluation of the major methodologies used in the research project. These will include microscopy, FTIR, protein digestibility, biodegradation and antioxidant activity determination. Reviews of methodologies used to determine film functional properties were discussed by Taylor (2003) and polyphenol-protein binding by Emmambux (2004) and so will not be discussed further here. Some thoughts on protein aggregation, the mechanism of kafirin microparticle formation and kafirin microparticle film formation will follow. Finally, there will be a short discussion of some potential applications of kafirin microparticles and suggestions for future work to improve kafirin microparticle properties.

5.1. METHODOLOGIES USED IN RESEARCH PROJECT

5.1.1. Microscopy

Extensive use of various microscopy techniques have been used to visualise kafirin microparticles and materials made from them. The major limitation of the microscopy used was the nature of the material itself rather than the types of microscopy techniques used.

5.1.1.1. Light Microscopy

Kafirin microparticles were formed in diluted organic acid solutions of various concentrations, which were by their nature corrosive. Any form of microscope is a precision optical instrument. They are very sensitive and very expensive to purchase and maintain. This thus limited the use of wet preparations to light microscopy and at very low magnifications in order to protect the optical system of the microscopes from corrosion. Obviously, it would have been preferable to use higher magnifications, increasing resolution and obtaining a more detailed view of the kafirin microparticle morphology in the form that they were prepared.

Removal of the acid from the kafirin microparticles would probably have changed the nature of the material examined and introduced artefacts. Whilst this was unavoidable for SEM and TEM, there was a need for as much information as possible on kafirin microparticle morphology in the 'native' state to facilitate the interpretation of more in

depth studies using SEM and TEM. Thus for light microscopy, sample preparation was kept to a minimum. Alterations to samples that occur during microscopy preparation must be considered when drawing conclusions from analytical results (Kaláb, Allan-Wojtas and Miller 1995). These workers recommend the use of a number of different imaging techniques in order to compare and confirm results.

The use of kafirin microparticle colloidal preparations was difficult. It was necessary to dilute the samples with acid of the specific concentration being investigated in order to see detail of the individual microparticles. The colloidal suspensions were subject to 'streaming' by convection and drying out as the sample was illuminated. As the samples dried, the acid was concentrated. This resulted in changes in the appearance of the sample, as the microparticles aggregated at higher acid concentrations. Working quickly and reducing evaporation of the liquid phase by sealing the cover slip in place using nail varnish minimised both these problems.

When film formation was studied, rapid evaporation of the liquid phase was needed and to facilitate this no cover slip was used. In order that optics of the microscope were not damaged by the corrosive nature of the acid as it evaporated an even lower magnification (x 100) was used than previously (x 400). This enabled the objective to be further away from the sample but resolution was sacrificed.

5.1.1.2. Electron Microscopy

Both scanning electron microscopy (SEM) and transmission electron microscopy (TEM) have far higher resolution than light microscopy (Kaláb et al., 1995). The illumination source is electrons focused with a magnetic lens. For comparison the wavelength of visible light covers the range 400-700 x 10^{-9} m whereas that of electrons in a 10 kV SEM is 12.3 x 10^{-12} m and in a 200 kV TEM is 2.5 x 10^{-12} m (Goodhew, Humphreys and Beanland, 2001). Since electrons are absorbed by air, electron microscopy is carried out under vacuum. Surface properties of kafirin microparticles and microparticle films were examined using SEM, whilst TEM was used to visualise the internal structure of kafirin microparticles. Little sample preparation was required for SEM of freeze dried microparticles and for films. They could be mounted directly on double sided tape before sputter-coating with gold. Wet microparticle samples for both SEM and TEM needed more extensive sample preparation. Dehydration of the samples prior to embedding in

resin for TEM or critical point drying for SEM was the most concerning. Commonly dehydration is achieved by washing sequentially with increasing concentrations of aqueous ethanol. Since kafirin is soluble in 70% aqueous ethanol, acetone was used for dehydration. Comparison of SEM of freeze dried kafirin microparticles which required minimal preparation with SEM of wet microparticle samples which required extensive preparation showed both preparations had similar surface morphologies. Thus, it appeared that the more extensive sample preparation technique was not damaging and did not change the sample morphology.

The visualisation of the success or otherwise of encapsulation of polyphenols within kafirin microparticles was difficult. Confocal laser scanning microscopy (CLSM) was potentially an attractive way of visualising both the polyphenol and the protein. This technique has been used successfully to characterise and estimate the rate of encapsulation of oil within gelatine and gum arabic microcapsules (Lamprecht, Schäfer and Lehr (2000). CLSM is capable of producing optical sections through a specimen in one focal plane. When the focal plane is changed by steps through the specimen, the information can be combined to give a three dimensional image (Dürrenberger, Handschin, Conde-Petit and Escher, 2001). Thus, if two components of a system fluoresce after excitation at different wavelengths, a composite picture can be obtained which visualises both the components. Substances may auto-fluoresce or be stained with fluorescent dyes, for example Acid Fuchin for protein (Dürrenberger et al., 2001). However, since the CLSM is a sophisticated form of light microscopy the resolution is low and the size of kafirin microparticles was too small (1-10 μm) for sufficient detail to be seen.

The technique of TEM after osmium staining was used to visualise whether encapsulation of polyphenols within kafirin microparticles was successful. Results obtained were not entirely satisfactory. The osmium stained the sorghum condensed tannins well. Dark areas could be clearly seen on the outside and interior of the microparticles. This implied that at least some of the sorghum condensed tannins became entrapped within the kafirin microparticles. The results of osmium staining of catechin were not as clearly visualised. Staining, when present, was lighter than with the sorghum condensed tannins and only a few areas of stain could be seen on the surface and in the interior of the microparticles. It appeared that the osmium stained protein-bound polyphenols (sorghum condensed

tannins) more intensely than those not bound to protein (catechin). Polyphenolic material which may have been encapsulated within the microparticle interior (in the vacuoles) could not be visualised by this method, as it would have been washed out of the microparticles during the rinsing stages of the sample preparation. However, by inference, since the condensed tannins appear to have been encapsulated at least in part within the kafirin microparticles then it may be expected that catechin would have penetrated the interior of the kafirin microparticles, even though it could not be clearly visualised by TEM.

5.1.1.3. Atomic Force Microscopy (AFM)

The nano-structure of zein was studied by atomic force microscopy (AFM) by Guo, Liu, An, Li and Hu (2005) and the results were used to help explain how cast zein films were formed. It was hoped that by using AFM, the nano-structure of kafirin microparticles and kafirin microparticle films could be studied in the same way. AFM is a form of probe microscope where the sample is 'felt' by scanning by a sharp probe attached to a cantilever (Morris, 2004). Changes in force between the sample and the probe are measured and amplified by an optical lever. The simplest imaging technique is when the probe is effectively touching the sample and is called contact mode (Kirby, Gunning and Morris, 1995). Additional imaging modes emphasise molecular structure, examine charge or elasticity. Guo et al. (2005) used the 'tapping' mode for their work. This is a non contact mode where the cantilever is vibrated above the sample in such a way that it periodically touches the sample (Kirby et al., 1995). This mode provides high resolution images. Unfortunately, the instrument available for this work could only operate in contact mode. This meant that although the topography of the film surfaces obtained gave useful information, no molecular structure could be seen.

5.1.2. Fourier Transform Infrared Spectroscopy (FTIR)

Fourier transform infrared spectroscopy is used to study protein secondary structure. Proteins and peptides consist of continuous chains of amino acids linked by amide bonds. These amide bonds vibrate at different frequencies due to differential hydrogen bonding among the amino acids contained in the proteins (reviewed by Singh, 2000). The differences in vibration along with geometric orientations of amide bonds in α-helix, β-sheet and random coil structures can be related to individual secondary structure folding. Three spectral regions have been identified for proteins, Amide I, II and III, of which the

Amide I region has the strongest signal. The Amide II protein band is not as sensitive as the Amide I and Amide IIII region and the Amide III region protein signal is generally weak. Peak assignment is difficult in the Amide I region as there is overlap of peaks representing different secondary structures and so care must be taken with interpretation of results. Another disadvantage of the Amide I region is -OH vibrations due to liquid water. Samples used in this study were solid powders or films and the attenuated total reflectance accessory (ATR) was used. The advantage of the ATR technique is the avoidance of solvent interference in the infrared spectra because the sample thickness is limited to a thin layer near the surface of the internal reflection element (reviewed by Singh, 2000). The major limitation of this study was the lack of sensitivity of the instrumentation. The narrowest band width possible to use without too much background noise was 8 cm^{-1} and the number of scans used was 32, instead of the more usual 2 cm^{-1} resolution and 128 or 256 scans. Whilst these conditions do not invalidate the data, it is not as good as it could be (Prof P.S. Belton, School of Chemical Sciences and Pharmacy, University of East Anglia, UK, personal communication, 2007).

5.1.3. Protein digestibility

The pepsin digestibility assay of Mertz, Hassen, Cairns-Whittern, Kirleis, Tu and Axtell (1984) was used throughout this study. The method was modified for use with small sample sizes. This is the most commonly used assay to determine sorghum protein digestibility. The method is long and involved, uses a relatively large sample size (200 mg) and few samples can be analysed at a time. The total protein of the grain must be determined. A two hour incubation is followed by centrifugation and determination of the residual protein. Residual protein may be lost during the removal of the supernatant after centrifugation which is a major source of error in the method and can result in falsely high protein digestibility estimations. This error is further magnified when small sample sizes was used.

The premise is made that all soluble nitrogenous material is protein and is digestible and all insoluble nitrogenous material is undigested proteins. This may not be a true assumption. Soluble material may include soluble proteins and low molecular weight nitrogenous substances such as free amino acids and peptides. This material can make up a large portion of the total protein of the grain and is variable in quantity according to

sorghum variety (Taylor and Taylor, 2002). These workers found levels of soluble protein between 9.9 - 21.8% of the total grain protein.

Another area for concern when considering in vitro protein digestibility methodology is the dependence of the assay on the use of an enzyme. There may be grain constituents present, specifically sorghum condensed tannins which are responsible for reduced sorghum protein digestibility values. Sorghum condensed tannins bind to proteins forming indigestible protein-tannin complexes (Butler, Riedl, Lebryk and Blytt, 1984) and also inhibit digestive enzymes (Nguz, Van Gaver and Huyghebaert, 1998). In this study sorghum condensed tannins were specifically added to modify protein digestibility of kafirin, kafirin films and kafirin microparticles used for encapsulation. Excess tannin may have bound to the enzymes used in the assay. Thus, it is not clear in this case whether the results obtained really reflect what would happen in the human digestive tract. However, Butler et al. (1984) suggested that although purified enzymes were inhibited by tannins, membrane bound enzymes were not. These workers concluded that sorghum tannins reduce dietary protein digestibility by binding with dietary protein not by forming complexes with digestive enzymes and inhibiting them.

5.1.4. Biodegradation

There is no commonly used standard method for determination of biodegradation of protein films. Le Tien, Letendre, Ispas-Szabo, Mateescu, Delmas-Patterson, Yu and Lecroix (2000) used digestion with pancreatin, measuring film weight loss with time to estimate biodegradation. Other workers have exposed films to microbial cultures in liquid media, measuring the increase in amount of soluble nitrogen present with time (Lacroix, Le, Outtara, Yu, Letendre, Sabato, Mateescu and Demas-Patterson, 2002) or by measuring the amount of carbon dioxide released by respirometry (Hoffmann, Řezničkova, Kozáková, Růžička, Alexy, Bakoš and Precnerová, 2003) or measuring the amount of oxygen consumed (Foulk and Bunn, 2007). Still other workers have estimated film biodegradation by simulated soil burial tests measuring carbon dioxide released by respirometry (Chiellini, Cinelli, Couti and Kenawy, 2001). Of these methods only the latter simulates the conditions found in landfills, which is the most common method of waste disposal.

In this study an adaptation of the American Society for Testing and Materials (ASTM) standard method D5512-96 for exposing plastic materials to a simulated compost environment (ASTM 1996) was used in order to try and simulate landfill conditions. The different kafirin films were buried in a mixture of well rotted horse manure and sawdust, at 37 or 60% moisture and incubated using a temperature regime for optimal composting conditions. It was anticipated that quantitative measurements could be made with time, for example changes in weight, size, nitrogen content and moisture. This was not possible as the film pieces became rapidly very fragile and so it was not possible to clean off all the compost adhering to the films. In spite of this, valuable visual information was obtained on the comparative biodegradation of the different film treatments.

A further potential drawback of the method used was that of repeatability. The micro organisms in the compost were not characterised and the microbial load was not known. Thus it was unknown whether the micro organisms present were acclimated to the protein films or not. It would be expected if the organisms were acclimated to the substrate then the protein films would be degraded more quickly. According to Mutabaruka, Hairiah and Cadisch (2007) certain soil micro organisms are more able to degrade polyphenol-protein complexes. When working on the microbial degradation of hydrolysable and condensed tannin polyphenol-protein complexes in soils, these workers found the ratio of fungi to bacteria increased in systems rich in condensed tannin complexes. They also found that fungal communities and high acidity were highly adapted to phenol rich soils especially those containing condensed tannin-protein complexes. Thus a microbial community containing more fungi than bacteria would probably biodegrade tannin cross-linked kafirin films faster than one containing more bacteria than fungi. Possibly a better approach would have been to sterilise the compost and then inoculate it with a known amount of specific soil micro organisms. It would then be easier to replicate the conditions of the test.

5.1.5. Antioxidant activity

Antioxidants can be defined in a number of ways. They can be considered as substances which reduce or retard oxidation of easily oxidised materials such as fats or as compounds able to accept radicals (Frankel and Meyer, 2000), or as substances which counteract free radicals, preventing damage caused by them (Ratnam, Ankola, Bhardwaj, Sahana and Kumar, 2006). Antioxidant activity can be measured by estimating the inhibition of

oxidation of a lipid substrate by an antioxidant or by using free radical trapping methods (Frankel and Meyer, 2000). The ABTS method used in this study falls into the latter category. The radical used in this method is 2,2'-azinobis-(3-ethyl-benzothiazoline-6-sulfonic acid) ($ABTS^{\bullet +}$). The $ABTS^{\bullet +}$ radical is generated by mixing the ABTS salt with potassium persulphate, a strong oxidising agent (Awika, Rooney, Wu, Prior and Cisneros-Zevallos, 2003). The method then measures the ability of an oxidising agent to scavenge the $ABTS^{\bullet +}$ radical generated in an aqueous phase as compared with a Trolox (a water soluble vitamin E analogue) standard. Results are expressed as Trolox equivalent antioxidant capacity (TEAC).

The ABTS method is one of the most commonly used methods for estimation of antioxidant activity. It is simple and rapid to perform and gives good repeatability (Awika et al., 2003). According to Awika et al. (2003) the method can be used over a wide pH range in aqueous and organic solvents. Pérez-Jiménez and Saura-Calixto, (2006), however, reported that there was an effect of solvent and pH on ABTS measurements both for Trolox and the polyphenols used by them. To minimise any potential solvent or pH effects in this work, separate calibration curves were prepared for each of the buffers (pH 2 and pH 7.6) used. Pérez-Jiménez and Saura-Calixto (2006) also found that certain food constituents, specifically proteins and amino acids gave positive ABTS results. They reported the amino acids, tyrosine and tryptophan give high ABTS values even at low concentrations. In this study kafirin microparticles gave positive antioxidant measurements which increased with both pepsin, trypsin and chymotrypsin digestion. However, since there was very little enzymic digestion of either the encapsulated catechin or sorghum condensed tannins it was thought unlikely that the kafirin was adding to the antioxidant activity released from the two encapsulated materials. An area of concern was that the purity of the catechin and sorghum condensed tannins was not known. In addition the degree of polymerisation of the sorghum condensed tannins was not known. Since both food constituents (Pérez-Jiménez and Saura-Calixto, 2006) and polymer chain length affect antioxidant activities (reviewed by Awika and Rooney, 2004), it is thus possible that both these factors could have influenced the amount of antioxidant activity measured by the ABTS assay.

The ABTS method's major drawback is the radical used is not found in biological systems and that it has not been correlated with in vivo studies. However, Awika et al. (2003) found that for sorghum polyphenols, ABTS method correlated well with the oxygen radical absorbance capacity (ORAC) method, which measures the ability of antioxidants to protect protein from damage from free radicals. The ORAC method uses the peroxyl radical which is generated by oxidative processes in the human body (Pérez-Jiménez and Saura-Calixto, 2006) and so is thought to better mimic polyphenol antioxidant activity in biological systems than other methods and combines both inhibition time and degree of antioxidant inhibition into a single quantity (Cao, Sofic and Prior, 1996). However, this method was not used in this study because it involves the use of expensive equipment which was not available.

5.2. KAFIRIN MOLECULAR AGGREGATION

Aggregation is involved in both kafirin microparticle formation and kafirin microparticle film formation. Recently Kim and Xu (2008) reported on zein aggregate formation, measuring aggregation number of zein molecules in solution by dynamic light scattering. These workers found that solutions of zein in aqueous ethanol showed increasing turbidity when the concentration of ethanol was decreased from just less than 90% to 70%, indicating increasing aggregate size with decreasing ethanol concentration, from approximately 60 molecules at 87% aqueous ethanol to 10 000 molecules at 70% ethanol. At even lower aqueous ethanol concentration the aggregates precipitated out. When the aqueous ethanol concentration was increased above 95% zein precipitation also occurred. It was assumed at around 90% aqueous ethanol each zein molecule exists as a freely moving particle. It was suggested that the zein aggregates form a macromolecular micelle. According to Kim and Xu (2008) if zein acts as an amphiphile, then depending on the solvent composition, the hydrophobic part of the molecule faces outwards when the solvent is hydrophobic or the hydrophilic part faces outwards in a hydrophilic solvent.

A different situation occurs with kafirin microparticle aggregation in aqueous acetic acid. At low (5.4%) acetic acid concentration microparticle size is between 3-4 µm, whereas as the acid concentration increases to 21.6% the particle size increases to 6 µm, as the aggregate number increases. Whilst it has not been possible in this study to measure aggregate number, it can be approximated by calculation using Avagadro's constant.

Firstly, we must make certain assumptions, that the average size of a kafirin microparticle is 5 μm in diameter, spherical, with half its volume consisting of holes (vacuoles) and that the average molecular weight of α-kafirin is 25,000. Then using Avagadros constant we can calculate that one average sized kafirin microparticle will contain approximately 800 million molecules. This is considerable greater than the range of aggregate number (60 to 10 000 molecules) that Kim and Xu (2008) measured. However, it should be noted that they did not measure the aggregate number of precipitated zein at lower than 70% or greater than 95% aqueous ethanol.

Depending on the interactions between protein monomers different structures can be formed during aggregation (Bolder, Hendrickx, Sagis and Van der Linden, 2006). Examples include fibril formation, microparticle formation and microparticle film formation. Protein aggregation has been identified as important in fibril formation of zein (Bugusu, Rajwa and Hamaker, 2002; Mejia, Mauer and Hamaker, 2007), whey protein (Bolder et al., 2006), soy protein (Akkermans, Van der Goot, Venema, Gruppen, Vereuken, Van der Linden and Boom, 2007), and amyloid fibrils (Gorbenko and Kinnunen, 2006). Amyloid fibrils are thought to be responsible for certain conformational diseases such as Parkinson's disease and Alzheimer's disease (reviewed by Gorbenko and Kinnunen, 2006). Gorbenko and Kinnunen (2006) state that there is good evidence that it is a generic property of polypeptide chains to have a propensity to form amyloid fibrils. In all these cases either low pH or heat is required for fibril formation. Gorbenko and Kinnunen (2006) list conditions which favour in vitro fibril formation, as acidic pH, elevated temperatures or the addition of organic solvents or denaturants. These conditions favour partial unfolding of natively folded proteins or refolding of unfolded proteins and may enable polymerisation into β-sheet structures, which is thought to be a universal energetic minimum for aggregated proteins (reviewed by Gorbenko and Kinnunen, 2006). Amyloid fibrils consist of a core of cross β-sheet structure with polypeptide chains oriented so that the β-sheets run perpendicular to the long axis of the fibril (Gorbenko and Kinnunen, 2006). Secondary structures of soy glycinin and soy protein isolate fibrils and zein fibrils have been reported to contain β-sheet structures (Mejia, et al., 2007; Akkermans et al., 2007 respectively).

Evidence of protein aggregation was seen by SEM and TEM and when size distribution of kafirin microparticles was examined with increasing acid concentration. FTIR of kafirin microparticles also showed an increase in the amount of β-sheet conformation as the acid concentration was increased when compared with that of the original kafirin from which the microparticles were made. This indicated protein aggregation had occurred (Mizutani, Matsumura, Imamura, Nakanishi, Mori, 2003). Other workers have found conformational changes in proteins as microparticles have been formed. Beaulieu, Savoie, Paquin and Subirade (2002) found the formation of microparticles from whey protein has an effect on the secondary structure of whey proteins, resulting in the formation of intermolecular bonded β-sheet structures. Secondary structure changes were also found when silk fibroin microparticles were made (Hino, Tanimoto and Shimbayashi, 2003). In its natural state silk fibroin occurs as a mixture of amorphous and β-pleated sheets conformation. When silk fibroin microparticles were made by dissolution in ethanol in the presence of calcium chloride and spray dried, they assumed an irregular structure. These workers also found that the secondary structure of the microparticles changed to a β-sheet conformation in a humid atmosphere of 89% RH. Different conformational changes were reported by Yeo, Lee, Lee and Kim (2003). They found that silk fibroin in aqueous solution at neutral pH was in a random coil conformation which changed to β-sheet on spray drying the silk fibroin microparticles. Similarly, Wang, Wenk, Matsumoto, Meinel, Li and Kaplan (2007) made silk fibroin microparticles using lipid vesicles as templates and found these microparticles also had a β-sheet conformation.

Protein aggregation was also observed during kafirin microparticle film formation as shown by light microscopy and by FTIR of microparticle films where the secondary structure was shown to have a large amount of β-sheet structure, which again is indicative of protein aggregation (Mizutani et al., 2003). This is in agreement with findings of Subirade, Kelly, Guéguen and Pézolet (1998), who, when working with the soybean protein, glycinin found conformational changes during film formation, resulting in the formation of intermolecular hydrogen bonded β-sheet structures. These workers also referred to similar changes in legumin from peas and wheat gluten proteins during film formation. Kafirin, like wheat gluten, is a prolamin protein, thus the finding that similar conformational changes occur during kafirin film formation is not surprising. Subirade et al. (1998) suggested that β-sheet structures might be essential for protein-protein

interactions and network formation in protein films from vegetable origins, intermolecular hydrogen bonding between β-sheets acting as junction zones stabilizing the film network.

Thus, there seems to be some commonality in the secondary structural changes that take place during fibril formation, microparticle formation and microparticle film formation. The mechanism for all seems to be a form of controlled aggregation and the specific differences in conditions dictate whether fibrils, microparticles or films are formed. Whilst it is interesting to speculate on what may be happening at a molecular level it is very difficult as it is a complex process. However, it is suggested that the mechanism involved may be similar to that reviewed by Gorbenko and Kinnunen (2006) for amyloid fibril formation and Bolder, Sagis, Venema and Van der Linden (2007) for whey protein fibril formation. Both proposed mechanisms are similar and begin with a slow activation and nucleation (aggregation) stage followed by a more rapid elongation and growth phase resulting in fibril formation and finally a termination phase. The initial stage involves a structural transformation of the protein. Low pH results in partial unfolding of the native protein, giving rise to a higher charge on the protein. This may enhance side chain charge repulsion resulting in a more open structure with exposed hydrophobic areas. This is thought to lead to self association by hydrophobic interactions of the non polar side chains. It may be suggested that this is how microparticle aggregates form. The more rapid growth phase results in fibril formation and occurs for amyloid fibrils as the local concentration of protein increases (Gorbenko and Kinnunen, 2006). Rogers, Venema, Van der Ploeg, Van der Linden, Sagis and Donald (2006) suggest that β-lactoglobulin fibrils form by monomers joining head to tail. Bolder et al. (2007) suggest termination occurs when the monomers are changed possibly by hydrolysis and so are no longer susceptible to assembly. It is suggested for kafirin film formation that the kafirin microparticle aggregates form into chains interlinking as the solvent evaporates and the local protein concentration increases. Finally as the film dries a complete matrix is formed.

5.3. MECHANISM OF KAFIRIN MICROPARTICLE FORMATION

Zein microspheres have been prepared using both solvent extraction/evaporation (Cook and Shulman, 1998) and phase separation/coacervation techniques (Demchak and Dybas, 1997; Dong, Sun and Wang, 2004; Lui, Sun, Wang, Zhang and Wang, 2005; Parris, Cooke and Hicks, 2005; Wang, Lin, Liu, Sheng, and Wang, 2005; Hurtado-López and

Murdan, 2005; Muthiselvi and Dhalathreyan, 2006). No references in the literature could be found for the preparation of kafirin microspheres/microparticles. However, since kafirin and zein show a high degree of homology (Belton, Delgadillo, Halford and Shewry, 2006) it would be expected that they would behave in the same manner during microparticles preparation. Most methods of producing zein microspheres involve dissolving the 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, 2005; Muthiselvi and Dhalathreyan, 2006).

An ethanol free method of making kafirin microparticles by phase separation was devised in this study. Kafirin was dissolved in glacial acetic acid and then water added, reducing the acetic acid concentration until the kafirin was no longer soluble and precipitated out as microparticles.

A factor which makes the kafirin microparticles formed in this study unique is the presence of holes (vacuoles), which results in their very large surface area. In the body of this work the formation of these vacuoles or holes was discussed. A theory was proposed that they are footprint of air bubbles entrapped during microparticle preparation. Experimental evidence, whilst not conclusive appeared to support this theory. Kafirin microparticles were made with the addition of gas by bubbling air through the solvents used to make the microparticles and by removal of gas from the solvents by boiling. When gas was added the matrix material had the appearance of an expanded foam suggesting that a large amount of gas was entrapped in the protein matrix as the kafirin precipitated. In contrast when gas was removed the protein matrix appeared more solid and the holes were much smaller than those in the foam matrix. What has not been considered in detail so far in this discussion is a possible mechanism of kafirin microparticle formation.

Hurtado-López and Murdan (2005) explained 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. It is possible a similar mechanism is involved when kafirin microparticles are made by using glacial acetic acid as the solvent for kafirin and water as the diluent. Acetic acid is polar and like ethanol can form hydrogen bonds with itself and other molecules such as water (Morrison and Boyd, 1992). Possibly the extended hairpin structure of kafirin when it is dissolved in acetic acid would fit into a three dimensional structure of acetic acid molecules held together by hydrogen bonds. As water is added, the solvent three dimensional structure would change due to the formation of hydrogen bonds between the acetic acid and water molecules, which would no longer accommodate the kafirin molecule. The kafirin would then precipitate out as microparticles. However, a factor not considered is the relative size of the molecules. Ethanol (MW 46), water (MW 18) and acetic acid (MW 60) are very small molecules relative to kafirin (MW 25 000). Even if we consider kafirin as an extended hairpin structure, and ethanol or acetic acid as extended polymers, it difficult to envisage the kafirin molecule fitting within this solvent structure. Also, if the theory of Hurtado-López and Murdan (2005) was true then one would expect that the kafirin/zein molecule would dissolve in almost 100% ethanol at ambient temperatures. However this does not happen. Full solvation of kafirin/zein will only occur in 100% ethanol at elevated temperature and pressure (Lawton, 2006) or in aqueous ethanol (70%) at elevated (70° C) temperature.

Shewry, Halford, Tatham, Popineau, Lafiandra and Belton (2003) suggest that solvation of prolamin proteins is more complex. They consider that elements of hydrophobicity (thus hydrophobic interactions), hydrophilicity (including the presence of polar groups and hydrogen bonding), secondary structure and protein-protein interactions may all play a part in determining whether a prolamin is soluble in a specific solvent. For solvation to occur there must be a net decrease in free energy of the protein and solvent on mixing (Shewry et al., 2003). According to these workers this takes place in two phases, firstly formation of a liquid from a solid phase (free energy change of the formation of a liquid solute designated ΔG_{fus}). This doesn't happen in practice since a complicated kinetic process takes place but the energies involved must be the same (Prof P.S. Belton, School of Chemical Sciences and Pharmacy, University of East Anglia, UK, personal communication, 2008). Secondly a mixing of the liquid solute and solvent (free energy

change of the formation of the mixture designated ΔG_{mix}). Both events have a change in free energy associated with them and the total free energy change (designated ΔG_{sol}) is the sum of the free energy change of both events. This can be expressed as follows:

$$\Delta G_{sol} = \Delta G_{fus} + \Delta G_{mix}$$

For solvation to occur ΔG_{sol} must be negative, ΔG_{fus} will be positive and depends on the strength of the intermolecular forces in the solid. For solution to occur ΔG_{mix} thus must be negative and greater than ΔG_{fus}.

Kafirin is considered highly hydrophobic and is insoluble in water (Belton et al., 2006). The degree of hydrophobicity of kafirin can be shown by considering the hydrophilic/hydrophobic balance of kafirin by calculating the sum of its free energies of hydration from its amino acid sequences. Compared with a 'typical protein' (-164 k cal mol^{-1}) (Shewry et al., 2003), kafirin has a low negative free energy of hydration for all its components (α-kafirin -140 k cal mol^{-1}; β-kafirin -123 k cal mol^{-1} and γ-kafirin -99 k cal mol^{-1}) (Belton et al., 2006). However, kafirin also has hydrophilic characteristics (Belton et al., 2006). Hydrophilic proteins like wheat gluten absorb water when heated, whereas hydrophobic proteins expel water under the same conditions (Grant, Belton, Colquhoun, Parker, Plijter, Shewry, Tatham, Wellner, 1999). When kafirin and zein are heated with deuterated water (D_2O), they absorb small amounts of water but less than gluten (reviewed by Taylor and Belton, 2002). This shows that kafirin and zein are more hydrophobic than gluten but because they absorbed water rather than expelling it, is indicative of some hydrophilic characteristics. These hydrophilic characteristics are in part, a result of the large amount of glutamine that kafirin and zein contain and would enable hydrogen bonding to occur between the polar $–NH_2$ groups of glutamine and water.

Shewry et al. (2003) suggest the solubility of prolamins in aqueous alcohols is possible when the balance of hydrophobic and hydrophilic forces are favourable. Applying this to the solvation of kafirin in aqueous ethanol, there will be interaction of the hydrophobic groups with ethanol. Also since ethanol is a polar solvent there will be some interaction with the amide groups of glutamine. This latter interaction will be less favourable than the interaction of the glutamine amide groups with water. If there is the correct balance of interactions with hydrophobic side chains resulting in a decrease in total free energy of the system then solubility with occur. As the amount of water increases there will be less

hydrophobic interactions with ethanol and more hydrogen bonding with water and thus kafirin will no longer be soluble and will precipitate out.

A model (Figure 5.3.1.) for the solvation of kafirin in glacial acetic acid and the precipitation of kafirin as microparticles on the addition of water is proposed. Glacial acetic acid has a low dielectric constant (6.1 at 20°C, Merck Chemical Data Base, 2005), is poorly dissociated and so considered a hydrophobic solvent. Since generally 'like dissolves like' a hydrophobic protein like kafirin will interact with glacial acetic acid by hydrophobic interactions. As long as there is a net decrease in the free energy of the system then solvation will occur.

Carboxylic acid molecules are polar and are capable of forming hydrogen bonds with each other, with water or with other substances and so show similar solubility behaviour to the alcohols (Morrison and Boyd, 1992). Thus a similar model to that proposed for precipitation of kafirin from an aqueous ethanol solution can be applied to the precipitation of kafirin from acetic acid. As water is added the acetic acid acts as a polar solvent and forms hydrogen bonds with water and would also interact with the amide groups of the kafirin glutamine residues. As more water is added, interaction of the amide groups with water would be more favourable than with the acetic acid. Acetic acid would also preferentially form hydrogen bonds with water. As the proportion of water increased the kafirin would no longer be soluble and would precipitate out. In addition, increased mobility of the kafirin molecules may be expected as water is added which may enable increased number of protein/protein interactions to occur resulting in larger molecules which are less easily soluble.

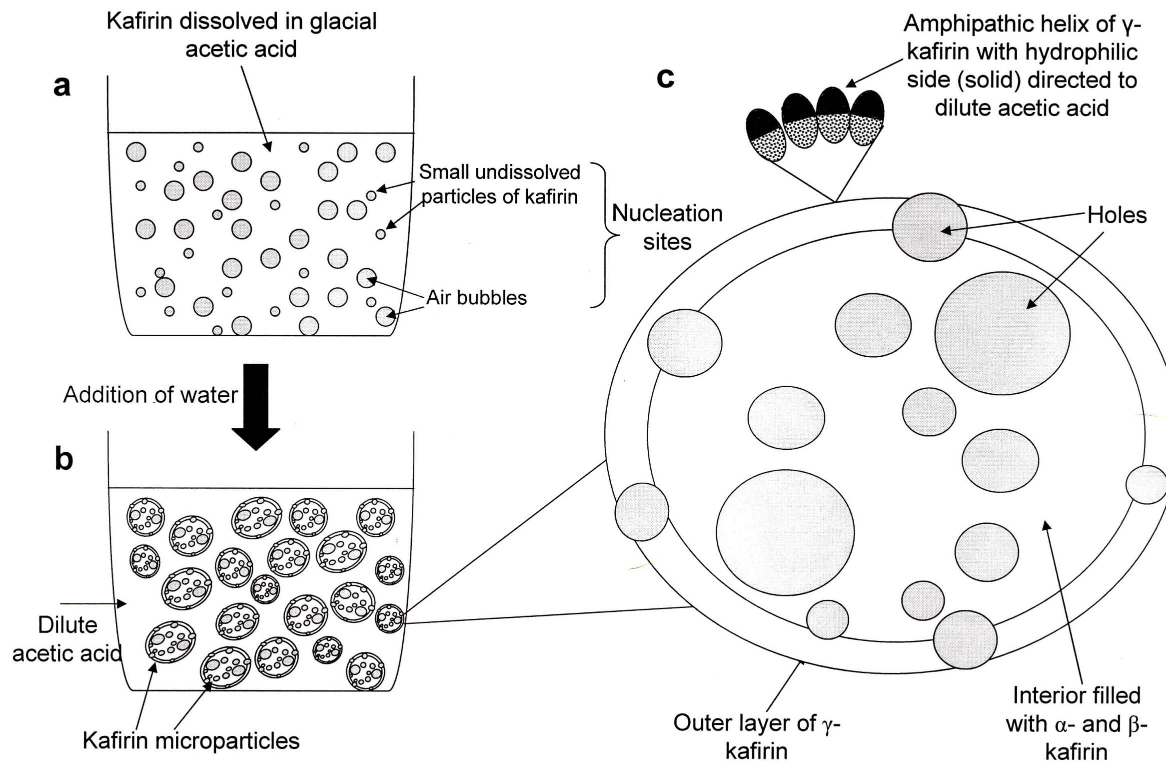

Figure 5.3.1: Hypothetical model to describe the formation of kafirin microparticles
a: Solution of kafirin in glacial acetic acid showing entrapped air bubbles and particles of undissolved kafirin, b: On addition of water, microparticles form as discrete particles, c: Expanded view of a single kafirin microparticle (not to scale). It is suggested that α-kafirin would precipitate out first around a nucleation site (as suggested previously, a small particle of undissolved kafirin), followed by β-kafirin which would then stabilise the α-kafirin. Finally it is suggested that the hexapeptide repeat of γ-kafirin would use a self assembly mechanism to coat the surface of the partially formed microparticle and thus stabilise the microparticle by disulphide bonds.

Almost identical kafirin microparticles can be formed by phase separation using other organic acids such as propionic and lactic acids. However, propionic acid formed aggregates at a lower acid concentration than kafirin microparticles made with acetic acid. This is probably because kafirin is more soluble in propionic acid than acetic acid under the same conditions. As stated, this is possible because propionic acid is a more hydrophobic solvent than acetic acid due to its longer chain length. On addition of water to a solution of kafirin in propionic acid, super saturation is reached with the addition of more water than with acetic acid. Protein precipitation and subsequently particle size growth occurs with the presence of more water with propionic acid than with acetic acid. Thus with propionic acid protein aggregation could be observed at a lower acid concentration, 5.4%, than with acetic acid approximately 20%. The general appearance of the kafirin microparticles made with the different acids was similar and further work was limited to the use of acetic acid as kafirin solvent.

Kafirin microparticles precipitate as spheres probably because a spherical shape results in the smallest surface area for a given volume and thus minimises the surface free energy. The sphere is thus always the preferred shape as long as no other factors are involved. It is suggested that kafirin dissolves in glacial acetic acid at a concentration of kafirin close to forming a saturated solution (Figure 5.3.1.a). When water is added the solution of kafirin in the acid becomes supersaturated. As more water is added the protein comes out of solution probably precipitating out on very tiny particles of undissolved kafirin or on the surface of tiny air bubbles which act as nucleation sites (Figure 5.3.1.b, c). This happens throughout the solution so there are many tiny protein spheres. These spheres will be in motion in the aqueous suspension and so will randomly collide with each other. On collision the protein particles will stick to the central core leaving an area of protein depletion around the growing protein sphere. This then reduces the probability of further protein collision and the size of the protein sphere is limited.

Hurtado-López and Murdan (2005) suggested that zein microspheres are formed by precipitation onto the surface of the original undissolved particles of zein. The undissolved particles of zein particles then acted as nuclei for particle growth. These authors also stated that zein microspheres could not be produced without the presence of Tween 20, a surfactant. Tween 20 was used as a stabilizer and aid to solubilisation of the zein. This suggests that other factors are involved besides the presence of suitable nuclei

for precipitation as spheres. Although kafirin at the concentration used, appeared to be completely dissolved in the glacial acetic acid, the presence of very tiny undissolved kafirin particles, dust or other particulate matter may act as nucleation sites for precipitation of kafirin.

The role played by the different kafirin species in kafirin microparticle formation has not yet been explored. Kafirin microparticles contain the same kafirin species as the original kafirin, that is α-, β-, and γ-kafirin. For zein, it has been shown that γ-zein contains a proline rich hexapeptide repeat (PPPVHL) at the N terminus (Kogan, Dalcol, Gorostiza, López-Inlesias, Pons, Pons, Sanz and Giralt, 2002). This repeat is also present in kafirin (Belton et al., 2006). Examination of synthetic peptides based on this repeat has shown its structure to be that of an amphipathic polyproline II type conformation in aqueous solution, with hydrophilic charged histidine side chains arranged on one side of the helix and hydrophobic residues on the other side (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 et al. (2002) using atomic force microscopy and transmission electron microscopy (TEM) has shown that these peptides are able to self assemble into cylindrical micelles. The surface properties and potential for self assembly has suggested a role of this octamer in protein body formation (Kogan et al., 2002).

The presence of this octamer in γ-kafirin with its specific surface properties and ability to self assemble may play a role in kafirin microparticle formation. If we consider an analogy with zein protein body formation, the specific zein species and order in which they are assembled is vital for normal spherical protein bodies to form (Coleman, Herman, Takasaki and Larkins, 1996). Without the presence of all the different zein species deformed protein bodies form. Thus it is suggested that all the kafirin species play a role in kafirin microparticle formation. Zein microparticles could not be formed from commercial zein using the method in this study. The resultant material was a sticky aggregate of zein 'taffy'. Commercial zein is predominately α-zein (Lawton, 2002) and so it is suggested that the lack of γ- and β-zein prevented microparticle formation.

Like protein bodies, kafirin microparticles are spherical and are of slightly larger size than kafirin protein bodies (0.4 -2.0 μm) (Taylor, Novellie and Liebenberg, 1984). It appears

that γ-zein synthesis is important for initiating protein body formation and possibly determining the size and number of protein bodies (Coleman et al., 1996). It is also thought that the hydrophilic nature of γ-zein is important in maintaining the spherical orientation of the protein body. Interactions of γ-zein and α-zein are thought to promote α-zein retention within the protein body and β-zein is thought to enhance the stability of α-zein (Coleman et. al., 1996). Although it is unlikely that the different kafirin species assemble into kafirin microparticles in the same way as the different zein species in protein bodies, it is suggested that the specific kafirin species fulfil a similar purposes in maintaining microparticle structure as those of their zein counterparts in protein bodies.

Zein protein body assembly occurs according to the theory of Kogan et al. (2001) by an initial interaction between the hexapeptide repeat and the endoplasmic reticulum (ER) membrane. The self assembly mechanism of this protein then coats the inner face of the ER membrane and is stabilised by intermolecular disulphide bonds (γ-and β-zein are both rich in cysteine residues capable of forming disulphide linkages). Subsequently, the hydrophobic α-zein deposits within the γ-zein coat and is stabilised by the β-zein. There is no membrane structure involved in kafirin microparticle formation and all the kafirin species are present rather than sequentially synthesised as in protein body formation. Thus initially for kafirin microparticle formation, the kafirin is dissolved in glacial acetic acid, resulting in a viscous solution with entrapped air bubbles (Figure 5.3.1.a). As water is added the microparticles form (Figure 5.3.1.b). It is suggested that α-kafirin would precipitate out first around a nucleation site (as suggested previously, a small particle of undissolved kafirin), followed by β-kafirin which would then stabilise the α-kafirin (Figure 5.3.1.c). Finally it is suggested that the hexapeptide repeat of γ-kafirin would use a self assembly mechanism to coat the surface of the partially formed microparticle and thus stabilise the microparticle by disulphide bonds (Figure 5.3.1.c).

5.4. MECHANISM OF KAFIRIN MICROPARTICLE FILM FORMATION

Few references to microparticle film formation and no description of the mechanism for film formation from microparticles could be found in the literature. It is suggested that a film made from microparticles would, in principle, form in a similar way to that which Guo et al. (2005) described for a cast zein film. According to these workers, the nano-structure of a cast zein film exists as small globules, which consist of aggregates of many zein molecules. When examined by AFM the zein globules were observed as interlinked

rods that appeared to form an extended meshwork. They suggested that as the zein film solution was dehydrated the concentration of the proteins increased until the zein molecules agglomerated and hydrogen bonding, disulphide bonding and hydrophobic interactions occurred maintaining a meshwork and resulting in the formation of a cohesive film.

According to Banker (1966) maximum solvation and polymer chain extension results in the best films with the greatest strength and cohesiveness. Kafirin is not soluble in dilute aqueous acetic acid solutions (Figure 5.4.1.a) but cohesive films could still be formed from such colloidal suspensions at specific minimum acid concentrations (21.6% acetic acid with 2% kafirin microparticles). Carboxylic acids, like acetic acid, are polar and miscible with water due to hydrogen bonding between water and the carboxylic acid resulting in high boiling points (Morrison and Boyd, 1992). Acetic acid is very soluble in water but poorly dissociated in aqueous solutions (Brown, Le May and Burnsten, 2006). Since the boiling point of water (100°C) is less than that of acetic acid (116-118°C) (Merck Chemical Data Base, 2005) it would be expected that there would be preferential evaporation of water from the film forming solutions as heat was applied. This would effectively concentrate the amount of acid in the film forming solution (Figure 5.4.1.c). As the solvent evaporated from the 21.6% kafirin microparticle film forming solution, the kafirin dissolved in the more concentrated acid and as the solvent completely evaporated a cohesive film formed (Figure 5.4.1.e). This did not happen when the same concentration of kafirin microparticles (2%) was used in a lower acid concentration (5.4%). With the lower acid concentration, as the water preferentially evaporated there was insufficient acetic acid remaining to dissolve the same amount of kafirin microparticles. This prevented a cohesive film being formed and resulted in the deposition of the kafirin microparticles as an opaque mesh on the slide.

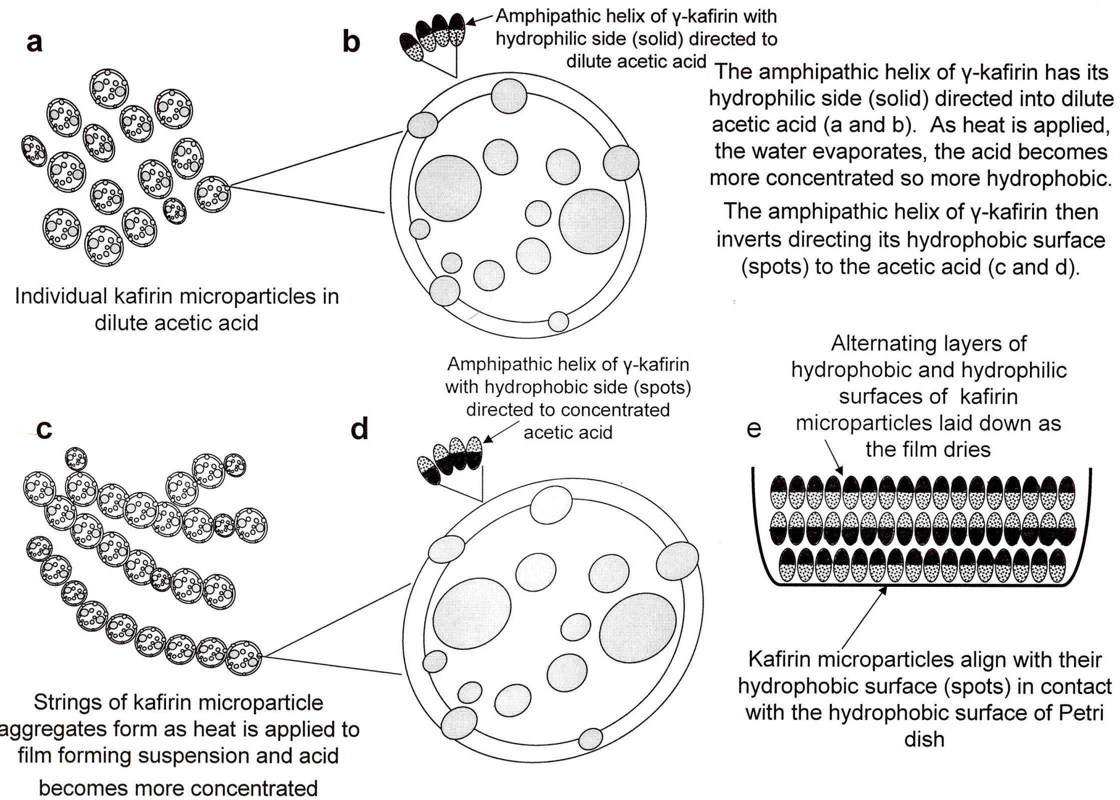

Figure 5.4.1.: Hypothetical model to describe the formation of a kafirin microparticle film
a: Individual kafirin microparticles in dilute acetic acid, b: Expanded view of a single kafirin microparticle (not to scale), showing the amphipathic helix of γ-kafirin with its hydrophilic side directed to the dilute acetic acid, c: Strings of kafirin microparticle aggregates formed as heat is applied and acetic acid becomes more concentrated and environment becomes more hydrophobic, d: Expanded view of a single kafirin microparticle (not to scale), showing the amphipathic helix of γ-kafirin inverted with its hydrophobic side directed to concentrated acetic acid, e: Alternating layers of hydrophilic and hydrophobic surfaces of kafirin microparticle film

Although observed on a much greater scale than the nanoscale observations of Guo et al. (2005), it is suggested that the chains of kafirin microparticles that were observed by light microscopy (Figure 5.4.1.c) to form when a kafirin microparticle film was drying were probably aggregates of very large numbers of kafirin molecules. These apparent similarities between a macro and microscale during kafirin film formation 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, Bouchet and Baldwin, 1997).

Possibly as the solvent evaporated during films formation, the aggregates of kafirin molecules bonded together by hydrogen bonding, disulphide bonding and hydrophobic interactions in a similar way to that which Guo et al. (2005) suggested for zein, resulting in a the formation of a cohesive film when all the solvent had evaporated. This theory does not, however, address how the secondary structure of the proteins change during film formation to facilitate inter- and intra-molecular bonding of the polymer chains.

It has been proposed that in its native form kafirin takes a hairpin form comprising of α-helices, β-sheet and turns with the α-helices interacting via hydrogen bonds between the glutamine residues in the turn regions as discussed in the literature review for zein (reviewed by Belton et al., 2006). The hydrophobic amino acids would then face to the centre of the structure and the hydrophilic amino acids would be on the surface. As determined by FTIR during film formation the secondary structure of the kafirin changes from the mainly α-helical form, becoming predominately of β-sheet conformation in the film. It is suggested that the previously hidden hydrophobic amino acids would be exposed as the protein conformation changed. As the solvent evaporated this more open structure would allow the polypeptide chains to become closer together enabling the formation of a film matrix stabilised by hydrophobic interactions, hydrogen bonds and electrostatic interactions. Thus it is suggested that the kafirin microparticle films with a greater proportion of β-sheet conformation than the glacial acetic acid cast film would be capable of forming more protein-protein interactions, by intermolecular hydrogen bonding and hydrophobic interactions between β-sheets than in glacial acetic acid cast kafirin films.

An extension of this idea for film formation is suggested by the ability of zein to assemble onto surfaces. Various workers have examined the ability of zein to form on surfaces of either hydrophilic or hydrophobic nature depending on the prevailing conditions of the experiment (Kogan et al., 2001; Wang, Giel and Padua, 2004). Kim and Xu (2008) demonstrated that zein can adhere to either hydrophilic or hydrophobic surfaces depending on the structural inversion of micelle-like zein particles. They suggest that under hydrophilic conditions, such as in solutions of less than 90% ethanol, zein micelles form with a hydrophilic end directed to the solvent. Under these conditions they showed zein particles adhering to a hydrophilic surface of glass spheres. Under hydrophobic conditions such as in solutions greater than 90% ethanol, zein micelles orientate themselves with the hydrophobic end directed to the solvent. In this case these workers demonstrated zein particles adhering to toner particles which are hydrophobic. It is suggested that something similar happens when free-standing kafirin films form from kafirin microparticles (Figure 5.4.1). As suggested above it is speculated that kafirin microparticles have a central core of α-kafirin stabilised by β-kafirin and surrounded by an outer layer of γ-kafirin. It is suggested that the properties of this outer layer is dictated by the hexapeptide repeat of γ-kafirin and so in dilute acid the amiphathic helix would have its hydrophilic side towards the solvent (Figure 5.4.1.b). As the water evaporates from the film forming suspension the environment becomes more hydrophobic and it is suggested that the amiphathic helix would invert and its hydrophobic face would be towards the solvent (Figure 5.4.1.d). Films are cast in plastic Petri dishes which have very hydrophobic surfaces. As the acid concentration increases further the secondary structure of the individual kafirin molecules would begin to unfold becoming a more open β-sheet conformation. The hydrophobic side of the molecule could then adhere to the hydrophobic surface of the Petri dish by hydrophobic interactions (Figure 5.4.1.e). 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. Thus, it is suggested that the kafirin molecules would be deposited on the surface of the Petri dish as a series of alternating hydrophobic and hydrophilic layers (Figure 5.4.1.e).

5.5. POTENTIAL APPLICATIONS OF KAFIRIN MICROPARTICLES

Potential uses for protein microparticles were reviewed in Chapter 2.5. In spite of there being many references in the scientific literature for use of microparticles made from proteins as potential drug delivery systems, there are few commercial applications (Sinha and Trehan, 2003). Low encapsulation efficiency, burst release, lack of consistency of microparticle size, and drug inactivation are listed as the main reasons for this (Radwick and Burgess, 2002, Sinha and Trehan, 2003). One of the anticipated uses for kafirin microparticles may be in the area of encapsulation of neutraceuticals. This was attempted by the encapsulation of sorghum polyphenols in order to maximize the health benefits of their antioxidant activity. This was found to be relatively successful, in that there was only a small burst release and antioxidant activity was released over a period of four hours.

Generally the conditions of microparticle preparation are often harsh, involving high temperatures, use of organic solvents and vigorous agitation resulting in physical and chemical degradation of the proteins themselves or the encapsulated material (Radwick and Burgess, 2002). These disadvantages also apply to any potential food applications for protein microparticles, for example fruit coatings. Since it is possible to form very thin coatings from kafirin microparticles it was thought that these may have a potential application as fruit coatings. Unfortunately, the acidic nature of the colloidal material burns the skin of the fruit as the coating dries (data not shown). Further work is required in order to overcome this problem. However some fruits such as lychees, which are prone to post harvest pericarp browning, undergo an acid dip as part of their post harvest processing without adversely affecting the fruit quality (Zauberman, Ronen, Akerman and Fuchs, 1990). Currently sulphur dioxide is used to prevent pericarp browning of lychees. Due to negative health connotations of the use of sulphur dioxide, exporters of lychees are looking for an alternative method of reducing post harvest pericarp browning of lychees. A kafirin microparticle coating could be used to reduce post harvest browning of lychees and possible have the added advantage of preventing or sealing pericarp micro-cracks, another post harvest defect of lychees which reduces shelf life.

Specific uses for kafirin microparticles in the biomedical area as substrates for tissue scaffolds or as artificial skin. The later two possibilities are suggested by the bone-like nature of the microstructure of kafirin microparticles when exposed to high shear and the

thinness and functional properties of kafirin microparticle films. However, one area of concern for the potential application of kafirin microparticles for tissue scaffolds is the lack of robustness of the material. Some form of strengthening of the microparticle structure, possibly by cross-linking as described below, would be necessary for this application.

5.6. IMPROVEMENT OF KAFIRIN MICROPARTICLE PROPERTIES AND FUTURE WORK

Kafirin microparticles made by the process used in this study are very fragile and their micro-structure is easily broken by the application of shear. A suggestion for potential improvement of kafirin microparticle properties is to expose either the original kafirin or the preformed kafirin microparticles or films made from them to cross-linking agents. Both physical, chemical and enyzmic methods have been used to cross-link protein microparticles and make them more resistant to degradation (Patil, 2003). 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.

Physical cross-linking by application of heat has the disadvantage 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, Remondetto and Subirade, 2006). Microwave energy has been used as an alternative to cross-link gelatin microparticles producing insoluble but swellable microspheres (Vandelli, Romagnoli, Monti, Gozzi, Guerra, Rivasi and Forni, 2004). Both kafirin and kafirin films have been shown to have improved functional properties when heated by microwave energy (Byaruhanga, Erasmus and Taylor, 2005; Byaruhanga, Erasmus, Emmambux and Taylor, 2007). Thus application of microwave energy may be expected to have a positive affect on kafirin microparticle and kafirin microparticle film properties.

Enzymes, for example transglutaminase, have also been used successfully as protein cross-linking agents (Gouin 2004). This is potentially a food compatible cross-linking method which could be used to improve kafirin microparticle properties.

Chemical cross-linking agents such as 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). Disadvantages include toxic side effects due to residual cross-linking chemicals and unwanted reactions between the encapsulated drug and the cross-linking agent (Burgess and Hickey, 1994; Chen et al., 2006). Additionally, these cross-linking agents are not suitable for food use. Strauss and Gibson (2004) suggested the use of plant derived polyphenols as alternative 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. Preliminary work in this study (Section 4.3.4.2.) and work by Emmambux, Stading and Taylor (2004) has shown that cross-linking with sorghum condensed tannins improved kafirin film properties by decreasing protein digestibility, biodegradation and oxygen permeability whilst improving tensile stress (Emmambux et al., 2004). Also when sorghum condensed tannins were encapsulated within kafirin microparticles, protein digestibility was greatly reduced. Consequently, it may be expected that cross-linking kafirin microparticles with sorghum condensed tannins would have a positive affect on their properties, potentially making them more resistant to enzymic digestion and increasing their mechanical strength and improving the functional properties of films made from them.

As previously stated, the main cause of poor protein digestibility of sorghum is thought to be protein cross-linking involving γ- and β-kafirins, which inhibit the digestion of the major storage protein α-kafirin (Duodu, Taylor, Belton, and Hamaker, 2003). In addition γ-kafirin is capable of binding more condensed tannins than other kafirin species (Taylor et al., 2007). Thus it would be expected that increasing the amount of γ-kafirin, in conjunction with gentle heating or addition of very small amount of sorghum condensed tannins as cross-linking agents would result in decreasing protein digestibility of both kafirin microparticles and kafirin microparticle films and improve tensile properties, oxygen barrier properties and biodegradation of microparticle films. Thus, another approach to improve kafirin microparticle and kafirin microparticle film properties is to isolate kafirin from sorghum grain enriched with γ-kafirin or to add isolated γ-kafirin when preparing kafirin microparticles. Sorghum grain with high levels of γ-kafirin could be identified by screening non tannin grain for very low protein digestibility.

The recent drive into production of bio-ethanol made by the dry-grind process from maize and sorghum has resulted in large volumes of by-products which are rich in protein and of relatively low value which are being used presently as animal feed. The major by-product, distillers dried grains with solubles (DDGS) has a protein content of 28-35% and its price is determined by its protein content (Kwiatkowski, McAloon, Taylor and Johnston, 2006). Currently it is produced at a rate of approximately 119 million kg/year. Kale, Zhu and Cheryan (2007) quote predictions of ethanol production from the dry-grind process at 30 billion litres by 2012. As more bio-ethanol plants come on line the amount of DDGS available will increase dramatically. In order to make the process of ethanol production cost effective high value addition products need to be produced from DDGS. Extraction of the prolamin proteins of sorghum and maize from these by-products have a potential use as protein microparticles for biomedical and food use and as bio-plastic materials for packaging. Not only would these products be a value addition from waste material they have potential to replace at least in part some of the packaging materials which are currently being made from non renewable, non biodegradable petroleum based sources. In order for this to come to fruition research will be needed into the extraction of these proteins from DDGS and of preparation techniques for microparticle and films in order to optimise their functional properties for specific applications.

Finally, further research is needed in the area of microparticle formation and film formation on a molecular level in order to better understand the processes and be able to manipulate the functional properties more effectively for specific end use purposes. This could be done by the use of a more powerful AFM than was available for this study. It would hopefully allow the visualisation the molecular arrangement within kafirin microparticles and films made from them. In addition it would be useful to try and determine the role of the individual kafirin species in kafirin microparticle formation and also in kafirin microparticle film formation. Initially electron microscopy, both SEM and TEM with immunolocalisation techniques would help to determine the position of each of the kafirin species relative to each other within the kafirin microparticle. Then, the individual kafirin species could be extracted and an attempt to make kafirin microparticles with them individually, in different combinations and using different proportions of each. Again electron microscopy, both SEM and TEM with immunolocalisation techniques and possibly powerful AFM would show if the use of different kafirin species affect the micro-structure and molecular arrangement of kafirin microparticles. When possible

these modified kafirin microparticles could be used to make films and the functional and structural properties of these films examined.

6. CONCLUSIONS AND RECOMMENDATIONS

A simple, novel, ethanol free method for making kafirin microparticles has been devised. It involves the dissolution of kafirin in glacial acetic acid followed by the precipitation of kafirin microparticles on the addition of water. Kafirin microparticles so formed have unique properties, including an extremely large internal surface area. Manipulation of the final acetic acid concentration results in kafirin microparticle with differing characteristics, which may have potential applications in the food, biomedical and pharmaceutical industry as agents for encapsulation, film formation and tissue engineering.

These same kafirin microparticles may be used to form very thin free standing films and coatings. There is a minimum amount of acid required before a free standing film can be formed from kafirin microparticles in relation to the amount of protein present. When acetic acid was the organic acid used, this relative amount was 10.8:1, percent organic acid to percent protein. Film formation appears to involve a controlled aggregation of the kafirin microparticles and then dissolution of the microparticles in the acid before drying into a cohesive film matrix. Some of the functional properties, e.g. film surface properties, WVP and reduced protein digestibility of these films are superior to those of conventionally cast kafirin films at the same protein content. However, the tensile properties of these films need to be improved before they can be used for practical applications. Cross-linking by physical or chemical agents is suggested as a potential means of improving kafirin microparticle film tensile properties.

The importance of disulphide cross-linking and sorghum condensed tannin protein interactions has been confirmed as major causal factors of the poor protein digestibility of sorghum. Gamma-kafirin has been found to bind the most condensed tannins compared to the α-and β-kafirins, probably due to its high proline content. As expected the protein digestibility of kafirin-tannin complexes is much lower than unbound kafirins. This influences the biodegradation of kafirin films made with bound tannins. They appear to have extended life due to a decrease in protein digestibility caused by kafirin-tannin binding.

Finally, a practical application of kafirin microparticles was attempted and found to be feasible. Encapsulation of catechin and sorghum condensed tannins within kafirin microparticles was found to be an effective way to exploit the binding properties of polyphenols with protein to enhance potential health benefits by controlled release of antioxidant activity within the stomach and gastrointestinal tract.

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