4. RESEARCH

4.1. FORMATION AND CHARACTERISATION OF KAFIRIN MICROPARTICLES BY PHASE SEPARATION FROM AN ORGANIC ACID

4.1.1. Abstract

Zein microparticles have potential for food and pharmaceutical applications. However, preparation involves aqueous ethanol as a solvent for the zein. Ethanol is flammable, needs a government license for its use and is unacceptable for food use by some religious communities. Thus an ethanol free method of making microparticles would be advantageous. Kafirin is more hydrophobic and less digestible than zein so may form microparticles with superior properties. An ethanol free method of making kafirin microparticles by phase separation was devised. Glacial acetic acid was used as kafirin solvent and the microparticles formed on addition of water. The kafirin microparticles were characterized by Light Microscopy (LM), Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM) and their size distribution were measured. The microparticles were mainly spherical, porous and between 1-10 μm in diameter. The kafirin microparticles had very large internal surface area probably due to entrapment of air during microparticle formation. This may have advantages for encapsulation of food ingredients or pharmaceuticals. Additionally, as the final concentration of acetic acid increased, the microparticles changed from porous spheres to an open matrix, coinciding with a change in secondary structure from α-helical to β-sheet, indicative of protein aggregation. Potentially, the open matrix structure may have medical applications for example tissue scaffolds.

4.1.2. Introduction

Microspheres, also known as microparticles, when not true spheres, can be made from proteins. Protein microparticles can be used for a variety of applications. Zein microparticles made from the prolamin protein of maize has potential for use as food coatings (Cook and Shulman, 1998), encapsulation of essential oils (Parris, Cooke and Hicks, 2005), drug delivery (Liu, Sun, Wang, Zhang and Wang, 2005), tissue engineering (Dong, Sun and Wang, 2004), drug eluting films (Wang, Lin, Liu, Sheng and Wang, 2005) and delayed release of pesticides (Demchak and Dybas, 1997).

Most processes for microparticle preparation are modifications of three basic techniques: spray drying, solvent extraction/evaporation and phase separation/coacervation (Whittlesay and Shea, 2004; Sinha and Trehan, 2003). Zein microparticles have been made by using 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). When phase separation is used for zein microparticle preparation the zein is generally dissolved in aqueous ethanol. Water or an acid is used as the second solvent, which may include a coacervation agent, for example an oil or another organic solvent (Coombes, Lin, O'Hagen and Davis, 2003). Demchak and Dybas (1997) used acetic acid (concentration unstated) as an alternative to ethanol as a zein solvent and then emulsified the resulting solution into lecithin.

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 or microparticles (Dong et al., 2004; Lui et al., 2005; Parris et al., 2005; Wang et al., 2005; Hurtado-Lopez and Murdan, 2006a; Muthiselvi and Dhalathreyan, 2006). Ethanol is flammable, needs a government license for its use and is unacceptable for food use by some religious communities. Thus an ethanol free method of making microparticles would be advantageous.

Kafirin, the prolamin protein of sorghum has potential for microparticle preparation and use. DeRose, Ma, Kwon, Hasnain, Klassy and Hall (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 (reviewed by Duodu, Taylor, Belton and Hamaker, 2003), a factor which may make kafirin microparticles less susceptible to bacterial attack, enabling delivery of the encapsulated agent to the desired site.

This study describes a simple, ethanol free method of making kafirin microparticles which have a very large internal surface area.

4.1.3. Materials and methods

4.1.3.1. Materials

A mixture of two condensed tannin-free, tan plant, white sorghum cultivars PANNAR PEX 202 and 206 were used for kafirin extraction using the method described by Emmambux and Taylor (2003). Decorticated, milled grain, particle size less than 8 mm was extracted with 70% (w/w) aqueous ethanol containing 5% sodium hydroxide (w/w) and 3.5% sodium metabisulphite (w/w) at 70°C for one hour with vigorous stirring. The extractant was recovered by centrifugation and the ethanol removed by evaporation. Kafirin was precipitated on pH adjustment of the protein suspension to approximately pH 5 and recovered by filtration under vacuum and freeze dried. The kafirin was defatted with hexane at ambient temperature and air dried. The protein content of the kafirin was 88% (N x 6.25) as determined by the Dumas combustion method (American Association of Cereal Chemists (2000).

4.1.3.2. Preparation of kafirin microparticles with acetic acid

Plasticiser (0.66 g 1:1:1 lactic acid, polyethylene glycol (400), glycerol-40% in relation to protein) was mixed with glacial acetic acid (4.34 g) and added to kafirin (1.8 g, 88% protein) with gentle stirring. The temperature, was slowly raised to 30°C to ensure full solvation of the kafirin. This solution was then allowed to 'rest' for 16 h. After this period distilled water, at ambient temperature was added slowly over a period of 5 min with stirring to a total weight of 80 g. On addition of the water microparticles formed. The concentration of protein was 2%, with an acetic acid concentration of 5.4%. Freeze dried microparticles were prepared by removing the acid by centrifugation and washing the pellet containing the microparticles with distilled water. This was repeated three times and finally the supernatant was removed before freeze drying the resultant pellet.

4.1.3.3. Preparation of kafirin microparticles with lactic acid or propionic acid

The method was followed as above substituting lactic acid or propionic acid for glacial acetic acid. When lactic acid was used all the lactic acid was absorbed by the kafirin. A further 5 g of lactic acid was needed before a solution was formed.

4.1.3.4. Preparation of kafirin microparticles without plasticiser

Kafirin microparticles were prepared as described above but without the addition of plasticiser.

4.1.3.5. Preparation of kafirin microparticles with gas saturated or degassed solvents

Glacial acetic acid and distilled water were degassed by boiling for 10 min and then sealed in gas tight bottles to cool. Glacial acetic acid and distilled water were saturated with gas by bubbling air through the solvents for 1 h and then sealed in gas tight bottles prior to use. Kafirin microparticles were then prepared by the method described above without the addition of a plasticiser, either using the degassed or gas saturated solvents.

4.1.3.6. Preparation of kafirin microparticles with aqueous ethanol

Kafirin (1.26 g, 88% protein) was dissolved in 70% (w/w) aqueous ethanol (15 ml) at 70°C. The solution was allowed to 'rest' for 16 h. On cooling the kafirin precipitated. The suspension was reheated to dissolve the kafirin before distilled water was added slowly over a period of 5 min with stirring to a total weight of 55 g. As with acetic acid method, on addition of water the microparticles formed. The concentration of protein was 2%.

4.1.3.7. Effect of shear on microparticle formation

Microparticles were prepared in acetic acid or aqueous ethanol as described above but water was added whilst samples were mixed with an Ultra Turrax (Janke and Kunkel, IKA Labortechnik, Staufen, Germany) at 13500 rpm for 2 min.

4.1.3.8. Size, shape and size distribution of kafirin microparticles

Suspensions of kafirin microparticles, 2% (w/w on protein basis) protein, in different acetic acid concentrations were prepared by centrifuging aliquots of microparticles suspension in 5.4% acetic acid at 3880 g for 5 min, decanting off the supernatant and replacing it with an equivalent weight of higher (10.8%, 21.6%, 30%, 40%) acetic acid concentration containing placticiser. Samples were mixed and left overnight before viewing and photographing under phase contrast conditions using a Nikon Optiphot light microscope (Kanagawa, Japan). Size of the microparticles was determined by comparing the microparticle images with that of a scale bar of the same magnification. At least 150 microparticles of each treatment were measured. These microparticles were compared with aqueous ethanol prepared kafirin microparticles.

4.1.3.9. Effect of storage on kafirin microparticle size distribution

Samples of kafirin microparticles (2% protein w/w basis) in suspension of 5.4% and 21.6% acetic acid were examined as above after storage at 8°C for at least one month.

4.1.3.10. Electron Microscopy of microparticles

Wet preparations of microparticles were prepared for Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM) by removing the liquid fraction and fixing in gluteraldehyde in pH 7.4 phosphate buffer before staining with osmium tetraoxide. Samples were dehydrated sequentially in acetone. TEM samples were infiltrated with Quetol resin and polymerized at 60°C. Sections were cut and stained with ural acetate and lead citrate. SEM samples were subjected to critical point drying before mounting on a stub and sputter coated with gold. Freeze dried microparticles were mounted on a stub with double sided tape and sputter coated with gold. TEM preparations were viewed with Philips EM301 Transmission Electron Microscope (Eindhoven, Netherlands). SEM preparations were viewed with a Joel JSM-840 Scanning Electron Microscope (Tokyo, Japan).

4.1.3.11. SDS-PAGE

Protein preparations were characterised by SDS-PAGE on a 4-18% acrylamide gradient both under reducing and non-reducing conditions. The gradient was prepared from a stock of 40% (w/v) acrylamide/bis (19:1) (Merck, Halfway House, South Africa) and allowed the separation of proteins with a molecular weight of 14 to 200 k. A vertical electrophoresis system (Protean II, Bio-Rad, Hercules, USA) was used with gels of 140 mm length and 1.5 mm thick according to Gallagher (1999). The different protein preparations were loaded to constant protein (≈15 μg) as determined by the Dumas combustion method (American Association of Cereal Chemists, 2000). Molecular weight markers (low-range protein marker, Roche Molecular Biochemicals, Indianapolis, IN, USA) were used. Proteins were stained with Coomassie Brilliant Blue R250.

4.1.3.12. Fourier Transform Infrared Spectroscopy (FTIR)

Samples were scanned using a Perkin Elmer Spectrum GX FTIR system, (Waltham, MA, USA), using 32 scans, 8 cm^{-1} band and an interval of 1 cm in the Attenuated Total Reflectance (ATR) mode. The FTIR spectra were Fourier-deconvoluted with a resolution enhancement factor of 2 and a bandwidth of 12 cm^{-1}.

4.1.4. Results and discussion

4.1.4.1. Morphology of kafirin microparticles

Preparation of kafirin microparticles by phase separation, using glacial acetic acid as a solvent for kafirin resulted in the formation of kafirin microparticles of different form and size distribution to those of kafirin microparticles prepared by a method similar to that of Parris et al. (2005) using aqueous ethanol as a solvent for kafirin and with gentle mixing (Figure 4.1.1.a, c, SEM outer surface, Figure 4.1.2.a, c, TEM inner surface). Generally the kafirin microparticles made using glacial acetic acid as kafirin solvent, were spherical or irregular in shape with a rough porous surface and numerous internal holes or vacuoles as shown by SEM (Figure 4.1.1.a, outer surface) and TEM (Figure 4.1.2.a, internal structure). This method of preparation resulted in the formation of microparticles with a very large surface area. As an example, the total surface area of the acetic acid prepared microparticle illustrated in Figure 4.1.2.a is approximately 116 μm^2 compared with that of a similar sized aqueous ethanol prepared microparticle illustrated in Figure 4.1.2.c, of 60 μm^2. Thus the holes in the acetic acid microparticle approximately doubled the microparticle surface area.

Aqueous ethanol prepared microparticles were mainly small, smooth spheres (Figure 4.1.1.c) with no or very few internal holes (Figure 4.1.2.c). The larger aqueous ethanol prepared microparticles did have more internal holes as illustrated in Figure 4.1.2.c. In the literature, SEM of zein microspheres shows them to be spherical with a smooth surface (Dong et al., 2004; Liu et al., 2005; Parris et al., 2005; Wang et al., 2005). The method and conditions of preparation affects the size and properties of microparticles (Sinha and Trehan, 2003). Since the conditions of preparation were the same except for the use of different solvents, the difference in size between acetic acid prepared microparticles and aqueous ethanol prepared microparticles appears to be due to the effect of the different solvents. Possibly the low pH in the presence of acetic acid may have resulted in the aggregation of protein molecules resulting in larger microparticles. This will be discussed in more detail later.

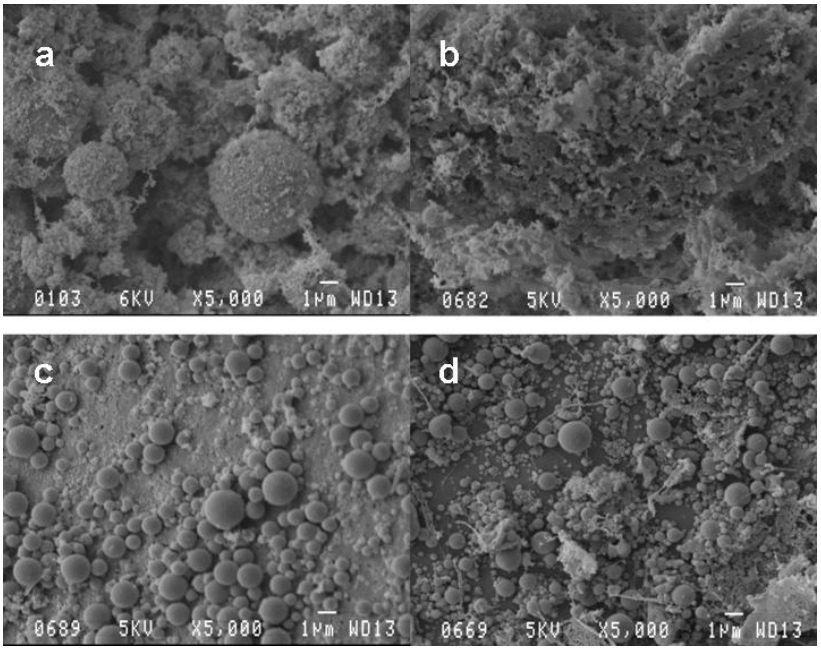


Figure 4.1.1: SEM of kafirin microparticles made with acetic acid or aqueous ethanol a-Acetic acid, low shear, b-Acetic acid, high shear, c-Aqueous ethanol, low shear, d-Aqueous ethanol, high shear

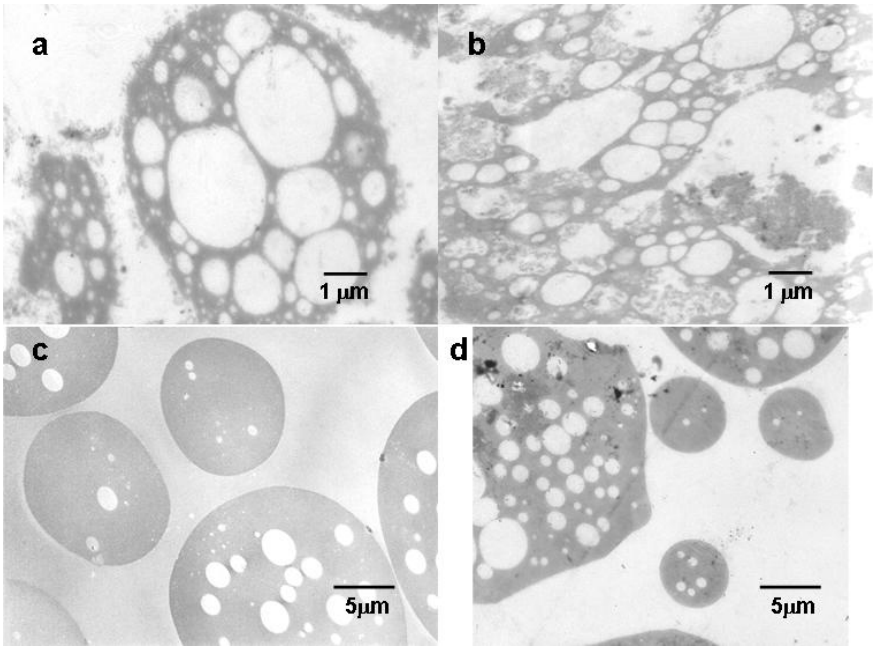


Figure 4.1.2: TEM kafirin microparticles made with acetic acid or aqueous ethanol a-Acetic acid, low shear, b-Acetic acid, high shear, c-Aqueous ethanol, low shear, d-Aqueous ethanol, high shear

4.1.4.2. Size distribution

The size distribution (mean diameter) of kafirin microparticles made using glacial acetic acid as the kafirin solvent was between 1-10 µm (Figure 4.1.3.). Kafirin microspheres made by the method of Parris et al. (2005) using aqueous ethanol as solvent were generally smaller than those made using glacial acetic acid as kafirin solvent, the majority being between 1-3 µm (Figure 4.1.3.). Parris et al. (2005) reported sizes of zein microspheres between 0.05-0.1 µm. The smaller size of these microparticles would be expected as high speed mixing using a Ultra Turrax homogenizer not gentle stirring was used during their preparation. Increasing mixing speed generally decreases microparticle size due to stronger shear forces and greater turbulence (Freitas, Merkle and Gander, 2005). This is generally supported by the fact that other workers who have used gentle stirring during zein microparticle preparation reported zein microparticle diameter to range between 0.25-2.5 µm (Dong et al., 2004), 0.25-1.88 µm (Hurtado-López and Murdan, 2005) and 1-1.7 µm (Muthuselvi and Dhathathreyan, 2006).

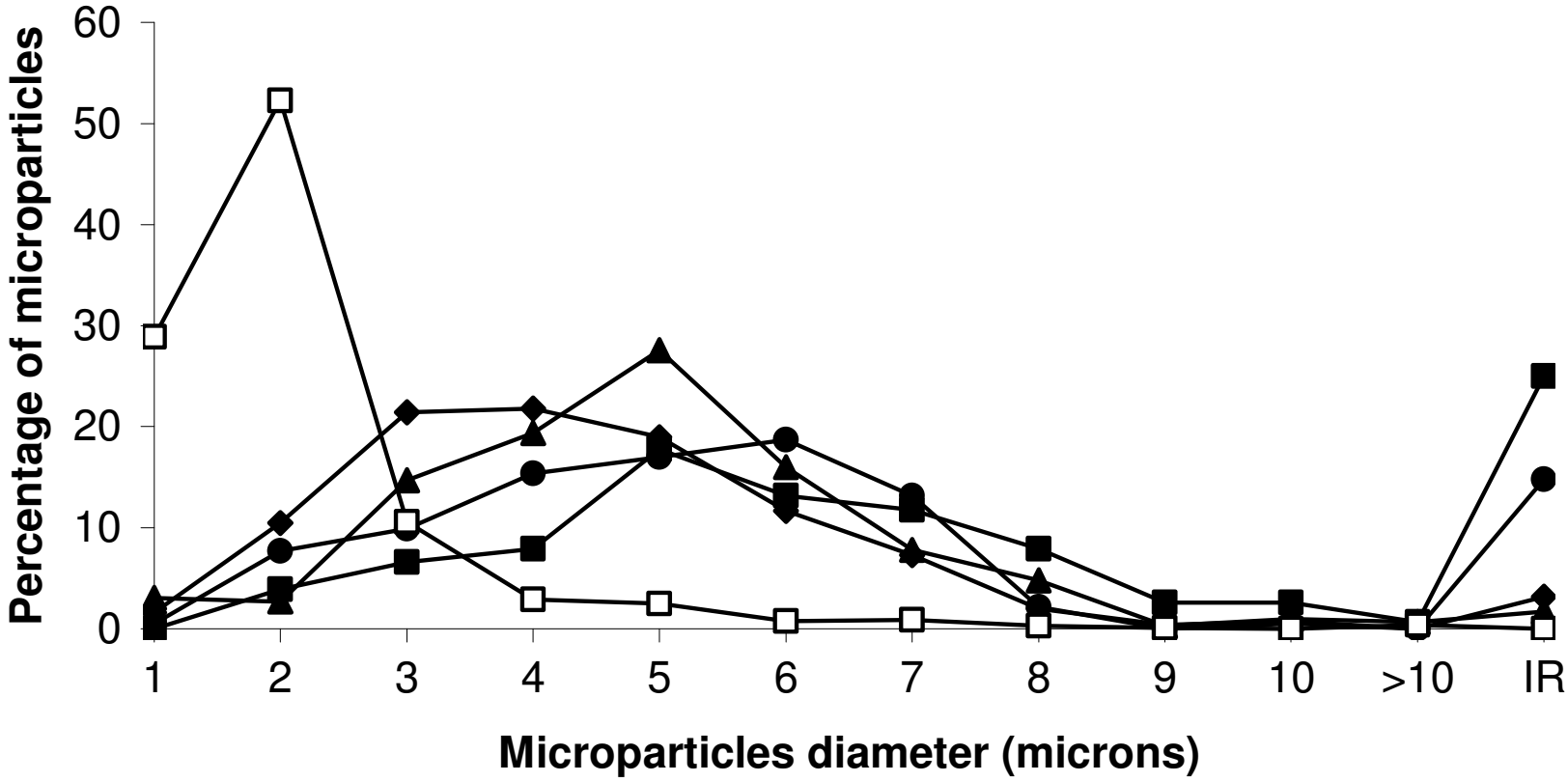


Figure 4.1.3: Particle size distribution of kafirin microparticles
5.4% acetic acid (diamond), 10% acetic acid (triangle), 21.6% acetic acid (circle), 30% acetic acid (square), aqueous ethanol (open square)

4.1.4.3. Effect of shear

The effect of shear was investigated on the preparation of karirin microparticles made with both glacial acetic acid and aqueous ethanol (Figures 4.1.1., 4.1.2.). Generally, kafirin microparticles made with aqueous ethanol resulted in more smaller microparticles and some continuous matrix (Figure 4.1.1.d) when higher shear (Ultra Turrax) was applied than when lower shear was used (magnetic stirring) (Figure 4.1.1.c). This is consistent with the concept that the speed and duration of mixing during droplet formation 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. TEM of aqueous ethanol formed kafirin microparticles showed that application of higher shear during preparation appeared to increase the size of holes in the microparticles. The increased turbulence with high shear would incorporate more air into the liquid phase, the bubbles possibly coalescing to form larger bubbles before being entrapped within the microparticles as the protein precipitated.

Acetic acid prepared microparticles behaved differently to those made with aqueous ethanol on the application of high shear. Instead of resulting in smaller microparticles, a continuous matrix formed (Figure 4.1.1., 4.1.2.). This may have been due to the high shear breaking the microparticles apart. The continuous matrix was possibly due to the aggregation of fragments of the microparticles. Thus it appears that because of their open porous structure microparticles made with acetic acid are more fragile and less able than aqueous ethanol produced microparticles to withstand high shear, consequently their sphere-like structure was completely destroyed.

4.1.4.4. Effect of changing the acetic acid concentration on microparticle size and morphology

When kafirin microparticles were made by dissolving the kafirin in glacial acetic acid, the final acetic acid concentration could be varied resulting in kafirin microparticles of different sizes and structure (Figure 4.1.4. a-e) and can be compared with those made with aqueous ethanol (Figure 4.1.4.f). At low acid concentration (5.4%) (Figure 4.1.4.a) the majority of microparticles had a diameter between 3-4 μm (Figure 4.1.3.). At 10.8% acid (Figure 4.1.4.b) the microparticle diameter had increased to 5 μm (Figure 4.1.3.) and at

21.6% (Figure 4.1.4.c) to 6 µm (Figure 4.1.3.). As the acetic acid concentration was increased further to 30%, the microparticles lost their spherical shape appearing to form aggregates of larger overall size (Figures 4.1.3., 4.1.4.d) than the spherical particles. When the acid concentration reached 40% the kafirin microparticles appeared to dissolve in the acetic acid (Figure 4.1.4.e).

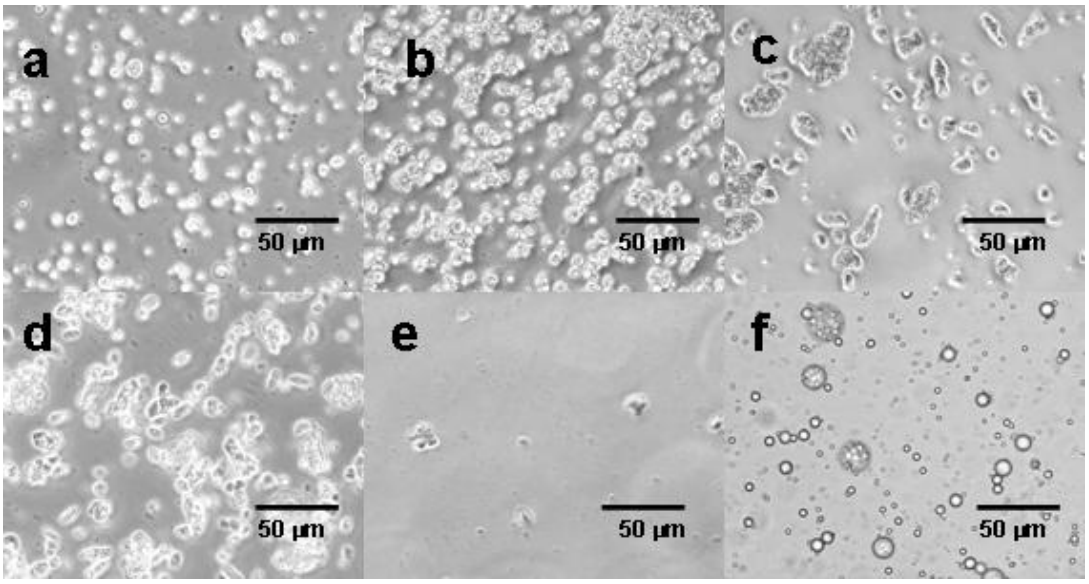


Figure 4.1.4: Light microscopy to illustrate the effect of increasing acetic acid concentration on preformed kafirin microparticles (Appearance of kafirin microparticles made with aqueous ethanol for comparison)
a- 5.4%, b-10.8%, c-21.6%, d-30% e-40% acetic acid concentrations respectively
f- aqueous ethanol prepared microparticles

Figure 4.1.5. illustrates how the external (SEM) and internal (TEM) appearance of the kafirin microparticles changes with increasing acetic acid concentration. At low acid (5.4%) concentration (Figure 4.1.5.a, d) the microparticles appear to be mainly spherical with a rough, porous surface. Internally there are a few holes or vacuoles. As the acetic acid concentration increases to 21.6%, the number of spherical microparticles decreases and there are more aggregated particles but the rough, porous surface remains (Figure 4.1.5.b). Internally these microparticles appear to have more holes or vacuoles of varying size (Figure 4.1.5.e). At 40% acetic acid the spherical microparticle structures have completely disappeared and have been replaced by an open matrix which resembles an expanded foam (Figure 4.1.5.c). The same matrix can be seen by TEM (Figure 4.1.5.f). Some spherical structures can also be seen embedded within the matrix by TEM.

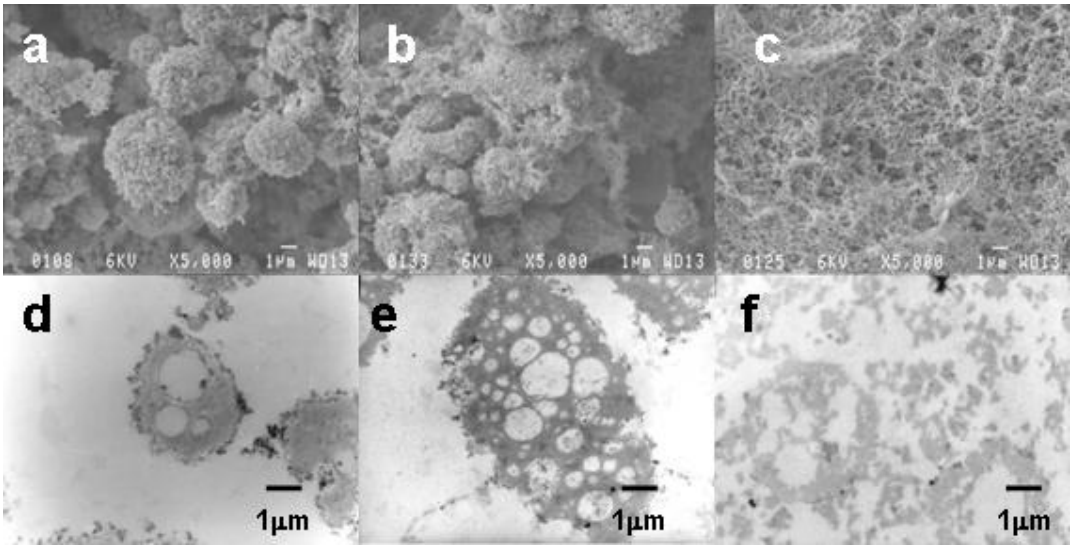


Figure 4.1.5: SEM and TEM of kafirin microparticles at different acetic acid concentrations
a-c SEM, d-f TEM, a, d-5.4%, b, e-21.6%, c, f-40% acetic acid

4.1.4.5. Effect of storage on microparticle size and size distribution

A comparison of stored and freshly prepared microparticle size distribution followed the same trend (data not shown). As the acid concentration increased, both sets of microparticles showed a similar increase in size and number of irregular shaped particles. Thus on storage at 8°C for at least one month there was no obvious change in microparticle size.

4.1.4.6. Effect of freeze drying on microparticle morphology

The microparticles could be freeze dried (Figure 4.1.6. b-e). They were similar in appearance to wet preparations of the same (Figure 4.1.1.) but had slightly smoother surfaces. Interestingly the original kafirin from which the microparticles were made also appears to contain some spherical microparticles (Figure 4.1.6.a). As stated this kafirin was extracted with aqueous ethanol (70%) at elevated temperature. The kafirin was recovered after the removal of ethanol by evaporation by precipitation on addition of cold water and pH adjustment. The presence of kafirin microparticles in this preparation is not surprising since it was similar to the method used by Parris et al. (2005) to make zein microparticles. Parris et al. (2005) dissolved zein in aqueous ethanol and precipitated microparticles on addition of water.

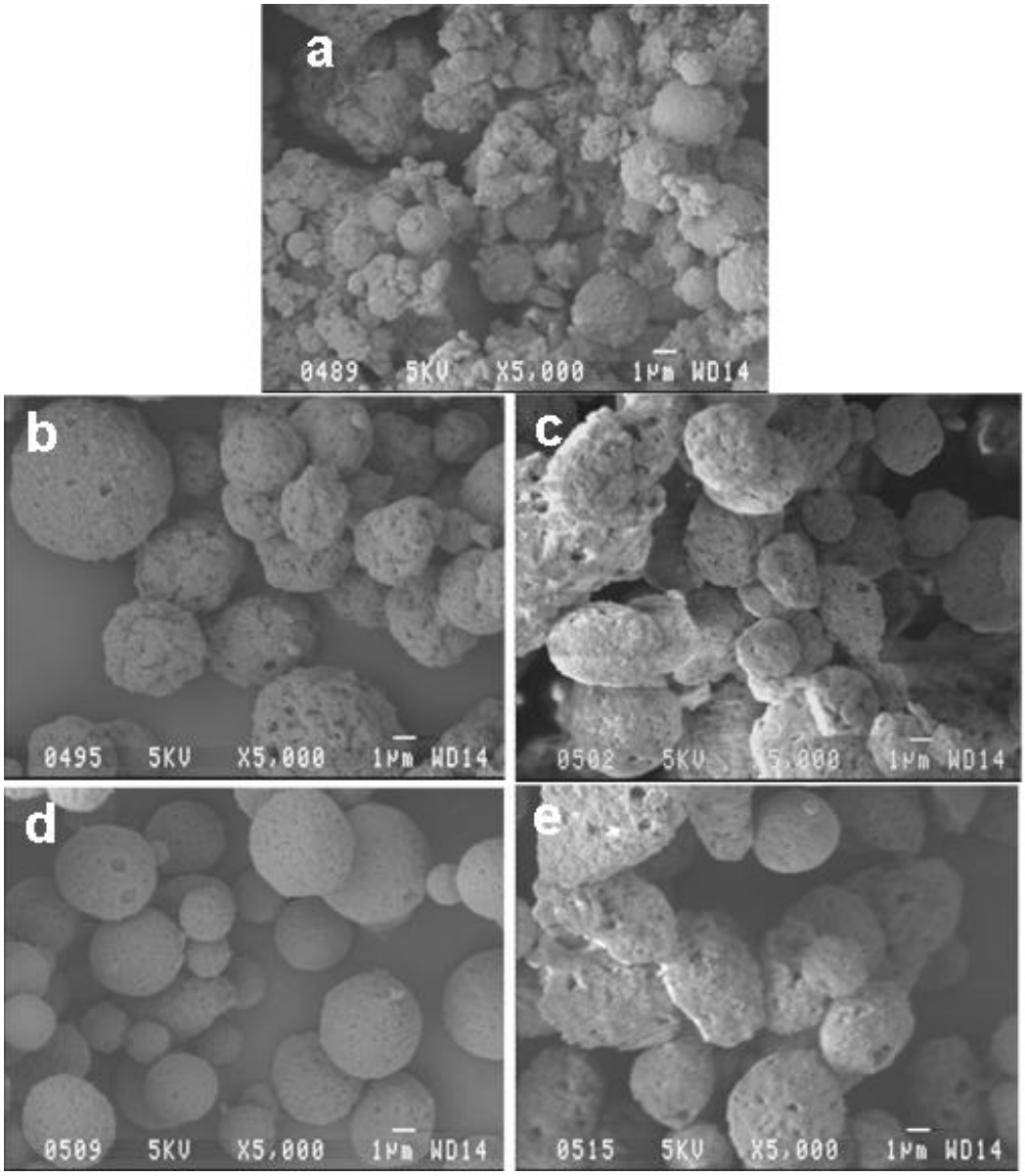


Figure 4.1.6: SEM freeze dried microparticles and kafirin used to prepare them
a kafirin, b,c newly prepared microparticles, 5.4% and 21.6% acetic acid concentration respectively, d,e stored microparticles, 5.4% and 21.6% acetic acid concentration respectively

4.1.4.7. SDS-PAGE of kafirin and kafirin microparticles

SDS-PAGE of kafirin and kafirin microparticles was run under reducing and non-reducing conditions (Figure 4.1.7.). In both cases the band pattern was the same for the original kafirin and the kafirin microparticles. The intensity of the bands for the original kafirin was slightly darker than for the kafirin microparticles. This may have been due to a difference in protein content of the material applied as the protein content of the microparticle preparation was estimated, whereas that of the original kafirin was based on a measured value. Under both reducing and non-reducing conditions bands with molecular weights of 22-26 k, 18 k, 14 k and 50 k were present. The broad band 22-26 k is probably equivalent to γ- (26 k), α1- (24 k), α2- (22 k) bands reported by El Nour, Peruffo and Curioni (1998). The bands probably did not resolve into individual bands because the gel was overloaded. This was done in order that the minor bands could be visualized. The 18 k band is consistent with the β- (18 k) kafirin reported by El Nour et al. (1998). The 14 k and 50 k bands were possibly γ-kafirins, equivalent to the 16 k and 50 k bands of γ-zein reported by Kim, Woo, Clore, Burnett, Carneiro and Larkins (2002).

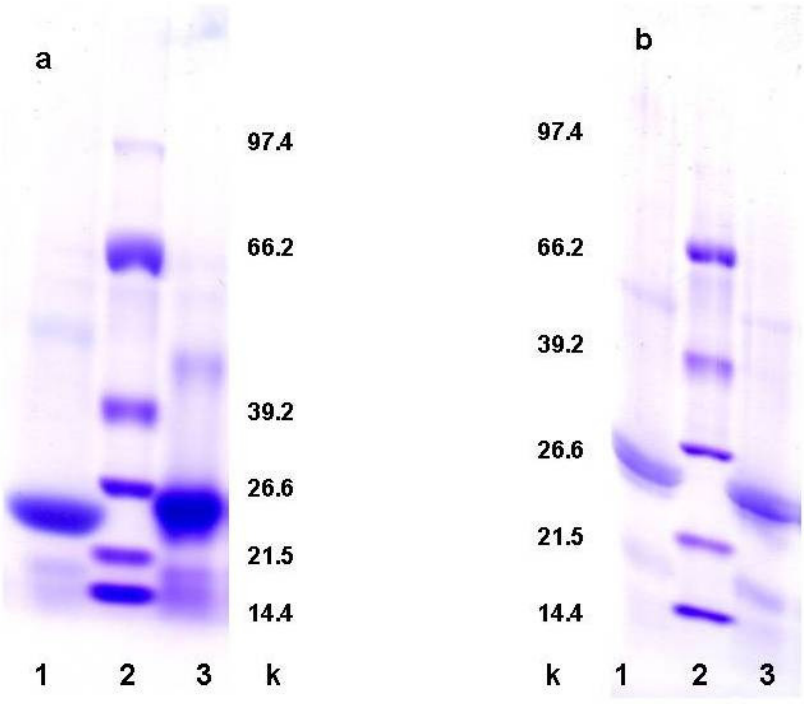


Figure 4.1.7: SDS-PAGE of kafirin microparticles (Track 1) and original kafirin (Track 3) under reducing (a) and non-reducing (b) conditions. Molecular weight marker (Track 2)

In spite of the severity of the conditions, there was no apparent effect on the kafirin proteins of the microparticle preparation, procedure. When kafirin was extracted with glacial acetic acid, Taylor, Taylor, Dutton and de Kock (2005) found typical kafirin SDS-PAGE band patterns under reducing conditions. Under non-reducing conditions, when dialysis or pH adjustment at 25°C was used for kafirin recovery, Taylor et al. (2005) reported the presence of a possible disulphide linked dimer of β-kafirin in addition to γ-, α1-, α2- and β-kafirin. Although the conditions used by Taylor et al. (2005) were similar to those used for microparticle preparation no dimer of β-kafirin was observed. This may be due to the difference in extraction solvents. Kafirin used for microparticle preparation was extracted with an aqueous ethanol based solvent whereas Taylor et al. (2005) used glacial acetic acid after a metabisulphite presoak.

4.1.4.8. Fourier Transform Infrared Spectroscopy (FTIR) of kafirin microparticles

FTIR spectra of freeze dried kafirin preparations prepared at different acid concentrations show changes in kafirin secondary structure (Table 4.1.1. and Figure 4.1.8.). Considering the amide I band of the FTIR spectra of the freeze dried preparations, two main peaks were observed at wavenumber 1650 and 1620 nm (Figure 4.1.8.). According to Duodu, Tang, Wellner, Belton and Taylor (2001) the peak around 1650 cm^{-1} can be assigned to α-helical conformations and 1620 cm^{-1} to anti-parallel β-sheet conformations. When the ratio of α-helical to intermolecular β-sheet conformation is calculated there is a significant difference between the original kafirin that the microparticles were formed from and kafirin microparticles at increasing acid concentrations (Table 4.1.1.). In its native state kafirin is approximately 60% α-helical (Duodu et al., 2001; Belton et al., 2006). The original kafirin and kafirin microparticles at low (5.4%) acid concentration had the highest ratio of α-helical to intermolecular β-sheet conformation of the preparations examined, 1.29 and 1.30 respectively (Table 4.1.1.). This indicates that these preparations were similar in secondary structure and contained more α-helices to intermolecular β-sheet than the other samples examined. This is slightly higher than the ratio of α-helical to intermolecular β-sheet conformation (1.10) reported by Gao, Taylor, Wellner, Byaruhanga, Parker, Mills, Belton (2005), for kafirin extracted and dried under the same conditions. These differences in ratios can be attributed to the use of different batches of kafirin. As the acetic acid concentration of the microparticles increased to 21.6%, the ratio of α-helical to intermolecular β-sheet conformation decreased indicating an increase

in the amount of β-sheet present. This change was observed regardless of the age of the microparticles and is indicative of protein aggregation (Mizutani, Matsumura, Imamura, Nakanishi, Mori, 2003). Protein aggregation was also illustrated by SEM and TEM (Figure 4.1.5.) and shown by kafirin microparticle size and size distribution at increasing acid concentrations (Figure 4.1.3.).

Table 4.1.1: Estimated ratio of α-helical to intermolecular β-sheet conformation of kafirin and kafirin microparticles

	Ratio of α-helices to β-sheets	
	Amide I[a]	**Amide II[b]**
Kafirin	1.29 (0.03)b[c]	1.28 (0.19)a[d]
Kafirin microparticle, stored, 5.4% acid	1.31 (0.01)c	1.21 (0.11)a
Kafirin microparticle, stored, 21.6% acid	1.08 (0.02)a	1.05 (0.16)a
Kafirin microparticle, freshly prepared, 5.4% acid	1.30 (0)bc	1.18 (0.18)a
Kafirin microparticle, freshly prepared, 21.6% acid	1.07 (0.01)a	1.04 (0.24)a

[a] Amide I: α-helices (1650cm^{-1})/ β-sheets (1620cm^{-1}). [c] Values for amide I with different letters are significantly different ($p<0.05$)
[b]. Amide II: α-helices (1545cm^{-1})/ β-sheets (1516cm^{-1}). [d]Values for amide II with different letters are significantly different ($p<0.05$)

Kafirin is rich in hydrophobic amino acids. Alpha-, β-, and γ-kafirins have 15.3, 12 and 8.3 mole % leucine, 14.9, 13.4 and 5.7 mole % alanine and 7.7, 9.7, and 23.3 mole % proline respectively (Taylor and Belton, 2002). Thus in suspension at low acetic acid concentration it is likely that the protein folds into a globular shape with the hydrophobic amino acids facing inwards. At higher acetic acid concentration it is possible that the kafirin secondary structure unfolds, changing from predominately α-helical structure to a more open β-sheet conformation, exposing previously hidden hydrophobic amino acids. This would then allow hydrophobic interactions between polypeptide chains. The more open structure would also expose other ionic and neutral amino acids which would then be available to form inter and intra molecular hydrogen bonds. The combination of hydrogen bonding and hydrophobic interaction between polypeptide chains would result in protein aggregation.

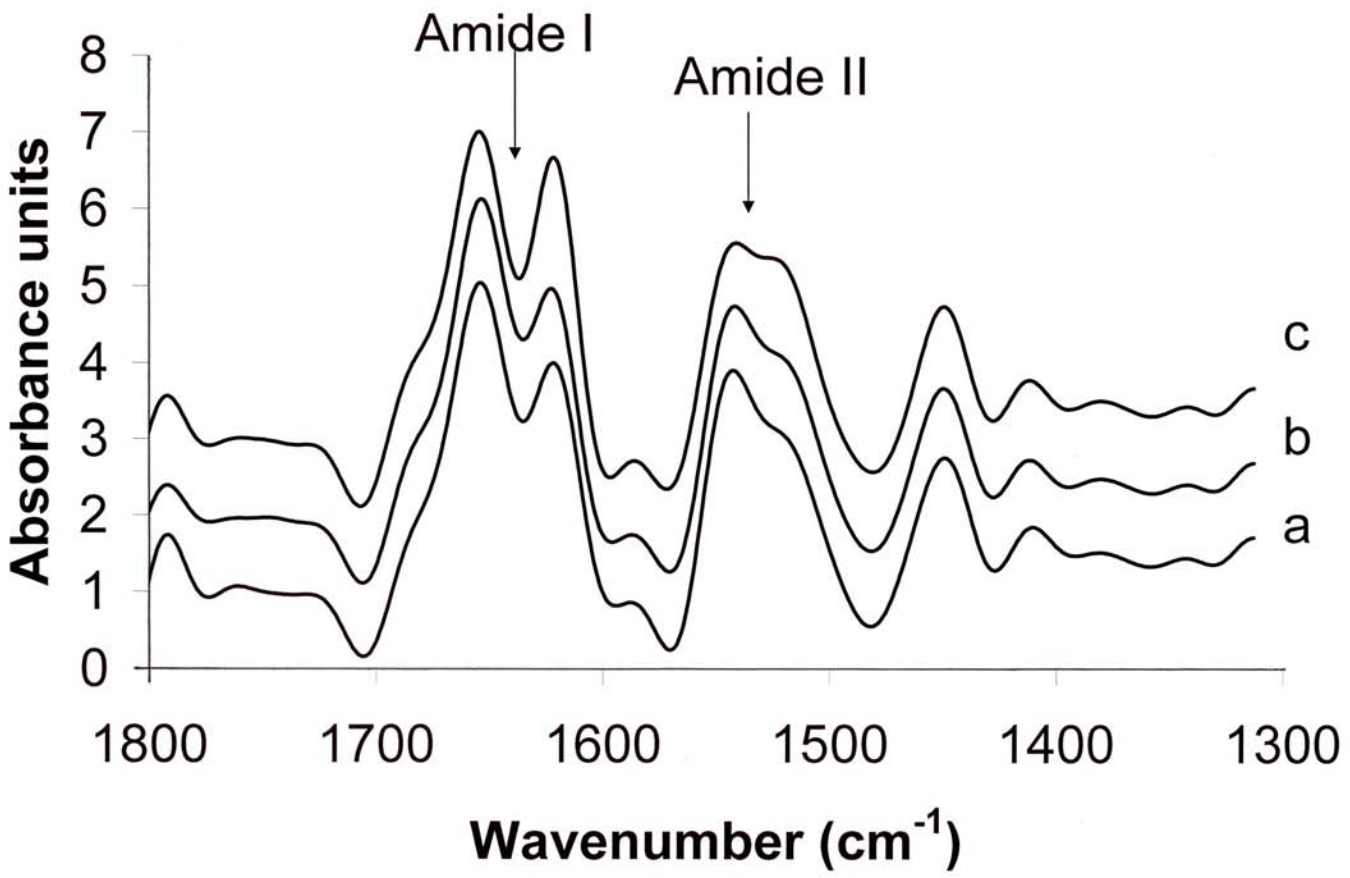


Figure 4.1.8: FTIR of original kafirin (a) and kafirin microparticles at different acid concentrations 5.4% acid (b), 21.6% acid (c)

4.1.4.9. Kafirin microparticle formation from other organic acids

It can be seen from Figure 4.1.9. that almost identical kafirin microparticles can be formed by phase separation using other organic acids such as propionic or lactic acids. However, propionic acid formed aggregates at a lower acid concentration than kafirin microparticles made with acetic acid (Figure 4.1.4.). This is probably because kafirin is more soluble in propionic acid than acetic acid under the same conditions (1.8 g kafirin (88% purity), 0.66 g plasticiser and 4.34 g solvent). This is possible because propionic acid is a more hydrophobic solvent than acetic acid due to its longer chain length. Thus protein precipitation and particle size growth occurs with the presence of more water with propionic acid than with acetic acid. Kafirin dissolved well in lactic acid but more lactic acid was needed before a solution could be formed because the kafirin initially absorbed the lactic acid.

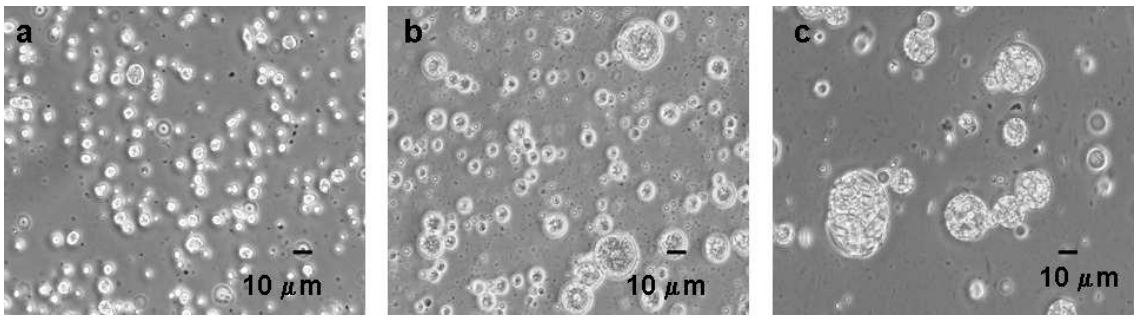


Figure 4.1.9: Light microscopy of kafirin microparticles made with acetic acid (5.4%, a), lactic acid (10.8%, b) and propionic acid (10.8%, c)

4.1.4.10. Presence of holes or vacuoles in kafirin microparticles

As can be seen kafirin microparticles made with acetic acid have numerous internal holes or vacuoles (Figure 4.1.2.a), whereas those made with aqueous ethanol had fewer holes or none at all (Figure 4.1.2.c). Whey protein microspheres prepared from pH 4.5 and 5.5 core-in-wall emulsions and used to encapsulate anhydrous milk fat shared some similarities with kafirin microparticles made with acetic acid although they were larger (10-100 μm) in size (Lee and Rosenberg 2000a). These whey protein microspheres had porous, wrinkled outer surfaces with irregularities and porous inner surfaces and were described as networks of large aggregates of proteins separated by voids of different size and shapes (Lee and Rosenberg 2000a, b). Similar whey microspheres made at pH 7.2 were described as having smooth dent-free outer surfaces with isolated core domains with no pores or channels connecting them to the outer surface.

Initially it was thought that the plasticiser added to the solvent may be responsible for the formation of holes or vacuoles (Figure 4.1.2.) observed. Possibly the kafirin would precipitate around a group of plasticiser molecules and on completion of microparticle formation the plasticiser would leach out of the microparticles leaving the observed holes or vacuoles. This is similar in principal to the use of soluble porogens for formation of poly(lactic-co-glycolic acid) (PGLA) matrixes (Song, Labhasetwar and Levy,1997; Kim, Chung and Park, 2006). A porogen is a water soluble additive present during microparticle preparation. After microparticle formation the porogen is leached out generating a highly porous matrix.

Kafirin microparticles were made in the presence and absence of plasticiser. Figure 4.1.10.a, c show kafirin microparticles made with plasticiser and Figure 4.1.10.b, d show kafirin microparticles made without plasticiser. The only apparent difference between the two preparations is that kafirin microparticles made without plasticiser appear larger than those made with plasticiser possibly due to a protein aggregation effect.

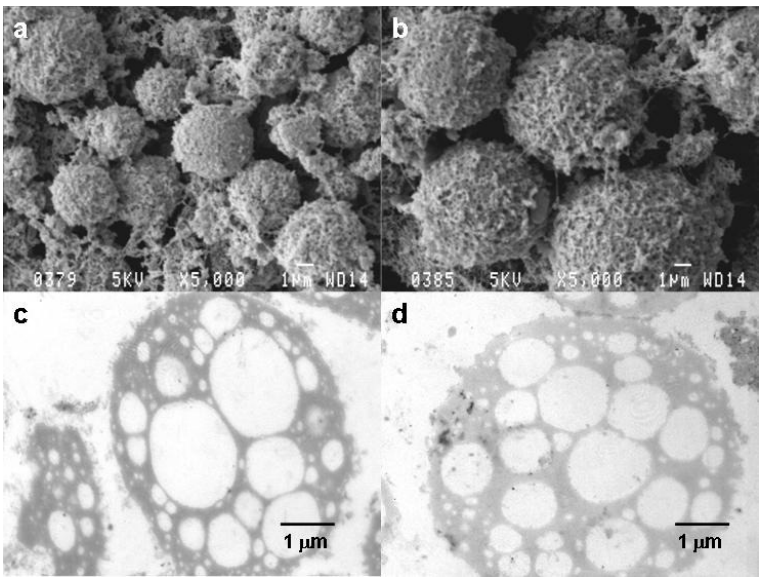


Figure 4.1.10: Kafirin microparticles prepared with and without plasticizer
With plasticiser (a SEM, c TEM), Without plasticiser (b SEM, d TEM)

It is possible that the structural features seen in kafirin microparticles made by dissolving the kafirin in glacial acetic acid may also be a result of protein-protein interactions resulting from low pH (pH 2). Certainly, when kafirin microparticles were made by the

method of Parris et al., (2005) using aqueous ethanol as kafirin solvent at neutral pH the microparticles had smooth surfaces as previously described (Figure 4.1.1.c). Lee and Rosenberg (2000a, b) attributed the structural differences of whey protein microparticles previously described to be a result of the effects of pH on the whey protein-protein interactions. However, kafirin microparticles still had internal holes or vacuoles regardless of the preparation pH, so it is unlikely that these features were caused by an effect of pH.

Lee and Rosenberg (2000a, b) also suggested the possibility that the large central voids they observed in whey protein microspheres may be due to the presence of air bubbles incorporated during microsphere preparation. An analogy can be drawn with the crumb structure of a cake. During preparation the cake batter is beaten to incorporate air and a foam is formed. On baking the foam is set and the air bubbles leave an imprint that becomes the crumb structure of the cake. When kafirin is dissolved in glacial acetic acid or aqueous ethanol at the initial concentration used to prepare the microparticles, the solution is very viscous and air bubbles are present. During the addition of water to precipitate out the microparticles there is a considerable amount of foaming. It is thus possible that air bubbles may be entrapped in the microparticles as they are formed and then appear as the holes or vacuoles in the hardened or 'set' microparticles as observed by TEM (Figure 4.1.2.a). In support of this theory is the fact that the kafirin microparticles made with aqueous ethanol had no or very few holes (Figure 4.1.2.c). Ethanol is known as a powerful degasser. Thus it would be expected that there would be less dissolved air in the aqueous ethanol solution that the microparticles were made from compared with the solution of kafirin in glacial acetic acid.

Figures 4.1.11. and 4.1.12. illustrate SEM and TEM of kafirin microparticles 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. It can be seen in both cases that there was a mixture of microparticles and matrix material. It was not possible to remove all the gas from the solvents and so it would be expected that some microparticles made with degassed solvents would still have holes. However, the matrix material appeared different for each treatment. When gas was added, the matrix material had the appearance of an expanded foam. This suggests 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. Whilst the evidence is not conclusive it does appear that the holes present in kafirin microparticles are possibly the footprint of air bubbles entrapped during microparticle preparation.

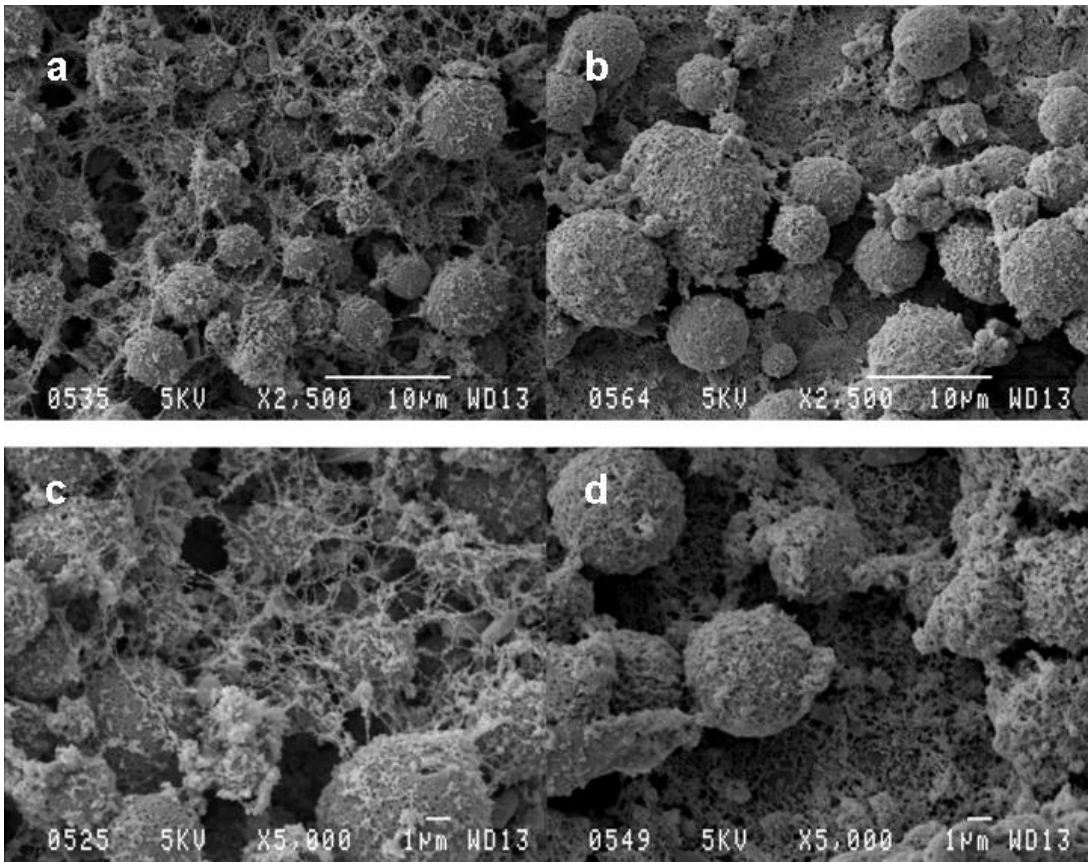


Figure 4.1.11: SEM kafirin microparticles made either with the addition of gas to the solvents (a, c), or made with degassed solvents (b, d)

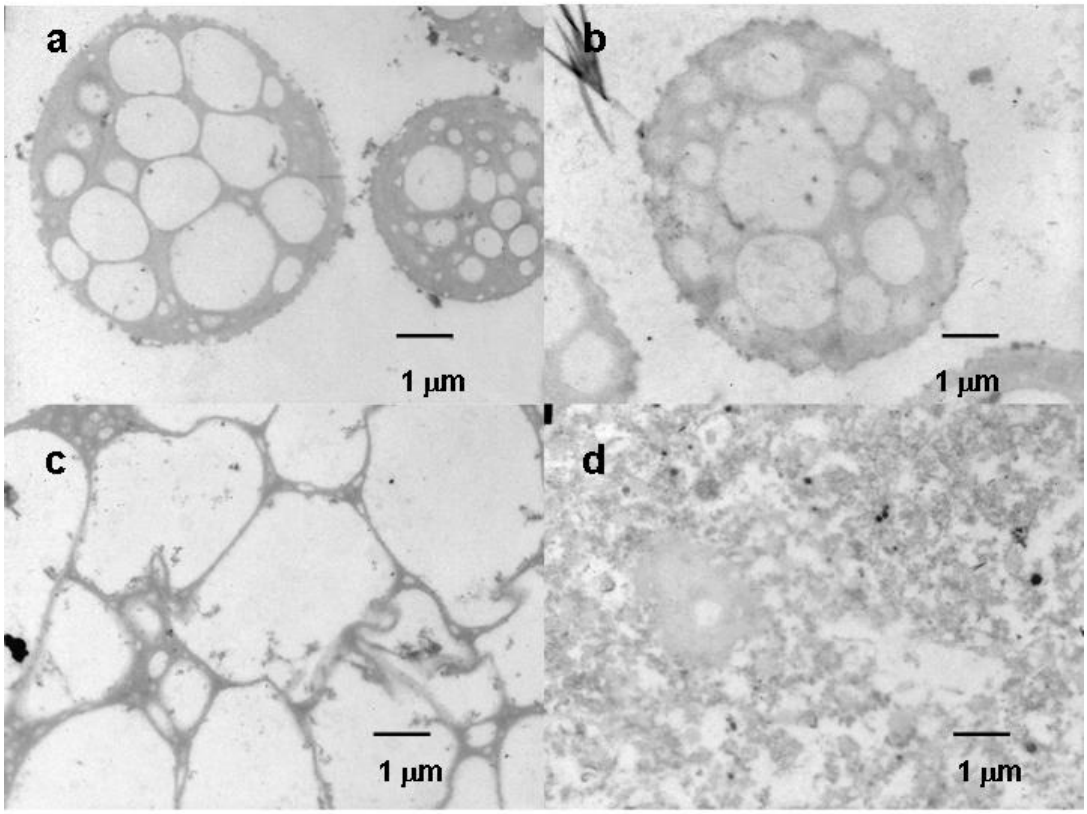


Figure 4.1.12: TEM kafirin microparticles made either with the addition of gas to the solvents (a, c), or made with degassed solvents (b, d)

4.1.5. Conclusions

In conclusion, the use of glacial acetic acid as solvent for kafirin followed by the addition of water is a simple ethanol free method for making microparticles from kafirin with unique properties, including an extremely large internal surface area. By varying the final acetic acid concentration the kafirin microparticle characteristics can be manipulated which may have potential applications in the food, biomedical and pharmaceutical industry as agents for encapsulation, film formation and tissue engineering.

4.1.6. References

American Association of Cereal Chemists, 2000. Crude protein-combustion, Standard Method 46-30. Approved Methods of the AACC. The Association, St Paul, MN.

Belton, P.S., Delgadillo, I., Halford, N.G., Shewry, P.R., 2006. Kafirin structure and functionality. Journal of Cereal Science 44, 272-286.

Chen, L., Remondetto, G.E., Subirade, M., 2006. Food protein-based materials as nutraceutical delivery systems. Trends in Food Science and Technology 17, 272-283.

Cook, R.B., Shulman, M.L., 1998. Colloidal dispersions of gluten, method of making and use therefor. United States Patent and Trademark Office 5736178.

Coombes, A.G.A., Lin, W., O'Hagen, D.T. Davis, S.S., 2003. Preparation of protein microspheres, films and coatings. United States Patent and Trademark Office 6592844.

DeRose, R., Ma, D.P., Kwon, I.S., Hasnain, S.E., Klassy, R.C., Hall, T., 1989. Characterisation of the kafirin gene family reveals extensive homology with zein from maize. Plant Molecular Biology 12, 245-256.

Demchak, R.J., Dybas, R.A., 1997. Photostability of abamectin/zein microspheres. Journal of Agricultural and Food Chemistry 45, 260-262.

Dong, J., Sun, Q., Wang, J.Y., 2004. Basic study of corn protein, zein, as a biomaterial in tissue engineering, surface morphology and biocompatibility. Biomaterials 25, 4691-4697.

Duodu, K.G., Taylor, J.R.N., Belton, P.S., Hamaker, B.R., 2003. Factors affecting sorghum protein digestibility. Journal of Cereal Science 38, 117-131.

Duodu, K.G., Tang, H., Wellner, N., Belton, P.S., Taylor, J.R.N., 2001. FTIR and solid state ^{13}C NMR spectroscopy of proteins of wet cooked and popped sorghum and maize. Journal of Cereal Science 33,261-269.

El Nour, N.A., Peruffo, A.D.B., Curioni, A., 1998. Characterisation of sorghum kafirins in relations to their cross-linking behaviour. Journal of Cereal Science 28, 197-207.

Emmambux, N. M., Taylor, J.R.N., 2003. Sorghum kafirin interaction with various phenolic compounds. Journal of the Science of Food and Agriculture 83, 402-407.

Freitas, S., Merkle, H.P., Gander, B., 2005. Microencapsulation by solvent extraction/evaporation: reviewing the state of the art of microsphere preparation process technology. Journal of Controlled Release 102, 313-332.

Gao, C., Taylor, J., Wellner, N., Byaruhanga, Y.B., Parker, M.L., Mills, E.N.C., Belton, P.S., 2005. Effect of preparation conditions on protein secondary structure and biofilm formation of kafirin. Journal of Agricultural and Food Chemistry 53, 306-312.

Gallagher, S.R., 1999. Electrophoretic separation of proteins, in: Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.E., Seidman, J.G., Smith, J.A., Strule, K. (Eds.), Current Protocols in Molecular Biology. John Wiley and Sons, New York, pp. 10.2A1-9.

Hurtado-López, P., Murdan, S., 2005. Formulation and characterization of zein microspheres as delivery vehicles. Journal of Drug Delivery Science and Technology 15, 267-272.

Kim, C.S., Woo, Y., Clore, A.M., Burnett, R.J., Carneiro, N.P., Larkins, B., 2002. Zein protein interactions, rather than the asymmetric distribution of zein mRNAs on endoplasmic reticulum membranes, influence protein body formation in maize endosperm. The Plant Cell 14**,** 655-672.

Kim, H.K., Chung, H.J., Park, T.G., 2006. Biodegradable polymeric microspheres with "open/closed" pores for sustained release of human growth hormone. Journal of Controlled Release 112, 167-174.

Lee, S.J., Rosenberg, M., 2000a. Whey protein-based microcapsules prepared by double emulsification and heat gelation. Lebensmittel-Wissenschaft und-Technologie 33, 80-88.

Lee, S.J., Rosenberg, M., 2000b. Preparation and some properties of water-insoluble, whey protein-based microcapsules. Journal of Microencapsulation 17, 29-44.

Liu, X., Sun, Q., Wang, H., Zhang, L., Wang, J.Y., 2005. Microspheres of corn protein, zein, for an ivermectin drug delivery system. Biomaterials 26, 109-115.

Mizutani, Y., Matsumura, Y., Imamura, K., Nakanishi, K., Mori, T., 2003. Effects of water activity and lipid addition on secondary structure of zein in powder systems. Journal of Agricultural and Food Chemistry 51, 229-235.

Muthuselvi, L., Dhathathreyan, A., 2006. Simple coacervates of zein to encapsulate gitoxin. Colloids and Surfaces B: Biointerfaces 51, 39-43.

Parris, N., Cooke, P.H., Hicks, K.B., 2005. Encapsulation of essential oils in zein nanospherical particles. Journal of Agricultural and Food Chemistry 53, 4788-4792.

Patil, G.V., 2003. Biopolymer albumin for diagnosis and in drug delivery. Drug Development Research 58, 219-247.

Shull, J.M., Watterson, J.J., Kirleis, A.W., 1991. Proposed nomenclature for the alcohol-soluble proteins (kafirins) of *Sorghum bicolor* (L. Moench) based on molecular weight, solubility and structure. Journal of Agricultural and Food Chemistry 39, 83-87.

Sinha, V.R., Trehan, A., 2003. Biodegradable microspheres for protein delivery. Journal of Controlled Release 90, 261-280.

Song, C.X., Labhasetwar, V., Levy, R.J., 1997. Controlled release of U-86983 from double-layer biodegradable matrices: effect of additives on release mechanism and kinetics. Journal of Controlled Release 45, 177-192.

Taylor, J., Taylor, J.R.N., Dutton, M.F., De Kock, S., 2005. Glacial acetic acid-a novel food compatible solvent for kafirin extraction. Cereal Chemistry 85, 485-487.

Taylor, J.R.N., Belton, P.S., 2002. Sorghum. In: Belton, P.S., Taylor, J.R.N., (Eds.), Pseudocereals and Less Well-Known Cereals, Berlin, Springer, pp. 25-38.

Wang, H.J., Lin, Z.X., Liu, X.M., Sheng, S.H., Wang, J.Y., 2005. Heparin-loaded zein microsphere film and hemocompatibility. Journal of Controlled Release 105, 120-131.

Whittlesey, K.J., Shea, L.D., 2004. Delivery systems for small molecule drugs, proteins, and DNA: the neuroscience/biomedical interface. Experimental Neurology 190, 1-16.

4.2. PREPARATION OF KAFIRIN MICROPARTICLE FILMS AND COATINGS AND MECHANISM OF FILM FORMATION AND FILM FUNCTIONAL PROPERTIES

4.2.1. Abstract

A novel approach to the preparation of free standing kafirin bioplastic films and coatings is described using kafirin microparticles which were made by phase separation from an organic acid. Film preparation involved the suspension of the kafirin microparticles in an organic acid solution. On evaporation of the organic acid a complete, smooth, transparent film was formed. It was found that there was a minimum concentration of acid required to form a cohesive film relative to the concentration of protein. This relative amount was 10.8:1, percent organic acid to percent protein when acetic acid was the organic acid used. The mechanism of 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. The functional properties of these films were compared with those of kafirin films at the same protein concentration cast from glacial acetic acid. The films made from kafirin microparticles were very thin (<15 μm), relatively strong but not extensible, with better water barrier properties and lower protein digestibility than conventionally cast kafirin films.

4.2.2. Introduction

At present the functional properties of protein bioplastic materials are not as good as those of synthetic polymers. For example, protein bioplastic films generally have much worse water barrier properties and lower tensile strength and elongation than synthetic polymers (reviewed by Krotcha, 2002). However, Krotcha (2002) mentions that bioplastic films made from zein (maize prolamins) have some of the best functional properties. Kafirin, the similar prolamin protein of sorghum is potentially a good choice for making bioplastics as it is more hydrophobic than zein (Belton, Delgadillo, Halford and Shewry, 2006; Duodu, Taylor, Belton and Hamaker, 2003) and it demonstrates reduced protein digestibility on wet heating (Duodu et al., 2003). These are both characteristics which may positively influence the barrier properties of the films.

Taylor, Schober and Bean (2006) reviewed research into kafirin bioplastics, quoting promising potential applications such as fruit coatings. Da Silva and Taylor (2005) showed that cast kafirin films could be made from kafirin extracted from different sorghum milling fractions including bran, a by-product of dry milling. In addition, research has been published describing methods of kafirin bioplastic film modification by cross-linking with condensed tannins (Emmambux, Stading and Taylor, 2004) and by heating using microwave energy (Byaruhanga, Erasmus and Taylor, 2005) to improve the functional properties of such films.

A novel approach for the preparation of kafirin bioplastics is the use of kafirin microparticles for their preparation. The major objective of this work was to prepare films from microparticles and compare their functional properties with those of conventionally cast kafirin films. Elucidation of some aspects of the microparticle film forming process was also attempted.

4.2.3. Materials and methods

4.2.3.1. Materials

A mixture of two condensed tannin-free, tan plant, white sorghum cultivars PANNAR PEX 202 and 206 were used for kafirin extraction as described in Chapter 4.1.

4.2.3.2. Preparation of kafirin microparticles

Kafirin microparticles were prepared with acetic acid, propionic acid or lactic acid as described in Chapter 4.1.

4.2.3.3. Preparation of films

Preparation of free standing kafirin films

Free standing kafirin films were cast using the method described by Taylor, Taylor, Dutton and De Kock (2005) using 2% kafirin, with 40% plasticiser in relation to protein and glacial acetic acid as casting solvent. The plasticiser was a 1:1:1 (w/w) mixture of glycerol, polyethylene glycol 400 and lactic acid. This plasticiser was used throughout this work. Films were dried overnight at 50°C in an oven (not forced draught).

Preparation of kafirin microparticle free standing films

Suspensions of kafirin microparticles in acetic acid (4 g) were mixed at various concentrations with 40% plasticiser in relation to protein and then cast in Perspex Petri dishes (9 cm) by drying overnight at 50°C in an oven (not forced draught). All free standing films were assessed visually and photographed using a flatbed scanner.

Preparation of kafirin microparticle free standing films cast with different acids

Suspensions of kafirin microparticles (2% protein) prepared in either acetic, propionic or lactic acid (4 g) were mixed with 40% plasticiser in relation to protein and then cast in Petri dishes by drying overnight at 50°C in an oven (not forced draught). Acid concentrations of 5.4, 10.8 and 21.6% were used. Acid concentrations were manipulated as described in Chapter 4.1.

Preparation of films from kafirin at low protein concentration using glacial acetic acid as casting solvent

Kafirin was dissolved in glacial acetic acid at concentrations of 0.5 and 1.0%, containing plasticiser (40% in relation to protein) and allowed to 'age' overnight. Aliquots (4 g) were cast into Petri dishes and dried overnight at 50°C in an oven (not forced draught).

Preparation of films from kafirin microparticles at low acid and low protein concentration

Kafirin microparticles were prepared as described in Chapter 4.1. Additional water was added to give a concentration of 1% protein in 10.6% acetic acid or 0.5% protein in 5.4% acetic acid. Aliquots (4 g) were cast into Petri dishes and dried overnight at 50°C in an oven (not forced draught).

4.2.3.4. Analysis of films

Effects of protein concentration and acid concentration on kafirin microparticle film formation

Acetic acid prepared microparticles were cast into free standing films at 2 and 4% protein in 5.4, 10.8, and 21.6% acetic acid. At 8% protein, 5.4 and 10.8% acetic acid was used. Plasticiser concentration was constant at 40% (plasticiser concentration in relation to protein) for all films cast.

Effects of plasticiser concentration and acid concentration on kafirin microparticle film formation

Acetic acid prepared microparticles were cast into free standing films at 2% protein in 5.4, 10.8, 15 and 21.6% acetic acid and with relative weights of 0, 20, 40, 60, 80 and 100% plasticiser in relation to the weight of protein.

Scanning Electron Microscopy (SEM) of free standing films

Kafirin films (2% protein, 40% plasticiser in relation to protein) cast in acetic acid and microparticle (2% protein, 40% plasticiser in relation to protein, 21.6% acetic acid) film surfaces were examined by mounting the top and under side of the films on a stub with double sided tape. Samples were then sputter coated with gold and SEM preparations were viewed with a JSM-840 Scanning Electron Microscope (Tokyo, Japan) and photographed.

Atomic Force Microscopy (AFM) of kafirin microparticle film surfaces

Samples were prepared by dropping kafirin microparticles (5µl) in either 5.4% or 21.6% acetic acid on to a piece of polished silica and allowing the solution to dry in a desiccator. Samples were viewed with a Topometrix TMX 2000 'Discoverer' AFM (Santa Clara, CA, USA) in contact mode and photographed.

Fourier Transform Infrared Spectroscopy (FTIR) of films

Film samples were scanned as a double layer using a Perkin Elmer Spectrum GX FTIR system, (Waltham, MA, USA), with a zinc selenide crystal using 32 scans, 8 cm^{-1} band width and an interval of 1 cm^{-1} in the Attenuated Total Reflectance (ATR) mode. The FTIR spectra were Fourier-deconvoluted with a resolution enhancement factor of 2 and a bandwidth of 12 cm^{-1}.

Water Vapour Transmission (WVT) and Water Vapour Permeability (WVP) of films

A modified method based on the ASTM method E96-97, (American Society for Testing and Materials, 1997a), was used as described by Taylor et al. (2005). The thickness of the films was measured in five places using a micrometer, after conditioning the films for 48 h in a 50% RH chamber. Circles (40 mm diameter) were cut from the cast films, which were mounted on top of modified Schott bottles containing distilled water to a level where the neck of the bottle was constant in diameter (120 ml). The screw top of the Schott bottle was modified by accurately drilling a 33 mm hole in the centre. A fibre tap washer (external diameter 39 mm) was placed between the screw top of the bottle and the film ensured a water-tight seal was maintained. The bottles were placed in a forced draught incubator at 24°C with an average relative humidity of 19.5% over the period of the test. Weight loss was recorded daily for 10 days. At least 3 replicates were performed for each treatment. A graph of water loss against time was plotted. The best fitting straight line was drawn with the first point at the Origin. The equation of the line and thus the gradient was recorded. WVT was calculated by dividing the gradient of the line by the area of the film.

Accounting for the thickness of the different films was attempted using a calculation to determine the Water Vapour Permeability (WVP):

$$WVP = \frac{\text{gradient (g/h) x thickness of film (mm)}}{\text{Area (m2) x Po (kPa) x (RH1-RH2)/100}}$$

Where:
Po (at 25˚C) = 3.17 kPa
RH1 = Relative humidity inside the bottle
RH2 = Relative humidity outside the bottle

Relative humidity inside the bottle was assumed to be 100%. Relative humidity outside the bottle was measured using a Kane-May KM 8006 Relative humidity meter (Welwyn Garden City, England).

Tensile properties of films

Film tensile properties were determined by a modified method based on ASTM D882-97 (American Society for Testing and Materials, 1997b), as described by Taylor et al. (2005) using a TA-XT2 Texture Analyser (Stable Micro Systems, Goldalming, UK) with tensile grips coated with abrasive paper. Strips (60 mm x 6 mm, sd ±0.05 mm with respect to the width) of film were cut with a sharp scalpel using a pre-marked cardboard template and conditioned for 48 h at 50% RH prior to analysis. The thickness of the strips was measured in 5 places using a micrometer, before mounting between the tensile grips (40 mm apart). Tension was applied with a cross-head speed of 0.4 mm/s. The maximum force and distance at break was recorded and the stress and strain calculated. At least 6 strips were tested from each film and at least 3 films were tested for each treatment.

Protein digestibility of films

A modified version of the pepsin method of Mertz, Hassen, Cairns-Whittern, Kirleis, Tu and Axtell, (1984) was used. Film samples were prepared by freeze fracturing in liquid nitrogen and then grinding with a mortar and pestle. Film particles (10 mg, protein basis) were digested for 2 h at 37°C with 998 units of pepsin P7000 (Sigma). Total protein and residual protein (N x 6.25) were determined by the Dumas combustion method (AACC standard method 46-30) (American Association of Cereal Chemists, 2000). Protein digestibility was calculated by the difference between the total protein and the residual protein after pepsin digestion, divided by the total protein and expressed as a percentage.

Biodegradation of films

Biodegradation of films was determined using a procedure based on ASTM Method D5512-96 (American Society for Testing and Materials, 1996). Films were not preconditioned before the test. They were cut and mounted into 35 mm plastic film slide frames and photographed using a flatbed scanner. The mounted films were then buried in well-fermented compost (horse manure and sawdust, moisture 60%) contained in plastic buckets with tight fitting lids. A low density polyethylene film (LDPE) was included for comparison. The intention was to follow an incubation protocol for optimal composting conditions. Incubation was to be in the dark at 35°C for 2 days, 58°C for 4 days, 50°C until day 26 and 35°C for the final 2 days of the test. The containers were to be aerated daily by removing the lids for 1 h and films removed at 5 day intervals, cleaned gently with a soft brush and scanned. However, all the protein films had degraded by day 3 and so the test was terminated.

Observation of microparticle film formation

Droplets of microparticles (2% kafirin in 5.4% acetic acid and 2% kafirin in 21.6% acetic acid) were placed on microscope slides with no cover slip. The microscope slide was then placed on a Linkam Scientific Instruments CO 102 hot stage (Tadworth, England) and viewed using a Reichert Neovar (Vienna, Austria) light microscope at x 100 magnification and photographed using a still camera. The temperature of the hot stage was set to heat up to a temperature of 50°C, the temperature used for drying free standing films. The film forming process was complete from start to finish in 2 min. Photographs were taken every 5 or 10 s in an attempt to record the film formation process.

4.2.3.5. Statistical analysis

One-way analysis of variance (ANOVA) was applied to the data on FTIR of films, film protein digestibility, tensile, water vapour transmission and water vapour permeability tests. In all cases tests were carried out at least in duplicate and repeated at least once giving a total of at least four results for each test.

4.2.4. Results and discussion

4.2.4.1. Effect of protein concentration and acid concentration on kafirin microparticle film formation

Preparations of kafirin microparticles suspended in acetic acid were found to dry into clear, transparent films under certain conditions. A protein concentration of 2% was chosen for initial work on film formation. This was the protein concentration used for fruit coatings in the Enviropak project (http://www.sik.se/enviropak). Fruit coatings were envisaged as a potential application for kafirin microparticles. At 2% protein, progressively increasing the acetic acid concentration from 5.4% to 10.8% and then 21.6% (Figure 4.2.1. a-c) had a strong influence on the completeness of film formation. At 21.6% acetic acid (Figure 4.2.1.c) a complete film was formed, which could be released from the Petri dish. At 10.8% acid (Figure 4.2.1.b) the film was almost complete and transparent, whereas at 5.4% acid (Figure 4.2.1.a) the film was incomplete, formed in small fragments and was opaque. At the higher protein concentrations the same pattern was observed (Figure 4.2.1.d-h). Increasing acid concentration increased the degree of completeness and clarity of the film. Increasing protein concentration also seemed to have an effect on film formation as "at a particular acid concentration" the fragments of film were larger with increased protein concentration. The protein concentration did not appear to affect the clarity of the film. However, as would be expected, increased protein concentration increased the thickness of the resultant films and the colour changed from clear (21.6 and 10.8% acid) or white (5.4% acid) at 2% protein to yellow at higher protein concentrations.

There are few references in the literature to the formation of films from microparticles and none found using them to make the type of free standing films made previously by workers in our laboratories (Taylor et al., 2005; Da Silva and Taylor, 2005; Byaruhanga et al., 2005). The patent of Cook and Shulman (1998) described zein colloidal dispersions similar to the microparticle suspensions used in this study and claimed that they could be dried into glossy coatings and 'films'. According to these workers a cohesive 'film' could be made from zein microparticles at a much lower acid concentration but higher protein concentrations than was found in this study. Details of the methods of preparation were insufficient to enable valid comparisons to be made. Also, no evidence was presented that indicated that the films could be released from the coating surface.

Dong, Sun and Wang (2004) and Wang, Lin, Liu, Sheng and Wang (2005) described the formation of zein microsphere 'films' cast from 40% aqueous ethanol, a different solvent system to that used in this study. Dong et al. (2004) showed by SEM that zein microspheres could agglomerate together to form a film at very low zein concentration (< 1% w/v). However, the illustrations of microsphere films made by both groups (Dong et al., 2004; Wang et al., 2005) showed very little fusion of the microspheres and so the films would not be considered as a continuous film. The apparent superiority of the kafirin microparticle films made in my study may have been due to either the higher protein concentration or the use of a better solvents system for film formation.

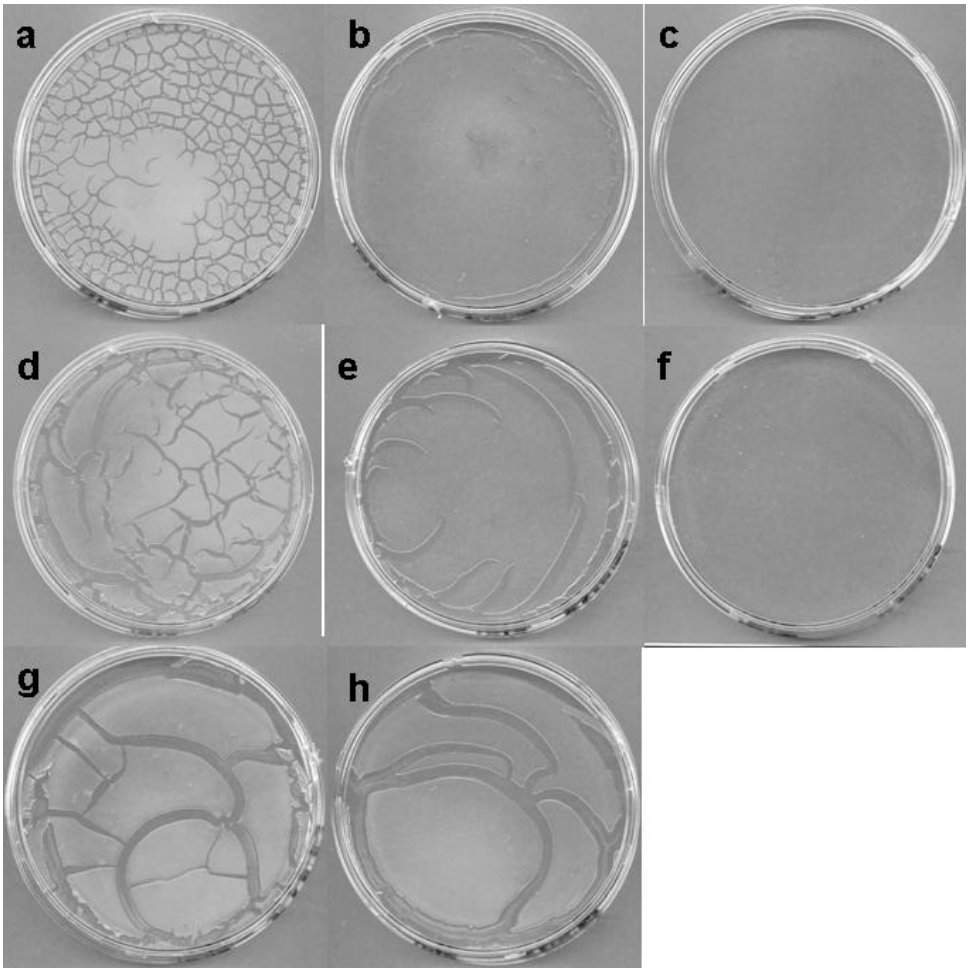


Figure 4.2.1: Effects of increasing protein concentration and acetic acid concentration on kafirin microparticle film formation
a-2% kafirin, 5.4% acetic acid, b-2% kafirin, 10.6% acetic acid, c-2% kafirin, 21.6% acetic acid, d-4% kafirin, 5.4% acetic acid, e-4% kafirin, 10.6% acetic acid, f-4% kafirin, 21.6% acetic acid, g-8% kafirin, 5.4% acetic acid, h-8% kafirin, 10.6% acetic acid.

4.2.4.2. Effects of plasticiser concentration and acid concentration on kafirin microparticle film formation

A plasticiser as defined by Banker (1966) is a non-volatile, non-separating substance, which when added to a polymer changes certain physical and mechanical properties of that polymer. Plasticisers are added to cast films to reduce brittleness, increase flexibility and extensibility by decreasing intermolecular forces between polymer chains (Padua and Wang, 2002). The plasticiser used in this work was a mixture of 1:1:1 (w/w) of glycerol, polyethylene glycol and lactic acid. This plasticiser combination has been found effective for cast kafirin films made in our laboratories (Gao, Taylor, Wellner, Byaruhanga, Parker, Mills and Belton, 2005; Da Silva and Taylor, 2005; Taylor et al., 2005).

Figure 4.2.2. illustrates the combined effect of varying plasticiser and acetic acid concentration on the formation of kafirin microparticle films cast at constant protein concentration (2%). Only films made with 21.6% acetic acid formed complete films regardless of the concentration of plasticiser. This is not surprising since as stated, addition of plasticiser decreases intermolecular forces which would have the effect of decreasing the cohesiveness of the microparticle suspension, which is necessary for film formation (Banker, 1966). At 21.6% acetic acid (Figure 4.2.2.t-x), all plasticiser concentrations except 0% (Figure 4.2.2.s) formed clear, transparent, flexible films with smooth surfaces. Films with 40% plasticiser (Figure 4.2.2.u) produced the best films sensorially. They were odourless, stronger and less extensible than films with higher levels of plasticiser. Films with 60, 80 and 100% plasticiser (Figure 4.2.2.v-x) in relation to the weight of protein, were sticky and over plasticised, appearing weaker and more extensible than those with lower levels of plasticiser. The stickiness was probably due to excess glycerol which is noted to migrate through a film matrix to the surface of the film resulting in loss of flexiblity (Padua and Wang, 2002). Gao, Stading, Wellner, Parker, Noel, Mills and Belton (2006) noted glycerol leaching out of kafirin films at a lower level of plasticisation (40%) than was found in this study. These workers suggested some phase separation was occurring at this level of glycerol plasticisation due to the presence of two glass transition temperatures, one of which corresponded to that of pure glycerol. Based on spectroscopic, rheological and calorimetric methods they found at low levels of glycerol plasticisation, glycerol was absorbed onto or into the kafirin. At low levels of glycerol plasticisation, most of the molecular interactions would be protein-protein or protein-glycerol with few glycerol-glycerol interactions. It is suggested that at higher

levels of glycerol plasticisation the number of glycerol-glycerol interactions would increase, changing the nature of the mechanical behaviour of the film. Possibly due to the different nature of kafirin microparticle films more glycerol could be absorbed onto the kafirin than with conventionally cast kafirin films, resulting in glycerol leaching occurring only at higher plasticiser levels. Certainly, kafirin microparticles films made with 40% plasticiser were still very flexible after several months with no apparent loss of plasticiser. On the basis of this observation, it is suggested that there must have been some intramolecular interaction between the kafirin microparticles and the plasticiser components.

At 21.6% acetic acid, the films with 0% plasticiser were complete but brittle and opaque (Figure 4.2.2.s). It is possible that the lactic acid component of the plasticiser helped the dissolution of the kafirin during film formation. According to Jackson and Shandera (1995) lactic acid is important for protein dispersion. Possibly at higher acid concentrations a complete film would have formed without additional lactic acid.

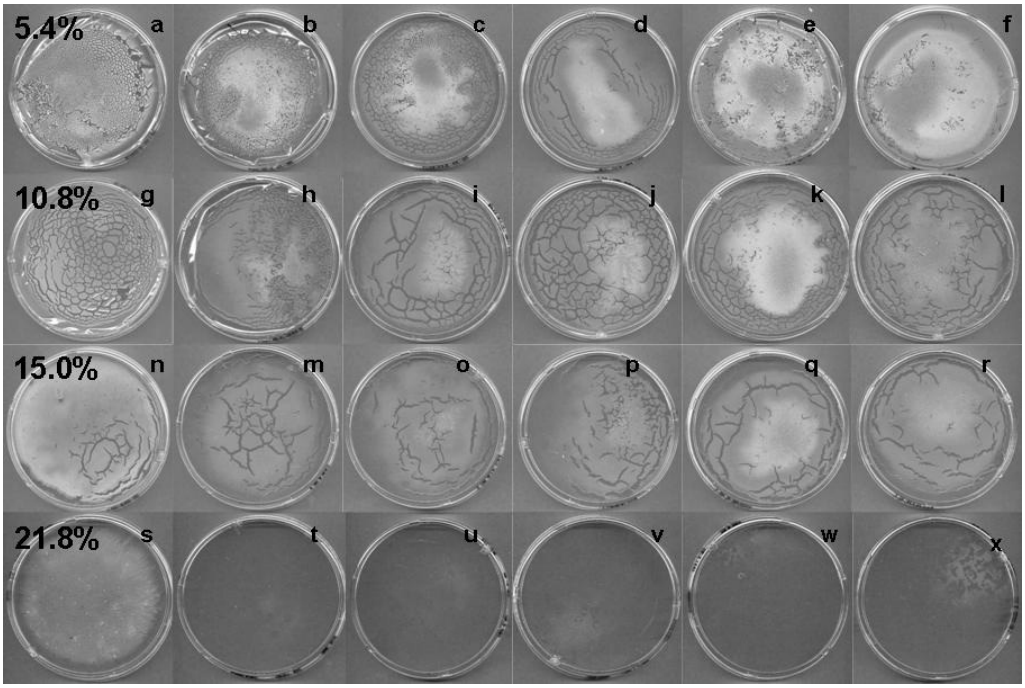


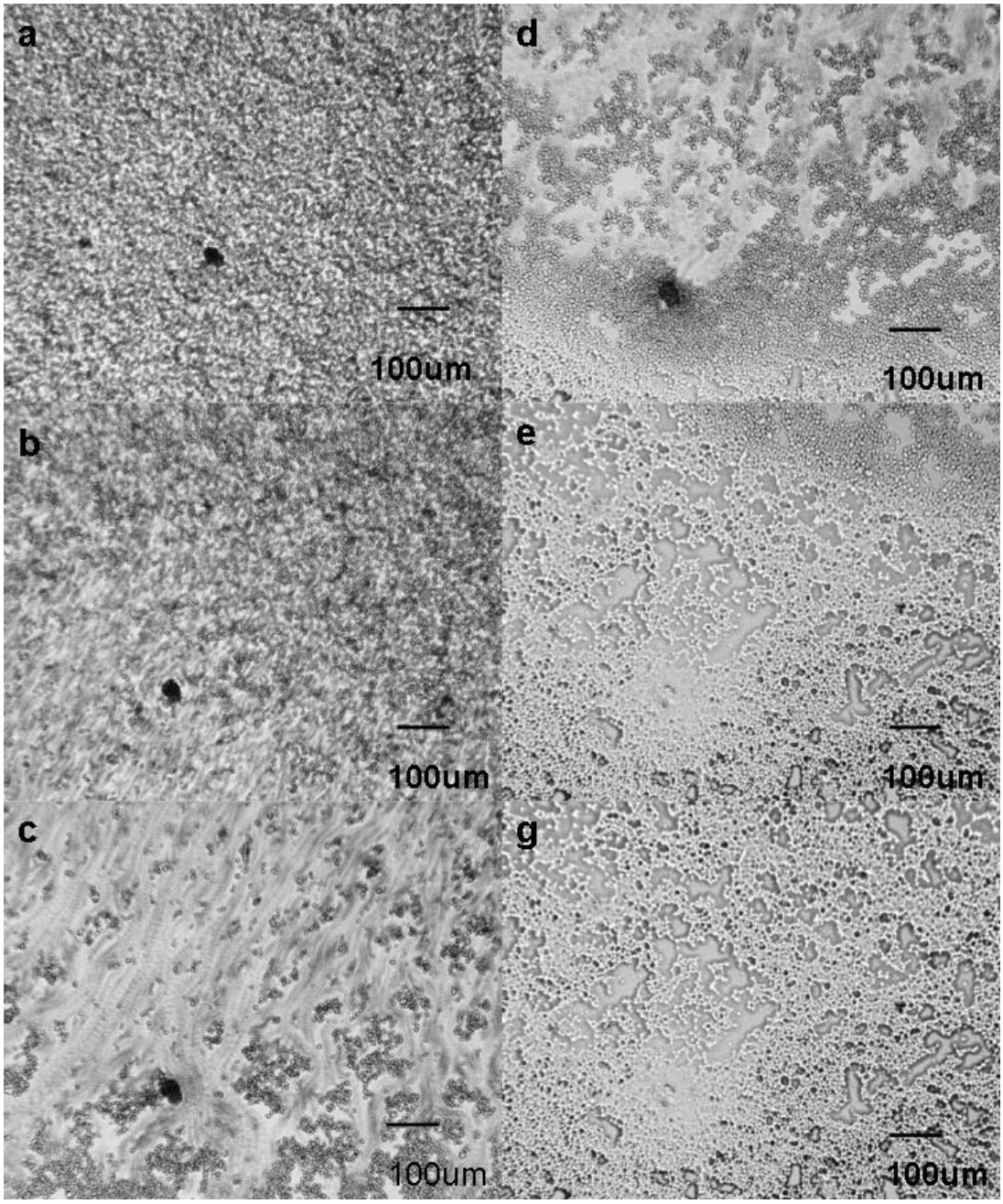
Figure 4.2.2: Effects of increasing acetic acid concentration and increasing plasticiser concentration on kafirin microparticle film formation (2% protein)
5.4% acetic acid, a-f, 0, 20, 40, 60, 80, 100% placticiser with respect to protein
10.8% acetic acid, g-h, 0, 20, 40, 60, 80, 100% placticiser with respect to protein
15% acetic acid, m-r, 0, 20, 40, 60, 80, 100% placticiser with respect to protein
21.6% acetic acid, s-x, 0, 20, 40, 60, 80, 100% placticiser with respect to protein

4.2.4.3. Microparticle film formation

Figure 4.2.1. illustrates that kafirin films can be cast from kafirin microparticles in acidic solutions at much lower protein concentration than in aqueous ethanol. It appears that a minimum amount of acid relative to the amount of protein is required in order to form a complete film. This was confirmed by following the film formation process with 2% kafirin microparticles in 5.4% and in 21.6% acetic acid using light microscopy (Figure 4.2.3.A. and B. respectively). Figure 4.2.3.A.a, shows the individual kafirin microparticles in 5.4% acid as small well defined spheres. As heat was applied to the film forming suspension via the hot stage on which the slide was mounted, the spheres moved across the slide probably due to convection. The spheres appeared to form into short interlinked chains and separated from the liquid phase (Figure 4.2.4.A.b-c). It is suggested that convection influenced the formation of the strings of spheres. This appears, similar to the effect of molecular combing during conventional zein film formation observed by AFM (Guo, Liu, An, Li and Hu, 2005). These workers described zein film as consisting of globules of non uniform size. When alignment by molecular combing was applied these workers suggested that the zein globules formed into joined zein rods. In this present work, as more heat was applied the interlinked chains of microparticles appeared to aggregate and merge together as some of the solvent evaporated (Figure 4.2.3.A.d). This process continued as more liquid evaporated forming an incomplete mesh arrangement (Figure 4.2.3.A.e). When all the solvent had evaporated (Figure 4.2.3.A.f) the mesh of microparticles was deposited on to the slide and viewed as a rough, opaque, incomplete and still to some extent particulate film.

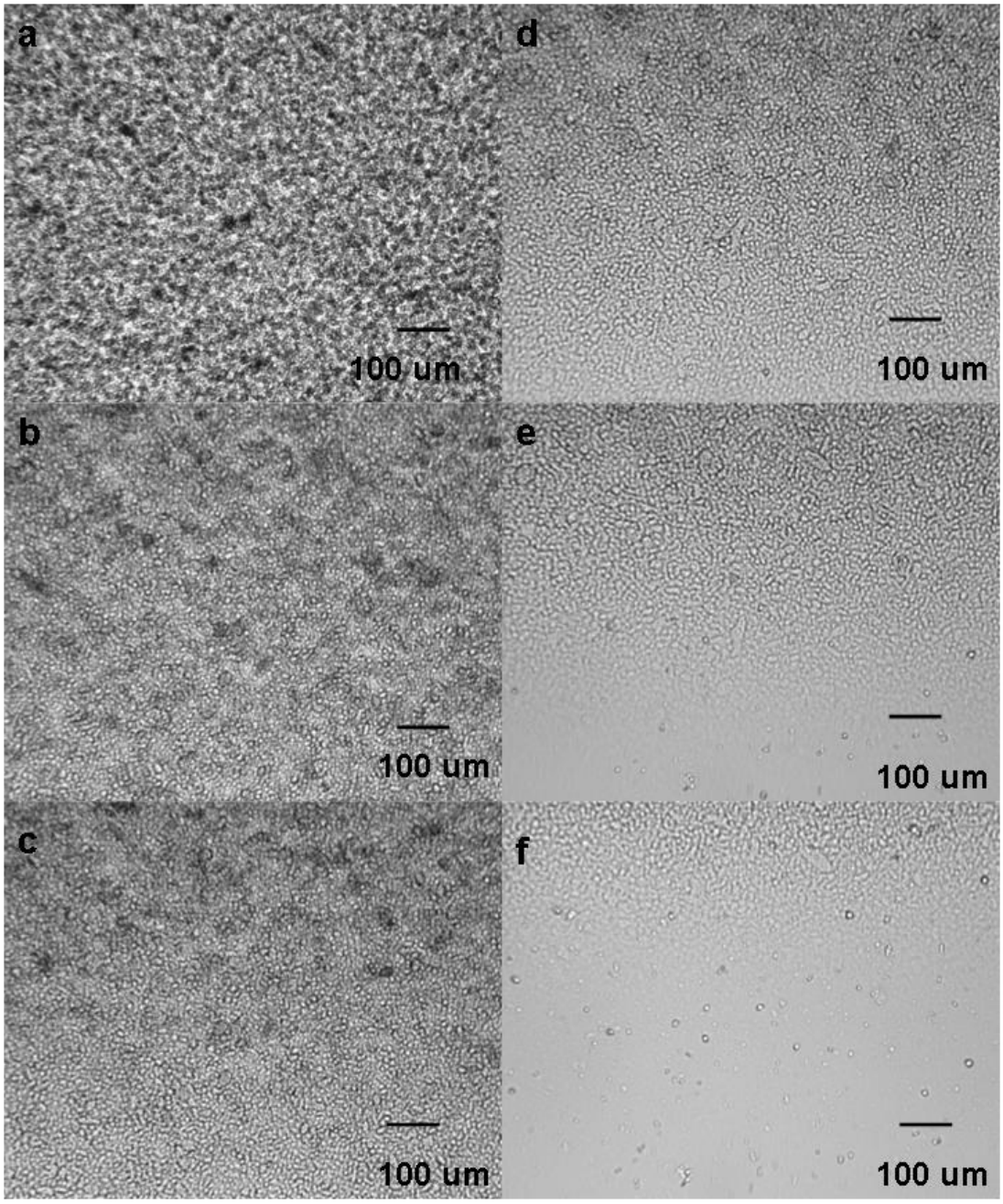
Figure 4.2.3.B. illustrates how a complete cohesive film is formed from 2% kafirin microparticles with the higher acetic acid concentration of 21.6%. Figure 4.2.3.B.(a) shows the individual kafirin microparticles in 21.6% acid were slightly larger than those in the 5.4% acetic acid, but still well defined spheres. The increase in microparticle size with increasing acid concentration was described and discussed in Chapter 4.1. As heat was applied to the film forming suspension, the spheres appeared to form into short interlinked chains in the same way as in the presence of 5.4% acid (4.2.3.B.b-c). However, at the higher acid concentration there did not seem to be a separation from the liquid phase (Figure 4.2.3.B.b-c). As more heat was applied the interlinked chains of microparticles appeared to aggregate in a controlled manor and merge together (Figure 4.2.3.B.d). As the solvent evaporated, the microparticle aggregates appeared to dissolve

in the remaining solvent (Figure 4.2.3.B.e). Finally as all the solvent evaporated a clear, cohesive, transparent film was deposited on the slide (Figure 4.2.3.B.f). Thus in order for a cohesive film to form it appears that the kafirin microparticles must dissolve in the solvent, in this case acetic acid.



A

Figure 4.2.3: Light microscopy, time lapse record of kafirin microparticle film formation
A: 5.4% acetic acid, a-0 seconds, b-40 seconds, c-80 seconds, d-100 seconds, e-110 seconds, f-120 seconds
B: 21.6% acetic acid, a-0 seconds, b-40 seconds, c-60 seconds, d-80 seconds, e-90 seconds, f-110 seconds



B

4.2.4.4. Preparation of films from kafirin microparticles at low acid and low protein concentration

It appears that a minimum amount of acid relative to the amount of protein is required in order to form a complete film. For kafirin, microparticles made with acetic acid this minimum acid concentration appears to be 21.6% when 2% protein is used. If the relationship between protein concentration and acid concentration holds it was thought that a complete film or at least a complete coating should form at 1% protein with 10.6% acetic acid and 0.5% protein and 5.4% acetic acid. This was found to be the case (Figure 4.2.4.). At the higher protein concentration, 1%, (Figure 4.2.4.b) a complete, transparent, smooth, flexible film was formed which could be released whole from the Petri dish. At the lower protein concentration, 0.5%, (Figure 4.2.4.a) a complete, transparent, smooth coating was formed but was too thin to be released from the Petri dish. At 1% and 0.5% kafirin films could also be cast from glacial acetic acid but they were not as good sensorially, having a rough surface texture.

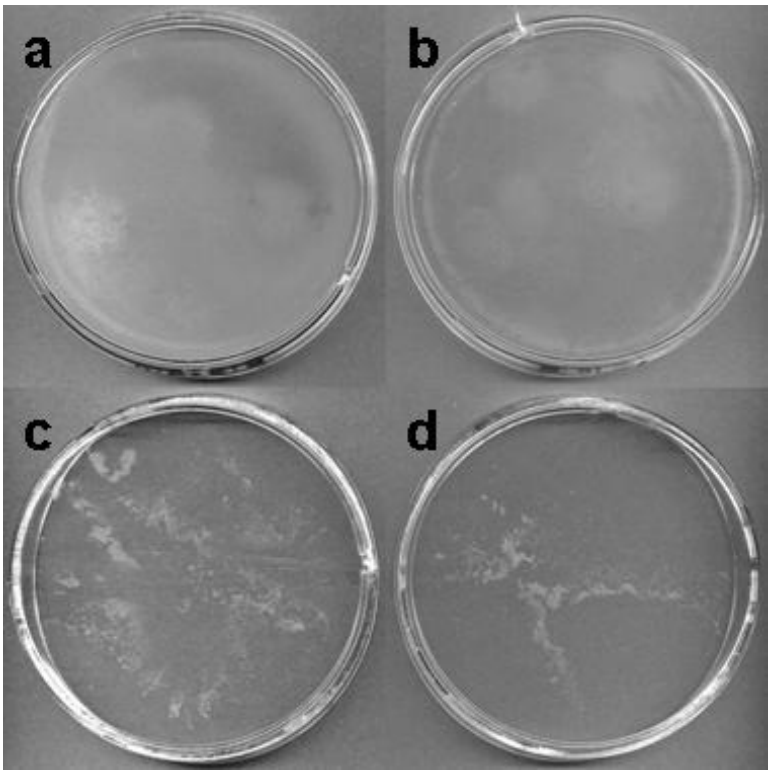


Figure 4.2.4: Effect of low protein concentration on kafirin film formation
Kafirin microparticle films, a- 0.5% kafirin, 5.4% acetic acid, b-1% kafirin, 10.8% acetic acid. Glacial acetic acid cast kafirin films, c-0.5% kafirin, d-1.0% kafirin

4.2.4.5. Microparticle film formation in organic acids other than acetic acid

The minimum acid concentration required to form a complete film at a particular kafirin microparticle concentration was found to be different for different acids. For acetic acid with 2% kafirin microparticles the minimum acid concentration for complete film formation was 21.6%, (Figure 4.2.5.a-c) whilst for propionic acid the minimum acid concentration was 10.8% (Figure 4.2.5.g-i). Figure 4.2.5.d-f illustrates that free-standing films could not be formed from lactic acid, probably due to its lack of volatility. However, free standing films could be made from lactic acid prepared microspheres if the lactic acid was removed by centrifugation and replaced by acetic acid (21.6%). Films made from propionic acid were as good sensorially as the acetic acid (21.6%) cast kafirin microparticle films being smooth, clear, flexible and with no odour. The ability of kafirin microparticles to form complete films at a lower acid concentration with propionic acid than acetic acid is probably related to the relative solubility of the kafirin in the different acids. Kafirin is more soluble in propionic acid than glacial acetic acid. 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% (Chapter 4.1. Figure 4.1.9.). Protein aggregation has been observed to occur before cohesive films form (Figure 4.2.3.). Also as the water evaporates the protein then appears to dissolve in the residual solvent, finally drying into a cohesive film. Thus if kafirin is more soluble in propionic acid than acetic acid and as shown, protein aggregation occurs at a lower acid concentration, then the same amount of protein would dissolve in less residual solvent than with acetic acid and subsequently a cohesive film would form at a lower acid concentration. This was found to be the case as a film could be formed from propionic acid at an acid concentration of 10.8 % (Figure 4.2.5.h).

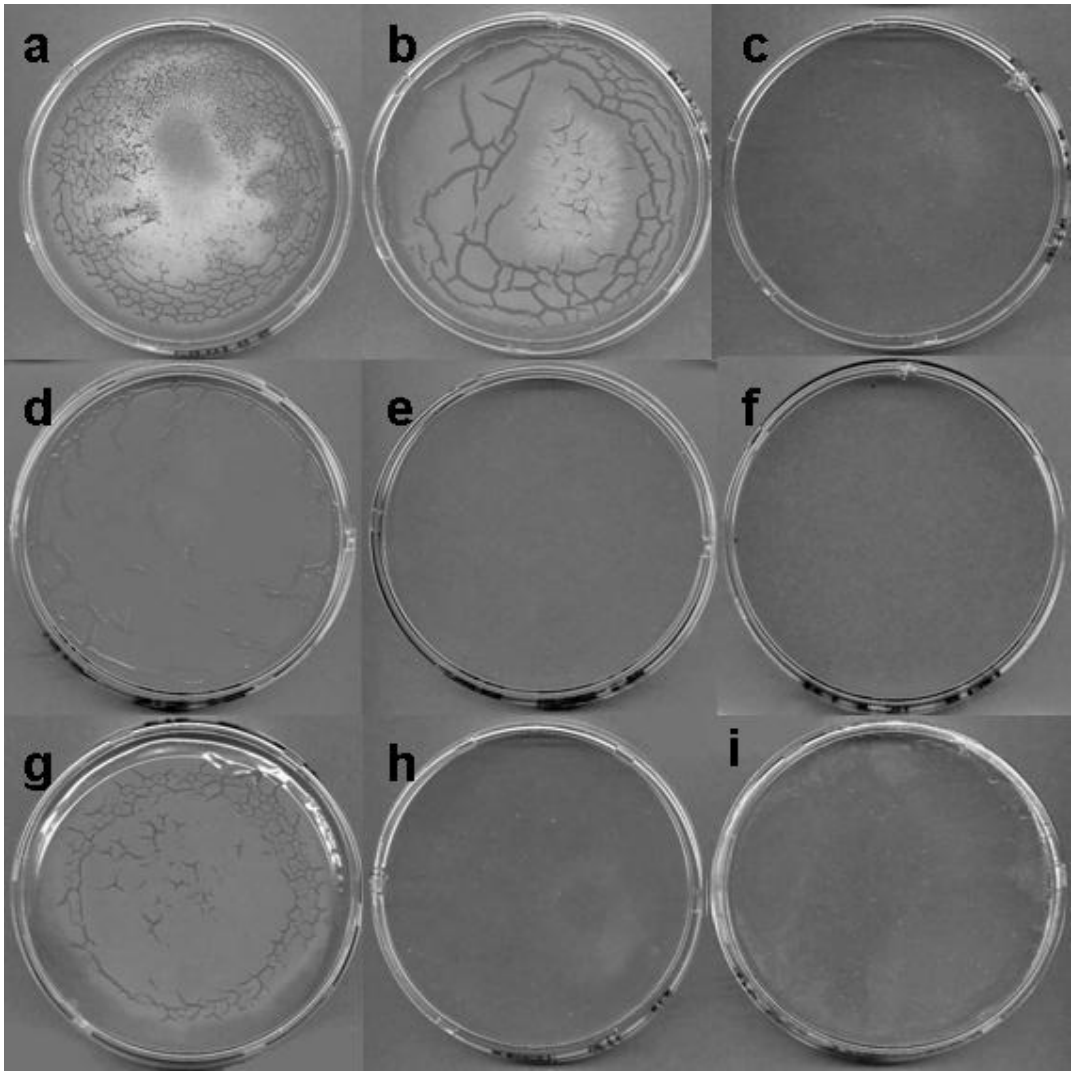


Figure 4.2.5: Effects of kafirin microparticles made with different acids with increasing acid concentration on film formation
Acetic acid, a-c, 5.4%, 10.6%, 21.8% acid
Lactic acid, d-f, 5.4%, 10.6%, 21.8% acid
Propionic acid, g-I, 5.4%, 10.6%, 21.8% acid
Note that use of lactic acid did not result in film formation

4.2.4.6. Fourier Transform Infrared Spectroscopy (FTIR) of kafirin films

FTIR spectra of cast kafirin films and cast microparticle kafirin films were compared and found to show different secondary structure between the two types of films (Table 4.2.1., Figure 4.2.6.). Considering the Amide I band of the FTIR spectra of the preparations, two main peaks were observed at wavenumbers 1650 and 1620 cm^{-1} (Figure 4.2.6.), the proportions of the peaks differing with the different films. As stated in Chapter 4.1., the peak around 1650 cm^{-1} can be assigned to α-helical conformations or random coils and 1620 cm^{-1} to anti-parallel β-sheet conformations (Singh, 2000; Duodu, Tang, Wellner,

Belton, and Taylor, 2001). The Amide I band vibrations are due mainly to C=O stretching, some C-N stretching, CNN deformation and in plane NH bends (Bandekar, 1992). In the Amide I region the glacial acetic cast film showed more α-helical to β-sheet conformation, whilst the microparticle film had almost equal proportions α-helical to β-sheet conformation. However, when the Amide II region was examined both films showed similar conformations of mainly α-helical with a smaller amount of β-sheet conformation. The difference between the Amide I and Amide II regions may have been due to the lower sensitivity of the Amide II region to variations in protein secondary structure (Singh, 2000) or due to interference from tyrosine at about 1515 cm^{-1} (Byaruhanga, Emmambux, Belton, Wellner, Ng and Taylor, 2006). Thus, only the Amide I region will be considered.

In order to quantify the differences in secondary structure between the two film preparations, the ratio of α-helical to intermolecular β-sheet conformation was calculated for the Amide I region (Table 4.2.1.). The glacial acetic acid cast film had a ratio of 1.21:1, whilst the ratio for the microparticle film was 1.02:1 in the Amide 1 region. Byaruhanga et al. (2006) reported a slightly higher ratio of 1.33:1 in the Amide I region for kafirin films cast from glacial acetic acid, indicating the presence of slightly more α-helical conformation than in found in films prepared in this study. Byaruhanga et al. (2006) and Gao et al. (2005) showed that for kafirin, differences in extraction and drying conditions resulted in slight differences in secondary structure. Thus, the higher ratio of α-helical conformation found by Byaruhanga et al. (2006) was probably a result of the different extraction procedures being used. No reference could be found in the literature of FTIR of protein microparticle films.

Table 4.2.1: Estimated ratio of α-helical to intermolecular β-sheet conformation of kafirin and kafirin films

Film type	**Ratio of α-helices to β-sheets**	
	Amide I[a]	**Amide II[b]**
Acetic acid microparticle, 21.6% acid, 40% plasticiser	1.02a[c] (0.07)	1.35a[d] (0.13)
Glacial acetic acid, kafirin 40% plasticiser	1.21b (0.02)	1.46a (0.12)

[a] Amide I: α-helices (1650 cm^{-1})/ β-sheets (1620 cm^{-1}). [c] Values for amide I with different letters are significantly different ($p<0.05$)
[b] Amide II: α-helices (1545 cm^{-1})/ β-sheets (1516 cm^{-1}). [d] Values for amide II with different letters are significantly different ($p<0.05$)

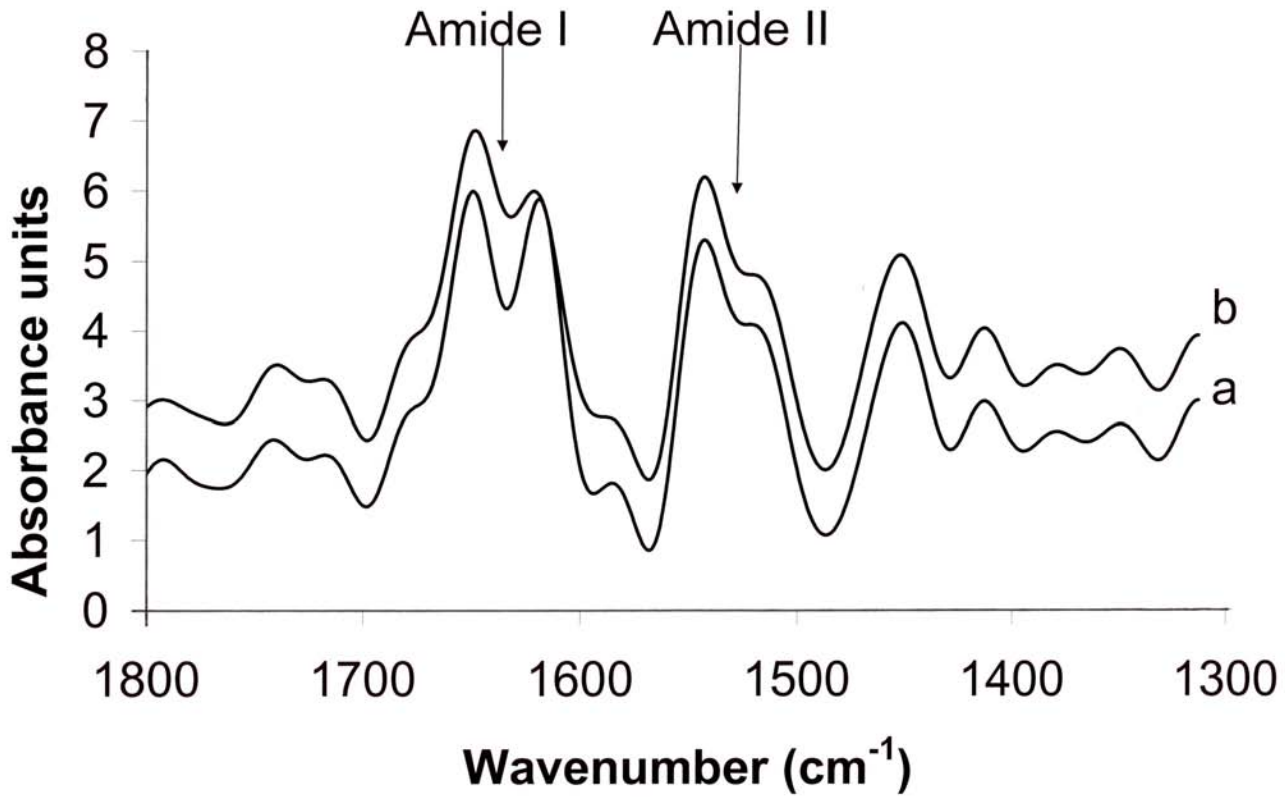


Figure 4.2.6: FTIR of kafirin films, a- kafirin microparticle film, b- cast in glacial acetic acid

The data indicate that the protein secondary structure of the kafirin microparticle film contained more β-sheet conformation than the glacial acetic acid film. According to Mizutani, Matsumura, Imamura, Nakanishi and Mori (2003) the presence of β-sheet conformation is indicative of protein aggregation. This is in agreement with protein aggregation observed when microparticle film formation was followed microscopically (Figure 4.2.3.A. and B.). Contrary to these findings, Hsu, Weng, Liao and Chen (2005) found the secondary structure of zein films to be predominately α-helical and concluded that hydrophobic interactions play an important role in film formation. According to the methodology of these workers, commercially prepared zein was used for film preparation and films were cast from 95% ethanol but details of temperature and degree of shear used to dissolve the zein was not given. In the author's experience commercial zein dissolves more readily than kafirin and so it is suggested that the conditions used for film formation in this study and those of Byaruhanga et al. (2006) were more severe than those used by Hsu et al. (2005) causing greater changes in secondary structure of the kafirin proteins on film formation.

The findings of this study are in agreement with those 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 conformational changes were not specific for glycinin since these workers referred to similar conformational changes occurring 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 it would be expected that the higher degree of intermolecular interactions which are present in β-sheet structures would result in the formation of films with better functional properties than films with a predominately α-helical secondary structure (Belton et al., 2006). In the case of kafirin microparticle films, this seems to be the case. Consequently this may result in the better film functional properties of kafirin microparticle films than glacial acetic acid cast kafirin films which are described below.

4.2.4.7. Functional properties of kafirin microparticle films

Film surface properties

Films cast from kafirin microparticles at 21.6% acetic acid were very smooth to the touch with no apparent imperfections. When observed by SEM, the top surface of the films appeared uniformly smooth with very few small holes (Figure 4.2.7.a). These were of approximately 1µm in diameter. When the underside of the same film was examined by SEM, it showed patches of roughness, possibly where the film adhered to the plastic of the Petri dish it was cast in (Figure 4.2.7.b). In comparison, films cast directly from kafirin at the same protein concentration using glacial acetic acid as casting solvent were slightly rough to the touch. When examined by SEM these films were excessively pitted on the surface with many holes, which were approximately 1-3µm in diameter (Figure 4.2.7.c). These holes may have been caused by air bubbles entrapped during the film casting process. The solution of kafirin in glacial acetic acid was very viscous compared to the kafirin microparticle suspension and so would be more likely to retain air bubbles incorporated by stirring during protein dissolution. During the drying process the film is heated to 50°C. With increasing temperature the viscosity of the kafirin solution would be reduced allowing escape of air bubbles from the film surface. As the film dried residual bubbles on the surface would have formed a circular footprint on the film surface, seen as holes by SEM. The underside of this film observed by SEM (Figure 4.2.7.d) showed more patches of roughness than the kafirin microparticle film but they were smaller in size. This may again have been due to adhesion to the plastic of the Petri dish and possibly the high viscosity of the solution may have increased the amount of adhesion. Byraruhanga et al. (2005) presented SEM micrographs of the top surface and underside of kafirin films cast from glacial acetic acid. They were similar in appearance to the kafirin films cast from glacial acetic acid of this study being pitted on the top surface and rough on the underside. These workers offered no explanation for the imperfections but noted that the number and size of the pores became less on modification by microwave heating.

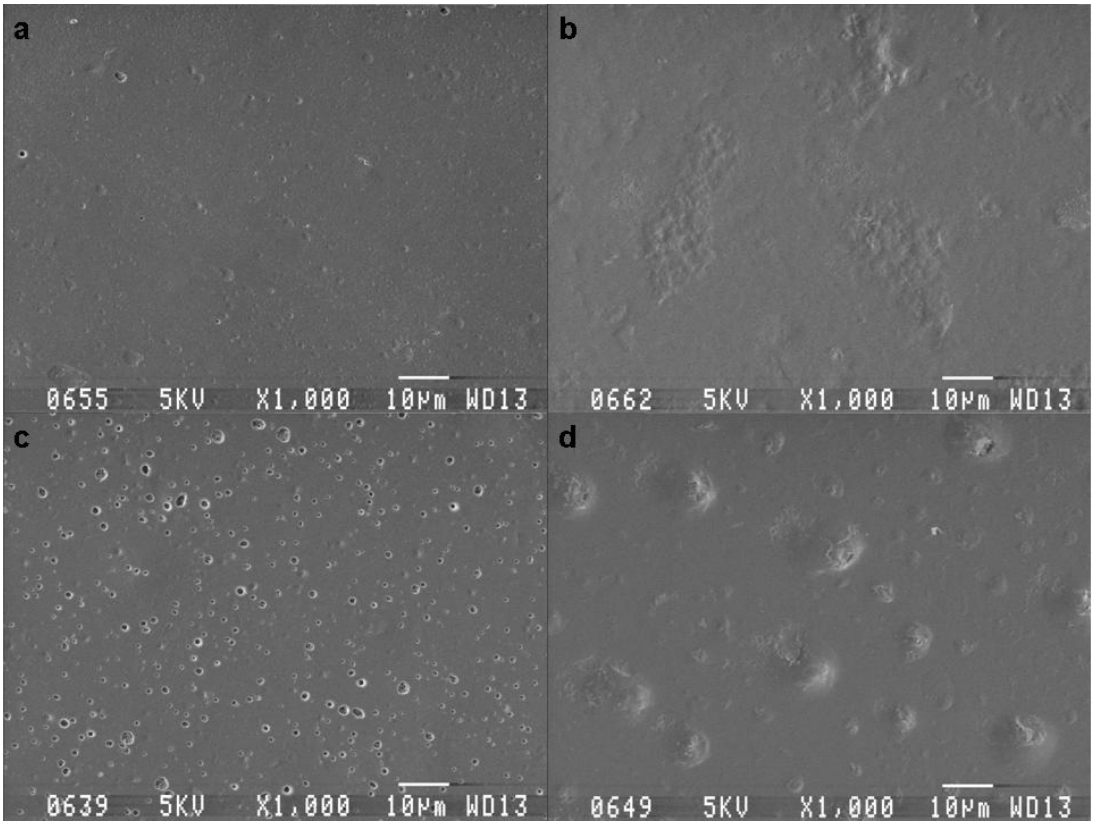


Figure 4.2.7: SEM of kafirin film surfaces
Kafirin microparticle film, a-top surface, b-underside
Glacial acetic acid cast kafirin film, c- top surface, d-underside

Concerning microparticle films, descriptions of acidified colloidal zein 'film' surfaces by Cook and Shulman (1998) appear similar to kafirin microparticle films made in this study. These authors describe the appearance of the colloidal 'films' (which by the definitions of this study were actually coatings), when visualised by SEM, as smooth and more dense and homogeneous than ethanol cast zein films, containing no void spaces or porosity. These coatings were said to have excellent resistance to moisture, lipid and gas permeation and excellent mechanical properties providing gloss and scruff resistance to substrates and were superior to zein films cast from aqueous ethanol.

Other workers have examined the surfaces of zein microparticle type films cast from microparticles made by various methods based on aqueous ethanol by SEM (O'Donnell, Wu, Wang, Wang, Oshlack, Chasin, Bodmeir and McGinity, 1997; Dong et al., 2004; Wang et al., 2005). Some film surfaces, such as those of described by O'Donnell et al. (1997) using zein pseudolatexes (6% protein) as coating material for tablet coatings appeared similar to kafirin microparticle films when plasticiser was used. However, when

no plasticiser was used these films were incomplete with many surface defects and cracks (O'Donnell et al., 1997). In contrast, none of the films cast from zein microparticles by Dong et al. (2004) or Wang et al. (2005) would be considered as complete cohesive films in terms of the present study. As stated, both Dong et al. (2004) and Wang et al. (2005) found that their films consisted of spherical particles agglomerated together to a greater or lesser extent.

Figure 4.2.8. illustrates AFM of kafirin microparticle films cast directly onto polished silica from 5.4% (Figure 4.2.8.a and b) and 21.6% acetic acid (Figure 4.2.8.c and d) at the same protein concentration (2%). As would be expected the two film casting formulations produced films with large differences, in keeping with already observed findings (Figure 4.2.3.). The film cast from the lower acid concentration shows in cross section many indentations, some of which were up to 250 nm in depth. The same film also had places of elevation of up to 100 nm. In comparison the kafirin microparticle films cast from 21.6% acetic acid was more uniform with fewer imperfections covering a maximum depth of 100 nm and elevation of 20 nm.

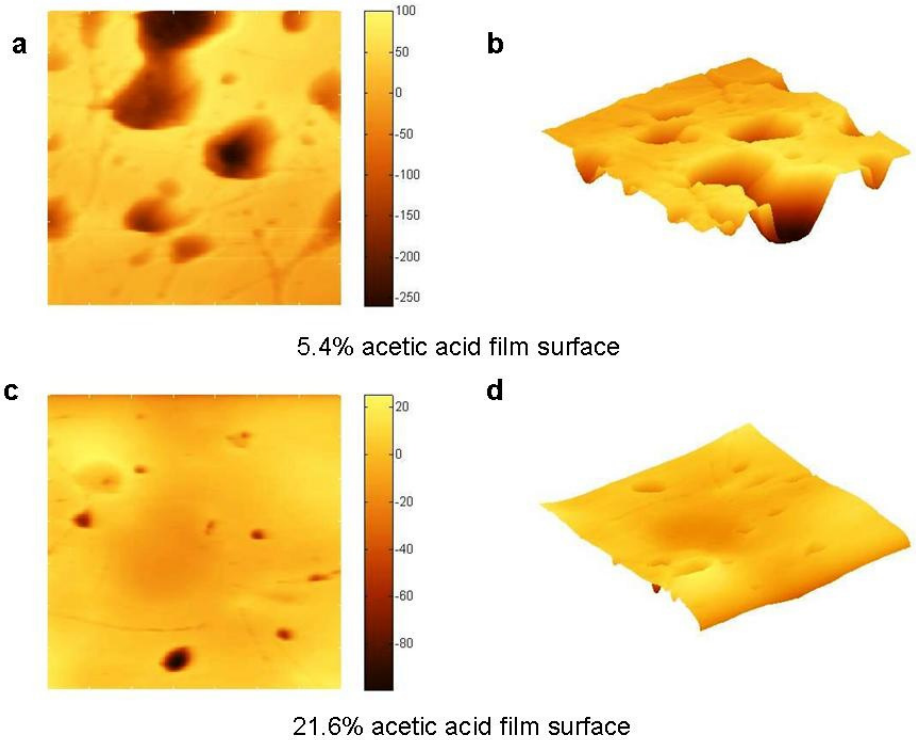


Figure 4.2.8: AFM of kafirin microparticle film surfaces
5.4% acetic acid microparticle film, a-top surface, b-3-D image of film surface
21.6% acetic acid microparticle film, c-top surface, d-3-D image of film surface
Scale bar in nm

Unfortunately the AFM used was not sufficiently sensitive to reveal information on the kafirin microparticle film structure at a molecular level. Guo et al. (2005) used AFM in the tapping mode to examine conventional zein films cast from 70% aqueous ethanol. They found the zein was present as globules of non uniform size. When a molecular combing procedure was used, rod-like structures were observed in shapes described as dumbbells, pole and branched structures. Under slightly different conditions these rods joined with each other forming doughnuts in an extended meshwork. These structures appeared similar to those observed during kafirin micoparticle film formation but the dimensions of the structures were vastly different.

Water Vapour Permeability (WVP) and Water Vapour Transmission (WVT)

The water vapour transmission (WVT) through all microparticle and glacial acetic acid cast films examined was similar except for kafirin microparticle films containing a low level of plasticiser (20%) (Table 4.2.2.). Here the WVT was slightly lower but not substantially so. Irrespective of the level of plasticiser the WVP through acetic acid microparticle films was much lower than through kafirin films of the same protein concentration cast with glacial acetic acid and propionic acid microparticle films (Table 4.2.2.). This is not surprising since WVP is a derived unit, which takes into consideration the thickness of the films. The acetic acid microparticle films were much thinner and more uniform than the acetic acid cast kafirin films. Propionic acid cast microparticle films were not significantly different in thickness to acetic acid kafirin microparticle films but had a WVP intermediate between acetic acid kafirin microparticle films and kafirin films cast with glacial acetic acid. Whilst apparently sensorially similar to the acetic acid microparticle films, it is possible that that there were imperfections in the film surface which resulted in more active diffusion through this film. However, since the surface properties of these films were not examined by SEM it is not possible to confirm this.

Kafirin films cast with glacial acetic acid were thicker than any of the microparticle films and had by far the highest WVP. These films were complete, clear but slightly yellow in colour with a rough and slightly uneven surface texture. The roughness and unevenness was possibly due to the kafirin being incompletely dissolved. There may also have been some phase separation of the plasticisers. Differences in film thickness at the same protein concentration were reflected in the film surface properties when viewed by SEM as previously described. The glacial acetic acid cast films were rougher than kafirin

microparticle films with many small holes. The different nature of the film forming solutions may have affected the rates at which the films dried. This may have resulted in variations in film thickness and the observed defects. Park and Chinnan, (1995) noted that the WVP through a film increases as film thickness increases due to differences in film structure. Since the mechanism of vapour flow through a film is predominantly by diffusion, any holes, cracks or imperfections as seen in the glacial acetic acid films would be expected to result in higher WVP as was observed. It is known that the permeability of zein films to water increases with increasing RH (reviewed by Lawton, 2002). This is thought to be due to the plasticising effect of water and to swelling of the film caused by absorbed water resulting in increased polymer chain mobility, which facilitates water vapour diffusion through the film (Roy, Gennadios, Weller and Testin, 2000). This is not supported by the data in this study since there is no apparent increase in film thickness at the end of the test.

Table 4.2.2: WVT and WVP of kafirin microparticle films compared with kafirin films cast in glacial acetic acid at the same protein concentration (2%)

Film type	Thickness (µm), start of test	Thickness (µm), end of test	WVT ($g/h/m^2$)	WVP (gmm/m^2hkPa)
Acetic acid microparticle, 21.6% acid, 20% plasticiser	15.9a (2.2)	13.1a (2.2)	36. 2a (1.0)	0.19a (0.03)
Acetic acid microparticle, 21.6% acid, 40% plasticiser	19.5ab (6.7)	14.1a (3.0)	39.4ab (3.7)	0.22a (0.05)
Acetic acid microparticle, 21.6% acid, 60% plasticiser	17.7ab (1.9)	15.4a (2.3)	41.7b (2.2)	0.25ab (0.03)
Propionic acid microparticle, 10.8% acid, 40% plasticiser	23.0ab (3.7)	22.9ab (6.9)	40.5b (1.9)	0.36b (0.11)
Glacial acetic acid, kafirin 40% plasticiser	26.4b (5.5)	31.0b (5.7)	40.8b (2.6)	0.50c (0.11)

Values in the same column but with different letters are significantly different ($p<0.05$).
Figures in parentheses indicate standard deviations

It is difficult to compare WVP for microparticle type films. Cook and Shulman (1998) in their patent described the water loss through zein colloidal films as 42 mg/hour. Film area was not considered and no control value was given. Film thickness, at an unspecified

solids content was given as 6 µm for colloidal films compared with 10 µm for ethanol cast zein films. Meaningful comparisons of these values with those obtained in this study are not possible. When comparisons are made with other published data for conventionally cast kafirin films, the kafirin microparticle films almost inevitably have much lower WVP since they are much thinner,. Buffo, Weller and Gennadios (1997) reported kafirin film WVP as 5.5 g mm/m^2 h kPa, whereas other workers reported lower values ranging from 0.4 to 0.8 g mm/m^2 h kPa (Gao et al., 2004; Da Silva and Taylor, 2005; Taylor et al., 2005; Byaruhanga et al., 2005). These values are similar to the control films cast from kafirin dissolved in glacial acetic acid. Kafirin films cast by the above workers contained 8 times more protein than the kafirin microparticle films and were consequently approximately 10 times thicker. Byraruhanga et al. (2005) showed SEM micrographs of the surface of kafirin films cast from glacial acetic acid. These films had many pores and imperfections and appeared similar to the control films cast from kafirin dissolved in glacial acetic acid in this study. It would then appear that the uniformity and lack of defects in the kafirin microparticle films are responsible for their better WVP than conventionally cast kafirin films.

Tensile Properties

Tensile properties of kafirin microparticle films were in the range of 2.4-8.5 N/mm^2 for stress at break (tensile strength) and 1.15-2.5% for strain (extensibility) (Table 4.2.3.). Not surprisingly, the strongest kafirin microparticle films were those containing the least amount of plasticiser (20%). There were no real difference in stress (tensile strength) between any of the films at the same plasticiser concentration and all the films had low percentage strain and were not extendable. It should be noted that standard deviations within a treatment were very high in most cases and so although statistically significant differences were shown in Table 4.2.3. between the different film treatments these results should be viewed with caution.

Stress (tensile strength) values for kafirin microparticle films (2.4-8.5 N/mm^2) and control kafirin films (5.4 N/mm^2) are similar to published values for kafirin films cast from aqueous ethanol, 6-8 N/mm^2 (Da Silva and Taylor, 2005) and 1.6-5.9 N/mm^2 (Gao et al., 2005) and 1.4 N/mm^2 for glacial acetic acid cast films (Byraruhanga et al., 2005) and 3.6-3.9 N/mm^2 (Taylor et al., 2005) but strain (extensibility) values were much lower, ranging

from 1.2-2.5% for microparticle films to 3.0% for the control films. Published strain (extensibility) values are 5-40% (Da Silva and Taylor, 2005) and 13.5-142% (Gao et al., 2005) for ethanol cast films both tested at ambient RH and 42-55.5% for glacial acetic acid cast films tested at ambient RH (Taylor et al., 2005) and 142% tested at 50% RH (Byraruhanga et al., 2005). The very low strain (extensibility) values of the kafirin microparticle films and the control kafirin films were probably due to their very low protein concentration. All the films in previously reported work contained more protein (i.e. 16%) than the kafirin microparticle films and the control kafirin films of this study. However, as noted above the kafirin microparticle films were more uniform and had fewer imperfections than conventionally cast kafirin films possibly accounting for the comparable stress (tensile strength) values.

Table 4.2.3: Tensile properties of kafirin microparticle films compared with kafirin films cast in glacial acetic acid at the same protein concentration (2%)

Film type	Thickness (μm)	Max Force (N)	Force at break (N)	Stress (N/mm^2)	Stress at break (N/mm^2)	Strain (%)
Acetic acid microparticle, 21.6% acid, 20% plasticiser	13.0a (3.0)	0.66b (0.34)	0.63b (0.33)	8.98c (4.71)	8.53c (4.48)	1.70ab (1.85)
Acetic acid microparticle, 21.6% acid, 40% plasticiser	14.0a (3.0)	0.38a (0.44)	0.33a (0.41)	4.18ab (5.25)	3.72ab (4.87)	2.53b (3.37)
Propionic acid microparticle, 10.8% acid, 40% plasticiser	14.0a (5.0)	0.22a (0.36)	0.16a (0.27)	3.26a (5.44)	2.39a (4.13)	1.15a (1.81)
Glacial acetic acid, kafirin 40% plasticiser	30.0b (3.4)	0.79b (0.26)	0.74b (0.25)	5.77b (2.15)	5.39b (2.07)	2.99b (1.79)

Values in the same column but with different letters are significantly different ($p<0.05$).
Figures in parentheses indicate standard deviations

Film Protein Digestibility

Protein digestibilities of the films ranged from 47.5% for acetic acid microparticle films with 60% plasticiser to 89% for kafirin films cast from glacial acetic acid (Table 4.2.4.). All microparticle films were significantly less digestible than glacial acetic acid cast kafirin films. Protein digestibilities of conventionally cast kafirin films is in agreement with values for films cast with glacial acetic acid at 16% protein concentration by Byaruhanga, Erasmus, Emmambux and Taylor (2007) and Byaruhanga et al. (2005). The lower protein digestibility of microparticle films compared with kafirin films cast in glacial acetic acid may have been due to their superior surface properties. They were smooth and uniform in contrast to kafirin films cast in glacial acetic acid which were rough and uneven, with some phase separation and incomplete protein solvation. The poor surface properties of these glacial acetic acid films would allow more ease of access for the pepsin enzyme than the microparticle films. Byaruhanga et al. (2005) explained the high protein digestibility of glacial acetic acid cast kafirin films by saying that the glacial acetic acid was thought to deamidate the gluatamine residues of the kafirin resulting in breakage of both hydrogen bonds and hydrophobic interactions, making the film readily accessible to pepsin digestion. Enzyme susceptibility is known to increase with deamidation of wheat gluten (Mimouni, Raymond, Merle-Desnoyers, Azana and Ducastaing (1994) and whey proteins (Lakkis and Villota, 1992). Since the microparticles were prepared by dissolving the kafirin in glacial acetic acid it is likely that that the kafirin would also have undergone deamidation in the same way. Thus it is more probable that only the poor surface properties of the glacial acetic acid films would be the cause of their higher protein digestibility when compared to kafirin microparticle films.

Acetic acid microparticle films (21.6% acid) with 60% plasticiser were the most resistant to pepsin digestion (Table 4.2.4.). According to Byaruhanga et al. (2007) addition of plasticisers to kafirin films would increase susceptibility to enzyme attack by increasing the free volume between the kafirin molecules. In the case of kafirin microparticle films, plasticiser concentration appeared to have an effect on the protein digestibility converse to that expected. Since this is counterintuitive it is difficult to explain unless the plasticiser bound to the protein in a way which protected it from the pepsin enzyme. Evidence described above on the apparent lack of leaching of glycerol from kafirin microparticle films indicates that there may be some binding of the plasticisers to the protein in microparticle films. It is possible that this would protect the films from pepsin digestion.

Table 4.2.4: Protein digestibility of kafirin microparticle films compared with kafirin films cast in glacial acetic acid at the same protein concentration (2%)

Film type	Protein digestibility (%)
Acetic acid microparticle, 21.6% acid, 20% plasticiser	71.9c (3.9)
Acetic acid microparticle, 21.6% acid, 40% plasticiser	65.7b (2.5)
Acetic acid microparticle, 21.6% acid, 60% plasticiser	47.5a (7.4)
Propionic acid microparticle, 10.8% acid, 40% plasticiser	69.9bc (6.7)
Glacial acetic acid, kafirin, 40% plasticiser	89.0d (1.3)

Values in the same column but with different letters are significantly different ($p<0.05$).
Figures in parentheses indicate standard deviations

Biodegradation of films

All films had completely biodegraded by day 3 except for the low density polyethylene (LDPE) control (Figure 4.2.9.). This faster rate of degradation than the 14 days found by Byaruhanga et al. (2005, 2007) was probably due to the very low protein concentration in the film casting solution (2% compared to 16% protein) and corresponding film thinness compared to films used by these workers. These results are not surprising when the tensile properties of the films at 50% RH are considered. The films were very thin and weak with little cohesive strength at high RH and so would expect to be readily degraded by microbial enzymes under moist conditions.

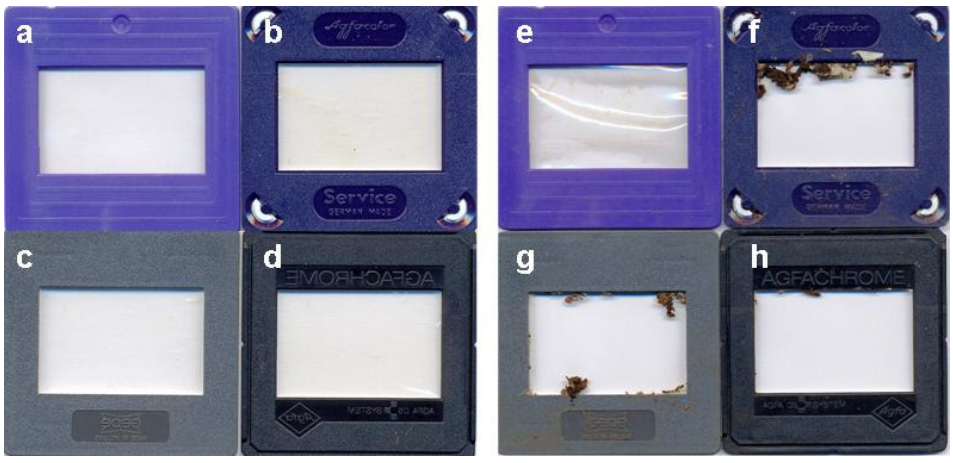


Figure 4.2.9: Biodegradation of kafirin films
Day 0, a-LDPE, b-glacial acetic acid cast kafirin film, c- propionic acid cast kafirin microparticle film, d-acetic acid cast kafirin microparticle film
Day 3, e-LDPE, f-glacial acetic acid cast kafirin film, g- propionic acid cast kafirin microparticle film, h-acetic acid cast kafirin microparticle film

4.2.5. Conclusions

Kafirin microparticles made by phase separation from an organic acid 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. This relative amount was 10.8:1, percent organic acid to percent protein when acetic acid was the organic acid used. The mechanism of 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 concentration, whilst others such as the tensile properties need to be improved before they can be used for practical applications. Investigation into cross-linking either by physical or chemical means as a potential means to improve kafirin microparticle film tensile properties is needed.

4.2.6. References

American Association of Cereal Chemists, 2000. Crude protein-combustion, Standard Method 46-30. Approved Methods of the AACC. The Association, St Paul, MN.

American Society for Testing and Materials, 1996. Annual Book of the American Society for Testing and Materials Standards. ASTM D5512-96. Standard practice for exposing plastics to a simulated compost environment using an externally heated reactor. The Society: West Conshohocken, PA.

American Society for Testing and Materials, 1997a. Annual Book of the American Society for Testing and Materials Standards. ASTM E96-97. Standard test methods for water vapour transmission of materials. The Society: West Conshohocken, PA.

American Society for Testing and Materials, 1997b. Annual Book of the American Society for Testing and Materials Standards. ASTM D882-97. Standard method for tensile properties of thin plastic sheeting. The Society: West Conshohocken, PA.

Bandekar, J., 1992. Amide modes and protein conformation. Biochimica et Biophysica Acta 1120, 123-143.

Banker, G.S., 1966. Film coating theory and practice. Journal of Pharmaceutical Sciences 55, 81-89

Belton, P.S., Delgadillo, I., Halford, N.G., Shewry, P.R., 2006. Kafirin structure and functionality. Journal of Cereal Science 44, 272-286.

Buffo, R.A., Weller, C.L., Gennadios, A., 1997. Films from laboratory-extracted kafirin. Cereal Chemistry 74, 473-475

Byaruhanga, Y.B., Erasmus, C., Taylor, J.R.N., 2005. Effect of microwave heating of kafirin on the functional properties of kafirin films. Cereal Chemistry 82, 565-573.

Byaruhanga, Y.B., Erasmus, C., Emmambux, M.N., Taylor, J.R.N., 2007. Effect of heating cast kafirin films on their functional properties. Journal of the Science of Food and Agriculture 87, 167-175.

Byaruhanga, Y.B., Emmambux, M.N., Belton, P.S., Wellner, N., Ng, K.G., Taylor, J.R.N., 2006. Alteration of kafirin film structure by heating with microwave energy and tannin complexation. Journal of Agricultural and Food Chemistry 54, 4198-4207.

Cook, R.B., Shulman, M.L., 1998. Colloidal dispersions of gluten, method of making and use therefor. United States Patent and Trademark Office 5736178.

Da Silva, L.S., Taylor, J.R.N., 2005. Physical, mechanical, and barrier properties of kafirin films from red and white sorghum milling fractions. Cereal Chemistry 82, 9-14.

Dong, J., Sun, Q., Wang, J.Y., 2004. Basic study of corn protein, zein, as a biomaterial in tissue engineering, surface morphology and biocompatibility. Biomaterials 25, 4691-4697.

Duodu, K.G., Taylor, J.R.N., Belton, P.S., Hamaker, B.R., 2003. Factors affecting sorghum protein digestibility. Journal of Cereal Science 38, 117-131.

Duodu, K.G., Tang, H., Wellner, N., Belton, P.S., Taylor, J.R.N., 2001. FTIR and solid state ^{13}C NMR spectroscopy of proteins of wet cooked and popped sorghum and maize. Journal of Cereal Science 33,261-269.

Emmambux, M.N., Stading, M., Taylor, J.R.N., 2004. Sorghum kafirin film property modification with hydrolysable and condensed tannins. Journal of Cereal Science 40, 127-135.

Enviropak (2003) http://www.sik.se/enviropak Date accessed 4 September, 2003.

Gao, C., Stading, M., Wellner, N., Parker, M.L., Noel, T.R., Mills, E.N.C., Belton, P.S., 2006. Plasticization of a protein-based film by glycerol: a spectroscopic, mechanical, and thermal study. Journal of Agricultural and Food Chemistry 54, 4611-4616.

Gao, C., Taylor, J., Wellner, N., Byaruhanga, Y.B., Parker, M.L., Mills, E.N.C., Belton, P.S., 2005. Effect of preparation conditions on protein secondary structure and biofilm formation of kafirin. Journal of Agricultural and Food Chemistry 53, 306-312.

Guo, Y., Liu, Z., An, H., Li, M., Hu, J., 2005. Nano-structure and properties of maize zein studied by atomic force microscopy. Journal of Cereal Science 41, 277-281.

Hsu, B.L., Weng, Y.M., Liao, Y.H., Chen, W., 2005. Structural investigation of edible zein films/coatings and directly determining their thickness by FT-Raman spectroscopy. Journal of Agricultural and Food Chemistry 53, 5089-5095.

Jackson, D.S., Shandera, J.R., 1995. Corn wet milling: Separation chemistry and technology. Advances in Food and Nutrition Research 38, 271-300.

Krochta, J.M., 2002. Proteins as raw materials for films and coatings: definitions, current status, and opportunities. In: Gennadios, A., (Ed.), Protein-Based Films and Coatings. CRC Press, Boca Raton, FL, pp. 1-42.

Lakkis, J., Villota, R., 1992. Comparative performance of chemically and enzymatically modified whey proteins. Food Chemistry 43, 93-105.

Lawton, J.W., 2002. Zein: a history of processing and use. Cereal Chemistry 79, 1-18.

Mertz, E.T., Hassen, M.M., Cairns-Whittern, C., Kirleis, A.W., Tu, L., Axtell, J.D., 1984. Pepsin digestibility of proteins in sorghum and other major cereals. Proceedings of the National Academy of Sciences of the United States of America 81, 1-2.

Mimouni, B., Raymond, J., Merle-Desnoyers, A.M., Azana, J.L., Ducastaing, A., 1994. Combined acid deamidation and enzymic hydrolysis for improvement of the functional properties of wheat gluten. Journal of Cereal Science 21, 153-165.

Mizutani, Y., Matsumura, Y., Imamura, K., Nakanishi, K., Mori, T., 2003. Effects of water activity and lipid addition on secondary structure of zein in powder systems. Journal of Agricultural and Food Chemistry 51, 229-235.

O'Donnell, P.B., Wu, C., Wang, J., Wang, L., Oshlack, B., Chasin, M., Bodmeir, R., McGinity, J.W., 1997. Aqueous pseudolatex of zein for film coating of solid dosage forms. European Journal of Pharmaceutics and Biopharmaceutics 43, 83-89.

Padua, G.W., Wang, Q., 2002. Formation and properties of corn zein films and coatings. In:Gennadios, A., (Ed.), Protein-Based Films and Coatings. CRC Press, Boca Raton, FL, pp. 43-68.

Park, H.J., Chinnan, M.S., 1995. Gas and water vapour barrier properties of edible films from protein and cellulosic materials. Journal of Food Engineering 25, 497-507.

Roy, S., Gennadios, A., Weller, C.L., Testin, R.F., 2000. Water vapor transport parameters of a cast wheat gluten film. Industrial Crops and Products 11, 43-50.

Singh, B.R., 2000. Basic aspects of the technique and applications of infrared spectroscopy of peptides and proteins. In: Singh, B.R., (Ed.), Infrared Analysis of Peptides and Proteins, Principles and Application. American Chemical Society Symposium Series, American Chemical Society, Washington, DC, pp. 2-37.

Subirade, M., Kelly, I., Guéguen, J., Pézolet, M., 1998. Molecular basis of film formation from a soybean protein: comparison between the conformation of glycinin in aqueous solution and in films. International Journal of Biological Macromolecules 23, 241-249.

Taylor, J., Taylor, J.R.N., Dutton, M.F., De Kock, S., 2005. Identification of kafirin film casting solvents. Food Chemistry 32, 149-154.

Taylor, J.R.N., Schober, T.J., Bean, S.B., 2006. Novel food and non-food uses for sorghum and millets. Journal of Cereal Science 44, 252-271.

Wang, H.J., Lin, Z.X., Liu, X.M., Sheng, S.H., Wang, J.Y., 2005. Heparin-loaded zein microsphere film and hemocompatibility. Journal of Controlled Release 105, 120-131.

4.3. TANNIN INTERACTIONS WITH SORGHUM PROTEINS, KAFIRIN FILMS AND MICROPARTICLES AND ITS INFLUENCE ON THEIR DIGESTIBILITY AND FUNCTIONALITY

This chapter has been published in part in the Journal of Cereal Science,
Taylor, J., Bean, S.R., Ioerger, B.P., Taylor, J.R.N., 2007. Preferential binding of sorghum tannins with γ-kafirin and the influence of tannin binding on kafirin digestibility and biodegradation. Journal of Cereal Science 46, 22-31.

4.3.1. Abstract

With the aim of exploiting the porous nature of kafirin microparticles for encapsulation of nutrient additives several factors were examined for their influence on sorghum protein digestibility. It was confirmed that disulphide cross-linking, especially on cooking and interaction of tannins with sorghum proteins reduce sorghum protein digestibility. It was found that γ-kafirin bound more condensed tannins than other kafirin species and that γ-kafirin bound tannins were aggregates of molecular size >200 k. Kafirins bound to tannins had lower digestibilities than unbound kafirins. Also, tannin-bound kafirin films had lower digestibility and were less biodegradable than films made from unbound kafirin. Tannin-kafirin interactions and low kafirin digestibility were exploited to develop a new delivery vehicle for dietary polyphenolic antioxidants. Catechin and sorghum condensed tannins were encapsulated within kafirin microparticles and the antioxidant release profiles investigated under simulated gastric conditions. Antioxidant activity of the catechin was 15327 μmoles Trolox equivalents/g and of the sorghum condensed tannins 5366 μmoles Trolox equivalents/g. Over a period of four hours, catechin and sorghum condensed tannin encapsulated kafirin microparticles showed virtually no protein digestion but released approximately 70% and 50% respectively total antioxidant activity. Thus, encapsulation of catechin and sorghum condensed tannins in kafirin microparticles has potential as an effective way to deliver dietary antioxidants.

4.3.2. Introduction

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). Interaction between tannins and sorghum proteins is another causal factor of poor protein digestibility of sorghum (reviewed by Serna-Saldivar and Rooney, 1995). This interaction is particularly important as a substantial proportion of sorghum varieties, the Types II and III, contain condensed tannins (reviewed by Awika and Rooney, 2004). This is important in both human (Salunkhe, Chavan and Kadam, 1990) and animal nutrition (Hancock, 2000). The formation of complexes between sorghum proteins and tannins is thought to render the proteins indigestible (Butler, Riedl, Lebryk and Blytt, 1984) as well as inhibit digestive enzymes (Nguz, Van Gaver and Huyghebaert, 1998). During the brewing process sorghum malt amylase enzymes are inhibited by tannin interaction (Daiber, 1975). However, tannin-protein interactions can be used in some cases to improve protein functionality. For example, a useful application of sorghum tannin-protein interaction is the improvement in tensile properties of kafirin bioplastic films by tannin binding (Emmambux, Stading and Taylor, 2004).

Proteins rich in proline bind more sorghum tannins than other proteins (reviewed by Spencer, Cai, Gaffney, Goulding, Magnolato, Lilley and Haslam, 1988). In addition, a protein containing more proline repeats will bind more tannin than one with less such repeats (Baxter, Lilley, Haslam and Williamson, 1997). Kafirin, the sorghum prolamin storage protein, is relatively rich in proline (11 mole %) (Evans, Schüssler and Taylor, 1987) and has been shown to form complexes with both sorghum condensed tannins and tannic acid (Emmambux and Taylor, 2003). Gamma-kafirin is the most proline rich of the kafirin polypeptide species (23 mole %) (reviewed by Shewry, 2002) and contains the most proline repeats (Swiss Prot, 2005) of the kafirins. In view of the importance of protein-tannin interactions in the nutritional and functional properties of sorghum, more in depth knowledge on the interactions between kafirins and tannins are needed.

Recently, there has been considerable interest in the positive health aspects of sorghum polyphenols since they exhibit considerable antioxidant activity (reviewed by Awika and Rooney, 2004). When compared to blueberries they have similar antioxidant levels and

higher levels than other cereals and fruits such as strawberry and plums (Dykes and Rooney, 2007). According to Awika and Rooney (2004) sorghum consumption reduces the risk of some types of cancer in humans and the high levels of sorghum phytochemicals are thought to be responsible. In addition, sorghum phytochemicals have been shown to promote cardiovascular health in animals. Whilst not proven in humans, reduced risk of cardiovascular disease is thought to be attributable to tannins and other polyphenols in red wine and tea (reviewed by Awika and Rooney, 2004; reviewed by Dykes and Rooney, 2006).

The antioxidant activity of polyphenols in the body is dependant on their degree of absorption and metabolism (reviewed by Bravo, 1998). Only partial amounts of food polyphenols are absorbed in vivo. This is thought to be due to factors such as poor solubility, inefficient permeability, instability due to food storage, first pass metabolism before reaching the systemic circulation and gastrointestinal degradation (Ratnam, Ankola, Bhardwaj, Sahana and Kumar, 2006). In order to take advantage of the potential therapeutic benefits of antioxidants, development of new methods of delivery are required.

Thus, the first objective of this work was to determine the effect of sorghum variety, tannin content, cooking and the effect of addition of a reducing agent to raw and cooked flour on in vitro sorghum protein digestibility. A second objective was to determine whether γ-kafirin (due to its high levels of proline and proline repeats) preferentially binds to sorghum condensed tannin and to determine the effect of the tannin binding on kafirin in vitro digestibility, kafirin film digestibility and biodegradation. The final objective was to exploit previously thought disadvantages of the interactions between sorghum polyphenols and proteins, such as cross-linking and the resulting decrease in protein digestibility to develop a potential new delivery vehicle for dietary antioxidants, such as catechin and sorghum condensed tannins by encapsulation within kafirin microparticles. Further, to determine the release profiles of these antioxidants and the enzymic degradation of the encapsulated materials within the kafirin microparticles under simulated gastric conditions.

4.3.3. Materials and methods

4.3.3.1. Materials

Eleven sorghum varieties used to determine the effect of a reducing agent on sorghum protein digestibility were from the University of Pretoria sorghum collection. Two additional sorghum varieties were obtained from the CSIR, Pretoria, South Africa. Protein contents (N x 6.25) ranged from 6-14%. One Type III and three Type II tannin sorghum lines were included. Sorghum samples were milled to particle size less than 0.5 mm using a laboratory hammer mill (Falling Number, Huddinge, Sweden). All reagents were analytical grade and obtained from Merck SA. Porcine pepsin (Merck 107190, 2000 FIP-U/g), trypsin (Sigma TO 303 14000 BAEE unit/mg) and chymotrypsin (Sigma C4129, 83.9 BTEE unit/mg) were used for the various protein digestibility assays as described in the methodology below.

4.3.3.2. Extraction of total kafirin and gamma-kafirin

A mixture of two condensed tannin-free, tan plant, white sorghum cultivars PANNAR PEX 202 and 206 was used for total kafirin extraction. The method of total kafirin extraction was as described by Emmambux and Taylor (2003). This total kafirin was also used to prepare kafirin microparticles as described in Chapter 4.1. and used for the encapsulation study.

Gamma-kafirin was isolated from total kafirin using 0.05 M sodium lactate containing 2% (v/v) 2-mercaptoethanol (Evans et al., 1987) at a protein to solvent ratio of 1:5. Extraction was carried out for 3 periods of 1 h with constant stirring at 25°C. After each period the mixture was centrifuged at 7200 g for 10 min. The supernatant was removed and the pellet re-extracted for a further period. Supernatants were combined and dialysed against distilled water over a period of 36 h with frequent changes of water. Dialysed material and the residual pellet were freeze dried and designated γ-kafirin and residual kafirin (i.e. total kafirin minus γ-kafirin), respectively. The identity of the γ-kafirin was confirmed by comparing its amino acid composition with that in the literature (Taylor and Belton, 2002) (Table 4.3.1.). It was also confirmed using reversed-phase high performance liquid chromatography (RP-HPLC) and free zone capillary electrophoresis (FZCE) by comparing the separation of the isolated γ-kafirin to previously published reports of the separation of the kafirin species (Taylor, Bean, Ioerger and Taylor, 2007).

Condensed tannin was extracted using the method of Emmambux and Taylor (2003) from a red Type III tannin sorghum (ex. Nola GH91) and used for the binding assay, preparation of tannin modified kafirin films and for encapsulation in kafirin microparticles. The condensed tannin content of the extract was 2730 mg/g (dry weight) (catechin equivalents). Catechin (Sigma C1788, St. Louis, MO, USA) was used for encapsulation in kafirin microparticles.

Table 4.3.1: Amino acid composition (moles %) of kafirin preparations

Amino acid	Total kafirin	γ-kafirin	Residual kafirin
Asparagine	4.3	0.9 (0)	4.5
Glutamine	20.1	12.8 (11.9)	20.4
Serine	6.3	5.7 (5.2)	6.3
Glycine	3.2	9.4 (8.8)	2.7
Histidine	1.4	7.0 (7.8)	1.2
Arginine	1.4	2.7 (2.1)	1.2
Threonine	3.2	5.1 (4.7)	3.0
Alanine	15.1	6.7 (5.7)	15.5
Proline	11.1	18.7 (23.3)	10.7
Tyrosine	3.7	2.3 (2.1)	3.7
Valine	5.0	6.1 (6.2)	5.0
Methionine	1.1	1.4 (1.0)	1.2
Cystine	0.4	7.8 (7.8)	0.4
Isoleucine	4.1	2.7 (2.6)	4.2
Leucine	14.7	8.3 (8.3)	15.0
Phenylalanine	4.8	1.8 (1.6)	4.9
Lysine	0.2	0.6 (0)	0.2

Figures in parenthesis are literature values from Taylor and Belton (2002)

4.3.3.3. Methods

Effect of reducing agent on sorghum protein digestibility

The method used was the in vitro protein digestibility assay of Mertz, Hassen, Cairns-Whittern, Kirleis, Tu, and Axtell (1984). Samples were analysed raw, raw reduced, cooked and cooked reduced. For raw samples under reducing conditions, approximately 200 mg of accurately weighed sample was suspended in 7 ml pH 2.0 citrate buffer without pepsin and 100 µl mercaptoethanol added with vigorous shaking. Citrate buffer (pH 2.0, 28 ml) containing 131 mg pepsin/100 ml buffer was then added and samples mixed thoroughly before incubating as described above. For samples cooked in the presence of a reducing agent, 200 mg of accurately weighed sample was suspended in 2 ml distilled

water and 100 µl mercaptoethanol added with vigorous shaking before continuing for cooked samples as described in Chapter 4.1.

Tannin content

The tannin content was determined using the Vanillin HCl method of Price, Van Scoyoc and Butler (1978) using catechin as a standard.

Tannin Type

Tannin Type was determined by visual assessment in combination with the Chlorox bleach test (Taylor, 2001).

Amino acid analysis

The amino acid composition of the protein preparations was determined using the Pico-Tag method (Bidlingmeyer, Cohen and Tarvin, 1984) and analysed by reversed phase High Performance Liquid Chromatography (HPLC).

Tannin binding assay

The method of Emmambux and Taylor (2003) was used with slight modification. Total kafirin, γ-kafirin and residual kafirin (1 mg protein (N x 6.25)/ml) and condensed tannin (0-400 µg/ml) (in terms of catechin equivalents) were dissolved individually in 75% (v/v) absolute ethanol containing 0.02 M (pH 4.0) phosphate buffer. One ml kafirin solution was mixed with 1 ml condensed tannin solution and incubated for 1 h at 60°C in a shaking waterbath. Samples were chilled overnight at 4°C before being vortexed and centrifuged at 2000 g for 5 min. The supernatant was then decanted and used for the determination of residual condensed tannin. The amount of condensed tannin bound to protein was determined by difference between the total amount of condensed tannin added and the amount remaining, as it was not possible to completely re-solubilise the precipitated condensed tannin bound protein. The International Organisation for Standardization (ISO) (1988) ferric ammonium citrate method was used to quantify tannins using catechin (Sigma, St. Louis, MO, USA) as a standard.

SDS-PAGE

Samples for determination of protein digestibility and characterisation by SDS-PAGE were prepared using a 10 mg/ml protein solution and a 23 mg/ml condensed tannin solution. After binding as described above and chilling overnight, samples were centrifuged at 2000 g for 15 min. The supernatant was retained and the pellet washed with the ethanol/buffer solution. This was repeated twice more to remove any unbound condensed tannin and the supernatants were bulked. Ethanol was allowed to evaporate off at room temperature before the supernatants and pellets were freeze dried. The supernatant was assumed to contain any remaining unbound protein as shown by SDS-PAGE, whereas the pellet contained the precipitated, insoluble tannin-protein complexes.

Protein preparations were characterised by SDS-PAGE on a 4-18% acrylamide gradient both under reducing and non-reducing conditions. A gradient system was used in order to separate high molecular weight oligomers present in the samples. The gradient was prepared from a stock of 40% (w/v) acrylamide/bis (19:1) (Merck, Halfway House, South Africa) and allowed the separation of proteins with a M_r of 14 to 200 k. A vertical electrophoresis system (Protean II, Bio-Rad, Hercules, CA, USA) was used with gels of 140 mm length and 1.5 mm thick according to Gallagher (1999). The different protein preparations were loaded to constant protein (≈15 µg) as determined by the Dumas combustion method, American Association of Cereal Chemists standard method 46-30, (AACC 2000). Special care was taken to completely resolubilise the condensed tannin bound samples. This was done by repeatedly heating and vortexing until solubilisation was obtained. Molecular weight markers (low-range protein marker, Roche Molecular Biochemicals, Indianapolis, IN, USA) were used. Proteins were stained with Coomassie Brilliant Blue R250.

Film formation

Total kafirin, kafirin bound to condensed tannin (20% with respect to protein) and zein (Sigma Z-3625, St. Louis, MO, USA) were used to cast free standing films as described by Emmambux and Taylor (2003).

Protein digestibility of protein preparations and films

The pepsin method of Mertz et al. (1984) was used as described in Chapter 4.2. Protein preparations and films (10 mg protein basis) were digested for 2 h at 37°C with pepsin as described above. Total protein and residual protein (N x 6.25) was determined by the Dumas combustion method.

Film biodegradation

Biodegradation of films was determined using a procedure based on American Society for Testing and Materials (ASTM) Method D5512-96 (American Society for Testing and Materials, 1996) as described in Section 4.2.3.4., Biodegradation of films, except that two moisture contents were used, 37% and 60% and the test was continued for a total of 28 days.

Preparation of kafirin microparticles for encapsulation

Freeze dried kafirin microparticles were prepared as described in Chapter 4.1.

Encapsulation of catechin and sorghum condensed tannins in kafirin microparticles

Two phenolic compounds were chosen for encapsulation by kafirin microparticles; catechin, which has not been found to complex with kafirin and an extract of sorghum condensed tannins, found to complex with kafirin (Emmambux and Taylor, 2003). Kafirin microparticles were used to encapsulate catechin and sorghum condensed tannins at a level of 20% polyphenol in relation to protein. Catechin and sorghum condensed tannin extract (400 mg) was dissolved separately in 10 ml 70% aqueous acetone. A portion (3.75 ml) of each polyphenol solution was mixed individually with 750 mg freeze dried kafirin microparticles. Samples were left for 1 h before drying overnight at ambient temperature (25°C). The resultant material was ground to a fine powder using a mortar and pestle. Two samples of each polyphenol were prepared.

Transmission Electron Microscopy (TEM) of kafirin microparticles, kafirin microparticle encapsulated catechin and kafirin microparticle encapsulated sorghum condensed tannins

Samples were prepared for TEM as described in Chapter 4.1.

Dissolution and release of antioxidant activity by simulated digestion

Catechin encapsulated within kafirin microparticles (20% in relation to protein), sorghum condensed tannins encapsulated within kafirin microparticles (20% in relation to protein) and freeze dried kafirin microparticles (control) were used. Total protein content of the encapsulated material and control was determined. Approx. 20 mg sample was accurately weighed into a 2 ml Eppendorf tube with cap. Sodium citrate buffer, 0.1M (0.75 ml), pH 2.0 was added, followed immediately with 1.0 ml pH 2.0 citrate buffer containing pepsin (367.5 mg pepsin/100 ml buffer) and suspended by swirling. Tubes were incubated at 37°C in a water bath with vortex mixing every 15 min. Samples were taken at 10 min, 30 min, 1h, 1.5 h and 2 h. Eppendorf tubes were centrifuged at 7200 g for 5 min to form a firm pellet and the clear supernatant very carefully pipetted off using a Pasteur pipette, making sure no sediment was removed. Supernatants were retained in clean Eppendorf tubes and immediately frozen and stored prior to determination of antioxidant activity. The pellets were washed once with 1.0 ml distilled water, centrifuged and the clear supernatant pipetted off and discarded. Residues were dried in the Eppendorf tubes at 100^{o}C overnight in a forced draft oven and protein content determined.

Additional samples were exposed to a 2 h pepsin digestion and then centrifuged at 7200 g for 5 min. and the supernatant discarded. Immediately 0.75 ml 0.1 M sodium phosphate buffer, pH 7.6 was added, followed by a further 1 ml 0.1 M sodium phosphate buffer, pH 7.6 containing a mixture of trypsin/chymotrypsin (117 mg trypsin and 138 mg chymotrypsin/30 ml 0.1 M sodium phosphate buffer, pH 7.6). Tubes were incubated as previously at 37°C in a water bath with vortex mixing every 15 min. Samples were taken at 30 min, 1 h, 1.5 h and 2 h, supernatants retained for determination of antioxidant activity and the pellet used for residual protein determination. Protein digestibilities were determined at each time interval for the pepsin digestion and the trypsin/chymotrypsin digestion. Samples were assayed in duplicate and 2 separate runs were performed, giving four results per sample. Another set of the same samples exposed to 2 h pepsin and 2 h pepsin followed by 2 h trypsin/chymotrypsin digestion were centrifuged and the pellets frozen prior to preparation for TEM as described above.

ABTS antiradical analysis

Antioxidant activity assays were performed on the frozen supernatant samples using the ABTS (2,2´-azino-bis(3-ethylbenz-thiazoline-6-sulphonic acid)) antiradical method (Awika, Rooney, Wu, Prior, and Cisneros-Zevallos, 2003b) with Trolox as a standard.

Equal volumes of 8 mM ABTS and 3 mM potassium persulphate were mixed and allowed to react for 12 h. This solution (5 ml) was then either diluted with 0.1 M sodium phosphate buffer solution (pH 7.6) (145 ml) or 0.1 M sodium citrate buffer (pH 2.0) (145 ml) depending on which samples were to be analysed to give a working solution. Standard curves were prepared with Trolox dissolved in either 5% methanol in 0.1 M sodium phosphate buffer solution (pH 7.6) or 5% methanol in 0.1 M sodium citrate buffer, (pH 2.0) to cover the range between 0 and 800 µM Trolox. The working solution (2900 µl) was added to 100 µl of the standard or sample, shaken and allowed to react for exactly 30 min. Timing was critical as the absorbance is not stable with time. The absorbance was read at 734 nm.

Calculation

Antioxidant activity (µM Trolox equivalents/g sample)

$$= \frac{\text{(slope x (abs reagent blk-abs sample) + C)}}{\text{g/l sample used in analysis}}$$

Where:

Slope = slope of standard curve

C = y intercept

The calculation is the equation of the standard curve which gives a value of µM Trolox Equivalants per 100µl of sample. Values were then corrected for sample dilutions.

Statistical analysis

One-way analysis of variance (ANOVA) was used to determine whether there was a significant ($P<0.05$ or <0.01, as stated in tables) effect of different factors on protein digestibility of sorghum flour, kafirin type on the amount of condensed tannin bound and whether there was any significant differences in protein digestibility between protein type, whether the protein was bound and between different film types.

4.3.4. Results and discussion

4.3.4.1. Effect of independent variables on sorghum protein digestibility

The total protein content of the sorghum varieties used to determine the effect of different variables on in vitro sorghum digestibility ranged from 6.3% (PAN 8564) to 14.0% (Type II Sudan) (Table 4.3.2.) which is similar to the range of average sorghum total protein values quoted by Serna-Saldivar and Rooney (1995) of 7.3-15.6%. Protein digestibility of raw sorghum ranged from 36.8% for Seredo, a Type III tannin sorghum to 75.0% for Macia, a white tan plant variety (Table 4.3.2.). The range of in vitro sorghum protein digestibilities quoted in the literature is highly variable, for example: 88.6-93% (Axtell, Kirleis, Hassen, D'Crox-Mason, Mertz and Munck, 1981), 80.7% (Hamaker, Kirleis, Mertz, and Axtell, 1986), 73.2% (Oria, Hamaker and Shull, 1995a) and 55.1-55.8% (Duodu, Nunes, Delgadillo, Parker, Mills, Belton and Taylor, 2002). The values obtained in this study fell within this range. The low protein digestibility of tannin sorghums is well documented (reviewed by Duodu et al., 2003) and in this case was 36.8% for Seredo, a Type III tannin sorghum, as stated and 41.1 to 53.7% for the Type II tannin sorghums. There appears to be no relationship between the total protein content of the sorghum grain and in vitro protein digestibility. PAN 8564 had the lowest protein content at 6.3% but had one of the highest protein digestibilities for raw grain, 70.9%. Conversely, 3442-22-OP had a relatively high protein content, 11.0% and a relatively low protein digestibility, 58.7%.

The decrease in sorghum in vitro protein digestibility on wet cooking has been well known since the early 1980's (Axtell et al., 1981; Mertz et al., 1984; Hamaker et al., 1986). Thus, the decrease in vitro protein digestibility on wet cooking of all thirteen sorghum varieties used in this study was expected (Table 4.3.2.). The decrease in protein digestibility ranged from 10-22%, the lowest value again being the Type III tannin sorghum Seredo, 21.8% and the highest again, Macia, 58.9%. This range is slightly lower than decreases in protein digestibility on wet cooking quoted by other workers, 38.3% (Axtell et al., 1981), 15.9% (Hamaker et al., 1986), 18.0% (Oria et al., 1995a) and 19.2-28.6% (Duodu et al., 2002). The three Type II tannin sorghums analysed all also showed a significant reduction in protein digestibility on wet cooking in spite of having a much lower tannin content than the Type III sorghum, Seredo (Table 4.3.2.).

Table 4.3.2: The effects of tannins, reducing agent, cooking and cooking in the presence of a reducing agent on the protein digestibility (PD) of 13 sorghum varieties

Variety	% Total Protein	Tannin Type	Tannin (mg catechin equi/100 g flour)	% PD Raw	% PD Cooked	% PD Raw, Reduced	% PD Cooked, Reduced
HD 1	10.4 (0.02)	Type I	0.01 (0.04)	68.6 h,B (2.3)	49.7 f,A (4.9)	65.7 d,B (2.4)	70.9 efg,B (2.4)
LD 5	10.6 (0.13)	Type I	None detected	48.1 c,B (1.9)	38.5 d,A (2.2)	50.4 bB (1.6)	67.6 cde,C (1.0)
Macia	9.3 (0.01)	Type I	None detected	75.0 j,B (1.3)	59.8 g,A (0.7)	75.2 e,B (2.2)	75.4 g,B (0.8)
Kari Mtama	7.6 (0.15)	Type I	None detected	64.2 g,B (2.3)	50.8 f,A (1.7)	67.0 dB (1.0)	72.7 fg,C (2.4)
Town	10.4 (0.00)	Type I	0.03 (0.04)	61.1 fg,B (2.7)	44.5 e,A (1.8)	60.9 c,B (1.3)	63.5 c,B (4.1)
3442-22-OP	11.0 (0.08)	Type I	None detected	58.7 ef,B (1.4)	47.0 ef,A (0.9)	62.0 c,B (2.1)	68.0 de,C (1.5)
PAN 8564	6.3 (0.09)	Type I	0.08 (0.06)	70.9 hi,B (1.8)	48.7 f,A (1.6)	72.6 e,B (1.4)	70.4 ef,B (1.4)
Sima	10.7 (0.15)	Type I	0.03 (0.02)	56.9 de,B (2.2)	43.8 e,A (0.3)	59.9 c,B (1.6)	65.4 cd,C (1.8)
NK 8828	6.8 (0.03)	Type I	0.02 (0.03)	73.5 ij,B (1.7)	57.3 g,A (2.3)	75.1 e,B (1.1)	73.3 fg,B (4.0)
Sudan 96	14.0 (0.15)	Type II	None detected	47.4 c,B (1.8)	33.7 c,A (1.4)	49.6 b,B (1.9)	57.06 b,C (2.3)
Parent	11.0 (0.01)	Type II	0.21 (0.03)	41.1 b (2.0)	25.7 ab (1.5)	ND	ND
ABS#1	12.1 (0.02)	Type II	1.39 (0.04)	53.7 d (0.6)	26.2 b (1.9)	ND	ND
Seredo	11.6 (0.09)	Type III	3.95 (0.34)	36.8 a,B (1.8)	21.8 a,A (2.7)	26.4 a,A (2.5)	24.6 a,A (1.7)

Values with different letters (lower case) in a column are significantly different ($p<0.01$). Values with different letters (upper case) in a row are significantly different ($p<0.01$). ND: Not determined
Standard deviations in parenthesis
Type I: non-tannin sorghum, Type II: tannin sorghum without spreader gene, Type III: tannin sorghum with spreader gene

This may be a reflection of the fact that a very small amount of tannin can bind to a large amount of protein. According to Butler et al. (1984) under optimal conditions, sorghum tannin is capable of binding at least 12 times its own weight of protein.

Causal factors involved in the reduction of sorghum protein digestibility on wet cooking are very complex. They are thought to include grain organisational structure, polyphenols, phytic acid, starch and non-starch polysaccharides, disulphide cross-linking, kafirin hydrophobicity and changes in protein secondary structure (reviewed by Duodu et al., 2003). Of these factors Duodu et al. (2003) proposed that protein cross-linking may be the strongest factor influencing sorghum protein digestibility.

There was no effect of the addition of a reducing agent on the protein digestibility of raw sorghum flour except for the Type III tannin sorghum, Seredo (Table 4.3.2.). This was in contrast to the findings of other workers who reported increased pepsin digestibility when a reducing agent was added to raw sorghum flour compared to raw flour with no reducing agent (Hamaker, Kirleis, Butler, Axtell and Mertz, 1987; Rom, Shull, Chandrashekar and Kirleis, 1992; Arbab and El Tinay, 1997). Hamaker et al., (1987) suggested that the reducing agent was responsible for breaking of disulphide linkages in the glutelin proteins which exist mainly as polymers bound by intermolecular disulphide bonds and form a matrix around the sorghum protein bodies which contain the kafirin proteins. Thus, the breaking up of the protein matrix would enable the pepsin enzymes more accessibility to the kafirin proteins contained in the protein bodies. Also, El Nour, Puruffo and Curioni (1998) demonstrated the presence of disulphide linked oligomers of the different kafirins in raw sorghum grain. On addition of a reducing agent the disulphide links would be broken and monomers formed. Kafirin monomers are more susceptible to pepsin digestion than their oligomers (Hamaker et al., 1987). Thus, possibly for both reasons, addition of a reducing agent to raw sorghum flour could result in an increase in protein digestibility. This was found by the workers mentioned. The reason that this increased digestibility was not found in this study may be due to several factors. The most influential factor was probably the length of exposure of the flour to the reducing agent. The workers mentioned soaked the sorghum flour for extended periods (at least 12 hours) before exposing the flour to pepsin digestion. This would have allowed more time for disulphide linkages to be reduced than in this study where the reducing agent was added directly before the pepsin digestion began. Other factors may be the nature and

concentration of the reducing agent and the variable quantity of oligomers found in different sorghum cultivars. According to Hamaker et al. (1987) 2-mercaptoethanol, as used in this study, was less effective than either sodium bisulphite (used by Rom et al., 1992 and Arbab and El Tinay, 1997) or dithiothreitol at improving pepsin digestibility of cooked sorghum flour over a range of different concentrations. In addition, Nunes, Correira, Barros and Delgadillo (2005) found that the quantity and nature of disulphide linked oligomers in raw sorghum flour was highly variable depending on the cultivar examined. Thus it is possible that the sorghum cultivars used in this study had low amounts of disulphide linked oligomers in the raw flour or contained more oligomers containing gamma kafirin which may be less susceptible to pepsin digestion than other kafirin species due to its greater hydrophobicity (Belton, Delgadillo, Halford and Shewry, 2006).

For Seredo (Type III sorghum), the digestibility was reduced from 36.8% in the raw flour to 26.4% with the addition of a reducing agent to the raw flour. It is suggested that the presence of a reducing agent would reduce the disulphide linked oligomers present in the raw flour to their monomers. These newly formed monomers would then cross-link with the tannins present in Seredo, making them unavailable for pepsin digestion. This would then result in the observed reduction in protein digestibility. No such reduction in protein digestibility was observed in Sudan 96, a Type II sorghum. It is suggested that there may have been insufficient tannin in this variety to further cross-link any additional kafirin monomers and so the protein digestibility was not decreased further. Arbab and El Tinay (1997) found that soaking raw sorghum flour in a reducing agent for 18 hours prior to pepsin digestion considerably improved the digestibility of a high tannin sorghum. It is not known why this should be if the sorghum did, as stated, contain large quantities of tannin. It is suggested that the level of tannin was not sufficiently high to cross-link the additional kafirin monomers released on reduction and consequently resulting in an increase in protein digestibility.

When a reducing agent was added during cooking, there was a significant increase in protein digestibility to at least the level in the uncooked flour in all the sorghum varieties analysed, except for Seredo (Table 4.3.2.). Cooking with a reducing agent improved the protein digestibilities of five of the sorghum varieties, LD5, Kari Mtama, 3442-22-OP, Sima and Sudan 96 to levels higher than that of the raw flour. These results are in

agreement with those of Hamaker et al. (1987), Rom et al. (1992) and Arbab and El Tinay (1997) who all found that the protein digestibilities of non tannin sorghum increased to levels higher than the raw grain on addition of a reducing agent. In this study not all the sorghum varieties analysed with reducing agent, on cooking had protein digestibilities higher than the raw grain. The possible reasons for this were thought to be the same as those discussed above when raw flour was treated with a reducing agent. When Arbab and El Tinay (1997) determined the protein digestibility of a high tannin sorghum flour cooked in the presence of a reducing agent they found an improvement in digestibility. This was contrary to what was found in this study. It is suggested that the further reduction in protein digestibility seen in Seredo, the Type III tannin sorghum, on cooking with a reducing agent was due to the same reasons described above for the raw tannin sorghum flour treated with a reducing agent.

4.3.4.2. Kafirin-tannin binding

The conclusion of Chapter 4.2. suggested a need to improve kafirin microparticle film functional properties by cross-linking either by physical or chemical means. Emmambux et al. (2004) showed that the functional properties of kafirin films could be modified by cross-linking with sorghum condensed tannins. With the aim of using kafirin microparticles as encapsulating agents preliminary investigations into the effect of sorghum tannin cross-linking on kafirin digestibility were undertaken using different kafirin species and kafirin films prepared as described by Emmambux et al. (2004).

Figure 4.3.1. shows that isolated γ-kafirin bound the most condensed tannin (70-77%), whereas total kafirin and residual kafirin bound similar, but far lower, amounts of condensed tannin, 35-45% and 35-40%, respectively. The fact that the percentage condensed tannin bound to the kafirin species remained the same from 10 to 40% of condensed tannin added with respect to kafirin implies that the kafirin was saturated with condensed tannin.

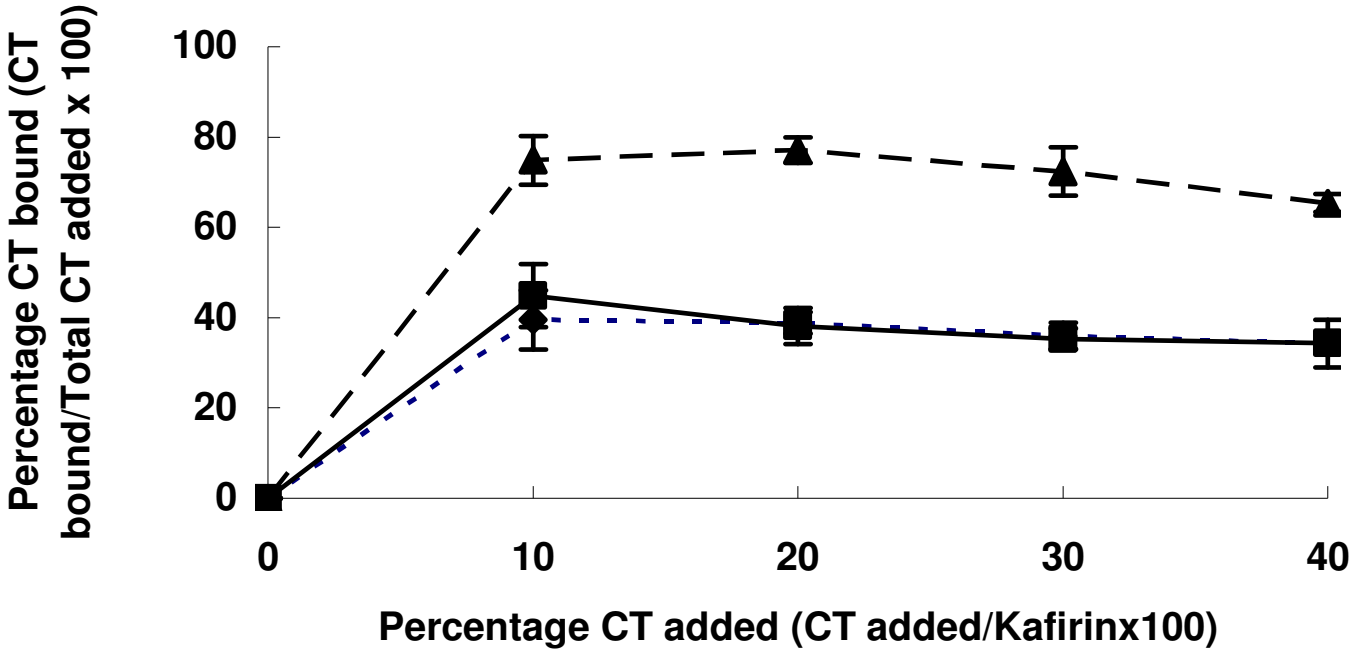


Figure 4.3.1: Percentage of sorghum condensed tannin (CT) bound to different kafirin species
Triangles: γ-kafirin, Squares: Total kafirin, Diamonds: Residual kafirin (kafirin after γ-kafirin removed)
Error bars represent +/- one standard deviation

Charlton, Baxter, Lockman Khan, Moir, Haslam, Davis and Williamson (2002) showed that intermolecular binding between peptides and tannins is dominated by the stacking of polyphenolic rings onto planar hydrophobic surfaces. This binding is strengthened by multiple binding of polyphenolic rings. Effectively the peptide becomes increasingly coated with polyphenol until polyphenol bridges occur and the peptide dimerises and precipitates out. Since up to 40% condensed tannin relative to the kafirins was added it would be expected that the precipitated condensed tannin-kafirin material would consist of very large molecular weight aggregates of kafirin polymers and condensed tannin molecules.

With regard to the relative amount of condensed tannin bound by the different kafirin species several factors may be involved. Awika, Dykes, Rooney and Prior (2003a) showed that the degree of interaction of procyanidins with food macromolecules increases with degree of polymerisation of the procyanidins. The composition of the aqueous acetone extract of condensed tannin was not determined. However, from the literature using a similar extraction procedure procyanidin profiles of two sorghum varieties were 14-31% oligomers of degree of polymerization (dp) 1-10 and the remainder were oligomers larger than dp 10 (Awika et al., 2003a). It is expected that the profile of the extract used in this study was similar. Since the same condensed tannin extract was used to bind each of the kafirin species the degree of protein polyphenol binding would only be affected by the type of kafirin bound. It has long been known that proteins containing high amounts of proline bind more sorghum tannins than those with less proline (reviewed by Spencer et al., 1988). Thus the fact that γ-kafirin bound most condensed tannin is not surprising since it contains far more proline (18.7 mole %) than the total kafirin (11.1 mole %) or residual kafirin (10.7 mole %) (Table 4.3.1.). Another important factor that determines the amount of tannin bound to protein is the number of proline repeats that the protein contains (Baxter et al., 1997). Gamma-kafirin has three repeats of two proline residues, three repeats of three residues, and one repeat of four residues (Swiss Prot, 2005). This would be expected to give γ-kafirin a more open structure than total kafirin, which is primarily α-kafirin and in its native form is mainly α-helical (Gao, Taylor, Wellner, Byaruhanga, Parker, Mills and Belton, 2005). According to Butler et al. (1984) proline residues disrupt the α-helix, breaking internal hydrogen bonds and so provide an opportunity for forming multiple hydrogen bonds between tannin

molecules and the peptide backbone and to also maximise non-polar interactions. The side chain of proline is a five membered pyrrolidine ring structure. As a result of this structure, proline residues cause the peptide chains to form a rigid and extended conformation (Edens, Van der Laan and Craig, 2005). Multiple proline residues give rise to a higher degree of rigidity and allow both hydrogen bonds and hydrophobic interactions to form between peptide chains and tannins. It may have been expected that residual kafirin would bind less condensed tannins than total kafirin since the γ-kafirin had been removed. This was not so (Figure 4.3.1.), possibly because the γ-kafirin represents only a small proportion (9-12%) of the total kafirin (Shewry, 2002). This is also reflected by the fact that the proline content of the residual kafirin (10.7 mole %) was found to be virtually the same as that for total kafirin (11.1 mole %) (Table 4.3.1.).

SDS-PAGE under non-reducing conditions (Figure 4.3.2.A) of the condensed tannin bound γ-kafirin (track 7) showed a band of 14-18 k possibly equivalent to the 16 k band of γ-zein shown by Kim, Woo, Clore, Burnett, Carneiro and Larkins (2002). The additional bands of 27 or 50 k for γ-zein observed by Kim et al. (2002) were not seen for γ-kafirin. In contrast, the unbound γ-kafirin (track 4) showed 14-18 k bands plus strong bands at 22-24 k, plus bands at 39, 60 and 74 k, the latter three presumably being disulphide linked γ-kafirin dimers, trimers and tetramers. However, under reducing conditions (Figure 4.3.2.B) unbound γ-kafirin (track 2) showed the expected strong band of slightly higher apparent molecular weight than the total kafirin (track 4), which is mainly α-kafirin (Shewry, 2002). Under reducing conditions (Figure 4.3.2.B) condensed tannin bound γ-kafirin (track 3) gave a much fainter band that the unbound γ-kafirin (track 2). This is evidence that much of the condensed tannin bound γ-kafirin was, in the form of aggregates of molecular size >200 k that were too large to enter into the separating gel. Sarni-Manchado, Cheynier and Moutounet (1999) working with grape seed tannins bound to salivary proline rich protein (PRP) obtained similar results in that bound material did not enter the electrophoresis separating gel, whereas the unbound material did.

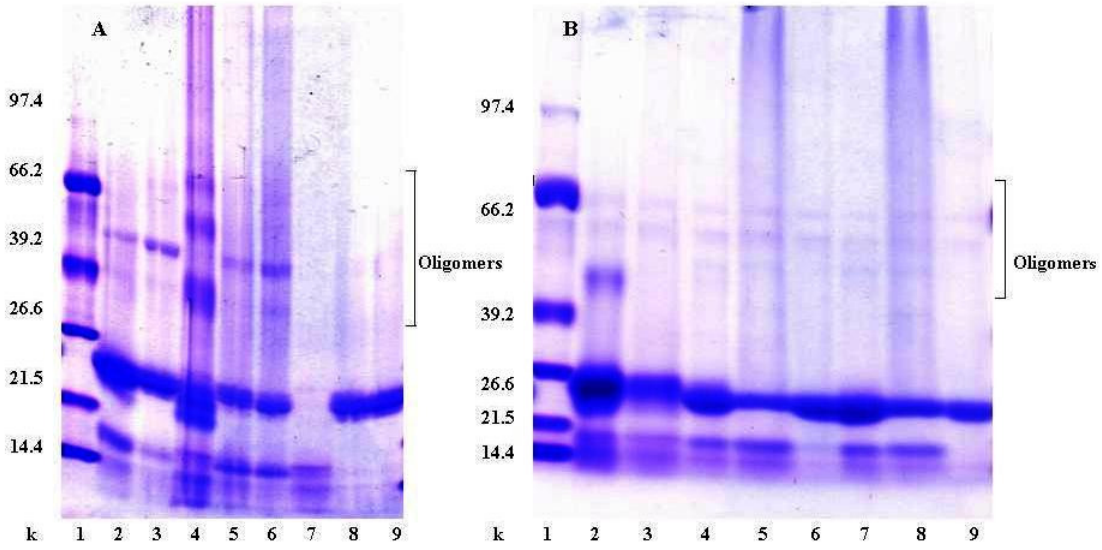


Figure 4.3.2.: SDS-PAGE of kafirin species and kafirin species bound to sorghum condensed tannins (CT) under non-reducing (A) and reducing conditions (B)

(A) 1-molecular weight standards, 2-total kafirin, 3-residual kafirin, 4-γ-kafirin, 5-total kafirin bound to CT, 6-residual kafirin bound to CT, 7-γ-kafirin bound to CT, 8-supernatant total kafirin bound to CT, 9-supernatant residual kafirin bound to CT

(B) 1-molecular weight standards, 2- γ-kafirin, 3-γ-kafirin bound to CT, 4-total kafirin, 5-total kafirin bound to CT, 6- supernatant total kafirin bound to CT, 7- residual kafirin, 8-residual kafirin bound to CT, 9-supernatant residual kafirin bound to CT

With regard to tannin binding by the total kafirin and residual kafirin, the intensity of the condensed tannin bound total kafirin (Figure 4.3.2.A track 5 and Figure 4.3.2.B track 5) and condensed tannin bound residual kafirin (Figure 4.3.2.A track 6 and Figure 4.3.2.B track 8) was somewhat reduced compared to the unbound protein species both under non-reducing and reducing conditions (Figure 4.3.2.A and 4.3.2.B, total kafirin tracks 2 and 4 and residual kafirin tracks 3 and 7 respectively), but not nearly to the same extent as with the γ-kafirin. This finding is in agreement with their lower level of tannin binding compared to γ-kafirin (Figure 4.3.1.). In this regard, there was some protein in the supernatant recovered when total kafirin and residual kafirin were bound to condensed tannin, but not with γ-kafirin. The amount of this material was not quantified. However, SDS-PAGE showed that it was mainly α-kafirin (Figure 4.3.2.A tracks 8 and 9, and Figure 4.3.2.B tracks 6 and 9, total and residual kafirin under reducing and non-reducing conditions respectively). It is probable that the majority of this material was kafirin

which did not bind to the condensed tannin. This was reflected by the higher intensities of these bands in comparison with the condensed tannin bound kafirins.

When the kafirin species were bound to condensed tannin their digestibilities were reduced (Table 4.3.3.). Kafirins bound to condensed tannin were far less digestible than unbound kafirins. Kafirin coated with a layer of tannins in the manner described by Charlton et al. (2002) would be expected to be less accessible to pepsin than unbound kafirin. It is of interest that unbound γ-kafirin was much less digestible than either total kafirin or residual kafirin (Table 4.3.3.). This may be due to the γ-kafirin existing primarily in the form of disulphide cross-linked oligomers, as shown by SDS-PAGE (Fig. 4.3.2.). Disulphide cross-linking of kafirins has been shown to reduce protein digestibility (Duodu et al., 2002).

When bound with condensed tannin, γ-kafirin as insoluble condensed tannin γ-kafirin complexes were slightly more digestible than either the total kafirin or residual kafirin condensed tannin insoluble complexes (Table 4.3.3.). It is not clear why this is so. It is speculated that the bound γ-kafirin may have a slightly more open structure due to the proline repeats it contains, as described above, than does bound total kafirin or bound residual kafirin, and additionally tannin binding may further open the structure and make it slightly more accessible for protein digestion. Duodu et al. (2003) reviewing sorghum protein digestibility refers to the importance of protein conformation and the extent to which the enzyme has accessibility to the protein on the determination of a protein's digestibility.

As anticipated, films made from condensed tannin bound kafirin had much lower protein digestibility than films made from unbound kafirin (Table 4.3.3.). The latter were slightly less digestible than the zein films. The kafirin used for film formation contained α-, β-, and γ-kafirin (Figure 4.3.2.). Beta- and γ-kafirin would be capable of forming disulphide cross-linkages during film formation because of their high cysteine content.

Table 4.3.3.: In vitro protein digestibility (%) of sorghum condensed tannin (CT) bound and unbound kafirin preparations and of films

Sample	Unbound	CT bound
Protein		
Total kafirin	59.1e (1.8)	8.4ab (1.2)
γ-kafirin	38.0d (2.1)	17.2c (1.9)
Residual kafirin	61.2e (3.9)	7.6a (1.8)
Films		
Total kafirin	41.5y (2.2)	16.7x (1.2)
Zein	54.4z (3.9)	Not determined

[a]Each value represents the mean of four replicate analyses with standard deviation in parenthesis

Values with different letters in a block are significantly different ($p<0.01$). Protein and film data statistically analysed separately.

The zein films were made from commercial zein, which consists of mainly α-zein (Lawton, 2002) and would be less able to form disulphide linkages because of its lower amount of cysteine residues. Hence, the kafirin structure would be less accessible to pepsin digestion than the zein, resulting in lower protein digestibility of the kafirin films than the zein films. Also of interest was the fact that kafirin films were somewhat less digestible than the kafirin they were made from (Table 4.3.3.). This is contrary to the findings of Byaruhanga, Erasmus and Taylor (2005) where kafirin films were much more digestible than the kafirin they were made from. This difference was probably due to the different casting solvents used. Byaruhanga et al. (2005) used glacial acetic acid as opposed to aqueous-ethanol in this work. Glacial acetic acid was thought to deamidate the glutamine residues of the kafirin resulting in breakage of hydrogen bonds, making the film readily accessible to pepsin digestion (Byaruhanga et al., 2005). Casting the kafirin films in aqueous ethanol at elevated temperatures, as in this study, would promote the formation of disulphide linkages, as previously discussed, resulting in the formation of less digestible kafirin oligomers.

Films made from unbound kafirin were biodegradable under aerobic conditions within 20 days under low moisture conditions and within 10 days under high moisture conditions (Figure 4.3.3.), a similar time to zein films. Films made from condensed tannin bound kafirin took up to 8 days longer to biodegrade under both high and low moisture conditions. Degradation under low moisture conditions followed a similar pattern but the degradation was slower (not shown). This was probably due to greater microbial growth

and subsequent metabolism at the higher moisture level. Film degradation followed a similar pattern for all films. As time progressed they became opaque and wrinkled. Subsequently the films developed pin-holes which progressively became larger as the film fragmented and finally completely degraded. The extended life of films made from condensed tannin bound kafirin is probably related to their lower protein digestibility. Since binding with condensed tannin reduced film protein pepsin digestibility, it would also be expected to reduce microbial degradation due to a lower accessibility of the proteins to the microbial hydrolytic enzymes. Inducement of protein cross-linkages has also been reported to slow biodegradation of heat-treated whey protein films (Le Tien, Letendre, Ispas-Szabo, Mateescu, Demas-Patterson, Yu and Lacroix, 2000), γ-irradiated soy protein films (Lacroix, Le, Ouattara, Yu, Letendre, Sabato, Mateescu and Demas-Patterson, 2002) and heat treated kafirin films (Byruhanga et al., 2005).

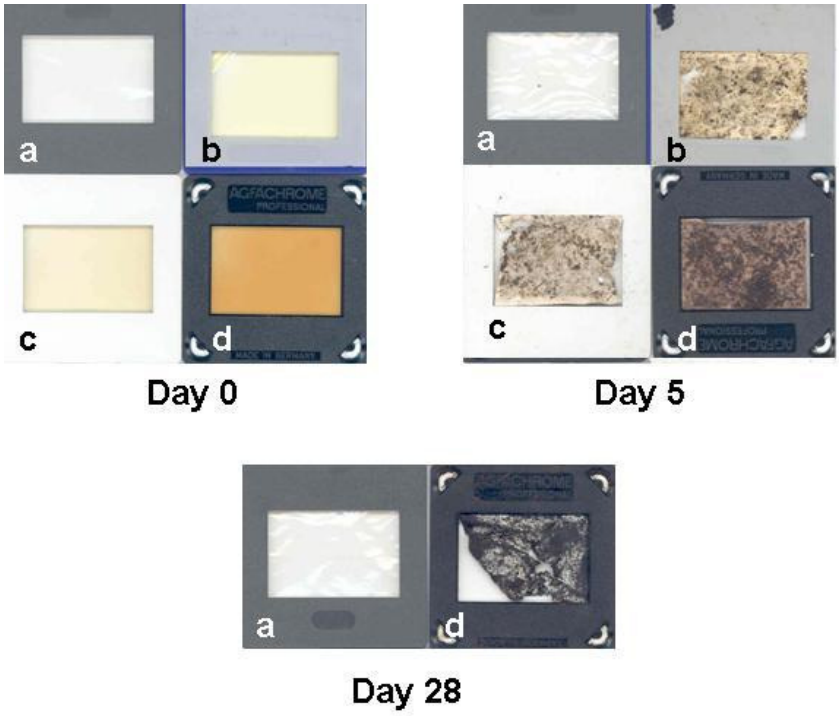


Figure 4.3.3: Biodegradation of films under high moisture conditions
a-LDPE, b-zein film, c-total kafirin film, d-film made with kafirin bound to CT

4.3.4.3. Efficiency of polyphenol encapsulation in kafirin microparticles

Having shown that sorghum condensed tannins bind to kafirin and reduces its digestibility, it was decided to exploit this property by using kafirin microparticles as encapsulating agents for the controlled or delayed release of dietary antioxidants.

An extract of sorghum condensed tannins which binds strongly with kafirin (Emmambux and Taylor, 2003) and catechin which does not bind with kafirin were chosen for encapsulation within kafirin microparticles. Both of these polyphenols exhibited good antioxidant activity, catechin 15327 µmoles Trolox equivalents/g and sorghum condensed tannins 5366 µmoles Trolox equivalents/g. These values are slightly higher than values quoted in the literature. Sikwese and Duodu (2007) reported an antioxidant activity of 4600 µmoles Trolox equivalents/g for a freeze dried crude phenolic extract from sorghum bran. Pérez-Jiménez and Saura-Calixto (2006) described antioxidant activities of a mixture of catechin:gallic acid (1M:1M) varying from 6764-30218 µmoles Trolox equivalents/g. Large variability in antioxidant values are a consequence of the method of analysis and the solvent used to dissolve the polyphenol (Pérez-Jiménez and Saura-Calixto, 2006). The raw material used for extraction also influences the amount of antioxidant activity measured. Sorghum condensed tannins are located in the pericarp and testa layers of sorghum grain and so are concentrated in sorghum bran (reviewed by Serna-Saldivar and Rooney, 1995). Thus, it is difficult to compare absolute values of antioxidant activity with those in published literature. However, for the purpose of this work, comparisons of relative antioxidant activity for the different treatments rather than absolute values are more important.

To determine the effectiveness of the encapsulation procedure, catechin and sorghum condensed tannins separately encapsulated within kafirin microparticles and the kafirin microparticles used for encapsulation were examined by TEM after osmium staining (Figure 4.3.4.). According to Morrall, Liebenberg and Glennie (1981) sorghum tannins are strongly osmiophilic and appear as darkly stained material. Encapsulation of catechin (Figure 4.3.4.b) or sorghum condensed tannins (Figure 4.3.4.c) appears to have caused changes to the microstructure of kafirin microparticles when compared with the kafirin microparticles used for the encapsulation process (Figure 4.3.4.a). A layer of darkly stained material could be seen clearly around the periphery of the kafirin microparticles

with encapsulated condensed tannins (Figure 4.3.4.c). The tannins appear to have linked the kafirin microparticles together, forming large irregular shaped structures. There was also some dark staining material on the inside of the microparticles, mainly on the edge of some of the vacuoles. This implies that at least some of the sorghum condensed tannins became entrapped within the kafirin microparticles. The kafirin microparticles encapsulated with catechin appeared generally to be much lighter stained than the tannin microparticles with very slight darkening around the periphery of the microparticles (Figure 4.3.4.b). This was expected as catechin is known not to bind to kafirin (Emmambux and Taylor, 2003). However, by implication, 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. Interestingly, the catechin encapsulated kafirin microparticles had a similar appearance to kafirin microparticles in 21.6% acetic acid (Chapter 4.1. Figure 4.1.5.e). The edges of the microparticles were eroded, and the general shape of the microparticles was irregular, with some apparent aggregation. In Chapter 4.1. it was noted that as the acetic acid concentration increased the kafirin microparticles appeared to aggregate. During film formation (Chapter 4.2.) it could be seen as the film forming solution evaporated, the acetic acid became more concentrated and the kafirin microparticles appeared to dissolve in the acetic acid. Thus, it is suggested that the catechin was actually acting as a solvent for the kafirin microparticles and the changes in the microstructure of the kafirin microparticles are due to a partial solvation effect of the catechin. Evans and Manley (1941) noted the importance of the presence of –OH, $-NH_2$, $-CONH_2$ or -COOH groups in solvents for zein. Since kafirin and zein are very similar this prediction should apply to kafirin. The structure of catechin contains many –OH groups and so it should be expected to be a good solvent for kafirin.

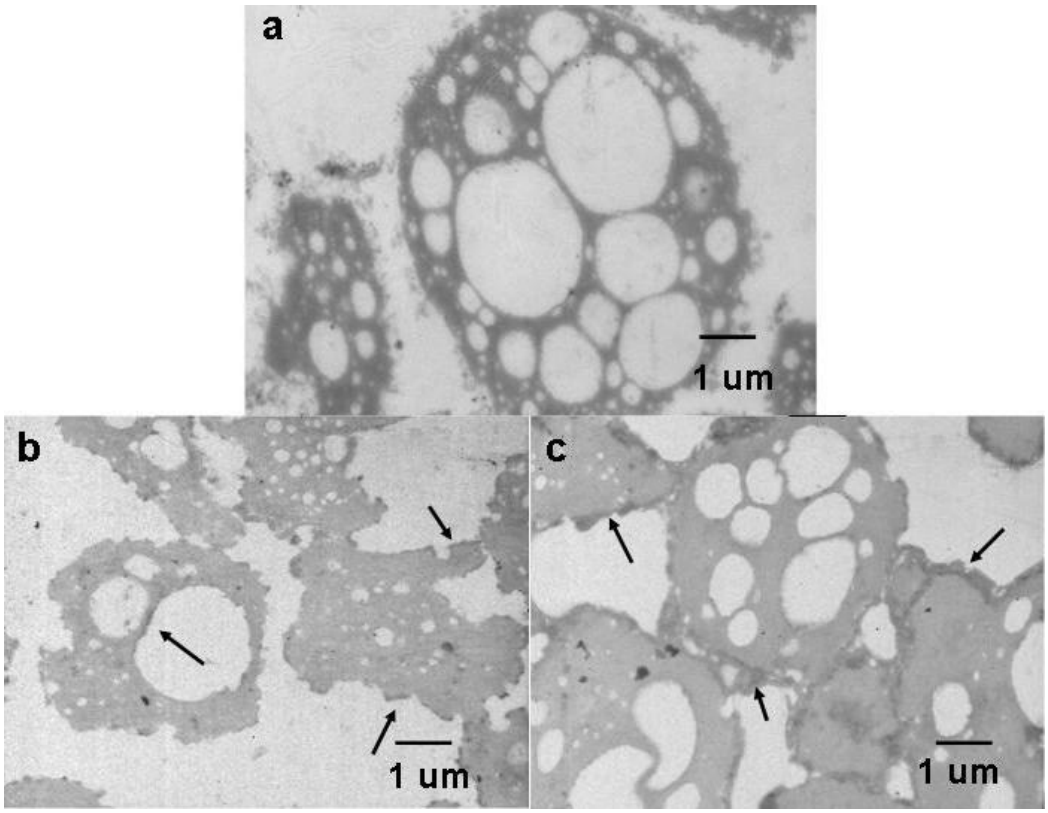


Figure 4.3.4: TEM of kafirin microparticles (a) and kafirin microparticles after encapsulation of catechin (b) and sorghum condensed tannins (c), arrows indicate probable bound phenolics

The release of antioxidant activity from unencapsulated kafirin microparticles, kafirin microparticle encapsulated catechin and kafirin microparticle encapsulated sorghum condensed tannins on progressive digestion with pepsin followed by trypsin and chymotrypsin is shown in Figure 4.3.5. The corresponding kafirin digestion for these samples is shown in Figure 4.5.6. and illustrated by TEM in Figure 4.3.7. Both catechin- and sorghum condensed tannins-encapsulated microparticles showed a 'burst release' of slightly less than 20% antioxidant activity after 10 minutes pepsin digestion (Figure 4.3.5.). It is known that the process of microparticle formation leaves some of the incorporated material on the microparticle surface (Whittlesey and Shea, 2004). The rapid release of this material in the release medium is known as 'burst release' (Huang and Brazel, 2001). The 'burst release' in this case was probably due to residual, unbound material on the outside or near the surface of the microparticles which was washed off with the buffer. This mechanism for the initial release of encapsulated material has been described for zein microparticles (Wang, Lin, Liu, Sheng and Wang, 2005; Liu, Sun,

Wang, Zhang and Wang, 2005; Muthuselvi and Dhathathreyan, 2006). Specific amounts of material released were not mentioned but it is known that the magnitude of the 'burst release' increases with higher loading of encapsulated material (Huang and Brazel, 2001).

Interestingly, the kafirin used for encapsulation also exhibited antioxidant activity (Figure 4.3.5.). The release of this antioxidant activity was progressive up until 90 minutes when a plateau was reached and approximately 500 µmol Trolox equivalents/g kafirin was measured. When the pepsin was replaced by trypsin and chymotrypsin in buffer at pH 7.6 there was a further release of antioxidant activity until 150 minutes when just less than 1000 µmol Trolox equivalents/g kafirin was measured. Beyond this point there was another plateau with essentially no further antioxidant activity released. Dlamini (2007) reported increased antioxidant activity when ground tannin free sorghum grain was treated with pepsin and α-amylase. The highest level of antioxidant activity was found in the supernatants of the hydrolysed material and was of the order of 200 µmol Trolox equivalents/g ground grain. Antioxidant activity has also been reported for zein powder at high water activity against the perioxidation of methyl linoleate but not for its papain hydrolysate (Wang, Fujimoto, Miyazawa and Endo, 1991; Wang, Miyazawa and Fujimoto, 1991). These workers thought that at least some of the zein antioxidant activity was a result of phenol compounds contaminating the protein. It is unlikely that this was the case for the kafirin microparticles used in this study as the original kafirin had a relatively high purity (94% dwb) and was extracted from tannin free sorghum. Protein hydrolysates from soy, (Chen, Muramoto, Yamauchi and Nokihara, 1996; Chen, Muramoto, Yamauchi, Fujimoto and Nokihara, 1998) elastin, (Hattori, Yamaji-Tsukamoto, Kumagai, Feng and Takahasi, 1998), whey, (Peña-Ramos, Xiong and Arteaga, 2004) casein, (Kim, Jang and Kim, 2007) and porcine myofibrillar protein, (Saiga, Tanabe and Nishmura, 2003) have all been shown to have antioxidant activity. Some amino acids have been reported to exhibit antioxidant activity, such as histidine, tyrosine, methionine and cysteine (Marcuse, 1960).

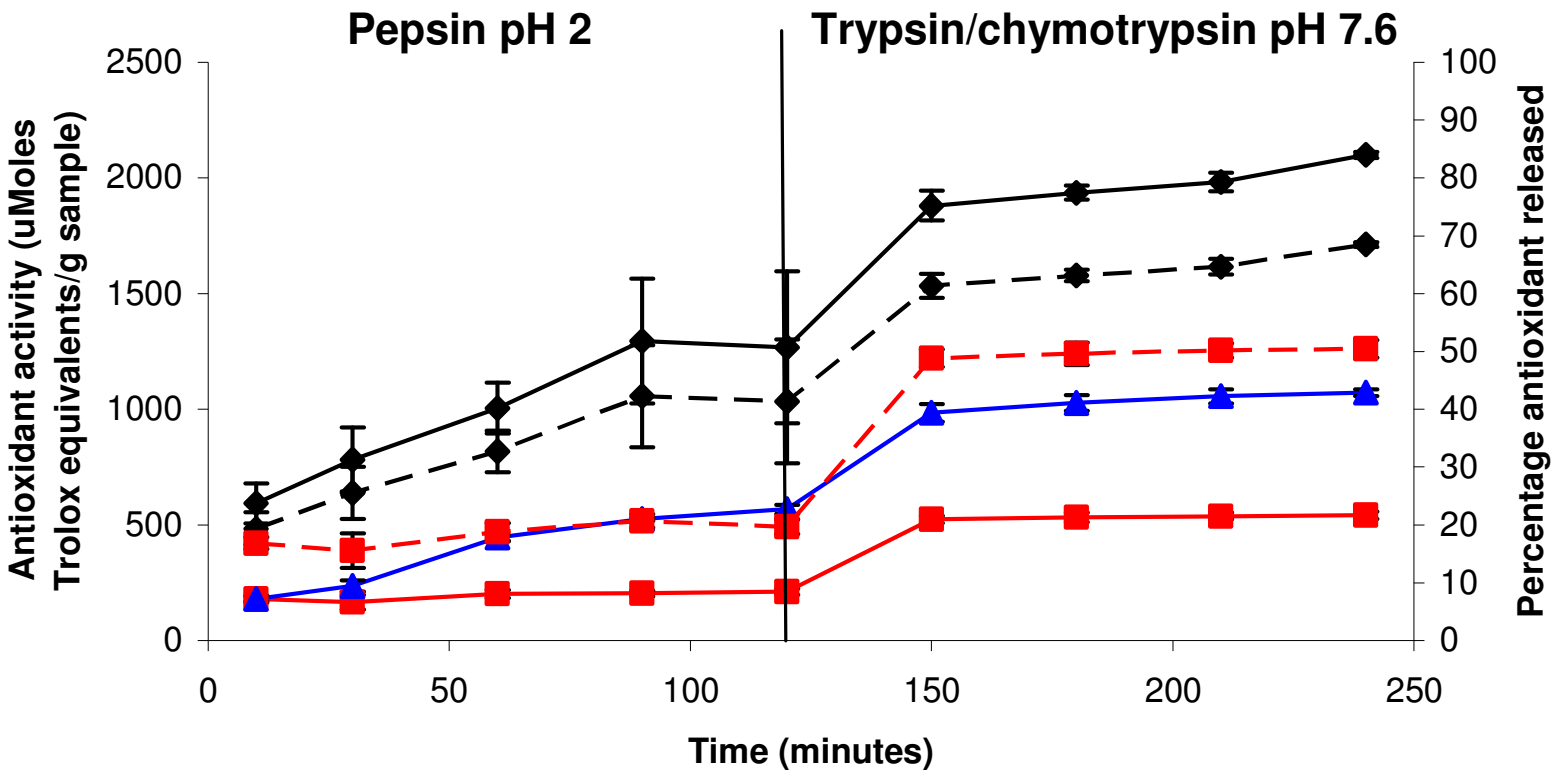


Figure 4.3.5: Effect of pepsin digestion followed by trypsin/chymotrysin digestion on antioxidant activity (solid line) and percentage antioxidant released (dotted line) from kafirin microparticles (triangles), kafirin microparticles with encapsulated catechin (diamonds) and kafirin microparticles with encapsulated sorghum condensed tannins (squares). Error bars represent +/- one standard deviation

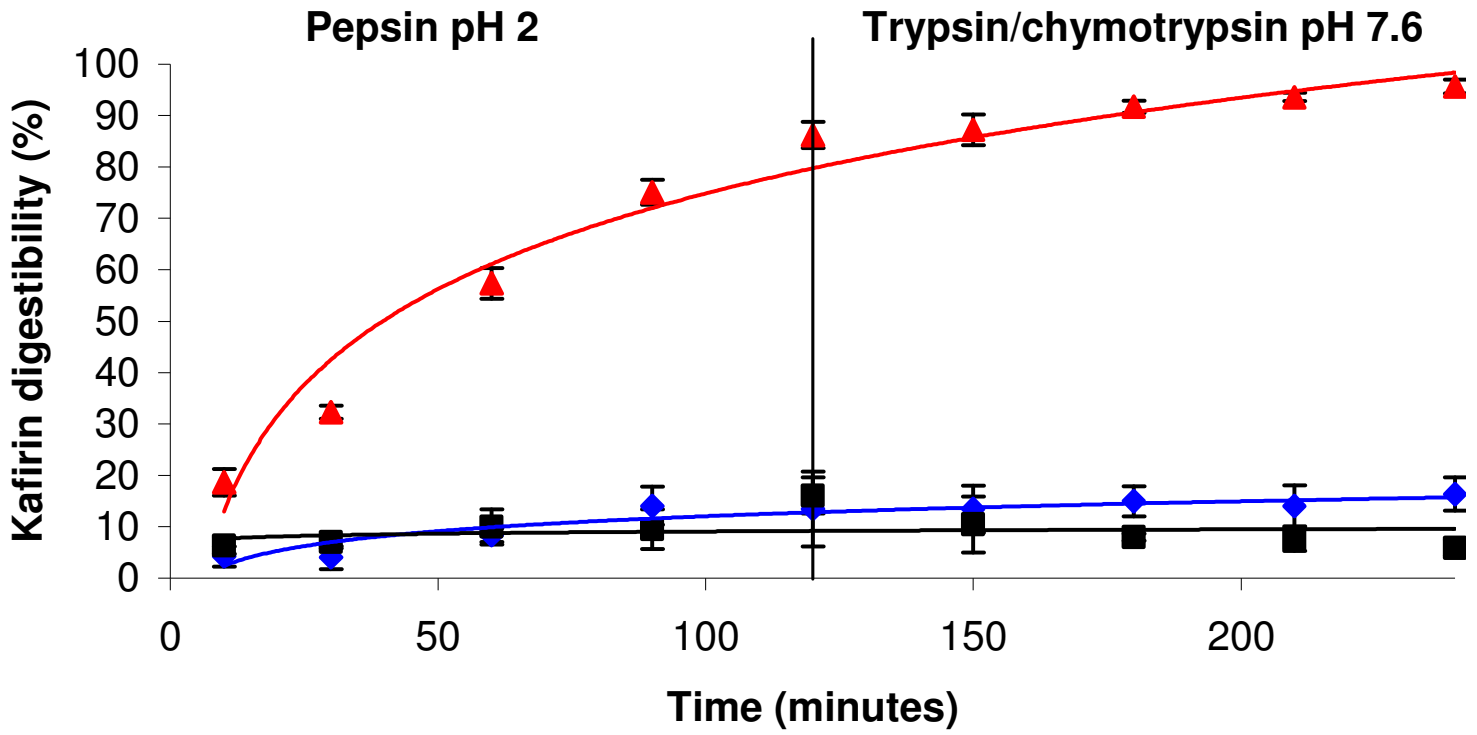


Figure 4.3.6: Effect of pepsin digestion followed by trypsin/chymotrysin digestion on kafirin digestibility of kafirin microparticles (triangles), kafirin microparticles with encapsulated catechin (diamonds) and kafirin microparticles with encapsulated sorghum condensed tannins (squares). Error bars represent +/- one standard deviation

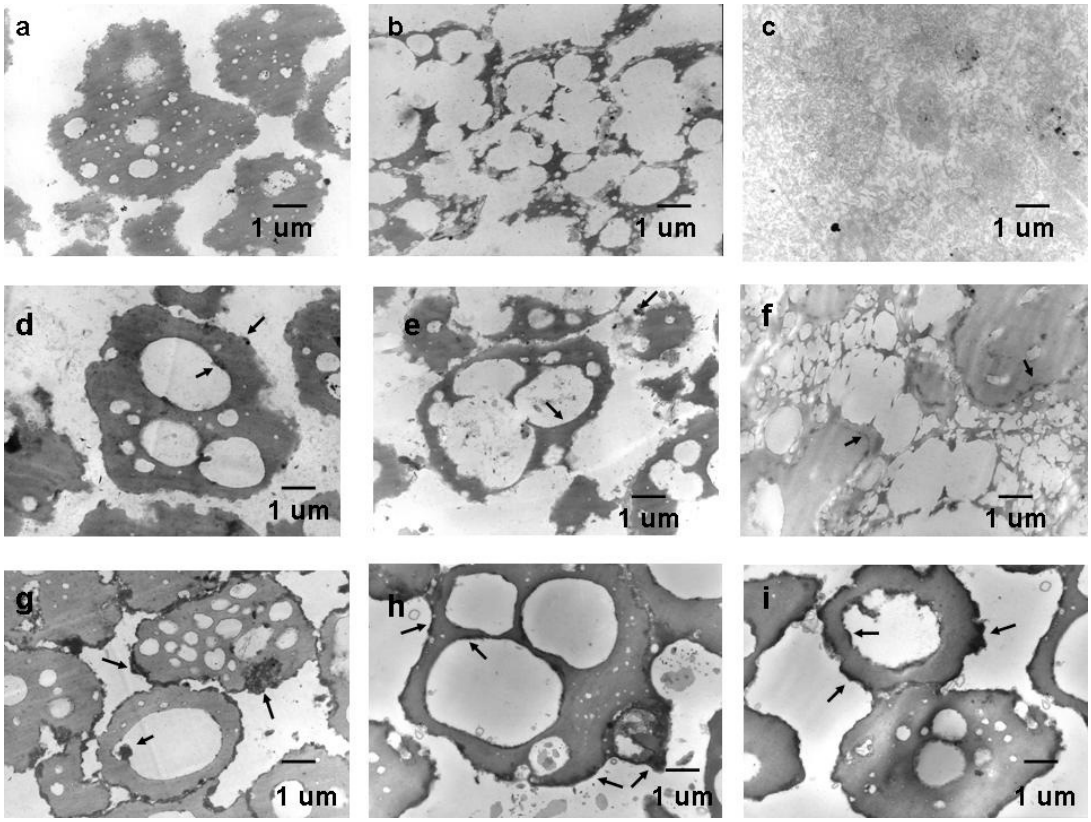


Figure 4.3.7: TEM illustrating the effect of pepsin digestion followed by trypsin/chymotrysin digestion on kafirin digestibility of kafirin microparticles (a-c), kafirin microparticles with encapsulated catechin (d-f), and kafirin microparticles with encapsulated sorghum condensed tannins (g-i), arrows indicate probable bound phenolics

However both Saiga et al. (2003) and Peña-Ramos et al. (2004) demonstrated that the peptides in protein hydrolysates seem to be important for high antioxidant activity. Peña-Ramos et al. (2004) stated that for whey protein hydrolysates in general high antioxidant activity was found with peptides with high concentrations of histidine and some hydrophobic amino acids. Kafirin is rich in hydrophobic amino acids (Belton et al., 2006) and γ-kafirin, specifically, is relatively rich in histidine (7.8 mole % of amino acid) (Taylor and Belton, 2002). It may be expected that on enzymic hydrolysis peptides containing hydrophobic amino acids and histidine would be released which may be responsible for the measured antioxidant activity.

The digestion of unencapsulated kafirin was progressive with time. After 120 minutes digestion with pepsin approximately 80% of the kafirin had been digested, rising to

approximately 95% after a further 120 minutes digestion with trypsin and chymotrypsin (Figure 4.3.6.). As the digestion of the kafirin progressed, the amount of peptides released would increase. If, as suggested, these peptides are responsible for antioxidant activity then the amount of antioxidant activity measured would also be expected to increase, as was found. There is little literature on the protein digestibility of isolated kafirins. Duodu et al. (2002) reported the pepsin digestibility of uncooked sorghum protein body enriched samples as about 75%. Table 4.3.3. shows the protein digestibility of total kafirin, lower at 59%. This lower value may have been due cross-linking, which could have occurred during extraction and drying and is known to reduce protein digestibility as already discussed (Duodu et al., 2003). The higher protein digestibility of the kafirin microparticles was probably a result of the large surface area of the microparticles available for pepsin attack.

The progressive digestion of kafirin by pepsin followed by trypsin/chymotrypsin was illustrated by TEM (Figure 4.3.7.a-c). Digestion appeared to take place initially both on the outer surface of the microparticles and also from the inside of the vacuoles in the microparticle. This would be expected as the enzyme molecules would be able to penetrate the microparticles via holes on the surface of the microparticles and possibly through interconnected channels to the interior vacuoles. This pattern of digestion appears to be similar to that of the degradation of sorghum protein bodies during germination as described by Taylor, Novellie and Liebenberg (1985). The protein bodies were degraded by progressive reduction in size from the surface and in some cases appeared to have holes in the central region. As digestion of kafirin microparticles progressed it appeared that some of the vacuoles merged forming larger structures (Figure 4.3.7.b). Finally at the end of digestion very little material was left and no specific ultra structure remained (Figure 4.3.7.c).

After the initial burst release encapsulated sorghum tannin showed essentially no further release of antioxidant activity during the period of pepsin digestion (Figure 4.3.5.). When the pepsin was replaced by trypsin and chymotrypsin in buffer at pH 7.6 there was a further release of antioxidant activity until 150 minutes, antioxidant activity release then levelled off. The total amount of antioxidant activity released was about 50%. There was essentially no kafirin digested over the whole period regardless of the enzyme used. This is not surprising since sorghum condensed tannins are known to bind to protein (Butler et

al., 1984). This may have had the effect of rendering the microparticles indigestible reducing enzyme accessibility (Butler et al., 1984) or the tannin may have bound to the digestion enzymes causing their inhibition (Nguz et al., 1998; reviewed by Bravo, 1998) or possibly both. After 2 hours of pepsin digestion, TEM of sorghum condensed tannin encapsulated kafirin reveals only very slight degradation (Figure 4.3.7.d and e) when compared to kafirin microparticles after the same period of pepsin digestion (Figure 4.3.7.a and b). After a further 2 hours digestion with trypsin and chymotrypsin there was still very little evidence of degradation (Figure 4.3.7.f), whereas, by this time there were essentially no kafirin microparticles remaining (Figure 4.3.7.c). The release in antioxidant activity that occurred for all the samples when pepsin was replaced by trypsin and chymotrypsin was probably due to the effect of a change in pH from pH 2 to pH 7.6 rather than any enzymic degradation of the protein allowing release of encapsulated material. Of the samples, only the unencapsulated kafirin microparticles showed progressive enzyme digestion. The binding of sorghum condensed tannins to protein is due to hydrophobic interactions and hydrogen bonding with no involvement of electrostatic interactions (Butler et al., 1984). Thus changes in pH would not affect binding of sorghum condensed tannins to protein. However, changes in pH are known to affect protein conformation (Cheftel, Cuq and Lorient, 1985). It is suggested that the change in pH resulted in a conformational change in the kafirin. Consequently, indirectly this may have resulted in a change in binding properties of the sorghum condensed tannins to the kafirin microparticles thus allowing the release of further antioxidant activity. There was also an increase in antioxidant release for the catechin encapsulated kafirin microparticles and the kafirin microparticles themselves. Here it is speculated that the kafirin conformational change may have exposed more amino acids responsible for antioxidant activity resulting in an increase in antioxidant activity as measured.

After an initial burst release, as digestion with pepsin continued a progressive release of antioxidant activity from the catechin encapsulated kafirin microparticles occurred up until 90 minutes digestion (Figure 4.3.5.). At this time a plateau was reached, approximately 40% of encapsulated antioxidant activity having been released. On change of pH to 7.6 and enzyme to trypsin and chymotrypsin a further release of antioxidant activity occurred up to 150 minutes digestion as described above. Beyond this period a further plateau was reached and very little further antioxidant activity was released. The

total antioxidant release from catechin encapsulated kafirin microparticles was just less than 70%.

When the pepsin digestion of the catechin encapsulated kafirin is considered there was, as stated, very little kafirin digested over the 2 hour period. However, TEM of this material revealed the start of some degradation from the outside of the microparticles (Figure 4.3.7.d and e). In addition there appeared to be some degradation from the inside of the microparticles causing some vacuoles to merge together as illustrated in Figure 4.3.7.e. There was also very little digestion of the catechin encapsulated kafirin microparticles by the tryspin and chymotrypsin. However, the TEM of these microparticles after an additional 2 hours digestion with trypsin and chymotrypsin showed extensive changes of their ultra-structure (Figure 4.3.7.f) when compared to the same microparticles just after pepsin digestion (Figure 4.3.7.d and e).

It is difficult to explain these results without concluding that catechin can at least partially or loosely bind to protein sufficiently to prevent enzymic degradation. It is known from the work of Emmambux and Taylor (2003) that catechin does not bind to kafirin. However, these workers also showed that at high catechin concentration there was a small amount of binding to bovine serum albumin. Butler et al. (1984) stated that the affinity of different proteins for sorghum tannins varied over four orders of magnitude. This may also be the case to a lesser extent for catechin, although catechin does not have as many binding sites as sorghum tannins, being considered ‘double ended’ rather than ‘multiple ended’ like sorghum tannins (Siebert and Lynn, 1998). Thus, it is suggested that the catechin may have bound to the pepsin, trypsin and chymotrypsin, preventing enzymic digestion of the catechin encapsulated kafirin microparticles.

The mechanism of release of active ingredients from microparticles is usually by diffusion but is influenced by the size, shape and core materials of the microparticles (reviewed by Radwick and Burgess, 2002). Release is also dependant on whether the active ingredient is bound to the core material and whether there is any enzymic degradation of the microparticle (reviewed by Patil, 2003). For catechin encapsulated kafirin microparticles there was no binding of catechin to the kafirin and there was effectively no enzymic digestion of the kafirin. Thus it is likely that the progressive release of antioxidant activity from the catechin encapsulated kafirin microparticles was

primarily by diffusion. The rate of diffusion was initially rapid and then decreased as the distance that the catechin had to diffuse through became greater requiring a longer diffusion time from the interior of the microparticle. It is suggested that the remaining catechin was physically entrapped within the kafirin microparticle and was only released when the conformation of the protein changed with the change in pH at 120 minutes.

The observed changes in the ultrastructure of the catechin encapsulated kafirin microparticles (Figure 4.3.7.f) is possibly due to physical break up of the microparticles, caused by the repeated shaking of the digestion mixtures throughout the incubation period, in conjunction with possible partial solvation, as described above. Previous work has shown the kafirin microparticles are relatively fragile and with shear, break up forming a continuous matrix (Chapter 4.1.). Although in this case the shear was not severe it is possible it was sufficient to cause the observed changes in the microstructure. It is likely that the same changes happened to the unencapsulated kafirin microparticles but the effect may have been enhanced by simultaneous enzyme digestion. The repeated shaking of the digestion mixtures appeared to have no effect on the ultrastructure of the sorghum condensed tannin encapsulated microparticles possibly due 'strengthening' of the kafirin microparticle structure caused by the sorghum condensed tannin binding. Cross-linking of gelatine-pectin microparticles with polyphenols resulted in microparticles with greater mechanical strength, more resistance to elevated temperatures and less stickiness and cohesiveness than similar uncross-linked material (Strauss and Gibson, 2004).

In spite of there being essentially no enzymic digestion of the polyphenol encapsulated kafirin microparticles the total release of antioxidant activity over an extended period of time was relatively high, being approximately 70% from the catechin encapsulated kafirin microparticles and approximately 50% from the sorghum condensed tannin encapsulated kafirin microparticles. This was much better than the reported 15 and 40% catechin released from catechin encapsulated within chitosan microparticles (Zhang and Kisaraju, 2007). In addition, Riedl and Hagerman (2001) demonstrated that sorghum tannins complexed with protein retained at least 50% of their antioxidant activity. These workers suggest that this bound material acts as a free radical sink within the gastrointestinal tract thus sparing other antioxidants. Thus, there is the possibility that an additional 25% of the antioxidant activity of sorghum condensed tannins encapsulated in kafirin

microparticles would not be lost but would be available to act as a free radical sink within the gastrointestinal tract.

4.3.5. Conclusions

This study confirms the importance of disulphide cross-linking and sorghum condensed tannin protein interactions as major causal factors of the poor protein digestibility of sorghum.

Gamma-kafirin binds the most condensed tannins compared to the α-and β-kafirin, probably due to its high proline content. The protein digestibility of kafirin-tannin complexes is much lower than unbound kafirins. However, kafirin films made with bound tannins appear to have extended life due to a decrease in protein digestibility caused by kafirin-tannin binding.

Encapsulation of catechin and sorghum condensed tannins within kafirin microparticles seems 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.

4.3.6. References

American Association of Cereal Chemists, (AACC), 2000. Crude protein-combustion, Standard Method 46-30. Approved Methods of the AACC. The Association, St Paul, MN.

Arbab, M.E., El Tinay, A.H., 1997. Effect of cooking and treatment with sodium bisulphite or ascorbic acid on the in vitro protein digestibility of two sorghum cultivars. Food Chemistry 59, 339-343.

American Society for Testing and Materials, 1996. Annual Book of the American Society for Testing and Materials Standards. ASTM D5512-96. Standard practice for exposing plastics to a simulated compost environment using an externally heated reactor. The Society: West Conshohocken, PA.

Awika, J.M., Rooney, L.W., 2004. Sorghum phytochemicals and their potential impact on human health. Phytochemistry 65, 1199-1221.

Awika, J.M., Dykes, L., Rooney, L.W., Prior, R.L., 2003a. Processing of sorghum (*Sorghum bicolor*) and sorghum products alters procyanidin oligomer and polymer distribution and content. Journal of Agricultural and Food Chemistry 51, 5516-5521.

Awika, J.M., Rooney, L.W., Wu, X., Prior, R.L., Cisneros-Zevallos, L., 2003b. Screening methods to measure antioxidant activity of sorghum (*Sorghum bicolor*) and sorghum products. Journal of Agricultural and Food Chemistry 51, 6657-6662.

Axtell, J.D., Kirleis, A., Hassen, M.M., D'Crox-Mason, N., Mertz, E.T., Munck, L., 1981. Digestibility of sorghum proteins. Proceedings of the National Academy of Sciences of the United States of America 78, 1333-1335.

Baxter, N.J., Lilley, T.H., Haslam, E., Williamson, M.P., 1997. Multiple interactions between polyphenols and a salivary proline-rich protein repeat result in complexation and precipitation. Biochemistry 36, 5566-5577.

Belton, P.S., Delgadillo, I., Halford, N.G., Shewry, P.R., 2006. Kafirin structure and functionality. Journal of Cereal Science 44, 272-286.

Bidlingmeyer, B.A., Cohen, S.A., Tarvin, T.L., 1984. Rapid analysis of amino acids using pre-column derivatization. Journal of Chromatography 336, 93-104.

Bravo, L., 1998. Polyphenols: chemistry, dietary sources, metabolism and nutritional significance. Nutrition Reviews 56, 317-333.

Butler, L.G., Riedl, D.J., Lebryk, D.G., Blytt, H.J., 1984. Interaction of proteins with sorghum tannins: Mechanism, specificity and significance. Journal of the American Oil Chemists Society 6, 916-920.

Byaruhanga, Y., Erasmus, C., Taylor, J.R.N., 2005. Effect of microwave heating of kafirin on the functional properties of kafirin films. Cereal Chemistry 82, 565-573.

Charlton, A.J., Baxter, N.J., Lockman Khan, M., Moir, A.J.G., Haslam, E., Davis, A.P., Williamson, M.P., 2002. Polyphenol/peptide binding and precipitation. Journal of Agricultural and Food Chemistry 50, 1593-1601.

Cheftel, J.C., Cuq, J.L., Lorient, D., 1985. Amino acids, peptides and proteins, in: Fennema, O.R., (Ed.), Food Chemistry. 2nd edition, Marcel Dekker, New York, pp. 245-369.

Chen, H.M., Muramoto, K., Yamauchi, F., Nokihara, K., 1996. Antioxidant activity of designed peptides based on the antioxidative peptide isolated from digests of a soybean protein. Journal of Agricultural and Food Chemistry 44, 2619-2623.

Chen, H.M., Muramoto, K., Yamauchi, F., Fujimoto, K., Nokihara, K., 1998. Antioxidative properties of histidine-containing peptides designed from peptides fragments found in the digests of a soybean protein. Journal of Agricultural and Food Chemistry 46, 49-53.

Daiber, K. H., 1975. Enzyme inhibition by polyphenols of sorghum grain and malt. Journal of the Science of Food and Agriculture 26, 1399-1411.

Dlamini, N.R.N., 2007. Effect of sorghum type and processing on antioxidant properties of sorghum (*Sorghum biocolor* (L.) Moench) based foods. PhD disertation, Texas A&M University, College Station, TX.

Duodu, K.G., Taylor, J.R.N., Belton, P.S., Hamaker, B.R., 2003. Factors affecting sorghum protein digestibility. Journal of Cereal Science, 38, 117-131.

Duodu, K.G., Nunes, A., Delgadillo, I., Parker, M.L., Mills, E.N.C., Belton, P.S., Taylor, J.R.N., 2002. The effect of grain structure and cooking on sorghum and maize *in vitro* protein digestibility. Journal of Cereal Science 35**,** 161-175.

Dykes, L., Rooney, L.W., 2006. Sorghum and millet phenols and antioxidants. Journal of Cereal Science 44, 236-251.

Dykes, L., Rooney, L.W., 2007. Phenolic compounds in cereals grains and their health benefits. Cereal Foods World 52, 105-111.

Edens, L. Van der Laan, J.M., Craig, H.D., 2005. Mechanisms and prevention by proline-specific proteases. Brauwelt International 3, 157-161.

El Nour, N.A., Peruffo, A.D.B., Curioni, A., 1998. Characterisation of sorghum kafirins in relations to their cross-linking behaviour. Journal of Cereal Science 28, 197-207.

Emmambux, M.N., Stading, M., Taylor, J.R.N., 2004. Sorghum kafirin film property modification with hydrolysable and condensed tannins. Journal of Cereal Science 20, 127-135.

Emmambux, N.M, Taylor, J.R.N., 2003. Sorghum kafirin interaction with various phenolic compounds. Journal of the Science of Food and Agriculture 83, 402-407.

Evans, C.D., Manley, R.H., 1941. Solvents for zein, primary solvents. Industrial and Engineering Chemistry 33, 1415-1417.

Evans, D.J., Schüssler, L., Taylor, J.R.N., 1987. Isolation of reduced-soluble protein from sorghum starchy endosperm. Journal of Cereal Science 5. 61-65.

Gallagher, S.R., 1999. Electrophoretic separation of proteins, in: Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.E., Seidman, J.G., Smith, J.A., Strule, K. (Eds.), Current Protocols in Molecular Biology. John Wiley and Sons, New York, pp. 10.2A1-9.

Gao, C., Taylor, J., Wellner, N., Byaruhanga, Y.B., Parker, M.L., Mills, E.N.C., Belton, P.S., 2005. Effect of preparation conditions on protein secondary structure and biofilm formation of kafirin. Journal of Agricultural and Food Chemistry 53, 306-312.

Hamaker, B.R., Kirleis, A.W., Mertz, E.T., Axtell, J.D., 1986. Effect of cooking on the protein profiles and *in vitro* digestibility of sorghum and maize. Journal of Agricultural and Food Chemistry 34, 647-649.

Hamaker, B.R., Kirleis, A.W., Butler, L.G., Axtell, J.D., Mertz, E.T., 1987. Improving in-vitro protein digestibility of sorghum with reducing agents. Proceedings of the National Academy of Science USA 84, 626-628.

Hancock, J.D., 2000. Value of sorghum and sorghum co-products in diets for livestock, in: Smith, C.W., Frederiksen, R.A. (Eds.), Sorghum: Origin, History, Technology and Production. John Wiley and Sons, New York, pp. 731-749.

Hattori, M., Yamaji-Tsukamoto, K., Kumagai, H., Feng, Y., Takahashi, K., 1998. Antioxidant activity of soluble elastin peptides. Journal of Agricultural and Food Chemistry 46, 2167-2170.

Huang, X., Brazel, C.B., 2001. On the importance and mechanism of burst release in matrix-controlled drug delivery systems. Journal of Controlled Release 73, 121-136.

International Organization for Standardization (ISO), 1988. Sorghum: determination of tannin content, ICS:67.060, Food Technology: Cereals, Pulses and Derived products. International Organization for Standardization, ISO 9648, Paris.

Kim, C.S., Woo, Y., Clore, A.M., Burnett, R.J., Carneiro, N.P., Larkins, B., 2002. Zein protein interactions, rather than the asymmetric distribution of zein mRNAs on endoplasmic reticulum membranes, influence protein body formation in maize endosperm. The Plant Cell 14**,** 655-672.

Kim, G.N., Jang, H.D., Kim, C.I., 2007. Antioxidant capacity of caseinophosphopeptides prepared from sodium caseinate using Alcalase. Food Chemistry 104, 1359-1365.

Lacroix, M., Le, T.C., Ouattara, B., Yu, H., Letendre, M., Sabato, S.F., Mateescu, M.A., Demas-Patterson, G., 2002. Use of γ-irradiation to produce films from whey, casein and soya proteins: structure and functional characteristics. Radiation Physics and Chemistry 63, 827-832.

Lawton, J.W., 2002. Zein: a history of processing and use. Cereal Chemistry 79**,** 1-18.

Le Tien, C., Letendre, M. Ispas-Szabo, P. Mateescu, M.A., Demas-Patterson, G., Yu, H.L., Lacroix, M., 2000. Development of biodegradable films from whey proteins by cross-linking and entrapment in cellulose. Journal of Agricultural and Food Chemistry 48, 5566-5575.

Liu, X., Sun, Q., Wang, H., Zhang, L., Wang, J.Y., 2005. Microspheres of corn protein, zein, for an ivermectin drug delivery system. Biomaterials 26, 109-115.

Marcuse, R., 1960. Antioxidant effect of amino acids. Nature 186, 886-887.

Mertz, E.T., Hassen, M.M., Cairns-Whittern, C., Kirleis, A.W., Tu, L., Axtell, J.D., 1984. Pepsin digestibility of proteins in sorghum and other major cereals, Proceedings of the National Academy of Sciences of the United States of America 81, 1-2.

Morrall, P., Liebenberg, N. VdW., Glennie, C.W., 1981. Tannin development and location in bird-resistant sorghum grain. Scanning Electron Microscopy 111, 571-576.

Muthuselvi, L., Dhathathreyan, A., 2006. Simple coacervates of zein to encapsulate gitoxin. Colloids and Surfaces B: Biointerfaces 51, 39-43.

Nguz, K., Van Gaver, D., Huyghebaert, A., 1998. *In vitro* inhibition of digestive enzymes by sorghum condensed tannins (*Sorghum bicolor* (L Moench)). Science Alimentaris 18**,** 507-514.

Nunes, A., Correia, I., Barros, A., Delgadillo, I., 2005. Characterization of kafirin and zein oligomers by preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Journal of Agricultural and Food Chemistry 53, 639-643.

Oria, M.P., Hamaker, B.R., Shull, J.M., 1995a. In vitro protein digestibility of developing and mature sorghum grain in relation to α-, β-, and γ-kafirin disulphide crosslinking. Journal of Cereal Science 22, 85-93.

Patil, G.V., 2003. Biopolymer albumin for diagnosis and in drug delivery. Drug Development Research 58, 219-247.

Peña-Ramos, E.A., Xiong, Y.L., Arteaga, G.E., 2004. Fractionation and characterisation for antioxidant activity of hydrolysed whey protein. Journal of the Science of Food and Agriculture 84, 1908-1918.

Pérez-Jiménez, J., Saura-Calixto, F., 2006. Effect of solvent and certain food constituents on different antioxidant capacity assays. Food Research International 39, 791-800.

Price, M.L., Van Scoyoc, S., Butler, L.G., 1978. A critical examination of the vanillin reaction as an assay for tannin in sorghum grain. Journal of Agricultural and Food Chemistry 26, 1214-1218.

Radwick, A.E., Burgess, D.J., 2002. Proteins as microencapsulating agents and microencapsulated active compounds in pharmaceutical applications. In:Gennadios, A., (Ed.), Protein-Based Films and Coatings. CRC Press, Boca Raton, pp. 341-366.

Ratnam, D.V., Ankola, D.D., Bhardwaj, A.V., Sahana, D.K., Ravi Kumar, M.N.V., 2006. Role of antioxidants in prophylaxis and therapy: a pharmaceutical perspective. Journal of Controlled Release 113, 189-207.

Riedl, K.M., Hagerman, A.E., 2001. Tannin-protein complexes as radical scavengers and radical sinks. Journal of Agricultural and Food Chemistry 49, 4917-4923.

Rom, D.L., Shull, J.M., Chandrashekar, A., Kirleis, A.W., 1992. Effects of cooking and treatment with sodium bisulfite on in vitro protein digestibility and microstructure of sorghum flour. Cereal Chemistry 101-104.

Saiga, A., Tanabe, S., Nishimura, T., 2003. Antioxidant activity of peptides obtained from porcine myofibrillar proteins by protease treatments. Journal of Agricultural and Food Chemistry 51, 3661-3667.

Salunkhe, D.K., Chavan, J.K., Kadam, S.S., 1990. Dietary Tannins: Consequences and Remedies. CRC Press. Boca Raton, FL, pp.113-135.

Sarni-Manchado, P., Cheynier, V., Moutounet, M., 1999. Interactions of grape seed tannins with salivary proteins. Journal of Agricultural and Food Chemistry 47**,** 42-47.

Serna-Saldivar, S., Rooney, L.W., 1995. Structure and chemistry of sorghum and millets, in: Dendy D.A.V. (Ed.), Sorghum and Millets: Chemistry and Technology. American Association of Cereal Chemists, St Paul, MN, pp. 69-124.

Shewry, P.R., 2002. The major seed storage proteins of spelt wheat, sorghum, millets and pseudocereals, in: Belton P.S., Taylor, J.R.N. (Eds.), Pseudocereals and Less Well-Known Cereals. Springer, Berlin, pp 1-24.

Siebert, K.J., Lynn, P.Y., 1998. Comparison of polyphenol interactions with polyvinylpolypyrrolidone and haze-active protein. Journal of the American Society of Brewing Chemists 56, 24-31.

Sikwese, F.E., Duodu, K.G., 2007. Antioxidant effect of a crude phenolic extract from sorghum bran in sunflower oil in the presence of ferric ions. Food Chemistry 104, 324-331.

Spencer, C.A., Cai, Y., Gaffney, R.M.S.H., Goulding, P.N., Magnolato, D., Lilley, T.H., Haslam, E., 1988. Polyphenol complexation-some thoughts and observations. Phytochemistry 27, 2397-2409.

Strauss, G., Gibson, S.M., 2004. Plant phenolics as cross-linkers of gelatin gels and gelatin-based coacervates for use as food ingredients. Food Hydrocolloids 18, 81-89.

Swiss Prot, UniProtKB/TrEMBL entry Q6Q299, (2005)
http:/us.expasy.org/uniprot/Q6Q299 accessed 26 October, 2005.

Taylor, J., Bean, S.R., Ioerger, B.P., Taylor, J.R.N., 2007. Preferential binding of sorghum tannins with γ-kafirin and the influence of tannin binding on kafirin digestibility and biodegradation. Journal Cereal Chemistry 46, 22-31.

Taylor, J.R.N. 2001. Methods to be used to identify and specify characteristics desired by industrial processors that use sorghum as an input, technical report #2, task order No 4.1. USAID, Gaborone, Botswana.

Taylor, J.R.N., Belton, P.S., 2002. Sorghum, in: Belton P.S., Taylor, J.R.N. (Eds.), Pseudocereals and Less Well-Known Cereals. Springer, Berlin, pp 25-38.

Taylor, J.R.N., Novellie, L., Liebenberg, N.VdW., 1985. Protein body degradation in the germinating starchy endosperm of germinating sorghum. Journal of Experimental Botany 36, 1287-1295.

Wang, H.J., Lin, Z.X., Liu, X.M., Sheng, S.H., Wang, J.Y., 2005. Heparin-loaded zein microsphere film and hemocompatibility. Journal of Controlled Release 105, 120-131.

Wang, J.Y., Fujimoto, K., Miyazawa, T., Endo, Y., 1991. Antioxidative mechanism of maize zein in powder model systems against methyl linoleate: effect of water activity and coexistence of antioxidants. Journal of Agricultural and Food Chemistry 39, 351-355.

Wang, J.Y., Miyazawa, T., Fujimoto, K., 1991. Inhibition of methyl linoleate peroxidation by maize zein in powder model system at high water activity. Agricultural and Biological Chemistry 55. 1531-1536.

Whittlesey, K.J., Shea, L.D., 2004. Delivery systems for small molecule drugs, proteins, and DNA: the neuroscience/biomedical interface. Experimental Neurology 190, 1-16.

Zang, L., Kosaraju, S.L., 2007. Biopolymeric delivery system for controlled release of polyphenolic antioxidants. European Polymer Journal 43, 2956-2966.