

Preparation, characterisation and functionality of kafirin microparticles

by

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DECLARATION

I hereby declare that this thesis submitted at the University of Pretoria for the award of PhD degree is my work and has not been submitted by me for a degree at any other University or Institution of Higher Education.

**Janet Taylor
April 2008**

ABSTRACT

Preparation, characterisation and functionality of kafirin microparticles

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Whilst working on a Masters degree on alternative solvents and extractants for the sorghum prolamin protein, kafirin, the author serendipitously found an ethanol-free method of making kafirin microparticles in dilute organic acid. Further, on drying a suspension of kafirin microparticles in dilute organic acid, a clear, transparent film was found to be formed. Microparticles from zein, the maize prolamin protein, have shown potential for food and pharmaceutical applications. Kafirin is more hydrophobic and less digestible than zein so it was hypothesised that it may form microparticles with superior properties. However, the structural and functional characteristics of kafirin microparticles and films made from them needed to be known before any potential applications could be exploited.

Kafirin microparticles were made by dissolution of kafirin in glacial acetic acid followed by precipitation on addition of water. They were characterized by Light microscopy (LM), Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM) and were found to be mainly spherical, porous and between 1-10 μm in diameter. The kafirin microparticles had very large internal surface area due to the presence of many smooth walled holes or vacuoles of variable sizes, probably caused by entrapment of air during microparticle formation. Increasing the final acetic acid concentration resulted in kafirin microparticles of increased size, with an increasing number of internal holes. At 40% acetic acid the spherical microparticle structures completely disappeared and were replaced by an open matrix which resembled an expanded foam.

The kafirin microparticles were found to form very thin ($<15 \mu\text{m}$) free standing films and coatings. A minimum concentration of organic acid (10.8 percent) is required to form a cohesive kafirin microparticle film relative to the concentration of protein (1 percent for acetic acid). Some functional properties, e.g. smooth film surface properties, low water

vapour permeability (WVP) and low protein digestibility of these films are superior to those of similar conventionally cast kafirin films.

With the aim of exploiting the porous nature of kafirin microparticles for encapsulation of nutrient additives, several factors were examined for their influence on retarding protein digestibility. Retardation of digestibility of kafirin microparticles would allow controlled release of the encapsulated agent in the stomach and gastrointestinal tract. The importance of disulphide cross-linking and sorghum condensed tannin protein interactions were confirmed as major causal factors of the poor protein digestibility of sorghum. Gamma-kafirin was found to bind the most condensed tannins compared to the α - and β -kafirins, probably due to its high proline content. As expected, the protein digestibility of kafirin-tannin complexes was much lower than unbound kafirins. This seems to slow the biodegradation of kafirin films made with bound tannins.

The antioxidants, catechin and sorghum condensed tannins were encapsulated within kafirin microparticles and the antioxidant release profiles investigated under simulated gastric conditions. Over a period of four hours, catechin and condensed tannin encapsulated kafirin microparticles showed virtually no protein digestion but released approximately 70% and 50% respectively total antioxidant activity.

The mechanism for the formation of kafirin microparticles and films formed from them seems to involve controlled aggregation of kafirin molecules. Models for the formation of both were proposed based on an analogy with protein body formation and the potential ability of γ -kafirin to undergo a structural inversion exposing either hydrophilic or hydrophobic ends depending on the prevailing conditions.

Research into cross-linking by physical or chemical agents is needed before practical applications can be exploited. However, encapsulation of catechin and sorghum condensed tannins within kafirin microparticles seems to be an effective way to use the binding properties of polyphenols with protein to enhance potential health benefits by controlled release of antioxidant activity within the stomach and gastrointestinal tract.

DEDICATION

To my family, with love. Without you all I would not have succeeded.

‘The outcome of any serious research can only be to make two questions grow where one question grew before’

Thorstein Veblen

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Think where man's glory most begins and ends, and say my glory was I had such friends
William Butler Yates

And with this in mind I would like to thank:

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TABLE OF CONTENTS

	ABSTRACT	iii
	DEDICATION	v
	ACKNOWLEDGEMENTS	vi
	TABLE OF CONTENTS	vii
	LIST OF TABLES	xi
	LIST OF FIGURES	xii
1.	INTRODUCTION	1
2.	LITERATURE REVIEW	2
2.1.	MICROPARTICLES	2
2.1.1.	Definitions	2
2.1.2.	Characteristics	3
2.1.3.	Microparticle preparation	3
2.1.3.1.	<i>Spray drying</i>	4
2.1.3.2.	<i>Solvent extraction/evaporation</i>	4
2.1.3.3.	<i>Phase separation/coacervation</i>	5
2.1.3.4.	<i>Methods of zein microparticle preparation</i>	5
2.1.4.	Suitability of different materials for microparticle preparation	7
2.1.4.1	<i>PLGA microparticles</i>	7
2.1.4.2	<i>Protein microparticles</i>	7
2.2.	KAFIRIN	10
2.2.1.	Kafirin chemical composition	10
2.2.2.	Kafirin secondary structure	12
2.2.3.	Kafirin hydrophobicity	16
2.2.4.	Kafirin protein digestibility	18
2.3.	PROPERTIES OF PROTEIN MICROPARTICLES	21
2.3.1.	Properties of zein microparticles	23
2.4.	ALTERATION OF PROTEIN MICROPARTICLE PROPERTIES	24
2.5.	POTENTIAL USES OF MICROPARTICLES	27
2.5.1.	Non food uses	27
2.5.2.	Food uses	28
2.6.	CONCLUSIONS	31
3.	HYPOTHESES AND OBJECTIVES	32
3.1.	HYPOTHESES	32
3.2.	OBJECTIVES	33
4.	RESEARCH	34
4.1.	FORMATION AND CHARACTERISATION OF KAFIRIN MICROPARTICLES BY PHASE SEPARATION FROM AN ORGANIC ACID	34
4.1.1.	Abstract	34
4.1.2.	Introduction	35
4.1.3.	Materials and methods	37
4.1.3.1.	<i>Materials</i>	37
4.1.3.2.	<i>Preparation of kafirin microparticles with acetic acid</i>	37
4.1.3.3.	<i>Preparation of kafirin microparticles with lactic acid or propionic acid</i>	37

4.1.3.4.	<i>Preparation of kafirin microparticles without plasticiser</i>	37
4.1.3.5.	<i>Preparation of kafirin microparticles with gas saturated or degassed solvents</i>	38
4.1.3.6.	<i>Preparation of kafirin microparticles with aqueous ethanol</i>	38
4.1.3.7.	<i>Effect of shear on microparticle formation</i>	38
4.1.3.8.	<i>Size, shape and size distribution of kafirin microparticles</i>	38
4.1.3.9.	<i>Effect of storage on kafirin microparticle size distribution</i>	39
4.1.3.10.	<i>Electron Microscopy of microparticles</i>	39
4.1.3.11.	<i>SDS-PAGE</i>	39
4.1.3.12.	<i>Fourier Transform Infrared Spectroscopy (FTIR)</i>	39
4.1.4.	Results and discussion	40
4.1.4.1.	<i>Morphology of kafirin microparticles</i>	40
4.1.4.2.	<i>Size distribution</i>	42
4.1.4.3.	<i>Effect of shear</i>	44
4.1.4.4.	<i>Effect of changing the acetic acid concentration on microparticle size and morphology</i>	44
4.1.4.5.	<i>Effect of storage on microparticle size and size distribution</i>	46
4.1.4.6.	<i>Effect of freeze drying on microparticle morphology</i>	46
4.1.4.7.	<i>SDS-PAGE of kafirin and kafirin microparticles</i>	48
4.1.4.8.	<i>Fourier Transform Infrared Spectroscopy (FTIR) of kafirin microparticles</i>	49
4.1.4.9.	<i>Kafirin microparticle formation from other organic acids</i>	52
4.1.4.10.	<i>Presence of holes or vacuoles in kafirin microparticles</i>	52
4.1.5.	Conclusions	57
4.1.6.	References	58
4.2.	PREPARATION OF KAFIRIN MICROPARTICLE FILMS AND COATINGS AND MECHANISM OF FILM FORMATION AND FILM FUNCTIONAL PROPERTIES.	62
4.2.1.	Abstract	62
4.2.2.	Introduction	63
4.2.3.	Materials and methods	64
4.2.3.1.	<i>Materials</i>	64
4.2.3.2.	<i>Preparation of kafirin microparticles</i>	64
4.2.3.3.	<i>Preparation of films</i>	64
	Preparation of free standing kafirin films	64
	Preparation of kafirin microparticle free standing films	64
	Preparation of kafirin microparticle free standing films cast with different acids	64
	Preparation of films from kafirin at low protein concentration using glacial acetic acid as casting solvent	65
	Preparation of films from kafirin microparticles at low acid and low protein concentration	65
4.2.3.4.	<i>Analysis of films</i>	65
	Effects of protein concentration and acid concentration on kafirin microparticle film formation	65
	Effects of plasticiser concentration and acid concentration on kafirin microparticle film formation	65
	Scanning Electron Microscopy (SEM) of free standing films	65

	Atomic Force Microscopy (AFM) of kafirin microparticle film surfaces	66
	Fourier Transform Infrared Spectroscopy (FTIR) of films	66
	Water Vapour Transmission (WVT) and Water Vapour Permeability (WVP) of films	66
	Tensile properties of films	67
	Protein digestibility of films	67
	Biodegradation of films	68
	Observation of microparticle film formation	68
4.2.3.5.	<i>Statistical analysis</i>	68
4.2.4.	Results and discussion	69
4.2.4.1.	<i>Effect of protein concentration and acid concentration on kafirin microparticle film formation</i>	69
4.2.4.2.	<i>Effects of plasticiser content and acid concentration on kafirin microparticle film formation</i>	71
4.2.4.3.	<i>Microparticle film formation</i>	73
4.2.4.4.	<i>Preparation of films from kafirin microparticles at low acid and low protein concentration</i>	77
4.2.4.5.	<i>Microparticle film formation in organic acids other than acetic acid</i>	78
4.2.4.6.	<i>Fourier Transform Infrared Spectroscopy (FTIR) of kafirin films</i>	79
4.2.4.7.	<i>Functional properties of kafirin microparticle films</i>	83
	Film surface properties	83
	Water Vapour Permeability (WVP) and Water Vapour Transmission (WVT)	86
	Tensile Properties	88
	Film Protein Digestibility	90
	Biodegradation of films	91
4.2.5.	Conclusions	92
4.2.6.	References	93
4.3.	TANNIN INTERACTIONS WITH SORGHUM PROTEINS, KAFIRIN FILMS AND MICROPARTICLES AND ITS INFLUENCE ON THEIR DIGESTIBILITY AND FUNCTIONALITY	98
4.3.1.	Abstract	98
4.3.2.	Introduction	99
4.3.3.	Materials and methods	101
4.3.3.1.	<i>Materials</i>	101
4.3.3.2.	<i>Extraction of total kafirin and gamma-kafirin</i>	101
4.3.3.3.	<i>Methods</i>	102
	Effect of reducing agent on sorghum protein digestibility	102
	Tannin content	103
	Tannin Type	103
	Amino acid analysis	103
	Tannin binding assay	103
	SDS-PAGE	104
	Film formation	104
	Protein digestibility of protein preparations and films	105
	Film biodegradation	105
	Preparation of kafirin microparticles for encapsulation	105

	Encapsulation of catechin and sorghum condensed tannins in kafirin microparticles	105
	Transmission Electron Microscopy (TEM) of kafirin microparticles, kafirin microparticle encapsulated catechin and kafirin microparticle encapsulated sorghum condensed tannins	105
	Dissolution and release of antioxidant activity by simulated digestion	106
	ABTS antiradical analysis	107
	Statistical analysis	107
4.3.4.	Results and discussion	108
4.3.4.1.	<i>Effect of independent variables on sorghum protein digestibility</i>	108
4.3.4.2.	<i>Kafirin-tannin binding</i>	112
4.3.4.3.	<i>Efficiency of polyphenol encapsulation in kafirin microparticles</i>	120
4.3.5.	Conclusions	132
4.3.6.	References	133
5.	GENERAL DISCUSSION	142
5.1.	METHODOLOGIES USED IN RESEARCH PROJECT	142
5.1.1.	Microscopy	142
5.1.1.1.	<i>Light Microscopy</i>	142
5.1.1.2.	<i>Electron Microscopy</i>	143
5.1.1.3.	<i>Atomic Force Microscopy (AFM)</i>	145
5.1.2.	Fourier Transform Infrared Spectroscopy (FTIR)	145
5.1.3.	Protein digestibility	146
5.1.4.	Biodegradation	147
5.1.5.	Antioxidant activity	148
5.2.	KAFIRIN MOLECULAR AGGREGATION	150
5.3.	MECHANISM OF KAFIRIN MICROPARTICLE FORMATION	153
5.4.	MECHANISM OF KAFIRIN MICROPARTICLE FILM FORMATION	161
5.5.	POTENTIAL APPLICATIONS OF KAFIRIN MICROPARTICLES	166
5.6.	IMPROVEMENT OF KAFIRIN MICROPARTICLE PROPERTIES AND FUTURE WORK	167
6.	CONCLUSIONS AND RECOMMENDATIONS	171
7.	REFERENCES	173
8.	PUBLICATIONS AND PRESENTATION MADE BASED ON THIS RESEARCH	197

LIST OF TABLES

Table 2.1.	Free energy of hydration of kafirin subunits compared with that of an 'average protein'. Adapted from Belton et al. (2006).	16
Table 4.1.1.	Estimated ratio of α -helical to intermolecular β -sheet conformation of kafirin and kafirin microparticles	50
Table 4.2.1.	Estimated ratio of α -helical to intermolecular β -sheet conformation of kafirin and kafirin films	80
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%)	87
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%)	89
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%)	91
Table 4.3.1.	Amino acid composition (moles %) of kafirin preparations	102
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	109
Table 4.3.3.	In vitro protein digestibility (%) of sorghum condensed tannin (CT) bound and unbound kafirin preparations and of films	118

LIST OF FIGURES

Figure 2.1	Scanning electron micrograph of zein microspheres (Parris et al., 2005)	6
Figure 2.2.	Structural models proposed for α -zein. A-Argos et al., B-Garratt, et al., C- Matsushima et al., D- Bugs et al., E- Momany et. al. (adapted from Belton et al., 2006).	13
Figure 2.3.	Alignment of amino acid sequences of γ -kafirins with those of γ -zein to demonstrate the number of hydrophobic amino acids present. Hydrophobic amino acids are highlighted (adapted from Belton et al., 2006).	17
Figure 4.1.1.	SEM of kafirin microparticles made with acetic acid or aqueous ethanol	41
Figure 4.1.2.	TEM kafirin microparticles made with acetic acid or aqueous ethanol	41
Figure 4.1.3.	Particle size distribution of kafirin microparticles	43
Figure 4.1.4.	Light microscopy to illustrate the effect of increasing acid concentration on preformed kafirin microparticles. Appearance of kafirin microparticles made with aqueous ethanol for comparison	45
Figure 4.1.5.	SEM and TEM of kafirin microparticles at different acetic acid concentrations	46
Figure 4.1.6.	SEM freeze dried microparticles and kafirin used to prepare them	47
Figure 4.1.7.	SDS-PAGE of kafirin microparticles	48
Figure 4.1.8.	FTIR of original kafirin	51
Figure 4.1.9.	Light microscopy of kafirin microparticles made with acetic acid, lactic acid and propionic acid	52
Figure 4.1.10.	Kafirin microparticles prepared with and without plasticizer	53
Figure 4.1.11.	SEM kafirin microparticles made either with the addition of gas to the solvents	55
Figure 4.1.12.	TEM kafirin microparticles made either with the addition of gas to the solvents or made with degassed solvents	56
Figure 4.2.1.	Effects of increasing protein concentration and acetic acid concentration on kafirin microparticle film formation	70
Figure 4.2.2.	Effects of increasing acetic acid concentration and increasing plasticiser concentration on kafirin microparticle film formation	72
Figure 4.2.3.	Light microscopy, time lapse record of kafirin microparticle film formation A: 5.4% acetic acid, B: 21.6% acetic acid	75
Figure 4.2.4.	Effect of low protein concentration on kafirin film formation	77
Figure 4.2.5.	Effects of kafirin microparticles made with different acids with increasing acid concentration on film formation	79
Figure 4.2.6.	FTIR of kafirin films	81
Figure 4.2.7.	SEM of kafirin film surfaces	84
Figure 4.2.8.	AFM of kafirin microparticle film surfaces	85
Figure 4.2.9.	Biodegradation of kafirin films	91

Figure 4.3.1.	Percentage of sorghum condensed tannin (CT) bound to different kafirin species	113
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)	116
Figure 4.3.3.	Biodegradation of films under high moisture conditions	119
Figure 4.3.4.	TEM of kafirin microparticles and kafirin microparticles encapsulated with polyphenols	122
Figure 4.3.5.	Effect of pepsin digestion followed by trypsin/chymotrysin digestion on antioxidant activity and percentage antioxidant released from kafirin microparticles, kafirin microparticles with encapsulated catechin and kafirin microparticles with encapsulated sorghum condensed tannins	124
Figure 4.3.6.	Effect of pepsin digestion followed by trypsin/chymotrysin digestion on kafirin digestibility of kafirin microparticles, kafirin microparticles with encapsulated catechin and kafirin microparticles with encapsulated sorghum condensed tannins	125
Figure 4.3.7.	TEM illustrating the effect of pepsin digestion followed by trypsin/chymotrysin digestion on kafirin digestibility of kafirin microparticles	126
Figure 5.3.1	Model for kafirin microparticle formation	158
Figure 5.4.1.	Model for kafirin microparticle film formation	163

1. INTRODUCTION

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

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

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

2. LITERATURE REVIEW

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

2.1. MICROPARTICLES

2.1.1. Definitions

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

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

2.1.2. Characteristics

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

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

2.1.3. Microparticle preparation

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

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

2.1.3.1. Spray Drying

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

2.1.3.2. Solvent extraction/evaporation

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

2.1.3.3. Phase separation/coacervation

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

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

2.1.3.4. Methods of zein microparticle preparation

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

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

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

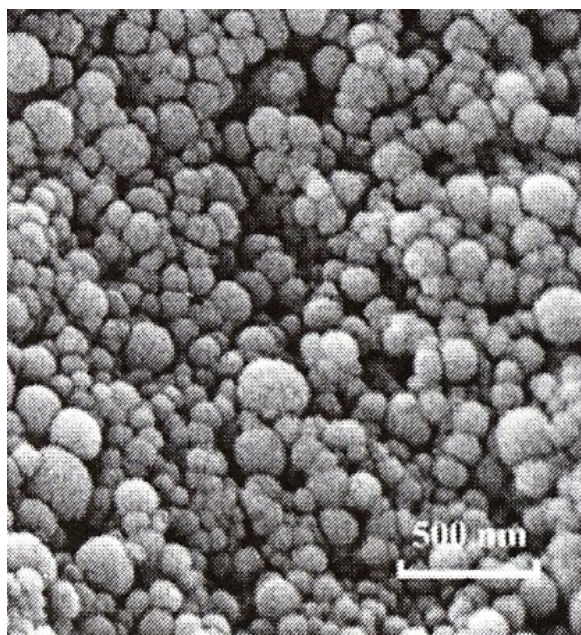


Figure 2.1: Scanning electron micrograph of zein microspheres (Parris et al., 2005)

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

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

2.1.4. Suitability of different materials for microparticle preparation

2.1.4.1. PLGA microparticles

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

2.1.4.2. Protein microparticles

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

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

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

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

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

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

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

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

2.2 KAFIRIN

2.2.1. Kafirin chemical composition

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

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

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

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

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

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

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

2.2.2. Kafirin secondary structure

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

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

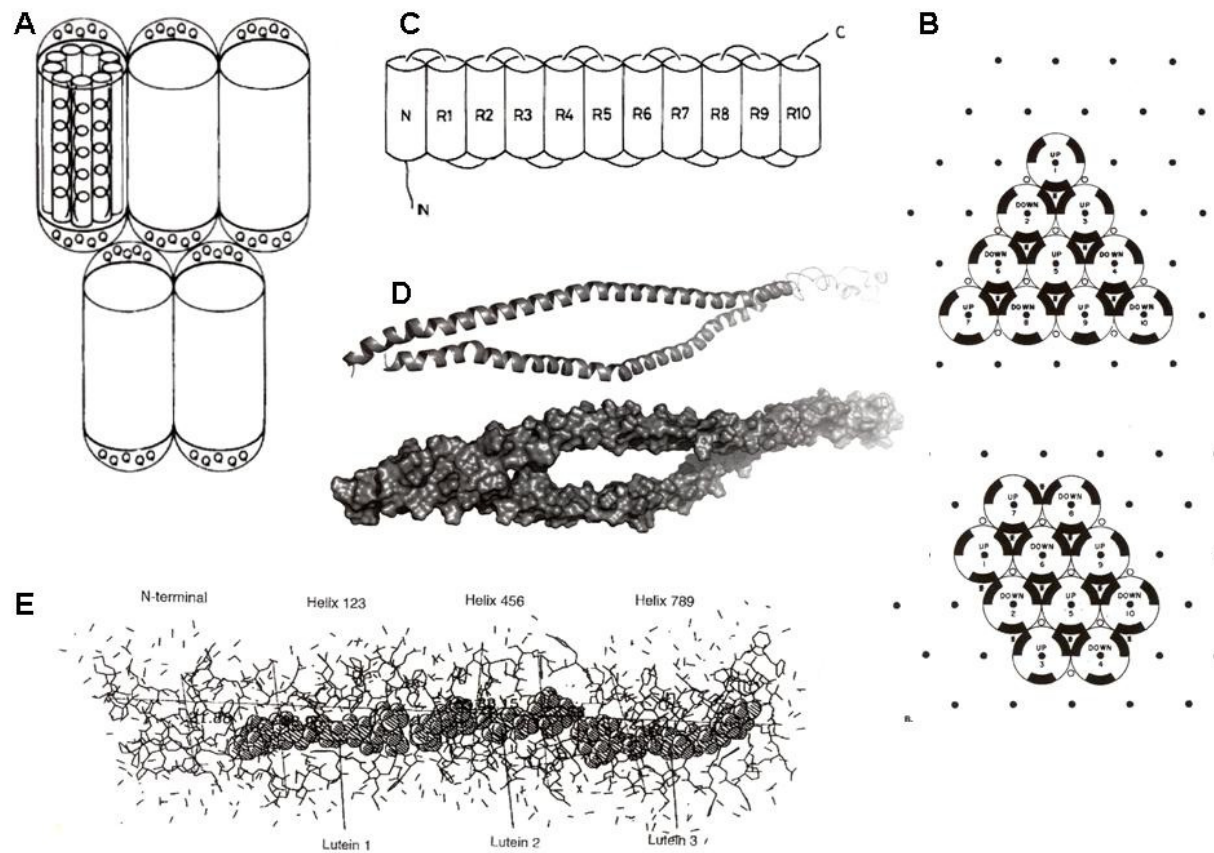


Figure 2.2: Structural models proposed for α -zein. A-Argos et al., (1982) B-Garratt, et al., (1993) C-Matsushima et al., (1997) D- Bugs et al., (2004) E- Momany et. al. (2006) (adapted from Belton et al., 2006)

The turns at the top and bottom of the cylinder of helices are rich in glutamine residues which allow hydrogen bonding between molecules in different planes. This model fitted the physical measurements available at the time of publication but as measurement techniques have become more sophisticated the Argos model has been modified to better fit the latest physical data.

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

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

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

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

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

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

2.2.3. Kafirin hydrophobicity

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

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

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

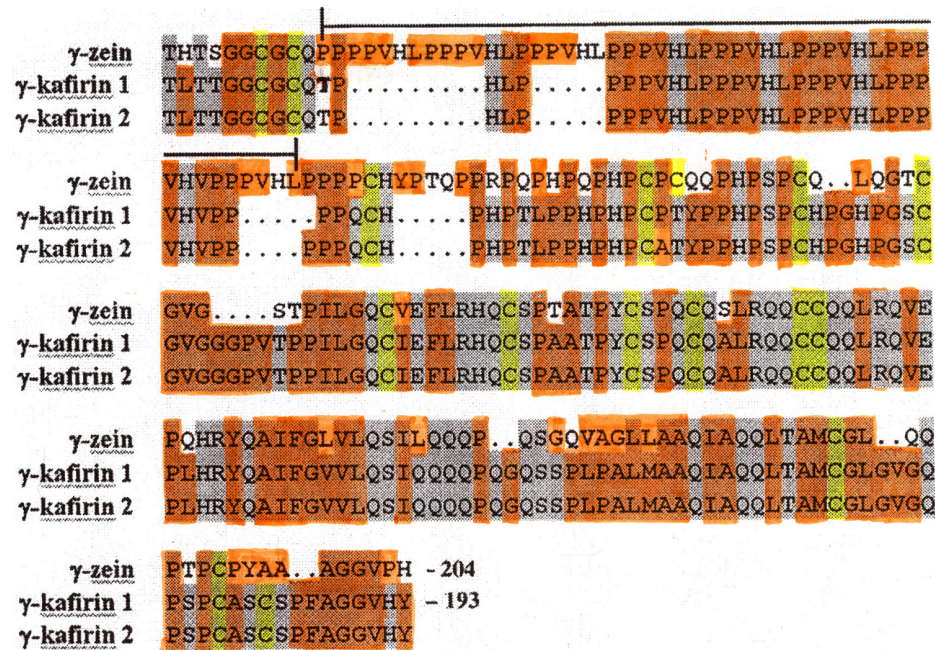


Figure 2.3: Alignment of amino acid sequences of γ -kafirins with those of γ -zein to demonstrate the number of hydrophobic amino acids present. Hydrophobic amino acids are highlighted (adapted from Belton et al., 2006)

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

2.2.4. Kafirin protein digestibility

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

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

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

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

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

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

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

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

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

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

2.3. PROPERTIES OF PROTEIN MICROPARTICLES

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

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

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

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

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

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

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

2.3.1. Properties of zein microparticles

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

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

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

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

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

2.4. ALTERATION OF PROTEIN MICROPARTICLE PROPERTIES

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

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

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

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

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

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

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

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

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

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

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

2.5. POTENTIAL USES OF MICROPARTICLES

2.5.1. Non food uses

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

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

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

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

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

2.5.2. Food Uses

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

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

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

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

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

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

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

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

2.6. CONCLUSIONS

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

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

3. HYPOTHESES AND OBJECTIVES

3.1. HYPOTHESES

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

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

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

3.2. OBJECTIVES

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

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

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

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 Labor Technik, 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 plasticiser. 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 glutaraldehyde in pH 7.4 phosphate buffer before staining with osmium tetroxide. Samples were dehydrated sequentially in acetone. TEM samples were infiltrated with Quetol resin and polymerized at 60°C. Sections were cut and stained with uranyl 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 ($\approx 15 \mu\text{g}$) 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 $116 \mu\text{m}^2$ compared with that of a similar sized aqueous ethanol prepared microparticle illustrated in Figure 4.1.2.c, of $60 \mu\text{m}^2$. 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.

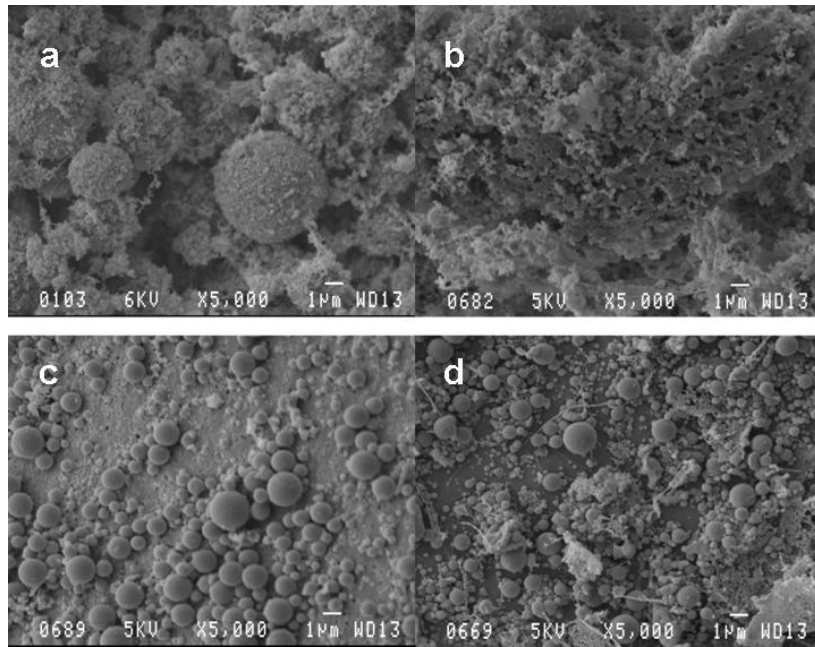


Figure 4.1.1: SEM of kafirin microparticles made with acetic acid or aqueous ethanol
 a-Acetic acid, low shear, b-Acetic acid, high shear, c-Aqueous ethanol, low shear,
 d-Aqueous ethanol, high shear

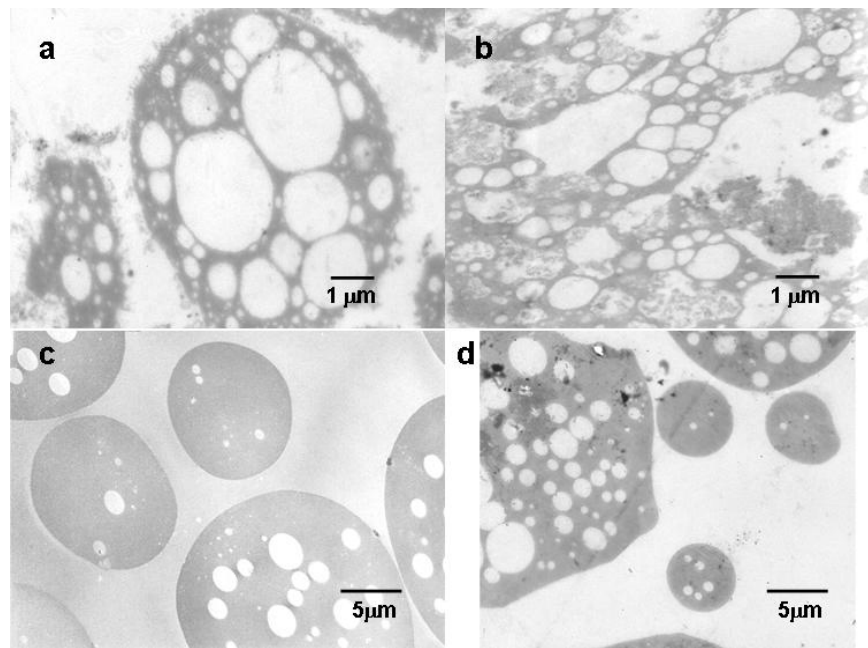


Figure 4.1.2: TEM kafirin microparticles made with acetic acid or aqueous ethanol
 a-Acetic acid, low shear, b-Acetic acid, high shear, c-Aqueous ethanol, low shear,
 d-Aqueous ethanol, high shear

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).

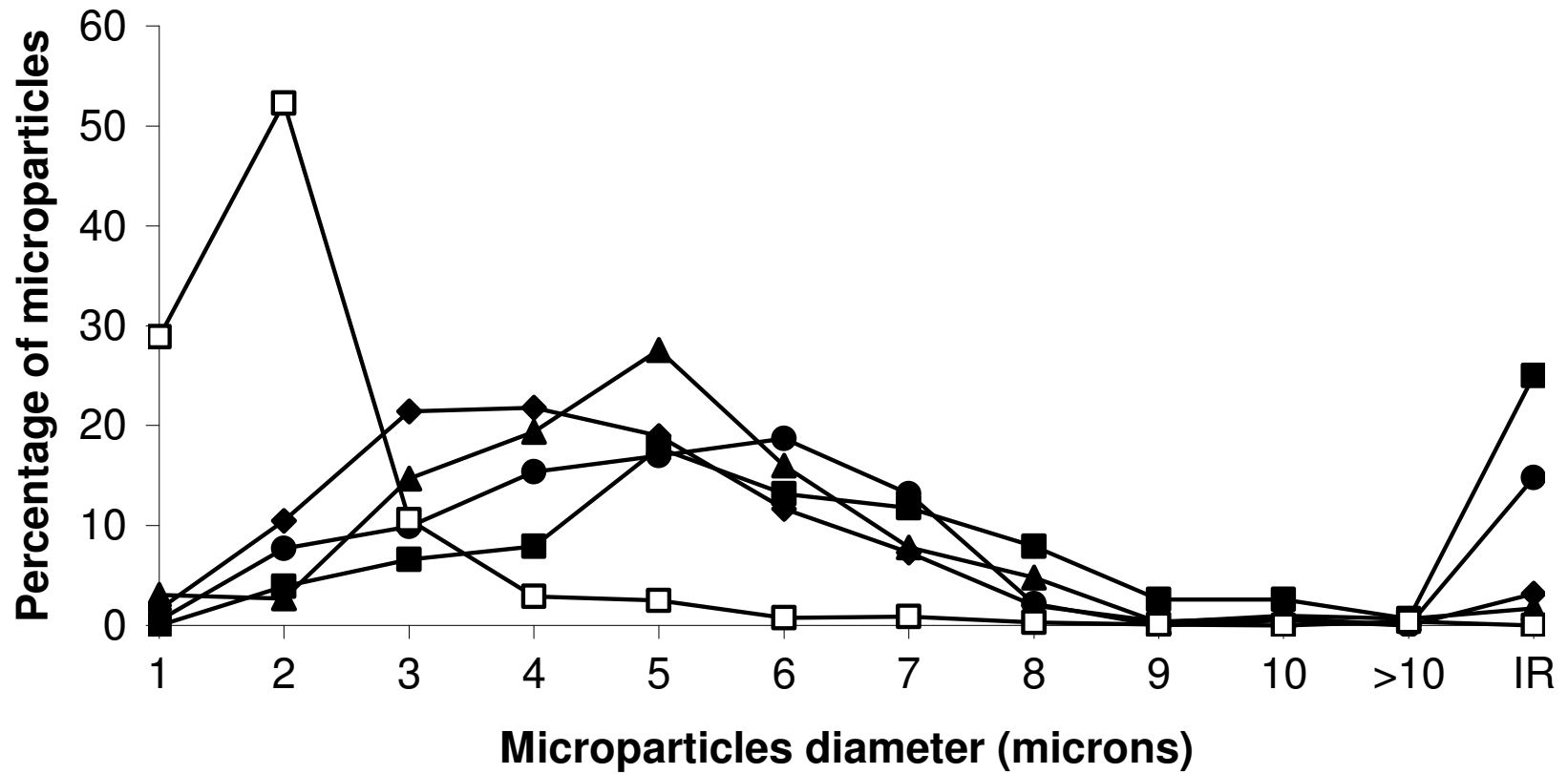


Figure 4.1.3: Particle size distribution of kafilin microparticles
 5.4% acetic acid (diamond), 10% acetic acid (triangle), 21.6% acetic acid (circle), 30% acetic acid (square), aqueous ethanol (open square)

4.1.4.3. Effect of shear

The effect of shear was investigated on the preparation of kafirin 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).

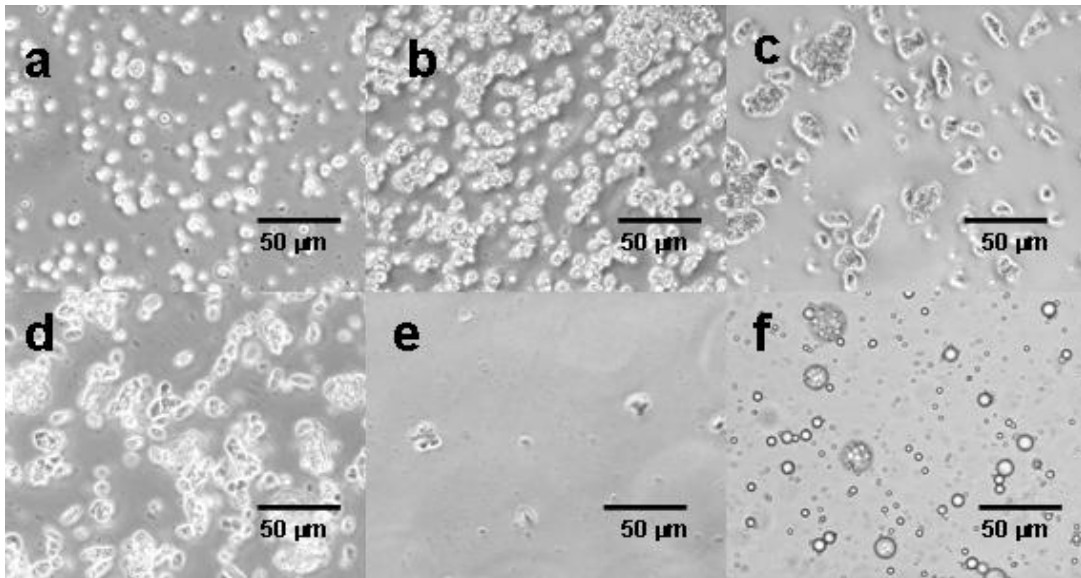


Figure 4.1.4: Light microscopy to illustrate the effect of increasing acetic acid concentration on preformed kafirin microparticles (Appearance of kafirin microparticles made with aqueous ethanol for comparison)
 a- 5.4%, b-10.8%, c-21.6%, d-30% e-40% acetic acid concentrations respectively
 f- aqueous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

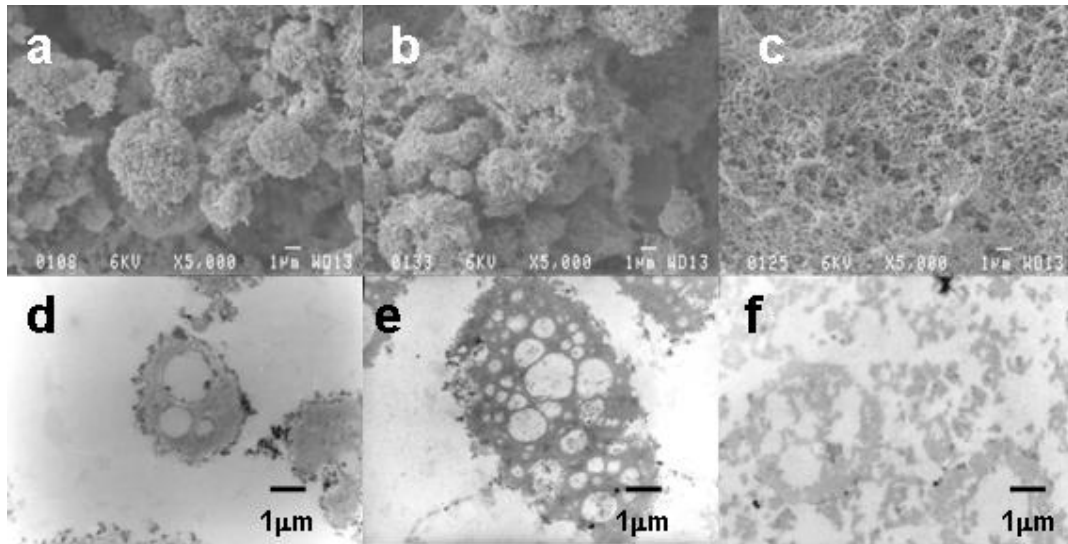


Figure 4.1.5: SEM and TEM of kafirin microparticles at different acetic acid concentrations

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.

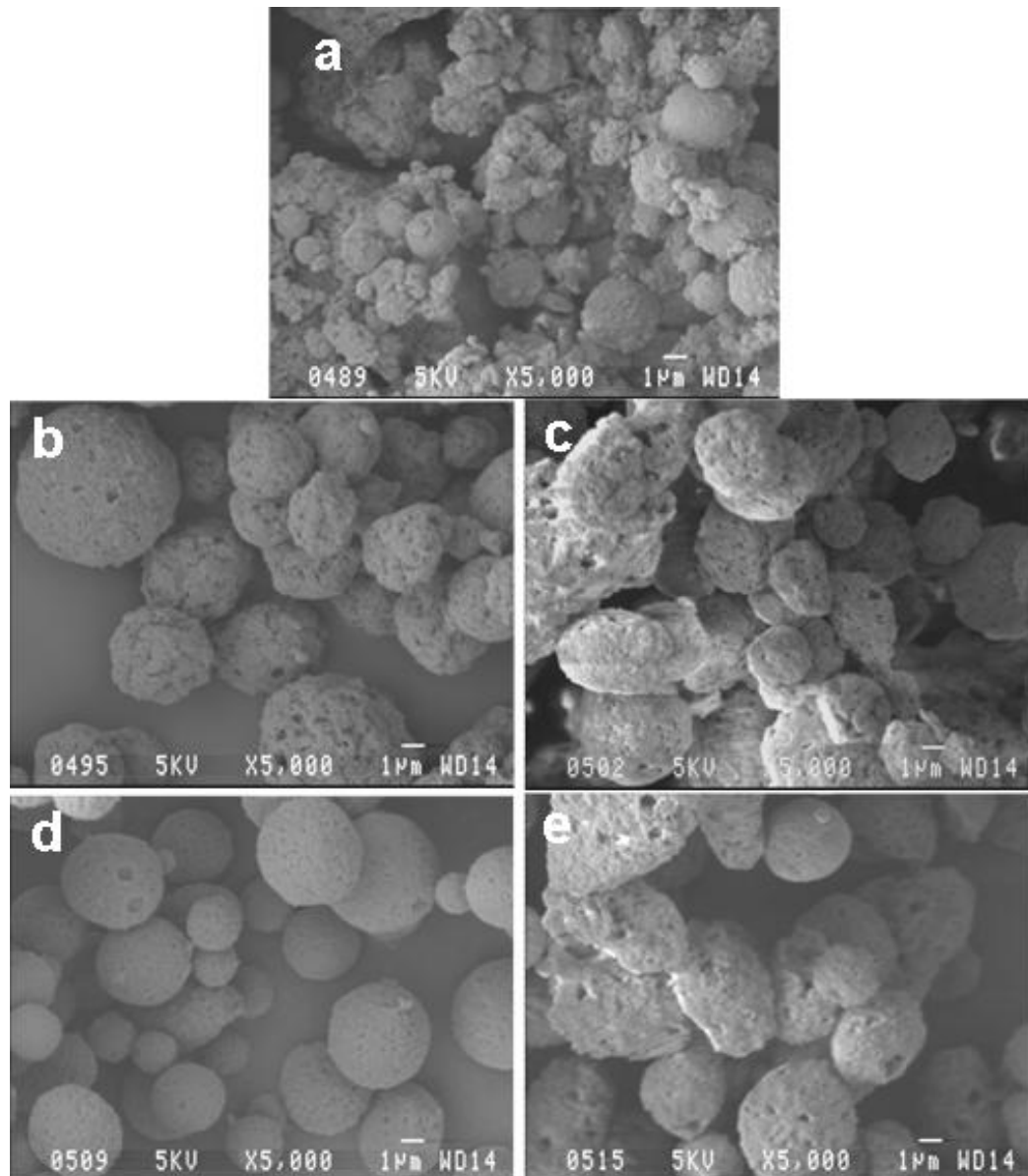


Figure 4.1.6: SEM freeze dried microparticles and kafirin used to prepare them
a kafirin, b,c newly prepared microparticles, 5.4% and 21.6% acetic acid concentration
respectively, d,e stored microparticles, 5.4% and 21.6% acetic acid concentration
respectively

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), α 1- (24 k), α 2- (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).

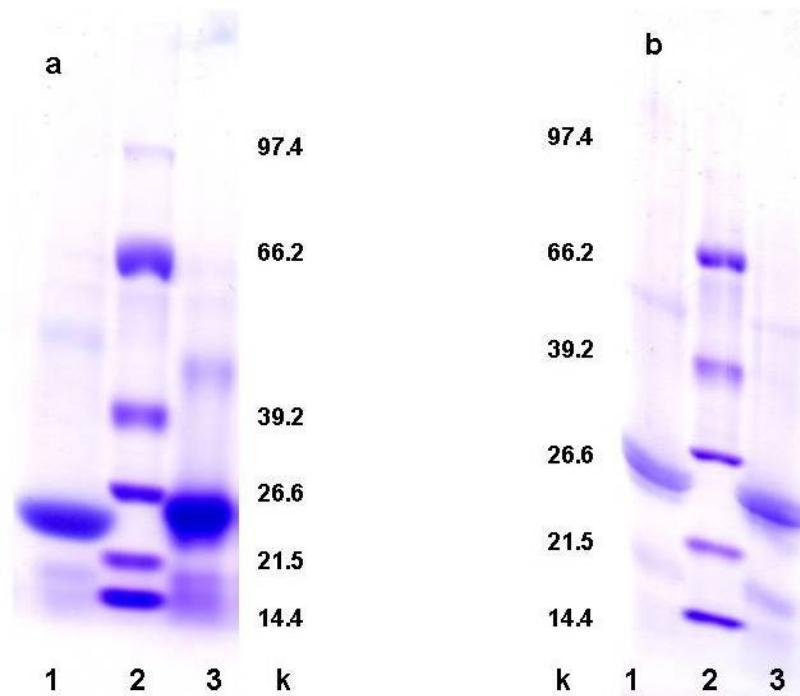


Figure 4.1.7: SDS-PAGE of kafirin microparticles (Track 1) and original kafirin (Track 3) under reducing (a) and non-reducing (b) conditions. Molecular weight marker (Track 2)

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 -, $\alpha 1$ -, $\alpha 2$ - 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}). ^dValues 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.

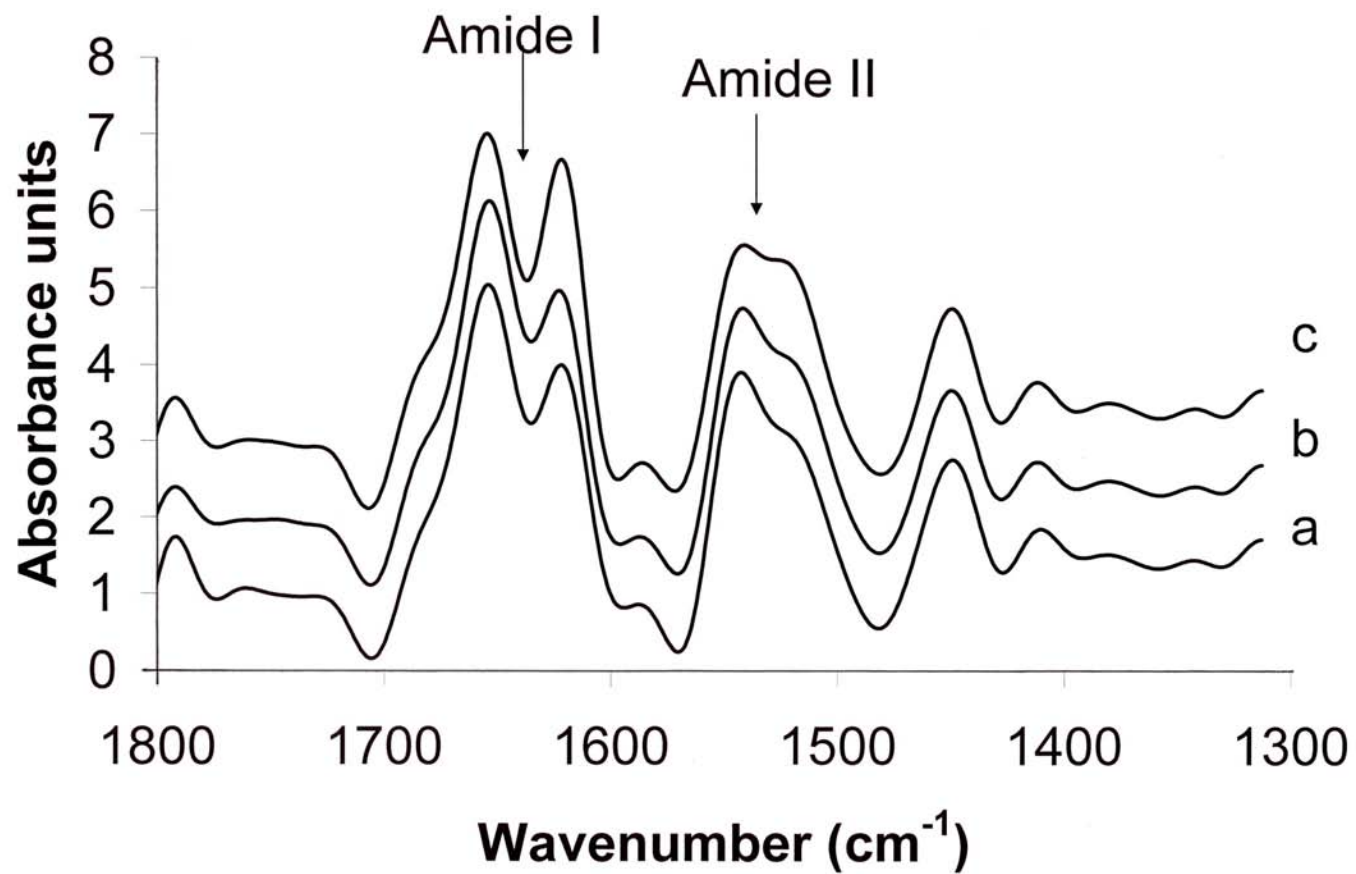


Figure 4.1.8: FTIR of original kafirin (a) and kafirin microparticles at different acid concentrations 5.4% acid (b), 21.6% acid (c)

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.

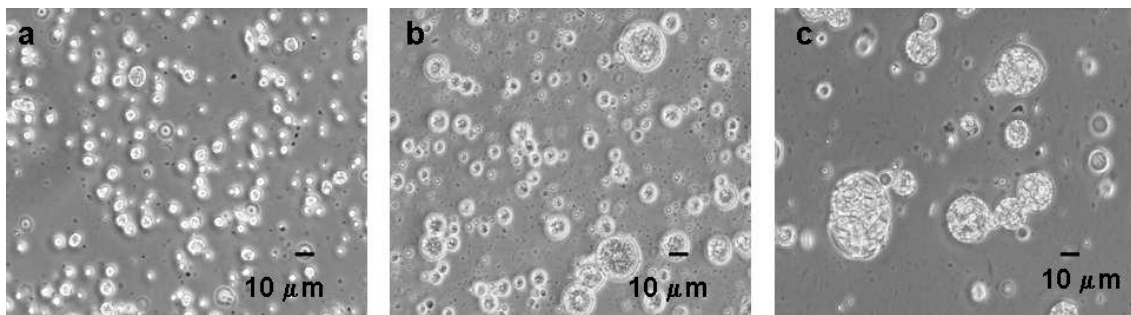


Figure 4.1.9: Light microscopy of kafirin microparticles made with acetic acid (5.4%, a), lactic acid (10.8%, b) and propionic acid (10.8%, c)

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.

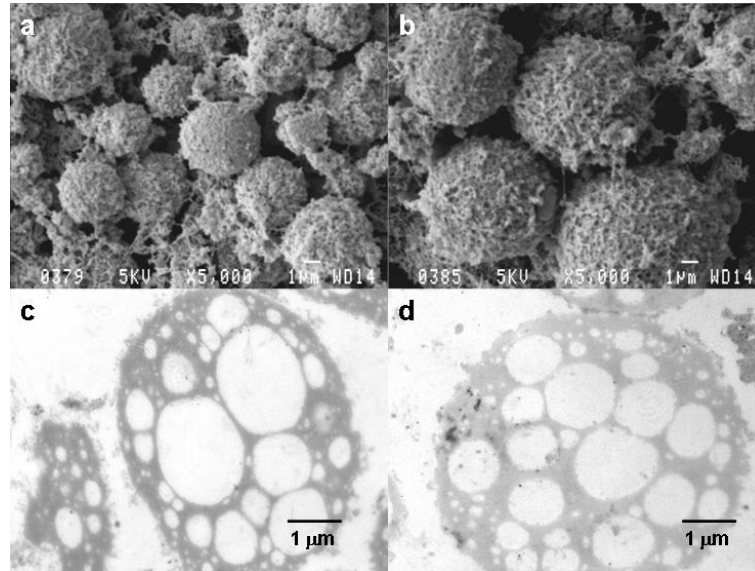


Figure 4.1.10: Kafirin microparticles prepared with and without plasticizer
 With plasticiser (a SEM, c TEM), Without plasticiser (b SEM, d TEM)

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.

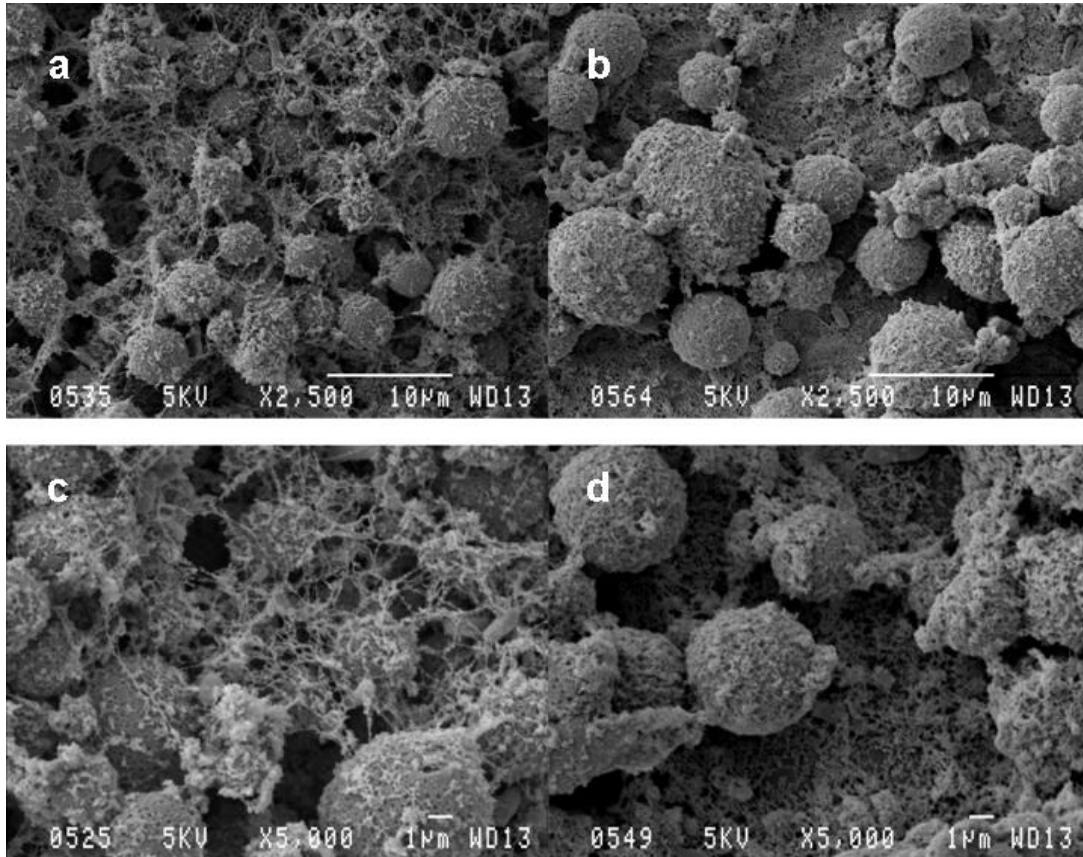


Figure 4.1.11: SEM kafirin microparticles made either with the addition of gas to the solvents (a, c), or made with degassed solvents (b, d)

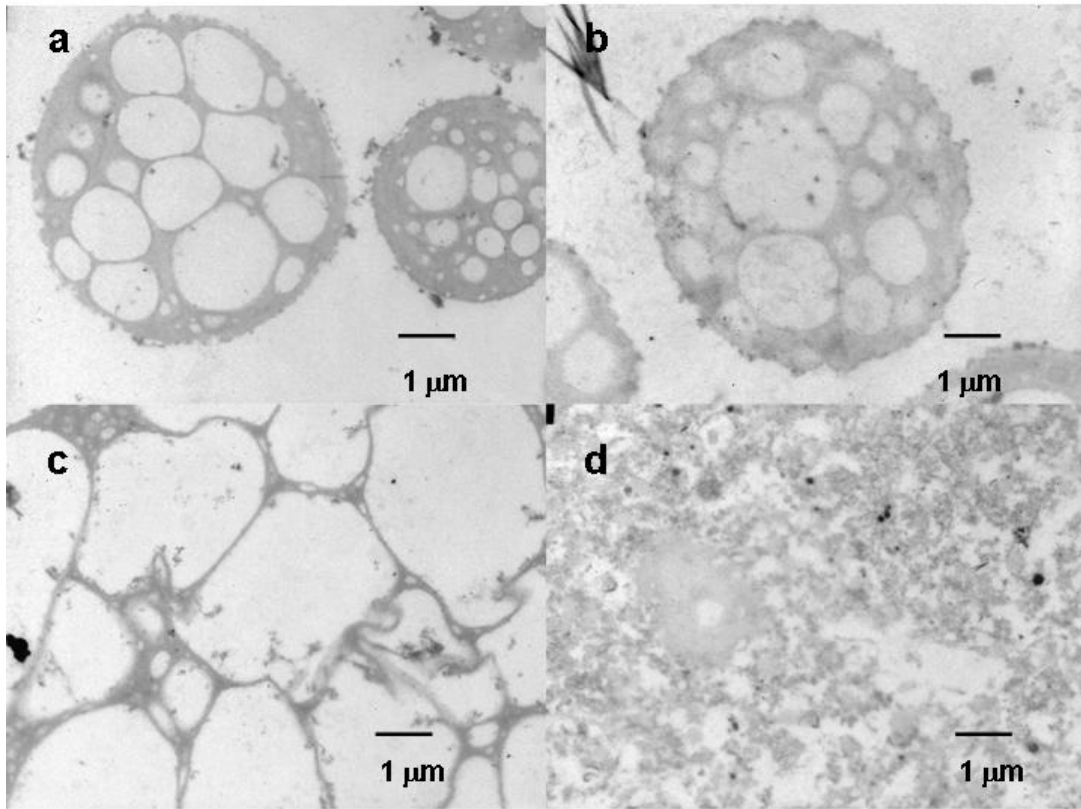


Figure 4.1.12: TEM kafirin microparticles made either with the addition of gas to the solvents (a, c), or made with degassed solvents (b, d)

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.

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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 prolamins 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 (5 μ l) 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):

$$\text{WVP} = \frac{\text{gradient (g/h)} \times \text{thickness of film (mm)}}{\text{Area (m}^2\text{)} \times P_o \text{ (kPa)} \times (\text{RH1-RH2})/100}$$

Where:

P_o (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.

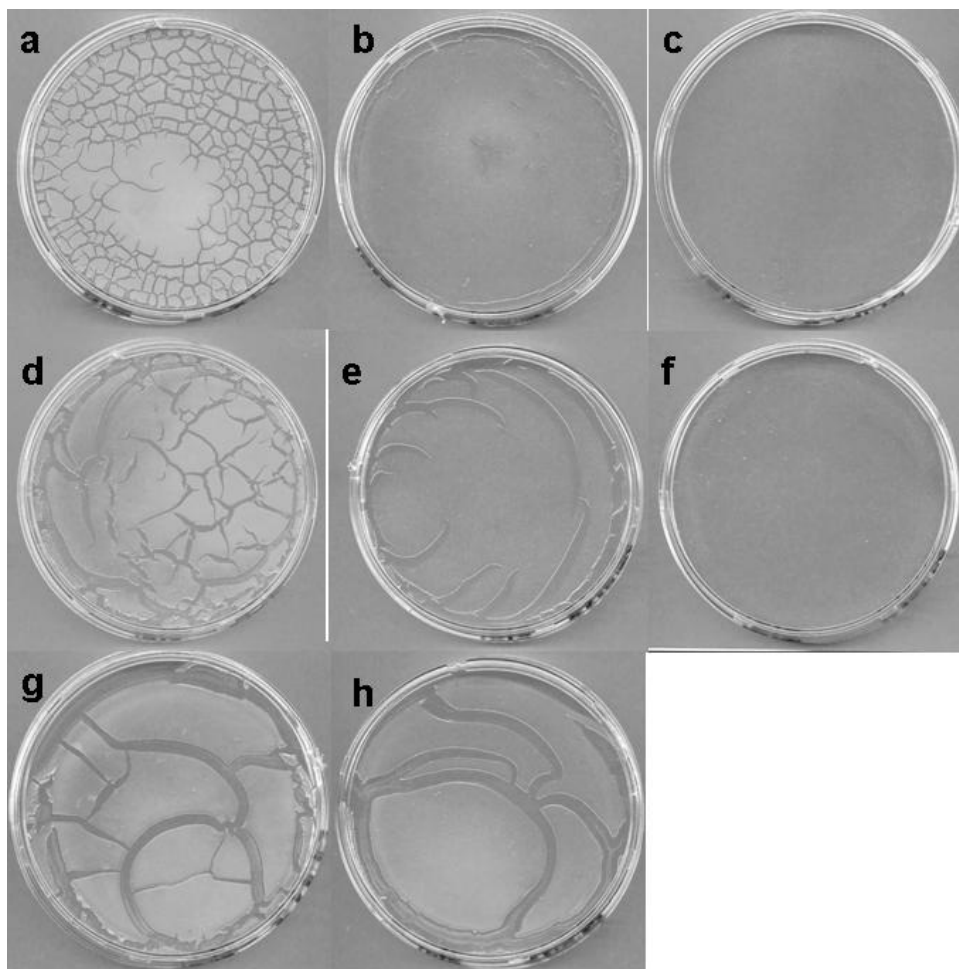


Figure 4.2.1: Effects of increasing protein concentration and acetic acid concentration on kafirin microparticle film formation
 a-2% kafirin, 5.4% acetic acid, b-2% kafirin, 10.6% acetic acid, c-2% kafirin, 21.6% acetic acid, d-4% kafirin, 5.4% acetic acid, e-4% kafirin, 10.6% acetic acid, f-4% kafirin,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

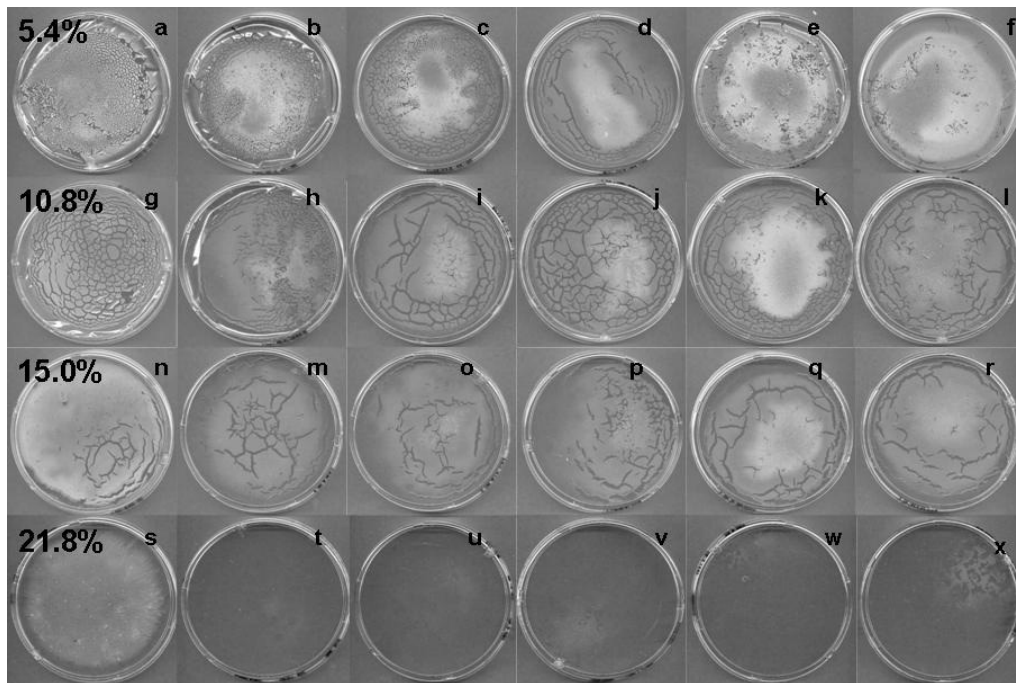


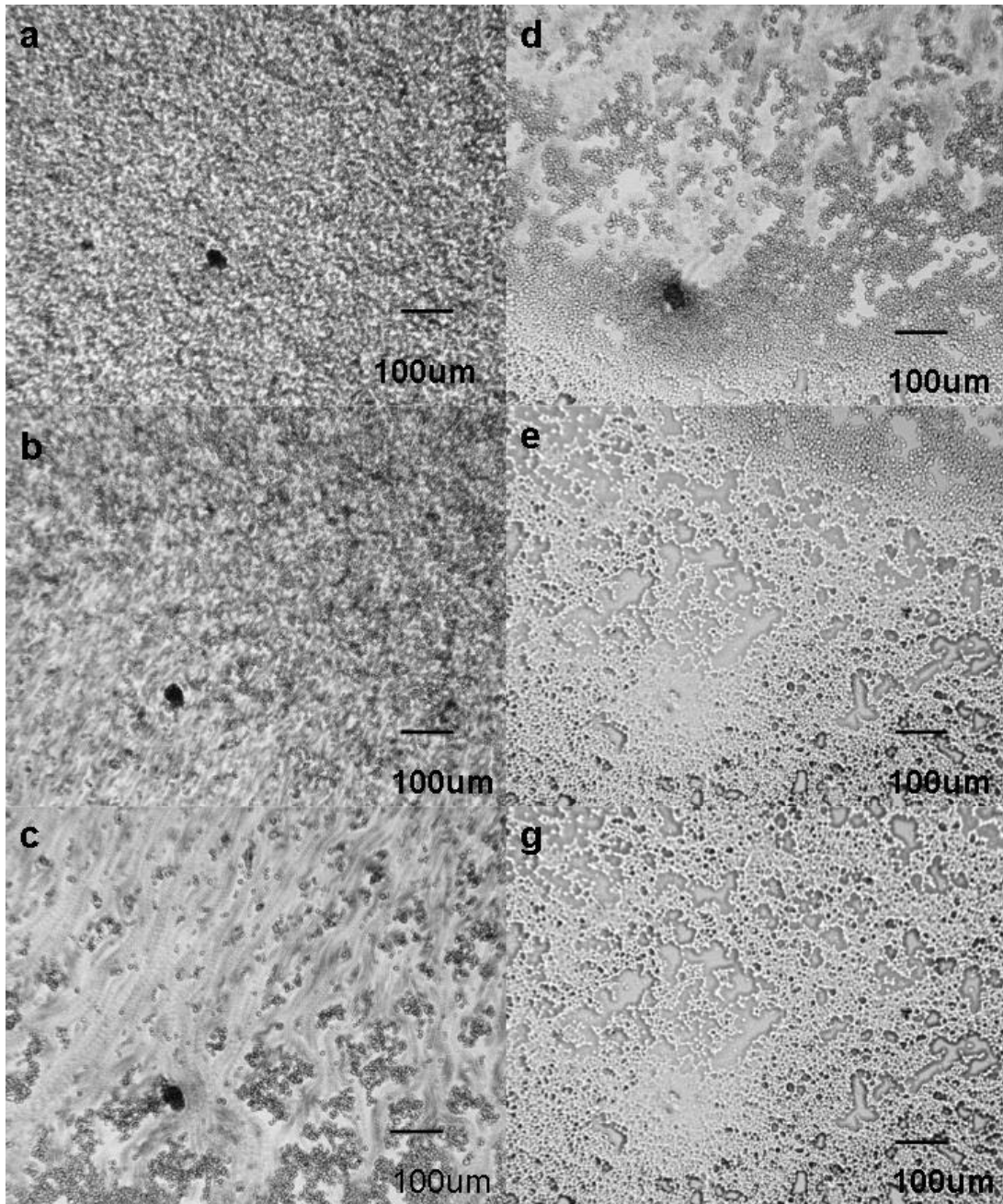
Figure 4.2.2: Effects of increasing acetic acid concentration and increasing plasticiser concentration on kafirin microparticle film formation (2% protein)
 5.4% acetic acid, a-f, 0, 20, 40, 60, 80, 100% plasticiser with respect to protein
 10.8% acetic acid, g-h, 0, 20, 40, 60, 80, 100% plasticiser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

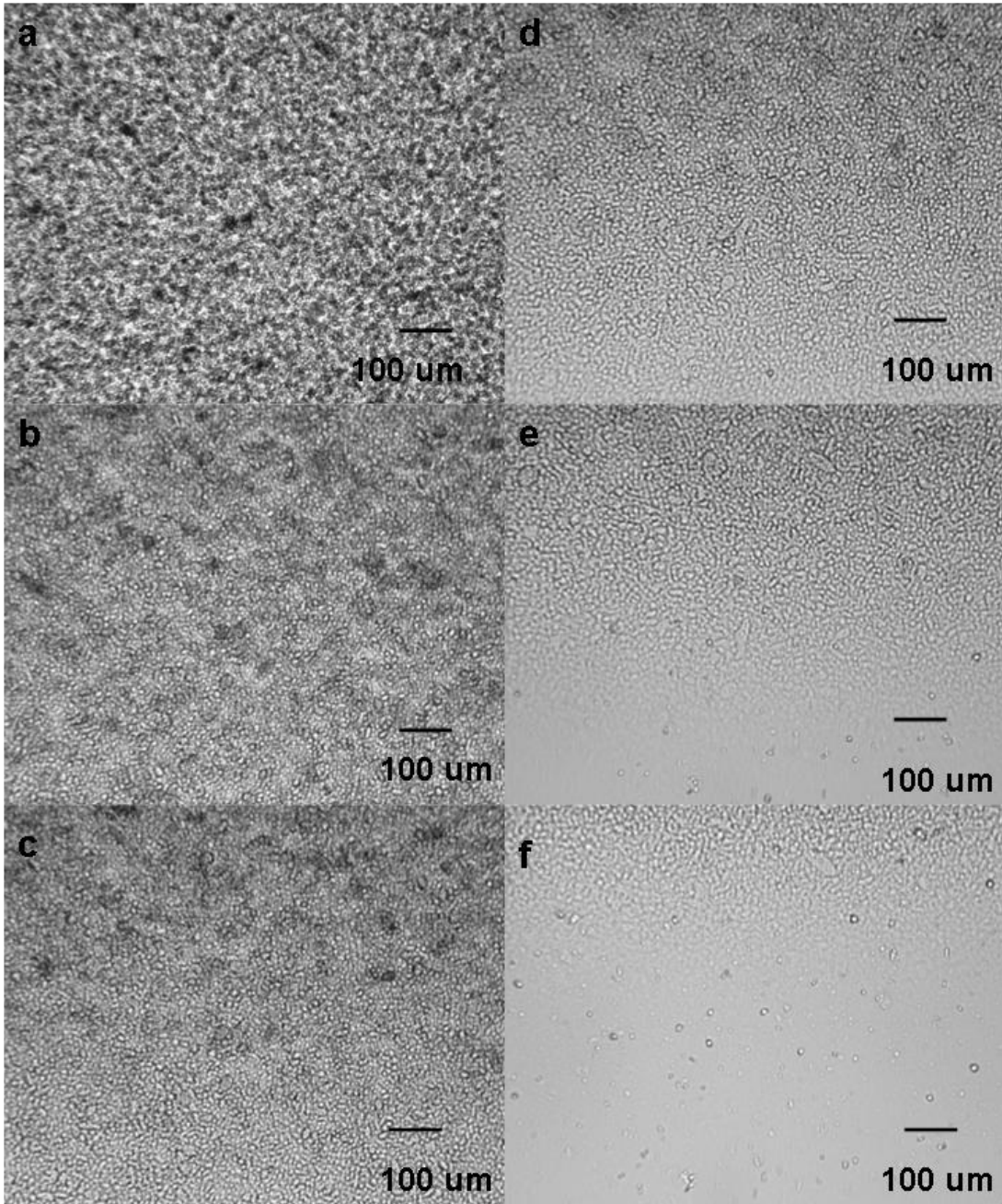
Figure 4.2.3.B. illustrates how a complete cohesive film is formed from 2% kafirin microparticles with the higher acetic acid concentration of 21.6%. Figure 4.2.3.B.(a) shows the individual kafirin microparticles in 21.6% acid were slightly larger than those in the 5.4% acetic acid, but still well defined 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.

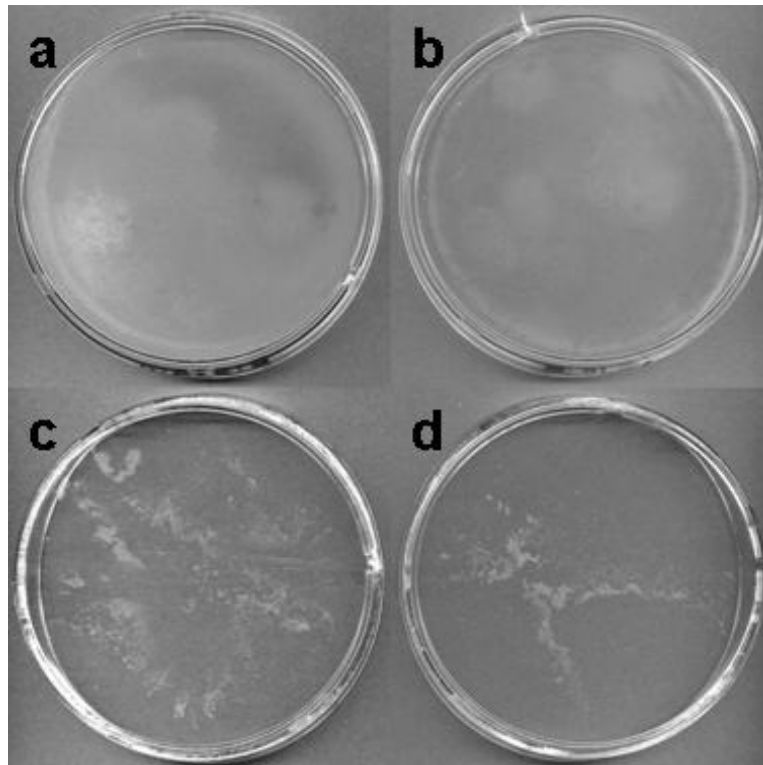


Figure 4.2.4: Effect of low protein concentration on kafirin film formation
 Kafirin microparticle films, a- 0.5% kafirin, 5.4% acetic acid, b-1% kafirin, 10.8% acetic acid. Glacial acetic acid cast kafirin films, c-0.5% kafirin, d-1.0% kafirin

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).

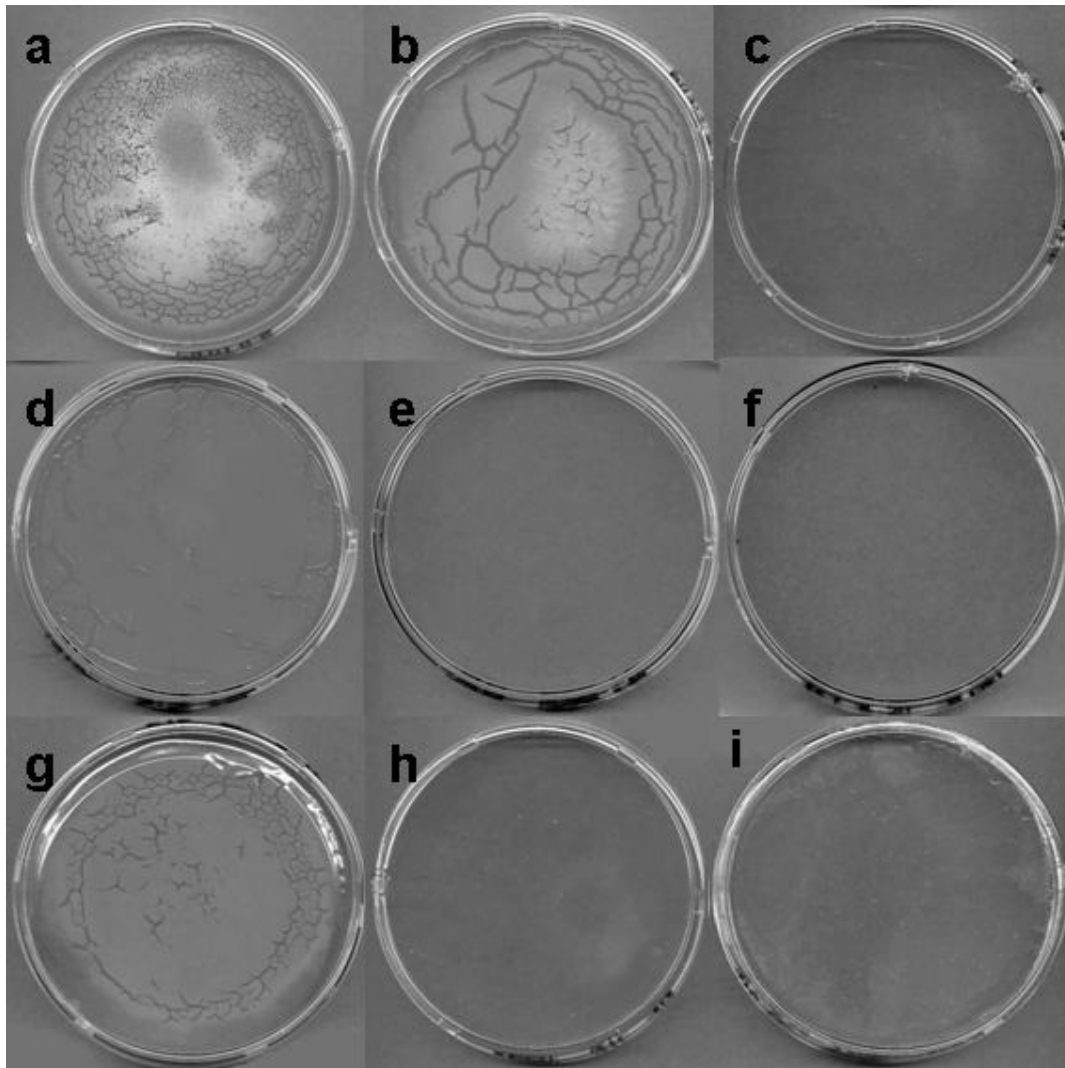


Figure 4.2.5: Effects of kafirin microparticles made with different acids with increasing acid concentration on film formation
 Acetic acid, a-c, 5.4%, 10.6%, 21.8% acid
 Lactic acid, d-f, 5.4%, 10.6%, 21.8% acid
 Propionic acid, g-i, 5.4%, 10.6%, 21.8% acid
 Note that use of lactic acid did not result 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 (Bandeekar, 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 I 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 ($p < 0.05$)

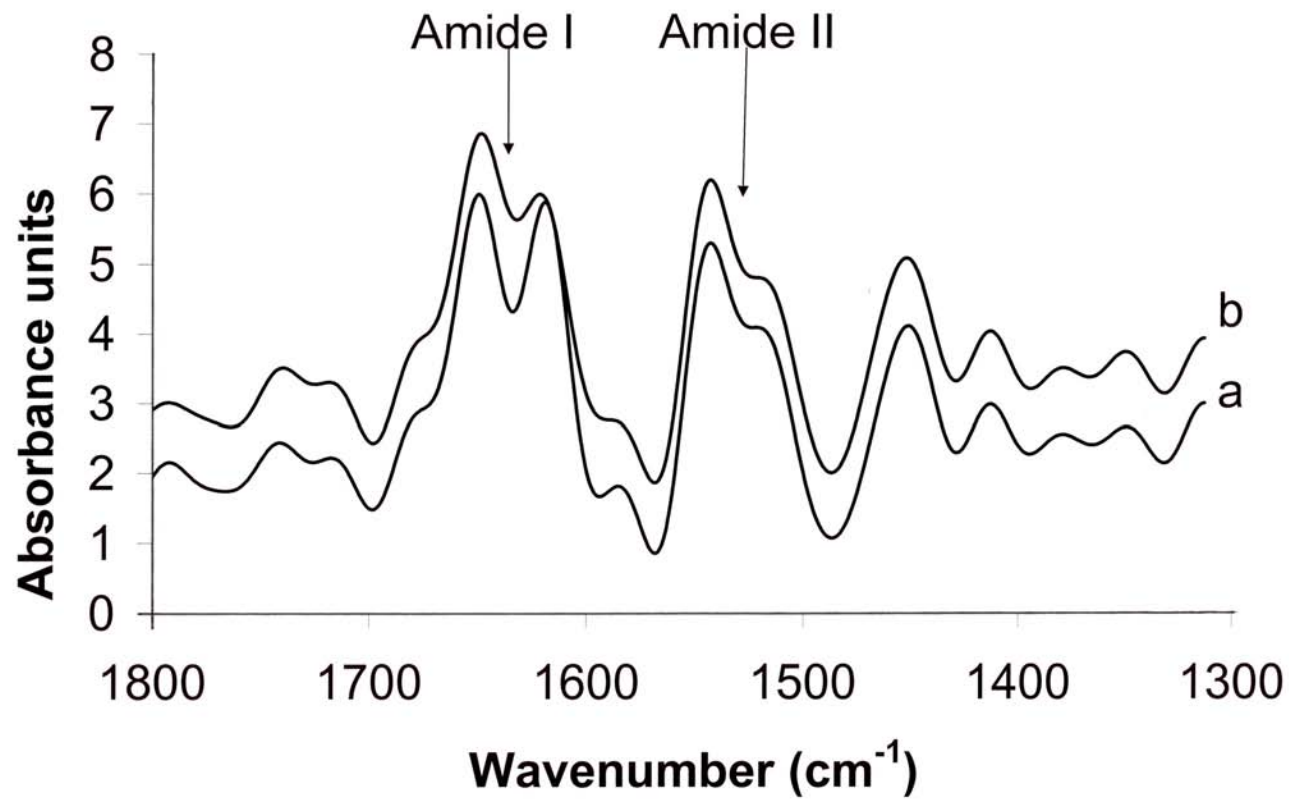


Figure 4.2.6: FTIR of kafirin films, a- kafirin microparticle film, b- cast in glacial acetic acid

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 1 μ m 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 1-3 μ m 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.

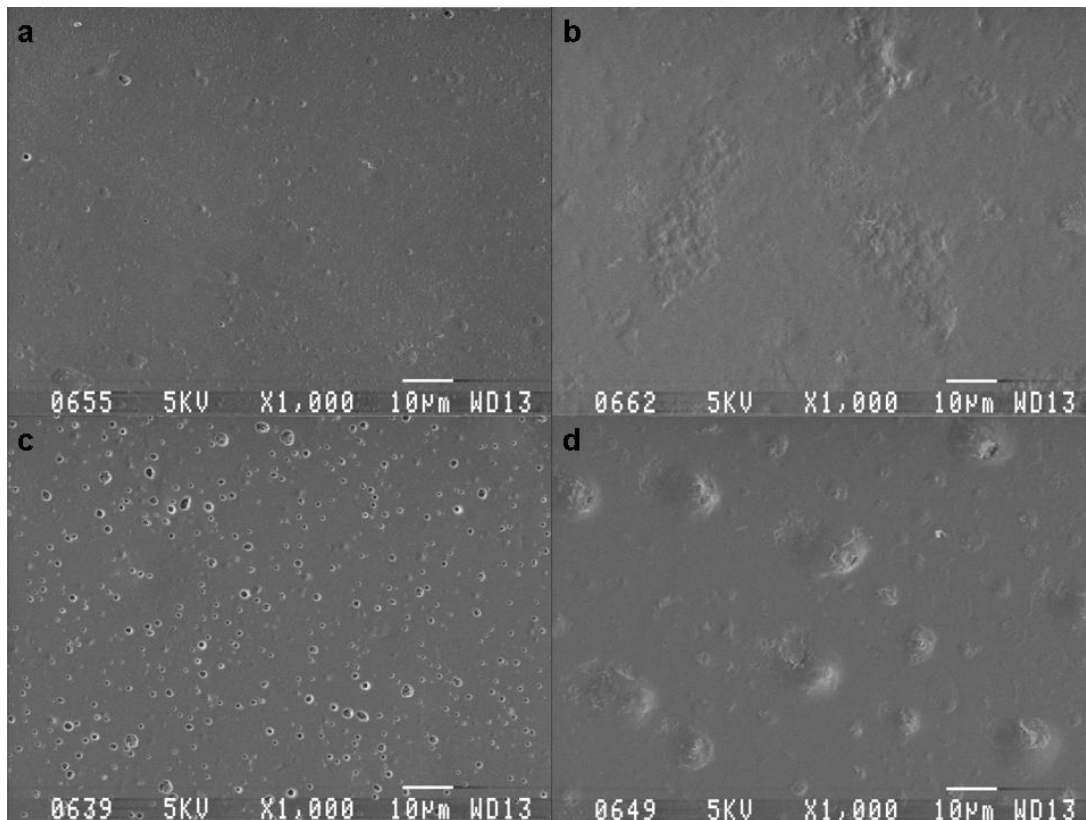


Figure 4.2.7: SEM of kafirin film surfaces
Kafirin microparticle film, a-top surface, b-underside
Glacial acetic acid cast kafirin film, c- top surface, d-underside

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.

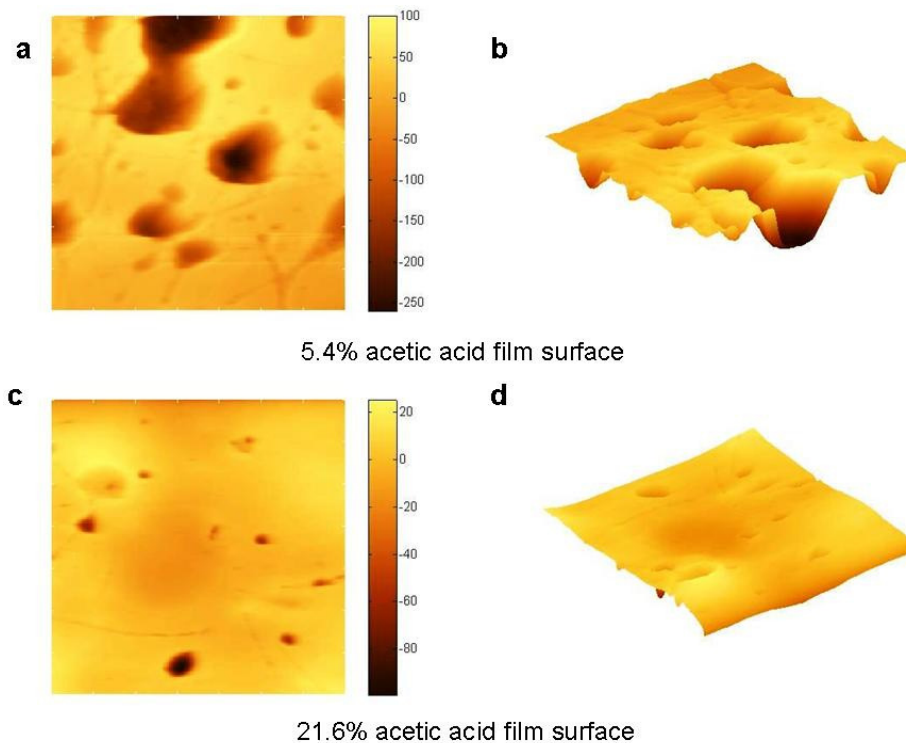


Figure 4.2.8: AFM of kafirin microparticle film surfaces
5.4% acetic acid microparticle film, a-top surface, b-3-D image of film surface
21.6% acetic acid microparticle film, c-top surface, d-3-D image of film surface
Scale bar in nm

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 microparticle 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 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 ² hkPa)
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 $\text{g mm/m}^2 \text{ h kPa}$, whereas other workers reported lower values ranging from 0.4 to 0.8 $\text{g mm/m}^2 \text{ h kPa}$ (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. Byaruhanga 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 (Byaruhanga 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/mm²)	Stress at break (N/mm²)	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 glutamine 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.

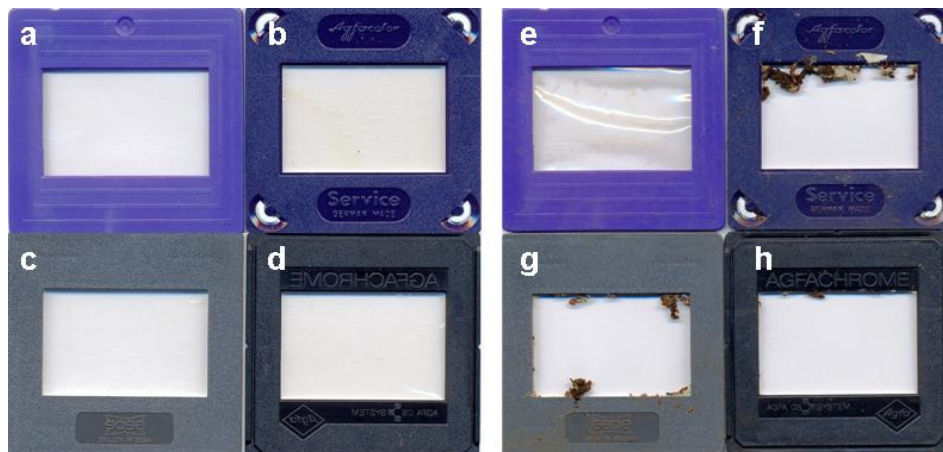


Figure 4.2.9: Biodegradation of kafirin films

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.

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

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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 μ moles Trolox equivalents/g and of the sorghum condensed tannins 5366 μ moles 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, Lebyrk 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 BAAE 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-Whittem, 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 μ l 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 μ l 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 μ g/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 ($\approx 15 \mu\text{g}$) 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°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 μ M Trolox. The working solution (2900 μ l) was added to 100 μ l 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 (μ M Trolox equivalents/g sample)

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

Where:

Slope = slope of standard curve

C = y intercept

The calculation is the equation of the standard curve which gives a value of μ M Trolox Equivalants per 100 μ l 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.

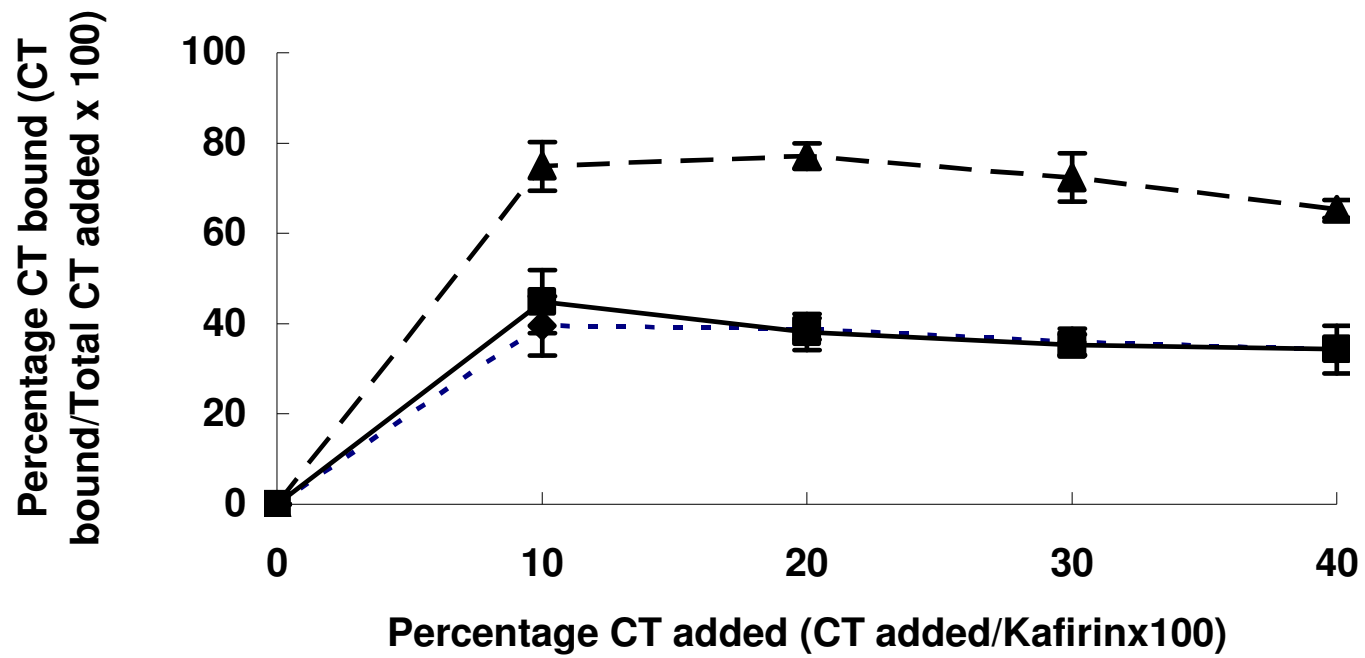


Figure 4.3.1: Percentage of sorghum condensed tannin (CT) bound to different kafirin species
 Triangles: γ -kafirin, Squares: Total kafirin, Diamonds: Residual kafirin (kafirin after γ -kafirin removed)
 Error bars represent +/- one standard deviation

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 than 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.

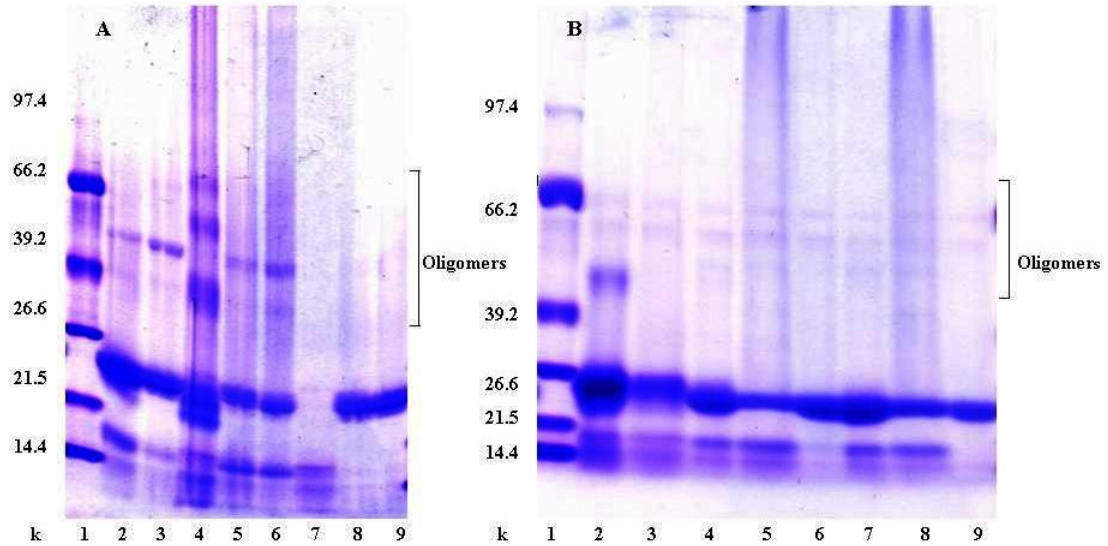


Figure 4.3.2.: SDS-PAGE of kafirin species and kafirin species bound to sorghum condensed tannins (CT) under non-reducing (A) and reducing conditions (B)

(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
<u>Protein</u>		
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)
<u>Films</u>		
Total kafirin	41.5y (2.2)	16.7x (1.2)
Zein	54.4z (3.9)	Not determined

^aEach 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).

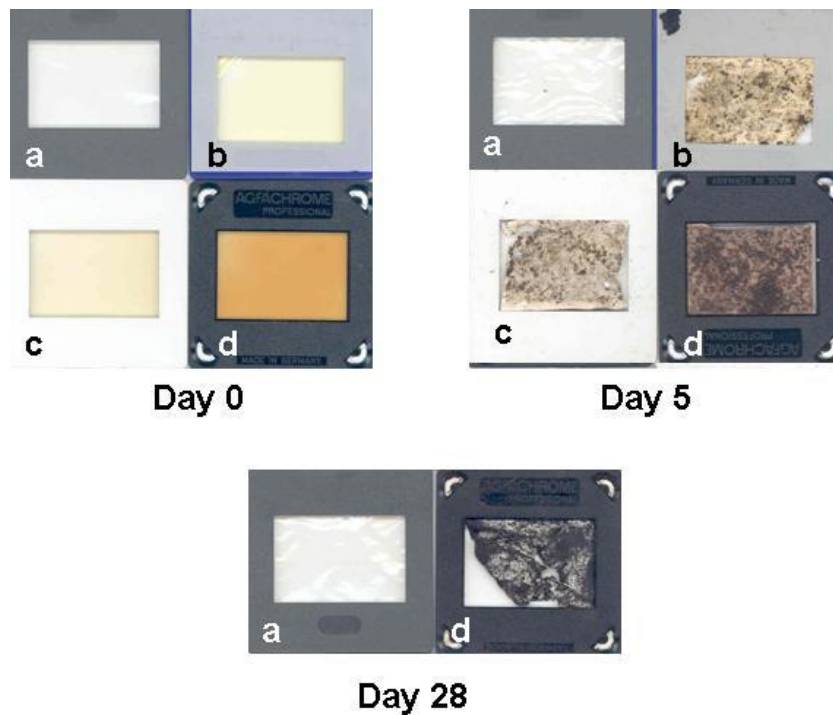


Figure 4.3.3: Biodegradation of films under high moisture conditions
a-LDPE, b-zein film, c-total kafirin film, d-film made with kafirin bound to CT

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 μ moles Trolox equivalents/g and sorghum condensed tannins 5366 μ moles Trolox equivalents/g. These values are slightly higher than values quoted in the literature. Sikwese and Duodu (2007) reported an antioxidant activity of 4600 μ moles 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 μ moles 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.

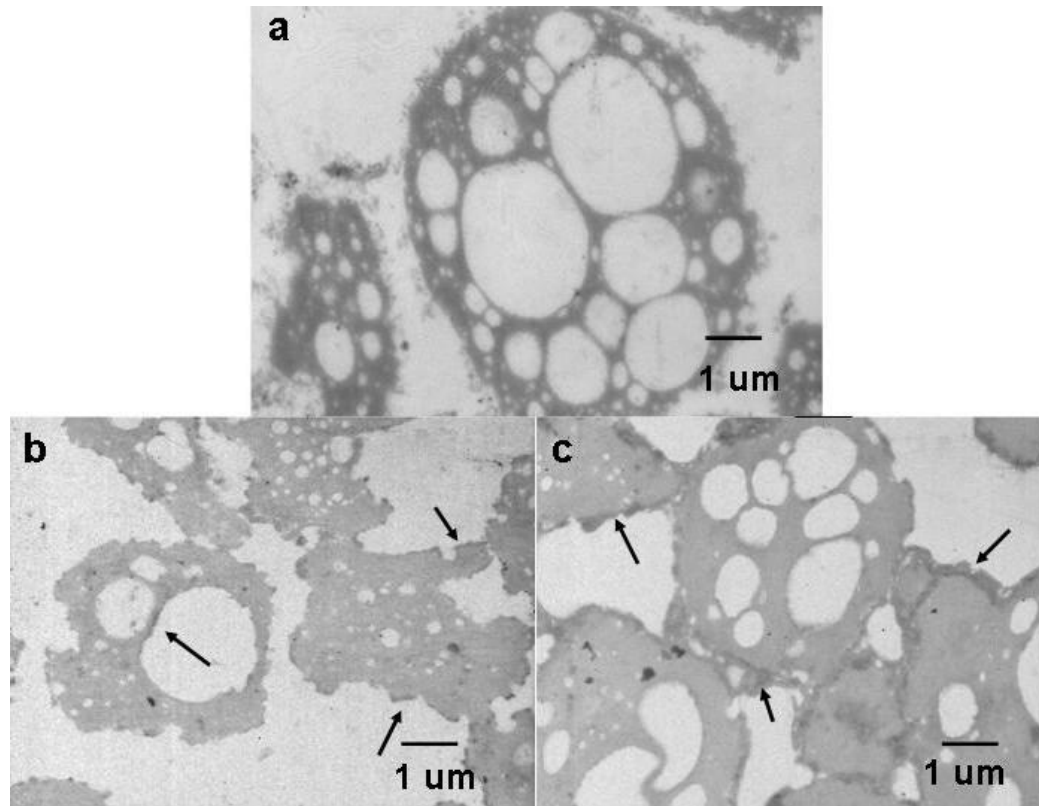


Figure 4.3.4: TEM of kafirin microparticles (a) and kafirin microparticles after encapsulation of catechin (b) and sorghum condensed tannins (c), arrows indicate probable bound phenolics

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 peroxidation 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).

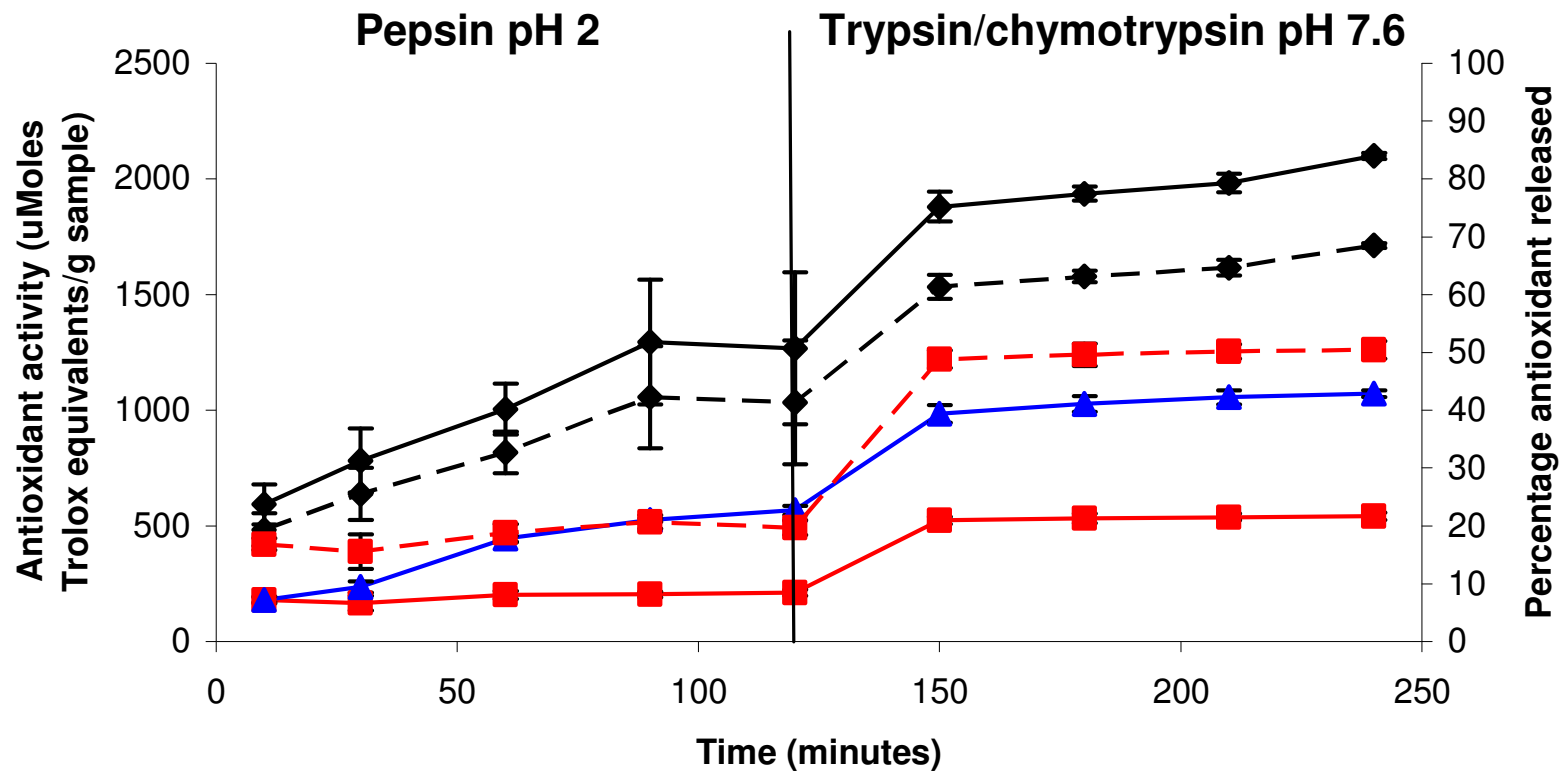


Figure 4.3.5: Effect of pepsin digestion followed by trypsin/chymotrypsin digestion on antioxidant activity (solid line) and percentage antioxidant released (dotted line) from kafirin microparticles (triangles), kafirin microparticles with encapsulated catechin (diamonds) and kafirin microparticles with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

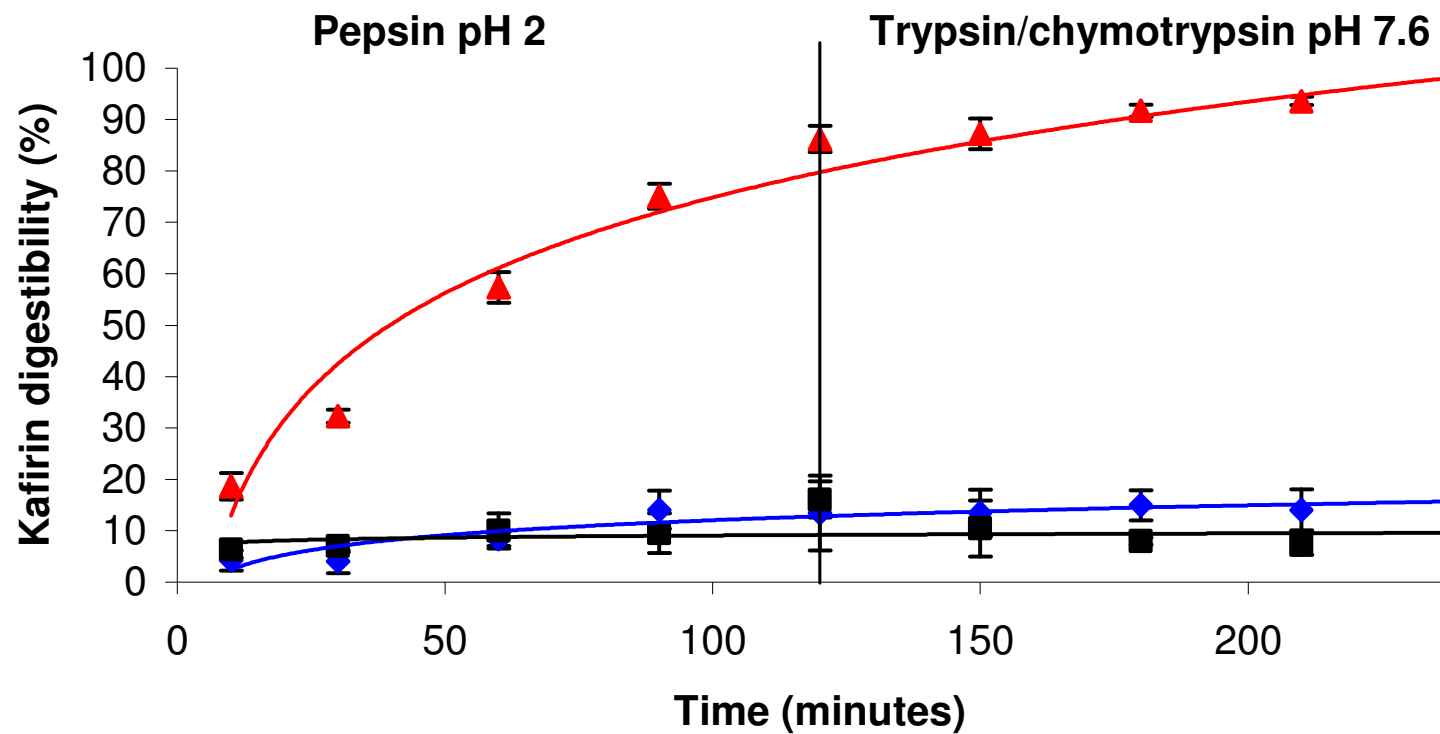


Figure 4.3.6: Effect of pepsin digestion followed by trypsin/chymotrypsin digestion on kafirin digestibility of kafirin microparticles (triangles), kafirin microparticles with encapsulated catechin (diamonds) and kafirin microparticles with encapsulated sorghum condensed tannins (squares). Error bars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

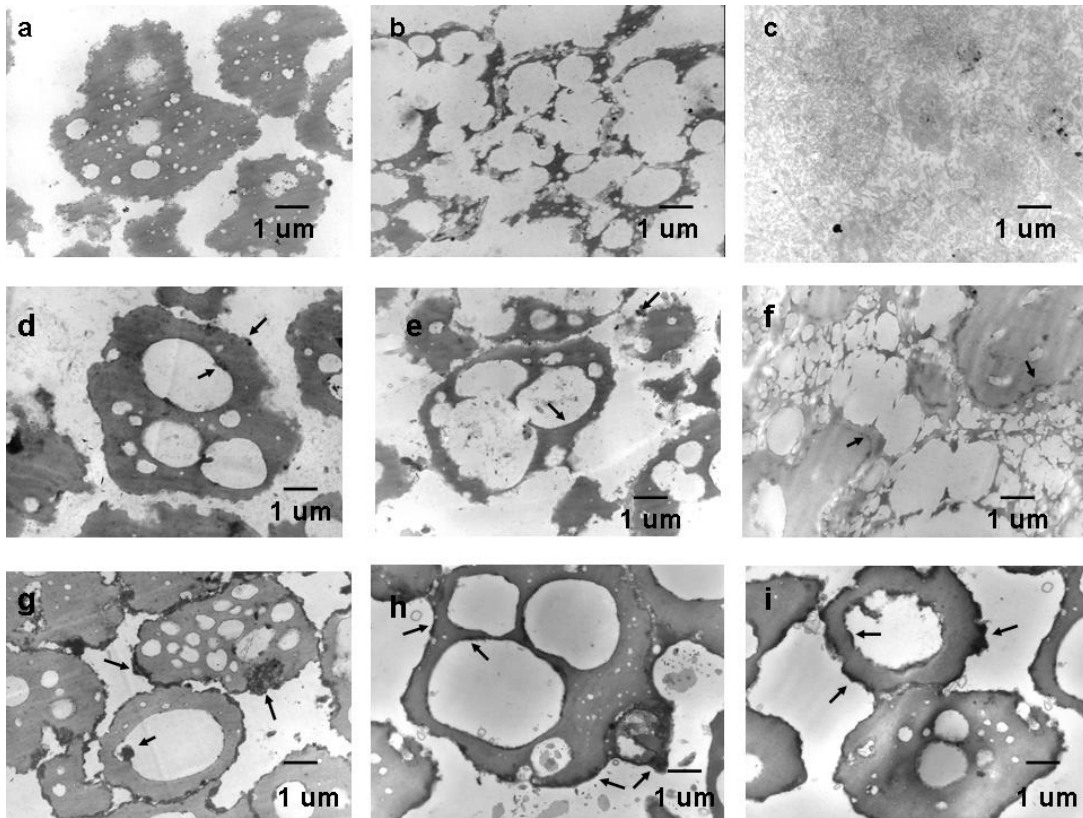


Figure 4.3.7: TEM illustrating the effect of pepsin digestion followed by trypsin/chymotrysin digestion on kafirin digestibility of kafirin microparticles (a-c), kafirin microparticles with encapsulated catechin (d-f), and kafirin microparticles with encapsulated sorghum condensed tannins (g-i), arrows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

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5. GENERAL DISCUSSION

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

5.1. METHODOLOGIES USED IN RESEARCH PROJECT

5.1.1. Microscopy

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

5.1.1.1. Light Microscopy

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

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

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

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

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

5.1.1.2. Electron Microscopy

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

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

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

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

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

5.1.1.3. Atomic Force Microscopy (AFM)

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

5.1.2. Fourier Transform Infrared Spectroscopy (FTIR)

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

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

5.1.3. Protein digestibility

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

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

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

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

5.1.4. Biodegradation

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

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

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

5.1.5. Antioxidant activity

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

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

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

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

5.2. KAFIRIN MOLECULAR AGGREGATION

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

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

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

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

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

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

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

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

5.3. MECHANISM OF KAFIRIN MICROPARTICLE FORMATION

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

Murdan, 2005; Muthiselvi and Dhalathreyan, 2006). No references in the literature could be found for the preparation of kafirin microspheres/microparticles. However, since kafirin and zein show a high degree of homology (Belton, Delgado, Halford and Shewry, 2006) it would be expected that they would behave in the same manner during microparticles preparation. Most methods of producing zein microspheres involve dissolving the zein in aqueous ethanol and then reducing the ethanol concentration until the zein is no longer soluble and precipitates out in the form of microspheres (Dong et al., 2004; Lui et al., 2005; Parris et al., 2005; Wang et al., 2005; Hurtado-López and Murdan, 2005; Muthiselvi and Dhalathreyan, 2006).

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

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

Hurtado-López and Murdan (2005) explained the precipitation of zein microspheres by combining what is known of zein solubility and structure. They suggested the zein dissolved by fitting its elongated α -helical structure into the three dimensional structure formed when 'polymers' of ethanol formed by intermolecular hydrogen bonds combined with the three dimensional clusters of water molecules. As the amount of water in the

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

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

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

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

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

Kafirin is considered highly hydrophobic and is insoluble in water (Belton et al., 2006). The degree of hydrophobicity of kafirin can be shown by considering the hydrophilic/hydrophobic balance of kafirin by calculating the sum of its free energies of hydration from its amino acid sequences. Compared with a 'typical protein' ($-164 \text{ k cal mol}^{-1}$) (Shewry et al., 2003), kafirin has a low negative free energy of hydration for all its components (α -kafirin $-140 \text{ k cal mol}^{-1}$; β -kafirin $-123 \text{ k cal mol}^{-1}$ and γ -kafirin $-99 \text{ k cal mol}^{-1}$) (Belton et al., 2006). However, kafirin also has hydrophilic characteristics (Belton et al., 2006). Hydrophilic proteins like wheat gluten absorb water when heated, whereas hydrophobic proteins expel water under the same conditions (Grant, Belton, Colquhoun, Parker, Plijter, Shewry, Tatham, Wellner, 1999). When kafirin and zein are heated with deuterated water (D_2O), they absorb small amounts of water but less than gluten (reviewed by Taylor and Belton, 2002). This shows that kafirin and zein are more hydrophobic than gluten but because they absorbed water rather than expelling it, is indicative of some hydrophilic characteristics. These hydrophilic characteristics are in part, a result of the large amount of glutamine that kafirin and zein contain and would enable hydrogen bonding to occur between the polar $-\text{NH}_2$ groups of glutamine and water.

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

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

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

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

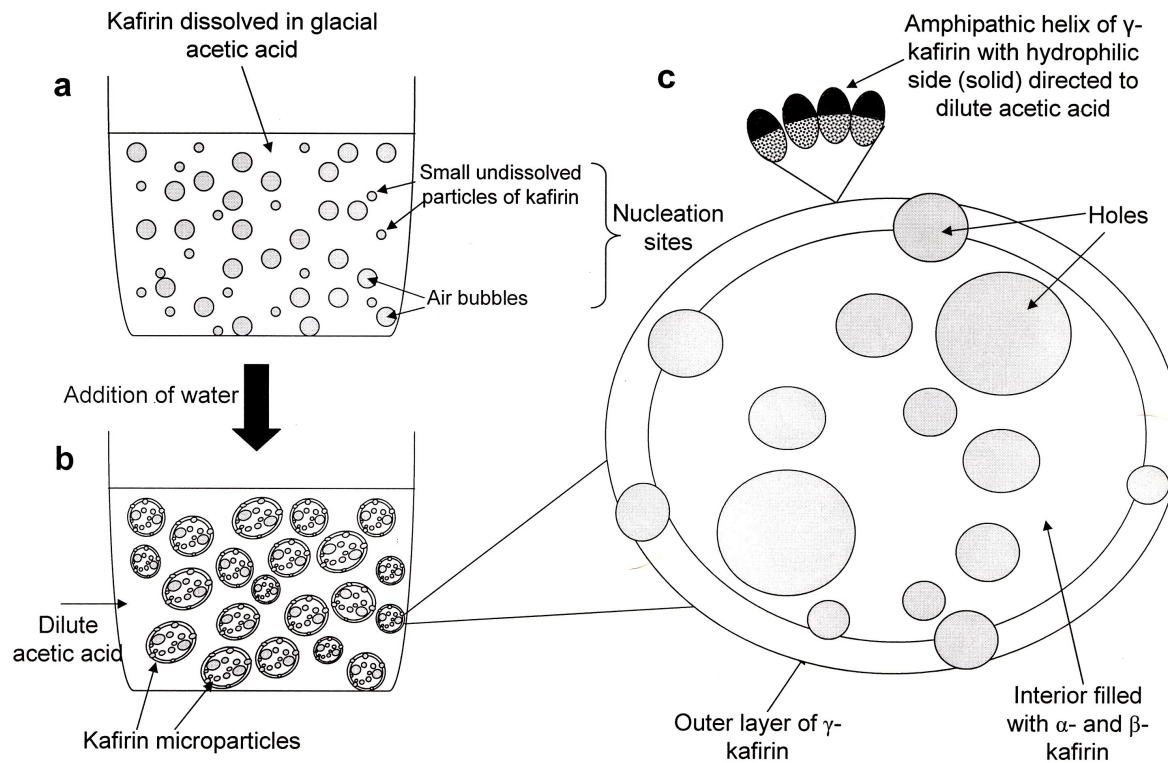


Figure 5.3.1: Hypothetical model to describe the formation of kafirin microparticles

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

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

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

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

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

The role played by the different kafirin species in kafirin microparticle formation has not yet been explored. Kafirin microparticles contain the same kafirin species as the original kafirin, that is α -, β -, and γ -kafirin. For zein, it has been shown that γ -zein contains a proline rich hexapeptide repeat (PPPVHL) at the N terminus (Kogan, Dalcol, Gorostiza, López-Inlesias, Pons, Pons, Sanz and Giralt, 2002). This repeat is also present in kafirin (Belton et al., 2006). Examination of synthetic peptides based on this repeat has shown its structure to be that of an amphipathic polyproline II type conformation in aqueous solution, with hydrophilic charged histidine side chains arranged on one side of the helix and hydrophobic residues on the other side (Pons, Feliz, Celma and Giralt, 1987; Dalcol, Pons, Ludevid and Giralt, 1996). Further work by Kogan, Dalcol, Gorostiza, López-Inlesias, Pons, Sanz, Ludevid and Giralt (2001) and Kogan et al. (2002) using atomic force microscopy and transmission electron microscopy (TEM) has shown that these peptides are able to self assemble into cylindrical micelles. The surface properties and potential for self assembly has suggested a role of this octamer in protein body formation (Kogan et al., 2002).

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

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

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

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

5.4. MECHANISM OF KAFIRIN MICROPARTICLE FILM FORMATION

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

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

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

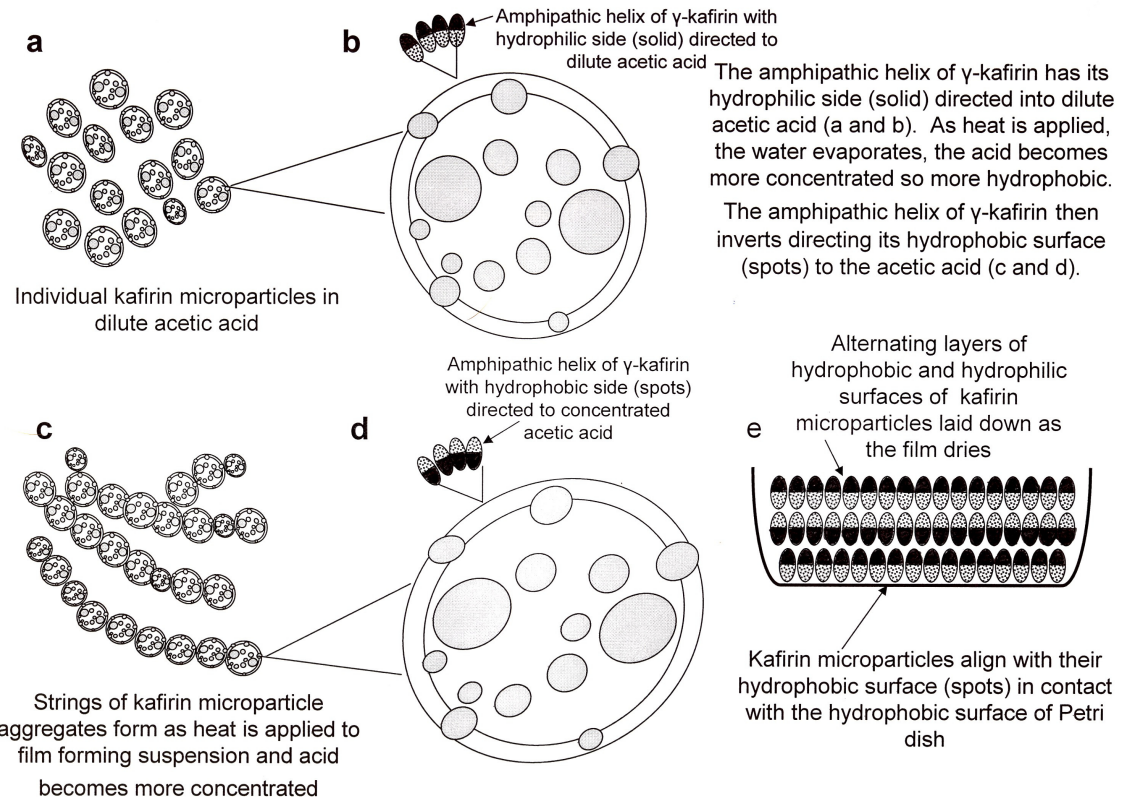


Figure 5.4.1.: Hypothetical model to describe the formation of a kafirin microparticle film
 a: Individual kafirin microparticles in dilute acetic acid, b: Expanded view of a single kafirin microparticle (not to scale), showing the amphipathic helix of γ -kafirin with its hydrophilic side directed to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

Although observed on a much greater scale than the nanoscale observations of Guo et al. (2005), it is suggested that the chains of kafirin microparticles that were observed by light microscopy (Figure 5.4.1.c) to form when a kafirin microparticle film was drying were probably aggregates of very large numbers of kafirin molecules. These apparent similarities between a macro and microscale during kafirin film formation may be considered to be analogous with the filaments of myosin and the structure of muscle (Alais and Linden, 1991) or the assembly of blocklets which make up the starch granule (Gallant, Bouchet and Baldwin, 1997).

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

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

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

5.5. POTENTIAL APPLICATIONS OF KAFIRIN MICROPARTICLES

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

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

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

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

5.6. IMPROVEMENT OF KAFIRIN MICROPARTICLE PROPERTIES AND FUTURE WORK

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

Physical cross-linking by application of heat has the disadvantage that heat sensitive compounds which are to be encapsulated by the microparticles may be damaged and lose efficacy by the use of high temperatures (Patil, 2003; Chen, Remondetto and Subirade, 2006). Microwave energy has been used as an alternative to cross-link gelatin microparticles producing insoluble but swellable microspheres (Vandelli, Romagnoli, Monti, Gozzi, Guerra, Rivasi and Forni, 2004). Both kafirin and kafirin films have been shown to have improved functional properties when heated by microwave energy (Byaruhanga, Erasmus and Taylor, 2005; Byaruhanga, Erasmus, Emmambux and Taylor, 2007). Thus application of microwave energy may be expected to have a positive affect on kafirin microparticle and kafirin microparticle film properties.

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

Chemical cross-linking agents such as glutaraldehyde, formaldehyde and D-glyceraldehyde have been used in an attempt to make protein microparticles more

hydrophobic (Burgess and Hickey, 1994; Latha, Rathinam, Mohanan and Jayakrishnan, 1995; Latha, Lal, Kumary, Sreekumar and Jayakrishnan, 2000). Disadvantages include toxic side effects due to residual cross-linking chemicals and unwanted reactions between the encapsulated drug and the cross-linking agent (Burgess and Hickey, 1994; Chen et al., 2006). Additionally, these cross-linking agents are not suitable for food use. Strauss and Gibson (2004) suggested the use of plant derived polyphenols as alternative chemical cross-linking agents which would be suitable for food use. They used grape juice and coffee containing unidentified phenolic acids and flavonoids directly to cross-link gelatin-pectin coacervates resulting in microparticles with greater mechanical strength and thermal stability than untreated material. Preliminary work in this study (Section 4.3.4.2.) and work by Emmambux, Stading and Taylor (2004) has shown that cross-linking with sorghum condensed tannins improved kafirin film properties by decreasing protein digestibility, biodegradation and oxygen permeability whilst improving tensile stress (Emmambux et al., 2004). Also when sorghum condensed tannins were encapsulated within kafirin microparticles, protein digestibility was greatly reduced. Consequently, it may be expected that cross-linking kafirin microparticles with sorghum condensed tannins would have a positive affect on their properties, potentially making them more resistant to enzymic digestion and increasing their mechanical strength and improving the functional properties of films made from them.

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

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

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

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

6. CONCLUSIONS AND RECOMMENDATIONS

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

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

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

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

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8. PUBLICATIONS AND PRESENTATION MADE BASED ON THIS RESEARCH

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