

# **Preparation, characterisation and functionality of kafirin microparticles**

by

**Janet Taylor**

**Submitted in partial fulfilment of the requirements for the  
Degree**

**PhD Food Science**

in the

**Department of Food Science  
Faculty of Natural and Agricultural Sciences  
University of Pretoria  
Pretoria  
South Africa**

**April 2008**

## **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

Janet Taylor

Supervisor: Prof A. Minnaar  
Co-supervisor: Prof P.S. Belton  
Co-supervisor: Prof J.R.N. Taylor

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  $\mu\text{m}$  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  $\alpha$ - and  $\beta$ -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  $\gamma$ -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**

## ACKNOWLEDGEMENTS

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:

Firstly my best friend, John, my husband for all his love, encouragement and constant support. Also for managing the difficult task of also being one of my co-supervisors so well.

Next my three daughters, Alice, Emma and Amy and my son-in-law, Daniel. All their love and hugs helped tremendously.

Prof Amanda Minnaar also needs a very special thankyou. As my first supervisor and Head of the Department of Food Science at the University of Pretoria, she could not have been more encouraging, helpful and supportive.

Prof Peter Belton from the University of East Anglia, UK. As my other co-supervisor, discussions with Peter always generated lots of new exciting ideas and potential explanations for my experimental results.

The staff and postgraduate students of the Department of Food Science at the University of Pretoria for all your friendship, encouragement, help and support.

For technical help with the microscopy, Alan Hall and Chris van der Merwe cannot be thanked enough. Their patience was endless. Also thanks to Dr Nic van der Berg for his help with the atomic force microscopy.

Finally, the Taylor family's youngest members, Oggie and Rosie for their constant companionship throughout the writing of my thesis. Oggie assisted in the only way a sausage dog knows how, by sitting on my lap and helping with the typing (not very well). Rosie, a good listener, now knows more than any other Labrador on the planet about sorghum prolamin proteins.

## TABLE OF CONTENTS

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

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.	<b>Results and discussion</b>	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.	<b>Conclusions</b>	57
4.1.6.	<b>References</b>	58
4.2.	<b>PREPARATION OF KAFIRIN MICROPARTICLE FILMS AND COATINGS AND MECHANISM OF FILM FORMATION AND FILM FUNCTIONAL PROPERTIES.</b>	62
4.2.1.	<b>Abstract</b>	62
4.2.2.	<b>Introduction</b>	63
4.2.3.	<b>Materials and methods</b>	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
	<b>Preparation of free standing kafirin films</b>	64
	<b>Preparation of kafirin microparticle free standing films</b>	64
	<b>Preparation of kafirin microparticle free standing films cast with different acids</b>	64
	<b>Preparation of films from kafirin at low protein concentration using glacial acetic acid as casting solvent</b>	65
	<b>Preparation of films from kafirin microparticles at low acid and low protein concentration</b>	65
4.2.3.4.	<i>Analysis of films</i>	65
	<b>Effects of protein concentration and acid concentration on kafirin microparticle film formation</b>	65
	<b>Effects of plasticiser concentration and acid concentration on kafirin microparticle film formation</b>	65
	<b>Scanning Electron Microscopy (SEM) of free standing films</b>	65



	<b>Atomic Force Microscopy (AFM) of kafirin microparticle film surfaces</b>	<b>66</b>
	<b>Fourier Transform Infrared Spectroscopy (FTIR) of films</b>	<b>66</b>
	<b>Water Vapour Transmission (WVT) and Water Vapour Permeability (WVP) of films</b>	<b>66</b>
	<b>Tensile properties of films</b>	<b>67</b>
	<b>Protein digestibility of films</b>	<b>67</b>
	<b>Biodegradation of films</b>	<b>68</b>
	<b>Observation of microparticle film formation</b>	<b>68</b>
4.2.3.5.	<i>Statistical analysis</i>	68
4.2.4.	<b>Results and discussion</b>	<b>69</b>
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
	<b>Film surface properties</b>	<b>83</b>
	<b>Water Vapour Permeability (WVP) and Water Vapour Transmission (WVT)</b>	<b>86</b>
	<b>Tensile Properties</b>	<b>88</b>
	<b>Film Protein Digestibility</b>	<b>90</b>
	<b>Biodegradation of films</b>	<b>91</b>
4.2.5.	<b>Conclusions</b>	<b>92</b>
4.2.6.	<b>References</b>	<b>93</b>
4.3.	<b>TANNIN INTERACTIONS WITH SORGHUM PROTEINS, KAFIRIN FILMS AND MICROPARTICLES AND ITS INFLUENCE ON THEIR DIGESTIBILITY AND FUNCTIONALITY</b>	<b>98</b>
4.3.1.	<b>Abstract</b>	<b>98</b>
4.3.2.	<b>Introduction</b>	<b>99</b>
4.3.3.	<b>Materials and methods</b>	<b>101</b>
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
	<b>Effect of reducing agent on sorghum protein digestibility</b>	<b>102</b>
	<b>Tannin content</b>	<b>103</b>
	<b>Tannin Type</b>	<b>103</b>
	<b>Amino acid analysis</b>	<b>103</b>
	<b>Tannin binding assay</b>	<b>103</b>
	<b>SDS-PAGE</b>	<b>104</b>
	<b>Film formation</b>	<b>104</b>
	<b>Protein digestibility of protein preparations and films</b>	<b>105</b>
	<b>Film biodegradation</b>	<b>105</b>
	<b>Preparation of kafirin microparticles for encapsulation</b>	<b>105</b>

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

## 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 $\alpha$ -helical to intermolecular $\beta$ -sheet conformation of kafirin and kafirin microparticles	50
Table 4.2.1.	Estimated ratio of $\alpha$ -helical to intermolecular $\beta$ -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 $\alpha$ -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 $\gamma$ -kafirins with those of $\gamma$ -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