

Kafirin and zein as coatings for the controlled release of amino acid supplements

by

Celeste Pretorius

**SUBMITTED IN PARTIAL FULFILMENT OF THE
REQUIREMENTS FOR THE DEGREE**

MSc Food Science

**IN THE
DEPARTMENT OF FOOD SCIENCE
FACULTY OF NATURAL AND AGRICULTURAL
SCIENCES
UNIVERSITY OF PRETORIA
SOUTH AFRICA**

APRIL 2008

DECLARATION

I declare that the dissertation herewith submitted for the degree MSc Food Science at the University of Pretoria, has not previously been submitted by me for a degree at any other university or institution of higher education.



For

Jared

and

my family, Leon, Hazel, Nikki and Jacques

ABSTRACT

Kafirin and zein coatings for the controlled release of amino acid supplements

by

Celeste Pretorius

Supervisor: Prof. J.R.N. Taylor

Degree: MSc Food Science

This experimental work investigated the development and testing of a controlled release system for methionine. Methionine is one of the limiting amino acids for the milk production in dairy cows. The quantities of methionine which reach the small intestine are affected by the bacteria in the rumen which utilize methionine. A controlled release system which will offer a protective barrier for methionine may ensure that the methionine reaches the small intestine in sufficient quantities. The work involved the development of a coating around methionine crystals, which would act as a barrier, protecting it from the rumen conditions. Zein and kafirin proteins from maize and sorghum, respectively, were used as the principal coating components for the controlled release system. Two different approaches were used in the development of the controlled release system. First, the zein and kafirin proteins were tested for their ability to act as barriers for the controlled release of methionine, and second, zein and kafirin microparticles were used as the controlled release agents.

Relatively successful, laboratory-scale methods were developed for coating the methionine with the proteins and the microparticles. Protein coatings were made by addition of methionine crystals to acid-dissolved proteins which led to the formation of a protein/methionine matrix. For coating the methionine with microparticles, glacial acetic acid was used to fuse microparticles around the methionine crystals. Dissolution assays were performed to test the release of methionine from the coatings under simulated rumen conditions. Both the zein and kafirin and microparticle coatings exhibited a barrier effect for methionine. The barrier effects of these coatings were influenced by several factors. Increasing the proportion of the coating agents led

to improved barrier properties. However, this only occurred until a certain proportion of coating agent was present (50%), after which the barrier properties no longer increased. Heat treatment of the coatings also increased the barrier properties of the coatings. This may be due to the formation of disulphide cross-links being formed during the application of heat. When a simple extrusion method was used to form the coatings, the barrier properties also improved in comparison to those coatings which were not formed using extrusion. When producing the microparticles, it was found that only the laboratory extracted kafirin preparation with 85% (db) protein formed microparticles. It was hypothesized that microparticle formation might be related to the purity of the protein preparations. Scanning electron microscopy of the coatings after the dissolution tests and pepsin digestion revealed pores on the surface of the coating. These were probably where the methionine leached from the coating into the dissolution medium.

The protein coatings did act as partial barriers, extending the release of methionine. From the release curves of methionine from the coatings, it could be seen that a sustained release of methionine occurred over a period of time, rather than a controlled release of methionine at a certain time. The aim of the application was thus only partially achieved as a complete protective barrier for methionine was not obtained from the protein coatings. No significant difference between the barrier properties of the coatings prepared from the proteins themselves and the microparticles were found. However, when based on equal protein purity the kafirin protein coatings showed the most effective barrier properties. Further research regarding kafirin coatings as a controlled release agent is recommended based on the results of the above named calculation. This research would entail investigating various coating technologies and methods.

ACKNOWLEDGEMENTS

I would sincerely like to thank:

My supervisor, Prof J.R.N. Taylor, for your guidance, valued advice, continual encouragement and patience during the course of my study. It was an honour being able to learn from you.

Janet Taylor for your friendship, kind ways, constant assistance and endless patience during the course of this study.

Allan Hall and Chris van der Merwe, Laboratory of Microscopy and Microanalysis, for your time and assistance regarding the microscope-related work for this study.

Prof Amanda Minnaar and Dr Riette de Kock for your friendship and sincere support throughout all my years at the Department of Food Science.

To all the staff members of the Department of Food Science for all your shared knowledge and support during all the years spent in this department.

To all the Masters, Doctoral and undergraduate students at the Department of Food Science, thank you for friendship and support.

To my family, Pappa, Mom, Nikki and Jacques, thank you for your enduring love and endless positive support; your encouragement was uplifting and inspirational.

Jared, your support at trying times kept me moving forward, thank you for your continual reassurance of my abilities, and for your unconditional love and faith in me.

Oumie, your life and who you are, is simply put, 'my aspiration'.

To Aunty Eileen, Kim, Hendrik, Mathew, Derek and Samantha thank you for your true friendship, sincere interest and support in the ways that only friends know how.

LIST OF TABLES	xi
LIST OF FIGURES	xii
CHAPTER 1	
1. INTRODUCTION	1
1.1 Statement of the problem	1
1.2 Literature review	3
1.2.1 Cattle stomach morphology and fate of proteins in the rumen	3
1.2.1.1 Compartments and physiology of the stomach of a cow	3
1.2.1.2 The fate of proteins in the rumen and the effect on milk yields	4
1.2.2 Definitions and the use of protein coatings as controlled release agents	6
1.2.2.1 What is a coating?	6
1.2.2.2 The use of proteins as coatings for controlled release	7
1.2.3 Characteristics of kafirin and zein	8
1.2.3.1 General structure and solubility characteristics	8
1.2.3.2 Secondary structure of kafirin and zein	10
1.2.3.3 Factors affecting protein digestion in the rumen and bacterial resistance properties of kafirin and zein	12
1.2.4 Formation of kafirin and zein coatings	14
1.2.4.1 Methods of coating and film formation	14
1.2.4.2 Interactions during the formation of a coating or film	15
1.2.4.3 Methods for applying coatings to nutrients	17
1.2.4.3.1 Mixing and solvent evaporation	17
1.2.4.3.2 Spray drying	17
1.2.4.3.3 Fluidized bed coating	18
1.2.4.3.4 The use of microparticles as coating agents	18
1.2.4.4 Additives used in coatings and films	20
1.2.4.4.1 Plasticizers	20
1.2.4.4.2 Cross-linking agents	21

1.2.5 Relevant uses of kafirin and zein as coatings for food additives	21
1.2.6 Conclusions	23
1.3 Objectives	25
1.4 Hypotheses	26
CHAPTER 2	
2. COATING METHIONINE WITH KAFIRIN AND ZEIN PROTIENS	27
2.1. Introduction	30
2.2 Materials and methods	
2.2.1 Kafirin and zein	31
2.2.2 Coating of methionine with Zein Standard, Zein Gold and kafirin	31
2.2.3 Dissolution test	33
2.2.4 Analyses	
2.2.4.1 Protein content	33
2.2.4.2 Moisture content	34
2.2.4.3 Fat content	34
2.2.4.4 SDS-PAGE	34
2.2.4.5 10-Phenanthroline-iron (III) assay	34
2.2.4.6 Ninhydrin assay	35
2.2.4.7 Stereomicroscopy	35
2.2.4.8 Statistical analysis	35
2.3 Results and discussion	
2.3.1 Composition of protein preparations	35
2.3.2 10-phenanthroline-iron (III) assay	36
2.3.3 Coating of methionine with Zein Gold, Zein Standard and kafirin	39
2.3.4 Dissolution tests	41
2.4 Conclusions	49
2.5 References	49
CHAPTER 3	
3 KAFIRIN AND ZEIN MICROPARTICLE COATINGS OF METHIONINE	53



3.1 Introduction	55
3.2 Materials and methods	
3.2.1 Raw materials	55
3.2.2 Kafirin extraction	55
3.2.3 Kafirin and zein microparticle preparation	55
3.2.4 Coating of methionine with microparticles	56
3.2.5 Analysis	
3.2.5.1 Dissolution test	57
3.2.5.2 Pepsin digestion assay	57
3.2.5.3 SDS- PAGE	58
3.2.5.4 Protein content	58
3.2.6 Microscopy	58
3.2.7 Statistical analysis	58
3.3 Results and Discussion	
3.3.1 Analysis of laboratory prepared kafirin	59
3.3.2 Formation of microparticles and coating around methionine	60
3.3.3 Coating methods and consequent observations	61
3.3.4 Dissolution tests and methionine release characteristics	65
3.3.5 Pepsin digestion of the microparticle coatings	74
3.4 Conclusions	80
3.5 References	80
CHAPTER 4	
GENERAL DISCUSSION	83
4.1 Methodological considerations	
4.1.1 Coating methods	83
4.1.2 Analytical methods	85
4.2 Formation and barrier properties of the prolamin coated methionine	88
4.3 Mechanism of methionine leaching from the coatings	92
4.4 Success of method and moving forward	93
CHAPTER 5	
CONCLUSIONS AND RECOMMENDATIONS	



5.1 Conclusions and recommendations	96
-------------------------------------	----

CHAPTER 6

LITERATURE CITED

6.1 Literature cited	97
----------------------	----

LIST OF TABLES

Table I:	Proximate composition of Zein Gold, Zein Standard and kafirin	35
Table II:	Proximate composition of laboratory prepared kafirin	59

LIST OF FIGURES

Fig. 1:	Morphology of the stomach of a cow and movement of food through digestive system.	4
Fig. 2:	The additional dietary crude protein needed to meet the animal's tissue needs at various milk yields when bacterial protein synthesis is at its maximum.	5
Fig. 3:	Forms of controlled release products where the active substances are coated.	8
Fig 4:	The nine-helix protein model for α -zein suggested by Argos et al.	11
Fig. 5:	The hairpin model for α -zein as described by Bugs et al.	11
Fig. 6:	Possible arrangements of zein molecules in the film.	16
Fig. 7:	Experimental design used for the preparation of nutrients coated with proteins and microspheres and determination of the effectiveness of the coatings.	28
Fig. 8:	Formation of methionine/zein paste using a hand mincer. (a) Hand mincer used to form the methionine/protein paste, (b) Dried extrudate formed by the hand mincer.	32
Fig. 9:	Formation of methionine/zein paste using a sieve. (a) Sieve used to form the methionine/protein paste, (b) Dried extruded clumps of zein coated methionine.	32
Fig. 10:	Sodium dodecyl sulphate polyacrylamide gel electro-phoresis (SDS-PAGE) of Zein Gold, Zein Standard and kafirin under reducing conditions.	36
Fig. 11:	Absorbance of ferrion measured for the 10-Phenanthroline-iron (III) assay on three different occasions.	38
Fig. 12:	Stereomicrographs of protein coated methionine (1:4) formed by a hand mincer. (a) Kafirin coated methionine, (b) Zein Gold coated methionine, (c) Zein Standard coated methionine.	40
Fig. 13:	Effect of Zein Gold, Zein Standard and kafirin coated methionine (4:1) on the FAN released at pH 2, 37°C.	42

- Fig. 14:** Effect of different proportions of Zein Gold used to coat methionine on methionine release at pH 5.5, 39°C. 44
- Fig. 15:** Effect of heat drying at 103°C on Zein Gold coated methionine on methionine released at pH 5.5, 39°C. 47
- Fig. 16:** Effect of pH on the release of methionine from Zein Gold coatings (1:4). 48
- Fig. 17:** Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions of laboratory prepared kafirin. Track 1- kafirin 2, track 3 -molecular weight standard. 59
- Fig. 18:** SEM of microparticles. a) Microparticles made from kafirin, b) Microparticles made from Zein Gold. Arrows indicate pores. 61
- Fig. 19:** SEM for air dried kafirin (a) and air dried kafirin microparticles (b). 63
- Fig. 20:** Stereomicrographs of kafirin microparticle coated methionine (1:1). a) Fused together with 20% acetic acid, b) fused together with glacial acetic. 64
- Fig. 21:** Stereomicrograph of methionine. 64
- Fig. 22:** Stereomicrographs of the kafirin microparticle coated methionine (1:1) fused with glacial acetic acid, extruded through a syringe. a) air dried at ambient temperature, b) heat treated at 60-70°C. Pores are indicated by arrows. 65
- Fig. 23:** SEM of the kafirin microparticle coated methionine (1:1) fused together with glacial acetic acid, extruded through a syringe. a) air dried at ambient temperature, b) heat treated at 60-70°C. 65
- Fig. 24:** Effect of Zein Gold microparticles mixed with methionine (1:4) and dried, on the release of methionine at pH 2, 39°C. 67
- Fig. 25:** Effect of fusing kafirin microparticles to form a coating around methionine using 20% acetic acid or glacial acetic acid to form a coating (1:1) on the release of methionine at pH 5.5, 39°C. 68
- Fig. 26:** Stereomicrographs of kafirin microparticle coated methionine (1:1) after dissolution test. a) Fused together using 20% acetic acid, b) Fused together with glacial acetic acid. Pores are indicated by arrows. 69

- Fig. 27:** Effect of heat treatment of the kafirin microparticle coated methionine (1:1) fused using glacial acetic acid and extruded on methionine released at pH 2, 39°C. 71
- Fig. 28:** Stereomicrographs of the kafirin microparticle coated methionine (1:1) fused together with glacial acetic acid, extruded through a syringe after dissolution test. a) air dried at ambient temperature, b) heat treated at 60-70°C. Pores are indicated by arrows. 72
- Fig. 29:** SEM of the kafirin microparticle coated methionine (1:1) fused together with glacial acetic acid, extruded through a syringe. a) air dried at ambient temperature, b) heat treated at 60-70°C. Pores indicate arrows. 72
- Fig. 30:** Comparison of the effect of Zein gold coated methionine (1:1) heat treated and not heat treated (from Chapter 2), kafirin microparticle coating (1:1) extruded/not extruded and heat treated/not heat treated on the release of methionine at pH 2, 39°C 73
- Fig. 31:** Effect of pepsin action on zein, kafirin and kafirin microparticle coated methionine (1:1) on the methionine released in buffer and the corrected value for the methionine released from pepsin 75
- Fig. 32:** Effect of pepsin on the digestion of Zein Gold, kafirin and kafirin microparticle coatings of methionine (1:1). 76
- Fig. 33:** SEM of Zein Gold coating with and without methionine before and after incubation in a pH 2 buffer and in a pH 2 buffer containing pepsin. Row A) Coating before pepsin digestion test, Row B) Coating after incubation in buffer, Row C) Coating after incubation in buffer containing pepsin. 78
- Fig. 34:** SEM of Zein Gold coating with and without methionine before and after incubation in a pH 2 buffer and in a pH 2 buffer containing pepsin. Row A) Coating before pepsin digestion test, Row B) Coating after incubation in buffer, Row C) Coating after incubation in buffer containing pepsin. 79
- Fig. 35:** The Wurster coating chamber. a) Air distributor, b) spray, c) cylindrical partition, d) bag filter, e) particle and air flow. 84
- Fig. 36:** Generic representation of “sustained” and “delayed” release

profiles.	89
Fig. 37: Model constructed for organization of methionine entrapped within the kafirin proteins (β -sheet conformation).	89
Fig. 38: Model constructed for organization of methionine particles entrapped within a kafirin microparticle matrix.	90
Fig. 39: Mechanism of methionine released from the coatings.	92

CHAPTER 1

1 INTRODUCTION

1.1 Statement of the problem

Controlled release is a process or technique by which one or more active ingredients are made available to a particular target at a specific time and rate (Kydonieus 1980, Koontz 2006). What then is the rationale for the use of controlled release systems in the food industry? Some examples of current and possible applications of controlled release in the food industry will motivate the use and need for these systems. Controlled release systems can be used for: the separation of reactive or incompatible compounds, limiting the loss of active ingredients during food processing, antimicrobial release packaging (Koontz 2006); individual protection to small substances until they need to be released in the gastrointestinal tract (Guilbert 1985), and the encapsulation of flavours either to mask a flavour or for its timely release.

The use of edible films and coatings as the control release agent is of great interest to the food industry as there are many benefits from its use. The films and coatings formed can be consumed (Guilbert 1985, Gennadios and Weller 1990) and the degraded end products have a low toxicity (Lui et al. 2005). Thus, they can be used for controlled release of substances in humans and animals. In addition, waste and environmental pollution is reduced as the materials used are produced from renewable sources and are biodegradable (Gennadios and Weller 1990, Shukla and Cheryan 2001).

An application for controlled release is in the dairy industry. In South Africa, the dairy industry plays an important economic role. In June 2003 it employed 4856 milk producers (Coetzee 2003). The average milk production between 2004-2005 was slightly less than 2000 million litres. Methionine and lysine are often the limiting components for production of milk (Polan et al. 1991). For high yielding cows these amino acids are often not in sufficient quantities and thus unfavourably affect milk yield as well as milk protein content (Yoshimaru et al. 2000). They are not in sufficient quantities because amino acids and proteins from the ruminant feed are utilized by bacteria in the rumen. If these amino acids could be increased in the diet of

cows and protected from degradation in the rumen there will be an increase in milk production and as a result there will be an increase in income through milk production.

Arshady (1993) gives important criteria for microencapsulation of food additives. In my opinion these criteria can be applied for coating of food additives. The following is thus adapted by applying the given criteria to coatings in place of microcapsules as given by the author.

1. The reason for coating should be clearly defined
2. The active ingredient should not deteriorate during the coating method
3. The polymer coat should be edible, non-reactive and tasteless
4. The concentration of the active ingredient should be optimized with respect to performance and cost
5. Core release should be studied and optimized against application parameters (dissolution, pH, temperature, pressure, etc.) as may be necessary
6. The cost of the polymer coat and the coating process should justify in terms of improved performance

Other properties of the coating agent which should be taken into consideration when coating an active ingredient for protection in the rumen are the microbial resistance of the coating agent to degradation and the stability of the coating agent at the various pH conditions in the stomach of the cow.

Potential materials which can be used as edible coatings are the proteins zein from maize (Padua and Wang 2002) and kafirin from sorghum (Park et al. 2002). The structural characteristics of kafirin (reviewed by Belton, Delgadillo, Halford and Shewry 2006, reviewed by Duodu, Taylor, Belton and Hamaker 2003) and zein (Shukla and Cheryan 2001; reviewed by Duodu, Taylor, Belton and Hamaker 2003) support their reasons for their uses as potential edible coatings. These relate to their hydrophobic nature, solubility in aqueous alcohols and disulphide bonding interactions of these proteins. If these protein coatings are found to be successful barriers to the conditions in the rumen they can be applied and adapted for human gastrointestinal applications.

1.2 Literature review

This review of the literature will give a brief overview of the cow stomach morphology and proteolysis in the cow stomach. A definition for coatings and films will be given and the use of proteins as a controlled release agent will be discussed. The general and secondary structure of kafirin and zein as well as their properties which confer bacterial resistance will be considered. The formation of kafirin and zein coatings including relevant examples of these proteins as coatings for controlled release will also be discussed.

1.2.1. Cattle stomach morphology and fate of proteins in the rumen

1.2.1.1 Compartments and physiology of the stomach of a cow

Schummer (1960) according to Hofmann (1973) defined the ruminant stomach as a compound, subdivided stomach lined by two morphologically and physiologically different mucous membranes. It is developed from a single sac but shows four defined compartments, namely the rumen, reticulum, omasum and the true stomach (abomasum). The rumen and reticulum are often thought of as one compartment, the reticulorumen, as there is no sphincter between the two separate compartments (Forbes and France 1993). According to these authors feed moves from the mouth into the reticulorum. In the reticulorum the feed is degraded by microorganisms and in addition is mixed by muscular contractions. These muscular contractions are responsible for the movement of the feed throughout the digestive tract. Figure 1 illustrates the passage of the digesta through the stomach of the cow. The digesta passes from the reticulum through a sphincter to the omasum, and then to the abomasum. The abomasum is similar to that of the monogastric stomach where acid and enzymes are mixed with the stomach. From here the digesta passes through to the small intestine.

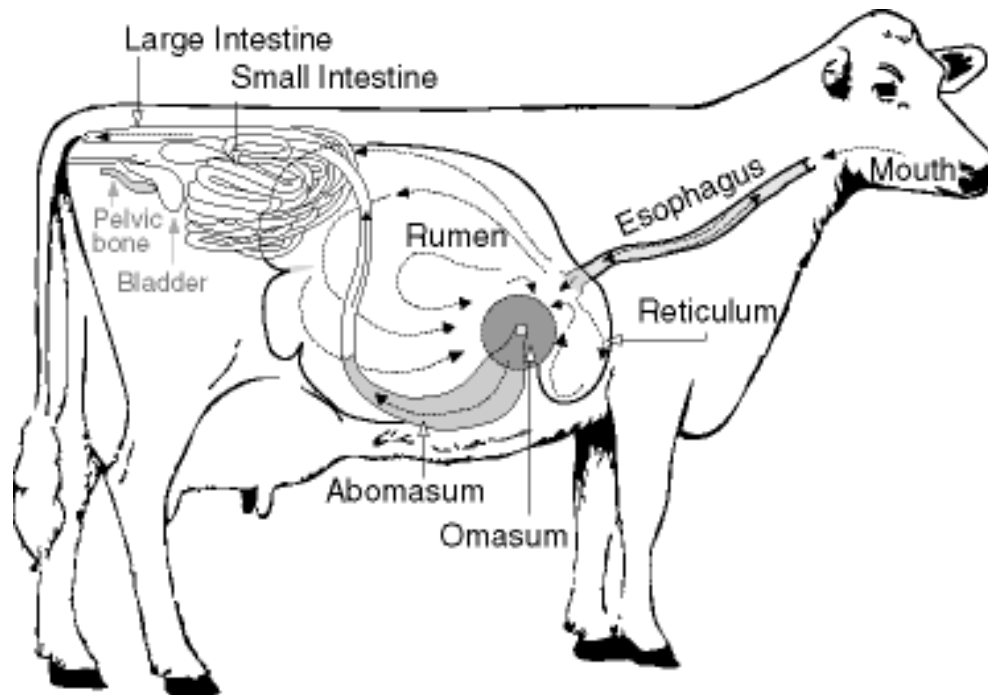


Figure 1: Morphology of the stomach of a cow and movement of food through digestive system (Wattiaux and Howard 2007)

1.2.1.2 The fate of proteins in the rumen and the effect on milk yields

The bacteria present in the rumen can be grouped according to their action, namely cellulolytic, amylolytic, those fermenting soluble sugars and proteolytic bacteria (Ørskov 1982). Rumen protozoa and anaerobic fungi are also present. The focus of the following discussion will be the proteolytic bacteria and their role in degradation of the ingested protein. A proportion of the protein intake of the feed of ruminants is degraded by the microorganisms present in the rumen (Schneider 2004). Some of the ingested proteins are digested by the bacterial extracellular enzymes and the resulting peptides and amino acids are absorbed by the ruminal bacteria (Bach, Calsamiglia, and Stern 2005). The absorbed peptides are hydrolysed to amino acids by the rumen bacterial enzymes. The energy available to the bacteria will determine the fate of the absorbed peptides and amino acids. If there is available energy the amino acids can be used in microbial protein synthesis. The amino acids which will reach the small intestine after digestion in the ruminant stomach are from two sources. These sources include the proteins and amino acids from microbial protein synthesis as well as from protein which is not degraded in the rumen (Polan et al. 1991). These authors draw

attention to the fact that the microorganisms do not provide adequate amino acids for cows producing milk

Under normal physiological conditions, according to Kaufmann and Lüpping (1982), between 60-70% and as reviewed by Bach et al. (2005) 50-80% of protein that reaches and is absorbed in the intestines of high-yielding cows is supplied by bacterial protein synthesis. Kaufmann and Lüpping (1982) state that to sustain milk yield above 20 litres/day more dietary protein than the flora in the rumen can utilize must be supplied in the diet.

In Figure 2 it can be seen that the more susceptible a protein is to degradation in the rumen the higher quantity of the protein is needed to yield the same amount of milk in comparison to protein which is less degradable.

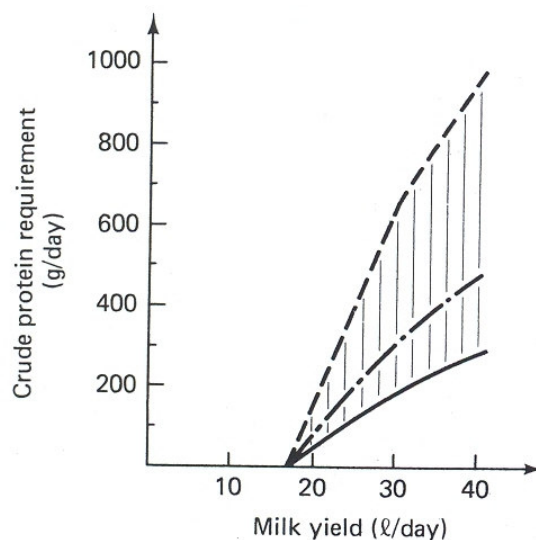


Figure 2: The additional dietary crude protein needed to meet the animal's tissue needs at various milk yields when bacterial protein synthesis is at its maximum (Kaufmann and Lüpping 1982). Crude protein requirement in the intestine — ; crude protein requirement at 40% degradability - - - - - ; crude protein requirement at 70% degradability

Kaufmann and Lüpping (1982) propose that to ensure an adequate amount of un-degraded protein arrives in the intestines, protein of low degradability can be fed to the ruminants. They suggest various methods of attaining a low degradability protein. These are the use of proteins which are naturally protected against degradation,

artificial protection and lastly physical protection against rumen microbial degradation. According to the authors an example of natural protection would be to lower the overall solubility of the cows diet by adding maize products, brewer's spent grain and oilseeds which will lead to an increase in milk yield. Artificial protection is also discussed where techniques can be employed to ensure protection of the proteins. The use of formaldehyde pre-treatment of soya protein is mentioned as an artificial method for rendering the proteins less degradable. Lastly, physical methods for reducing degradability are mentioned, the chief technology being the application of heat. Other than the above named specific examples another potential physical method to increase the amount of un-degraded protein, amino acids and vitamins that reach the intestine, is to use a substance which has a low degradability as a coating, which could function as a barrier for protection (Ardaillon and Bourrain 1991, Drouillard, Herald, and Greenquist 2005).

1.2.2 Definitions and the use of protein coatings as controlled release agents

1.2.2.1 What is a coating?

The Dictionary of Food Science and Technology defines coatings as “materials which form thin continuous layers or coverings over the surface of foods. The coatings are used to enclose and/or protect the food, and may be eaten along with the food or removed before consumption” (International Food Science Information Service 2005).

The words coating and film are often used interchangeably. Gennadios and Weller (1990) state that there is no distinct difference between the terms. However, they describe each of the terms separately. This literature review pertains to the use of coatings as controlled release agents and to avoid confusion between the two terms the definitions given by Gennadios and Weller (1990) will be used. According to these authors, a coating is directly applied and formed on the surface of a product, whereas a film is formed separately as a thin sheet and then applied to the product.

1.2.2.2 The use of proteins as coatings for controlled release

Krochta (2002) lists some of the possible functions of protein films and coatings. These include: a barrier to moisture, oxygen, aroma, oil, carriers of antimicrobials or antioxidants, carrier of flavour, colours and nutrients, resistance to mechanical forces and can also be used as a product appearance enhancer (gloss, colour).

The film forming properties of proteins have been used in industrial applications since ancient times (Gennadios, McHugh, Weller and Krochta 1994). According to Lawton (2002), the first commercial use of zein (the prolamin protein of maize) was as a replacement for shellac in lacquers, varnishes and coatings. Shukla and Cheryan (2001), Gennadios et al. (1994) and Dong, Sun and Wang (2004) mention many industrial applications of zein and zein-based coatings, for example paper coatings for glossy magazine covers, in toilet cleansing blocks, grease-proof paper, floor coatings, laminated boards, in cosmetic products, composite wound dressings, biodegradable films and plastics, and microspheres to delay the release of drugs, to name but a few.

There are many research articles and patents using proteins such as zein, wheat gluten, soy protein, gelatine, milk casein and whey proteins as coatings and films. However, proteins such as egg albumin, barley hordein and sorghum kafirin have received less attention (Gennadios et al. 1994).

According to Kydonieus (1980) there are two main components in a controlled release system: the active agent and the polymer matrix or matrices that regulate release of the active agent. The protein coatings mentioned above can be used as the controlled release agent. The premise is that the coatings will potentially form a continuous layer or enclosure around the active substance. Controlled release devices may assume many different shapes and sizes. Kydonieus (1980) illustrates some of the forms in which controlled release systems where a coating is used as the agent for controlling release (Figure 3).

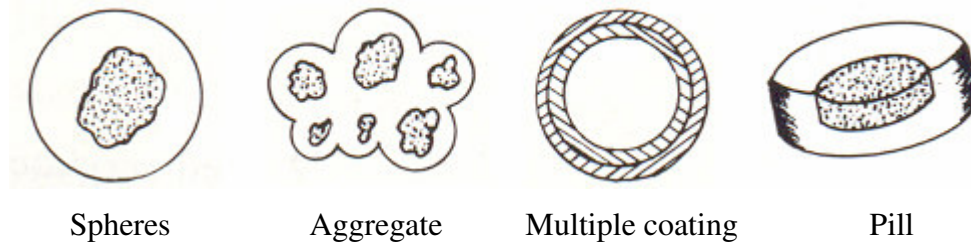


Figure 3: Forms of controlled release products where the active substances are coated (adapted from Kydonieus 1980).

The characteristics of kafirin and zein which will enable the use of these proteins as edible coatings will be discussed below.

1.2.3 Characteristics of kafirin and zein

1.2.3.1 General structure and solubility characteristics

The main storage proteins in sorghum and maize are kafirin and zein, respectively. Kafirin and zein are located in protein bodies in the starchy endosperm of the grain (Shukla and Cheryan 2001) accounting for 50% of the protein in these grains (Duodu, Tang, Grant, Wellner, Belton, and Taylor 2001). DeRose, Din-Pow, Kwon, Hasnain, Klassy, and Hall (1989) showed that there is extensive homology between kafirin and zein. According to Belton et al. (2006) the more extensively studied zein proteins can serve as a basis for the examination of kafirins. The kafirin polypeptides have been grouped based on their similarity to the zein polypeptides (Belton et al. 2006). The zein and kafirin polypeptides are divided into α - , β - , γ - , and δ - fractions. The δ -kafirin fractions have, however, not yet been characterised at the protein level. The α -fractions form the largest group of prolamins, followed by the γ -fractions and the β -fractions, respectively (Shukla and Cheryan 2001; Belton et al. 2006).

The solubility characteristics of these proteins can be used to classify them. Kafirin and zein are classified as prolamins. Prolamins are proteins which are insoluble in water and only soluble in aqueous alcohol (Duodu et al. 2001). Prolamins are hydrophobic proteins containing a large number of nonpolar hydrophobic amino acids. A review by Wall and Paulis (1978) discusses the amino acid composition of

zein and kafirin. Zein has high levels of leucine, alanine, proline, phenylalanine and glutamine. Kafirin has higher levels of glutamine and leucine in comparison to zein and had higher amounts of the non-polar, hydrophobic amino acids proline, alanine and valine. The β - and γ -zeins have a high content of the sulphur containing amino acids methionine and cysteine (Shull, Watterson and Kirleis 1991, El Nour, Peruffo, and Curioni 1998), whereas the α -kafirins have low amounts of cysteine (El Nour et al. 1998). The free energy of hydration is the interaction free energy between a substance and water and can be used to evaluate the hydrophobic or hydrophilic characteristics of a substance (Faibish, Yoshida and Cohen 2002). Duodo et al. (2003) discuss the free energy of hydration of kafirin and zein. The α -fractions of these proteins have approximately the same free energy of hydration and thus the same level of hydrophobicity. However, the free hydration energy of γ -kafirin shows that this fraction of the kafirin protein is more hydrophobic than the γ -zein fraction. This, as well as the higher amounts of hydrophobic amino acids in kafirin, agrees with that of the review article by Wall and Paulis (1978) in that kafirin is more hydrophobic than zein.

Shull et al. (1991) determined the molecular weights of kafirin polypeptides using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and the solubility characteristics of kafirin. These were compared to the known molecular weights of the zein fractions. Zein polypeptides are characterised as α -zein ($M_r = 23$ k and 25 k), β -zein ($M_r = 18$ k, 17 k and 15 k), γ -zein ($M_r = 27$ k) and δ -zein ($M_r = 10$ k). The polypeptides of kafirin are classified as follows: α -kafirin ($M_r = 23$ k and 25 k), β -kafirin ($M_r = 16$ k, 18 k and 20 k) and the γ -kafirins ($M_r = 28$ k). The extraction of zein can be performed using aqueous 2-propanol. However, kafirin is a more hydrophobic protein and thus a more hydrophobic solvent such as 2-methyl-2-propanol is used (Belton et al. 2006). The greater hydrophobicity of kafirin is associated with the γ -fraction, this fraction being the most hydrophobic prolamin fraction of kafirin and zein. The α -prolamins are extracted at high alcohol concentrations of between 50-95% and the β -fractions can be extracted with lower proportions of alcohol (10-60%). The γ -fractions are extracted at a wide range of alcohol concentrations (10-80%) in the presence of a reducing agent (Shull et al. 1991). According to Lawton (2002), a reducing agent must be added to the alcohol to

extract the β - , γ -fractions. This is due to the disulphide bonds present in these fractions.

1.2.3.2 Secondary structure of kafirin and zein

In the review by Belton et al. (2006) the authors state that the major components of kafirin have a secondary structure which is similar to that of the α -zeins. There is 40-60% α -helices present in the secondary structure of α -zein and small amounts of β -sheet conformation are present when dissolved in aqueous ethanol (Shewry and Tatham 1990, Belton et al. 2006). According to Belton et al. (2006) the relatively wide range of α -helical structure (Fig. 4A) may be due to various factors. These include differences in fractions used, the solvents and methods used for deconvolution. There have been no detailed studies of the secondary structure of the kafirin subunits. The secondary structure of zein is used as a basis for comparison of the secondary structure of kafirins.

Shewry and Tatham (1990) and Belton et al. (2006) review the structural model given by Argos, Pedersen, Marks and Larkins (1982) for α -zein. The model entails nine homologous repeat units in the M_r 19k and M_r 22k zeins. Each repeat unit forms an α -helix separated by a turn region. The model of Argos et al. (1982) further suggests that the neighbouring α -helices run antiparallel and group together to form a warped cylinder (Figure 4A). The antiparallel helices are stabilized by intramolecular hydrogen bonds between polar residues on adjacent residues. Depicted in Figure 4B is the arrangement of the α -zeins within the protein body. The arrangement results from the interaction via hydrogen bonds between glutamine residues in the turn regions of the helices.

According to Belton et al. (2006) the model of Argos et al. (1982) was modified by Garratt, Olivia, Caraceli, Leite and Arruda (1993) which allowed for a general model for all α -prolamins by making provision for a varying number of α -helices.

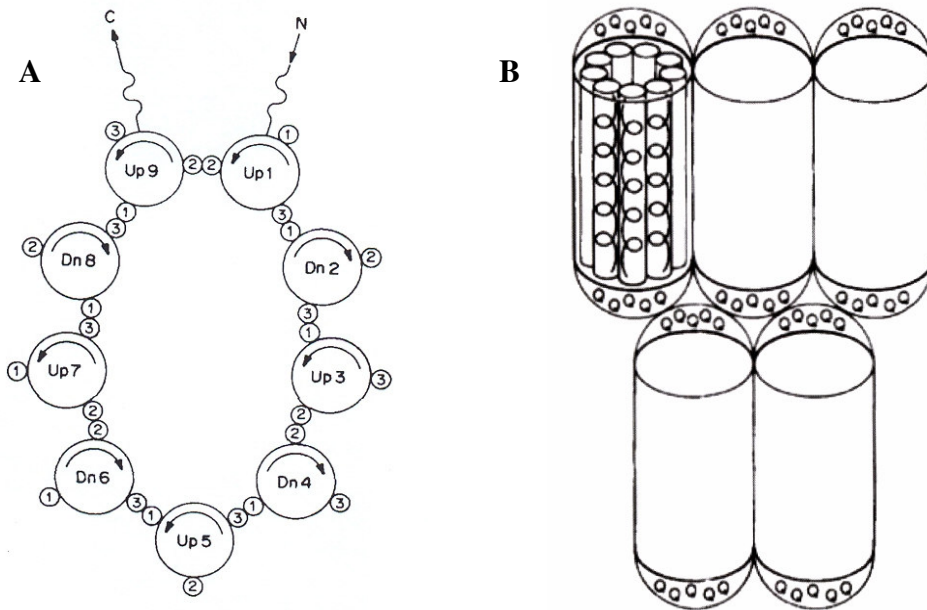


Figure 4: The nine-helix protein model for α -zein suggested by Argos et al. 1982. (A) The axis is orthogonal to the plane of the figure. The small circles indicate the hydrogen – bonding polar residue segments. ‘Up’ and ‘down’ indicate the direction of the antiparallel helices. (B) A model for the arrangement for zein proteins in and between planes. Hydrogen bonding occurs between Gln residues in the turn regions between helices.

Elongated or extended structures of the α -helices have been proposed by various authors (as reviewed by Belton et al. 2006). The more recent models describe a hairpin model consisting of α -helices, β -sheets and turns folding back on itself. Figure 5 depicts the hairpin model proposed by Bugs, Forato, Bortoleto-Bugs, Fischer, Mascarenhas, Ward and Colnago (2004).

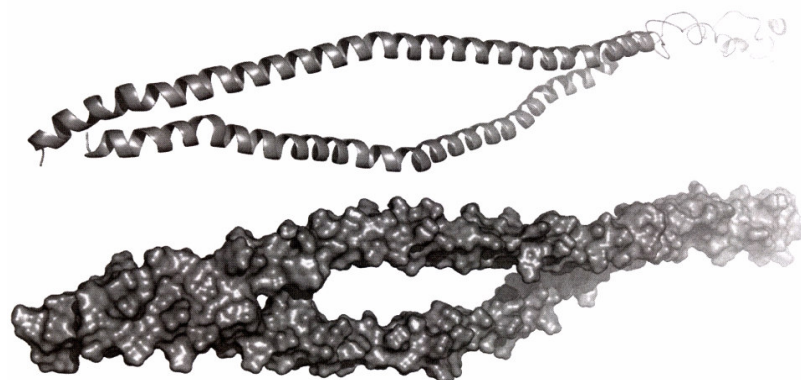


Figure 5: The α -zein hairpin model of Bugs et al. (2004).

The β -zeins have a small amount of α -helix conformation but are mostly present in β -sheets (Shewry and Tatham 1990). According to these authors the β -zeins do not contain repeated sequences, which is unusual for prolamins. The amount of α -helix and β -sheets in γ -zeins are approximately the same ratio. The smallest portion of all the zeins, δ -zein, does not have any repeat sequences or a clear cut domain structure. Studies regarding the conformation of δ -zein have not yet been performed.

1.2.3.3 Factors affecting protein digestion in the rumen and bacterial resistance properties of kafirin and zein

In the review article by Duodu et al (2003) protein digestibility is described as the measure of the susceptibility of a protein to proteolysis. In this review the factors that influence the digestibility of kafirin from sorghum are divided into two groups namely, exogenous and endogenous factors. In my opinion, several of these factors can also be applied to zein. Exogenous factors occur from the interaction of cereal proteins with non-protein components, whereas endogenous factors occur from changes which take place within the cereal proteins themselves. The exogenous factors for sorghum include grain organizational structure, polyphenols, phytic acid, cell wall components, and starch. The endogenous factors include protein cross-linking, racemization and isopeptide formation, disulphide cross-linking, hydrophobicity of the proteins and secondary structure. It would seem that the less digestible the protein, the better it would function as a coating agent.

Lawton (2002) states that zein has microbial resistance properties. The bacterial resistance properties of zein and presumably kafirin contribute to the motivation for using these proteins as coatings. The resistance of a protein to microbial proteases is highly dependant on the structure and solubility of the protein (Bach et al. 2005). Romagnolo, Polan and Barbeau (1994) state that digestibility of cereal and legume proteins is related to their amino acid composition. They found that zein is relatively resistant to ruminal degradation. This was determined by incubating samples in lactating dairy cows for 72 hours after which electrophoretic and densitometric analysis was performed to analyse the protein fractions remaining. From their results they suggest that the resistance of protein to degradation in the rumen will increase

with an increase in hydrophobicity of the protein. As explained, kafirin has a higher hydrophobicity than zein and may be less digestible in the rumen. In the article by Duodu et al. (2003) it is stated that enzymes function in an aqueous environment. Due to the higher hydrophobicity of kafirin it may be less accessible to enzymatic attack and consequently rendering it less digestible than zein.

The disulphide cross-linking that occurs in kafirin and zein is related to their cysteine content. The mole % of the amino acid cysteine in the α -fractions of zein and kafirin is 0.4, β -kafirin has a slightly higher mole % of 4.9 in comparison to β -zein which has a value of 4.4. The γ -fractions have the highest mole % of cysteine. For γ -kafirin the mole % is 7.8 and that for γ -zein is 7.4 (reviewed by Taylor and Belton 2002). El Nour et al. (1998) found that extraction of sorghum flour using 60% 2-methyl-2-propanol resulted in a high amount of protein in the form of SS-linked oligomers. Under reducing conditions these oligomers were broken down into α_1 -, α_2 - and γ -kafirin. Beta-kafirin was not present in these oligomers. Only sonication led to the presence of β -kafirin in the polymers. From their findings the authors suggest that β -kafirin can act as a bridge linking together oligomers of α_1 - and γ -kafirin. Hence, these oligomers become too large to be extracted without reduction. Shull, Watterson and Kirleis (1992) suggest that γ - and β -kafirins may exist in the protein body as cross-linked polymers due to the high cysteine content of these proteins, which can form disulphide bonds.

From the research of various authors it is evident that disulphide crosslinking increases during the cooking process. Oria, Hamaker and Schull (1995) performed *in vitro* protein digestibility assays for cooked and uncooked sorghum flour. Cooked sorghum showed a decrease in the protein digestibility. The authors found that the protein bodies in cooked sorghum were generally unaffected by pepsin digestion. They suggested that in cooked sorghum enzymatically resistant protein polymers are formed through disulphide bonding between the γ - and β -kafirins. There may possibly be disulphide bonding between other proteins located in the periphery of the protein body. They also observed an increase in digestibility when a reducing agent was added to the cooking water. However, the digestibility was not fully reversed to that of uncooked flour. The authors suggest that this may be due to the presence of inaccessible disulphide bonds. Nunes, Correia, Barros and Delgadillo (2004) found

that for uncooked sorghum and maize flours the protein digestibility was similar, one of the sorghum varieties had a higher digestibility than that of maize. However, on cooking the sorghum became less susceptible to protein digestion than the maize samples. These results correlate with the protein digestibility assay performed by Hamaker, Kirleis, Butler, Axtell and Mertz (1987). They showed that sorghum's digestibility was reduced by cooking and when reducing agents were added the digestibility increased. These authors concluded that formation of disulphide bonds during the cooking process renders the sorghum less digestible.

According to Bach et al. (2005) pH and the microorganisms present are the two main factors which affect protein degradation in the rumen. In high-forage fed dairy cattle there is a directly proportional relationship between the pH of the rumen and the proteolytic activity. As the pH decreases the proteolytic activity decreases. A pH of between 5-7 is ideal for the rumen proteolytic enzymes. At this pH the bacterial resistance properties of the kafirin and zein will play a role in preventing the microbial degradation of the coatings. According to the patent of Autant, Cartillier and Pigion (1989) when the nutrient coated with zein reaches the abomasums (pH < 3.5), the protein coating must either become soluble, dispersed or swollen so as to release the active substance which it is coating.

1.2.4 Formation of kafirin and zein coatings

1.2.4.1 Methods of coating and film formation

According to Debeaufort, Quezada-Gallo and Voilley (1998) there are a number of processes which will result in the formation of a coating or film.

1. Melting and solidification of solid fats, waxes and resins
2. Simple coacervation. An aqueous solution containing a dispersed hydrocolloid is precipitated or gellified by various methods. The removal of the solvent, addition of a non-electrolyte solute in which the polymer is not soluble, addition of an electrolyte substance inducing a "salting out" effect, or by modification of the pH of the solution.
3. Complex coacervation. Two hydrocolloids with opposite charges are combined, inducing interactions and the precipitation of the polymer mixture

4. Thermal gelation or coagulation. Heating of macromolecule solutions which involves denaturation, gelification, precipitation, or by a rapid cooling of the hydrocolloid solution that for example induces a sol-gel transition

In addition, Donhowe and Fennema (1994) give another method of film formation, namely solvent removal. In my opinion this is the most widespread method used for film and coating formation. Film formation using a solvent involves dispersing/dissolving the film forming component in the solvent after which the solvent is removed, normally by evaporation.

1.2.4.2 Interactions during the formation of a coating or film

According to Cuq, Gontard, Guilbert (1998) for proteins to form a macromolecular network three steps are required. First, the low energy intermolecular bonds that stabilize polymers in their native state must be broken. Once the bonds are broken the polymer chains arrange and orientate themselves. Lastly, there is the formation of a three-dimensional network stabilized by new interactions and bonds. The formation of a new protein network only occurs once the agent that caused the intermolecular bonds to break has been removed.

To form a coating or film the formation of a matrix with cohesive properties is important (Debeaufort et al. 1998). During the formation of a zein film, hydrogen and hydrophobic interactions occur in the film matrix (Gennadios et al. 1994). According to Belton et al. (2006) the exact protein-protein interactions responsible for the development of protein films are unknown. However, Guilbert (1985) and Banker (1966) stated that two forces are in operation when a film coating is formed. First cohesion forces act between the polymer molecules and are responsible for forming the film and secondly, adhesion forces acting between the film and the substance to be coated. These forces are affected by the chemistry and organization of the polymer. Molecular weight, branching, polarity, regularity of chain structure can affect these forces. The adhesive property depends on the amount and type of interactions between the film or coating and the material being coated (Debeaufort et al. 1998). Film properties such as compactness, permeability and brittleness are affected by cohesion forces (Guilbert 1985). Other factors which may influence film properties are the

concentration of the protein in solution, the method of application and the amount of layers applied (Cook and Shulman 1998). For example, a thinner coating layer requires a smaller amount of the coating material upon application. The concentration of the protein in solution can also be decreased for a thinner coating layer. Yamada, Takahashi and Noguchi (1995) stated that there can be a number of zein protein arrangements in a film (Figure 6). They suggested an arrangement of zein proteins in a film where the zein molecules will gather forming an aggregated pile. The aggregated pile will expose its hydrophobic region towards the outer surface depending on the solvent used. They suggested that these aggregates will gather together to form the film during drying. The ellipse in Figure 6 corresponds to the zein molecule and the shadowing on the ellipse shows the hydrophobic region of zein. As stated, the solvent used for the formation of the zein film will affect the arrangement depending on its hydrophobic or hydrophilic nature. In an acetone and water solution, for example, the zein molecules will be aligned to form tubular piles where the hydrophilic solvent (water) will be entrapped, stabilizing the hydrophilic region. The outer surface of the pile will be stabilized through hydrophobic interactions with the other more hydrophobic solvent (for example, acetone).

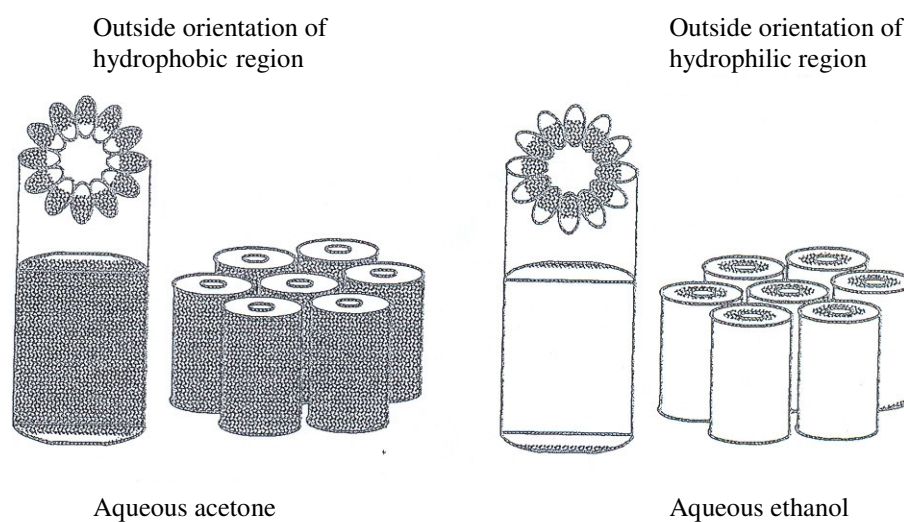


Figure 6: Possible arrangements of zein molecules in the film. (Yamada et al. 1995).

It is important to note that free standing zein and kafirin films are brittle and could possibly pose a problem during coating of substances. Plasticizers are generally added to improve the flexibility of the film (Gennadios et al. 1994) or coating. Lai, Padua, Wei (1997) found that the structure of zein sheets without fatty acids (a plasticizer)

were amorphous and had a cracked appearance. When the concentration of fatty acid increased a layered structure was formed where the fatty acid was situated in between protein layers. They state that the formation of cracks is usually due to fracture of the polymers where there is a localised stress concentration. With the use of Atomic Force Microscopy (AFM) they found that the zein sheets without plasticizers had an amorphous sponge-like structure and those containing plasticizers had a rearrange oriented protein matrix.

1.2.4.3 Methods for applying the coatings to nutrients

Coating solutions can be applied to the substance to be coated in a variety of different methods. Autant et al (1989) state that the coated substances can consist of a central core covered by a continuous layer of the coating, or the active substances can be dispersed in the coating composition.

Techniques used for applying coatings:

- Extrusion (Autant et al. 1989)
- Spraying of solutions or emulsions (an emulsifier may be needed to decrease the viscosity when spraying solutions) in a fluidized bed (Autant et al. 1989; Arshady 1993, Lin and Krochta 2006)
- Coacervation and encapsulation in a molten medium can be used (Autant et al. 1989) to apply the coatings.
- Spray and drip applications are mentioned by Grant and Burns (1994).

1.2.4.3.1 Mixing and solvent evaporation

The material used as a coating is solubilised and the active ingredient is incorporated into the solution. The solvent is then evaporated off leaving a film of coated particles (Donhowe and Fennema 1994).

1.2.4.3.2 Spray drying

The basic principle of spray drying involves the use of an atomizing nozzle through which a solution or suspension (the coating agent and the active ingredient) are forced

into a hot chamber. Small droplets are formed and fall to the bottom of the chamber while the solvent will evaporate from the droplets in the hot surroundings (Tang, Chan, and Heng 2005). Yoshimaru et al. (2000) used spray drying to coat microcapsules with a secondary coating of zein. The microcapsules were made by mixing porous starch with *l*-lysine and sonicating the solution. The solution was freeze-dried and coated with a synthetic acrylic copolymer. The microcapsules were then coated with zein.

1.2.4.3.3 Fluidized bed coating

In the process of fluidized air coating the core material is suspended by air, while the liquid coating material is sprayed onto the suspended cores (Tang et al. 2005). Different coating materials will result in different methods of film formation. For example, when polymers (such as kafirin and zein) are used as the coating agent an increase in concentration of the polymer will occur as the solvent evaporates. This will result in an immobile polymer particle which forms a film over the core or active ingredient (Tang et al. 2005). Lin and Krochta (2006) used a fluidized bed process to coat peanuts with whey protein isolate. They found virtually complete coverage of the peanuts with the whey protein (with an added surfactant) as well as a consistent coating efficiency. A disadvantage of fluidized bed coating is that some of the coating solution may dry before reaching the particles to be coated leading to a loss of the coated material (Lin and Krochta 2006).

1.2.4.3.4 The use of microparticles as coating agents

Coombes, Lin, O'Hagen and Davis (2003) define microspheres as colloidal, sphere-shaped particles with diameters between 10 nm–2 mm. In the literature this definition seems to apply to the terms microspheres, microparticles and microcapsules which are used interchangeably. The term microparticle will be used in this literature review as to avoid terms (such as '-sphere') which will distinctly define the word into a specific physical form.

Radwick and Burgess (2002) divide microparticles into two types, microcapsules and micromatrices or micropheres. Microcapsules and micromatrices are both used for

microencapsulation, a term defined as enclosing a solid, liquid or gas inside a polymeric coating. The authors describe microcapsules as an encapsulated drug or protein core centrally located as a reservoir completely surrounded by a distinct capsule wall. Micromatrices are described as a separate or homologous dispersion of the encapsulated drug or protein throughout the microsphere matrix. For the purposes of this literature review the use of microparticles as a coating agent will pertain to the micromatrices described above.

The use of microparticles for encapsulation is described in many research articles. However, there seems to be a gap in literature for the use of microparticles as a coating agent. Given that there is no found literature on the microparticles as coating agents the use of microparticles for encapsulation will be reviewed. Various methods of microencapsulation exist. These include coacervation, extrusion, spray drying, fluidized bed coating, and solvent evaporation (Radwick and Burgess 2002, Gouin 2004, Freitas Merkle and Gander 2005). The terms coacervation and phase separation seem to be used interchangeably by many authors. For the purpose of this literature review coacervation will be discussed as it is the process which will be used to form the micromatrices used as a coating agent for this dissertation. Gouin (2004) describes microencapsulation by coacervation as a phase separation of one or many hydrocolloids from the initial solution and the subsequent deposition of the newly formed coacervate phase around the active ingredient suspended or emulsified in the same reaction media. Gouin (2002) refers to method devised by Arneodo where a phase separation of the hydrocolloids occurs due to the adjustment of the pH of the solution.

Some examples of the use of coacervation to encapsulate various substances are will be discussed briefly below. Lui et al. (2005) used zein to produce microspheres for the controlled delivery of ivermectin, a parasiticide, for cattle, sheep, pigs and dogs. The zein microspheres containing the ivermectin were prepared by a phase separation technique. An encapsulation efficiency of 60% was attained where the ivermectin loading was nearly 20%. The *in vitro* release of the ivermectin in a pH 7.4 buffer resulted in a biphasic sustained release curve which after the first day showed a 40% release and after nine days approximately a 90% release. Zein was used to encapsulate Gitoxin, a cardiac glycoside (Muthuselvi and Dhathathreyan 2006). Coacervation was

used to prepare the encapsulated Gitoxin. The authors found satisfactory encapsulation efficiency when the concentration of Gitoxin and zein was 1.1:16, respectively, in which the encapsulation efficiency of Gitoxin was the highest. The release kinetics of the drug were examined across a spectrapor membrane and the drug concentration released in the phosphate buffer was measured spectrophotometrically. The results showed a biphasic curve. At 40 hours there was approximately a 25% release and after 80 hours approximately 80% was released. Hurtado-López and Murdan (2005) formed zein microspheres containing ovalbumin as a model antigen. Coacervation was used to form the microspheres. The release kinetics of the ovalbumin loaded microspheres was examined in a buffer at pH 7.4 for seven days. They found that no significant ovalbumin was released in the first four days. However, on the fifth day 20% of the ovalbumin has detected in the release medium. Scanning electron microscopy of the microspheres after day seven showed that the morphology of the microspheres was similar to the microspheres before the incubation period indicating that the release possibly arose from slow erosion of the zein microspheres.

1.2.4.4 Modifications of coatings and films

Various additives and treatments can be used to alter the coating or film and may have an impact on the coating as a controlled release system. The effect which plasticizers, cross-linking agents and heat may have on the coating or film will be discussed briefly in the following section.

1.2.4.4.1 Plasticizers

Plasticizers are small molecular weight hydrophilic compounds which may be added to alter the properties of the film or coating (Donhowe and Fennema 1994, Krochta 2002). The plasticizer will improve the flexibility of the coating or film. The plasticizer competes for the hydrogen bonding and electrostatic interactions with the protein chains and thus decreasing intermolecular forces between neighbouring protein chains (Kester and Fennema 1986, Krochta 2002). This leads to various changes to the protein film or coating. These changes include a reduction of the glass transition temperature, increase of film flexibility and ductility, and a decrease of film

strength and the moisture and oxygen barrier properties (Kester and Fennema 1986, Krochta 2002).

1.2.4.4.2 Cross-linking agents

A film or coating may not exhibit the barrier properties that are needed for a specific application. For example, a protein may not act as a sufficient barrier when used to coat an active substance for a controlled release application. The barrier properties of the protein can be improved by the use of cross-linking agents. Tannins can be used to cross-link proteins, the advantage of using tannins is that they are cross-linkers naturally found in plant foods. Emmambux, Stading and Taylor (2004) used tannic acid and sorghum condensed tannins to cross-link kafirin films. They found that the tannins caused the films to become stiffer, however less plastic. The authors also found a decrease in water uptake of the films containing the tannin but no change in the water vapour permeability of the films.

Heat can be used as a means of cross-linking the proteins in kafirin and zein. Heating kafirin with microwave energy was used by Byaruhanga, Emmambux, Belton, Wellner, Ng and Taylor (2006) to induce cross-linking in kafirin powder which was used to form kafirin films. They found that microwave heating induced the formation of disulphide bonds. A patent by Pelosi (1997) describes a method for cross-linking zein. The method involves heating zein in the presence of water to a temperature of at least 130°C which will result in cross-linking of the protein.

1.2.5 Relevant uses of kafirin and zein as coatings for food additives

There are a number of patents and journal articles that refer to the use of kafirin and zein as protein coatings for additives. Those which are relevant will be discussed. A patent by Autant, Cartillier and Pigeon (1989) describes the coating of a biologically active substance, for example methionine or lysine, where zein and zein-based components are named as one of the film-forming components. The coated nutrients are added to the feed of ruminants. The coated substance is either surrounded by a continuous skin of coating or a microcapsule consisting of a central core. According

to the patent, various techniques can be used to form these pellets. For example, extrusion or spraying of solutions or emulsions in a fluidized bed. Techniques of encapsulation in a molten or semi-molten medium, and coacervation can be used to form these pellets.

Ardaillon and Bourrain (1991) describe an enzymatically degradable coating for granules which can be fed to ruminants. The coating is claimed to be stable in the rumen (pH 6) and the abomasum (pH 2). Apparently, coated substance is only released when it reaches the intestine (pH 7). Here it is degraded by enzymes present between the abomasum and ileum. The active substance to be coated can be a medicine, vitamins or amino acids. The coating consists of zein, plus a water-insoluble polymer (not named) and an optional plasticizer. It can also be composed of zein, a hydrophobic substance (not named) and a water-insoluble polymer (not named). The methods for coating the active substance are the same as those mentioned in the patent by Autant et al. (1989).

A recent patent application by Drouillard et al. (2005) also involves coating micronutrients for ruminants. Various proteins are used as the proteinaceous film-forming component where zein is named as one of the proteins. The protein source is dissolved in a solute after which the substance to be coated (vitamins, minerals, amino acids) is mixed with the barrier solution. To remove excess solute the mixture is then either vacuum dried, spray-dried, freeze-dried or oven dried, which results in the particulate matter being surrounded by a proteinaceous film. The protein can also be modified to form and improve cross-links. According to the authors, at low pH the film coating the particulate will solubilise and release the coated substance. This proteinaceous coating will render the coated particles more resistant to microbial degradation in the rumen of animals such as cattle and sheep.

A deodorized and decolorized zein product, Kobayashi Zein DP is produced by CBC Europe (Kobayashi Zein DP, 2004). According to the data sheet the coating is applied by spraying onto materials and then dried. As an example of a coating application, Kobayashi Zein DP was used to coat a placebo tablet. The coating resulted in a controlled release of the tablet. In a gastric resistance test a 6 % coating resulted in

less than 10% release after 2 hours. After 2 hours of the intestinal fluid test almost 20% of the placebo tablet was released.

There is a need to develop aqueous solvents for proteinaceous substances as to eliminate the risks of using organic solvents. O'Donnell, Wu, Wang, Wang, Oshlack, Chasin, Bodmeier and McGinity (1997) developed an aqueous pseudolatex of zein for coating purposes. To form the aqueous pseudolatex, zein was dissolved in ethanol, and then poured into purified water and agitated. They found that a spontaneous dispersion was formed. The ethanol and some of the water was evaporated off, this solution was then be used to coat substances. They used this aqueous pseudolatex to coat Acetaminophen (APAP) tablets and Nu-Core sugar spheres. Various plasticizers and antimicrobial agents were added. Dissolution studies to determine the release kinetics, were performed using the United States Pharmacopeia (USP) dissolution method II with distilled-deionized water as the dissolution medium. Dissolution as used in this context can be defined as the separation or disintegration of something in to smaller or more basic constituents, or dissolving into smaller parts or elements. The dissolution studies show that the APAP coated with 5% zein, 0.05% methyl paraben and 0.01% propyl paraben released 100% of the drug with in 5 hours, and when the methyl paraben was increased to 0.1%, the entire drug was released in 12 hours. The parabens' primary function was to act as antimicrobials, they were, however, found to also have a plasticizing effect.

1.2.6 Conclusions

It is evident from the above literature review that kafirin and zein may be used as coating agents to exhibit a protective effect, forming a barrier around the active agents and thus enabling their controlled release. Zein and zein-based compositions have been used as the coating material in many patents and experimental applications to coat diet supplements and drugs for their controlled release. There is an apparent gap in published literature and patents regarding the use and applications of kafirin as a coating material with the same application. Given the homology between kafirin and zein, there is great possibility that kafirin can have similar and possibly superior coating applications in contrast to zein. This is because of the greater hydrophobicity

and disulphide cross-linking of kafirin compared to that of zein. Also an effective, simple and inexpensive method for coating the methionine with kafirin and zein is needed. The optimum concentration of coating material needs to be determined as well the effectiveness of the coating in terms of barrier or protective properties under simulated rumen conditions. Methods for improving the barrier properties of the proteins also need to be investigated.

1.3 Objectives

1. To determine whether kafirin and zein proteins can form a protective barrier coating for methionine for its controlled release in the gut.

1.1 To determine the optimum ratio between methionine to be coated and the coating materials kafirin and zein.

1.2 To compare the effectiveness of kafirin and zein as coatings.

1.3 To determine the effect of heat drying the coated methionine in comparison to air drying

2. To determine whether kafirin and zein protein microparticles can form a protective barrier coating for methionine for the controlled release in the gut.

2.1 To devise a method for coating the methionine using the protein microparticles.

2.2 To compare the effectiveness of kafirin and zein microparticles as coatings

2.3 To determine the effect of heat drying the microparticle coated methionine in comparison to air drying

1.4 Hypotheses

The hydrophobic nature and extensive cross-links in kafirin and zein (Gao, Taylor, Wellner, Byaruhanga, Parker, Clare Mills, Belton 2005) will enable coatings made from these proteins to exhibit resistance to the conditions and proteolytic enzymes present in the rumen. This will enable the protein films to have a protective effect on the nutrients they coat.

The protective coating formed by laboratory extracted kafirin is expected to have a superior protective effect in comparison to commercial zein. This is because laboratory kafirin contains higher amounts of γ -prolamin. The γ -kafirin component has a higher degree of cross-linking and is a more hydrophobic component of the kafirin protein (Belton et al. 2006), whereas commercial zein constitutes mainly of α -zein (Shukla and Cheryan 2001).

When heat treated, the kafirin protein or the kafirin microparticles, will show an increase in the barrier properties and become less digestible in comparison to the zein or zein microparticles used to coat the methionine. This is because Nunes et al. (2004) found that on cooking the sorghum becomes less susceptible to protein digestion than maize. This is due to the formation of disulphide bonds (Hamaker et al. 1987). Dry heating may induce disulphide bonds (Byaruhanga, Erasmus, Emmambux and Taylor 2007) in the nutrient coated samples which will in turn render the protein coatings less digestible.

CHAPTER 2

2 Coating methionine with kafirin and zein proteins

The following research chapters are written in the style of scientific papers for the journal *Cereal Chemistry*. A flow diagram of the experimental design used in this research is given in Figure 7.

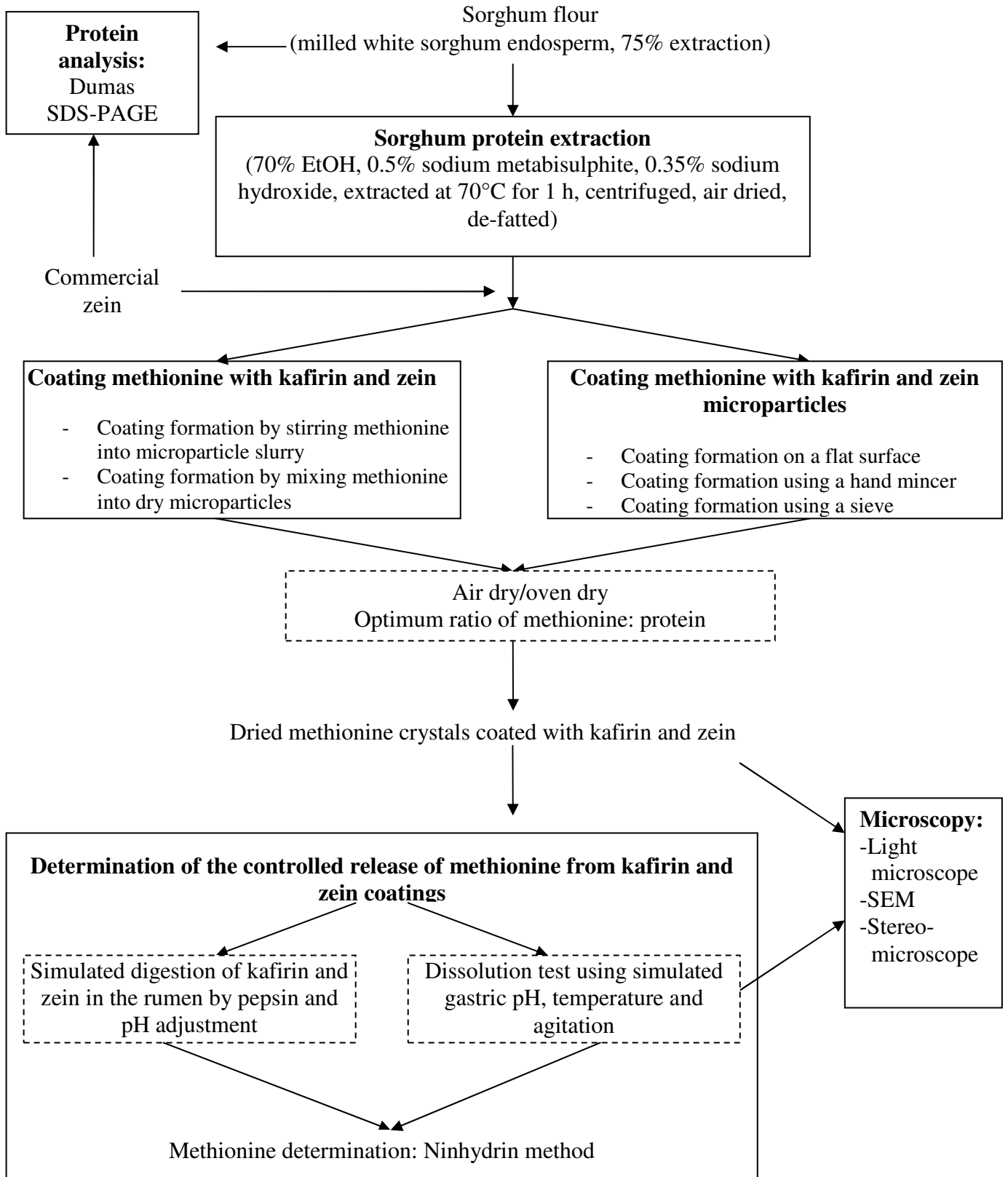


Figure 7: Experimental design used for the preparation of methionine coated with zein and kafirin proteins and microparticles and determination of the effectiveness of the coatings

2. COATING METHIONINE WITH KAFIRIN AND ZEIN PROTEINS

Abstract

In several patents zein, the prolamin of maize, has been mentioned as one of the proteins which is used as a protective barrier for active substances. The protective barrier enables the active substance to pass through the gastrointestinal tract of ruminants without being used by the ruminal bacteria. Due to the extensive homology between zein and the sorghum prolamin, kafirin, there is potential for kafirin to exhibit similar barrier properties. Methionine was selected as a model active substance to be coated with zein and kafirin. It is a limiting amino acid for the production of milk in dairy cows because bacteria present in the rumen utilize the methionine before it reaches the small intestine. A simple coating procedure was developed and tested for methionine release. Protein coatings were made by addition of methionine to acid-dissolved proteins to form a protein/methionine matrix. The results showed that both the zein and kafirin exhibited a barrier effect on the release of methionine. The barrier properties of coatings were evaluated as methionine released from the coating during a dissolution test. The barrier properties of the coating materials were improved by two methods: increasing the concentration of the protein used as the coating agent, and by heat treatment of the coated samples. It was determined that the kafirin coating had superior barrier properties in comparison to the zein coating based on equal protein purity. The better barrier effect of the kafirin may possibly be due to the fact that kafirin is a more hydrophobic protein than zein.

2.1. INTRODUCTION

Protecting vitamins, minerals and other compounds from the cattle rumen environment to ensure absorption at a later stage in the gastrointestinal tract has been the subject of several patents dating back some 19 years, for example Autant et al (1989). Most patents describe the use of the prolamin storage protein of maize, zein, as a protective barrier for the active substances (Autant et al. 1989, Ardaillon et al. 1991, Drouillard et al. 2005). Kafirin, the sorghum prolamin has extensive homology with zein (DeRose et al. 1989). However, the literature reveals no information concerning the use of kafirin for the above named application, even though the two proteins have similar characteristics. Accordingly, kafirin can potentially be used as coating agent to enclose and protect the active substances.

A kafirin coating may have superior barrier and protective properties in comparison to a zein coating as kafirin is more hydrophobic (Wall and Paulis 1998, Duodu et al. 2003). This is one reason why kafirin is less susceptible to enzymatic attack and thus less digestible in comparison to zein (Duodu et al. 2003). Another reason relates to the more extensive disulphide cross-linking which occurs in kafirin. Various authors have found that cooked kafirin is less digestible than cooked zein (Hamaker et al 1987, Oria et al 1995, Duodu, Nunes, Delgadillo, Parker, Mills, Belton & Taylor 2002, Nunes et al 2004) and propose it is due to the formation of disulphide cross-links.

It is essential that the method for coating the nutrients must be industrially feasible and economical. Simplicity may play a key role in attaining this. Zein (reviewed by Shukla & Cheryan 2001) and kafirin (reviewed by Taylor et al. 2006) have film forming properties. A simple method of film formation involves dissolving or dispersing the film materials in a solvent, after which the solvent is evaporated off (Donhowe & Fennema 1994). This process can be applied to form a coating (personal communication with Mrs Janet Taylor, Research Officer, University of Pretoria). Ardaillon and Bourrain vaguely mention such a process of coating formation. Taylor, Taylor, Dutton & De Kock (2005) found that glacial acetic acid can be used as a solvent, instead of aqueous ethanol, to form free-standing kafirin films. An important

advantage of using glacial acetic acid is that it can be used at much lower temperatures (25°C) in comparison to aqueous ethanol.

Methionine, an essential amino acid, is one of two amino acids which can limit milk production in cows (Polan et al. 1991), the shortage of which can lead to economic losses. The objectives of this work were to use methionine as a model active substance to be coated. A suitable coating procedure and release kinetics evaluation assays were developed. This work also compares kafirin and zein as controlled release coating agents for methionine.

2. 2 MATERIALS AND METHODS

2. 2.1 Kafirin and zein

Kafirin was extracted from decorticated white, tannin free sorghum grain on large scale by the Council for Scientific and Industrial Research (CSIR), Modderfontein, South Africa. The extraction method followed was essentially that given by Emmambux & Taylor (2003). These authors extracted kafirin in 70% (w/w) absolute ethanol, 0.5% (w/w) sodium metabisulphite and 0.35% sodium hydroxide at 70°C for 1 hr. Two commercial zein samples of variant purity, ZPP Gold and ZPP Standard were obtained from Zein Protein Products, Marina, CA.

2. 2.2 Coating of methionine with Zein Standard, Zein Gold and kafirin

The prolamin preparations were dissolved in glacial acetic acid (1:5) w/w using an orbital kitchen mixer with continuous stirring. Once dissolved, methionine was added to the prolamin solutions and thoroughly mixed. The ratios of prolamin to methionine investigated were 80:20, 50:50 and 20:80. Three different coating formation and drying procedures were investigated.

Formation on flat surface: Once the methionine was well mixed into the prolamin solution the mixture was poured (approximately 4 mm thickness) onto a flat stainless steel tray and left to air dry at ambient temperature. Once dry, the coating was removed from the tray using a spatula to loosen the coating and broken into pieces

using a mortar and pestle.

Formation using a hand mincer: The methionine/prolamin mixture was blended for 3-4 hr in a Hobart mixer to allow some of the glacial acetic acid to evaporate and a paste to form. The paste was then forced through a hand mincer (Fig. 8a). The extrudate was placed on a tray in a fume hood overnight at ambient temperature to allow the glacial acetic acid to evaporate off (Fig. 8b).

Formation using a sieve: The methionine/protein mixture was blended for 3-4 hr to allow some of the glacial acetic acid to evaporate off and a paste to form. The paste was then forced through a sieve (Fig. 9a). The extrudate was dried as described above.

Half of the coatings were first partially air dried for 5 hr, after which they were subjected to heat treatment in a force draft oven at 100°C.

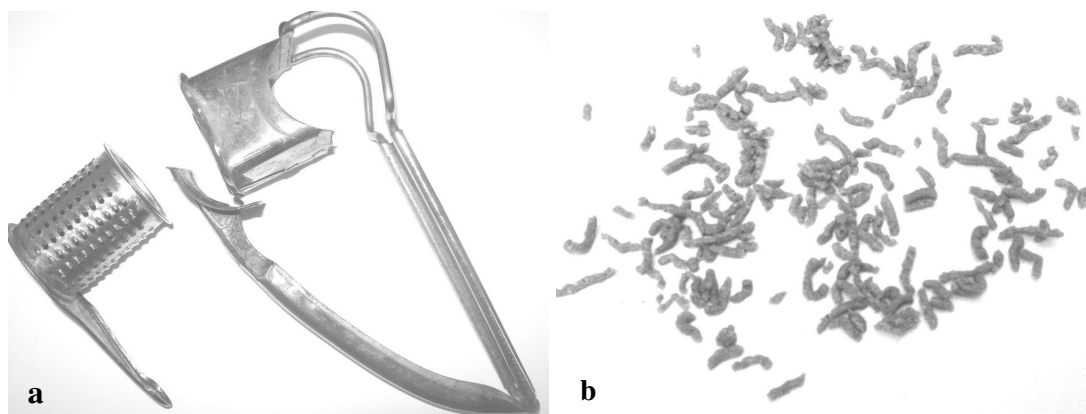


Figure 8: Formation of methionine/zein paste using a hand mincer. (a) Hand mincer used to form the methionine/protein paste, (b) Dried extrudate formed by the hand mincer.

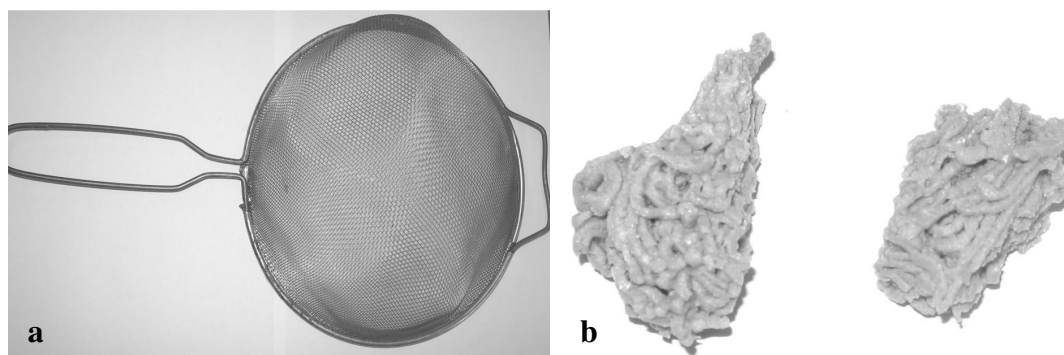


Figure 9: Formation of methionine/zein paste using a sieve. (a) Sieve used to form the methionine/protein paste, (b) Dried extruded clumps of zein coated methionine.

2.2.3 Dissolution test

A dissolution test was designed on the basis of the conditions of the dissolution studies of Oneda & Ré (2003) and Gunasekaran, Xiao & Ould Eleya (2006). It was determined that a concentration of 1% (w/w) methionine dissolved in the chosen buffer. However, it had to be diluted to be read spectrophotometrically in the ninhydrin colour range.

The dissolution test was performed as follows: Coated samples (0.5 g) and uncoated methionine (weight equal to methionine in the coated sample) were weighed into 50 mL plastic centrifuge tubes. Two different buffers were used, namely a 0.1M pH 2.0 sodium citrate-phosphate buffer and a 0.1M pH 5.5 citric acid-sodium citrate buffer. Buffer (50 mL) was added and the contents were mixed by vortex mixing. The centrifuge tubes were placed in a shaking water bath at 37°C or 39°C for 1.5 or 8 hr, respectively. Aliquots (3 mL) were withdrawn at thirty minute time intervals (including time zero). The aliquots were centrifuged at 12580 g for 20 min to separate any undissolved methionine or suspended coated particles. The methionine content of the supernatant was determined to calculate percentage dissolution.

Various treatments were investigated including different concentrations of protein and heat treatment. For the dissolution tests the methionine (uncoated) was added in the same concentration or treated in the same manner as the coated sample. The percentage methionine (uncoated) depicted in the graphs is thus an average of the various concentrations or treatments for a specific experiment.

2. 2.4 Analyses

2.2.4.1 Protein content

The Dumas combustion method, AACC Standard Method 46-30, (American Association of Cereal Chemists 2000) was used to determine the protein content in triplicate (N x 6.25) using a Leco Protein/Nitrogen Analyzer FP528 (Leco, St Joseph, MI).

2.2.4.2 Moisture content

Moisture Air Oven Method 44-15A (American Association of Cereal Chemists 2000) was used where samples were analysed in triplicate.

2.2.4.3 Fat content

Fat was determined (in triplicate) using the AACC Standard Method 30-35 by the Soxhlet extraction method with petroleum ether (American Association of Cereal Chemists 2000).

2.2.4.4 SDS-PAGE

The zein and kafirin prolamin preparations were characterized by SDS-PAGE under reducing conditions using the method described by Byaruhanga et al. (2006). A vertical electrophoresis system (XCell SureLock™ Mini-Cell, Version H, Invitrogen, Carlsbad, CA) with a 4-12% polyacrylamide gradient gel (NuPAGE® Novex Gels, Invitrogen™, Carlsbad, CA) of 8 cm x 8 cm and 1.0 mm thick was used. Ten micrograms of protein was loaded into the wells. Low-range premixed protein molecular weight markers were used with $M_r = 97.4$ k, 66.2 k, 39.2 k, 26.6 k, 21.5 k and 14.4 k (Roche Diagnostics Corporation, Indianapolis, IN). Electrophoresis was conducted at a current of 100 mA and a voltage of 200 V. The protein gels were stained with Coomassie Brilliant Blue R-250, destained and scanned using a flat bed scanner.

2.2.4.5 10-Phenanthroline-iron (III) assay

The method of El-Brashy and Al-Ghannam (1995) was used to determine the methionine released from the kafirin and zein coatings. It involves the oxidation of methionine with iron (III) to form methionine sulphone and iron (II). In an acidic medium the iron (II) formed combines with 1, 10-phenanthroline to form ferroin. Ferroin content was measured spectrophotometrically at 512 nm.

2.2.4.6 Ninhydrin assay

The ninhydrin colorimetric method of the European Brewery Convention (1987), Method 8.8.1 was used to determine the methionine released from the kafirin and zein coatings, samples were analysed in triplicate. Ninhydrin reacts with ammonia and primary and secondary amines to form a deep blue or purple colour read at 570 nm. The assay was performed using methionine as a standard.

2.2.4.7 Stereomicroscopy

Coated samples were viewed under the Carl Zeiss Stemi DV4/DR stereomicroscope (Carl Zeiss, Germany).

2.2.4.8 Statistical analysis

All experiments were repeated twice for both kafirin and zein. One way analysis of variance (ANOVA) with Fisher's Least Significant Difference (LSD) test was performed.

2.3 RESULTS AND DISCUSSION

2.3.1 Composition of protein preparations

Table I: Proximate composition of Zein Gold, Zein Standard and kafirin

	Protein (g/100 g)	Fat (g/100 g)	Moisture (g/100 g)
Zein Standard	70.7 ^b ± 0.8 (74.7)	7.6 ^c ± 0.2 (8.0)	5.4 ± 0.2 ^a
Zein Gold	91.0 ^c ± 0.7 (96.0)	0.2 ^a ± 0.1 (0.2)	5.2 ± 0.1 ^a
Kafirin	64.7 ^a ± 0.8 (68.1)	3.6 ^b ± 0.1 (3.8)	5.0 ± 0.3 ^a

1. Dry basis indicated in parentheses
2. ± Indicates one standard deviation
3. Values are means and standard deviations of three replicates. Means with different superscripts within a column differ significantly (p<0.05)

The purity of the different prolamins varied (Table I). The commercial Zein Gold had the highest purity. This is because it had the highest percentage protein and a very low fat content. Da Silva & Taylor (2004) found similar results. Among

the extracted kafirin and commercial zein samples they used, the commercial zein was of highest purity. They suggested that this may be because the zein was possibly prepared from corn gluten which is high in protein. Maize gluten is a by-product of maize wet milling (reviewed by Shukla & Cheryan 2001). Zein Standard and kafirin had much lower protein contents. This is because they contained higher amounts of fat and other compounds. Da Silva & Taylor (2004) suggested that the other components present in such preparations may be ash and non-starch polysaccharides.

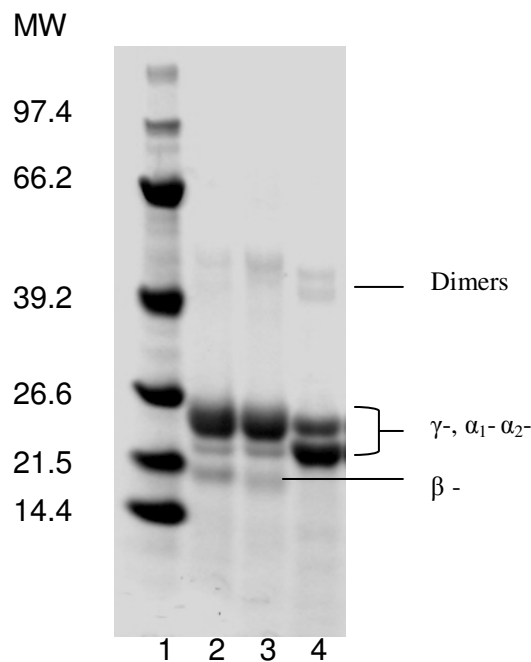


Figure 10: Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of Zein Gold, Zein Standard and kafirin under reducing conditions. Track 1 - molecular weight standards, track 2 - Zein Gold, track 3 - Zein Standard and track 4 - kafirin.

Comparison of the SDS-PAGE patterns for zein and kafirin from various works (Shewry 2002, Nunes et al 2005) shows that the α -, β - and γ -fractions were present in both the commercial zein and laboratory extracted kafirin preparations (Fig. 10). No other bands were present on the SDS-PAGE. This indicates that with respect to protein, the zein and kafirin preparations were pure.

2.3.2 10-phenanthroline-iron (III) assay

The absorbance of methionine at different concentrations was measured using the 10-phenanthroline-iron (III) assay. The assay was performed on three separate days (Fig.

11). It was evident that the assay was not repeatable as the absorbance values varied at each concentration for the repeated experiments. In addition, the absorbance readings did not increase linearly with an increase in the concentration of methionine, but instead gave an essentially horizontal line. This assay, in the author's hands, was not reliable and was not used further.

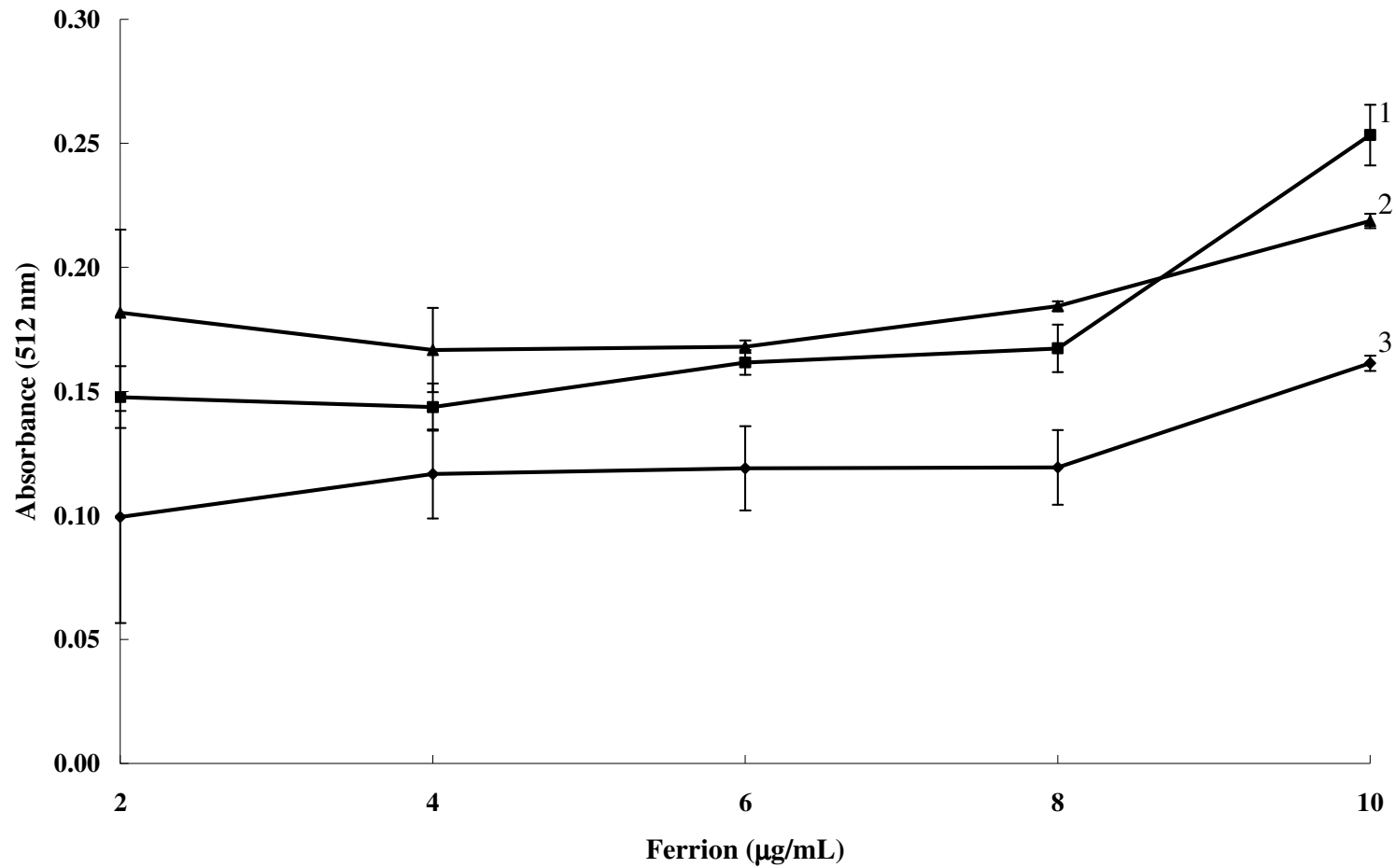


Figure 11: Absorbance of ferrion measured for the 10-Phenanthroline-iron (III) assay on three different occasions. Numbers indicate experiments performed on different days.
*Error bars indicate \pm one standard deviation

2.3.3 Coating of methionine with Zein Gold, Zein Standard and kafirin

Various methods were investigated to form a coating around the methionine. First, formation of the methionine/prolamin coating on a flat surface (stainless steel) was examined. Once the glacial acetic acid had evaporated off, a dry, hard methionine/prolamin sheet formed. This sheet proved extremely difficult to remove from the tray. In the review by Lawton (2002) the use of zein as a floor coating for steamship engine rooms is mentioned. Zein was used for its grease resistance and durability. These zein floor coatings could not, however, be removed from the steamship engine room floors. When removed from the flat surface, the coatings broke into pieces and were not uniform in size or shape. A concern was that breaking the sheet into pieces would result in exposed, uncoated methionine on the edges of the individual broken pieces. This could possibly result in a burst release of methionine during the dissolution test. This is undesirable as too much methionine will initially be released from the coating. A further problem was that the sheet took approximately 3 days to dry completely. To avoid the problems of the long drying time and exposed methionine on the edges, another method of forming the coating around methionine was investigated.

Extrusion using a hand mincer was used to shape the methionine/protein paste into pellets (Fig. 8b). All the dry extrudates had a rough, irregular string-like appearance (Fig. 12). They were, however, all similar in cross-sectional diameter ranging from 1.2 to 2.5 mm. There were a number of problems in using the hand mincer to form the extrudates. First, the consistency of the paste affected extrudate formation. A soft paste did not hold the shape of the extrudate but a paste that was too firm could barely be forced through the hand mincer. The hand mincer was cleaned with difficulty as the methionine/prolamin paste was extremely sticky. After 36 hr the extrudates were dry but a glacial acetic acid odour could still be detected. Drouillard et al 2005 state that oven drying, spray drying and vacuum drying can be used to remove excess solvent from the protein film of the coated sample. The Zein Gold coated methionine had a dull yellow colour, while the Zein Standard had a dark orange-yellow colour. The colour of the kafirin coated methionine was grey. The colours of the zein coated methionine were probably as a result of the carotenoid, xanthophylls and other pigments present in the zein (Shukla and Cheryan 2001). Since the Zein Standard was

more impure, more carotenoids were expected to be present and hence the darker yellow colour. The colour of the kafirin coated methionine was affected by the aluminium blade used to blend the sample. This blade was not used for the zein samples. This extrusion method of shaping the methionine/prolamin paste was an improvement in comparison to formation on a flat surface, as there was a shorter drying time and it did not result in exposed methionine of the edges of the coating. Forcing the methionine/zein paste through a sieve was then attempted in order to improve the hand mincer method. The sieve was used as smaller quantities of the methionine/prolamin paste was needed in comparison to the hand mincer. It was also thought that the sieve would be easier to clean. However, the methionine/prolamin paste formed a clump and failed to separate into individual extrudates (Fig. 9b) after being forced through a sieve. Similar difficulties as with the hand mincer arose when using the sieve. In addition, the extrudate took longer to dry, approximately 3 days, due to clumping of the extrudate. The methionine/prolamin clump which was pushed through the sieve had a smaller surface area in comparison to the extrudate formed by the hand mincer. The glacial acetic acid evaporated off more slowly from the smaller surface area and led to a longer drying time.

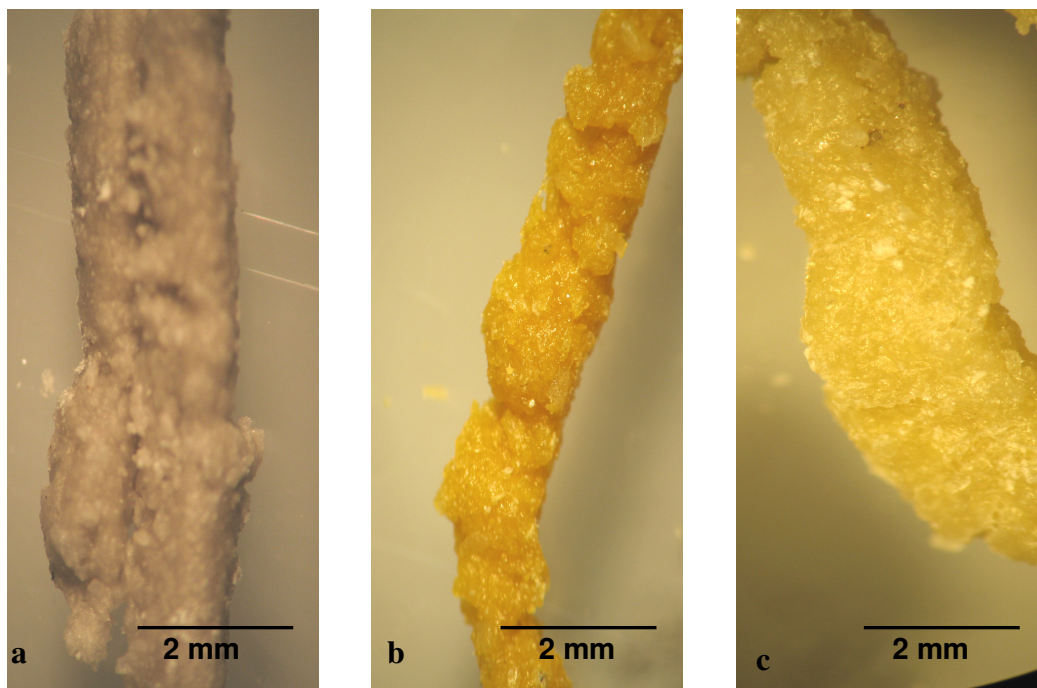


Figure 12: Stereomicrographs of protein coated methionine (1:4) formed by a hand mincer. (a) Kafirin coated methionine, (b) Zein Gold coated methionine, (c) Zein Standard coated methionine.

All coatings used in the dissolution tests were formed using the hand mincer as this method, even though not perfect, was the most effective.

2.3.4 Dissolution tests

Figure 13 shows the release of methionine from Zein Gold, Zein Standard and kafirin coatings. The dissolution test conditions were selected to simulate the abomasum division in the stomach of a cow (Ardaillon & Bourrain 1991). The incubation buffer and protein preparations without methionine released insignificant amounts of free amino nitrogen (FAN). These results were expected as no degradation of the protein coatings in the buffer was anticipated. The FAN from the methionine (uncoated) and the methionine coated with protein was thus solely from the methionine present. The amount of FAN that solubilized for the methionine (uncoated) control increased during the first 30 min of the dissolution test. After 30 min all the methionine (uncoated) had dissolved in the buffer and remained at a relatively constant level. The FAN solubilised for the methionine (uncoated) control showed a slightly higher value than the actual amount of methionine which was added to the buffer (8 mg/mL) after 30 min. This anomaly was because during the dissolution test aliquots were removed at time intervals. This decreased the amount of buffer present before all the methionine was dissolved.

The FAN released from the prolamin coated methionine increased over the total incubation period of 1.5 hr. This indicated that there was a slower release of methionine from the prolamin coating in comparison to the methionine (uncoated) control. These results showed that the prolamin coatings had a barrier effect on the release of methionine. This finding was in accordance with literature and patents which state that prolamins have barrier properties and can be used as coatings for the controlled release of substances (for example, Ardillon & Bourrain 1991, Cuq et al 1998, Drouillard et al 2005). After 30 min, Zein Standard and kafirin coated methionine started to show an asymptotic tendency towards the methionine (uncoated) control after 1.5 hr. The Zein Standard coated methionine had the most rapid release of FAN, where approximately 7.5 mg/mL of FAN was released after 1.5 hr. Zein Gold coated methionine showed the slowest release of FAN at approximately 5.7 mg/mL after 1.5 hr. The kafirin coated methionine showed an intermediate release of approximately 7.2 mg/mL.

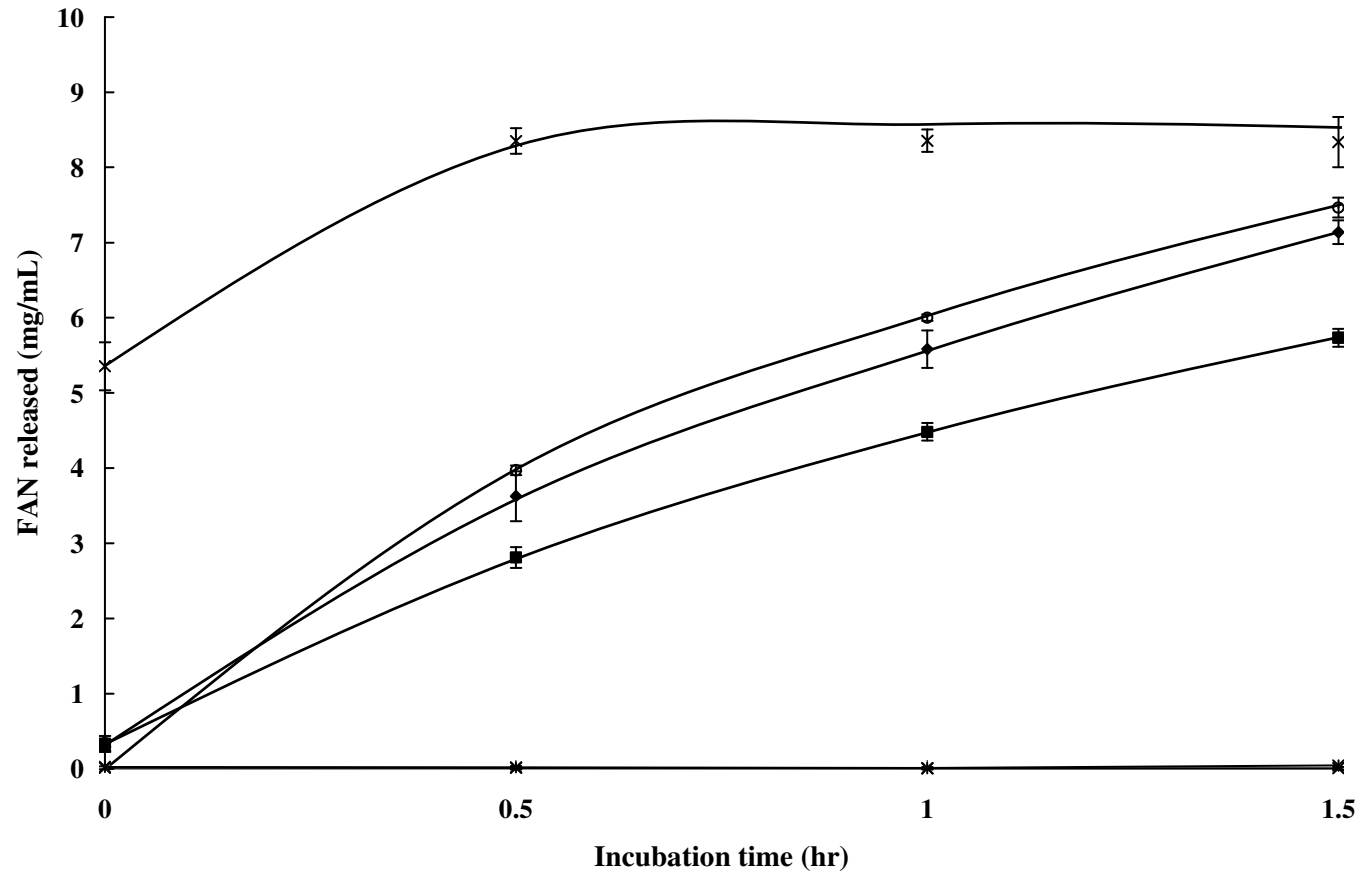


Figure 13: Effect of Zein Gold, Zein Standard and kafirin coated methionine (4:1) on the FAN released at pH 2, 37°C. * Buffer only, kafirin only, Zein Gold only, Zein Standard only; ■ Zein Gold coated methionine; ♦ kafirin coated methionine; ○ Zein Standard coated methionine; x methionine (uncoated).

* Error bars indicate \pm one standard deviation

The protein with the highest purity, Zein Gold (91%), seemed to be the most effective barrier as it gave the slowest release of methionine. However, the methionine released from the coating can be recalculated to give adjusted values, assuming the protein preparations were pure protein. The calculation showed that kafirin would be the most effective barrier if it was pure protein. Extraneous matter present in the kafirin protein preparations probably interferes with the barrier properties of the coating.

Even though kafirin may possibly result in a coating with better barrier properties, it would have to undergo purification to render an essentially pure protein. In the available state of the proteins, Zein Gold showed the best barrier properties, and consequently further experiments were done using this protein as the coating agent. The effect of protein coating to methionine ratio on methionine released was determined for Zein Gold (Fig. 14). In contrast to the previous dissolution test, this test was performed at pH 5.5 to simulate the rumen division in the stomach of a cow (Ardaillon & Bourrain 1991). This was done as these are the conditions the coated substance will first be exposed to in the stomach of the cow. As with the previous test the percentage methionine (uncoated) released was slightly above the maximum amount added to the buffer. The lowest ratio of Zein Gold to methionine (1:4) released the most methionine. After approximately 6 hr all the methionine was released. The release trend of methionine exceeded the maximum amount present after 6 hr, resulting in a methionine value which was apparently higher than was actually present. This can again be explained by the fact that the buffer was removed at time intervals before all the methionine had dissolved. An increase in the ratio of zein used for the coating led to a decrease in the methionine released. After 1.5 hr the Zein Gold coated methionine at ratios of 1:1 and 4:1 had released two and a half times less methionine than the Zein Gold coated methionine (1:4). Zein Gold coated methionine at ratios of 1:1 and 4:1 released approximately the same amount of methionine, about 80% over 8 hr. Thus an improvement in barrier properties with an increase in coating material appears to only occur until a certain point, or threshold, where the coating agent is at an optimum ratio to the methionine. The optimum coating ratio may be less than 50% Zein Gold. However, it is evident that the optimum coating ratio is well above 1:4. The trend of a decreased release of an active

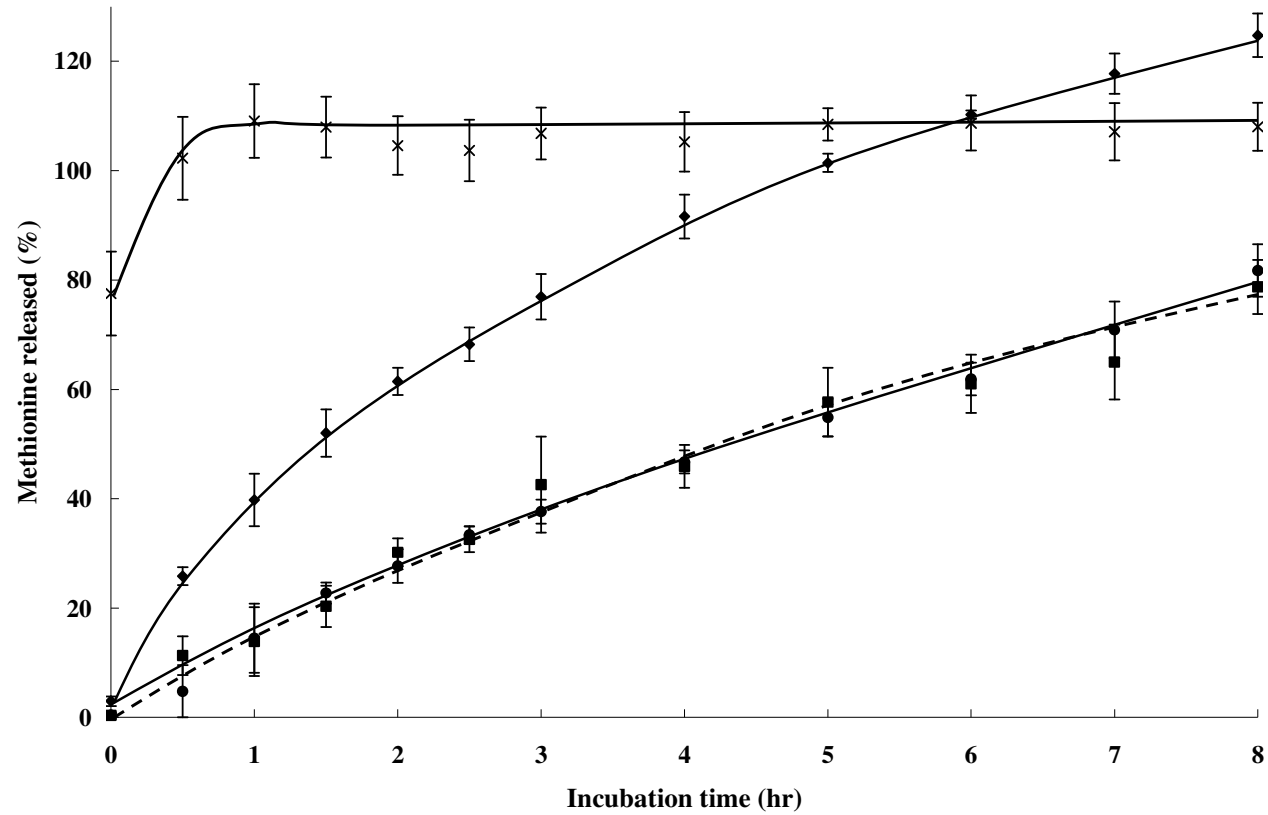


Figure 14: Effect of different proportions of Zein Gold used to coat methionine on methionine release at pH 5.5, 39°C. ■ Zein Gold coated methionine (4:1); ● Zein Gold coated methionine (1:1); ♦ Zein Gold coated methionine (1:4); x methionine (uncoated).

*Error bars indicate \pm one standard deviation

substance due to an increased coating concentration was observed by O'Donnell et al. (1997) and also shown on the data sheet of Kobayashi Zein DP (2004). O'Donnell et al. (1997) used different concentrations of a zein pseudolatex to coat tablets. Kobayashi Zein PD, a deoderized and decolourized zein protein, was used to coat a placebo tablet at different concentrations. Both coatings contained various other additives and were used at low coating material ratios, where not more than 10% of the coating material was used.

As Zein Gold coated methionine at a ratio of 1:4 was the least effective barrier the Zein Gold coated methionine at ratios of 1:1 and 4:1 were used in the subsequent experiment. The coated methionine was heat treated in an attempt to increase the barrier properties of the coating. The effect of heat treatment was demonstrated by Byaruhanga et al. (2007). They found that kafirin films which were heat treated with microwave energy were stronger and less extensible. The effect of heat treatment of the Zein Gold coating on the methionine released was determined (Fig. 15). A temperature of 103°C was used to heat treat the Zein Gold coated methionine, Zein Gold on its own and methionine. This temperature was chosen as Byaruhanga et al. (2006) used a temperature of 96°C to heat treat kafirin powder in water using microwave heating. Their aim was to determine the effect of the heat treated kafirin on the molecular causes for changes in film tensile properties. They found that heat treatment of kafirin cause cross-links of kafirin to form by disulphide bonding. Thus, a temperature of approximately 100°C appeared to be suitable for improving the barrier properties of the zein coating. Pelosi (1997) heated zein protein at 130°C to induce cross links in the presence of water with the aim of producing a stable, water resistant zein without the use of chemical cross-linking agents. This author claimed that the heat treatment rendered the zein insoluble in 50% acetic acid and highly water resistant. In the work reported here a dry heat treatment was applied rather than a wet heat treatment. A dry heat treatment was used because it was assumed that a wet heat treatment would result in leaching out of the methionine from the coating as occurred during the dissolution tests. After heat treatment the coated methionine was a dark brown colour. This may have been an indication that the temperature was too high and possibly resulted in a scorched or scorched sample. Byaruhanga, Erasmus, Emmambux & Taylor (2007) also found colour change when they heat treated cast kafirin films by microwave heating. They found that a high power (120 W) gave the

films a burnt colour. They stated that the colour change was the primary negative effect of heat treatment of the films. At both ratios of zein to methionine, the heat treated coatings released methionine at a slower rate than the coatings which were not heat treated. In fact, the heat treated coatings released methionine at a steady rate. The reason for the increased barrier properties of the heat treated samples may be due to formation of disulphide cross-links between the protein molecules of the zein. Byaruhanga et al. (2006) found that when kafirin was heated in water with microwave energy disulphide cross-links of kafirin were induced. This was due to a change in secondary structure of the kafirin from α -helical to β -sheet conformation, resulting in oligomers of kafirin. For the heat treated coatings there was no difference in the methionine released during the first hour of the dissolution test. After 1 hr the heat treated Zein Gold coated methionine with the higher ratio of protein (4:1) begun to release methionine more slowly and was thus the better barrier. It released 20% less methionine than the heat treated Zein Gold methionine with a lower protein ratio (1:1) and released 40% less methionine than the coating which were not heat treated.

Figure 16 combines the data from two separate experiments to show the effect of pH on methionine released from the Zein Gold coated methionine (20:80). Methionine was released from the Zein Gold coating slightly faster at pH 2 than at pH 5.5. O'Donnell et al. (1997) reported similar results with aqueous zein pseudolatex used to coat a drug in a tablet form. They found that as the pH of the dissolution media increased, the drug release decreased. According to these authors the faster release at a lower pH was due to the greater solubility of zein in an acidic environment.

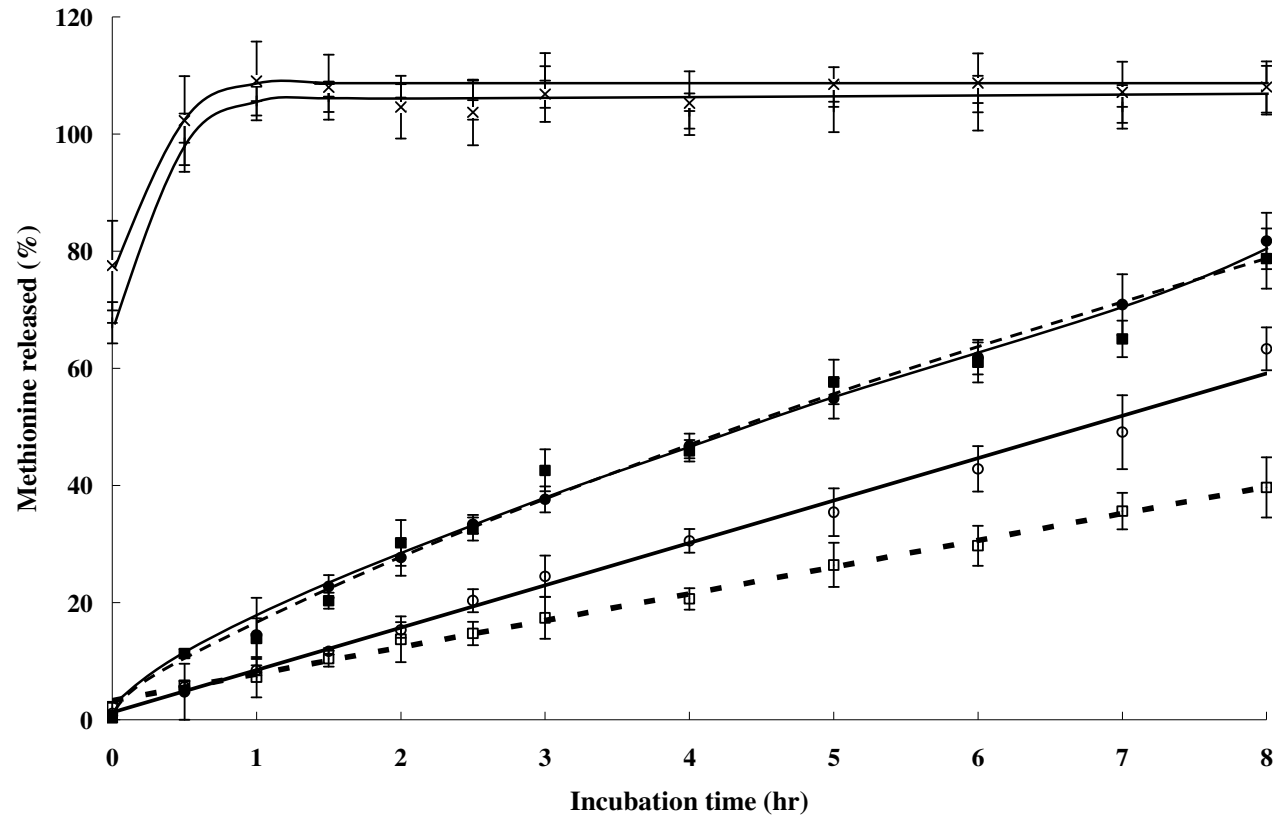


Figure 15: Effect of heat drying at 103°C on Zein Gold coated methionine on methionine released at pH 5.5, 39°C. ■ Unheated Zein Gold coated methionine (4:1); □ Heat treated Zein Gold coated methionine (4:1); ● Unheated Zein Gold coated methionine (1:1); ○ Heat treated Zein Gold coated methionine (1:1); + Heat treated methionine (uncoated), x Unheated methionine (uncoated).

* Error bars indicate \pm one standard deviation

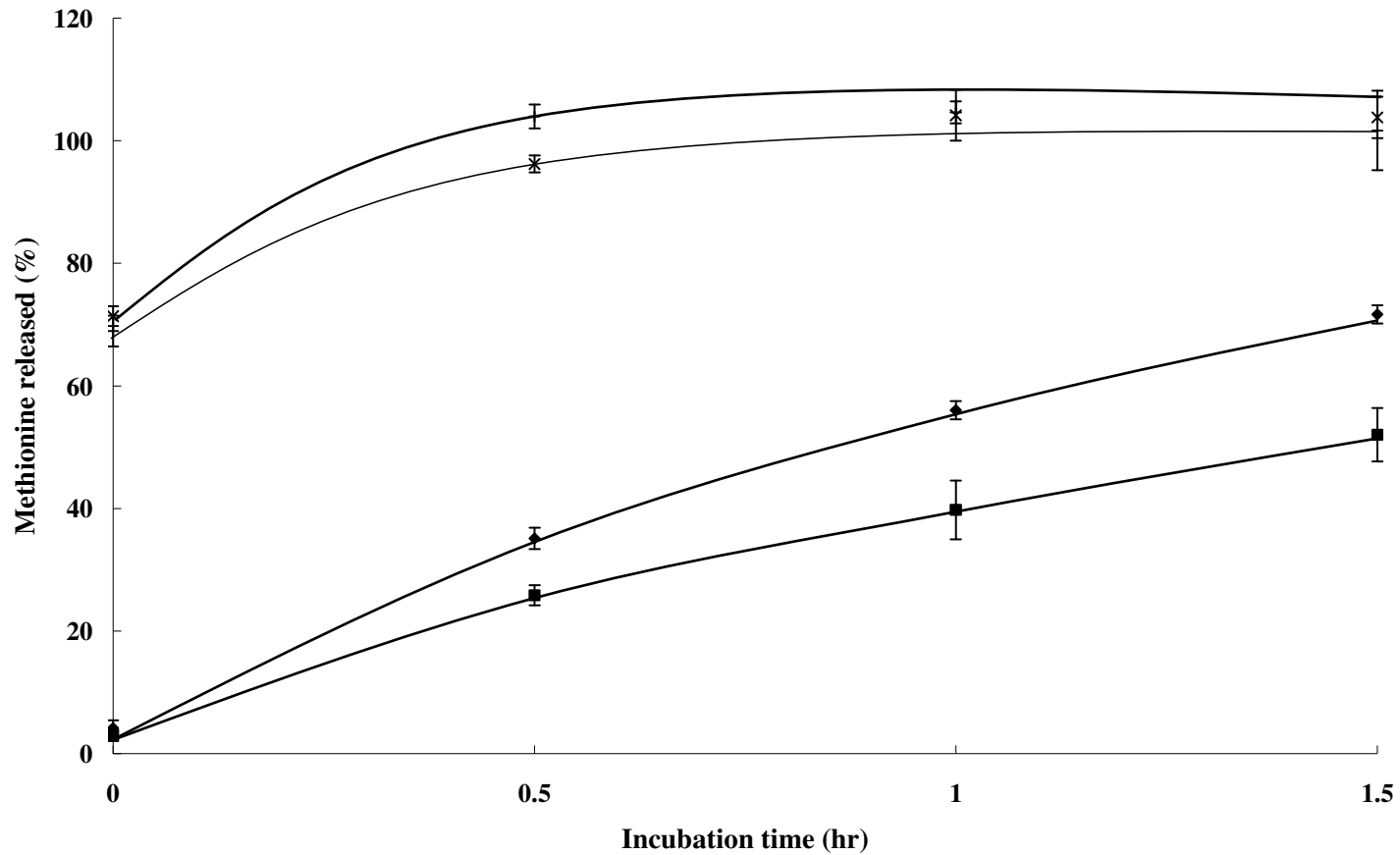


Figure 16: Effect of pH on the release of methionine from Zein Gold coatings (1:4). ■ Zein Gold coated methionine in pH 5.5 buffer; ◆ Zein Gold coated methionine in pH 2 buffer; + Methionine (uncoated) in pH 2 buffer; x Methionine (uncoated) in pH 5.5 buffer.

* Error bars indicate \pm one standard deviation

2.4 CONCLUSIONS

Zein Gold, Zein Standard and laboratory extracted kafirin all acted as barriers to methionine when used as coating agents. There is much potential for these proteins as controlled release barriers. However, the barrier properties of the protein coatings must be improved. This may be done by further research regarding heat treatments of the protein coatings as well as possible additives which may be incorporated into the coatings to increase the barrier properties. An alternative option for improving the barrier properties of the coatings is to reassess the approach used for the formation of the protein coatings by using protein microparticles as coating agents.

2.5 REFERENCES

- AACC International. 2000. Approved Methods of the American Association of Cereal Chemists, 10th Ed. Approved Methods 30-25, 44-15A and 46-30. The Association. St. Paul, MN.
- Ardailon, P., and Bourrain, P. 1991. Granules for feeding ruminants with enzymatically degradable coating. United States Patent and Trademark Office. Patent number: 4983403.
- Autant, P., Cartillier, A., and Pigeon, R. 1989. United States Patent and Trademark Office. Patent number: 4876067.
- Byaruhanga, Y.B., Emmambux, M.N., Belton, P.S., Wellner, N., Ng, K.G., and Taylor, J.R.N. 2006. Alteration of kafirin and kafirin film structure by heating with microwave energy and tannin complexation. *J. Agric. Food Chem.* 54: 4198-4207.
- Byaruhanga, Y.B., Erasmus, C., Emmambux, M.N., and Taylor, J.R.N. 2007. Effect of heating cast kafirin films on their functional properties. *J. Sci. Food Agric.* 87: 167-175.
- Da Silva, L.S., and Taylor, J.R.N. 2004. Sorghum bran as a potential source of kafirin. *Cereal Chem.* 81: 322-327.

De Rose, R.T., Din-Pow, M., Kwon, I., Hasnain, S.E., Klassy, R.C., and Hall, T.C. 1989. Characterization of the kafirin gene family from sorghum reveals extensive homology with zein from maize. *Plant Mol. Biol* 12: 245-256.

Donhowe, I.G., and Fennema, O. 1994. Edible films and coatings: Characteristics, formation, definitions, and testing methods. Pages 1-24 in: *Edible coatings and films to improve food quality*. J.M. Krochta, E.A. Baldwin, and M. Nisperos-Carriedo, eds.. Technomic Publishing Company: PA, Lancaster.

Drouillard, J.S., Herald, T.J., and Greenquist, M. 2005. Method for encapsulation of orally ingested materials to alter the site of digestion, site of action, or stability. United States Patent and Trademark Office. Patent Application number: PCT/US2005/011898.

Duodu, K.G., Nunes, A., Delgadillo, I., Parker, M.L., Mills, E.N.C., Belton, P.S., and Taylor, J.R.N. 2002. Effect of grain structure and cooking on sorghum and maize *in vitro* protein digestibility. *J. Cereal Sci.* 35: 161-174.

Duodu, K.G., Taylor, J.R.N., Belton, P.S., and Hamaker, B.R. 2003. Factors affecting sorghum protein digestibility. *J. Cereal Sci.* 38: 117-131.

El-Brashy, A.M., and Al-Ghannam, S.M. 1996. Atomic absorption determination of some amino acids containing a sulphur group. *Anal Lett.* 29: 2713-2726.

El Nour, I.N., Peruffo, A.D.B., and Curioni, A. 1998. Characterisation of sorghum kafirins in relation to their cross-linking behaviour. *J. Cereal Sci.* 28: 197-207.

European Brewery Convention. 1987. Method 8.8.1. Ninhydrin colorimetric method (International Method). *Analytica- EBC*, 4th Ed., Brauerei- und Getranke-Rundschau, Zurich, p. E 141.

Gunasekaran, S., Xiao, L., and Ould Eleya, M.M. 2006. Whey protein concentrate hydrogels as bioactive carriers. *J. Appl. Polym. Sci.* 99: 2470-2476.

Hamaker, B.R., Kirleis, A.W., Butler, L.G., Axtell, J.D., and Mertz, E.T. 1987. Improving the in vitro protein digestibility of sorghum with reducing agents. P. Natl. Acad. Sci. USA. 84: 626-628.

Kobayashi Zein DP, 2004. CBC - Europe, www.cbc-europe.it , accessed 2006.

Nunes, A., Correia, I., Barros, A., and Delgadillo, I. 2005. Sequential in vitro pepsin digestion of uncooked and cooked sorghum and maize samples. J. Agric. Food Chem. 52: 2052-2058.

O'Donnell, P.B., Wu, C., Wang, J., Wang, L., Oshilack, B., Chasin, M., Bodmeier, R., and McGinity, J.W. 1997. Aqueous pseudolatex of zein for film coating of solid dosage forms. Eur. J. Pharm. Biopharm. 43: 83-89.

Oneda, F., & Ré, M.I. 2003. The effect of formulation variables on the dissolution and physical properties of spray-dried microspheres containing organic salt. Powder Technol. 130 (1-3): 377-384.

Pelosi, L.F. 1997. Crosslinking processing/agent for zein. United States Patent and Trademark Office. Patent number: 5596080.

Polan, C.E., Cummins, K.A., Sniffen, C.J., Muscato, T.V., Vicini, J.L., Crooker, B.A., Clark, J.H., Johnson, D.G., Otterby, D.E., Guillaume, B., Muller, L.D., Varga, G.A., Murray, R.A., and Peirce-Sandner, S.B. 1991. Responses of dairy cows to supplemental rumen-protected forms of methionine and lysine. J. Dairy Sci. 74: 2997-3013.

Shewry, P.R. 2002. The major seed storage proteins of spelt wheat, sorghum, millets and pseudocereals. Pages 1-24 in: P.S. Belton and J.R.N. Taylor, eds. Pseudocereals and Less Common Cereals – Grain Properties and Utilization Potential. Springer: Berlin.

Shukla, R., and Cheryan M. 2001. Zein: the industrial protein from corn. Ind. Crop. Prod. 13:171-192.

Taylor, J., Taylor, J.R.N., Dutton, M.F., and De Kock, S. 2005. Identification of kafirin film casting solvents. Food Chem. 90: 401-408.

Taylor, J.R.N., Schober, T.J., and Bean, S.R. 2006. Novel food and non-food uses for sorghum and millets. *J. Cereal Sci.* 44: 252-271.

Wall, J.S., and Paulis, J.W. 1978. Corn and Sorghum grain proteins. Pages 135-219 in: Y. Pomeranz, ed. *Advances in Cereal Science and Technology*. Vol II. American Association of Cereal Chemists: St. Paul, MN.

CHAPTER 3

3. KAFIRIN AND ZEIN MICROPARTICLE COATINGS OF METHIONINE

ABSTRACT

Zein and kafirin proteins proved to be good barriers for the controlled release of methionine. This barrier effect can possibly be increased if a different physical form of the proteins were used to coat methionine. In this work, the use of microspheres/microparticles made from kafirin and zein to coat methionine was investigated. The objective of the coating agents was to form a protective barrier for the amino acid methionine. The use of maize zein protein to make microparticles has specifically been mentioned in literature, however, no mention has been made of sorghum kafirin protein microparticles. Motivation for the used of kafirin to form microparticles stems from the fact that kafirin is more hydrophobic than zein. The higher hydrophobicity of kafirin may lead to improved barrier properties. It was found that microparticles could not be formed from commercial zein and thus only kafirin microparticles were investigated as a coating agent. A coating procedure which used microparticles was developed. The barrier effect of the microparticle coating was tested using dissolution assay and pepsin digestion. The kafirin microparticle coating around the methionine showed barrier properties, leading to a sustained release of methionine over time. Heat treatment and an extrusion process to form the kafirin microparticle coatings improved the barrier properties of the microparticle coatings by approximately 18%. Pepsin digestion of the kafirin microparticle coated methionine revealed that only 36 % of the coating was digested. This may have been due to the closely fused coating structure which was formed around the methionine. When compared to the kafirin and zein protein coatings, the microparticle coatings did not have significantly improved barrier properties. The kafirin microparticle coating which was heat treated and extruded had the same barrier properties as the heat treated Zein Gold coated methionine (Chapter 2).

3.1 INTRODUCTION

From the research in Chapter 2 it is evident that Zein and kafirin proteins proved to be good barriers for the controlled release of methionine. To possibly increase this barrier effect of the proteins, the physical form of the proteins was altered by the creation of microparticles from the proteins. Microspheres or microparticles are colloidal, generally sphere-shaped particles with diameters between 10 nm–2 mm (Coombes et al 2003). Microencapsulation involves enclosing a solid, liquid or gas inside a polymeric coating (Radwick & Burgess 2002). There are a number of research articles that relate to the formation of microparticles (microspheres/microcapsules) from zein protein. In these articles the function of the microparticles was to encapsulate various substances (Muthuselvi and Dhathathreyan 2006, Liu et al 2005, Hurtado-López & Murdan 2005). The microparticles in this research chapter were investigated for their use as coating agents. Coatings are described as materials which form thin continuous layers or coverings over the surface of foods (International Food Science Information Service 2005). The difference between microencapsulation and a coating may be difficult to distinguish. However, to clarify the difference, here a coating will be considered as the formation of a microparticle polymer matrix around the active substance whereas, encapsulation would involve incorporating the active substance into the microparticle.

Although there is no published information on kafirin microparticles as either encapsulation or coating agents this protein will also be considered as it is homologous to zein (DeRose et al 1989). It was postulated that the spherical structure of the microparticles may lead to improved barrier properties in comparison to that of the protein. The spherical nature of the microparticles may present a ordered structure for the formation of a matrix for the active substance to be entrapped.

There is a gap in literature regarding the use of microparticles as coating agents. As mentioned in Chapter 1, the only example of microparticles used as a coating agent found in literature was by O'Donnell et al. (1997). The microparticles (referred to as a zein pseudolatex) were used as coating agents where the maximum ratio of coating agent to material to be coated was 1:9. The coating was applied to Nu-core sugar spheres and

Acetaminophen tablets by using a fluid bed coater and a Freund mini hi-coater (a tablet film coating apparatus), respectively.

One of the objectives of this research was to develop a process for the formation of a coating of zein and kafirin microparticles around particulate methionine. The methionine release kinetics from the microparticle coating was evaluated. Protection of the methionine from the conditions in the rumen was evaluated by the pepsin digestion assay.

3.2 MATERIALS AND METHODS

3.2.1 Raw materials

Grain of a mixture of white, tannin-free sorghum cultivars PANNAR PEX 202 and 206 was dehulled and hammer milled through a sieve with a aperture size opening of 800 μm . Two commercial zein products: ZPP Gold and ZPP Standard were obtained from Zein Protein Products, Marina, CA. Kafirin was extracted from decorticated white, tannin free sorghum grain on large scale by the Council for Scientific and Industrial Research (CSIR), Modderfontein, South Africa.

3.2.2 Kafirin extraction

Kafirin was extracted from the sorghum endosperm flour using the method of Emmambux and Taylor (2003) with 70% (w/w) aqueous ethanol containing 0.5% (w/w) sodium metabisulphite and 0.35% (w/w) sodium hydroxide.

3.2.3 Kafirin and zein microparticle preparation

The method of Taylor and Taylor (2006) was used to form the microparticles. Kafirin or zein (1.64 g protein), respectively was weighed into a 125 mL Erlenmeyer flask and mixed with 5 g ambient temperature glacial acetic acid. The temperature of the protein was slowly raised to 30°C to ensure full solvation. The protein solution was then allowed to 'rest' for 16 hr in a fume hood. This gave a 32% (w/w) protein solution in glacial acetic acid. The solution was then diluted 1:5 by slowly adding distilled water with a dropper,

while the solution was continuously stirred to give 6.4% (w/w) protein in 20% (w/w) acetic acid. Upon addition of water the microparticles were formed. The microparticle suspension was then allowed to rest for 16 hr.

The microparticles were separated from the acetic acid solution by centrifugation at 350 g for 5 min at ambient temperature and the supernatant removed. The microparticles were either used as is (in a slurry) or washed three times with distilled water before air drying. The microparticles were stored in 20% acetic acid at 5°C.

3.2.4 Coating of methionine with microparticles

Two different coating procedures were investigated.

Methionine stirred into microparticle slurry: Methionine powder was stirred into wet microparticles from which the acetic acid had been pipetted off, at a ratio of 4:1 (w/w). The material was placed on a flat tray and dried in fume hood at ambient temperature or at 103°C. Once dry, the protein coated methionine was broken into small pieces (3-5 mm diameter).

Mixing methionine into dry microparticles: Microparticles separated by centrifugation and washed with distilled water, were either air dried at ambient temperature in a fume hood. Once dry, the microparticles were ground into pieces using a pestle and mortar. The dried microparticles were mixed with methionine powder at a ratio of 1:1 (w/w). The powder mixture was then either mixed with 20% acetic acid (1:2) w/w or glacial acetic acid (1:2) w/w to form a paste with the aim of fusing together the microparticles around the methionine. The paste was then either dried directly on a flat surface or first underwent simple extrusion in a small syringe (approx. 1 mm diameter orifice, 3 mL volume) and cut while damp with a scalpel blade into pieces 5-7 mm in length. All preparations were either air dried or partially air dried and then heat treated in a force draft oven at 60-70°C overnight. This temperature was lower than that used previously (103°C) (Chapter 2) as that temperature seemed to have scorched the material. Once dry, the coated material was broken up into pieces (5-7 mm diameter) using a mortar and pestle (if dried on a flat surface) or separated into individual pieces if it underwent extrusion. The microparticle coated methionine preparations were then subjected to dissolution and simulated pepsin digestion assays to determine methionine release.

3.2.5 Analysis

2.4.5.1 Dissolution assay

As per Chapter 2

3.2.5.2 Pepsin digestion assay

A modification of the pepsin *in-vitro* protein digestibility assay of Hamaker et al. (1986) was used. The microparticle coated methionine material (100 mg) was weighed into a 50 mL centrifuge tube. Microparticles treated in the same manner but without methionine, were used as the controls (50 mg). Pepsin (1.07190.0100 2000 FIP –U/g, Merck, Halfway House, South Africa) was added to the pH 2.0 citrate buffer solution to give 105 mg pepsin/100 mL buffer. Thirty five mL of the pepsin solution was added to the microparticle coated methionine and controls which were suspended by swirling. Microparticle coated methionine and control samples in the buffer solution without pepsin were included as additional controls. The tubes were incubated for 2 hr at 37°C in a shaking water bath with vortex mixing every 15 min. At time intervals of 15 min (before vortexing) aliquots were withdrawn (including time zero) and placed in boiling water (10 min) to inactivate the enzyme. The same amount of buffer was then added back to the sample being incubated. After 2 hr the reaction was stopped by the addition of 2 mL 2M sodium hydroxide. Centrifugation was not necessary to separate the coated material and controls from the buffer as the samples were already in a pellet form which settled easily. Clear supernatant was carefully pipetted off and the residue washed with distilled water. The distilled water was carefully pipetted off. Residues were dried in the centrifuge tubes at 100°C overnight. The aliquots which had been withdrawn every 15 min were then diluted and methionine content determined using the ninhydrin assay (Chapter 2). Samples were analysed in duplicate and each experiment was repeated at least once to give a total of four values per sample.

$$\text{Protein digestibility} = \frac{(\text{OP} - \text{RP})}{\text{OP}} \times 100$$

Where: Original weight of protein (OP) = g coating before digestion x % protein

Residual weight of protein (RP) = (g sample after drying – RM) x % protein

Weight of residual material (RM) = OM – MR

Where: OM = Original material

MR = Weight of methionine released

3.2.5.3 SDS-PAGE

As per Chapter 2.

3.2.5.4 Protein content

As per Chapter 2.

3.2.6 Microscopy

Stereomicroscopy

Samples were viewed using a Carl Zeiss Stemi DV4/DR stereomicroscope (Carl Zeiss, Germany).

Scanning electron microscopy (SEM)

The microparticles in acetic acid solution were fixed in glutaraldehyde in a pH 7.4 phosphate buffer and rinsed sequentially three times with the phosphate buffer. The sample was then fixed in aqueous osmium tetroxide. Ethanol was used to dehydrate the sample sequentially with increasing concentrations. Once dehydrated, the samples were subjected to critical point drying from liquid CO₂ after which the sample was mounted on stub and sputtered coated with gold.

Dry samples were simply mounted on a stub and sputter coated with gold before viewing with JEOL-JSM 840 scanning electron microscope at 5 kV (JEOL, Tokyo, Japan). Some of the dry samples were sectioned using a scalpel blade.

3.2.7 Statistical analysis

Experiments were repeated twice for both kafirin and zein microparticle coatings. One way analysis of variance (ANOVA) and Fisher's LSD test was performed.

3.3 RESULTS AND DISCUSSION

3.3.1 Analysis of laboratory prepared kafirin

The kafirin extracted by the CSIR will be designated as CSIR kafirin and the kafirin extracted as described in the Materials and Methods in 2.2.2.3 will be designated at laboratory prepared kafirin.

Table II: Proximate composition of laboratory prepared kafirin

	Protein (g/100 g)	Moisture (g/100 g)
Laboratory prepared Kafirin	78.5 ± 0.6 (85.0)	8.98 ± 0.10

1. Dry basis indicated in parentheses
2. ± Indicates one standard deviation

Laboratory prepared kafirin which was extracted as described by Emmambux and Taylor (2003), had a higher protein purity in comparison to the CSIR kafirin (Chapter 2). Protein purity (db) of the protein preparations used in this experimental work were, in ascending order: CSIR kafirin (68.1%), Zein Standard (74.7%), laboratory prepared kafirin (85.0%) and Zein Gold (96.0%).

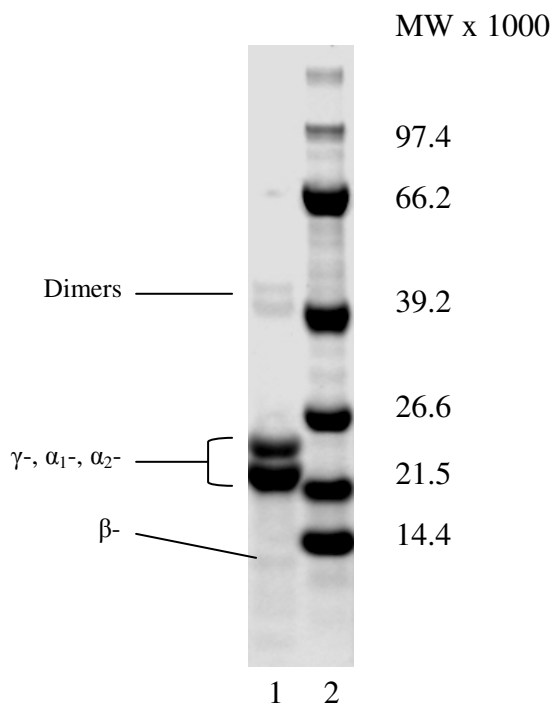


Figure 17: Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions of laboratory prepared kafirin. Track 1- laboratory prepared kafirin, Track 2 -molecular weight standard.

SDS-PAGE (Fig. 17) for the laboratory prepared kafirin showed the presence of α -, β - and γ -fractions of the protein (Shewry 2002). According to the SDS-PAGE results the laboratory extracted kafirin was essentially the same as the CSIR kafirin (Chapter 2). For further explanation regarding the SDS-PAGE results refer to Chapter 2. No bands other than the above named were visible on the SDS-PAGE in the laboratory prepared kafirin. This indicated that the kafirin preparation was pure in terms of protein composition.

3.3.2 Formation of microparticles and coating around methionine

Zein Gold, Zein standard, CSIR kafirin and laboratory prepared kafirin were evaluated for their ability to form microparticles. After approximately one quarter of the distilled water was added to the dissolved proteins in glacial acetic acid, Zein Standard and the CSIR kafirin aggregated. Protein particles were observed to aggregate into fibres and clumps. This formation of ‘Taffy’ by zein is mentioned by Lawton (2002). Thus, Zein Standard and CSIR kafirin failed to form microparticles. This may have been due to the lower protein contents of these preparations in comparison to Zein Gold (74.7% db) and the laboratory prepared kafirin (68.1% db). Research papers which pertain to zein microparticles do not mention the protein purity of the zein used in their preparations. However, two research articles which relate to whey protein microparticles give the protein content of the whey used to make them as being above 95% protein (Lee & Rosenburg 1999, Lee & Rosenburg 2002). No aggregation of the Zein Gold and the laboratory prepared kafirin protein occurred when distilled water was added to form microparticles. Cloud formation occurred with every drop of water added which was then dispersed through the stirring action of the magnetic stirrer. Once all the water was added the outcome was an opaque mixture. When the mixture was left to stand, a distinct separation was visible. It was assumed that these were the microparticles settling out. Microscopy was then used to confirm microparticle formation. Due to their inability to form microparticles Zein Standard and the CSIR kafirin were not used further. Hence for simplicity, the laboratory prepared kafirin will simply be referred to as kafirin for the remainder of this research chapter.

The kafirin microparticles were mostly spherical shaped and had a wide size distribution of between 1.5-9.0 μm (Fig. 18a). The kafirin microparticles had a rough surface and pores of varied shape and size (0.01-0.1 μm diameter). Few protein fibres on and between the kafirin microparticles were observed. Large holes (1-3 μm diameter)

appeared in some of the microparticles. In contrast, very few microparticles were formed from the Zein Gold (Fig. 18b). Instead, a mat of protein fibres with randomly distributed microparticles was observed. A reason for the failure of Zein Gold to form microparticles may be the small proportions of β - and γ -zein fractions present as commercial zein contains mainly α -zein (Lawton 2002). There are, however, various authors which have formed microparticles from commercial zein (Hurtado-López & Murdan 2005, Lui et al. 2005, O'Donnell et al. 1997, Muthuselvi and Dhathathreyan 2006).

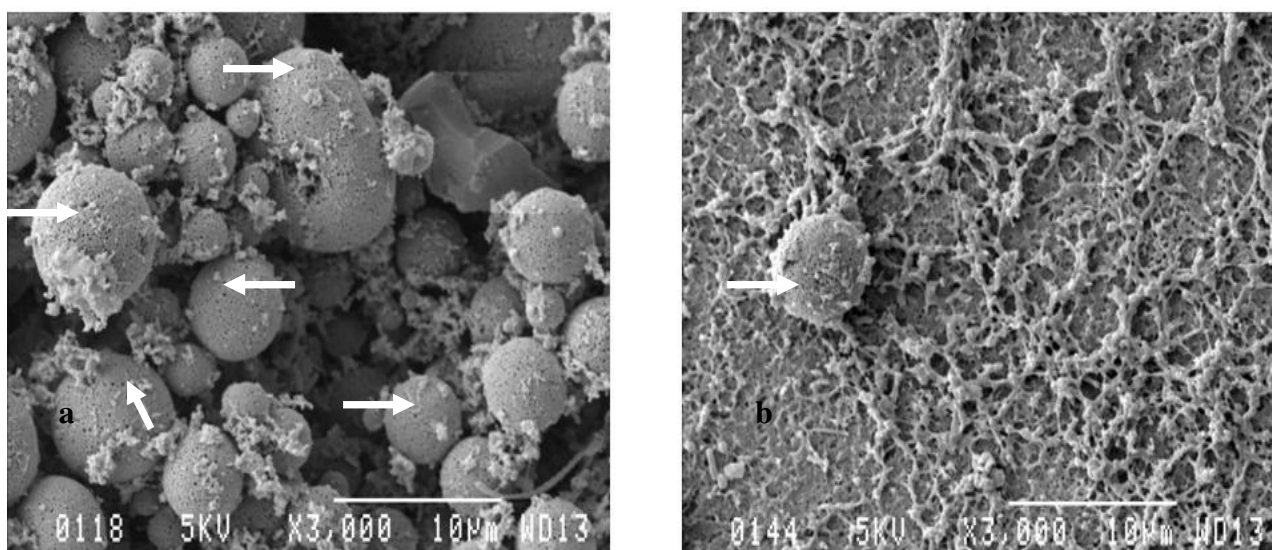


Figure 18: SEM of microparticles. a) Microparticles made from kafirin, b) Microparticles made from Zein Gold. Arrows indicate pores.

3.3.3 Coating methods

Two procedures to coat methionine using microparticles were investigated. The coating procedures and resulting coated material will first be discussed in the following section. The results of the dissolution tests and release characteristics of methionine from the various formed coating will then be discussed in 3.3.4.

Methionine stirred into microparticle slurry:

To form a film from kafirin microparticles by solvent evaporation, the microparticles must be suspended in a minimum of 20% acetic acid (Taylor and Taylor 2006). It was postulated that when the solvent evaporated, the protein microparticles would fuse together to form a coating around the methionine. In fact, however, when the methionine powder was added to the microparticle dispersion, the protein microparticles aggregated (not shown). It was visually

evident that very little, if any, of the microparticles formed a coating around the methionine. This is probably because addition of methionine powder changed the pH of the microparticle dispersion, causing aggregation and precipitation of the microparticles. It was found that the pH of zein microparticles in 20% acetic acid was approximately 1.8. The pH increased to 2.5 when methionine was added at a ratio of 1:1. This change in pH may have caused the protein microparticles to aggregate. Another possible reason for the microparticle aggregation may have been interference of methionine with the forces that stabilize the microparticles. According to Kawaguchi (2000) there are three factors that affect the stability of polymeric microspheres. These are electrostatic repulsive forces, Van der Waal's forces and steric repulsive forces among the microparticles. It is possible that the addition of methionine interfered with these forces.

As a result of this unsuccessful attempt to coat the methionine it was decided to remove the microparticles from the 20% acetic acid before using them as coating material. Centrifugation resulted in separated kafirin microparticles which could be re-suspended in water. However, not all of the Zein microparticles separated from the acetic acid solution at low centrifugal force (350 *g*). At higher centrifugal force (620 *g*) the Zein Gold microparticles formed a hard pellet which did not re-suspend. SEM of the Zein Gold microparticles (Fig. 18b) provides an explanation for the hard pellet formation after centrifugation. As there was a low proportion of microparticles the centrifugation may have initiated the formation of stronger bonds and interactions between the protein fibres causing the formation of a hard pellet.

As the kafirin formed microparticles, the microparticles formed from this protein preparation were used to investigate it as a coating for methionine in subsequent experiments. Once the kafirin microparticles were separated by centrifugation they were air dried at ambient temperature. The air dried microparticles (Fig. 19b) differed significantly from the air dried kafirin protein (Fig. 19a). The dried kafirin protein formed large uneven, misshaped clumps of protein. The structure of the air dried microparticles was similar to that of the microparticles prepared for SEM (Fig. 18). The microparticles retained their spherical shape, porous nature and wide size distribution after air drying. Thus, air drying had no visible effect on the structure of the microparticles.

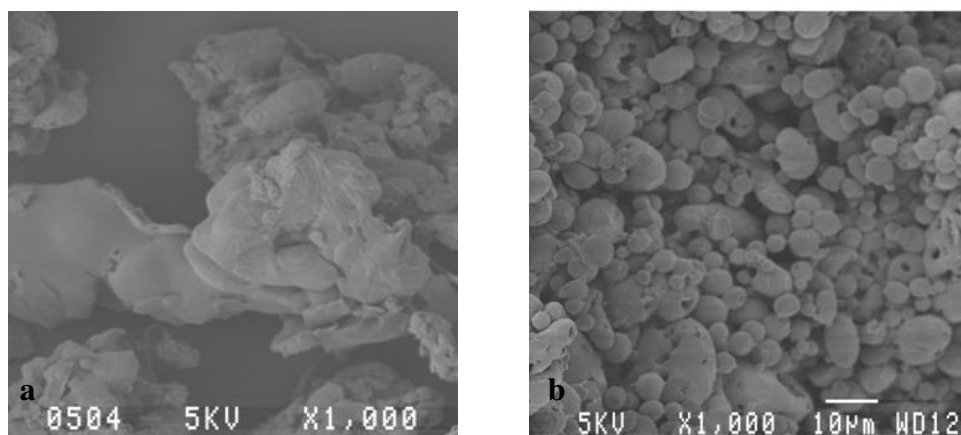


Figure 19: SEM of kafirin preparations (a) air dried kafirin protein, (b) air dried microparticles.

Mixing methionine into dry microparticles:

In the next attempt to coat methionine with kafirin microparticles an even dispersion of the kafirin microparticles and methionine powder was mixed with glacial acetic acid or 20% acetic acid. The objective of adding the solvent to the dry powder mixture was to fuse the protein microparticles together to form a coating. It was thought that the methionine powder dispersed between the kafirin microparticles would be entrapped in the newly formed microparticle matrix, forming a coating around the methionine. Once the solvent had evaporated from the microparticle matrix, it was broken into small pieces. A similar concern arose as reported in Chapter 2 that when breaking the dried microparticle coated methionine, methionine may be exposed on the edges where the coating was broken. It was postulated that the exposed methionine would result in a faster release of methionine from the coating. This will later be discussed when the methionine dissolution tests are described.

The microparticle coating formed by fusion using 20% acetic acid had a coarse, flaky, brittle appearance, while the microparticle coated methionine formed by fusion using glacial acetic acid had a slightly smoother and firmer appearance (Fig. 20). Both coatings had a glossy outer appearance. This was perhaps due to the uncoated, exposed methionine crystals, as the methionine crystals have a glossy appearance. The glossy appearance of methionine is shown in Figure 21. Another possible reason for the glossy appearance of the kafirin coating may simply be from the kafirin protein itself. Zein protein has been used for glossy protective coatings (reviewed by Shukla and Cheryan 2001, reviewed by Lawton 2002). Thus if zein has a glossy appearance when used as a coating then kafirin will probably also display this characteristic.

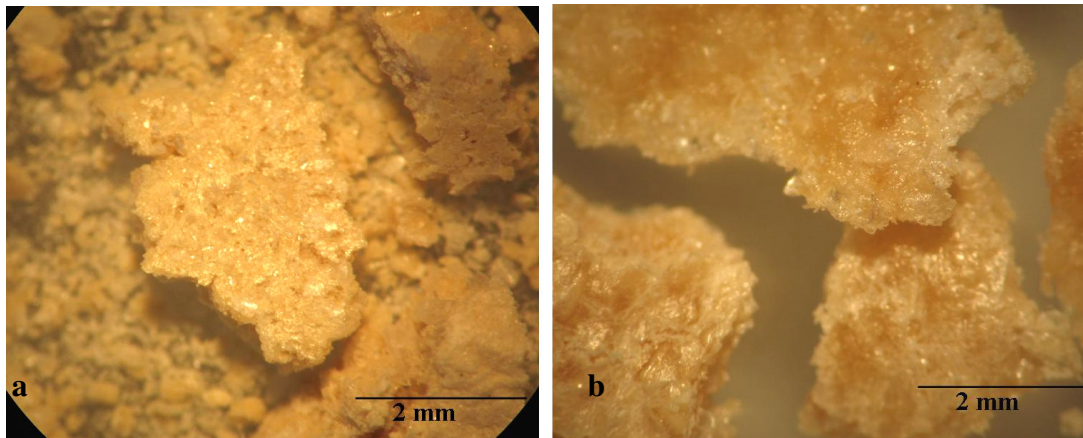


Figure 20: Stereomicrographs of kafirin microparticle coated methionine (1:1). a) Fused together with 20% acetic acid, b) fused together with glacial acetic.

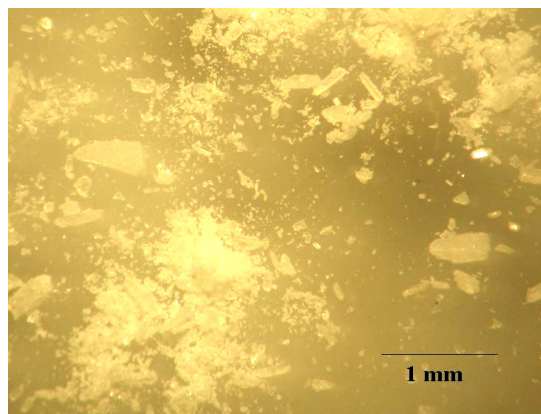


Figure 21: Stereomicrograph of methionine crystals

To avoid possible exposed methionine on the edges of the coating due to manual breaking, a process for the pellet formation of the kafirin microparticle and methionine fusion (with glacial acetic acid) was investigated. Glacial acetic acid was stirred into the microparticle/methionine mixture, the mixture was placed in a syringe and extruded onto a flat tray. Some of the samples were then heat treated because it had been found that heating of the material improved the barrier properties (Chapter 2). As proposed in Chapter 2, this may be due to the formation of heat induced disulphide bonds which form cross-links between the proteins. Visually there was no difference in external appearance between the heated and unheated samples (Fig. 22 and 23). When the coated material was examined, no exposed methionine on the edges of the coating was visible (Fig. 22 and 23). However, pores were visible on the underside (the side which was in contact with the flat surface) of the coated material (Figure 22), while none were seen on the upper side of the coated material. The pores may have been due to kafirin stabilized air bubbles. During

the mixing of the glacial acetic acid into the particulate mixture air was incorporated. Air could also have been incorporated when the wetted powders were placed in the syringe. As the wetted particulate mixture left the syringe the entrapped air bubbles may have burst due to a drop in pressure, creating the pores seen on the underside of the coating.

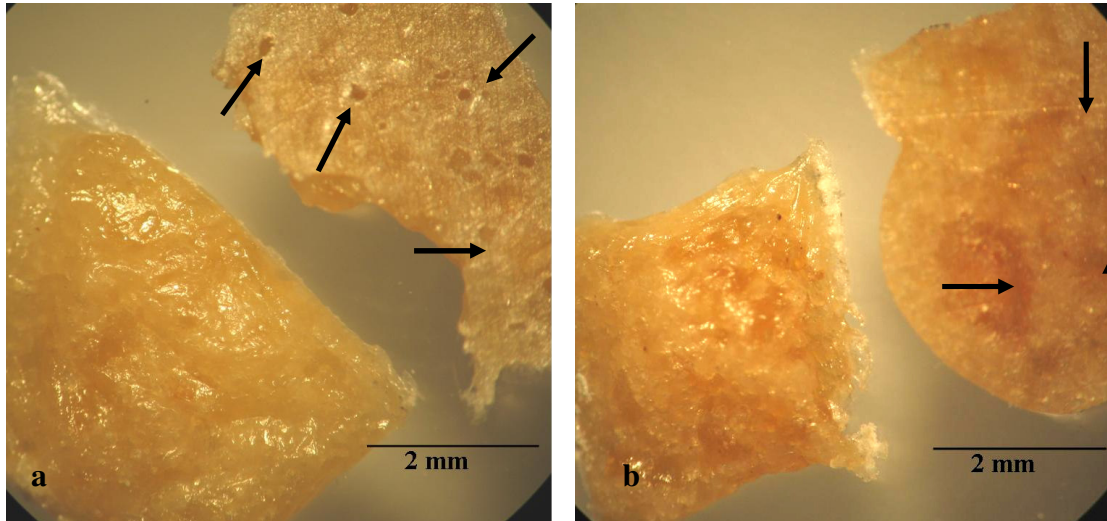


Figure 22: Stereomicrographs of the kafirin microparticle coated methionine (50:50) fused with glacial acetic acid, extruded through a syringe. a) air dried at ambient temperature, b) heat treated at 60-70°C. Pores are indicated by arrows.

* Note: the left pellet on each stereomicrograph represents the upper side of the pellet while the right represents the underside.

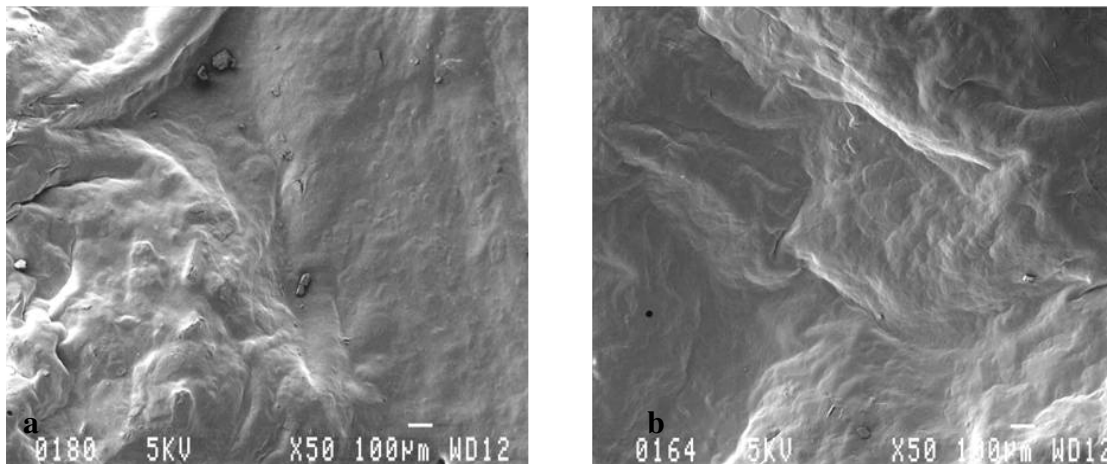


Figure 23: SEM of the kafirin microparticle coated methionine (1:1) fused together with glacial acetic acid, extruded through a syringe. a) air dried at ambient temperature, b) heat treated at 60-70°C.

3.3.4 Dissolution tests and methionine release characteristics

Dissolution results of coating prepared by stirring methionine into microparticle slurry:

The dried, aggregated microparticle-methionine material was subjected to the dissolution test. After the first 30 min the aggregated microparticle-methionine material had released all the methionine present (Fig. 24). The release of methionine from the aggregated Zein Gold-methionine and kafirin microparticle-methionine seemed to be slightly slower than the methionine (uncoated) during the first 30 min of the dissolution test. Possibly some of the methionine may have adhered to the outside of the aggregated Zein Gold and kafirin microparticles during the solvent evaporation. Such adherence could have slightly delayed the dissolution of the methionine into the buffer. The adhesive properties of zein are mentioned in the review of Lawton (2002), and Parris & Dickey (2003) demonstrated the adhesive properties of zein to glass. As previously mentioned, zein and kafirin have similar characteristics in terms of hydrophobicity and disulphide cross linking. Methionine may also have adhered to the kafirin microparticles which resulted in a similar, slight delay in the release of methionine as the Zein Gold aggregates. The insignificant difference of release between the microparticle-methionine and the methionine (uncoated) indicated that there was no formation of a barrier around the methionine. This was not expected as it was thought that when the solvent evaporated from the microparticles, it would lead to adherence of the microparticles to each other as well as round the methionine. The total methionine released from the methionine-aggregated protein was apparently slightly higher than the methionine (uncoated). This anomaly may have been because during the dissolution test aliquots were removed at time intervals (including time zero). This decreased the amount of buffer present before all the methionine was dissolved. This anomaly also occurred during the subsequent dissolution tests, and will not be mentioned again.

The effect on methionine release from a coating of microparticles fused together by glacial acetic acid or 20% acetic acid is shown in Figure 25. Incubation conditions for this dissolution test were chosen to simulate the first conditions which the kafirin microparticle coated methionine will encounter in the cow. The first section is the rumen with a pH of approximately 5.5 (Ardailon & Bourrain 1991). The kafirin microparticle coating created with the glacial acetic acid released the methionine more slowly than the microparticle coating created with the 20%

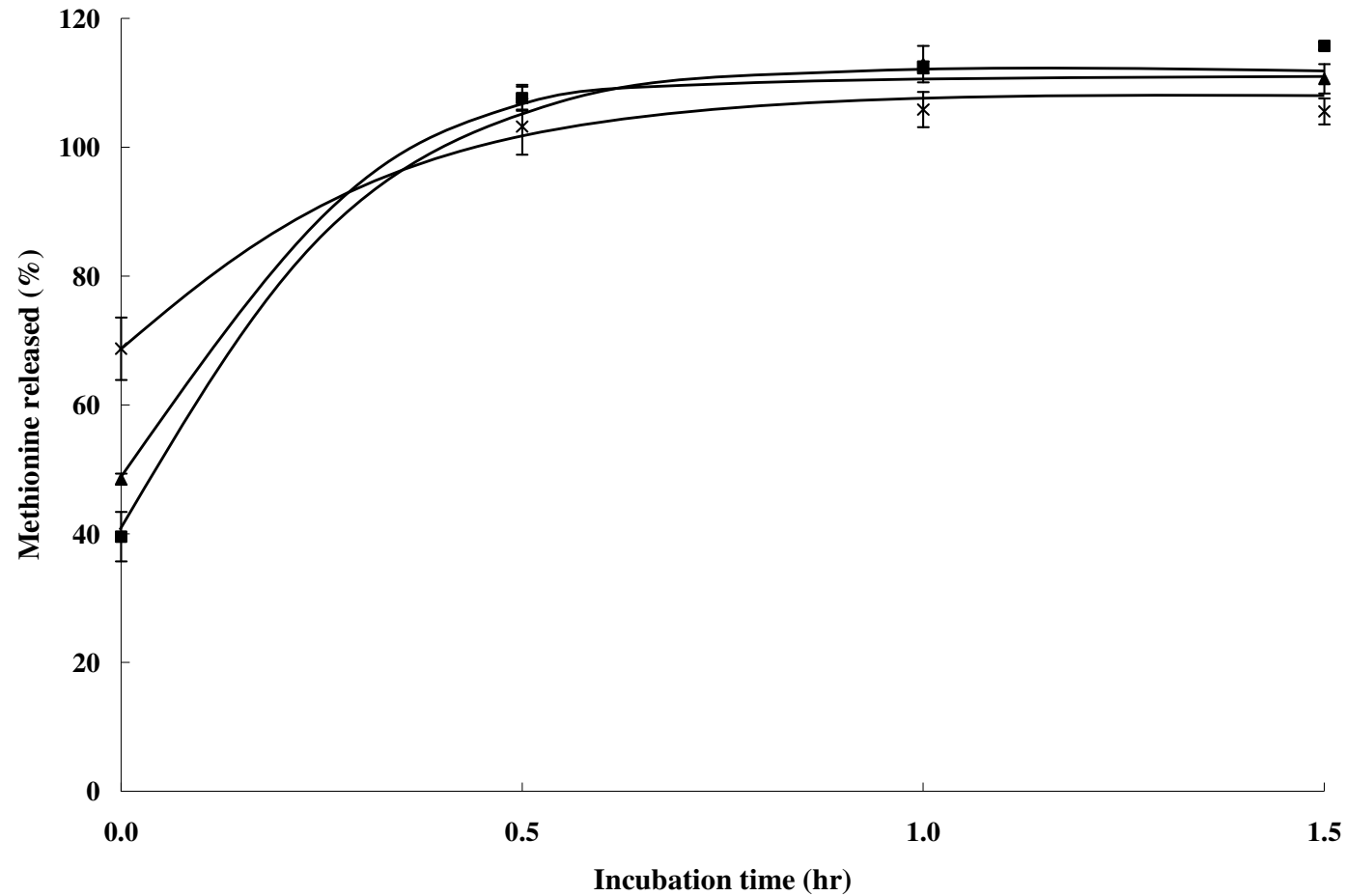


Figure 24: Effect of Zein Gold and kafirin microparticles mixed with methionine (1:4) and dried, on the release of methionine at pH 2, 39°C. —x— Methionine (uncoated), —■— Zein Gold microparticle-aggregated material, —▲— kafirin microparticle-aggregated material.

* Error bars indicate \pm one standard deviation

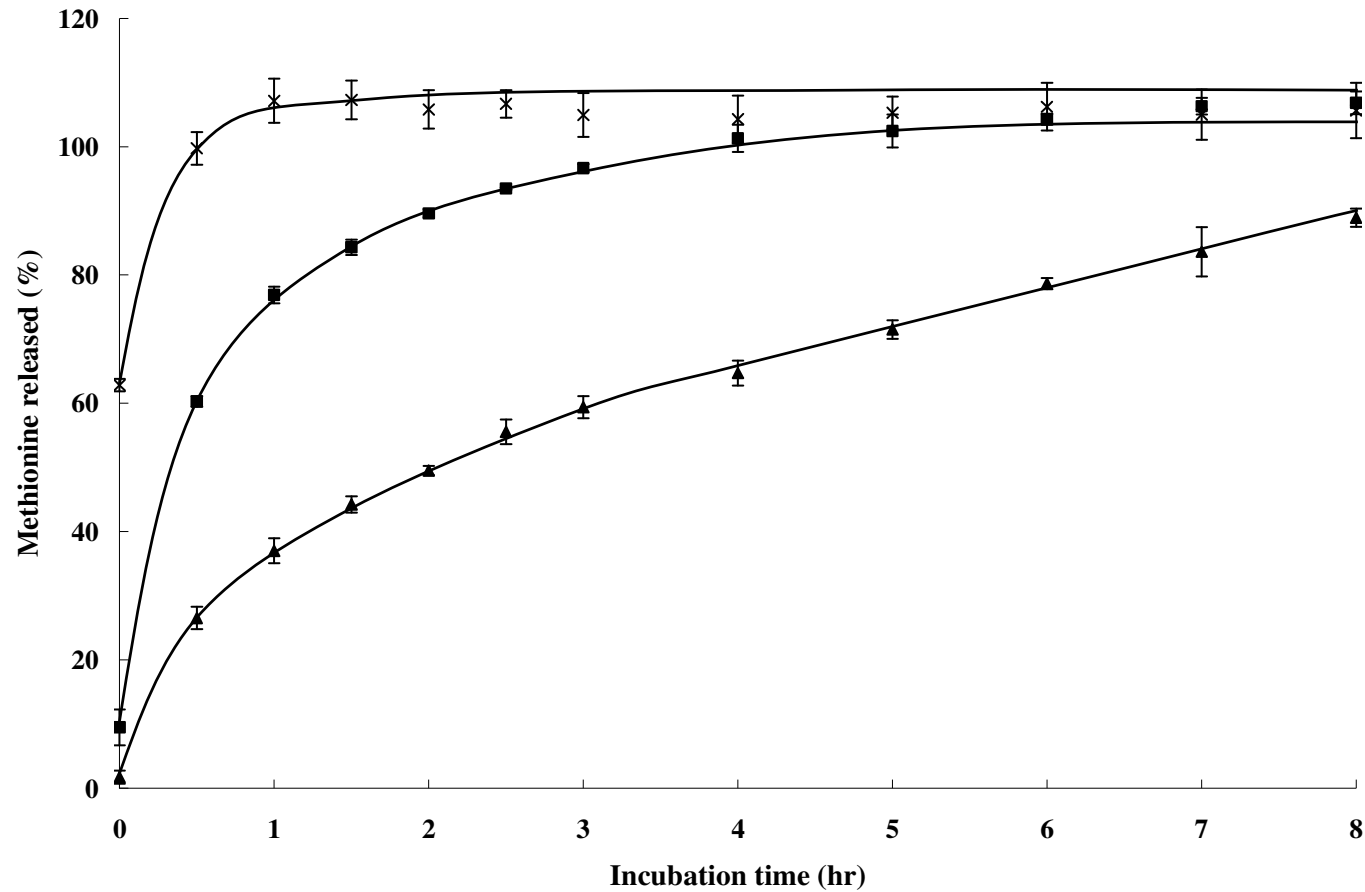


Figure 25: Effect of fusing kafirin microparticles to form a coating around methionine using 20% acetic acid or glacial acetic acid to form a coating (1:1) on the release of methionine at pH 5.5, 39°C. —x— Methionine (uncoated),—■— Kafirin microparticle coated methionine fused with 20% acetic acid, —▲— Kafirin microparticle coated methionine fused with glacial acetic acid.

- Error bars indicate \pm one standard deviation

acetic acid. After 1 hr the microparticle coating created with glacial acetic acid released approximately 50% less methionine in comparison to the microparticle coating created with 20% acetic acid. The more concentrated glacial acetic probably promotes superior fusion and adherence of the microparticles to each other. Taylor et al (2005) found that glacial acetic acid is a better solvent for kafirin than ethanol. The glacial acetic acid may have partly dissolved the outer surface of the microparticles creating “sticky edges” which were able to fuse together. After 5 hr all the methionine from the microparticle coating formed with 20% acetic acid was released, whereas approximately 80% of the methionine was released from the glacial acetic acid formed microparticle coating after 8 hr. Examination of the kafirin microparticle coatings after the dissolution test showed the disappearance of the coatings’ glossy surface and revealed the presence of pores (Fig. 26). Thus the pores must have formed during the dissolution test. It is possible that the methionine leached out of the coating through these pores. If the methionine crystals were responsible for the glossy appearance (as discussed previously), disappearance of the gloss would be expected, as the methionine entrapped close to the surface of the coating would have been released first.

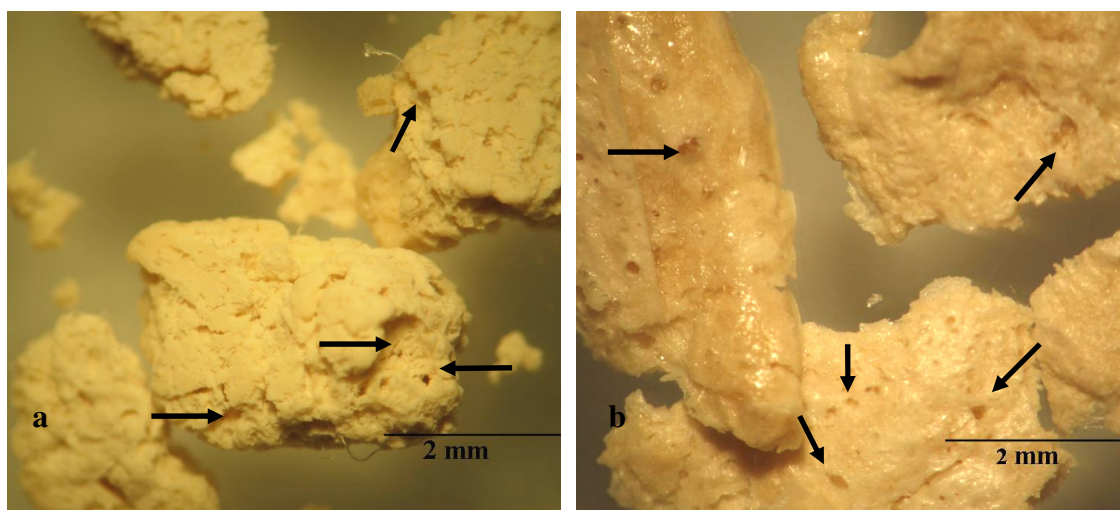


Figure 26: Stereomicrographs of kafirin microparticle coated methionine (50:50) after dissolution test. a) Fused together using 20% acetic acid, b) Fused together with glacial acetic acid. Pores are indicated by arrows.

As the kafirin microparticle coated methionine formed using glacial acetic acid showed superior barrier properties compared to that formed using 20% acetic acid, glacial acetic acid was used in the subsequent work to form microparticle coated material

Figure 27 shows that the heat treated microparticle coated methionine released methionine at a slower rate than the coating which was not heat treated. As stated in Chapter 2, it was thought that the improved barrier properties of the heat treated coatings was due to the formation of disulphide cross-links between the protein microparticles. After 8 hr the heat treated sample released approximately 15% less methionine than the sample that was not heat treated. As previously stated, no exposed methionine on the edges of the coating was visible. The dissolution test confirmed this as there was no burst release effect of methionine from the kafirin microparticle coatings (Fig. 27). After the dissolution test pores were seen on the upper and under sides of the coated material and the outer surface had a rough appearance (Figs. 28 and 29). The rough outer appearance of the coating may indicate that the buffer solution slightly corroded the external surface of the coating.

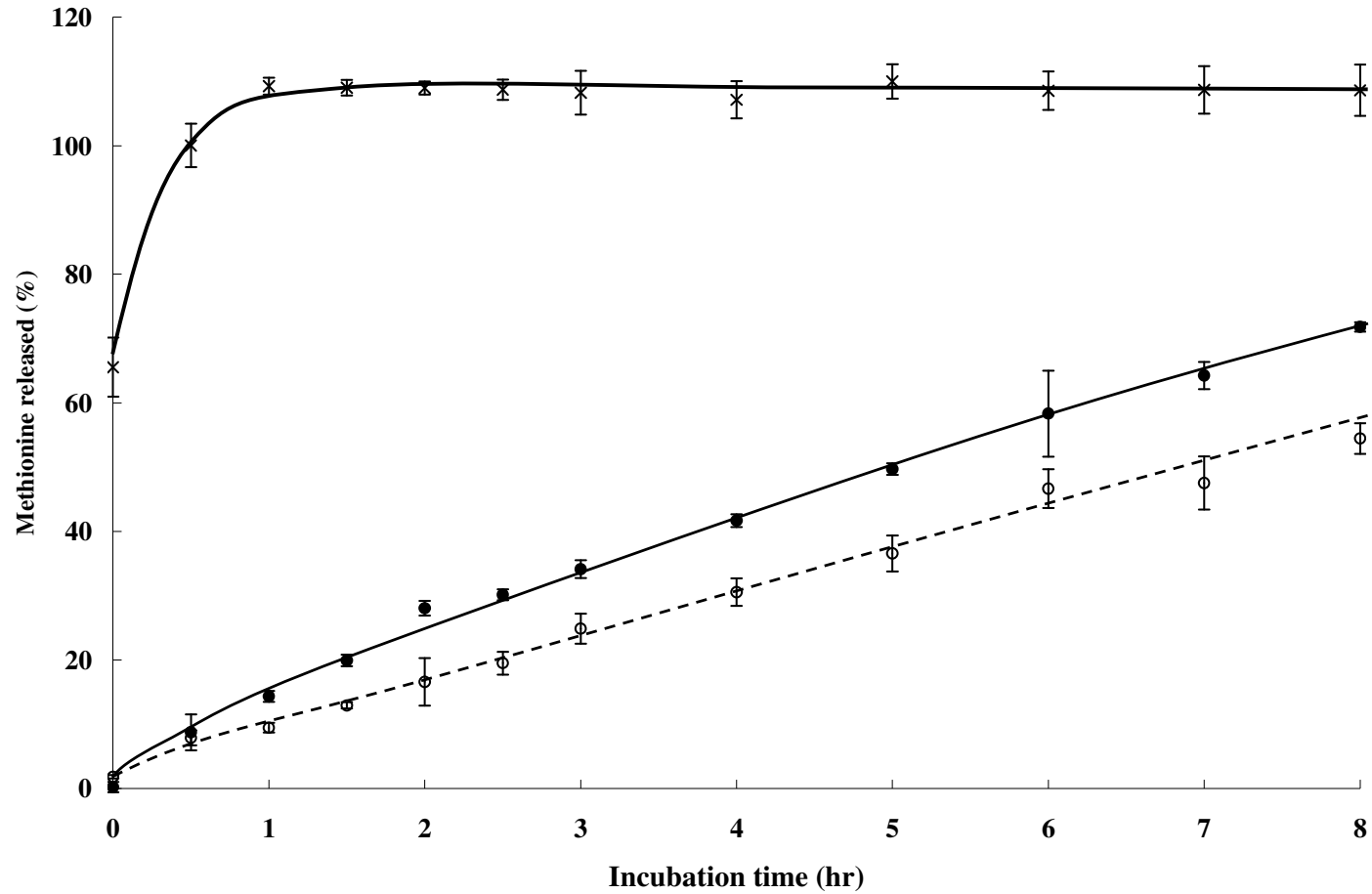


Figure 27: Effect of heat treatment of the kafirin microparticle coated methionine (1:1) fused using glacial acetic acid and extruded on methionine released at pH 2, 39°C. —x— Methionine (uncoated), —●— Kafirin microparticle coated methionine, not heat treated, —○— Kafirin microparticle coated methionine, heat treated.

- Error bars indicate \pm one standard deviation

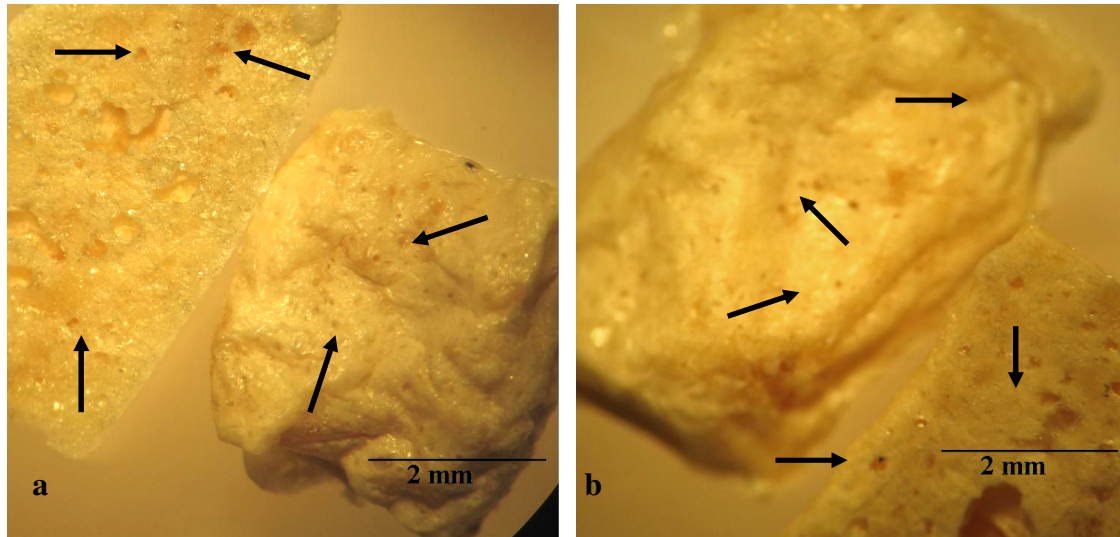


Figure 28: Stereomicrographs of the kafirin microparticle coated methionine (1:1) fused together with glacial acetic acid, extruded through a syringe after the dissolution test. a) air dried at ambient temperature, b) heat treated at 60-70°C. Pores are indicated by arrows.

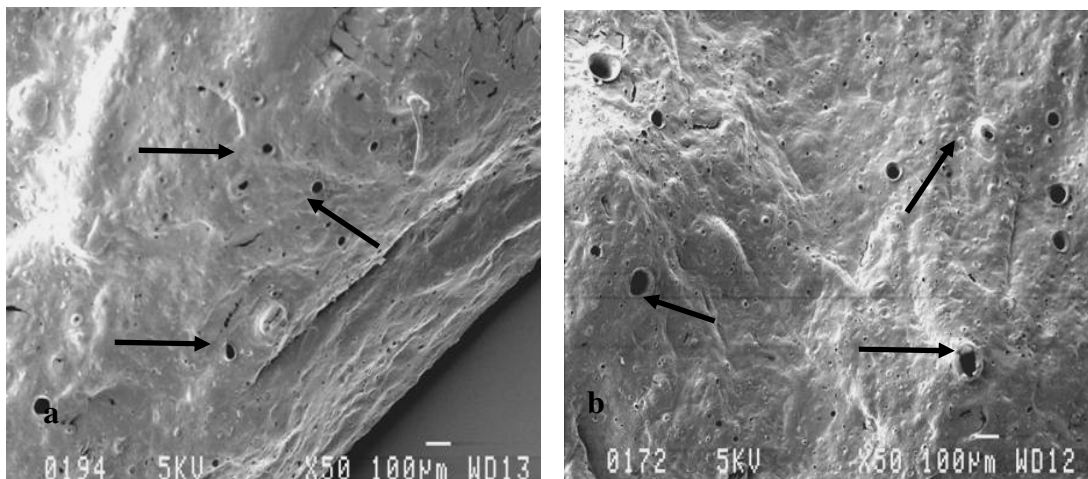


Figure 29: SEM of the kafirin microparticle coated methionine (1:1) fused together with glacial acetic acid, extruded through a syringe after the dissolution test. a) air dried at ambient temperature, b) heat treated at 60-70°C. Pores indicate arrows.

Figure 30 shows combined data from various experiments (Fig. 15, Fig. 25. and Fig. 27) for comparison. The kafirin microparticle coating which was heat treated and extruded had the same barrier properties as the heat treated Zein Gold coated methionine (Chapter 2). The extruded kafirin microparticle coating and the Zein Gold coated methionine which were heat treated released less methionine compared to the coating which was not heat treated. The kafirin microparticle which was not extruded nor heat treated had the worst barrier properties.

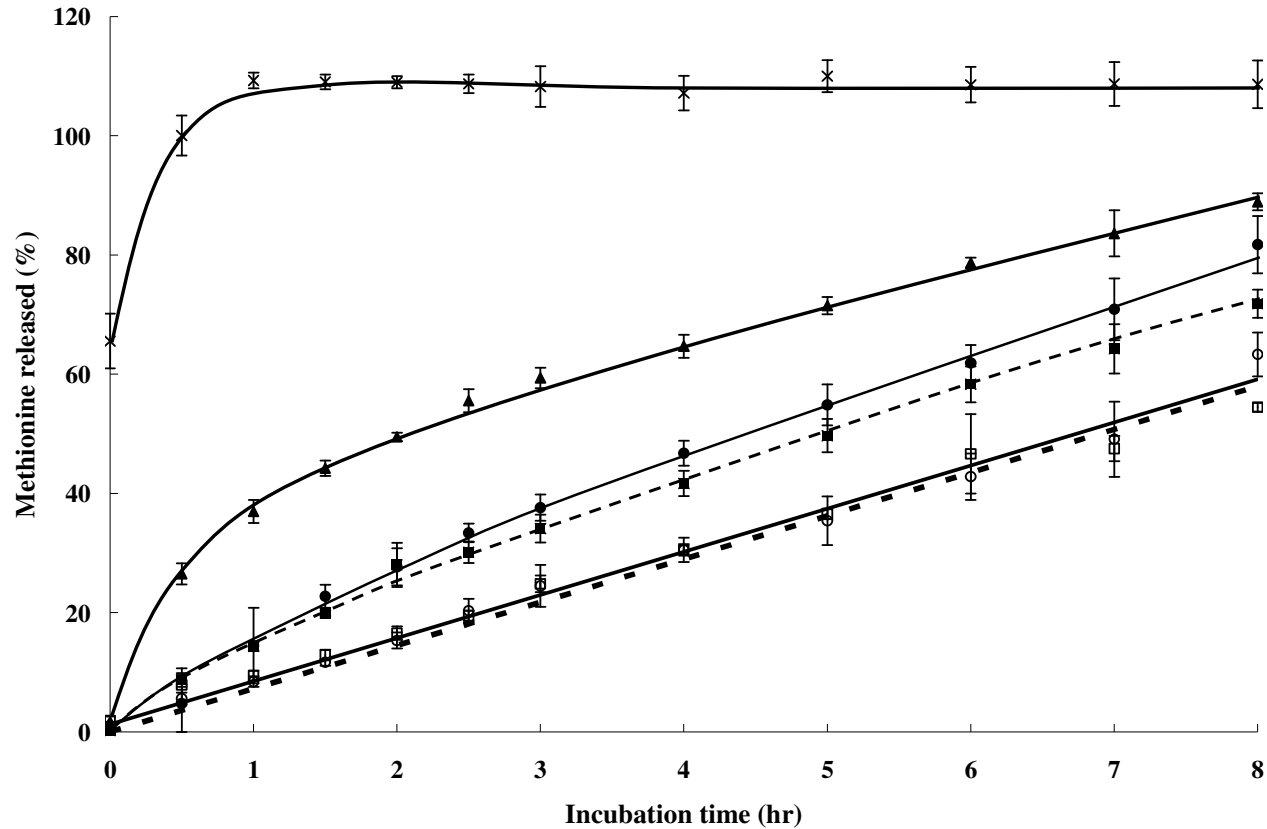


Figure 30: Comparison of the effect of Zein gold coated methionine (1:1) heat treated and not heat treated (from Chapter 2), kafirin microparticle coating (1:1) extruded/not extruded and heat treated/not heat treated on the release of methionine at pH 2, 39°C. —x— Methionine (uncoated), —■— Kafirin microparticle coated methionine, extruded, air dried, —□— Kafirin microparticle coated methionine, extruded, heat treated, —●— Zein Gold coated methionine, formed in Chapter 2 hand mincer, heat treated, —○— Zein Gold coated methionine formed in Chapter 2, not heat treated, —▲— Kafirin microparticle coated methionine, not extruded, not heated.
* Error bars indicate \pm one standard deviation

3.3.5 Pepsin digestion of the microparticle coatings

Figure 31 shows the methionine released from the prolamin coatings with pepsin digestion. As stated, the kafirin microparticle, kafirin protein and Zein Gold coating controls were prepared in the same manner as the coated sample without any addition of methionine. There seemed to be interference by the pepsin present in the buffer during the ninhydrin assay as the buffer containing pepsin had in a higher blank value. The percentage methionine determined for the microparticle coatings and controls in the buffer containing pepsin was corrected. The free terminal amino groups of pepsin may have reacted with the ninhydrin used to determine free amino nitrogen (FAN).

The data from the pepsin digestion assay were used to analyze two different criteria. The methionine released during pepsin digestion was evaluated as well as the amount of coating digested by the pepsin enzyme. There was no FAN detected for the aliquots withdrawn during the digestion test for the control coatings (those without methionine) in the pH 2 buffer or in the buffer containing pepsin. Thus, these are not indicated in Figure 31. This showed that there was very little hydrolysis of the control coatings by the pepsin enzyme. This is not as expected as various other authors have shown that raw and cooked sorghum and maize are digestible by pepsin, whereas cooked samples are far less digestible (Hamaker, Kirleis, Mertz & Axtell 1986, Mertz et al 1984, Oria et al 1995). It is postulated that the low digestibility of the microparticle coatings were due to the fusing of the microparticles with the glacial acetic acid which has been found to be a better solvent than ethanol (Taylor & Taylor 2006) leading to a coated material which is not accessible to enzymes. The release of methionine from the coatings in the pH 2 buffer and the buffer containing pepsin did not differ significantly. This would suggest that pepsin did not have a significant effect of the digestibility of the coatings and consequently on the release of methionine. The reasons for the low digestibility are similar to those discussed previously regarding the form and structure of the coatings. However, it should be noted that the zein coated methionine in the pH 2 buffer and the buffer containing pepsin released the most methionine. The kafirin microparticle coating in the pH 2 buffer released the least amount of methionine.

Figure 32 shows the percentage digestion of the coatings during the pepsin digestion assay. The control coatings (those without methionine) had a protein digestibility of roughly

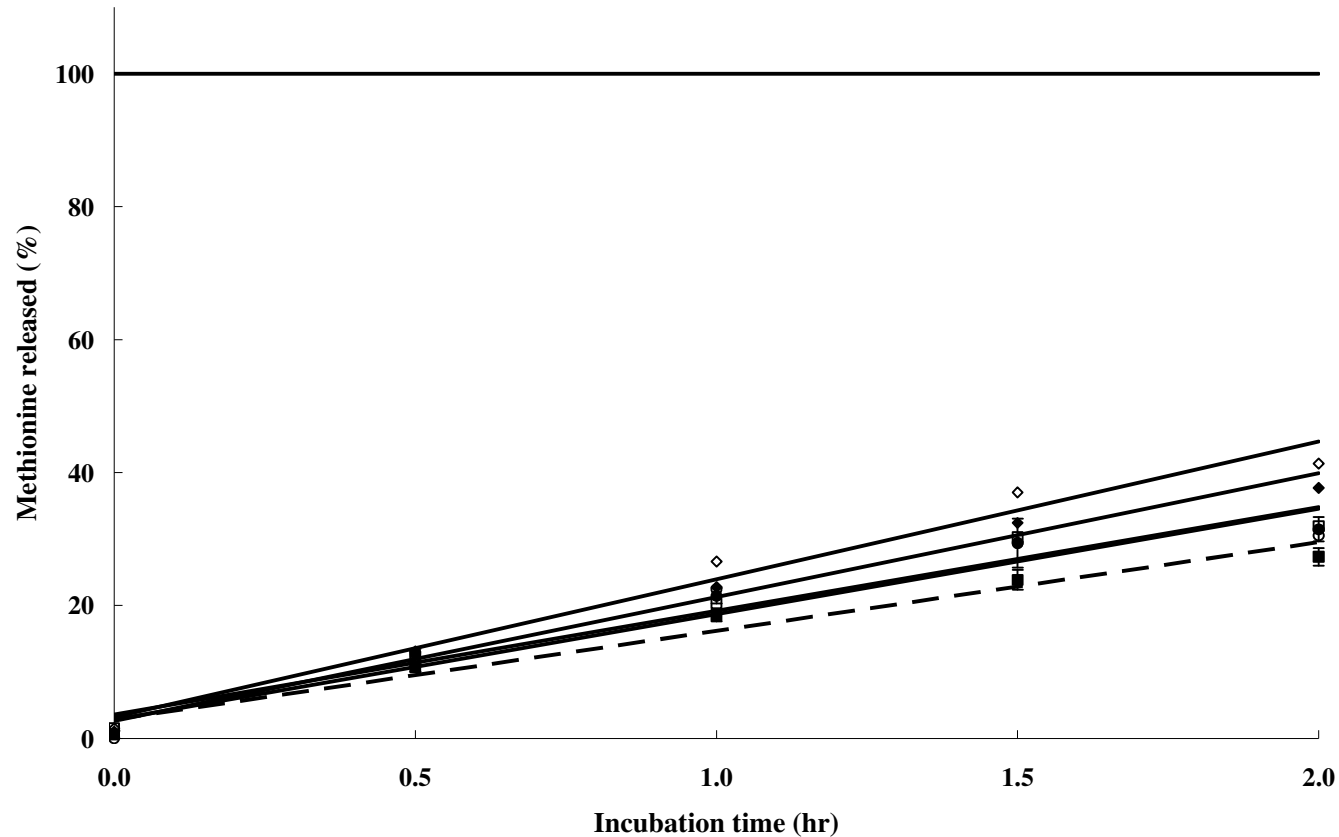


Figure 31: Effect of pepsin action on zein, kafirin and kafirin microparticle coated methionine (1:1) on the methionine released in buffer and the corrected value for the methionine released from pepsin. — Projected methionine, —◇— Zein Gold coated methionine + pepsin, - -○- - kafirin coated methionine + pepsin, - -□- - kafirin microparticle coated methionine + pepsin, —◆— Zein Gold coated methionine + buffer, - -●- - kafirin coated methionine + buffer, - -■- - kafirin microparticle coated methionine + buffer.

* Error bars indicate \pm one standard deviation

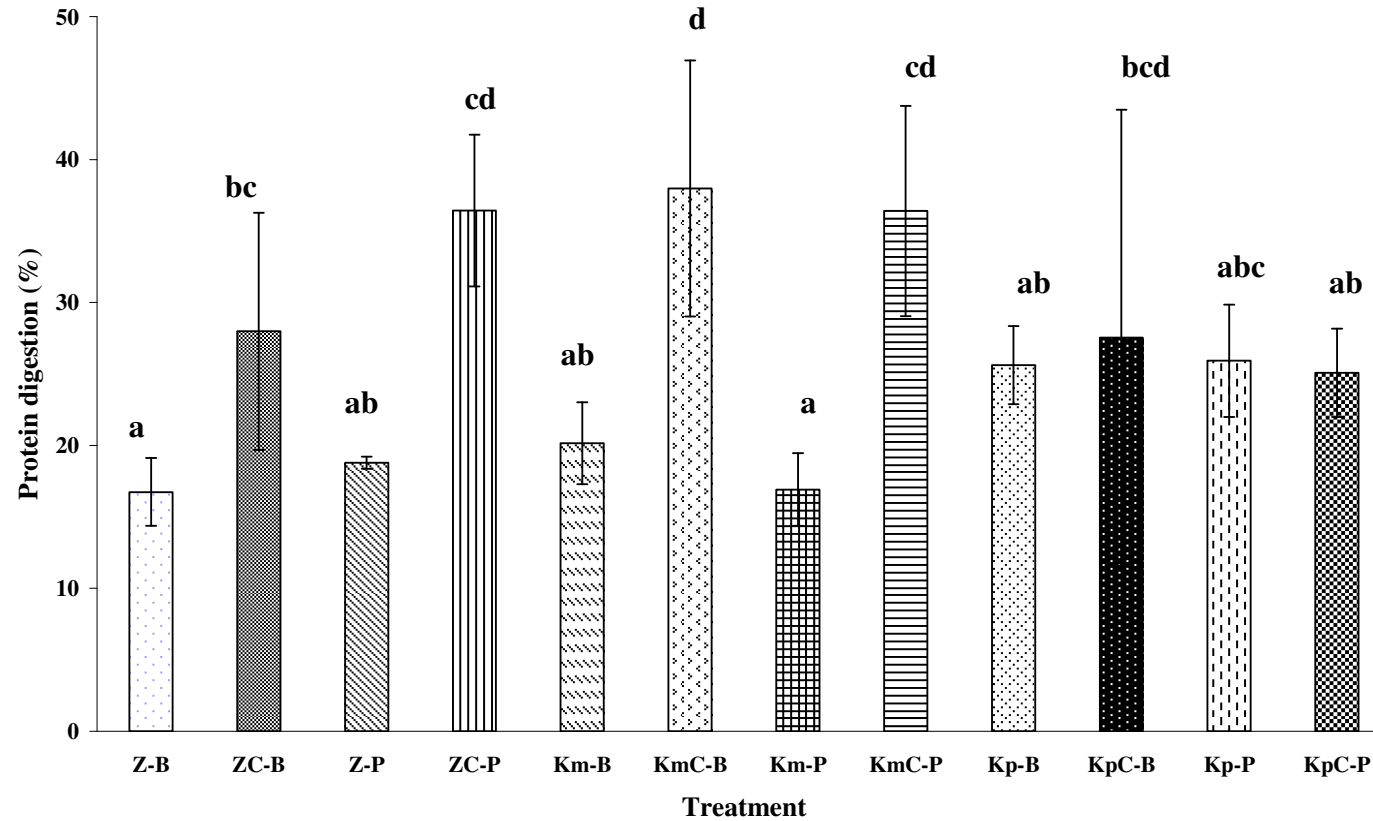


Figure 32: Effect of pepsin on the digestion of Zein Gold, kafirin and kafirin microparticle coatings of methionine (1:1).

Z-B – Zein Gold in buffer, ZC-B – Zein Gold coated methionine in buffer, Z-P–Zein Gold in pepsin, ZC-P–Zein Gold coated methionine in pepsin, Km-B–Kafirin microparticle in buffer, KmC-B–Kafirin microparticle coated methionine in buffer, Km-P–Kafirin microparticle in pepsin, KmC-P–Kafirin microparticle coated methionine in pepsin, Kp-B–Kafirin protein in buffer, KpC-B–Kafirin protein coated methionine in buffer, Kp-P–Kafirin protein in pepsin, KpC-P– Kafirin protein coated methionine in pepsin.

* Error bars indicate \pm one standard deviation

** Columns with different letters differ significantly ($p < 0.05$)

between 17% and 27%. When comparing this to the results from the dissolution test (Fig. 31) it would seem that the protein digestibility (% digestion of the coating) was relatively high since no FAN was detected when the withdrawn aliquots were analysed (data not shown). A reason for this may be that the hydrolysis products of the protein coatings were peptides which are not detected by the ninhydrin assay.

The digestibility of the coatings containing methionine was higher than that of the control coatings (Fig. 32). This may be due to the methionine entrapped in the coating. It is possible that the methionine interferes with the fusing of the protein or microparticles to each other, leading to a coating which is not as tightly fused or compacted as the one without methionine. This suggestion is supported by SEM observations (Figs. 33 and 34). The Zein Gold control coating (Fig. 33A) sectioned through the middle had a smooth continuous appearance, while the Zein Gold coating containing methionine had visible irregular sections. The irregular sections may either be the microparticles which did not fuse together properly, due to the interference of methionine, or it can simply be the embedded methionine. These differences were also seen with the kafirin microparticle control (without methionine) coating and the kafirin microparticle coating containing methionine (Fig. 34A). It should be noted that the Zein Gold coating (Fig. 33) which released more methionine than the kafirin microparticle coating had a more extensive and larger pore structure (inside and on surface) after incubation in the buffer (pH 2) and the buffer containing pepsin than the kafirin microparticle coated methionine (Fig. 34). This supports the previous suggestion that the methionine was leaching from the pores, as a more porous structure would result in a higher methionine release. There appears to be no distinct difference between the coatings which were incubated in the buffer (pH 2) and the buffer containing pepsin for both the Zein Gold coated methionine and the kafirin microparticle coated methionine. The appearance of the coatings suggests that there is very little effect of the pepsin on the digestion of the coatings. This supports the results of the protein digestion assay for the control samples as there was very little digestion of them.

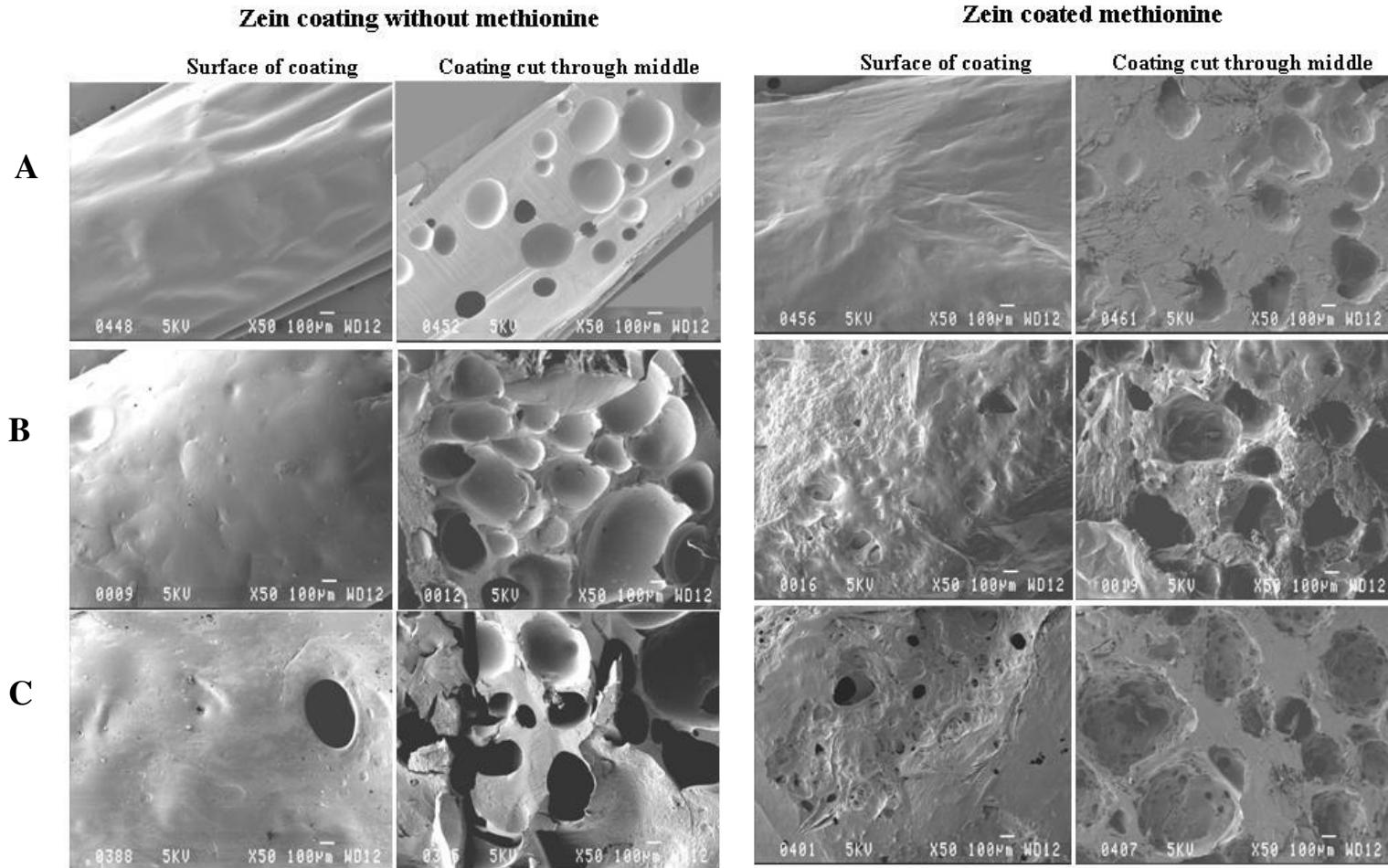


Figure 33: SEM of Zein Gold coating with and without methionine before and after incubation in a pH 2 buffer and in a pH 2 buffer containing pepsin. Row A) Coating before pepsin digestion test, Row B) Coating after incubation in buffer, Row C) Coating after incubation in buffer containing pepsin.

Kafirin microparticle coating without methionine

Kafirin microparticle coated methionine

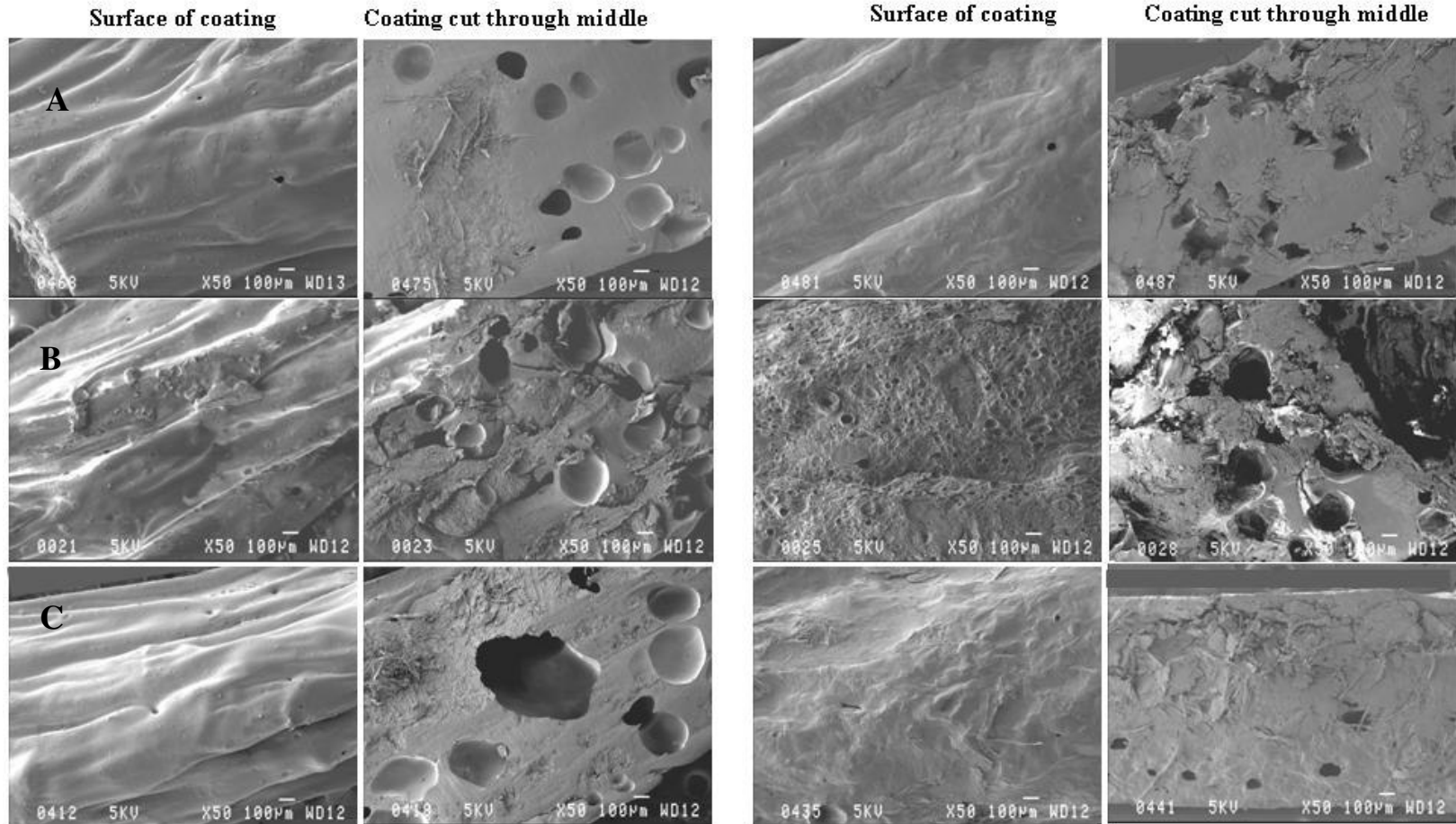


Figure 34: SEM of kafirin Gold coating with and without methionine before and after incubation in a pH 2 buffer and in a pH 2 buffer containing pepsin. Row A) Coating before pepsin digestion test, Row B) Coating after incubation in buffer, Row C) Coating after incubation in buffer containing pepsin.

3.4 CONCLUSIONS

Kafirin microparticles can make successful coating agents for active substances such as methionine. The microparticles form a coating around the methionine when the kafirin microparticles are fused together using glacial acetic acid, entrapping the methionine in the microparticle matrix. Extrusion and heat treatment of the microparticle coating improve the barrier properties and give the slowest release of methionine as well as a coating which is slowly digested by pepsin.

3.5 REFERENCES

- Ardaillon, P., and Bourrain, P. 1991. Granules for feeding ruminants with enzymatically degradable coating. US patent 4,983,403.
- Coombes, A.G.A., Lin, W., O'Hagen, D.T., and Davis, S.S. 2003. Preparation of protein microspheres, films and coatings. US patent 6,592,844.
- DeRose, R.T., Din-Pow, M., Kwon, I., Hasnain, S.E., Klassy, R.C., and Hall, T.C. 1989. Characterisation of the kafirin gene family from sorghum reveals extensive homology with zein from maize. *Plant Mol. Biol.* 12: 245-256.
- Emmambux, N.M., and Taylor, J.R.N. 2003. Sorghum kafirin interaction with various phenolic compounds. *J. Sci. Food Agric.* 863: 402-407.
- Hamaker, B.R., Kirleis, A.W., Mertz, E.T., and Axtell, J.D. 1986. Effect of cooking on the protein profiles and in vitro digestibility of sorghum and maize. *J. Agric. Food Chem.* 34: 647-649.
- Hurtado-López, P. and Murdan., S. 2005. Formulation and characterisation of zein microspheres as delivery vehicles. *J. Drug Deliv. Sci. Tech.* 15: 267-272.
- International Food Science Information Service, ed. 2005. *Dictionary of Food Science and Technology*. Blackwell Publishing: Oxford

Kawaguchi, H. 2002. Functional polymer microspheres. *Prog. Polym. Sci.* 25: 1171-1210.

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

Lee, S.J., and Rosenberg, M. 1999. Preparation and properties of glutaraldehyde cross-linked whey protein-based microcapsules containing theophylline. *J. Control. Release* 61:123-136.

Lee, S.J., and Rosenberg, M. 2002. Whey protein-based microcapsules prepared by double emulsification and heat gelation. *Lebensm-Wiss. Technol.* 33: 80-88.

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

Mertz, E.T., Hassen, M.M., Cairns-Whittern, C., Kirleis, A.W., Tu, L., and Axtell, J.D. 1984. Pepsin digestion of proteins in sorghum and other major cereals. *Proc. Natl. Acad. Sci. USA* 81: 1-2

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

O'Donnell, P.B., Wu, C., Wang, J., Wang, L., Oshlack, B., Chasin, M., Bodmeier, R., and McGinity, J.W. 1997. Aqueous pseudolatex of zein for film coating of solid dosage forms. *Eur. J. Pharm. Biopharm.* 43: 83-89.

Oria, M.P., Hamaker, B.R., and Schull, J.M. 1995. In vitro protein digestibility of developing and mature sorghum grain in relation to α -, β -, and γ -kafirin disulphide cross-linking. *J. Cereal Sci.* 22: 85-93.

Parris, N., and Dickey, L.C. 2003. Adhesive properties of corn zein formulations on glass surfaces. *J. Agric. Food Chem.* 51: 3892-3894.

Radwick, A.E., and Burgess, D.J. 2002. Proteins as microencapsulating agents and microencapsulated active compounds in pharmaceutical applications. Pages 341-366 in: Protein-based Films and Coatings. A. Gennadios, ed. CRC Press, Boca Raton, FL.

Shewry, P.R. 2002. The major seed storage proteins of spelt wheat, sorghum, millets and pseudocereals. Pages 1-24 in: Pseudocereals and Less Common Cereals – Grain Properties and Utilization Potential. P.S. Belton, and J.R.N. Taylor, eds. Springer: Berlin.

Shukla, R., and Cheryan, M. 2001. Zein: the industrial protein from corn. *Ind. Crop. Prod.* 13:171-192.

Taylor, J., and Taylor, J.R.N. 2006. Process for producing protein microparticles. South African patent draft application. Patent Application number: PA143050

Taylor, J., Taylor, J.R.N., Dutton, M.F., and De Kock, S. 2005. Identification of kafirin film casting solvents. *Food Chem.* 90: 401-408.

CHAPTER 4

4. DISCUSSION

This section will discuss the important methodologies used during this study. A number of methods were used during this study. The important methods will be discussed. The principles, strengths, limitations and modifications will be considered. The mechanisms and potential of kafirin and zein proteins as well as microparticles to act as coatings for the controlled release of nutrients will then be discussed. Then the implications the findings of this research will be discussed. Lastly, future potential of these proteins and microparticles as coatings will be considered.

4.1 Methodological considerations

4.1.1 Coating methods

Kafirin was extracted by the method of Emmambux and Taylor (2003). The extraction procedure was relatively simple. However, an observation during the extraction procedure must be highlighted. The kafirin became solubilised in the extraction mixture and was separated from the un-dissolved flour components by centrifugation. During the centrifugation process the kafirin began to precipitate out of the extraction mixture. This occurred when the mixture began to cool during the centrifugation process since not all the material could be centrifuged at once. This would result in a loss of the kafirin protein as it would be removed by centrifugation with the un-dissolved components of the flour. To prevent precipitation of the proteins from occurring, glacial acetic acid may be used as a solvent during extraction instead of ethanol, as it can be used at 25°C (Taylor et al 2005).

The principle for coating methionine was to form a protective covering around the methionine crystals by entrapping the methionine in a protein matrix. A simple method was used to coat methionine using zein & kafirin protein during this experimental work. Methionine was mixed into the dissolved proteins and formed through a hand mincer. Cleaning the mixer and hand mincer was difficult as zein and

kafirin are sticky substances when dissolved in glacial acetic acid. The stickiness of zein and handling difficulties were reported by Padua, Rakotonirainy, and Ha (2001). The formed pellets varied in shape and size. A continuous system for this coating process may be a more practical approach. For example, a system where methionine is mixed into the dissolved protein and pumped through an extruder to form pellets may be used. Another possible way for coating the particulate methionine may be in a fluidized bed coater. Fukumori, Fukuda, Hanyu, Takeuchi, Osaka (1987) used a fluidized bed process to coat pharmaceutical powders with an aqueous coating of methacrylate acid-ethylacrylate copolymer. The authors state that a problem with the coating of powders is the particle size. The small particle size of methionine requires a large amount of coating material to cover the surface area. The authors used a Wurster process (Fig. 35) to coat lactose with the above named coating material. If the Wurster process could be applied to coat substances such as amino acids using prolamin proteins, the consistency of the coating material would have to be altered as it would be too thick to use in the Wurster process. Another factor which would need to be considered when using this process is the sticky nature of these acid-wetted proteins. It may cause the coating material itself or the particles to be coated to adhere to the walls of the coating chamber resulting in a loss of coating material or material to be coated. To minimize cost during the coating process the glacial acetic acid which is evaporated off during the mixing and drying processes should be recovered.

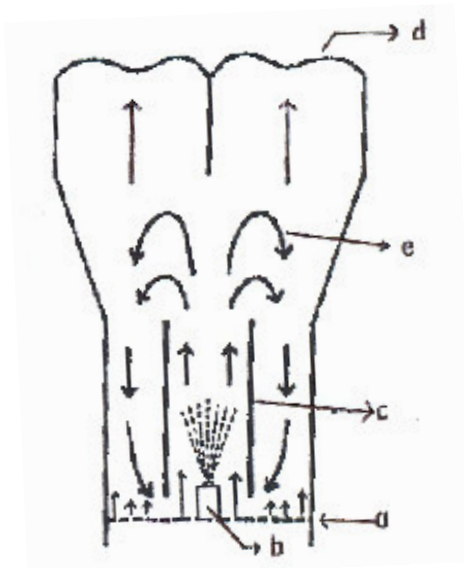


Figure 35: The Wurster coating chamber. a) Air distributor, b) spray, c) cylindrical partition, d) bag filter, e) particle and air flow (Fukumori et al 1987).

Kafirin microparticles in 20% acetic acid were also used as a coating material. When methionine was added to the microparticle suspension, the microparticles aggregated. If the Wurster process was used to coat methionine with microparticles in 20% acetic acid aggregation of the microparticles may also occur. The microparticles were separated from the 20% acetic acid solution by centrifugation before they were dried. Hurtado-López & Murdan (2005) first removed the solvent by rotatory evaporation before the remaining aqueous liquid was centrifuged off. If the acetic acid was first removed by rotatory evaporation before the centrifugation step, it might have resulted in a more effective separation of the kafirin microparticles.

The dried microparticles were mixed with the methionine and fused together with glacial acetic acid. This coating method resulted in the successful coating of methionine. The dried microparticles and methionine were fused with glacial acetic acid and formed into pellets using a syringe as a mini-extruder. As discussed above, a continuous process where mixing and extrusion takes place in the same machine or process would be more practical to create a faster, easier to clean process which can produce evenly shaped pellets. Both methods employing protein and microparticles as coating agents to coat methionine were successful coating procedures on a laboratory scale. The methods were simple and provided an understanding of the coating potential of the zein and kafirin proteins and microparticles. However, if these processes were up-scaled for industrial applications a continuous process employing large-scale equipment will be needed which ensures ease of cleaning, evenly shaped pellets and recovery of solvent.

4.1.2 Analytical methods

A dissolution test was developed for the evaluation of methionine released from the coatings. The principles of a dissolution test involves measuring the amount of active substance dissolved in a known volume of liquid at a predetermined time, using a specified apparatus designed to control the parameters of the dissolution test (Cox, Douglas, Furman, Kirchoefer, Myrick, & Wells 1978). The pH, temperature and agitation employed during the dissolution test should be analogous to the conditions to be simulated.

The ninhydrin assay is a relatively simple method of analysis. The ninhydrin reaction is based on the spectrophotometric estimation of the purple chromophore, Ruhemann's purple, which is formed when ninhydrin reacts with free amino groups (McSweeney & Fox 1995). It requires a relatively short time to perform the analysis. However heat is necessary for the reaction to occur. An advantage of the ninhydrin assay is that it is an internationally accepted method (European Brewery Convention 1987). A disadvantage of using this method is that it gives an estimate of amino acids, ammonia, terminal alpha-amino nitrogen groups of peptides and proteins, but is not specific for methionine (the model substance which was coated). Another disadvantage is that the ninhydrin method may be influenced by contaminants (Matsumura, Shin & Murao 1987).

A control test was done to ensure that the dissolution test and subsequent ninhydrin analysis evaluated only the methionine released from the coatings and not other free amino nitrogen which may be present. No methionine was added to coatings (control coatings) which were prepared in the same manner as the coatings that contained methionine and tested under the same conditions. Because no free amino nitrogen was detected in the control coatings, the specificity of the test was solely for the methionine released from the coating and not from the coating degradation products.

The coated material in test tubes was placed in a shaking water bath. This form of agitation was done to simulate the peristaltic movements of the stomach. When referring to the dissolution tests in literature (Li, Yang, Ferguson, Hudson, Watanabe, Katsuma, & Fix 2002, Oneda & Ré 2003, Menegola, Steppe, and Schapoval 2007), it is apparent that most dissolution tests are performed using specific dissolution test equipment. Dissolution test methods and equipment are given by the United States Pharmacopeia (USP). Qureshi (1996) stated that most drug dissolution studies are performed with a basket or a paddle. The agitation source in this experiment was from the water bath (an external agitation source), whereas dissolution tests in literature use paddle apparatus, an internal agitation source. One of the limitations of the dissolution test used in this work may have been that the shaking water bath did not produce enough agitation to imitate the peristaltic movements of the gastrointestinal tract of the cow.

During this dissolution test aliquots were withdrawn at several time intervals. This caused a reduction in the volume of the dissolution medium resulting in an apparently higher amount of methionine being measured than was actually present. This anomaly occurred in all the dissolution tests during the experimental work. Cox et al. (1978) suggest that the volume of each aliquot removed should be taken into consideration when calculating the final release of the active agent. Menegola et al (2007) replaced the same volume of dissolution medium when aliquots were withdrawn to maintain a constant volume. The samples were mixed with a vortex mixer to ensure an even dispersion of dissolved methionine before the aliquots were withdrawn. Aliquots were withdrawn at constant time intervals between different samples as to eliminate timing errors.

The dissolution test used in this work simulated conditions for only one division of the cows' stomach. The release of methionine would have been better predicted if each section in the stomach was consecutively simulated. Intestinal conditions could also be simulated after testing the methionine release in the stomach. A dissolution method where release at gastric and intestinal conditions was determined was done by Agrawal and Panchagnula (2004). Oneda and Ré (2004) used 3 different pH values consecutively to simulate in vivo conditions of the gastrointestinal tract.

The pepsin digestion assay involves incubating samples in a buffer (pH 2) containing pepsin (Hamaker et al. 1987, Mertz et al. 1984). The protein digestibility is measured by determining the proportion of nitrogen that goes into solution. Two assumptions, which may not be correct, are made. First, if the material is solubilised, it has been digested, and secondly, if it is not solubilised, then it has not. The assay is commonly used to determine the protein digestibility of sorghum and maize (Duodu et al 2003, Nunes et al 2004). The results showed that very little digestion of the coatings by pepsin occurred. It was postulated that the reason for this may be the tightly fused coating structure which does not allow the enzymes to access the coating enough to digest it. Another method to determine whether the coating is effective is to place the material to be tested in a bag in the rumen. Romagnolo et al. (1994) tested the ruminal degradability of maize and corn gluten meal by using the in situ Dacron bag technique for incubation in the cow's stomach. After the incubation period the bags were removed and the residue dried. The contents were analysed for the amount of protein

which remained. The authors used electrophoretic analysis of the maize protein fractions in order to provide an *in vitro* system for monitoring protein degradation in the rumen. They found that the zein fraction was relatively resistant to ruminal proteolysis. However, the albumins, globulins and glutelins were degraded and contributed very little to the protein residues. These authors suggest that the hydrophobic nature of the zein may be the reason for the low utilization by microorganisms. This supports the data from the pepsin digestion during this work, the low digestibility values may be due to the hydrophobic nature of kafirin and zein. A similar method can be used to determine the effect of enzyme degradability of the coated methionine.

Various changes to that of the method of Hamaker et al. (1986) were done. During the pepsin digestion, aliquots at time intervals of 0, 30, 60, 90 and 120 min were withdrawn. The aliquots were used for the dissolution analysis of the methionine from the coatings. The same amount of buffer containing pepsin was added back to maintain a constant volume. Centrifugation was not done as it was not necessary to separate the control and coated sample from the buffer. Because of the large pellet size, the buffer could be pipetted off without removing any of the residual samples after digestion.

4.2 Formation and barrier properties of the prolamin coated methionine

Figure 36 depicts two principle methods of controlled release namely, sustained release and delayed release. Delayed release is defined by Lakkis (2002) as a mechanism whereby the release of an active substance is delayed from a finite “lag time” up to a point when/where its release is favoured and no longer hindered. Sustained release is defined as a mechanism designed to maintain constant concentration of an active substance at its target site. All the graphs shown in this experimental work follow the trend of a sustained release curve shown in Fig. 36. In my opinion, the ideal trend of a release curve for this application (controlled release of amino acids in ruminants) would be similar to that of the delayed release curve. A delayed release curve would ensure that most of the coated substance will pass through the rumen, avoiding hydrolysis of the active substance by ruminal bacteria, and be released in the intestine. However, the results in Chapters 2 and 3 show that all

the coatings tested show a sustained release curve. Thus, some of the active substance will be released from the coating while passing through the rumen. The objective of forming a controlled/delayed release coating was therefore not met, but instead a sustained release of the active substance was created.

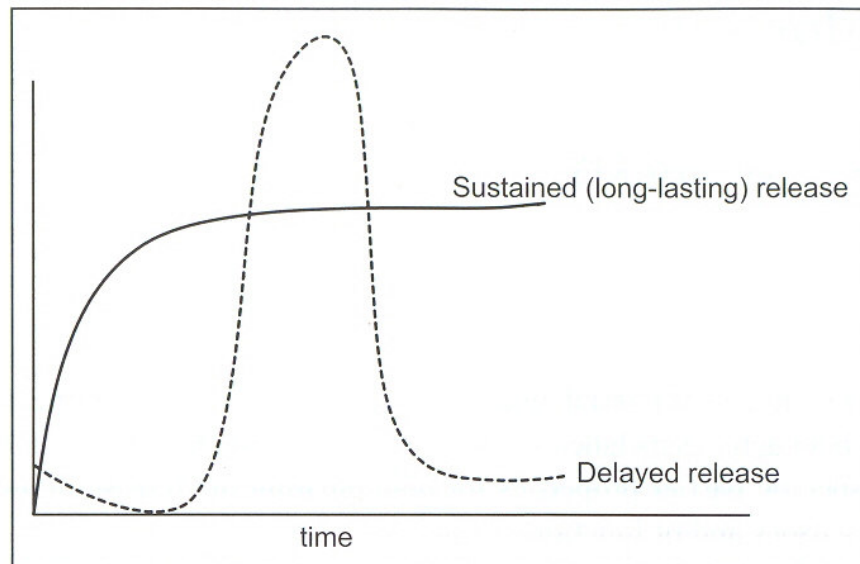


Figure 36: Generic representation of “sustained” and “delayed” release profiles (Lakkis 2007).

The initial coatings were formed using the kafirin and zein proteins which were dissolved in glacial acetic acid. A model was constructed for the protein coated methionine. Byaruhanga et al. (2006) used fourier transform infrared spectroscopy (FTIR) to investigate the structure of kafirin protein and its films. They found that heat treated kafirin was mainly in the α -helical conformation, while the films which were made from the heat treated kafirin were mainly in the β -sheet conformation. They suggested that the β -sheet conformation of the films was possibly due to the α -helices unfolding to a certain degree and then aggregating. Based on this a simple model for the structure of the prolamin coated methionine was suggested where the methionine crystals become embedded in layers of β -sheet proteins.

Microparticles were then used as the coating material. Glacial acetic acid was added to the dried kafirin microparticles and methionine which were mixed together and extruded through a syringe to form pellets. The glacial acetic acid fuses the

microparticles together leading to the formation of a microparticle matrix surrounding the methionine crystals. It is possible that the glacial acetic acid caused the formation of “sticky-ends” on the microparticles. This model is based on the findings by Taylor et al (2005). They found that glacial acetic acid is a suitable solvent for kafirin and can be used at room temperature. Addition of the glacial acetic acid into the kafirin microparticle–methionine dispersion may initiate the solvation of the microparticles around the microparticle surface. This solvation of the surface of the microparticles may cause “sticky-ends” of individual microparticles which fuse with the “sticky-ends” of the surrounding microparticles and forming a coating (Fig. 38). The methionine particles would then be entrapped between the microparticles, which are fused together via the “sticky-ends”. The microparticle coating serves as a barrier to protect the methionine from the buffer environment. With time, the methionine molecules begin to dissolve in the buffer causing the methionine to leach out from the microparticle coating. As stated, the release mechanism of methionine will be described later. SEM of the microparticle coated methionine showed the presence of pores in the coating. This is probably due to the presence of air bubbles which are incorporated in the microparticle-methionine dispersion during the addition of glacial acetic acid. The incorporated air becomes entrapped between the fused microparticles.

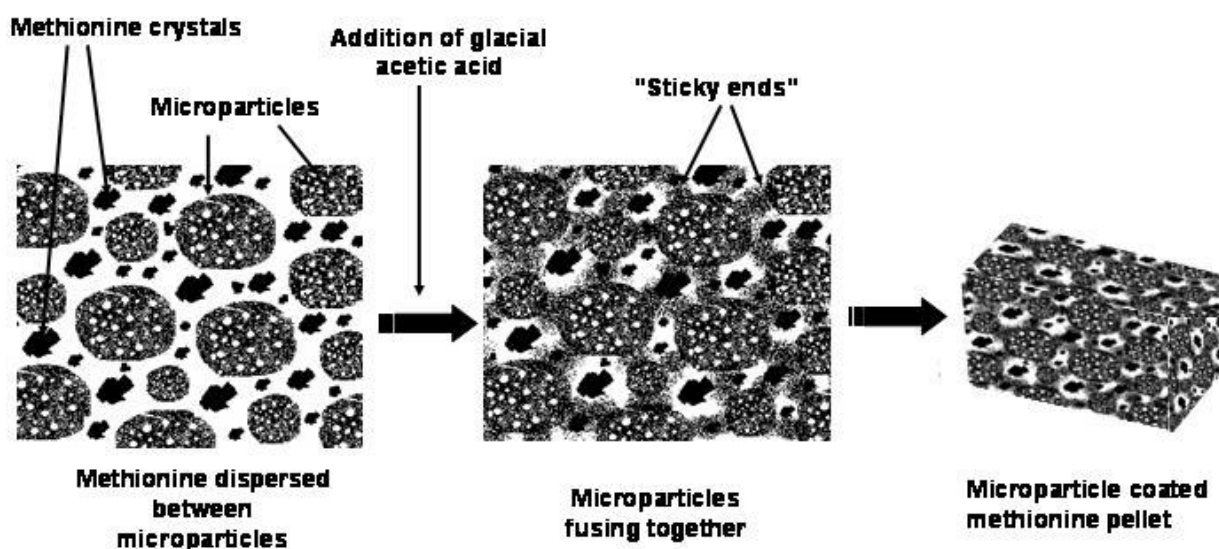


Figure 38: Proposed model for organization of methionine crystals entrapped within a kafirin microparticle matrix.

The results showed that the kafirin microparticle coatings formed by extrusion had improved barrier properties than those which were not extruded (Chapter 3).

Extrusion through a syringe was performed to prevent any exposed methionine on the edges of the coating. The formation of a more compact structure may have been the reason for improved barrier properties. Wang and Padua (2004) investigated the water sorption properties of extruded zein films and compared it to zein powder. The extruded zein films were prepared by forming a zein resin. The zein resin was prepared by dissolving zein in 75% ethanol. The mixture was then precipitated and kneaded in a Farinograph. This was then extruded using a slit die extrusion method at room temperature. The extruded films and zein powder were then subjected to moisture sorption isotherm testing. The moisture sorption isotherms showed that the extruded products adsorbed less water than the zein powder. The authors attributed the difference in water adsorbed to structural differences between the extruded films and the zein powder. It was hypothesized that the resin formation and extrusion modified the morphological structure of the zein. They suggested that the zein was compressed into a compact solid and pores and capillaries were removed, leading to a lower moisture sorption capacity. The coatings in Chapter 3 all showed relatively compact structures. However, they all contained pores. The improved barrier properties of the extruded coatings may thus solely be attributed to a compact structure of the coating. Further research regarding various extrusion methods to form the coatings may be done as well as the effect on barrier properties of the various extrusion methods can be investigated. The relatively low protein digestibility values reported in Chapter 3 may also be explained by the theory that the coatings have a compact structure which does not allow the pepsin enzymes to hydrolyse the protein coating. Hurtado-López and Murdan (2006) subjected zein microspheres to pepsin (simulated gastric conditions). After 1 hr of incubation in pepsin the zein particles disappeared. After centrifugation of the mixture a small pellet was formed, this was shown to be the undigested dimer which was present in the zein microparticles. These results show that pepsin successfully degraded the microparticles which were not present in compact structures, supporting the hypothesis that the compact structure or the extruded coatings hindered pepsin digestion. Further research can also be done to determine if extrusion cooking has an effect on the protein digestibility of prolamin coated amino acids on characteristics such as the structure (compatibility) and uniformity of the coatings.

4.3 Mechanism of methionine leaching from the coatings

A model for the release of methionine from the coatings will follow (Fig. 39). As shown in Chapter 2, the higher the proportion of protein used to coat the methionine, the better barrier properties the coating will have. The likelihood of methionine being entrapped close to the edge of the coating will increase with the increase in the proportion of methionine (decrease in the coating proportion). It is proposed that the coating becomes slightly degraded by the buffer. This leads to the exposure of the methionine crystals which are entrapped close to the edge of the coating. The buffer comes into contact with the methionine and the methionine molecules begin to dissolve.

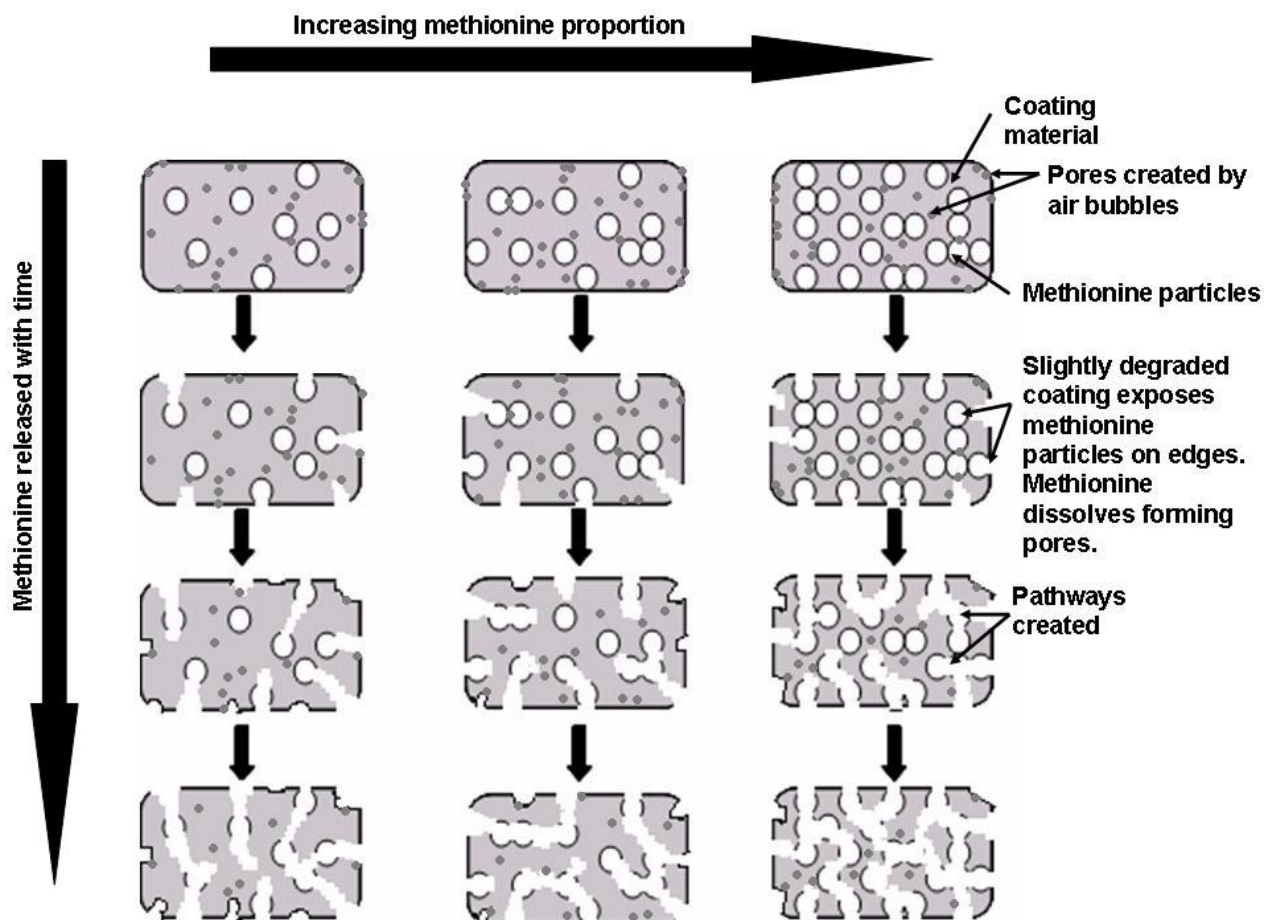


Figure 39: Proposed mechanism of methionine release from the coatings.

Once the methionine is dissolved, a pore will form where the methionine was entrapped. The buffer enters the pore and may slightly degrade the coating. This

creates a pathway for the buffer to move to the air bubble or entrapped methionine. The buffer may also enter the coating via air bubbles in the coating in the same manner described above. Beck, Tomka and Waysek (1996) state that even though zein is not soluble in water at neutral pH, it is highly swellable in comparison to typical structure proteins such as silk and keratins. Due to the homology between kafirin and zein (DeRose et al 1989) this swelling may also occur for kafirin. It may also be possible that the pores and channels in the coating increase in size and number due to possible swelling of the coating. This may contribute to the manner in which methionine is released from the kafirin and zein coatings.

4.4 Success of method and moving forward

It would appear that moving forward with research regarding proteins and microparticles as coating agents would be somewhat limited. It is evident from the results obtained in this experimental work that both zein and kafirin proteins are potentially effective coating barriers for methionine and consequently for amino acids. The current status of the coating will have to be improved if they are to be used as controlled release agents in ruminants. This may primarily be due to the hydrophobic nature and disulphide bonding interactions of kafirin (reviewed by Belton et al 2006, reviewed by Duodu et al 2003) and zein (Shukla & Cheryan 2001; reviewed by Duodu et al 2003). From the calculations done in Chapter 2, it seems that if the protein percentage of the kafirin and zein was equal, then kafirin would form a coating with better barrier properties. This may be because kafirin is more hydrophobic and has more potential for disulphide bonding interactions due to the higher proportion of γ -fraction present in the protein (Belton et al. 2006). Further research regarding these prolamins as coatings may involve alternative coating procedures to that used in Chapter 2. A variation of the Wurster process may be investigated. Further research regarding the use of proteins as coating agents could also involve investigating the use of a cheaper source of protein, which would result in a more economical process. Maize gluten meal is an example of a cheaper source of protein. Maize gluten meal is the by product of maize wet milling which produces starch, oil and fibre (Beg, Pickering, & Weal 2005). Unrefined maize gluten meal has 50%-70% moisture, and contains approximately 60% protein, 15-18% fat, 20-25% carbohydrate, 3.5% fibre and 1-2% ash on a dry basis (Ponte, Doğan, & Kulp 2000).

Maize gluten meal (60% protein) is sold for approximately \$557/ tonne, where \$1=R7.90, (personal communication with African Products, South Africa 2007), whereas zein is sold for approximately \$15/ kg (personal communication with Dr J.W Lawton, Scientist Poet Energy, USA 2007). Using approximate conversions the value of CGM is approximately 24 times cheaper (per kg) than zein. A suggestion for further research would be to test the barrier properties of the cheaper source of protein in combination with zein and kafirin or on its own. To fully evaluate the barrier properties of the coating I propose that in vivo testing be performed in the final analysis of the coated material. The desired effect of the coated protein should also be analysed. For example, in this instance it was to increase the milk production in cows. To increase the barrier properties of the coating material, techniques other than heat treatment or increasing the proportion of the coating agent can be investigated. For example, further research to improve the barrier properties of the kafirin and zein coatings can be investigated using tannins. Emmambux et al. (2004) showed that kafirin films containing tannic acid and sorghum condensed tannins had a decreased effect on the water absorption, oxygen permeability and tensile strain. It was hypothesized by these authors that the reason for the observed effects was that the tannins interacted with the kafirin polypeptide chains by cross-linking causing a decrease in protein chain mobility.

Using microparticles as the coating material requires a more complicated and costly process as the microparticles must be produced first. When forming the microparticles, it was observed that not all the protein preparations resulted in microparticle formation. The quality of the proteins which were used as the raw material for the formation of microparticles would have to be monitored to be consistent and of a pure standard of protein to always lead to microparticle formation. It was thought that zein microparticles formed however, only when they were viewed by SEM it could be seen that essentially there was no microparticle formation. A rapid method for determining whether or not the microparticles are present may need to be developed and implemented. There may be some research required regarding the protein characteristics which are required for the formation of microparticles. This information may aid in optimizing the process of making and possibly increasing the applications of microparticles. For example, to determine the extent of protein purity and specific α -, β -, γ -fractions (Shewry 2002) which need to be present to facilitate

microparticle formation. The economic impact of the process has to be considered as it involves two additional processing steps which are not required when the proteins are used as the coating agents. These processing steps include formation of the microparticles and subsequent drying. These additional processes will result in an extended coating procedure and will also increase the cost of the process. Developing a process to coat the methionine with the microparticles was a challenging task and many preliminary trials were investigated without success. Further studies may be done to find a more efficient coating procedure using microparticles. However, the time and cost involved to produce the microparticles will have to be weighed against simply using the proteins as coating agents. The experimental work showed that the barrier properties of the coatings which were produced using microparticles were slightly better than the coatings made from the proteins. However, in my opinion it is not worth further pursuing the use of microparticles as coating agents for this specific application of controlled release in ruminants as the improved barrier properties of the microparticle coating will not outweigh the cost and time involved to produce this coating. Brownlie (2007) discusses the cost of microencapsulation, this outlook can be applied to the use of microparticles as coating agents. The statement made simply emphasizes that the extra cost associated with microencapsulation should be justified in terms of improvement in performance of that ingredient within the food product. Brownlie (2007) also states that the application of this work, the microparticle coating needs to show a clear improvement of the barrier properties in comparison to using the protein coating. However, the microparticle coatings only show a slight improvement on the barrier properties.

CHAPTER 5

5.1 CONCLUSIONS AND RECOMMENDATIONS

Zein and kafirin proteins have effective barrier properties when used as a coating for methionine. Barrier properties are improved by an increase in concentration of coating material as well as heat treatment of the coating. The improved barrier properties of the heat treated coatings are probably due to the formation of disulphide cross-links, more so in the kafirin than the zein. Based on a calculation it was determined, if kafirin and zein had the same protein purity, kafirin protein would be a better barrier for methionine. This is probably due to kafirin being a more hydrophobic protein.

The commercial zein samples and CSIR kafirin used in this dissertation did not form microparticles. Further research regarding the factors which may influence microparticle formation may be done. Factors such as protein purity and proportion of protein fractions which need to be present may be investigated.

Kafirin microparticles made from laboratory kafirin proved to be a successful coating for methionine. The barrier properties of the microparticle coating were the same as the kafirin protein. It would be more beneficial to use the proteins as the coating agent for methionine as the method used is far more simple and faster in comparison to the method using microparticles as coating agents.

The coatings which were formed using a simple extrusion technique showed better barrier properties, than those coatings which were poured as a film and manually broken into pieces. For both the proteins and the microparticles use as coating agents, the coating procedures used to coat methionine can be improved. Further research can be done using various blending equipment and extrusion techniques to improve the process.

CHAPTER 6

6.1 LITERATURE CITED

AACC International. 2000. Approved Methods of the American Association of Cereal Chemists, 10th Ed. Approved Methods 30-25 Crude fat in Wheat, Corn, and Soya Flour, Feeds, and Mixed Feeds, 44-15A Moisture – Air-Oven Methods and 46-30 Crude Protein – Combustion Method. The Association. St. Paul, MN.

Agrawal, S., and Panchagnula, R. 2004. Dissolution test as a surrogate for quality evaluation of rifampicin containing fixed dose combination formulations. *Int. J. Pharm.* 287: 97-112.

Ardaillon, P., and Bourrain, P. 1991. Granules for feeding ruminants with enzymatically degradable coating. US patent 4,983,403.

Argos, P., Pedersen, K., Marks, D., Larkins, B.A., 1982. A structural model for maize zein proteins. *The Journal of Biological Chemistry* 257, 9984-9990.

Arshady, R. 1993. Microcapsules for food. *J. Microencapsul.* 10: 413-435.

Autant, P., Cartillier, A., and Pigeon, R. 1989. Compositions for coating feeding stuff additives intended for ruminants and feeding stuff additives thus coated. US patent 4, 876, 067.

Bach, A., Calsamiglia, S., and Stern, M.D. 2005. Nitrogen metabolism in the rumen. *J. Dairy Sci.* 88 (1): E9-E22.

Banker, G.S. 1966. Film coating theory and practise. *J. Pharm. Sci.* 55 (1): 81-89.

Beck, M.I., Tomka, I., and Waysek, E. 1996. Physio-chemical characterisation of zein as a film coating polymer. A direct comparison with ethyl cellulose. *Int. J. Pharm.* 141:137-150.

- Beg, M.D.H., Pickering, K.L., and Weal, S.J. 2005. Corn gluten meal as a biodegradable matrix material in wood fibre reinforced composites. *Mat. Sci. Eng. A* 412: 7-11.
- Belton, P.S., Delgadillo, I., Halford, N.G., and Shewry, P.R. 2006. Kafirin structure and functionality. *J. Cereal Sci.* 44: 272-286.
- Brownlie, K. 2007. Marketing perspective of encapsulation technologies in food applications. Pages 213-233 in: *Encapsulation and Controlled Release Technologies in Food Systems*. J.M. Lakkis, ed. Blackwell Publishing: Ames, IA.
- Bugs, M.R., Forato, L.A., Bortoleto-Bugs, R.K., Fischer, H., Mascarenhas, Y.P., Ward, R.J., Colnago, L.A., 2004. Spectroscopic characterisation and structural modeling of prolamin from maize and pearl millet. *European Biophysics Journal* 33, 335-343.
- Byaruhanga, Y.B., Erasmus, C., Emmambux, M.N., and Taylor, J.R.N. 2007. Effect of heating cast kafirin films on their functional properties. *J. Sci. Food Agric.* 87: 167-175.
- Byaruhanga, Y.B., Emmambux, M.N., Belton, P.S., Wellner, N., Ng, K.G., and Taylor, J.R.N. 2006. Alteration of kafirin and kafirin film structure by heating with microwave energy and tannin complexation. *J. Agric. Food Chem.* 54: 4198-4207.
- Coetzee, K. 2003. Statistics. *Lacto Data* 6: 68-71.
- Cook, R.B., and Shulman, M.L. 1998. Colloidal dispersions of gluten, method of making and use thereof. US patent 5,736,178.
- Coombes, A.G.A., Lin, W., O'Hagen, D.T., and Davis, S.S. 2003. Preparation of protein microspheres, films and coatings. US patent 6,592,844.
- Cox, D.C., Douglas, C.C., Furman, W.B., Kirchhoefer, R.D., Myrick, J.W., and Wells, C.E. 1978. Guidelines for dissolution testing. *Pharm. Tech.* 2 (4):40-82.

- Cuq, B., Gontard, N., and Guilbert, S. 1998. Proteins as agricultural polymers for packaging production. *Cereal Chem.* 75: 1-9.
- Da Silva, L.S., and Taylor, J.R.N. 2004. Sorghum bran as a potential source of kafirin. *Cereal Chem.* 81: 322-327.
- Debeaufort, F., Quezada-Gallo, J., and Voilley, A. 1998. Edible films and coatings: Tomorrow's packagings: A review. *Crit. Rev. Food Sci.* 38: 299-313.
- DeRose, R.T., Din-Pow, M., Kwon, I., Hasnain, S.E., Klassy, R.C., and Hall, T.C. 1989. Characterisation of the kafirin gene family from sorghum reveals extensive homology with zein from maize. *Plant Mol. Biol.* 12: 245-256.
- Dong, J., Sun, Q., and Wang, J. 2004. Basic study of corn protein, zein, as a biomaterial in tissue engineering, surface morphology and biocompatibility. *Biomaterials* 25: 4691-4697.
- Donhowe, I.G., and Fennema, O. 1994. Edible films and coatings: Characteristics, formation, definitions, and testing methods. Pages 1-24 in: *Edible Coatings and Films to Improve Food Quality*. J.M. Krochta, E.A. Baldwin, and M. Nisperos-Carriedo, eds. Technomic Publishing Company: Philadelphia, PA.
- Drouillard, J.S., Herald, T.J., and Greenquist, M. 2005. Method for encapsulation of orally ingested materials to alter the site of digestion, site of action, or stability. US Patent Application number: PCT/US2005/011898.
- Duodu, K.G., Taylor, J.R.N., Belton, P.S., and Hamaker, B.R. 2003. Factors affecting sorghum protein digestibility. *J. Cereal Sci.* 38: 117-131.
- Duodu, K.G., Nunes, A., Delgadillo, I., Parker, M.L., Mills, E.N.C., Belton, P.S., and Taylor, J.R.N. 2002. Effect of grain structure and cooking on sorghum and maize *in vitro* protein digestibility. *J. Cereal Sci.* 35: 161-174.

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

El-Brashy, A.M., and Al-Ghannam, S.M. 1996. Atomic absorption determination of some amino acids containing a sulphur group. *Anal Lett.* 29: 2713-2726.

El Nour, I.N., Peruffo, A.D.B., and Curioni, A. 1998. Characterisation of sorghum kafirins in relation to their cross-linking behaviour. *J. Cereal Sci.* 28: 197-207.

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

Emmambux, N.M., and Taylor, J.R.N. 2003. Sorghum kafirin interaction with various phenolic compounds. *J. Sci. Food Agric.* 863: 402-407.

European Brewery Convention. 1987. Method 8.8.1. Ninhydrin colorimetric method (International Method). *Analytica- EBC*, 4th Ed., Zurich: Brauerei- und Getranke-Rundschau: E 141.

Faibish, R.S., Yoshida, W., and Cohen, Y. 2002. Contact angle study on polymer-grafted silicon wafers. *J. Colloid Interf. Sci.* 256: 341-350.

Forbes, J.M., and France, J. 1993. Introduction. Pages 1-12 in: *Quantitative Aspects of Ruminant Digestion and Metabolism*. J.M. Forbes and J. France, eds. C.A.B International: Wallingford, UK.

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

Fukumori, Y., Fukuda, T., Hanyu, Y., Takeuchi, Y., and Osaka, Y. 1987. Coating of pharmaceutical powders by fluidized bed process. I. Aqueous Enteric coating with

methacrylic acid-ethylacrylate copolymer and the dissolution behaviour of products. *Chem. Pharm. Bull.* 35: 2949-2957.

Gao, C., Taylor, J., Wellner, N., Byaruhanga, Y.B., Parker, M.L., Clare Mills, E.N., and Belton, P.S. 2005. *J. Agric. Food Chem.* 53: 306-312.

Gennadios, A. and Weller, C.L. 1990. Edible films and coatings from wheat and corn proteins. *Food Technol.* 44 (10): 63-69.

Gennadios, A., McHugh, T.H., Weller, C.L., and Krochta, J.M. 1994. Edible films and coatings based on proteins. Pages 201-277 in: *Edible Coatings and Films to Improve Food Quality*. J.M. Krochta, E.A. Baldwin, and M. Nisperos-Carriedo, eds. Technomic Publishing Company: Philadelphia, PA.

Gouin, S. 2004. Microencapsulation: industrial application of existing technologies and trends. *Trends Food Sci. Tech.* 15: 330-347.

Grant, L.A., and Burns, J. 1994. ApplicationS of coatings. Pages 189-200 in: *Edible Coatings and Films to Improve Food Quality*. J.M. Krochta, E.A. Baldwin, and M. Nisperos-Carriedo, eds. Technomic Publishing Company: Philadelphia, PA.

Guilbert, S. 1985. Technology and application of edible protective films. Pages 371-394 in: *Food Packaging and Preservation: Theory and Practice*. M. Mathlouthi, ed. Elsevier Applied Science Publishers: London.

Gunasekaran, S., Xiao, L., and Ould Eleya, M.M. 2006. Whey protein concentrate hydrogels as bioactive carriers. *J. Appl. Polym. Sci.* 99: 2470-2476.

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

- Hamaker, B.R., Kirleis, A.W., Butler, L.G., Axtell, J.D., and Mertz, E.T. 1987. Improving the in vitro protein digestibility of sorghum with reducing agents. Proc. Natl. Acad. Sci. USA 84: 626-628.
- Hofmann, R.R. 1973. The ruminant stomach: Stomach structure and feeding habits of East African game ruminants. Pages 12-37 in: East African Monographs in Biology. II. Introduction to the Functional Anatomy of the Ruminant Stomach. East African Literature Bureau: Nairobi.
- Hurtado-López, P. and Murdan., S. 2005. Formulation and characterisation of zein microspheres as delivery vehicles. J. Drug Deliv. Sci. Tec. 15: 267-272.
- Hurtado-López, P. and Murdan., S. 2006. Zein microspheres as drug/antigen carriers: A study on their degradation and erosion, in the presence and absence of enzymes. J. Microencapsul. 23 (3): 303-314.
- International Food Science Information Service, 2005. Dictionary of Food Science and Technology. Blackwell Publishing: Oxford
- Kaufmann, W., and Lúpping, W. 1982. Protected proteins and protected amino acids for ruminants. Pages 36-75 in: Protein Contributions for Feedstuffs in Ruminants. E.L. Miller, I.H. Pike, and A.J.H. Vanes, eds. Butterworth Scientific: London.
- Kawaguchi, H. 2002. Functional polymer microspheres. Prog. Polym. Sci. 25: 1171-1210.
- Kester, J.J., and Fennema, O.R. 1986. Edible films and coatings: A review. Food Techol. 40 (12): 47-59.
- Kobayashi Zein DP, 2004. CBC - Europe, www.cbc-europe.it. Obtained from Labworld, Centurion, South Africa, January 2007.

Koontz, J.L. 2006. Controlled release of active ingredients from food and beverage packaging. Italian Packaging Technology Award (IPTA) Paper. Institute of Packaging Technology. www.iopp.org, March 2008.

Krochta, J.M. 2002. Proteins as raw materials for films and coatings: definitions, current status, and opportunities. Pages 1-41 in: Protein-based Films and Coatings. A. Gennadios, ed. CRC Press: Boca Raton, FL.

Kydonieus, A.F. 1980. Fundamental concepts of controlled release. Pages 1-19 in: Controlled Release Technologies: Methods, Theory and Applications. A.F. Kydonieus, ed. CRC Press: Boca Raton, FL.

Lai, H., Padua, G.W., and Wei, L.S. 1997. Properties and microstructure of zien sheets plasticized with palmitic and stearic acids. *Cereal Chem.* 74: 83-90.

Lakkis, J.M. 2007. Introduction. Pages 1-11 in: Encapsulation and Controlled Release Technologies in Food Systems. J.M. Lakkis, ed. Blackwell Publishing: Ames, IA.

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

Lee, S.J., and Rosenberg, M. 1999. Preparation and properties of gluteraldehyde cross-linked whey protein-based microcapsules containing theophylline. *J. Control. Release* 61:123-136.

Lee, S.J., and Rosenberg, M. 2002. Whey protein-based microcapsules prepared by double emulsification and heat gelation. *Lebensm-Wiss. Technol.* 33: 80-88.

Li, J., Yang, L., Ferguson, S.M., Hudson, T.J., Watanabe, S., Katsuma, M., and Fix, J.A. 2002. In vitro evaluation of dissolution behaviour for a colon-specific drug delivery system (CODESTM) in multi-pH media using United States Pharmacopeia Apparatus II and III. American Association of Pharmaceutical Scientists. *Pharm. Sci. Tech.* 3 (4): article 33.

- Lin, S.Y., and Krochta, J.M. 2006. Fluidized-bed system for whey protein film coating of peanuts. *J. Food Process Eng.* 29: 532-546.
- Lui, X., Sun, Q., Wang., H., Zhang, L., and Wang, J. 2005. Microspheres of corn protein, zein, for an ivermectin drug delivery system. *Biomaterials* 26: 109-115.
- McSweeney, P.L.H., and Fox, P.F. 1995. Cheese: Methods of chemical analysis. Page 353 in: *Cheese: Chemistry, Physics and Microbiology*. P.F. Fox, ed. Springer: Berlin.
- Matsumura, E., Shin, T., and Murao, S. 1987. A new screening method for microorganisms which produce carboxypeptidases. *Agric. Biol. Chem.* 51: 2863-2864.
- Menegola, J., Steppe, M., and Schapoval, E.E.S., 2007. Dissolution test for citalopram in tablets and comparison of in vitro dissolution profiles. *Eur. J. Pharm. Biopharm.* 67: 524-530.
- Mertz, E.T., Hassen, M.M., Cairns-Whittern, C., Kirleis, A.W., Tu, L., and Axtell, J.D. 1984. Pepsin digestion of proteins in sorghum and other major cereals. *Proc. Natl. Acad. Sci. USA* 81: 1-2
- Muthuselvi, L., and Dhathathreyan, A. 2006. Simple coacervates of zein to encapsulate Gitoxin. *Colloid Surface. B.* 51: 39-43.
- Nunes, A., Correia, I., Barros, A., and Delgadillo, I. 2005. Sequential in vitro pepsin digestion of uncooked and cooked sorghum and maize samples. *J. Agric. Food Chem.* 52: 2052-2058.
- O'Donnell, P.B., Wu, C., Wang, J., Wang, L., Oshlack, B., Chasin, M., Bodmeier, R., and McGinity, J.W. 1997. Aqueous pseudolatex of zein for film coating of solid dosage forms. *Eur. J. Pharm. Biopharm.* 43: 83-89.

- Oneda, F., and Ré, M.I. 2003. The effect of formulation variables on the dissolution and physical properties of spray-dried microspheres containing organic salt. *Powder Technol.* 130 (1-3): 377-384.
- Oria, M.P., Hamaker, B.R., and Schull, J.M. 1995. In vitro protein digestibility of developing and mature sorghum grain in relationship to α -, β -, and γ -kafirin disulfide crosslinking. *J. Cereal Sci.* 22: 85-93.
- Ørskov, O.R. 1982. *Protein Nutrients in Ruminants*. Academic Press: London. pp. 19-39.
- Padua, G. W. and Wang, Q. 2002. Formation and properties of corn zein films and coatings. Pages 43-68 in: *Protein-based Films and Coatings*. A. Gennadios, ed. CRC Press: Boca Raton, FL.
- Padua, G.W., Rakotonirainy, A.M., and Ha, T.T. 2001. Method of manufacturing corn zein resin films, sheets, and articles. World Intellectual Property Organisation. Publication number: WO/2001/083597.
- Parris, N., and Dickey, L.C. 2003. Adhesive properties of corn zein formulations on glass surfaces. *J. Agric. Food Chem.* 51: 3892-3894.
- Park, H.J., Rhim, J.W., Weller, C.L., Gennadios, A., and Hanna, M.A. 2002. Pages 305-328 in: *Protein-based Films and Coatings*. A. Gennadios, ed. CRC Press: Boca Raton, FL.
- Pelosi, L.F. 1997. Crosslinking processing/agent for zein. US patent 5,596,080.
- Polan, C.E., Cummins, K.A, Sniffen, C.J., Muscato, T.V., Vicini, J.L., Crooker, B.A., Clark, J.H., Johnson, D.G., Otterby, D.E., Guillaume, B., Muller, L.D., Varga, G.A., Murray, R.A., and Peirce-Sandner, S.B. 1991. Responses of dairy cows to supplemental rumen-protected forms of methionine and lysine. *J.Dairy Sci.* 74: 2997-3013.

Ponte, J.G. Jr., Doğan, E.S., and Kulp, K. 2000. Special food ingredients from cereals. Page 764 in: Handbook of Cereal Science and Technology. K. Kulp and J.G. Ponte Jr., eds. Marcel Dekker: New York.

Qureshi, S.A., 1996. Calibration – The USP dissolution apparatus suitability test. Drug Inf. J. 30: 1055-1061.

Radwick, A.E., and Burgess, D.J. 2002. Proteins as microencapsulating agents and microencapsulated active compounds in pharmaceutical applications. Pages 341-366 in: Protein-based Films and Coatings. A. Gennadios, ed. CRC Press, Boca Raton, FL.

Romagnolo, D., Polan, C.E., and Barbeau, W.E. 1994. Electrophoretic analysis of ruminal degradability of corn proteins. J. Dairy Sci. 77: 1093-1099.

Schneider, K. 2004. Evaluation of grain legumes as a protein and energy feed for ruminants. PhD dissertation, Universität Hohenheim, Germany.

Shewry, P.R. 2002. The major seed storage proteins of spelt wheat, sorghum, millets and pseudocereals. Pages 1-24 in: Pseudocereals and Less Common Cereals – Grain Properties and Utilization Potential. P.S. Belton, and J.R.N. Taylor, eds. Springer: Berlin.

Shewry, P.R., and Tatham, A.S. 1990. The prolamin storage proteins of cereal seeds: structure and evolution. Biochem. J. 267: 1-12.

Shukla, R., and Cheryan, M. 2001. Zein: the industrial protein from corn. Ind. Crop. Prod. 13:171-192.

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

- Shull, J.M., Watterson, J.J., and Kirleis, A.W. 1992. Purification and immunocytochemical localisation of kafirins in *Sorghum bicolor* (L. Moench) endosperm. *Protoplasma* 171: 64-74.
- Tang, E.S.K., Chan, L.W., and Heng, P.W.S. 2005. Coating of multiparticulates for sustained release. *Am. J. Drug Deliv.* 3: 17-28.
- Taylor, J., and Taylor, J.R.N. 2006. Process for producing protein microparticles. South African patent draft application. Patent Application number: PA143050
- Taylor, J., Taylor, J.R.N., Dutton, M.F., and De Kock, S. 2005. Identification of kafirin film casting solvents. *Food Chem.* 90: 401-408.
- Taylor, J.R.N., and Belton, P.S. 2002. Sorghum. Pages 26-45 in: *Pseudocereals and Less Common Cereals – Grain Properties and Utilization Potential*. P.S. Belton, and J.R.N. Taylor, eds. Springer: Berlin.
- Taylor, J.R.N., Schober, T.J., and Bean, S.R. 2006. Novel food and non-food uses for sorghum and millets. *J. Cereal Sci.* 44: 252-271.
- Wall, J.S., and Paulis, J.W. 1978. Corn and Sorghum grain proteins. Pages 135-219 in: *Advances in Cereal Science and Technology*. Vol II. Y. Pomeranz, ed. American Association of Cereal Chemists: St. Paul, MN.
- Wang, Y., and Padua, G.W. 2004. Water sorption properties of extruded zein films. *J. Agric. Food Chem.* 52: 3100-3105.
- Wattiaux, M.A., and Howard, W.T. 2007. Digestion in the dairy cow. Chapter 1 in: *Nutrition and Feeding*. The Babcock Institute for International Dairy Research and Development. http://babcock.cals.wisc.edu/downloads/de_html/ch01.en.html , 24 April 2007.

Yamada, K., Takahashi, H., and Noguchi, A., 1995. Improved water resistance in edible zein films and composites for biodegradable food packaging. *Int. J. Food Sci. Tech.* 30: 599-608.

Yoshimaru, T., Takahashi, H., and Matsumoto, K., 2000. Microencapsulation of L-Lysine for improving the balance of amino acids in ruminants. *J. Fac. Agric. Kyushu U.* 44: 359-365.