CHAPTER 1

INTRODUCTION

Cereal grains have been a major food source and staple for humankind since the birth of civilisation. They are consumed in a very wide variety of either home-made or industrially processed food products. The importance of cereals among the food groups stems from the fact that they are dense, nutritious food packages which can be produced and traded economically in large quantities. If moisture and insect infestation are controlled, cereals can be stored for long periods.

In many parts of Africa, Asia and indeed, the semi-arid tropics worldwide, sorghum (*Sorghum bicolor* (L.) Moench) is an important basic food cereal. According to Doggett (1988), the wild forms of sorghum were confined to Africa, and the cultivated crop was domesticated on the African continent. Its food uses include various kinds of porridges, flatbreads, beer and other beverages. In fact, sorghum acts as a principal source of energy, protein, vitamins and minerals for millions of the poorest people living in these regions (reviewed by Klopfenstein & Hoseney, 1995).

Sorghum is said to be a warm-season, annual crop which is favoured by high day and night temperatures (reviewed by Rooney & Serna-Saldivar, 1993). In the semi-arid tropics, sorghum has the distinct advantage (compared to maize) of being drought-resistant and many subsistence farmers in these regions cultivate sorghum as a staple food crop for consumption at home (reviewed by Murty & Kumar, 1995). Therefore sorghum is crucial to the world food economy because it contributes to household food security in many of the world's poorest, most food-insecure regions (ICRISAT, 1996).

A limitation to the use of sorghum as food is the poor digestibility of sorghum proteins when cooked. *In vivo* studies (Maclean, Lopez, De Romana, Placko & Graham, 1981), and *in vitro* studies (Axtell, Kirleis, Hassen, D'Croz Mason, Mertz & Munck, 1981) indicate that the proteins of wet cooked sorghum are significantly less digestible than the proteins of other similarly cooked cereals like wheat and maize.

A great deal of research has been conducted by different workers into the possible reasons for this poor quality characteristic of sorghum protein. It is not surprising therefore, that diverse hypotheses have been proposed. Condensed tannins (oligomers of phenolic compounds) in certain sorghum varieties impart astringency to the grain and give a degree of bird- and mould-resistance (Hahn, Rooney & Earp, 1984). Astringency is caused by binding and precipitation of proteins by condensed tannins (Hahn *et al.*, 1984). This protein binding and precipitation reduces digestibility.

However, the problem of low sorghum protein digestibility also occurs in varieties which do not contain condensed tannins (Maclean *et al.*, 1981). Disulphide cross-linking of sorghum proteins on wet cooking (Hamaker, Kirleis, Butler, Axtell & Mertz, 1987) has been proposed as a possible cause of reduced sorghum protein digestibility. It has also been suggested that a strong association of protein with indigestible fibre components (Bach Knudsen & Munck, 1985) could cause lowered sorghum protein digestibility.

Thus, knowledge and comprehension of the reasons for poor sorghum protein digestibility remain far from complete and many important questions still remain unanswered. For example, there is no clear picture of what the nature of the problem is at various levels of structural organisation of the grain, for example, whole grain, endosperm, protein body and protein levels. The fact that sorghum and maize proteins exhibit extensive homology (De Rose, Ma, Kwon, Hasnain, Klassy & Hall, 1989) makes the superior protein digestibility of wet-cooked maize difficult to understand. More puzzling is the observation that disulphide cross-linking on cooking, believed to be one of the factors contributing to poor protein digestibility of cooked sorghum, has been shown to occur in cooked maize as well (Batterman-Azcona & Hamaker, 1998).

Cereals will remain a fundamental component in human diets and this puts sorghum in sharp focus as an important grain in areas where it is used for human consumption. World population is projected to increase by about nine million people per year over the coming decades and much of this growth is expected to be in the developing countries of Africa, Asia and Latin America (Kennedy & Haddad, 1993). According to the World Health Organisation, large populations of children and adults, especially in Africa, subsist on inadequate food supplies in times of drought (WHO, 1990). Sub-Saharan Africa, Southeast Asia and central America are listed as some of the areas having the greatest proportion of children with low

weight-for-age, a characteristic indicator of protein-energy malnutrition (Brown & Solomons, 1993). Therefore, the improvement of sorghum nutrient availability is critical for food security in these regions.

Cereal scientists and sorghum food processors are thus faced with the challenge of identifying the factors which adversely affect, and developing processing procedures which improve sorghum protein digestibility.

CHAPTER 2

LITERATURE REVIEW

2.1 Sorghum and maize: Origin, physical characteristics and chemical composition

Sorghum (*Sorghum bicolor* (L.) Moench), and maize (*Zea mays* L.) are grains produced by members of the grass family Poaceae (FAO, 1995). Sorghum belongs to the tribe Andropogonae (FAO, 1995) and maize, to Maydae (Winton & Winton, 1932). Sorghum is believed to have originated in Ethiopia (reviewed by House, 1995). Due to its drought-resistant nature, it is grown primarily in semi-arid parts of the world in harsh environments where other crops grow or yield poorly (FAO, 1995). Maize, on the other hand, is native to the Americas, with Mexico considered as its centre of origin (reviewed by Johnson, 1991). Today, every continent, except Antarctica produces maize and it ranks as the second most widely produced cereal crop worldwide (reviewed by Johnson, 1991).

Sorghum and maize kernels are botanically classified as naked caryopses (dry, indehescent, single-seeded fruit) (reviewed by Winton & Winton, 1932; reviewed by Johnson, 1991), though sorghum may be partially covered with glumes (reviewed by Serna-Saldivar & Rooney, 1995). Sorghum kernels are generally spherical and vary in size (between 4 to 8 mm in diameter) (reviewed by FAO, 1995). Sorghum kernel weight also varies widely, from 3 to 80 g per 1000 kernels but between 25 and 30 g in majority of varieties. On the other hand, maize kernels tend to be flat seeds due to pressure during growth from adjacent kernels on the cob (reviewed by Johnson, 1991). They have a blunt crown and a conical tip cap. Maize kernels are the largest cereal grains, weighing 250-300 mg each.

Both cereals are widely consumed in Africa as staples and are therefore important sources of nutrients. Maize occupies a more dominant position, as it is generally, the most suitable field crop for the growing conditions in Africa (Cownie, 1993). Sorghum production tends to be restricted to the drier areas. In most of the developing world where sorghum is grown by local farmers on a subsistence level for human consumption, the crop plays a major role in contributing to household food security (ICRISAT, 1996). It is estimated that more than 70 percent of the sorghum crop is consumed as food in the main production areas of Africa and Asia (ICRISAT, 1996). This makes the role of sorghum and maize as nutrient sources crucial.

The proximate compositions of sorghum and maize are very similar, as shown in Table 1 below:

Table 1. Proximate composition of sorghum and maize grain in g per 100 g edible portions at 12% moisture*

	Sorghum	Maize
Protein	10.9	9.2
Fat	3.2	4.6
Carbohydrate	73	73
Crude fibre	2.3	2.8
Ash	1.6	1.2

*Data from Klopfenstein & Hoseney (1995)

2.1.1 Proteins of sorghum and maize

In the areas where sorghum and maize are consumed as staples, protein from animal sources tend to be expensive or even unaffordable. As a result, these rural communities rely on these grains for their protein supply. Therefore the quality and quantity of protein from sorghum and maize is important from the point of view of these rural communities.

Seed proteins in general are composed of three groups namely, storage proteins, structural proteins and biologically active proteins (enzymes) (reviewed by Fukushima, 1991). The storage proteins are quantitatively major ones and are thought to function as a mobilisable source of carbon and nitrogen to support seedling growth and development during germination. In fact, the storage proteins have been described as a sink for surplus nitrogenous compounds required for physiological processes (Tsai, Huber & Warren, 1978).

Osborne (1924) described a method by which cereal proteins can be fractionated and categorised. Osborne's classification includes albumins (soluble in water), globulins (soluble in saline solution), prolamins (soluble in alcohol) and glutelins (soluble in dilute alkali). This procedure has provided the basis for, and been most useful in structural and functional investigations of cereal proteins. According to Taylor, Schüssler and Van der Walt (1984a), protein fractionation in sorghum has been used for many purposes. These include:

determination of their chemical composition, comparison of the composition of proteins from different sorghum varieties, explanation of different responses of rats fed high- and low-tannin sorghum, determination of which protein are increased in high lysine varieties, determination of which proteins are affected when sorghum grain is dehulled and micronized among other things.

One of the major problems of Osborne's fractionation procedure was its low yield of extracted protein. Skoch, Deyoe, Shoup and Bathurst (1970) reported extraction of only 26-40% of total proteins in sorghum using Osborne's method. The procedure was subsequently modified by Landry and Moureaux (1970) to yield five fractions. According to Taylor *et al.* (1984a), two important changes were introduced which resulted in much improved protein extraction. These changes were the use of aqueous alcohol plus reducing agent after the aqueous alcohol extraction and a final extraction with basic buffer containing a detergent and a reducing agent.

The Osborne protein fractions are summarised in Table 2 below.

Table 2 Osborne protein fractions of sorghum* and maize**

	Protein fraction	
Extractant	Sorghum	Maize
Saline	Low molecular weight nitrogen, albumins and globulins	Low molecular weight nitrogen, albumins and globulins
Alcohol	Kafirin 1	Zein 1
Alcohol with reducing agent	Crosslinked kafirin (Kafirin 2)	G1 glutelins*** (Zein 2)
Buffer with reducing agent	Glutelin-like proteins	G2 glutelins
Buffer with reducing agent and detergent	Glutelin	G3 glutelins

* Guiragossian, Chibber, Van Scoyoc, Jambunathan, Mertz & Axtell (1978).

** Landry & Moureaux (1970).

*** The G1 glutelins are zeins in a disulphide crosslinked form.

The prolamins are the major alcohol-soluble cereal proteins and make up about 50% of the total grain protein (Paulis & Wall, 1979, Lending, Kriz, Larkins & Bracker, 1988). It was Osborne who originally coined the term "prolamin" during his work on seed proteins. His criteria for a protein to be referred to as a prolamin was extractability in aqueous alcoholic solvents (but not in aqueous buffers or water), and high proline and amide nitrogen (glutamine and/or asparagine) (Esen, 1987). The prolamins have been given different names in different cereals like the gliadin of wheat, hordein of barley, secalin of rye, zein of maize, panicin of millet and the kafirin of sorghum (Hulse, Laing & Pearson, 1980). Zeins and kafirins are found in protein bodies in the endosperm (Taylor *et al.*, 1984a) and are structurally related (Hamaker, Mohamed, Habben, Huang & Larkins, 1995).

A system of nomenclature has been proposed for the zein polypeptides (Esen, 1987). In this system, the zeins are separated into three distinct classes, α-, β, and γ-zeins based on differences in molecular weight, solubility and amino acid composition. According to Esen (1987), α-zein constitutes 75-85% of the total zein in maize, depending on the genotype and is made up of polypeptides of molecular weight in the range 21-25 kDa. Beta-zein constitutes 10-15% of total zein and includes two methionine-rich polypeptides in the molecular weight range 17-18 kDa. Gamma-zein constitutes 5-10% of total zein and is made up of a one-size class, a 27 kDa proline-rich polypeptide. There has since been a revision of this nomenclature system in which the 18 kDa polypeptide is removed from the β-zein class and designated γ-zein$_2$, whilst the 27 kDa polypeptide (formerly γ-zein) is referred to as γ-zein$_1$ (Esen, 1990). Esen (1987) also reported the presence of a group of minor low molecular weight (9-10 kDa) zeins which may be referred to as δ-zeins. He proposed that one of them could be included in the α-zein class on the basis of its solubility in 90% 2-propanol and slight immunological cross-reactivity with a 22 kDa α-zein.

Shull, Watterson and Kirleis (1991) have reported that the kafirin polypeptides in sorghum could be extracted under conditions similar to those used for corresponding zeins and so could be named in an analogous fashion. Alpha-kafirins comprise 66-71% and 80-84% of the total kafirin in the opaque and vitreous kernel sections respectively. They are two groups of polypeptides of molecular weight 25 and 23 kDa, may be extracted with 40-90% *tert*-butyl alcohol plus 2-mercaptoethanol and show immunological cross-reactivity with α-zein. Beta-kafirin (extractable with 10-60% *tert*-butyl alcohol plus 2-mercaptoethanol) comprises 7-8%

of sorghum prolamin (Hamaker *et al.*, 1995), consists of a 20 kDa polypeptide and shows immunological cross-reactivity with β-zein antiserum (Shull *et al.*, 1991). Shull *et al.* (1991) also found two other polypeptides with molecular weights 18 and 16 kDa which did not give a positive reaction with β-zein antiserum. However, because these polypeptides displayed similar solubility properties to the 20 kDa protein, they suggested that the 18 and 16 kDa proteins be added to β-kafirin class. Of the total kafirin, γ-kafirin (extractable in 10-80% *tert*-butyl alcohol plus 2-mercaptoethanol), comprises 9-12% and consists of a polypeptide of molecular weight 28 kDa.

2.1.2 Structural organisation of sorghum and maize grains

Sorghum and maize are remarkably similar in the type and organisation of their anatomical parts (Figures 1 and 2). The principal anatomical components in both cereals are the pericarp, germ or embryo and the endosperm (reviewed by Johnson, 1991; reviewed by Serna-Saldivar & Rooney, 1995). Maize is considered to have a fourth component, the tip cap, which provides the point of attachment between the cob and the kernel (reviewed by Johnson, 1991). The distribution by weight of these components in sorghum is on the average, pericarp 6%, endosperm 84% and germ 10% (FAO, 1995). In maize, it is pericarp 5.2%, endosperm 82%, germ 12% and tip cap 0.8% (Eckhoff, 1995).

2.1.2.1 Pericarp

The pericarp is the outermost structural component in both kernels and is arranged in distinct sub-layers namely, epicarp, mesocarp and endocarp (Eckhoff, 1995). In sorghum, the epicarp is considered to be composed of the epidermis and the hypodermis (reviewed by FAO, 1995). Epidermal cells in both cereals are generally thick-walled and covered with a layer of a waxy substance called cutin (Eckhoff, 1995, reviewed by FAO, 1995) which restricts the entry of water, water vapour and other gases and liquids (Eckhoff, 1995). The mesocarp appears to be the thickest layer of the pericarp in both cereals, consisting of several layers of elongated, thin-walled cells. Sorghum mesocarp may contain starch granules, unlike other cereals (reviewed by Serna-Saldivar & Rooney, 1995). The endocarp is the innermost sub-layer of the pericarp and consists of cross and tube cells. They are large, open cells and allow for diffusion of gases and liquids into the kernel (Eckhoff, 1995).

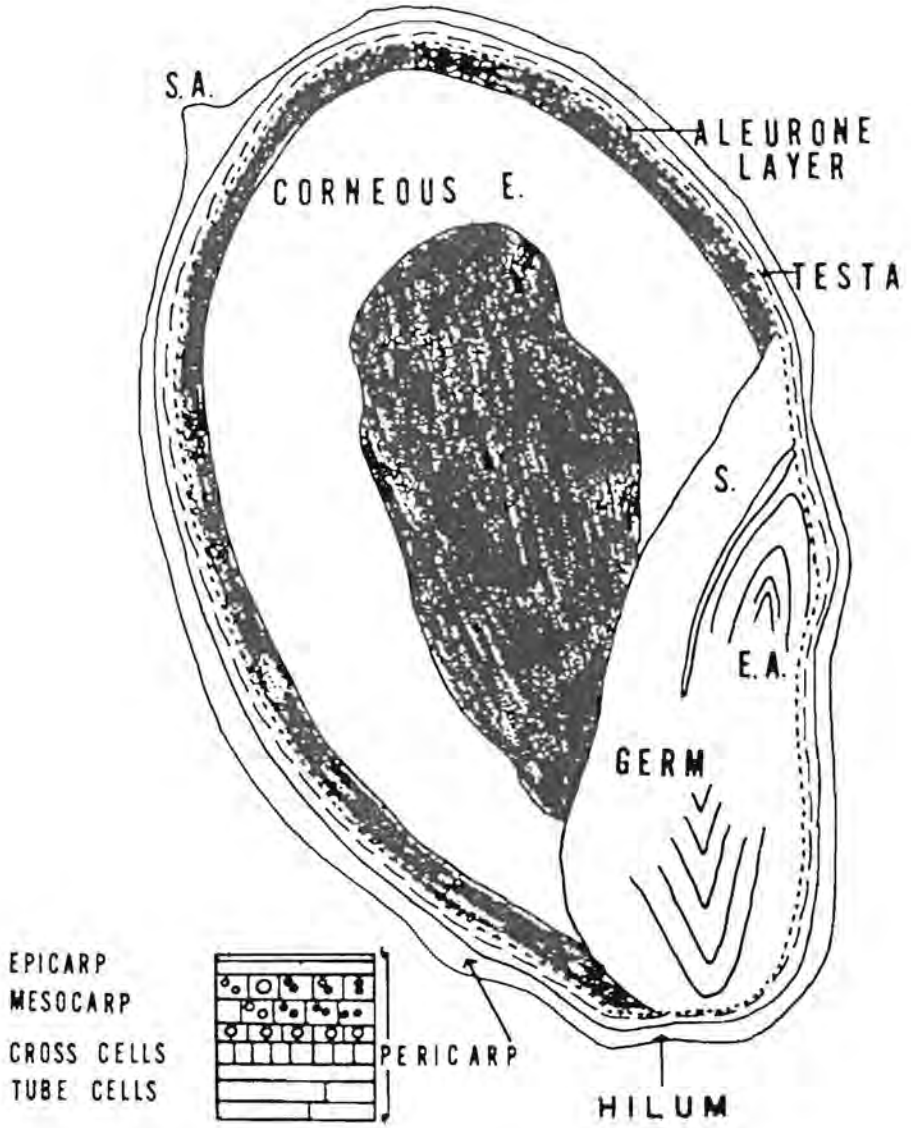

Figure 1: A sorghum kernel. S.A., stylar area; E., endosperm; S., scutellum; E.A., embryonic axis. (Hoseney, 1994).

Just underneath the pericarp layers is the seed coat or testa layer. The testa in maize is considered to be a semi-permeable membrane, which restricts movement of macromolecules into and out of the kernel (Eckhoff, 1995). In sorghum, the testa may be highly pigmented, a characteristic which is genetically controlled (reviewed by Serna-Saldivar & Rooney, 1995). Such sorghums with pigmented testa contain condensed tannins and are referred to as type II or type III sorghums (the latter contain the greater amount of condensed tannins) (reviewed by Serna-Saldivar & Rooney, 1995).

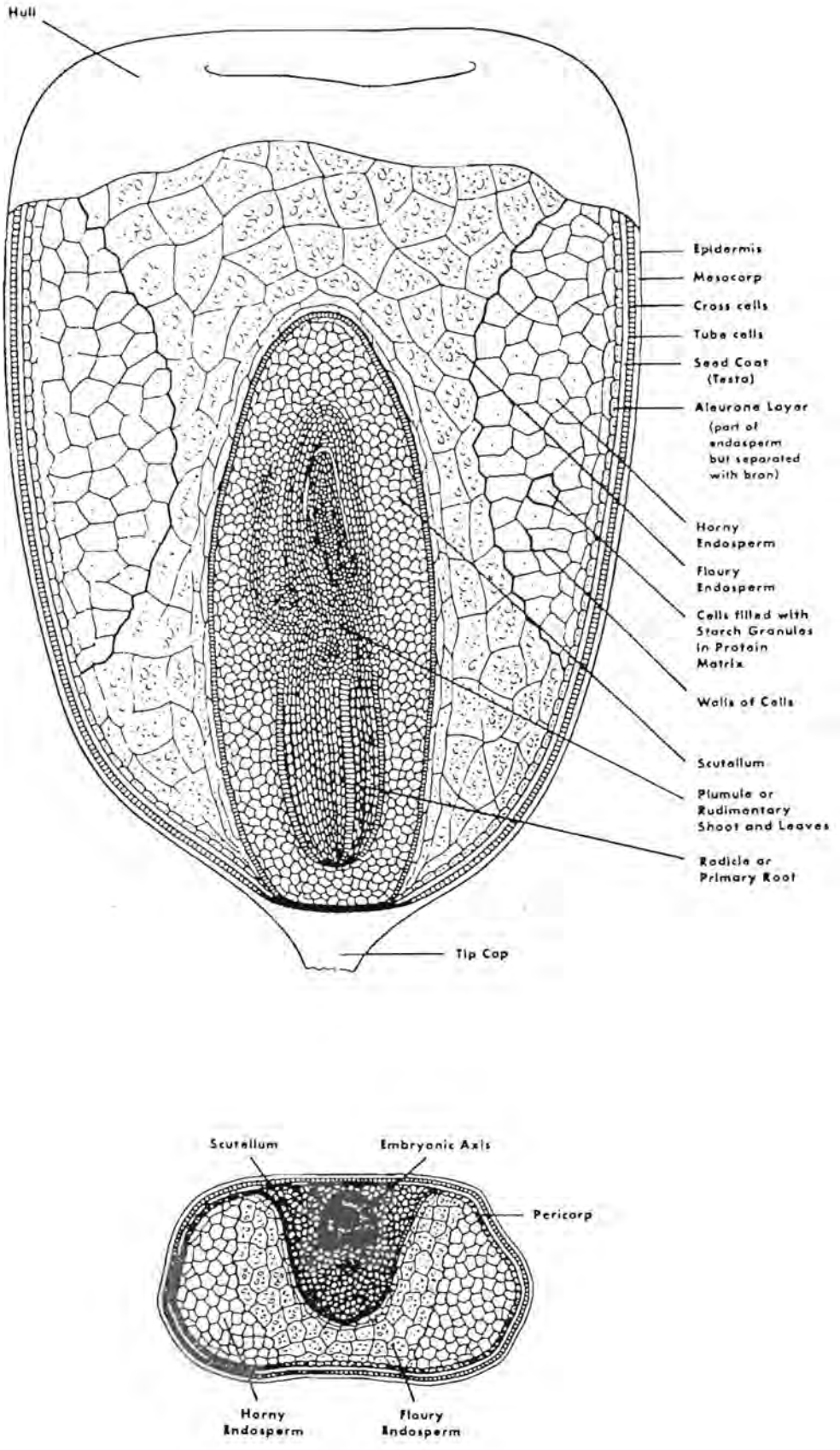

Figure 2: Longitudinal and cross sections of a maize kernel. (Hoseney, 1994)

2.1.2.2 Germ

The germ tissue is rich in lipids, protein, enzymes and minerals (FAO, 1995). This store of enzymes and nutrients is important to the plant from a reproductive standpoint (Eckhoff, 1995). The oil in the germ of both cereals is very similar, rich in polyunsaturated fatty acids (FAO, 1995).

2.1.2.3 Endosperm

The endosperm is composed of two parts, an outer single layer of cells known as the aleurone, and the starchy endosperm. The aleurone layer lies just interior to the testa and its cells are rich in minerals, vitamins, oil and contain hydrolysing enzymes (FAO, 1995). The starchy endosperm is the major storage tissue and the largest component of the kernel.

Electron microscopic techniques have revealed that the ultra-structure of sorghum and maize endosperm bear considerable similarity to each other. The main structural organelles in the endosperm are cell walls, starch granules, protein bodies and a protein matrix. The endosperm is considered to comprise of two visually and physically distinct regions; soft (floury) and hard (horny) endosperm (Hoseney, Davis & Harbers, 1974). In sorghum, the outermost region of the starchy endosperm, just beneath the aleurone layer has been described as the peripheral endosperm (reviewed by Serna-Saldivar & Rooney, 1995).

Duvick (1961) used an analogy to describe the arrangement of organelles in maize endosperm. Light microscopy of a section through a mature, horny endosperm cell, revealed that it had "somewhat the appearance of a section through a box of white marbles (starch grains) in which buckshot (protein bodies) has been used as packing between the marbles. The whole boxful is then filled with a transparent glue (clear, viscous cytoplasm or protein matrix) which surrounds the marbles and buckshot and makes the ensemble, when dry, a rigid conglomerate". This model aptly describes the structural organisation of the endosperm in sorghum and maize; starch granules amongst which numerous protein bodies embedded in a protein matrix are scattered.

Various workers have described this mode of organisation in the endosperm of the two cereals. Khoo and Wolf (1970) in examining mature maize kernels, observed a network in which protein bodies and starch granules were scattered in an amorphous matrix of protein. Working on sorghum endosperm ultrastructure, Seckinger and Wolf (1973) observed that the

subaleurone and horny endosperm portions of the grain contained protein bodies with an average diameter of 2 μm and were tightly packed within a network of matrix protein. In the more inner floury endosperm, protein bodies were not so tightly packed and much smaller in size, ranging from 0.3 to 1.5 μm in diameter. The development of sorghum endosperm has been investigated using electron microscopy (Shull, Chandrashekar, Kirleis & Ejeta, 1990). As the seed matured, there was expansion of the endosperm due to cell enlargement. At 25 days after pollination, the pericarp cells were compressed by the expanding endosperm and the starch granules assumed a polygonal shape in the outer endosperm, leading to tight cell packing in the outer endosperm. Protein bodies became buried in a protein matrix. The combination of large starch granules, numerous protein bodies, and protein matrix in the outer endosperm formed a continuous structure. The central endosperm on the other hand, was less packed with more spherical starch granules. At 40 days after pollination, cell packing was tight and the protein bodies caused deep indentations on the surface of the starch granules. Central endosperm cells remained loosely packed with a discontinuous matrix.

2.1.3 Localisation of proteins in the various anatomical parts of sorghum and maize

Protein distribution is uneven between the different anatomical portions of sorghum and maize. The most comprehensive investigations into protein compositions of the anatomical parts of maize and sorghum include those of Landry and Moureaux (1980) on maize and Taylor and Schüssler (1986) on sorghum. These studies indicate that sorghum and maize are very similar with regard to localisation of proteins in the various parts of the grains.

Approximately 3% of the total grain nitrogen is found in sorghum pericarp (Taylor & Schüssler, 1986) but most of this pericarp protein was not extractable using the modified Osborne fractionation procedure of Landry and Moureaux (1970), possibly due to association with cell walls. Landry and Moureaux (1980) reported a similar content of protein in maize pericarp, 25% of which could be extracted with water and saline. The remaining protein was not subjected to further extraction with alcohol. Sorghum pericarp protein is rich in glycine, lysine and arginine. Small quantities of protein extracted with alcohol from sorghum pericarp had relatively low quantities of glutamic acid and rich in lysine compared to similarly extracted endosperm proteins, suggesting that they were not kafirins (Taylor & Schüssler, 1986).

Sorghum germ contains approximately 16% of grain nitrogen (Taylor & Schüssler, 1986) whilst two maize varieties studied had protein concentrations of 20.1% and 14.9% in the germ (Landry & Moureaux, 1980). Most of the germ protein occurs as low molecular weight nitrogen and albumin and globulin proteins and were rich in essential amino acids, especially lysine (Landry & Moureaux, 1980; Taylor & Schüssler, 1986).

Sorghum endosperm contains the highest proportion of grain nitrogen, approximately 80% and more than 60% of this protein is prolamin, rich in glutamic acid, proline, alanine and leucine but poor in lysine (Taylor & Schüssler, 1986). Maize endosperm had a similar protein profile (Landry & Moureaux, 1980). The kafirins and zeins are the most abundant proteins of sorghum and maize grains and they are endosperm-specific (Landry & Moureaux, 1980; Taylor & Schüssler, 1986).

The G3-glutelin protein (extracted with buffer, reducing agent and detergent) was the second most important fraction in sorghum endosperm. It was poor in glutamic acid and rich in lysine compared to the kafirins. Taylor and Schüssler (1986) suggest that the G3-glutelins may comprise the glutelin matrix surrounding the protein bodies in sorghum endosperm.

The protein compositions of the vitreous (horny) and opaque (floury) portions of the endosperm are different. Work on sorghum revealed that vitreous endosperm contains 1.5-2 times more total protein than opaque endosperm (Watterson, Shull & Kirleis, 1993). Opaque endosperm also contained less kafirin (2.0-2.4%) compared to vitreous endosperm (5.8-8.5%). In contrast, opaque endosperm had higher levels of albumin and globulin proteins whilst the amount of glutelin protein was similar in both vitreous and opaque endosperm (Watterson *et al.*, 1993).

The mechanisms of prolamin synthesis in sorghum and maize are believed to be the same. Prolamins are synthesised on membrane-bound polyribosomes of the rough endoplasmic reticulum as higher molecular weight precursors containing signal peptides which are discharged into and cleaved off as the proteins enter the lumen of the rough endoplasmic reticulum (Miflin, Burgess & Shewry, 1981; Taylor, Schüssler & Liebenberg, 1985a). The resultant polypeptides, once inside the lumen of the rough endoplasmic reticulum, associate through interactions including disulphide bond formation, to form dense, insoluble masses which causes the endoplasmic reticulum to become distended to form the deposits known as

protein bodies (Larkins, Pedersen, Marks & Wilson, 1984). In this respect, the protein bodies of sorghum and maize differ from other cereals such as wheat (Parker & Hawes, 1982) and barley (Cameron-Mills & Von Wettstein, 1980) where the protein bodies occur in the vacuole.

Study of degradation and hydrolysis patterns of zeins in maize and kafirins in sorghum endosperm during germination and have shown that these proteins are hydrolysed in a sequential manner and that the protein bodies are degraded progressively from their surface inwards (Taylor, Novellie & Liebenberg, 1985b; Taylor & Evans, 1989; Torrent, Geli & Ludevid, 1989; Mohammad & Esen, 1990). In addition, immunocytochemical techniques have been used to determine the localisation of zeins and kafirins within protein bodies.

Staining with uranyl acetate and lead citrate reveals with transmission electron microscopy, light- and dark-staining regions of protein bodies with the darker stain predominating at the periphery and the lighter stain in the central region (Lending *et al*, 1988; Shull, Watterson & Kirleis, 1992). The light-staining central region may contain dark-staining inclusions. Alpha-zeins and α-kafirins are generally limited to the light-staining regions within the core of maize and sorghum protein bodies (Lending *et al*, 1988; Shull *et al*, 1992). Beta- and γ-zeins and kafirins are found mainly in the dark-staining regions in a peripheral band around the core of the protein bodies, and also in the dark-staining central inclusions (Lending *et al*, 1988; Shull *et al*, 1992). Work by Esen and Stetler (1992) has shown that δ-zein is localised to the core region of maize protein bodies.

Lending and Larkins (1989) proposed a descriptive model for the pattern of zein deposition during protein body formation (Figure 3). Initially, dark-staining deposits of β- and γ-zeins build up within the rough endoplasmic reticulum with little or no α-zein. Subsequently, α-zein begins to accumulate and is observed as discrete, light-staining deposits within the β- and γ-zeins. The deposits of α-zein fuse and aggregate to form a central core whilst some smaller locules of α-zein remain and are interspersed in the outer region of the protein body. The dark-staining region containing β- and γ-zein forms a continuous layer around the periphery of the protein body. In the final stages of protein body maturation, α-zein fills most of the core of the protein body and is surrounded by a thin layer of β- and γ-zeins. Small, dark-staining patches of β-zein and, more commonly, γ-zein may occur within the interior region (Lending & Larkins, 1989).

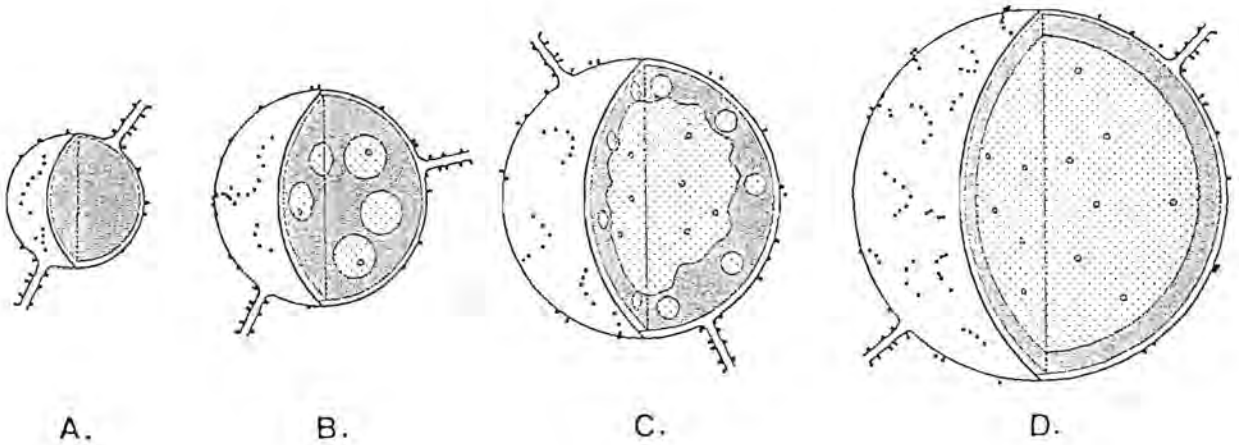

Figure 3: Development of protein bodies in maize endosperm as proposed by Lending and Larkins (1989). Dark shaded regions are rich in β- and γ-zeins and the light shaded regions are rich in α-zein. The dark dots represent ribosomes.

However, Taylor, Schüssler and Liebenberg (1984b) presented evidence which seemed to suggest that in maize, the β- and γ-zeins were less peripheral and did not seem to form a layer or shell at the protein body periphery as suggested above by the Lending and Larkins model. Transmission electron micrographs showed that extraction of maize and sorghum protein bodies with alcohol resulted in removal of most of the material within the protein bodies (Taylor *et al.*, 1984b). The unextracted material in maize appeared to be fairly randomly distributed throughout the protein bodies whilst this material appeared to be mainly in the form of thin layers on the inside surfaces of the sorghum protein bodies with some deposits in the middle. The reason for these apparent differences in prolamin distribution within sorghum and maize protein bodies is not clear. Perhaps this could be due to protein bodies in different stages of development.

2.2 Food uses of sorghum and maize

In many parts of Africa, the food uses of sorghum and maize are still mostly traditional and their methods of processing may involve the use of wet or dry heat (reviewed by Murty & Kumar, 1995).

Porridges appear to be the most common types of food prepared from sorghum and maize using wet heat treatment. A range of porridges of varying consistencies (soft or thick) may be prepared from fermented or non-fermented sorghum or maize meal (reviewed by Murty & Kumar, 1995). Porridge preparation involves cooking the meal with boiling water and the process varies considerably depending on the type of porridge being produced (Taylor, Dewar, Taylor & Von Ascheraden, 1997).

Sorghum and maize grains are also popped and consumed as snacks or delicacies. Traditionally, popping is carried out by heating the grain in a hot pan or bowl over a steady fire with popping occurring within a minute accompanied with a hissing and splitting noise (reviewed by Murty & Kumar, 1995).

2.3 Protein nutritional value of sorghum and maize

2.3.1 Amino acid composition

One of the indicators of protein nutritional value is amino acid composition. Generally, a protein may be considered as of good nutritional value if it is a good source of essential amino acids. Sorghum and maize appear to have similar amino acid compositions as shown in Table 3 below. Like cereals in general, sorghum and maize grains, in comparison with a high quality animal protein like egg, are very poor sources of essential amino acids, in particular, lysine and the sulphur-containing amino acids. The germ and pericarp, normally removed during processing, are two to three times richer in lysine than the endosperm (Taylor & Schüssler, 1986). Therefore decortication of sorghum or degerming of maize leads to a product with reduced lysine content (Taylor & Schüssler, 1986). Supplementation of sorghum- or maize-based diets with legumes helps to alleviate this problem. This is of particular importance for infants who have a high essential amino acid requirement (reviewed by Serna-Saldivar & Rooney, 1995).

Table 3 Essential amino acid composition (mg/g crude protein) of maize and sorghum whole grain* in comparison with suggested amino acid requirements (mg/g crude protein) for infants and adults and amino acid composition of egg as a high quality animal protein**.

Amino acid	Maize	Sorghum	Infant requirement	Adult requirement	Egg
Lysine	33.9	25.2	66.0	16.0	70.0
Histidine	30.4	21.4	26.0	16.0	22.0
Threonine	45.7	42.7	43.0	9.0	47.0
Valine	59.7	56.3	55.0	13.0	66.0
Isoleucine	50.4	56.3	46.0	13.0	54.0
Leucine	142.8	132.0	93.0	19.0	86.0
Methionine + Cystine	48.6	50.1	42.0	17.0	93.0
Phenylalanine + Tyrosine	98.4	67.0	72.0	19.0	47.0

*Values re-calculated from Scherz & Senser (1989) based on crude protein contents of 8.5% for maize and 10.3% for sorghum.

**FAO/WHO/UNU (1985)

2.3.2 Protein digestibility

Digestibility may be used as an indicator of protein availability. It is essentially a measure of the susceptibility of a protein to proteolysis. A protein with high digestibility is of better nutritional value than one of low digestibility because it would provide more amino acids for absorption on proteolysis. The protein digestibility of sorghum and maize has been a subject of extensive research and many *in vivo* and *in vitro* studies have been conducted in this regard.

It has been suggested that the protein digestibility of raw (uncooked) sorghum grain is lower than for other cereals (Hamaker, Kirleis, Mertz & Axtell, 1986; Hamaker *et al*, 1987; Oria, Hamaker & Shull, 1995a). A closer look at the literature suggests that this might not necessarily be the case. *In vitro* protein digestibilities of uncooked sorghum and maize reported by different workers are shown in Table 4 below. Marginally lower protein digestibilities for uncooked sorghum compared to uncooked maize have been reported

(Hamaker *et al.*, 1986; Hamaker *et al.*, 1987). However, protein digestibility values for uncooked sorghum show a lot of variation with very high results (92.9%) in some cases. In comparing the values in Table 4 though, the possibility of environmental factors affecting protein digestibility in different years must be borne in mind.

Table 4 *In vitro* protein digestibility, IVPD (%) of uncooked sorghum and maize reported by different workers

Test sample	(IVPD)	Reference
High-tannin sorghum variety BR64; 37% dehulled	70.8	Chibber, Mertz & Axtell (1980).
Condensed tannin-free sorghum variety P-721N; whole grain	92.9	Axtell, *et al.* (1981).
Condensed tannin-free sorghum variety P-721N; whole grain	80.7	Hamaker, *et al.* (1986).
Condensed tannin-free sorghum variety P-721N; whole grain	80.8	Hamaker, *et al.* (1987).
Maize, whole grain	81.5	Hamaker, *et al.* (1986).
Maize, whole grain	83.4	Hamaker, *et al.* (1987).

It is generally agreed though, that cooking reduces the protein digestibility of sorghum significantly *in vivo* (Kurien, Narayanarao, Swaminathan & Subrahmanyan, 1960) and *in vitro* (Axtell *et al*, 1981; Hamaker *et al.*, 1986; Hamaker *et al.*, 1987). Other cereals like maize, barley, rice and wheat may show some decrease in protein digestibility after cooking. However it appears this is not nearly to the same degree as sorghum. Hamaker *et al.* (1987) observed a 24.5% decrease in sorghum protein digestibility *in vitro* on cooking compared to a 4.1% decrease for maize, 13.0% for barley, 9.1% for rice and 5.4% for wheat. The problem of poor sorghum protein quality due to its low content of the essential amino acids is therefore exacerbated by reduction of sorghum protein digestibility on cooking.

2.4 Factors affecting protein digestibility of sorghum and maize

2.4.1 Starch and cell walls

The association of proteins with components of the grain like starch and cell walls appears to have an influence on protein digestibility. Significant amounts of protein have been found associated with total dietary fibre and acid detergent fibre fractions in uncooked and cooked sorghum which differed significantly in this respect from other cereals like wheat, rye, barley and maize. Higher amounts of protein were associated with dietary fibre fractions of cooked sorghum (Bach Knudsen & Munck, 1985).

An important factor governing bio-availability of nutrients is the physical form in which foods are consumed (Tovar, De Fransisco, Björck & Asp, 1991). It has been shown in legumes that the cotyledon tissue structure and the presence of thick cell walls represent a physical barrier for starch digestion (Tovar *et al.*, 1991; Tovar, Granfeldt & Björck, 1992) and also limits protein digestibility (Melito & Tovar, 1995). In germinating barley seeds, endosperm cells with intact cell walls, starch granules and storage protein were observed adjacent to degraded endosperm tissue and appeared identical to endosperm cells of ungerminated seeds (Gram, 1982). Isolated sorghum endosperm cell walls were found to have 46% protein associated with them (Glennie, 1984). These observations suggest that the endosperm cell wall could form a barrier against enzymes hydrolysing starch and protein within the endosperm (Gram, 1982; Melito & Tovar, 1995).

As described earlier, starch granules and protein bodies in sorghum and maize endosperms are in very close association with each other. In the horny endosperm of both grains, the largely polygonal, tightly packed starch granules have cellular spaces in which numerous, largely spherical protein bodies embedded in a protein matrix are scattered (Khoo & Wolf, 1970; Shull *et al.*, 1990). The implication of such a close association between starch and protein may be that the starch, especially when gelatinised after cooking could reduce accessibility of proteolytic enzymes to the protein bodies and therefore reduce protein digestibility. However, Oria, Hamaker and Shull (1995b) found that the protein digestibility of decorticated sorghum flour cooked with heat-stable α-amylase was approximately the same as that cooked without

The opposite effect of protein on starch gelatinisation and digestibility has been investigated. Chandrashekar and Kirleis (1988) found more kafirin-containing protein bodies in sorghum grains with lower capacities for starch gelatinisation. Additionally, the manner in which protein bodies were organised around the starch granule appeared to act as a barrier to starch gelatinisation (Chandrashekar & Kirleis, 1988). Hamaker and Griffin (1993) reported similar results from their study on rice. They observed that addition of reducing agent (2-mercaptoethanol) to cooking media increased the degree of gelatinisation of rice starch. The reducing agent presumably cleaved disulphide bonds linking protein polymers surrounding the starch granules thus leading to an increase in degree of starch gelatinisation. Zhang and Hamaker (1998) have reported that when sorghum flour was treated with pepsin before cooking, or cooked with a reducing agent there was in increase in starch digestibility, suggesting that protein had an influence on starch digestibility.

2.4.2 Polyphenols

Phenolic compounds in sorghum may be divided into three major categories: phenolic acids, flavonoids and tannins (Hahn *et al*, 1984). Maize contains phenolic acids and flavonoids but not tannins. The fact that some sorghum cultivars produce tannins makes it unique among major cereals (reviewed by Serna-Saldivar & Rooney, 1995). According to Gupta and Haslam (1978), barley is the only other cereal in which tannins are found. However, rye has also been mentioned as another cereal containing tannin (Butler, Riedl, Lebryk & Blytt, 1984).

Phenolic acids are derivatives of cinnamic or benzoic acid with hydroxyl (OH) and methoxy (OCH_3) groups substituted at various points on the aromatic ring (Figure 4 Xi) and ii)). They may occur as free acids, soluble esters or insoluble esters in cereals and are concentrated in the outer layers of the grain (pericarp, testa and aleurone) (Hahn *et al.*, 1984). Only the bound, insoluble ester forms are found in the endosperm and appear to be associated with the endosperm cell walls. Ferulic acid (3-methoxy-4-hydroxycinnamic acid) is the major bound phenolic acid of sorghum (Hahn *et al.*, 1984). High levels of bound *trans*-ferulic acid have been reported in maize (Sosulski, Krygier & Hogge, 1982).

Flavonoids consist of two units: a C6-C3 fragment from cinnamic acid and a C6 fragment from malonyl-coenzyme A (Figure 4Y) (reviewed by Serna-Saldivar & Rooney, 1995). Major flavonoids include anthocyanidins, catechins and leucoanthocyanidins and they are pigments in many flowers, stalks and leaves (Hahn *et al.*, 1984). Sorghum pericarp colour is said to be

due to a combination of anthocyanin (glucoside form of anthocyanidin) and anthocyanidin pigments and other flavonoid compounds. Such pigments from pericarp of red and white sorghum varieties have been characterised (Nip & Burns, 1969; Nip & Burns, 1971).

Tannins are so-named because of their use in tanning hides into leather by binding proteins such as collagen in animal skins (Butler *et al.*, 1984). They consist of two classes. The first class known as hydrolysable tannins, are phenolic carboxylic acids (like gallic acid or tannic acid) esterified to sugars such as glucose (Butler *et al.*, 1984; Hahn *et al.*, 1984). The phenolic acid and sugar are released upon acid, alkali or enzymic hydrolysis (Hahn *et al.*, 1984). The second class known as non-hydrolysable tannins (condensed tannins) are polymers resulting from condensation of flavan-3-ol (catechin) units and are the only tannins reported in sorghum (Butler *et al.*, 1984; Hahn *et al.*, 1984). They are also referred to as proanthocyanidins because they release anthocyanidins on treatment with mineral acid (Hahn *et al.*, 1984). Sorghum tannins are localised in the pericarp and testa layers and in some glumes (reviewed by Serna-Saldivar & Rooney, 1995).

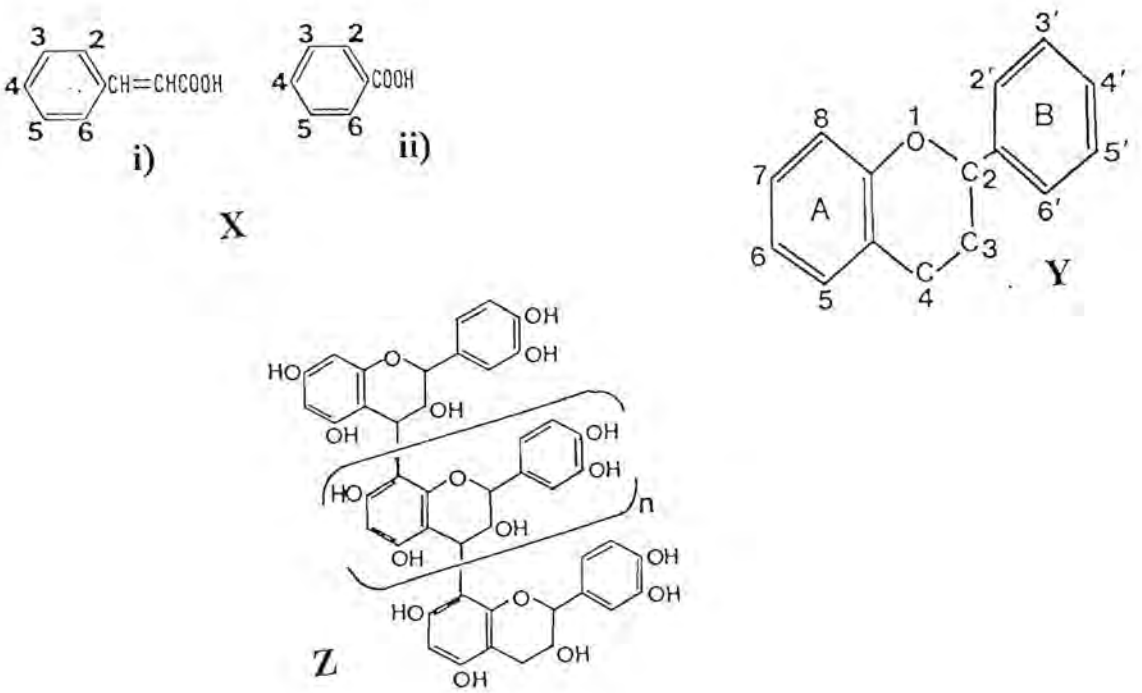

Figure 4: X) Basic structure of phenolic acids i) cinnamic acid; ii) benzoic acid.
Y) Basic flavonoid ring structure.
Z) Structure of proanthocyanidin (tannin) polymer; (n = 5-7). (Hahn *et al.*, 1984).

Whilst tannins protect the grain against insects, birds and weathering, this agronomic advantage is accompanied with nutritional disadvantages and reduced food qualities (reviewed by Serna-Saldivar & Rooney). According to Butler *et al.*, (1984), under optimal conditions, sorghum tannin is capable of binding and precipitating at least 12 times its own weight of protein. The tannin-protein interaction in sorghum is believed to involve hydrogen bonding and non-polar hydrophobic associations (Butler *et al.*, 1984). Sorghum grain contains approximately 10% protein and therefore in theory, high-tannin cultivars would contain more than enough tannin (2-4%) to bind all the seed protein (Butler *et al.*, 1984). Daiber and Taylor (1982) obtained lower protein yield for high-tannin compared with low-tannin sorghum on subjecting both grains to Landry-Moureaux protein fractionation. This was due to interactions between tannin and the albumin, globulin and prolamin proteins, rendering most of the proteins insoluble. Furthermore, electrophoresis indicated that proteins extractable from high-tannin sorghum were bound to tannins.

In high-tannin sorghum varieties, formation of indigestible protein-tannin complexes is a major limiting factor to protein utilisation (Chibber *et al*, 1980). *In vivo* studies have demonstrated this antinutritional effect of tannins in uncooked and cooked sorghum (Armstrong, Featherston & Rogler, 1973; Rostagno, Featherston & Rogler, 1973; Armstrong, Featherston & Rogler, 1974a). The protein-tannin complex problem was found to occur *in vitro* as well (Armstrong, Featherston & Rogler, 1974b; Schaffert, Lechtenberg, Oswalt, Axtell, Pickett & Rhykerd, 1974; Butler *et al.*, 1984). Electrophoretic analyses indicated that the indigestible residue of high-tannin sorghum consisted mainly of prolamins (Butler *et al.*, 1984).

Sorghum tannins have been reported to inhibit enzymes like amylases (Daiber, 1975). However, it has been suggested that the antinutritional effect of sorghum tannins lies in their ability to form less digestible complexes with dietary protein and not by inhibition of digestive enzymes (Butler *et al.*, 1984). Grinding, cooking and other processing methods of high-tannin sorghum enhance the opportunity for interaction of tannin with dietary protein before it encounters digestive enzymes (Butler *et al.*, 1984). Because of their high degree of hydroxylation, low-molecular weight phenols are unable to precipitate protein (Bravo, 1998). Oligomers must contain at least three flavonol subunits (like the condensed tannins) to effectively precipitate protein (Bravo, 1998).

Protein precipitation however, may not necessarily always lead to reduction in protein digestibility. Denaturation of proteins (sometimes characterised by protein precipitation) may lead to improvement in protein digestion (Cheftel, Cuq & Lorient, 1985). One of the main determinants of how digestible a protein will be is the conformation in which it is and to what extent that conformation allows enzymes access to the protein. Phenolic acids, flavonoids and condensed tannins, due to their hydroxyl groups, may all interact with and form complexes with proteins and this may lead to protein precipitation in the case of the tannins because of their large size. However, it is not this precipitation *per se* which causes reduction in protein digestibility. In addition to a possible change in protein conformation (which may not favour enzyme accessibility), the tannins may also exert steric effects (due to their large size) and prevent enzymes access to the proteins.

The antinutritional effects of sorghum tannin may be alleviated by treating grain with dilute aqueous ammonia (Price, Butler, Rogler & Featherston, 1979), strong alkalis (Chavan, Kadam, Ghonsikar & Salunkhe, 1979; Muindi, Thomke & Ekman, 1981), formaldehyde (McGrath, Kaluza, Daiber, Van der Riet & Glennie, 1982) or by decortication (Chibber *et al.*, 1980).

2.4.3 Phytic acid

Phytic acid (*myo*-inositol hexaphosphoric acid) usually occurs in seeds as mixed potassium, magnesium and calcium salts (phytins or phytates) (Ryden & Selvendran, 1993). It is believed to serve primarily as a storage compound for phosphorus, inositol and inorganic phosphate ions which are used in the energy metabolism of the plant, especially during germination (Johnson & Southgate, 1994; reviewed by Serna-Saldivar & Rooney, 1995). Therefore germination or malting significantly reduces the amount of phytates due to production of phytases (reviewed by Serna-Saldivar & Rooney, 1995). In sorghum, the highest phytate concentration is found in the germ (Hulse *et al*, 1980; Ali & Harland, 1991).

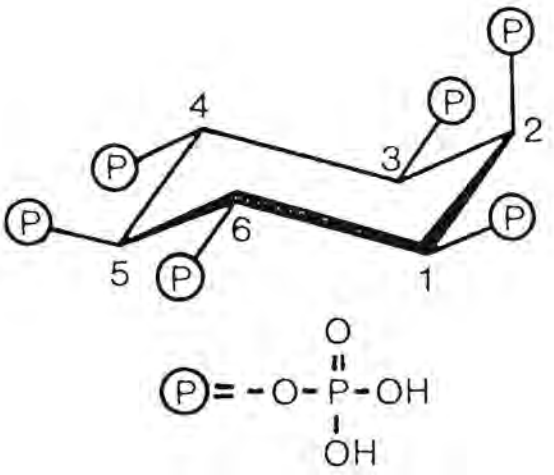

Figure 5: Structure of phytic acid. (Hoseney, 1994).

The phytate molecule is highly charged with six phosphate groups and so is an excellent chelator, forming insoluble complexes with mineral cations and proteins (Ryden & Selvendran, 1993). This leads to reduced bioavailability of trace minerals and reduced protein digestibility. Processing methods used to reduce phytate levels in sorghum include germination or malting, milling and decortication (reviewed by Serna-Saldivar & Rooney, 1995) and gamma-irradiation (Duodu, Minnaar & Taylor, 1999).

2.4.4 Protein crosslinking

During processing, the physical and chemical conditions proteins encounter can result in changes ranging from subtle changes in the hydration of the protein to thermal destruction (pyrolysis) with potential formation of mutagens (Figure 6) (Finley, 1989). The main chemical reactions which occur are the formation of derivatives of special amino acids or their crosslinking with other amino acids in the same or in another protein molecule (Erbersdobler, 1989). Such protein crosslinks may bring about a decrease in the digestibility and biological value of the food proteins.

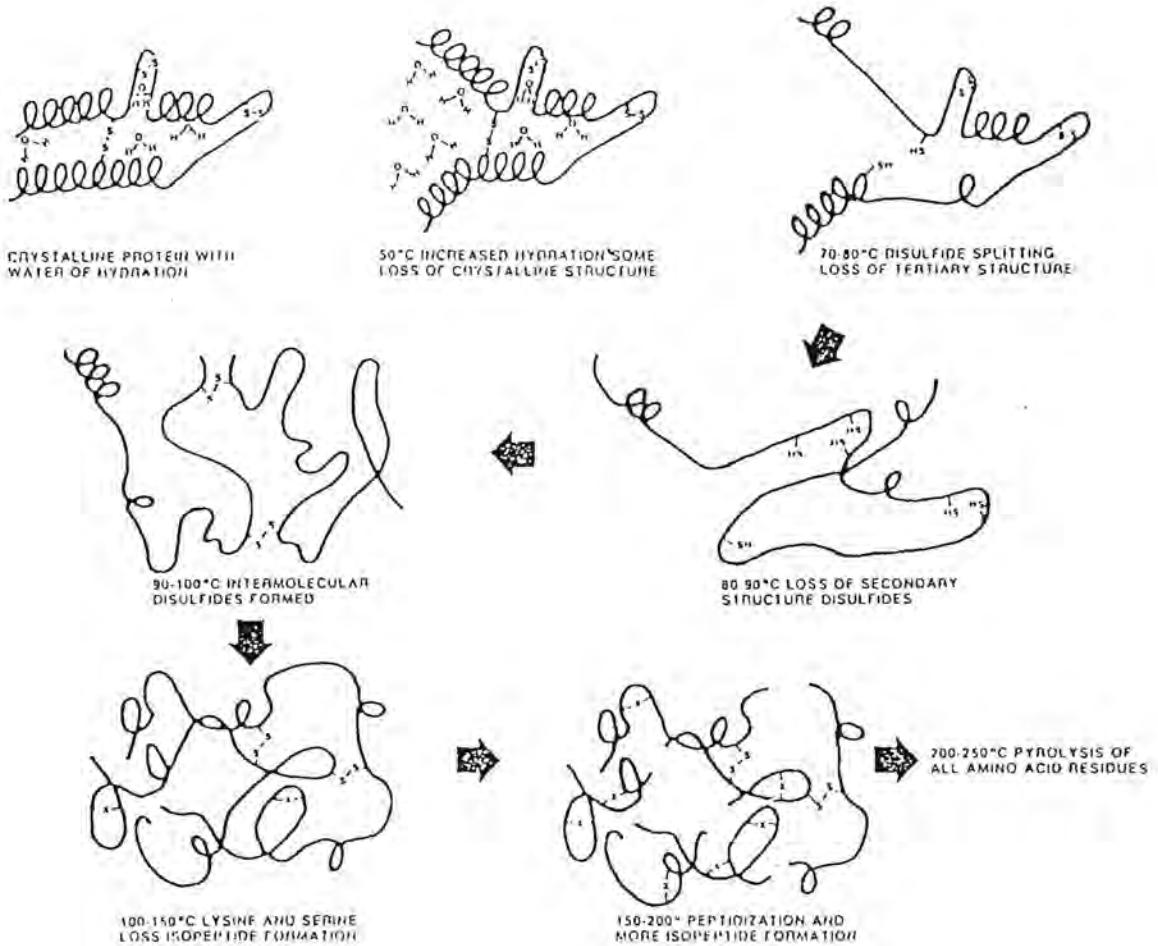

Figure 6: Changes a protein undergoes during heat treatment. (Finley, 1989).

2.4.4.1 Disulphide crosslinking and kafirin solubility

Using *in vivo* and *in vitro* approaches, Elkin, Freed, Hamaker, Zhang & Parsons (1996) showed that sorghum cultivars with similar tannin contents may vary greatly in their uncooked protein digestibilities. This provided an indication that tannins may not always be associated with depression in sorghum protein digestibility and that other components besides tannins could be at play. Furthermore, the lowering of sorghum protein digestibility on cooking has been shown to occur with low-tannin (condensed tannin-free) varieties also. This was demonstrated *in vivo* (Maclean *et al*, 1981) and *in vitro* (Axtell *et al.*, 1981) thus implying that formation of protein-tannin complexes may not be the only factor affecting sorghum protein digestibility.

Cooking ground, whole wheat gruel and ground, whole maize gruel did not decrease their (uncooked) protein digestibility values (Axtell *et al.*, 1981), therefore suggesting that the observed reduction of protein digestibility on cooking might be unique to sorghum. Mertz, Hassen, Cairns-Whittern, Kirleis, Tu and Axtell (1984) observed that wheat, maize and rice have digestion values about 25 percentage points higher than that of normal sorghum. Other workers (Hamaker *et al.*, 1986; Hamaker *et al.*, 1987) have reported superior protein digestibility of cooked maize compared to cooked sorghum.

The literature seems to indicate that in uncooked sorghum, Landry-Moureaux fraction 3 proteins (kafirin 2) are more than fraction 2 (kafirin 1) whilst the opposite is the case for the zein 1 and zein 2 fractions of uncooked maize. Table 5 below gives values of Landry-Moureaux fractions 2 and 3 obtained by different workers from sorghum and maize.

Table 5 L-M fractions 2 and 3 proteins in sorghum and maize (% of total protein)

Sorghum		Maize	
Kafirin 1	Kafirin 2	Zein 1	Zein 2
19.9*[a]	35.1*[a]	52.8 *[c]	7.9 *[c]
9.9[b]	15.3[b]	39.4 *[c]	9.4 *[c]
20.0[d]	44.0[d]	45.0[d]	21.8[d]
20.0*[e]	33.0*[e]	34.0*[e]	10.0*[e]

[a] Jambunathan & Mertz (1973)

[b] Guiragossian *et al.*, (1978)

[c] Landry & Moureaux (1980)

[d] Vivas, Waniska & Rooney, (1992)

[e] Hamaker, Mertz & Axtell (1994)

* % of total nitrogen.

Hamaker *et al.* (1986) reported that protein solubility properties of sorghum was altered on cooking. First of all, non-extractable proteins increased significantly from 11.5% to 25.8% after cooking for sorghum as against 6.6% to 14.2% for maize. Secondly, in sorghum, there appeared to be a shift in alcohol-soluble proteins (fractions 2 and 3) to the higher fractions, namely fraction 5 (extracted with pH 10 buffer, 2-mercaptoethanol and sodium dodecyl

sulphate) and fraction 6 (defined as non-extractable). Electrophoretic analysis showed that prolamin-type proteins were present in fraction 5 of sorghum after cooking.

There seems to be a potential relationship between kafirin solubility and protein digestibility. Landry-Moureaux (L-M) fractionation showed that in cooked sorghum, the amount of indigestible protein was significantly larger than in uncooked while there was essentially no difference in cooked and uncooked maize. (Hamaker *et al.*, 1986). This indicated that indigestible sorghum proteins are increased during cooking while maize proteins are not. Sorghum prolamins become much less soluble and much less pepsin-digestible than maize prolamins on cooking.

The observed lowering of kafirin solubility on cooking appears to be as a result of disulphide crosslinking. *In vitro* studies indicate that cooking sorghum with reducing agents improves its protein digestibility (Hamaker *et al.*, 1987; Rom, Shull, Chandrashekar & Kirleis, 1992; Oria *et al.*, 1995b; Arbab & El Tinay, 1997). These observations point to disulphide crosslinking as a possible factor affecting sorghum protein digestibility.

Cooking sorghum and maize with reducing agents, namely, 2-mercaptoethanol, dithiothreitol, sodium bisulphite and L-cysteine resulted in enhanced protein digestibility of cooked and uncooked sorghum and maize (Hamaker *et al.*, 1987). The enhanced protein digestibility was more pronounced in sorghum than in maize. It was proposed that on cooking, kafirin proteins may form polymeric units bound by intermolecular disulphide bonds which may be less susceptible to digestion. Protein aggregation through disulphide crosslinking on thermal processing is also believed to occur in wheat semolina (Ummadi, Chenoweth & Ng, 1995), maize (Batterman-Azcona & Hamaker, 1998) and rice (Mujoo, Chandrashekar & Ali, 1998). In terms of how this disulphide bond formation affects sorghum protein bodies, it was suggested that on cooking, a disulphide-bound protein coat may be formed by proteins surrounding the protein body and this could reduce accessibility of the protein bodies to enzymatic attack (Hamaker *et al.*, 1987). There may also be an interior "toughening" of the periphery of the protein body because of disulphide bond formation.

Electron microscopic techniques have been used to investigate the effect on protein body structure on treatment with reducing agents. Using scanning electron microscopy (Rom *et al.*, 1992) and transmission electron microscopy (Oria *et al.*, 1995) it was observed that on subjecting uncooked sorghum flour to pepsin digestion, protein bodies were digested by pitting from the outside. This is in agreement with earlier observations from germination experiments in sorghum (Taylor *et al.*, 1985b; Taylor & Evans, 1989) and maize (Torrent *et al*, 1989; Mohammad & Esen, 1990). Most of the protein bodies from cooked sorghum did not show any pitting on pepsin digestion (Rom *et al.*, 1992). However on treating with a reducing agent, most of the protein bodies from cooked sorghum were pitted (Rom *et al.*, 1992; Oria *et al.*, 1995). The progress of protein digestion was monitored using enzyme-linked immunosorbent assay (ELISA) and this showed that α-kafirins took longer to digest as observed earlier in maize (Torrent *et al*, 1989; Mohammad & Esen, 1990) and sorghum (Shull *et al.*, 1992), an indication of its more central location within protein bodies (Hamaker *et al.*, 1995).

From these observations, a hypothesis was proposed to explain the role played by the various kafirins during disulphide bonding. When sorghum is cooked, enzymatically resistant protein polymers are formed through disulphide bonding of the β- and γ- kafirins, which contain unusually high proportions of the sulphur-containing amino residue cysteine (Shull *et al.*, 1992), and possibly other proteins which are located to the outside of the protein body. The disulphide cross-linked proteins thus formed would then prevent access to and restrict digestion of the more digestible and centrally located α-kafirin within the protein body (Hamaker *et al.*, 1987; Rom *et al.*, 1992; Hamaker *et al.*, 1994; Oria *et al.*, 1995; Hamaker *et al.*, 1995).

Perhaps one of the shortcomings of the disulphide bonding hypothesis, as presented, is that it does not explain the reason for the fact that cooking does not reduce protein digestibility of maize even though formation of disulphide bonds is reported to occur on cooking maize. Batterman-Azcona and Hamaker (1998) have reported from electrophoretic analysis that during cooking of maize there was extensive disulphide-mediated polymerisation of α-zein.

The identification of some sorghum genotypes with high uncooked and cooked *in vitro* protein digestibility has been reported (Weaver, Hamaker & Axtell, 1998). Though cooking brings about a decrease in their digestibilities, this decrease is much less compared to normal sorghum. This is probably because protein bodies of the highly digestible genotype are highly invaginated and contain deep folds rather than a typical spherical shape. Gamma-kafirin is located at the base of the folds in protein bodies of the highly digestible genotype as opposed to the periphery in normal protein bodies (Weaver *et al.*, 1998; Oria, Hamaker, Axtell & Huang, 2000). As a result, α-kafirin in the highly digestible sorghum is more exposed to digestive enzymes than in normal protein bodies and this improved accessibility accounts for the overall higher protein digestibility.

2.4.4.2 Racemization and isopeptide formation

The amino acids of proteins are members of the L-series. Whilst D-amino acids occur in nature, they are not constituents of proteins (Coultate, 1990). The process whereby L-amino acids are converted to the D form is known as racemization. This conversion is of importance nutritionally because D-amino acids are absorbed much slower than the corresponding L form and even if digested and absorbed, most D isomers of essential amino acids are not utilised by man (Liardon & Hurrell, 1983). In addition, L-D, D-L and D-D peptide bonds introduced during the racemization process would resist attack by proteolytic enzymes which function best with L-L bonds (Friedman, Zahnley & Masters, 1981). Amino acid racemization occurs most readily after alkaline treatments (Masters & Friedman. 1979; Liardon & Hurrell, 1983; Jenkins, Tovar, Schwass, Liardon & Carpenter, 1984), but can also occur to a lesser extent in acid conditions (Ikawa, 1964; Manning, 1970; Jacobsen, Willson & Rapoport, 1974), and during severe heat treatment and roasting of proteins (Hayase, Kato & Fujimaki, 1975; Liardon & Hurrell, 1983).

Racemization of amino acids is believed to be a prelude to the formation of isopeptide bonds in proteins (Friedman *et al.*, 1981). The racemized amino acid forms a dehydroprotein (also called a dehydroalanyl residue) by elimination of nucleophilic species like the disulphide group of cystine or hydroxyl group of serine (Friedman *et al.*, 1981; Erbersdobler, 1989; Otterburn, 1989). The isopeptide linkage is then formed when the dehydroprotein reacts with other amino acids. These amino acids may include cystine to form a lanthionine crosslink, lysine to form a lysinoalanine crosslink, arginine to form an ornithinoalanine crosslink and

histidine to form a histidinoalanine crosslink (Friedman *et al.*, 1981; Erbersdobler, 1989; Otterburn, 1989). Isopeptide crosslinks can impair the nutritional quality of foods by decreasing the amount of essential L-amino acids and decreasing digestibility and bioavailability of proteins (Friedman *et al.*, 1981; Erbersdobler, 1989; Otterburn, 1989).

From a study on various processed foods, Bunjapamai, Mahoney and Fagerson (1982) concluded that it is unlikely that conventional processing or cooking methods will cause extensive racemization of protein amino acids in foods. According to Fay, Richli and Liardon (1991), significant isomerization or racemization of amino acids only occurs under excessive conditions of temperature, alkaline pH and/or treatment time. Temperature and pH prevailing under normal food processing conditions produce negligible amounts of D-amino acids (Fay *et al.*, 1991). The likelihood of amino acid racemization and the extent thereof in cooked sorghum and maize porridge have not been investigated. From the observations that racemization occurs on alkali or severe heat treatment, it may be speculated that if it occurs in sorghum and maize porridge, it is not likely to be extensive. Perhaps during cooking of sorghum and maize porridge, the likelihood of racemization is greatest at the bottom of the cooking vessel where proteins are closest and exposed for a longer time to the heating source.

2.5 Analytical methods for protein digestibility and protein conformation

2.5.1 *In vitro* protein digestibility assays

Ideally, the best way to determine protein digestibility would be by conducting *in vivo* experiments using animal and human subjects. However, one major drawback to the use of *in vivo* methods is that it raises questions about ethics. Moreover, these procedures are time-consuming and expensive. Therefore much effort has been expended in developing *in vitro* procedures. A desirable *in vitro* method would be expected to be rapid, repeatable, reproducible and most importantly, correlate with *in vivo* studies. According to Pedersen and Eggum (1983), a good *in vitro* method should be simple, accurate and applicable to a wide variety of protein sources.

Several *in vitro* methods for estimation of protein digestibility have been developed and these include both single and multiple-enzyme assays. Multiple-enzyme systems which have been used include pepsin-pancreatin (Akeson & Stahmann 1964; Youssef, 1998), pepsin-trypsin (Saunders, Conner, Boother, Bickoff & Kohler, 1973; Elmaki, Babikar & El Tinay, 1999) and trypsin-chymotrypsin-peptidase (Hsu, Vavak, Satterlee & Miller, 1977).

It is considered that compared to a single-enzyme system, multiple-enzyme systems could reduce the effects of endogenous inhibitors specific for a single enzyme. In addition, a single-enzyme system that attacks at a specific peptide bond may give different results for proteins containing different concentrations of the specific amino acid (Hsu *et al.*, 1977). However multiple-enzyme methods tend to be complicated and time-consuming, involving multiple digestions and washings (Hahn, Faubion, Ring, Doherty & Rooney, 1982). In addition, multiple-enzyme systems are more expensive. Therefore a rapid and accurate single-enzyme system which exhibits good correlation with *in vivo* studies would be desirable.

Hahn *et al.* (1982) developed a semiautomated single-enzyme system using pronase. The motivation for the use of pronase was that it shows no hydrolytic specificity and releases amino acids from both the carboxyl and amino ends of peptides. Therefore pronase can hydrolyse all available protein into amino acids and peptides, thus giving a true index of the total digestibility of the protein. This method proved more sensitive than that of Hsu *et al.* (1977) in demonstrating differences in digestibility among sorghums of varying kernel structures and compositions (Hahn *et al.*, 1982). However, pronase is a proteolytic enzyme preparation from the fungus *Streptomyces griseus* (Laskowski & Sealock, 1971). Therefore its use would not give a true reflection of sorghum protein digestibility in humans since the enzyme is not of human origin.

A more appropriate single-enzyme assay is the pepsin system used by Chibber *et al.* (1980) since this enzyme is found in humans unlike pronase. These authors investigated the *in vitro* protein digestibilities of high tannin sorghums at different stages of dehulling using the single-enzyme system pepsin and compared it to a trypsin-chymotrypsin mixture. They observed that solubilisation of nitrogen in sorghum was achieved much more effectively by the action of pepsin than by the trypsin-chymotrypsin combination. In addition their results with pepsin supported the earlier *in vivo* results obtained by Armstrong *et al.*, (1974) working with the same high tannin sorghums.

The pepsin method used by Chibber *et al.* (1980) involves incubating the sample-pepsin mixture for 2 h at 37°C and analysing the supernatant for solubilized nitrogen. Mertz *et al*, (1984) modified it by analysing the residue for residual nitrogen. This improvement in the method also agreed with *in vivo* findings of Maclean *et al.* (1981). The simplicity of the

pepsin method coupled with the fact that it agrees with *in vivo* observations makes it very useful as a rapid screening procedure for determining the biological value of sorghum grain varieties (Chibber *et al.*, 1980). It is not surprising therefore that it has subsequently been used by many workers in estimating *in vitro* protein digestibility of various cereals, including sorghum, maize, wheat and rice (Axtell *et al.*, 1981; Hamaker *et al.*, 1986; Lorri & Svanberg, 1993; Elkin *et al.*, 1996; Weaver *et al.*, 1998).

2.5.2 Fourier Transform Infrared spectroscopy

The infrared region of the electromagnetic spectrum is that with wavelength (λ) in the range 2.5-25 μm or 400-4000 cm^{-1} in terms of wavenumbers. On passing infrared light through a sample, some frequencies are absorbed while others are transmitted through the sample without being absorbed. A plot of percent absorbance or transmittance against frequency is an infrared spectrum (Kemp, 1987).

The atoms in a molecule are in constant vibrational motion which may be stretching or bending vibrations. Different bonds of different functional groups (for example C-C, C=C, C≡C, C=O, O-H etc) have different vibrational frequencies and are capable of absorbing infrared radiation of that frequency. Therefore the presence of these bonds in a molecule can be detected by identifying this characteristic frequency as an absorption band in the infrared spectrum.

Several spectroscopic methods including infrared, produce interferograms (interference patterns) that are complex and difficult to explain because they are in the time domain (changes in intensity versus time). Interferograms in the frequency domain (plot of intensity versus frequency) are less complex and easier to explain. The conversion of one form to the other is known as Fourier Transformation (Kemp, 1987). Modern infrared spectrometers are equipped with computer programs which perform the Fourier transformation in a few seconds to generate the infrared spectral plots of intensity versus frequency (Kemp, 1987). Hence the name Fourier transform infrared spectroscopy (FTIR).

In infrared spectroscopy of proteins, the vibrational modes of the protein molecule are sensitive to changes in chemical structure, conformation and environment and therefore their measurement is of potential value to the protein chemist (Fraser & Suzuki, 1970). Each normal mode of vibration of a protein molecule involves simultaneous motions of all the atoms in the molecule. It is found in practice, however, that some modes involve significant atomic motions only in the main chain, while others are highly localised in individual side chains.

Main chain vibrations are sensitive to changes in the chain conformation and to the nature of the coupling between amide groups. A study of the frequencies associated with these vibrations could yield information about conformation, orientation and regularity of the main chain (Fraser & Suzuki, 1970). Protein infrared spectra are dominated by the absorption bands of the N-substituted amide groups in the polypeptide backbone (Fraser, 1956). This is because of the high relative concentration of this group and the intense absorption associated with its vibrational modes. As a result the spectra of proteins, synthetic polypeptides and small peptides are remarkably similar.

Strong main chain absorption bands around 1550 cm^{-1} (Amide II), 1650 cm^{-1} (Amide I) and 3300 cm^{-1} (Amide A), have been identified with NH bond bending and CO and NH bond stretching vibrations respectively (Ambrose & Elliot, 1951; Fraser, 1956). These modes however, (particularly the Amide I and II) cannot be described as pure bond-bending and bond-stretching vibrations. They involve more complex motions of the atoms. In simple amides, the Amide II band involves a mixture of CN stretching (40%) and in-plane NH bending (60%) contributions while the Amide I band involves 80% CO stretching, 10% CN stretching and 10% in plane NH bending contributions.

The amide bands of proteins are conformation-sensitive and are composites of overlapping component bands of different protein structures such as α-helices, β-strands, turns and non-ordered polypeptide fragments (Surewicz & Mantsch, 1988). These bands due to each type of conformation are too broad and overlap too extensively and therefore only unresolved features are observed (Kauppinen, Moffatt, Mantsch & Cameron, 1981; Yang, Griffiths, Byler & Susi, 1985; Surewicz & Mantsch, 1988). The most effective procedure of narrowing infrared bands for resolution enhancement is Fourier self-deconvolution which is a

mathematical operation based on Fourier transforms (Kauppinen *et al.*, 1981; Yang *et al.*, 1985; Byler & Susi, 1986; Surewicz & Mantsch, 1988). Surewicz and Mantsch (1988) point out that the key to meaningful Fourier self-deconvolution lies in selecting the conditions that give the maximum band narrowing while keeping the increase in noise and the appearance of side lobes at a minimum. It is important for the spectroscopist to bear in mind that all the sharp, though often weak features in the spectra originating from random noise or uncompensated water vapour will be greatly amplified by the deconvolution operation. These may show up in the resolution-enhanced spectrum as artifacts that are often indistinguishable from the real protein amide bands. Therefore there is a need for complete elimination of water vapour bands and for a high signal-to-noise ratio.

Amide band frequencies for proteins in the β-conformation are generally lower by approximately 30 cm^{-1} than frequencies for the α-form (Kretschmer, 1957). Amide I components centred between 1650 and 1658 cm^{-1} are believed to represent α-helical segments (Lavialle, Adams & Levin, 1982; Surewicz & Mantsch, 1988; Bandekar, 1992), whilst bands between 1620 and 1640 cm^{-1} (Jakobsen, Brown, Hutson, Fink & Veis, 1983; Surewicz & Mantsch, 1988; Bandekar, 1992), and also between 1675 and 1680 cm^{-1} (Timasheff, Susi & Stevens, 1967; Lavialle *et al.*, 1982), indicate the presence of antiparallel, intermolecular β-sheet structure. Bands at 1545 cm^{-1} and 1547 cm^{-1} in the amide II region have been assigned to α-helical proteins and bands at 1524 cm^{-1} to β-sheet components (Surewicz & Mantsch, 1988; Bandekar, 1992).

Due to the conformation sensitivity of the amide bands, infrared spectroscopy has been an attractive technique for studying changes in protein structure and conformation during the process of denaturation. A general hypothesis was that globular proteins consisted of polypeptide chains folded to form a compact, approximately ellipsoidal molecule. During denaturation, this structure is unfolded to yield extended molecules that can be oriented in the β-configuration (Senti, Copley & Nutting, 1945; Kretschmer, 1957). Infrared studies of proteins seemed to bear out this hypothesis.

Ambrose and Elliott (1951) observed during an infrared study of various globular proteins that heat precipitation involved a change from the intra-chain hydrogen bonds of the folded state (α-configuration) to the inter-chain hydrogen bonds of the extended state (β-

configuration). An FTIR study of the protein CaATPase from rabbit skeletal muscle, showed that the native protein contained mainly α-helical and random coil structures with moderate contributions from β-sheet (Jaworsky, Brauner & Mendelsohn, 1986). Thermal denaturation produced a large increase in the β antiparallel-pleated sheet content. Similar effects of thermal denaturation have been reported from FTIR studies of lipophilin, a protein from the human central nervous system (Surewicz, Moscarello & Mantsch, 1987). Solvent-denatured globular proteins were reported to contain large amounts of a special kind of β-strands (Purcell & Susi, 1984).

The application of infrared spectroscopy to the study of cereal proteins is a growing area of research. Kretschmer (1957) found that the amide I band of zein film heated in steam consisted of a component at 1660 cm^{-1} due to the α-form and a shoulder at 1630 cm^{-1} due to the β-form which was not evident in the spectrum of the unheated steam. This suggested heat denaturation transforms part of the zein from the α to the β-form. Wu, Cluskey and Jones (1971) reported an absorption maximum between 1645 and 1651 cm^{-1} in the infrared spectrum of sorghum prolamin in 60% *tert*-butanol in D_2O. They deduced that this indicates that the protein is a mixture of α-helix and unordered structures. FTIR spectroscopy has also been used to study secondary structural changes induced in cereal proteins on hydration. Generally, it is reported that hydration brings about an increase in extended β-sheet secondary structures in a high molecular weight subunit of wheat glutenin (Belton, Colquhoun, Grant, Wellner, Field, Shewry & Tatham, 1995), wheat ω-gliadins (Wellner, Belton & Tatham, 1996) and wheat gluten (Grant, Belton, Colquhoun, Parker, Plijter, Shewry, Tatham & Wellner, 1999).

2.5.3 Nuclear Magnetic Resonance (NMR) spectroscopy

Nuclear magnetic resonance (NMR) is concerned with the magnetic properties of atomic nuclei and NMR spectroscopy may be defined as the absorption and emission of electromagnetic radiation by the nuclei of certain atoms when placed in a magnetic field (Field, 1989). It is one of the most powerful techniques which can be used to study the chemical and physical structure of complex, heterogeneous materials in a non-invasive manner (Ablett, 1992).

Atomic nuclei behave as tiny spinning bar magnets because they possess both electric charge and mechanical spin. As a result, under the influence of an external magnetic field, the nuclei will tend to align themselves with that field. The alignment may be either with (parallel to) the field (the lower energy state) or opposed to (antiparallel to) the field (the lower energy state) (Kemp, 1987). The nuclei also perform a type of motion known as precession round the axis of the applied external magnetic field. The precessional frequency, ν, is directly proportional to the strength of the external magnetic field $\mathbf{B}_o$, ($\nu \propto \mathbf{B}_o$) (Kemp, 1987).

Nuclei precessing in the aligned orientation (low energy state) may absorb energy and pass into the opposed orientation (high energy state) and *vice versa* (Figure 7). If the precessing nuclei are irradiated with a beam of radiofrequency energy of the right frequency, low energy nuclei may absorb this energy and move to a higher energy state. A nucleus will only absorb energy from the radiofrequency source if the precessing frequency (of the nucleus, ν) is the same as the frequency of the radiofrequency beam. When this occurs, the nucleus and the radiofrequency beam are said to be *in resonance*; hence the term *nuclear magnetic resonance*. The NMR phenomenon is exhibited only by those nuclei whose spin quantum number I is greater than zero. Such nuclei include ^{1}H, ^{13}C, ^{15}N and ^{31}P.

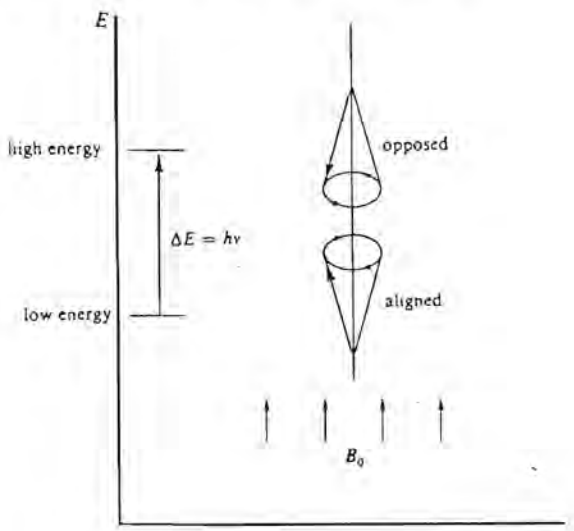

Figure 7: Representation of precessing nuclei and the energy transition between the aligned and opposed conditions. (Kemp, 1987).

In a magnetic field, nuclei are shielded or screened from the field by the electrons which surround the nuclei. The degree of screening depends on the electron density and thus on the type of bonding in the molecule in which the nuclei reside. Nuclei in different chemical environments are shielded to different extents and therefore have different resonance frequencies. The different screening experienced by nuclei in different chemical environments is called the *chemical shift*. Resonance frequencies (or shieldings) are measured relative to the frequency of a standard compound, taken as a reference and chemical shifts are expressed in units of parts per million (ppm, given the symbol δ) of that reference compound. Such reference compounds include tetramethylsilane ($Si(CH_3)_4$) and glycine.

Customarily, in NMR spectral plots, the direction of increasing resonance frequency is to the left. The more shielded a nucleus from the applied magnetic field, the lower the effective magnetic field acting on the nucleus and hence, the lower is its resonance frequency. High resonance frequency corresponds to high δ values and vice versa.

The shielding of a nucleus depends on the orientation of a molecule and its bonds with respect to the external magnetic field. In liquids, molecular reorientation is rapid enough to ensure that shielding is averaged over all orientations (Field, 1989). However, many food systems contain solids, crystalline materials or polymers which associate to form solid state motional restriction (Lillford & Ablett, 1999). Unlike liquids, molecular motion is restricted in solids and therefore the chemical shift of a nucleus in a solid depends on the orientation of the molecule in the magnetic field. In the NMR spectrum of a single crystal of a solid, the chemical shifts vary with the orientation of the crystal (Field, 1989). There is therefore overlap of spectra from molecules with all possible orientations with respect to the magnetic field resulting in broadening of the observed resonances (Baianu & Förster, 1980; Field, 1989; Ablett, 1992). Another contribution to these broad resonances is the nuclear dipolar interaction between ^{1}H and ^{13}C nuclei (Baianu & Förster, 1980; Ablett, 1992). This problem is rectified by the use of the technique known as Magic Angle Spinning (MAS) where the magic angle is 54.74°. Rapid spinning of solids at this angle cancels out variations in chemical shift and suppresses dipole-dipole interactions caused by the effects of molecular orientation (Ablett, 1992).

One of the weaknesses of NMR is its low sensitivity. The ^{13}C nucleus for instance, is regarded as one with dilute spin due to its low natural abundance (1.108% compared to 99.985% for ^{1}H) (Field, 1989). The proton therefore, has far greater sensitivity than the ^{13}C nucleus and this is a major reason why proton NMR has been predominantly used in many studies of food systems (Ablett, 1992). The sensitivity of ^{13}C NMR may be improved by use of the technique known as Cross Polarisation (Pines, Gibby & Waugh, 1973). In simple terms, this involves initially exciting the ^{1}H nuclei followed by the ^{13}C nuclei. The strength of the magnetic fields of both nuclei are adjusted such that a so-called Hartmann-Hahn condition (Hartmann & Hahn, 1962) is reached. This condition implies that the protons and carbons precess at equal rates and their effective energies are comparable. The protons then pass some of their magnetisation on to adjacent ^{13}C nuclei.

In a similar manner to conventional solution-state spectra, solid-state NMR can be used to elucidate chemical structure. It can also provide information on the physical structure of solid materials thus opening up the possibility of studying the microdynamics of specific molecular regions within a complex food structure (Ablett, 1992). According to Schofield and Baianu (1982), high-resolution solid-state NMR can be used to identify specific chemical groups in proteins and also to determine their mobilities, degree of ordering and dynamics.

Carbon-13 NMR spectra of proteins generally show signals from aliphatic amino acids, aromatic amino acids and the carbonyl carbon (C=O) in the peptide bond. The characteristic chemical shifts of these carbons are shown in Table 6 below.

Table 6. Characteristic chemical shifts of protein carbons in ^{13}C NMR spectra

Carbon type	Chemical shift, δ (ppm)
$C_{\beta,\gamma,\delta}$ of aliphatic amino acids	20-40[a,b]
C_{α} of aliphatic amino acids	45-58[a,b]
Carbons of aromatic amino acids	120-130[c]
Carbonyl (C=O) carbon	170-180[c]

[a] Kricheldorf & Muller (1984).

[b] Kricheldorf, Muller & Ziegler (1983).

[c] Schofield & Baianu (1982).

In proteins, the carbonyl group as part of the peptide bond is an intrinsic part of the protein backbone, and hence has influence on protein secondary structure. The α-carbons by reason of their close proximity to peptide bonds are also important for protein secondary structure. The usefulness of NMR as a technique for the study of proteins is that chemical shifts seem to have strong correlations with protein secondary structure (Pastore & Saudek, 1990; Wishart, Sykes & Richards, 1991). In ^{13}C NMR, (as in proton NMR), the α-carbons (and the α-protons) and the carbonyl carbons experience a downfield shift (towards higher δ values) when the protein is in a helical conformation and an upfield shift (lower δ values) for a β-strand or extended configuration (Pastore & Saudek, 1990; Wishart *et. al.*, 1991).

Whilst high-resolution solution-state NMR has found extensive application in the elucidation of the chemical structures of organic compounds, ^{13}C NMR solution and solid state spectroscopy has been used to study various proteins of both non-cereal and cereal origin. Baianu and Foster (1980) used solid-state ^{13}C NMR in an attempt at a physicochemical characterisation of wheat flour, gluten and wheat protein powders. They reported NMR spectra containing basic chemical shift information directly related to the molecular components of these systems. A lot of the ^{13}C NMR studies have been aimed at characterisation of proteins with regard to their structure and conformation and attempts have been made to assign chemical shifts to various amino acid residues within the proteins.

Tatham, Shewry and Belton (1985) studied the structure of C hordein of barley by a combination of solution and solid-state ^{13}C NMR spectroscopy. They reported that the repetitive structure of C hordein resulted in simple spectra in which it was possible to assign majority of the resonances to the five major residues of the protein. The spectra also provided evidence for a β-turn-rich conformation. Carbon-13 NMR spectra for maize zein in solution have been reported (Augustine & Baianu, 1986; Augustine & Baianu, 1987). These workers proposed spectral assignments for the amino acids in zein and observed spectral differences in zeins extracted with different organic solvents. They attributed these differences in spectra to possible changes in zein conformation caused by treatment with alcohol. Fisher, Marshall and Marshall (1990a & 1990b) have reported ^{13}C NMR solution spectra for soybean glycinin and β-conglycinin and also studied the effects of gelation and heat and chemical denaturation on the proteins. Solution spectra and spectral assignments for soybean 7S globulin have been reported (Kakalis & Baianu, 1990). Solid-state ^{13}C NMR spectra have been reported for wheat

proteins (Baianu & Förster, 1980; Schofield & Baianu, 1982; Gil, Alberti, Tatham, Belton, Humpfer & Spraul, 1997; Gil, Alberti, Naitô, Okuda, Saitô, Tatham & Gilbert, 1999) and hordein (Tatham *et al.*, 1985; Gil, Naitô, Tatham, Belton & Saitô, 1997).

2.6 Gaps in knowledge

Various factors affecting protein digestibility of sorghum and maize have been proposed. These include association of proteins with starch, cell walls, antinutritional factors and protein crosslinking. However, there still remain unanswered questions about sorghum protein digestibility in comparison to maize.

Investigations into sorghum protein digestibility have been carried out on either whole grain, decorticated grain or some other undefined fraction. As a result there is no clear picture about what exactly the nature of the protein digestibility problem is at different levels of structural organisation of the grain. This has, in part, contributed to the apparent confusion in the literature about whether uncooked sorghum protein digestibility is lower than maize. As the grain is progressively taken apart from whole grain, through endosperm to protein bodies, interfering factors in grain parts like the pericarp and the germ would be eliminated. The effect of this on sorghum protein digestibility and how it compares with maize has not been investigated.

In their investigation into *in vitro* digestibility of sorghum proteins, Axtell *et al.* (1981) observed that particle size of the ground sorghum sample is important in the pepsin test. Sample ground in a coffee grinder at the finest setting gave a protein digestibility value of 34.3%, compared with a value of 46.7% after the sample from the coffee grinder was reground in a mill. Based on this, it could be hypothesised that accessibility of the enzyme to the protein substrate could be a factor influencing protein digestibility. Disrupting the close association between protein and starch either mechanically or by the use of enzymes would improve accessibility and hence protein digestibility. There has not been adequate investigation into this.

Disulphide crosslinking in sorghum and maize on cooking suggest a change in protein secondary structure. As mentioned earlier, this still leaves the question unanswered as to the better protein digestibility of cooked maize compared to cooked sorghum. Sophisticated spectroscopic methods like NMR and FTIR provide information about protein conformation

and secondary structure. Therefore the use of NMR and FTIR in the study of protein secondary structural changes in maize and sorghum on cooking is an attractive prospect.

A recent report shows that popping does not decrease sorghum protein digestibility as wet cooking does (Parker, Grant, Rigby, Belton & Taylor, 1999). These authors suggest that the explosive popping process leads to fragmentation of endosperm cell walls hence improved accessibility of endosperm protein to enzymes. Whether a different kind of protein secondary structural change occurs in popped grain compared to wet cooked is not known.

2.7 Objectives and hypotheses

The broad objective of this project was to investigate the effects of grain structural organisation on the digestibility of sorghum protein on cooking.

In pursuit of this objective, experiments were carried out to test the following hypotheses:

- Accessibility of digestive enzymes to sorghum grain protein may influence the protein digestibility. Improvement of accessibility by enzymatic digestion of starch may improve protein digestibility.

- Interfering factors in parts of the sorghum grain like the pericarp and germ may bind proteins and render them indigestible. Investigating protein digestibility at the whole grain, endosperm and protein body levels of organisation will give insight into this.

- Secondary protein structural change on processing between sorghum and maize and between wet cooked and popped grain may differ qualitatively and quantitatively.

CHAPTER 3

MATERIALS AND METHODS

3.1 Grain samples

Five condensed tannin-free sorghum varieties, NK 283, a red hybrid (ex. Nola, Randfontein, South Africa), KAT 369 (a white Kenyan variety, grown in Cheplambus, Baringo, Kenya), Kenyan local white sorghum, two sorghum lines derived from crosses containing high-lysine mutants namely, P850029 and P851171 (kindly supplied by Prof. B.R. Hamaker, Purdue University, USA), a high tannin sorghum hybrid, DC 75 (ex. Sorghum Board, South Africa), a white maize hybrid (PAN 6043, grown in Vryburg, South Africa) and white maize grits (a commercial variety) were used in this work.

3.2 Sample preparation

3.2.1 Whole grain meal

Clean whole grain samples of NK 283, KAT 369 and PAN 6043 were milled with a laboratory hammer mill (Falling Number AB, Huddinge, Sweden) fitted with an 800 µm screen.

3.2.2 Decorticated sorghum and degermed maize

Whole grain sorghum (NK 283, KAT 369 and Kenyan local white) was decorticated by passing twice through a rice pearler (Miag Braunschweig, Germany). Approx. 20% of the grain (mainly pericarp and germ) was removed during the decortication process.

Maize grain was conditioned for 30 min in a conditioner (Miag Braunschweig, Germany) with water to a final moisture content of approx. 14%. The conditioned maize grain was then degermed in a Beall-type degerminator.

3.2.3 Endosperm meal

Decorticated sorghum (NK 283 and KAT 369) and degermed maize (PAN 6043) grain was carefully screened to select grain without germ and pericarp. Selected pieces of endosperm were milled into a fine powder with a laboratory hammer mill fitted with an 800 µm sieve.

3.2.4 Preparation of protein body-enriched samples

This was carried out using a modification of the method described by Taylor, Novellie & Liebenberg (1984c). Approx. 200 g decorticated grain of NK 283 sorghum, KAT 369 sorghum, P851171 sorghum mutant, P850029 sorghum mutant and PAN 6043 maize were suspended in 1 litre distilled water and stirred occasionally for 2 h. The mixture was then passed four or five times through a Fryma wet stone mill (Rheinfelden, Germany) to break the starch-protein complex. The slurry was then passed sequentially through a 250 µm and 75 µm screen, each time discarding the residue remaining on the screen. The resulting slurry was centrifuged for 10 min at 2000 *g* and the supernatant discarded. The layer on top of the white starch, containing the protein bodies, was scraped off, bulked, resuspended in distilled water and recentrifuged for 10 min at 2000 *g*. This fraction was filtered through a 35 µm sieve and the residue on the sieve retained. The sieving process was repeated several times, each time retaining the residue on the sieve and examining under the microscope to check for starch contamination until the protein body preparation was largely free of starch. This protein body fraction retained on the sieve is essentially networks of protein bodies held together by protein matrix. Individual protein bodies (normally 1-2 µm in diameter) are not retained and pass through the sieve. Protein body preparations were freeze-dried and then milled.

3.2.5 Preparation of unalkylated and alkylated total kafirin and zein

Milled samples (decorticated Kenyan local white sorghum and maize grits) were defatted by extraction with petroleum ether (40-60°C) (300 g flour in 1.5 l petroleum ether), stirred for 20 h at room temperature, centrifuged at 23000 x *g* for 15 min and the supernatant discarded. This process was repeated once. The defatted flour was dried in a fume cupboard overnight and then extracted for total kafirin and zein using *tert*-butanol (60% v/v) containing 50 mM dithiothreitol (DTT) (125 g defatted flour in 500 ml *tert*-butanol/DTT solution). Extraction was carried out by stirring at ambient temperature for 5 h and the mixture centrifuged as described above. The supernatant was rotary evaporated to remove most of the solvent and the remainder freeze-dried. The freeze-dried solid (total kafirin or zein) was dialysed against distilled water at 1°C for approx. 5 days and freeze-dried again to obtain the dry protein. The alkylation procedure involved preparing a 20 mg/ml mixture of total kafirin or zein in an 8 M urea solution containing 50 mM Tris HCl, pH 7.5 and 1% (v/v) mercaptoethanol. The mixture was stirred for 1 h under nitrogen before adding 4-vinylpyridine (1.5% v/v) and the reaction allowed to continue for 20 min in the dark. Reaction was terminated by dialysis against

frequent changes of ice-cold distilled water for 7 days. The resultant alkylated protein was then freeze dried.

3.2.6 Cooked whole grain meal, cooked endosperm meal, cooked protein body-enriched samples and cooked protein fractions (unalkylated and alkylated kafirin or zein)

Distilled water (33 ml) was brought to a boil in a beaker. Whole grain or endosperm meal (10 g) was made into a slurry with 17 ml distilled water. The slurry was added to the boiling water and cooked with constant stirring for 10 min at approx. 90°C to obtain the porridge. For protein body-enriched samples and extracted protein fractions, 17.5 ml distilled water was added to 20 mg sample in an Erlenmeyer flask and pressure cooked at 100 kPa for 10 min.

3.2.7 Alpha-amylase-treated samples

To determine the effect of gelatinised starch on *in vitro* protein digestibility, wet cooked whole grain and wet cooked endosperm samples were subjected to alpha-amylase treatment prior to pepsin digestion. Aliquots (1 mg/5 ml) of alpha-amylase (from *Bacillus amyloliquefaciens*, Boehringer Mannheim, Cat no. 161 764), prepared in distilled water, were added to the cooked samples and incubated in a shaking water bath at 37°C for 1h to thin the starch before incubation with pepsin.

3.2.8 Popped grain

Whole kernels of NK 283 sorghum, KAT 369 sorghum and PAN 6043 maize were popped separately in a domestic hot-air popcorn maker (Prima, model PCM001, China) as described by Parker *et. al.* (1999). Popped grains were selected visually, ground in a blender and sieved through a mesh of size 500 µm. Approx. percentage of kernels that popped were 75% for NK 283 sorghum, 65% for KAT 369 sorghum and 40% for PAN 6043 maize.

3.2.9 Starch hydrolysis prior to Fourier Transform Infrared (FTIR) spectroscopy

To enhance the protein concentration to make the protein peaks more visible in FTIR spectroscopy, uncooked whole grain, wet cooked whole grain and popped grain of NK 283 sorghum and PAN 6043 maize were subjected to amylase treatment to reduce their starch content. Enzymes used were amyloglucosidase (Sigma, Cat. no. A7420; 0.83 mg/g substrate), α-amylase (Boehringer Mannheim, Cat. no. 161 764; 0.15 mg/g substrate) and pullulanase (Megazyme, Cat. no. E-PULKP; 50 µl/g substrate), prepared in substrate solution containing

5 mM calcium chloride, 100 mM sodium acetate and 0.01% (w/v) sodium azide (pH 5.0). Substrate concentration was 10% (w/v) and incubation was carried out in a shaking water bath at 37°C for 7 days. Samples were then centrifuged at 2000 *g* for 10 min. Pellets obtained were freeze-dried before spectroscopic analyses.

3.3 Analytical methods

3.3.1 Protein content

Protein content was determined as total nitrogen using the Kjeldahl method. Sample is digested with concentrated sulphuric acid in the presence of a catalyst to convert nitrogen to ammonium hydrogen sulphate. The digest is neutralised with concentrated sodium hydroxide and volatile ammonia is distilled off into a solution of boric acid. An amount of borate anions equivalent to the ammonia is formed which is then titrated against standard hydrochloric acid (Christian, 1986). The distillation and titration steps were performed using an automated Büchi 322 Distillation Unit (Flawil, Switzerland). Total nitrogen was then converted to total protein using a conversion factor of 6.25. Protein assays were performed in triplicate.

3.3.2 In vitro protein digestibility (IVPD)

In vitro protein digestibility was determined using a modified form of the pepsin method of Hamaker *et al.*, (1987). The method involves the determination of residual nitrogen after a fixed period of pepsin digestion. From the total amount of nitrogen present prior to pepsin digestion, the amount of nitrogen digested is calculated and expressed as a percentage of total nitrogen.

Uncooked whole grain or endosperm meal (200 mg as is) or cooked whole grain or endosperm (500 mg as is) (all samples contained approximately 20 mg protein) were suspended by swirling in 35 ml pepsin solution (105 mg pepsin (from porcine stomach mucosa, Sigma, Cat Number P-7000) per 100 ml pH 2.0, 0.1M sodium citrate buffer) in 250 ml conical flasks. For the protein body preparations and the extracted kafirins and zeins (unalkylated and alkylated), 17.5 ml pepsin solution prepared with 0.2 M sodium citrate buffer was added to the sample (20 mg as is) already suspended in or cooked in 17.5 ml distilled water. The flasks were incubated at 37°C in a shaking water bath after which reaction was stopped by adding 2 ml 2M sodium hydroxide. The incubation mixture was filtered using Whatman No. 4 filter paper (diameter 12 cm). This modification gave better separation and recovery of insoluble protein than the centrifugation procedure of Hamaker *et al.* (1987). The

residue on the filter paper was analysed for nitrogen using the Kjeldahl procedure described above. Preliminary work had shown that the Whatman No. 4 filter paper was nitrogen-free according to the same Kjeldahl analysis. Protein digestibility was calculated by expressing the difference between total nitrogen and residual nitrogen as a percentage of total nitrogen. IVPD assays were performed in triplicate.

3.3.3 Total polyphenols

Total polyphenols was determined for whole grain, endosperm and protein body samples in triplicate using a modified Jerumanis ferric ammonium citrate (FAC) method, as described by Daiber (1975). FAC reacts with phenolic compounds under alkaline conditions and the absorbance of the reaction products is linearly related to concentration of the phenolic compounds (Daiber, 1975).

A 5% extract was prepared by shaking 250 mg finely milled material with 5 ml 75% (v/v) dimethylformamide (DMF) solution prepared in distilled water for 1 h at room temperature. The suspension was centrifuged at 2000 *g* for 5 min, the pellet discarded and the supernatant used in the absorbance measurements described below. Standard tannic acid (Merck) (50 mg) was dissolved in DMF extractant and made up to 5 ml. A 2 ml aliquot of this stock standard solution was diluted to 10 ml with DMF extractant to give a working standard of 4% tannic acid. Further dilutions of 2% and 1% tannic acid standard solutions were prepared from the working standard.

Reagents were mixed in a test tube in the following order: 5 ml distilled water, 1 ml carboxymethylcellulose/ethylenediaminetetraacetate (CMC/EDTA; containing 1% (w/v) CMC and 0.2% (w/v) EDTA in distilled water), 0.2 ml DMF extract or tannic acid standard, 0.2 ml 1.75% (w/v) FAC (containing 16% Fe) and 0.2 ml 28.8% (w/v) ethanolamine. For each extract and each tannic acid standard, a blank was prepared by replacing the FAC reagent with 0.2 ml distilled water.

Samples, blanks and standards were left to stand for 10 min and absorbances were read at 525 nm against distilled water. Absorbances for the blanks were subtracted from the individual sample or standard absorbances and a calibration curve plotted using the tannic acid standards. The tannic acid equivalent of each sample was read off the standard curve and results expressed as % total polyphenols (dry basis).

3.3.4 Enzyme inhibition by whole grain

This was determined using the malt amylase inhibition method of Daiber (1975). It involves incubation of ground whole grain with an enzyme extract from malt. The treated enzyme extract is then incubated with starch under standard conditions of temperature, time and pH and amylase activity determined by ferricyanide reduction by the products of starch hydrolysis. This involves initial reduction of Fe^{3+} ions to Fe^{2+} by reducing substances produced by the starch hydrolysis. The Fe^{2+} ions then oxidise iodide (I^-) ions (from potassium iodide) to iodine (I_2) which forms a dark purple complex with the excess, unhydrolysed starch. The iodine is then titrated against standard thiosulphate solution to a white endpoint. The amylase activity, referred to as Diastatic Power (DP), is expressed as Sorghum Diastatic Units per gram (SDU/g). One SDU per gram is taken as the amount of enzymatic activity which, under the conditions of the test, produces a quantity of sugar equivalent to a fixed volume of standard thiosulphate solution (South African Bureau of Standards, 1970).

Finely milled sorghum malt of diastatic power higher than 20 Sorghum Diastatic Units per gram was milled in an Ultra Turrax T25 (Janke & Kunkel, Germany) at highest speed for 5 min in 100 ml distilled water. The sample was centrifuged at 2000 *g* for 5 min and the clear supernatant (enzyme extract) used for the enzyme inhibition study.

Enzyme inhibition was calculated as the difference between the DP without and with added whole grain sample (to the enzyme extract), expressed as a percentage of DP without added whole grain.

3.3.5 Transmission electron microscopy

Samples (uncooked protein body preparations of all five grain varieties) were fixed in 3% glutaraldehyde in 0.05M cacodylate buffer, pH 7.2 for 2 h. Fixed samples were washed three times in the same buffer and post-fixed in 1% osmium tetroxide for 1 h. Tissues were dehydrated in a graded ethanol series, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100% ethanol followed by 100% acetone. Tissues were then transferred to 25, 50, 75% and finally pure Spurr epoxy resin before polymerisation in an oven overnight at 60°C. Sections were cut with a diamond knife, collected on copper grids and stained with uranyl acetate and lead citrate before examination using a JEOL 1200EX/B transmission electron microscope.

3.3.6 Fourier Transform Infra-Red (FTIR) Spectroscopy

The FTIR experiment involves monitoring the absorption frequencies associated with the vibrations of the functional groups in the sample being studied. For proteins, the absorption bands of the amide groups in the protein backbone are of particular interest since these yield information on protein conformation and secondary structure (Fraser & Suzuki, 1970).

FTIR analyses were done on protein body preparations of NK 283 sorghum, KAT 369 sorghum, P851171 sorghum mutant, P850029 sorghum mutant, PAN 6043 maize and uncooked whole grain, cooked whole grain and popped grain samples (of NK 283 sorghum, KAT 369 sorghum and PAN 6043 maize). Protein body preparations (uncooked and cooked) were defatted in hexane prior to FTIR analysis.

FTIR spectra were obtained using an FTS6000 Spectrometer (Bio-Rad) by Horizontal Attenuated Total Reflectance (HATR) in the dry state (256 scans at 2 cm^{-1} resolution) using a Ge ATR crystal (Specac) with 45° angle of incidence. A drop of distilled water was placed on the crystal and approximately 5 mg sample spread out evenly on the crystal in the water. The evenly spread sample was then carefully dried out completely with dry air at ambient temperature before the spectrum was recorded. To ensure complete elimination of the effect of water, a spectrum of water (collected by spreading out a thin film of water on the crystal) was subtracted from the sample spectra.

3.3.7 ^{13}C Nuclear Magnetic Resonance (NMR) Spectroscopy

In ^{13}C NMR spectroscopy of proteins, different carbon types from aliphatic amino acids, aromatic amino acids and the carbonyl functional group (C=O) have characteristic chemical shifts. The carbonyl group, being part of the peptide bond in the protein backbone has influence on protein secondary structure. The α-carbons of aliphatic amino acids, being close to the peptide bonds are also important for protein secondary structure. Therefore the chemical shifts of these carbons provide information on protein conformation and structure (Pastore & Saudek, 1990).

^{13}C NMR spectroscopy was done on uncooked and wet cooked (both defatted) samples of NK 283 sorghum, KAT 369 sorghum, PAN 6043 maize and P850029 sorghum. All magic angle spinning (MAS) experiments were carried out at 300 K on approximately 500 mg sample placed in an NMR glass tube with a Bruker MSL-300 spectrometer operating at 300.13 and 75.46 MHz for ^{1}H and ^{13}C respectively. A Bruker double bearing magic-angle spinning (DBMAS) probe-head and a 7 mm zirconia rotor were employed with typical sample spinning rate of about 4 kHz. CPMAS (cross polarisation magic angle spinning) spectra were recorded with a single contact time of 1.2 ms following a 90° proton pulse of 4 μs. Hartman-Hahn matching (Hartmann & Hahn, 1962) was set up using adamantane (Sigma). The strength of radio-frequency power in both proton and carbon channels was optimised by careful tuning of the probehead for both frequencies. Although perfect Hartman-Hahn matching was difficult to verify for each individual sample, there was no obvious problem of matching loss experienced for any sample studied. Glycine was used as an external chemical shift reference (176.03 ppm for the carbonyl peak).

3.3.8 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

The electrophoretic process involves the movement of charged species under the influence of an external electric field. The anionic detergent sodium dodecyl sulphate (SDS) is used to solubilise and to give the proteins a uniform charge distribution. The proteins are then loaded onto a polymer matrix, in this case, polyacrylamide gel which acts as a support and the electric field applied. The proteins then diffuse through the gel and are separated based on their relative molecular sizes since they have a uniform charge distribution (Hawcroft, 1997).

Uncooked and cooked protein body-enriched samples and the residues of these after pepsin digestion (3.3.2) were examined using SDS-PAGE. Samples studied were NK 283 sorghum, KAT 369 sorghum, PAN 6043 maize and P850029 sorghum mutant.

Cooked samples were centrifuged at 2000 *g* for 10 min and pellets freeze-dried for SDS-PAGE. For *in vitro* pepsin digestion, an amount of sample equivalent to 50 mg protein for each variety was used. After pepsin digestion as described above, samples were centrifuged at 2000 *g* for 10 min, supernatants discarded and pellets (pepsin-indigestible residue) freeze-dried for SDS-PAGE.

Electrophoresis was carried out under non-reducing and reducing conditions using 12 cm long and 1 mm thick gels on a Hoeffer/Pharmacia Biotech vertical electrophoresis system (SE600), with an EPS500 power supply. The separating gel was 15% acrylamide prepared from a stock solution of 40% (w/v) acrylamide and 2% (w/v) N,N′-bis-methyleneacrylamide in 0.125 M Tris/borate buffer (pH 8.9) and 0.1% (w/v) SDS. The stacking gel of 3% (w/v) acrylamide was prepared in 0.12 M Tris/HCl buffer (pH 6.8) and 0.1% (w/v) SDS. Separating and stacking gels were polymerised with 0.1% (w/v) ammonium persulphate and tetramethylethylenediamine (TEMED).

The protein body preparations of the different grain varieties had different protein contents. Therefore different amounts of sample of each variety were weighed out (8 mg NK 283 sorghum, 8 mg KAT 369 sorghum, 12 mg PAN 6043 maize and 5 mg P850029 sorghum mutant, uncooked and cooked) into Eppendorf tubes to give 3 mg protein content in each sample for protein extraction. Weighed samples were extracted with 0.5 ml sample buffer (3.33% (w/v) SDS, 0.067 M Tris-HCl pH 6.8, 10% (v/v) glycerol and 0.001 (w/v) Pyronin Y) to give protein extracts of concentration 6 µg/µl. For experiments under reducing conditions, protein extracts were prepared with 50, 100 and 200 mM dithiothreitol (DTT) added to the buffer. Molecular weight markers (3.5 mg mixture of bovine albumin, egg albumin, glyceraldehyde-3-phosphate dehydrogenase, carbonic anhydrase, trypsinogen, soybean trypsin inhibitor, alpha-lactalbumin) (Sigma, SDS-7) was dissolved in 0.5 ml sample buffer with 100 mM DTT in an Eppendorf tube. The extraction mixtures in the Eppendorf tubes were boiled in distilled water for 3 min to ensure complete protein extraction. For all protein body samples, 40 µg protein was loaded onto the gel. Loading of pepsin-indigestible residues was done in approximately inverse proportion to the protein digestibility of the grain variety, with a maximum loading of approximately 30 µg protein. For molecular weight standards, 10 µl (70 µg protein) was loaded. Electrophoresis was conducted at 13 mA per gel and 120 V for about 1 h until the tracker dye had run into the separating gel and subsequently at 25 mA per gel and 250 V for a further 3 h at ambient temperature.

Proteins were stained with 0.25% (w/v) Coomassie Brilliant Blue R-252 in 10% (w/v) trichloroacetic acid (TCA) and 40% (v/v) methanol. Gels were destained with 10% (w/v) TCA and photographed.

3.4 Statistical analyses

Analysis of variance by the least significant difference test (LSD-test) was performed on the results obtained from the *in vitro* protein digestibility, total polyphenol and enzyme inhibition assays to determine whether a significant difference existed ($p < 0.05$) between means of treatments.