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ROLE OF GRAIN ORGANISATIONAL STRUCTURE IN SORGHUM PROTEIN DIGESTIBILITY

BY

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

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ABSTRACT

ROLE OF GRAIN ORGANISATIONAL STRUCTURE IN SORGHUM PROTEIN DIGESTIBILITY

by

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Sorghum (*Sorghum bicolor* (L.) Moench) is a drought-tolerant basic food cereal in many parts of Africa and Asia. Wet cooking decreases the digestibility of sorghum proteins significantly and this is a limitation to the use of sorghum as food. Uncooked sorghum protein digestibility is also considered lower than other cereals.

In vitro protein digestibility of uncooked and cooked condensed-tannin-free sorghum varieties and a white maize variety was examined at four levels of grain organisational structure: whole grain, endosperm, protein body preparations and isolated proteins. The possibility of polyphenols and gelatinised starch having an effect on *in vitro* protein digestibility of sorghum and maize was also examined. Fourier transform infrared (FTIR) and solid-state ^{13}C NMR spectroscopic methods were used to investigate changes in protein secondary structure on thermal processing. Electrophoresis (SDS-PAGE) was used to investigate the possibility of protein crosslinking on thermal processing by examining molecular weight differences.

Uncooked and cooked sorghum protein digestibility improved from whole grain, through endosperm to protein body preparations. However, uncooked and cooked maize protein digestibility was essentially the same at these levels. Uncooked sorghum protein digestibility was lower than uncooked maize at the whole grain level, same as maize at the endosperm level and higher than maize at the protein body-enriched level. Isolated kafirins and zeins from sorghum and maize had similar uncooked protein digestibility. Total polyphenol content

of sorghum decreased from whole grain to endosperm and increased from endosperm to protein body preparations.

As expected, cooking reduced protein digestibility of sorghum whole grain, endosperm, protein body preparations and extracted kafirins. Cooked sorghum whole grain and endosperm had similar protein digestibilities but cooked protein body preparations were more digestible. There was overall improvement in sorghum protein digestibility with change in organisational level. Protein digestibilities of uncooked and cooked maize were essentially the same at all the organisational levels.

Treating cooked whole grain and endosperm samples of sorghum and maize with alpha-amylase before pepsin digestion improved protein digestibility. In the protein body preparations where the proportion of starch was lower, such treatment had no effect on protein digestibility.

SDS-PAGE under non-reducing and reducing conditions of uncooked and cooked protein body preparations from normal sorghum, maize and sorghum mutants (of known high protein digestibility) showed oligomers of M_r 45, 66 and >66 kDa and monomeric kafirins and zeins. Sorghum had more 45-50 kDa oligomers than maize. In comparison with maize, more of these oligomers were resistant to reduction in cooked normal sorghum.

SDS-PAGE also showed that residues of the protein body preparations remaining after pepsin digestion consisted mainly of α-zein (uncooked and cooked maize) or α-kafirin (uncooked normal sorghum), whilst cooked normal sorghum had in addition, β- and γ-kafirin and reduction-resistant 45-50 kDa oligomers.

FTIR and solid-state ^{13}C NMR spectra of normal sorghum, maize and sorghum mutants indicated a change in protein secondary structure from α-helical to antiparallel intermolecular β-sheet conformation on cooking. The extent of secondary structural change seemed to be greater in sorghum than in maize.

Grain organisational structure does influence sorghum protein digestibility. Interfering factors in the grain outer layers, namely pericarp and germ may be responsible. The decrease in sorghum total polyphenol content from whole grain to endosperm accompanied with an increase in uncooked and cooked sorghum protein digestibility suggests that polyphenols may affect sorghum whole grain protein digestibility. In contrast to earlier reports, uncooked sorghum protein digestibility may not always be lower than that of maize. It depends on the nature of the material being assayed. Gelatinised starch, probably by reducing accessibility of pepsin to protein, reduced digestibility of sorghum and maize whole grain and endosperm.

It appears that cooking reduces protein digestibility in sorghum by unravelling of prolamin polypeptides in the α-helical conformation which re-associate either through disulphide or non-disulphide crosslinks to form the antiparallel intermolecular β-sheet conformation. This conformation may be less digestible due to restricted enzyme access to the protein. Such crosslinking may occur to a greater extent in sorghum than in maize perhaps due to subtle differences in prolamin tertiary structure between the two cereals, contributing to the worse digestibility of cooked sorghum proteins.

Do not be afraid of walking slowly

Be afraid only of standing still

- Old Chinese proverb

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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).

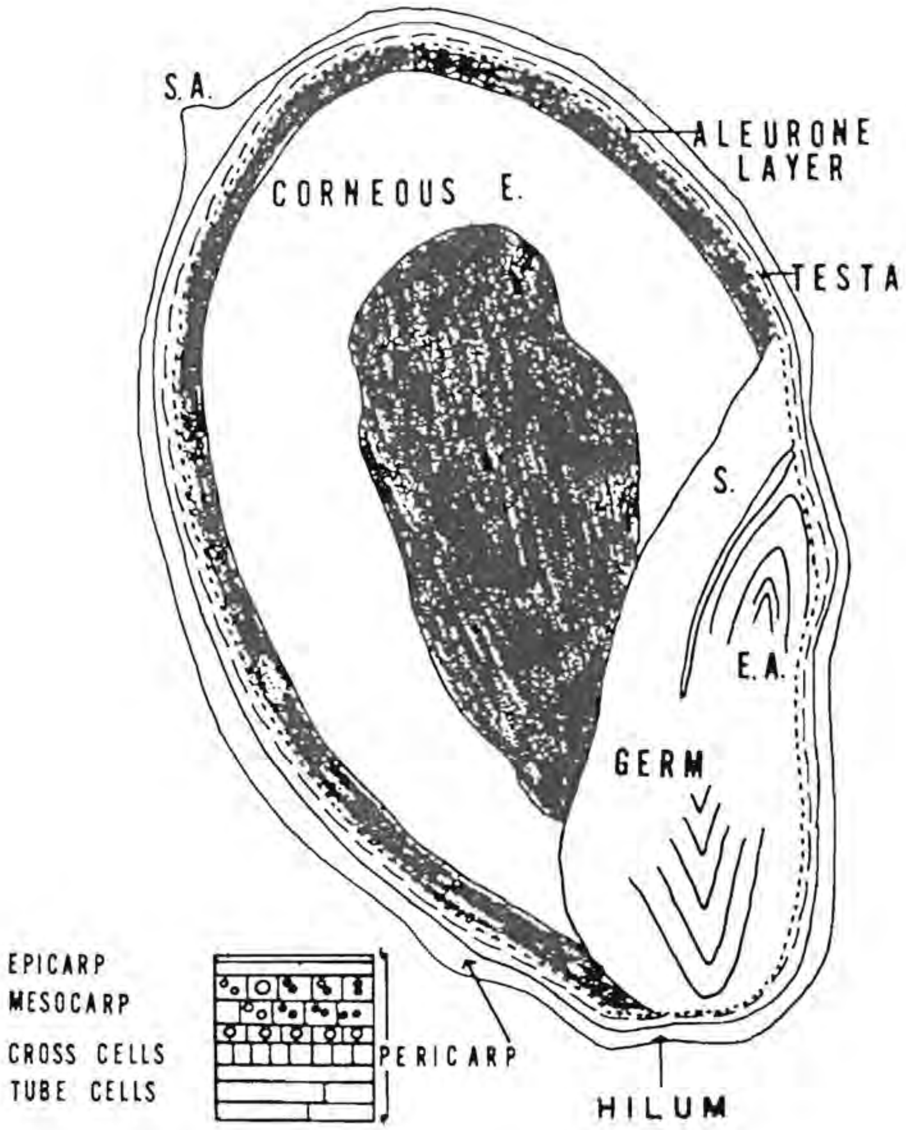
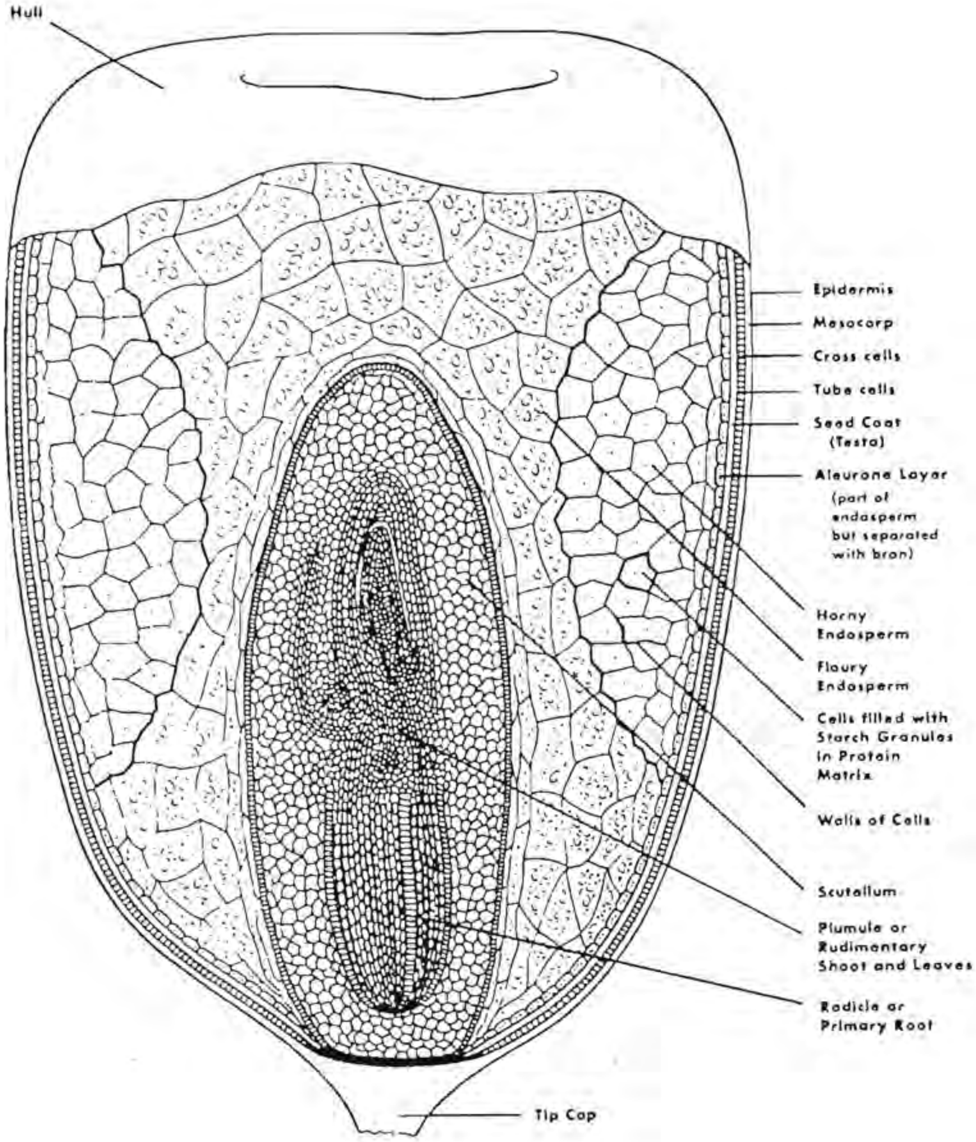


Figure 1: A sorghum kernel. S.A., stylar area; E., endosperm; S., scutellum; E.A., embryonic axis. (Hoseney, 1994).

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).



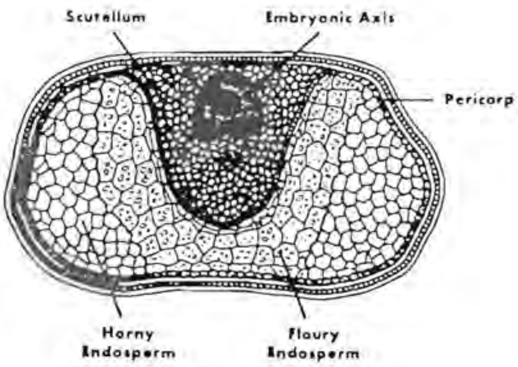


Figure 2: Longitudinal and cross sections of a maize kernel. (Hoseney, 1994)

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).

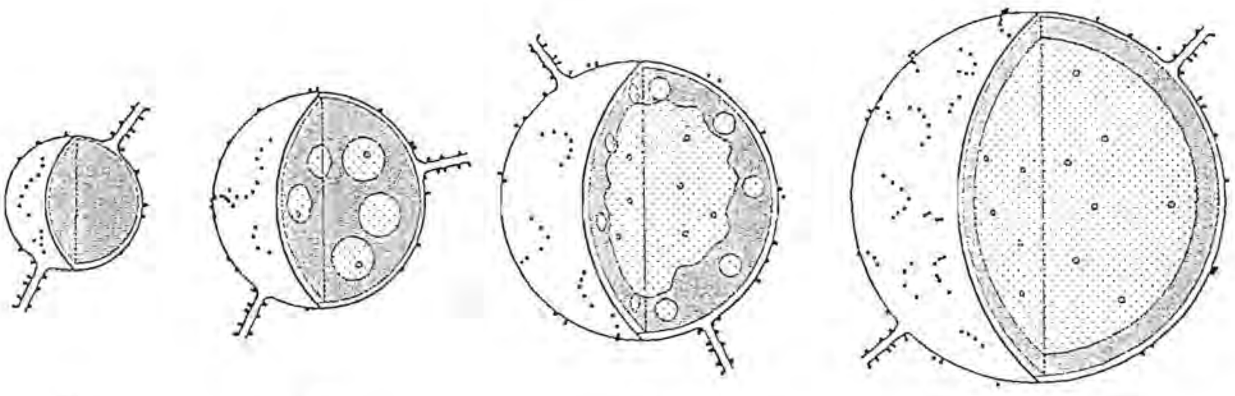


Figure 3: Development of protein bodies in maize endosperm as proposed by Lending and Larkins (1989). Dark shaded regions are rich in β- and γ-zeins and the light shaded regions are rich in α-zein. The dark dots represent ribosomes.

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 X(i) 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).

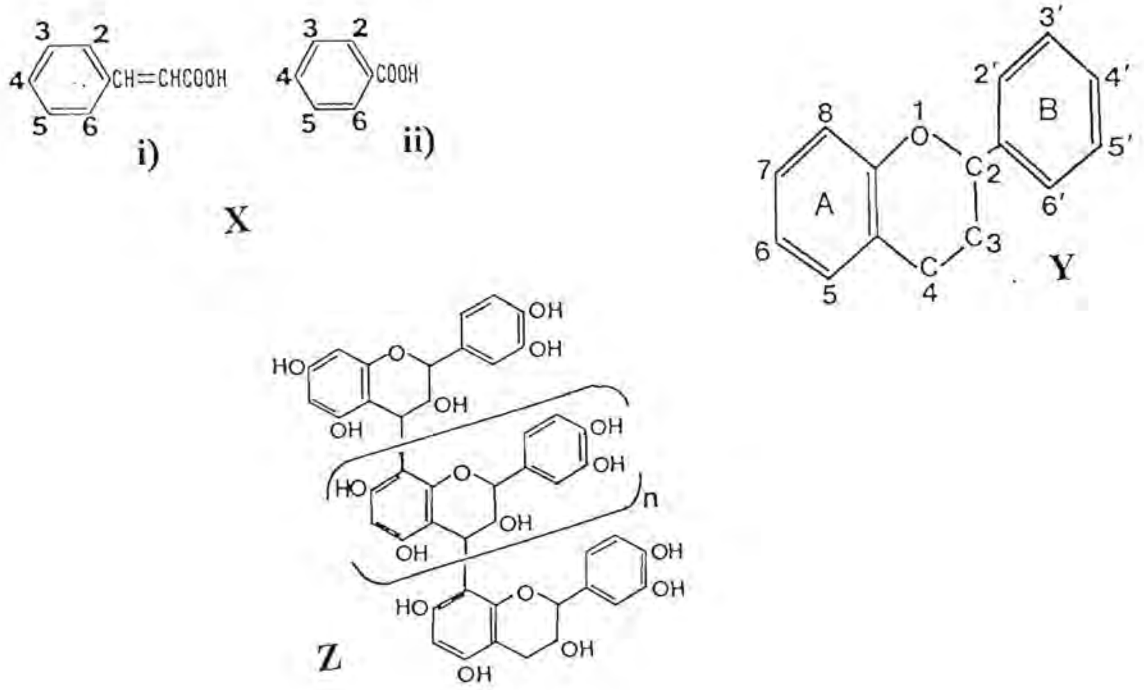


Figure 4: X) Basic structure of phenolic acids i) cinnamic acid; ii) benzoic acid.

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).

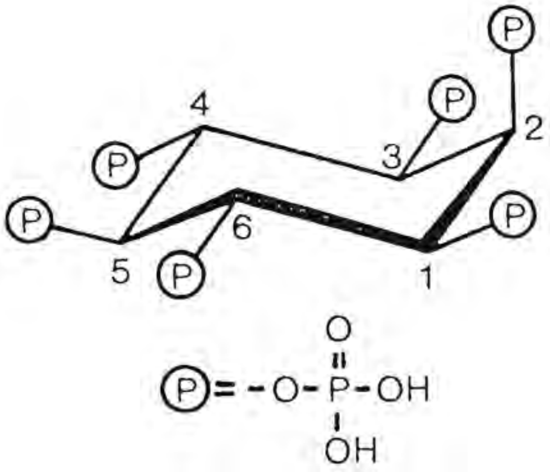


Figure 5: Structure of phytic acid. (Hoseney, 1994).

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.

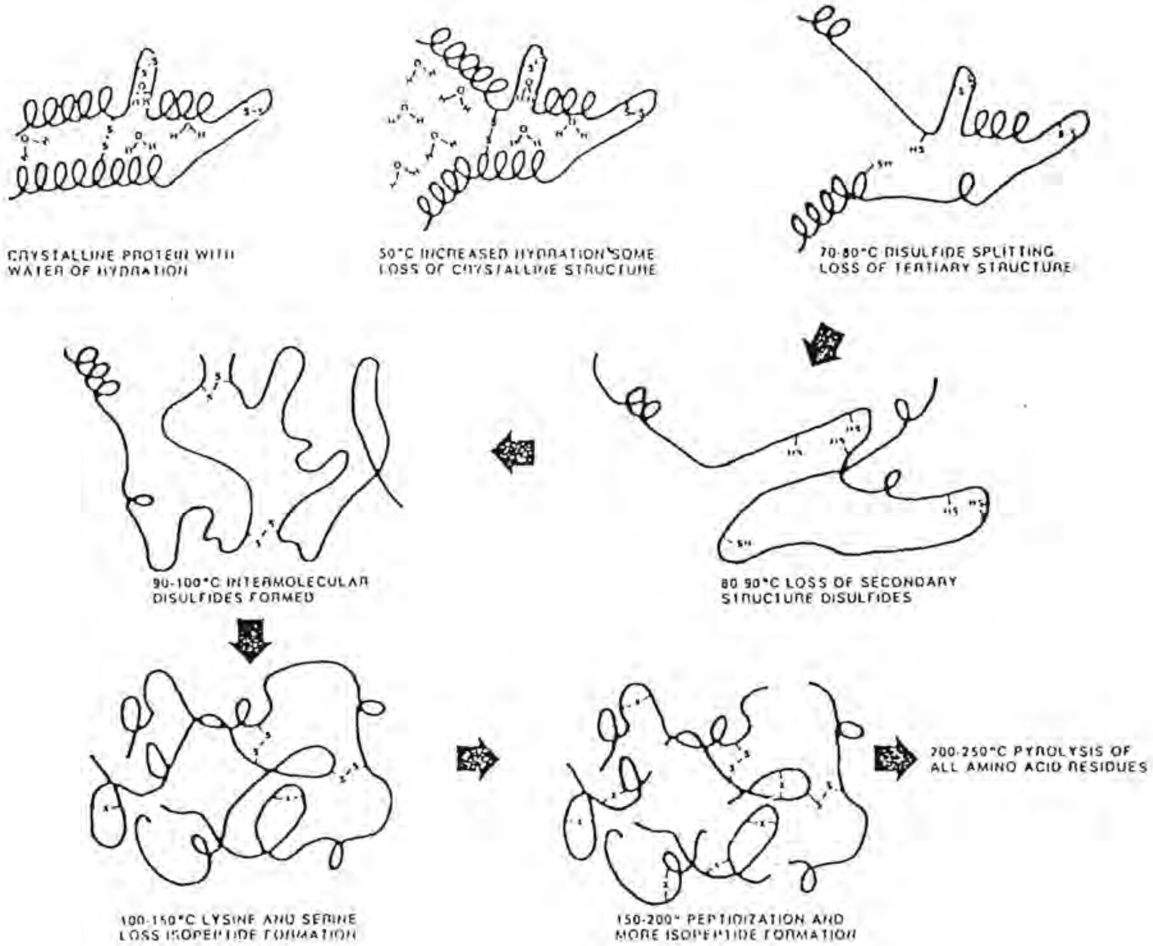


Figure 6: Changes a protein undergoes during heat treatment. (Finley, 1989).

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.

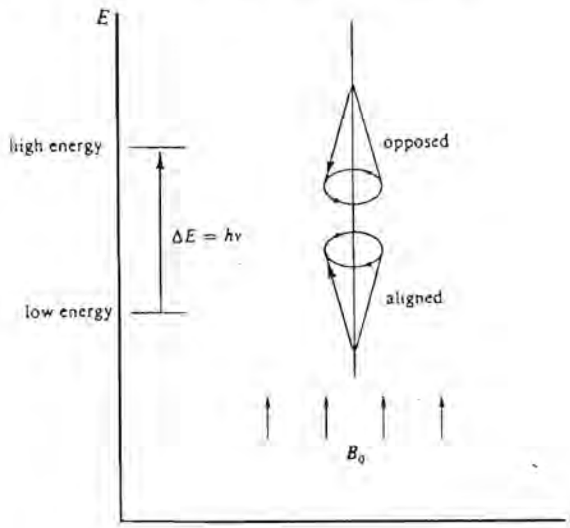


Figure 7: Representation of precessing nuclei and the energy transition between the aligned and opposed conditions. (Kemp, 1987).

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.

CHAPTER 4

RESULTS

4.1 Total protein and polyphenol contents of sorghum and maize samples

Table 7. Total protein contents (g/100 g dry basis) of whole grain, endosperm and protein body-enriched samples of sorghum (NK 283, KAT 369), maize (PAN 6043) and protein body-enriched samples of P851171 and P850029 sorghum mutants and total polyphenol contents (% tannic acid equivalents dry basis) of whole grain, endosperm and protein body-enriched samples of sorghum (NK 283, KAT 369) and maize (PAN 6043).

	NK 283 sorghum (red)		KAT 369 sorghum (white)		PAN 6043 maize		P851171 sorghum Mutant		P850029 sorghum mutant	
	Total protein	Total polyphenol	Total Protein	Total polyphenol	Total protein	Total Polyphenol	Total Protein	Total polyphenol	Total protein	Total polyphenol
Whole Grain	10.8	0.28	9.0	0.21	10.0	0.19	ND[2]	ND	ND	ND
Endosperm	8.5	0.17	7.3	0.10	9.1	0.21	ND	ND	ND	ND
PB[1]	33.7	0.24	32.0	0.17	22.9	0.19	58.3	ND	53.9	ND

[1]Protein body-enriched sample

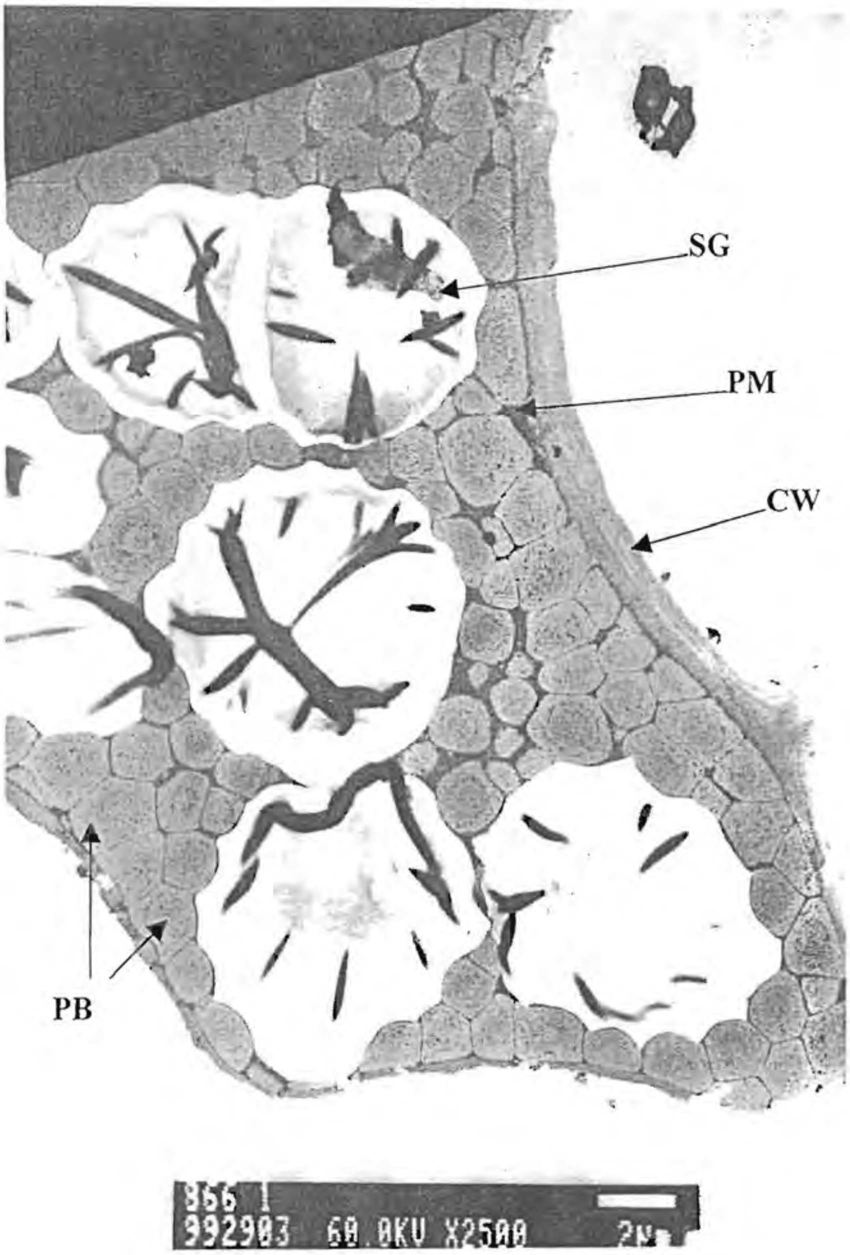
[2]Not determined

For whole grain, NK 283 sorghum had the highest protein content followed by PAN 6043 maize and then KAT 369 sorghum. They all had significantly higher protein content in the whole grain than endosperm. For the endosperm, PAN 6043 maize had the highest protein content followed by NK 283 sorghum and KAT 369 sorghum. The protein body-enriched samples of the two normal sorghums and the maize had much higher protein content (approximately 2-4 times greater) than their corresponding whole grain and endosperm. Of the protein body-enriched samples, PAN 6043 maize had the lowest protein content followed by the two normal sorghums (NK 283 and KAT 369) with the sorghum mutants (P851171 and P850029) having the highest protein contents.

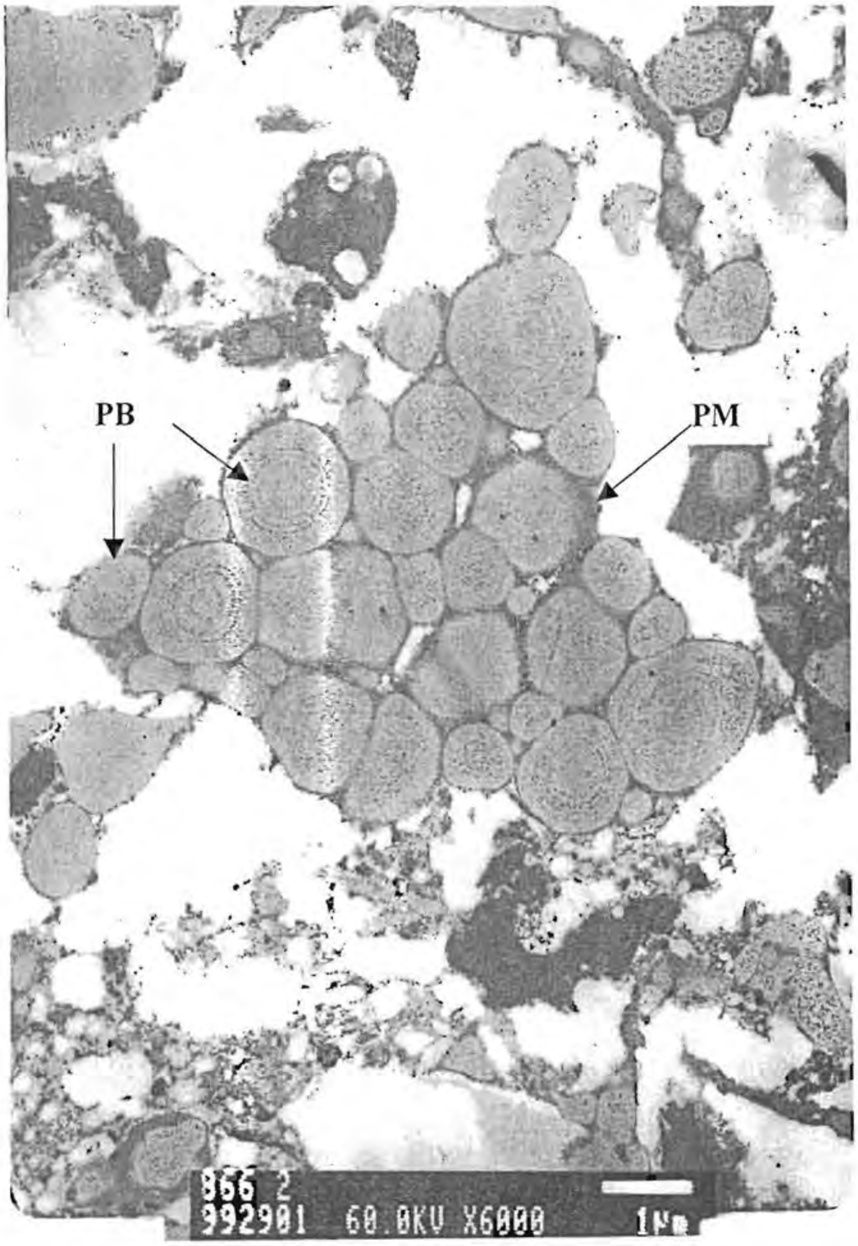
The NK 283 red sorghum grain contained more total polyphenols than the KAT 369 white sorghum and the white maize. For the two sorghums, the endosperm had lower total polyphenol content than whole grain. The protein body-enriched samples of the sorghums had similar total polyphenol content to whole grain. The PAN 6043 maize, in contrast to the sorghums, had similar total polyphenol contents at whole grain, endosperm and protein body levels.

4.2 Ultrastructure of protein body-enriched samples

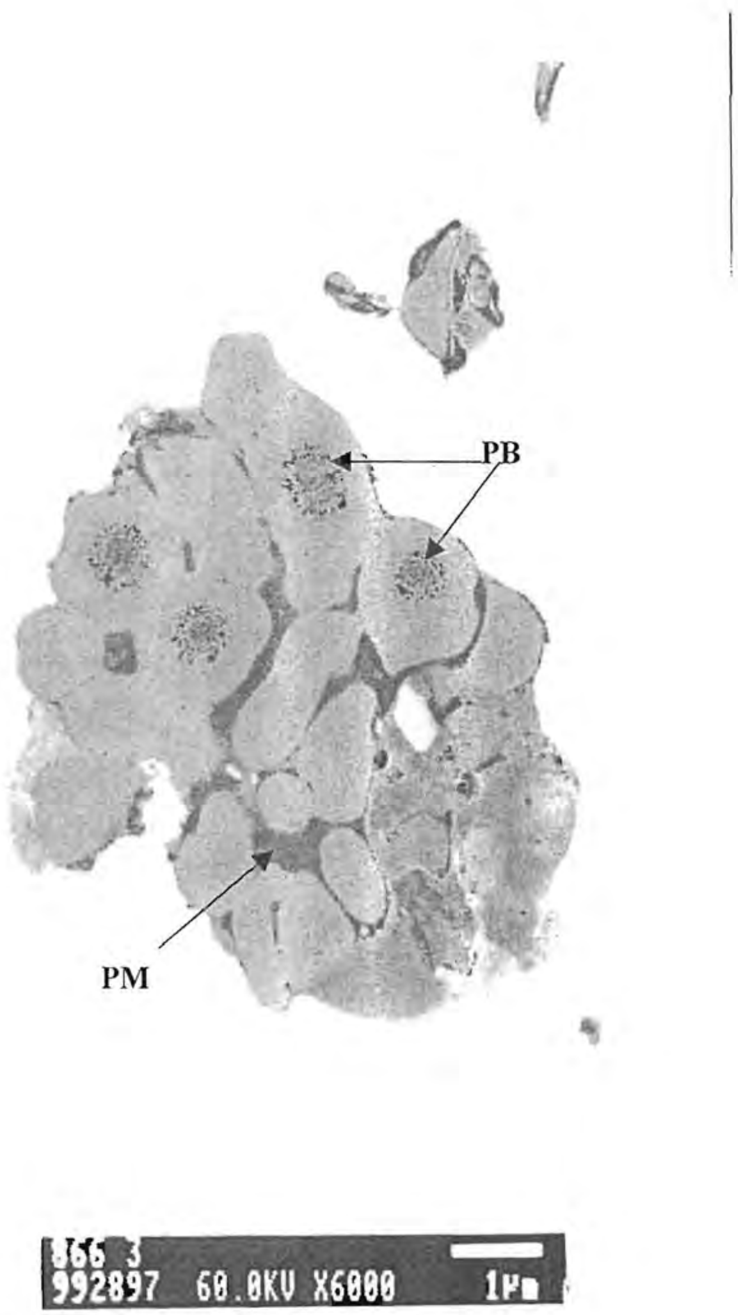
Figure 8. Transmission electron micrographs of uncooked protein body-enriched samples of sorghum (NK 283 and KAT 369), maize (PAN 6043) and sorghum mutants (P851171 and P850029).



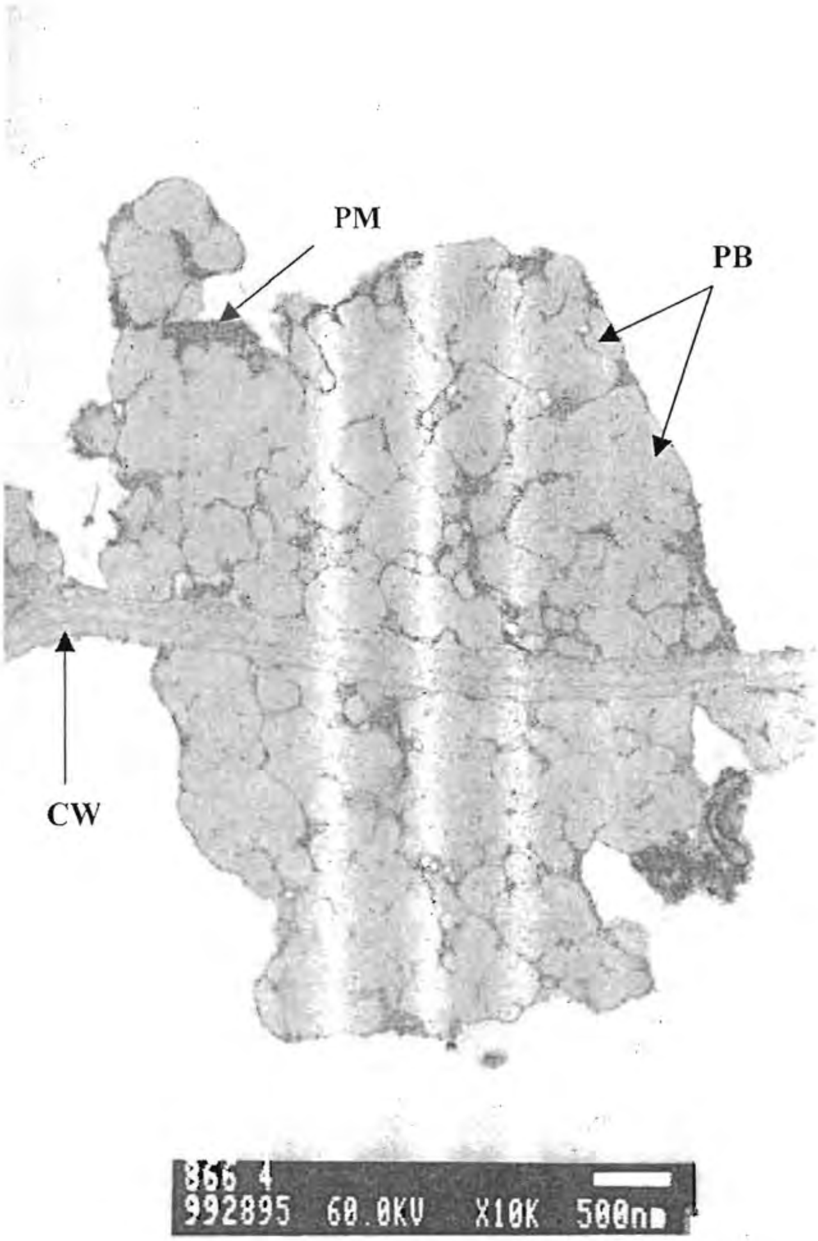
A) Transmission electron micrograph of uncooked NK 283 sorghum protein body-enriched sample. PB – protein body; PM – protein matrix; CW – cell wall; SG – starch granule.



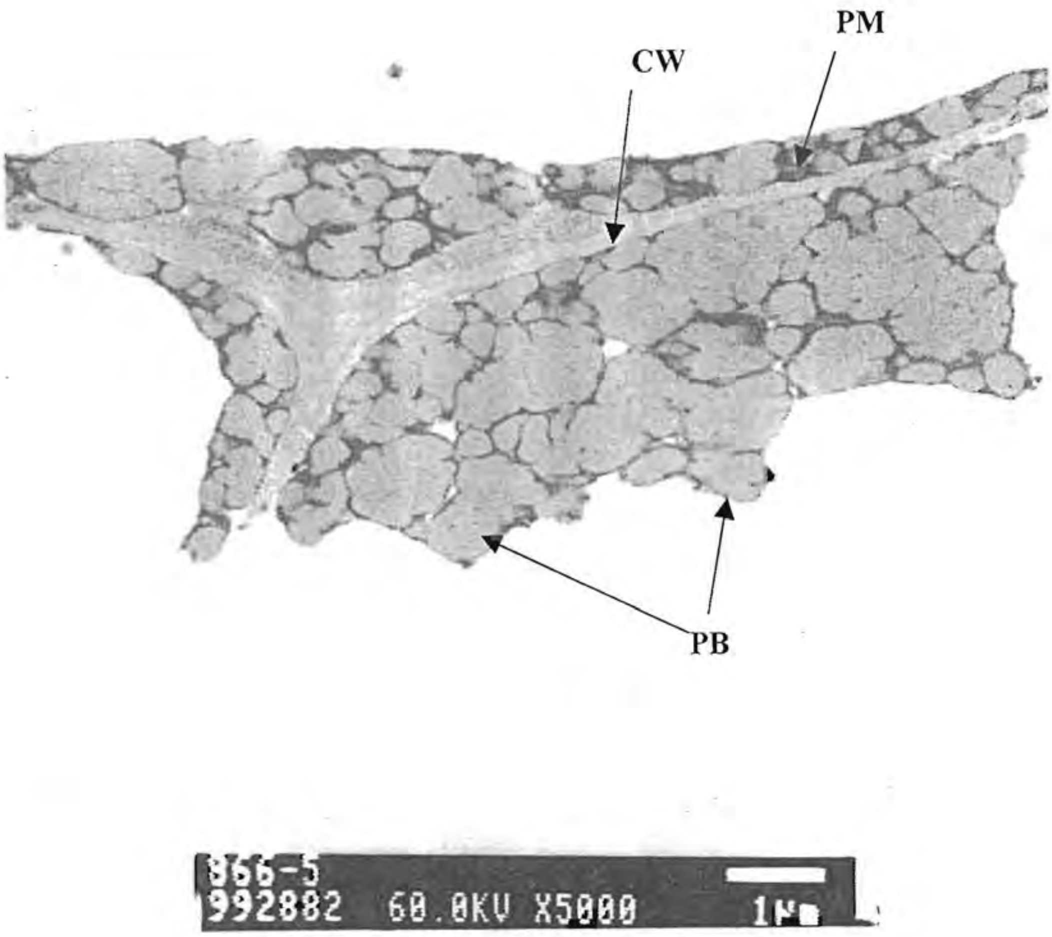
B) Transmission electron micrograph of uncooked KAT 369 sorghum protein body-enriched sample. PB – protein body; PM – protein matrix.



C) Transmission electron micrograph of uncooked PAN 6043 maize protein body-enriched sample. PB – protein body; PM – protein matrix.



D) Transmission electron micrograph of uncooked P851171 sorghum mutant protein body-enriched sample. PB – protein body; PM – protein matrix; CW – cell wall.



E) Transmission electron micrograph of uncooked P850029 sorghum mutant protein body-enriched sample. PB – protein body; PM – protein matrix; CW – cell wall.

The protein bodies of the normal sorghums (NK 283 and KAT 369) and the maize (PAN 6043) were mostly round-shaped compared to those of the sorghum mutants (P851171 and P850029) which appeared to be irregularly-shaped with many invaginations. For all five samples, the protein bodies appeared to be embedded in a dark staining matrix. The interior of the protein bodies had some dark-staining deposits which occurred in the form of concentric rings. The NK 283 sorghum and the mutant sorghum micrographs showed the presence of cell wall material. Starch granules were present in the NK 283 sorghum micrograph.

4.3 *In vitro* protein digestibility of whole grain, endosperm and protein body-enriched samples and enzyme inhibition by whole grain

Table 8 Effect of cooking and addition of alpha-amylase after cooking on percentage *in vitro* protein digestibility of whole grain, endosperm and protein body-enriched samples of NK 283 red sorghum

	Whole grain		Endosperm		Protein body-enriched sample		Mean treatment
Treatment	PD[1]	% of uncooked	PD	% of uncooked	PD	% of uncooked	
Uncooked	59.1 c[2] ± 3.7[3]	100	65.7 c ± 0.9	100	72.8 b ± 2.5	100	65.8 γ
Cooked	30.5 a ± 1.6	52	35.9 a ± 5.1	55	44.2 a ± 3.2	61	36.9 α
Cooked/ α-amylase	36.5 b ± 1.8	62	49.0 b ± 4.3	75	45.3 a ± 3.4	62	43.6 β
Mean OL[4]	42.0 x		50.2 y		54.1 z		

[1]Protein digestibility.

[2]Mean values with different letters in the same column or row are significantly different from each other ($p < 0.05$).

[3]Standard deviation.

[4]Organisational level.

Overall, there was progressive increase in protein digestibility with change in organisational level from whole grain, through endosperm to protein body-enriched samples. Cooking decreased protein digestibility whilst alpha-amylase treatment of cooked samples prior to pepsin digestion improved protein digestibility above the level of the cooked samples. There was progressive increase in protein digestibility of uncooked and cooked NK 283 red sorghum with change in organisational level from whole grain, through endosperm to protein body-enriched samples. Cooking reduced protein digestibility at all three levels of grain organisation. Treating cooked samples with alpha-amylase prior to pepsin digestion increased protein digestibility above the level of cooked samples at the whole grain and endosperm levels but not at the protein body-enriched level.

Table 9 Effect of cooking and addition of alpha-amylase after cooking on percentage *in vitro* protein digestibility of whole grain, endosperm and protein body-enriched samples of KAT 369 white sorghum

	Whole grain		Endosperm		Protein body-enriched sample		Mean treatment
Treatment	PD[1]	% of uncooked	PD	% of uncooked	PD	% of uncooked	
Uncooked	55.8 c[2] ± 0.9[3]	100	67.4 c ± 1.2	100	74.3 b ± 4.7	100	65.9 γ
Cooked	36.6 a ± 2.8	66	39.4 a ± 4.4	58	63.5 a ± 1.7	85	46.5 α
Cooked/ α-amylase	42.2 b ± 2.0	76	43.7 b ± 2.9	65	62.7 a ± 3.9	84	49.6 β
Mean OL[4]	44.9 x		50.2 y		66.8 z		

[1]Protein digestibility.

[2]Mean values with different letters in the same column or row are significantly different from each other ($p < 0.05$).

[3]Standard deviation.

[4]Organisational level.

Overall, there was progressive increase in protein digestibility with change in organisational level from whole grain, through endosperm to protein body-enriched samples. Cooking decreased protein digestibility, whilst alpha-amylase treatment of cooked samples prior to pepsin digestion improved protein digestibility above the level of the cooked samples. There was progressive increase in protein digestibility of uncooked and cooked KAT 369 white sorghum with change in organisational level from whole grain, through endosperm to protein body-enriched samples. Cooking reduced protein digestibility at all three levels of grain organisation. Treating cooked samples with alpha-amylase prior to pepsin digestion increased protein digestibility above the level of cooked samples at the whole grain and endosperm levels but not at the protein body-enriched level. Uncooked and cooked KAT 369 white sorghum had similar protein digestibility to uncooked and cooked NK 283 red sorghum at the whole grain and endosperm levels. However, the cooked protein body-enriched sample of KAT 369 was 19.3% more digestible than cooked protein body-enriched sample of NK 283 (see Table 8) and 17.4% more digestible when the cooked protein body-enriched sample was treated with alpha-amylase.

Table 10 Effect of cooking and addition of alpha-amylase after cooking on percentage *in vitro* protein digestibility of whole grain, endosperm and protein body-enriched samples of PAN 6043 maize

	Whole grain		Endosperm		Protein body-Enriched sample		Mean treatment
Treatment	PD[1]	% of uncooked	PD	% of uncooked	PD	% of uncooked	
Uncooked	66.6 b[2] ± 1.3[3]	100	67.4 a ± 1.2	100	68.8 a ± 2.3	100	67.6 β
Cooked	62.0 a ± 3.2	93	63.6 a ± 2.3	94	67.4 a ± 4.1	98	64.3 α
Cooked/ α-amylase	72.5 c ± 3.3	109	72.2 b ± 2.3	107	68.2 a ± 3.8	99	67.6 β
Mean OL[4]	67.0 x		67.7 x		68.1 x		

[1]Protein digestibility.

[2]Mean values with different letters in the same column or row are significantly different from each other ($p < 0.05$).

[3]Standard deviation.

[4]Organisational level.

Overall, PAN 6043 protein digestibility remained the same at the three organisational levels. Cooking reduced protein digestibility whilst alpha-amylase treatment of cooked samples improved protein digestibility back to the level of uncooked. The protein digestibility of uncooked PAN 6043 maize at the three organisational levels was essentially the same. Cooking reduced protein digestibility slightly at the whole grain level (4.6% reduction) but not at the endosperm and protein body-enriched levels. In contrast, cooking reduced protein digestibility of NK 283 red sorghum (see Table 8) and KAT 369 white sorghum (see Table 9) at the whole grain, endosperm and protein body-enriched levels. For NK 283 red sorghum, this reduction was by 28.6% at the whole grain level, 29.8% at the endosperm level and 28.6% at the protein body-enriched level, whilst for KAT 369 white sorghum, reduction on cooking was by 19.2% at the whole grain level, 28.0% at the endosperm level and 10.8% at the protein body-enriched level. For cooked PAN 6043 maize samples, alpha-amylase treatment prior to pepsin digestion improved protein digestibility above the level of uncooked and cooked samples at the whole grain and endosperm levels but not at the protein body-enriched level.

Table 11 Effect of cooking and addition of alpha-amylase after cooking on percentage *in vitro* protein digestibility of protein body-enriched samples of P851171 and P850029 sorghum mutants in comparison with red sorghum NK 283, white sorghum KAT 369 and maize PAN 6043

	P851171		P850029		NK 283[4]		KAT 369[4]		PAN 6043[4]	
Treatment	PD[1]	% of uncooked	PD	% of uncooked	PD	% of uncooked	PD	% of uncooked	PD	% of uncooked
Uncooked	83.1 a[2] ± 2.0[3]	100	80.0 b ± 1.7	100	72.8	100	74.3	100	68.8	100
Cooked	80.3 a ± 1.5	97	74.3 a ± 3.0	93	44.2	61	63.5	85	67.4	98
Cooked/ α-amylase	78.8 a ± 1.9	95	78.3 b ± 2.1	98	45.3	62	62.7	84	68.2	99

[1]Protein digestibility.

[2]Mean values with different letters in the same column or row are significantly different from each other ($p < 0.05$).

[3]Standard deviation.

[4]Values from Tables 2, 3 and 4.

The protein body-enriched samples of the two sorghum mutants had higher protein digestibilities (uncooked) than the two normal sorghums and the maize. Cooking reduced the protein digestibility of P850029 sorghum mutant very slightly but not P851171. This reduction was low (5.7%) compared to the 10.8% reduction for white KAT 369 sorghum and 28.6% reduction for red NK 283 sorghum. The cooked protein body-enriched samples of the sorghum mutants had higher protein digestibility than the cooked normal sorghums and maize protein body-enriched samples. Treating cooked samples with alpha-amylase prior to pepsin digestion slightly increased protein digestibility of P850029 sorghum mutant by 4.0% but had no effect on the digestibility of P851171.

Table 12. Percentage of enzyme (amylase) inhibition caused in whole grain of NK 283 sorghum, KAT 369 sorghum and PAN 6043 maize in comparison with DC 75 sorghum (a high-tannin hybrid)

	NK 283 sorghum	KAT 369 Sorghum	PAN 6043 maize	DC 75 High-tannin sorghum
Enzyme inhibition (%)	5.7 b[1]	0.6 a	2.5 ab	64.9 c

[1]Mean values with different letters in the same row are statistically different from each other ($p < 0.05$).

The high-tannin sorghum very substantially inhibited amylase activity, while the condensed-tannin-free sorghums and the maize had a negligible effect on amylase activity.

4.4 *In vitro* protein digestibility of reduced/alkylated and reduced/non-alkylated kafirin and zein

Table 13 Effect of cooking and alkylation on *in vitro* protein digestibility (PD) of total kafirin (kafirin 1 and kafirin 2) and total zein (zein 1 and zein 2).

	Kafirin		Zein	
	% PD	% of uncooked	% PD	% of uncooked
Unalkylated, uncooked	53.2 c[1] ± 1.5[2]	100	54.5 b ± 2.7	100
Unalkylated, cooked	32.7 a ± 3.5	62	47.9 a ± 2.8	88
Alkylated, uncooked	60.6 d ± 1.2	100	58.9 b ± 1.9	100
Alkylated, cooked	43.1 b ± 3.2	71	54.3 b ± 1.8	92

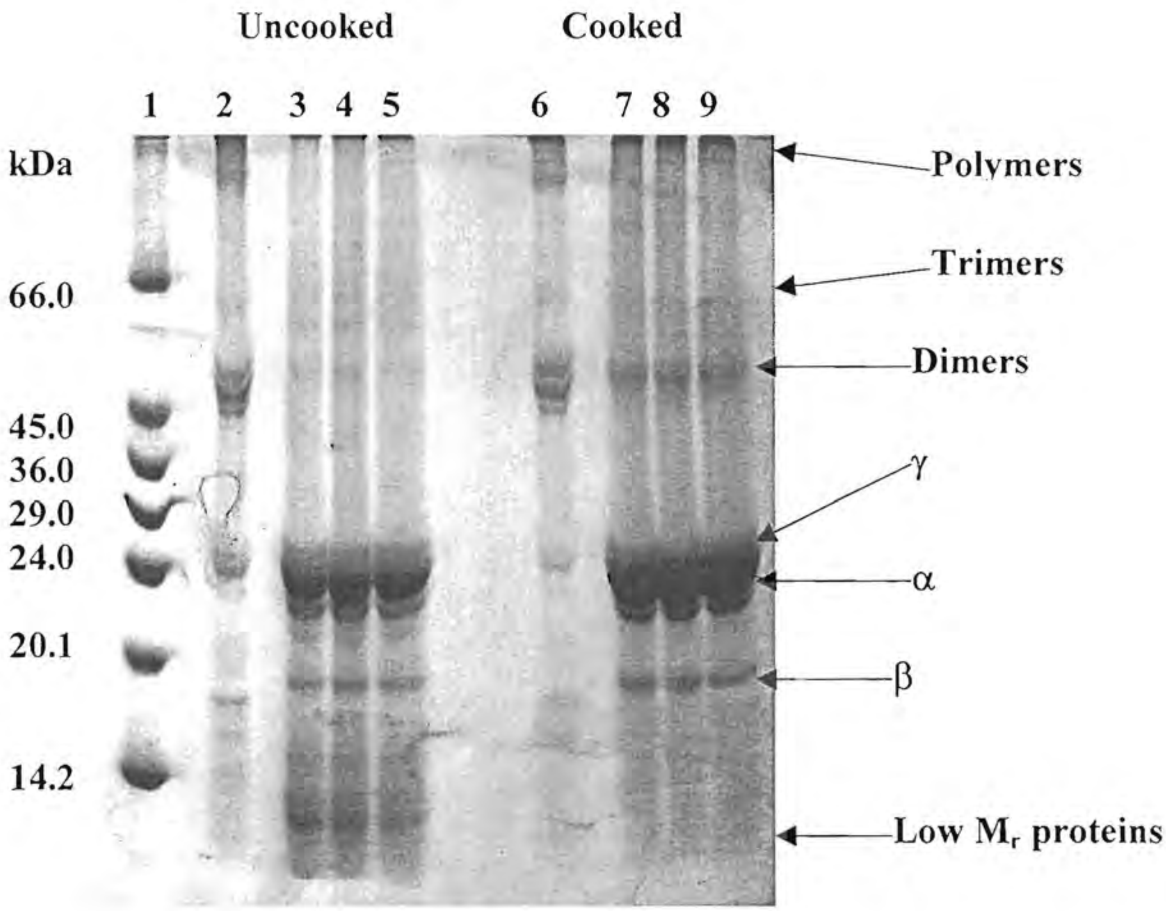
[1]Mean values with different letters in the same column are significantly different from each other ($p < 0.05$).

[2]Standard deviation.

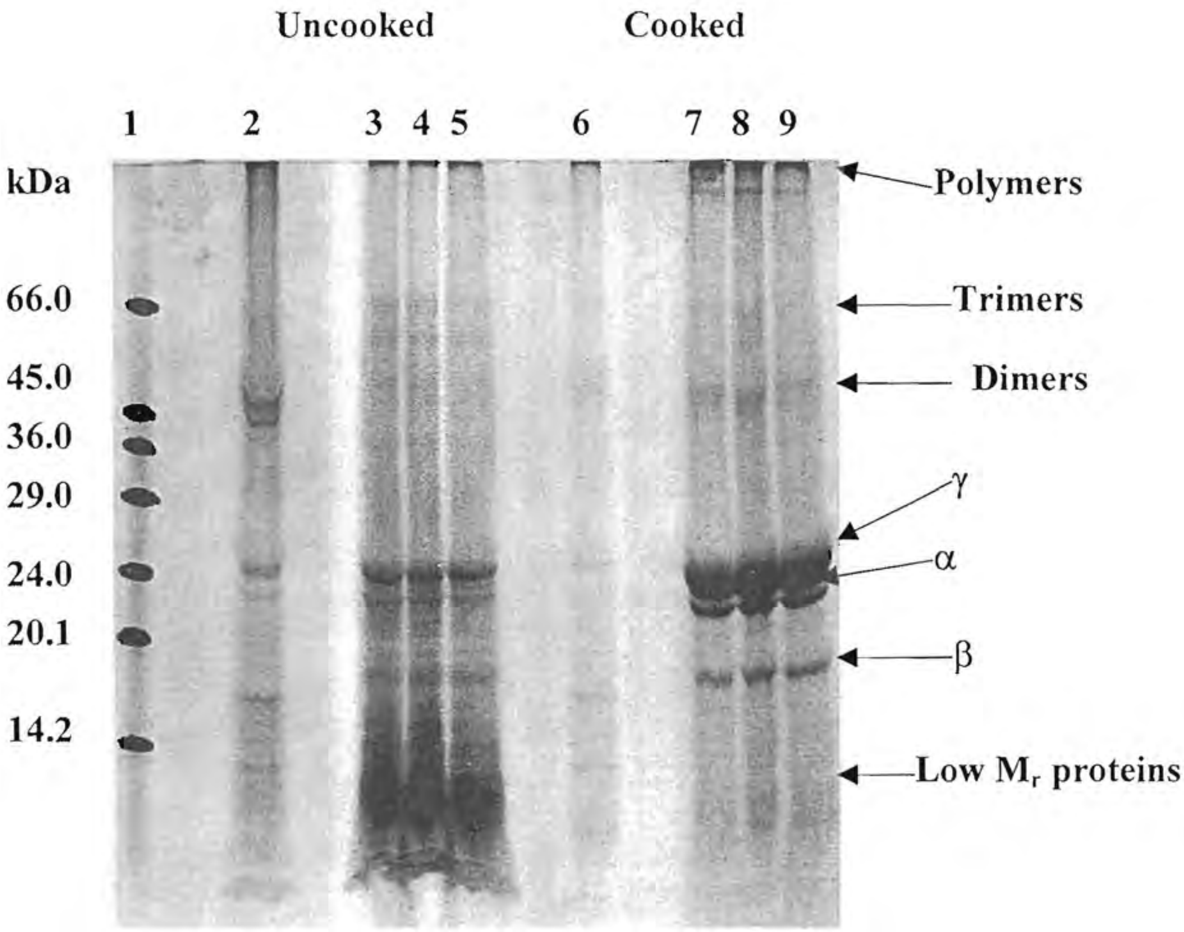
Cooking reduced protein digestibility of both unalkylated and alkylated kafirin and zein. However the drop in digestibility on cooking was more pronounced for the kafirins (by 20.5% for unalkylated kafirin and 17.5% for alkylated kafirin) than for the unalkylated zeins (by 6.6% for unalkylated zein). There was no significant drop in digestibility on cooking the alkylated zein. Unalkylated, uncooked kafirin and zein had the same digestibility as did alkylated, uncooked kafirin and zein Cooked zein (unalkylated and alkylated) was more digestible than cooked kafirin. Alkylated samples (uncooked and cooked) for both kafirin and zein were more digestible than the unalkylated.

4.5 SDS-PAGE of protein body-enriched samples of sorghum and maize under non-reducing and reducing conditions

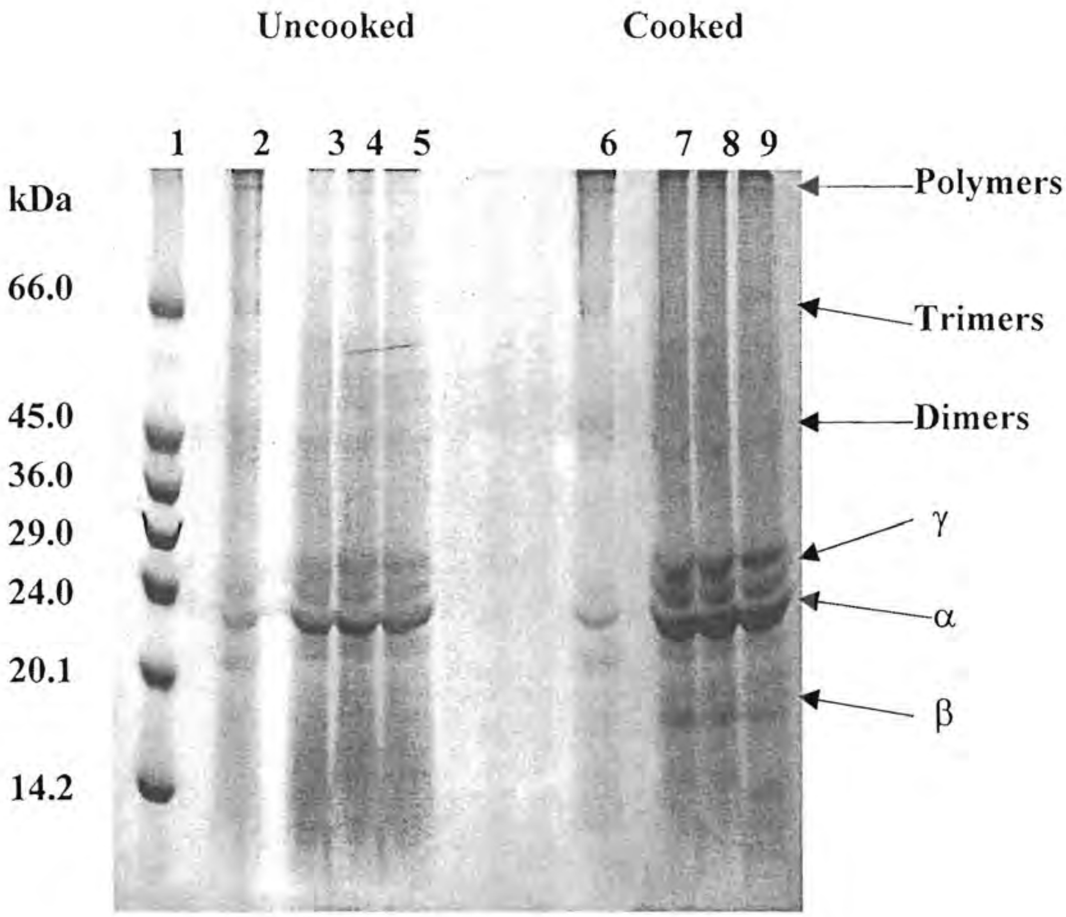
Figure 9: SDS-PAGE of uncooked and cooked protein body-enriched samples of sorghum (NK 283 and KAT 369), maize (PAN 6043) and sorghum mutant (P850029)



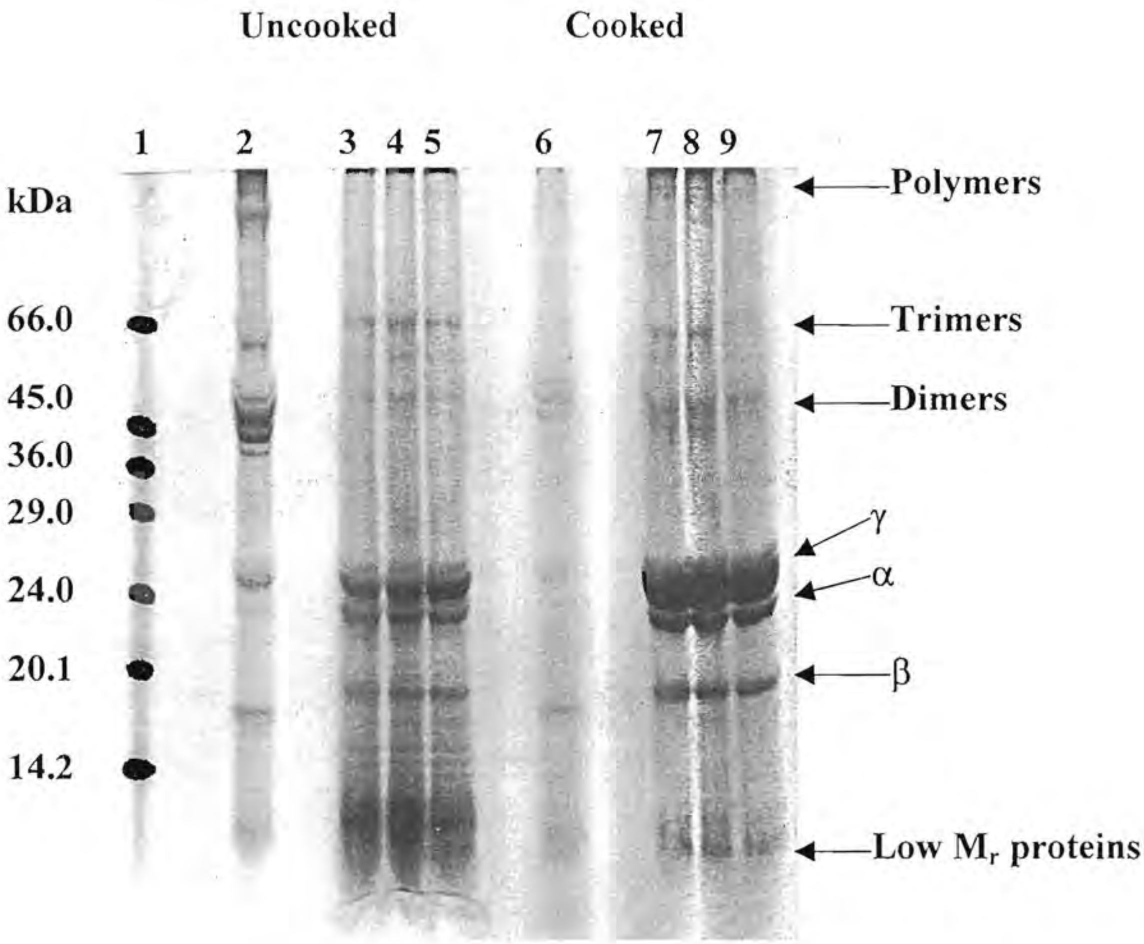
A) SDS-PAGE of uncooked and cooked protein body-enriched samples of sorghum (NK 283) under non-reducing and reducing conditions. Track 1, molecular weight standards; 2, uncooked, non-reduced; 3, uncooked, 50 mM DTT; 4, uncooked, 100 mM DTT; 5, uncooked, 200 mM DTT; 6, cooked, non-reduced; 7, cooked, 50 mM DTT; 8, cooked 100 mM DTT; 9, cooked, 200 mM DTT.



B) SDS-PAGE of uncooked and cooked protein body-enriched samples of sorghum (KAT 369) under non-reducing and reducing conditions. Track 1, molecular weight standards; 2, uncooked, non-reduced; 3, uncooked, 50 mM DTT; 4, uncooked, 100 mM DTT; 5, uncooked, 200 mM DTT; 6, cooked, non-reduced; 7, cooked, 50 mM DTT; 8, cooked 100 mM DTT; 9, cooked, 200 mM DTT.



C) SDS-PAGE of uncooked and cooked protein body-enriched samples of maize (PAN 6043) under non-reducing and reducing conditions. Track 1, molecular weight standards; 2, uncooked, non-reduced; 3, uncooked, 50 mM DTT; 4, uncooked, 100 mM DTT; 5, uncooked, 200 mM DTT; 6, cooked, non-reduced; 7, cooked, 50 mM DTT; 8, cooked 100 mM DTT; 9, cooked, 200 mM DTT.



D) SDS-PAGE of uncooked and cooked protein body-enriched samples of sorghum mutant (P850029) under non-reducing and reducing conditions. Track 1, molecular weight standards; 2, uncooked, non-reduced; 3, uncooked, 50 mM DTT; 4, uncooked, 100 mM DTT; 5, uncooked, 200 mM DTT; 6, cooked, non-reduced; 7, cooked, 50 mM DTT; 8, cooked 100 mM DTT; 9, cooked, 200 mM DTT.

The tracks of both uncooked and cooked samples under non-reducing and reducing conditions of the three sorghum varieties (NK 283, KAT 369 and P850029) were essentially identical. There was stained material at the origin of the gels of the three sorghums under both non-reducing and reducing conditions. Under non-reducing conditions, bands appeared in the 66 kDa and 45-50 kDa regions and at 24, 23, 22 and 18 kDa. Under reducing conditions, there were increases in the intensities of the bands at 24, 23, 22 and 18 kDa accompanied with decreases in intensities of the bands at 66 kDa and 45-50 kDa. For the three concentrations of reducing agent used, some bands in the region 45-96 kDa appeared to be resistant to reduction in both uncooked and cooked sorghum samples. However, there seemed to be more of these reduction-resistant bands in cooked than in uncooked sorghum samples especially in the 45-50 kDa region. Increasing the concentration of reducing agent (DTT) did not seem to decrease the intensity of persistent bands in the 45-50 kDa region. There were low molecular weight protein bands ($\leq$14 kDa) under reducing conditions in uncooked and cooked protein-body-enriched samples of the three sorghums. However these bands were fainter for the cooked samples than the uncooked.

For uncooked and cooked maize (PAN 6043) under non-reducing conditions, bands appeared in the 45-50 kDa and 66 kDa regions and also above 66 kDa, towards the origin of the gel. There were also bands at 25, 22 and 19 kDa. No bands appeared in the 14-16 kDa region. After reduction, the intensities of the bands between 19 and 25 kDa increased and bands appeared at 14 and 16 kDa in both uncooked and cooked maize protein body-enriched samples. As observed for the sorghums, bands in the region between 45 and 96 kDa persisted following reduction of both uncooked and cooked samples of maize protein-body-enriched samples.

Under non-reducing conditions, it appears the proportion of bands in the 45-50 kDa region were much lower for the maize than the three sorghums.

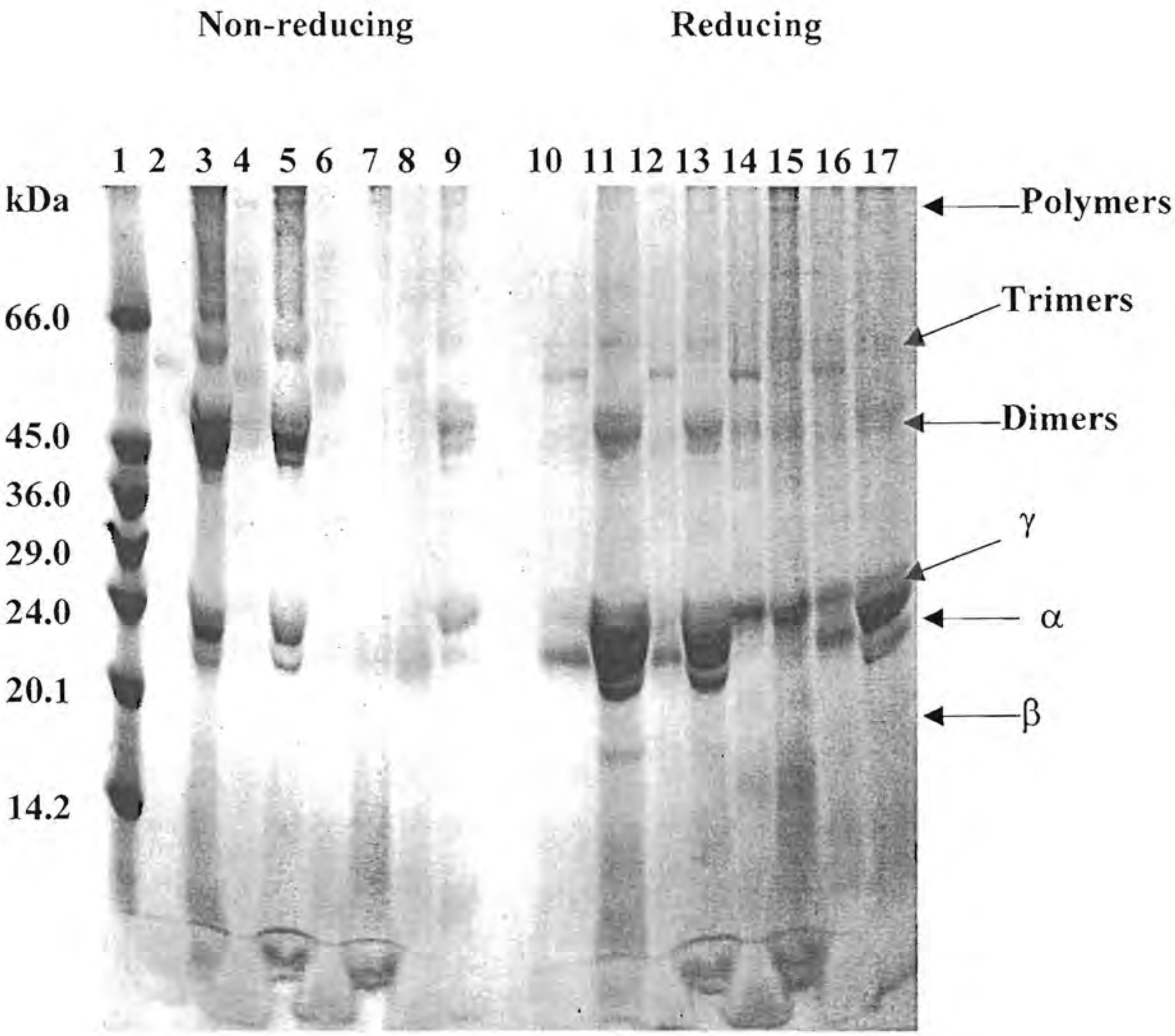


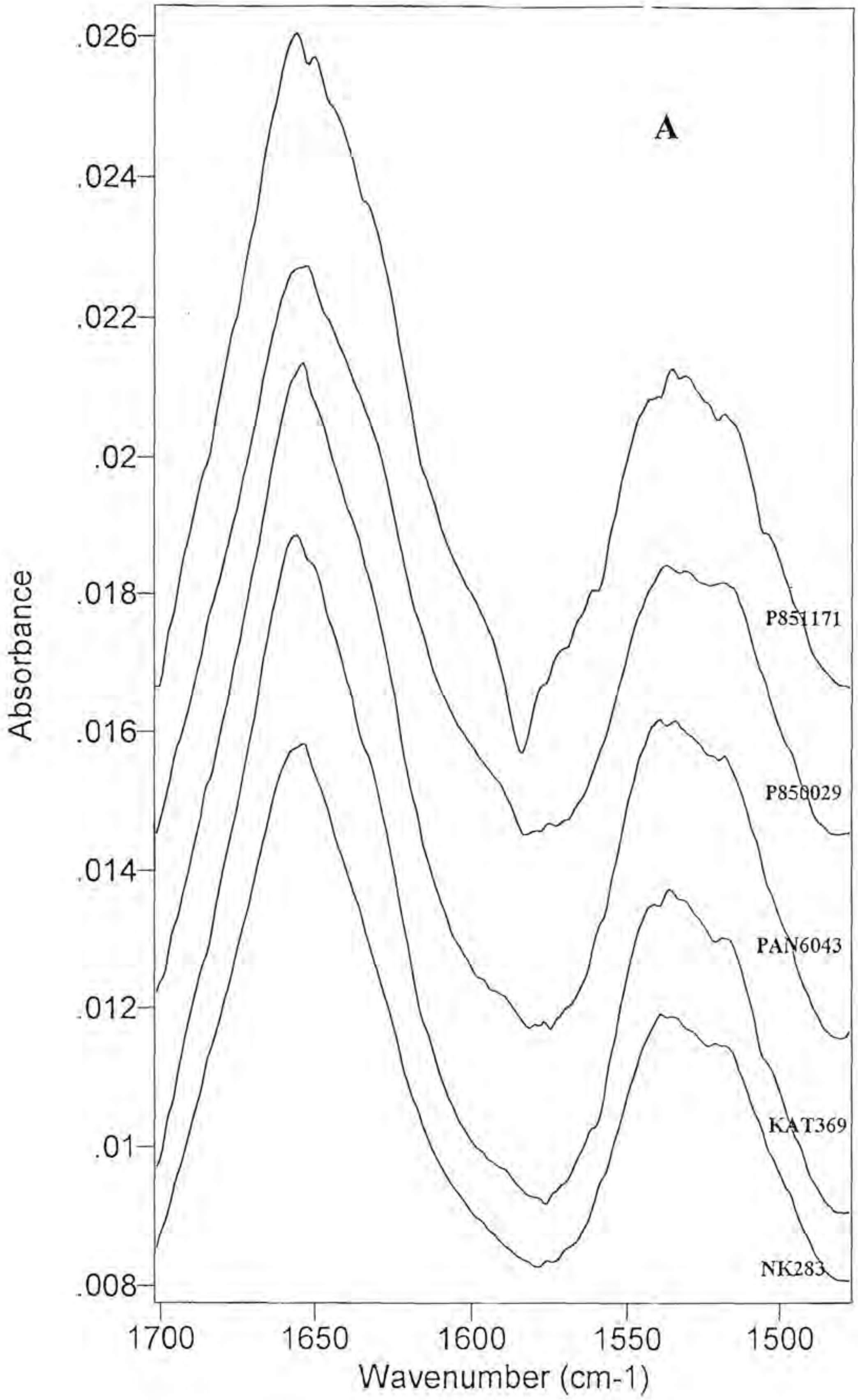
Figure 10. SDS-PAGE of pepsin-indigestible residues of sorghum (NK 283 and KAT 369), maize (PAN 6043) and sorghum mutant (P850029) protein body-enriched samples under non-reducing and reducing conditions (200 mM DTT).

1:- molecular weight markers
2:- uncooked NK 283, non-reduced
3:- cooked NK 283, non-reduced
4:- uncooked KAT 369, non-reduced
5:- cooked KAT 369, non-reduced
6:- uncooked PAN 6043, non-reduced
7:- cooked PAN 6043, non-reduced
8:- uncooked P850029, non-reduced
9:- cooked P850029, non-reduced
10:- uncooked NK 283, reduced
11:- cooked NK 283, reduced
12:- uncooked KAT 369, reduced
13:- cooked KAT 369, reduced
14:- uncooked PAN 6043, reduced
15:- cooked PAN 6043, reduced
16:- uncooked P850029, reduced
17:- cooked P850029, reduced

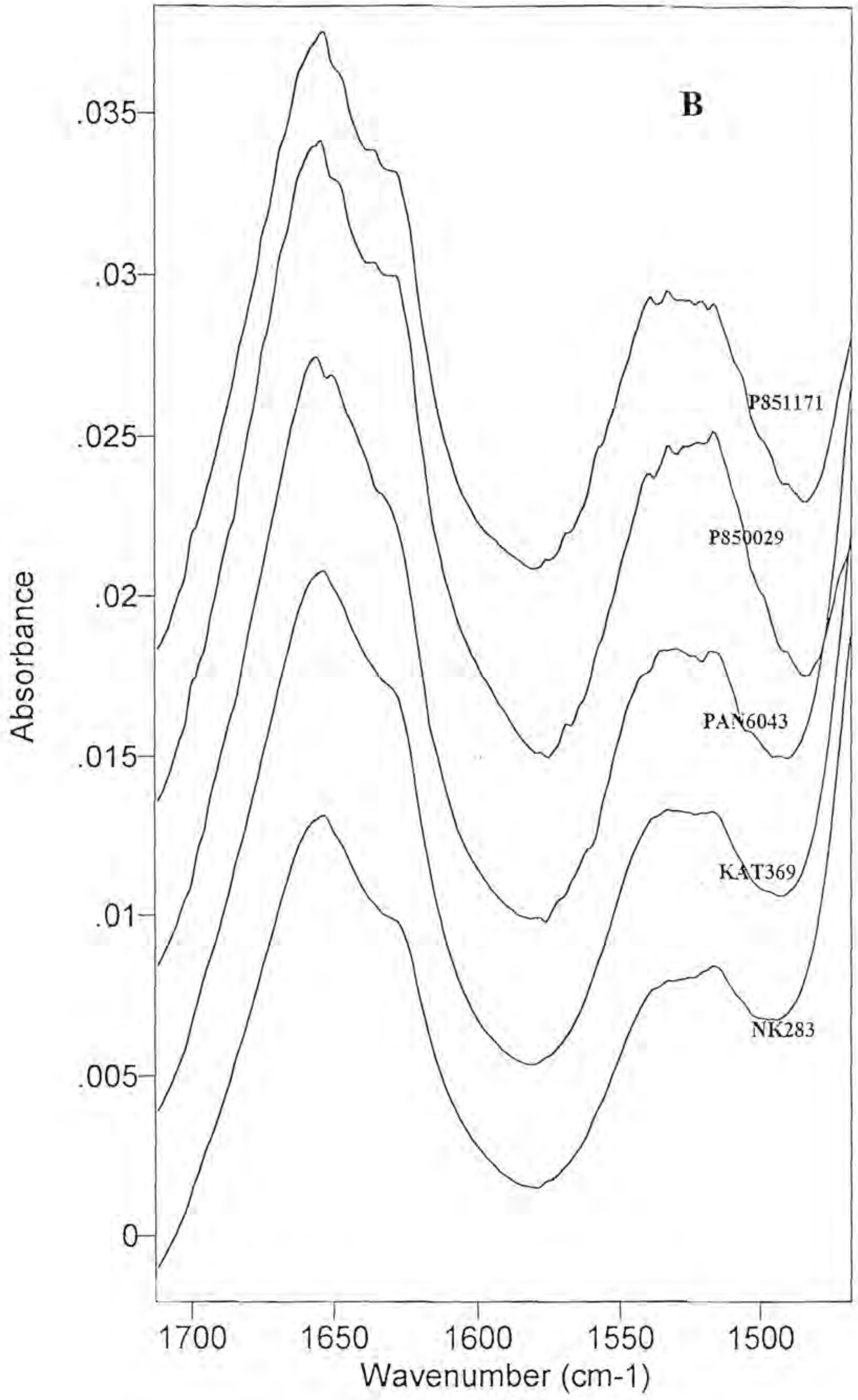
For the pepsin-indigestible sorghum and maize under non-reducing conditions, tracks for uncooked samples were very faint with bands barely visible at 60 kDa. The cooked, pepsin-indigestible sorghum samples under non-reducing conditions showed bands at the top of the gel (96 kDa), the 66 kDa region, 45-50 kDa and at 24, 23 and 22 kDa. These bands were much fainter for the sorghum mutant (P850029) compared to the normal sorghums. The cooked maize residue under non-reducing conditions showed a very faint band at 19 kDa. Under reducing conditions, there were increases in intensities of bands between 18 and 24 kDa for both uncooked and cooked sorghum and maize pepsin-indigestible residues. There were bands in the region 50-60 kDa which appeared to be resistant to reduction in uncooked and cooked sorghum and maize. These bands were fainter for uncooked samples, cooked maize and cooked sorghum mutant compared to the cooked normal sorghums.

4.6 FTIR and ^{13}C NMR spectroscopy of uncooked and cooked protein body-enriched samples of sorghum and maize

Figure 11: FTIR spectra of uncooked and cooked protein body-enriched samples of sorghum (NK 283 and KAT 369), maize (PAN 6043) and sorghum mutants (P851171 and P850029)



A) FTIR spectra of uncooked protein body-enriched samples of sorghum (NK 283 and KAT 369), maize (PAN 6043) and sorghum mutants (P851171 and P850029)



B) FTIR spectra of cooked protein body-enriched samples of sorghum (NK 283 and KAT 369), maize (PAN 6043) and sorghum mutants (P851171 and P850029)

In general, all samples (uncooked and cooked) gave similar spectra. There were two broad bands, one between 1675-1620 cm^{-1} with an absorption maximum at 1660 cm^{-1} and another between 1550-1500 cm^{-1}. For cooked samples, the 1675-1625 cm^{-1} band became broadened and had a shoulder at 1635 cm^{-1} in addition to the peak at 1660 cm^{-1} (Figure 11b). The shapes of the 1550-1500 cm^{-1} bands for all samples were altered on cooking with a peak forming at approximately 1520 cm^{-1} in all cases.

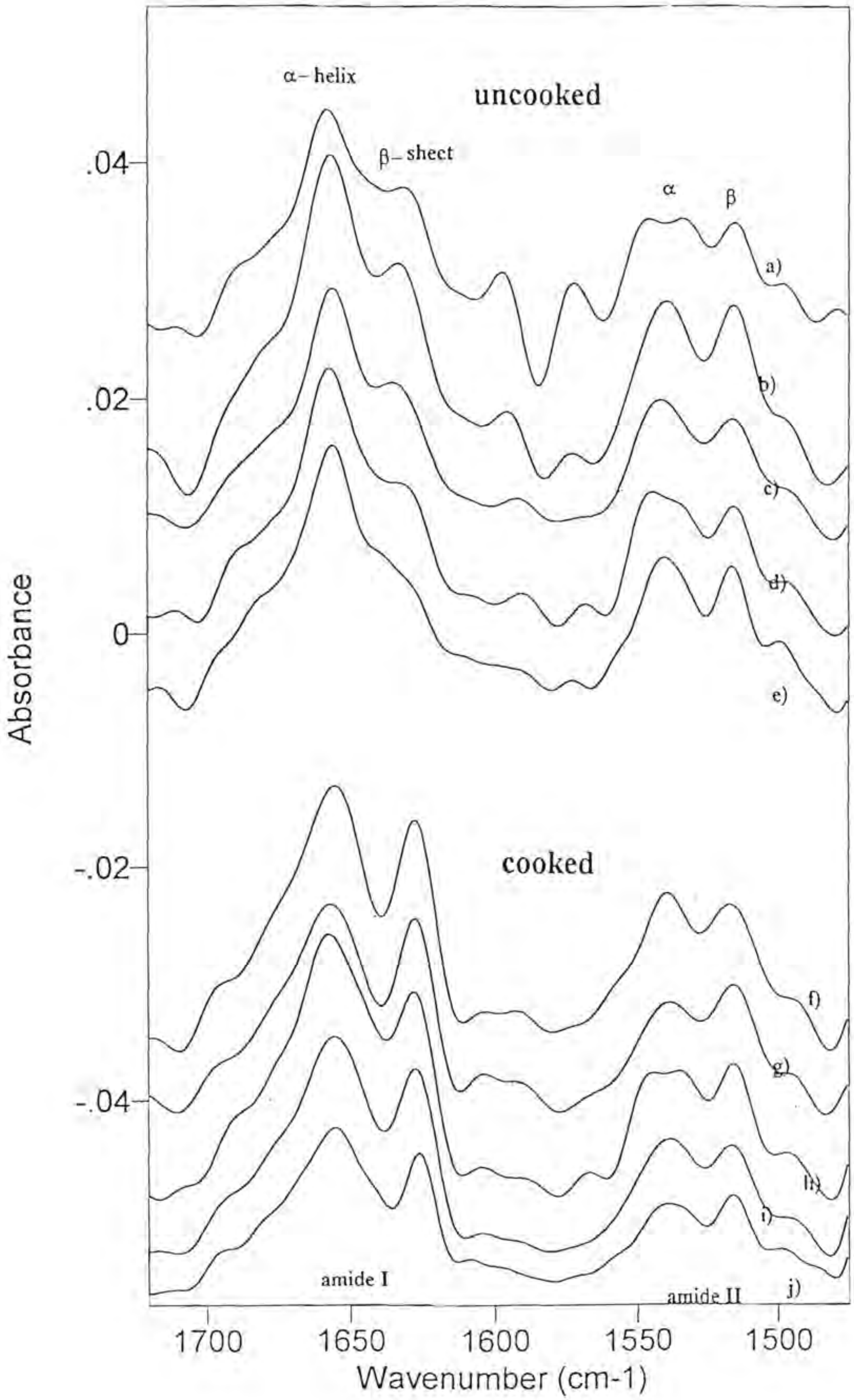
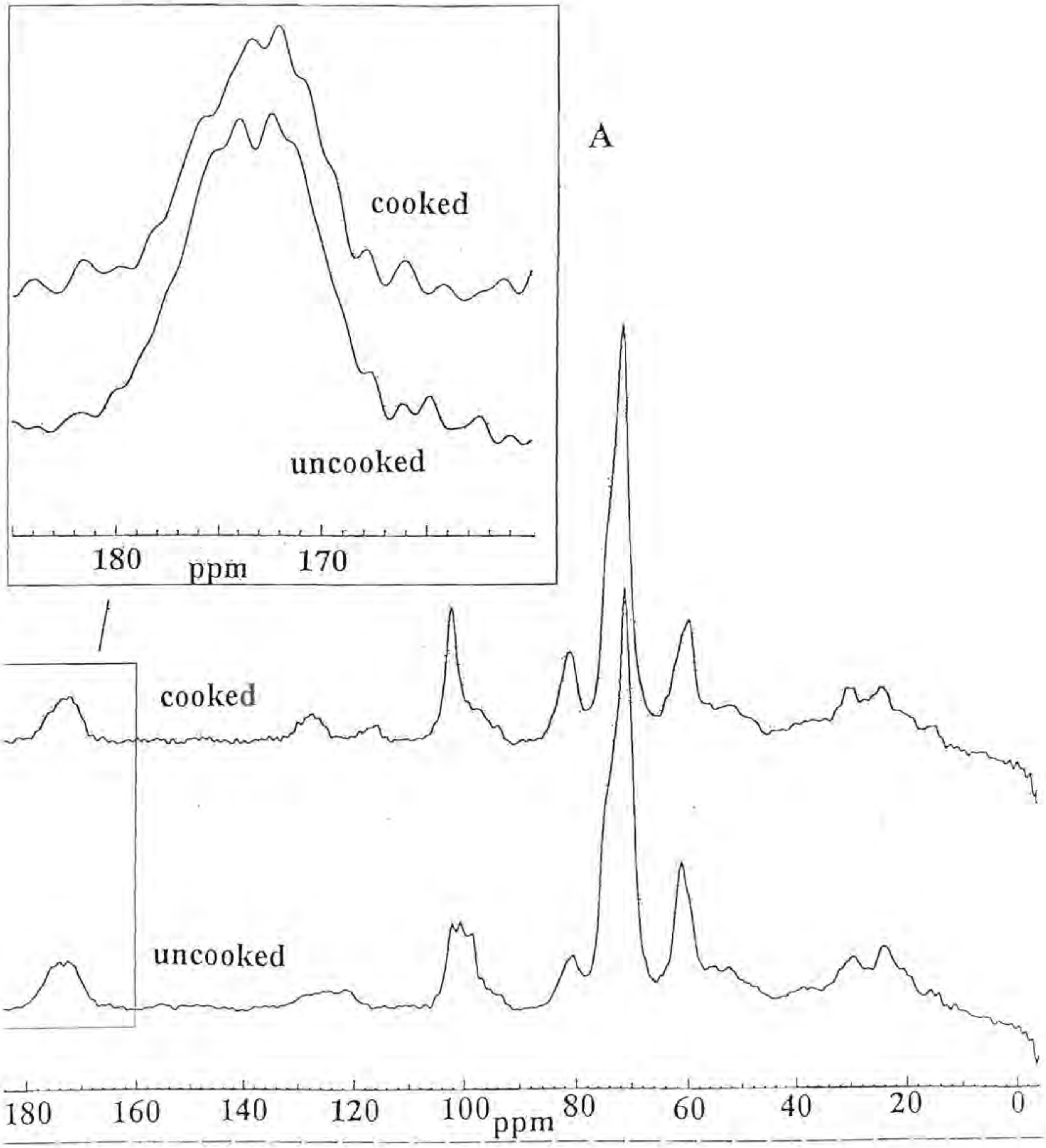


Figure 12: Fourier deconvoluted FTIR spectra of uncooked and cooked protein body-enriched samples of sorghum and maize varieties

a) uncooked P851171 sorghum mutant b) uncooked P850029 sorghum mutant c) uncooked PAN 6043 maize d) uncooked KAT 369 white sorghum e) uncooked NK 283 red sorghum f) wet cooked P851171 sorghum mutant g) wet cooked P850029 sorghum mutant h) wet cooked PAN 6043 maize i) wet cooked KAT 369 white sorghum j) wet cooked NK 283 red sorghum

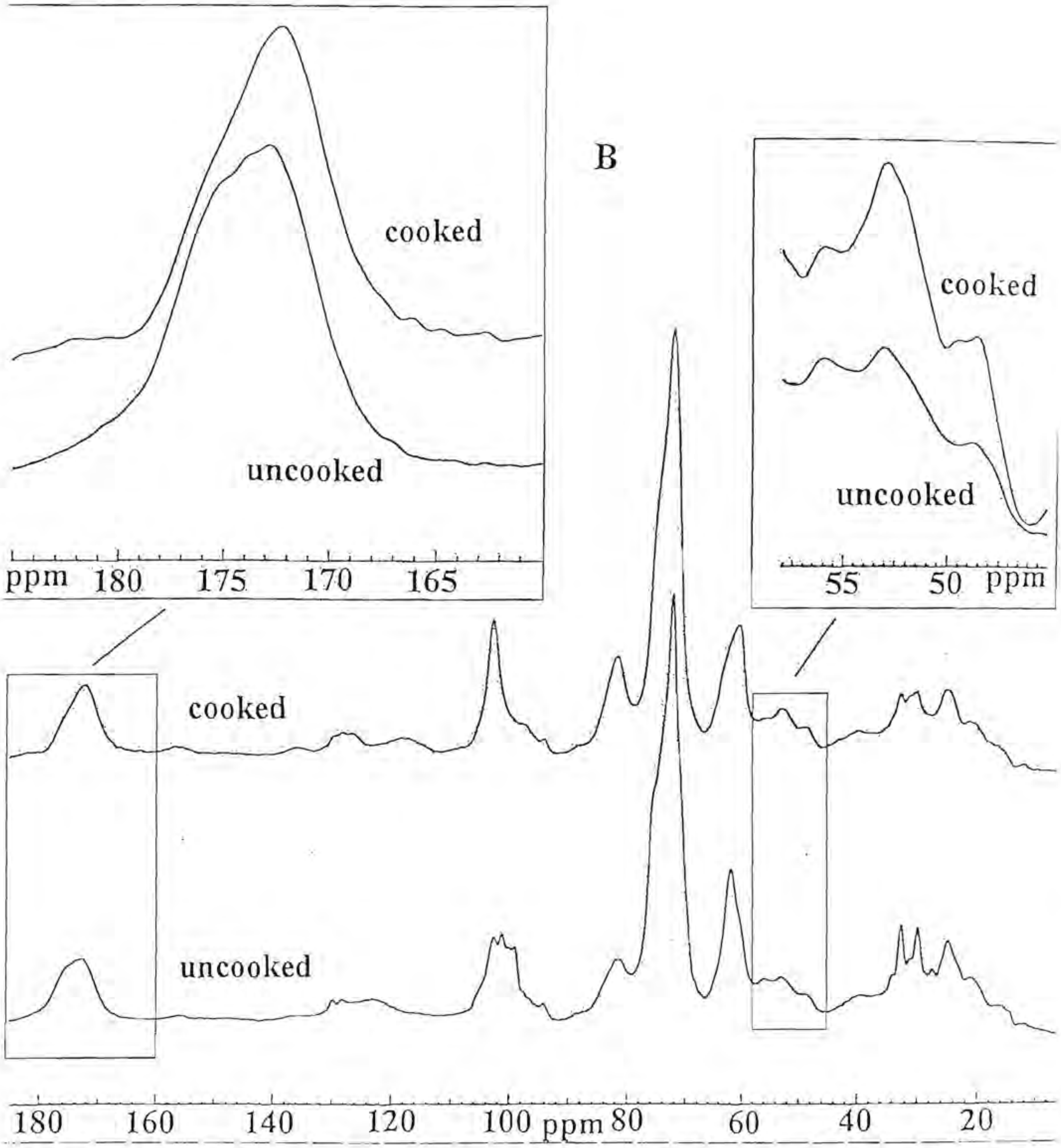
Fourier deconvolution brought about resolution enhancement of the bands at 1675-1625 cm^{-1} and 1550-1500 cm^{-1}. The spectra of all uncooked samples for all grain varieties were similar and so were the spectra of wet cooked samples. The band at 1675-1625 cm^{-1} had peaks at 1660 cm^{-1} and 1635 cm^{-1} for uncooked and wet cooked samples. Cooking increased the intensity of the 1635 cm^{-1} peak for all samples. The 1550-1500 cm^{-1} band produced peaks at 1540 cm^{-1} and 1520 cm^{-1} for both the uncooked and wet cooked samples. The 1540 cm^{-1} peak appeared to be split into two components at 1545 cm^{-1} and 1535 cm^{-1} for uncooked P851171 sorghum mutant, uncooked KAT 369 sorghum and cooked PAN 6043 maize. Wet cooked NK 283 sorghum, PAN 6043 maize and P850029 sorghum mutant appeared to have their absorption maxima for the 1550-1500 cm^{-1} band shifted towards the peak at 1520 cm^{-1}.

Figure 13: ^{13}C CPMAS NMR spectra of uncooked and wet cooked protein body-enriched samples of sorghum and maize

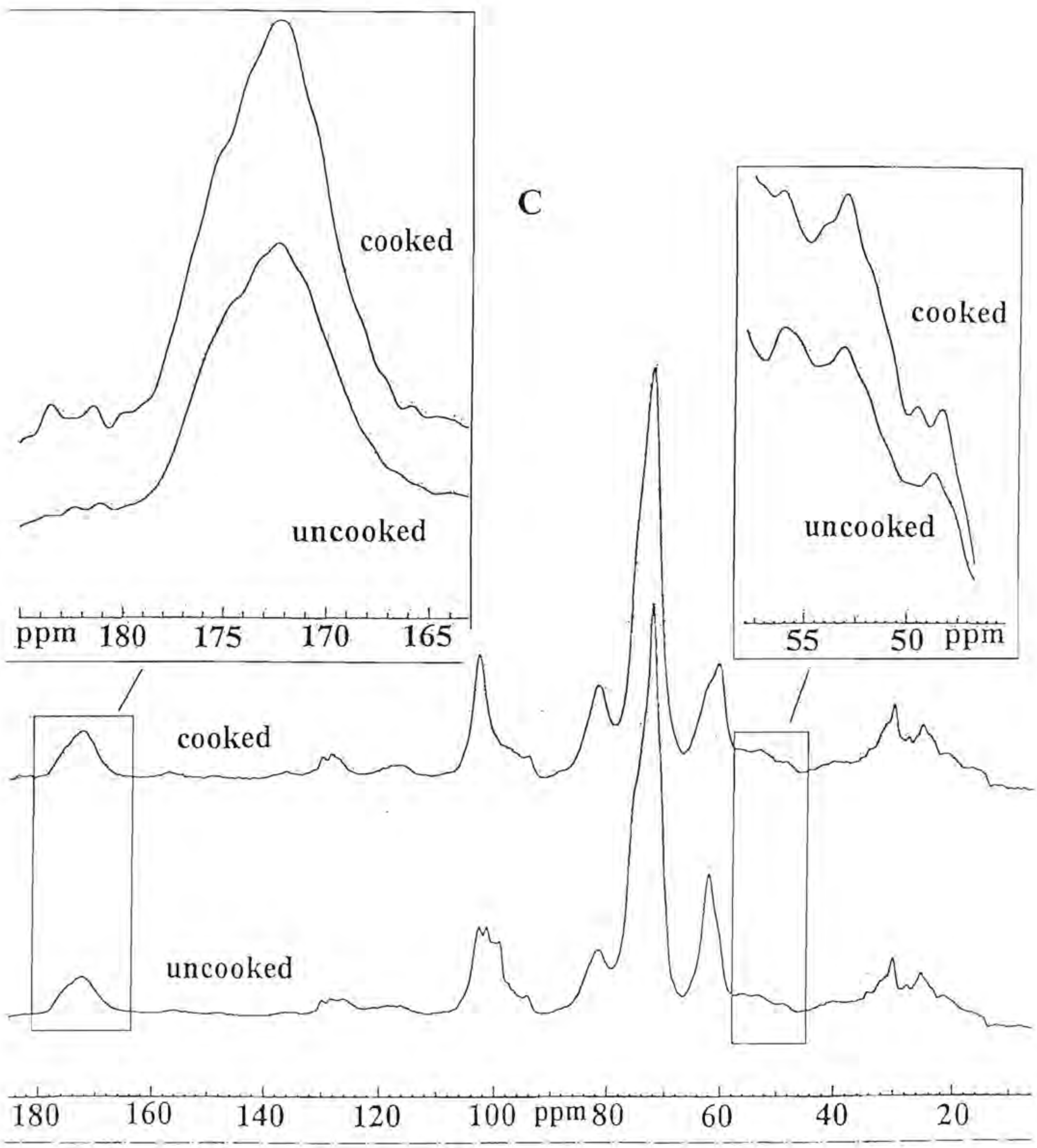


A) ^{13}C CPMAS NMR spectra of uncooked and wet cooked protein body-enriched samples of NK 283 sorghum

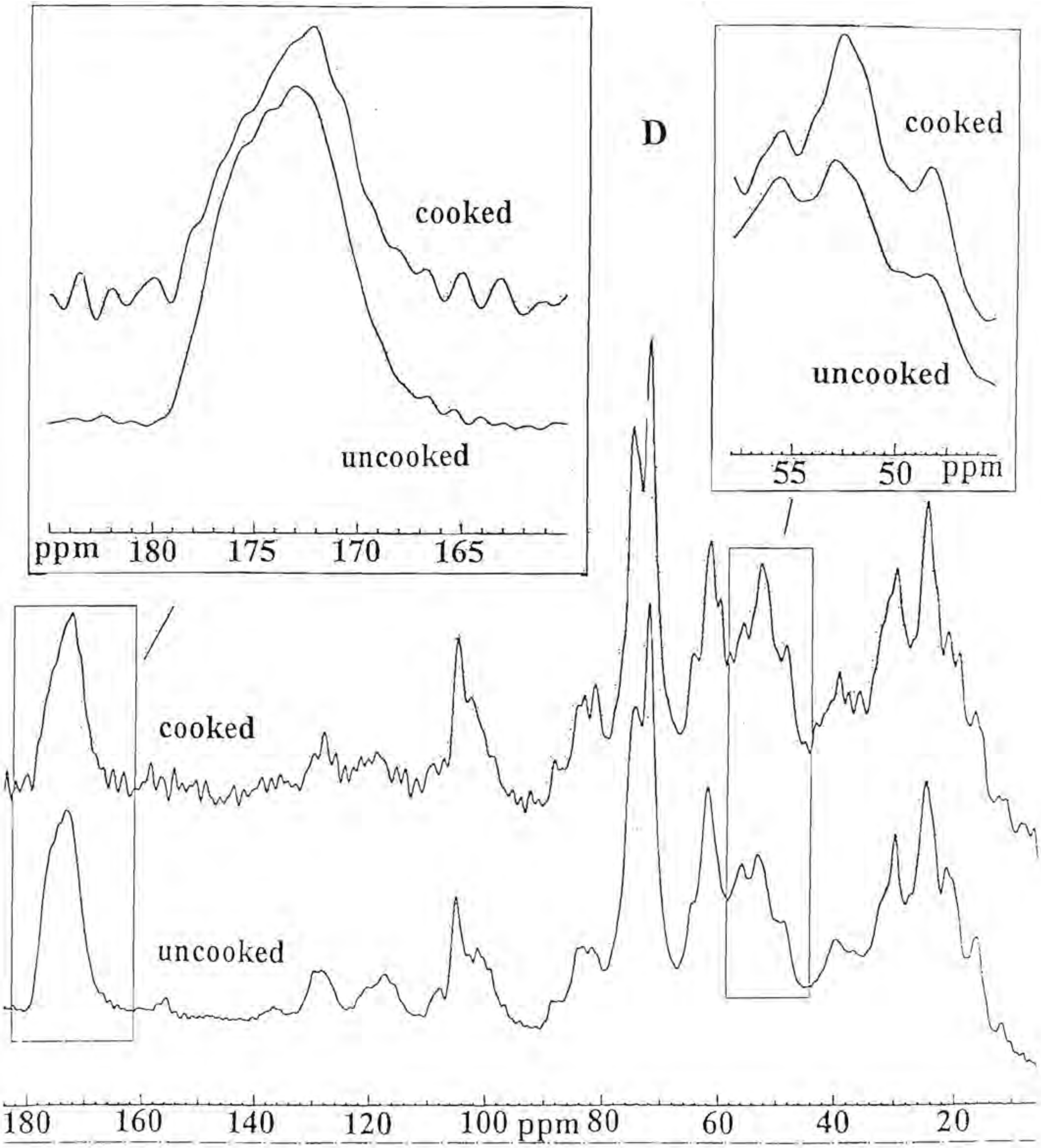
(NB: The 45-58 ppm region is not shown inset due to high signal-to-noise ratio)



B) ^{13}C CPMAS NMR spectra of uncooked and wet cooked protein body-enriched samples of KAT 369 sorghum



C) ^{13}C CPMAS NMR spectra of uncooked and wet cooked protein body-enriched samples of PAN 6043 maize



D) ^{13}C CPMAS NMR spectra of uncooked and wet cooked protein body-enriched samples of P850029 sorghum mutant

Peaks were obtained in the 20-58 ppm, 58-110 ppm, 120-130 ppm and 170-180 ppm regions of the spectra of all samples, uncooked and cooked. The effect of wet cooking on the spectra of the normal sorghums, maize and the highly digestible sorghum mutant appeared to be the same for all samples. There were alterations in shapes and intensities of the peaks in the above-named regions. In addition to changes in shape and intensity, peaks in the 45-58 ppm and 170-180 ppm regions were shifted upfield (towards the right or lower ppm) in all samples on wet cooking. This is illustrated in the insets of the spectra of the four samples.

4.7 *In vitro* protein digestibility and FTIR spectroscopy of popped sorghum and maize

Table 14. *In vitro* protein digestibility of popped NK 283 sorghum and PAN 6043 maize in comparison with uncooked and wet cooked whole grain

	NK 283	PAN 6043
Popped	41.3 a[1]	53.3 b
	± 0.5[2]	± 2.1
Uncooked	(59.1)[3]	(66.6)
Wet cooked	(30.5)	(62.0)

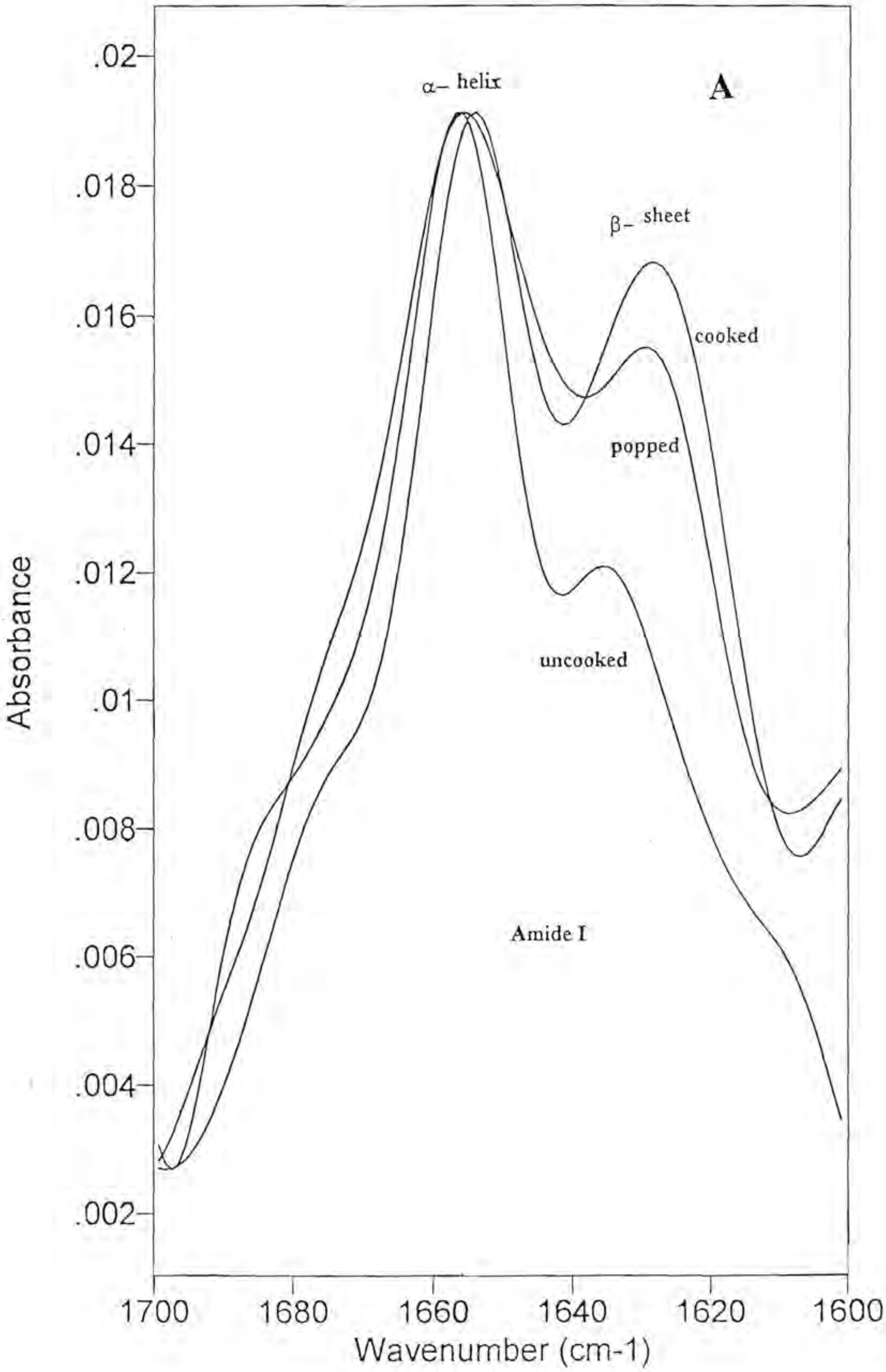
[1]Mean values with different letters in the same row are significantly different from each other ($p < 0.05$).

[2]Standard deviation.

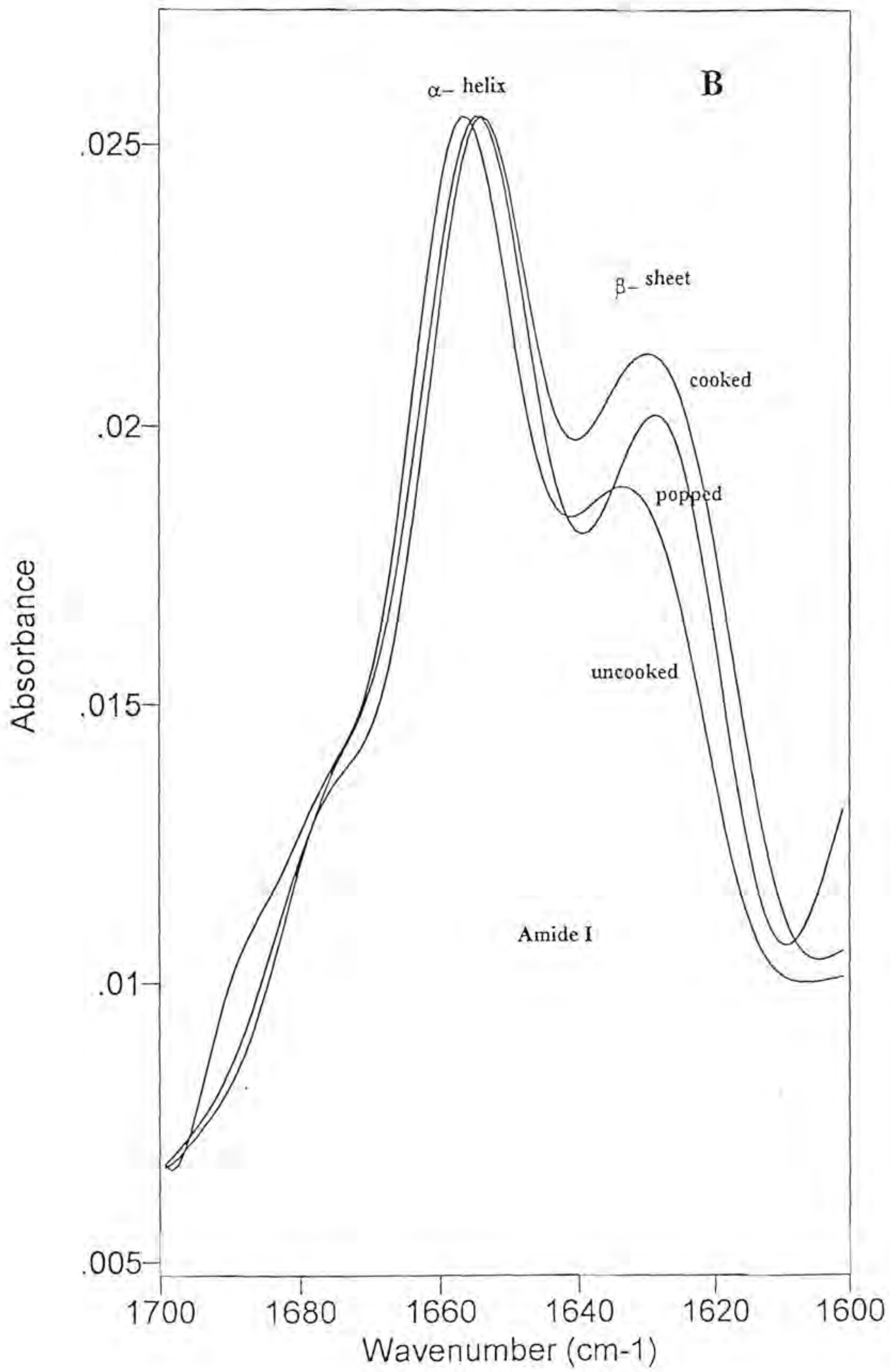
[3]Values in parenthesis from Table 3

Popped maize had higher protein digestibility compared to popped sorghum. Uncooked whole grain of both sorghum and maize were more digestible than the popped whole grain. Wet cooked sorghum was less digestible than popped. However, wet cooked maize was more digestible than popped.

Figure 14: Fourier deconvoluted FTIR spectra of uncooked, wet cooked and popped whole grain of sorghum and maize



A) Fourier deconvoluted FTIR spectra of uncooked, wet cooked and popped whole grain of NK 283 sorghum



B) Fourier deconvoluted FTIR spectra of uncooked, wet cooked and popped whole grain of PAN 6043 maize

Uncooked sorghum and maize samples produced two peaks at approximately 1655 cm^{-1} and 1635 cm^{-1}. Popping and wet cooking brought about shifts of these peaks towards lower wavenumbers in addition to increase in intensity of the peak at 1635 cm^{-1} for both sorghum and maize. There was a greater increase in intensity of the 1635 cm^{-1} peak of wet cooked sorghum and maize compared to popped sorghum and maize. The increase in intensity of the 1635 cm^{-1} peak on popping and on wet cooking was greater in sorghum than in maize

CHAPTER 5

DISCUSSION

It has been suggested that sorghum in the uncooked state has lower protein digestibility than uncooked maize (Hamaker *et al.*, 1986; Hamaker *et al.*, 1987; Oria *et al.*, 1995a). The slightly lower digestibility of the uncooked sorghums compared to uncooked maize at the whole grain level agrees with this suggestion. However, the fact that this investigation showed that at the endosperm level, protein digestibility of the uncooked sorghums was essentially the same as uncooked maize, whilst at the protein-body-enriched level, the two sorghums (uncooked), had higher protein digestibility than the maize shows that this is not always the case. Protein digestibility values of uncooked sorghum in the literature show a lot of variation. Marginally lower protein digestibilities for uncooked sorghum than uncooked maize have been reported by Hamaker *et al.* (1986) (80.7% for sorghum and 81.5% for maize) and Hamaker *et al.* (1987) (80.8% for sorghum and 83.4% for maize). Values as low as 65.8% for uncooked, decorticated sorghum (Weaver *et al.*, 1998) have been reported. Axtell *et al.* (1981) reported 92.9% protein digestibility for uncooked sorghum. One of the reasons for such variations is that different sorghum cultivars or varieties may show different protein digestibilities. Equally importantly, uncooked sorghum protein digestibility also depends on the nature of the material being assayed or the form in which it is. Protein digestibility assays have been conducted on either whole grain (Axtell *et al.*, 1981; Hamaker *et al.*, 1986; Hamaker *et al.*, 1987; Rom *et al.*, 1992; Oria *et al.*, 1995a), decorticated grain (Axtell *et al.*, 1981; Mertz *et al.*, 1984; Oria *et al.*, 1995b; Weaver *et al.*, 1998) or some undefined commercial grain fraction (Bookwalter, Kirleis & Mertz, 1987). These different types of grain material have differing proportions of pericarp, endosperm and germ. This investigation shows that protein digestibility of uncooked sorghum is improved as the proportion of pericarp and germ material becomes less. Similar results have been reported by Bradbury, Collins and Pyliotis (1984) who observed an improvement in uncooked rice protein digestibility from whole grain to endosperm.

The observed improvement of protein digestibility of uncooked and cooked sorghum with change in organisational level from whole grain to endosperm but not with maize suggests that certain factors that interfered with protein digestibility in sorghum were either not present in maize or present in lower quantities in maize compared to sorghum. Phenolic compounds are a possible candidate. The pericarp colour of sorghum has been attributed to flavonoid-type phenolic compounds (Hahn *et al.*, 1984) like the anthocyanin and anthocyanidin pigments which have been found in the pericarp of red sorghum (Nip & Burns, 1969) and white sorghum (Nip & Burns, 1971). The antinutritional effect of tannins in sorghum by formation of indigestible protein-tannin complexes *in vivo* (Armstrong *et al.*, 1973; Rostagno *et al.*, 1973; Armstrong *et al.*, 1974a) and *in vitro* (Armstrong *et al.*, 1974b; Schaffert *et al.*, 1974; Butler *et al.*, 1984) is well known. Though the red NK 283 sorghum hybrid and the white KAT 369 sorghum variety used in this investigation are condensed-tannin-free, a possible involvement of polyphenols (flavonoids and phenolic acids in this case) in lowering protein digestibility of uncooked and cooked whole grain sorghum may be inferred from the above results. If polyphenols do play a role in lowering protein digestibility, it would be expected that a reduction in polyphenol content would bring about an improvement in protein digestibility.

The maize cultivar PAN 6043 in contrast to the sorghums, had similar total polyphenol contents at whole grain flour, endosperm flour and protein-body-enriched levels. The protein digestibility of the uncooked maize at these three levels was essentially the same. The higher total polyphenol content of the red sorghum NK 283 whole grain compared to the white sorghum KAT 369 whole grain and the white maize whole grain was expected. Total polyphenol contents reported for sorghum and maize are 0.17-10.23% dry basis (sorghum) and 0.03% dry basis (maize) (reviewed by Bravo, 1998). Since grain pericarp colour is attributable to flavonoid-type phenolic compounds, the red sorghum variety would be expected to have a higher total polyphenol content. The decrease in total polyphenol content from whole grain to endosperm for the two sorghums is due to removal of the pericarp which is rich in phenolic compounds (Nip & Burns, 1969; Nip & Burns, 1971). The accompanying increase in protein digestibility of the uncooked and cooked sorghums from whole grain to endosperm may suggest possible involvement of polyphenols in lowering protein digestibility. This observation appears to be at odds with the current view regarding the absence of effect of the smaller molecular weight polyphenols, namely, phenolic acids and flavonoids on protein digestibility. Though these polyphenols have been reported to hinder iron absorption in the gastro-intestinal lumen (Brune, Rossander & Hallberg, 1989), they are not known to have any adverse effects on protein digestibility (reviewed by Serna-Saldivar & Rooney, 1995).

The sorghum protein-body-enriched samples had similar total polyphenol content to whole grain. These sorghum protein-body-enriched samples were coloured and this is a reflection of the ability of non-tannin polyphenols to bind to protein. In addition, the protein-body-enriched samples contained endosperm cell wall material (see Figure 8) which is a source of phenolic compounds such as ferulic acid (Hahn *et al.*, 1984). This increase in total polyphenols going from endosperm to protein-body-enriched samples did not bring about a decrease in protein digestibility of the uncooked and cooked sorghums at the protein-body-enriched level; an observation which does not support the hypothesis that the polyphenols of the sorghum varieties used here may be involved in reducing protein digestibility. From the observation that there is improvement in uncooked and cooked sorghum protein digestibility in going from whole grain to endosperm, a possible role of polyphenols of the condensed-tannin-free sorghums reducing protein digestibility at the whole grain level may not be discounted totally. According to Damodaran (1996), polyphenols in several plant proteins may be oxidised to quinones by molecular oxygen at neutral to alkaline pH. The quinones may go on to form peroxides which are highly reactive oxidising agents and could bring about oxidation of several amino acid residues and polymerisation of proteins. Polyphenols may influence sorghum protein digestibility at the endosperm level but not as phenolic acids or flavonoids. An alternative mechanism may be through protein crosslinking with ferulic acid in endosperm cell walls. This may explain why the uncooked and cooked sorghum protein body-enriched samples, which have a lower proportion of cell wall material, have better digestibility than the endosperm samples.

Condensed tannins inhibit enzymes (Daiber, 1975). However, the antinutritional effect of sorghum condensed tannins is believed to lie in their ability to form complexes with dietary protein rather than inhibition of digestive enzymes (Butler *et al.*, 1984). These authors argued that grinding, cooking or other processing of high-tannin sorghum enhances the opportunity for interaction of tannin with dietary protein before it encounters digestive enzymes *in vivo*. Nevertheless, the likelihood of enzyme inhibition in the *in vitro* system used in this investigation contributing to reduction in sorghum protein digestibility was investigated. The high-tannin sorghum very substantially inhibited amylase activity while the condensed-tannin-free sorghums and the maize had a negligible effect on amylase activity. It may be concluded therefore, that the polyphenols of the condensed-tannin-free sorghums and the maize are not enzyme inhibitory. Therefore if polyphenols are involved in reducing protein digestibility, the mechanism would likely be through interaction of these polyphenols with substrate protein and not with the pepsin enzyme.

The reduction in protein digestibility on cooking for both sorghum varieties but not to any great extent with maize (and other cereals) is an effect well documented in literature (Axtell *et al.*, 1981; Mertz *et al.*, 1984; Hamaker *et al.*, 1986; Hamaker *et al.*, 1987). This has been shown to occur for whole grain (Axtell *et al.*, 1981; Hamaker *et al.*, 1986; Hamaker *et al.*, 1987; Rom *et al.*, 1992; Oria *et al.*, 1995a) and decorticated grain (Axtell *et al.*, 1981; Mertz *et al.*, 1984; Bookwalter *et al.*, 1987; Oria *et al.*, 1995b; Weaver *et al.*, 1998). This investigation shows that cooking reduces sorghum protein digestibility at the endosperm and protein-body-enriched levels and at the level of the extracted proteins (Table 13). Considering uncooked and cooked samples together, the overall improvement in protein digestibility of the two sorghums from whole grain, through endosperm to protein-body-enriched samples is an indication that grain organisational structure has an effect on protein digestibility. Removal of the outer layers of the grain, namely the pericarp and germ improved protein digestibility in the sorghum grain but not the maize. This indicates interaction between sorghum protein and pericarp and germ components which leads to a reduction in protein digestibility.

Dietary fibre refers to the polysaccharide fraction of plant foods, particularly cereals, that are not digested by the human alimentary tract (Johnson & Southgate, 1994). In cereals, this fraction is derived from the pericarp and endosperm cell walls with the major constituents being cellulose and non-cellulosic polysaccharides mainly, heteroxylans and variable amounts of β-glucans (Johnson & Southgate, 1994; Verbruggen, 1996). The binding of protein to these non-starch polysaccharides is believed to be one of the factors which impairs protein digestion (Cheftel *et al*, 1985). Various workers have reported results that show an association between protein and pericarp or endosperm cell walls (Gram, 1982; Glennie, 1984; Bach Knudsen & Munck, 1985), the latter two with sorghum. It is possible that such an association could lower protein digestibility either by reduction of access to enzymes or formation of indigestible complexes. Studies conducted by Gram (1982) on germinating barley seeds showed that some endosperm cells of ungerminated and germinated seeds appeared to be identical. They both contained intact cell walls and storage protein, indicating that the cell walls constituted a barrier to proteases. Within sorghum (Shull *et al.*, 1990) and maize (Khoo & Wolf, 1970) endosperm, starch granules and protein bodies are surrounded by cell walls. Glennie (1984) observed that isolated sorghum endosperm cell walls had 46% protein associated with them. Bach Knudsen and Munck (1985) found significant amounts of protein associated with total dietary fibre and acid detergent fibre fractions in uncooked and cooked sorghum. The amino acid composition of the sorghum proteins associated with acid detergent fibre resembled that of kafirins. Choct and Annison (1992) have reported that addition of wheat pentosans (arabinoxylans) to the diets of chicken broilers decreased protein digestibility by 18.7% in the ileum. In legumes, it has been shown that cell walls represent a physical barrier to protein digestion, thus limiting protein digestibility (Melito & Tovar, 1995).

The nature of this protein-cell wall adhesion is not very clear. The cell wall itself has been described as a biphasic structure, consisting of a rigid skeleton of cellulose microfibrils held together by a gel-like matrix (reviewed by Fry, 1986). The matrix is very complex in chemical terms, and is built up of noncellulosic polysaccharides (like the arabinoxylans), glycoproteins and phenolic compounds (Brett & Waldron, 1990). Saulnier and Thibault (1999) have proposed a model for the cell walls of maize bran in which highly crosslinked heteroxylans (of the cell wall matrix) constitute a network in which cellulose microfibrils may be embedded (Figure 15). Endosperm cell walls would be composed of the heteroxylans crosslinked with ferulic acid and glycoproteins. Crosslinks within the cell wall are important in maintaining its integrity. After extraction from the wall, most of the matrix polysaccharides are soluble, being polyhydroxy, hydrophilic molecules. However, within the cell wall, they are water-insoluble and it makes the wall matrix very coherent, a characteristic feature of the cell wall, which may be attributed to the existence of crosslinks between the cell wall polymers (reviewed by Fry, 1986; Brett & Waldron, 1990). These crosslinks may be of a non-covalent or covalent nature. Hydrogen bonds between heteroxylans and cellulose microfibrils and ionic bonds between positively charged glycoproteins and negatively charged wall polysaccharides are two examples of non-covalent crosslinks within the cell wall (Fry, 1988).

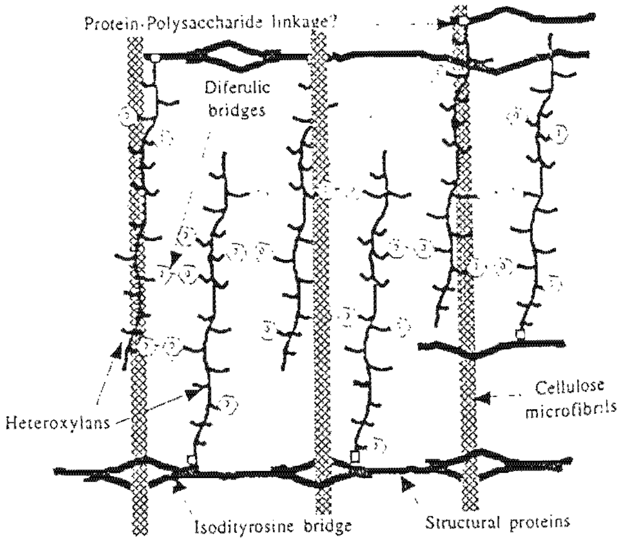
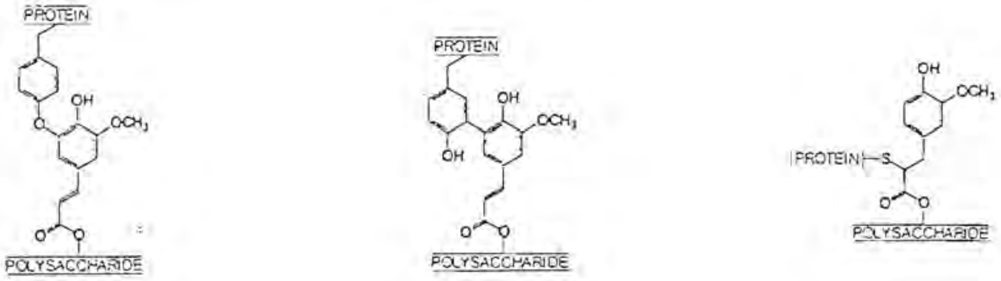


Figure 15: Proposed model for maize bran cell walls. (Saulnier & Thibault, 1999).

Phenolic acids have been identified as being involved in covalent crosslinks within the cell wall. Cell wall arabinoxylans of the Poaceae may be esterified with phenolic acids such as *p*-coumaric and ferulic acid (Nishitani & Nevins, 1989; Saulnier, Vigouroux & Thibault, 1995; Ng, Greenshields & Waldron, 1997). Ferulic acid has the ability to couple oxidatively to another ferulic acid of an arabinoxylan to form a diferulate crosslink. The formation of phenolic crosslinks is dependent on the synthesis of the phenol-bearing arabinoxylan, the presence of peroxidase enzyme and a supply of hydrogen peroxide or an equivalent oxidising agent (Fry, 1988). Therefore it may be assumed that in general, oxidising conditions could promote formation of phenolic or specifically, ferulic crosslinks. Hypothetically, phenolics-mediated crosslinking of proteins within the cell wall is considered possible. It has been suggested that dimerisation may occur between tyrosine residues in proteins and ferulic acid residues on arabinoxylans (Bacic, Harris & Stone, 1988). From a study of oxidative gelation of wheat flour pentosans, Hoseney and Faubion (1981) suggested that the esterified ferulic acid may be crosslinked to the sulphydryl group of cysteine residues in proteins (see Figure 16).

It could be hypothesised therefore, that the cooking process, which is conducted in the presence of oxygen, could lead to the formation of such ferulic acid crosslinks between proteins and arabinoxylans and in so doing, bring about adhesion between proteins and the cell wall. Removing the outer layers of the grain would reduce the amount of cell wall material and hence, less protein-cell wall adhesion and improved protein digestibility. Van Sumere, De Pooter, Ali and Degrauw-Van Bussel (1973) have suggested an alternative mode of ferulic acid crosslinking in which the carboxylic acid group of the phenolic is attached to the amino group of the N-terminal amino acid of a polypeptide forming a so-called pseudopeptide bond.



A B C

Figure 16: Structures of proposed covalent crosslinks between polysaccharides and proteins in cell walls. A) and B) Tyrosyl-feruloyl crosslinks (Bacic *et al.*, 1988); C) Feruloyl-sulphydryl crosslink (Hoseney & Faubion, 1981).

Another mode of protein-cell wall adhesion could be by direct attachment of the protein to carbohydrate moieties. Cell walls are known to contain a variety of different proteins and most of these are glycosylated (Brett & Waldron, 1990). The best characterised are the structural cell wall glycoproteins known as hydroxyproline-rich glycoproteins (HRGPs) or extensins. Their presence in sorghum (Raz, Crétin, Puigdomènech & Martínez-Izquierdo, 1991) and maize (Kieliszewski & Lamport, 1987; Hood, Shen & Varner, 1988; Hood, Hood & Fritz, 1991) have been reported. The amino acid compositions of sorghum and maize HRGPs are similar; rich in hydroxyproline, proline, lysine, tyrosine and threonine (Kieliszewski, Leykam & Lamport, 1990; Raz *et al.*, 1991). The hydroxyproline residues normally serve as attachment points for arabinose oligosaccharides (Figure 17) (Kieliszewski & Lamport, 1987; Kieliszewski, Kamyab, Leykam & Lamport, 1992). The polypeptide-carbohydrate linkage is thought to be an O-glycosidic bond in which the reducing terminus of the carbohydrate is attached to an –OH group on the polypeptide (Fry, 1988). These structural glycoproteins are highly resistant to most proteases especially when the oligoarabinose side chains are still attached. By analogy, it may be suggested that such enzyme-resistant protein-carbohydrate linkages may be formed in sorghum and maize on cooking through formation of O-glycosidic bonds between proline residues of sorghum and maize proteins and the arabinose residues of the cell wall. Such an event is made more likely by the fact that the kafirins of sorghum and the zeins of maize are rich in proline (Evans, Schüssler & Taylor, 1987).

Though normal sorghum and maize both contain pericarp and endosperm cell walls, it appears that the protein-cell wall adhesion is stronger and occurs to a greater extent in sorghum than in maize. Bach Knudsen and Munck (1985) observed that higher amounts of protein were associated with dietary fibre fractions of cooked sorghum than other cereals like wheat, rye, barley and maize. This might explain the superior protein digestibility of cooked maize compared to cooked sorghum.

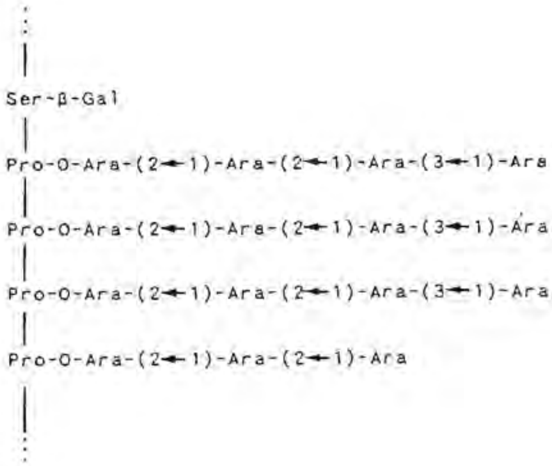


Figure 17: Structure of the hydroxyproline-rich region of plant cell wall glycoproteins. (Brett & Waldron, 1990).

Treatment of the cooked sorghum and maize whole grain and endosperm flours with *alpha*-amylase improved protein digestibility. This indicates that gelatinised starch reduces protein digestibility, a finding supported by the fact that in the protein-body-enriched samples, where the proportion of starch was much lower, there were no differences in protein digestibility between cooked and cooked plus *alpha*-amylase treatments. *Alpha*-amylase breaks α-1,4 glycosidic bonds of starch at random, forming glucose and dextrins leading to a reduction in viscosity (Belitz & Grosch, 1999). This would allow the pepsin greater access to the protein in the cooked grain. However, Oria *et al.*, (1995b) found that the protein digestibility of decorticated sorghum flour cooked with heat-stable *alpha*-amylase was approximately the same as that cooked without, which appears to contradict the findings of this investigation. The mode of treatment with *alpha*-amylase was different in the present work as samples were treated with *alpha*-amylase after cooking, before pepsin digestion. Protein appears to have an effect on starch gelatinisation and digestibility in sorghum. Chandrashekar and Kirleis (1988) observed that sorghum grains with lower capacities for starch gelatinisation had more kafirin-containing protein bodies. Addition of 2-mercaptoethanol to cooking media increased degree of rice starch gelatinisation (Hamaker & Griffin, 1993). Furthermore, Zhang and Hamaker (1998) reported that treating sorghum flour with pepsin before cooking led to an increase in starch digestibility. It would be expected therefore, that the presence of starch could in turn influence protein digestibility. Improvement of protein digestibility of sorghum and maize porridge after treatment with *alpha*-amylase has important nutritional implications. This scenario is similar to the order of events in the *in vivo* situation where food is first attacked by salivary amylase in the mouth before digestion with pepsin in the stomach and with other proteases in the duodenum.

From the foregoing, it appears that polyphenols may affect protein digestibility of uncooked and cooked sorghum in going from whole grain to endosperm. Protein-cell wall adhesion affects protein digestibility of cooked sorghum, whilst gelatinised starch reduces accessibility of enzymes to protein in sorghum and maize thus limiting protein digestibility. However, unlike maize, the protein digestibility of cooked sorghum remained far lower than uncooked at the protein-body-enriched level where other components like cell walls and starch occurred in much lower proportions. This suggests that changes occur in the proteins themselves on cooking which makes them less digestible. It has been proposed that higher molecular weight, disulphide-bonded protein polymers are formed in sorghum (Hamaker *et al.*, 1986) and maize (Batterman-Azcona & Hamaker, 1998) on wet cooking. Such an event would indicate an alteration in protein secondary structure as a result of the wet cooking process.

The disulphide crosslinking hypothesis is supported by the observations in this investigation from SDS-PAGE. For uncooked and cooked protein-body-enriched samples of sorghum and maize, large amounts of stained material with extremely low mobility at the origin of gels run under non-reducing conditions indicate the existence of substantial amounts of high molecular weight oligomers (Figure 9). For the sorghums, (uncooked and cooked under non-reducing and reducing conditions; Figure 9A, B and D), bands appearing at approximately 24, 23, 22 and 18 kDa are identified as the monomeric γ-, α1-, α2- and β-kafirins respectively (Shull *et* *al*., 1991). For maize (Figure 9C), the band at 25 kDa is identified as γ-zein and those at 22 and 19 kDa as α-zein (Esen, 1987). The β-zeins (14-16 kDa) appeared only under reducing conditions, an indication that they are involved in disulphide crosslinking in maize. The appearance of monomeric kafirins and zeins in uncooked sorghum and maize under non-reducing conditions indicates that some of the prolamins exist as monomers in the sorghum and maize seed, a similar finding to that observed by El Nour *et al*. (1998) in sorghum. Under reducing conditions, the increases in monomer band intensities for both uncooked and cooked protein-body-enriched samples of sorghum and maize accompanied with decreases in oligomer bands (≥ 45 kDa) was expected. This is as a result of oligomers comprising of disulphide-linked monomeric units of the α-, β- and γ-kafirins or zeins being separated into their constituent monomers (El Nour *et al*., 1998).

The appearance of bands in the 45-50 kDa and 66 kDa regions and also above 66 kDa, towards the origin of the gels indicate the presence of high molecular weight protein oligomers in uncooked and cooked sorghum and maize protein-body-enriched samples. Such oligomeric proteins in the 44 kDa to 97 kDa region have been found in maize (Landry, Paulis & Wall, 1987) and sorghum (El Nour *et al*., 1998) and have been designated dimers (45 kDa), trimers (66 kDa) and polymers (97 kDa) (Landry *et al*., 1987; El Nour *et al*., 1998).

The observation that for SDS-PAGE of the pepsin-indigestible residues (Figure 10) under reducing conditions, uncooked tracks were much fainter than cooked is an indication that though some disulphide-bonded oligomers are present in the uncooked samples, more of such oligomeric protein species are formed on cooking in both sorghum and maize. This is in agreement with the proposal that on cooking or thermal processing, higher molecular weight, disulphide-bonded protein polymers are formed in sorghum (Hamaker *et al*., 1986), wheat (Ummadi *et al*., 1995), rice (Mujoo *et al*., 1998) and maize (Batterman-Azcona & Hamaker, 1998). On electrophoretic examination of proteins in Landry-Moureaux fractions 4 (extracted with pH 10 buffer and 2-mercaptoethanol) and 5 (extracted with pH 10 buffer, 2-mercaptoethanol and SDS) of pepsin-indigestible residue of cooked sorghum, Hamaker *et al*. (1986) reported the presence of monomeric kafirins in these fractions. This is confirmed by the observation in this investigation that the cooked pepsin-indigestible residues consisted of monomeric kafirins and zeins on reduction.

Concerning α-, β- and γ-kafirins and sorghum protein bodies, Oria *et al.*, (1995b) have proposed that during cooking of sorghum, enzymatically-resistant protein polymers are formed by disulphide crosslinking of the β- and γ-kafirins which are located at the periphery of the protein body. Such crosslinking would restrict digestion of the more centrally located α-kafirin. The absence of β-kafirin from the uncooked sorghum pepsin-indigestible residue (Figure 10, track 10) and its appearance in the cooked sample after reduction (Figure 10, track 11) indicates that it was involved in disulphide cross-linking during the cooking process. The reduction of rice protein digestibility on cooking has also been attributed to formation of enzyme-resistant, disulphide-bonded protein polymers which in contrast to sorghum, are believed to occupy the core of rice protein bodies (Resurreccion, Li, Okita & Juliano, 1993). However this hypothesis has been challenged by Barber, Lott and Yang (1998) who found no apparent concentration of sulphated prolamin polypeptides in the central region of rice protein bodies in faecal protein particles.

The observation that sorghum (uncooked and cooked) contains more oligomers in the 45-50 kDa region than maize might indicate that the extent of oligomer formation differs in the two cereals in both the uncooked and cooked states. It appears that in uncooked sorghum, the degree of crosslinking is much more than in maize. Various workers have reported results which indicate that in uncooked sorghum, the more crosslinked proteins of Landry-Moureaux fraction 3 (extracted with alcohol and reducing agent) occur in higher quantities than fraction 2 (extracted with alcohol) (Jambunathan & Mertz, 1973; Guiragossian *et al.*, 1978; Hamaker *et al.*, 1986; Vivas *et al.*, 1992; Hamaker *et al.*, 1994), whilst the opposite is the case for maize (Landry & Moureaux, 1980; Hamaker *et al.*, 1986; Vivas *et al.*, 1992; Hamaker *et al.*, 1994) (see Table 5). It is possible that whilst cooking may lead to oligomer formation involving the monomeric prolamins, this might be more extensive in sorghum than in maize. As a result, more enzyme-resistant protein oligomers would be formed in sorghum than in maize which may explain the superior digestibility of cooked maize proteins compared to cooked sorghum.

The occurrence of protein oligomers around 45-50 kDa resistant to reduction in pepsin-indigestible residues of cooked sorghum protein-body-enriched samples in higher quantities compared to maize lends weight to this hypothesis and is supported by the work of Hamaker *et al.* (1986). Landry-Moureaux fractionation of uncooked and cooked sorghum and maize conducted by Hamaker *et al.* (1986) showed that non-extractable proteins were 25.8% for cooked sorghum compared to 14.2% for cooked maize. Their results showed a more pronounced shift in alcohol-soluble proteins (towards the more crosslinked fractions) in sorghum than in maize. They also reported that cooked sorghum had a higher amount of indigestible protein (35.2%) compared to uncooked sorghum (19.3%) while there was essentially no difference between cooked (18.1%) and uncooked maize (18.5%).

Oria *et al.* (1995b) reported that cooking sorghum flour with a reducing agent improved protein digestibility but not to the level of uncooked flour. These authors attributed this to the possible presence of inaccessible disulphide bonds. In this light therefore, occurrence of reduction-resistant protein oligomers in pepsin-indigestible residues of cooked sorghum (Figure 10) is not surprising. Perhaps these oligomers are in such a conformation which does not allow easy access of reducing agent to disulphide bonds. The likelihood that cooking may also bring about protein crosslinking not involving disulphide bonding could be considered. Initially, the hydroxyproline-rich glycoproteins (HRGPs) within the cell wall are secreted in soluble form and bind ionically to acidic wall polysaccharides. Later they become more firmly bound in the wall and this is thought to be as a result of oxidative coupling of tyrosine residues to form a crosslinking dimer known as isodityrosine (IDT, made up of two tyrosine units linked by a diphenyl ether bridge) (Fry, 1982). It is believed that IDT may form both intra-polypeptide loops (Epstein & Lamport, 1984) and inter-polypeptide crosslinks (Biggs & Fry, 1990) and such crosslinks may contribute to the insolubility and indigestibility of HRGPs within the cell wall (Fry, 1988). Brady, Sadler and Fry (1996) showed the existence of a tetramer of tyrosine (di-isodityrosine) in plant cell wall proteins. They suggested that due to steric factors, this tetramer is incapable of forming an intra-polypeptide loop in which all four tyrosine units are neighbour residues within a single polypeptide chain. They therefore concluded that this tetramer may participate in inter-polypeptide crosslinking. In similar vein, it could be proposed that the oxidising conditions of the cooking process could lead to formation of more of such non-disulphide crosslinks in sorghum proteins than in maize proteins and this may account for the existence of the reduction-resistant oligomers. Perhaps other non-disulphide crosslinks may be formed by esterification of amino acid residues between different polypeptide chains.

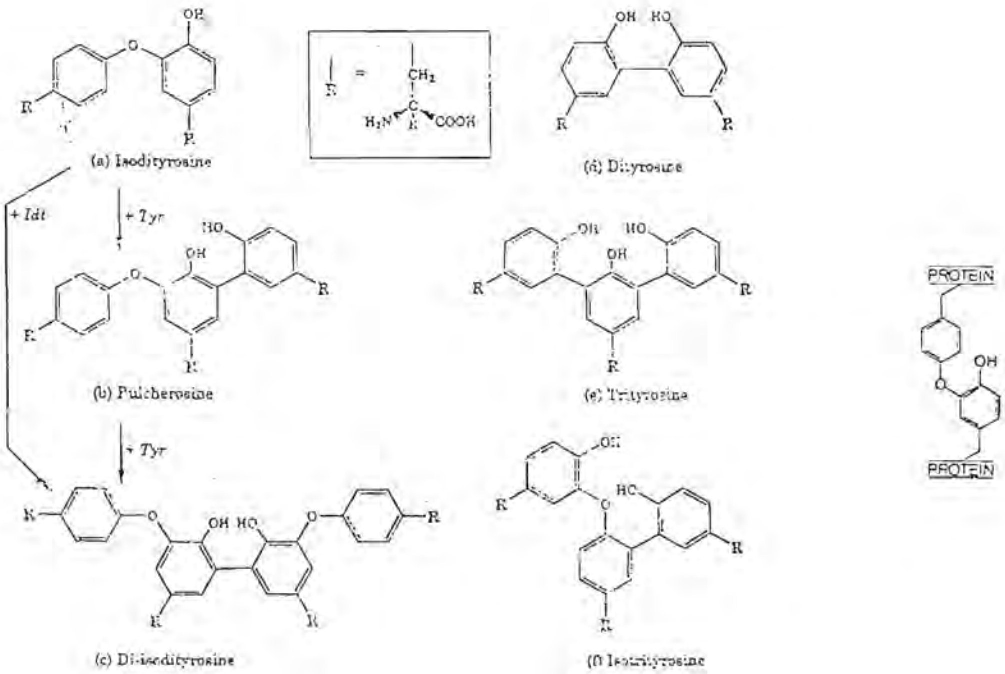


Figure 18: Proposed structures of oxidatively coupled products of tyrosine which may lead to formation of non-disulphide crosslinks in proteins (Brady, Sadler & Fry, 1998) and possible structure of a tyrosyl-tyrosyl crosslink between proteins (Bacic *et al.*, 1988).

Formation of non-disulphide crosslinks is made more likely by the finding that cooked, alkylated sorghum kafirin did not have as high protein digestibility as uncooked kafirin (Table 13). This also confirms the finding by Oria *et al.*, (1995b) that cooking sorghum flour with reducing agent did not improve digestibility to the level of uncooked flour. The alkylation process involves using a reducing agent to cleave disulphide bonds and then free thiols thus generated are trapped with an alkylating agent to prevent their re-oxidation (Hollecker, 1997). If protein crosslinking on cooking is exclusively through disulphide bond formation, it would be expected that alkylation would very significantly improve the protein digestibility of cooked samples. The observation that alkylated and cooked kafirin still had much lower protein digestibility than uncooked suggests that there could be crosslinking not involving disulphide bonds.

However, there is no reason why non-disulphide crosslinking should not occur in maize zein as well. The better digestibility of the alkylated and cooked zein as compared to alkylated, cooked kafirin is a reflection of the possibility that the extent of crosslinking may be lower in maize compared to sorghum. This observation also agrees with those from SDS-PAGE in this present investigation, where pepsin-indigestible residues of cooked sorghum appeared to have protein oligomers in higher quantities compared to maize. A possible role played by polyphenols is also brought in focus by this observation. Like the sorghum protein body-enriched samples, the kafirin samples were coloured compared to the zein samples; a reflection of polyphenols binding to protein. Such polyphenol-protein interaction which may not be occurring to the same extent in zein may also explain the better digestibility of the zein samples.

In FTIR spectroscopy, absorption bands due to the N-substituted amide groups in the polypeptide backbone dominate protein spectra (Fraser, 1956). The amide I (1650 cm^{-1}) and amide II (1550 cm^{-1}) are two of such characteristic amide absorption bands in protein FTIR spectra. The amide I vibration mode is primarily a C=O stretching vibration coupled to contributions from the CN stretch, CCN deformation and in-plane NH bending modes whilst the amide II vibration mode is considered to be an out-of-phase combination of CN stretch and in-plane NH deformation modes of the peptide group (Lavialle *et al.*, 1982; Bandekar, 1992). The absorption bands in the FTIR spectra may be considered to arise predominantly from the kafirins and zeins of sorghum and maize respectively, since these are the major storage proteins in the mature seed. Further, Taylor *et al.* (1984c) reported that in sorghum, the kafirins make up about 85% of the proteins in the type of protein body preparation used in this present investigation. For the normal sorghums, maize and the sorghum mutants (Figures 11 and 12), the band appearing in the 1670-1620 cm^{-1} region is identified as the amide I and that in the 1550-1500 cm^{-1} region as amide II.

The amide bands of proteins are sensitive to the conformation in which the protein is (Surewicz & Mantsch, 1988). Bands in the amide I region centred between 1650 and 1658 cm^{-1} show the presence of proteins in α-helical conformation (Lavialle *et al.*, 1982; Jakobsen *et al.*, 1983; Surewicz & Mantsch, 1988; Bandekar, 1992), whilst bands between 1620 and 1640 cm^{-1} (Jakobsen *et al.*, 1983; Surewicz & Mantsch, 1988; Bandekar, 1992), and sometimes at 1675 cm^{-1} (Lavialle *et al.*, 1982), are due to antiparallel, intermolecular β-sheet structure. In the amide II region, bands at 1545 cm^{-1} and 1547 cm^{-1} arise from α-helical proteins and bands at 1524 cm^{-1} from β-sheet components (Surewicz & Mantsch, 1988; Bandekar, 1992). Consequently, the amide I peak located at 1660 cm^{-1} is assigned to α-helical components and that at 1625 cm^{-1} to antiparallel intermolecular β-sheet. The amide II peak at 1540 cm^{-1} is assigned to α-helical components and that at 1520 cm^{-1} to β-sheet (Figure 12). These peaks were common to the normal sorghums, maize and the sorghum mutants.

It is clear from the absorption maxima in the amide I band, that there was a predominance of α-helical structures in the uncooked samples. This is in agreement with earlier observations from circular dichroism and optical rotatory dispersion experiments which indicated that the zeins contained an average α-helical content of 50-60% (Argos, Pedersen, Marks & Larkins, 1982). The kafirins of sorghum may have similar α-helical content as they have been shown to have extensive homology with the zeins of maize (DeRose *et al.*, 1989). Estimated α-helical contents of the uncooked samples from peak heights in the spectra were 54.0% for P851171 mutant sorghum, 54.5% for P850029 mutant sorghum, 58.5% for KAT 369 sorghum, 57.5% for NK 283 sorghum and 57.5% for PAN 6043 maize. The amide I band shapes of uncooked and cooked sorghum and maize whole grain confirm the spectral observations from the protein-body-enriched samples. The effect of cooking was to bring about increases in intensity of bands attributable to antiparallel intermolecular β-sheet structures and this effect was observable in the normal sorghums, maize and the sorghum mutants. In the amide II region, the apparent shift of absorption maxima towards the β-sheet peaks at 1520 cm^{-1} for NK 283 sorghum, PAN 6043 maize and P850029 sorghum, might indicate that this increase in β-sheet character may have been accompanied by a loss in some α-helical conformation.

The transition from the α-helical conformation to antiparallel intermolecular β-sheet on cooking is supported by the observations from the ^{13}C NMR spectra (Figure 13). Peaks in the 20-58 ppm region as observed in the normal sorghums, maize and the sorghum mutant arise from resonances due to carbons of aliphatic amino acid side chains (Schofield & Baianu, 1982) such as leucine, glutamine, alanine and proline. Peaks at 20-40 ppm may be assigned to β-, γ- and δ-carbons (Kricheldorf *et al.*, 1983; Kricheldorf & Muller, 1984) and peaks at 45-58 ppm to α-carbons of the aliphatic amino acids (Kricheldorf *et al.*, 1983; Kricheldorf & Muller, 1984). Resonances due to aromatic amino acids like phenylalanine, histidine and tyrosine are identified within the 120-130 ppm region (Schofield & Baianu, 1982). The resonances in the region 170-180 ppm are due to the carbonyl carbon (C=O) in the peptide bond whilst those in the 58-110 ppm are due to carbohydrate carbons (starch or non-starch polysaccharides) (Chinachoti, White, Lo & Stengle, 1991). The chemical shifts of these signals are sensitive to protein secondary structure (Saito, Tabeta, Shoji, Ozaki & Ando, 1983; Shoji, Ozaki, Saito, Tabeta & Ando, 1984). In general, the peaks at 56 and 53 ppm (shown inset for all samples except NK 283 sorghum) are associated respectively with proteins in α-helical and β-sheet conformations (Kricheldorf *et al.*, 1983; Kricheldorf & Muller, 1984; Wishart *et al.*, 1991; Wishart & Sykes, 1994), as are signals at 176 and 172 ppm. Signals at 174 ppm correspond to carbons in disordered structures (Wishart & Sykes, 1994).

The alterations in shapes and intensities of the peaks attributable to carbohydrates (58-110 ppm) is likely due to starch gelatinisation (Chinachoti *et al.*, 1991). The upfield shift for both carbonyl and α-carbon signals observed on wet cooking is related to secondary structure changes from α-helical to β-sheet conformation (Kricheldorf *et al.*, 1983; Kricheldorf & Muller, 1984; Wishart *et al.*, 1991; Wishart & Sykes, 1994). The carbonyl group forms part of the peptide bond and hence, an intrinsic part of the protein backbone. The proximity of the α-carbons to the protein backbone also indicates that they would have an influence on protein secondary structure.

The FTIR and ^{13}C NMR spectra show that similar secondary structural changes occur on wet cooking in the normal sorghums, maize and the highly digestible sorghum mutants. The protein assumed more intermolecular β-sheet structure, perhaps at the expense of some α-helical conformation. Such changes have been reported to be characteristic of heat- or solvent-denatured and aggregated proteins (Kretschmer, 1957; Surewicz & Mantsch, 1988) and have previously been observed in zein (Kretschmer, 1957).

Concerning the mutant sorghums of known high protein digestibility (Weaver *et al.*, 1998), the observations from spectroscopy that their proteins underwent the same type of secondary structural change as in the normal sorghums on wet cooking supports those from SDS-PAGE where tracks for the cooked sorghum mutant under non-reducing and reducing conditions though fainter, were identical to those for the normal sorghums. This suggests that alternative factors other than change in protein secondary structure may be at play in the sorghum mutants. These mutant sorghums have been reported to have highly invaginated protein bodies (Weaver *et al.*, 1998) compared to the spheroid-shaped protein bodies of normal sorghum and maize. Additionally, highly disulphide-bound γ-kafirins at the periphery of normal sorghum protein bodies, are found at the base of folds in the mutant protein bodies (Oria *et al.*, 2000). The invaginated form and changed location of γ-kafirin should allow greater accessibility of proteases to the protein bodies (in particular, the highly digestible α-kafirin) of the sorghum mutants and hence their very high digestibilities as observed in this investigation and reported earlier by Weaver *et al.*, (1998). These sorghum mutants contain cell wall material and protein-cell wall adhesion may well occur. A consequence of the invaginated structure of their protein bodies is that the α-kafirins will be much closer to the cell wall than the β- and γ-kafirins which are more likely to form crosslinks because of their high content of cysteine residues. This, in addition to the better accessibility of proteases to α-kafirins may account for the high digestibilities of the mutants in spite of possible protein-cell wall adhesion.

The observed shifts in the α-helix and β-sheet peaks of popped sorghum and maize towards lower wavenumbers and increase in intensity of the β-sheet peak at 1630 cm^{-1}, signify an increase in β-sheet character of the protein when the grains are popped. This indicates that popping brings about the same type of change in the spectra (for sorghum and maize) as observed for wet cooking. It is clear from the spectra that even though the same type of secondary structural change occurred in the protein by either wet cooking or popping, the increase in β-sheet components on thermal processing occurred to a greater extent by wet cooking than by popping in both sorghum and maize. This secondary structural change which may contribute to lowered protein digestibility on thermal processing, occurs to a greater extent in wet cooked than in popped samples and may explain the better protein digestibility of popped grain compared to wet cooked as observed with sorghum in this investigation and also by Parker *et al.*, (1999). The effect of popping on microstructure of the grain is also believed to contribute to the better protein digestibility of popped grain. During the popping process, the pericarp acts as a pressure vessel which allows the moisture in the kernel to turn into superheated steam (Hoseney, Zeleznak & Abdelrahman, 1983). Rupture occurs eventually when the hull can no longer withstand the internal pressure. Hoseney *et al.*, (1983) suggest that in the horny endosperm the superheated steam vaporises into the hilum of the starch granule and then gelatinise and expand the starch into a thin film. According to Parker *et al.*, (1999), the explosive popping process leads to fragmentation of the cell walls of the vitreous endosperm. This appears to improve accessibility of protein components within the endosperm to enzymes hence better protein digestibility of popped compared to wet cooked sorghum.

However, the observation that popped PAN 6043 maize had lower protein digestibility than the wet cooked PAN 6043 maize whole grain was an anomaly. This appears to contradict the observations from the FTIR spectra where the extent of protein secondary structural change in popped maize was less than wet cooked. The PAN 6043 maize is a dent maize variety and therefore does not pop as effectively as normal popcorn would. Popcorn is a flint-type maize which consists predominantly of horny endosperm with tightly-packed starch granules (Pordesimo, Anantheswaran & Mattern, 1991). This contrasts with dent maize varieties which contain a higher proportion of floury endosperm with many intergranular spaces. The floury endosperm with its intergranular spaces, provide channels of escape for the superheated steam generated during the popping process. As a result the starch granules are not expanded and retain their birefringence. It may be that less effective popping of the PAN 6043 dent maize variety could lead to less cell wall fragmentation, hence less accessibility of protein components within the endosperm to enzymes. In addition, popped grains were selected visually for the *in vitro* protein digestibility assay. It is likely that the popped maize grains selected for FTIR spectroscopy and for the digestibility assay were not representative of each other.

The better digestibility of popped compared to wet cooked sorghum may be related to findings from studies on the effect of extrusion on solubility and digestibility of sorghum kafirin from the perspective of improved accessibility of enzymes to proteins. Hamaker *et al.*, (1994) reported that extrusion improved cooked sorghum protein digestibility to the level of uncooked flour and prevented the decrease normally observed. They proposed that the extrusion process disrupted the structure of the protein bodies due to the heat and shearing action involved, thus permitting easy access to α-kafirin by digestive enzymes. The same concept of improved accessibility is applicable in the popping situation where the explosive process fragments cell walls and brings about greater exposure of protein components within the endosperm to digestive enzymes.

Whilst the SDS-PAGE results provide information about protein secondary structural change on cooking through disulphide and other forms of crosslinking, the spectra (FTIR and ^{13}C NMR) provide such information from the perspective of α-helical to antiparallel intermolecular β-sheet conformational transitions. In order to relate these two forms of protein secondary structural change, a hypothesis may be proposed.

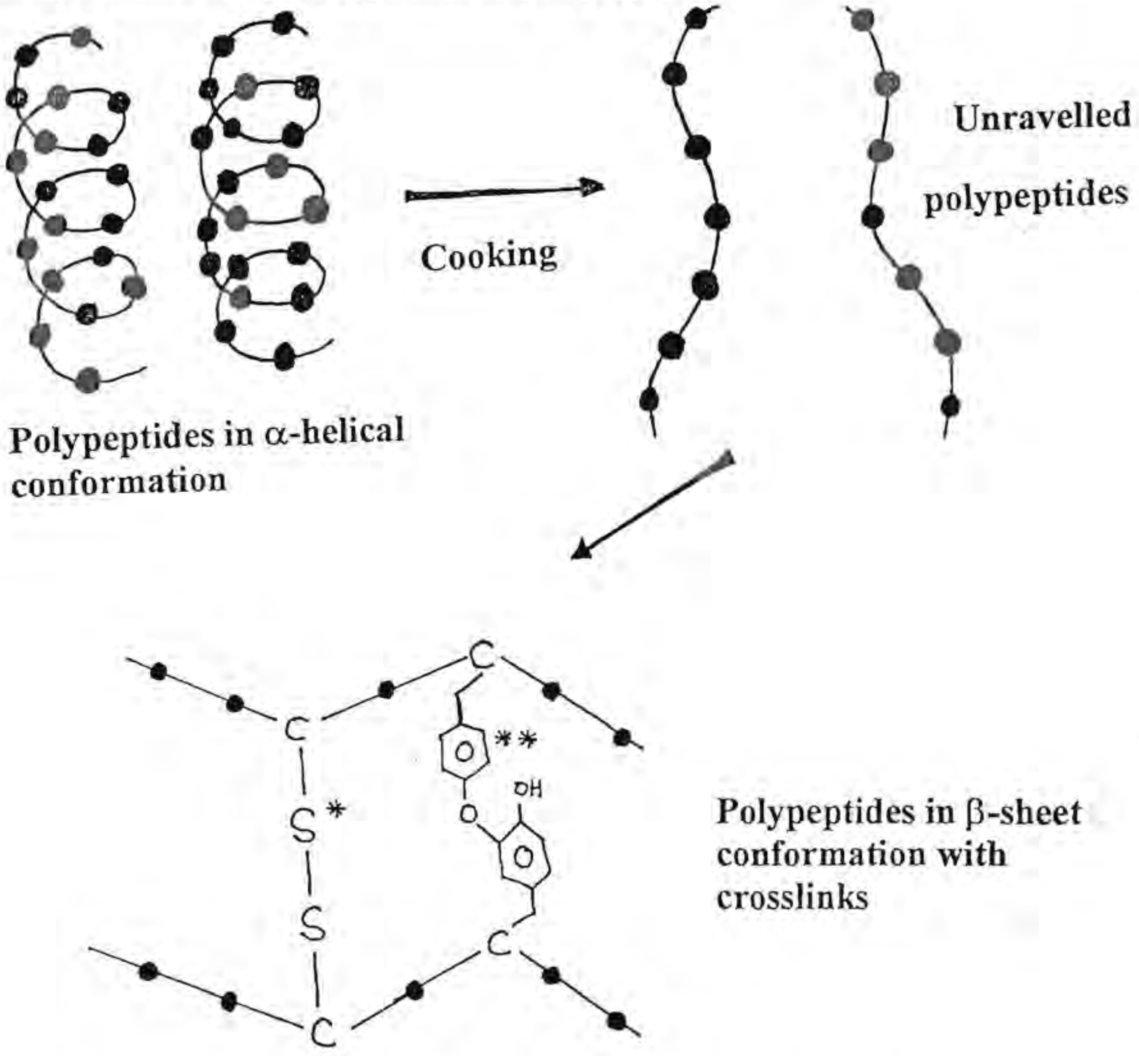


Figure 19: Representation of α-helix to β-sheet conformational change on cooking followed by crosslink formation between polypeptide chains. -●-●-, amino acid residues.
* disulphide crosslink; ** tyrosyl crosslink.

A scenario whereby the application of energy during the wet cooking process breaks intra-chain hydrogen bonds that would otherwise stabilise the α-helices could be imagined. Polypeptides (most likely β- and γ-kafirins or zeins) forming these helices may thus become unravelled and aligned next to each other to form the intermolecular β-sheet conformation. It is then not inconceivable that disulphide and other forms of crosslinking could occur between polypeptides closely aligned to each other in this manner (Figure 19). This would result in a compact structure in which accessibility of enzymes to the protein is restricted, thus leading to a reduction in protein digestibility. The SDS-PAGE results show that protein crosslinking on cooking (which brings about aggregation and change in secondary structure) occurs to a greater extent in the normal sorghums than in maize. This indicates that though changes in protein secondary structure may be similar qualitatively between sorghum and maize, they may differ quantitatively, being more extensive in the normal sorghums.

It is not clear from this investigation the reasons why thermal processing leads to more extensive protein crosslinking in sorghum than in maize. Sorghum kafirins and maize zeins are believed to bear extensive homology to each other (DeRose *et al.*, 1989). However, this may not mean that they are exactly identical. It is possible that subtle differences in tertiary structure between sorghum kafirins and maize zeins may be responsible for the observed different extents of protein crosslinking on thermal processing between sorghum and maize.

CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

The protein digestibility of sorghum is influenced by a number of factors. Some factors are more important than others depending on whether one is dealing with uncooked or cooked grain or the nature of the grain, that is, whole grain, endosperm, protein bodies or the extracted proteins.

The present study showed that whilst uncooked sorghum whole grain has lower protein digestibility than uncooked maize whole grain, at the endosperm level, uncooked sorghum protein digestibility is essentially the same as maize and higher than maize at the protein body-enriched level and the same as maize at the level of extracted kafirins and zeins. This is an indication that in contrast to what has been suggested by other workers, uncooked sorghum may not always have lower protein digestibility than uncooked maize. The protein digestibility of uncooked sorghum depends on the nature of the material being assayed.

The protein digestibility of uncooked and cooked sorghum is improved with change in organisational level from whole grain to endosperm and this is accompanied with a decrease in total polyphenol content. This suggests that polyphenols (phenolic acids and flavonoids) may influence protein digestibility of uncooked sorghum at the whole grain level, which is at odds with the current view that the smaller molecular weight polyphenols are not known to have any adverse effects on protein digestibility. However, in going from the endosperm to the protein body-enriched level, an increase in total polyphenol content does not bring about a decrease in uncooked and cooked sorghum protein digestibility which is in agreement with the current view regarding the effect of smaller molecular weight polyphenols on protein digestibility. This is an indication that at the endosperm and protein body-enriched levels, polyphenol involvement in influencing protein digestibility is more likely through an alternative mechanism such as protein crosslinking with ferulic acid in cell walls rather than with phenolic acids and flavonoids. Thus the lower proportion of cell wall material in the protein body-enriched samples explains the better uncooked and cooked protein digestibility of the protein body-enriched samples compared to the endosperm.

The condensed-tannin-free sorghums and the maize used in this study have a negligible effect on amylase activity in contrast with the high-tannin sorghum variety which substantially inhibits amylase activity. It is therefore proposed that if polyphenols are involved in reducing protein digestibility, the mechanism is likely to be through interaction with substrate protein rather than with the pepsin enzyme.

Cooking reduces sorghum protein digestibility at the whole grain level but not maize, which is in agreement with what is documented in literature. This study shows that reduction of sorghum protein digestibility on cooking also occurs at the endosperm, protein body-enriched levels and at the level of extracted kafirins. In contrast, the protein digestibilities of uncooked and cooked maize remain essentially the same at these levels of organisation.

It is suggested that a possible interaction between sorghum protein and cell walls leads to reduction in protein digestibility on cooking. This is in agreement with the observations of other workers who have reported an association of cereal and legume proteins with dietary fibre or cell wall components.

Some mechanisms are proposed to explain the nature of this protein-cell wall adhesion which may bring about reduction in protein digestibility on cooking.

- The oxidising conditions of the cooking process could promote phenolics-mediated crosslinking of proteins within the cell wall. Dimerisation may occur between tyrosine residues in proteins and ferulic acid residues esterified to arabinoxylans of the cell walls. Ferulic acid may also be crosslinked to the sulphydryl group of cysteine residues in proteins.
- There may be direct attachment of protein to carbohydrate moieties of the cell wall. In an analogous fashion to the structure of plant cell wall glycoproteins, O-glycosidic bonds may be formed between proline residues of sorghum and maize proteins (if hydroxylated) and the arabinose residues of the cell wall on cooking. This could result in enzyme-resistant protein-carbohydrate linkages.

It is suggested that the superior protein digestibility of cooked maize compared to sorghum may be because the protein-cell wall adhesion is stronger and occurs to a greater extent in sorghum than in maize.

Treatment of cooked sorghum and maize whole grain and endosperm flours with alpha-amylase improves protein digestibility, which is an indication that gelatinised starch, probably by reducing accessibility of pepsin enzyme to protein substrate, reduces protein digestibility. In support of this observation, in the protein body-enriched samples where the proportion of starch is much lower, there are no differences in protein digestibility between cooked and cooked plus alpha-amylase treatments in both sorghum and maize.

Reduction of sorghum protein digestibility as a result of cooking may also be through an alteration in protein secondary structure. Disulphide crosslinking on cooking (a form of protein secondary structural change) has been proposed by other workers as responsible for the lowered protein digestibility of sorghum. This hypothesis is confirmed by observations in this investigation from SDS-PAGE. Sorghum (uncooked and cooked) contains more oligomers than maize and therefore, it is proposed that formation of disulphide-bonded protein oligomers may be more extensive in sorghum than in maize possibly due to subtle differences in tertiary structure between sorghum kafirins and maize zeins. More enzyme-resistant oligomers would then be formed in sorghum than in maize and hence the superior digestibility of cooked maize proteins compared to cooked sorghum.

Some protein oligomers in cooked sorghum and maize are resistant to reduction. More of such reduction-resistant oligomers occur in sorghum than in maize. It is suggested that these oligomers may be in such a conformation which does not allow easy access of reducing agent to disulphide bonds. It is also proposed that these reduction-resistant oligomers may not have been formed through disulphide crosslinking. This proposal is made more likely by the finding that alkylation of cooked sorghum kafirin does not improve protein digestibility to the level of uncooked kafirin. A hypothesis is that such non-disulphide-bonded protein oligomers may be formed by

- oxidative coupling of tyrosine residues between polypeptide chains to form the crosslinking dimer known as isodityrosine or tetramer known as di-isodityrosine,
- esterification of amino acid residues between polypeptide chains.

The FTIR and ^{13}C NMR spectra provide information about protein secondary structural change on cooking from the perspective of α-helical to antiparallel intermolecular β-sheet conformational transitions. Cooking appears to bring about a change in protein secondary structure from the α-helical conformation to antiparallel intermolecular β-sheet in both sorghum and maize. The extent of this structural change appears to be more in sorghum compared to maize which explains the higher digestibility of maize proteins. Proteins of sorghum mutants of known high protein digestibility undergo the same kind of secondary structural change on cooking as the normal sorghums namely, α-helix to antiparallel intermolecular β-sheet.

In agreement with recent published results, popped sorghum has higher protein digestibility than wet cooked. Similar to wet cooking, popping also brings about secondary structural changes from α-helical to antiparallel intermolecular β-sheet conformation. This structural change which is associated with reduction in protein digestibility, occurs to a greater extent in wet cooked than in popped samples and may explain the better protein digestibility of popped grain compared to wet cooked. Improved accessibility of protein components in the endosperm to enzymes as a result of endosperm cell wall fragmentation due to the explosive popping process may also explain the better digestibility of popped grain.

From a broad perspective, this investigation provides evidence that grain organisational structure does affect protein digestibility of sorghum. Emanating from this, four main factors namely, polyphenols (phenolic acids and flavonoids), cell walls, gelatinised starch and protein crosslinking may be identified as affecting protein digestibility. In Table 15 below, these proposed factors and their levels of importance at the three levels of organisation of sorghum and maize are summarised.

Table 15. Proposed factors affecting protein digestibility of uncooked and cooked sorghum and maize and their levels of importance at the whole grain, endosperm, protein body and extracted protein levels.

	Sorghum				Maize			
Factors	Whole grain	Endosperm	Protein bodies	Extracted proteins	Whole grain	Endosperm	Protein bodies	Extracted proteins
Uncooked								
Polyphenols	♦♦	♦	♦	♦	♦	♦	♦	♦
Cell walls	♦♦♦	♦♦	♦	♦	♦♦	♦♦	♦	♦
Gelatinised starch	♦	♦	♦	♦	♦	♦	♦	♦
Disulphide crosslinking	♦♦	♦♦	♦♦	♦♦	♦♦	♦♦	♦♦	♦♦
Non-disulphide crosslinking	♦♦	♦♦	♦♦	♦♦	♦♦	♦♦	♦♦	♦♦
Cooked								
Polyphenols	♦♦	♦	♦	♦	♦	♦	♦	♦
Cell walls	♦♦♦	♦♦♦	♦	♦	♦♦	♦♦	♦	♦
Gelatinised starch	♦♦	♦♦	♦	♦	♦♦	♦♦	♦	♦
Disulphide crosslinking	♦♦♦♦	♦♦♦♦	♦♦♦♦	♦♦♦♦	♦♦	♦♦	♦♦	♦♦
Non-disulphide crosslinking	♦♦	♦♦	♦♦	♦♦	♦♦	♦♦	♦♦	♦♦

♦ Not involved; ♦♦ Involved; ♦♦♦ More important; ♦♦♦♦ Most important

It appears that for uncooked sorghum, polyphenols (phenolic acids and flavonoids) and cell walls appear to be important factors affecting protein digestibility whilst cell walls and not polyphenols are important for uncooked maize. Cell walls, protein crosslinking and gelatinised starch are the main factors affecting protein digestibility in cooked sorghum and maize. Association of protein with cell walls and protein crosslinking appear to occur to a greater extent in sorghum than in maize and may explain the worse digestibility of cooked sorghum compared to cooked maize.

Further research is required to investigate the possible formation of non-disulphide crosslinks on cooking. A possible line of investigation would be to conduct a more detailed structural characterisation of the pepsin-indigestible residues of both sorghum and maize. These may be purified and subjected to spectroscopic analysis. Protein oligomers which are resistant to reduction may also be examined in this way to gain an understanding of their structure and conformation. X-ray crystallography and spectroscopy may also be used to investigate possible differences in tertiary structure between kafirins and zeins. This would provide a better understanding of the reasons why sorghum proteins have the tendency to form more oligomers on cooking compared to maize. The possibility of polyphenols influencing protein digestibility of cooked condensed-tannin-free sorghum could be investigated by extraction of polyphenols from the isolated proteins (kafirins and zeins) and determining protein digestibility after adding them back.

CHAPTER 7

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