1 INTRODUCTION AND LITERATURE REVIEW

1.1 STATEMENT OF PROBLEM

Sorghum (*Sorghum bicolor* (L.) Moench) and maize (*Zea mays* L.) are staple food crops for many of the world's poorest people and constitute a major source of energy and proteins for millions of people in Africa and Asia (Taylor & Belton, 2002; Eckhoff, Paulsen & Yang, 2003). Sorghum and maize can therefore be considered as significant contributors to ensuring food security in these regions.

Sorghum contains about 9-14% proteins (Rooney & Serna-Saldivar, 2003) and maize 8-12% (Eckhoff *et al*., 2003). Sorghum and maize proteins are limiting in the essential amino acid lysine (Rooney & Serna-Saldivar, 2003; Wilson, 1983). However, sorghum is more limiting in its use as a protein source, in that its proteins become less digestible when cooked into porridge. This has been demonstrated in both *in vitro* and *in vivo* studies (reviewed by Duodu, Taylor, Belton & Hamaker, 2003). The reduction in the digestibility of sorghum proteins with cooking is attributed to the formation of proteolytic enzyme resistant protein polymers, through disulphide bonding of β- and γ-kafirins at the periphery of the protein body with themselves and with matrix proteins, which restricts digestion of α-kafirin at the interior of the protein body (Oria, Hamaker & Shull, 1995b). This suggests that the conformation of sorghum proteins could be an important factor influencing its digestibility, as this may affect enzyme access to the proteins.

The poor protein digestibility of sorghum porridges could perhaps be related to the prevalence of malnutrition in sorghum consuming areas. It was estimated that in 2000 33% (182 million) children (<5 year) in developing countries were stunted, with 70% of these children in Asia, 26% in Africa and 4% in Latin America and the Caribbean (de Onis, Frongillo & Blössner, 2000). In Cameroon, in the early 1990s, malnutrition in children under the age of 5 years was estimated to be at 24% (Balépa, Fotso & Barrère 1992). The situation was most acute in the three northern provinces of Cameroon where malnutrition was estimated at 30%, and infant mortality rates in these regions was 2.5 times higher than elsewhere in the country (Balépa *et al*., 1992). Sorghum, together with

pearl millet and maize are the staple foods in these areas, and are consumed mainly as porridges. Given that protein digestibility of sorghum decreases substantially when cooked into porridge, it is therefore not unlikely that this could contribute to the higher prevalence of malnutrition in these regions. More digestible proteins in sorghum porridges may assist towards reducing the incidence of malnutrition.

Irradiation is a technology used in food processing. In cereal grains, it is recommended for grain disinfestations to eliminate spoilage microorganisms (Institute of Food Science and Technology, IFST, 1998). As radiation energy passes through foods, it interacts with food molecules converting them to free radicals that can then react with each other or other unchanged molecules in the food. These reactions can bring about changes in the food components. Radiation energy can cleave disulphide bonds, hydrogen bonds and other bonds involved in stabilizing protein structure (Garrison, 1987; Di Simplicio, Cheeseman & Slater, 1991; Köksel, Sapirstein, Celik & Bushuk, 1998). This could lead to denaturation or breakdown of the proteins to smaller peptides (Davies, Delsignore & Lin, 1987a; Garrison, 1987; Kempner, 1993). However, with increasing irradiation dose, the denatured proteins or smaller peptides may polymerise to form high molecular weight protein polymers (Kempner, 1993; Cieśla, Roos & Głuszewski, 2000). This notwithstanding, some proteins have been reported to become more susceptible to proteolysis after irradiation (Davies, 1987; Davies, Lin & Pacifici, 1987b).

Disulphide bonds occur in mature sorghum (Oria, Hamaker & Shull, 1995a) and maize (Larkins, Pedersen, Marks & Wilson, 1984) and as indicated before, their, additional formation during cooking is associated with the lower digestibility of wet cooked sorghum (Hamaker, Kirleis, Butler, Axtell & Mertz, 1987; Rom, Shull, Chandrashekar & Kirleis, 1992). Considering that irradiation has the potential to break disulphide and other bonds, and consequently to modify protein structure, both of which are important factors influencing sorghum wet cooked protein digestibility, it was important to investigate the effects of irradiation of sorghum flour before cooking into porridge on its protein digestibility, in comparison with maize.

1.2 LITERATURE REVIEW

This review will examine the concept of protein digestibility, relevant enzymatic methods of determining protein digestibility in sorghum and maize together with protein interactions that affect their digestibility. It will also examine sorghum and maize proteins and the effect of wet cooking on their digestibility. The effect of irradiation on proteins and in particular cereal proteins and how this could affect the digestibility of sorghum and maize proteins will also be reviewed.

1.2.1 Protein Digestibility

The protein digestibility of a food is one of the determinants of its nutritional quality. Protein quality in essence refers to the ability of proteins to meet human nutritional requirements for essential amino acids (Walker, 1983). Before proteins can be utilized by man, they must be digested by the gastric, pancreatic and intestinal proteolytic enzymes in the digestive tract to yield small peptides and amino acids which can then be absorbed into the bloodstream to be utilized by the consuming organism (Swaisgood & Catignani, 1991). To obtain information about the digestibility and nutritional quality of a protein intended for human consumption, it would be ideal to feed it to humans. However, ethical issues involved with using humans for such experiments limit their use. Also, *in vivo* determination of protein digestibility is time-consuming and costly (Pederson & Eggum, 1983). To overcome these shortcomings, rapid *in vitro* assays, which use enzymes similar to those found in the human intestinal tract and carried out under conditions that simulate physiological conditions, have been developed (Walker, 1983). The following section will briefly consider some relevant *in vitro* enzymatic methods that have been used to determine the protein digestibility of cereals, with particular reference to sorghum and maize, and their relationship to *in vivo* digestibility.

1.2.1.1 In Vitro Enzymatic Methods of Determining Protein Digestibility

In vitro enzymatic assays for determining protein digestibility usually require treating the sample with proteolytic enzymes, either singly or in combination. Protein digestibility is then measured as a change in protein solubility, amino nitrogen or free amino acids (Phillips, 1997). These assays either measure the initial rate of hydrolysis, which is then extrapolated to obtain total digestibility, or they measure maximal digestibility values (Boisen & Eggum, 1991).

In single enzyme systems the sample is incubated with one enzyme, usually pepsin, trypsin, pronase, papain or rennin (Boisen & Eggum, 1991). Single enzyme systems using pepsin have been widely used to determine *in vitro* protein digestibility in sorghum and maize (Chibber, Mertz & Axtell, 1980; Mertz, Hassen, Cairns-Whittern, Kirleis, Tu & Axtell, 1984; Hamaker, Kirleis, Mertz & Axtell, 1986; Duodu, Nunes, Delgadillo, Parker, Mills, Belton & Taylor, 2002) as well as of barley (Bhatty & Whitaker, 1987). Digestibility in this case is measured as the amount of nitrogen solubilized by pepsin over a given period and expressed as a percentage of the total nitrogen in the sample (Mertz *et al*., 1984). However, *in vitro* results can only be of practical significance if they correlate with *in vivo* results. In the case of sorghum, Axtell, Kirleis, Hassen, D'Croz-Mason, Mertz & Munck (1981) using *in vitro* pepsin assay, showed that the protein digestibility of cooked sorghum gruel (50%) was similar to that obtained by MacLean, Lopez de Romana, Placko & Graham (1981) using children (47%). Significant correlations have also been obtained between *in vitro* pepsin (Mertz *et al.* 1984) and *in vivo* (MacLean, Lopez de Romana, Gastanaduy & Graham, 1983) protein digestibility with extruded sorghum, and with barley (Bhatty & Whitaker, 1987).

Incubation with two enzymes, trypsin-chymotrypsin in one step has also been used to determine changes in protein digestibility in sorghum (Chibber *et al*., 1980; Hamaker *et al*., 1986) and maize (Hamaker *et al*., 1986) with processing. Chibber *et al*. (1980) investigated the effect of sequential decortication of condensed tannin sorghum on its protein digestibility, whereas Hamaker *et al*. (1986) studied the effect of wet cooking on protein digestibility. In both studies, the digestibility of proteins using these two enzymes

was compared with pepsin digestibility, and it was found that more proteins were solubilized using pepsin enzyme alone than when a combination of trypsin and chymotypsin was used. Chibber *et al*. (1980) concluded that pepsin was more effective in solubilizing sorghum proteins than trypsin and chymotrypsin. Pepsin preferably cleaves bonds containing hydrophobic residues (Huang & Tang, 1968), and has been reported to cleave peptide bonds between Phe-Val, Phe-Thr, Leu-Met, Asp-Tyr, Val-Ala, Leu-Val, Ala-Leu, Leu-Tyr, Tyr-Leu, Phe-Phe, Phe-Tyr, Glu-His, Glu-Ala, Gly-Phe residues (Ryle & Porter, 1959; Huang & Tang, 1968; Etherington & Taylor, 1971). Trypsin cleaves peptide bonds at residues of arginine and lysine, whereas chymotrypsin cleaves peptide bonds at aromatic amino acid residues such as tyrosine, phenylalanine and tryptophan (Brody, 1994). In sorghum and maize, the predominant proteins are the hydrophobic prolamins (Guiragossian, Chibber, Van Scoyoc, Jambunathan & Mertz, 1978; Wilson, 1983; Taylor, Novellie & Liebenberg, 1984b). This could explain why Chibber *et al.* (1980) and Hamaker *et al.* (1986) observed a higher protein digestibility in sorghum and maize using pepsin alone compared to using a combination of trypsin and chymotrypsin. However, a predigestion with pepsin followed by trypsin-chymotrypsin solubilized more proteins, giving a higher digestibility than when either pepsin or trypsin-chymotrypsin was used alone (Hamaker *et al.,* 1986). This could probably be due to the fact that trypsin and chymoytypsin cleaved additional peptide bonds not cleaved by pepsin. Notwithstanding, pepsin used alone, or trypsin and chymotrypsin used together, or a combination of these three enzymes, were all able to differentiate between uncooked and cooked protein digestibility in sorghum and maize

Protein digestibility in cereals has also been determined using a multi-enzyme assay with three enzymes: namely, trypsin, chymotrypsin and peptidase (Pederson & Eggum, 1983; Weaver, Hamaker & Axtell, 1998; Aboubacar, Axtell, Huang & Hamaker, 2001). This assay monitors the rate of peptide release over a 10 min period, and correlates this to the protein digestibility of the sample. The rate of peptide release is determined either by a drop in pH, the pH drop method (Hsu, Vavak, Satterlee & Miller, 1977), or by measuring the amount of sodium hydroxide needed to maintain the pH constant over the assay period, the pH stat method (Pederson & Eggum, 1983). The pH drop method was used to estimate protein digestibility in barley (Bhatty & Whitaker, 1987), but it did not seem to

be as sensitive as the pepsin method, being unable to distinguish differences in protein digestibility between barley genotypes, which the pepsin method did. The inability of the multienzyme assay to differentiate between barley genotypes was attributed to a strong buffering effect by the barley meal, which may have affected pH changes. Rombo (2002) determined protein digestibility in maize porridge prepared from irradiated flour using both the pepsin and pH drop methods. The methods gave opposite results at 2.5 kGy irradiation, with pepsin showing an increase in digestibility and the pH drop multienzyme a reduction in protein digestibility. A low multienzyme digestibility implies that the pH of the sample decreased only slightly under the test conditions. This could suggest that there was some buffering of the pH by the sample. However, the reasons for these differences in digestibility between pepsin and pH drop assays in barley and maize are not quite clear and need to be investigated further.

The pH stat method is less influenced by the buffering capacity of the test material compared to the pH drop method (Pederson & Eggum, 1983), and could therefore be better suited for determining protein digestibility in barley and maize, since it appears that these cereals have a strong buffering capacity that may hinder the determination of their digestibility using the pH drop method. Aboubacar *et al*. (2001) used the pH stat and pepsin assays to determine uncooked protein digestibility in normal and highly digestible sorghum lines and found a strong correlation ($r = 0.86$) between the two methods. Weaver *et al*. (1998) used the pH stat assay together with the pepsin assay to determine differences in digestibility between uncooked normal and high-lysine sorghum and maize. Both methods showed differences between the more digestible high-lysine mutants and the less digestible normal ones. It shows that the pH stat assay can be used and may indeed be better than the pH drop assay in determining digestibility differences of uncooked sorghum flours. It is therefore likely that it could also discriminate between digestibility of uncooked and cooked sorghum and maize proteins. This, however, still needs to be investigated.

1.2.1.2 Protein Interactions that Affect Digestibility

While cooking or heating of food generally denatures proteins and improves their digestibility (Mostafa, 1987; Farag, 1999), these thermal treatments sometimes result in the formation of protein crosslinks that are less digestible and consequently reduce the digestibility of the protein (Erbersdobler, 1989).

When protein foods are heated in the presence of carbohydrates, such as reducing sugars, Maillard reactions can occur between the ∈-amino group of lysine and the carbonyl group of the reducing sugar (Baltes, 1982; Rizzi, 1994). The participation of lysine in Maillard browning results in the destruction of lysine (Mauron, 1982). Products of Maillard reactions are resistant to proteolysis (Öste, Dahlqvist, Sjöström, Norén & Miller, 1986; Öste, Miller, Sjöström & Norén, 1987), and may result in reduced digestibility of proteins in which such crosslinks are present. Other amino acids such as cysteine and leucine may be affected through their reaction with products from the Maillard reaction to form enzyme-resistant crosslinks (Hurrell, 1984). Sorghum and maize are poor in lysine, which means they may be less likely to be affected by Maillard reactions. High-lysine mutants, on the other hand, may be a cause for concern because of their higher lysine content (Mertz, 1992; Weaver *et al.,* 1998). Maillard reactions can also occur during irradiation of foods (Krumhar & Berry, 1990; Cunha, Sgabieri & Damasio, 1993). This will be discussed in the section on irradiation (1.2.5).

Severe heating and/or alkaline treatment of proteins can destroy some essential amino acids through racemization, which is the conversion of amino acids from the L- to the D-form (Hurrell, 1984; Friedman, 1999a, 1999b). The D-form in addition to not being easily hydrolysed by enzymes, is absorbed more slowly and even when absorbed is not utilised by humans (Hurrell, 1984; Cheftel, Cuq & Lorient, 1985). The amino acids most likely to undergo racemization include aspartic acid, tyrosine, glutamic acid, alanine, lysine and leucine (Erbesdobler, 1989). Sorghum and maize prolamin proteins contain high levels of asparagine, glutamine, alanine and leucine (Taylor & Schüssler, 1986; Wilson, 1983) and can therefore be expected to undergo racemization during cooking. Cheftel *et al.* (1985), however, stated that the heat and alkali treatments employed during domestic cooking of

foods, such as solubilization of vegetable proteins and cooking of corn in lime, would result in the formation of only small amounts of lysinoalanine and its derivatives. Racemised amino acids can subsequently undergo β-elimination to form dehydroalanine derivatives, which can then crosslink with the ∈-amino group of lysine residues to form the isopeptide, lysinoalanine (Liardon & Hurrell, 1983; Friedman, 1999a). Other amino acids that can be destroyed by forming isopeptides include cysteine, ornithine, and histidine (Friedman, 1999a).

Amino acids of the same type within a food may crosslink with one another during processing to form less digestible complexes. Cysteine residues within a protein food can become oxidised and crosslink with one another through disulphide bonds (Gerrard, 2002). Heat treatment can enhance formation of disulphide linkages, as observed in sorghum during wet cooking, which in turn would produce less digestible protein polymers (Hamaker *et al*., 1987; Duodu *et al*., 2002) (see below, 1.2.3). Tyrosine amino acids may also become linked through a phenolic coupling of two or three phenoxy radicals of tyrosine to form di- or tri-tyrosine (Otterburn, 1989; Brady, Sadler & Fry, 1996). Crosslinking of tyrosine amino acids with each other has been reported in irradiated foods (Mezgheni, D'Aprano & Lacroix, 1998). More details are provided in the section on irradiation (1.2.5).

1.2.2 Sorghum and Maize Proteins

Sorghum and maize proteins, like other seed proteins, may be classified according to solubility as proposed by Osborne into the albumins, globulins, prolamins and glutelins (Landry & Moureaux, 1970; Guiragossian *et al*., 1978; Taylor, Schüssler & Van der Walt, 1984a).

1.2.2.1 Albumins and Globulins

The albumins and globulins are the salt-soluble proteins in sorghum and maize. They are usually the first fraction to be extracted in a sequential extraction. The proteins extracted

in salt solution contain low molecular weight nitrogen compounds including small peptides and free amino acids, in addition to the albumins and globulins (Wilson, 1983; Taylor *et al* 1984a). To eliminate the salt and low molecular weight nitrogen components in this extract, it is recommended to dialyse them against water (Taylor *et al*., 1984a).

The albumins and globulins together make up about 23% of total proteins in sorghum whole grain flour (Taylor *et al*., 1984a; El Nour, Peruffo & Curioni, 1998) and 19% of total protein in maize whole grain flour (Landry & Moureaux, 1970). However, the proportion of these proteins is much lower in the endosperm, about 7.5% in sorghum (Watterson, Shull & Kirleis, 1993) and about 3.5% in maize (Yau, Bockholt, Smith, Rooney & Waniska, 1999). This is because the albumin and globulin proteins are abundant in the germ (Taylor & Schüssler, 1986; Wilson, 1987), which is removed during the decortication and degerming processes to produce endosperm flour (Table 1.1). The extent of reduction in albumin and globulin content in endosperm flour will therefore depend on the extent of removal of the germ. This fraction is nutritionally important, being rich in the essential amino acid lysine, which happens to be the first limiting amino acid in these cereals (Guiragossian *et al*., 1978; Van Scoyoc, Ejeta & Axtell, 1988; Yau *et al*., 1999).

The nutritional importance of albumins and globulins derives from the fact that the nutritional quality of a protein depends among other factors on its ability to meet human needs for essential amino acids (Walker, 1983). The enhanced lysine content of high-lysine mutant types results primarily from an increase in relatively lysine-rich glutelins plus albumin and globulins and a decrease in the lysine-deficient prolamin proteins (Guiragossian *et al*., 1978; Van Scoyoc *et al.,* 1988; Yau *et al.,* 1999). This makes them nutritionally superior to the normal lines. Albumin and globulin proteins in sorghum consist of a mixture of low molecular weight proteins of M_r 15 and 18 k, and high molecular weight proteins with M_r 38 and 44 k on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Watterson *et al*., 1993).

Table 1.1. The distribution of different protein fractions in the different anatomical parts of normal sorghum and maize types (% of total protein)

Protein fractions	Whole grain		Endosperm		Germ		Pericarp	
	Sorghum[1]	Maize[2]	Sorghum[1]	Maize[2]	Sorghum[1]	Maize[2]	Sorghum[1]	Maize
Albumins + globulins	18.3	12	5.8	6	33.3	52	10.4	N/A
Prolamins	44.0	52	68.3	60	8.7	5	11.6	N/A
Glutelins[3]	18.5	25	14.0	26	6.9	18	18.9	N/A

[1]Average values for 2 sorghums (Taylor & Schüssler, 1986) (% of total protein)

[2]Wilson, 1987 (% of total N)

[3] Extracted glutelins

N/A Not available

1.2.2.2 Prolamins

The prolamins (aqueous-alcohol soluble proteins) are the major protein fraction in sorghum and maize. They are called kafirins in sorghum and zeins in maize, where they are found as protein bodies within the starchy endosperm (Taylor *et al*., 1984b, Lending & Larkins, 1989). Their main function is as nitrogen reserve for the plant (Taylor & Schüssler, 1986; Fukushima, 1991; Shewry, 2002). The prolamins in sorghum and maize cannot all be extracted by aqueous alcohol, alone as some prolamins exist crosslinked with themselves and other proteins and thus require a reducing agent to cleave these crosslinks and assist in their efficient extraction (Esen, 1987; El Nour *et al*., 1998). This gives rise to the uncrosslinked prolamins (prolamin I) extracted with aqueous alcohol alone and the crosslinked prolamin (prolamin II) requiring in addition a reducing agent. The combination of these two fractions makes up the total prolamins. The prolamin I is rich in monomeric α-prolamin with some oligomeric and polymeric components, whereas

prolamin II is rich in β- and γ-prolamins as well as in δ-zeins in maize (Watterson *et al.,* 1993; Shewry, 2002).

Hamaker, Mohamed, Habben, Huang & Larkins (1995) using aqueous alcohol plus reducing agent together with SDS as extracting solvent, reported total zein and kafirin contents of about 50-56% and 68-73% respectively in maize and sorghum whole grain flour. El Nour *et al.* (1998) and Taylor *et al*. (1984a), however, reported lower total prolamin protein contents (50.8 and 48% respectively) for sorghum whole grain flour using a sequential extraction with alcohol without and with reducing agent. The differences in prolamin contents reported by these authors could result from the differences in the extraction procedures used. The addition of SDS probably allowed for a more complete extraction of the prolamins, hence the higher values reported by Hamaker and co-workers. With endosperm flour, Taylor & Schüssler (1986) reported kafirin contents of 67-69% for sorghum, while (Yau *et al*., 1999) obtained zein contents of 52-61% for maize. Prolamin contents of endosperm flour are higher than those from whole-grain flour. This is so, because the removal of the germ entails loss of protein material mostly albumin and globulins, but not prolamins which, as stated, are located in protein bodies in the starchy endosperm (Taylor *et al*., 1984b; Lending & Larkins, 1989). This would then give a higher concentration of prolamin when it is expressed as a percentage of total protein. The prolamins generally contain a high proportion of the amino acids proline, asparagine, glutamine, leucine and alanine, but are low in lysine (Table 1.2) (Wilson, 1983; Taylor & Schüssler, 1986; Larkins, Lending & Wallace, 1993).

Zeins have been separated into α-, β-, γ- (Esen, 1987) and δ-zeins (Esen & Stetler, 1992) based on their solubility differences, amino acid composition, electrophoretic, chromatographic and immunological properties (Table 1.3). The kafirins, based on their similarity to zein (DeRose, Ma, Kwon, Hasnain, Klassy & Hall, 1989) and their solubility differences have been separated into the α-, β- and γ-kafirins (Shull, Watterson & Kirleis, 1991). Delta-kafirin homologues have recently been reported by Chandrashekar & Venkatesha (2001). Table 1.3 summarizes the solubility, sequence features and molecular weight properties of the different prolamin fractions (α-, β-, γ- and δ-) in maize and sorghum proteins.

Table 1.2. Amino acid content (mole % of amino acid) of the different kafirins and zeins

Amino acid	Total zein[1]	α-zein[2]	β-zein[2]	γ-zein[2]	Total kafirin[3]	α-kafirin[2]	β-kafirin[2]	γ-kafirin[2]
Asparagine (N)	4.1	5.3	2.5[a]	0	4.8	6.0	3.3[a]	0
Aspartic acid (D)	N/A	0	N/A	0	N/A	0.4	N/A	0
Threonine (T)	3.1	2.8	2.5	4.4	2.8	4.0	4.6	4.7
Serine (S)	6.5	6.9	5.0	3.9	4.7	6.0	4.6	5.2
Glutamine (Q)	16.4	20.7	18.1[b]	14.7	20.0	24.6	17.8[b]	11.9
Glutamic acid (E)	N/A	0.8	N/A	1.0	N/A	0.4	N/A	1.0
Proline (P)	10.7	8.9	8.8	25	11.2	7.7	9.7	23.3
Glycine (G)	2.5	0.8	8.8	6.4	2.7	1.6	6.8	8.8
Alanine (A)	14.5	13.8	13.8	4.9	15.6	14.9	13.4	5.7
Cysteine (C)	1.0	0.4	4.4	7.4	0.7	0.4	4.9	7.8
Valine (V)	4.0	6.9	1.9	7.4	5.6	4.4	5.2	6.2
Methionine (M)	1.5	2.0	11.3	0.5	1.7	0.8	5.7	1.0
Isoleucine (I)	3.7	4.5	0.6	2.0	4.1	5.6	2.3	2.6
Leucine (L)	19.4	17.1	10.0	9.3	15.4	15.3	12.0	8.3
Tyrosine (Y)	3.6	2.8	8.8	2.0	3.0	2.8	3.0	2.1
Phenylalanine (F)	5.6	3.3	0	1.0	4.7	2.4	1.9	1.6
Histidine (H)	1.1	1.2	0	7.8	1.6	1.2	0.9	7.8
Lysine (K)	0.1	0	0	0	0.2	0	0.5	0
Arginine (R)	1.3	1.6	3.1	2.5	1.2	0.8	2.7	2.1
Tryptophan (W)	N/A	0	N/A	0	N/A	0.4	N/A	0

[1] Esen, Bietz, Paulis & Wall. (1985)

[2] Taylor & Belton (2002)

[3] Evans Schüssler & Taylor (1987)

[a] Asparagine + Aspartic acid are expressed as Asparagine

[b] Glutamine + Glutamic acid are expressed as Glutamine

N/A Not available

Table 1.3. Characterisation of the protein species of zein and kafirin

Name	Molecular weight (k)	Optimum solvent for extraction[a]	Consensus repeat motifs / sequence features[b]	% of total prolamin protein
Maize[1,2,3,4]				
α- zein	21-25	50-95% propan-2-ol	AALQQFPAQL	75-85
β-zein	17, 18	30-85% propan-2-ol + 2-ME	Methionine-rich	10-15
γ-zein	27	Water plus reducing agent and up to 80% propan-2-ol plus reducing agent	8 repeats of PPPVHL; cysteine-rich	5-10
δ-zein	10	Water plus reducing agent and up to 80% propan-2-ol plus reducing agent	No repeats	N/A
Sorghum[4,5,6,7]				
α-kafirin	23, 25	40-90% t-butanol + 2-ME	AALQQFPAQL ANSYLQQ	80
β-kafirin	20, 18, 16	10-60% t-butanol + 2-ME		7-8
γ-kafirin	28	Water plus reducing agent or 10-80% t-butanol plus reducing agent	4 repeats of PPPVHL; cysteine-rich C-terminal domain	9-12
δ-kafirin	15	N/A	Methionine-rich	N/A

[a] 2-ME, 2-mercaptoethanol ; N/A not available

[1] Esen (1987), [2] Esen & Stetler (1992), [3] Lending & Larkins (1989)

[4] Shewry (2002), [5] Shull *et al*. (1991), [6] Watterson *et al*. (1993) [7] Chadrashekar & Venkatesha (2001)

[b] Refer to Table 1.2 for the single letter abbreviation for the amino acids

The amino acid composition of zein and kafirin are very similar; as are those of α-, β-, and γ-zein to their kafirin homologues α-, β-, and γ-kafirin (Table 1.2) (Wilson, 1983; Taylor & Belton, 2002). Detailed sequence analysis of the sorghum kafirin and maize zein using cDNA has revealed extensive sequence homology between the two proteins (DeRose *et al*., 1989) indicating structural similarity between them. Argos, Pederson, Marks & Larkins (1982) studied the M_r 19 and 22 k α-zein proteins using circular dichroism and found them to consist mainly of α-helical and β-turn structures with no β-sheet structure. They proposed a model for the structure of zein based on circular dichroism data and amino acid sequences. In this model (Fig. 1.1), it is proposed that zein consists of a collection of nine helical rods packed in a capsular shaped array with the end pieces of the capsule consisting of a glutamine repeat sequence, which serve to join the capsules together by hydrogen bonding. The helical structures themselves are held together by hydrogen bonds and Van der Waals interactions. However, only the α-zeins were considered in this model, and possibly explains why only α-helical structures were observed. Given the similarities between zein and kafirin proteins (DeRose *et al*., 1989), this model could be applied to the α-kafirin species as well.

Duodu, Tang, Grant, Wellner, Belton & Taylor (2001) using Fourier Transform Infra Red (FTIR) and solid state ^{13}carbon nuclear magnetic resonance (^{13}C NMR) spectroscopy with protein body-enriched flour samples showed that the prolamin proteins in both sorghum and maize as well as high-lysine sorghum mutants contained an abundance of α-helical structures together with β-sheet structures. Alpha-helical structures represented about 55-59% of secondary structure in these samples. These results agree with earlier reports from circular dichroism (CD), optical rotatory dispersion (ORD) and infra red (IR) spectroscopy (Wu, Cluskey & Jones, 1971), which indicated that kafirins had an abundance of α-helical and unordered structures with some β-turns. The kafirins studied by Wu *et al.* (1971) were dissolved in 60% *t*-butanol without a reducing agent, which could mean that mainly the α-kafirins were solubilized (Shull, Watterson & Kirleis, 1992). As stated, α-zein was shown to consist mainly of α-helices and β-turns (Argos *et al*., 1982), and this could explain the observation of only α-helices and β-turns in the kafirins analysed by Wu *et al.* (1971). The α-helical and β-sheet structures reported by Duodu *et al*. (2001) would appear to be more representative of the secondary structure

composition of sorghum and maize prolamin proteins in general, since all the different types of prolamins were taken in to account through the use of protein body enriched samples. Recent studies by Forato, Doriguetto, Fischer, Mascarenhas, Craievich & Colnago (2004), analysed the conformation of Mr 19 zein polypeptide using FTIR and reported 46% α-helix, 22% β-sheets, 23% β-turns and other undefined structures making up 13% of this protein.

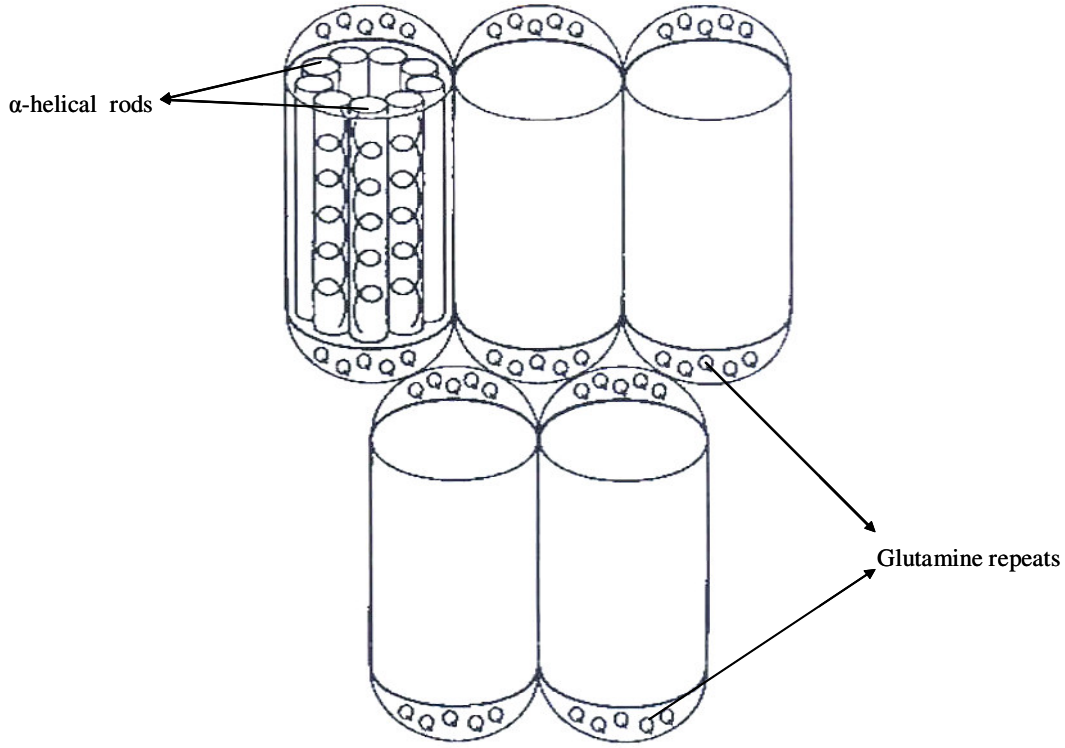

Figure 1.1. Structural model for maize zein (Argos *et al*., 1982)

1.2.2.2.1 Alpha-prolamins

Alpha-kafirin has high concentrations of glutamine together with the hydrophobic amino acids phenylalanine, leucine and isoleucine (Table 1.2) compared to β- and γ-kafirins

which explains the need for higher concentrations of alcohol for their extraction (Shull *et al*., 1992). It is, however, low in sulphur-containing amino acids and lysine. It occupies the central portion of the protein body (Shull *et al*., 1992). The alpha-zeins like their kafirin homologues are located at the centre of the protein body and are rich in proline, glutamine, and in the non-polar amino acids alanine and leucine (Lending & Larkins, 1989; Dombrink-Kurtzman & Bietz, 1993; Shewry, 2002). Alpha-kafirins are the most digestible of all the kafirins when isolated, but in intact protein bodies they are the least digested, because of their central location. This is so because protein bodies are digested from the outside inwards (Oria *et al*., 1995b).

1.2.2.2.2 Beta-prolamins

The β-kafirins compared to the α-kafirins, have low amounts of the hydrophobic amino acids phenylalanine, leucine and isoleucine but have a high content of the hydrophilic amino acid glycine (Table 1.2), which makes them soluble in low concentrations of alcohol (10-60% *tert*-butanol plus 2-mercaptoethanol) (Shull *et al.,* 1992). The β-kafirins and β-zeins are rich in methionine and cysteine with some of the cysteine residues forming interchain disulphide bonds. The latter explains the need for a reducing agent to efficiently extract them (Esen, 1987; Shull *et al*., 1992; Shewry, 2002). Unlike the α-kafirins, the β-kafirins do not contain repeated sequences or have a clear domain structure, but the methionine residues tend to be clustered (Shewry, 2002). Esen *et al*. (1985) found no N-terminal sequence homology between β-zeins and the other zeins and suggested that they may have a unique primary structure. Of all the zein proteins, β-zein is lowest in proline, valine, isoleucine, histidine and phenylalanine, but has high levels of methionine, glycine, alanine, tyrosine and arginine with four repeats each of Ala-Gly and Gly-Leu (Esen *et al.,* 1985). β-kafirins (Shull *et al.,* 1992) and β-zeins (Ludevid, Torrent, Martinez-Izquierdo, Puigdomenech & Palau, 1984) are located at the periphery of the protein body and also occur as inclusions within the protein body. Beta-kafirins in intact protein bodies are more digestible compared to α-kafirins, because of their peripheral location in the protein body that exposes it to proteolytic enzymes before α-kafirin (Oria *et al*., 1995b).

1.2.2.2.3 Gamma-prolamins

They are rich in the hydrophilic amino acids glycine and histidine (Table 1.2) and this could be the reason why they are extractable in low concentrations of alcohol (Esen, 1987; Shull *et al*., 1992) or even in water containing a reducing agent (Esen, 1986; Taylor, Von Benecke & Carlsson, 1989). Gamma-kafirin contains high levels of cysteine and proline with low levels of lysine and aspartic acid (Watterson, Shull, Mohamed, Reddy & Kirleis, 1990). The high amounts of cysteine in both γ-zein (Esen, 1987) and γ-kafirin (Shull *et al*., 1992) makes them prone to forming intermolecular disulphide bonds, hence the need for a reducing agent during their extraction. Gamma-kafirins contain the highest levels of disulphide crosslinked compounds compared to α- and β-kafirin (Oria *et al*., 1995b). The γ-prolamins differ from the other prolamins in being soluble in water when reduced, although a reducing agent is required for efficient extraction (Esen, 1986; Watterson *et al.,* 1993). They contain an N-terminal domain comprising highly conserved hexapeptide repeats (PPPVHL) with eight copies being present in γ-zein and four in γ-kafirin (Shewry, 2002). Gamma-kafirin (Shull *et al.,* 1992) and γ-zein (Ludevid *et al*., 1984) are found at the periphery of the protein bodies, together with the β-prolamins, and also as inclusions within the protein body. Gamma-kafirin, like β-kafirin, is more digestible than α-kafirins in intact protein bodies, also due to its peripheral location (Oria *et al.,* 1995b).

1.2.2.2.4 Delta-prolamins

Delta-zein comprises polypeptides with M_r of about 10 k and may be present at the protein body core interspersed with α- and γ-zein (Esen & Stetler, 1992). Delta-kafirin on the other hand has a reported M_r of about 15 k, and is rich in methionine. Its amino acid sequence consists of 134 amino acids with no repeat sequence (Chandrashekar & Venkatesha, 2001). Delta-zeins also do not contain repeated amino acid sequences but resembles the β-zeins in its amino acid composition (Shewry, 2002). It shows extensive immunological cross reactivity with α- and γ-zein (Esen & Stetler, 1992).

1.2.2.3 Glutelins

The glutelins are classified as proteins extractable in dilute alkali or the residue left after extraction of the salt soluble and aqueous alcohol soluble proteins (Wilson, 1983). Glutelins have been quantified as the amount of proteins remaining after sequential extraction with salt and aqueous alcohol without and with a reducing agent and found to represent less than 30% of total proteins in sorghum whole grain flour (Taylor *et al*., 1984a) and 24–29 % in sorghum endosperm flour (Taylor & Schüssler, 1986; Watterson *et al.*, 1993). The glutelins account for about 30-40% of maize proteins (Landry & Moureaux, 1970). They are the second largest protein fractions in sorghum after the prolamins (Taylor & Schüssler, 1986). The endosperm glutelins are high molecular weight proteins and are primarily located in the matrix surrounding the protein bodies (Taylor *et al*., 1984b). These glutelin polymers are crosslinked by intermolecular disulphide bonds (Wall, 1971; Wilson, 1983). Their amino acid pattern is similar to that of the albumins and globulins, both of which are high in lysine and thus are of good nutritional quality (Wilson, 1983).

1.2.3 Effect of Wet Cooking on Sorghum and Maize Protein Digestibility

Sorghum in the uncooked state has been reported to have a lower protein digestibility when compared to maize and other cereals such as rice and wheat (Axtell *et al*., 1981; Mertz *et al*., 1984; Hamaker *et al*., 1987; Duodu *et al*., 2002). The lower digestibility of sorghum proteins in the uncooked state is thought to be due to the formation of less digestible disulphide bonded protein polymers at the later stages of grain development up until maturity (Oria *et al*., 1995b). Duodu *et al*., (2002) however, stated that uncooked sorghum protein digestibility may not always be lower than that of uncooked maize but depends on the origin and physical form of the uncooked sorghum fractions. They observed a progressive increase in protein digestibility of uncooked sorghum flour as its composition varied from whole grain, through endosperm to protein body enriched flour (Table 1.4).

Table 1.4. Effect of wet cooking on the *in vitro* protein digestibility of sorghum and maize

Flour Sample	Protein Digestibility (%)		Enzyme Assay	Reference
	Uncooked	Cooked		
Sorghum whole grain	80.7	64.8	Pepsin	Hamaker *et al*. (1986)
	72.7	57.9	TC[1]	Hamaker *et al*. (1986)
	87.6	70.5	P-TC[2]	Hamaker *et al*. (1986)
Maize whole grain	81.5	81.9	Pepsin	Hamaker *et al*. (1986)
	79.4	87.7	TC	Hamaker *et al*. (1986)
	88.3	90.7	P-TC	Hamaker *et al*. (1986)
Sorghum whole grain	80.8	56.3	Pepsin	Hamaker *et al*. (1987)
Maize whole grain	83.4	79.3	Pepsin	Hamaker *et al*. (1987)
Sorghum whole grain	79.0	58.0	Pepsin	Rom *et al*. (1992)
Sorghum decorticated	69.2	43.6	Pepsin	Oria *et al*. (1995b)
Sorghum whole grain	73.2	55.2	Pepsin	Oria *et al*. (1995a)
Red sorghum whole grain	59.1	30.5	Pepsin	Duodu *et al*. (2002)
Red sorghum endosperm	65.7	35.9	Pepsin	Duodu *et al*. (2002)

Table 1.4 continues on next page

Table 1.4 continued

Red sorghum protein body preparation	72.8	44.2	Pepsin	Duodu *et al*. (2002)
White sorghum whole grain	55.8	36.6	Pepsin	Duodu *et al*. (2002)
White sorghum endosperm	67.4	39.4	Pepsin	Duodu *et al*. (2002)
White sorghum protein body preparation	74.3	63.5	Pepsin	Duodu *et al*. (2002)
Maize whole grain	66.6	62.0	Pepsin	Duodu *et al*. (2002)
Maize endosperm	67.4	63.6	Pepsin	Duodu *et al*. (2002)
Maize protein body preparation	68.8	67.4	Pepsin	Duodu *et al*. (2002)
Red sorghum whole grain	68.6	32.7	Pepsin	Nunes *et al*. (2004)
Maize whole grain	66.0	64.5	Pepsin	Nunes *et al*. (2004)
High lysine sorghums				
P721Q decorticated	86.7	71.9	Pepsin	Weaver *et al*. (1998)
P851171 decorticated	88.3	72.5	Pepsin	Weaver *et al*. (1998)
P850029 decorticated	89.4	75.1	Pepsin	Weaver *et al*. (1998)

[1]TC, Trypsin Chymotrypsin; [2]P-TC, Pepsin, Trypsin, Chymotrypsin

Upon wet cooking, as in porridge making, the digestibility of sorghum proteins decreases substantially compared to that of maize. This observation has been made both in human studies (Kurien, Narayanarao, Swaminathan & Subrahmanyan, 1960; MacLean *et al*., 1981, 1983) and *in vitro* protein digestibility studies (Axtell *et al*., 1981; Hamaker *et al.*, 1986, 1987; Oria *et al.,* 1995a, 1995b; Duodu *et al*., 2002). Kurien *et al*. (1960) observed a progressive reduction in digestibility of rice diets of schoolboys aged 10-11 years with increasing sorghum substitution. Digestibility decreased from 74.9% in an all rice diet to 55.2% in an all sorghum diet. In another study with Peruvian children MacLean *et al*. (1981) reported low *in vivo* digestibility (46%) for gruels made from sorghum, whereas rice, maize and wheat gruels had a higher digestibility, 66, 73 and 81% respectively. In *in vitro* studies using pepsin, wet cooking brought about a 24.5% reduction in protein digestibility of sorghum, while digestibility of maize only decreased by 4.1% (Hamaker *et al*., 1987). Numerous *in vitro* studies have established the reduced protein digestibility of wet cooked sorghum. Table 1.4 summarises several studies showing the effect of wet cooking on sorghum and maize protein digestibility.

The reduction in protein digestibility of sorghum on wet cooking has generally been attributed to the formation of disulphide linked protein polymers during cooking, which are resistant to proteolysis and as such could lead to a reduction in digestibility (Hamaker *et al*., 1987; Rom *et al.,* 1992; Oria *et al*., 1995b; Duodu *et al.,* 2003). Oria *et al*. (1995b) postulated that during wet cooking, β- and γ-kafirins could crosslink with themselves or with matrix proteins through disulphide bonds to form polymers that are resistant to proteolytic digestion.

Since the majority of β- and γ-kafirin proteins are found at the periphery of the protein body (Shull *et al.,* 1992), crosslinks between them could form a barrier around the protein body which could in turn restrict access of proteolytic enzymes to α-kafirins which are located within the protein body. The α-kafirins in isolation are more digestible than the β- and γ-kafirins (Oria *et al.,* 1995b) and represent the bulk (80%) of the kafirins (Watterson *et al.,* 1993). If the digestibility of α-kafirin is impaired, it could therefore result in a reduction in protein digestibility of sorghum. Pepsin indigestible residues from cooked sorghum comprised mainly α-kafirin (Oria *et al.,* 1995b) together with β- and γ-kafirin

(Oria *et al.,* 1995b; Nunes, Correia, Barros & Delgadillo, 2004), indicating that the reduced digestibility of wet cooked sorghum proteins may indeed be related to poor digestion of α-kafirin.

It has been demonstrated in uncooked sorghum, using scanning electron microscopy (SEM), that protein bodies are digested by pitting from the outer surface towards the centre (Rom *et al.,* 1992) as has been observed during germination using transmission electron microscopy (TEM) (Taylor, Novellie & Liebenberg, 1985). This would imply that β- and γ-kafirin at the protein body periphery would be degraded before the α-kafirins at the interior. Using TEM, Oria *et al.* (1995b) observed that in cooked sorghum the majority of the protein bodies remained undigested after treatment with pepsin. As stated, it has been postulated that during cooking β- and γ-kafirin located at the periphery of the protein body form less digestible disulphide bonded protein polymers with themselves and matrix proteins to form a protective coat around the protein body which is resistant to digestive enzymes (Oria *et al.,* 1995b). The TEM observations by Oria *et al.* (1995b) are therefore consistent with the formation of disulphide bonds as proposed by Hamaker *et al.* (1987).

A problem with this theory is that disulphide bonds are also formed in maize during wet cooking, but the digestibility of maize is not reduced to the same extent as that of sorghum (Hamaker *et al.*, 1987; Duodu *et al.*, 2002). It, however appears, that more disulphide-bonded oligomers are formed in sorghum during cooking compared to maize, which may in part explain the lower digestibility of sorghum proteins (Duodu *et al.*, 2002). However, the small differences in the amount of disulphide bonds formed cannot completely explain the differences in reduction in digestibility in sorghum and maize on wet cooking (Duodu *et al.*, 2003). The possibility of non-disulphide crosslinks participating in reducing protein digestibility of wet cooked sorghum has been suggested. Alkylation of uncooked kafirin and zein points to the possible formation of non-disulphide crosslinks during cooking (Duodu, 2000). In the alkylation process, free thiol groups are reacted with an alkylating agent such as iodoacetamide or iodoacetate to prevent their oxidation to form disulphide bonds (Hollecker, 1997). Alkylated uncooked and cooked kafirin and zein samples were found to be more digestible compared to

unalkylated samples (Duodu, 2000). However, alkylated and cooked kafirin had a much lower digestibility than alkylated uncooked kafirin, suggesting that non-disulphide crosslinks may also be involved in reducing protein digestibility of wet cooked sorghum. High molecular weight kafirin oligomers of M_r 45–60 k (Duodu *et al.,* 2002; Nunes *et al.,* 2004) have been identified in wet cooked sorghum, and in the pepsin indigestible residues from cooked sorghum. These oligomers were not completely cleaved by a reducing agent, also suggesting that disulphide bond formation may not be the only reason for the reduction in sorghum protein digestibility with wet cooking.

The kafirins and the zeins are known to be hydrophobic proteins, but the kafirins appear to be slightly less soluble, hence more hydrophobic than the zein (Wall & Paulis, 1978). The slightly hydrophobic nature of kafirin could also contribute to the difference in its protein digestibility compared to zein, since enzymes function in an aqueous environment. Therefore, if kafirins are more hydrophobic than zeins, they may be less accessible to the enzymes and consequently less digestible (Duodu *et al*., 2003). In fact, α-zein and α-kafirin have the same level of hydrophobicity, whereas it is the γ-kafirin that is slightly more hydrophobic than γ-zein (Duodu *et al*., 2002). Considering that the γ-kafirin and γ-zein are located primarily at the periphery of the protein bodies and come in contact with the enzyme first, a more hydrophobic γ-kafirin could compound the problem of an already low digestible disulphide bonded γ-kafirin (Oria *et al*., 1995b), and hence, reduce the digestibility of the protein even further.

Duodu *et al*. (2001) studied the changes brought about in the secondary structure of sorghum and maize proteins by wet cooking using FTIR and ^{13}C NMR. They found that during wet cooking the proteins assume a more antiparallel intermolecular β-sheet structure, accompanied by a reduction in α-helices. It is possible therefore that the change to β-sheet conformation during cooking could encourage the formation of disulphide crosslinks between polypeptides in close proximity, resulting in a rigid, less digestible structure (Duodu *et al*., 2001). The same structural changes have been observed in high lysine sorghums and also in popped sorghum (Duodu *et al*., 2001). However, the maize, high lysine sorghum and popped sorghum were more digestible than the normal sorghum

when cooked (Parker, Grant, Rigby, Belton & Taylor, 1999; Duodu *et al*., 2001; 2002). The changes in protein secondary structure, as with the reduction in digestibility, occurred to a slightly greater extent in normal sorghum than in maize, high-lysine sorghum and popped sorghum. It is possible therefore, that the formation of β-sheet structure could be related to the decrease in protein digestibility of wet cooked sorghum and maize. Notwithstanding this, it is difficult to believe that these small structural differences could alone account for the large decreases in sorghum protein digestibility. In spite of all the possible reasons advanced for the observed differences between sorghum and maize cooked protein digestibility, the exact reasons for this are still not known.

Besides wet cooking, the condensed tannins present in some sorghum cultivars can bind the sorghum proteins and render them less digestible (Butler, Riedl, Lebryk & Blutt, 1984). The kafirin (Emmambux & Taylor, 2003) together with the salt-soluble proteins (Jambunathan & Mertz, 1973; Chibber, Mertz & Axtell, 1978) are the ones most affected by tannin binding. Cell walls may also form a barrier restricting access of proteolytic enzymes to proteins within the endosperm cells (Duodu *et al*., 2002) contributing to reducing digestibility.

In high-lysine sorghum mutants, higher uncooked and cooked protein digestibility (Table 1.4) was found to be a result of more rapid digestion of the main sorghum storage protein α-kafirin (Weaver *et al*., 1998). In these sorghum mutants (Fig. 1.2), the protein body structure is not spherical but highly invaginated and α-kafirin is exposed at the surface of the protein body with γ-kafirin located at the base of the invaginations (Oria, Hamaker, Axtell & Huang, 2000). The more digestible α-kafirin is thus directly exposed to proteolytic enzymes during digestion, accounting for the higher protein digestibility in these mutants. It therefore appears that the structure of sorghum protein bodies influences the access of digestive enzymes to the kafirin proteins and accordingly its digestibility. However, one drawback with these high-lysine mutants is that their endosperms of are of a floury type (Mertz, 1992; Weaver *et al*., 1998) making them more prone to insect damage (Mertz, 1992).

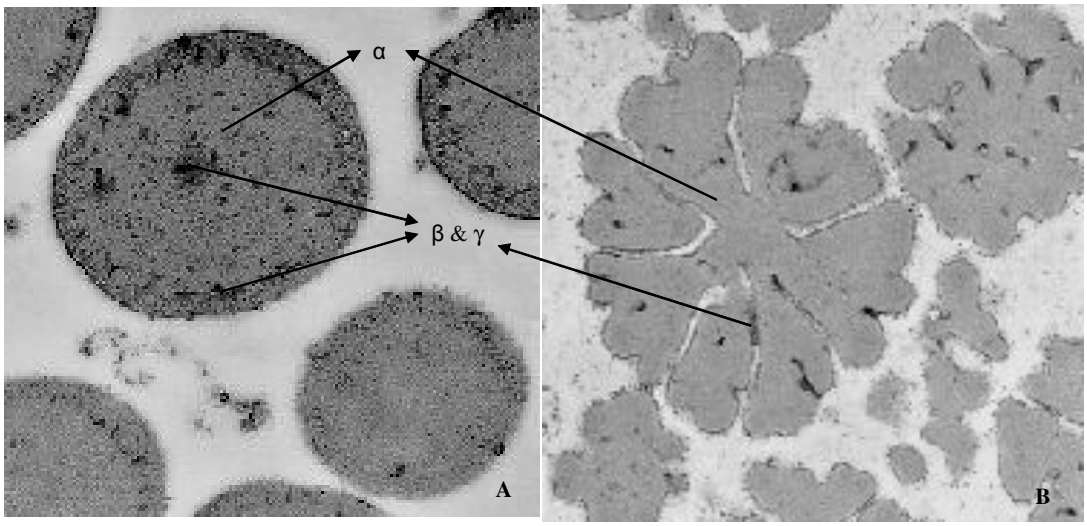

Figure 1.2. Sorghum protein bodies in a protein matrix. (A) Spherical protein body of a normal sorghum. (B) Protein body of a high digestibility sorghum mutant with invaginations (Oria *et al*., 2000)

1.2.4 Effect of Some Processing Methods on Sorghum and Maize Protein Digestibility

The reduction in protein digestibility obtained with wet cooking in sorghum has not been observed when dry heat treatments were applied to sorghum. Popping is a dry heat treatment that expands the starch granules in the grain to produce an expanded grain (Parker *et al*., 1999). *In vitro* protein digestibility of sorghum and maize grains was not reduced by popping (Parker *et al*., 1999). Popping induces fragmentation of the cell walls that will in turn allow proteolytic enzymes greater access to the proteins, resulting in a high digestibility (Parker *et al*., 1999).

Attempts have been made to improve wet cooked digestibility of sorghum through processing with varying degrees of success. When the reducing agents, sodium

metabisulphite, ascorbic acid or L-cysteine (El Khalifa, Chandrashekar, Mohamed & El Tinay, 1999), sodium bisulfite (Rom *et al*., 1992; Arbab & El Tinay, 1997), and 2-mercaptoethanol, sodium bisulphite, dithiotheitol or L-cysteine (Hamaker *et al*., 1987) were mixed with sorghum flour before cooking they prevented the reduction in protein digestibility observed on wet cooking. Protein digestibility of sorghum cooked under reducing conditions was 25% higher than the digestibility of sorghum cooked in water alone (Hamaker *et al.,* 1987). When maize flour was cooked with reducing agent, protein digestibility increased by only 5% (Hamaker *et al*., 1987). According to these authors, the fact that reducing agents could prevent a reduction in sorghum protein digestibility on wet cooking supports the proposition that the formation of disulphide bonds during cooking plays a major role in making sorghum gruels less digestible.

Extrusion cooking has been found to improve cooked sorghum protein digestibility (MacLean *et al*., 1983; Mertz *et al*., 1984; Fapojuwo, Maga & Jansen, 1987; Hamaker, Mertz & Axtell, 1994). In fact, the proteins of extruded sorghum were found to be well digested *in vitro* (79%) (Mertz *et al*., 1984) and *in vivo* (81%) (MacLean *et al*., 1983) compared to the wet cooked sorghum where *in vitro* protein digestibility was only 57% (Mertz *et al*., 1984). The extrusion cooking process employs heat and shearing action that could disrupt the structure of the protein bodies, possibly breaking disulphide and other bonds. This would in turn facilitate access by digestive enzymes to the more digestible α-kafirin at the centre of the protein body, hence the increase in digestibility (Hamaker *et al*., 1994).

Fermentation gives some minimal improvement in cooked sorghum protein digestibility (Au & Fields, 1981; Kazanas & Fields, 1981; El Khalifa & El Tinay, 1995; Hassan & El Tinay, 1995; Taylor & Taylor, 2002). A modification in protein structure rather than breakdown of proteins to smaller components was proposed as the mechanism by which fermentation improved protein digestibility (Taylor & Taylor, 2002).

From the foregoing it appears that the hindering of α-kafirin digestion by crosslinking involving the γ- and β-kafirins with themselves and with matrix proteins in conjunction with their peripheral location in the protein body is the most important factor influencing

protein digestibility in sorghum. It would appear therefore, that, if kafirin structure is modified to impede crosslinking of γ- and β-kafirins with themselves and matrix proteins, this could allow proteolytic enzymes better access to α-kafirins and other proteins, and contribute towards enhancing cooked sorghum protein digestibility.

1.2.5 Food Irradiation

Food irradiation involves treating foods with ionising radiation (γ-rays, X-rays and electron beam) (WHO, 1988). It is called ionising radiation because it removes electrons from atoms and molecules and in the process converts them into ions (Thakur and Singh, 1994). However, not all kinds of ionising rays may be used with foods. The α–particles for example are not used because of their low penetrating power, whereas high-energy electrons and X–rays generated above certain energy levels may induce radioactivity in the irradiated food. In view of these constraints a joint FAO/IAEA/WHO expert committee meeting recommended the following types of ionising radiation for use in foods (FAO/IAEA/WHO, 1981):

Gamma rays from ^{60}Co and ^{137}Cs at energy levels of 5 MeV (1 eV = 1.6 x 10^{-19}J)

X–rays generated from machine sources at or below energy levels of 5 MeV

Electrons generated from machine sources at or below an energy level of 10 MeV.

1.2.5.1 Mechanism of Action of Ionizing Radiation

When ionising radiation penetrates food, the atoms and molecules in the food absorb its energy. Depending on the amount of energy absorbed these atoms and molecules could either become excited (I), lose an electron (II) or dissociate (III) to form ions or free radicals. These ions and excited species are unstable and chemically reactive. They can react with each other or with other components in the food to form stable products and in the process modify food components (Urbain, 1986, Thakur & Singh, 1994).

$RH \rightarrow {}^{\cdot}RH$ Excitation (I)

$RH \rightarrow {}^{\cdot}RH^{+} + e^{-}$ Ionization (II)

$RH \rightarrow {}^{\cdot}R + {}^{\cdot}H$ Dissociation (III)

In dry food systems, the ions and excited species formed are not completely free to move about and thus combine with free radicals and ions in their vicinity to form stable products (Thakur & Singh, 1994). In such systems therefore, food molecules would be modified mostly through direct interaction between ionising radiation and the food molecule (Garrison, 1987; Kempner, 1993; Thakur & Singh, 1994).

On the other hand, in moist systems, the water molecules are ionised to produce reactive species that can move about and react with other ions and free radicals causing extensive modification in food macromolecules (Kempner, 1993; Thakur & Singh, 1994). The effects of irradiation in foods will therefore be expected to be more pronounced in moist than in dry systems given the additional effects of water radiolysis products (Kempner, 1993) and the increased accessibility of the free radicals (Cho, Yang & Song, 1999).

Radiolysis of water

$H_2O \rightarrow {}^{\cdot}OH + e^{-}_{aq} + {}^{\cdot}H + H_2O_2 + H_3O^{+}$ (Thakur & Singh, 1994)

1.2.5.2 Effect of Irradiation on Proteins

Proteins are made up of amino acids linked together by peptide bonds (Cheftel *et al.*, 1985). Changes in proteins brought about by irradiation will therefore be reflected as changes in their primary, secondary and tertiary structures (Kempner, 1993).

Some amino acids such as the sulphur containing and aromatic amino acids are reported to be more susceptible to radiation damage than the others (Khattak & Klopfenstein, 1989; Diehl, 1990; Swallow, 1991). When maize grain was irradiated at 5 kGy methionine and cysteine contents both decreased by about 18% (Khattak & Klopfenstein, 1989). In another study by Hooshmand & Klopfenstein (1995) maize grains were surface

sterilised in 80% aqueous methanol, followed by 2% sodium hypochlorite (NaOCl), washed with distilled water and air dried. The dried grains were then milled into flour and irradiated. At low doses of 5 kGy there was no significant difference in cysteine and methionine contents. When the irradiation dose was increased to 20 kGy, methionine content decreased by about 18%, whereas cysteine content was not significantly affected. The reason for the differences in the effects of irradiation on cysteine and methionine contents in maize in these two studies is not clear, but could be related to variability that occurs in the amino acid analysis (Finley, 1982). In both studies the proteins were hydrolysed with *p*-toluenesulphonic acid and amino acids determined chromatographically using an amino acid analyser. As explained earlier, the β- and γ-prolamin proteins of sorghum and maize that have been proposed to form disulphide crosslinks during cooking and cause the reduction in protein digestibility in wet cooked sorghum (Hamaker *et al*., 1987) contain high levels of cysteine (Shewry, 2002). If this amino acid is affected by irradiation, then these proteins will also be affected and this may influence digestibility.

Destruction of amino acids during irradiation can occur through dimerisation, which is the combination of two amino acid radicals to form dimers or trimers (Swallow, 1991). An example is bityrosine, a covalently bound biphenol, produced by the reaction of two tyrosyl radicals or a tyrosyl radical and a tyrosine molecule (Davies *et al.,* 1987a; Tuce, Janata, Radojčić & Milosavljević, 2001). Irradiation of caseinates to produce edible film enhanced crosslinking of tyrosine residues (Mezgheni *et al.,* 1998). Bityrosine formation in these films increased with increasing radiation dose from 8 to 128 kGy. Intermolecular bityrosine formation has been implicated in the crosslinking of proteins during irradiation (Davies, 1987; Davies & Delsignore, 1987). Such crosslinks may impair the digestibility of food proteins (Davies *et al*., 1987b). Cysteine molecules in foods could also crosslink during irradiation to form disulphide crosslinks, which leads to polymerisation of proteins (Swallow, 1991). As discussed, disulphide crosslinks are the major cause of reduced cooked protein digestibility in wet cooked sorghum. If such crosslinks are formed during irradiation of sorghum, it may further compound the problem of reduced digestibility when sorghum is wet cooked.

Aggregation of proteins is common when food proteins are irradiated in the absence of oxygen. However, in oxygenated systems, irradiation of proteins yields mainly fragmentation products (Schuessler & Schilling, 1984; Puchala & Schuessler, 1993; Tuce *et al*., 2001). Using bovine serum albumin (BSA) as a model protein, Schuessler & Schilling (1984) proposed a mechanism for the fragmentation of proteins in oxygenated systems. This begins with an attack on the protein by the $OH^{\cdot}$ radical to form a protein radical (1), which then reacts with oxygen to form a peroxy radical (2). Two peroxy radicals can combine to form an oxyradical (3), which can later decompose into fragments (F) (4). If oxygen is absent the protein radicals can combine to form aggregates also known as protein polymers or protein crosslinks.

$$PH + OH^{\cdot} \rightarrow P^{\cdot} + H_2O \qquad (1)$$

$$P^{\cdot} + O_2 \rightarrow PO_2^{\cdot} \qquad (2)$$

$$2PO_2^{\cdot} \rightarrow 2PO^{\cdot} + O_2 \qquad (3)$$

$$PO^{\cdot} \rightarrow F_1 + F_2 \qquad (4)$$

However, in practice foods will not normally be irradiated under oxygen or nitrogen. In practical food irradiation therefore the effects of oxygen may be minimal because the oxygen present in the system becomes depleted as irradiation proceeds (Swallow, 1991). This appears to suggest that at higher doses of irradiation aggregation of proteins may be favoured because of the absence of oxygen, if the oxygen in the system gets used up and is not replenished. However, before either fragmentation or aggregation can take place the proteins first have to be denatured, as the unfolding of the proteins facilitates both fragmentation and aggregation (Davies & Delsignore, 1987). Thus, at low doses of irradiation fragmentation of proteins is more likely to occur, whereas as the dose is increased the unfolded or fragmented proteins may then aggregate to form high molecular weight protein polymers. This has been demonstrated with irradiated (0.5-10 kGy) solutions of egg white proteins (ovalbumin, ovomucoid, ovotransferrin) (Kume & Matsuda, 1995; Moon & Song, 2000), BSA and lactoglobulin solutions irradiated at 0.5-10 kGy (Cho *et al*., 1999) and in irradiated (1-10 kGy) bovine and porcine plasma protein solutions (Lee, Lee & Song, 2003). In these studies, the formation of high molecular

weight polymers was negligible at low doses, but increased with increasing irradiation dose. Fragmentation and aggregation of proteins may lead to changes in protein structure. Such changes in proteins have been shown to influence their susceptibility to proteolysis (Davies, 1987; Davies *et al*., 1987b).

Proline residues have been identified as possible targets for peptide chain scission because tertiary amide bonds involving proline are easier to oxidise than secondary amide bonds that are formed by the other amino acids (Schuessler & Schilling, 1984). Gamma-prolamin proteins of sorghum and maize are high in proline and could be fragmented by irradiation. Fragmentation of proteins would perhaps expose more peptide bonds to proteolytic attack. Splitting of disulphide bonds could also cause fragmentation of the proteins (Di Simplicio *et al*., 1991).

Lysine is a limiting amino acid in most cereals including sorghum and maize (Van Scoyoc *et al*., 1988; Yau *et al*., 1999) and it may be destroyed by irradiation. Lysine content decreased by 7 and 13% respectively, when maize and wheat flours were irradiated at 7.5 kGy (Hooshmand & Klopfenstein, 1995). Irradiation (5 kGy) of maize and wheat grains reduced lysine content by 12 and 11%, respectively (Khattak & Klopfenstein, 1989). On the other hand, the lysine contents of isolated wheat gluten and free lysine content of wheat flour were not affected by irradiation at 10 kGy (Srinivas, Ananthaswamy, Vakil & Sreenivasan, 1972). The mechanism of lysine loss or destruction by irradiation is not certain but may be similar to what occurs when food is heated, given that the chemical effects of irradiation on foods are similar to those of heat treatment (Diehl, 1990; Kume & Matsuda, 1995). When protein foods are heated, they may undergo Maillard reactions in which the $\in$-amino group of lysine reacts with the carbonyl group of a reducing sugar resulting in destruction of lysine (Hurrell, 1984). Similar reactions may occur during irradiation of proteins and cause loss of lysine. Evidence of Maillard reactions occurring during irradiation of proteins has been found by Krumhar & Berry (1990) in BSA and bovine lactalbumin and also in beans by Cunha *et al.* (1993). Irradiation of maize flour (Rombo, Taylor & Minnaar, 2001) and of rice grains (Wootton, Djojonegoro & Driscoll, 1988) resulted in darkening of these products and was attributed to Maillard reactions.

In spite of the fact that irradiation may cause loss of some essential amino acids it has also been reported to improve absorption of some amino acids and proteins. Lysine availability in maize grains increased by 7.5% following irradiation at 5 kGy (Khattak & Klopfenstein, 1989). Irradiation (30-90 kGy) of coarsely ground barley and oats improved amino acid absorption and feed utilisation in chicks (Campbell, Classen & Ballance, 1986). It was suggested that increased feed absorption and utilisation in this case was not linked to a direct effect of irradiation on the amino acids or the proteins but rather to the depolymerisation of β-glucans that reduced the viscosity of the feed, thereby improving protein absorption by chicks. Apparently, the observed effects of irradiation on proteins in food systems, as opposed to pure systems, may not always be directly related to the effect of irradiation on the proteins themselves but could be a consequence of irradiation effects on different components of the food. As stated, some sorghums contain condensed tannins that can complex with proteins and render them less digestible (Butler *et al*., 1984). Reduction of tannins with irradiation has been reported in sorghum (Abu-Tarboush, 1998) and could improve the protein digestibility of condensed tannin sorghums.

Moon & Song (2000) used circular dichroism CD to measure changes in protein secondary structure of egg white proteins, ovalbumin, ovomucoid and ovotransferrin with irradiation up to 10 kGy. Ovalbumin and ovomucoid have mostly α-helical and β-sheet structures, respectively. When solutions of these proteins were irradiated at 10 kGy there was an increase in unordered structures and random coils, suggesting that the covalent bonds holding the proteins together in their native conformation had been cleaved leading to denaturation and unfolding of the proteins. Sorghum and maize proteins both have α-helical and β-sheet structures in the uncooked state (Duodu *et al*., 2001). Irradiation could have similar effects on these proteins (denaturation and unfolding), that may prevent disulphide bond formation during subsequent cooking and thus prevent the reduction in protein digestibility with wet cooking; this, together with the fact that irradiation can cleave disulphide bonds (Di Simplicio *et al.*, 1991; Köksel *et al*., 1998), which also occur in the proteins of mature sorghum and maize (Vivas-Rodriguez, Waniska & Rooney, 1992; Hamaker *et al*., 1994).

Glutenin proteins of wheat are polymeric proteins linked by intermolecular disulphide bonds (Stauffer, 1998). Srinivas *et al.* (1972) found that irradiation of wheat flour at 10 kGy resulted in a reduction in the glutenin chromatographic peaks, and an increase in the low molecular weight proteins, suggesting that disulphide bonds of glutenin may have been cleaved to fragment proteins. Similar observations have been made by Köksel *et al.* (1998) who irradiated wheat at 2.5-20 kGy and extracted the 50% 1-propanol insoluble glutenin proteins. This fraction consists of HMW-GS (high molecular weight-glutenin subunits) that are believed to be directly related to dough quality (Stauffer, 1998). Using CD, size exclusion HPLC and SDS-PAGE, Köksel *et al.* (1998) investigated the effects of gamma irradiation on the 50% 1-propanol insoluble glutenin and gliadin fractions. Wheat gliadins are single chain proteins with no crosslinks (Stauffer, 1998), and these were not affected by gamma irradiation. On the other hand, the concentrations of HMW-GS and LMW-GS (low molecular weight-glutenin subunits) in the 50% 1-propanol insoluble glutenin fraction were both reduced by irradiation at 10 and 20 kGy (Köksel *et al.*, 1998). The decrease was evident both on SDS-PAGE and HPLC. Irradiation, however, had a greater effect on the HMW-GS, which may be associated with the disruption of disulphide bonds. This seems to suggest that disulphide bonds are susceptible to irradiation damage. Working with BSA that has 17 disulphide residues per molecule, Di Simplicio *et al.* (1991) also demonstrated that irradiation could cleave disulphide bonds leading to fragmentation of these proteins. Other reports of radiation cleaving disulphide bonds appear in the literature (Doguchi, 1969; Garrison, 1987; Swallow, 1991)

1.2.6 Conclusions

Wet cooking causes a reduction in the digestibility of sorghum and maize proteins. The reduction is more acute in sorghum than in maize. The major cause for this reduction is believed to be the formation of disulphide bonded polymers between γ- and β-prolamin proteins. These disulphide bonded protein polymers are resistant to proteolysis, hence, the reduction in digestibility. Irradiation can cleave disulphide bonds in proteins (Di

Simplicio *et al.*, 1991; Köksel *et al.*, 1998). Information is, however, lacking about the effects of irradiation on sorghum and maize proteins. Considering that sorghum prolamin proteins like the glutenin proteins of wheat, contain disulphide bonds, and have α-helical and β-sheet secondary structures, and that the disulphide bonds in wheat are cleaved by irradiation (Srinavas *et al.*, 1972; Köksel *et al.*, 1998), it could be expected that irradiation might cleave the disulphide bonds in sorghum and maize prolamin proteins and cause changes in protein structure. Modification of the primary, secondary and tertiary structure of proteins by irradiation has been shown to improve susceptibility of proteins to proteolytic enzymes, possibly by exposing more peptide bonds to enzymatic hydrolysis (Davies, 1987; Davies *et al.*, 1987b). How these structural changes would affect protein digestibility of sorghum and maize is not known.

1.3 Objectives

The goal of this study was to establish whether irradiation could be used to alleviate the reduction in protein digestibility of sorghum proteins when cooked into porridge, in comparison with maize

The specific objectives were to:

Determine the effects of irradiation in wet and dry systems with and without cooking on the *in vitro* protein digestibility of sorghum and maize flours.

Compare the pepsin and multi-enzyme methods of determining *in vitro* protein digestibility in sorghum and maize.

Determine the effects of irradiation and cooking of sorghum and maize flours on disulphide bond concentration, and how this affects the extractability of their prolamins and their protein digestibility.

Determine changes brought about by irradiation and cooking on some molecular properties of sorghum and maize proteins using techniques such as sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Fourier transform infrared spectroscopy (FTIR).

Determine the fundamental cause of the effects of irradiation on sorghum and maize protein digestibility.

1.4 Hypotheses

Irradiation will cleave disulphide bonds in sorghum and maize prolamin proteins, as has been observed with the disulphide bonds in wheat gluten proteins, resulting in a change in protein structure.

A change in protein structure brought about by irradiation can in turn improve susceptibility of the sorghum and maize proteins to proteolytic enzymes by exposing additional peptide bonds to proteolysis.

Irradiation in wet medium would generate more free radicals through the radiolysis of water, and as such bring about more changes in sorghum and maize proteins.

High dose of irradiation would also bring about greater changes in the proteins of sorghum and maize, since high doses generate more radiation energy that could produce more free radicals.