3 DISCUSSION

This section will first discuss some of the methodologies used in this study. Subsequently, it will go on to examine the main finding of this research, which is that irradiation of sorghum flour before cooking into porridge partially alleviated the reduction usually observed in pepsin protein digestibility when sorghum is cooked into porridge. A mechanism by which this occurs is proposed, and reasons are advanced as to why the relief in reduction of digestibility is higher at low dose (10 kGy dry) of irradiation, but decreases when the dose is increased to 50 kGy and when the flour samples are irradiated in wet medium.

3.1 Methodological Considerations

Various methods were used in this study. The key methods will be discussed here; their principles, strengths and limitations, as well as modifications made to original methods, and why. A discussion of how good the methods were in measuring the desired effects and what could have been done to improve their performance where necessary will also be provided.

This study set out to investigate the effects of irradiation and cooking on the protein digestibility of sorghum in comparison with maize. It has been established that the effects of irradiation on proteins, as well as on any food component, are influenced by the moisture content of the sample (Cieśla *et al*., 2000; Lee *et al*., 2003). This is so because radiolysis of water generates free radicals with high oxidizing and reducing potentials. In a liquid environment these free radicals are free to move about and react with food components, thus causing extensive modification to the proteins compared to when irradiation is carried out on dry solids or flour samples with low moisture content (Cieśla *et al*., 2000). Irradiation was therefore carried out additionally in wet medium, as this was expected to heighten the effects of irradiation on sorghum and maize proteins for clearer understanding. High dose (50 kGy) of irradiation was also employed for similar reasons. The use of 30% solids content for the wet irradiated and porridge samples was prompted by the fact that it approximates the concentration at which stiff porridges are prepared

from sorghum and maize (Murty & Kumar, 1995). From the results obtained, it was apparent that the effects of irradiation were more intense at high dose and also in the wet irradiated samples, and this was particularly evident in the cooked samples, where the problem of sorghum protein digestibility resides.

Whole grain sorghum and maize flour are commonly used for preparing stiff porridges in the northern and western parts of Cameroon respectively (Fombang, personal observation). Hence, the choice of whole grain flours. It was an added advantage that the sorghum grains used were condensed tannin-free cultivars, which avoids the complication of tannin-protein interactions interfering with digestibility (Chibber *et al,* 1980).

In order to be able to determine the effects of irradiation combined with cooking on proteins at the molecular level, it was essential to concentrate the proteins in the samples. Given that the problem of reduced protein digestibility in sorghum occurs when it is cooked into porridge, it was decided to consider only the cooked samples for molecular studies, with the unirradiated cooked samples serving as the control. This decision may not have been the best as it was later discovered that it would have been helpful to have the unirradiated uncooked control samples to be better able to judge if the observed effects were due to irradiation alone or a combination of irradiation and cooking.

Protein concentration was carried out according to the wet milling procedure of Taylor *et al*. (1984b). Given that the prolamin proteins are the ones that influence protein digestibility of sorghum and maize significantly (Hamaker *et al*., 1987; Oria *et al*., 1995a; Duodu *et al*., 2003), a decision was made to first extract the salt soluble proteins before wet milling. Wet milling mechanically disrupts the protein and starch complex and separates cell wall fibres from the starch and proteins (Eckhoff *et al*., 2003). The fibres are then removed by sieving. In the normal wet milling procedure uncooked samples are used. Because the samples used in this study were cooked porridges, the gelatinized starch made sieving the wet milled products impossible. To overcome this problem, the porridges were treated with α-amylase to break down gelatinized starch before wet milling. This treatment thinned the slurry and it could then be sieved.

The wet milling did not, however, produce pure proteins as would have been desired. The low protein concentration (35-50%) of the protein preparations suggests that the proteins were not completely separated from the starch and fibres. Starch-protein interactions that occur during cooking (Zhang & Hamaker, 1998) may hinder the effective separation of starch from proteins and thus contribute to the low purity of the protein preparations. Nonetheless, the samples were sufficiently protein rich that they could be used for the proposed analyses.

Concerning assaying for protein digestibility, the pepsin assay is a single enzyme assay that has been widely used in estimating protein digestibility of sorghum and maize proteins and especially in determining the effects of wet cooking on sorghum and maize protein digestibility (Mertz *et al*., 1984; Hamaker *et al*., 1987; Duodu *et al*., 2002). It involves incubating the sample with pepsin at acidic pH and measuring the amount of insoluble protein. One of the drawbacks of this method is that it could underestimate protein digestibility for proteins that are resistant to pepsin because of their primary structure or acid stability of their tertiary structure (Swaisgood & Catignani, 1991). However, in the case of sorghum and maize it could be expected to give a good indication of protein digestibility given that pepsin preferably hydrolyses bonds containing hydrophobic amino acid residues (Huang & Tang, 1968). In sorghum and maize, the predominant proteins are the prolamins (Guiragossian *et al*., 1978; Taylor *et al*., 1984b), which are high in the hydrophobic amino acids proline, alanine and leucine (Esen *et al*., 1985, Esen, 1987; Taylor & Belton, 2002). Added to this is the fact that results obtained in human studies with sorghum (MacLean *et al*., 1981, 1983) paralleled those obtained with pepsin (Axtell *et al*., 1981; Mertz *et al*., 1984).

The multienzyme pH stat assay on the other hand, determines protein digestibility using a combination of three enzymes; trypsin, chymotrypsin and amino-peptidase (Pederson & Eggum, 1983). It is based on the principle that during proteolysis protons are released from the cleaved peptide bonds resulting in a decrease in pH of the suspension (Boisen & Eggum, 1991). Assuming a correlation between the initial rate of peptide release and protein digestibility, the later can be predicted by measuring the amount of base needed to maintain the pH of the reaction mixture constant for a period of 10 min. It has been used

to estimate protein digestibility of uncooked sorghum and maize (Weaver *et al*., 1998; Aboubacar *et al*., 2001) and found to correlate (r = 0.86) well with pepsin digestibility (Aboubacar *et al*., 2001) under those conditions. With extruded sorghum (Gomez, Waniska, Rooney & Lusas, 1988), however, the pH-stat multienzyme method indicated that digestibility of protein was less affected by extrusion than was indicated by the pepsin assay. It is, however, difficult to draw any conclusion from these two studies about the performance of the multienzyme assay, as the problem of protein digestibility in sorghum is with the wet cooked sorghum, and not with the uncooked or extruded samples (reviewed by Duodu *et al*., 2003).

Given that the pH-stat assay correlated well with pepsin in determining protein digestibility of uncooked sorghum and maize, and has the advantage over pepsin of being relatively more rapid, its performance was investigated with wet cooked sorghum and maize. The results obtained with the pH-stat method in this study do not show a reduction in protein digestibility of sorghum with cooking as has repeatedly been reported in literature (reviewed by Duodu *et al*., 2003), and suggests therefore, that, this assay may not be suitable for determining the effects of wet cooking on sorghum protein digestibility.

As stated, in section 2.2, the amount of extractable prolamins in sorghum and maize is believed to be somehow related to their protein digestibility (Hamaker *et al*., 1986; Hamaker *et al*., 1994). The prolamin proteins of the sorghum and maize flour samples were extracted first without and then with reducing agent to obtain the uncrosslinked (prolamin I) and crosslinked prolamins (prolamin II) (Guiragossian *et al*., 1978; Shewry, 2002), and the extracts freeze-dried. The lower kafirin and zein content obtained in this study for the unirradiated uncooked samples in comparison with reported values (Taylor *et al*., 1984a; Hamaker *et al.,* 1995) indicates that not all of the kafirins and zeins were extracted. The low extractability of the prolamins was due to some problems encountered while carrying out the extraction. It was impossible to determine the protein content of the liquid extracts using the Dumas method because their protein concentration was too dilute (~0.09% N) to be detected using the Leco protein/nitrogen analyzer. It was then resolved to freeze-dry the extracts before determining their protein content. The extracts

were poured into weighed aluminium plates for freeze-drying. After drying, the plates together with the samples were weighed before transferring the powder samples to bottles for storage at 4ºC. The dry samples were stored for about three weeks before determining their protein content, and during this period they probably picked up moisture that would have diluted their nitrogen content. Because of the small quantity of the samples (< 1 g) their moisture content could not be determined before analysis, and the protein content was calculated relative to the weight of the samples obtained immediately after freeze-drying, thus giving a low protein content for these proteins. Losses encountered during handling may have additionally contributed to the low recoveries. Determining the protein content in the liquid extracts would most certainly have improved accuracy. This shortcoming, notwithstanding, the results obtained could be expected to reflect general trends in prolamin extractability with irradiation and cooking.

Polyphenols were determined according to the International Organization for Standardization (ISO, 1988) method for the determination of tannins in sorghum grain. It is based on the interaction of ferric ions with phenols in the presence of ethanolamine to give a coloured complex (Mole & Waterman, 1987). This method is not specific for one single type of polyphenol, which makes it appropriate for our study as the sorghum samples used were condensed tannin-free sorghums. However, such sorghums can contain phenolics such as phenolic acids and flavonoids (Hahn *et al*., 1984; Serna-Saldivar & Rooney, 1995), which would give a positive reaction with this assay.

To establish whether or not the effects of irradiation were influenced by the presence of polyphenols, which are known to possess antioxidant activity (AOA) (Rice-Evans, Miller & Paganga, 1997), AOA was measured using the ABTS radical cation decolorization assay (Re *et al*., 1999). The $ABTS^{\cdot+}$ radical is generated by oxidation of ABTS with potassium persulphate and the radical is then reduced in the presence of hydrogen-donating antioxidants. The percent inhibition of absorbance at 734 nm is a measure of the AOA. The ABTS method is suitable for use with samples where pigmentation is present (Awika, 2002) as in the red sorghum BR7, since the pigmentation would not interfere with the results. The method also has the advantage of being applicable to antioxidants

including flavonoids and carotenoids (Re *et al*., 1999), the former being present in sorghum (Hahn *et al*., 1984; Serna-Saldivar & Rooney, 1995).

Concerning the determination of sulphydryl groups and disulphide bonds, the solid-state direct colorimetric assay for disulphide bond determination is said to be a rapid and convenient method for determining cysteine and cystine content of suspensions that contain a mixture of soluble and insoluble proteins (Thannhauser *et al*., 1987). This assay was developed to avoid the tedious extraction procedure that could not solubilise all the proteins and required removal of the reducing agent before assay by colorimetric means (Thannhauser *et al*., 1987). Ellman's reagent, 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) reacts specifically with thiol groups and $NTSB^{2-}$ (disodium-2-nitro-5-thiosulphobenzoate) reacts with cysteine and thiol groups formed after reduction of disulphide bonds with sodium sulphite (Thannhauser *et al*., 1987; Chan & Wasserman, 1993; Pöyri, Mikola, Sontag-Strohm, Kaukovirta-Norja & Home, 2002). To maximize reaction of the protein with DTNB and $NTSB^{2-}$, a combination of urea and SDS are added to the reaction mixture to expose any thiol or disulphide bonds that may be buried within the hydrophobic protein matrix (Chan & Wasserman, 1993). This was particularly important for the porridge samples used in this study, as it is known that the kafirins and zeins are hydrophobic (Wall & Paulis, 1978; Wilson, 1983) and crosslink through disulphide bonds during cooking (Hamaker *et al*., 1987). Some of these disulphide bonds may become buried in the protein matrix, hence the need for SDS and urea to expose them. However, it appears from my results that not all of the disulphide bonds, particularly in the case of sorghum, were exposed for assay possibly because the proteins were extensively crosslinked and folded after cooking. Studies by Chan & Wasserman (1993) and Narayan *et al.,* 2002 had shown that formation of stable tertiary structure in proteins may result in disulphides bonds being 'locked in' and therefore not detectable. It is possible that crosslinking of sorghum during cooking may have caused folding of the protein that may in turn have prevented some of the disulphide bonds from being detected. This poses a limitation to the use of this assay, especially with highly crosslinked products, since it appears that not all of the disulphide bonds may be accessed for determination. In view of this limitation, therefore, the results of this assay only represent apparent values and should therefore be treated with caution.

One major advantage of this assay though, is that regardless of whether DTNB and $NTSB^{2-}$ reacts with soluble or insoluble proteins, the product of this reaction, the chromophore NTB^{2-} (2-nitro-5-thiobenzoate) is soluble in aqueous solution (Chan & Wasserman, 1993). The solids can then be separated by centrifugation and the absorbance of the supernatant read. In addition, the reaction of NTSB with thiols and disulphides can be carried out in the presence of dissolved oxygen and the reducing agent, sodium sulphite, which eliminates the inaccuracy and inconvenience associated with the necessity to work under an oxygen free atmosphere as well as the need to remove the reducing agent (Thannhauser *et al*., 1987). The reliability of this assay was tested with a reference protein, bovine serum albumin (BSA), and the measured cysteine content was 35.6 mol %, which agrees with that calculated from the amino acid sequence of BSA (35 mol %). Different concentrations of sulphite were also tested to ensure sufficient sulphite was available to reduce all the disulphide bonds, and the 0.1M sulphite reported in this method was found to be optimal.

Electrophoresis describes the migration of a charged particle under an electric field (Wilson & Walker, 1995). SDS-PAGE separates proteins according to subunit size (Smith, 1994) and has been used to determine changes in the molecular weight of proteins following irradiation (Bhatty & MacGregor, 1988; Puchala & Schuessler, 1993; Nićiforović, Radojčić & Milosavljević, 1999).

In SDS-PAGE, the protein is saturated with SDS, which masks the natural charge of the protein. SDS also denatures the protein, degrading its secondary, tertiary and quaternary structure and aids solubilisation (Otter, 2003). Reducing agent is added to break disulphide bonds when electrophoresis is carried out under reducing conditions. Polyacrylamide gel is used because it has pore sizes similar to that of protein molecules and so contributes a molecular sieving effect (See & Jackowski, 1989; Smith, 1994). The proteins bind SDS, become negatively charged and are separated based on size alone (Smith, 1994). It is, however, important that the protein standards and unknown proteins bind equal amounts of SDS per gram of protein, which will ensure identical mobility on SDS-PAGE gels. If this does not happen, it will result in differential mobility on the gel and give an unreliable estimation of molecular weight (Bunce, White & Shewry, 1985).

The lower molecular weight estimations obtained for the γ-prolamins in this study (22-26 k) as opposed to 27-28 k (Shewry, 2002) is believed to be a problem associated with the Laemmli method used. It has been observed that this method underestimates the molecular weight of γ-prolamins in sorghum and maize (Prof JRN Taylor, Department of Food Science, University of Pretoria, personal communication).

The molecular weight of the proteins in this study was initially determined using the uniform gel system, but because band separation was not clear on this system, gradient gels were subsequently used. Gradient gels because of their decreasing pore sizes give increased protein sieving effect at high polyacylamide concentrations, leading to the formation of sharper bands at lower molecular weights, since the proteins are about the same size as the sieve. Gradient gels also allow determination of polypeptides with a wider range of molecular weights (Bollag, Rozycki & Edelstein, 1996).

Infrared spectra are increasingly utilized for the analysis of peptides and proteins because they detect the amide (peptide) bond, which display distinct IR signals for differently folded peptides and proteins (Singh, 2000). Proteins are composed of a mixture of different structural elements, including α-helical, β-sheets, turns and unordered strands. Each of these has a characteristic hydrogen bonding pattern involving amide C=O and N-H groups, and therefore the amide C=O groups associated with each structural element may be expected to have a characteristic electron density. This in turn will provide a characteristic absorption frequency (Jackson & Mantsch, 1993). It is this separation of the amide absorptions that underlies the determination of protein secondary structure by IR spectroscopy

In the infrared spectra, the secondary structure of proteins is most clearly reflected by the Amide I and II regions, particularly the former which absorbs around 1620-1690 cm^{-1} (Byler & Susi, 1986, Bandekar, 1992). Despite the clarity of protein secondary structure in the Amide I region, it has the disadvantage of absorbing water bands at 1640 cm^{-1} interfering with protein bands at 1650 cm^{-1} and the difficulty of assigning peaks correctly due to serious overlapping of the random coil and the α-helix bands (Singh, 2000). Water interference was not expected to be a problem in this study because the samples used

were freeze-dried. Despite its disadvantages, the Amide I region is, however, considered useful for monitoring structural changes caused by protein denaturation (Christy Yukihiro & Gregoriou, 2001). The Amide II region, on the other hand, is not as sensitive as the Amide I and III regions to variations in secondary structure content and is not by itself used for secondary structure estimation (Singh, 2000). The Amide I and II bands were used in this study to determine secondary structure components of the FTIR spectra because they gave high signal bands.

The Amide III region (1200-1350 cm^{-1}) does not have interfering OH vibrations from water and no overlapping bands arising from different secondary structures (Singh, 2000). It is, however, limited for secondary structure analysis because of a lower signal band that requires higher signal-to-noise ratio spectra for accurate analysis, and interference from CH vibrations of amino acid side chains and other non-protein groups (Singh, 2000). The Amide III band was not used in this study because of low signal bands.

FTIR was carried out in this study using high pressure diamond optics and a microscope, ATR (Attenuated Total Reflectance) Zinc selenide cell and KBr pellets. High pressure diamond optics had the advantage of needing smaller samples (about 10 mg) and gave better resolution spectra compared to ATR Zinc selenide cell and KBr pellets. ATR requires perfect contact between the ATR crystal and the sample for good intensity spectra (Spectrum, 2.0 Users Guide, 1998), and uses large quantities of sample (about 0.3 g), which was a handicap in this study due to limited protein samples. In addition, ATR did not give good intensity spectra compared to the diamond cell possibly due to poor contact between the sample and the ATR cell. With KBr pellets, it is possible that the samples were unevenly distributed within the pellet, and this may have caused the lower resolution of the spectra obtained with this method.

Compared to other methods of protein secondary structure analysis such as nuclear magnetic resonance (NMR), circular dichroism (CD) and Raman spectroscopy, FTIR spectroscopy has the advantage that it can be used with protein preparations of low protein concentration (Jackson & Mantsch, 1995). High molecular weight proteins can also be studied using FTIR (Singh, 2000). CD analysis requires an accurate protein

concentration and is limited to clear protein solutions. NMR requires protein preparations of high protein concentration and is limited to small proteins of about 200 amino acids (Jackson & Mantsch, 1993; Singh, 2000). However, FTIR alone is not sufficient to unequivocally establish protein secondary structure without verification by other methods such as NMR and X-ray diffraction (Wilder, Friedrich, Potts, Daumy & Francoeur, 1992; Li-Chan, 1998).

Raman spectroscopy has the advantage of having a high signal-to-noise ratio, but its use is impaired by the fact that it requires samples with high protein concentrations; otherwise there is excessive fluorescence (Jackson & Mantsch, 1993; Singh, 2000; Thygesen, Lokke, Micklander & Engelsen, 2003). Raman spectroscopy was attempted in this study, but, because of the low purity of the samples, there was too much fluorescence and no reasonable spectra could be obtained.

3.2 Sorghum and Maize Proteins and the Effects of Cooking on their Protein Digestibility

The aim of this study was to determine if irradiation prior to cooking could alleviate the reduction observed in protein digestibility when sorghum flour is cooked into porridge. To assess the effects of these treatments on protein digestibility, the *in vitro* pepsin and multienzyme assays were used. Results of sorghum protein digestibility obtained using the pepsin assay has previously been shown to correlate with *in vivo* protein digestibility (Axtell *et al.,* 1981; MacLean *et al.,* 1981). In this study, the results of sorghum protein digestibility as determined by the multienzyme assay did not correlate with the pepsin assay (Table 3.1). Because pepsin digestibility has been shown to correlate with *in vivo* digestibility, this discussion will focus on the effects of irradiation and cooking on protein digestibility as determined by the pepsin assay.

Table 3.1. Correlation matrix showing the relationship between pepsin and multienzyme digestibilities with prolamin extractability for sorghum and maize flours

Sorghum BR7	Prolamins	Pepsin	pH stat
Prolamins	1		
Pepsin	0.83**	1	
pH stat	0.20	-0.05	1
Sorghum Madjeri	Prolamins	Pepsin	pH stat
Prolamins	1		
Pepsin	0.75*	1	
pH stat	0.17	0.42	1
Maize PAN 6043	Prolamins	Pepsin	pH stat
Prolamins	1		
Pepsin	0.32	1	
pH stat	0.49	0.62	1

* $p<0.05$; ** $p<0.01$

Sorghum and maize prolamin proteins in the uncooked state contain disulphide bonds (Esen, 1987; Oria *et al*., 1995a). Argos *et al*. (1982) proposed a model for α-zeins that shows the presence of intra- and inter-molecular hydrogen bonds within and between α-zein polypeptides. According to Koppelman, Nieuwenhuizen, Gaspari, Knippels, Penninks, Knol, Hefle & De Jongh (2005), disulphide bonds contribute to the stability of protein structure on the secondary and tertiary folding levels. Likewise, in soybean proteins hydrogen and disulphide bonds are considered to be important in maintaining network structure (Marsman, Gruppen, de Groot & Voragen, 1998). One can therefore presume that the disulphide bonds (Esen, 1987; Oria *et al*., 1995a) and hydrogen bonds (Argos *et al*., 1982), present in sorghum and maize prolamins are involved in stabilising the protein structure.

During wet cooking of sorghum, the formation of disulphide bonds between prolamin proteins and possibly other proteins may cause the protein structure to become more compact and folded (Fig 3.1b), considering that disulphide bonds contribute to the folded structure of proteins (Koppelman *et al*., 2005).

Because disulphide bonds can crosslink proteins polypeptides to form macropolymers, they could cause folding of the protein, with the result that some peptide bonds susceptible to proteolysis could become inaccessible to proteolytic enzymes, thus rendering these macropolymers less digestible (Carbonaro, Bonomi, Iametti, Cappelloni & Carnovale, 1998). This, is so, because, the digestibility of a protein is dependent on the accessibility of susceptible peptide bonds to specific proteolytic enzymes (Carbonaro *et al*., 1998). Crosslinking of proteins, as suggested by Hurrell & Finot (1982), reduces their digestibility by preventing enzyme access to peptide bonds or by masking sites of enzyme attack. Thus in wet cooked sorghum, crosslinking of proteins through the formation of disulphide bonds may result in folding of the proteins, preventing enzyme access to them, hence, the reduction in digestibility.

As in the case of wet cooked sorghum, reduced proteolysis as a result of the formation of disulphide-bonded macropolymers during processing has also been reported in whey proteins (Carbonaro *et al*., 1998) and in soy bean proteins (Hager, 1984). In maize, on the

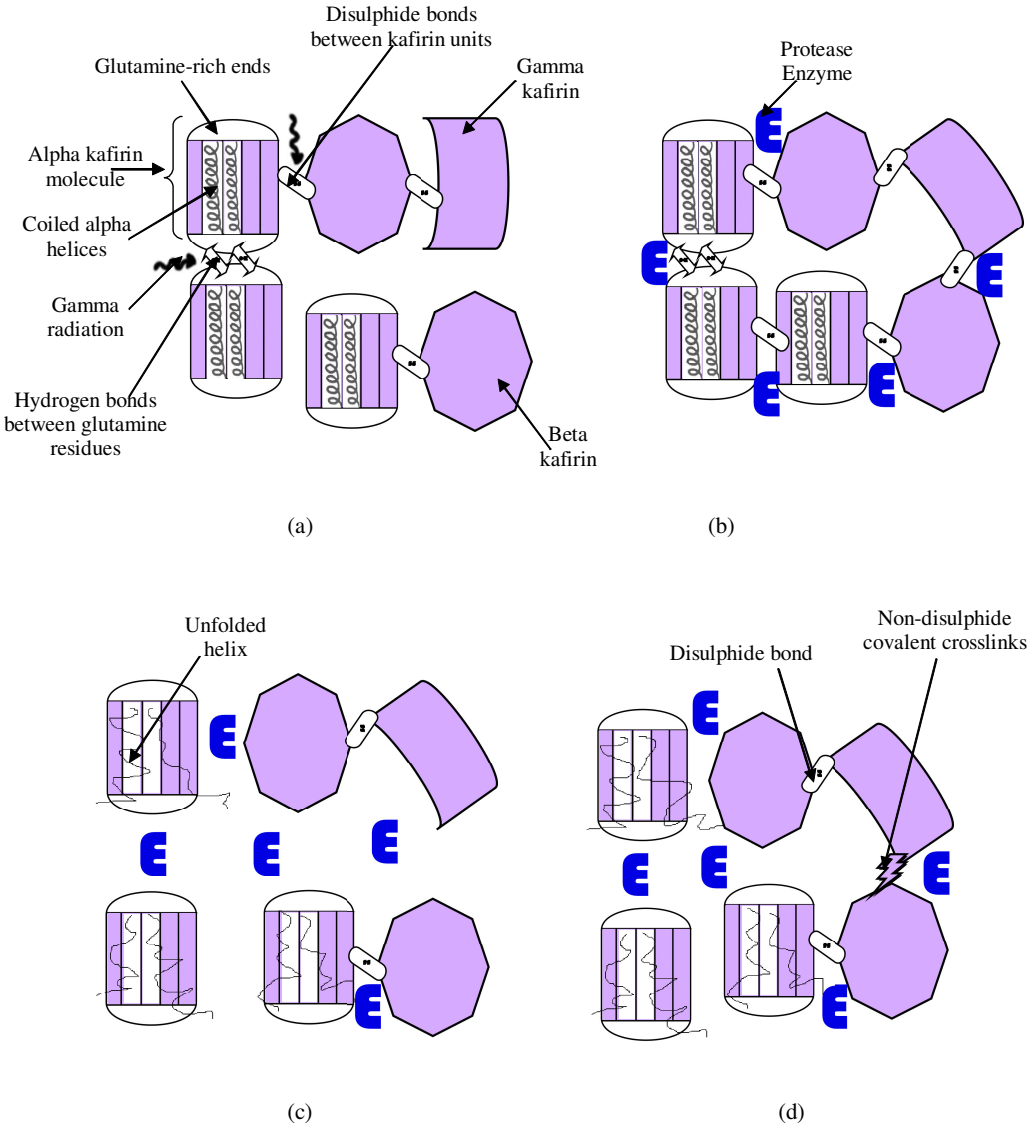

Fig 3.1 Model showing sorghum kafirin proteins, and proposed changes in sorghum kafirin crosslinking patterns with cooking alone and irradiation combined with cooking, and the effects of these crosslinks on protease enzyme access to the proteins; (a) kafirins in uncooked flour showing possible sites of attack by irradiation, (b) porridge from unirradiated flour, (c) porridge from 10 kGy dry irradiated flour, (d) porridge from 50 kGy wet irradiated flour.

other hand, disulphide bond formation during wet cooking is less extensive compared to sorghum, and has been advanced as the reason for the lower reduction in maize protein digestibility on cooking (Duodu *et al*., 2002). The finding that porridges from unirradiated sorghum flour had more dimers compared to their maize counterparts (section 2.2) supports this proposition. However, the results of disulphide bonds did not agree with this proposition. The results, showed similar levels of disulphide bonds in sorghum and maize porridges. Possible reasons for this discrepancy have been discussed in section 3.1.

Pepsin hydrolyses peptide bonds between hydrophobic amino acids such as phe-val and phe-ala residues, which frequently occupy the interior of the proteins (Brody, 1994). Such amino acids and peptide bonds occur in kafirin (DeRose *et al*., 1989) and zein (Viotti, Cairo, Vitale & Sala, 1985). It is possible therefore that during cooking, with the formation of disulphide bonds, the proteins become more folded enclosing the hydrophobic residues further inside the protein core, thus preventing them from coming into contact with the enzyme. This may contribute to the reduced protein digestibility of sorghum when cooked into porridge.

3.3 Effects of Irradiation and Cooking on Protein Digestibility of Sorghum and Maize Flours

Irradiation generates free radicals that can attack protein molecules. Radiation energy can disrupt non-covalent bonds (hydrogen bonds and hydrophobic interactions) and covalent (disulphide) bonds (Garrison, 1987; Swallow, 1991; Kempner, 1993) that stabilise protein structure. Hydrogen bonds, such as those in the Argos model between glutamine residues, can be disrupted when the glutamine residues are deamidated following irradiation (Venkatachalam & Sathe, 2003). Cleaving of disulphide bonds by irradiation can occur either, through the direct action of ionising radiation on disulphide bonds or through the secondary effects of water radiolytic products on disulphide bonds (Di Simplicio *et al*., 1991). Direct action will most likely have dominated during irradiation of the dry flours, where radiation energy reacts directly with the protein with the ejection of electrons to

form free radicals (Swallow, 1991). On the other hand, during wet irradiation, secondary effects will be more prevalent. In wet systems, radiation energy is absorbed by water, which then dissociates to give hydroxyl radical, hydrated electrons and other radical species, which are very reactive, and will in turn react with proteins producing free radicals. The reaction below shows the possible radiolytic products that can be obtained from the radiolysis of water:

$$H_2O \longrightarrow \cdot OH + \acute{e}_{aq} + H\cdot + H_2 + H_2O_2 \qquad \text{(Thakur \& Singh, 1994)}$$

Energy not taken up by water is taken up directly by the protein and other molecules present with the ejection of electrons to form radicals. Irradiation in wet medium will therefore achieve greater radiolytic damage because of the combination of both direct and indirect effects. The finding that the protein digestibility of porridges from dry sorghum flours irradiated at 50 kGy was similar to digestibility of porridges from wet flours irradiated at 10 kGy, and that protein digestibility of maize porridges was only significantly affected in wet irradiated flour samples, demonstrates the enhanced effects of radiation damage in wet medium. Similar reports of the enhanced effects of radiation damage in wet compared to dry medium appear in the literature (Cieśla *et al*., 2000; Lee *et al*., 2003).

Since disulphide and hydrogen bonds are involved in stabilising protein structure, their breaking can result in loss of conformational or structural integrity that could expose additional peptide bonds and enhance proteolysis (Kananen, Savolainen, Mäkinen, Perttilä, Myllykoski & Pihlanto-Leppälä, 2000; Venkatachalam & Sathe, 2003; Koppelman *et al*., 2005). Working with dry beans, Reddy, Pubois & McGinnis (1979) concluded that irradiation of beans at 210 kGy prior to autoclaving could cause changes in their protein structure that enhanced denaturation of the protein during subsequent heating, and improved its digestibility. It is possible that in sorghum, following irradiation, the protein structure may have been changed, such that it impaired the formation of disulphide bonds during cooking. The observation of fewer disulphide bonds in proteins of porridges from irradiated flour compared to unirradiated flour lends support to this argument.

In an attempt to explain the mechanism by which irradiation alleviated the reduction in cooked sorghum protein digestibility, this discussion will focus on the prolamin proteins (kafirins), which are the majority of the proteins in sorghum, and are the proteins directly implicated in reduced protein digestibility in wet cooked sorghum (reviewed by Duodu *et al*., 2003). Figure 3.1 shows a graphical representation of the proposed mechanism for the alleviation of the reduction in cooked sorghum protein digestibility, when cooking was preceded by irradiation of flour.

As stated, kafirin, like zein, can be divided into three species α-, β- and γ-kafirins, based on similarities in their solubilities, molecular weight and cross reactivity of kafirins with zein antisera (Shull *et al*., 1991). Amongst these, the α-kafirins are the major group, representing about 80% of the total kafirins, while β- and γ-kafirins account respectively for about 8 and 12% of total kafirins (Shull *et al*., 1991). Argos *et al*. (1982) proposed a structural model for the zein 19 and 22 k protein (the α-zein polypeptide species) based on CD data and their amino acid sequences. Given the sequence homology between zein and kafirin (DeRose *et al*., 1989), the model can also be applied to α-kafirin. The model shows α-zein as consisting of a collection of nine helical rods packed in a cylinder, with the top and bottom of the cylinder populated by glutamine residues. Polar and hydrophobic residues distributed along the helical surfaces will allow intra- and intermolecular hydrogen bonds and van der Waals interaction among neighbouring helices such that the rod-shaped zein molecule could aggregate in molecular planes which can then stack through glutamine interactions (hydrogen bonds) at the cylinder caps (Fig 3.1a). This model does not show any intra- or intermolecular disulphide bonds, possibly because of the low cysteine content of the α-zein, about 1 or 2 cysteine residues (Shewry, 1995). The structure of β- and γ-zeins are, however not known.

It has been proposed that during wet cooking of sorghum, disulphide crosslinks are formed between the different kafirin species with themselves and possibly with matrix proteins, to form protein polymers that are less digestible, thus, accounting for the reduction in protein digestibility when sorghum is cooked into porridge (Oria *et al.,* 1995b). Fig 3.1b shows proposed disulphide bonding between kafirin species when sorghum is cooked into porridge, and how this affects enzyme access to the proteins.

However, sorghum kafirins in the uncooked flour contain disulphide bonds (Oria *et al*., 1995a) as well as hydrogen bond and van der Waals interaction, as seen in the Argos model. When sorghum flours are subjected to irradiation therefore, these disulphide and hydrogen bonds may become cleaved by radiation energy, as discussed.

Considering the kafirin proteins, it is conceivable therefore, that, breaking of the disulphide bonds between the kafirin species and between these and other proteins, and breaking of hydrogen bonds within and between the α-kafirin helices, would lead to unfolding of the helices within the α-kafirin molecule, and dissociation of the helices and kafirins species from one another. With the individual kafirins species now more free to move, this would increase the intermolecular distances between them and create an open protein network. Unfolded and free polypeptides, together with a more open protein network would reduce the chances of the cysteine residues coming together during subsequent cooking to form disulphide bonds or other crosslinks. Consequently fewer disulphide bonds would be expected in porridges from irradiated flours compared to porridges from unirradiated flours (Fig 3.1c). This is in agreement with the data for disulphide bonds and SDS-PAGE summarized in Table 3.2. With an open protein network following irradiation and cooking, it is probable that additional peptide bonds were exposed to proteolysis and the enzymes had better access to the proteins, hence, the alleviation in the reduction in protein digestibility.

As stated earlier, pepsin hydrolyses peptide bonds between hydrophobic amino acids. Although protein hydrophobicity was not measured in this study, work done by Dogbevi, Vachon & Lacroix (2000) with red kidney beans, whose proteins are also globular, indicated that irradiation at doses up to 8 kGy increased hydrophobicity through exposure of hydrophobic amino acids following unfolding of the protein structure. With a more open protein network in the irradiated cooked porridges, it is possible that the pepsin susceptible hydrophobic amino acid peptide bonds buried within the protein core were exposed, resulting in an increase in protein digestibility.

Table 3.2. Summary of results showing changes in pepsin protein digestibility of sorghum and maize flours with some protein attributes when unirradiated and irradiated (mild and severe) flour samples are cooked in to porridge

Sorghum BR7	Pepsin digestibility	Prolamin extractability	NSI	AG solubility	Free SH groups	SS bonds	SDS PAGE dimer	L-colour	b-colour
Unirradiated flour	Control	Control	Control	Control	ND	ND	ND	Control	Control
Porridge from unirradiated flour	↓↓	↓↓	-	↓	Control	Control	Control	↓	↓
Porridge from 10 kGy dry irradiated flour	-	↓	-	↓	↑	↓	↓	↓	↑
Porridge from 50 kGy wet irradiated flour	↓	↓↓	-	↓↓	↑↑	↓↓	↓	↓	↑

Sorghum Madjeri	Pepsin digestibility	Prolamin extractability	NSI	AG solubility	SH groups	SS bonds	SDS PAGE dimer	L-colour	b-colour
Unirradiated flour	Control	Control	Control	Control	ND	ND	ND	Control	Control
Porridge from unirradiated flour	↓↓	↓↓	↓	↓	Control	Control	Control	↓	↓
Porridge from 10 kGy dry irradiated flour	-	↓	-	↓	↑	↓	↓	↓	↑
Porridge from 50 kGy wet irradiated flour	↓	↓	↓	↓↓	↑↑	↓↓	↓	↓	↑

Table 3.2 Cont'd

Maize PAN 6043	Pepsin digestibility	Prolamin extractability	NSI	AG solubility	SH groups	SS bonds	SDS PAGE dimer	L-colour	b-colour
Unirradiated flour	Control	Control	Control	Control	ND	ND	ND	Control	Control
Porridge from unirradiated flour	↓	↓	-	↓	Control	Control	Control	-	↓
Porridge from 10 kGy dry irradiated flour	↓	↓	↓	↓	-	-	-	↓	↑
Porridge from 50 kGy wet irradiated flour	↓↓	↓↓	↓	↓↓	↑	-	↓	↓	↑

- Same as control
Arrows indicate degree of increase (↑) or decrease (↓) relative to control
ND Not determined
NSI Nitrogen solubility index
AG Albumin and globulin proteins
SH Sulphydryl groups
SS Disulphide bonds

As the prolamins crosslink through disulphide and other bonds during cooking, their extractability also decreases in parallel with protein digestibility (Hamaker *et al.*, 1986). Thus, as stated, Hamaker *et al* (1994) proposed that the extractability of prolamins could somehow be related to their protein digestibility. It was therefore interesting to observe that the alleviation in the reduction in sorghum protein digestibility with cooking of dry flour irradiated at 10 kGy paralleled a relief in the reduction of the extractability of its prolamin proteins, compared to prolamins of porridges from unirradiated flour. Furthermore, there were significant positive correlations between pepsin protein digestibility and prolamin extractability for sorghum BR7 ($r = 0.83$; $p<0.01$) and Madjeri ($r = 0.75$; $p<0.05$) (Table 3.1). Maize prolamin extractability was, however, not significantly correlated with pepsin protein digestibility. This finding is not surprising considering that the extractability of maize prolamins, like its protein digestibility, was less affected by cooking and the combination of irradiation and cooking, compared to sorghum (Table 3.2).

The flour samples prepared for irradiation were not packaged under vacuum, implying that some oxygen would have been present in the system at the onset of irradiation. It has been demonstrated that irradiation in the presence of oxygen leads to breakdown of proteins to smaller peptides, whereas in the absence of oxygen, polymerisation of proteins occurs (Davies, 1987; Puchala & Schuessler, 1993; Kume & Matsuda, 1995; Tuce *et al*., 2001). As discussed in the literature review (section 1.2.5.1), Schuessler & Schilling (1984) proposed a mechanism by which protein breakdown occurs during irradiation in an oxygenated system. It begins with an attack on the peptide bond of the protein by the hydroxyl radical to form a protein radical, which then reacts with oxygen to form a peroxy radical. The two peroxy radicals can combine to form an oxyradical which can later decompose into peptides in the presence of oxygen. If, on the other hand, oxygen is absent, the protein radicals can combine to form polymers.

During irradiation, the oxygen present in the system is being used up and may become depleted with time (Swallow, 1991). The flour samples for irradiation were packaged in polyethylene bags that were placed in polystyrene boxes. Both types of packaging material have poor gas barrier properties (Mauer, 2003). Thus the samples had access to

oxygen during irradiation. It is, however, not certain whether the rate of oxygen usage during irradiation equalled the rate of oxygen diffusion into the polyethylene bags. Nonetheless, it appears that under severe conditions of irradiation, protein polymers were formed as suggested by the reduction in the solubility of the albumin and globulin (AG) proteins, as well as the reduction in the alleviation in protein digestibility. It is possible therefore, that oxygen could have become limiting under severe conditions of irradiation and favoured protein polymerisation. This would, however, need to be investigated.

Protein polymers, often referred to as aggregates, formed during irradiation are proposed to be linked by disulphide (Garrison, 1987), bityrosine (Davies, 1987; Davies *et al*., 1987a; Mezgheni *et al*., 1998; Ressouany, Vachon & Lacroix, 1998), and other crosslinks involving protein radicals (Tuce *et al*., 2001; Lee *et al*., 2003). The absence of an increase in disulphide bonds with irradiation and cooking (Table 3.2) may suggest that disulphide bonds did not contribute significantly to polymer formation in porridges from irradiated flours. Presumably, other crosslinks may have been involved.

The reduction in the solubility of AG proteins under severe conditions of irradiation, and the reductions observed in lysine content in porridges from 50 kGy wet irradiated samples could suggest the formation of protein complexes involving lysine, such as lysinoalanine and Maillard products. Lysinoalanine is formed when a racemised amino acid (D-amino acid) undergoes β-elimination to form a dehydroalanine derivative, which can later crosslink with the ∈-amino group of a lysine side chain to form lysinoalanine (Cheftel *et al*., 1985; Friedman, 1999a). Irradiation of proteins can lead to racemization of amino acids and subsequently to lysinoalanine formation (Friedman, 1999b). Lysinoalanine formation results in reduced protein digestibility and in the destruction of amino acids involved in the crosslinking (Liardon & Hurrell, 1983). The AG proteins of sorghum, like those of maize, are rich in lysine (Van Scoyoc *et al*., 1988; Yau *et al*., 1999), and thus can participate in the formation of lysinoalanine. Proteins containing lysine can also undergo Maillard reactions with reducing sugars, resulting in the formation of insoluble polymers and a decrease in nitrogen solubility (Yen, Lee & Chichester, 1989). The occurrence of Maillard reactions was assessed by measuring the lightness and yellowness of the flour colour, since Maillard reactions produce products with brown and yellow coloured

pigments (Whistler & Daniel, 1985; Yen *et al*., 1989). The results summarised in Table 3.2 indicate that Maillard reactions may have occurred in porridges from irradiated flour.

Browning in irradiated foods has been associated with Maillard reactions (Wootton *et al*., 1988), and/or the oxidation of phenolics, and aromatic amino acids (phenylalanine and tyrosine) to produce melanin-type pigments (Wootton *et al*., 1988; Giroux & Lacroix, 1998). Oxidation of phenylalanine produces tyrosine and hydroxyl derivatives. Oxidation of tyrosine produces 3,4-dihydroxyphenylalanine (DOPA), which can be oxidised further to produce crosslinking reactions which provoke melanin-type pigment formation (Diehl, 1990; Giroux & Lacroix, 1998). Flour colour became darker and yellowish in porridges from irradiated flour (Table 3.2). The darkening of porridges with irradiation of flour, together with the decrease in solubility of AG proteins, suggests that Maillard browning and other crosslinks such as bityrosine may be involved in protein polymerisation under severe conditions of irradiation.

Another possible indication of Maillard browning was the slight increase in antioxidant activity in 50 kGy dry irradiated sorghum and maize flours, as described in section 2.1. Products from Maillard reactions have been shown to possess antioxidant activity (Baltes, 1982; Eiserich & Shibamoto, 1994), with the antioxidant effects being greatest at the beginning of the browning reactions, due to Maillard intermediates such as the reductones (Baltes, 1982). These Maillard intermediates give rise to yellow coloured products (Whistler & Daniel, 1985), and the yellow colouration was highest in uncooked dry sorghum and maize flours irradiated at 50 kGy, which could represent the onset of Maillard browning. With Maillard reaction initiated in the irradiated flour, cooking will most likely accentuate the process. Products from Maillard reactions can inhibit proteolytic enzymes (Öste *et al*., 1986, 1987), and cause a reduction in protein digestibility (Hafez, Mohamed, Hewedy & Singh, 1985). Maillard reactions may therefore have contributed to the reduced alleviation in protein digestibility of sorghum porridges from severely irradiated flours, through the formation of crosslinks that prevent enzyme access to peptide bonds and/or by inhibiting the enzyme (Hurrell & Finot, 1982).

As mentioned earlier, bityrosine crosslinks could also be involved in protein polymerisation during irradiation, and this could adversely affect its digestibility (Davies, 1987; Davies & Delsignore, 1987). Davies *et al* (1987a) reported increased formation of bityrosine in bovine serum albumin (BSA) with increased exposure to hydroxyl radical produced by gamma irradiation that decreased its susceptibility to proteolysis. Ressouany *et al*., (1998) also found that the amount of bityrosine crosslinks increased with increasing irradiation dose when calcium caseinate solution was irradiated with doses of up to 64 kGy. Bityrosine is a covalently bonded biphenol produced by reaction of two tyrosyl radicals or a tyrosyl radical plus a tyrosine molecule. The tyrosyl radicals are produced as a result of hydrogen abstraction by hydroxyl radicals (Davies *et al*., 1987a). Bityrosine crosslinks are more likely to form between two protein molecules (intermolecular) than within the same protein molecule (intramolecular), thus leading to polymerisation (Davies, 1987). Although bityrosine levels were not measured in this study, the possibility of their formation under severe conditions of irradiation could be indicated by the decreases observed in tyrosine content with irradiation. The formation of bityrosine crosslinks may therefore have contributed to the lower protein digestibility of these porridges, and needs to be investigated further.

Speculating further using the model proposed in Fig 3.1, it is possible that under severe conditions of irradiation of sorghum flour, the increased formation of covalent non-disulphide crosslinks (Maillard, bityrosine, lysinoalanine), as discussed above, may have caused some of the unfolded and free polypeptides to become crosslinked once again, with the result that the protein network closed up (Fig 3.1d). A tighter protein network would restrict enzyme access to peptide bonds and cause the subsequent reduction in the alleviation in protein digestibility of porridges from flour samples irradiated under severe conditions (Table 3.2).

With reference to the solubility and extractability of the sorghum proteins (NSI, AG solubility and prolamin extractability), the red sorghum BR7 behaved differently from the white sorghum Madjeri (Table 3.2). The reduction in prolamin extractability of porridges of sorghum Madjeri was alleviated by irradiation of flour, both under mild and severe conditions, before cooking, whereas, in sorghum BR7 the alleviation only occurred under

mild irradiation conditions. NSI was not significantly affected by irradiation and cooking in sorghum BR7, but it decreased in sorghum Madjeri with cooking alone. This decrease was relieved by irradiation of flour under mild conditions, but not under severe condition. Although, the behaviour of the AG was similar for sorghum BR7 and Madjeri with cooking alone or with irradiation and cooking, when flour samples were irradiated without cooking, the solubility of sorghum BR7 AG proteins decreased more with irradiation compared to Madjeri (Table 2.1.5). These differences in the solubility properties of sorghum BR7 and Madjeri were attributed to the higher levels of polyphenols in sorghum BR7, which could act as antioxidants (Awika & Rooney, 2004) and offer some protection against the effects of irradiation. Because the sorghums used were condensed tannin free cultivars, the values obtained for phenols could therefore have been due in part to phenolic acids, which are present in all sorghums (Hahn *et al*., 1984). The higher levels of phenols in sorghum BR7 may have been due to flavonoids (Hahn *et al*., 1984), which caused its red pigmentation. The flavonoids found in sorghum are the anthocyanidins, with the most common being the 3-deoxyanthocyanidins, which include apigeninidin and luteolinidin. Of these two, apigeninidin was found to be most prevalent in red sorghum bran (reviewed by Awika & Rooney, 2004).

The effect of irradiation on proteins depends on free radicals being freely available to react with the proteins. Polyphenols such as the flavonoids can act as antioxidants by directly scavenging free radicals such as superoxide, and peroxyl radicals (Rice-Evans *et al*., 1997; Velioglu *et al*., 1998), generated during irradiation. In so doing, they act as hydrogen or electron donors and in the process become oxidised themselves to *o*-quinones, which can react with available nucleophilic groups (XH) on proteins such as the ϵ-amino groups of lysine, thiol groups of cysteine and N-terminal amino groups, to bring about crosslinking and ultimately polymerisation (Haslam, 1989). Figure 3.2 shows a schematic representation of the mechanisms by which flavonoids can act as antioxidants and become oxidised to *o*-quinone radicals that can react with proteins to polymerise them, using apigeninidin, the flavonoid found in red sorghum bran.

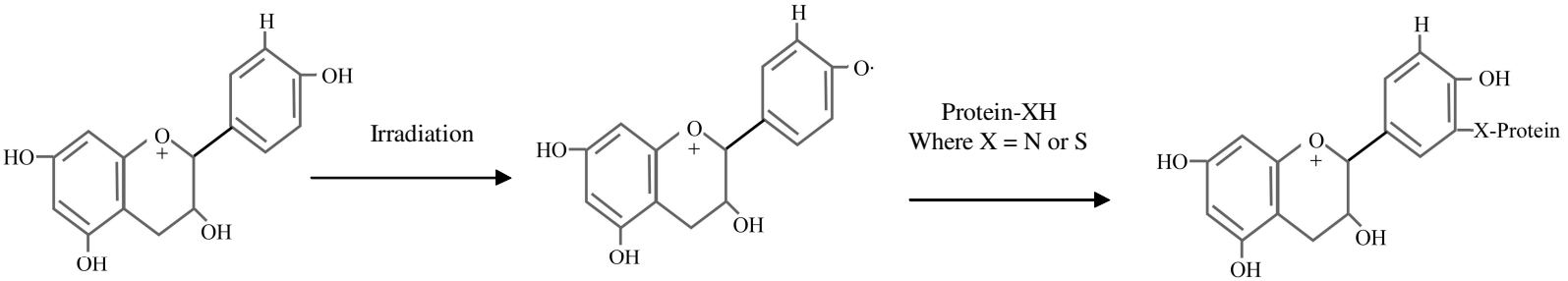

Fig 3.2. Model showing the proposed mechanism of crosslinking between oxidized polyphenols (*o*-quinone radical) with proteins through the amino group of lysine or the sulphur group of cysteine

The absence of a significant change in nitrogen solubility of sorghum BR7 with irradiation, without and with cooking (Table 3.2), may be a consequence of the protective antioxidant effects of BR7 polyphenols, which could have scavenged free radicals and thus reduced the effects of irradiation. However, in the case of AG solubility, which decreased more with irradiation alone in sorghum BR7 compared to Madjeri, the protective effects of the polyphenols may have been counterbalanced by the reaction of the *o*-quinones radical with the ϵ-amino groups of lysine (Fig 3.2) that is abundant in this fraction (Van Scoyoc *et al*., 1988; Yau *et al*., 1999), to polymerise the AG proteins and reduce their solubility. The possibility of the *o*-quinones complexing with cysteine sulphydryl groups (Haslam, 1989) may also be implicated in the reduced alleviation in the reduction of prolamin extractability in porridges from sorghum BR7 flours irradiated under severe conditions, compared to sorghum Madjeri.

In maize, cooked protein digestibility was only significantly reduced in porridges from wet irradiated flour (Table 3.1). In the case of sorghum, it was suggested that cleaving of disulphide and hydrogen bonds by irradiation may have effected a change in protein structure that limited the formation of disulphide crosslinks during cooking, and hence relieved the reduction in protein digestibility. As stated, maize zein proteins are very similar to sorghum kafirins, with the difference that zeins apparently have fewer disulphide bonds in the uncooked flour than kafirins (Hamaker *et al*., 1986). The amounts of disulphide bonds in uncooked sorghum and maize flours were, however, not measured in this study.

According to Swallow (1991), and based on rate constants, the sulphur-containing and aromatic amino acids are more susceptible to radiation damage. Therefore a protein that has more disulphide bonds would be expected to undergo greater conformational changes following irradiation. This has indeed been observed with soybean proteins (Byun *et al.,* 1994). These authors noted that soybean 11S proteins with 21 disulphide bonds and 2 free sulphydryl groups underwent greater conformational changes during irradiation at 20 kGy, compared to the 7S proteins that had neither disulphide bonds nor sulphydryl groups. By comparison therefore, one would expect less conformational changes in zeins compared to kafirins. The fact that the amount of disulphide bonds in zein were not

significantly affected by irradiation and cooking, whereas in sorghum disulphide groups decreased significantly with irradiation and cooking, suggests that protein structure of zein may not have been significantly affected by irradiation. The absence of a significant change in protein digestibility of maize porridges from dry irradiated flour compared to the unirradiated flour seems to agree with the above suggestion.

However, under wet conditions of irradiation, where non-disulphide crosslinks may have been formed, as in the case with sorghum, this may have caused the reduction in the protein digestibility of maize porridges from wet irradiated flours. It would therefore, appear that, because of the presence of fewer disulphide bonds in zeins, irradiation had no beneficial effect on the protein digestibility of their porridges, but rather under wet conditions of irradiation there was a direct negative effect, owing to the enhanced effects of irradiation in wet medium.